Food Bioactive Ingredients

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Analytical Methods in the Determination of Bioactive Compounds and Elements in Food

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Chapter 1
Introduction: Bioactive Compounds and Elements in Human Nutrition

Agnieszka Zgoła-Grześkowiak and Tomasz Grześkowiak

Abstract The bioactive compounds and elements, as the name implies (the Greek "bios" means life, and the Latin "activus" means dynamic or full of energy), are characterized by a certain biological activity that has a direct impact on the living organisms. The interaction can have a positive or negative effect, depending on the nature of the substance, its dose, and bioavailability. The bioactive compounds can act as antioxidants, enzyme inhibitors and inducers, inhibitors of receptor activities, and inducers and inhibitors of gene expression, among other actions. The essential trace elements play a vital role in many biochemical reactions and are important building blocks of some enzymes and proteins. Nevertheless, although the bioactive components of the human diet are not those necessary to meet the basic nutritional needs as the nutrients do, they still play an important role in human nutrition.

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Bioactive Compounds

The bioactive compounds are found in numerous plant foods such as fruits, vegetables, grains, and legumes but typically occur in small amounts. Among the active compounds found in plants, a few important groups can be mentioned: flavonoids, anthocyanins, tannins, betalains, carotenoids, plant sterols, and glucosinolates. Moreover, some non-plant foods such as meat, fish, milk, eggs, and others are also considered as sources of bioactive compounds. For example, meat, although not widely studied, contains important bioactive compounds. These include amino acids and peptides derived from meat proteins by enzymatic hydrolysis. The concentrations of these bioactive substances in meat and meat products contribute to the quality and taste of meat products.

An impressive number of known bioactive compounds are constantly increasing. All these compounds were identified as showing potentially significant health benefits. The main challenge in this area, however, is not only the identification of bioactive compounds and linking the health effects to them but also finding the underlying biological mechanisms of their action. The flavonoids are one of the groups that have been studied extensively, which has the greatest amount of evidence about their therapeutic potential in health, related to the reduction in the risk of developing chronic/degenerative diseases.

Flavonoids

Flavonoids are the largest group of bioactive compounds. Their structures are based on the flavan skeleton and, depending on the differences in their central ring, may be grouped into flavan-3-ols, flavanones, flavanonols, flavones, isoflavones, flavonols, anthocyanidins, and their sugar derivatives anthocyanins as well as chalcones possessing an opened central flavan ring. Each of the subgroups of flavonoids contains numerous active compounds that can be found in different plants including fruits, vegetables, and tea (Jeszka-Skowron and Zgoła-Grześkowiak 2014; Stalikas 2007; Walia et al. 2019). Most of these compounds are known to neutralize free radicals, have anti-inflammatory properties, and reduce the risk of cancer. There were also reports presenting their antidiabetic properties, preventing cardiovascular diseases, and even fighting influenza A virus (Sattanathan et al. 2010; Vaidya et al. 2016; Walia et al. 2019).

Phenolic Acids

Phenolic acids are the derivatives of hydroxybenzoic and cinnamic acids differing in the number and positions of the hydroxy and methoxy groups on the aromatic ring (Stalikas 2007). They often occur in the bound form – the hydroxybenzoic acid derivatives are bounded into hydrolyzable tannins, and the hydroxycinnamic acid derivatives occur as esters with glucose or quinic acid including caffeoyl and dicaffeoylquinic acids (Walia et al. 2019). The unbounded phenolic acid can be found in fruits, cereals, and beverages, including the most widely used tea and coffee, and are known for their anticancer properties and ability to scavenge radicals (Arai et al. 2015; Eroglu et al. 2015; Jeszka-Skowron and Zgoła-Grześkowiak 2014; Robins 2003; Terao et al. 1993).

Tannins

Tannins contain three or more phenol subunits and are classified into condensed and hydrolyzable. The condensed tannins are chemically condensed proanthocyanidins that are composed of flavonoids, while the hydrolyzable ones contain a central sugar esterified with gallic acid and condensed into gallotannins and ellagitannins depending on the type of bonds connecting the acidic units. Tannins can be found in many different berries, spices, cocoa, and tea (Walia et al. 2019). They have a positive influence on the human organism including the improvement of the urinary tract, reduction of cardiovascular diseases, and antioxidant properties (Kontiokari et al. 2001; Walia et al. 2019; Ziberna et al. 2010).

Methylxanthines

Methylxanthines including caffeine, theophylline, and theobromine are synthesized by many plant species and widely studied due to a variety of health benefits. Caffeine stimulates the central nervous system – it improves motor activity and enhances attention, vigilance, activity, and performance. Although it generally improves mood and lessens aggressiveness, chronic administration of high dosages of caffeine (and also theophylline) induces aggressive behavior. Reports on arm and hand tremors induced by caffeine were also published (Nehlig et al. 1992). Theophylline and theobromine also stimulate the central nervous system, but to a lesser extent. On the other hand, they are better cardiac stimulants (Beale Jr 2011). Moreover, theophylline is used in chronic obstructive pulmonary disease, asthma, and as a diuretic (Barnes 2013; Salihović et al. 2014). Caffeine can be found in tea and coffee, which are the most widely used beverages, while both caffeine and theobromine are present in cocoa powder and products containing it, mainly chocolate (Arai et al. 2015;

Fang et al. 2019; Franco et al. 2013; Monteiro et al. 2016; Zhang et al. 2019). Although the negative effects of caffeine and theobromine were observed at high doses, as described above, typical levels of tea, coffee, and chocolate consumed by humans do not pose a threat.

Carotenoids

Carotenoids are composed of eight isoprene units linked together. The conjugated double bonds make them yellow, orange, and red pigments often used in the industry. There are a few hundred carotenoids that are classified into two groups: carotenes and xanthophylls (Kulczyński et al. 2017; Zakynthinos and Varzakas 2016). Carotenes contain only the carbon and hydrogen atoms in their molecules and include lycopene, α-carotene, and β-carotene, while xanthophylls also have the oxygen atoms in the hydroxy and carbonyl groups located on one or both ends of the molecules and include lutein and zeaxanthin, among other compounds. Carotenoids can be found in many plants, in algae, and also in some animals. For example, carotene is found in a considerable amount in carrots, dill, kale, parsley, spinach, and sweet potatoes; lycopene in tomatoes, papayas, and watermelons; and both lutein and zeaxanthin in dill, spinach, and hens' egg yolk (Kulczyński et al. 2017; Takaichi 2011; Zaheer 2017). It has been shown that carotenoids have antioxidant properties and reduce the risk of atherosclerotic disease. β-carotene is a precursor of vitamin A, whereas lutein is important in the prophylaxis of age-related eyesight deterioration (Rodriguez-Concepcion et al. 2018; Walia et al. 2019; Widomska et al. 2016; Zaheer 2017).

Capsaicinoids

Capsaicinoids, mainly capsaicin and dihydrocapsaicin, can be found in plants belonging to the *Capsicum* genus and are responsible for their pungency (Lu et al. 2017). Capsaicin has numerous therapeutic properties, including treatment of obesity, diabetes, hypertension, and other diseases (Antonious 2018). Its anticancer properties are also studied, including the suppressing of carcinogenesis in the prostate, skin, breast, colon, lung, and human bladder, but some reports suggest its carcinogenic and co-carcinogenic activity as well (Lu et al. 2017; Surh and Lee 1996). What is more, oral exposure to high dosages of capsaicin can cause cardiovascular dysfunction and respiratory depression, eventually leading to death (Glinsukon et al. 1980). The susceptibility of different tested mammals to capsaicin differs, but the lethal dose or lethal concentration values are generally at a few dozen mg per kg (Antonious 2018; Glinsukon et al. 1980). These values are high but indicate that the supplementation with capsaicinoids must be done with some caution.

Peptides

Peptides are important because they are crucial materials required for the biosynthesis of proteins and are also used as a source of energy. They can be obtained from food proteins by enzymatic hydrolysis, microbial fermentation, or food processing. Alternatively, they can be synthesized from amino acids. Among them, the bioactive peptides can be found; they are generally short peptides consisting of 2–20 amino acids exerting a biological activity surpassing that of its expected nutritional value (Sánchez and Vázquez 2017). Abundant amounts of proteins can be found in plant and animal material. Rich sources of bioactive peptides include cereals, soybeans, eggs, meat, and milk (Sánchez and Vázquez 2017). Both isolated bioactive peptides and hydrolysates containing them exert positive actions that may be anticancer, antioxidant, antihypertensive, antimicrobial, antithrombotic, antidiabetic, and osteoprotective (Bhandari et al. 2020). However, there is also a risk connected with the use of the bioactive peptides because proper dosage and frequency of administration cannot be established in the absence of solid pharmacokinetic data. Moreover, the hydrolysates can contain immunogenic proteins and peptides that may cause allergic reactions in some humans (Chakrabarti et al. 2014).

Bioactive Elements

In recent years, the interest in bioactive elements in medical science as well as in nutrition has increased. Elements occur in the environment in different forms, and their bioavailability and transport properties strongly depend on them. It is possible that some elements, such as chromium, may be toxic or essential for the human body depending on their form. It is well known that trace elements act as key components of essential enzyme systems or other proteins that perform important biochemical functions. It is estimated that 98% of the human body weight consists of 9 non-metallic elements and 4 main metals (sodium, magnesium, potassium, and calcium) are present at 1.89%, while the rest, or approximately 0.02%, are made up of 11 typical trace elements (Prashanth et al. 2015). However, the latter group of elements has a significant influence on all body functions. Conducted observations and studies show that in the adult human body there are at least 29 different types of non-metallic and metallic elements. Among them, there are trace elements, the daily requirement of which is less than 100 mg, but their deficiency leads to disorders and may be fatal. These elements include copper, iron, zinc, chromium, cobalt, iodine, molybdenum, and selenium. However, there are also some trace elements whose role is not clear (nickel, silica, tin, vanadium, and aluminum) and those that cause toxicity, like mercury and lead (Prashanth et al. 2015).

Bioactive Elements Needed for the Proper Functioning of Humans

There are a few trace elements needed for the proper working of human organisms. They have important roles in our metabolism, including appropriate functioning of the copper-ATPase enzyme (copper) and glutathione peroxidase enzymes (selenium), blood functioning (iron), control of diabetes (chromium), and other functions (Anderson 2000; Harris 2001; Prashanth et al. 2015; Rotruck et al. 1973). However, it must be taken into account that the trace elements must be supplied in appropriate form and amount. For example, hexavalent chromium is carcinogenic (Prashanth et al. 2015), and an increased selenium intake may cause Keshan syndrome (Chen et al. 1980). What is more, for selenium the concentration range between deficiency and toxicity is very narrow, i.e., from 0.1 mg kg^{-1} to 1 mg kg^{-1} in the ingested food (Pedrero and Madrid 2009). This makes supplementation very difficult and enforces it to be done strictly according to the doctor's instructions as at too-high doses it can even cause death (Kenfield et al. 2015).

Bioactive Elements Negatively Influencing Living Organisms

The toxic elements (e.g., mercury and arsenic) were initially determined in food and the environment without any speciation. However, studies have shown that their effect on the human and animal organisms differs depending on the form in which they exist. The most widely known example of mercury poisoning is the Minamata disease, which was caused by poisoning due to consuming a large number of aquatic organisms containing organic mercury compounds – for years known as the methylmercury poisoning. It was believed that methylmercury found in local aquatic organisms was discharged to the bay from a nearby chemical plant, although it can also be formed from inorganic mercury compounds (Hamdy and Noyes 1975). The latest study has shown, however, that the most probable compound involved in the contamination of the Minamata Bay was α-mercuri-acetaldehyde (James et al. 2020). Nonetheless, the disclosure of the Minamata disease contributed to the growing interest in speciation of mercury.

Speciation is important also in the determination of other metals and semimetals. Toxicity of arsenic depends on many factors including its valence, with As(III) compounds being more toxic than As(V) compounds. In opposition to mercury, organoarsenic compounds are less toxic than inorganic arsenic compounds. The practically non-toxic arsenobetaine is present in substantial amounts in some foods and excreted in the urine mainly unchanged. Therefore, the determination of total arsenic in urine may lead to obtaining wrong conclusions, and speciation should preferably be applied (Mandal and Suzuki 2002).

Summary

The bioactive compounds and elements are very important in maintaining health. However, it must be taken into account that not only their deficiency but also an excess can be harmful. For example, eating food and drinking beverages containing antioxidants should be beneficial for scavenging the free radicals formed in the organism and decrease reactive oxygen species. Thus, the reduction of oxidative stress is possible. However, the oxidative processes are necessary for life as they provide energy to the organism. Therefore, although the basic processes taking place in the human body seem to be well known, there is still a lot of knowledge to acquire. The latest study on the gut microbiome of mice has shown that certain antioxidants (i.e., gallic acid) may be harmful and cause cancer. In the study, the authors have proven that the mutant p53 protein exerts the tumor-suppressive effects in the proximal mouse gut and that it was completely abolished and instead converted to oncogenic gain-of-function activity in the presence of the native microbiota in the distal part of the gut or specifically by supplementation with gallic acid (Kadosh et al. 2020). Therefore, studies on the role of bioactive compounds and elements in human nutrition and on the mechanisms involved in their activities are so important in understanding the advantages of their supplementation and threats connected with their inadequate levels in the human organisms.

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Chapter 2
Application of Liquid Chromatography for the Analysis of Flavonoids in Food: An Overview

Aleksandra Sentkowska

Abstract Flavonoids are a known group of organic compounds found in plants, where they act as dyes and natural fungicides. Together with the food of plant origin, they are delivered to the human body.

The chapter focuses on discussing the various modes of liquid chromatography used in the analysis of flavonoids in the food of plant origin. The most commonly used reversed-phase chromatography (RP-HPLC) has been compared with quite new hydrophilic interaction liquid chromatography (HILIC). Also, the potential of using two-dimensional chromatography in the analysis of polyphenolic compounds in the samples of plant origin is discussed. A separate subsection is devoted to chromatography combined with online antioxidant activity measurement. The whole chapter is completed by a short review of the detection methods used in the chromatographic determination of flavonoids.

Introduction

Phenolic compounds are secondary plant metabolites, which are intensively studied for their health-promoting properties. Their antimutagenic, anticancer, anti-inflammatory properties as well as the ability to prevent many oxidative-stress-associated diseases have been confirmed in several publications (Jafari et al. 2014; Mojzer et al. 2016; Shahidi and Ambigaipalan 2015; Soto-Vaca et al. 2012). The main groups of phenolic compounds are flavonoids, phenolic acids, tannins, stilbenes, and lignans. Flavonoids, which are crucial from the point of view of this chapter, are low-molecular-weight compounds, consisting of 15 carbon atoms arranged in C6-C3-C6 configuration, which is shown in Fig. 2.1. Modifications within the heterocyclic C ring lead to the formation of various flavonoid compounds

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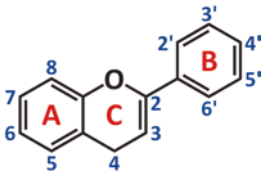


Fig. 2.1 Scheme of flavonoid structure

such as flavonols, flavones, flavan-3-ols, isoflavones, flavanonols, anthocyanidins, and chalcones. Variation in flavonoid structure arises from the scale and pattern of hydroxylation, alkylation, and glycosylation reactions that alter the basic molecules.

Moreover, flavonoids can exist as monomers (catechins) and proanthocyanidins (polymers of catechin and epicatechin subunits). In plant tissues, most flavonoids occur (in addition to free aglycons) in the form of glycosides. In the sugar part, glucose is most common, but there are also combinations with galactose, rhamnose, xylose, arabinose, and glycuronic acid. The sugars are linked to a hydroxyl group at the C7 position in flavones and isoflavones, C3 and C7 positions in flavonol structures, and C3 and C5 for anthocyanins (Rice-Evans et al. 1997). Glycosides are characterized by greater polarity than aglycons, which explains their good solubility in water and easier passage through cell membranes. The division of flavonoids with examples is given in Fig. 2.2.

Flavonoids in Food

Due to many health-promoting properties, flavonoids should be present in the daily diet. According to various sources, the mean daily dose of flavonoids ranges from 189.7 mg per day for both sexes to 780 mg for women and 1058 mg for men (Chun et al. 2007; Stevenson and Hurst 2007). The mentioned 189.7 mg was mainly from flavan-3-ols (83.5%), followed by flavanones (7.6%), flavonols (6.8%), anthocyanidins (1.6%), flavones (0.8%), and isoflavones (0.6%). The greatest daily mean intake of flavonoids was from the following foods: tea (157 mg), citrus fruit juices (8 mg), wine (4 mg), and citrus fruits (3 mg) (Chun et al. 2007). Examples of flavonoid-rich products are summarized in Table 2.1. Vegetables generally contain less polyphenolic compounds than fruit, and the way the vegetables are grown has a significant effect on the content of polyphenolic compounds (Wawer et al. 2012). Similar conclusions were done for fruits. The results showed that organic fruits tend to have higher hydrolyzable polyphenol contents than conventional ones, with values being 11.5% in orange peels, to 72.6% in papaya peels, higher for hydrolyzable polyphenols (Faller and Fialho 2010). In recent years, flavonoids are increasingly becoming ingredients in dietary supplements. However, it should be remembered that the action of flavonoids depends not only on their daily intake but also on the degree of their absorption and further metabolic changes in the body.

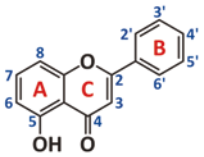
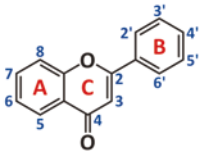
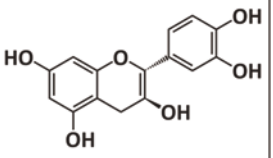
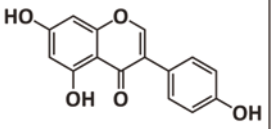
Structure	Subgroup	Examples						
	Flavones		3	7	2′	3′	4′	5′
		Chrysin	H	OH	H	H	H	H
		Apigenin	H	OH	H	H	OH	H
		Luteolin	H	OH	H	OH	OH	H
	Flavon-3-ols (flavonoles)	Kaempferol	OH	OH	H	H	OH	H
		Morin	OH	OH	OH	H	OH	H
		Myricetin	OH	OH	OH	H	OH	OH
		Rhamentin	OH	OCH_3	H	OH	OH	H
		Quercetin	OH	OH	H	OH	OH	H
		Rutin	O-rutinoside	OH	H	OH	OH	H
		Quercitrin	O-rhamnoside	OH	H	OH	OH	H
	Flavanons		7	5	3′		4′	
		Naringenin	OH	OH	H		OH	
		Naringin	O-rutinoside	OH	H		OH	
		Hesperetin	OH	OH	OH		OCH_3	
		Hesperidin	O-rutinoside	OH	OH		OCH_3	
	Flavan-3-ols (catechins)	Catechin						
	Isoflavones	Genistein						

Fig. 2.2 Division of flavonoids into subclasses with examples

Chromatographic Determination of Flavonoids

High-performance liquid chromatography is a commonly used technique for separation and quantification of flavonoids. Different types of stationary phases and eluents are used for this purpose. The most typical chromatographic columns contain C18 or C8 stationary phases and the mobile phase consists of mixtures of water and methanol or acetonitrile. Mass spectrometry is increasingly used as a detection technique due to the possibility of assigning polyphenolic compound structures in samples with selected matrices.

Table 2.1 Food source of flavonoids

Subgroup	Representative flavonoids	Food sources
Anthocyanins	Cyanidin	Fruit, flowers
Flavanols	Catechin, gallocatechin, epicatechin, epigallocatechin gallate, procyanidin	Tea, beer, apples, hops, wine, fruit juice, nuts
Flavanones	Hesperidin, naringenin	Citrus fruit, chick peas, licorice, cumin, peppermint
Flavones	Apigenin, chrysin, luteolin	Cereals, fruits, herbs, vegetables
Flavonols	Quercetin, kaempferol, rutin, myricetin	Onions, cherries, apples, broccoli, tomatoes, buckwheat
Isoflavones	Genistein	Soybeans

Based on Peterson and Dwyer (1998) with changes

A very common problem in the analysis of polyphenolic compounds is their coelution, even with the use of high-resolution columns. This issue is particularly important when it is planned to determine a large number of polyphenolic compounds during a single run. For the determination of only a small number of selected compounds, coelution is not so burdensome. Liquid chromatography coupled with tandem mass spectrometry was used to analyze selected polyphenolic compounds in infusions of *Forsythia supensa* leaves collected at various vegetation periods (Dai and Mumper 2010). The authors of this work, however, focused on specific five selected polyphenolic compounds, so the problems encountered in the chromatographic analysis of polyphenolic compounds have been kept to a minimum. Another disadvantage of the reversed-phase mode is a long time of the single run, which is in the range from 30 min to an hour. Harly et al. (2006) presented the separation of flavonoids extracted from fruit and nuts, using a mixture of methanol, acetonitrile, and trifluoroacetic acid as the mobile phase. The authors managed to separate 20 polyphenolic compounds in 60 min. Sakakibara et al. (2003) developed a method that allows the separation and quantification of several classes of polyphenolic compounds in food samples; however, the time of a single analysis was as much as 95 min. Lee et al. (2014) analyzed polyphenolic compounds in chokeberry juice. The time of a single analysis in this case was about 70 min. Evaluation of the polyphenolic composition of fresh-cut purple Poligano carrots took 65 min in a gradient elution (Pace et al. 2020).

One of the ways to shorter the time of the single analysis is the application of Ultra-Pressure Liquid Chromatography (UHPLC). The basis of this technique is the use of columns packed with particles of very small diameters (<2 μm). This generates very high pressure, but the obtained results are characterized by higher resolution and sensitivity as well as shorter retention times compared to the conventional system (Jing et al. 2013; Motilva et al. 2013). Schwarz et al. (2009) compared the separation of phenolic compounds in brandy using UHPLC and conventional HPLC (both separations performed in reversed-phase mode). Application of UHPLC instrumentation enables to separate polyphenols in 6.5 min in comparison to 60 min in the case of HPLC. The UHPLC using the Chromolith Fast Gradient Monolithic RP-18e column enables the separation and quantification of selected flavonoids

present in different herbal dietary supplements in time less than 4 min using DAD detection method (Magiera et al. 2015). The chromatograms obtained from the separation of the dietary supplements extracts are presented in Fig. 2.3. The selectivity of the developed method was confirmed by comparison of the results with UHPLC-MS/MS analysis.

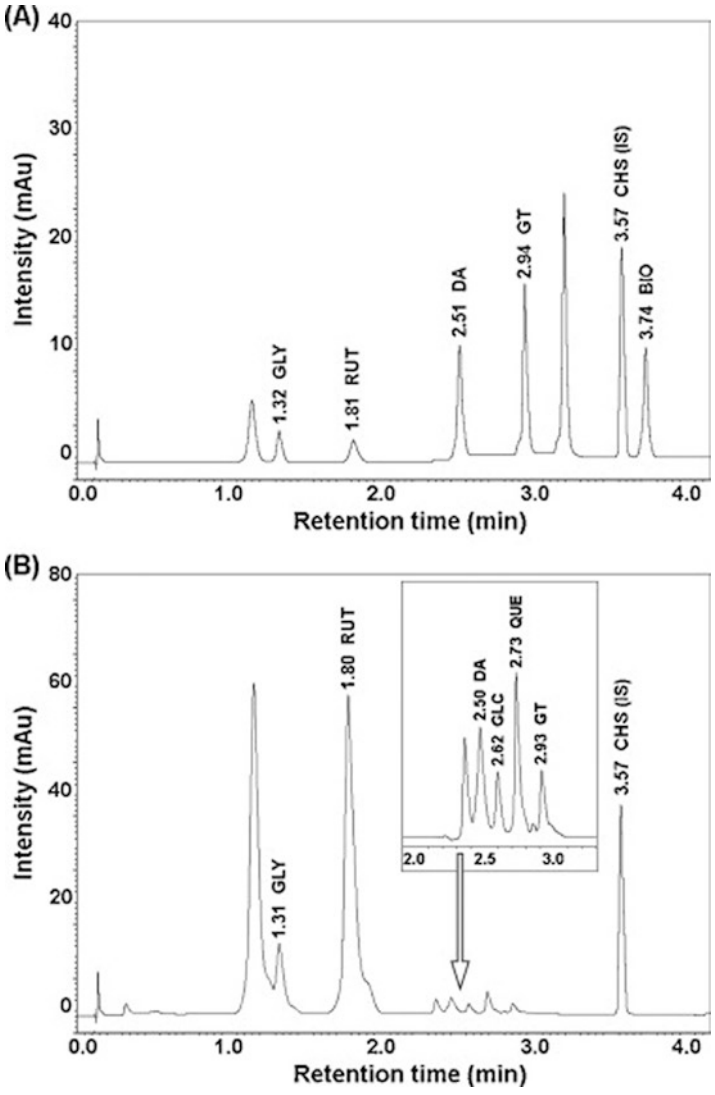


Fig. 2.3 Representative chromatograms obtained for a dietary supplement sample: (**a**) sample 2 and (**b**) sample 6 using the UHPLC-UV method. (Magiera et al. (2015). Reproduced by permission of Elsevier)

The separation and quantification of several isoflavones simultaneously with phenolic acids was possible using the BEX C18 column and DAD detection (Klejdus et al. 2009). The time of the single run was 1.9 min.

Lei et al. (2019) presented the plant flavonoid analysis protocol involving UHPLC and photo-diode array (PDA) as well as MS/MS detection methods. The protocol consists of four steps: sample preparation and extraction, chromatographic separation, mass spectrometric analyses, and data analyses. In the chromatographic step, flavonoids are separated on a reverse-phase column based on their different properties (mainly on their polarity resulting from their hydroxylation, methylation, and glycosylation). Then the detection of flavonoids using a PDA detector with a wavelength range of 190–400 nm is performed. The last step of detection involves mass spectrometry detection to confirm the results obtained from the previous detection step. Due to the similar structure and polarity sometimes it is very difficult to obtain the baseline separation of analyzed flavonoids. The application of MS/MS detection eliminates this problem. In recent years UHPLC-MS/MS has been used in several publications to separate the food phenolics (Epriliati et al. 2010; Oszmianski et al. 2013; Silva et al. 2011; Slimestad et al. 2020).

High-Temperature Liquid Chromatography

During the discussion concerning UHPLC, it is worth mentioning a slightly less popular variant of liquid chromatography, namely, the High-Temperature Liquid Chromatography. According to the theory of chromatography, the temperature at which separation is carried out affects its selectivity and retention of the analyzed compounds, but also entails a change in the viscosity of the mobile phase that is used. As the temperature increases, the viscosity of the eluent decreases. Thus, when separation is performed at high temperatures, higher eluent flow rates can be used. That procedure results in a reduction of the time of a single analysis but also causes an increase in the value of backpressure. That is why it is recommended to use instrumentation that is dedicated to UHPLC. Nevertheless, HTLC also found use in flavonoid analysis. Klejdus et al. (2007) performed the separation of 13 isoflavones from soy preparations and other plant extracts operating Zorbax C18 column at 80 °C. The time of the separation was 1 min. The same authors (Klejdus et al. 2009) compared the separation efficiency of isoflavones and polyphenolic acids using different types of stationary phases dedicated to UHPLC. They concluded that the best separation efficiency was obtained when the stationary phase with a particle diameter of 1.8 μm was used and the separation was performed at 58 °C. The application of such conditions enables one to separate 19 compounds in time less than 2 min.

Hydrophilic Interaction Chromatography

Hydrophilic Interaction Liquid Chromatography was first introduced by Alpert in 1990 (Alpert 1990) and since then its popularity is still increasing. That is because HILIC is a technique between normal and reverse-phase chromatography. In general, it combines polar stationary phases with eluents with a high content of organic solvent. HILIC stationary phases can be divided into three subgroups: neutral (*e.g.*, diol), charged (*e.g.*, bare silica) and zwitterionic (*e.g.*, sulfobetaine) (Jandera and Janas 2017). The basis for this division is the presence or absence of electrostatic interactions between the stationary phase and the analyte. Neutral stationary phases are not able to interact electrostatically with the analyte, while charged columns strongly electrostatically interact with the analyte. Zwitterionic stationary phases exhibit only weak electrostatic interactions with analytes. In general, retention mechanism in HILIC mode is based on the partitioning of the analyte between the water layer adsorbed on the surface of the polar stationary phase and the highly organic mobile phase. It is known that other interactions like hydrogen bonding, dipole–dipole, and, as mentioned before, electrostatic interaction can significantly influence the retention in this chromatographic mode (Jovanovic et al. 2014; McCalley 2017). HILIC mode gives a possibility for the separation of compounds that were not retained on hydrophobic stationary phases used in the reverse phase HPLC (eluted in the dead volume) or were retained too strongly on polar stationary phases in normal phase mode. HILIC chromatography was also used in the separation of polyphenols. Robbins et al. (2009) showed the potential of several diol-bonded HILIC columns under the same condition of the mobile phase in the separation of flavanols and procyanidins in cocoa and chocolate samples. Although all the columns used contained the same bed (diol stationary phase), they came from different manufacturers, which significantly affected the results. The authors observed the significant differences between the columns in retention times, peak shapes, and resolution. The best separation was achieved using the Develosil diol column. Another diol column (Luna HILIC) was evaluated in the separation of procyanidins in an apple extract by Hollands et al. (2017). The authors compared the separation obtained on different columns using the same mobile phase, which consisted of acidic acetonitrile and acidic aqueous methanol, which is shown in Fig. 2.4. The differences in separation between the signals, peak resolution, and time of the single run were significant. What is more, using a shorter column resulted in a lack of the separation of monomeric forms (catechin and epicatechin), while the time of the single run shortened to 45 min. It should be highlighted that a diol column was introduced to minimize the problem with adsorption caused by free silanol groups on bare silica stationary phases (Hemstrom and Irgum 2006). The overall polarity of diol stationary phases is similar to those of silica ones (West and Lesseltier 2006). A diol stationary phase was used in the separation of flavonoids, however, the separation efficiencies were better when the zwitterionic stationary phase was used, that is why the analysis of natural samples was carried out using ZIC-HILIC column (Sentkowska et al. 2015a, b, 2016a, b).

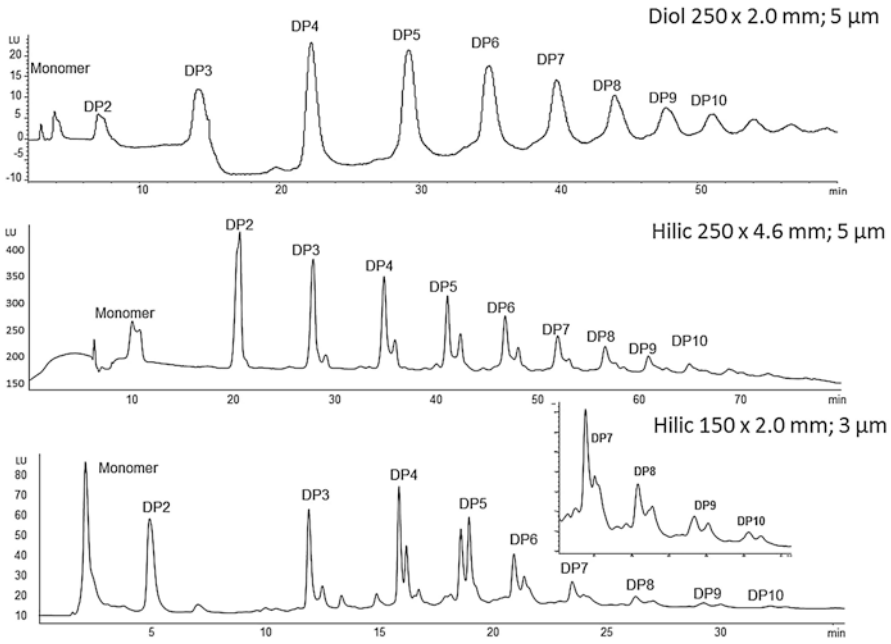


Fig. 2.4 Chromatographic separation of monomeric and oligomeric procyanidins in apple extract obtained on different chromatographic columns. (Hollands et al. (2017). Reproduced by permission of Elsevier under Creative Commons CC-BY license)

The main types of phases used in flavonoids analysis are sulfobetaine stationary phases. This type of bed contains both positive (quaternary ammonium) and negative (sulfonic acid) groups separated by a short alkyl spacer. Such groups are present in the whole stationary phase in 1:1 molar ratio. According to the theory, the primary retention mechanism onto this stationary phase is a mixed-mode but with the domination of a hydrophilic partition. That is because this phase has a strong ability to adsorb water onto its surface. On the other hand, adsorption also can play an important role when this stationary phase is used (Sentkowska et al. 2013). ZIC-HILIC stationary phase was used in the analysis of flavonoids in extracts of St John's worth and Lemon balm (Sentkowska et al. 2015a, b) as well as from *Genista tinctoria* (Sentkowska et al. 2016a, b). Another advantage of HILIC mode is the enhanced sensitivity of MS detection due to the high content of organic solvent, which is shown in Fig. 2.5. For the same concentration of apigenin in the sample, the intensity of the signal was significantly higher in HILIC mode in comparison to the RP mode. This phenomenon was observed even if the same stationary phase was used and only the mode (mobile phase composition) was changed as it was done for the diol column. Such specific solvent composition can be very useful in the sample preparation step. As acetonitrile and other organic solvents are weak in HILIC mode, samples prepared in such solvents can be directly injected into the HPLC system.

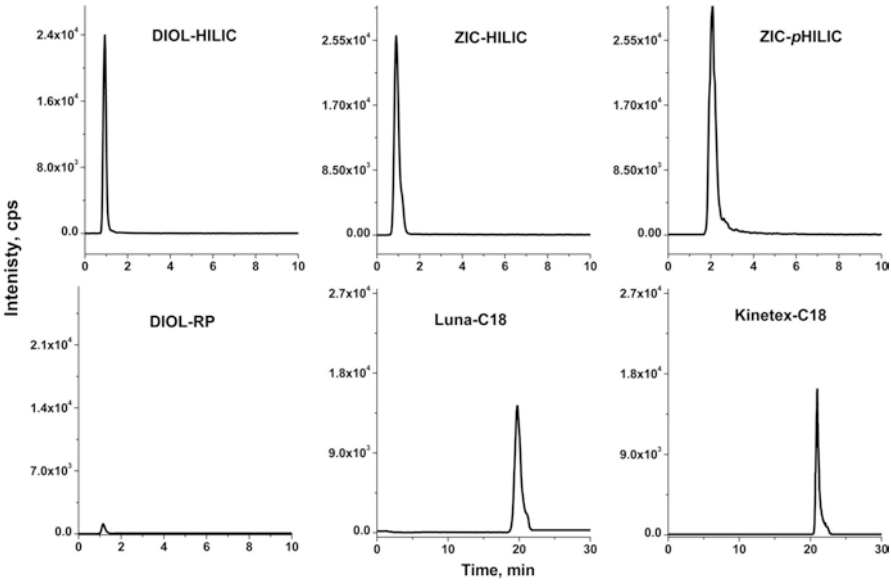


Fig. 2.5 Extracted ion chromatograms of apigenin on HILIC and RP columns under isocratic elution. Mobile phase for the HILIC column: ACN/H_2O (95/5% v/v), for DIOL-RP: (45/55% v/v) and for C18: (20/80% v/v). (Taken from Sentkowska et al. (2015a, b). Reproduced by permission of Oxford University Press)

Two-Dimensional Liquid Chromatography

The requirements for chromatographic separation of polyphenolic compounds are high. Improvements that can be done by increasing the length of the column or by decreasing the diameters of the particles of the stationary phase are limited by pressure constrains. The analytical task to identify and determine all flavonoids in the sample is difficult. Meyer et al. (2008) draw attention to the fact that only dimers of procyanidins based on catechin and epicatechin rings can be found in 48 variants. If all of the procyanidins and other polyphenolic compounds are planned to be separated in one single run, the potential of the one-dimensional liquid chromatography is not sufficient. In such a case, multidimensional liquid chromatography can be used as an alternative method. Such instrumentation is characterized by higher resolution power in comparison to the one-dimensional technique, that is why it is recommended for the separation of complex samples (Cacciola et al. 2017; Serrut et al. 2014). Since the 2D variant is a combination of separations of the same sample on two different columns, the apparatus solutions may look different. Thus, LC × LC separation can be performed online, offline, or under stop-flow mode. The scheme of instrumentation for on-line and stop-flow is presented in Fig. 2.6. In the online configuration, fractions from the first dimension are directly transferred to the second dimension. The peak capacity in online LC × LC is usually less and the time of the analysis is similar to the one-direction chromatography. Two-dimensional online

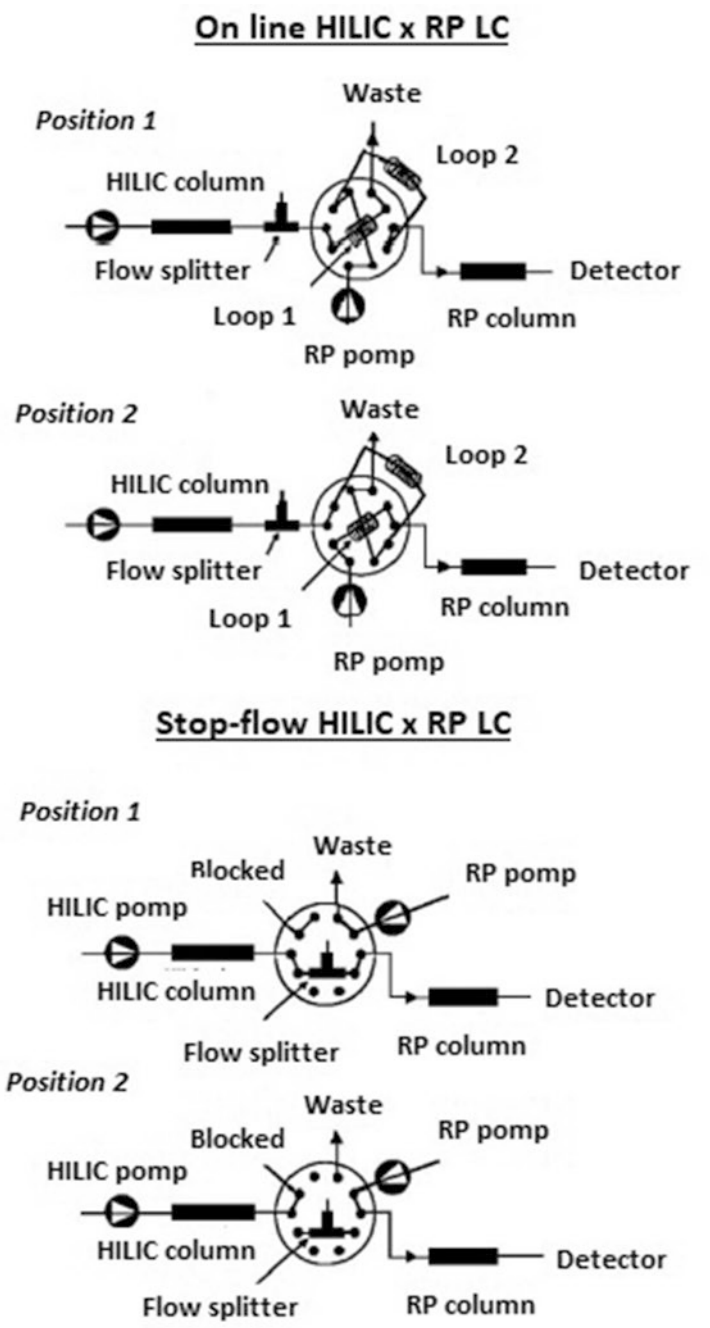


Fig. 2.6 Schematic illustration of the instrumental configuration used for online and stop flow HILIC × RP-LC. (Kalili and de Villiers (2013). Reproduced by permission of Elsevier)

chromatographic analysis was used in the analysis of beer, wine, and herbal infusions samples (Cesla et al. 2009; Dugo et al. 2008a, b; Hajek et al. 2008). In all cited publications, reverse phase chromatography (RP-LC) was used in both dimensions. This action is mainly aimed to increase the peak capacity for a given separation. However, this is not the only possible connection. A combination of hydrophilic interaction chromatography (HILIC) and reverse phase chromatography (RP-LC) was also used in the analysis of the previously mentioned procyanidins in the cocoa sample and apple extract (Kalili and de Villiers 2009). The authors draw attention to the use of orthogonality of these two chromatographic techniques. When separating procyanidins in the first dimension (using HILIC chromatography), the compounds are separated based on their polarity. In the case of procyanidins, this means longer retention of compounds containing more hydroxyl groups in their structure. It can, therefore, be said that at this stage the individual isomers are separated. When the analytes are separated in the second dimension (using reverse phase chromatography), the compounds are separated based on their hydrophobicity. Thus, compounds having more hydroxyl groups in the molecule are poorly retained on the column, which translates into their short retention times (inversely than in the first dimension analysis).

The offline approach involves the collection of the fractions from the first dimension, which are subsequently injected on the second column. The HILIC-RPLC connection in off-line mode has also been used in the analysis of polyphenolic compounds in green tea (Kalili and de Villiers 2010). It has been noticed that the first dimension retention of compounds using hydrophilic interaction chromatography rises with the increase in the number of sugar substituents in the molecule, while mono-, di-, and triglycosides are separated in the second dimension using reverse phase chromatography. Offline configuration was also used in the separation of flavonoids from complex matrices of Chinese medicines (Zheng et al. 2012), and extracts of *Lobelia chinensis Lour* (Wang et al. 2019) as well as in the purification of flavonoids from licorice extract (Fan et al. 2016). In the stop flow approach, two columns are coupled without trapping or sample loops. Following the transfer of fractions from the first column to the second one, the flow in the first dimension is stopped, until the separation in the second dimension is completed. Such an approach is also presented in Fig. 2.6. In general, the offline configuration provides the highest overall peak capacity, however, it requires very long times. The peak capacity in online LC × LC is usually less and analysis time is similar to that of a one-dimensional approach. It should be highlighted that the choice of configuration depends mainly on the availability of the proper instrumentation. On the other hand, some problems with system compatibility may occur mainly when HILIC with HPLC is planned to be combined. The eluents with a high content of organic solvent, which are used in the first dimension, are not good in reversed-phase mode. The use of the same mobile phase in both dimensions can result in a decrease in the retention times as well as asymmetric peaks in the second dimension. To overcome this problem the weak solvent can be introduced into collected fraction or evaporation of organic solvent after the first dimension can be done. Similar effect can be achieved by collecting and transferring a very small fraction from the first dimension

to the second one. That is why low flow rates and capillary columns in the first dimension and high flow rates in the combination with core-shell columns with larger diameters in the second mode are recommended (Jandera et al. 2012). Despite apparatus difficulties and analytical challenges, two-dimensional chromatography is used in the analysis of flavonoids and polyphenolic compounds in general. Examples of the applications of 2D liquid chromatography are shown in Table 2.2.

HPLC Combined with Online Antioxidant Activity Measurement

Flavonoids are well known for their antioxidant activity. Nowadays, many different methods are used to determine the antioxidant capacities of plant products and food. Although these methods are simple and easy, they can only be used for the evaluation of the total antioxidant activity of polyphenols. It should be highlighted that other colored compounds existing in plant extracts affect the result of antioxidative properties measurement. As a consequence, the development of analytical methods for the estimation of antioxidant activity of purified compounds has taken place. The progress in the development of chromatographic techniques and detection methods enabled to solve this problem by coupling the HPLC system with, e.g., free radical scavenging methods. Simultaneous determination of the content of the flavonoids in the sample followed by determination of its antioxidant capacity is successfully used (Burnaz et al. 2017; Kusznierewicz et al. 2011; Malherbe et al. 2012; Shi et al. 2009; Zhao et al. 2017). Usually, postcolumn measurement of antioxidant activities of extracts from plants, fruits, or vegetables is performed (Burnaz et al. 2017; Pedan et al. 2016; Shahidi and Zhong 2015; Zhao et al. 2017). Parameters such as reagent concentration, buffering the solution as well as reaction time and temperature must be optimized to estimate the reliable results (Malherbe et al. 2012). Flavonoids give negative peaks in DPPH (measured at 517 nm) or ABTS (414 nm) method and positive in CUPRAC (450 nm) or FRAP (595 nm) after treatment with a postcolumn reagent. The HPLC-DAD and on-line HPLC-DAD-ABTS profiles of *Athrixia phylicoides* extract are shown in Fig. 2.7 (De Beer et al. 2011).

Detection of Flavonoids

Due to the ability of polyphenolic compounds to absorb UV-Vis radiation, spectrophotometric detection is the most commonly used technique for their determination (Biesaga et al. 2006; de Rijke et al. 2006; Michalkiewicz et al. 2008; Wach et al. 2007). Phenolic acids are determined at 280 nm. However, each flavonoid subgroup is determined at a different wavelength, *e.g.*, flavonols and isoflavones in the 330–370 nm range, flavonones at 280 nm, and anthocyanins at 530–580 nm. However, due to the similar structure and the possibility of occurrence of isomeric

Table 2.2 Examples of application of two-dimensional LC methods in the analysis of polyphenolic compounds

		Chromatographic column		
Sample	Compounds	1st dimension	2nd dimension	Ref.
Off-line mode				
Traditional Chinese medicinal plants	Flavonoids	XTerraMS C18 column (150 × 2.1 mm, 5 μm)	Homemade Click OEG (150× 4.6 mm, 5 μm)	Zheng et al. (2012)
Licorice extract	Flavonoids	Unitary C18 (250 × 4.6 mm, 5 μm)	UHPLC HSS C18 (100 × 2.1 mm, 1.7 μm)	Fan et al. (2016)
Apple, cocoa	Procyanidins	HILIC Diol-1000 (150 × 4.6 mm, 5 μm)	RP Zorbax SB SB-C18 (50 × 4.6 mm, 1.8 μm)	Kalili and de Villiers (2009)
Green tea	Proanthocyanidins, flavonols, phenolic acids	Devosil Diol-1000 (250 × 1.0 mm, 5.0 μm)	RP Zorbax SB SB-C18 (50 × 4.6 mm, 1.8 μm)	Kalili and de Villiers (2010)
Beer, tea	Phenolic acids, flavones	Luna HILIC 200A (100 × 2.0 mm, 5.0 μm)	Discovery HS PEG (150 × 2.1 mm, 5 μm)	Skerikova and Jandera (2010)
Blueberries, black beans, red radish, grapes	Anthocyanins	HILIC XBridge BEH Amide (150 × 4.6 mm, 2.5 μm)	Kinetex C18 (50 × 4.6 mm, 2.6 μm)	Willemse et al. (2014)
On-line mode				
Lemon oil	Coumarins	NP Betasil Diol (250 × 1.0 mm, 5 μm)	Zorbax SB SB-C18 (50 × 4.6 mm, 3.5 μm)	Francois et al. (2008)
Wines	Rutin, catechin, gallic acid	Ascentis Phenyl (250 × 1.0 mm, 5.0 μm)	Ascentic Express C18 (300 × 4.6 mm, 2.7 μm)	Dugo et al. (2009)
Rooibos tea	Flavones, flavonones, flavonols	HILIC Diol-1000 (250 × 1.0 mm, 5 μm)	Zorbax SB SB-C18 (50 × 4.6 mm, 1.8 μm)	Beeiders et al. (2012)
Wine	Polyphenols	Ascentis Es-Cyano (250 × 1.0 mm, 5.0 μm)	Ascentic express C18 (30 × 4.6 mm, 2.7 μm)	Donato et al. (2016)
Heart cutting				
M. ilicifolia leaves extract	Flavonol glycosides	SEC Ultrahydrogel-120 (300 × 7.8 mm, 5 × 10^3 Da)	SupelCosil LC-18 (250 × 4.6 mm, 5.0 μm)	De Souza et al. (2009)

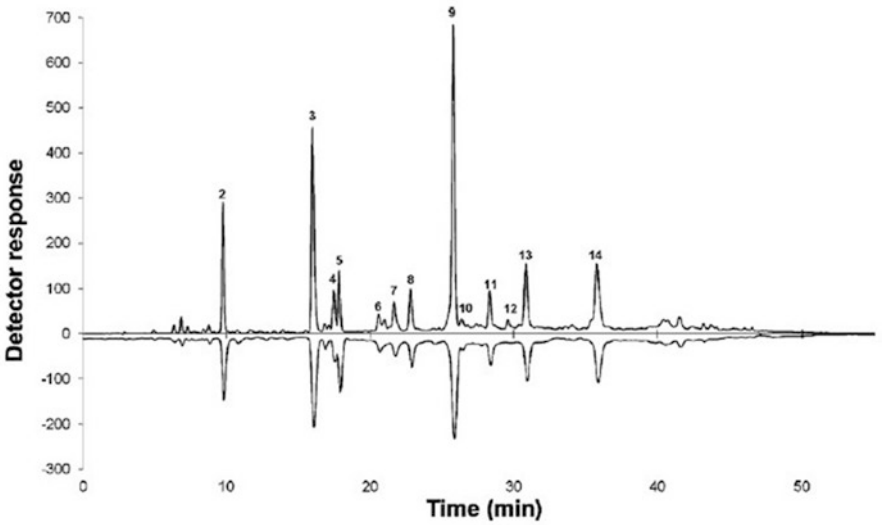


Fig. 2.7 HPLC-DAD (positive peaks) and on-line ABTS scavenging (negative peaks) profiles of 50% ethanol *Athrixia phylicoides* extract. Column: Gemini C18 (150 × 4.6 mm, 5 μm); mobile phase: CAN/0.1% HCOOH in gradient elution. Post-column reaction: ABTS reagent added at 0.5 mL min^{-1} with reaction time of 0.5 min followed by detection at 430 nm. (Taken from de Beer et al. (2011). Reproduced by permission of Elsevier)

forms of polyphenolic compounds (e.g., different positions of the hydroxyl substituent not causing significant differences in the UV-VIS spectrum), the obtained spectra were not always unambiguous to interpret. Among other things, spectrophotometric methods cannot be described as selective. In the analysis of samples with complex matrices, especially natural samples, this is extremely important. Fluorimetric detection is more sensitive than UV-VIS detection (Michalkiewicz et al. 2008; Surowiec et al. 2003). When a fluorimetric detector was used, the detection limit was obtained by an order of magnitude smaller in the case of vanillic, syringic, and 4-coumaric acids (Michalkiewicz et al. 2008). Many flavonoids form complexes with metal ions such as Al(III), Mg(II), Be(II), Zn(II), Sc(III), Ga(III), and In(III) that show fluorescence. Such reactions were used to detect fluorimetric flavonoids containing the 3-hydroxy-4-keto system using a post-column reaction (Surowiec et al. 2003). Due to the ability of polyphenolic compounds to undergo redox reactions, it is possible to detect them using an electrochemical detector (Bolarinwa and Linseisen 2005). A significant limitation of these detection methods is the lack of information on the structure of the compound. Hence, in recent years there has been an increase in the use of mass spectrometry as a method of detection of polyphenolic compounds in combination with separation techniques. The identification of analytes is made not only based on retention time but primarily based on mass spectra or fragmentation spectra (tandem MS/MS systems). That is why mass spectrometry detection is widely used in the analysis of flavonoids and all the

polyphenolic compounds (Jandera et al. 2010; Sentkowska et al. 2015a, b, 2016a, b; Stander et al. 2013; Steevensz et al. 2012; Yanagida et al. 2007).

Conclusion

As the flavonoids and other phenolic compounds are the markers of food quality, the techniques which enable their analysis are very important. High-Performance Liquid Chromatography is one of such techniques. In recent years UHPLC as well as HILIC became increasingly popular. HILIC offers convenient sample preparation (organic solvent extraction) and enhanced mass spectrometry detection, while UHPLC requires small solvent consumption, but needs more sophisticated instrumentation. Due to the number and diversity of flavonoids, it can be predicted that many new methods based on 2D chromatography will be developed in the nearest future.

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Chapter 3
Phenolic Compounds in Coffee and Tea Beverages

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Abstract Phenolic compounds are bioactive metabolites determined in plants which can be divided into six main groups: phenolic acids, flavonoids, tannins, stilbenes, lignans, and coumarins. These compounds possess not only high antioxidant activity but also many other bioactive properties, with anticancer activity as well. Very important sources of phenolic compounds in the human diet are coffee brew and tea leaves infusion. In this chapter, modern high pressure liquid chromatography (HPLC) and liquid chromatography coupled with mass spectrometry (LC-MS) methods are described for the determination of chlorogenic acids (CGAs) in coffees as well as catechins and theaflavins (TFs) in white, green, yellow, oolong, black, and pu-erh teas. Nowadays, the consumption of hot beverages is still common, and some other special coffees have become more and more popular. Apart from the roasting process, other special treatment processes of coffee beans such as steaming, decaffeination, or natural fermentation (Kopi Luwak coffee) influence the composition of chlorogenic acids and/or other phenolic compounds of coffee brew. Instant coffees and "ready-to-drink" coffee beverages are available on the market as well. In addition, coffee brews prepared by different methods contain a wide and dissimilar level of total chlorogenic acids and their lactones. Coffee leaves infusions, drunk from ages, also possess phenolic compounds and bioactive properties. Moreover, the preparation of tea leaves and/or buds (non-oxidized, semi-oxidized, fully oxidized, and post-fermented) influences the catechins, theaflavins, and other bioactive compounds present in tea infusions. Furthermore, the antioxidant activity of tea can be improved by the addition of spices (e.g., cayenne pepper), flowers (e.g., jasmine), herbs (mint or basil), fruits (e.g., lemon or dried fruits), bergamot oil, and also chocolate. The most common flavored black tea types are Earl Grey and masala. Other teas such as rooibos, yerba mate, and lapacho, best known and drunk in the places of origin, become more popular in the USA and Europe. They

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also contain phenolic acids and other phenolic compounds among other bioactive compounds.

Introduction

Phenolic compounds (polyphenols and phenols) are widely distributed in plants including fruits and vegetables, as well as plants used to prepare beverages, mainly tea and coffee. These secondary plant metabolites possess antioxidant activity, especially the free radical scavenging, and the mechanism of their action depends on their structure and the place where the reactive oxygen and nitrogen species (ROS and RNS) are found (Naczk and Shahidi 2004, 2006; Tung et al. 2007). The term "plant phenolics" is very wide and includes such compounds as phenolic acids, flavonoids, tannins, stilbenes, lignans, and coumarins (Shahidi and Ambigaipalan 2015; Robbins 2003). They can be found in all parts of plants: fruits, nuts, seeds, leaves, roots, and the bark (Naczk and Shahidi 2004, 2006; Wanasundara et al. 1996). Therefore, a diet based on food containing phenolic compounds is often connected with antimutagenic, anticarcinogenic, anti-inflammatory, antimicrobial, and other positive effects (Aviram et al. 2000; Bose et al. 2008; Lu et al. 2012; McSweeney and Seetharaman 2015; Morton et al. 2000; Scalbert et al. 2005; Yang et al. 2000). As a result, these compounds are popular ingredients of dietary supplements and functional food (AFC 2004).

The consumption of phenolic compounds is often low among people whose diet is based mainly on highly processed food, meat products, and fast foods. However, tea and coffee that contain large amounts of plant-based phenols are still the most widely consumed beverages. The fruit teas based on blends of green or black tea with many different fruits are also becoming popular due to a wide variety of taste offered. Therefore, studies on phenolic compounds present in these beverages are so important for the consumers, giving information on their positive influence. Also, the manufacturers will be able to formulate better products and the authorities to control their adulteration.

The Chemistry and Biological Activity of Phenolic Compounds

Phenolic acids are phenols that possess one carboxylic acid functionality. Among plant metabolites, there are two main groups of these acids, which are based on hydroxybenzoic C6–C1 and hydroxycinnamic C6–C3 acid skeletons (Fig. 3.1), but hydroxyphenylacetic and hydroxyphenylpropanoic acid derivatives are also found (Motilva et al. 2013). The numbers and positions of the hydroxy and methoxy groups on the aromatic ring of these compounds can be different (Table 3.1).

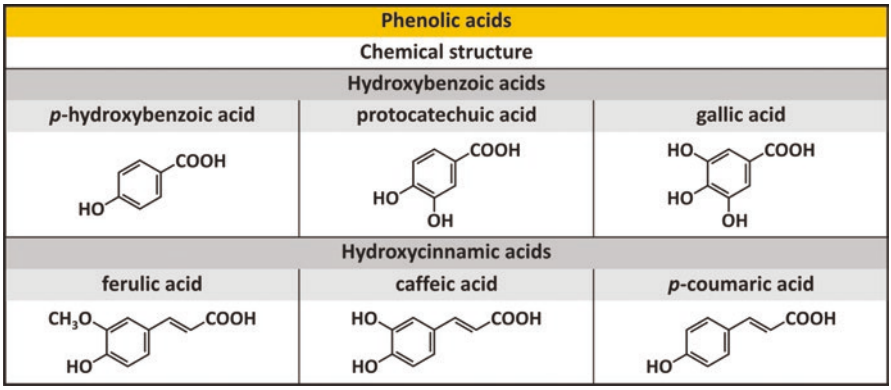

Fig. 3.1 Structures of phenolic acids

Hydroxybenzoic acids and their derivatives are mainly present in foods in the form of glucosides, and they are bounded with other compounds such as lignins or hydrolyzable tannins. Vanillic, *p*-hydroxybenzoic, and protocatechuic acids are most frequently determined in food products of plant origin (Mattila and Hellström 2007; Naczk and Shahidi 2004, 2006; Shahidi and Ambigaipalan 2015). Among hydroxycinnamic acids, the most common are caffeic, *p*-coumaric, and ferulic acids, which often occur in food and plants as simple esters with quinic acid or glucose. A very important group of hydroxycinnamic acid derivatives consists of chlorogenic acids that are the esters and diesters of hydroxycinnamic and quinic acids. There are numerous derivatives of chlorogenic acid (CGA) arising from isomers in the quinic acid part and substitutions in the cinnamic acid moiety (Mattila and Hellström 2007; Naczk and Shahidi 2004). It was recently revealed that consumption of chlorogenic acid in the diet could reduce the risk of developing neurodegenerative conditions (Heitman and Ingram 2017; Ochiai et al. 2019), which is of great importance because these compounds belong to the main phenolic compounds found in coffee (Jeszka-Skowron et al. 2016a).

Flavonoids are compounds with at least two phenol subunits. The flavan nucleus, also known as the diphenylpropane skeleton, contains 15 carbon atoms arranged in three rings (C6–C3–C6) that are labeled by the letters A, B, and C. The group consists of flavones, flavanones, isoflavones, flavonols, flavanonols, flavan-3-ols, anthocyanidins, and anthocyanins (acyl glycosides and glycosides of anthocyanidins) (Table 3.1). They differ in structure (Fig. 3.2) because of the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation, as well as the lack of the heterocyclic C-ring, which is opened in chalcones (Stalikas 2007). Considerable changes in their chemical, physical, and biological properties are caused by glycosylation, which also influences their absorption and bioavailability in the human organism (Scalbert and Williamson 2000).

The compounds possessing three or more phenol subunits are referred to as tannins, which are commonly classified into two groups: condensed and hydrolyzable (Fig. 3.3). The condensed tannins are oligomeric and polymeric proanthocyanidins

Table 3.1 Phenolic compounds groups, main sources and beverages, and health benefits

Group of phenolic compounds	Main sources and/or beverages	Main compounds from groups	Potential health benefits[a]	References
Phenolic acids				
Hydroxybenzoic acids	Black tea (*Camellia sinensis*), onion	*p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, vanillic acid	Scavenge free radicals: superoxide radical, hydroxyl radical, and peroxy radical, anticancer activities	Hajipour et al. (2016)
Hydroxycinnamic acids	Coffee, valerian herbal tea (*Valeriana),* rooibos herbal tea	*p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, chlorogenic acids		Eroglu et al. (2015) and Terao et al. (1993)
Flavonoids				
Anthocyanidins/ anthocyanins	Fruits, dried fruits (cranberries, red grapes)	Cyanidin, leucocyanidin, delphinidin, propelargonidin	Scavenge free radicals, anti-inflammatory, antimicrobial activities, reduce risk of cancer in cells	Du et al. (2015) and Yoon et al. (2013)
Flavan-3-ols	Green tea (*Camellia sinensis*), *Cistus incanus* herbal tea, cocoa, grapes	(+)-catechin, (−)-epicatechin, (−)-gallocatechin, (−)-epigallocatechin	Neutralize free radicals: superoxide and hydroxyl radicals, reduce risk of cancer, displays protective effects against oxidative damage of the erythrocyte, cardiovascular diseases, and inflammation	Ferrali et al. (1997), Hertog et al. (1993), Kashima (1999), Seeram et al. (2009), and Xu et al. (1992)
Flavanones	Figs, citrus fruits, tomatoes	Morin, naringenin, eriodictyol	Neutralize free radicals, reduce risk of cancer	Kuzu et al. (2018) and Sharma et al. (2018)
Flavanonols	Fruits, white grape skin, wine	Astilbin, taxifolin	Neutralize free radicals, anti-inflammatory properties anti-diabetic nephropathy properties	Li et al. (2009), Meng et al. (2016), and Xueyan et al. (2018)
Flavones	Fruits, fruit skin vegetables (parsley, celery), rosemary	Apigenin, chrysin, lutein	Neutralize free radicals, reduce risk of cancer	He et al. (2015)

Group of phenolic compounds	Main sources and/or beverages	Main compounds from groups	Potential health benefits[a]	References
Flavonols	Black tea (*Camellia sinensis*), buckwheat, fruits (grapes, cranberries), vegetables (onion, kale)	Quercetin, kaempferol, isorhamnetin, rutin	Neutralize free radicals, anti-inflammatory, antiulcer, anti-allergy, and anti-proliferative properties, prevention of cardiovascular diseases, fights with influenza A virus, regulation of gene expression, reduce the risk of chronic diseases, cancer, diabetes	Al-Jabban et al. (2015), Huang et al. (2010), Sattanathan et al. (2010), Sharma et al. (2007), and Vaidya et al. (2016)
Isoflavones (isoflavonoids)	Soybean, flaxseed	Daidzein, genistein	Prevention of cancer, atherosclerosis, ameliorate muscle wasting	Hirasaka et al. (2016), Liu et al. (2014), and Tan et al. (2016)
Chalcones	Fruits, vegetables, oregano	Phloridzin, xanthohumol, isoliquiritigenin	Anti-inflammatory, anticancer, cytotoxic, anti-immunosuppressive potential	Orlikova et al. (2011)
Tannins				
Condensed tannins (proanthocyanidins)	Cranberries and cranberry products, cocoa, chocolate	Monomers, dimers, trimers, 4–6mers, 7–10mers, polymers	Improve urinary tract health, reduce risk of cardiovascular disease	Howell and Foxman (2002), Kontiokari et al. (2001), and Ziberna et al. (2010)
Hydrolyzable tannins	Tea (*Camellia sinensis*), fruits (raspberry, blackberry, grapes)	Ellagitannins, gallotannins	Protein-precipitating agents, biological antioxidants	Cerda et al. (2005)
Other				
Lignans	Buckwheat, flaxseed, sesame seeds, rye	Secoisolariciresinol, pinoresinol, matairesinol, enterodiol, enterolactone	Display anti-hormone-dependent cancer properties, prevent cardiovascular diseases, and have anti-diabetic properties	Chang et al. (2018), Durazzo et al. (2014), Peñalvo and Lopez-Romero (2012), and Sun et al. (2014)

(continued)

Table 3.1 (continued)

Group of phenolic compounds	Main sources and/or beverages	Main compounds from groups	Potential health benefits[a]	References
Stilbenes	Grapes, wine	*Trans*-resveratrol, pterostilbene	Prevention of cancer, inhibition of vascular endothelial growth factor production in human breast cancer cells, mild in dementia	Lange and Li (2018), McCormack and McFadden (2012), and Sanghani et al. (2013)
Coumarins	*Citrus* genus, noni fruit juice (*Morinda citrifolia*), fenugreek (*Trigonella foenum-graecum),* horse chestnut	Umbelliferone, aesculetin, psoralen, scopoletin, esculetin	Scavenge free radicals, anti-inflammatory activity, anticancer activity in cells	Dussossoy et al. (2016), Girennavar et al. (2007), Panno et al. (2009), Stanley and Jurd (1971), and Wang et al. (2000a)

[a]Potential health effects were usually found in culture cells and/or animals

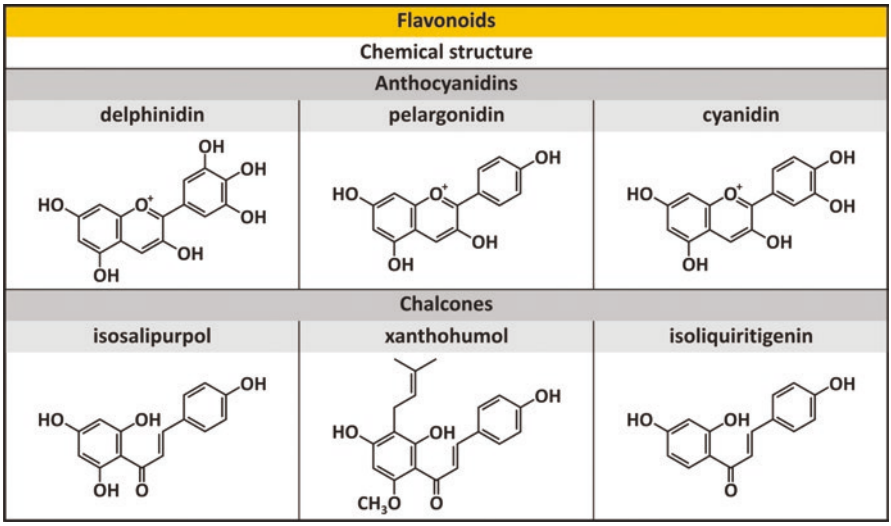

Fig. 3.2 Structures of flavonoids

that can possess different inter-flavanyl coupling and substitution patterns, while the hydrolyzable ones contain a central sugar esterified with gallic acid (gallotannins) and ellagic acid (ellagitannins). But according to the definition of tannins described by Khanbabaee and van Ree (2001), due to their structural characteristic tannins should be divided into four groups: (1) gallotannins are all those tannins in which galloyl units or their metadepsidic derivatives are bound to diverse polyol-, catechin-, or triterpenoid units; (2) ellagitannins are those tannins in which at least two galloyl units are C–C coupled to each other and do not contain a glycosidically linked catechin unit; (3) complex tannins are tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit; (4) condensed tannins are all oligomeric and polymeric proanthocyanidins formed by linkage of the C-4 unit of one catechin with the C-8 or C-6 unit of the next monomeric catechin. According to many studies, tannins positively influence human health; for example, proanthocyanidins, especially from cranberries, help in the urinary tract diseases (Table 3.1).

Lignans contain two phenylpropanoid units coupled by a β,β′-bond between their propane side chains; hence, they belong to the group of diphenolic compounds (Willför et al. 2006). They may be divided into eight structural subgroups (according to how oxygen is incorporated and the pattern of cyclization): dibenzylbutyrolactol, dibenzocyclooctadiene, dibenzylbutyrolactone, dibenzylbutane, arylnaphthalene, aryltetralin, furan, and furofuran (Fig. 3.4). Each subgroup can be further subdivided according to the lignan molecule oxidation level, including their carbon skeletons, the way in which oxygen is incorporated into the skeletons, and the cyclization pattern (Teponno et al. 2016). Lignans show the antioxidant and

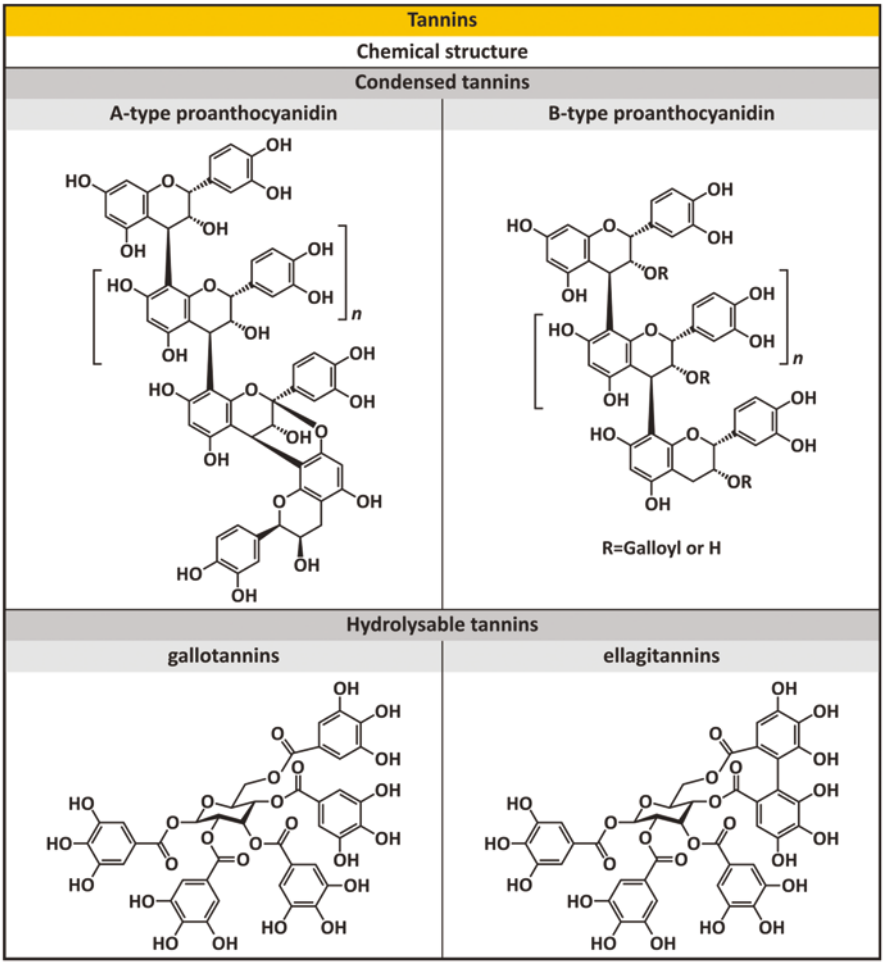

Fig. 3.3 Structures of tannins

antitumor properties in the human organism and also possess other biological activities (Table 3.1).

Stilbenes with the C6–C2–C6 skeleton and various hydroxylation patterns (Fig. 3.5) are highly bioactive phenolic compounds; however, their low bioavailability limits their potential benefits for human health (Gao and Hu 2010). Trans-resveratrol exhibiting anti-inflammation, anti-tumorigenesis, cardioprotective, and neuroprotective effects is mostly found in red wine and grapes, and sometimes in raisins (Baur and Sinclair 2006; Han et al. 2006) (Table 3.1). Pterostilbene, a derivative of resveratrol with two methoxy groups, is found to be equally or even more bioactive than resveratrol itself (Chang et al. 2012; Cichocki et al. 2008).

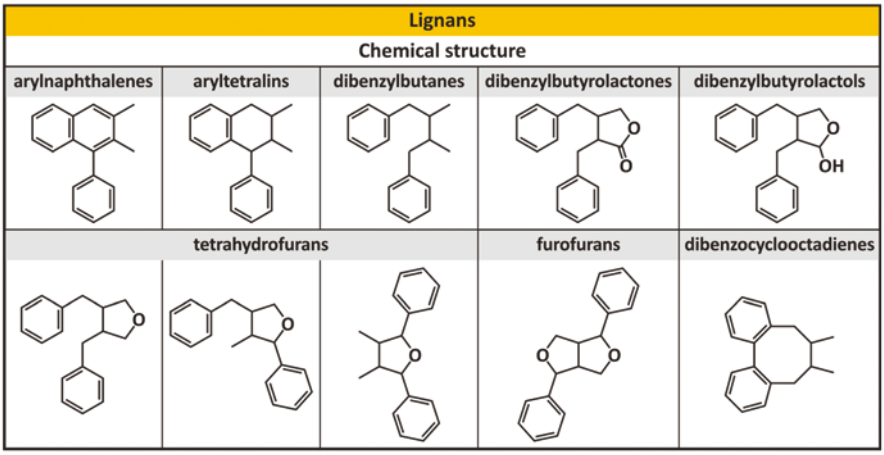

Fig. 3.4 Structures of lignans

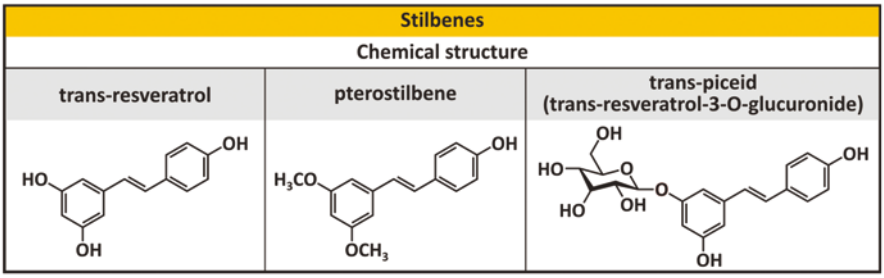

Fig. 3.5 Structures of stilbenes

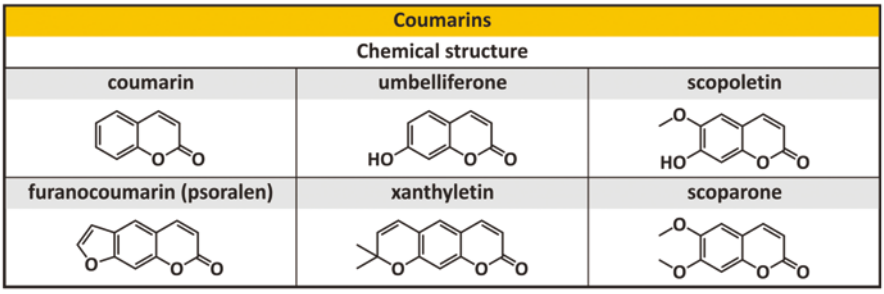

Fig. 3.6 Structures of coumarins

Coumarins are benzopyran-2-ones and have a large diversity in their chemical structures (Fig. 3.6). They are classified from simply substituted coumarins to poly-substituted polycyclic coumarins (Sandhu et al. 2014). Till now over 1300 coumarins have been identified in green plants, fungi, and bacteria (Revankar et al. 2017).

In the 1980s these compounds were suspected of genotoxic and carcinogenic activities (AFC 2004). However, later studies suggested that coumarin is not a genotoxic agent, and the International Agency for Research on Cancer (IARC) has classified it into group 3—not classifiable as to its carcinogenicity in humans (Lake 1999). At a later time, the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) established a tolerable daily intake 0.1 mg kg^{-1} body weight (bw) and revealed the non-observed-adverse-effect level for hepatotoxicity in animal experiments (AFC 2004). Afterward, the human exposure to coumarin from the diet was calculated and the 0.02 mg kg^{-1} per day level was determined (Lake 1999; AFC 2004). Also, the theoretical maximum daily intake of coumarin from food and plant products was assessed to be 4.085 mg per day (0.07 mg kg^{-1} bw per day) (AFC 2004), showing its safety to humans. It was recently revealed that coumarins possess anti-inflammatory, antimicrobial, antiviral, antioxidant, antitumor, antiasthmatic, antidepressant, anti-HIV, and anti-Alzheimer's activities (Anand et al. 2012; Klenkar and Molnar 2015; Kostova et al. 2011; Nitiema et al. 2012; Ramírez-Pelayo et al. 2019) (Table 3.1).

Phenolic Compounds in Coffee (*Coffea*)

Coffee (*Coffea)* belongs to the *Rubiaceae* family and is an evergreen shrub or a small tree (Fig. 3.7) that grows natively in the mountains of South West Ethiopia (Ukers 1922). There are about 100 *Coffea* species, which grow in tropical and subtropical areas, especially in the equatorial regions. *Coffea arabica* L., *Coffea*

Fig. 3.7 Growing coffee. (Photo by: Piotr Werner)

canephora L. (commonly known as *Coffea robusta*), and *Coffea liberica* (*C. liberica*) represent the most significant coffees in the world, but the most commercialized are *Coffea arabica* L. and *Coffea robusta* (Toci et al. 2016). Among these species, the Typica and Bourbon varieties of Arabica coffees are widespread throughout the world; others are specific to the region: Mundo Novo and Catuai (Brazil), Jimma and Harar (Ethiopia), and Villa Sarchi (Costa Rica) (Toci et al. 2016). The annual coffee consumption rate is approximately 10 million tons, and the main producers of coffee in the world are Brazil, Vietnam, and Colombia (ICO 2020).

Coffea arabica and *canephora* (Robusta) brews are a great source of phenolic compounds in the human diet, especially phenolic acids and their derivatives. These widespread beverages of roasted coffee beans are drunk not only because of the taste and aroma but also for their beneficial health properties (Fig. 3.8). Also, green coffee brews from unroasted beans have become more and more popular. Coffee brews contain not only chlorogenic acids (CGA), especially caffeoylquinic acids (CQA), feruloylquinic acids (FQA) and dicaffeoylquinic acids (diCQA) (Fig. 3.9), their lactones, and other derivatives, but also several other bioactive compounds such as methylxanthines (caffeine, theophylline, and theobromine), diterpenes (cafestol and kahweol), nicotinic acid (niacin) and its precursor trigonelline, amino acids, and carbohydrates (Farah et al. 2005; Farah and de Paula Lima 2019a, b, c; Jeszka-Skowron et al. 2015a, 2016a, b, 2020; Jeszka-Skowron and Zgoła-Grześkowiak 2017; Rodrigues and Bragagnolo 2013). Recently, three lignans—secoisolariciresinol, lariciresinol, and matairesinol—were found in espresso coffees

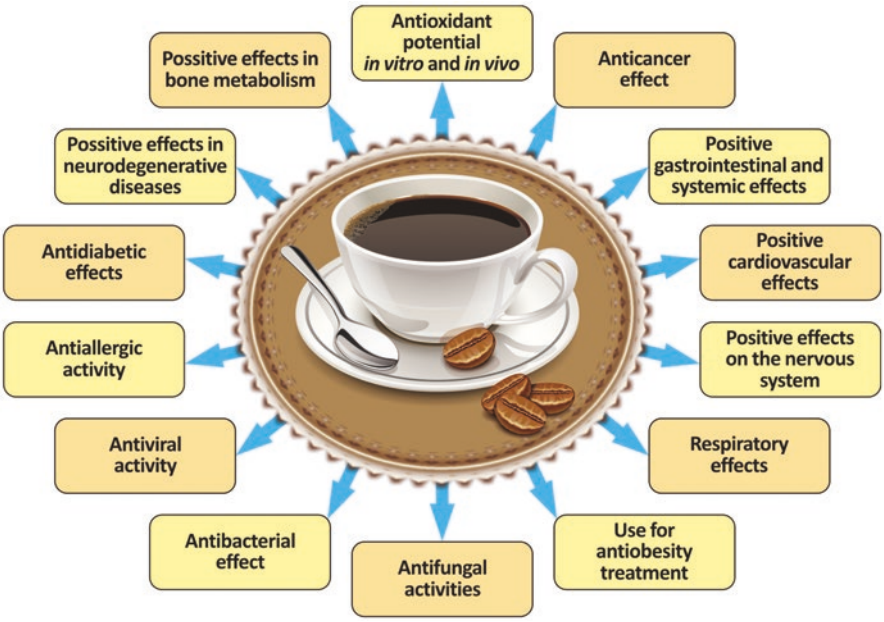

Fig. 3.8 Beneficial health properties of coffee

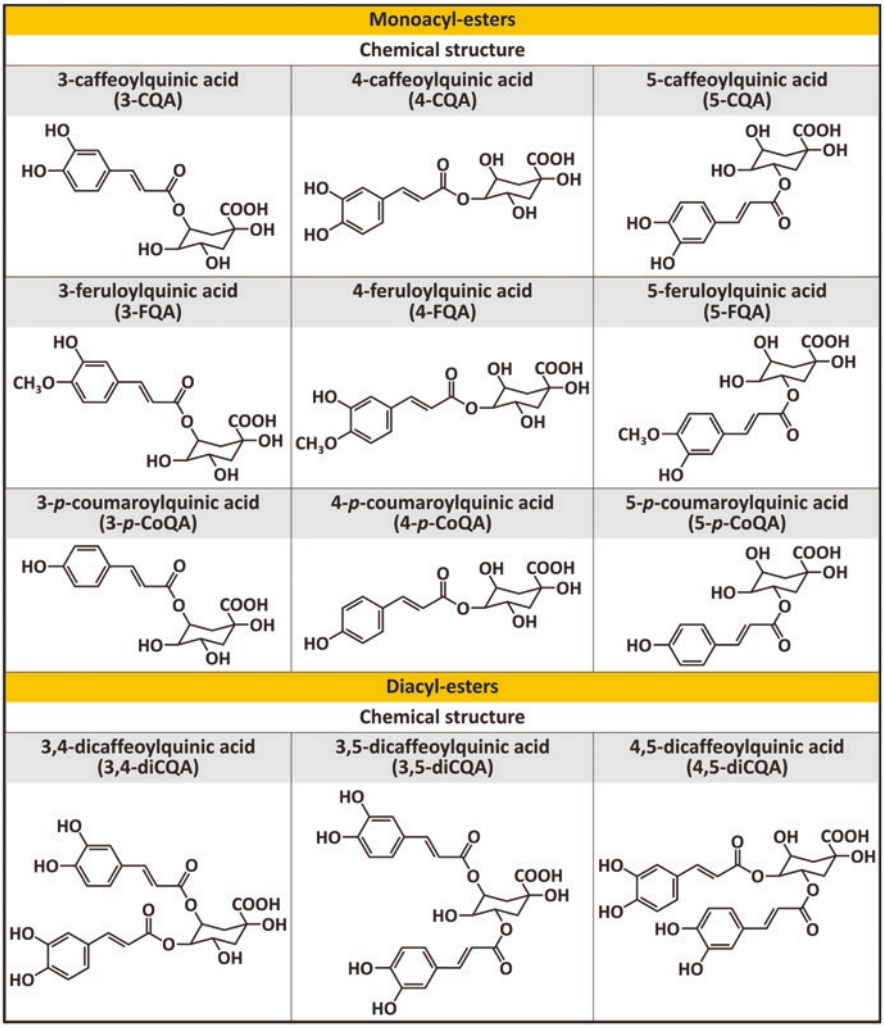

Fig. 3.9 Structures of quinic acids present in coffee

with the use of high-performance liquid chromatography coupled with tandem mass spectrometry (Angeloni et al. 2018, 2020).

The number of different chlorogenic acids in coffee is huge and arises from isomerization in the quinic part and substitutions in the cinnamic acid moiety. Other hydroxycinnamoyl esters of caffeic, *p*-coumaric, ferulic, sinapic, dimethoxycinnamic, and trimethoxycinnamic acids conjugate with quinic acid, shikimic acid, amino acids, or sugar molecules and form hydroxycinnamoyl esters, amides, or glycosides were revealed in coffee beans (Asamenew et al. 2019; Clifford et al. 2003, 2006a, b). The determination of the main CGA isomers is ordinarily

performed using high-performance liquid chromatography with a UV-Vis detector. However, for improved selectivity, modern techniques and methods are employed. The determination of CGA and related compounds in silver skin of coffee cherry, coffee beans, brews, capsules, and spent coffee grounds is often performed using liquid chromatography coupled with tandem mass spectrometry (Table 3.2).

In general, Robusta green coffee beans contain a higher level of chlorogenic acids than Arabica coffees: 7.6–8.6% and 5.2–7.1% of the dry mass, respectively; and the chemical composition of coffee depends on genetic factors, variety, environment, growing conditions, degree of ripeness, processing conditions, as well as pre- and post-harvest practices. The dominating compounds from this group are caffeoylquinic acids—88.2–95.0% of all phenolic compounds including 5-caffeoylquinic acid (5-CQA) that was determined in the highest amount from 48.1% to 63.4% in both species (Baeza et al. 2016; Mehari et al. 2016; Perrone et al. 2008). Moreover, three caffeoylquinic acids, that is, 3-CQA, 4-CQA, and 5-CQA, are the main phenolic compounds of green beans and reaching 83% of the total CGA (Farah et al. 2006). Other CGA compounds in green *C. arabica* and *C. canephora* samples include 3-FQA, 4-FQA, 5-FQA, *p*-coumaroylquinic acids (3-p-CoQA, 4-p-CoQA, 5-p-CoQA), 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, feruloylquinic acid (3,4-diFQA) and di-*p*-coumaroylquinic acid (3,4-dip-CoQA), and six caffeoylferuloylquinic acids (CFQA) isomers (Perrone et al. 2008). The differences in phenolic acid (and/or other compounds) content due to various origin, types of cultivar, and processing of coffees were also shown with the use of the principal component analysis (PCA) (Jeszka-Skowron et al. 2016a; Kuhnert et al. 2011; Mehari et al. 2016).

Green coffee beans contain a higher level of chlorogenic and other phenolic acids than roasted coffee beans. During roasting of the beans, chemical reactions and transformations take place, such as hydrolysis, transesterification (acyl migration), lactonization, and isomerization of phenolic acids. A part of hydroxycinnamic acids is also incorporated into the melanoidins during the Maillard reaction. Lactone compounds that are determined in roasted coffee are responsible mainly for coffee aroma and other attributes such as bitterness, body, acidity, and stringent (Clifford et al. 2008). Until now 137 different hydroxycinnamoyl derivatives have been identified from green and roasted beans of *C. arabica* and *C. robusta* (Asamenew et al. 2019). In this group of compounds three isomers of caffeoylquinic acids (3-CQA, 4-CQA, 5-CQA), dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA), feruloylquinic acids (3-FQA, 4-FQA, 5-FQA), p-coumaroylquinic acids (3-p-CoQA, 4-p-CoQA, 5-p-CoQA), and caffeoylferuloylquinic acids (CFQA) were usually determined in roasted coffees (Alonso-Salces et al. 2009; Angelino et al. 2018; Clifford et al. 2003).

The levels of chlorogenic acids including their lactones in coffee brews vary from 4.4 mg 100 mL^{-1} in ready-to-drink coffees (including the three main CQA isomers in reported studies) to 1030 mg 100 mL^{-1} in concentrated espresso coffees (Table 3.3). The common amounts of CGA, including caffeoylquinic, feruloylquinic, and dicaffeoylquinic compounds, and their main lactones (3-O-caffeoyl-1,5-quinide and 4-O-caffeoyl-1,5-quinide) range from 50 to 200 mg 100 mL^{-1} (Farah and de

Table 3.2 Modern methods of determination (identification and/or quantification) of the main phenolic acids and in green and roasted coffee beans, capsule-brew coffees, coffee silver skin and spent coffee grounds

Samples	Extraction method	Technique and column	Eluent (gradient analysis)	CGA: 3-, 4- and 5-O-caffeoylquinic acid (% dry basis) and other quinic acid derivatives or phenolics	References
Silver skin of green and roasted *C. arabica*	Methanol extract (E1), water extract (E2), methanol/water (50:50) extract (E3), and ethanol/water (70:30) extract (E4)—all with sonication	LC-MS/MS Kinetex PFP (100 mm × 2.1 mm, 2.6 μm)	A: 0.1% formic acid B: 0.1% formic acid in methanol	3-CQA: 0.27–0.40% 5-CQA: 0.03–0.20% 3,5-diCQA: 0.004–0.020%	Nzekoue et al. (2020)
Green and roasted *C. arabica* and *C. robusta* beans	Extraction with methanol:water: formic acid (80:15:5) and Sep-Pak C18 cartridge	UPLC-DAD-QToF/MS Cortexs® UPLC® T3 C18 (150 × 2.1 mm, 1.6 μm)	A: 0.1% formic acid B: 0.1% formic acid in acetonitrile	57 hydroxycinnamoyl derivatives 5.6–8.6% in green coffee beans 0.79–1.9% in roasted coffee beans	Asamenew et al. (2019)
Coffee and spent coffee grounds (*C. arabica*)	Extraction with ethanol:water (25:75)	Capillary LC-DAD[a] Synergi fusion-C18 (150 × 0.3 mm, 4 μm)	A: 0.1% trifluoroacetic acid adjusted to pH 3.24 B: acetonitrile	Chlorogenic acid n.d. to 0.48% *p*-coumaric acid n.d. to 0.147%	Ramon-Goncalves et al. (2019)
Green *C. arabica* beans	Extraction with methanol:water (95:5)	UPLC–MS Acquity UPLCBEH C18 (column dimensions not given, 1.7 μm)	A: 0.1% formic acid B: acetonitrile	Eight main CGA and derivatives 5.2–6.8%	Mehari et al. (2016)
Green and roasted beans, silver skin, spent grounds (*C. arabica*)	Extraction with ethanol:water (70:30)	HPLC-UV[a] Kinetex C-18 (75 × 2.1 mm, 2.6 μm)	A: 0.1% of formic acid with 0.02% trifluoroacetic acid B: 0.1% formic acid in acetonitrile with 0.02% trifluoroacetic acid	26 CGA and CGA lactones 7.71% in roasted coffee 21.20% in green coffee beans 8.32% in spent coffee 20.00% in coffee silver skins 22.75% in silver skins from decaffeinated coffee	Regazzoni et al. (2016)

Samples	Extraction method	Technique and column	Eluent (gradient analysis)	CGA: 3-, 4- and 5-O-caffeoylquinic acid (% dry basis) and other quinic acid derivatives or phenolics	References
Green *C. arabica* beans	Extraction with 2 M HCl in methanol:water (50:50)	LC-DAD[a] Suphersper RP18 (250 × 4 mm, 4 µm)	A: 1% formic acid B: acetonitrile C: methanol	Total polyphenols Colombia 6.4% Brazil 6.1% Ethiopia 7.1% Kenya 6.4%	Baeza et al. (2016)
Green *C. arabica* and *C. robusta* beans	Soxhlet extraction with methanol: water (70:30) and clarification with Carrez reagent	LC-TOF-MS Diphenyl (150 × 3 mm, 5 µm)	A: 0.05% formic acid B: methanol	Identification of 69 CGA and other quinic acids derivatives without a quantitative analysis	Kuhnert et al. (2011)
Green *C. arabica* and *C. robusta* beans	Ultrasound-assisted extraction with methanol:water:acetic acid (30:67.5:2.5) containing 2 g L^{-1} ascorbic acid	HPLC-DAD[a] Symmetry C18 (250 × 4.6 mm, 5 µm)	A: 0.2% acetic acid B: methanol	*C. arabica* CGA 2.58–5.41% *C. robusta* CGA 2.79–5.54%	Alonso-Salces et al. (2009)
Green *C. canephora* beans	Extraction with methanol:water (4:6)	MEC-DAD Fused silica capillary (22.5 cm × 0.75 µm)	SDS (70 mM)–phosphate (17.6 mM)–methanol (5%) buffer system, pH 2.5	3-CQA: 0.37–0.79% 4-CQA: 0.37–0.74% 5-CQA: 1.34–3.58%	Risso et al. (2007)
Green *C. robusta* beans	Extraction with methanol:water (70:30) and clarification with Carrez reagent	LC-MS Luna Phenylhexyl (150 × 3 mm, 5 µm)	A: water/acetonitrile/acetic acid (980:20:5, pH 2.68) B: acetonitrile/acetic acid (1000:5)	Identification of 18 CGA and isomers without a quantitative analysis	Clifford et al. (2003)

[a]Mass spectrometry was used for additional confirmation of phenolic acids and their derivatives or for additional qualitative studies, also using a separate chromatographic separation method; *UPLC-DAD-QToF/MS* ultra-performance liquid chromatography with diode array detection and quadrupole time-of-flight mass spectrometry; *LC-DAD* liquid chromatography with diode array detection; *LC-TOF-MS* liquid chromatography with quadrupole time-of-flight mass spectrometry; *MEC-DAD* micellar electrokinetic chromatography with diode array detection; *SDS* sodium dodecyl sulfate

Table 3.3 Main chlorogenic acids CGA (3-, 4-, 5-CQA, FQA, and diCQA) and major lactones in coffee brews prepared by different methods

Coffee samples	Preparation (time and temperature of brewing/ pressure)		CGA and lactones (mg in 100 mL)	References
Espresso	2 or 3 min	90 or 92 °C	34–1030	Angeloni et al. (2019), Bastos (2012), Bravo et al. (2012), Crozier et al. (2012), Farah and de Paula Lima (2019a), and Moeenfard et al. (2014)
Italian coffee brews prepared by moka pot	7 or 10 min	90, 93, or 95 °C	55–267	Angeloni et al. (2019), Bastos (2012), Bravo et al. (2012), Farah and de Paula Lima (2019a), and Moeenfard et al. (2014)
Brew from aeropress	1 min	93 °C	154	Angeloni et al. (2019)
French press brews	2.5, 5, or 6 min	95, 98, or 100 °C	46–281	Angeloni et al. (2019), Fuller and Rao (2017), and Moeenfard et al. (2014)
Manually dripped (filtered) brews	2, 2.5, or 3 min	95, 98, or 100 °C	24–157	Bastos (2012), Farah and de Paula Lima (2019a), and Jeon et al. (2017)
Electric dripper (filtered) brews	2.5, 3, 4, or 6 min	90, 95, or 100 °C	34–167	Bastos (2012), Bravo et al. (2012), Farah and de Paula Lima (2019a), and Moeenfard et al. (2014)
Turkish coffee brews	5 min	90 or 98 °C	110–201	Niseteo et al. (2012) and Farah and de Paula Lima (2019b)
Boiled coffees	2, 4, or 5 min	100 °C	14–296	Bastos (2012), Farah and de Paula Lima (2019a), Jeon et al. (2019), Moeenfard et al. (2014), and Tfouni et al. (2014)
Americano coffees (from local shops)	Unreported		9.8–43	Jeon et al. (2019)
Cold dripped brews	6, 7, 12, or 24 h	25 °C (or 10 °C)	36–319	Rao and Fuller (2018)
Instant coffees (100% coffee) Green coffee	Unreported	100 °C	8.7–77 121	Jeon et al. (2019), Mills et al. (2013), and Moeenfard et al. (2014)
"Ready-to-drink" cold coffee beverage with non-milk added	Unreported		7.8–53	Jeon et al. (2019) and Moeenfard et al. (2014)
"Ready-to-drink" cold coffee beverage with milk added	Unreported		4.4–38	Jeon et al. (2019) and Moeenfard et al. (2014)

Paula Lima 2019b). The average of these compounds in coffee brews are in decreasing order: 5-CQA (41–48%), 4-CQA (20–25%), 3-CQA (17–20%), 5-FQA (4–8%), 4-FQA (2–5%), 3-FQA (1–4%), 3,4-diCQA (1–2.5%), 3,5-diCQA (1–1.5%), 4,5-diCQA (~1%), and others (<1%) (Farah and de Paula Lima 2019a, b).

The main factors determining the composition of the chlorogenic acids in coffees are (after harvest):

- wet, semi-dry, and dry post-harvest methods (or hybrid methods),
- decaffeination process,
- steaming process,
- roasting process,
- instant coffees.

After the coffee fruits are harvested, the pulp must be extracted to produce green coffee seeds. The most common methods of pulp extraction are known as wet and dry methods. In the dry method, the cherries are exposed to the sun or air dryers until the moisture content is 10–12%, and low rainfall during harvest is needed to ensure a good-quality coffee (Toci et al. 2006). After drying, the fruits are cleaned and dehulled (the dried skin and pulp are detached), leaving silver skin adhering to the seed surface. For the best quality beverage, the good seeds are separated from any defective seeds. This method is commonly used in Brazil and Africa. The wet process is more sophisticated, but it generally produces a higher quality brew. Before dehulling and separating the seeds, the cherry selection takes place in flotation tanks, followed by soaking and fermentation. During fermentation, when enzymes may be added, the silver skin is removed and acidity increases; the pH may even be reduced to 4.5. The seeds (parchment coffee) are then extensively washed, polished, and sun-dried and/or air-dried. The wet processing is frequently used where coffee is harvested by picking, like in Colombia, Asia, and Central America (Farah 2012). Usually, the wet-processed Arabica seeds had lower levels of total chlorogenic acids compared to the dry-processed seeds (Balylaya and Clifford 1995). On the other hand, there are minor differences of CGA classes in samples (hybrid-Robusta and Arabica coffee samples) treated by the semi-dry method and wet method, but coffees processed by the wet method presented significantly higher total CGA (excluding for the diCQA) content than those processed by the semi-dry method (Duarte et al. 2010). This difference between the results acquired for Arabica and Robusta/hybrid samples may be attributed to differences in their cell-wall constitutions (Toci and Farah 2008).

The decaffeination process is performed before roasting. The cheaper methods for caffeine extraction use organic solvents (dichloromethane or ethyl acetate) and water/vapor before and after extraction to wash the seeds and open the pores. Then, after caffeine removal, the seeds are dried. In a more expensive method, the supercritical carbon dioxide technique is used and carbon dioxide is held at high pressure (approximately 200 atm) and temperature above 31 °C. Apart from the high cost of decaffeination with supercritical carbon dioxide, this method better preserves the original chemical composition of regular coffee and its flavor (Farah et al. 2006, 2010). The level of CGA in decaffeinated coffees depends on the solvent used. The

decrease of CGA was 16% in *C. arabica* and 11% in *C. canephora* after treatment with dichloromethane and 1.5% more CGA was found in green *C. arabica* seeds after water decaffeination (Farah et al. 2006; Toci et al. 2006). On the other hand, in another study, the water decaffeination method used for the *C. arabica* led to a 20% decrease of the total CGA in green coffee seeds, and treatment with supercritical carbon dioxide removed even 1.2% more (Farah et al. 2011).

The steaming process is used for the Robusta coffee that may be steam-treated before roasting to make coffee less irritating to the stomach and give better flavor and aroma. It has been attributed to the reduction of chlorogenic acid content during steam treatment (Farah and de Paula Lima 2019a). However, it was also reported that the roasting process had a greater impact on the CGA decrease than the steaming process (Kalschne et al. 2019).

Roasting of coffee is one of the most important steps of coffee processing, and it usually takes from 5 to 15 min at temperature 185–250 °C. When the temperature is high (230 °C for even 15 min of the roasting process), it causes breakage of the carbon–carbon bonds in CGA, resulting in isomerization and degradation (Farah et al. 2005). The roasting usually affects the CGA composition and average concentrations were found 0.4–2.9% (dry mass) for *C. arabica* and 0.4–5.9% (dry mass) in *C. canephora* seeds (Farah et al. 2005; Farah and de Paula Lima 2019a). For the medium-roasted coffees, CGA composition oscillates from about 1.7 to 3.5% (dry mass) for *C. arabica* and from 1.0 to 4.3% (dry mass) for *C. canephora* (Farah and de Paula Lima 2019a). This method of roasting also achieves a maximum content of lactones (14% weight loss), and less than 10% of the total CGA and about 30% of the possible precursors in green coffee were converted to lactones (Farah et al. 2005). Total CGAs in espresso coffee made from light-, medium-, and dark-roasted coffee beans were 1060 mg 100 mL^{-1}, 517 mg 100 mL^{-1}, and 340 mg 100 mL^{-1}, respectively (Ludwig et al. 2014). Differences in CGA composition and pH were also found between medium-roasted and medium–dark-roasted coffees. The level of CGA was at least threefold lower in medium–dark-roasted and pH was higher than in dark-roasted coffees due to the high concentration of CGA isomers (the acidic components of coffee) in the medium-roasted coffees (Jeon et al. 2017). It is worth mentioning that isomers 4-CQA and 3-CQA enlarged in some varieties of the light-roasted coffee beans, and it is attributed to isomerization of CGAs brought on by milder heat treatment (Moon et al. 2009). The CGA isomer content in roasted coffee beans has been characterized in decreasing order to be: CQA > diCQA > FQA > *p*-CoQA (Perrone et al. 2008).

After roasting, the coffee brews may be prepared to instant/powder coffee. The production normally includes treating of the ground-roasted coffee with hot water and high pressure to extract the water-soluble compounds (soluble material is cooled and centrifuged, and dried through freeze-drying to decrease the moisture to 5%). What is more, producers use different techniques to improve the appearance and taste of soluble coffee. The ground-roasted coffee consists mainly of Arabica species alone or a high percentage of Arabica. Robusta coffee is also used in blends designated for instant coffee production because Robusta seeds contain higher amounts of soluble solids, which increases the yield of soluble coffee. It was

revealed that instant coffee suffers an additional thermal extraction treatment at high temperatures after roasting, which decreased their antioxidant capacity (Vignoli et al. 2011). These additional processes may affect the CQAs content due to the interaction of CGAs with Maillard reaction intermediates (Mills et al. 2013). Generally, instant coffees contain a lower level of CGA. It is also worth mentioning that ready-to-drink coffee, iced coffee, and iced cappuccino may contain additional ingredients such as milk which result in increased pH of beverages and afterward degradation of CQAs. Also, the lower ratio of coffee in these products than in other brews that are prepared only from pure coffee powder may influence the level of CQAs in the products (Moeenfard et al. 2014).

Special Coffee Brews

The Kopi Luwak is a special coffee obtained from coffee berries eaten and excreted by the Asian palm civet (*Paradoxurus hermaphroditus*), a small mammal native to Southern and Northern Asia (Fig. 3.10). Indonesian Kopi Luwak coffee beans were discovered by plantation workers in Luwak droppings in nearby forests. They have found that the aroma and flavor of this specific coffee is superior to regular coffee brews (Smith 2014). The civets living in wild jungles select Arabica or Robusta coffee cherries having the best flavored beans. When the bean is ingested, the coffee polycarp is digested, and the beans are excreted. The improved distinct flavor of the coffee beans is attributed to the internal fermentation inside the civet's gut (Jumhawan et al. 2016). After the feces are collected, the beans are brought out and

Fig. 3.10 The Asian palm civet (*Paradoxurus hermaphroditus*). (Photo by: Piotr Werner)

cleaned, wet-fermented, and then sun-dried. The major colonizing bacteria in the digestive tract of civets are the lactic acid bacteria, which are also preferred in wet processing systems in a very similar natural neutral fermentation (Marcone 2004). The Kopi Luwak coffees created in the process described above possess high amounts of chlorogenic acids. The total CGA found in the Indonesian *C. arabica* amounts to 4.8%, which is the sum of 3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA (Farah and de Paula Lima 2019b).

This civet coffee is very expensive due to the low supply of the authentic product, its special production regime, and a unique flavor (earthy, musty, syrupy, smooth, and rich with both jungle and chocolate undertones). Although it was revealed that the African civet cats also eat coffee fruits (Marcone 2004), the selling price of this coffee is still approximately a hundred times higher than that of a regular coffee (ICO 2020; Marcone 2004). To reduce costs and increase the production, the civets are often kept in cages and fed with coffee cherries (Carder et al. 2016). The Kopi Luwak is, however, not the only coffee that undergoes a special treatment procedure. There are also other ways to improve coffee flavor by treating green beans with microorganisms and enzymes (Burns and Walker 2019). An interesting alternative is the special Jacu bird-depulped coffee (*C. arabica*) containing 4.7% of the above-mentioned CGAs isomers—similarly to the Kopi Luwak coffee (Farah and de Paula Lima 2019b).

Apart from the very common usage of the coffee fruit, the dried coffee leaves are consumed like a tea. In Indonesia, this brew is called "copi daon" and in Ethiopia "jeno, jenuai." It is prepared from dried Arabica leaves and drunk for headaches as well as control of HIV/AIDS in Uganda (Chen et al. 2018; Patay et al. 2016). Also, the coffee leaves infusion is applied against fever and for the stimulation of prolactin production in Mexico, as cough suppressant in Peru, and a decoction for headache and stomach pain in Nicaragua, and traditionally dried leaves are used as a substitute of tea in Jamaica or India (Campa et al. 2012). The latest studies showed that the leaves are a source of bioactive compounds, similar to that found in coffee: alkaloids (caffeine, trigonelline, and theobromine), chlorogenic acids (5-CQA [the most abundant chlorogenic acid], 3-CQA, 4-CQA, 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA) as well as xanthones (mangiferin [1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside] and isomangiferin), other organic acids, carotenoids (carotene and xanthophyll), sitosterol, and *ent*-kaurane diterpenoids (Acidri et al. 2020; Chen et al. 2018; Monteiro et al. 2020; Ngamsuk et al. 2019). The *ent*-kaurane diterpenoid derivatives, however, might be toxic, and the level of them should be monitored in any *Coffea* leaf extracts and infusions that are used for human consumption (Stewart and Steenkamp 2000).

The total contents of CGAs found in the young coffee leaves (expressed on the dry matter basis) were reported as 7.4% in *C. arabica*, between 3.6% and 4.7% in *C. canephora*, from 4.4% to 6.2% in *Coffea eugenioides*, and 4.1–8.1% in *Coffea racemosa*. The leaves also contain 3-p-CoQA, 4-p-CoQA, 5-p-CoQA, 4-FQA, 5-FQA, cis 3-caffeoylquinic acid (cis 3-CQA), cis 3-p-coumaroylquinic acid (cis 3-p-CoQA), cis 4-caffeoylquinic acid (cis 4-CQA), cis 4-p-coumaroylquinic acid (cis 4-p-CoQA), cis 5-caffeoylquinic acid (cis 5-CQA), cis 5-p-coumaroylquinic

acid (cis 5-p-CoQA), and one of the cis isomers of 3,5-diCQA with low concentration (Monteiro et al. 2020). The presence of the *cis* isomers of CGAs in coffee leaves is characteristic, although they are also found in very low amounts in coffee beans. This is caused by the fact that leaves are naturally more exposed to ultraviolet light than coffee beans (Campa et al. 2012; Clifford et al. 2008). Moreover, content of CGAs is ten times higher in young leaves in comparison to old *C. canephora* leaves (Mondolot et al. 2006). During aging of the *C. arabica* var. Bourbon leaves, the decrease of 5-CQA and 3,5-diCQA was 74% and 97%, respectively (Monteiro et al. 2020). Other phenolic compounds were also determined in Arabica coffee leaves: catechin and epicatechin, procyanidin B and C, quercetin-3-*O*-glucoside, and rutin (Ngamsuk et al. 2019). Furthermore, Ratanamarno and Surbkar (2017) reported presence of such flavanols as epigallocatechin gallate (EGCG): 550–1640 mg 100 g^{-1}, epicatechin gallate: 26–48 mg 100 g^{-1}, epicatechin: 27–40 mg 100 g^{-1}, and catechin: 5–18 mg 100 g^{-1} in *C. arabica* fresh leaves.

When the leaves are processed as a tea using the white, green, or black tea processing methods, the level of 5-CQA may decrease. This decrease strongly depends on the type of processing. For Japanese-style green tea process, the young coffee leaves may contain even 20 times more 5-CQA than the black-tea-processed mature coffee leaves (Chen et al. 2018).

Antioxidant Activity of Coffee Bean Brews and Coffee Leaves Tea

For decades, the coffee brew was drunk not only for its stimulating effect, aroma, and flavor but also because of its high antioxidant activity. This activity is important due to formation of oxidative stress in humans resulting in degenerative diseases, cancer development, aging, and death. The antioxidant properties of brews, other beverages, and food are usually measured with spectrophotometric techniques and sometimes with the use of HPLC. The other methods, apart from clinical trials, are cells-based assays and experiments on laboratory animals. Coffee brews have antioxidant activities in 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, chelating activities, and radical scavenging by transition metals such as Fe^{2+}, and they interrupt free radical chain reactions (Gómez-Ruiz et al. 2007; Vignoli et al. 2011; Niseteo et al. 2012; Jeszka-Skowron et al. 2016a, b; Jeszka-Skowron and Zgoła-Grześkowiak 2017; Rao and Fuller 2018). Interestingly, the antioxidant activity of young leaves measured by ABTS, DPPH, and ferric reducing Fe(III) antioxidant potential (FRAP) methods was similar to that of the roasted coffee beans (Acidri et al. 2020).

In general, the green coffee brews normally possess higher antioxidant activities than roasted coffees, and it depends on chemical-based assay used, coffee origin, coffee preparation, and other factors (Gómez-Ruiz et al. 2007; Jeszka-Skowron and Zgoła-Grześkowiak 2017). On the contrary, the light- or medium-roasted Arabica

coffee brews were more effective in ABTS and oxygen radical-scavenging assays (Liang et al. 2016). In addition, Robusta coffees generally show higher antioxidant activity than Arabica coffees from different countries and regions (Jeszka-Skowron et al. 2016a, b; Jeszka-Skowron and Zgoła-Grześkowiak 2017).

Moreover, Arabica coffee brew extracts protect Caco-2 cells against AAPH-induced intracellular oxidative stress (AAPH - 2,2-azobis(2-amidinopropane) dihydrochloride) (Liang and Kitts 2016). The most active were green coffee brew extracts and the least dark-roasted coffees. Coffee extracts exhibited protective activity against free radical-induced DNA damage, and the most potent being the less-roasted sample of *C. arabica* (roasting conditions: 3 min 52 s; 215 °C) (Priftis et al. 2015). The coffee extract of *C. arabica* with the highest antioxidant activity also improves the antioxidant mechanisms in myoblast cells by increasing the glutathione levels. Treatment with green coffee bean extract significantly decreased reactive oxygen species production by the HepG2 cells (Baeza et al. 2014). Also, pre-treatment with green coffee bean extract or only 5-CQA and 3,5-diCQA returns glutathione levels and the activity of antioxidant enzymes to the values similar to those observed for the control cells. These enzymes show significant defense against oxidative stress in vitro.

5-CQA in the human keratinocyte cell line (HaCaT cells) protects against H_2O_2-induced and UVB-mediated oxidative stress (ultraviolet B-rays) (Cha et al. 2014). 5-CQA has been shown to protect against NH_2Cl-induced plasmid DNA breakage in cultured neutrophils (Shibata et al. 1999). Moreover, 5-CQA and both caffeinated and decaffeinated coffees also prevented apoptosis in primary cortical neurons by upregulating the antioxidant enzymes such as NADPH (nicotinamide adenine dinucleotide phosphate - reduced form):quinine oxidoreductase 1 (Kim et al. 2012). The main compounds of coffee CGAs—3-CQA, 4-CQA, and 5-CQA—were found to protect cells against hydrogen peroxide-induced apoptosis by suppressing the mitochondrial membrane depolarization caused by oxidative stress (Park 2013). What is more, chlorogenic acid in the diet of diabetic rats effectively reduced lipid hydroperoxide production and increased the level of non-enzymatic antioxidants: reduced glutathione and vitamins C and E (Karthikesan et al. 2010; Pari et al. 2010). In other study, it has been shown that chlorogenic acid can alleviate the oxidative stress induced by methamphetamine in rats by restoring liver superoxide dismutase and glutathione peroxidase activities and by preventing the accumulation of lipid peroxidation (Koriem and Soliman 2014).

The coffee leaves infusions contain high amounts of phenolics content and demonstrate antioxidant and anti-inflammatory activities. Black tea processing of mature coffee leaves has dual effects on inflammation as evidenced by the induction of NO (nitric oxide), iNOS (inducible NO synthase), COX-2 (cyclooxygenase-2), and pro-inflammatory cytokines such as IL-1α, IL-1β (interleukins), GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), and TNF-α (tumor necrosis factor) under basal conditions, and inhibited NO under induction conditions. Therefore, consumption of coffee leaves brews has different health benefits when they are processed and have lower amounts of CQA than in fresh leaves (Chen 2019; Chen et al. 2018). The

beverages from coffee leaves subjected to black tea fermentation may decrease high blood pressure, leading to the reduction of cardiovascular disease risk, and they also demonstrate antimicrobial activities. On the other hand, coffee leaves prepared as green tea possess antioxidant and anti-inflammatory properties.

Phenolic Compounds in Tea (*Camellia sinensis*)

One of the most popular drinks next to coffee is tea. Its consumption is rapidly increasing, and an average 5% growth per year was noted between 2007 and 2016, leading to world tea consumption over 5.5 million tones (FAO 2018a). Tea is prepared from the leaves of *Camellia sinensis* that is native to East Asia and most probably originates in the borderlands of China, India, and Myanmar (Fig. 3.11). Among different varieties of *Camellia sinensis*, there are two that gained the widest popularity: *Camellia sinensis* var. *sinensis* and *Camellia sinensis* var. *assamica*. The evergreen tea shrubs are grown in tropical and subtropical climates. For their growth, they prefer acidic soil and frequent rainfall. The higher they grow, the more intense their taste is. Depending on the size of the leaves, the tea bushes can be divided into three groups. The largest leaves come from Assam, followed by Cambodia, which are medium-sized, and the Chinese type has the smallest leaves.

The fresh tea leaves picked from *Camellia sinensis* are subjected to different processing that leads to white, green, yellow, oolong, black, and pu-erh teas (Fig. 3.12). White tea comes from the buds and immature leaves picked by hand that are processed in the most natural way possible. Its manufacturing consists of

Fig. 3.11 Growing tea. (Photo by: Piotr Werner)

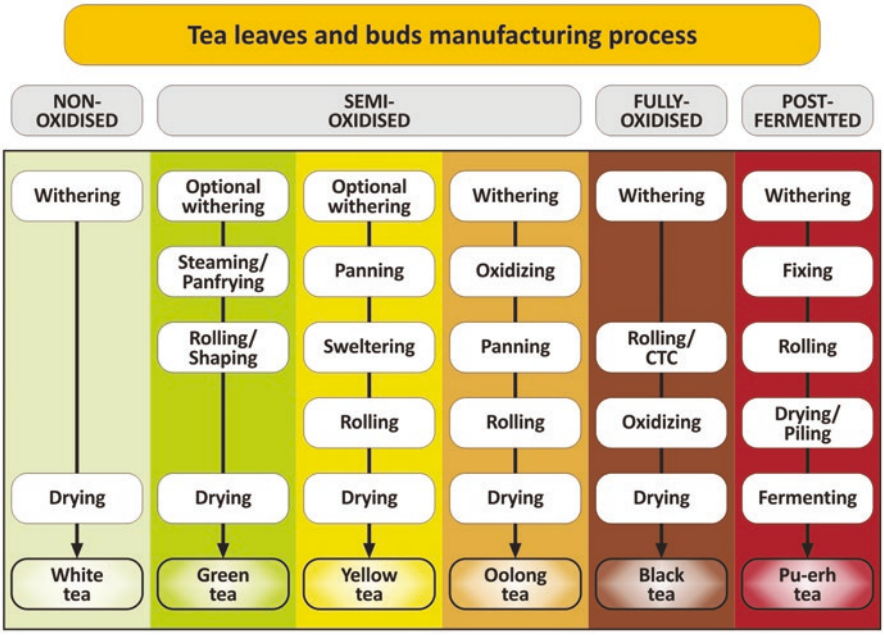

Fig. 3.12 Types of tea processing

withering and drying only, and there is no oxidation step. Therefore, the leaves must be handled with care to avoid damage to their structure that could result in release of enzymes causing oxidation (Xu et al. 2018). Unlike white tea, green tea is subjected to withering followed by deactivation of polyphenol oxidase and peroxidase enzymes during fixing. The fixing preserves all the healthy properties and natural components contained in the fresh tea leaves. It can be done in two ways, by steaming or panfrying. During steaming, the leaves immediately after harvesting are treated with steam at 95–100 °C for 30–45 s reducing enzyme activity while keeping the green color of the leaves. The panfrying, which is a traditional Chinese fixing method, is done in a large wok or in a panning machine. This process unlike steaming causes slight discoloration and oxidation of the leaves. After fixing, tea is rolled and dried (Hayat et al. 2015; Pradhan and Dubey 2019; Subramanian et al. 1999; Xu et al. 2018). The manufacturing of yellow tea is similar to that of green tea with one additional step, that is wrapping or wet-piling, called sweltering or yellowing. During this step, the leaves are wrapped in paper or heaped in a bamboo basket covered with a cloth which induces slight oxidation. Yellow tea in comparison to green tea is more sweet and mellow. It is also free of the grassy notes of green tea (Xu et al. 2018). The black tea leaves are plucked from the bushes, withered, rolled, or subjected to the CTC procedure (including mechanical crushing, tearing, and curling), oxidized and dried. The rolling is a traditional process which for ages was done by hand. It is used to damage the structure of leaves and release the essential oils that react with oxygen to develop flavor and aroma. The alternative CTC

procedure uses machines equipped with hundreds of small, sharp teeth. These sharp points break down the tea leaves into smaller pieces and the shredded tea leaves and granular pellets are obtained. The rolled or CTC-fragmented leaves are subjected to enzymatic oxidation by polyphenol oxidase and peroxidase, which is often wrongly referred to as fermentation. During this step, the enzymes facilitate oxidation and formation of polyphenols (Hayat et al. 2015; Pradhan and Dubey 2019; Subramanian et al. 1999; Xu et al. 2018). Oolong tea, gaining its increasing popularity, is partially oxidized tea, and it situates between green and black teas. During the manufacturing the withered leaves are subjected to bruising. This unique process damages only the edges, leaving the inner part of the leaves intact. While the leaves are exposed to the air, the edges turn dark because of enzymatic oxidation taking place. This is stopped by fixing that deactivates the enzymes. Tea with inactivated enzymes is then rolled to develop flavor, dried, and backed (Ng et al. 2017; Xu et al. 2018). Pu-erh tea comes from the Chinese Yunnan province, and it is made from the large-leaf tea variety, *Camellia sinensis* var. *assamica*. These tea plants are rather trees often growing in the middle of the forests than shrubs in plantations. The initial process of pu-erh tea manufacturing includes withering and fixing like for green tea. The fixing stage, however, is not a panning in a wok, but the leaves are sun-dried. This drying in a shaded sunlight is characteristic for all pu-erh teas, and the left-over enzymes have enough time for causing slight oxidation. Then the damaged dry leaves are removed, and the remaining leaves are compressed to form the pu-erh cake, usually in the form of disks. The disks produced in this process are then dried and subjected to fermentation, which is a microbial process. This traditional raw fermentation is natural and may last for years leading to Sheng Cha. The alternative is a wet fermentation, where the sun-dried leaves are stacked in a pile (heaped), sprayed with water, and covered with a sheet of paper or plastic. The temperature in the pile increases, accelerating the fermentation process, and the final product Shu Cha is obtained within weeks or months (Lv et al. 2013). However, even though there are so many types of tea, the world production is divided mainly between black and green teas. The estimated black tea production in 2017 was 3.3 million tones and for green tea, the actual 2015–2017 yearly production was 1.8 million tones. The projected production growth for black and green teas in the 2027 perspective is 2.2% per year and 5.0% per year, respectively (FAO 2018b).

Teas, including both green and black types, are a great source of phenolic compounds, but different production procedures lead to considerable differences in the content of particular flavonoids. Green tea contains catechins that account for 60–80% of total flavonoids, while black tea has 63–74% of thearubigins and only 6–24% of catechins (Higdon and Frei 2003). There are many different catechins present in green tea (Fig. 3.13), including (+)-catechin, (−)-epicatechin, (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), and their gallate esters: (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG), as well as gallic acid (Aucamp et al. 2000; Haslam 2003; Wang et al. 2000b; Zuo et al. 2002). Among these catechins, EGCG is the main one and can be found in green tea at a concentration ranging from 5.1% to 7.9% dry mass. EGC was determined from 2.8% to 3.8% dry mass and ECG in the range 1.1–2.2% dry

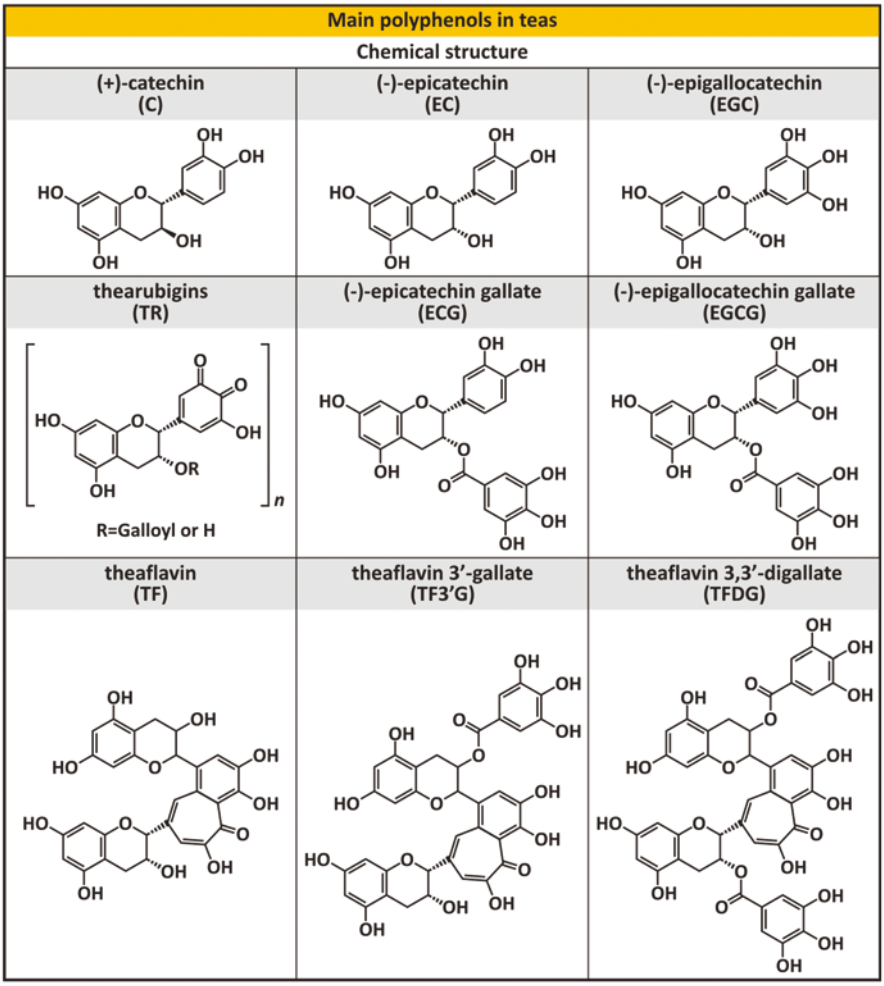

Fig. 3.13 Structures of catechins and theaflavins

mass (Aucamp et al. 2000; Zuo et al. 2002). The green tea brew prepared from 3 g leaves in 150 mL water contains about 30 mg per 100 mL of both EGCG and EGC and about 5 mg per 100 mL ECG (Wang et al. 2000b). Apart from catechins green tea also contains oxidized catechins formed during withering, which represent 20–30% of total flavonoids and flavonols accounting for 7–9% of total flavonoids (Higdon and Frei 2003).

The flavonols found in green tea are not considerably affected by oxidation and are present in similar quantities in green, black, and oolong teas, while the catechins are severely influenced by this process. Therefore, during the manufacturing of the black tea, catechins form dimers and polymers that are known as theaflavins and thearubigins (Fig. 3.13) (Higdon and Frei 2003). The four main theaflavins are

formed from the main catechins present in green tea; that is, theaflavin (TF) comes from EC and EGC, theaflavin-3-gallate (TF-3-G) from EC and EGCG, theaflavin-3′-gallate (TF-3′-G) from ECG and EGC, and theaflavin-3,3′-digallate (TF-3,3′-G) from ECG and EGCG (Wright et al. 2002). Therefore, simultaneously with the formation of theaflavins and thearubigins, the concentration of catechins drops considerably—EGCG was found in the ranges 2.2–2.8% dry mass and 0.4–1.1% dry mass in the oolong and black teas, respectively. EGC was reported from 1.1% to 1.6% dry mass in oolong teas and from 0.1% to 6.6% dry mass in black teas. ECG was found at a concentration of 0.6% dry mass in oolong teas and from 0.4% to 0.8% dry mass in black teas (Aucamp et al. 2000; Zuo et al. 2002). The black tea brew contains only about 1.0–1.1 mg per 100 mL EGCG, 0.9–1.8 mg per 100 mL EGC, and 1.2–2.9 mg per 100 mL ECG, that is, up to 30 times less than in green teas (Wang et al. 2000b). Changes in the content of theaflavins caused by the tea oxidation were presented for green, oolong, and black teas. These compounds were not found in any of five tested green teas. However, they were found already in partially oxidized oolong teas. The average concentrations from five samples were 0.943 mg g^{-1}, 0.867 mg g^{-1}, 0.517 mg g^{-1}, and 0.612 mg g^{-1} for TF, TF-3-G, TF-3′-G, and TF-3,3′-G, respectively. The amount of theaflavins were considerably higher in ten tested black teas, that is, 2.387 mg g^{-1}, 2.392 mg g^{-1}, 2.383 mg g^{-1}, and 2.422 mg g^{-1} for TF, TF-3-G, TF-3′-G, and TF-3,3′-G, respectively. However, the difference between various black tea samples was considerable for all four analytes. As a result, the total amount of theaflavins ranged from 2.02 to 17.64 mg g^{-1} (Zhang et al. 2019). Similarly high range between theaflavin content in different black tea samples was also noted in other studies. Nishimura et al. (2007) analyzed only four tea samples and noted the total content of TF, TF-3-G, TF-3′-G, and TF-3,3′-G ranging between 0.63 and 19.07 mg g^{-1} dry mass. Friedman et al. (2006) analyzed ethanolic (80%) and water tea extracts from 32 black tea samples and also found considerable differences. The sum of theaflavins in ethanolic extracts ranged from 3.71 to 20.7 mg g^{-1} dry mass and in water extracts from not detected to 8.8 mg g^{-1} dry mass. Moreover, Wright et al. (2002) found considerable differences between content of theaflavins in good-quality and poor-quality black teas. The average content of four theaflavins for good-quality tea was 20.9 ± 5.0 μmol g^{-1} dry mass and for poor-quality teas 14.1 ± 3.5 μmol g^{-1} dry mass. The highest difference between good and poor teas was noted for TF and the lowest for TF-3,3′-G. It is not clear, however, if the poor quality of black teas was due to the low quality of the unprocessed leaves or low quality of the manufacturing process.

Apart from polyphenols, tea also contains caffeine and related compounds including theobromine. Typically, both green and black tea beverages contain 3% w/w of caffeine in extracted solids; however, the amount varies between 2% and 5% depending on the variety (Harbowy and Balentine 1997). The exception is *Camellia irrawadensis* that lacks this compound due to the absence of the methylation path transforming 7-methylxanthine into theobromine and caffeine (Negishi et al. 1985; Roberts et al. 1958). This tea, however, is not produced commercially because of the poor quality of the finished products. Instead, decaffeinated teas are available for those who are sensitive to caffeine (Harbowy and Balentine 1997).

Table 3.4 Modern methods used for the determination of catechins and theaflavins in white, green, yellow, oolong, black, and pu-erh teas

Extraction method	Technique and column	Eluent (gradient analysis)	Gallic acid, catechins, and theaflavins (mg g^{-1})		References
White tea					
Extraction with tetrahydrofuran, methanol–acetic acid–water (50:3.7:46.3, v/v/v), diethyl ether–ethyl acetate (1:1, v/v)	HPLC-PAD Zorbax Extend-C18 (250 mm × 4.6 mm, 5 μm)	A: methanol B: 0.1% formic acid in water	C: nd EC: nd–1.31 GC: nd EGC: nd–8.42 CG: nd	ECG: 3.27–3.84 GCG: nd EGCG: 3.54–8.54 GA: 2.02–2.49 TF: nd	Tang et al. (2019b)
Extraction with water	LC-MS/MS Kinetex Evo C18 (150 mm × 2.1 mm, 2.6 μm)	A: 0.1% formic acid in water B: acetonitrile	GC: 1.85 C: 0.8 EC: 6.72	EGCG: 39.72 GCG: 1.29 ECG: 9.85	Jeszka-Skowron et al. (2018)
Green tea					
1. Step—extraction with 80% methanol 2. Step—extraction with 80% methanol containing 0.15% HCl	HPLC-PAD Alltech adsorbosil C18 (250 mm × 4.6 mm, 5 μm)	A: water:acetic acid (97:3, v/v) B: methanol	GA: 0.37–0.74 EGC: 27.7–30.8 EGCG: 51.1–52.7 EC: 7.25–10.3	ECG: 11.3–21.8 CG: nd	Zuo et al. (2002)
Extraction with 80% ethanol	HPLC-UV Inertsil ODS-3v (250 mm × 4 mm, 5 μm)	A: 20 mM KH_2PO_4 B: acetonitrile	EGC: nd–13.9 C: nd–4.8 EC: tr–3.1 EGCG: 7.0–74.0 GCG: 0.2–9.0 ECG: 2.4–40.5	CG: nd–1.3 TF: nd TF3G: nd TF3′G: nd TF33′G: nd	Friedman et al. (2006)
Extraction with water	HPLC-UV Inertsil ODS-3v (250 mm × 4 mm, 5 μm)	A: 20 mM KH_2PO_4 B: acetonitrile	EGC: nd–14.2 C: nd–7.9 EC: 0.1–2.6 EGCG: 2.2–53.6 GCG: 0.3–6.3 ECG: 1.2–27.1	CG: nd–0.4 TF: nd–0.2 TF3G: nd TF3′G: nd TF33′G: nd	Friedman et al. (2006)
1. Step—extraction with 80% methanol 2. Step—extraction with 80% methanol containing 0.15% HCl	HPLC-PAD Spherisorb S10 ODS 2 (250 mm × 4.5 mm, 5 μm)	A: water:acetic acid (97:3, v/v) B: methanol	EGCG: 73.3–103.5 EGC: 24.3–45.3 ECG: 10.4–23.0	EC: 8.1–14.1 GA: 0.3–1.5	Cabrera et al. (2003)

Table 3.4 (continued)

Extraction method	Technique and column	Eluent (gradient analysis)	Gallic acid, catechins, and theaflavins (mg g^{-1})		References
Extraction with methanol: water: formic acid (50:45:5, v/v/v)	UHPLC-QToF/MS Kinetex XB C18 (150 mm × 2.1 mm, 1.7 µm)	A: 0.5% formic acid in water B: 0.5% formic acid in acetonitrile	EGC: 1.56 EC: 2.98 EGCG: 60.86 ECG: 23.18	TF: nd TF3G: nd TF3′G: nd TF33′G: nd	Lee et al. (2019)
Extraction with tetrahydrofuran, methanol–acetic acid–water (50:3.7:46.3, v/v/v), diethyl ether–ethyl acetate (1:1, v/v)	HPLC-PAD Zorbax Extend-C18 (250 mm × 4.6 mm, 5 µm)	A: methanol B: 0.1% formic acid in water	C: nd–1.32 EC: 5.38–8.70 GC: 1.82–4.00 EGC: 13.09–100.68 CG: nd–0.64	ECG: 7.60–35.39 GCG: nd–5.84 EGCG: 33.10–59.35 GA: 0.70–1.43 TF: nd	Tang et al. (2019b)
Extraction with 50% methanol	HPLC-UV XTerra RP18 (150 mm × 4.6 mm, 3.5 µm)	A: 0.2% H_3PO_4 B: methanol	C: 0.99–2.48 EC: 11.84–12.59 GC: 2.28–3.65 EGC: 30.52–40.34	ECG: 38.14–41.19 GCG: 5.61–6.76 EGCG: 103.95–112.86	Lee et al. (2014)
Extraction with water	LC-MS/MS Kinetex Evo C18 (150 mm × 2.1 mm, 2.6 µm)	A: 0.1% formic acid in water B: acetonitrile	GC: 0.05–3.48 C: 0.07–4.87 EC: 0.23–15.02	EGCG: 1.25–37.14 GCG: tr–0.22 ECG: 0.18–22.14	Jeszka-Skowron et al. (2018)
Yellow tea					
Extraction with tetrahydrofuran, methanol–acetic acid–water (50:3.7:46.3, v/v/v), diethyl ether–ethyl acetate (1:1, v/v)	HPLC-PAD Zorbax Extend-C18 (250 mm × 4.6 mm, 5 µm)	A: methanol B: 0.1% formic acid in water	C: nd–2.04 EC: 0.97–10.06 GC: 2.74–11.86 EGC: 13.66–45.48 CG: nd–1.61	ECG: nd–30.49 GCG: 1.39–24.71 EGCG: 17.21–57.23 GA: 0.75–3.82 TF: nd	Tang et al. (2019b)

(continued)

Table 3.4 (continued)

Extraction method	Technique and column	Eluent (gradient analysis)	Gallic acid, catechins, and theaflavins (mg g^{-1})		References
Oolong tea					
1. Step—extraction with 80% methanol 2. Step—extraction with 80% methanol containing 0.15% HCl	HPLC-PAD Alltech adsorbosil C18 (250 mm × 4.6 mm, 5 µm)	A: water:acetic acid (97:3, v/v) B: methanol	GA: 1.42–1.67 EGC: 10.0–15.9 EGCG: 22.2–28.2	EC: 2.63–2.96 ECG: 6.06–6.45 CG: nd–0.27	Zuo et al. (2002)
1. Step—extraction with 80% methanol 2. Step—extraction with 80% methanol containing 0.15% HCl	HPLC-PAD Spherisorb S10 ODS 2 (250 mm × 4.5 mm, 5 µm)	A: water:acetic acid (97:3, v/v) B: methanol	EGCG: 11.8 EGC: 7.3 ECG: 3.1	EC: 3.5 GA: 1.2	Cabrera et al. (2003)
Extraction with 70% methanol	HPLC-UV Chromolith RP-18 column (100 mm × 4.6 mm × 2.0 µm)	A: 2% (v/v) acetic acid in water B: acetonitrile: ethyl acetate (7:1, v/v)	TF: 0.4–1.36 TF3G: 0.67–1.24	TF3′G: 0.09–1.15 TF33′G: 0.34–1.04	Zhang et al. (2019)
Extraction with tetrahydrofuran, methanol–acetic acid–water (50:3.7:46.3, v/v/v), diethyl ether–ethyl acetate (1:1, v/v)	HPLC-PAD Zorbax Extend-C18 (250 mm × 4.6 mm, 5 µm)	A: methanol B: 0.1% formic acid in water	C: nd–0.77 EC: 4.34–13.72 GC: 3.93–11.52 EGC: 31.25–139.85 CG: nd–0.98	ECG: 3.68–8.43 GCG: nd–2.26 EGCG: 20.21–36.70 GA: 0.29–3.28 TF: nd–0.54	Tang et al. (2019b)
Extraction with 50% methanol	HPLC-UV Wonda Cract ODS-2 (250 mm × 4.6 mm, 5 µm)	A: 0.5% acetic acid B: acetonitrile	C: 152.1–376.5 EC: 4.7–19.2 EGC: 19.5–85.3	EGCG: 39.4–181.9 ECG: 15.4–64.2 GCG: 10.0–74.3	Xie et al. (2017)

Table 3.4 (continued)

Extraction method	Technique and column	Eluent (gradient analysis)	Gallic acid, catechins, and theaflavins (mg g^{-1})		References
Extraction with water	HPLC-DAD PartiSphere 5 C18 (110 mm × 4.6 mm, 5 μm) CE-DAD Light path capillary (40 cm × 50 mm I.D.)	A: 5% (v/v) acetonitrile with 0.035% (v/v) TFA B: 50% (v/v) acetonitrile with 0.025% (v/v) TFA 200 mM H_3BO_3 (pH 7.2), 10 mM KH_2PO_4 (pH 4.2), 4.5 mM of β-CD, and 27.5% (v/v) of acetonitrile	EC: 1.75 ECG: 3.58 EGC: 7.70 EC: 1.88 ECG: 3.07 EGC: 3.57	EGCG: 8.99 TFs: 0.66 EGCG: 7.36 TFs: 3.63	Lee and Ong (2000)
Black tea					
1. Step—extraction with 80% methanol 2. Step—extraction with 80% methanol containing 0.15% HCl	HPLC-PAD Alltech adsorbosil C18 (250 mm × 4.6 mm, 5 μm)	A: water:acetic acid (97:3, v/v) B: methanol	GA: 2.06 EGC: 5.71 EGCG: 3.79	EC: 1.36 ECG: 4.45 CG: nd	Zuo et al. (2002)
Extraction with 80% ethanol	HPLC-UV Inertsil ODS-3v (250 mm × 4 mm, 5 μm)	A: 20 mM KH_2PO_4 B: acetonitrile	EGC: nd–6.1 C: nd–4.0 EC: 0.6–7.0 EGCG: 0.6–47.8 GCG: 0.8–5.8 ECG: 3.8–67.1	CG: tr–2.0 TF: 0.4–9.1 TF3G: nd–5.8 TF3′G: nd–3.8 TF33′G: 0.6–10.9	Friedman et al. (2006)

(continued)

Table 3.4 (continued)

Extraction method	Technique and column	Eluent (gradient analysis)	Gallic acid, catechins, and theaflavins (mg g^{-1})		References
Extraction with water	HPLC-UV Inertsil ODS-3v (250 mm × 4 mm, 5 µm)	A: 20 mM KH_2PO_4 B: acetonitrile	EGC: nd–10.3 C: nd–8.1 EC: 0.3–6.3 EGCG: 0.5–28.2 GCG: 0.9–6.7 ECG: 1.7–26.8	CG: nd–1.5 TF: nd–5.4 TF3G: nd–2.1 TF3′G: nd–0.9 TF33′G: nd–6.4	Friedman et al. (2006)
1. Step—extraction with 80% methanol 2. Step—extraction with 80% methanol containing 0.15% HCl	HPLC-PAD Spherisorb S10 ODS 2 (250 mm × 4.5 mm, 5 µm)	A: water:acetic acid (97:3, v/v) B: methanol	EGCG: 12.3–85.1 EGC: 3.9–41.7 ECG: 4.4–20.6	EC: 4.0–11.4 GA: 2.5–4.5	Cabrera et al. (2003)
Extraction with water	MEKC-UV Fused-silica capillary (570 mm × ID 50 µm)	Buffer 100 mM SDS, 25 mM phosphate, 6% methanol pH 7.0	C: 1.21 EGCG: 10.62 EGC: 1.14	ECG: 7.51 EC: 2.17 GA: 2.29	Aucamp et al. (2000)
MeOH: water: formic acid (50:45:5, v/v/v)	UHPLC-QToF/MS Kinetex XB C18 (150 mm × 2.1 mm, 1.7 µm)	A: 0.5% formic acid in water B: 0.5% formic acid in acetonitrile	EGC: 0.79 EC: 0.89 EGCG: 9.45 ECG: 6.90	TF: 0.65 TF3G: 1.68 TF3′G: 0.98 TF33′G: 3.06	Lee et al. (2019)
Extraction with 40% ethanol	LC-MS Shimazu ODS 80Ts (250 mm × 2 mm)	A: 15% acetonitrile with 0.5% acetic acid in water B: 80% acetonitrile with 0.5% acetic acid in water	TF: 1.24–2.26 TF3G: 0.97–3.59	TF3′G: 0.63–2.27 TF33′G: 1.54–11.31	Nishimura et al. (2007)
Extraction with tetrahydrofuran, methanol–acetic acid–water (50:3.7:46.3, v/v/v), diethyl ether–ethyl acetate (1:1, v/v)	HPLC-PAD Zorbax Extend-C18 (250 mm × 4.6 mm, 5 µm)	A: methanol B: 0.1% formic acid in water	C: nd EC: nd–0.79 GC: nd–1.10 EGC: nd–8.48 CG: nd	ECG: nd–3.51 GCG: nd–0.51 EGCG: 0.54–3.80 GA: 1.74–3.55 TF: 0.49–0.56	Tang et al. (2019b)

Extraction method	Technique and column	Eluent (gradient analysis)	Gallic acid, catechins, and theaflavins (mg g^{-1})		References
Extraction with 40% ethanol	HPLC-UV Discovery RP-Amide C16 (250 mm × 4.6 mm, 5 µm)	A: H_3PO_4 (85%) and water (0.05:99.95, (v/v)) B: acetonitrile	C: nd–6.92 EC: nd–2.11 GC: nd–0.99 EGC: 0.13–10.47 CG: nd–0.66 ECG: 0.7–9.49 GCG: nd–14.69	EGCG: 0.17–18.24 GA: 0.95–5.80 TF: tr–4.43 TF3G: 0.56–4.43 TF3′G: 0.27–2.03 TF33′G: 0.47–3.61	Wang et al. (2010)
Extraction with 70% methanol	HPLC-UV Chromolith RP-18 column (100 mm × 4.6 mm × 2.0 µm)	A: 2% (v/v) acetic acid in water B: acetonitrile:ethyl acetate (7:1, v/v)	TF: 0.78–5.04 TF3G: 0.60–4.68	TF3′G: 0.18–4.17 TF33′G: 0.37–4.99	Zhang et al. (2019)
Pu-erh tea					
1. Step—extraction with 80% methanol 2. Step—extraction with 80% methanol containing 0.15% HCl	HPLC-PAD Alltech adsorbosil C18 (250 mm × 4.6 mm, 5 µm)	A: water:acetic acid (97:3, v/v) B: methanol	GA: 5.53 EGC: 6.23 EGCG: 1.99	EC: 3.24, ECG: 132 CG: nd	Zuo et al. (2002)
1. Step—extraction with 80% methanol 2. Step—extraction with 80% methanol containing 0.15% HCl	HPLC-PAD Spherisorb S10 ODS 2 (250 mm × 4.5 mm, 5 µm)	A: water:acetic acid (97:3, v/v) B: methanol	EGCG: 1.4 EGC: 5.8 ECG: 0.2	EC: 0.6 GA: 0.04	Cabrera et al. (2003)
Extraction with tetrahydrofuran, methanol–acetic acid–water (50:3.7:46.3, v/v/v), diethyl ether–ethyl acetate (1:1, v/v)	HPLC-PAD Zorbax Extend-C18 (250 mm × 4.6 mm, 5 µm)	A: methanol B: 0.1% formic acid	C: nd EC: 1.57 GC: nd EGC: nd CG: nd	ECG: 0.51 GCG: nd EGCG: 0.58 GA: 1.64 TF: nd	Tang et al. (2019b)

(continued)

Table 3.4 (continued)

Extraction method	Technique and column	Eluent (gradient analysis)	Gallic acid, catechins, and theaflavins (mg g^{-1})		References
Extraction with water	HPLC-DAD PartiSphere 5 C18 (110 mm × 4.6 mm, 5 μm) CE-DAD Light path capillary (40 cm × 50 mm I.D.)	A: 5% (v/v) acetonitrile with 0.035% (v/v) TFA B: 50% (v/v) acetonitrile with 0.025% (v/v) TFA 200 mM H_3BO_3 (pH 7.2), 10 mM KH_2PO_4 (pH 4.2), 4.5 mM of β-CD, and 27.5% (v/v) of acetonitrile	EC: 0.49 ECG: 0.07 EGC: 0.6 EC: 0.33 ECG: 0.11 EGC: 0.12	EGCG:0.3 TFs: 1.03 EGCG: 0.12 TFs: 0.68	Lee and Ong (2000)

GA Gallic acid, *EGC* (−)-epigallocatechin, *C* (−)-catechin, *EC* (+)-epicatechin, *EGCG* (−)-epigallocatechin-3-gallate, *GCG* (−)-gallocatechin-3-gallate, *ECG* (−)-epicatechin-3-gallate, *CG* (−)-catechin-3-gallate, *TF* theaflavin, *TF3G* theaflavin-3-gallate, *TF3′G* theaflavin-3′-gallate, *TF33′G* theaflavin-3,3′-digallate, *GC* gallocatechin, *β-CD* β-cyclodextrin, TFA trifluoroacetic acid, *PAD* photodiode array detector, *MEKC* micellar electrokinetic capillary chromatography, *nd* not detected, *tr* trace, *UHPLC-QToF/MS* Ultra-high-performance liquid chromatography coupled with time of flight mass spectrometry,*CE-DAD* Capillary electrophoresis with diode array detection

The determination of phenolic compounds in tea is performed using a few different techniques, but HPLC in the reversed-phase mode with DAD or mass spectrometric detection is the most widely used (Table 3.4). Especially convenient is the HPLC coupled with a tandem mass spectrometer that can be used for the simultaneous determination of a number of catechins and theaflavins present in teas (Jeszka-Skowron et al. 2018; Tao et al. 2016). Nevertheless, a large number of active compounds present in tea samples often leads to their co-elution in the reverse-phase HPLC, which makes it impossible to use the DAD detector and may restrict usage of mass spectrometers due to possible co-elution of isomers having similar fragmentation patterns. As a convenient solution, a two-dimensional HPLC method can be used. Such a method was proposed with a size-exclusion column in the first dimension and an octadecyl silica column in the second dimension. The developed method enabled the determination of about a hundred analytes including catechins, theaflavins, kaempferol and its derivatives, quercetin and its derivatives, caffeine, theobromine, lipids, and other compounds (Scoparo et al. 2012). Other techniques used for the determination of phenolic compounds in teas include micellar electrokinetic capillary chromatography (Aucamp et al. 2000) and capillary electrophoresis (Horie et al. 1997; Horie and Kohata 1998). The presented methods were successfully used for the determination of catechins, caffeine, theanine, and ascorbic acid in tea samples. Nevertheless, it must be taken into account that electromigration techniques like capillary electrophoresis cannot be used for quantitation of apolar compounds that cannot migrate under the influence of an electric field.

Antioxidant Activity of Tea Brews

Tea contains various bioactive compounds such as catechins, polyphenols, and alkaloids, possessing multiple health functions. Therefore, tea has numerous positive effects on human health including cardiovascular protection, anti-diabetic and anti-obesity properties, gastrointestinal protection, and so on (Fig. 3.14) (Tang et al. 2019a). The antioxidant activity of tea is known since the twentieth century, and it is connected with the presence of such compounds as polyphenols, polysaccharides, and pigments that can scavenge free radicals, decrease reactive oxygen species, and enhance enzyme activities (Tang et al. 2019a). In humans, the tea brews display protecting effects against the oxidative stress-related injury, for example, by preventing the oxidative stress induces by increased physical activity (Jówko et al. 2015) or protecting against cutaneous oxidative stress (Megow et al. 2017). However, the antioxidant activity is usually measured by in vitro tests that include both biological and chemical tests. The biological test was presented on tea brews and their components, evaluating using cellular antioxidant activity, erythrocyte hemolysis, and plasma oxidation assays (Liu and Huang 2015; Peluso et al. 2016; Zeng et al. 2016). These methods are more biologically relevant than the chemical assays because they take into account uptake, metabolism, and distribution of antioxidant compounds in cells. However, due to their simplicity, the chemical methods

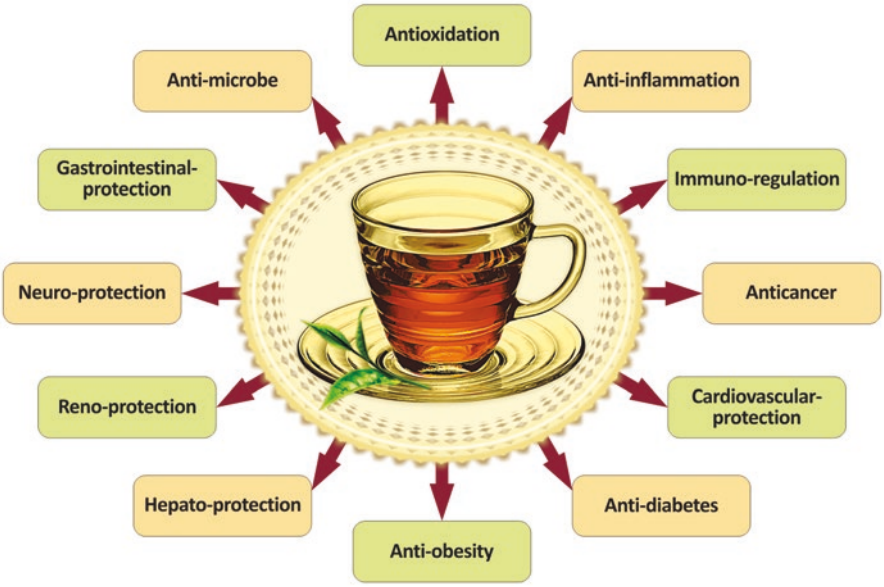

Fig. 3.14 Beneficial health properties of tea

also found a wide interest in the scientific community. These include mainly the ABTS and DPPH methods used to test free radical-scavenging ability, the FRAP assay utilized to determine the reducing ability, as well as the Folin–Ciocalteu method for assessment of the total phenolic content. Although these methods measure different properties, there is a high correlation between the results obtained using them for the tea samples (Zhao et al. 2019), except for fruit samples (Rotta et al. 2016).

The results presented for different tea types show a general trend of higher antioxidant activity of green teas than that of black teas. It was confirmed in both the DPPH test (Jeszka-Skowron and Zgoła-Grześkowiak 2014; Jeszka-Skowron et al. 2015b; Pereira et al. 2014) and the ABTS test (Jeszka-Skowron et al. 2015b; Pereira et al. 2014; Zhao et al. 2019). However, the radical-scavenging ability of white teas was similar to that found for green teas (Jeszka-Skowron and Zgoła-Grześkowiak 2014; Pereira et al. 2014) or to that found for black teas (Zhao et al. 2019). This discrepancy may be explained by different quality of teas used in the studies—the highest results are sometime two to five times greater than the lowest results (Pereira et al. 2014; Zhao et al. 2019). This is clearly visible in the study presented by Jeszka-Skowron et al. (2015b), who found white tea samples with radical-scavenging activity similar to both green and black teas. Also, previously mentioned low amount of theaflavins in poor-quality black teas (Wright et al. 2002) shows that antioxidant activity may be strongly affected by the quality of tea.

The antioxidant activity of tea is to a great extent caused by catechins and theaflavins that have similar antioxidant properties and also the standard one-electron

reduction potential (Higdon and Frei 2003; Łuczaj and Skrzydlewska 2005). However, the amount of catechins found in green teas is considerably higher than the sum of catechins and theaflavins in black teas (Table 3.4). This supports finding of many authors presenting higher antioxidant activity of green teas (Jeszka-Skowron et al. 2015b; Jeszka-Skowron and Zgoła-Grześkowiak 2014; Pereira et al. 2014; Zhao et al. 2019).

Special Tea Brews

Traditional black and green teas are often enriched with flavors or fruit. Among the most widely used flavors are spices (cayenne pepper, cinnamon, ginger, and cardamom), flowers (jasmine, rose, hibiscus, and cornflower), herbs (mint or basil), fruits (lemon, dried fruits including mango, pineapple, strawberry, etc.), bergamot oil, and chocolate. Such additives are known for their health benefits and are added to tea to enhance organoleptic properties and provide healthy drinks (Kumar Gupta et al. 2014; Kundu et al. 2014). There are two most common flavored tea types, that is, Earl Grey and masala. Both are based on black tea but contain different additives. Earl Grey is enriched with bergamot oil and sometimes other ingredients: for example, the Lady Gray tea type contains also orange or lemon peel and sometimes cornflowers. Masala is based on black tea with ginger but may also include other spices like cardamom, cinnamon, star anise, fennel, cloves, and peppercorn. Lapsang Souchong is another variant of black tea which is distinct from other teas because it is smoke-dried over pinewood fire, which considerably changes taste and aroma of the final product.

These additives (dried fruit, spices, and herbs) include biologically active components which may add health benefits to humans. Among these, the most important are the phenolic compounds, carotenoids, vitamins, minerals, and so on (Vicente et al. 2009), and it is worth mentioning that they are an integral part of the human diet (Wootton-Beard and Ryan 2011). Fruit phenolics include a wide range of compounds such as flavonols, flavan-3-ols, hydroxycinnamic acids, gallic acid derivatives, and anthocyanins (Lachman et al. 2000). The functionality of these compounds is mainly expressed in their ability to scavenge free oxygen radicals, which are involved in many pathological conditions (Briviba and Sies 1994; Hassan and Abdel-Aziz 2010; Tadić et al. 2008). However, combining different teas with herbs, fruit, and spices containing various bioactive constituents has been shown to result in both synergistic and antagonistic effects to the organisms. Synergistic effects can appear when the combined extracts of two or more plant species exhibit greater effects than the same extracts applied separately. The holistic action is therefore greater than the sum of effects resulting from the separate extracts. On the other hand, the antagonism between constituents of two extracts can lower the final result of their usage.

Black tea enriched with lemon, bergamot, clove, cinnamon sticks, and ground cinnamon increased the antioxidant activity of tea (Büyükbalci and El 2008). The

combination of black tea, black pepper, ginger, and tulsi showed high antioxidant activity compared to that of the individual herb, and lower synergistic effect was also found for the mixture of tea, ginger, and tulsi (Kumar Gupta et al. 2014). Similar synergistic effect was observed in green tea using its mixture with grape seed, amla, pomegranate, Chinese cinnamon, and *Ginkgo biloba* (Jain et al. 2011). However, fruit tea can also have low antioxidant properties, and this can be caused by poor quality of tea used to manufacture the final product. Examples were presented in the literature including tea with guava and lychee, quince, and mixed fruit which were purchased as commercial tea-bags (Jeszka-Skowron and Zgoła-Grześkowiak 2014). Also, it must be taken into account that some fruit teas containing high amount of total phenols have total flavonoids at lower level than pure green or black teas (Veljković et al. 2013). On the other hand, antagonistic effects were observed when both sweeteners and milk were added to tea, which resulted in reduced antioxidant activity of both black and green teas (Korir et al. 2014).

Apart from tea produced from the *Camelia sinensis* leaves, there are some other brews commonly known as teas. These include rooibos, yerba mate, and lapacho. Rooibos is obtained from fermented leaves of the *Aspalathus linearis* shrub that grows natively in South Africa. It is also known as bush tea, red tea, or redbush tea. This herbal brew gained popularity because it is caffeine-free and rich in phenolic compounds. However, it contains considerably lower amounts of catechins that are typical of traditional tea (Jeszka-Skowron et al. 2018; McKay and Blumberg 2007). Rooibos is traditionally consumed in its oxidized form, known as fermented (Joubert and Schultz 2006). To obtain oxidized rooibos, the harvested plant tops are allowed to wilt, machine-cut, spread on concrete platforms and sprayed with water, bruised to activate enzymes, wetted and stacked in heaps, and covered with bags. During 8–24 h the heaps are frequently turned until the color changes from green to brown (Morton 1983). The main phenolic compounds found in rooibos are glucosides of luteolin, quercetin, and apigenin, reaching up to a few milligrams per gram in oxidized rooibos (Joubert and de Beer 2011; Stander et al. 2017). However, amounts of these compounds differ considerably between plants, depending on their growing location and origin—the cultivated *Aspalathus linearis* may contain different main phenolic compounds than the wild one (Joubert and Schultz 2006; Stander et al. 2017). Similarly, to tea, there are some differences between the oxidized and green product. The concentrations of the main phenolic compounds belonging to dihydrochalcones, flavones, and flavonols decrease after oxidation. Amount of aspalathin drops from 25.59 to 4.21 mg g^{-1} dry mass, orientin from 2.63 to 2.06 mg g^{-1} dry mass, isoorientin from 4.50 to 3.29 mg g^{-1} dry mass, and rutin from 2.15 to 1.73 mg g^{-1} dry mass (Joubert and de Beer 2011).

Yerba mate is a traditional tea prepared from leaves and stems of *Ilex paraguariensis* shrub from the *Aquifoliaceae* family. The plant is native to South America, and the yerba mate brew is consumed mainly in Argentina, Paraguay, Uruguay, and Brazil. However, in recent years, it is gaining popularity in the USA and Europe due to its health-promoting effects including antioxidant, anti-inflammation, DNA-protecting, and other properties. These benefits are attributed to the presence of such compounds as methylxanthines, phenolic compounds, and saponins (Burris et al.

2012; Heck and de Mejia 2007). The yerba mate brew is prepared by using much greater amount of leaves and stems in proportion to water than in traditional green and black teas, and it is also infused many times. In South America, it is often served with the addition of different herbs. Therefore, it is very difficult to assess the amount of active compounds ingested by its consumers. The main active compounds found in yerba mate include saponins, chlorogenic acid, dicaffeoylquinic acids (4,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 3,4-dicaffeoylquinic acid), caffeic acid, gallic acid, gallocatechin, caffeine, and theobromine (Bremer Boaventura et al. 2013; Burris et al. 2012; Colpo et al. 2016). The amount of these compound found in the brews are strongly dependent on the amount of infusions prepared and also on the origin of the product. The brews prepared from Brazilian herbs contain considerably less chlorogenic acid, caffeic acid, caffeine, and theobromine than those prepared from Argentinean and Uruguayan herbs. The reason is attributed to the fact that the Brazilian brands contain more twigs (Colpo et al. 2016).

Red lapacho tea is a decoction prepared from the inner bark and heartwood of the *Tabebuia impetiginosa* tree or several related species (Lübeck 1999; Taylor 2005). *Tabebuia impetiginosa* is an evergreen canopy tree with pink or purple flowers, growing not only in the Amazon rainforest but also in other tropical regions of South and Latin America. Lapacho, also known as pau d'arco, tahuari, or Inca tea, is widely used in traditional medicine in South America. It has gained wide publicity due to its anticancer properties. Studies demonstrated *Tabebuia impetiginosa* activity against several cancer lines, including prostate, breast, cervical, and other types. Modern scientific research confirms also many of the traditionally recognized properties, such as antimicrobial, antifungal, antiviral, and anti-inflammatory (Gómez Castellanos et al. 2009). Red lapacho tea has also been registered as a dietary supplement, that is, a "herb used to alleviate conditions and symptoms of cancer" by the US Food and Drug Administration (FDA 1999). The chemical composition of lapacho has been studied extensively, and numerous compounds have been identified, including naphthoquinones (lapachol, α-lapachone, β-lapachone, and dehydro-α-lapachon), anthraquinones (1-hydroxyanthraquinone, 1-methoxyanthraquinone, 2-methylanthraquinone, and 2-acetoxymethylanthraquinone), furanonaphthoquinones, iridoids, and benzoic acids (Gómez Castellanos et al. 2009; Pires et al. 2015; Steinert et al. 1995).

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Chapter 4
Methylxanthines in Food Products

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Abstract This chapter covers methylxanthines, with a focus on caffeine, theophylline, and theobromine. The key topics of the chapter cover the chemical structure of methylxanthines, their potential in nutrition, and a broad spectrum of biological functions for pharmacological applications. The content of methylxanthines in food and their bioavailability are also discussed, as well as growing applications in functional food products and dietary supplements. The final section covers briefly the current analytical methodologies and techniques applied in the analysis of methylxanthines.

Introduction

Methylxanthines are very often and carefully studied compounds, which reflect the prevalence of high-frequency consumption in today's society. Methyl derivatives of xanthine are biosynthesized by many botanical species to protect themselves against insects and pathogens (Frischknecht et al. 1986; Kim et al. 2010; Monteiro et al. 2016; Sledz et al. 2015). Humans, however, have found that consuming methylxanthines brings a variety of health benefits, including mild stimulation on various organ systems such as central nervous and cardiovascular systems, and skeletal muscles (Table 4.1). The most popular food products containing methylxanthines are brewed coffee and tea, kola nuts beverages, and chocolate products. Nevertheless, other products are rapidly gaining popularity and therefore deserve special attention, i.e., energy drinks and dietary supplements containing high levels of methylxanthines.

The most relevant methylxanthines available from natural sources are caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine). They are produced by many plants, e.g., coffee (*Coffea*

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Table 4.1 Relative pharmacological potency of the methylxanthines

Methylxanthine	Caffeine	Theophylline	Theobromine
Central nervous system stimulation	✓✓✓	✓✓	✓
Respiratory stimulation	✓✓✓	✓✓	✓
Diuresis	✓	✓✓✓	✓✓
Coronary dilatation	✓	✓✓✓	✓✓
Cardiac stimulation	✓	✓✓✓	✓✓
Skeletal muscle stimulation	✓✓✓	✓✓	✓

Table adapted from Beale Jr (2011)
✓- weak potency; ✓✓- moderate potency; ✓✓✓- strong potency

sp.), tea (*Camellia sinensis* L.), and cacao (*Theobroma cacao* L.). It should be noted that another methylxanthine, that is not produced by plants but is a byproduct of caffeine metabolism in humans is paraxanthin (1,7-dimethylxanthine) (Cano-Marquina et al. 2013; Nehlig 2016; Orru et al. 2013; Zhang et al. 2019).

The investigations on the electric dipole moments confirmed the polarity of methylxanthines, among which theobromine is the most polar molecule (Salihović et al. 2014). It was confirmed that caffeine is a lipophilic molecule and therefore can easily diffuse through the cell membranes and blood–brain barrier. Methylxanthines belong to heterocyclic organic compounds containing coupled rings of pyrimidinedione and imidazole, thus constituting methylated xanthine derivatives (Talik et al. 2012). The differences in the structure of methylxanthines are based on different numbers and spatial location of N-CH_3 groups (Fig. 4.1).

Methylxanthine properties are related to the imino nitrogen at position 9. Theophylline has no methyl group at position 7 unlike the other methylxanthines, containing instead at that position a proton that can be donated. Whereas caffeine has electrophilic sites at positions 1, 3, and 7, theophylline possesses the same electrophilic predisposition (Beale Jr 2011; Monteiro et al. 2016). In consequence, even if both compounds are donors of the electron pair, only theophylline is a proton donor in most pharmaceutical systems. Caffeine differs from theobromine with a methyl group at position 1, which is enough to give caffeine various physicochemical properties that have been found to cause a significant increase in some physiological effects (Gardenhire 2016). Chemical structures of individual methylxanthines are presented in Fig. 4.1.

Methylxanthines are adenosine receptor antagonists in the brain which increases arousal, mood, and concentration levels (Chen and Chern 2011). Those substances are adsorbed in the gastrointestinal tract and further enter the central nervous system, exerting psychostimulant actions, which are more evident at high levels of intake (Onatibia-Astibia et al. 2017). Considering the toxicity of methylxanthines in humans, it is relatively low; however, a reasonable consumption introduces some health benefits. Their consumption has some side effects, depending on the dose of the substance, such as, e.g., cardiovascular and metabolic effects, bronchial relaxation, diuretic effect, and increased secretion of gastric acids (Monteiro et al. 2019).

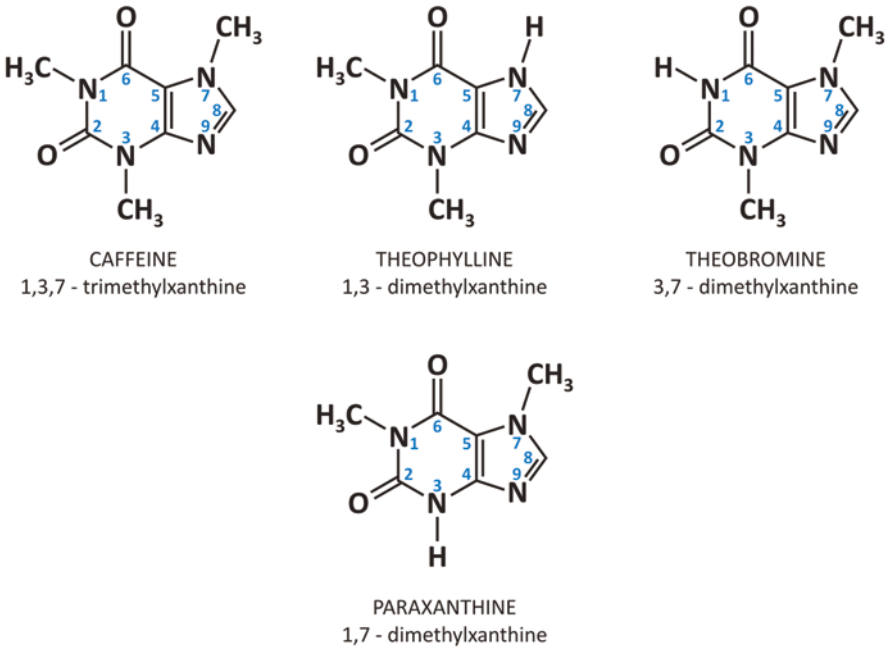

Fig. 4.1 Chemical structures of methylxanthines

Sources of Methylxanthines

Methylxanthines are present in plants and food products daily ingested worldwide (Table 4.2). The principal sources of caffeine are globally consumed coffee and tea, which can contain considerable and equal amounts of caffeine. Although caffeine content in different types of tea leaves depends on its production techniques and leaves maturity, it is generally found that the more oxidized types of tea have increased caffeine content (Gramza-Michalowska 2014). Pedan and others examined the fingerprint of bioactive components in teas and found that the fermentation process did not affect the contents of methylxanthines (Pedan et al. 2018). Analyzed pu-erh tea contained caffeine (30.8 mg g^{-1}) and theobromine (1.2 mg g^{-1}) regardless of tea maturity (young and ripened pu-erh tea).

Theobromine is the predominant methylxanthine found in cocoa beans, and some *Camellia* species (Ashihara et al. 2008; Fang et al. 2019; Franco et al. 2013; Jalil and Ismail 2008). It constitutes about 4% on a fat-free basis in cocoa, while the caffeine content is about 0.2%, and theophylline is present in trace amounts. Methylxanthine's content depends on the bean type and the fermentation process applied (Arunkumar and Jegadeeswari 2019; Todorovic et al. 2015). The unfermented cacao beans contain theobromine bound to tannins, which are hydrolyzed by acetic acid during the fermentation process, and theobromine is released in order to be diffused into the cacao shell (do Carmo Brito et al. 2017). Dark chocolate is

Table 4.2 Methylxanthines content in food and drinks

Product		Caffeine	Theobromine	Theophylline
Cacao powder (mg kg^{-1})		489–2400[a]	4621–26000[a]	b.l.–n.d.[a]
Milk chocolate (mg kg^{-1})		56[a]; 0.5–1.7[b]	1004[a]; 0.9–2.8[b]	b.l.[a]; 50[b]
Dark chocolate (mg kg^{-1})		625–875[a]; 2.2–3.1[b]	5000–7500[a]; 2.6–11.6[b]	n.d.[a]; 0–90[b]
Tea leaves (mg g^{-1})	Green	34.86[c]; 20–60[d]	1.29[c]	n.d.[c]
	White	27.17[c]; 10–50[d]	0.52[c]	n.d.[c]
	Yellow	24.49–33.32[c]	0.91[c]	n.d.[c]
	Oolong	19.67[c]; 20–60[d]	0.37[c]	n.d.[c]
	Black	28.54[c]; 20–60[d]	1.06[c]	n.d.[c]
	Dark	31.78[c]	1.42[c]	n.d.[c]
Tea leaves brew (mg 200–235 mL^{-1})	Green	31.0 (20.2-–42.8 range)[b]	0.2–0.7[b]	n.d.[b]
	Black	55.7 (41.6–71.2 range)[b]	1.8–3.6[b]	<1.0[b]
Coffee brew (mg 200–235 mL^{-1})	Espresso	140 (51–532 range)[b]	b.l.[b]	b.l.[b]
	Decaffeinated	3.0–15.8 range[b]	b.l.[b]	b.l.[b]
Soft drinks commercial (mg 100 mL^{-1})	Pepsi	10.7	n.d.	n.d.
	Coca-Cola	9.6	n.d.	n.d.
	Mountain Dew	15.2	n.d.	n.d.
Energy drinks commercial (mg 100 mL^{-1})	Red Bull	32	n.d.	n.d.
	Monster Energy	33.8	n.d.	n.d.

Soft and energy drinks methylxanthines content were set according to manufacturer's label specification

n.d. not determined, *b.l.* below the limit of detection

[a]Franco et al. (2013)

[b]Monteiro et al. (2016)

[c]Zhang et al. (2019)

[d]Fang et al. (2019)

high in theobromine and caffeine due to the addition of a greater amount of cocoa solids than that of other chocolates (Barišić et al. 2019). The amount of theobromine is higher in cocoa and cocoa products, compared to caffeine, which dominates in coffee and tea. Saeed and others presented results indicating that methylxanthines are cumulative, or even synergic, with other compounds (Malongane et al. 2017; Saeed et al. 2017; Zheng et al. 2004). It was found that tea catechins, or flavan-3-ols and L-theanine are the most bioactive components in tea next to methylxanthines.

Theophylline is also naturally present in plants; however, its content is rather vestigial (Barnes 2013). Although tea leaves, coffee beans, and cacao are the well-known plants containing methylxanthines, there are other sources such as guarana (*Paullinia* sp.), cola nuts (*Cola* sp.), and mate tea (*Ilex paraguariensis*) (Carrageta et al. 2018; dePaula and Farah 2019; Klein et al. 2015; Sosa et al. 2010). The seeds of guarana plant (*Paullinia cupana*) contain theobromine, theophylline, and a

significant amount of caffeine, with 40 mg of caffeine being equivalent to 1 g of guarana (Heckman et al. 2010). It has become a popular natural additive in energy drinks, as a result of its stimulatory effect and slower release rate compared to caffeine due to water insolubility and the presence of saponins and tannins (Scholey and Haskell 2008). Another plant commonly incorporated into energy drink formulations is yerba mate (*Ilex paraguariensis*), a strong central nervous system stimulant due to its high caffeine concentration. It was found that caffeine concentration in 240 mL of yerba mate beverage is equivalent to 78 mg, and is comparable to commercial energy drink (Heckman et al. 2010). Yerba mate contains a high amount of phytochemicals possessing various health benefits. It was stated that yerba mate shows antioxidant, anti-inflammatory and antidiabetic activity; cytotoxicity to cancer cells; supports obesity management; and improves the serum lipid parameters (de Morais et al. 2009; Heck and de Mejia 2007; Martins et al. 2009; Pang and Choi 2008).

Over the last few decades, the addition of functional ingredients into popular beverages and drinks became a very important element of the manufacturers' marketing strategy. Energy drinks contain a combination of calories, with sugar as a source of rapid energy, caffeine, as an active ingredient due to its stimulatory effect on the central nervous system, and other ingredients such as plant extracts, taurine, and B vitamins (Heckman et al. 2010; Nazir et al. 2019). Therefore, the energy drinks have become particularly popular with added caffeine contents ranging from 85 to 1200 mg L^{-1} (Corbo et al. 2014; Reissig et al. 2009). Energy drinks are sources of substances that reduce mental fatigue, increase physical endurance, reaction time, and improve mood. Despite this, however, concerns have been raised over their safety, considering its intake and the combination with other products, e.g., alcohol (O'Brien et al. 2008).

Pharmacology of Methylxanthines

Methylxanthines are pharmacologically active substances that have been consumed by humans for centuries. They display numerous biological activities that could be beneficial for the treatment of different disease conditions. Numerous reviews reveal the convergence that methylxanthines exhibit a stronger pro-cognitive effect when their natural composition of phytonutrients is maintained, and better in the plants compared to the processed product (McLellan et al. 2016; Schuster and Mitchell 2019). Studies have shown that some of the phytochemicals enhance the neurocognitive function of caffeine alone. Research shows the psychostimulant power of caffeine, theobromine, theophylline, and their metabolites to combat central nervous system diseases. It was found that sustained consumption of methylxanthines leads to a reduced risk of neurodegenerative diseases involving cell death in the nervous system, such as Alzheimer's and Parkinson's disease (Onatibia-Astibia et al. 2017).

Caffeine after consumption is absorbed rapidly within approximately 45 min and the peak plasma concentrations are being reached 30 min following oral consumption, whereas the half-life of caffeine, meaning the time taken for the body to eliminate 50% of the caffeine content, is approximately 2.5–5 h (Arnaud 2011; Einöther and Giesbrecht 2013). In adults, the half-life of caffeine varies and depends on age, body mass, sex, physiological status, medical treatment, and liver health (Aguiar et al. 2016; Cano-Marquina et al. 2013). Once ingested, caffeine is rapidly absorbed from the gastrointestinal tract and undergoes demethylations, getting mostly metabolized into paraxanthine (78%), theobromine (14%), and theophylline (9%) (Arnaud 2011). The stimulatory effects begin a few minutes after ingestion and last several hours. Nevertheless, methylxanthines after ingestion are not expected to accumulate in organs or tissues and are posteriorly metabolized in the liver (Arnaud 2011).

Due to caffeine lipophilic profile, it is capable of crossing the blood–brain barrier and blocking adenosine receptors, acting as a phosphodiesterase inhibitor, affecting the regulation of calcium levels and gamma-aminobutyric acid (GABA) neurotransmission (Schuster and Mitchell 2019). Caffeine exerts an antagonistic effect on the adenosine receptors (ARs) in the brain, through the structural similarity to adenosine, allowing binding to all adenosine receptors (Huang et al. 2005). Caffeine also increases the secretion of epinephrine, leading to a beneficial effect on physical or mental performance (Graham 2001).

As reported by Smit, theobromine is absorbed slower than caffeine and peak plasma concentrations are reached after 150 min, still, it depends on its medium. Administration of theobromine ingested as or with chocolate would require 2 h to reach peak plasma concentration, and the clearance rate is approximately half of the caffeine clearance. Theobromine, so as caffeine gets distributed throughout the total body water (Smit 2011). Moreover, the studies on mice have found theobromine in the brain after ingestion, but these concentrations were only 10% of those in plasma (Yoneda et al. 2017).

Caffeine is usually present at the highest levels in foods and beverages, however, moderate daily caffeine consumption is not associated with adverse health effects in healthy adult populations (Monteiro et al. 2019). The recommendations indicate that a daily intake of caffeine above 500–600 mg brings a significant health risk and may, therefore, be regarded as overdose (Bioh et al. 2013). Considering the natural levels of caffeine present in foods and beverages, toxic effects only tend to appear upon an excessive consumption of those with high caffeine content, caffeine-enriched dietary supplements, or if combined with drugs (Pendleton et al. 2013; Sanchez 2017). Thus, moderate consumption of coffee is recommended for obtaining the most positive outcomes without negative effects. Principles for the labeling of energy drinks comply with UE Regulation 1169/2011 requiring additional labeling for beverages containing more than 150 mg L^{-1} of caffeine. Special product labeling is also required as follows: “High caffeine content. Not recommended for children or pregnant or breast-feeding women.” The wording must be placed where the name of the product appears, followed by a reference in brackets and to the caffeine content expressed in mg per 100 mL. It was proven that short-term adverse effects of caffeine consumption include issues related to the central nervous system,

including anxiety, interrupted sleep, or even cardiovascular problems. Other actions comprise diuresis and metabolism activation (Cano-Marquina et al. 2013).

Theophylline is a promising stimulant of the central nervous system, however, it is primarily used in chronic obstructive pulmonary disease, asthma, and as a diuretic (Barnes 2013; Salihović et al. 2014). More recently it has been shown to have anti-inflammatory effects in asthma and chronic obstructive pulmonary disease.

Theobromine is present at high levels in chocolate and cocoa-based food products (Jalil and Ismail 2008; Trognitz et al. 2013). It represents higher cardiac stimulant potential than caffeine; however, its effect on behavior and mood varies according to dose, but oral doses above 1000 mg of theobromine were found to have negative effects (Baggott et al. 2013). It was suggested that the psychological effects of chocolate might be due to theobromine interactions with caffeine and probably polyphenols (Barišić et al. 2019; Franco et al. 2013). Theobromine among other methylxanthines has a very low influence on the central nervous system, probably due to poor physicochemical properties necessary for distribution in that system (Beale Jr 2011). Results of Eteng et al. showed that theobromine-rich cocoa powder induces weight loss and changes in the lipid profile of obese rats (Eteng et al. 2006). Other results suggested that theobromine inhibits adipocyte differentiation during the early stages of adipogenesis (Jang et al. 2015).

Determining human exposure to methylxanthines is complex and may not be accurate due to the assessment of their content in coffee or tea beverages and chocolate because the results can vary significantly with the origin and processing techniques. The commercial products are labeled for the content of individual ingredients, and the results indicate that values are fairly consistent. Sanchez found that none of the foods evaluated reached the recommended daily intake limit of 400 mg of caffeine with a single dose (Sanchez 2017). This limit however could be reached with approximately five doses of coffees and energy drinks. Although caffeine has a long history of safe use and promising scientific evidence it should be ingested in a moderate amount (300–400 mg d^{-1} $adult^{-1}$), and then no adverse effects should occur.

Methylxanthines Intake and Human Health

Numerous fruit, vegetables, and medicinal plants have been used in different anti-obesity products as food supplements to support weight loss (Stuby et al. 2019). Methylxanthines became a group of naturally occurring molecules with growing potential in the management of obesity. Research is mainly focused on their nutraceutical properties and the ability to support fat depletion through the regulation of adipogenesis and lipolysis in the adipose tissue (Carrageta et al. 2018). Caffeine acts as a brain cortex stimulant, facilitates awareness and wakefulness, improves clear thinking, promotes the ability to focus on the given task, reduces fatigue, and enhances the exercise activities relying on explosive strength and high-intensity exercise (Pickering and Grgic 2019). Furthermore, when caffeine consumption is associated with physical exercise, the effects are impressive. Results of Schubert

and others showed that lean individuals taking 3 mg kg^{-1} of caffeine 90 min before, and 30 min after performing physical activity increased the energy expenditure and fat loss (Schubert et al. 2014). Moreover, the exercise was perceived as less difficult and more enjoyable. Currently, the effect of caffeine on increasing performance in many sports and exercises is perfectly established by the results of metaanalysis (Raya-González et al. 2020).

General guidelines recommend the consumption of 3–6 mg of caffeine per 1 kg, usually an hour before the start of exercise; however, it must be noticed that there are variations in response to such a standardized protocol, e.g., genetic variation, muscular abilities, such as strength, training status, time of day, sex, or habitual caffeine consumption (Goldstein et al. 2010; Grgic and Pickering 2019; Pickering and Kiely 2018; Shen et al. 2019; Southward et al. 2018). Research indicated that the potential expected effects of caffeine were found, suggesting the psychological effect of caffeine consumption. It was suggested that if the individuals believe they have consumed caffeine, perceived as ergogenic, they are likely to experience the performance profits, even though caffeine was not consumed (Saunders et al. 2017; Shabir et al. 2018). It should be underlined that there are numerous nuances of caffeine and performance interactions, which remain incomplete and need further research.

Davoodi and others have demonstrated that caffeine improves weight loss in individuals with a caloric restriction diet, and prevents weight regain (Davoodi et al. 2014). The molecular activity of methylxanthines leads to the stimulation of lipolysis and inhibition of adipogenesis, by disturbing the adipocyte signaling and inhibiting the main adipogenic transcription factors, simultaneously decreasing the extent of the adipose tissue (Carrageta et al. 2018). Methylxanthines play a part in antagonizing the adenosine receptors, stimulation of neural catecholamines release, inhibition of phosphodiesterases, and decreasing the expression of important transcription factors. Methylxanthines are also phosphodiesterases inhibitors with a promising therapeutic anticancer activity against glioblastoma, regulating various cellular processes related to cancer progression (Pérez-Pérez et al. 2019). However, the mechanisms that govern these processes are not yet fully understood and further investigations are needed. The influence of caffeine on human health is summarized in Fig. 4.2.

The interactions of methylxanthines with other components, e.g., polyphenols are particularly interesting because of their conjunction in nature. These phytochemicals can contribute to the positive effects of methylxanthines in two ways: probable synergy with caffeine or theobromine, or might alleviate certain negative effects of caffeine (Schuster and Mitchell 2019). The combination of L-theanine and caffeine is among the most daily-consumed beverages as tea infusions. Results of many researchers concluded that the combinations of caffeine and L-theanine could increase subjective alertness, attention switching accuracy, and unisensory visual and auditory attention in healthy subjects (Camfield et al. 2013; Kahathuduwa et al. 2017). It was found that the overall effect of L-theanine on attentional performance is to modify caffeine's activity; moreover, it could also cancel out caffeine's

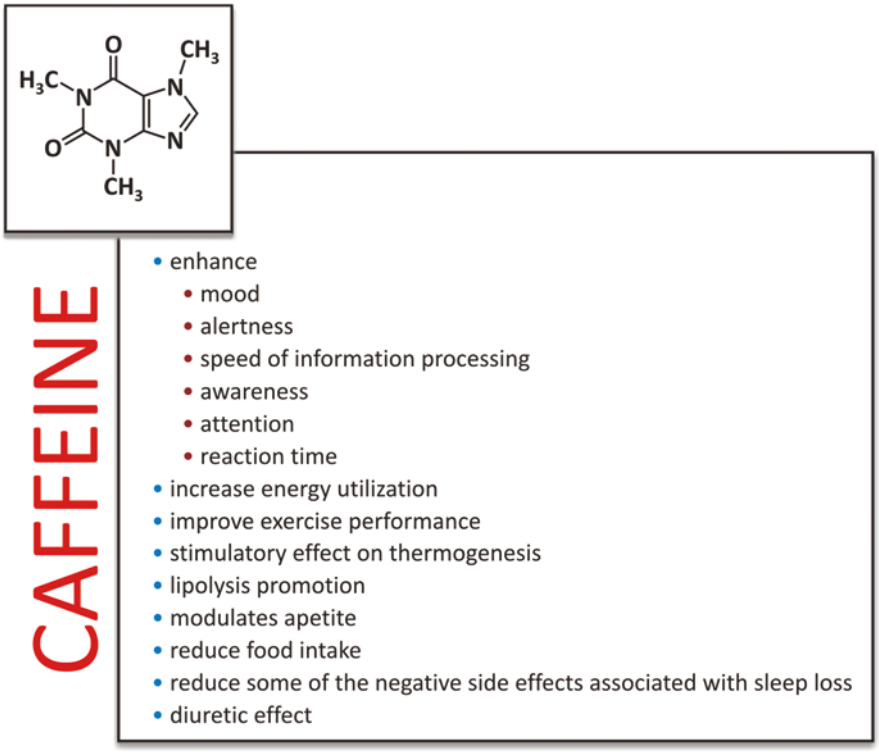

Fig. 4.2 Influence of caffeine on human health

efficacy (Foxe et al. 2012). Results of Giles and others found that caffeine and L-theanine caused adverse effects on attention under emotional arousal (Giles et al. 2017). Studies examining the effect of caffeine and L-theanine combined as an equivalent to 1–2 cups of tea found the elimination of the vasoconstrictive effect and behavioral effects of caffeine (Dodd et al. 2015).

Theobromine has significantly weaker arousing properties in comparison to caffeine, probably due to lower affinity to adenosine receptor and therefore lower blood–brain barrier permeability (Baggott et al. 2013). It is mainly associated with peripheral effects, associated with an increased heart rate, a decrease in blood pressure, self-reported calmness, and reduced reaction time at a dose of 700 mg theobromine (Beaudoin and Graham 2011; Mitchell et al. 2011). However, Smit and others found that the combination of theobromine and caffeine exerted effects on reaction time and attention at very low doses of caffeine, suggesting that theobromine increases selectively caffeine psychoactivity (Smit et al. 2004). Previously outlined research of Yoneda and others suggested that isolated doses of caffeine and theobromine exert differential effects than its preparations from plants providing many other phytochemicals as well (Yoneda et al. 2017). Considering theobromine there is no evidence that it has an effect on attention by itself (Judelson et al. 2013).

However, the study by Boolani and others found that consumption of caffeinated cocoa, containing 70 mg of caffeine, 179 mg of theobromine, and 499 mg flavanols, resulted in rarer omission errors and better accuracy in healthy subjects compared to cocoa alone (Boolani et al. 2017). A recent prospective cohort study showed a significant protective effect of chocolate intake on a lower risk of cognitive decline (Moreira et al. 2016). It was also found that a combination of cocoa extract and caffeine attenuated the anxiety-provoking effects of caffeine alone. The influence of methylxanthines on human health is summarized in Fig. 4.3. Marsh and others conducted research on postmenopausal women and found that consumption of dark chocolate resulted in improvement of vascular endothelial function and reduction of

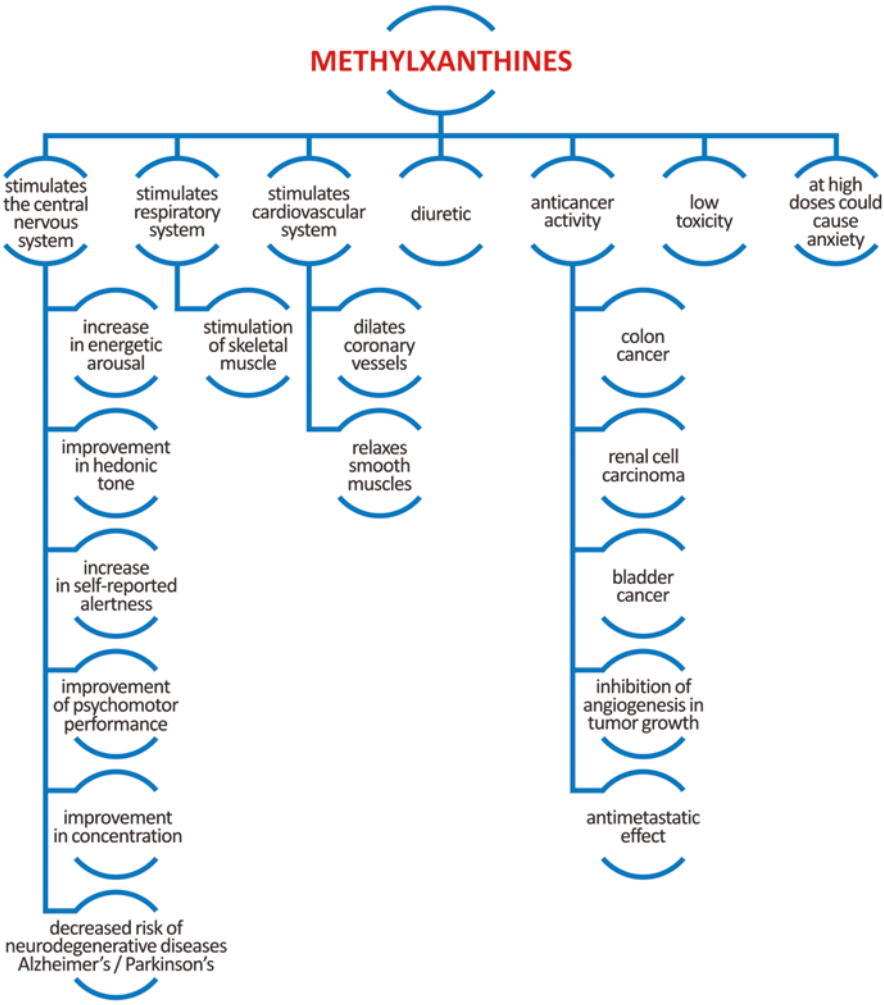

Fig. 4.3 Influence of methylxanthines on human health

cerebral blood flow responses, however, no parallel changes in cognitive performance were reported (Marsh et al. 2017). Methylxanthines may also increase the positive cardiovascular effects of cocoa flavanols via accelerated absorption (Sansone et al. 2017).

Caffeine is well known for its antimicrobial properties against human pathogens like the gram-positive bacteria: *Staphylococcus aureus*, *Bacillus cereus,* and gram-negative bacteria as *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* (Mohammed and Al-Bayati 2009). Therefore it could be used in foods as a natural preservative to control their growth. It was also confirmed by the research of Sledz and others, who indicated caffeine as a potential tool for the control of diseases caused by plant-pathogenic bacteria (Sledz et al. 2015).

The conclusions that can be drawn from latest results show that consumption of methylxanthines and polyphenol in, e.g., coffee, tea, cocoa, is safe and correlates with a reduced risk of mortality and certain illnesses (Arab et al. 2013; Feng et al. 2016; Magrone et al. 2017; Poole et al. 2017). It was found that coffee intake decrease mortality, the risk of Parkinson's disease, depression, and could protect against cognitive decline. Research of Panza suggested that gender appears to play a considerable role in the beneficial effects of caffeine, which are stronger in women (Panza et al. 2015).

Methylxanthines: Extraction and Determination Methods

There are many ways to prepare samples for the determination of methylxanthines. Their use varies depending on the type of sample: food and drinks (e.g., chocolate, cocoa, guarana, coffee, tea, mate, soft drinks, energetic drinks), biological fluids (e.g., blood serum and plasma, whole blood, saliva, gastric contents, sweat, urine, breast milk). Isolation of methylxanthines for the analysis is carried out by various extraction methods including solid-phase extraction (SPE), liquid–liquid extraction (LLE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), or solid-phase microextraction (SPME) (Andreeva et al. 2012; Monteiro et al. 2016; Sanchez 2017). The solvents used for extraction are both inorganic and organic including water, aqueous sulfuric acid, acetate buffer, borate buffer, ethyl acetate, methanol, aqueous methanol, ethanol, aqueous ethanol, and aqueous acetonitrile. Water is considered a good solvent for methylxanthines, but with low selectivity, therefore in the next step(s) additional extraction with other solvents is used, e.g., with chloroform, dichloromethane. Lipophilic solvents such as chloroform, dichloromethane, petroleum ether, and hexane are also used to degrease the sample.

In supercritical fluid extraction, supercritical carbon dioxide is used as a solvent, which, due to its low polarity, is used with the addition of polar solvents: water, ethanol, methanol, or isopropanol, increasing the extraction efficiency (Andreeva et al. 2012; Monteiro et al. 2016, 2019; Talik et al. 2012).

The most common method for the analysis of methylxanthines is high-performance liquid chromatography (HPLC) with the reversed-phase separation (RP) on a C18 column. More precise identification of the tested compounds is achieved by additional application of mass spectrometry (MS). Other methods used for the determination of methylxanthines are capillary gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), thin-layer chromatography (TLC), spectrophotometry, spectrofluorimetry, Fourier transform-Raman spectrometry, voltammetry (VA), radioimmunoassay (RIA), ion-exchange chromatography (IES), capillary electrophoresis, micellar electrokinetic chromatography (MEKC), and solid-phase ultraviolet sensing (Monteiro et al. 2016).

Research continues to develop new methods to ensure efficient separation, identification, and quantification of methylxanthines. Kaltbach et al. (2020) used high-performance thin-layer chromatography-mass spectrometry (HPTLC-MS) for the determination of bioactive ingredients (including caffeine and theobromine) from mate tea (*Ilex paraguariensis*). It seems that current research on the development of new methods will be focused on the search for easy, fast, noninvasive/minimally invasive and cheap to conduct analysis methods. Rapid methods for obtaining analytical results comparable to HPLC can be used in industry for online quality control and safety evaluation.

Bartella et al. (2019) developed a rapid method using paper spray tandem mass spectrometry (PS-MS/MS) to determine theobromine, theophylline, and caffeine in different types of food. The advantage of this method was the ability to generate ions for MS analysis from the raw samples. Quelal-Vásconez et al. (2020) described a method based on reflectance near-infrared spectroscopy (NIRS) as reliable, non-destructive, and fast for the routine assessment of theobromine and caffeine in the cocoa. Front-face fluorescence spectroscopy was proposed by Tan et al. (2019) as an easy and fast nondestructive method to determine theobromine and caffeine in chocolate.

Summary

The prevalence of noncommunicable diseases could be prevented by an adequate application of plant components and lifestyle improvement. Recent studies confirm that methylxanthines influence human health offering a wide spectrum of health-promoting properties. The research discussed in the chapter indicates that moderate and regular consumption of products rich in methylxanthines may contribute to the inhibition of a wide range of diseases development.

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Chapter 5
Carotenoids as Food Products Components and Health Promoting Agents

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Abstract This chapter covers carotenoids, a class of over 700 lipid-soluble bioactive compounds. The key topics of the chapter are carotenoids chemical structure, potential in nutrition, and a broad spectrum of biological functions for health benefits and therapeutic applications. The properties of lycopene β-carotene, astaxanthin, lutein, and zeaxanthin are discussed in detail. The content of carotenoids in food and their bioavailability are also discussed, as well as the growing interest of food designers in the development of functional food products and dietary supplements. The final section covers briefly the current analytical techniques and methodologies used in the analysis of carotenoids in foods and human samples.

Introduction

Carotenoids are a class of over 700 lipid-soluble compounds with coloring power and wide application in the food industry (Zakynthinos and Varzakas 2016). Carotenoids occur widely in nature; however, they are also chemically synthesized by the industry (Böhm 2019; Mezzomo and Ferreira 2016). Considering a consumer preference for natural compounds and clean label, there is a global trend in the production of foods containing natural ingredients, especially that the results of many studies indicate a relationship between increased consumption of food rich in carotenoids and lowering the risk of various diseases (Eldahshan and Singab 2013; Gryszczyńska et al. 2011; Juturu and Gormley 2013; Kulczyński et al. 2017; Rao and Rao 2007).

Due to the coloring properties of carotenoids, they are extensively used as colorants and also for food fortification due to possible activity as provitamin A and their biological functions for health benefits. Among the best-known properties are the

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following: anticancer, antiobesity, hypolipidemic, antioxidant properties or improvement of immunity response, and protective role in conditions, such as diabetes (Chisté et al. 2014; Sandmann 2019; Tanumihardjo 2013). Humans cannot synthesize carotenoids, thus they have to be supplied with food. Since carotenoids are produced only by photosynthetic organisms, e.g., algae, certain microorganisms and fungi, fruits, and vegetables are still their best source (Gong and Bassi 2016; Kandlakunta et al. 2008; Matos et al. 2017; Maiani et al. 2009; Rao and Rao 2007; Santana and Macedo 2019). Contents of individual carotenoids in selected plant origin food are given in Table 5.1.

Carotenoids are lipophilic water-insoluble compounds, however soluble in solvents such as chloroform, acetone, or alcohol. They are pigments synthesized by plants, algae, and microorganisms, characterized by yellow, orange, and red color, widely distributed in nature with broad utility in the food industry (Saini et al. 2018). The protection of the photosynthesis process from damage under light conditions is the main function of carotenoids (Young and Lowe 2001). Carotenoids are used as restoring colorants or to standardize the food products' color (e.g., fruit products, sweets, margarine). Each carotenoid is characterized by a particular light spectrum absorption. The chromophore group is the structure responsible for light absorption, which in carotenoids is characterized by conjugated double bonds. This group of fat-soluble pigments, found in animal and plant kingdoms, is classified to polyisoprenoids, containing 8 isoprene units composed of 40 carbon atoms, occurring in acyclic, monocyclic, or bicyclic forms (Baranska et al. 2006; Igielska-Kalwat et al. 2015). Due to the chemical structure carotenoids are found in nature in two groups of carotenes and xanthophylls. The carotenoids consist of linear hydrocarbons that can be cyclized at one end or both ends of the molecule (β-carotene), while the xanthophylls are oxygenated derivatives of carotenes such as lutein, astaxanthin, zeaxanthin, and β-cryptoxanthin (Britton and Khachik 2009; Eldahshan and Singab 2013; Gryszczyńska et al. 2011). The chemical structure of selected carotenes and xanthophylls are presented in Figs. 5.1 and 5.2.

Carotenoids occur in the form of acyclic, monocyclic, and bicyclic compounds, and are synthesized in the plastids of plants. Carotenoids also include apocarotenoids, i.e., compounds containing less than 40 carbon atoms, but retaining the central fragment of the carotene molecule with 4 methyl groups, e.g., crocetin. Their main precursor is a 5-carbon isopentenyl diphosphate molecule, which further transformations lead to a direct precursor of carotenoids – phytoene, which contains 40 carbon atoms and 9 double bonds in the trans configuration (Rodriguez-Concepcion et al. 2018). The most valuable biological property of carotenoids is their provitamin activity. However, vitamin A activity is only shown by the compounds having in their molecule a fragment with a retinol-like structure, i.e., β-ionone (e.g., β-carotene with two β-ionone rings). Main dietary sources of vitamin A range include plant- and animal-based foods, fortified food, and dietary supplements. Vitamin A includes retinol, retinal, retinoic acid as well as the retinyl esters. The vital biological roles of vitamin A compounds include growth, development, and maintenance of epithelial tissues, and vision by participating in the regeneration of photoreceptors, immunology, and reproduction. The status of vitamin A

Table 5.1 Carotenoid contents of selected foods (mg 100 g^{-1} fresh weight)

Carotenoids					
Source	Lycopene	β-carotene	α-carotene	Lutein	Zeaxanthin
Apple	0.06–0.31	0.15	–	–	–
Apricot	0.54	0.58–1.37	0.04	0.12–0.19	0.04
Avocado	–	0.05–0.08	0.02–30	0.21–0.36	0.008–0.018
Banana	0.02–0.25	0.01–0.13	0.06–0.16	0.01–0.19	–
Fig	0.32	0.04	0.02	0.08	–
Grapes, green	–	0.04	n.d	0.05	0.01
Guava	0.77–1.82	0.10–2.67	–	–	–
Mango	0.01–0.72	0.11–1.20	–	–	–
Papaya	4.30–7.56	0.32–0.66	n.d	0.01–0.32	–
Pineapple	0.26–0.61	0.05–0.35	–	n.d	–
Watermelon, red	1.35–8.73	0.31–0.78	–	–	–
Broccoli	n.d	0.29–0.56	n.d	0.71–1.0	n.d
Brussel sprouts, frozen	n.d	0.22–0.29	n.d	0.38–0.70	n.d
Cabbage, green, frozen	n.d	2.37–3.36	n.d	4.25–7.04	n.d
Carrots	n.d	4.35–8.84	2.84–4.96	0.25–0.38	n.d
Chicory	–	3.53–3.63	–	5.37–5.70	–
Cress	–	3.69	–	7.54	–
Dill	n.d	5.45	0.09	13.82	
Egg plant	n.d	1.11	n.d	0.17	
Kale, cooked	–	4.69	0.87	8.88	n.d
Leek	n.d	1.34–3.19	n.d	3.68	
Lettuce	n.d	1.47	n.d	1.61	
Parsley	n.d	4.44–4.68	n.d	8.34–10.65	n.d
Peas	n.d	0.52	n.d	1.91	
Pepper, green	n.d	0.25 –0.27	n.d	0.54	
Pepper, hot	n.d	0.35	n.d	0.78	
Pepper, orange	–	1.02	0.10	0.21	1.67
Pepper, red	n.d	1.63	–	0.35	–
Pumpkin	0.50	0.07	n.d	0.07	
Radish sprouts	n.d	2.1	–	3.73	–
Arugula	–	2.84 –3.30	–	5.00–5.20	–
Scallion	n.d	2.99	–	4.85	–
Spinach	n.d	4.81–5.10	n.d	5.93–7.22	
Spring onion	n.d	1.28	n.d	0.32	–
Summer squash	0.046	0.47	n.d	2.26	
Sweet potatoes	–	7.83	–	0.05	–
Tomato juice	8.2–11.0	–	–	–	–
Tomato ketchup	4.71–100.87	0.13–1.11	n.d	n.d	n.d
Tomato mash	0.37–0.64	1.63–2.83	–	–	–
Tomato puree	53.36–138.60	0.40–2.80	–	–	–
Tomatoes	0.85–60.40	0.23–0.78	n.d	0.21	

(continued)

Table 5.1 (continued)

Carotenoids					
Source	Lycopene	β-carotene	α-carotene	Lutein	Zeaxanthin
Tomatoes, canned	8.48–11.82	0.22–0.28	n.d	n.d	n.d
Amaranth	n.d	3.3	n.d	13	–
Coriander leaves	n.d	3.17	n.d	1.14	–
Eggs	n.d	n.d	n.d	0.18	n.d

Results adapted from Kulczyński et al. (2017)
n.d, not detected

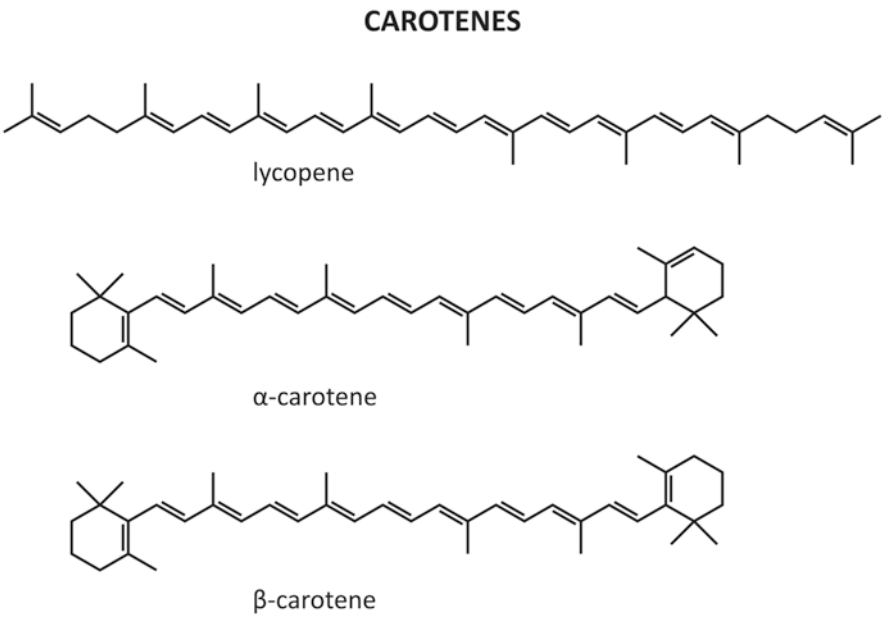

Fig. 5.1 Chemical structures of selected carotenes

is monitored in humans to prevent the occurrence of both subclinical deficiency and excess toxicity leading to various health symptoms (e.g., xerophthalmia, growth disorders, corneal ulcers, congenital malformation during pregnancy). Carotenoids bioconversion efficiency into vitamin A is diversified and depends on the body's requirements, and the source of pro-vitamin (Rodriguez-Concepcion et al. 2018).

The most important feature of carotenoids, the consequences of which are visible in the prevention and treatment of many diseases, are strong antioxidant properties based on quenching singlet oxygen and free radical scavenging. The biological activity of carotenoids is summarized in Fig. 5.3. Free radicals inhibition occurs based on three mechanisms. Hydrogen atom transfer leads to the formation of carotenoid cation radicals, or addition, which results in radical adduct formation. Carotenoids can act directly as antioxidants in biological systems through different

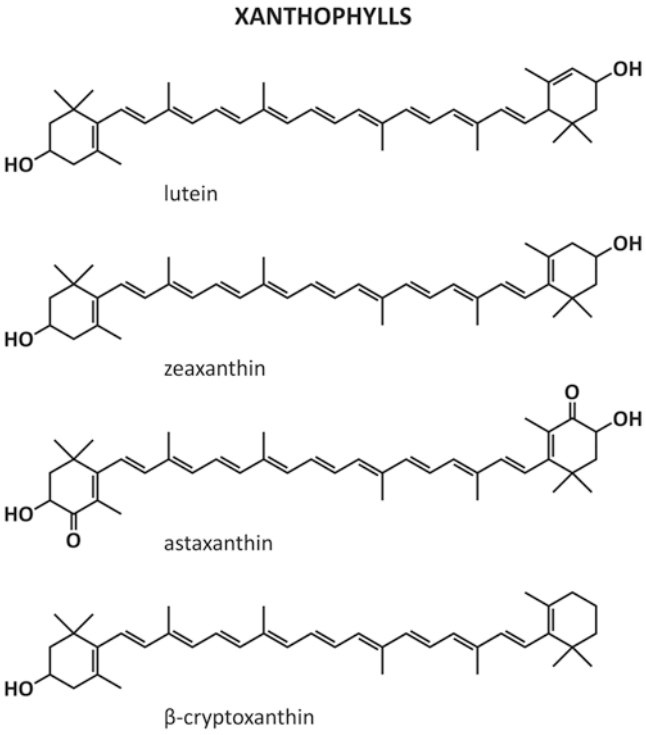

Fig. 5.2 Chemical structures of selected xanthophylls

mechanisms of action, e.g., neutralizing free radicals, preventing or reducing cell destruction caused by these compounds, or indirect involvement in the enzyme systems that have antioxidant activity (Maiani et al. 2009). There are numerous researches showing that carotenoids from both groups of vitamin A precursors and the non-precursors (e.g., lycopene, lutein, and zeaxanthin) protect cells against cancer (Hou and Cui 2018). Due to the high degree of carotenoids' unsaturation, the light, heat, acids, and enzymes are the main factors that cause their isomerization from the trans- form, which is the most stable, to the cis- form, promoting color loss and pro-vitamin activity. The carotenoid activity coefficient has been determined as follows: 1 retinol activity equivalent RAE equals 1 μg of retinol; 12 μg β-carotene; 24 μg β-cryptoxanthin or 24 μg α-carotene (Kulczyński et al. 2017; Tang 2010; Weber and Grune 2012). The β-carotene dioxygenase enzyme and molecular oxygen convert the β-carotene molecule into two retinol molecules, which in subsequent stages are reduced by the retinal reductase to retinal. Numerous factors influence the degree of β-carotene conversion, including the presence of lipids and antioxidants in the diet, the food matrix, the raw material processing technique, and individual variability, meaning that the enzyme conversion activity varies among individuals (Lintig et al. 2005; Nagao 2004; Tang 2010).

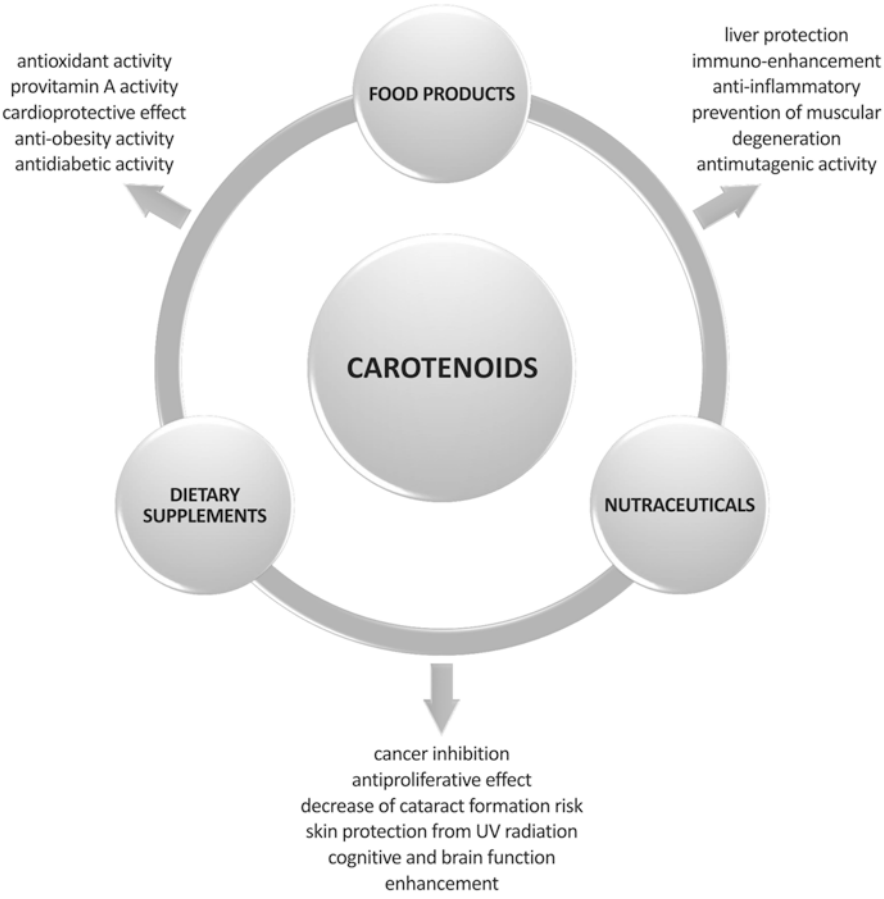

Fig. 5.3 Biological activity of carotenoids

The antioxidative properties of carotenoids allow them to prevent oxidative changes in the plasma lipoprotein structure thus limiting their proatherogenic properties. Carotenoids take part in the intensification of platelet aggregation, stimulation of leukocyte adhesion, and their transendothelial migration, reducing the nitrogen oxide bioavailability and increasing the growth factor expression leading to the reconstruction of blood vessel walls. Carotenoids as free radical scavengers inhibit the formation of advanced oxidation protein products, the amount of which is associated with increased common carotid artery intima-media thickness (CCA-IMT) (Bryk et al. 2017; Fiedor and Burda 2014; Piwowar 2014; Touyz and Briones 2011; Wójcicka et al. 2004).

There are numerous bioactive compounds responsible for neuroprotection, e.g., phenolics, cannabinoids, and also carotenoids. However, there are limiting factors that restrict the health benefits of plant-based bioactive compounds, including low stability in environmental conditions, which implies lower bioavailability,

permeability, and higher degradation during digestion processes (Ahmad et al. 2019; Boon et al. 2010; Priyadarshani 2017; Xavier and Mercadante 2019).

Lycopene

Lycopene is an unsaturated aliphatic hydrocarbon, composed of 40 carbon atoms, containing 11 conjugated and 2 unconjugated double bonds, and having an acyclic structure. It belongs to the group of carotenes and does not exhibit the activity of provitamin A (Kawata et al. 2018). As a natural pigment, lycopene is found in a large amount in fruit and vegetables. The main sources of lycopene are tomatoes and their products, such as juices, pulps, concentrates, or sauces, which provide a predominant amount with the diet. Significantly lower quantities of lycopene deliver pepper, carrot, apricot, or watermelon (Holden et al. 1999; Igielska-Kalwat et al. 2015; Palozza et al. 2012; Skiepko et al. 2015).

Estimations indicate that only 5–7 mg of lycopene is consumed with the Western diet daily, of which only 10–30% is absorbed in the body (Palozza et al. 2012; Petyaev 2016). Lycopene of natural origin is found mainly in the all-trans configuration (Palozza et al. 2012). Research showed that high temperature or light causes the isomerization of double bonds from the all-trans position to mono- or poly-cis isomers, which results in higher availability of lycopene (Belter et al. 2011; Igielska-Kalwat et al. 2015). When consumed, lycopene is found in the stomach, and the duodenum enters the lipid phase and forms liposomes under the influence of bile salts and pancreatic. Afterward, the liposomes are absorbed through the intestinal walls and transported in the form of chylomicrons from the alimentary tract to the liver, where their complexes with very low-density lipoproteins are formed and are thus transported to target tissues. Studies of Zaripheh and Erdman showed that lycopene is deposited in the liver, seminal vesicles, and prostate tissues (Periago et al. 2013; Zaripheh and Erdman 2005).

Research of Singh and others examined type 2 diabetes patients who consumed 4 mg powdered lycopene daily in the form of capsules for 3 months. It was observed that lycopene lowered malondialdehyde (MDA) plasma levels and increased levels of serum antioxidant enzymes by approximately 30%, e.g., glutathione – GSH, glutathione peroxidase – GPx, glutathione reductase – GR, and superoxide dismutase – SOD (Singh et al. 2012). Carotenoids are responsible for the induction of antioxidant defense resulting in lowering the levels of substances related to lipid oxidation. Results of Zhao et al. confirmed an increase in total superoxide dismutase, glutathione, and catalase levels in mice consuming the encapsulated form of lycopene. Apart from that they also reported higher effectivity of encapsulated lycopene in comparison to free form (Zhao et al. 2018).

Results of Bose and Agrawal showed that daily administration of 200 g of cooked tomatoes reduced lipid peroxidation rate in patients with arterial hypertension. Also, the increase of the antioxidant enzymes' concentrations, e.g., glutathione peroxidase, glutathione reductase, glutathione, and also superoxide dismutase was found.

However, there were no changes in the blood lipid profile, in particular the total serum cholesterol, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), high-density lipoproteins (HDL), and triglycerides (TG) levels (Bose and Agrawal 2007a, b). Similar results were found by Engelhard, who recorded a reduction for systolic (by 10 mmHg) and diastolic blood pressure (by 4.0 mmHg), and decrease in the concentration of lipid peroxidation products (AAPH-induced TBARS) in patients consuming 15 mg of lycopene daily (Engelhard et al. 2006). In turn Collins and others found no effect of lycopene consumption on blood plasma lipid levels and the concentration of malondialdehyde and glutathione peroxidase (Collins et al. 2004). Other research showed an increased serum lycopene concentration in patients after kidney transplants supplemented with tomato juice for 4 weeks (Sutherland et al. 1999). Observations of Ok and others showed that a high level of blood serum carbamylated LDL cholesterol could be responsible for the development of cardiovascular disease in patients with chronic kidney failure (Ok et al. 2005). Other research showed that lycopene inhibits carbamylation of LDL cholesterol, and thereby reduces the risk of atherosclerotic lesions in a group of patients with kidney failure (Ghaffari and Shanaki 2010).

The clinical trial conducted by Ried and others showed that the daily intake of tomato extract with lycopene (15 mg) for 8 weeks did not result in a significant reduction of systolic and diastolic arterial blood pressure in prehypertensive patients (Ried et al. 2009). Contrary to these studies, the study of Paran and others showed that consumption of lycopene (15 mg daily for 6 weeks) significantly reduced systolic and diastolic blood pressure (Paran et al. 2009).

It is believed that inflammations play a significant role in the development of atherosclerosis, which is a cause of approximately 80% of cases of sudden cardiac death. Studies indicate a relationship between a high concentration of blood plasma proinflammatory cytokines and the incidence of cardiac failure (Mann 2005). Chauhan and Shah investigated the influence of lycopene supplementation on the level of an inflammation marker, C-reactive protein, in the blood plasma of 60 cardiac failure patients (Chauhan and Shah 2015). The results indicated that both women and men noted a significant reduction of blood plasma C-reactive protein concentration. Thies and coworkers analyzed the effect of lycopene consumption (10 mg daily for 12 weeks) by healthy participants and found no effect on changes in plasma concentration of analyzed lipids, and the level of apolipoprotein A-I and apolipoprotein B-100 (Thies et al. 2012). Other research determined the effect of lycopene supplementation on oxidative stress and the markers of endothelial function, the levels of which are correlated with the incidence of cardiovascular risk factors (Kim et al. 2011a). Researchers found an increase in the activity of serum superoxide dismutase, the reduction of C-reactive protein concentrations, vascular cell adhesion molecule 1, and intercellular cell adhesion molecule (Blankenberg et al. 2003; Kim et al. 2011b).

The research of Cheng and coworkers found that both, the consumption of tomatoes and lycopene supplementation, were beneficial for lowering the cardiovascular risk factors in patients aged over 18 years. They showed a reduction in LDL cholesterol and interleukin 6 levels, the systolic blood pressure, and improved artery

flow-mediated dilation (FMD). Simultaneously, there were no changes in total cholesterol, HDL cholesterol, triglycerides, C-reactive protein concentration (CRP), and endothelial activation marker (ICAM-1) level and the aortic pulse wave velocity (PWV) (Cheng et al. 2017). Other research showed a correlation between a high concentration of lycopene in blood and reduced risk of cerebral stroke in men (Karppi et al. 2012a, b); however, the results of Jacques et al. did not confirm that relationship (Jacques et al. 2013). Also, administration of lycopene encapsulated with nano-liposomes reduced neurological deficits in individuals with ischemia induced by middle cerebral artery occlusion surgery (Zhao et al. 2018).

β-Carotene

β-carotene is the yellowish-orange carotenoid, which is composed of 40 carbon atoms and 11 conjugated and 2 unconjugated double bonds. It can be synthesized by α- and β-lycopene cyclase conversion of lycopene. This carotenoid occurs in nature in the form of all-trans β-carotene and 9-cis β-carotene, while low concentrations were also found in the 13-cis β-carotene form (Zakynthinos and Varzakas 2016).

β-carotene supplied with food comes first of all from carrot, kale, red pepper, pumpkin, apricot, and mango (Holden et al. 1999; Igielska-Kalwat et al. 2015). It is a precursor of vitamin A in human and animal tissues and could be transformed into retinol, followed by retinal and retinoic acid (Grune et al. 2010; Staniaszek and Goździcka-Józefiak 2008). Research found that only 1.3–2.9 mg of β-carotene is consumed with a daily diet. Also, the source of β-carotene matters since β-carotene from natural sources is absorbed to a limited degree (<14%), and β-carotene provided with dietary supplements is absorbed in approximately 70% (Gul et al. 2015; Staniaszek and Goździcka-Józefiak 2008). Research does not confirm the strong antioxidant activity of β-carotene in in vitro systems as lipids or in vivo, without significantly affecting the level of antioxidant enzymes concentration, including glutathione, glutathione peroxidase, and superoxide dismutase.

Shaish and others examined the effect of β-carotene intake on patients under the fibrates therapy for hypercholesterolemia and found that this combination increased the HDL cholesterol level. Considering the other components of the lipid profile as triglycerides, total cholesterol, apolipoprotein A and B no changes were found between the examined groups (Shaish et al. 2006). The above results were confirmed by Ribaya-Mercado team investigating the influence of β-carotene supplementation on changes in the lipid profile of women (Ribaya-Mercado et al. 1995). Results of Hennekens and others revealed that long-term supplementation with β-carotene (50 mg, every second day) did not reduce significantly the mortality due to cardiovascular disease or cardiac infarction (Hennekens et al. 1996). In turn, Karppi and others investigated the effect of β-carotene on the incidence of cardiovascular disease death of adult men and found a greater risk for individuals with lower plasma β-carotene concentrations (Karppi et al. 2012b). Moreover, it was also

found that a greater risk of sudden cardiac death and ischemic heart disease is associated with a low level of β-carotene in the blood (Karppi et al. 2013a, 2013b).

The U.S. National Cancer Institute and the Finnish National Public Institute jointly sponsored research on the influence of vitamin E and β-carotene supplementation on the reduction of lung cancer incidence in male smokers aged 50–69 years. Results showed a higher incidence of lung cancer in the group receiving β-carotene and also slightly increased mortality due to ischemic heart disease and cerebral stroke (The Alpha-Tocopherol; Beta Carotene Cancer Prevention Study Group 1994).

Lutein and Zeaxanthin

Xanthophyll compounds lutein and zeaxanthin are responsible for the physiological function of the vision organ. Both compounds play an important role in the prevention of cataract and age-related macular degeneration (AMD) development (Gruszecki 2009; Leermakers et al. 2015). Lutein belongs to unsaturated polyenic hydrocarbons and consists of 8 isoprene residues that form the carbon chain with 40 carbon atoms and 2 hydroxyl groups in β-ion rings. Zeaxanthin is an isomer of lutein and has a similar chemical formula, the only difference is in the position of the double bond in one of the terminal β-ion rings: between C5′ and C6′ in zeaxanthin and between C4′ and C5′ in lutein structure. Both lutein and zeaxanthin are not the precursors of vitamin A (Edwards 2016).

The main sources of lutein are green leafy vegetables, and orange-yellow fruits and vegetables, e.g., kale, cabbage, spinach, or avocado, and also egg yolks and egg products as a result of plant feed (Perry et al. 2009). Zeaxanthin supplied with food comes first of all from pepper and corn (Abdel-Aal et al. 2013; Holden et al. 1999). In the human body, lutein reacts with free radicals and is essential in the prophylaxis of age-related eyesight deterioration such as age-related macular degeneration (AMD) and cataracts (Johnson 2014; Raman et al. 2019). Research of Abdel-Aal and others found that lutein from eggs has three times higher bioavailability in humans, compared to lutein from other sources (Abdel-Aal et al. 2013; Blesso et al. 2013).

The study of Wang and others examined the influence of lutein administration on the lipid-lowering and antioxidant properties in healthy smokers and found no differences in terms of cholesterol and triglycerides concentration, as well as in antioxidant enzymes activity (CAT, SOD, GPx) (Wang et al. 2013). Simultaneously, they observed an increase in serum total antioxidant capacity and a decrease of malondialdehyde and C-reactive protein levels.

Xu and coworkers examined the effect of lutein supplementation on the level of inflammatory cytokines and the lipid profile in patients with atherosclerosis (Xu et al. 2013). The administration of 20 mg of lutein daily for 3 months reduced the concentration of LDL cholesterol and triglycerides, did not affect the activity of GPx and SOD, the antioxidant enzymes presented in erythrocytes (Hininger et al. 2001). Results of Polidori and others showed no correlation between low plasma

lutein level and high concentration of malondialdehyde in patients with ischemic cerebral stroke (Polidori et al. 2002). Research indicated that lutein plays a role in protection against atherosclerosis, especially in patients at an early stage. Zou and others reported that lutein concentration in plasma was inversely correlated with the carotid intima-media thickness (CIMT), while zeaxanthin concentration was inversely correlated with cardiovascular risk assessment markers as pulse wave velocity (PWV) and rigidity of the right common carotid artery (Zou et al. 2011).

Promising results were observed following supplementation of lutein with lycopene (Zou et al. 2014). Similar results were obtained Graydon and others, who conducted studies in which healthy participants received powdered spinach (15 mg lutein and zeaxanthin daily) or dietary supplement (10 mg lutein and 5 mg zeaxanthin) for 8 weeks of dietary intervention. No effect of carotenoid consumption was found on C-reactive protein and isoprostanes concentrations, as well as on the endothelial activation markers levels (Graydon et al. 2012). In the study on atherosclerotic patients, it was found that serum lutein concentration was inversely correlated with the concentration of interleukin 6 (Xu et al. 2012). Other studies showed no relationship between plasma lutein and zeaxanthin concentrations and cardiovascular risk among examined women and the incidence of atherosclerosis (D'Odorico et al. 2000; Sesso et al. 2002). Also, Tavani and others showed no beneficial effect of lutein and zeaxanthin consumption on the risk of acute myocardial infarction (Tavani et al. 2006). Hirvonen et al. (2000) confirmed no correlation between the risk of cerebral stroke in smokers and lutein and zeaxanthin intake.

Astaxanthin

One of the best known carotenoid, belonging to xanthophylls is astaxanthin, the lipophilic compound with two hydroxyl groups and two carbonyl groups, which does not contain at least one unsubstituted β-ionone ring, and therefore is not provitamin A. Main sources of astaxanthin are salmon, shrimp, trout; however, animals cannot synthesize it, so it has to be supplied with food (Fassett and Coombes 2009; Stachowiak 2014). Astaxanthin is found in large amounts in algae and yeast (Fassett and Coombes 2011; Igielska-Kalwat et al. 2015). Research showed that a microalga *Haematococcus pluvialis* is capable of accumulating astaxanthin at 4–5% dry cell mass. Commercially 1 kg of dry algal biomass produces 40 g of astaxanthin (Guerin et al. 2003; Riccioni et al. 2011).

Astaxanthin is characterized by low bioavailability. Its absorption in the duodenum is followed by bounding with lipoproteins and transporting to organs throughout the body; however, it is also capable of penetrating the blood–brain barrier (Fakhri et al. 2018; Gammone et al. 2015; Hussein et al. 2006). Research has shown that astaxanthin accumulation is highest in muscles, liver, and kidney tissues (Aoi et al. 2003). In the research by Tominaga et al. (2017) and others concerning safety, no adverse events were found during oral supplementation of astaxanthin.

Astaxanthin possesses high antioxidant activity acting as reactive oxygen species (ROS) and free radicals quencher (Fassett and Coombes 2012). The use of astaxanthin includes the food industry, with an application as a natural pigment or dietary supplement (Ambati et al. 2014; Seabra and Pedrosa 2010). Research conducted by Yoshida and others showed that astaxanthin intake significantly reduced triglyceride concentration and increased high-density lipoprotein (HDL) cholesterol levels; however, no changes in total cholesterol and low-density lipoprotein (LDL) cholesterol levels occurred (Yoshida et al. 2010). Karppi and others in their research examined a cohort of healthy men and confirmed that astaxanthin supplementation reduces fatty acids oxidation (Karppi et al. 2007). Also, the results of Iwamoto showed that astaxanthin inhibits low-density lipoproteins oxidation (Iwamoto et al. 2000).

Choi and others conducted a dietary intervention on overweight patients, who were administered 20 mg astaxanthin daily for 12 weeks. Results showed a decrease in serum concentrations of LDL cholesterol, apolipoprotein B, malondialdehyde, and isoprostane, and an increase of superoxide dismutase (SOD) level; however no differences in the levels of triglycerides, HDL cholesterol, and total cholesterol were found (Choi et al. 2011). In another study, it was found that the administration of astaxanthin (6 mg daily for 10 days) improved rheological properties of blood in male adults (Miyawaki et al. 2008). Results of Pan and others reported that astaxanthin exhibits protective effects against free radical damage and ischemia or reperfusion-induced neurodegeneration (Pan et al. 2017). Coombes with coworkers found that administration of 12 mg astaxanthin daily for a year to patients with kidney transplants did not affect oxidative stress, inflammatory condition, or the rigidity of arterial vessels (Coombes et al. 2016). Chintong and others suggested that shrimp astaxanthin would be a promising dietary supplement for skin health applications (Chintong et al. 2019). Other in vivo study demonstrated the biological activities of astaxanthin to achieve effective skin cancer chemoprevention (Rao et al. 2013).

Carotenoids – Extraction and Determination Methods

To date, many different methods of extracting carotenoids have been published. The most commonly used include Soxhlet extraction; supercritical fluid extraction (SPE) with carbon dioxide (CO_2); microwave-assisted extraction (MAE), including microwave-assisted solvent extraction (MASE) and microwave solvent-free extraction (MSFE); enzyme-assisted extraction (EAE) with the use of enzymes such as cellulose, hemicellulose, pectinase; ultrasound-assisted extraction (UAE); pulsed electric field (PEF)-assisted extraction; pressurized liquid extraction (PLE); and atmospheric liquid extraction with maceration (Saini and Keum 2018). These methods are characterized by different qualities. For example, pulsed electric field

extraction is characterized by high efficiency, microwave-assisted extraction is a very fast and simple technique, and the use of supercritical fluid extraction allows to obtain carotenoids of very high purity (without residues of other chemical compounds). In turn, enzyme-based extraction is characterized by reduced extraction time. The most common disadvantages of the extraction methods include thermal degradation and isomerization of carotenoids (Soxhlet extraction, microwave-assisted extraction), high cost of application (pulsed electric field extraction, supercritical fluid extraction, enzyme-assisted extraction), as well as time-consuming (Soxhlet extraction) (Adadi et al. 2018).

Carotenoids are lipophilic compounds and are therefore extracted with the use of organic solvents. The most commonly used solvents are n-hexane, methanol, ethanol, acetone, chloroform, isopropanol, diethyl ether, dichloromethane. Mixtures of the solvents mentioned are also commonly used, e.g., acetone: petroleum ether (50:50), n-hexane: acetone (60:40), n-hexane: acetone: ethanol (50:25:25) (Machmudah and Goto 2013).

The choice of the appropriate solvent is dictated by several factors, including the length of the carotenoid chain, the polarity of the carotenoids, the water content of the sample, as well as the type of sample/matrix (Saini and Keum 2018). Carotenoids containing polar functional groups include, among others lutein, zeaxanthin, astaxanthin, canthaxanthin, violaxanthin, and neoxanthin. In turn, beta-carotene and lycopene are classified as nonpolar carotenoids. For the simultaneous extraction of nonpolar and polar carotenoids, a mixture of hexane: acetone: ethanol is usually used. This mixture of solvents is most often used in the extraction of carotenoids from plant products. Ethanol is recommended for the extraction of polar carotenoids, and hexane is a good option for the extraction of nonpolar carotenoids. To neutralize the organic acids that may be in the test samples, the addition of magnesium carbonate, calcium carbonate, or sodium bicarbonate is usually used during the extraction of carotenoids (Saini and Keum 2018). The carotenoids present in the extracts are believed to be generally less stable than the carotenoids found in food. Therefore, it is recommended that the prepared samples (after extraction) are immediately subjected to further analysis (Kopec et al. 2012).

UV-Visible spectrophotometry or chromatographic methods are most often used to identify and determine the carotenoid content. Spectrophotometry is one of the simple methods of identifying carotenoids. Unfortunately, this technique does not distinguish between individual carotenoids and their isomers (cis and trans). The advantage of this method is low cost and simplicity in use. High-performance liquid chromatography (HPLC) is commonly used for the qualitative and quantitative determination of carotenoids. In some cases, however, this technique may be costly. HPLC coupled with ultraviolet/visible light detector (HPLC-UV-Vis), photodiode array detector (HPLC-PDA), or mass spectrometry (HPLC-MS) is more frequently used. Of these techniques, the most common analytical method is HPLC-UV-Vis. However, because the spectra of some carotenoids are very similar, mass spectrometry detectors are increasingly employed (Amorim-Carrilho et al. 2014).

Summary

Studies conducted in recent years indicate that carotenoids may influence human health offering a wide spectrum of health-promoting properties. The research discussed in the chapter indicates that consumption of products rich in carotenoids may contribute to the inhibition of numerous diseases development. Adequate nutrition and changes in a lifestyle can prevent the development of noncommunicable diseases that constitutes a serious public health problem.

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Chapter 6
Tocochromanols

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Abstract In food and biological systems, the main function of tocochromanols is antioxidant action – they deactivate free radicals and protect lipids from peroxidation (autoxidation). In addition to the antioxidant role of vitamin E and protection against oxidative stress, the "non-antioxidant" functions of vitamin E, including cell signaling and antiproliferation, is also described. Tocopherols and tocotrienols are not the only compounds classified as tocol derivatives. This chapter also includes an overview of the properties and occurrence of other forms of tocols (including: tocomonoenols, tocodienols, and plastochromanol-8). Content of tocochromanols in food may be determined using a wide range of analytical techniques. Capillary gas chromatography and high performance liquid chromatography coupled with various detection systems and mass spectrometers enable identification of individual compounds. In the analysis of these compounds, spectroscopic methods are also being developed. The principles of these techniques are discussed in the chapter and examples of their applications are also provided.

Introduction

Tocochromanols are native antioxidants widespread in many raw materials and food products, especially of plant origin. Research in tocopherols and tocotrienols properties as well as identification of new tocol derivatives, such as tocomonoenols or tocodienols, present in samples in small quantities, requires the development of separation, identification, and quantitative determination methods. Defining an analytical problem, choosing a suitable method, taking and preparing samples for analysis, measuring and analyzing the results are crucial stages of the properly conducted analytical procedure. In the case of tocochromanols, the lack of compounds distribution uniformity in food products and matrices diversity is very important from the

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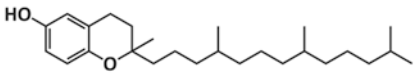
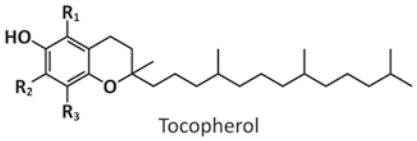
analytical point of view. For this reason, the preparation of samples for analysis is based on different methodologies for various foodstuffs.

Chemical Structure of Tocochromanols

The term tocopherols (−T) refers to a group of four compounds, composed of a chromanol ring with differently arranged methyl ligands and a 16-carbon saturated side chain. They are derivatives of tocol (Fig. 6.1). Tocopherols have three asymmetric carbon atoms at positions 2 (in the chromanol ring) and 4′ and 8′ (in the side chain), thus each homolog has eight optic isomers (Eitenmiller and Lee 2004; Schneider 2005). Chirality of these compounds is of significance in biological studies or clinical trials, since receptors and enzymes in living organisms are highly selective. As a result, one enantiomer may have a positive influence on the organism, while another may be neutral or cause an adverse effect (Colombo 2010). The

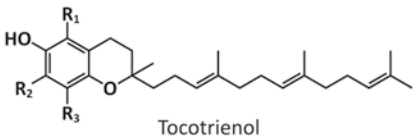


Tocol
2-methyl-2-(4`,8`,12`-trimethyltridecyl)chroman-6-ol



Tocopherol

Ring position			Name (Abbreviation)	Chemical name
R_1	R_2	R_3		
CH_3	CH_3	CH_3	α-tocopherol (α-T)	5,7,8-trimethyltocol
CH_3	H	CH_3	β-tocopherol (β-T)	5,8-dimethyltocol
H	CH_3	CH_3	γ-tocopherol (γ-T)	7,8-dimethyltocol
H	H	CH_3	δ-tocopherol (δ-T)	8-methyltocol



Tocotrienol

Ring position			Name (Abbreviation)	Chemical name
R_1	R_2	R_3		
CH_3	CH_3	CH_3	α-tocotrienol (α-T3)	5,7,8-trimethyltocotrienol
CH_3	H	CH_3	β-tocotrienol (β-T3)	5,8-dimethyltocotrienol
H	CH_3	CH_3	γ-tocotrienol (γ-T3)	7,8-dimethyltocotrienol
H	H	CH_3	δ-tocotrienol (δ-T3)	8-methyltocotrienol

Fig. 6.1 Structures of tocopherols and tocotrienols

naturally found RRR-α-T configuration exhibits the greatest biological activity. The synthetic forms are racemic mixtures of eight geometric combinations. Synthetic vitamin E is used to fortify foodstuffs, to enrich oils, and it is applied in the pharmaceutical industry. A particular role is played by α-tocopheryl acetate, which exhibits low activity compared to the naturally found forms, but the ester bond considerably improves the stability of this compound (Rupèrez et al. 2001).

Tocochromanols are synthesized by photosynthesizing organisms, such as plants, cyanobacteria, and algae. The type and amounts of tocochromanols vary in different plants or their tissues. They are found in all anatomical parts of plants. The presence of tocochromanols in the plant world is essential during vegetation and maturation, thus they have been detected in roots, tubers, stems, leaves, and flowers (Siger 2011). In green parts of plants (mainly chloroplasts) α-T is the dominant homolog, protecting the photosynthesizing organs against reactive oxygen and nitrogen species (ROS and RNS). The other tocopherol homologs are found outside these organelles. The γ-T homolog, mainly contained in seeds, ranks second in terms of its amounts. In mature seeds tocopherols also protect polyenic fatty acids against oxidation, particularly during storage and seed germination (Hofius and Sonnewald 2003; Trela and Szymańska 2019). Tocopherols are found in large amounts in wheat germs (total tocopherol 225.2 mg 100 g^{-1} oil) (Trela and Szymańska 2019) and in popular edible oils such as palm (42.5 mg 100 g^{-1} oil), rapeseed (46.8 mg 100 g^{-1} oil), sunflower (60.9 mg 100 g^{-1} oil), and corn oil (82.9 mg 100 g^{-1} oil) (Gliszczyńska-Świgło et al. 2007; Shahidi and De Camargo 2016).

Apart from tocopherols the tocochromanol group also includes tocotrienols (–T3) (Fig. 6.1). They have identical formulas as tocopherols in terms of the distribution of methyl groups in the chromanol ring, while they differ in the isoprenoid side chain, containing unsaturated bonds (at positions 3′, 7′, and 11′) (Schneider 2005). Tocotrienols have only one asymmetric carbon at position 2 of the ring. Thanks to three double bonds their chain is much more flexible compared to tocopherols (Sen et al. 2007). Tocotrienols, mainly α-T3 and γ-T3, are found first of all in non-photosynthesizing tissues, e.g., in seeds (husks and germs of maize, wheat, rice, barley, etc.). Their small amounts have also been detected in fruit (mainly α-T3). Tocotrienol sources particularly rich in γ-T3 include rice bran oil (total tocotrienols 46.6 mg 100 g^{-1}) and palm (*Elaeis guineensis*) oil (total tocotrienols 36.4 mg 100 g^{-1}) (Drotleff et al. 2015). Also, barley oil contains large amounts of tocotrienols, predominantly α-T3 (46.5–76.1 mg 100 g^{-1} oil) and γ-T3 (8.50–18.6 mg 100 g^{-1} oil) (Shahidi and De Camargo 2016). A general regularity has been observed, indicating greater amounts of tocotrienols in monocotyledonous rather than dicotyledonous plants (Horvath et al. 2006; Sen et al. 2007).

Total tocopherol contents in plants fluctuate during the maturation cycle of plants or even in their diurnal cycle, depending on light conditions, temperature, availability of water, and UV-B radiation (Szymańska et al. 2009). In turn, the total content of tocochromanols depends on the analyzed raw material/product (being greater in plant origin products and lower in animal origin products), variety, year of culture, climate and soil conditions, postharvest seed processing, and raw material storage

conditions (Gawrysiak-Witulska et al. 2016, 2020; Górnaś and Siger 2015; Górnaś et al. 2019; Munné-Bosch 2005; Rękas et al. 2017; Siger et al. 2018).

Tocochromanols are found in the form of a viscous, thick liquid resembling oil. They are readily soluble in organic solvents. They exhibit high resistance to the action of acids, bases, and high temperatures in an anaerobic environment, while they are sensitive to the action of oxygen, ultraviolet radiation, or transition metal ions (Eitenmiller and Lee 2004).

Antioxidant Activity of Tocopherols and Tocotrienols

An antioxidant is a substance delaying or inhibiting undesirable oxidation reactions through the reduction or neutralization of reactive oxygen or nitrogen species (Apak 2019). The main biological function of tocopherols and tocotrienols relates to the scavenging of free radicals and the protection of lipids against peroxidation. Antioxidant properties of tocochromanols depend on their concentration, type of substrate, other chemical compounds exhibiting prooxidative and synergistic action, pH, the presence of metal ions, solvent, light, temperature and/or regenerating substances such as ascorbic acid, chitosan, glutathione, flavonoids, carotenoids, melanoidins, and phospholipids (De Camargo et al. 2019; Kamal-Eldin and Appelqvist 1996). An important aspect determining the antioxidant properties of tocopherols is also related to the concentration, in which they are found. The concentration at which tocopherols exhibit optimal antioxidant activity amounts to 100–250, 250–500, and 500–1000 μg g^{-1} for α-, γ-, and δ-tocopherol, respectively (Carrera and Seguin 2016). This has been confirmed by numerous studies conducted on various fat substrates under diverse conditions (Gottstein and Grosch 1990; Jung and Min 1990; Lampi et al. 1999; Yanishlieva-Maslarova 2001). The efficacy of individual homologs also depends on the ligands in the chromanol ring as well as properties of the side chain. In a homogeneous solution, the reaction rate constant depends mainly on the number of methyl groups in the ring (Azzi and Stocker 2000). Antioxidant activity of homologs tocopherols in vivo may be ordered as follows: α-T > β-T > γ-T > δ-T, while in the case of their activity in vitro it is in the opposite order α-T < β-T ≈ γ-T < δ-T (Eitenmiller and Lee 2004; Munteanu et al. 2004; Yanishlieva-Maslarova 2001). Tocopherols are the most important inhibitors of the free radicals, which mediate in chain reaction, taking place during lipid oxidation (Schneider 2005). They inhibit lipid oxidation through the elimination of peroxide radicals before they damage fatty acid molecules or membrane proteins (Wang and Quinn 1999; Yang et al. 2018). Additionally, they are also capable of repairing oxidatively damaged biomolecules, again using their hydrogen atom or through the electron transfer mechanism. Moreover, tocochromanols provide enzymatic protection and repair pathways with transcriptional and kinetics effects, while in the process of lipid oxidation they affect enzymatic processes (Torquato et al. 2020). Their antioxidant activity results primarily from the capacity of supplying an

electron or hydrogen atom to free lipid radicals (Schneider 2005). The heterocyclic chromanol ring has an optimal form to maintain resonance stability of unpaired electrons of the α-tocopheroxyl radical, while electron donor ligands, e.g., methyl groups, enhance this effect (Azzi and Stocker 2000; Burton et al. 1983). According to Denisov and Afanas'ev (2005), the reaction rate constant for the reaction of α-T with radicals is 2×10^5 L mol^{-1} s^{-1}. This compound reacts both with peroxide and alkoxy radicals. Its antioxidant properties are provided by the hydroxyl group in its molecule. In the case of α-tocopherol, substitution of three electron donor methyl groups to the chromane ring promotes abstraction of hydrogen from the –OH group, as a result, α-tocopherol exhibits its optimal activity. According to Wijtmans et al. (2003), α-T is the most active known fat-soluble antioxidant. As reported by Ross et al. (2003), α-T exhibits the greatest capacity to donor hydrogen atom k_{ab}, the so-called antioxidant capacity amounting to 5.12×10^{-3} [M^{-1} s^{-1}]. For the other homologs this value is 2.24×10^{-3} [M^{-1} s^{-1}] for β-T, 2.42×10^{-3} [M^{-1} s^{-1}] for γ-T, 1.00×10^{-3} [M^{-1} s^{-1}] for δ-T, and 0.56×10^{-3} [M^{-1} s^{-1}] for tocol (Ross et al. 2003). When α-T is attacked by peroxide radicals of fatty acids, it is transformed into the α-tocopheroxyl radical (Azzi et al. 2001). Each molecule of α-T scavenges two free radicals, thus it simultaneously ends two oxidation reactions, since apart from tocopherol also tocopheroxyl radicals participate in reactions of peroxide radicals inactivation (Burton 1994). Tocopheroxyl radicals reduce peroxide radicals to hydroperoxides, while they themselves oxidize to α-tocopheryl quinone (α-T = O). Cell membranes contain one α-T molecule per 2000 phospholipid molecules. This fact explains the reaction rate of the peroxide radical with α-T, which is 10,000-fold greater than that of the reaction of the radical with a fatty acid (Bramley et al. 2000). Tocopherols also exhibit certain prooxidative properties. During the reduction of Fe^{+3} to Fe^{+2} or Cu^{+2} to Cu^{+1}, they stimulate the formation of hydroxyl radicals (Ross et al. 2003). Under some conditions, such as the presence of oxygen, high temperature, or high concentration of α-T/T3, their efficacy as antioxidants may deteriorate or they may even exhibit prooxidative action (become pro-oxidants) (Drotleff et al. 2015). Yanishlieva and Marinova (2003) explained this phenomenon by the participation of tocopherols and/or their radicals in the series of peroxide side reactions.

Tocopherols and tocotrienols inhibit the formation of lipid peroxidation products, such as trans,trans-hydroperoxyoctadecadienoates, which are specific to free radical oxidation reactions and are used as specific biomarkers for free radical peroxidation of lipids. Yoshida et al. (2003) showed that reactivity of tocochromanols toward oxygen radicals decreases in the order α > β, γ > δ and that respective tocopherols and tocotrienols exhibit identical reactivity. Serbinova et al. (1991) reported that α-T3 possesses 40–60 times higher antioxidant activity than α-T against Fe(II) + ascorbate and Fe(II) + nicotinamide adenine dinucleotide phosphate (NADPH)-induced lipid peroxidation in rat liver microsomal membranes. Also, Schaffer et al. (2005) expressed an opinion that tocotrienols show excellent antioxidant properties in vitro, while they also limit the action of reactive oxygen species more effectively than tocopherols. However, results concerning the antioxidant activity of tocotrienols are dependent on the adopted study model. The activity of tocotrienols toward radicals generated by metal ions in the solution or micelles of

SDS (sodium dodecyl sulfate) varies for individual homologs in the following order: α-T3 > β-T3 > γ-T3 > δ-T3 (Schauss 2009). In turn, in studies on liposomes, α- and γ-T3 inhibited the formation of 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) radicals (MeO-AMVN); however, γ-T3 was a more effective antioxidant (Yoshida et al. 2003). Kamat and Devasagayam (1995) showed that γ-T3 is a more effective antioxidant than α- and δ-T3 in hepatic microsomes and brain mitochondria of rats. Those authors used various sources of free radicals, such as the ascorbate-Fe system or photosensitization. During in vitro studies (isolated rat liver cells), Palozza et al. (2006) showed that δ-T3 is the most effective homolog inhibiting lipid peroxidation and generation of free radicals using 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), *tert*-butyl hydroperoxides (*t*-BOOH), and hydrogen peroxide (H_2O_2).

When considering the oxidation process one may not focus only on the radical reactions taking place, but also on the location of molecules participating in the autoxidation reactions, particularly at the oil/water interface in the nanoemulsion. According to Budilarto and Kamal-Eldin (2015), the formation of micellar structures has a considerable effect on the rate of autoxidation reactions and the action of antioxidants. Vegetable oils apart from triacylglycerols contain also amphiphilic compounds such as phospholipids, sterols, monoacylglycerols, free fatty acids, etc. After the so-called critical micelle concentration (CMC) is exceeded, these compounds accumulate at the oil–water interface forming association colloids – reverse micelles considered to be active oxidation centers (Rokosik et al. 2020). Tocopherols, thanks to their amphiphilic character, are capable of accumulating at the microemulsion interface. Through polar hydroxyl groups targeted toward the water phase, the formed reverse micelles may scavenge free radicals (scavenge aqueous peroxyl radicals) (De Camargo et al. 2019). According to this new understanding, the lipophilicity of tocopherols/tocotrienols (expressed as log P value or the octanol-water partition coefficient) is another important determinant of antioxidant activity, next to the dissociation energy of the phenolic O-H bond (Chaiyasit et al. 2008). The radical scavenging capacity exhibited by tocopherols is dependent on their bond dissociation energies and may be ordered as follows: α > β ≥ γ > δ (Kamal-Eldin and Appelqvist 1996). Antioxidant properties of tocotrienols may also be influenced by the presence of double bonds in the side chain, which affects their lipophilicity and molecule size (Serbinova et al. 1991).

Health Benefits of Tocochromanols

The above-mentioned tocopherol and tocotrienol homologs are classified by most researchers as active vitamin E compounds. However, according to Azzi (2019), the term “vitamin E” is used for several similar molecules (both tocopherols and tocotrienols) that have never been shown to have vitamin property, i.e., a protective effect against the human deficiency disease. In fact, the name “vitamin E” should only be used to define molecules that prevent the human deficiency disease “Ataxia with Vitamin E Deficiency” (AVED). Only one such molecule is known, α-tocopherol.

Although all tocopherol forms are adsorbed in the human organism, α-tocopherol is considered to be the most biologically active compound in the tocochromanol group. β-, γ- and δ-tocopherol are by 60, 90, and 98–99% less active than α-tocopherol (Carrera and Seguin 2016). The advantage of α-tocopherol over other forms is connected mainly with the transfer protein (α-TTP). It readily binds to α-tocopherol molecules and through lipoproteins it is transported over the body (Peh et al. 2016).

Studies conducted on the health-promoting effect of tocochromanols showed among other things that they exhibit anti-inflammatory and cardioprotective action (Colombo 2010; Trela and Szymańska 2019). Evidence is also available on their neuroprotective and even anticancer effects (Drotleff et al. 2015). The health-promoting activity of vitamin E may be explained first of all as prevention of oxidation in low-density lipoproteins, which cause atherosclerosis, and prevention of ROS formation, related to cancer initiation and progression (Azzi 2018). The positive effect of tocochromanols in the prevention of cardiovascular disease may be explained similarly. This is related mainly to the disruption of the chain reaction of lipid peroxidation, particularly the prevention of LDL cholesterol oxidation (Shahidi and De Camargo 2016). Atherosclerosis is an inflammatory disease, thus it leads to the formation of inflammatory mediators: prostaglandins and leukotrienes, triggered by such enzymes as cyclooxygenases (COX-2) and lipoxygenases (5-LOX). Tocopherols, particularly γ-tocopherol, by inhibiting the activity of the above-mentioned oxygenases reduce the amount of inflammatory mediators exacerbating atherosclerosis (Devaraj and Jialal 1999; Jiang et al. 2000).

Apart from the antioxidative effect of tocopherols consisting of the inhibition of lipid peroxidation, caused by reactive oxygen species, tocopherols and tocotrienols are also capable of scavenging reactive nitric oxide forms. Definitely, the greatest capacity to detoxify nitric peroxide is observed for γ-tocopherol, which is caused by the presence of nucleophilic carbon C5 in the ring unsubstituted by the methyl group. Tocopherols have found applications in the prophylaxis and treatment of cardiovascular disease by preventing the generation of peroxynitrite, which promotes endothelium dysfunction. The capacity of peroxynitrite inactivation is also exhibited by α- and γ-tocopherol. Only γ-T, naturally found in food, binds irreversibly the active nitric dioxide generated in this reaction. This reaction produces 5-NO_2-γ-tocopherol and ortho-quinone (tocopherol red). A marked increase in the amount of 5-nitro-γ-tocopherol, a product formed as a result of lipid nitration, was also observed in the brain of Alzheimer's patients, which confirms the protective effect of γ-tocopherol in relation to nitrogen compounds (Bloodsworth et al. 2000; Christen et al. 1997).

There are numerous studies stating that tocotrienols exhibit better health-promoting properties compared to tocopherols due to their closer location in the lipid layer of cell membranes and increased access to free radicals (Fairus et al. 2020). Tocotrienols are characterized by neuroprotective properties and they are capable of inhibiting cholesterol biosynthesis. Moreover, compared to α-T, the

antioxidant activity of α-T3 in liver microvilli is 40- to 60-fold greater (Trela and Szymańska 2019).

Considerable differences were also observed in the health-promoting activity within the tocopherol group. Although α- and β-tocopherol have similar antioxidant properties, β-tocopherol shows no capacity to inhibit the activity of the C protein kinase and does not inhibit cell proliferation and gene expression. In turn, as it is reported in studies comparing α- and γ- tocopherol, only γ-tocopherol shows anticancer action, preventing hormone-dependent breast cancer progression, colon carcinogenesis, lung tumorigenesis, and prostate cancer cell growth (Azzi 2019). The direct anticancer action of tocopherols and tocotrienols results from their capacity to induce apoptosis of malignant tumor cells (Neuzil et al. 2004). Esterification of the phenolic group in the chromane ring, e.g., by succinic or maleic acid reduces antioxidant properties of the molecule provided by the free hydroxyl group and the esterified molecule of α-tocopherol gains strong apoptogenic properties. In this respect, γ-tocotrienol is an exception, since it does not require modification (Wang et al. 2006). However, it is not clear how the food matrix affects its absorption and bioavailability (Bernhardt and Schlich 2006).

Other Forms of Tocochromanols

Tocopherols and tocotrienols are not the only compounds classified as tocol derivatives. Literature data report on other tocol derivatives. In 1997 Qureshi et al. (1997) described two new tocotrienols isolated from rice bran. These are desmethyltocotrienol and didesmethyltocotrienol (Fig. 6.2), which differ from the other tocotrienols by a lack of methyl groups in the chromanol ring. It was shown that they exhibit much greater antioxidant properties, reducing blood cholesterol level, and anticancer properties compared to other forms of vitamin E (Qureshi et al. 1997). Moreover, didesmethyltocotrienol in experiments on mice reduced atherosclerotic lesions (Qureshi et al. 2002).

Matsumoto et al. (1995) discovered α-tocomonoenol in palm oil, which accounts for approximately 3–4% (around 40 ppm) of tocochromanol contents in crude palm oil. Structural verification by 1H NMR spectroscopy verified that the double bond was located in the 11′-position (11′-α-tocomonoenol). The trace impurity detected in the isolate was identified to be 12′-α-tocomonoenol, a compound detected in marine samples (Müller et al. 2018). Butinar et al. (2011) assayed contents of α- and γ-tocomonoenol in pumpkin seeds and oil. Fiorentino et al. (2009) in kiwi fruits identified δ-tocomonoenol. The skin contained almost two-fold greater amounts of this compound than the fruit pulp, in contrast to α-T, which levels in the skin and the entire fruit are identical. This compound has one double bond in the isoprenoid chain at carbon 11 (Fig. 6.2). Its antioxidant capacity is markedly lower than that of α-T, which chemically and biologically is the most active homolog of vitamin E. δ-Tocomonoenol has properties comparable to those of δ-T. Its presence in kiwi

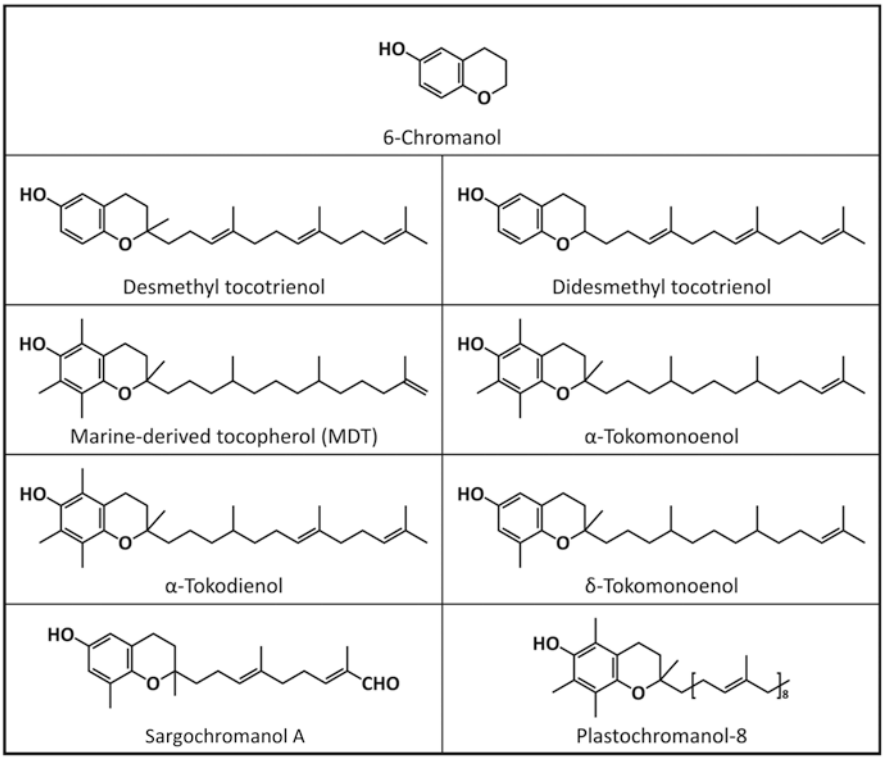


Fig. 6.2 Structures of other tocols

fruits considerably increases the total antioxidant capacity of this fruit (Fiorentino et al. 2009). Kruk et al. (2011) from leaves of *Kalanchoe daigremontiana* and *Phaseolus coccineus* isolated new tocochromanol forms, identified as β-, γ-, and δ-tocomonoenols, and proposed their biosynthesis pathways. In turn, Gee et al. (2016) in palm oil assayed tocopherols, tocotrienols, and α-tocomonoenol, while additionally they also identified a new compound, α-tocodienol (Fig. 6.2). Those studies confirmed the existence in nature of all four α-forms of vitamin E (α-tocopherol [α-T], α-tocomonoenol [α-T1], α-tocodienol [α-T2], and α-tocotrienol [α-T3]). Content of α-tocodienol in palm oil accounted for 0.2% of the total vitamin E content in the tocotrienol-rich fraction (Gee et al. 2016).

Yamamoto et al. (1999) isolated an isomeric and chemically distinct α-tocomonoenol from the lipophilic fraction of salmon eggs ("Marine-derived tocopherol"– MDT) having an unusual methylene unsaturation at the isoprenoid-chain terminus (Fig. 6.2). MDT shows an identical antioxidant action as α-T at a high concentration of free radicals in methanol or liposomal suspensions at a temperature of 37 °C. It is commonly found in muscle tissue of marine fish, particularly those living in cold waters. It is capable of faster diffusion in lipids, which are highly viscous at low temperatures. It prevents oxidation of polyenic fatty acids

(polyunsaturated fatty acids – PUFA), of which marine fish are rich sources. MDT inhibits the peroxidation of cholesterol-containing phosphatidylcholine liposomes to a greater extent than α-tocopherol does at 0 °C (Yamamoto et al. 2001). MDT has also been detected in the human blood plasma (Yamamoto et al. 2001), which indicates that MDT is recognized by α-TTP in the human liver, similarly as other vitamin E homologs. The relative bioavailability of vitamin E for MDT was established at approx. 49 (α-T has a value of 100) (Gotoh et al. 2009).

The literature on the subject also mentions compounds from the class of chromanols isolated from other marine organisms (sponges, macroalgae, tunicates, and coelenterata), which have a polyprenyl side chain bound to hydroquinone or a similar molecule to chromanol. Several such compounds have been discovered and described in brown algae (*Sargassum siliquastrum*) (Jang et al. 2005). One of them (sargochromanol A) has a chromanol ring identical to that of δ-tocotrienol, while the side chain has a substituted aldehyde group (Fig. 6.2). Those authors reported that the compounds exhibit good antioxidant properties (Jang et al. 2005). Other compounds found in algae (*Sargassum fallax*) have a more complex polyprenyl chain, substituted with various chemical groups (hydroxyl, ethyl, carboxyl) (Reddy and Urban 2009). Examples of such compounds include sargachromenol and fallachromenoic acid.

Plastochromanol-8 (PC-8) was discovered in 1965 and together with tocopherols and tocotrienols belongs to the group of tocochromanols. The chromanol ring in structure of tocochromanols and PC-8 is responsible for thier spectral and antioxidant properties (Kruk et al. 2014). It differs from γ-tocotrienol by having a longer side chain, thus it is also called γ-toco-octenol (Fig. 6.2) (Moreau and Lampi 2012). However, it needs to be stressed that tocochromanols and PC-8 have different biosynthesis pathways. Plastochromanol-8 is a fat-soluble, universal antioxidant and protects intratissue lipids. Within chloroplasts, it acts as the main antioxidant next to α-T. PC-8 is found in seeds, leaves, and other organs of higher plants (Martinis et al. 2011). It is essential in plant seeds during their prolonged storage or drying. It affects proper development and maturation of the seed coat; in contrast, in fresh seeds, it does not play such an important role (Gruszka et al. 2008; Mène-Saffrané et al. 2010). This compound participates in sugar transport in non-photosynthesizing plant organs (Strzałka et al. 2009). Studies have shown that the content of PC-8 in those plant parts is not determined by insolation, in contrast to the other tocochromanols, where light stimulates their synthesis (Szymańska and Kruk 2010). Plastochromanol-8 is widely distributed in the plant kingdom and it may be found in bryophytes, gymnosperms, and angiosperms, e.g., radish (Mène-Saffrané et al. 2010), as well as latex produced by rubber trees. It is also found in certain vegetable oils, e.g., linseed, rapeseed, camelina, mustard, corn, and soybean oils (Goffman and Mollers 2000; Gruszka et al. 2008; Kruk et al. 2014; Schwartz et al. 2008; Strzałka et al. 2009). The free radical scavenging capacity does not depend on the length of the side chain, but on the number of methyl groups attached to the chromanol ring; as a result, the antioxidant activity of PC-8 is comparable to that of γ-T3 (Gruszka et al. 2008). However, other sources reported that the unsaturated side chain also participates in the scavenging of singlet oxygen. This is evidenced by the

existence of oxidized forms of plastochromanol (Szymańska and Kruk 2010). According to Olejnik et al. (1997), antioxidant properties of PC-8 are 1.5-fold greater than that of α-T. In contrast to tocopherols, oxidation products of PC-8 are capable of natural fluorescence. Gruszka et al. (2008) detected two peaks of PC-8 oxidation products, which were identified using mass spectrometry. One of them was PC(OH)-8 (m/z 805.8) containing one hydroxyl group in the side chain, while the other compound was a PC-8 derivative containing three hydroxyl groups in the side chain ($PC(OH)_3$–8).

From the analytical point of view, the increasing number of compounds identified as tocol derivatives means that it is crucial to properly select and streamline assays for the presented compounds.

Analytical Methods for the Analysis of Tocochromanols

Instrumental methods applied in food analysis make it possible to assay investigated food components precisely and accurately, at adequately low limits of detection and limits of quantification. The selection of an appropriate separation method in food analysis depends to a considerable extent on the character of the assayed analyte, particularly its volatility, boiling point, solubility, polarity, thermal stability, or stability in a medium with a different pH. Separation techniques, i.e., liquid and gas chromatography, are dominant techniques in assaying tocochromanols in food. A combination of chromatographic techniques with mass spectrometry provides analysts with tools facilitating very good separation of multicomponent mixtures and ensures proper identification of their components. Content of tocochromanols in food may be determined using a wide array of analytical techniques, such as thin-layer chromatography (TLC) (Gogolewski et al. 1997), capillary gas chromatography (cGC) (Bartosińska et al. 2019), supercritical fluid chromatography (SFC) (Snyder et al. 1993), or high-performance liquid chromatography (HPLC). The most commonly applied technique is normal phase HPLC coupled with UV or fluorescent detection (AOCS 1990; Górnaś et al. 2014c; Górnaś and Siger 2015; ISO 2016; Rokosik et al. 2019; Siger et al. 2015, 2017; Siger and Michalak 2016).

Sample Preparation for Chromatography

Tocochromanols are strongly bound with lipophilic components of the food matrix. Several methods of sample preparation may be used to release tocochromanols and transfer them to the solvent appropriate for the chromatographic system. At each stage of analysis, it is essential to avoid oxidation-related losses of investigated compounds. Thus it is recommended to use laboratory glassware of darkened brown or amber color, which protects against ultraviolet and infrared radiation (Castanheira

et al. 2006). When contents of tocochromanols are analyzed in vegetable oils, sample preparation consists of weighing oil and its dissolution in *n*-hexane. After thorough mixing, such a prepared sample may be analyzed in the normal-phase HPLC (Górnaś et al. 2014a, b; Górnaś and Siger 2015; Siger et al. 2014). However, caution needs to be exercised in the case of oils produced from roasted seeds, since in such cases frequently an increase is observed in contents of γ-T and PC-8 (Siger et al. 2015, 2017; Wroniak et al. 2016). Most authors explain this fact by cell damage during roasting and the increasing extraction capacity of tocopherols, since heat disrupts bonds linking tocopherols with proteins or phospholipids (Vujasinovic et al. 2012). Wijesundera et al. (2008) analyzed the effect of rapeseed and mustard seed 5-min roasting at a temperature of 165 °C on the contents of tocochromanols. Those researchers reported practically no effect of roasting on contents of the α-T homolog. In turn, for γ-T they showed its higher content on average by approximately 10% for tested rape and mustard cultivars. According to those authors, this apparent increase in the concentration of γ-T after roasting is possibly due to its co-elution in HPLC with another component generated after roasting.

Since tocochromanols are bound with the lipoprotein complex in many food matrices, the protein-fat bonds have to be broken to release vitamins and for this purpose, hydrolysis is commonly used. This is typically alkaline hydrolysis, the so-called saponification. Saponification is generally run with the addition of antioxidants, such as ascorbic acid, butylated hydroxytoluene, or pyrogallol, together with nitrogen wash in order to reduce losses caused by oxidation (Blake 2007). This process is connected with the disruption of ester bonds of fatty acids with glycerol (leading to the formation of soap) and bonds of tocochromanols with the matrix. Also, esterified forms of tocopherols, e.g., α-T acetate, in the saponification process are hydrolyzed to the free form of α-T (Current Protocols in Food Analytical Chemistry 2003). Most frequently 60 or 80% (w/v) aqueous KOH solutions are used in the saponification process. The amount of KOH used in saponification depends on the content of fat in the product under analysis. An equally important factor is connected with the addition of ethanol, which is required to stabilize the saponified solution and prevent soap precipitation. Typically it is assumed that the ratio of KOH, ethanol, and the amount of fat during saponification should be 3 g–15 ml–1 g; however, these proportions need to be adapted to the type of the analyzed material (Eitenmiller and Landen 1999). In the literature on the subject and various international standards (AOAC International, European Committee for Standardization, International Dairy Federation, US Pharmacopeia, and International Organization for Standardization) one may find different saponification conditions depending on the type of matrix, sample size, and fat content in the sample. It was found that overnight saponification in the presence of methanol or ethanol at room temperature provides optimal conditions (Blake 2007). Siger et al. (2014) when analyzing contents of tocopherols in rapeseeds stated that all parameters of saponification, such as the addition of 60% KOH, the addition of pyrogallol, and saponification time have a statistically significant effect on the final result. It was found that each of the single parameters affects the contents of tocochromanols and statistically significant ($p < 0.05$) interactions were recorded between investigated

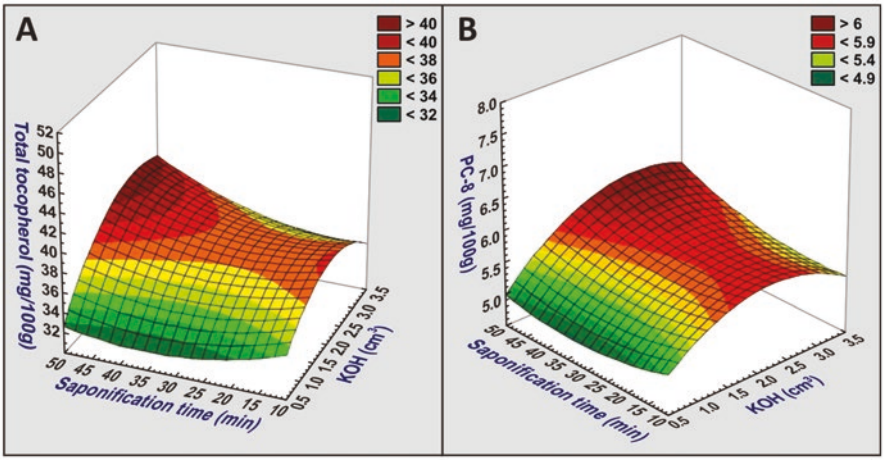


Fig. 6.3 Effect of 60% KOH concertation during saponification and saponification time on tocopherol and PC-8 contents (sample – seed of rape)

parameters. When optimizing the saponification process for rapeseeds it was shown that the best system is composed of 250 mg pyrogallol and 2.5 ml 60% KOH at saponification time of 45 min (Fig. 6.3). In the case of dietary preparations for infants and adults at simultaneous assays of vitamin A and total vitamin E (DL-α-tocopherol and DL-α-tocopherol acetate), it is proposed to prepare samples by enzymatic digestion. Papain was used to hydrolyze hydrophilic proteins before direct isooctane extraction of tested analytes. In contrast to many presently applied official methods (AOAC Method 992.03.2012 2012; AOAC Method 992.06.2012 2012; ISO 2016), alkaline saponification was avoided (Gilliland 2016).

Upon the completion of the saponification process, unsaponifiable substances need to be extracted. However, in the case of some foodstuffs, it is recommended to perform lipid extraction before saponification. This refers to raw materials and products rich in carbohydrates and proteins. This results from the fact that the contained components equally readily undergo hydrolysis in the alkaline environment and products of this hydrolysis may disturb later extraction of unsaponifiable substances and their further chromatographic analysis. This pertains also to products with high water content and low contents of analyzed compounds (tocochromanols) (Eitenmiller and Lee 2004).

After saponification is complete the mixture is supplemented with water and an organic solvent. This results in the formation of two phases: aqueous (glycerol, soap, proteins, sugars, fiber, etc.) and organic (unsaponifiable substances: tocochromanols, carotenoids, sterols, etc.). The most commonly used organic solvents are diethyl ether, petroleum ether, and *n*-hexane (De Camargo et al. 2019). In the case of diethyl ether typically an emulsion is formed and the separation into two phases is not complete. Additionally, such extraction needs to be repeated three times in different separators and the combined extracts need to be dehydrated using

anhydrous sodium sulfate. This process is labor- and time-intensive, while at the same time burdened with the risk of losses in analyzed compounds through sample transfer from the vessel to vessel. It is a better solution to use *n*-hexane in extraction with the addition of ethyl acetate (Current Protocols in Food Analytical Chemistry 2003; ISO 2016). Ueda and Igarashi (1987) reported that *n*-hexane itself is a good solvent to extract α-T, while the other homologs are not 100% extracted. A change in polarity of the solvent through the addition of ethyl acetate improves the extractability of the other homologs from the mixture following saponification. In order to prevent the formation of emulsion instead of water, it is recommended to use a saline solution (NaCl), exhibiting demulsification properties. Siger et al. (2014) optimized the extraction process for unsaponifiable substances. Analyses were conducted on the effect of the amount of solvent (50–100 mL) and extraction time of unsaponifiable substances (30–60 min.). Extraction was run using *n*-hexane and a mixture of *n*-hexane with a 10–20% addition of ethyl acetate. It was stated that both the percentage share of ethyl acetate in the extracting mixture and the amount of used solvent have a statistically significant effect on the obtained results. Moreover, a statistically significant interaction was found between these two parameters. Additionally, no effect of extraction time (within the range of 30–60 min.) was observed on the values of results concerning the contents of tocopherols. An optimal addition of ethyl acetate is 10%, while the amount of solvent is 50 ml (Fig. 6.4). Panfili et al. (2003) compared three methods: "hot" alkaline hydrolysis (saponification followed by extraction with a solvent), extraction of the solvent without saponification, as well as extraction using methanol. These studies showed that the most efficient sample preparation method is provided by alkaline saponification followed by extraction with an organic solvent. Extraction without saponification releases many additional substances together with the lipid fraction, which in turn requires

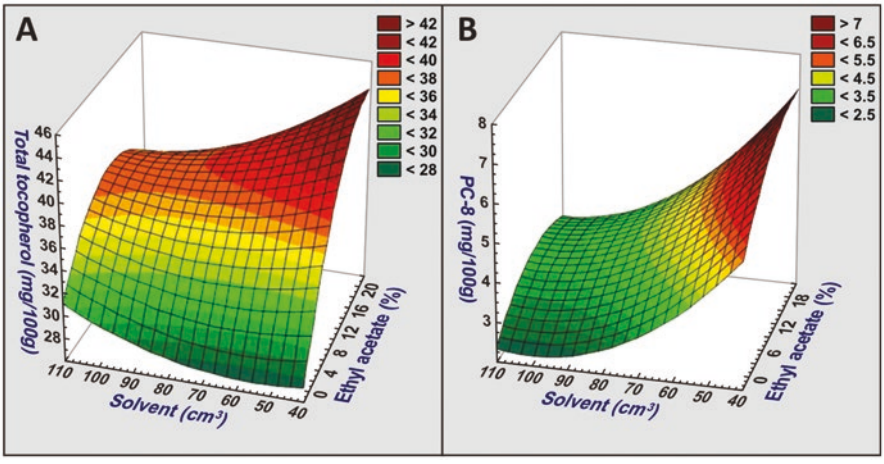


Fig. 6.4 Effect of the amount of solvent (*n*-hexane) and % share of ethyl acetate in the extraction mixture on the tocopherol and PC-8 contents (sample – seed of rape)

additional purification stages. The stage of alkaline hydrolysis releases tocochromanols from ester bonds or links with the experimental matrix. Moreover, all substances potentially disrupting the analysis are removed, e.g., soaps (Panfili et al. 2003). Also, *n*-hexane is used to extract tocotrienols, although – as it is reported by Choi and Lee (2009) – in the case of fractions rich in these compounds the use of methanol may be a better solution.

Attempts to reduce the consumption of solvents and time intensity of solvent extraction techniques have produced numerous modifications of conventional liquid–liquid extraction methods and solvent extraction from solid products. Proposed techniques include solid-phase extraction (SPE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), and matrix solid-phase dispersion (MSPD). These techniques have also been applied in the extraction of tocochromanols from raw materials and foodstuffs. SPE is an established technique of lipids extraction for their determination in different samples. It is nondestructive, which enables selective extraction preventing any chemical changes of the sample and analytes (Nolet 2012). In the case of tocochromanol extraction, various stationary phases have been used, e.g., silica cartridges, coarse-grained kieselguhr, C18, C8, C4, $C_6H_{11,}$ and HLB (Blake 2007; Fedder and Plöger 2005; Grigoriadou et al. 2007; Iwase 2000; Lechner et al. 1999; Papadoyannis et al. 1997; Sunarić et al. 2017). In turn, Sunarić et al. (2017) proposed solid-phase extraction for simultaneous and selective HPLC determination of α-T and α-T acetate in dairy products, plant milk, and dietary supplements. They analyzed several different available stationary phases (Oasis HLB, C18, C18ec, and HR-X). They showed that among the used substrates it was C18 that ensured the highest efficiency and thus it is the most popular sorbent for the analysis of fat-soluble vitamins. In contrast to saponification, the proposed SPE method facilitates the simultaneous assay of α-T and its ester. In comparison to liquid extraction, the proposed method does not require a large volume of harmful organic solvents and the evaporation stage. In the study by Capote et al. (2007) concerning the determination of fat-soluble vitamins in the human serum three sorbents were compared, including unterminated and terminated C18, and C8. As it was shown, the C8 bed was the most effective stationary phase to recover fat-soluble vitamins. Koštál et al. (2013) used an aminopropyl silica column to determine tocopherols. In turn, Beldean-Galea et al. (2010) investigated the efficiency of solid-phase extraction and its selectivity toward tocopherols from vegetable oils using four porous polymers (Porapak P, Porapak Q, Porapak QS, and Porapak N). The tocopherol elution from SPE cartridges was performed using several hexane:ethyl acetate mixtures (100:0, 95:5, 90:10, 85:15, v/v). Those authors showed that nonpolar and moderately polar polymers such as Porapak P, Porapak Q, and Porapak QS may be successfully used in solid-phase extraction of tocopherols. Polar polymers such as Porapak N provided less reliable results due to a very strong absorption taking place at their surface. The results confirmed that porous polymers represent promising SPE alternatives for the extraction of tocopherols from oils.

Shammugasamy et al. (2013) proposed a simple technique of sample preparation coupled with reverse-phase high-performance liquid chromatography. The sample

preparation procedure included small-scale hydrolysis of 0.5 g cereal samples through saponification, followed by extraction and condensation of tocopherols and tocotrienols from the saponified extract using dispersive liquid–liquid microextraction (DLLME). The developed DLLME method has been successfully applied in the case of cereals: rice, barley, oats, wheat, maize, and millet. Ultrasound-assisted extraction was applied by Nielsen and Hansen (2008) when analyzing tocochromanol contents in cereal grain. The extraction of bioactive compounds, which are sensitive, thermolabile, and found at low concentrations in food, indicated low efficiency of traditional techniques. Pressurized liquid extraction improves the efficiency of extraction by increasing solubility and mass transfer properties. PLE utilizes the effect of increased temperature and pressure. An advantage of pressurized extraction results from the fact that the temperature of extraction is not limited by the boiling point of the solvent. Thanks to the special design of the pressurized extraction apparatus the extracts are purer than those provided by conventional extraction. Delgado-Zamarreño et al. (2009) used this technique to extract tocopherols and tocotrienols from cereal grain and palm oil. Extraction was run under the following conditions: oven temperature 50 °C, pressure 1600 psi, and one static cycle with a static time of 5 min. The extraction solvent depends on the nature of the sample to be extracted. The sample was mixed with a desiccating agent before being placed in the extraction chamber to prevent the aggregation of sample particles and ensure optimal phase equilibrium between the sample matrix and the extraction solvent. The dependence between the amount of the sample and the analytical signal has to be linear. Those authors showed that it is linear up to 4.0 g for cereals and up to 0.8 g for palm oil. In PLE the use of solvents at elevated temperatures increases both the capacity of solvents to solubilize analytes and the diffusion rate. Also, high temperatures decrease the viscosity of solvents and debilitate strong solute–matrix interactions. We also need to take into consideration the thermal stability of the investigated compounds. The coupling of various sample preparation techniques is the latest strategy applied in analytical chemistry. A combination of PLE with a miniaturized analytical technique such as DLLME facilitates the extraction and determination of tocopherols and tocotrienols following the priorities of green chemistry. Such an analytical procedure was proposed by Viñas et al. (2014) to assay these compounds in plant origin foods. In turn, Müller et al. (2018) applied countercurrent chromatography (CCC) to isolate and purify 11′-α-tocomonoenol from the vitamin E extract of palm oil. That technique is successfully used to isolate and purify various natural and synthetic compounds from a mixture. In CCC the liquid stationary phase is held in a hollow tube wound around a coil, which rapidly rotates around a central axis while the liquid mobile phase is pushed through. The absence of a solid stationary phase in CCC leads to several benefits, i.e., high sample loads may be injected in a CCC system without time-consuming sample preparation steps (Ito 2005). Recently CCC has also been applied to isolate tocotrienols (Vetter et al. 2017).

Gas Chromatography

In the case of gas chromatography, it is required for the analyzed compounds to be volatile and stable at the temperature of analysis, thus limitations for the applicability of this technique are connected with volatility and thermal stability of analyzed compounds. The use of capillary gas chromatography (cGC) to assay minor components in oil matrices requires sample pretreatment consisting in the removal of the bulk lipid material. Sample purification is typically attained either through chemical modification, such as saponification, followed by column chromatography and thin-layer chromatography, or by applying sophisticated instruments, e.g., on-line LC–GC. Lechner et al. (1999) ran solid-phase extraction (SPE) before capillary gas chromatography. They developed a simple and reliable procedure to quantify both tocopherols and sterols within one analytical cycle. Ballesteros et al. (1996) employed cGC to determine cholesterol, α-tocopherol, and α-tocopherol acetate in edible oils, while triacylglycerols disturbing the assay were automatically discarded thanks to continuous on-line transesterification. The derivatization process is run in order to transform analytes through the introduction of chemical groups appropriate for a specific type of detection. Du and Ahnn (2002) when simultaneously assaying cholesterol, tocopherols, and sterols showed that derivatization is required to ensure adequate selectivity. Thus simultaneous derivatization of tocopherols, cholesterol, and phytosterols using Sylon BFT is a key element in sample preparation as it replaces active hydrogen in these compounds with a trimethylsilyl group improving their volatility and thermal stability while decreasing polarity and improving analysis of these compounds using GC. The derivatization process is performed in order to transform analytes through the introduction of chemical groups appropriate for a specific detection type. In the GC-MS analysis, derivatization is also carried out to enhance the sensitivity of mass detectors (Wells 1999). In the case of tocochromanol assays using gas chromatography, it is of considerable importance to ensure an anhydrous environment in derivatized samples, e.g., by adding pyridine. Water content in derivatized samples prevents an appropriate chemical conversion of the hydroxyl group in tocols and it may lead to hydrolysis of derivatizing reagents (Orata 2012). Analysis of tocopherols using GC requires the formation of silyl derivatives (Snyder et al. 1993). Silylation is commonly applied, particularly thanks to its simplicity and relatively short duration of the process; additionally, it is dedicated to compounds containing active hydrogen, including phenolic groups (Abidi 2000). Silylating agents, including derivatizing substances providing stable derivatives, include trimethylsilylate (TMS), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), MSTFA with trimethylchlorosilane (TMCS), and N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA). The latter in combination with TMCS ensures effective silylation of α- and γ-T, as well as their carboxyethyl hydroxychroman metabolites. Quantification of α-T and its derivatives in the human plasma may be performed using the Sylon BTZ mixture (Bartosińska et al. 2019). However, it needs to be done with caution, since at the application of GC in the analysis to derivatize TMCS, the peaks of α-T3 and campesterol partly overlap (Kim et al.

2012). At present, capillary columns are primarily used, as they provide superior resolution (precise separation of tocopherols) (Abidi 2000). Due to their lipophilic properties, tocol derivatives have to be separated using relatively nonpolar chromatographic stationary phases. In most cases, it is melted silica with bound dimethyl and phenyl groups. The sequence of elution for compounds separated on stationary phases depends on their boiling points and the same principle is true for tocochromanols and their derivatives (Stashenko and Martínez 2012). When a column with a nonpolar stationary phase is used, elution of tocopherols is run in the following order: δ-T, β-T, γ-T, and α-T (Melchert and Pabel 2000). Detectors used in the identification and quantification of tocochromanols for gas chromatography include flame ionization detectors (FID) and gas chromatography coupled with mass spectrometry (GC-MS) (Kadioglu et al. 2009). The former (FID) are universal detectors reacting with a signal to the presence of organic compounds. This is based on a change in electric conductivity of the flame atmosphere (hydrogen-air) at the appearance of an organic compound in the flame, which is formed in the process of carbon ion combustion. A signal from this detector is proportional to the number of carbon atoms unbound with oxygen, i.e., to the mass of the substance. This makes it possible to detect 10^{-12} g analyte. In the case of FID, nitrogen and helium are the most appropriate carrier gases (Szczepaniak 2008). Thanks to the advances in mass spectrometry (MS), in recent years the coupled techniques (hyphenated techniques) begin to predominate in food analysis. Coupled techniques constitute a combination of separation techniques, e.g., gas chromatography with detection methods of separated analytes, such as MS. Together with the development of capillary gas chromatography the GC-MS systems have become very common. Ionization in GC-MS is the ionization of the analyte in the gas phase and is run in a vacuum. The most commonly used type of ionization, electron ionization (EI), consists of bombarding analyzed molecules with a stream of electrons with specified energy (typically 70 eV). The main advantage of this ionization type is connected with the repeatability of spectra, which has facilitated the creation of free-access mass spectral libraries (NIST Wiley). As a result, GC-MS is a common tool in the identification of unknown organic compounds (Plutowska and Jeleń 2017). Fragmentation of underivatized tocopherols apart from precursor molecular ions ($M^{+\bullet}$) makes it possible to observe several diagnostic fragment ions. In a study by Fiorentino et al. (2009) α-T, apart from the molecular ion at m/z 430, fragment ions were also identified at m/z 205 and m/z 165 originating from 6-hydroxy-2,5,7,8-tetramethylchroman-2-ylium and (2,5-dihydroxy-3,4,6-trimethylphenyl)methylium cations. In the case of δ-T, a molecular ion was detected at m/z 402. As a result of the fragmentation of the generated molecular ion, peaks were identified at m/z 177 and m/z 137 coming from the 6-hydroxy-2,8-dimethylchroman-2-ylium cation (formed as a result of the fragmentation of the C2-C1' bond) and from the (2,5-dihydroxy-3-methyl-phenyl)methylium cation. Both in the case of α-T and δ-T, those authors identified fragment ions at m/z 43 originating from the isopropylium cation – the result of the fragmentation of the C11'-C12' bond. In the case of a new tocol derivative, which they identified as δ-tocomonoenol, the precursor ion was found at m/z 400. The fragmentation of the molecular ion provided identical peaks at m/z 177 and m/z 137, similarly as in

the case of δ-T, due to the chromane moiety of the molecule. In turn, those researchers also identified a diagnostic fragment ion at m/z 69 generated by the allylic 3-methylbut-2-en-1-ylium cation (cleavage of the C9'-C10' bond) (Fiorentino et al. 2009). When we analyze derivatized tocopherols, e.g., trimethylsilylated ethers, in mass spectra a characteristic feature will appear as a result of ion fragmentation at m/z 73, corresponding to the molecular mass of the trimethylsilyl group. The precursor molecular ions (m/z) in the case of silylated tocopherols are as follows: 502 (α-T), 488 (β-T and γ-T), and 474 (δ-T) (Bartosińska et al. 2019). In turn, using GC-MS Butinar et al. (2011) identified γ-tocomonoenol and α-tocomonoenol in pumpkin seed oil. A comparison of the MS spectrum of γ-tocomonoenol with that of γ-T shows that the only difference is its value of the molecular (precursor) ion, which is by 2 Da lower (m/z 486). The above-mentioned authors when analyzing mass spectra confirmed that the first double bond positions in gamma-tocomonoenol, gamma-tocodienol and alpha-tocomonoenol are found at C11'-C12' (generated as a result of ion fragmentation at m/z 69). In the analysis of tocochromanols, quadruple filters (Q) are the most commonly used analyzers in GC-MS sets (Butinar et al. 2011; Fiorentino et al. 2009; Lytovchenko et al. 2009; Melchert et al. 2002; Roessner et al. 2000). Other relatively popular analyzers include ion traps (IT), while recently also the time of flight analyzers (TOF). Kim et al. (2012) developed a rapid method to monitor bioactive compounds (including tocopherols and tocotrienols) in rice. Gas chromatography coupled to time-of-flight mass spectrometry was applied. That method was characterized by high efficiency, as 14 different compounds were identified in the course of a 13-min analysis. Although tocopherol derivatives yield almost identical mass spectra, the differences in retention are generally sufficient to provide chromatographically separated peaks. Peaks of α-T3 and campesterol were also completely separated based on the differences in the mass spectrum. The calibration curves of 14 lipophilic compounds measured under optimized saponification conditions were linear ($R^2 = 0.9958–0.9999$), while respective limits of detection (LOD) fell within the range of 0.01–0.05 μg. The recovery rates from rice flour ranged from 76% to 109%. The precision (RSD – relative standard deviation) of the method amounted to 1.5% for δ-T, 2.1 (β-T), 1.8 (g-T), 4.4 (δ-T3), 4.1 (β-T3), 3.1 (γ-T3), 11.1 (α-T), and 1.1 (α-T3). In turn, Melchert and Pabel (2000) used an ion trap as an analyzer with a simultaneous recovery using a complete MS scan and the selected ion monitoring (SIM) mode. The ions that can be used in identification and quantification procedures include: for TMS-α-T: m/z 236, m/z 237, m/z 277, and m/z 502; for TMS-β-T and TMS-γ-T m/z 222, m/z 223, m/z 263, and m/z 488; and for TMS-δ-T: m/z 208, m/z 249, and m/z 474. The limit of detection for all the investigated TMS-tocopherol derivatives was 40 pg in the SIM mode.

Liquid Chromatography

High-performance liquid chromatography has become the most commonly used technique in the quantification of tocochromanols in food. In liquid chromatography, molecules of the analyte are passed through a liquid mobile phase that participates in the separation mechanism. Knowing the composition and properties of the mobile phase one may influence the retention of analytes and the order of elution. As a consequence, a greater number of substances may be assayed using liquid chromatography compared to gas chromatography. Available literature contains an extensive body of data concerning chromatographic systems for the separation of tocopherols, tocotrienols, or both these groups jointly. Generally, in food analysis applying chromatography, it is rather the reverse phase system (C18), in which the mobile phase is more polar than the stationary phase. The reverse-phase system is characterized by greater stability of results and enhanced column stability. However, in the analysis of tocochromanols a drawback of the conventional reverse phase system (C18) is connected with the fact that homologs β- and γ-T as well as β-T3 and γ-T3 may not be separated (Current Protocols in Food Analytical Chemistry 2003; Pyka and Sliwiok 2001). For this reason, a normal phase system is applied (with the mobile phase being less polar than the stationary phase), where all the four homologs of tocopherols and tocotrienols may be separated (Amaral et al. 2005; Kamal-Eldin et al. 2000; Panfili et al. 2003). Using chromatography in the normal phase system, the sequence of tocochromanol elutions results first of all from their polarity. The least polar among all the homologs of tocopherols and tocotrienols are their α homologs and it is them that are eluted from the column as the first ones. The polarity of tocols is influenced primarily by the number of methyl groups in the chromanol ring, while it is modified to a lesser extent by steric effects of the methyl groups and slightly increased polarity of the unsaturated side chains of tocotrienols compared to those of tocopherols (Kamal-Eldin et al. 2000). The order of elutions in the normal phase chromatography is as follows: α-T, α-T3, β-T, γ-T, β-T3, γ-T3, δ-T, and δ-T3 (Kamal-Eldin et al. 2000). PC-8 is a compound with a long side chain and it may be easier to separate in the normal phase system. In the reverse-phase system, this chain reacts strongly with the substrate (e.g., C18) and it is difficult to elute it from the column. An additional advantage of the normal phase system is connected with the use of organic solvents ensuring high solubility of lipids, which facilitates direct analysis of oils and fats (Barros et al. 2008; Kamal-Eldin et al. 2000). Sanagi et al. (2006) analyzed the separation of tocochromanols (without PC-8) in the normal phase system using the silica and the amine columns while testing the mobile phase of *n*-hexane with different additions of more polar reagents (diethyl ether, ethyl acetate, isopropanol, 1,4-dioxane, and 1,4-dioxane with isopropanol and 1,4-dioxane with ethyl acetate) at concentrations ranging from 0.2% to 5%. They showed a greater efficiency for the column with a silica bed, while the most distinctly separated peaks were obtained using the mobile phase of *n*-hexane with 1,4-dioxane 96:4 (v/v) at a flow rate of 1 mL min^{-1}. Other authors confirmed that the silica column is most effective for the separation of tocochromanols

(Kamal-Eldin et al. 2000). Seven columns with the silica bed, three columns with diol packing, and an amine column with various mobile phases were compared. The best separation parameters and the shortest time of analysis were recorded for three silica columns, i.e., Altima SI 5 U (Alltech, 250 × 4.6 mm, 5 μm), Inertsil SI (Chrompack, 250 × 4.6 mm, 5 μm), and Genesis silica (Jones, 250 × 4.6 mm, 5 μm). In all these cases the best mobile phase consisted of a mixture of *n*-hexane with 1,4-dioxane 96:4 (v/v) (Kamal-Eldin et al. 2000). A similar analysis was conducted by Amaral et al. (2005) using the mobile phase of *n*-hexane with 1,4-dioxane at concentrations of 2.5–5%. A short separation time (22 min.) and good peak resolution were obtained using 3.5% 1,4-dioxane on the Inertsil 5 SI column (250 × 3 mm, Middelburg, Holland) at room temperature and for such parameters, validation was performed. The coefficient of variation ranged from 4.5% to 5.64% depending on tocopherol homologs. Similarly, the recovery rate fell within the range of 93–104%. The normal phase system has certain limitations resulting from poor stability of silica phases, inadequate repeatability of chromatography conditions, mainly as a result of long equilibration times, as well as the application of volatile and toxic mobile phases. Also, the nonaqueous mobile phases are not compatible with electrochemical detection (Abidi 1999, 2000). However, as reported by Gotoh et al. (2011), in NP-HPLC separation of α-T and marine-derived tocopherol (MDT), is not possible due to the identical structure of the chromanol ring. Yamamoto et al. (1999) separated α-T and MDT using reverse-phase liquid chromatography (RP-LC) on an analytical column (Supelcosil LC-18, 5 μm, 250 × 4.6 mm, Supelco) and measured by amperometric electrochemical detection. The oxidation potential in electrochemical detection (ECD) was maintained at +600 mV (vs. Ag/AgCl) on a glassy carbon electrode.

An example separation of tocopherols from actual samples (vegetable oils) applying HPLC in the reverse phase system (C18) was conducted by Warner and Mounts (1990). As a result of optimization conducted on the column, the authors selected separation parameters: the column thermostated at 25 °C, the mobile phase of acetonitrile/tetrahydrofuran/water (60:25:15 v/v) at a flow rate of 0.7 mL min^{-1}. While they obtained separate peaks for β-T and γ-T, the retention times of these compounds were too similar (19 and 21 min, respectively). Since this separation was run in the reverse phase, actual samples required additional purification processes before injection onto the column, as otherwise they could not be dissolved directly in the organic solvent being at the same time also the separation medium for tocopherols. Pyka and Sliwiok (2001) in their studies compared the two separation methods, i.e., the reverse and normal phase systems. In the former the mobile phase consisted of a mixture of water with ethanol (solvent gradient 0–10%) as well as water with methanol (10%), the flow rate was established at 1.5 mL min^{-1}. In the case of the normal phase, the mobile phase was *n*-hexane with isoamyl alcohol 99.5:0.5 (v/v) at a flow rate of 2 mL min^{-1}. At the same time, those authors stated that for quantitative and qualitative analyses of tocochromanols the normal phase system is optimal (peaks are adequately separated). The reverse-phase system using conventional column packings (ODS – octadecylsilane) is used for certain practical reasons, such as easy equilibrium of the stationary phases, repeatability of analyses,

low volatility of solvents used as the mobile phase, and limited use of toxic solvents (Abidi 2000). In this system using standard C8 or C18 columns, the phases may not guarantee complete selectivity (no separation of β and γ homologs). These homologs may not be separated using standard solvents such as an aqueous solution of methanol or acetonitrile (Gruszka and Kruk 2007). ODS columns may be used in the so-called nonroutine applications, where isopropanol and water are used as the mobile phase. Under such conditions these two homologs were separated; however, separation lasted very long and the system generated very high pressure at a very low flow rate of the mobile phase through the bed on the column (Satomura et al. 1992; Abidi 2000). Homologs β and γ in the reverse phase system may be separated in the reverse phase system using new stationary phases: silica modified by long alkyl chains (C30), beds not based on the silica gel, and polyvinyl alcohol, perfluorine silica gel modified by phenyl groups (Abidi and Mounts 1997; Richheimer et al. 1994; Stöggl et al. 2005). Commercially available columns with the C18 stationary phase, despite identical characteristics (length, diameter, grain size, and pore size), differ greatly in terms of their specific surface area, which may range from 150 to 400 $m^2\ g^{-1}$. Irakli et al. (2012) optimized separation on the PerfectSil Target ODS3 column (250 × 4.6 mm, 3 μm, MZ-Analysentechnik, Mainz, Germany) in the solvent concentration gradient (isopropanol/water 25–10% – in order to reduce the time of analysis) and at different thermostat temperatures of the column (5–25 °C). They used the stationary phase composed of ultra-pure silica gel (>99.999%) of ideal chemical and mechanical stability guaranteeing highly symmetric peaks, with grain specific surface area of 450 $m^2\ g^{-1}$ (Irakli et al. 2012). The temperature of 7 °C proved to be optimal and it was selected as a compromise between efficiency, resolution, and time of analysis. Despite optimal parameters, the separation of all homologs of tocopherols and tocotrienols lasted 1 h.

Stöggl et al. (2001) compared the potential of NP and RP-HPLC coupled with UV and fluorescent detection for qualitative and quantitative analyses of vitamin E in various samples. They showed greater selectivity of NP-HPLC in comparison to RP-HPLC. It was shown that the fluorescent detector (λ_{ex} = 295 nm; λ_{em} = 330 nm) is more selective and ten-fold more sensitive than the spectrophotometric detector (λ = 295 nm). In the case of the reverse phase system (RP), the order of elutions results from their different polarity and the differences in the saturation of the side chain (Stöggl et al. 2001). The selection of an appropriate detector is of considerable importance due to the chemical characteristics of vitamin E derivatives, their structural similarity, and variable concentration levels. Most analytical procedures assaying tocochromanol contents are based on liquid chromatography coupled with versatile detection types. To date, numerous publications have been devoted to assays of tocopherols or tocotrienols using diode array (DAD) or fluorescence detectors (FLD) (Górnaś et al. 2014a, b, c, 2019; Li et al. 2017; Nielsen and Hansen 2008; Siger et al. 2014, 2015, 2018; Sunarić et al. 2017). Ultraviolet detection is generally characterized by low sensitivity as well as limited selectivity. Tocochromanols absorb UV light at λ = 290–300 nm, but maximum absorbances are so small that UV absorption may be used only to detect and quantify tocochromanols in these samples, in which their content is high, e.g., vegetable oils (AOCS

1990). Recently UV detection has been of much lesser importance due to the much higher sensitivity of such detection techniques as fluorescence or mass spectrometry. Better sensitivity and higher selectivity are provided by fluorescence detectors and this is the technique recommended for most biological samples. In the case of tocochromanols, the excitation wavelength is 290–296 nm and the emission wavelength is 325–330 nm (Schwartz et al. 2008). The electrochemical detection was applied in the studies on tocochromanols by Delgado-Zamarreño et al. (2009) and Yamamoto et al. (1999). ECD is a useful technique for the selective detection of antioxidants, because such molecules may be oxidized at a low potential with high molar sensitivity (Yamamoto et al. 1999). Lee et al. (2018) presented a method to assay tocopherols, tocotrienols, and their chain-degradation metabolites using HPLC coupled with the Coulochem Electrode Array System (CEAS). The CEAS detection system provides high sensitivity, comparable to MS detection or fluorescence, but it is much more sensitive than UV absorption. A limitation of CEAS is connected with the fact that it does not supply structural information, which may be provided by MS, while the electrochemical technique may detect only active redox forms of vitamin E and their metabolites. The described procedure is very sensitive, easy to perform, and relatively inexpensive method for routine analyses of tocopherols, tocotrienols, and their metabolites for a large number of samples in the laboratory and epidemiological studies. In turn, Cunha et al. (2006) in the quantification of tocopherols and tocotrienols in olive oils applied the HPLC system with three different detection systems: fluorescence and diode array coupled with series, ultraviolet, and evaporative light scattering (ELSD). They stressed the importance of an appropriate detection method to be selected for the analyses. Those authors showed that the HPLC/diode array/fluorescence detection seems to be a useful tool in the evaluation of tocopherol and tocotrienol profiles of oils. Thanks to the combination of a more universal detector (diode array), with a more specific one (fluorescence detector) more information is obtained from one analysis and thus the identity of tocopherols and tocotrienols may be directly confirmed. At the same time, those authors were of an opinion that quantification of tocopherols using ELSD in oil samples was considerably hindered by the presence of other compounds co-eluting with analytes. Also, Rupèrez et al. (2001) reported poor sensitivity and selectivity of the ELSD detector in the analysis of tocochromanols. Mass spectrometry is also of considerable importance in tocochromanol analyses. The rapid development of LC-MS has been accomplished, thanks to advances in atmospheric pressure ionization (API) methods. The most commonly applied techniques include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). Negative ion APCI is considered a method of choice for the detection and quantification of tocopherols (Lanina et al. 2007). Those authors compared ESI and APCI ionization techniques both in the negative and positive ion mode for simultaneous LC-MS analysis of four tocopherol homologs (α-, β-, γ-, and δ-T). Both ESI and APCI ionization of tocopherols in the positive ion mode showed low efficiency and involved two competitive mechanisms using protonated molecules and molecular ions, which had an adverse effect on the repeatability of the MS signal. Ionization in the negative ion mode proved to be

more efficient both in ESI and APCI, as it generated only target deprotonated molecules. The negative ion APCI showed a greater range of linearity, lower limits of detection, and lesser sensitivity to differences in the chemical structure of tocopherols and the nature of applied solvents in comparison to the negative ion ESI method. The base separation of tocopherols was run on the Fluophase PFP (pentafluorophenyl silica-based) column. The limit of quantification was 9 ng mL^{-1} for α-T, 8 ng mL^{-1} for β-T, and γ-T as well as 7.5 ng mL^{-1} for δ-T. This method has been successfully applied to assay tocopherols in sunflower oil and milk (Lanina et al. 2007). Viñas et al. (2014) confirmed that the combination with LC using a dimethylpentafluorophenylpropyl stationary phase provides separation of all the isomers with good resolution. The agreement between fluorescence spectra, the expected retention time, and APCI-MS spectra ensures reliable identification of different vitamin E forms in food samples. The limits of detection were within the range of 0.2–0.3 ng mL^{-1} for tocopherols with base-line resolution. Tocochromanols cause problems in ionization since they do not have polar groups capable of protonation. The number of methyl groups in chromanol seems to be crucial for the effectiveness of the ionization process in the case of ESI (Lanina et al. 2007), in contrast to APCI sources, where ionized compounds are influenced by various mechanisms (Hao et al. 2005). For this reason, the head structure of tocol may not be significant for the ionization process (Lanina et al. 2007). The temperature of the vaporizer is the primary parameter responsible for the quality of the analytical signal during the ionization of tocopherols and tocotrienols. Inadequate adaptation of this parameter may lead to degradation of the analyte. Electrospray ionization requires the introduction of an electrolyte into the mobile phase or as a post-column addition, in order to enable detection of natural tocochromanols (Bartosińska et al. 2019). With ESI the ionization of tocopherols may be improved by a post-column addition of silver ions, which brings about Ag^+ adducts of vitamin E constituents with little fragmentation (Rentel et al. 1998). Moreover, the fragment-ion spectra of the tocopherols investigated may be used as "fingerprints" for the elucidation of complex samples of biological origin (Stöggl et al. 2001). Quadrupole analyzers are most commonly used in LC-MS systems for tocochromanol analysis. This type of analyzer acts as a "mass filter," facilitating the complete scanning mode of a given sample, while it also operates in the SIM mode. The former type of MS scanning makes it possible to find molecular or pseudo-molecular ions generated during ionization, while the latter ensures sensitive quantification (Delgado-Zamarreño et al. 2009; Lanina et al. 2007). With the available standards of tested compounds, it is very easy to optimize the chromatographic system for analysis of these compounds. Recently studies have been published on the identification of new tocol derivatives – in this case, to confirm the structure of investigated compounds, analytical tools are required to confirm and identify their molecular formula. Lately, many systems have been designed for tandem mass spectrometry, where the second analyzer is a high-resolution device (40000–240,000), typically a TOF or Orbitrap (Plutowska and Jeleń 2017). In turn, Tanno et al. (2020) used liquid chromatography-tandem mass spectrometry (quadrupole/linear ion-trap tandem mass spectrometer – QTRAP) to analyze oxidation products of α-tocopherol in extra virgin olive oil. Montero et al. (2012)

developed a method for the simultaneous determination of lipoic acid and/or Trolox methyl ether, along with α-, γ-, and δ-tocopherol using liquid chromatography-tandem mass spectrometry with negative electrospray ionization (HPLC-ESI-MS/MS) in an ion-trap mass spectrometer. Detection and quantification were accomplished by a multiple reaction monitoring method, using specific transitions from the precursor ion to the product ion for each analyte. Those authors showed that ion reactions and fragmentation of γ- and δ-tocopherols are dependent on the methylation of the phenol ring. Gee et al. (2016) for the separation of tocochromanols contained in palm oils applied ultra-performance convergence chromatography (Acquity® UPC^2) with a chromatographic runtime of 5.5 min. Apart from tocopherols and tocotrienols they also identified α-tocomonoenol and α-tocodienol. For this purpose, they also used liquid chromatography-tandem mass spectrometry (Xevo G2-S quadrupole time-of-flight (Q-TOF)). The spectrum obtained from high-resolution mass spectrometry made it possible to clearly distinguish three fragments of m/z 137 (exact masses m/z 137.0603, 137.0966, and 137.1330) and two fragments of m/z 69 (exact masses m/z 69.0704 and 69.0340). Those authors claimed that the position of the double bond in α-tocodienol may not be determined using low-resolution mass spectrometry because these instruments are not capable of separating ion fragments of similar mass.

Mass spectrometry identification of PC-8 has been carried out mostly by researchers studying plant development metabolites. Zbierzak et al. (2010) examined the PC-8 isolated from linseed oil as a natural source of this compound during studies of the plastoquinol metabolic pathways. PC-8 was identified in that work using Q-TOF by direct infusion of this compound dissolved in chloroform/methanol/ammonium acetate (300:665:35). Those authors obtained the cation radical molecular ion $M^{+\bullet}$ at *m/z* 750.63 and its fragmentation yielded product ions at *m/z* 191.10 and 151.07, respectively. This fragmentation pattern corresponded to the release of the side chain and the chromanol ring degradation. Martinis et al. (2011) used the APCI mode for the PC-8 mass spectrometric analysis in prenylquinone profiling of plant tissues. PC-8 was initially separated on the RP-UPLC system equipped with a C18 column using water/methanol (90–100%) as solvents. They obtained ions of protonated/deprotonated molecules ($[M + H]^+$ and $[M-H]^-$) at *m/z* 751.63 and 749.62, respectively. Only fragmentation of the negatively charged ions generated ions at *m/z* 734.60 and 149.06, which were used for confirmation of PC-8. In contrast to both the above-mentioned examples of mass spectrometry identifications of PC-8, in another study by Siger et al. (2014) this compound was dissolved in *n*-hexane before the direct infusion to the spectrometer. Similarly to PC-8 identification presented by Martinis et al. (2011), they obtained only the m/z 751.6 ion in the positive ionization mode, whereas in the negative mode in addition to the ions at m/z 749.4 and 734.4 they obtained the product ions corresponding to the stepwise release of 8 isoprene units (successive losses of neutral fragments of 68.0 Da) from the PC-8 side chain.

Since tocochromanols exhibit similar structural characteristics, in the course of analysis employing tandem mass spectrometry, the fragmentation processes prevent

their erroneous identification. Nevertheless, fragmentation may be dependent and characteristic to a given apparatus, ion sources, and ionization mode.

Spectroscopic Methods

Although the determination of tocopherols using HPLC is the "gold standard" in the analysis of these compounds, spectroscopic methods are also being developed. Among the spectroscopic methods, the most popular is the near-infrared spectrometry (NIR). Infrared radiation (IR) is an electromagnetic wave with wavelengths longer than visible light (Vis) and shorter than microwaves. In IR spectroscopy wavelengths in the range 0.8–100 μm are used, wherein this value can be expressed as frequency. A molecule can absorb infrared radiation if its charge distribution (electric dipole moment) changes during vibration. In IR spectrometers Fourier transform (FT) is applied. In this technique, all wavelengths reach the detector simultaneously and mathematical calculations (Fourier transform) are used to convert results into a typical infrared spectrum. Thus, spectra may be obtained faster, with a better signal-to-noise ratio in comparison to dispersive instruments. The near-infrared (NIR) spectral region (0.8–2.5 μm) is often used in the quantitative analysis of food compounds. The main advantage of NIR spectroscopy is connected with its ability to measure the content of substances directly in solid products using the diffuse reflection technique. Radiation that penetrates through the surface of the product is reflected off several sample particles before leaving it. Each time the NIR interacts with molecules in the sample, a part of the radiation is absorbed. Based on the amount of energy absorbed at a specific wavelength, the concentration of a compound in the sample may be calculated (Wehling 2010). Using the diffuse reflection technique the content of tocopherols in different food products and raw materials may be rapidly determined. This method requires either no extraction or other sample preparation procedures, or sample preparation is minimal (e.g., milling of oilseeds). On the other hand, the accuracy of these methods is lower than that of HPLC. Therefore, the results obtained are sometimes only an estimation of tocopherol content. For this reason, the reflection technique is useful in the determination of tocopherol contents in solid samples, for quality assessment in raw materials, as well as for screening tests. Due to the labor-intensive and time-consuming calibration (different matrices require separate calibration), this method is well suited for the determination of tocopherol in large quantities of the same type of material (e.g., in industrial laboratories). However, when the calibration is ready, the measurements of the samples take much less time than, e.g., HPLC assays. Also, no solvents or chemicals are consumed. Gotor et al. (2007) developed a near-infrared reflectance spectrometry method to determine tocopherol in sunflower seeds. To develop this method, the authors used approximately 1000 samples of ground seeds. Milled samples were scanned using a NIR spectrometer at 2 nm intervals from 400 to 2500 nm and tocopherols were determined using a standard reference method (HPLC coupled with a fluorescence detector). For NIR calibration, prediction

equations were calculated with a modified partial least-squares regression (MPLS) model after four outlier elimination passes. To predict tocopherol contents, an 860-spectra database was used for the calibration set and the content of total tocopherol ranged from 175 to 1005 mg kg^{-1} of oil. The developed NIR reflectance spectrometry method showed a relatively good correlation (R^2 = 0.64) between the content of total tocopherol predicted by NIR and determined using HPLC. That is why this method may be useful to classify seeds with high and low tocopherol contents. González-Martín et al. (2006) applied NIR spectroscopy to determine tocopherols in animal feeds. The apparatus was equipped with a remote reflectance fiber-optic probe. Before measurement, the samples were not subjected to any pretreatment. As a reference method liquid chromatography was used. The samples before HPLC analysis were hydrolyzed and tocopherols were extracted from the unsaponifiable material. The determined tocopherol content ranged between 0.06 and 1.87 mg 100 g^{-1}. In the discussed study, the MPLS method was applied to obtain NIR equations for all studied parameters. The NIR residuals at each wavelength (obtained after each factor was calculated) are standardized (divided by standard deviations of the residuals at each wavelength) before the next factor is calculated. The MPLS regression models accounted for 98% of the variance in the samples with eight principal components. The difference found between the chromatographic reference method and NIR spectroscopy in the external validation was 20% for α- and (β- + γ-) tocopherols and 15% for δ-tocopherol, when the tocochromanol content range was similar to that in the samples of the calibration group. NIR reflectance spectroscopy was applied by Xu et al. (2019) to distinguish variations of tocopherol contents in rapeseed (*Brassica napus*) genotypes of different origins. As in the case of the above-mentioned studies, the MPLS model was used for calibration. The determination coefficient of cross-validation R^2, showing the correlation between predicted and measured values for total tocopherols in rapeseed, was 0.64. The authors concluded that the presented NIR reflectance method has the potential to evaluate tocopherol contents in rapeseed seeds with sufficient accuracy and may be used as a screening tool in *Brassica napus* breeding programs designed to increase tocopherol contents in seeds. Cayuela and García (2017) used near-infrared spectroscopy to grade olive oil quality based on the tocopherol content. The partial least squares (PLS) model proved to be suitable to estimate α-tocopherol as well as total tocopherol content in olive oils. This model provided 99.9% success in the olive oil sorting test, in which the samples were divided into two classes depending on the α-tocopherol concentration. The content of α-tocopherol in commercial edible oils (sunflower, soybean, corn, rapeseed, grapeseed, olive oil) and a mixture of vegetable oils (a mix of rapeseed, soybean, and sunflower oils) after extraction with ethanol was determined using NIR and the PLS model (Szłyk et al. 2005). Based on the spectral region of 6500–4500 cm^{-1} a chemometric calibration model for standard solutions of α-tocopherol (0.54–53.54 mg mL^{-1}) was prepared. The determined contents of natural α-tocopherol in different samples of oils fell within the range of 17.53–57.10 mg 100 g^{-1}. The proposed NIR method was characterized by the following validation parameters: limit of detection (0.12 mg mL^{-1}), the limit of quantification (0.40 mg mL^{-1}), sensitivity (0.045 mg mL^{-1}), and selectivity (between

0.24% and 0.54% of the measured reflectance signal). Precision (RSD = 0.68–2.80%) and accuracy (recovery of 97.2–102.4%) for the applied NIR method was comparable to the reference HPLC method (0.79–3.06% and 96.8–103.2%, respectively).

Tocopherol exhibits intrinsic fluorescence at about 325 nm, with an excitation wavelength at 296 nm (Dwiecki et al. 2007). This property may be used to determine tocopherol levels in different matrices. It is worth mentioning that methods based on fluorimetric measurements are characterized by higher sensitivity compared to UV-Vis spectrophotometry. Demirkaya-Miloglu et al. (2013) applied spectrofluorimetric measurements for the rapid determination of α-tocopherol in pharmaceutical capsules and human plasma. Tocopherol was extracted from plasma with a hexane:dichloromethane mixture, whereas capsules were dissolved in ethanol, and the samples were sonicated and filtered. Fluorescence was measured at 334 nm with excitation at 291 nm. The calibration curve was linear in the concentration range of 0.25–2.5 μg mL^{-1} of α-tocopherol in both standard solutions and plasma samples. The precision of the assay was adequate because the relative standard deviation (RSD%) values for standard solutions and human plasma samples were below 6.76% and 7.94%, respectively. The recovery values of α-tocopherol from human plasma and pharmaceutical capsules for intraday and interday analyses were higher than 89.3% and 100.5%, respectively. The developed method was applied to determine α-tocopherol in the plasma samples of healthy volunteers and different types of bladder and stomach cancer patients, as well as in pharmaceutical capsules.

According to Escuderos et al. (2009), the band of the fluorescence emission spectrum, which is most accurately associated with the presence of α-tocopherol, extends in the range of 380–420 nm with excitation at 350 nm. In their study, virgin olive oil (cv. Cornicabra) was spiked with several quantities of α-tocopherol ranging from 25 mg kg^{-1} to 1200 mg kg^{-1}, and a ridge regression model (adjusted R^2 of 0.99) based on five wavelengths attributed to tocopherol fluorescence (370 nm, 371 nm, 378 nm, 414 nm, and 417 nm) was built. The tentative model was validated (adjusted R^2 of 0.87) with eight samples of virgin olive oil (cv. Picual) spiked with α-tocopherols ranging from 25 mg kg^{-1} to 250 mg kg^{-1}. The final model was successfully validated with seven monovarietal virgin olive oil samples from various countries (adjusted R^2 of 0.92). Presented results showed that the luminescent spectra of virgin olive oils may be used as the basis for a rapid evaluation method of α-tocopherol contents in olive oil samples. However, observed differences in the spectra of the right-angle measurement technique used, which may be not only due to the intrinsic fluorescence of α-tocopherol but also to light absorption together with artifacts associated with the technique, would explain a lack of linearity in the response of some samples at some wavelengths of the selected bands. It may be expected that these results could be improved using the front-face technique of fluorescence measurements.

Górnaś et al. (2006) measured the fluorescence intensity of tocopherols present in cold-pressed sunflower oil at different degrees of autoxidation in the presence of the 1,2- dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) membrane. It turned out that tocopherol fluorescence intensity depends on the amount of oil as well as

the membrane (phospholipid) concentration in the sample. The addition of oil with an increasing peroxide value caused a decrease in tocopherol fluorescence due to its disappearance during the oxidation process. At a constant DPPC concentration the chromatographically determined amount of tocopherol in oil samples correlated with peroxide value and fluorescence emission intensity. Thus a calibration curve could be plotted, which may be used for fast and accurate determination of peroxide value using the fluorescence technique.

Tocopherol may be applied as a fluorescent probe sensitive to its microenvironment (Dwiecki et al. 2007). Fluorescence parameters (fluorescence emission intensity, shifts in the emission maxima, fluorescence lifetimes, fluorescence anisotropy) may vary depending on properties of the molecule microenvironment (viscosity, presence of hydrogen bonds, the dielectric constant of the microenvironment, formation of dimers or aggregates, etc.); (Dwiecki et al. 2007; Jemioła-Rzemińska et al. 2003; Kruk et al. 2006). For this reason, intrinsic fluorescence may be applied to determine the location of tocopherol in heterogeneous media or interactions with surrounding molecules. Dwiecki et al. (2007) applied α-tocopherol intrinsic fluorescence and its quenching to estimate the tocopherol position in the egg phosphatidylcholine membrane. In the lipid membrane, concentration-dependent tocopherol fluorescence quenching was observed. Fluorescence coming from the α-tocopherol dimer is much weaker than that arising from the monomer. The observed fluorescence quenching suggests that the emission originates from the structures with high local tocopherol concentrations and may arise from α-tocopherol domains forming in the lipid membrane. According to Ausili et al. (2017) quenching of α-tocopherol intrinsic fluorescence by acrylamide in membranes made from different unsaturated phosphatidylcholines was ineffective, indicating that the chromanol ring responsible for emission was poorly accessible for this hydrophilic quencher. The authors claimed that α-tocopherol is located in the chromanol ring within the hydrophobic part of the bilayer, but not far from the lipid–water interface. Based on fluorescence measurements carried out in organic solvents and fluorescence lifetimes of α-tocopherol in liposomes, Kruk et al. (2006) estimated the relative distance of its fluorescent ring from the lipid (liposome) membrane surface. In a study performed by Jemioła-Rzemińska et al. (2003), the steady-state fluorescence anisotropy measurements of α-tocopherol incorporated into phospholipid liposomes revealed its motional freedom in the membrane. It was found that tocopherol shows very low mobility in the lipid bilayer. Intrinsic fluorescence of α-tocopherol was used by Aranda et al. (1989) as a tool to study its location and dynamics in phospholipid vesicles. It was concluded from the absorption spectra that a majority of α-tocopherol molecules are hydrogen-bonded, although the aggregates formed are fluorescent. Based on fluorescence parameters calculated in different solvents it was found that α-tocopherol is situated in a polar region of the membrane. Fluorescence quenching and resonance energy transfer showed that chromanol moiety of tocopherol is located in a position close to that occupied by the probes: 7-(9-anthroxyloxy)stearic acid (7-AS) and 5-(*N*-oxy-4,4-dimethyloxazolidine-2-yl)stearic acid (5-NS) in the bilayer. Additionally, quenching of tocopherol fluorescence by the 5-NS spin probe

was used to calculate its lateral diffusion coefficient in the membrane. The obtained value of $4.8 \cdot 10^{-6}$ cm^2 s^{-1} shows a very high lateral diffusion of α-tocopherol.

Tocopherols, tocotrienols, chlorophylls, and phenolic compounds are natural fluorophores present in vegetable oils. Emission derived from these compounds is used to prepare synchronous fluorescence spectra. These spectra are collected by simultaneously scanning the excitation and emission monochromators. During measurements, a constant difference between excitation and emission wavelengths is maintained. Synchronous fluorescence spectroscopy combined with chemometrics/statistical methods, e.g., multivariate data analysis, is used for the qualitative and quantitative determination of adulteration in vegetable oils. Applications of these methods include classification of oils produced from various raw materials such as soybean, sunflower, rapeseed, peanut, olive, grapeseed, linseed, and corn (Sikorska et al. 2005), authentication of adulteration in vegetable oil with refined spent frying oil (Tan et al. 2017), or identification of adulteration in cold-pressed grapeseed oil with refined soybean oil (Karuk Elmas et al. 2019).

Spectroscopic methods are relatively simple and fast, but their drawback is connected with their low specificity. In such a case interfering substances present in the sample matrix may influence determination results. This is primarily due to the additive nature of the Lambert-Beer law, on which spectroscopic quantitative measurements are based. Such a phenomenon is particularly evident in the case of NIR methods, where the results obtained are sometimes only an estimation of tocopherol contents. High-performance liquid chromatography is usually characterized by high specificity, facilitating the simultaneous determination of different tocopherol homologs. However, a drawback of chromatographic methods is connected with the relatively long analysis time, the use of expensive equipment, and large amounts of solvents. For this reason, new specific methods for the determination of tocopherols are still being developed. Such methods should combine the advantages of spectroscopic and chromatographic techniques. A promising solution is provided by the use of quantum dots as sensors in fluorimetric methods. Quantum dots (QD) are semiconductor nanocrystals with a diameter of 1–20 nm, which mainly consist of elements from groups II–IV, II–V, and IV–VI of the periodic table. The most commonly synthesized quantum dots are those composed of CdSe and CdTe (Dwiecki et al. 2015; Llorent-Martínez et al. 2013). Due to their small size, quantum dots have discrete energy levels similar to those found in atoms. Photoluminescence of these structures depends on their size, therefore it is relatively easy to obtain dots emitting electromagnetic radiation of different wavelengths. In comparison with organic fluorescent probes, quantum dots are characterized by relatively narrow photoluminescence emission bands, high quantum yield, long emission lifetime, high stability, and photobleaching resistance. Due to the small particle size, the area to volume ratio for quantum dots is relatively high. With the decreasing size of dots, an increasing number of atoms is present on their surface. Interactions between atoms on the QD surface and surrounding molecules in solutions may significantly affect photoluminescence (PL). This property is the basis for the use of quantum dots in chemical analytics (Zhang et al. 2010). In analytical methods based on

photoluminescence of quantum dots, interactions between the analyte and QD surface lead to a measurable change of PL intensity.

A sensor based on a molecularly imprinted polymer (MIP) linked to CdSe/ZnS quantum dots was applied by Liu et al. (2014) to determine tocopherol content in rice. Molecular imprinting is a simple and efficient method for the formation of selective recognition sites in a stable polymer matrix. Thanks to this method a MIP may be obtained. The molecularly imprinted polymer induces highly specific binding sites by template-directed cross-linking of functional monomers. A molecularly imprinted polymer based on quantum dots as a sensor for the detection of analytes from complex matrices has combined advantages of a highly selective MIP and sensitive QD. In the discussed study, MIP was linked to CdSe/ZnS quantum dots by simple embedding. Then the carboxyl group of the functional monomer (methacrylic acid) reacted with the phenolic hydroxyl group of template molecules (tocopherol) through hydrogen bond interactions. After the tocopherol molecule was extracted from the composites using methanol to decompose the hydrogen bond, distinct binding sites of tocopherol molecules were formed. The relative fluorescence intensity of the obtained sensor decreased linearly ($R^2 > 0.99$) with the increasing tocopherol concentration in the range of 1.16×10^{-7}–1.74×10^{-3} mol L^{-1} and the detection limit was 5.80×10^{-8} mol L^{-1}. Precision for five replicate detections of 1.0×10^{-4} mol L^{-1} tocopherol was 2.17% (RSD – relative standard deviation). Recoveries in the range of 90.4–100.2% were provided by direct detection when a QD sensor was used for selective determination of tocopherol in rice samples.

Finally, a hybrid "capture−detection" molecularly imprinted polymer−surface-enhanced Raman spectroscopic biosensor (MIPs-SERS) was developed by Feng et al. (2013) to assay α-tocopherol in vegetable oil. Tocopherol was used as a template for MIP synthesis, whereas methacrylic acid was formed as a functional monomer. Ethylene glycol methacrylate served as the cross-linking agent and 2,2′-azobisisobutyronitrile was the initiator. The obtained MIP was capable of fast and selective absorption and separation of α-tocopherol from oil components. A dendritic silver nanostructure synthesized by the displacement reaction was used as a SERS substrate to enhance the Raman signal. Second-derivative transformations and chemometric models based on SERS spectral features were applied for a rapid (15 min or less) and precise determination of tocopherol in four different sources of vegetable oils.

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Chapter 7
Cyclitols – Determination in Food and Bioactivity in the Human Organism

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Abstract Cyclitols are high polarity polyols, belonging to secondary metabolites, which are easily isolable from plant material. These compounds are biosynthetically derived from glucose, and occur in all living cells. They form a group of biologically active compounds and participate in many cellular processes such as: membrane biogenesis, signal transduction, ion channel physiology, osmoregulation, antioxidation, and others. The methods for the separation and determination of cyclitols include: preliminary sample preparation (drying, grinding, freezing,

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homogenization), sample extraction (optional: maceration, Soxhlet, ASE, MAE, SFE, UAE), isolation and purification (LLE, SPE, and others), analysis (GC-MS, HPLC, MALDI), and interpretation (identification, quantification, statistical analysis). Some other additional steps may be required, which are specific for the method chosen for the analysis. For example GC-MS analysis requires a derivatization step, while MALDI involves a laborious procedure for sample preparation. Cyclitols shows several health-promoting and therapeutic properties as follows: improving lipid profile in decreasing of serum triglycerides and total cholesterol, as well as having an insulin-mimetic effect. The beneficial properties of cyclitols are that they can be used for therapeutic purposes, particularly in neutralization of the results of chronic inflammation, which leads to increased risk of developing such metabolic disorders as diabetes, hypertension, atherosclerosis, etc. A notable factor is also good tolerance of cyclitols and their low toxicity, due to which they can be successfully used in treating pregnant women and children. Many benefits of cyclitols properties offer the possibility that these polyols may be candidates for discovery of novel drugs, food supplements and they can be used as well in cosmetic industry.

Introduction

Due to the important characteristics of widely unexplored cyclitols, a comprehensive review including their sources, functions, properties, and bioactivity in the human organism is required. From the point of view of the chemical structure, cyclitols are cycloalkanes with a hydroxyl group on three or more atom rings (hexahydroxycyclohexanes), also called cyclic polyols or sugar alcohols, naturally occurring in plants, tissues of mammals, fungi, and in some bacteria (Al-Suod et al. 2017; Owczarczyk-Saczonek et al. 2018a). Probably the cyclitols were described for the first time in 1955 in the French journal "*Helvetica Chimica Acta*" where the authors described their separation on paper chromatography using various solvents (Posternak et al. 1955).

In plants, cyclitols have no fundamental role in life processes maintenance; however, they play a crucial role in plant adaptation, self-defense, and interactions with its surrounding environment. Consequently, a large body of literature reported cyclitols as agents conferring protection against unfavorable environmental conditions such as ionic or osmotic stress (Al-Suod et al. 2019a; Hossain et al. 2017; Ncube et al. 2012; Orthen and Popp 2000).

Normally, cyclitols are found in vegetal sources in less than 1 mg g^{-1} of dried plant, but the amount produced is largely dependent on plant species, developmental stage, and morphological parts investigated (Al-Suod et al. 2018a; Ratiu et al. 2018, 2019). However, as a response to the environmental stress synthesis of all secondary metabolites (including cyclitols) is increasing, because when the growing process is inhibited, the surplus of carbon produced during photosynthesis is predominantly allocated to secondary metabolites instead of primary metabolites (Kido et al. 2013; Sengupta et al. 2008; Sengupta and Majumder 2010). For exam-

ple, when the quantification of cyclitols in roots, bacteroids, and cytosol of *Medicago sativa* nodules was investigated, the obtained results highlighted that D-pinitol concentration was slightly increased in roots, but strongly modified in cytosol and bacteroids under salt stress. Moreover, ononitol increased as well in all three investigated fractions (Fougere et al. 1991). In the case of *Lupinus luteus* L. and *Lemna minor* L., an increasing trend of three monitored cyclitols (D-pinitol, D-*chiro*-inositol, and *myo*-inositol) was observed when the salinity level was supplemented with different NaCl concentrations between 0 and 400 mmol L^{-1} (Sikorski et al. 2013). Increase of glycolate, inositol, and mannitol at both cellular and subcellular levels associated with salinity tolerance in sugar beet were highlighted as well (Hossain et al. 2017).

As mentioned before, the inositol's biosynthesis is related to both ionic and osmotic stress. *Myo*-inositol is the only naturally occurring cyclitol, while the others are derivatives of it. Cyclitol synthesis in plants occurs in three major steps. Firstly, the synthesis of *myo*-inositol 1-phosphate from glucose occurs and from this free *myo*-inositol is generated (because *myo*-inositol phosphate + H_2O ⇌*myo*-inositol + phosphate). In the second step, methylation of *myo*-inositol takes place, resulting in the formation of ononitol. Finally, ononitol is epimerized one more time and pinitol is generated, through the action of two enzymes, inositol-O-methyltransferase and ononitol epimerase, respectively (Sengupta et al. 2008; Sengupta and Majumder 2010). The resulted compound, pinitol, accumulates in high salinity conditions and is a well-known osmoprotectant in many plant species (Sengupta et al. 2008).

Cyclitols' biological activity, reported as anti-inflammatory, antidiabetic, or anticancer agents was described by different research groups (Hernandez-Mijares et al. 2013; Ligor et al. 2018; Rengarajan et al. 2015). Moreover, *myo*-inositol effectiveness in panic attacks, obsessive-compulsive behavior, or depression, was reviewed (Al-Suod et al. 2017). D-pinitol proved its efficiency in the treatment of rheumatoid arthritis, hypertension, cardiovascular *diseases*, and neurological disorders (Zheng et al. 2017). The benefits of *myo*-inositol in thyroid-stimulating hormone, follicle-stimulating hormone, improving osteogenesis and bone mineral density, and many other beneficial effects on the metabolism of animals and humans are well documented (Alberts et al. 2013; Bizzarri and Carlomagno 2014; Croze and Soulage 2013; Dai et al. 2011). *Myo*-inositol, D-c*hiro*-inositol, and D-pinitol were also reported as possessing health-promoting properties for metabolic syndrome and diabetes within a comprehensive review (Antonowski et al. 2019). Both *myo*-inositol and D-*chiro*-inositol had promising results in improving ovary functionality in women affected by the polycystic ovary (Cheang et al. 2008; Costantino et al. 2009). The potential beneficial effect of cyclitols on inflammatory processes in psoriasis is well documented as well (Owczarczyk-Saczonek et al. 2018b). Moreover, cyclitols play many other important roles including good functioning of cells by being involved in signal transduction, membrane biogenesis, cell wall formation, ion channel physiology, phosphate storage, and osmoregulation (Al-Suod et al. 2017; Antonowski et al. 2019; Donahue et al. 2010).

Due to the evidence presented above, and considering the multiple benefits of cyclitols, as secondary metabolites easily isolable from plant material, their bioactive properties, and the fact that they have no side effects on the human body, cycli-

tols may become in the near future the perfect candidates for the discovery of novel drugs and food supplements and they can be used in the cosmetic industry as well.

Division of Polyols

Polyols are low-calorie sugar replacers approved for human consumption all over the world. They are hydrogenated carbohydrates known as sugar alcohol with the general formula $H(HCHO)_{n+1}H$. However, polyols are neither sugars nor alcohols, but they are the hydrogenated form of carbohydrate, in which the carboxyl group of aldehyde or ketone has been reduced to a primary or secondary hydroxyl group. Generally, the polyols are divided into three classes:

- Hydrogenated monosaccharides including xylitol (Fig. 7.1), erythritol, mannitol, and sorbitol.
- Hydrogenated disaccharides including isomalt, lactitol, and maltitol.
- Hydrogenated starch hydrolysates (HSH), or polyglycitols such as maltitol and sorbitol syrups.

Cyclitols are a special class of polyols, bio-synthesized in plants from glucose via the Loewus pathway. As a result of the mentioned pattern formation, free *myo*-inositol is generated, which can be further converted via repeated methylation and epimerization reactions to other cyclitols (Ligor et al. 2018). Table 7.1 includes some physicochemical properties of selected polyols.

Isomerism and Nomenclature of Cyclitols

Cyclitols are the hydroxylated form of cycloalkanes containing one hydroxyl group on each of three or more carbon ring atoms. Inositols are the most widespread cyclitols in eukaryotic cells, with empirical formula $C_6H_{12}O_6$ (1,2,3,4,5,6-cyclohexanol). Inositols may occur in nine possible stereoisomers. Five of them: *myo*-, *muco*-, *scyllo*-, *neo,* and D-*chiro*-inositol, are naturally occurring in plants, while the other

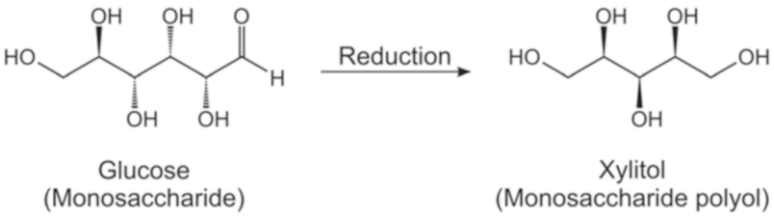

Fig. 7.1 The formation of xylitol from glucose

Table 7.1 Physicochemical properties of polyols

Polyols	Sugar source	Molecular mass (g mol^{-1})	Sweetness Sucrose = 100	Melting point (°C)	Solubility at 25 °C (g 100 g^{-1} H_2O)	Calories per gram	Impact on blood sugar	Ref.
Xylitol	D-glucose	152.15	100	93–94.5	64	2.4	Very low	Awuchi and Echeta (2019); Nabors and Hedrick (2001)
Sorbitol	D-glucose	182.17	50–60	93–112	72	2.6	Low	
Mannitol	Fructose	182.17	50–60	165–168	18	1.6	Low	
Lactitol	Lactose	344.31	30–40	94–97	206	2.0	None	
Maltitol	Maltose	344.31	80–90	149–152	Very soluble	2.1	Low	

four: L-*chiro*-, *allo*-, *epi*-, and *cis*-inositol are derivatives of *myo*-inositol. Cyclitols can also exist in other forms:

- When they contain phosphate such as phosphatidylinositol (PI) and their various phosphates, the phosphatidylinositol phosphate (PIP) lipids) or.
- In methylated forms like: 5-o-methyl-*myo*-inositol (sequoyitol), 3-o-methyl-D-*chiro*-inositol (D-pinitol), 1-O-methyl-*myo*-inositol (bornesitol), 4-o-methyl-*myo*-inositol (ononitol), 5-o-methyl-*allo*-inositol (brahol), di-o-methyl-(+)-*chiro*-inositol (pinpollitol), and 1 L-2-o-methyl-*chiro*-inositol (L-quebrachitol).

Myo-inositol can be divided into two mirror images along the plane of symmetry between C2 and C5 because it is a symmetrical compound. The carbon atoms numbering for unsubstituted *myo*-inositol can be in counter-clockwise (D-*myo*-inositol) or clockwise direction (L-*myo*-inositol), which represents the same compound that is called *myo*-inositol. In the case of phosphate or methylated cyclitols, a hydroxyl group is substituted by another substituent (phosphate or O-methyl) at a pro-chiral position. Consequently, an asymmetry is created, resulting in the configuration to follow D or L designation with the lowest-numbered chiral atom representing the substituent. *Chiro*-inositol is an asymmetrical compound with two enantiomers, D-*chiro*-inositol where the carbon atoms are numbered counter-clockwise and L-*chiro*-inositol where the carbons of L-*chiro*-inositol ring are numbered clockwise. Figure 7.2 illustrates the structures of inositol isomers and some other methylated cyclitols found in plants.

Sources of Cyclitols in Food

Cyclitols are minor constituents of food naturally present in vegetables, including plants with green leaves, fruits, beans, grains, pods, and nuts. Moreover, they can be contained by other nonedible plants. *Myo*-inositol and D-pinitol are probably the most widespread cyclitols in food. A comprehensive study investigated the *myo*-inositol content in 487 food sources (Clements and Darnell 1980). The authors concluded that in milk, constituents of meat, fat, commercially prepared beverages, and in miscellaneous foods relatively low *myo*-inositol content was found. Instead, foods that consist of seeds (beans, grains, and nuts) provided the most concentrated sources of *myo*-inositol. In the vegetables, higher contents were found in beans when compared with leafy vegetables. Generally, the fresh vegetables presented higher *myo*-inositol contents than frozen, canned, or salt-free products. Among the investigated fruits, cantaloupe and citrus fruits (except lemons) presented the highest amount of *myo*-inositol. Similar to the case of vegetables, the canned fruits contained less *myo*-inositol than the unprocessed fruit. Moreover, grain bread was found to contain more *myo*-inositol than white bread, while from the cereals, oats and bran contained more *myo*-inositol than did cereals derived from other grains (Clements and Darnell 1980). Besides the sources mentioned above, *myo*-inositol

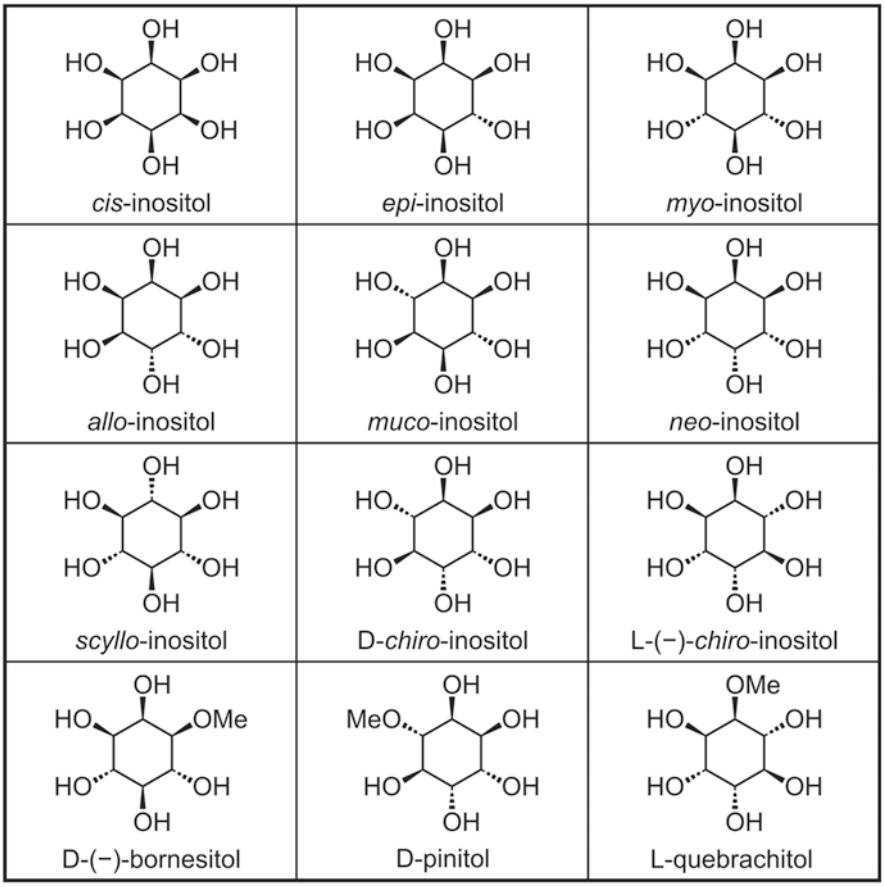

Fig. 7.2 The structures and nomenclature of inositols isomers and some methylated inositols

was isolated from *Medicago sativa L, Solidago genus, Phacelia tanacetifolia, Vaccinium myrtillus, Lactuca sativa, Cinnamomum verum*, etc. (Al-Suod et al. 2018a, 2018b; Ratiu et al. 2018, 2019).

Leguminosae families are the major natural source of D-pinitol. Within them, *Ceratonia siliqua L.* (Carob), an evergreen tree was found to contain higher amounts of D-pinitol than other legumes. Other species from which D-pinitol was isolated are *Pinaceae, Asteraceae, Caryophyllaceae, Zygophyllaceae, Cupressaceae, Aristolochiaceae,* and *Sapindaceae* (López-Sánchez et al. 2018). Many other studies also reported the carob as a very important source of D-pinitol (Nasar-Abbas et al. 2016; Ruiz-Aceituno et al. 2013; Tetik et al. 2011; Turhan 2014). For example, D-pinitol was quantified in 13 samples of carob syrup in concentrations ranging from 65.71 ± 4.60 mg g^{-1} up to 77.72 ± 5.44 mg g^{-1}, while in carob flesh samples the determined amount ranged between53.20 ± 3.72 mg g^{-1} and 54.58 ± 3.82 mg g^{-1} (Christou et al. 2019). In carob pods, the concentration of D-pinitol may vary

between 5% (Baumgartner et al. 1986) and more than 10% as detected in some Spanish varieties (Nasar-Abbas et al. 2016). Other important sources of D-pinitol are chickpea, lentil, soybean, jojoba seed, alfalfa, enological tannins from gall plant, chestnut, and quebracho (Al-Suod et al. 2017; Lein et al. 2002; Murakeözy et al. 2002; Sanz et al. 2008).

A total number of seven cyclitols (D-pinitol, *myo*-inositol, chiro-inositol, bornesitol, scyllo-inositol, *allo*-inositol, and ononitol), were identified and quantified in 52 vegetal sources, coming from 40 species of plants: *Allium ursinum, Sorbus aucuparia, Trigonella foenum-graecum, Carumcarvi, Myristica fragrans, Laurus nobilis, Elettaria cardamomum, Eugenia caryophyllus, Taraxacum officinale, Solidago virgaurea, Sambucus nigra, Rosa canina, Chamomilla recutita, Salvia officinalis, Vaccinium myrtillus, Calendulae anthodium, Mentha piperita, Hypericum perforatum, Solanum tuberosum, Anethum graveolens, Beta vulgaris, Petroselinum crispum, Allium sativum, Daucuscarota subsp. sativus, Lactuca sativa, Agaricus bisporus, Curcuma longa, Zingiber officinale, Oryza sativa, Capsicum annuum, Brassica oleracea var. acephala, Brassica oleracea, Allium cepa, Ceratonia siliqua* L.*, Ipomoea batatas, Arachis hypogaea, Phacelia tanacetifolia, Medicago sativa* L.*, Camelina Sativa* L., and *Lupinus perennis*. From the 52 mentioned sources, the authors reported that 37 sources were not investigated before. Figure 7.3 illustrates a snapshot of 7 detected cyclitols in 52 vegetal sources, while in Table 7.2, 10 the most reach sources of cyclitols are presented (Ratiu et al. 2019).

Bornesitol (the methyl ether of D-*myo*-inositol) is another cyclitol frequently appearing in plants. It was detected in all aerial parts of *Solidagogenus*; however, the highest amount was quantified in goldenrod flowers. Other important sources of bornesitol were reported to be white onion, red paprika, carrot, parsley roots, carob pods, Mountain Ash fruits, elder fruits, sweet potato, wild rose flowers, curcuma seeds, and beetroot (Ratiu et al. 2018, 2019). *Allo*-inositol is a rare cyclitol, one of the four possible isomers derived from *myo*-inositol, whose amount of 10.8 ± 0.2 mg g^{-1} of dried material was detected in blueberries fruits (Ratiu et al. 2019). Ononitol (a methyl *myo*-inositol) is another rare cyclitol detected in blueberries, wild garlic, garlic, kale, mint, and dill (Ratiu et al. 2019). An important source of *scyllo*-inositol is cinnamon (1.5 ± 0.01 mg g^{-1}). However, *scyllo*-inositol was detected in many other sources among which are carrot, goldenrod, caraway seeds, parsley roots, laurel leaves, marigold flowers, etc. (Ratiu et al. 2019). D-*chiro*-inositol was detected in Mountain Ash fruits (*Sorbus aucuparia*) containing 1.44 ± 0.07 mg g^{-1} of dried plant. Other important sources reported are chamomile and goldenrod flowers, lettuce leaves, carob pods, dandelion root, and curcuma seeds (Ratiu et al. 2019). In Table 7.2 the top 10 sources of cyclitols investigated in 52 vegetal sources are presented. The classification was made based on the total amount of cyclitols quantified in the investigated sources.

The accumulation of cyclitols was revealed in *Viscumalbum*, which parasitized 16 different species of trees. Consequently, 7 host-specific cyclitols were stored in mistletoe in such high concentrations that it was evaluated that 22.6–43.0% of mistletoe carbon was coming from the host (Richter and Popp 1992).

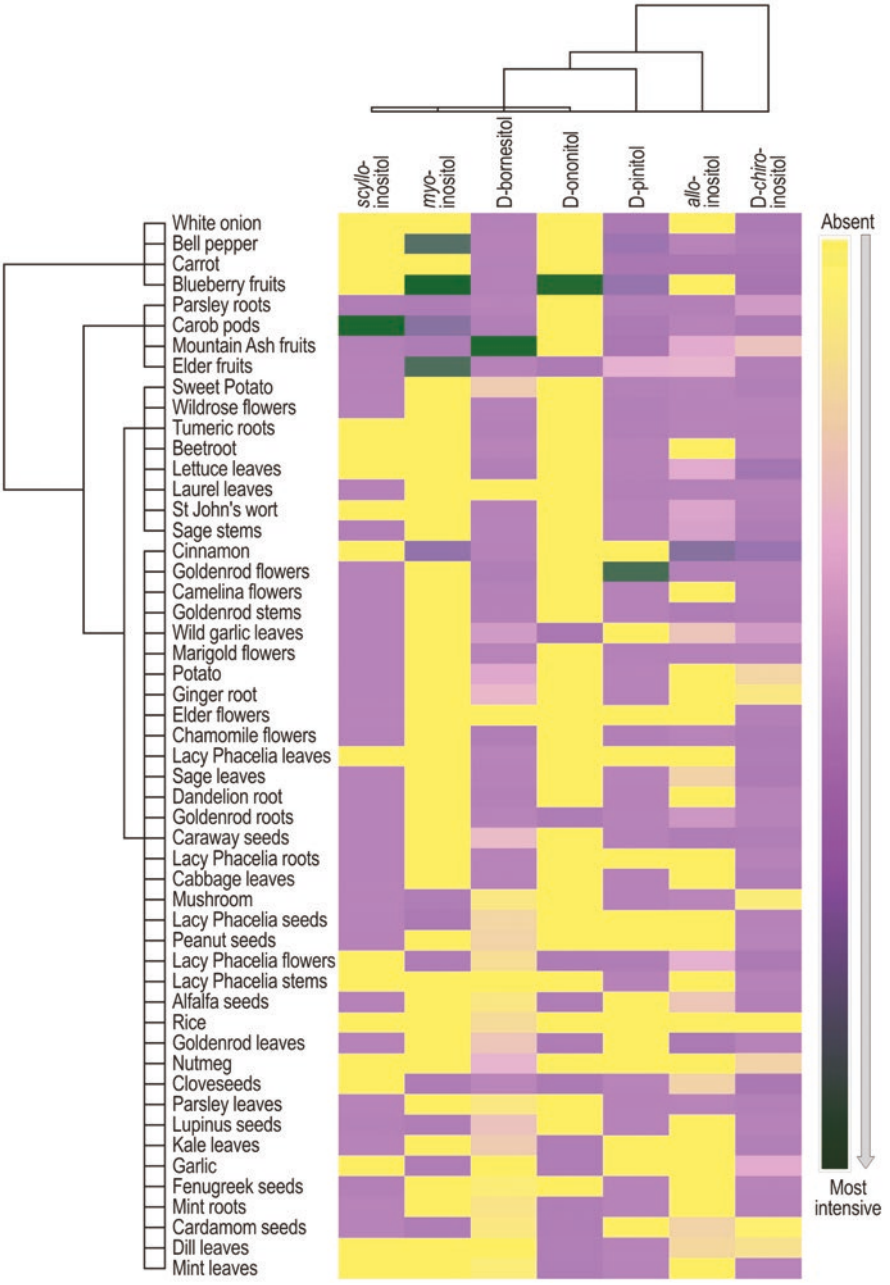

Fig. 7.3 A selection of seven cyclitols detected in edible plants and spices

Table 7.2 Top 10 sources of cyclitols investigated in 52 vegetal sources (Ratiu et al. 2019)

	D-pinitol[a]	Allo-inositol[a]	Chiro-inositol[a]	Ononitol[a]	Bornesitol[a]	Scyllo-inositol[a]	*Myo*-inositol[a]	Total amount[a]
Blueberry fruits	0	10.84	0.203	3.25	1.26	0	0.96	16.52
Carob pods	9.5	1.48	0.32	0	0.7	0.07	0.59	12.66
Bell pepper	0	3.342	0.104	0	1.159	0.052	0.505	5.162
Elder fruits	0.11	3.49	0.11	0.59	0.03	0.02	0.22	4.56
Cinnamon	0	1.31	0.11	0	0	1.51	1.21	4.14
Goldenrod flowers	0.08	0	0.4	0	2.59	0.2	0.12	3.38
Mountain ash fruits	0.21	0.51	1.44	0	0.82	0.027	0.02	3.04
Carrot	0	0	0.17	0	0.89	0.81	0.8	2.67
Lacy Phacelia flowers	0	0.52	0.01	0.54	0.44	0.03	0.67	2.21
Clove seeds	0	0.57	0.04	0.7	0.05	0.02	0.77	2.15

[a]The quantities presented are expressed in mg g^{-1} of the dry weight of the sample; each value represents the mean of three replicates

Cyclitols were documented as minor constituents of honey as well (Ratiu et al. 2020; Sanz et al. 2004). Quercitol, pinitol, 1-O-methyl-muco-inositol, and muco-inositol appear to have been reported for the first time in 28 edible honey samples in 2004, by detecting their O-trimethylsilyl derivatives using a GC-MS system (Sanz et al. 2004). A correlation study between 18 types of honey, originating from 10 countries, collected during 4 years and summarizing a total of 38 samples has reported the presence of 11 cyclitols. Generally, the authors reported that *epi*-inositol, *cis*-inositol, bornesitol, D-pinitol, and *chiro*-inositol were detected in lower amounts compared with other cyclitols, or not detected at all in some samples. However, ononitol, neo-inositol, quebrachitol and muco-inositol sequoyitol, and *allo*-inositol were present in all 38 investigated samples (Ratiu et al. 2020). It is worth mentioning that honey contains more frequently rare cyclitols which either were detected in plants in small quantities, or they were not detected at all.

Sample Preparation, Extraction, Purification, and Analytical Methods for the Separation and Determination of Cyclitols

Metabolites and secondary metabolites occur in plants as a result of the life cycle in a complex mixture of many organic compounds of a wide range of hydrophobicity and polarity. In plant material, three main groups of components can be found: low-polar, semi-polar, and high-polar.

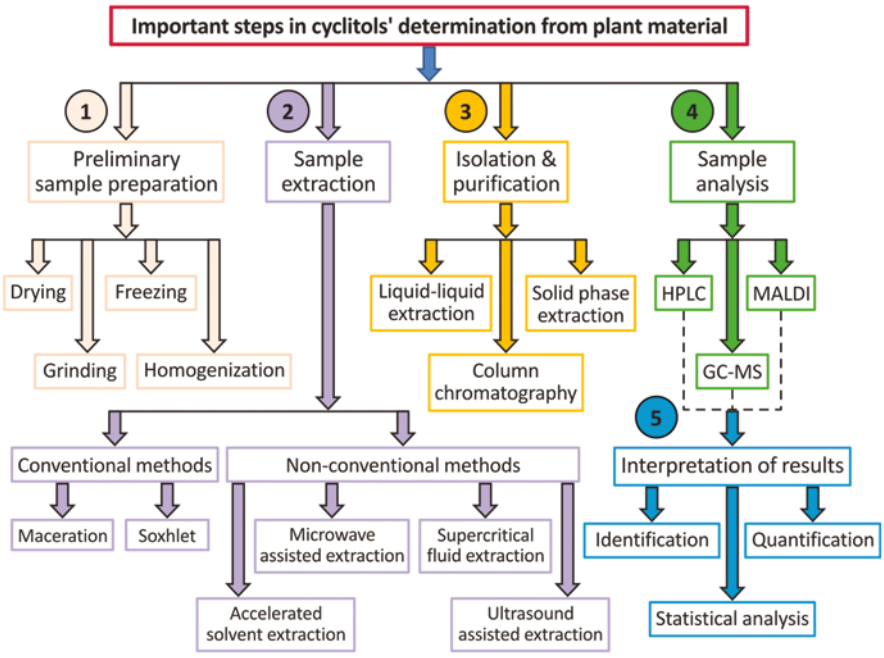

Fig. 7.4 Available options to be used in cyclitols' identification and quantification, according to the main five steps required

The sample preparation procedure for the detection and quantification of cyclitols (secondary metabolites, nonvolatile with high polarity) from plant material includes at least five steps: (1) preliminary sample preparation, (2) sample extraction, (3) isolation and purification, (4) analysis, and (5) interpretation. Some other additional steps may be required, which are specific for the method chosen for the analysis. For example, GC-MS analysis requires a derivatization step, while MALDI involves a laborious procedure for sample preparation. In Fig. 7.4 the main steps and options for the determination of cyclitols from plant material are illustrated.

Preliminary Sample Preparation

Preliminary sample preparation starts with plant selection and collection. In analytical chemistry, the most important requirement is that a selected sample must be representative. This supposes that the plants should be collected, treated, and stored without altering the chemical composition, while the final sample should be similar to the average composition of the total material. According to the aim of the study, the whole plant or just some morphological parts can be sampled. Herbaceous, small plants and cotyledons are usually collected whole. Higher plants are generally collected during blooming; the leaves during the growing period, and the needles after a period of growth from the outer part of the middle of the tree. The seeds and

straw are generally considered separate samples (Zygmunt and Namieśnik 2003). Once the desired matrix has been chosen, the plant material is prewashed (often not required), dried or freeze-dried, ground, and implicitly homogenized (necessary for improving the analyte extraction). The drying of plant material can be performed in different ways: in the oven, at 30 °C, from 24 to 48 h, depending on the plant material (Ratiu et al. 2019), stacked in a crate with a perforated bottom, in airflow (Al-Suod et al. 2019b) or in darkness at room temperature (Al-Suod et al. 2018a). The grinding is carried out in laboratory mills, which provide a good homogenization of the sample.

Sample Extraction

Conventional and nonconventional extraction techniques used for cyclitols' isolation have been reviewed before (Al-Suod et al. 2017; Ligor et al. 2018). Nowadays, some of the conventional extraction techniques such as maceration for several-days and Soxhlet extraction are being replaced with other modern techniques, which present the advantages of being more efficient, less time consuming, requiring a low amount of solvents, and allowing ready automatization of the apparatus.

Ultrasound-assisted extraction (UAE) is a modern extraction technique suitable for cyclitols' extraction, with some advantages (solvent amount reduction, low energy consumption, and time-saving) compared to classical methods. The principle of UAE is the utilization of ultrasound in order to create cavitation bubbles inside the liquid solvent meant to induce disruption of the plant cell wall and speed up the solvent penetration in plant material (Tetik and Yüksel 2014).

Supercritical fluid extraction (SFE) is a fast, selective, and environmentally friendly extraction technique, which uses carbon dioxide as a solvent. For extraction of cyclitols, a polar modifier cosolvent is required to increase the solubility. Consequently, the combination of solvent and cosolvent in the supercritical phase will effuse through the solid material like a gas and dissolve the components like a liquid, allowing this way for the extraction of the targets (Wang and Weller 2006).

Accelerated solvent extraction (ASE) is one of the most efficient techniques for cyclitols' extraction. It possesses high efficiency, low-cost equipment, and simple operation. The technique operates with solvents under high temperature and pressure without reaching the critical point.

A comparative study in terms of extraction efficiency by using different solvents and five extraction techniques was realized. The recovery of sugars from different *Solidago genus* was targeted. ASE proved its superiority beside the other techniques used (Soxhlet, maceration, UAE, and SFE), while water leads to the highest amounts obtained compared with other solvents used (96 and 70% ethanol) (Ratiu et al. 2018). However, comparative results were obtained when inositols extraction was realized by using microwave-assisted extraction (MAE) and ASE (Ruiz-Aceituno et al. 2016).

Isolation and Purification

In the case of any extraction technique involvement, the crude extract resulted will consist of a mixture of organic components both polar and nonpolar. Purification of the extract is necessary before analysis in order to remove the unwanted fractions. Three main purification methods are available: liquid-liquid extraction (LLE), solid-phase extraction (SPE), and column chromatography.

A consecutive liquid–liquid partitioning was used in the separation of D-pinitol extracted from *Agyrolobium roseum* (Chauhan et al. 2011). The crude ethanolic extract was mixed with petroleum ether, and the polar fraction obtained was again extracted with chloroform. The resulted residuum solution was again partitioned between n-butanol and water. The target was obtained this way in the aqueous phase. In another study, the removal of nonpolar components was realized by washing the crude extract with n-butanol in order to isolate the d-pinitol (Sharma et al. 2016).

Solid-phase extraction (SPE) using C18 cartridges was proved to be a very efficient method that can be used for purification of cyclitols (Al-Suod et al. 2018a, 2019a; Ratiu et al. 2018, 2019). The method implies passing a solution through a cartridge filled with octadecylsilane-bonded to silica (using irregular silica particles) that retains the nonpolar components.

Column chromatography, also more time consuming compared with the first two presented (LLE and SPE) was successfully used for fractionation of extracts in order to purify the cyclitols. Silica is the most used adsorbent, while methanol, ethyl acetate, chloroform, water, or their combination in different proportions are commonly used as eluents (Chauhan et al. 2011; Sharma et al. 2016).

Samples Analysis

High-performance liquid chromatography (HPLC) using various detectors, columns, and mobile phases maybe the most commonly used for both separation and quantification of cyclitols. The method is sensitive, time-saving, and normally does not involve a derivatization step. HPLC with an ELSD (evaporative-light scattering detector) was successfully used in the detection of cyclitols extracted from *Phacelia tanacetifolia* (Al-Suod et al. 2018b). In case HPLC with a UV detector is used, a derivatization step is however required in order to introduce a chromophore group. Thus, bornesitol isolated from *H. speciose* was detected using HPLC with a UV detector (at the wavelength of 230 nm) by inserting tosyl groups, which acted as chromophores. The groups were introduced by treating the extract with p-toluenesulfonyl chloride (Pereira et al. 2012). Liquid chromatography-mass spectrometry (LC-MS) is suitable as well in analyzing sugar alcohols and cyclitols; however, the required instrumentation is not cheap, matrix effects can appear, and a laborious sample pretreatment is necessary (Ghfar et al. 2015).

Gas chromatography with mass spectrometry (GC-MS) is a powerful technique widely used for the detection and quantification of cyclitols offering high resolution and low limits of detection. Because cyclitols are nonvolatile components, their determination using GC-MS or GC-FID systems requires a derivatization step. Silylation is a very useful derivatization technique that serves as a purification step as well, letting just the target derivatives become semi-volatile, without involving others in the analysis. Attaching of silyl groups to the targets can also cause a more favorable ion fragmentation pattern used for structure investigations (Ligor et al. 2018). GC-MS analyses were successfully used for cyclitols detection and quantification (Al-Suod et al. 2018a, 2019b; Ratiu et al. 2019, 2020).

Three cyclitols extracted from *Medicago sativa* L. were identified using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOF-MS). The technique proved to be useful and sensitive for identification, but quantification is not possible (Al-Suod et al. 2018c).

Interpretation of Results

For fast identification of components detected, important databases such as NIST and Wiley are available. They offer the possibility to predict the identity of targets with a certain probability. However, for confirmation and quantification, the analysis of pure standards followed by drawing of the calibration curves is required. Retention index usage based on known straight-chain hydrocarbons from the retention index standard is required for sample alignment and inter-laboratory comparison. In this case, the values assigned to each of the components are plotted against their respective retention times to produce a linear retention ladder. This is known as Kovat's retention index.

Besides the identification and quantification of components, sometimes statistical tools are necessary in order to check the relation between different variables. For example, the Mann-Whitney test can be performed in order to check the significance level between different groups compared with a control. Correlation analysis, including both nonparametric (Spearman correlation) and parametric tests (Pearson correlation), is often involved. The decision regarding the method chosen is made based on the available data set. In a study regarding the correlation between different types of honey, both approaches were used. Thus, Spearman correlation was involved to check the relation between dissimilar variables with different measurement units. The Pearson test was used as a parametric statistical tool expecting a linear correlation between investigated variables coming from the same source, and they have the same measure unit (Ratiu et al. 2020).

Therapeutic Action of Cyclitols

Cyclitols are substances important for the functioning of numerous living organisms. They are present in tissues of such organisms as mammals, embryophytes, fungi, and some bacteria, and can be considered as biologically active compounds. They play an important role in the regulation of cellular processes, signal transduction, and osmoregulation, and are a component of the cytoplasmic membrane (Owczarczyk-Saczonek et al. 2018a). The available literature confirms that cyclitols have several applications in the treatment of many lifestyle-related diseases. Importantly, they can also be used in the prevention of many conditions. The possible applications of cyclitols in the prevention and treatment of lifestyle diseases are presented schematically in Fig. 7.5.

Fig. 7.5 Applications of cyclitols with regard to their biological activity

Insulin Resistance and Diabetes

Diabetes mellitus is a chronic illness afflicting over 415 million people in the world. The incidence of diabetes in developed countries is on the rise due to the growing problem of obesity. Diabetes and its complications are among the leading causes of death as the illness leads to heart damage, loss of sight, and renal function impairment, which in turn is a serious burden on the state economy in the form of healthcare costs. As cyclitols can be used in the prevention or even treatment of diabetes, research on this group of compounds can have great practical significance. They influence human organisms in a way similar to insulin, by lowering blood sugar levels. Particularly important here are D-chiro-inositol and its derivatives (D-pinitol, L-quebrachitol, pinpollitol, and D-ononitol), present i.a. in carob pods, soybean, buckwheat, figs, mung bean, and fig-leaf gourd (Owczarczyk-Saczonek et al. 2018a; Ratiu et al. 2019). Insulin functions must be regulated through mediators, which are inositols combined with hexosamines, hexoses, organic phosphorus, and ethanolamine to make inositol phosphoglycans (Larner 2002; Shashkin et al. 2002). Phosphoglycans containing inositol are intercellular transducers, participate in the process of glucose intake by cells, and – as confirmed by in vivo studies – are putative mediators of insulin action (Larner 2002; Larner et al. 2010). It has been confirmed that patients with diabetes have lower levels of D-chiro-inositol due to significant bioactivity of this substance in tissues; its levels increase significantly after administration of insulin (Shashkin et al. 2002). D-chiro-inositol has been noticed to influence glucose uptake regulation and glycogen synthesis (Saleem and Rizvi 2017). In turn, under the influence of epimerase, *myo*-inositol transforms into D-chiro-inositol. It has been observed that in humans and animals with type-2 diabetes, the levels of D-chiro-inositol excreted with urine decrease while the levels of *myo*-inositol rise (Bizzarri et al. 2016; Croze and Soulage 2013; Larner 2002; Lin et al. 2013; Sacchi et al. 2016). It is thus postulated that diabetes can lead to secondary epimerase deficiency, which prevents the physiological conversion of *myo*-inositol to D-chiro-inositol, stimulated by insulin. This phenomenon has been observed in vivo in rats, and it has been demonstrated that physiological conversion decreases significantly in insulin-sensitive tissues (liver, muscles, fat), which can correspond to the level of insulin resistance (Larner 2002; Özturan et al. 2019).

D-pinitol and *myo*-inositol influence the activation of glucose-transporting protein GLUT4, which has an important role in regulating glucose transportation to skeletal muscles and fat tissue, stimulated by insulin. Binding insulin to its receptor leads to translocation of GLUT4 from the inside of the cell to its surface with the involvement of the phosphoinositide kinase (PI3K) signaling pathway (Dang et al. 2010; Gao et al. 2015; Paul et al. 2016). D-pinitol lowers glucose levels and decreases insulin resistance, but in the presence of PI3K inhibitor this process is blocked (Altaf et al. 2015; Bates et al. 2000). Meanwhile, *myo*-inositol phosphoglycan plays a key role in lowering the release of free fatty acids from fat tissue by blocking the adenylate cyclase enzyme. In turn, free fatty acids stimulate insulin resistance and contribute to increased synthesis of triglycerides. Furthermore,

myo-inositol and D-*chiro*-inositol promote the synthesis of glycogen, inducing conversion of glucose to glycogen, stored in the cells (Bizzari et al., 2016; Unfer et al. 2017). There are reports suggesting that D-chiro-inositol specifically stimulates insulin secretion in the pancreas (Lazarenko et al. 2014). Cyclitols also inhibit the storing of fats in fat tissue cells, which results in better penetration of glucose into cells and decreased insulin resistance (Bates et al. 2000).

Summing up, the significance of cyclitols in prevention and treatment of diabetes can result from:

- Their role in the normalization of blood glucose levels, connected with the activity of glucose transporting protein GLUT4, insulin sensitivity, stimulation of the pancreas β cells, and inhibiting storage of fats in fat tissue.
- Their antioxidant properties and their role in protecting cells from oxidative stress.

Polycystic Ovary Syndrome and Fertility

The polycystic ovary syndrome (PCOS) affects as many as 5–10% of women and is one of the most frequent reasons for menstrual cycle disorders and infertility. This disease can also be associated with metabolic disorders, progressive insulin resistance, and resulting hyperinsulinemia, which in turn contributes to the development of hyperandrogenism, another feature of PCOS (Genazzani et al. 2008). Ovary cells are stimulated by insulin to produce greater amounts of androgens; insulin also inhibits the synthesis of SHBG (sex hormone-binding protein) that takes place in the liver, thus indirectly increasing the levels of free androgens, particularly free testosterone (Bizzarri et al. 2016; Cheang et al. 2008; Croze and Soulage 2013; Sacchi et al. 2016). An obvious result of insulin resistance is the development of type-2 diabetes in women, particularly those with a family history of such problems (Bizzarri et al. 2016). In cases of hyperinsulinemia in women with PCOS, active substances sensitizing tissues to insulin are used, such as metformin, pioglitazone, and troglitazone, which result in decreased hyperinsulinemia and hyperandrogenism, and consequently regulate the menstrual cycle and ovulation (Rouzi and Ardawi 2006).

There are studies confirming the beneficial effect of cyclitols in polycystic ovary syndrome (Colazingari et al. 2013). The mechanism of cyclitol action in PCOS not only involves the regulation of insulin resistance but also of antiandrogen activity, and as a consequence influences fertility (Owczarczyk-Saczonek et al. 2018a). Increased epimerization with *myo*-inositol and D-*chiro*-inositol, as well as transport of D-*chiro*-inositol phosphoglycan into cells leads to a disturbed ratio of stereoisomers of *myo*-inositol and D-*chiro*-inositol phosphoglycans. Consequently, these cells are increasingly stimulated to produce androgens, with the simultaneous signal from the insulin receptor (Heimark et al. 2014).

The use of cyclitols in the treatment of PCOS can also result in decreased body mass index, even without introducing significant changes to lifestyle (Genazzani et al. 2008; Saleem and Rizvi 2017). D-*chiro*-inositol contributes to the normalization of the levels of LH (lutropin, luteinizing hormone, gonadotropin glycoprotein hormone), LH/FSH (lutropin/follitropin, luteinizing hormone/follicle-stimulating hormone), and androstenedione. Administration of *myo*-inositol is more effective in obese female patients with high fasting serum insulin levels and in patients with PCOS-related hyperinsulinemia with a family history of diabetes (Genazzani et al. 2008; Saleem and Rizvi 2017). *Myo*-inositol has a beneficial influence on metabolic profile, while D-*chiro*-inositol decreases hyperandrogenism more efficiently than *myo*-inositol (Saleem and Rizvi 2017). No side effects of such therapies have been observed, and the general results provide class 1A evidence (Nestler et al. 2012; Sortino et al. 2017). The value and effectiveness of cyclitol supplementation have been noted and the method is recommended by the Polish Gynecological Society (Oszukowski et al. 2014).

Hypertension

A dangerous condition, hypertension leads to heart muscle damage, strokes, and kidney function impairment. Pathophysiology of this condition includes a complex interaction of many vascular effectors, including activation of the sympathetic nervous system, the renin-angiotensin-aldosterone system, and inflammatory mediators. Progressive narrowing of blood vessels and inflammation lead to changes in blood vessel walls and ultimately to atherosclerotic plaque build-up in advanced stages of the disease. Inflammation is accompanied by oxidative stress, which limits the bioavailability of the natural vasodilatant nitric oxide and causes endothelial dysfunctions (Schulz et al. 2011). Silva et al. studied the influence of the *Hancornia speciosa Gomes* (*Apocynaceae*) plant, common to Brazil, on the cardiovascular system of rats. It was demonstrated that ethanol extract from the leaves of this plant species has vasodilating properties, influencing the muscular layer of the aorta and mesenteric vessels due to the production of nitric oxide (NO) in endothelium through activation of PI3K. Following administration of this plant extract, the activity of ACE (angiotensin-converting enzyme) and the levels of angiotensin II were significantly decreased in comparison with the control group receiving captopril, a traditional antihypertension medicine. The extract from *Hancornia speciosa* leaves contains flavonoids and bornesitol (Ferreira et al. 2007; Silva et al. 2011). Subsequent studies by the same authors confirmed earlier discoveries. Hypertension was induced in mice by surgical removal of a kidney and deoxycorticosterone administration. Vasodilatation in mesenteric vessels was measured with a myograph. The plant extract lowered blood pressure and induced vasodilatation of arteries, depending on the dose, together with an increase of nitrites in serum (Silva et al. 2016).

Atherosclerosis

The antiatherosclerotic activity of cyclitols has been demonstrated as well. Oxidized particles of low-density lipoproteins (ox-LDL) are consumed by macrophages and make foam cells, forming atherosclerotic plaque that narrows the vessel diameter or ruptures, causing, e.g., myocardial infarction. D-pinitol inhibits the creation of foam cells, thus limiting the build-up of the plaque (Choi et al. 2009).

Choi et al. studied the influence of D-pinitol of soy origin on the formation of foam cells, with a variety of THP-1 macrophages used. It was confirmed that depending on the dose, D-pinitol inhibits the creation of ox-LDL stored by macrophages. Ox-LDL is an atherosclerosis antigen, stimulating the immunological mechanism of inflammation. Limitation of the release of TNF-α and chemotactic protein was observed for monocytes-1 (MCP-1) after administration of D-pinitol in the concentration range of 0.05–0.5 mM, while production of IL-1β, IL-8 and the expression of matrix metalloproteinase-9 gene decreased significantly even after low doses of mentioned (0.05 and 0.1 mM) in comparison with cells without added D-pinitol, which confirms its strong antiatherosclerotic properties (Choi et al. 2007). An important aspect of preventing atherosclerosis development is improved lipid profile. After oral administration (all procedures involving laboratory animals) of D-pinitol (Geethan and Prince 2008) or pinitol (Choi et al. 2009), improvements in lipid profiles were observed, including lowered serum concentrations of triglycerides and total cholesterol.

Neurological Diseases

Inositol and its derivatives have signaling and regulatory functions in many cellular processes, so their influence on the functioning of the nervous system can be analyzed as well. In mammals, the serum concentrations of inositol are between 25 and 100 μM, while intracellular concentrations are several times as high, with the highest concentration observed in the brain (Frej et al. 2017). Disruption of *myo*-inositol transformation to phosphates and its secondary accumulation is present, e.g., in bipolar disorder, Alzheimer's disease, and epilepsy (Frej et al. 2017). Decreased levels were observed in patients undergoing treatments for the bipolar disease as their condition improved (Frej et al. 2017). Furthermore, therapy with lithium and valproic acid led to changes in concentrations of inositol in frontal and temporal lobes, cingulate cortex, and basal ganglia, resulting in limitation of its biosynthesis (Frej et al. 2017; Silverstone et al. 2005). Imbalance in inositol concentrations is also connected with a number of neurodegenerative diseases, including Alzheimer's disease. It was discovered that the brains of affected patients have higher concentrations of *myo*-inositol in comparison with healthy individuals despite different pathogenesis of this condition, which involves accumulation of amyloid A, toxic to neural cells (Frej et al. 2017; Griffith et al. 2008). Liu et al. assessed changes in mice brains

after 6-month supplementation of *scyllo*-inositol; they also noted lower content of amyloid A. In comparison to untreated mice, higher activity of microglia was observed as well as increased phagocytosis capability. *Scyllo*-inositol binds and stabilizes small, soluble conformations of amyloid Aβ, which are phagocyted by microglia (Liu et al. 2018).

An increased concentration of inositol was also discovered in the brain regions involved in epilepsy episodes (in the hippocampus even 20% higher), which suggests a seizure-induced change of inositol signaling in the brain (Frej et al. 2017; Pascente et al. 2016). Pascente et al. observed a temporary rise in *myo*-inositol concentration in the hippocampus of rats with pilocarpine-induced epilepsy, with an accompanying loss of astrocytes (Pascente et al. 2016). Another study describes newly identified antibodies connected with cerebellum disorders. The serum of 15 patients with cerebellar ataxia was tested on mice brain substrate. An auto-antibody of receptor 1 for inositol was identified in Purkinje neurons. As mice with such inborn deficiency have symptoms of ataxia and epilepsy, this auto-antibody may have a functional role. This offers a chance to develop specific immunotherapy for these disorders (Fouka et al. 2017).

At present, folic acid supplementation during gestation to limit neural tube defects in embryos is an important element of perinatal care. Embryonal defects can also be caused by inositol deficiency. Based on studies involving the administration of inositol together with folic acid, it was demonstrated that this scheme offers greater protection of the fetal neural system than supplementation of folic acid alone (Greene et al. 2017).

Cancer Prevention

Normal cells in an organism are subject to natural, programmed death (apoptosis). A number of cell death processes in the human body are accompanied by worsening inflammation. Cyclitols originated from plants have a beneficial effect on the regulation of progressive inflammatory processes in the human body (Zhang et al. 2018). The scheme of the anti-inflammatory action of D-chiro-inositol is shown in Fig. 7.6.

Furthermore, the apoptosis process does not occur in cancer cells, so they are "immortal," which contributes to the fast progression of cancer disease. Numerous studies demonstrated the anticancer effect of D-pinitol (Rengarajan et al. 2015). This substance stimulates apoptosis mechanisms in cancer cells. A study by Al-Daghri and coworkers assessed the influence of fenugreek extract on Jurkat cell lines (a model of immortalized human T lymphocytes, useful in assessing the susceptibility of cancers to drugs and radiation), and the cytotoxic effect was observed, depending on dose and time of exposure (Al-Daghri et al. 2012). A subsequent study used fenugreek extract to determine its in vitro influence on a panel of cancer cell lines, including T-cell lymphoma, and normal cells. Different concentrations (100 μg mL^{-1}, 200 μg mL^{-1}, 300 μg mL^{-1}) were used for a specific time (24 h, 48 h, 72 h, and 96 h). Selective cytotoxic effect was observed, and apoptosis was induced

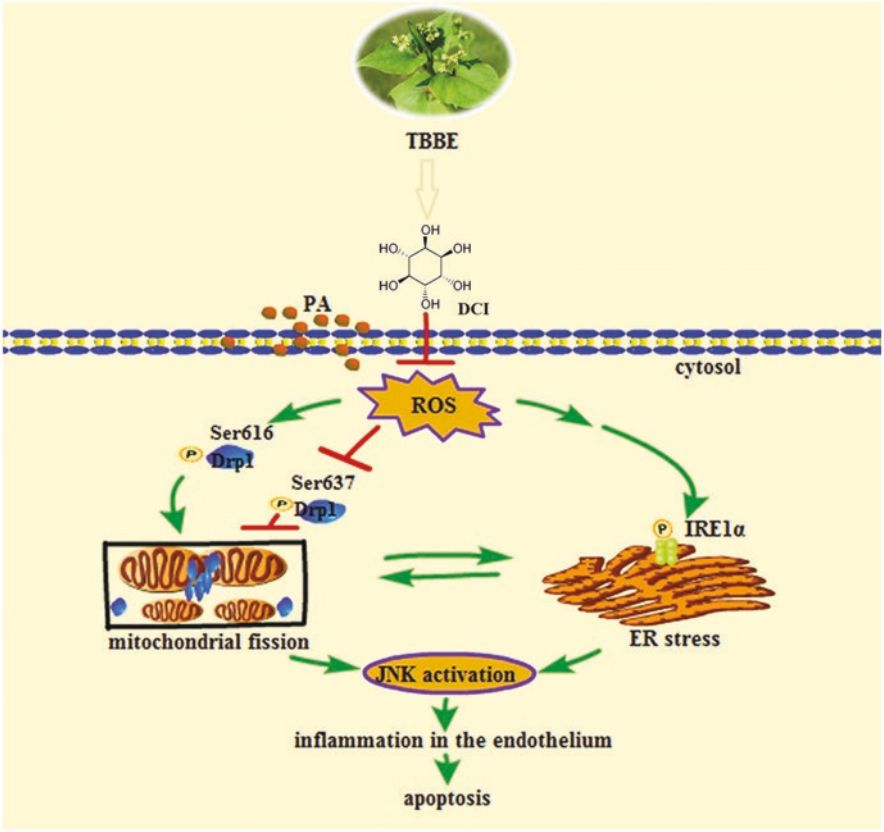

Fig. 7.6 The suggested pathway for the suppressive effect of Tartary buckwheat bran extract (TBBE) on inflammation in the endothelium and the effects of DCI-enriched extracts on superoxide anion generation; *where*: DCI D-chiro-inositol, Drp1 dynamin-related protein 1, ER endoplasmic reticulum, IRE-1α inositol-requiring enzyme-1α, JNK Jun n-terminal kinase, P phosphate group – phosphorylation, PA palmitate acid, ROS reactive oxygen species, Ser616, Ser637 polyclonal antibodies (Phospho-DRP1), TBBE tartary buckwheat bran extract, according to (Zhang et al. 2018)

only in abnormal cells. Normal lymphocytes showed no sign of apoptosis (Alsemari et al. 2014). Apoptosis induced by fenugreek extract was described for many different lines of cancer cells: colon cancer, osteosarcoma, leukemia, breast cancer, and liver cancer (Alsemari et al. 2014; Raju et al. 2004). Administration of 10–15 μg mL^{-1} fenugreek extract inhibited for 72 h the growth of cell lines of breast, pancreas, and prostate cancer (Shabbeer et al. 2009).

Rangarajan et al. confirmed the concentration-dependent antiapoptotic activity of D-pinitol of soybean origin through inducing expression of p53 and Bax proteins, and inhibition of antiapoptotic proteins (Bcl-2 iBcl-XL) in the MCF-7 cell line, a breast cancer model. A decreased amount of glutathione and increased concentra-

tion of LDH in MCF-7 was observed, which confirms the cytotoxic activity of D-pinitol (Rengarajan et al. 2015). Inhibition of glutathione production in cancer cells lowers its resistance to cytostatic drugs as this enzyme protects cells from oxidative stress. Its deficiency results in cellular damage. Deficiency of endogenous glutathione may stimulate apoptosis by inducing proapoptotic factors (Rengarajan et al. 2015). Similar results were obtained by Lin et al.; in their research on the prostate, they observed inhibition of the metastasizing process. Administration of D-pinitol caused the reduction of mRNA and expression of αvβ3 integrins on the cell membrane. Integrins are the main adhesive particles participating in the creation of metastases. Furthermore, D-pinitol acted as a suppressor, decreasing phosphorylation of focal adhesion kinase (FAK), and influenced the activity of c-Src kinase and activation of NF-kB (pathways responsible, e.g., for cell mobility) (Lin et al. 2013).

Cyclitols and Allergic Disorders as Well as Skin Diseases

As it has been already mentioned cyclitols exhibit and possess numerous antioxidative, anti-inflammatory, and anticancer properties. Some available studies concern the potential beneficial effects of cyclitols on inflammatory processes in psoriasis. The potential anti-inflammatory properties of cyclitols affecting the individual elements of the pathophysiology of psoriatic lesions include: inhibiting the expression of MHC class I and MHC class II (major histocompatibility complex (MHC) molecules) of mature dendritic cells, and resulting in a decrease of IL-12, IL-23, which are necessary to the differentiation of naive lymphocytes in Th1 and Th17 line. Moreover, it inhibits NF-βB (nuclear factor-kappa B, signaling molecule involved in inflammation and immune responses), a major signaling pathway activated by TNF-α (tumor necrosis factor α) with reducing of inflammation as well inhibits the angiogenic factor VEGF (vascular endothelial growth factor), with reducing epidermal hyperproliferation and enhances of the activity of antioxidants (Owczarczyk-Saczonek et al. 2018b).

Anti-inflammatory properties of cyclitol and the ability of D-pinitol to strengthen the expression of the transcription protein GATA 3 (a key particle in the regulation of Th1/Th2 balance) can be used in the treatment of allergic disorders such as atopic dermatitis, asthma, and others (Bae et al. 2012). Bae et al. (2012) demonstrated the effectiveness of fenugreek extract in mice with induced contact dermatitis and hypersensitivity to chicken egg albumins. The mice were administered fenugreek extract in the dose of 250 mg kg^{-1} of body mass for 7 days after allergization. The study demonstrated decreased inflammation, decreased number of eosinophils and mast cells in the infiltration as well as inhibition of production of IL-4, IL-5, IL-13, and IL-1β. Fenugreek extract prevented the differentiation of Th2 cells in splenocytes of the allergized mice, resulting in lower secretion of IL-4 and decreased expression of mRNA GATA-3, the transcription factor of IL-4. The secretion of

IFN-γ and the number of Th1 cells producing IFN-γ was normalized (Bae et al. 2012).

In turn, D-pinitol in the extract from a Himalayan plant *Argyrolobium roseum*, depending on the dosage, inhibited the production of antibodies and delayed hypersensitivity reactions through decreasing the number of CD3, CD19, CD4, and CD8 lymphocytes and the expression of Th1/Th2 cytokines in splenocytes. D-pinitol had also good safety profile and displayed lack of toxicity despite its immunosuppressive properties, both in vitro and in vivo studies on mice and rats which received oral doses of D-pinitol between 300–1200 mg kg^{-1} body mass, with the assessment after 4 h (Chauhan et al. 2011).

Possible Side Effects of Using Cyclitols

In adults, administering even high doses of inositol (6–18 g) did not cause any significant side effects except mild bloating or diarrhea in a small number of patients. No serious side effects were reported (Croze and Soulage 2013; Greene et al. 2017). In pregnant women, inositol supplementation (2 × 2 g daily) starting from the first trimester until the end of pregnancy did not cause side effects or increased risk of harmful effects (Croze and Soulage 2013; Greene et al. 2017).

Summary

Cyclitols belonging to the increasingly well-known compounds are biosynthetically derived from glucose, and occur in all living cells. They form a group of biologically active compounds and participate in many cellular processes such as membrane biogenesis, signal transduction, ion channel physiology, osmoregulation, antioxidation, and others. This group of compounds shows several health-promoting and therapeutic properties as follows: improving the lipid profile in decreasing serum triglycerides and total cholesterol, as well as having an insulin-mimetic effect. The beneficial properties of cyclitols can be used for therapeutic purposes, particularly in the neutralization of the results of chronic inflammation, which leads to an increased risk of developing such metabolic disorders as diabetes, hypertension, atherosclerosis, etc. A notable factor is also good tolerance of cyclitols and their low toxicity, due to which they can be successfully used in treating pregnant women and children.

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Chapter 8
Capsaicinoids – Properties and Mechanisms of Pro-health Action

Justyna Werner

Abstract Chili peppers (*Capsicum*) are among the most popular spice with culinary qualities because of the pungent taste of the fruit. From more than 30 varieties of the *Capsicum* genus, only 5 of them are domesticated: *Capsicum pubescens, Capsicum baccatum, Capsicum annuum, Capsicum chinense,* and *Capsicum frutescens*. The compounds which give a spicy taste in *Capsicum* fruit are capsaicinoids, which mainly include capsaicin (69%), dihydrocapsaicin (22%), as well as nordihydrocapsaicin (7%), homocapsaicin (1%), and homodihydrocapsaicin (1%). There is a direct correlation between the total content of capsaicinoids and *Capsicum* fruits pungency, and this pungency is measured in Scoville Heat Units (SHU). For example, a popular variety of Jalapeño measures from 2500 to 8000 SHU, while the value for pure capsaicin is equal to 16,000,000 SHU.

On the other hand, owing to capsaicinoids, *Capsicum* fruits are an interesting subject of scientific research, especially in terms of pro-health properties. It is worth noting that the mechanisms proposed by scientists confirm the analgesic effect of capsaicin and its effect on thermoregulation. Based on these mechanisms, the beneficial effect of capsaicinoids in the treatment of obesity, hypertension, diabetes, cardiovascular diseases, gastroprotective, or anti-cancer activity was investigated and partially or completely confirmed. However, especially in the case of the anticancerogenic activity of capsaicinoids, there are too many conflicting or incomplete studies to clearly confirm its activity in this regard.

In anticipation of specific scientific evidence which will confirm the mechanisms of action of capsaicinoids in living organisms as well as their pro-health properties in the treatment of various diseases, it is worth introducing *Capsicum* fruits to the daily diet because, in addition to their taste, they possess many properties that can have a positive effect on the human health.

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Introduction

Hot chili peppers that belong to the *Capsicum* plant genus are among the most popular and frequently consumed spices throughout the world. In total, there are more than 30 members of the *Capsicum* genus, but only 5 of them are domesticated (*Capsicum pubescens, Capsicum baccatum, Capsicum annuum, Capsicum chinense*, and *Capsicum frutescens*), and the last three species possess the most pungent fruits. The compounds which give a spicy taste in *Capsicum* fruits are capsaicinoids, which mainly include capsaicin (CAP) as well as dihydrocapsaicin (DHC), nordihydrocapsaicin (NDC), homocapsaicin (HC), and homodihydrocapsaicin (HDC). There is a direct correlation between the total level of capsaicinoids and *Capsicum* fruits pungency, and this pungency is measured in Scoville Heat Units (SHU). For example, a popular variety of Jalapeño measures from 2500 to 8000 SHU, while in the case of Habanero the pungency ranges from 100,000 to 350,000 SHU. For comparison, the value for pure capsaicin is equal to 16,000,000 SHU (Chapa-Oliver and Mejía-Teniente 2016; Kehie et al. 2014; Rezende Naves et al. 2019).

Capsicum fruits have been used in medicine for centuries, but in recent decades, they have been extensively studied for antioxidant, anti-inflammatory, cardiometabolic, and analgesic properties. Therefore, *Capsicum* fruits containing capsaicinoids can have therapeutic properties and use in the treatment of diseases such as obesity, diabetes, atherosclerosis, hypertension, fatty liver, and gastric disorders, as well as treatment of pain of various origins. The anticancer activity of capsaicin has been broadly reviewed for a variety of cancer types; however, the mechanisms of capsaicin activity against cancer cells have not been thoroughly understood and confirmed yet. Furthermore, some scientific reports suggest carcinogenic and co-carcinogenic effects of capsaicin (Antonious 2018; Surh and Lee 1996). Considering the many years of scientific research focused on capsaicinoids and their already established pro-health effects, it can be expected that their popularity will continue to increase.

Historical View of Chili Peppers and Capsaicin

The culinary and pro-health properties of chili peppers have been known for centuries. The first mention of the use of chili peppers comes from Mexican Indians. This is confirmed by the finding of pepper seeds dating from approximately 7500 BC in Mexico by the American archaeologists (Kaplan and MacNeish 1960). Furthermore, not only culinary but also medicinal and protective use of chili peppers by Native Americans was first referred by Chanca, a physician who accompanied Columbus on his second voyage to West Indies (Bernstein 1991; Chanca 1484). Irritant smoke formed by the burning of chili peppers was a weapon used by Native Americans

against the aggressors (Barceloux 2008; Bode and Dong 2011; Govindarajan and Salzer 1985; Perry et al. 2007).

Scientific research regarding the chemical ingredients in chili peppers began only in the nineteenth century. In 1846, Thresh crystallized the active substance in chili peppers and named it capsaicin (Thresh 1846). Next, Buchheim found that capsaicin caused a burning sensation when contacting mucous membranes and also increased the secretion of gastric juice (Buchheim 1873). Then, Nelson and Dawson identified the chemical structure of capsaicin in 1919 (Nelson 1919; Nelson and Dawson 1923), while Späth and Darling synthesized capsaicin in 1930 (Späth and Darling 1930). Capsaicin derivatives have been isolated from chili peppers by Japanese chemists, who referred to them as capsaicinoids (Kosuge et al. 1961).

The *Capsicum* Fruit and Their Pungency

Peppers which originate from humid and tropical zones of Central and Southern America biologically belong to the *Capsicum* genus (*Solanaceae* family). There are more than 30 members of the *Capsicum* genus. Major domesticated species and their taxonomic varieties include *Capsicum pubescens* (i.a. Rocoto), *Capsicum baccatum* (i.a. Bishop's Crown and Lemon Drop), *Capsicum annuum* (i.a. Sweet Bell, Anaheim, Jalapeño, Serrano, De Arbol, Bird's Eye, Cayenne, and Chiltepin), *Capsicum chinense* (i.a. Habanero, Red Savina, Bhut Jolokia, Naga Viper, Carolina Reaper, and Trinidad Scorpion "Butch T"), and *Capsicum frutescens* (i.a. Tabasco and Siling Labuyo) (Tainter and Grenis 2001). The last three species are widely spread and possess the most pungent fruits (Barceloux 2008; Johnson 2007; Stoica et al. 2016). The varieties of peppers are classified as "hot" or "sweet" based on Scoville Heat Units (SHU). The hotter the pepper is, the higher the SHU value. The scheme of the SHU values of the most popular varieties of *Capsicum* fruit and pure capsaicin is presented in Fig. 8.1 (Arora et al. 2011).

The Scoville Heat Units (SHU) measurement is used to determine the highest dilution of a chili pepper extract at which heat can be detected by taste (Bosland and Baral 2007; Cordell and Araujo 1993; Stoica et al. 2016). The scale was developed by Scoville in 1912 (Scoville 1912) and is related to the amount of capsaicinoids present in the sample (Chapa-Oliver and Mejía-Teniente 2016; Kehie et al. 2014). SHU is an arbitrary unit and depends on the palate of the taster. It is known that the human palate can detect it, even at a ratio as diluted as 1:17,000,000. According to this scale, varieties Naga Viper (900,000–1,380,000 SHU), Trinidad Scorpion Butch T (800,000–1,460,000 SHU), Trinidad Scorpion Moruga (1,200,000–2,000,000 SHU), Carolina Reaper (1,400,000–2,200,000 SHU), Dragon's Breath (approximately 2,480,000 SHU), and Pepper X (approximately 3,180,000 SHU) belonging to the *Capsicum chinense* species have been acknowledged as the hottest chili peppers in the world (Antonious et al. 2009; Chapa-Oliver and Mejía-Teniente 2016; Kehie et al. 2014; Sanatombi and Sharma 2008). Sweet bell peppers rank lowest at 0 SHU, Jalapeño range is within 2500–8000 SHU, in case of Habanero the values

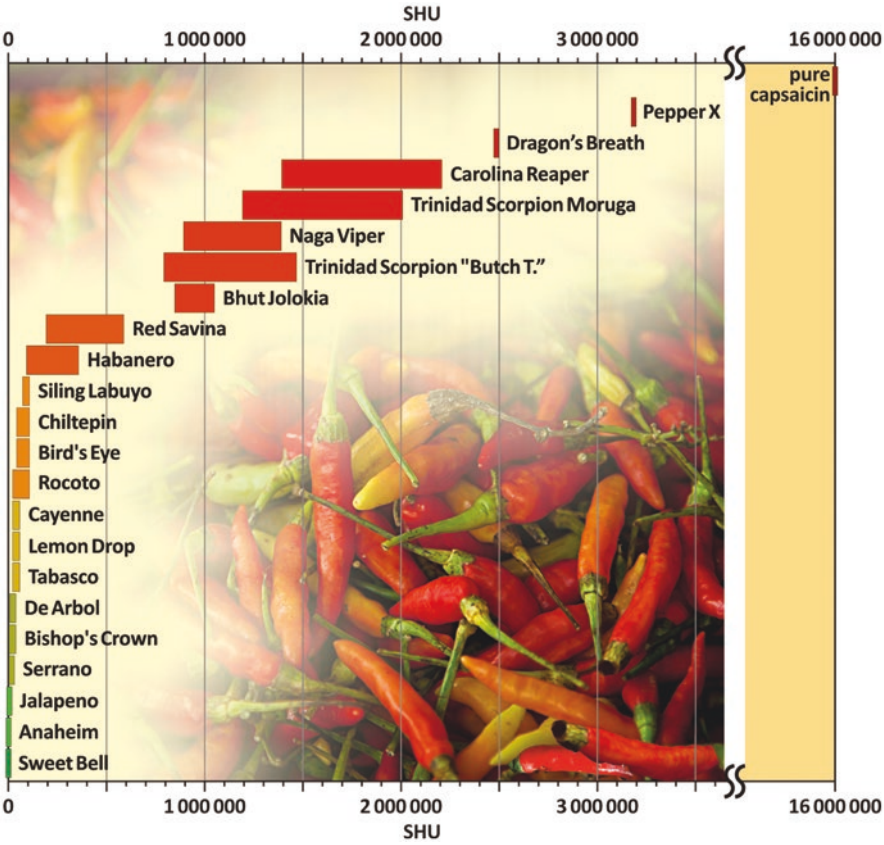


Fig. 8.1 The scheme of the SHU values of the most popular varieties of *Capsicum* fruit and pure capsaicin

range from 100,000 to 350,000 SHU, and for comparison pure capsaicin amounts to 16,000,000 SHU, whereas pure dihydrocapsaicin to 15,000,000 SHU (Bode and Dong 2011; Luo et al. 2011; Rezende Naves et al. 2019).

There is a direct correlation between the total level of capsaicinoids in *Capsicum* fruits and their pungency. Five levels of pungency are classified using the SHU and can be categorized into

1. non-pungent (0–700 SHU),
2. mildly pungent (700–3000 SHU),
3. moderately pungent (3000–25,000 SHU),
4. highly pungent (25,000–70,000 SHU), and
5. very highly pungent (>80,000 SHU) (Antonious 2018; Bosland and Baral 2007).

Currently, the SHU organoleptic test is increasingly being replaced by chromatographic techniques which are found to be more consistent and accurate compared to

the SHU scale which depends on subjective feelings of sensory organs (Gonzalez-Zamora et al. 2013; Masada et al. 2006; Popelka et al. 2017).

The Capsaicinoids in the *Capsicum* Fruits

Capsaicinoids are mainly represented by capsaicin and dihydrocapsaicin which constitute 69% and 22% of total capsaicinoids, respectively, and are the most irritant active compounds in *Capsicum* fruits which cause a burning sensation. Nordihydrocapsaicin accounts for approximately 7% of the total capsaicinoids. Homocapsaicin and homodihydrocapsaicin amount to 1% of total capsaicinoids and exhibits approximately a half of capsaicin pungency (Antonious 2018; Barceloux 2008; Bley et al. 2012; Cordell and Araujo 1993; Govindarajan and Sathyanarayana 1991; Stoica et al. 2016).

The capsaicinoids content of chili peppers ranges from 0.1% to 1% w/w. Research studies indicate that capsaicinoids are mostly located in vesicles or vacuole such as sub-cellular organelles of epidermal cells of the placenta in the pod. The highest concentrations of capsaicinoids are found in the ovary and the lower flesh (tip), whereas the lowest content of capsaicin can be found in the seeds. The seeds are not the source of pungency, but they occasionally absorb capsaicinoids because they are close to the placenta. No other plant part produces capsaicinoids. The majority (approximately 89%) of the capsaicin is associated with the placental partition of the fruit and nearly 5–6% with the pericarp and the seed. The composition of capsaicinoids may vary among different varieties of the same species and within the fruit of a single variety. The pungency is influenced by the weather conditions such as heat wave, and it increases with the maturation of the fruit (Andrews 1984; Arora et al. 2011; Cheema and Pant 2011).

Only *Capsicum* pepper fruits synthesize these compounds in nature. Although the proposed biosynthetic pathways for capsaicin and dihydrocapsaicin in *Capsicum* fruits were developed in 1968 (Bennett and Kirby 1968; Leete and Louden 1968), many enzymes involved in the biosynthesis of capsaicinoids are not yet well characterized, and regulation of the pathway is not fully understood. It is now known that there are two main routes for the biosynthesis of capsaicinoids. The first depends on the route of the phenylpropanoid, where L-phenylalanine derivatives such as cinnamic, *p*-coumaric, caffeic, and ferulic acid as well as vanillin lead to the formation of vanillylamine for subsequent condensation of capsaicin through capsaicin synthase. The second route involves the metabolism of branched fatty acids, mainly derived from valine or leucine, ending in the formation of 8-methyl-6-nonenoyl-CoA. Finally, capsaicin is generated through a condensation reaction between vanillylamine and 8-methyl-6-nonenoyl-CoA catalyzed by the coenzyme A-dependent acyltransferase (Arora et al. 2011; Baskaran et al. 2019; Chapa-Oliver and Mejía-Teniente 2016). Currently proposed pathways for biosynthesis of capsaicin in *Capsicum* fruits are shown in Fig. 8.2 (Aza-Gonzalez et al. 2011; Bennett and Kirby 1968; Kehie et al. 2015; Leete and Louden 1968).

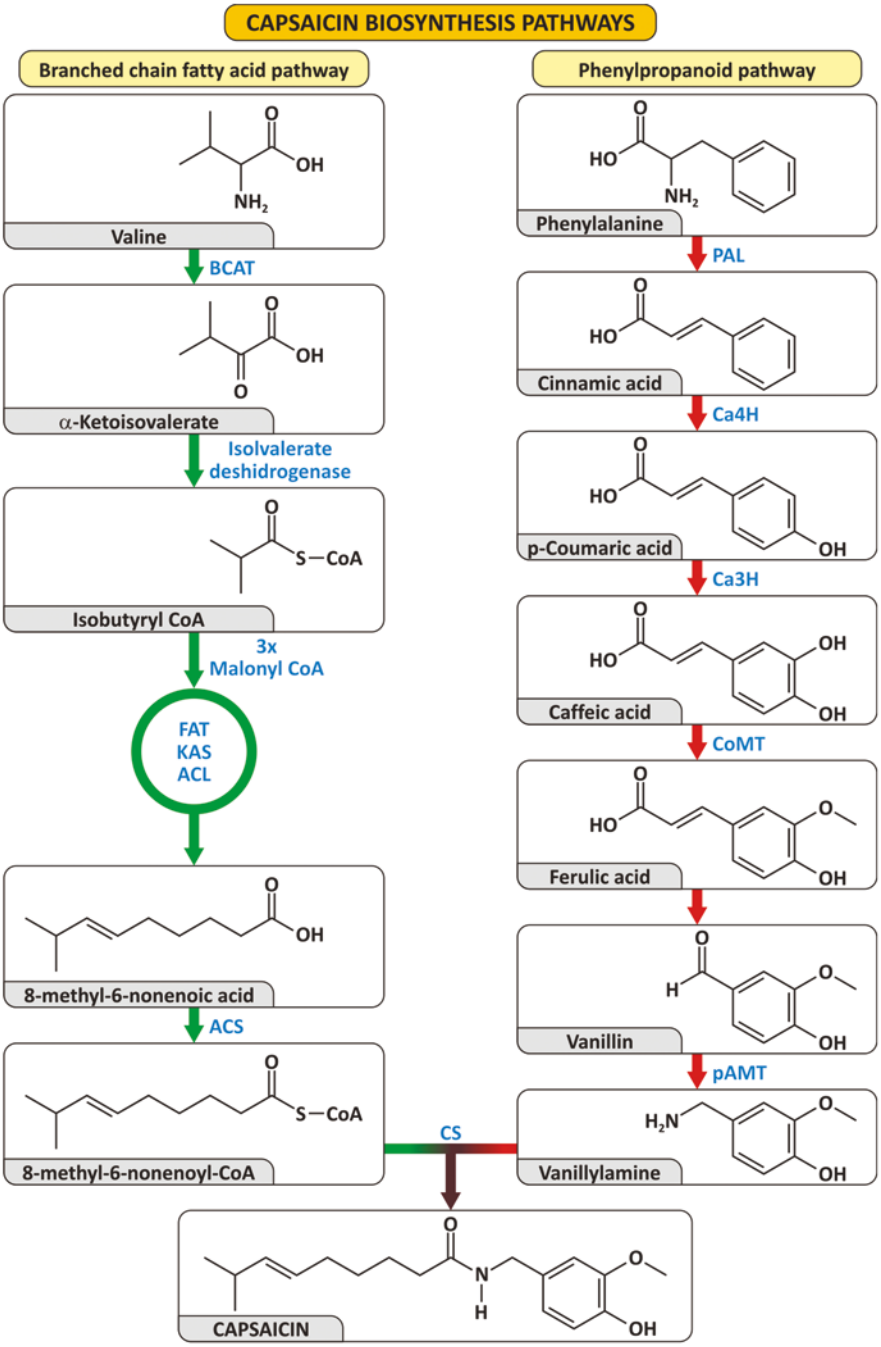


Fig. 8.2 The pathways for biosynthesis of capsaicin in *Capsicum* fruit (Aza-Gonzalez et al. 2011; Kehie et al. 2015; Leete and Louden 1968): *BCAT* branched-chain amino acid transferase, *FAT* fatty acid thioesterase, *KAS* β-ketoacyl synthase, *ACL* acyl carrier protein, *ACS* acyl-CoA synthetase, *PAL* phenylalanine ammonia lyase, *Ca4H* cinnamic acid 4-hydroxylase, *Ca3H* coumaric acid 3-hydroxylase, *CoMT* caffeic acid O-methyltransferase, *pAMT* *p*-aminotransferase, *CS* capsaicin synthase

The Chemistry of Capsaicinoids

Capsaicinoids are a naturally occurring group of compounds including capsaicin (CAP, *trans*-8-methyl-N-vanillyl-6-nonenamide), dihydrocapsaicin (DHC, 8-methyl-N-vanillylnonanamide), nordihydrocapsaicin (NDC, 7-methyl-N-vanillyloctanamide), homocapsaicin (HC, *trans*-9-methyl-N-vanillyl-7-decenamide), and homodihydrocapsaicin (HDC, 9-methyl-N-vanillyldecanamide) extracted from the fruit of the *Capsicum* genus plants. Capsaicinoids are derivatives of vanilloid same as vanillin from vanilla, eugenol from bay leaves and cloves, and zingerone from ginger. The vanilloids possess a vanillyl (4-hydroxy-3-methoxybenzyl) moiety which contributes to their biological activity. In addition to the vanilloid group, they contain a long hydrocarbon chain attached through a polar amide group (Arora et al. 2011; Hayman and Kam 2008; Ilie et al. 2019; Luo et al. 2011).

Capsaicin is a crystalline, lipophilic, colorless, and odorless alkaloid with a melting point of 62–65 °C and a molecular weight of 305.4 g mol^{-1}. Capsaicin is soluble in ethanol, acetone, and fatty oils, but is not soluble in cold water. As it is not water-soluble, alcohols and other organic solvents are used to solubilize capsaicin in topical preparations and sprays (Arora et al. 2011; Barceloux 2008; Hayman and Kam 2008; Reyes-Escogido et al. 2011).

Capsaicin and homocapsaicin display *cis/trans*-isomerism because the double bond prevents internal rotation. These capsaicinoids are always found as the *trans*-isomer because in the *cis* form the iso-propyl group and the longer chain on the other side of the double bond will be close together, causing them to repel each other slightly. This type of steric hindrance does not exist in the *trans*-isomer. This additional strain imposed causes the *cis*-isomer to be a less stable arrangement than the *trans*-isomer (Reyes-Escogido et al. 2011). Civamide (*cis*-8-methyl-N-vanillyl-6-nonenamide) is a synthetic and less stable *cis*-isomer of capsaicin which has been tested for treatment of a variety of conditions associated with ongoing nerve pain, including herpes simplex infections, cluster headaches, and migraine, as well as knee osteoarthritis (Barceloux 2008; Saper et al. 2002). Nonivamide (NVA, vanillyl-N-nonylamide) is a chemical structurally similar to capsaicin and sometimes termed "synthetic capsaicin," which possesses slightly less pungent properties compared with capsaicin but exhibits strong anti-inflammatory properties (Walker et al. 2017). Chemical structures of natural and synthetic capsaicinoids and their corresponding SHU values are presented in Fig. 8.3 (Barceloux 2008; Lu et al. 2017).

The Toxicity and Pharmacokinetics of Capsaicin

The widespread use of chili peppers in the food industry, as well as the health benefits of capsaicin contained in them, forced the need to study the degree of capsaicin toxicity. According to various sources, the lethal dose of capsaicin was estimated to

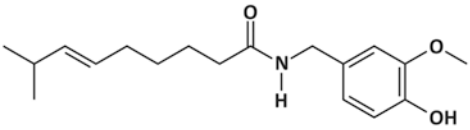
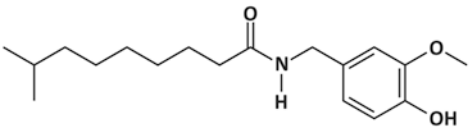
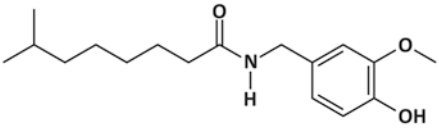
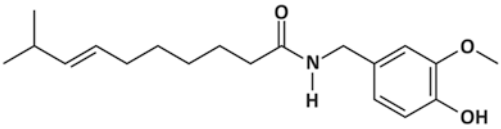
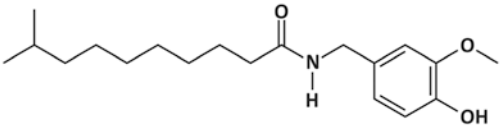
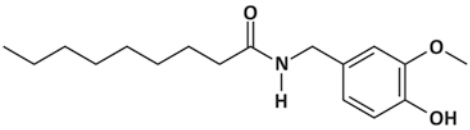
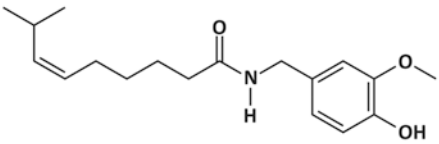
Capsaicinoid (abbreviation) Chemical structure	Relative amount [%]	Scoville Heat Units
CAPSAICIN (CAP) trans-8-methyl-N-vanillyl-6-nonenamide 	69	16 000 000
DIHYDROCAPSAICIN (DHC) 8-methyl-N-vanillylnonanamide 	22	15 000 000
NORDIHYDROCAPSAICIN (NDC) 7-methyl-N-vanillyloctanamide 	7	9 100 000
HOMOCAPSAICIN (HC) trans-9-methyl-N-vanillyl-7-decenamide 	1	8 200 000
HOMODIHYDROCAPSAICIN (HDC) 9-methyl-N-vanillyldecanamide 	1	8 600 000
NONIVAMIDE (NVA) vanillyl-N-nonylamide 	synthetic	9 200 000
CIVAMIDE cis-8-methyl-N-vanillyl-6-nonenamide 	synthetic	-

Fig. 8.3 Chemical structures of natural and synthetic capsaicinoids and their corresponding SHU values

be about 56–512 mg kg^{-1} body weight, which classifies it as a toxic substance. Capsaicin may cause histopathological and biochemical changes of gastric mucosa and lesions of liver and kidneys after too much oral dose ingestion. Also, oral exposure to too much dose of capsaicin is a cause of respiratory disorders which can lead to death (Antonious 2018; Baskaran et al. 2019; Johnson 2007; Reilly et al. 2003; Saito and Yamamoto 1996; Surh and Lee 1995).

Capsaicin is absorbed effectively and reaches maximum concentration if it is applied topically on the skin. The half-life of the capsaicin is equal to 24 h. During the metabolism of capsaicin, three major metabolites have been identified: 16-hydroxycapsaicin, 17-hydroxycapsaicin, and 16,17-dihydrocapsaicin (Chanda et al. 2008; Pershing et al. 2004; Szolcsányi 2004). In vitro studies regarding human skin suggest that most capsaicin remained unchanged while a small fraction was metabolized to vanillylamine and vanillyl acid, which further suggested the role of cytochrome P450 enzymes in the transformation of capsaicin in human skin. Capsaicin is mainly eliminated by the kidneys with a small untransformed fraction excreted in the feces and urine (Arora et al. 2011; Chanda et al. 2008; Ilie et al. 2019; Reyes-Escogido et al. 2011; Sharma et al. 2013).

Capsaicin has been widely investigated and is an important molecule in the area of medicinal research, but its clinical applications are still very limited due to its toxicity and not fully understood pharmacokinetics (Antonious 2018; Baskaran et al. 2019; Johnson 2007; Reilly et al. 2003; Saito and Yamamoto 1996; Surh and Lee 1995).

Role of Capsaicinoids in Disease Therapy

Capsicum fruits have been described for centuries as a source of compounds with therapeutic and pro-health properties. One of the reasons is the fact that *Capsicum* fruits are an excellent source of antioxidant compounds including anthocyanins, vitamins, phenolic acids, flavonoids, and carotenoids, as well as capsaicinoids. As a result, chili and red pepper fruit rank high in terms of antioxidant activity compared with other vegetables (Ionică et al. 2017; Materska and Perucka 2005; Surh 2002; Szolcsányi 2004; Zimmer et al. 2012).

However, when analyzing the chemical composition of *Capsicum* fruit, special attention should be paid to the properties of capsaicinoids, mainly capsaicin, the action of which is largely based on interaction with the sensory receptor of TRPV1, named transient receptor potential vanilloid 1 (Cortright and Szallasi 2004).

In the past decades, many research articles reported that capsaicinoids have been extensively studied for their analgesic and anti-obesity properties and play an important role in thermoregulation as well as in the metabolism of glucose and lipids. Importantly, in most cases, the action of capsaicin is based on TRPV1 activation. Therefore, the pro-health applications of capsaicin have been studied in the treatment of diseases such as obesity, diabetes, atherosclerosis, hypertension, and

fatty liver, as well as treatment of pain of various origins. There are also many scientific studies which confirm the anticancer activity of capsaicin; however, there are also researches that suggest the carcinogenic or co-carcinogenic activity of this compound (Antonious 2018; Chapa-Oliver and Mejía-Teniente 2016; Fattori et al. 2016; Sharma et al. 2013). It is believed that the main factor which inhibits the implementation of new uses of capsaicin, especially in medicine and pharmacology, is its significant toxicity.

Mechanism of TRPV1 Activation by Capsaicin

Ion channels of the transient receptor potential (TRP) are a group of over 30 structurally related channels, among others: TRPC (canonical), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin), and TRPV (vanilloid). The physiological role of TRP channels ranges from ion channels to thermo- and chemosensors (Cseko et al. 2019).

Transient receptor potential vanilloid receptor subtype 1 (TRPV1) is a cationic channel, the best permeable to divalent cations, mainly calcium. This receptor occurs in large quantities on nociceptive neurons in the peripheral nervous system but also occurs in tissues of the central nervous system, bladder, kidneys, intestines, and liver (Cortright and Szallasi 2004; Tominaga and Tominaga 2005).

TRPV1 contains 838 amino acids and consists of six transmembrane domains (TD) with a short pore-forming region between the fifth and sixth transmembrane

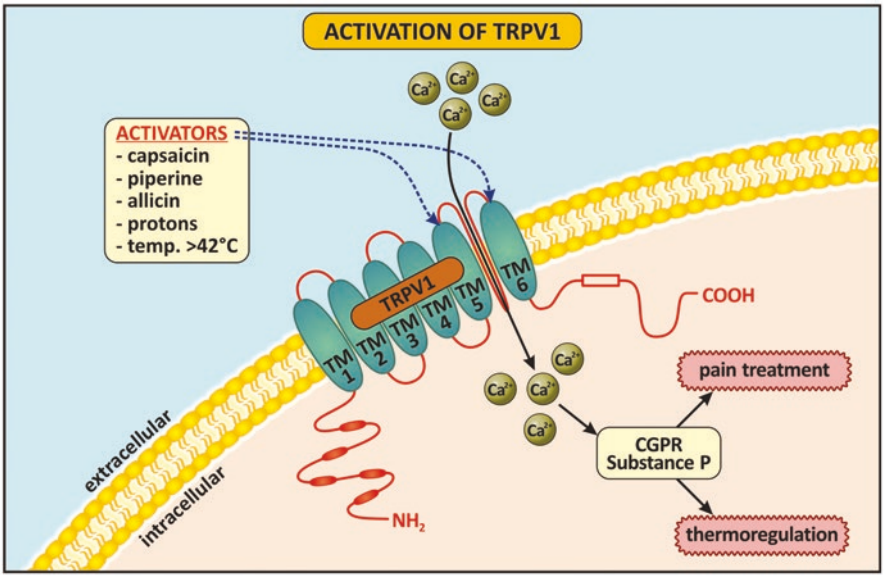


Fig. 8.4 The structure and mechanism of activation of receptor TRPV1

domains (Tominaga and Tominaga 2005). The structure of the transient receptor potential vanilloid receptor subtype 1 is presented in Fig. 8.4 (Leonelli et al. 2011).

In recent decades, the mechanism of action of capsaicinoids, mainly capsaicin, in living organisms has been extensively studied. It was repeatedly confirmed that capsaicin binds to the vanilloid receptor, TRPV1 (Cortright and Szallasi 2004). However, the TRPV1 receptor not only binds accordingly with capsaicin but is sensitive to a very wide range of factors, such as zingerone from ginger (Yin et al. 2019), allicin from garlic (Macpherson et al. 2005), piperine from black pepper (McNamara et al. 2005), and curcumin from turmeric (Yeon et al. 2010), and also to temperature above 42 °C and the presence of protons (at low pH).

Capsaicin, after binding to the receptor located at the ends of neurons, opens the channel and inflows of calcium ions inside the cell, which leads to the depolarization of the cell membrane. Thus, formed action potential is transmitted into the spinal cord and causes the feeling of pain and warmth, among others. Activation of nerve endings is also manifested by the release of pro-inflammatory neuropeptides, such as substance P (important in pain transduction processes) or the calcitonin gene-related peptide (CGRP). The CGRP plays an important role in maintaining the integrity of the duct mucosa tract. Activation of TRPV1 causes calcium influx in certain tissues what is associated with the increased activation or expression of key proteins, such as endothelial nitric oxide synthase (eNOS), uncoupling protein 2 (UCP2), peroxisome proliferation-activated receptors (PPARα, PPARγ, and PPARδ), as well as liver X receptor α (LXRα), which are important for the proper metabolism and functioning of the living organisms (Arora et al. 2011; Cortright and Szallasi 2004; Hayman and Kam 2008; Kárai et al. 2004; McCarty et al. 2015; Reyes-Escogido et al. 2011; Tominaga and Tominaga 2005). The mechanism of activation of TRPV1 by capsaicin (and other activating factors) is presented in Fig. 8.4 (Anand and Bley 2011).

It is also important that capsaicin not only interacts with the TRPV1 receptor but also affects many other metabolic pathways in the living organism, which is why the research on its pro-health properties is so comprehensive.

The Analgesic Effect of Capsaicin

Capsaicin may cause pain and hyperalgesia, but paradoxically, it has established clinical use as an analgesic. For this reason, capsaicin is an important tool in the study of mechanisms related to pain. Stimulation of receptor TRPV1 at the ends of the nerves by capsaicin causes the release of various neuropeptides, among others, discussed in the previous subsection, substance P. Its release causes pain and burning as a consequence (Tominaga and Julius 2000).

However, the analgesic effect associated with TRPV1 desensitization is more important for pro-health use. Long-term use of capsaicin causes the TRPV1 receptor hypersensitive; therefore, pain stimuli can no longer cause the release of inflammatory neuropeptides (substance P) into the synaptic cleft. The blockage of stimuli

conduction of pain and reduction of feeling pain in the spinal cord occurs as a consequence (Basith et al. 2016; Brederson et al. 2013; Mason et al. 2004).

Understanding the mechanism associated with the effect of capsaicin on reducing the sensation of pain has allowed its introduction to the medicinal use, i.e., in creams containing capsaicin. The topical creams with capsaicin are mainly used to treat pain from postherpetic and diabetic neuropathy (0.075% cream three to four times daily for 8 weeks), osteoarthritis (0.025% cream, four times daily), and rheumatoid arthritis. Capsaicin can also be used to treat pain due to psoriasis, bladder disorders, and cluster headaches (Arora et al. 2011; Brederson et al. 2013; Fattori et al. 2016; Mason et al. 2004).

The oral consumption of capsaicin with food is also safe, but it does not have pharmacologic effects on pain. For these reasons, the majority of studies regarding the pharmacokinetics of capsaicin in humans are carried out after topical application to the skin (Antonious 2018).

The Effect of Capsaicin on Thermoregulation

Capsaicin plays an important role in thermoregulation, which is also associated with the activation of the TRPV1 receptor and depends, inter alia, on the time since admission (immediate or delayed effects), the frequency of administration (single or regular), and the size of the dose. After a single application of a small amount of capsaicin, a decrease in body temperature (hypothermia) was found (Romanovsky et al. 2009; Szolcsányi 2015). It is associated with release of pro-inflammatory peptides (substance P and CGRP), which cause the expansion of peripheral blood vessels, which in turn increases the heat exchange surface with the surrounding, and thus the heat loss also increases. Also, there is a decrease in the metabolic rate, which is expressed as reduced oxygen consumption, which in turn results in reduced heat production. However, if capsaicin is administered regularly for a long time, the result is the opposite. This involves the inactivation of the TRPV1 receptor with prolonged exposure to capsaicin. An increase in body temperature, i.e., hyperthermia, is observed (Romanovsky et al. 2009; Wang and Siemens 2015).

Another example of capsaicin-assisted thermoregulation is a change of the activity of calcium transporters in muscles leading to increased thermogenesis (heat release). The ATP-dependent calcium transporter is a protein that transfers calcium ions from the cytoplasm to the endoplasmic reticulum of muscle cells, consuming energy from the breakdown of high-energy bonds contained in adenosine triphosphate (ATP). As a result of capsaicin attachment, this transporter stops transferring calcium ions, but still breaks down ATP. The energy is dissipated in the form of heat (Szolcsányi 2015). The role of capsaicin in thermoregulation is closely related to lipid metabolism and the reduction of body fat.

Anti-obesity Action of Capsaicin

Obesity is a major risk factor for various diseases, including coronary heart disease, hypertension, diabetes, and cancer. Therefore, searching for various possibilities to fight against obesity is of high importance.

Peroxisome proliferation-activated receptors (PPARs) are ligand-activated transcription factors involved in the regulation of lipid metabolism, energy production, and inflammations. Activation of TRPV1 by capsaicin stimulates the action of PPARα and PPARγ, which inhibit the adipogenesis (the process of adipose accumulation), and stimulates the thermogenesis (the process of heat production in the organism), which leads to a reduction of body fat and thus weight loss (Antonious 2018; Arora et al. 2011; Fattori et al. 2016; Leung 2014; Zheng et al. 2017).

Capsaicin also affects the transcription of genes responsible for the production of enzymes that catalyze lipid reduction reactions. Studies regarding the effect of capsaicin on weight reduction were initially conducted on animals and then also on humans, and most of the results confirmed its slimming effect. Among others, they involve increased fat oxidation, increased energy consumption associated with the activation of the sympathetic nervous system by catecholamines, and decreased appetite (Fattori et al. 2016; Panchal et al. 2018; Sharma et al. 2013; Zheng et al. 2017). Due to the confirmed effect on thermogenesis and reduction of body fat, capsaicin is increasingly used in dietary supplements supporting weight loss.

The Effect of Capsaicin on Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic lipids deposition as simple steatosis, steatohepatitis, and cirrhosis and is often connected with other cardiometabolic diseases. The results of studies of capsaicin intake show that TRPV1 activation reduces free fatty acids (FFAs) and prevents fatty liver. Long-term use of capsaicin accelerates the lipolysis process by increasing hepatic phosphorylated hormone-sensitive lipase (phospho-HSL), carnitine palmitoyltransferase 1 (CPT1), and peroxisome proliferator-activated receptor δ (PPARδ).

It is believed that the most important factor in the fight against fatty liver disease is the activation of PPARδ, which regulates lipogenesis in hepatocytes and significantly increases the expression of proteins associated with autophagy (Li et al. 2013).

The Effect of Capsaicin on Hypertension

Endothelial cells (ECs) are specialized cells localized at the interface between blood and tissue and are critical for several aspects of cardiovascular homeostasis, including thrombosis, blood flow control, and delivery of oxygen and nutrients throughout

the body. Accordingly, the dysfunction of ECs is a common cause of multiple cardiovascular diseases such as hypertension or atherosclerosis. One cause of ECs dysfunction is the inability of blood vessels to produce or respond to nitric oxide (NO), which is a vasodilatation factor. In blood vessels, NO is produced by the enzyme endothelial nitric oxide synthase (eNOS) (Pober and Sessa 2007).

TRPV1 is present in endothelial cells, and activation of this receptor, among others, by capsaicin enhances phosphorylation of protein kinase A (PKA) by increasing the activity of eNOS, thus the production of nitric oxide in endothelial cells. This process is calcium ion-dependent. Nitric oxide is a vasorelaxant factor; i.e., it causes vasodilatation. Therefore, TRPV1-mediated increase in NO production represents a promising and still widely studied mechanism in the treatment of hypertension (Adams et al. 2009; Pober and Sessa 2007; Sharma et al. 2013; Sun et al. 2016; Yang et al. 2010).

The cardiovascular system is rich in capsaicin-sensitive sensory nerves, which play a major role in regulating the function of this system by releasing neurotransmitters. One such neurotransmitter is CGRP, which is considered as one of the strongest vasodilators and plays an important role in regulating blood pressure. Capsaicin can stimulate the release of CGRP by activating TRPV1, thereby reducing blood pressure (Sharma et al. 2013; Yang et al. 2010).

The Effect of Capsaicin on Atherosclerosis

Atherosclerosis is a disease that is mainly characterized by vascular inflammation and lipids accumulation within the blood vessel walls and especially in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs).

Capsaicin activates the TRPV1, which then induces autophagy and inhibits foam cell transformation from VSMCs. This mechanism occurs through the regulation of the AMP-activated protein kinase (AMPK) pathway and increase of cholesterol efflux as well as reduction of cholesterol uptake through the regulation of ATP-binding cassette transporter (ABCA1). Capsaicin through activation of TRPV1 effects also on eNOS, which inhibits the expression of inflammatory cytokines and adhesion of molecules in ECs (Arora et al. 2011; Fattori et al. 2016; Sun et al. 2016).

Also, atherosclerosis is initiated by endothelial cell damage and the attachment of oxidized low-density lipoproteins (oxLDL) on the inner wall of an artery. In vitro studies indicated that capsaicin can increase the resistance of LDL to oxidation by delaying the initiation of oxidation or slowing the rate of oxidation. Regular consumption of *Capsicum* fruits for 4 weeks has been found to increase the resistance of serum lipoproteins to oxidation, so is a promising effect in the treatment of atherosclerosis (Adams et al. 2009; Fattori et al. 2016; Ma et al. 2011; Peng and Li 2010; Sun et al. 2016).

The Effect of Capsaicin on Diabetes

Capsaicin has been extensively investigated because of its role in improving glucose and insulin homeostasis and alleviating diabetes. The pathogenesis of diabetes is complex and involves oxidative stress and inflammation, which facilitate insulin resistance.

Capsaicin (through TRPV1 activation) may promote insulin secretion through the calcium influx in cells. Activation of TRPV1 alleviates insulin resistance and regulates glucose homeostasis by suppressing inflammation. Also, capsaicin may reduce obesity-induced insulin resistance. This beneficial effect of capsaicin was due to the attenuation of inflammatory phenotypes and enhanced adiponectin expression in adipose tissue and liver, which are important peripheral tissues for insulin sensitivity (Antonious 2018; Panchal et al. 2018).

Studies determined that long-term capsaicin intakes are related to the lower blood glucose level and preservation of the high insulin level, which is another positive aspect of this compound (Antonious 2018; Fattori et al. 2016; Narang et al. 2017; Panchal et al. 2018).

The influence of capsaicin on cardiometabolic diseases and related target organ damage was summarized in Fig. 8.5.

Influence of Capsaicin on the Gastrointestinal Diseases

In the treatment of gastrointestinal diseases, it is suggested to avoid spicy foods in the daily diet. However, the results of scientific studies indicate the potential gastroprotective effect of capsaicin contained in the *Capsicum* fruit. Reports indicate that regular use of low doses of capsaicin in a group of healthy people reduced the risk of stomach ulcers, the etiology of which is associated with increased ethanol consumption or aspirin intake. The anti-ulcer effect is combined with the activation of TRPV1 receptors present in sensory neurons of the gastric mucosa. It causes the release of neuropeptides, such as CGRP, somatostatin, or neurokinin A as well as nitric oxide, which stimulate the expansion of blood vessels, and thus increases blood microcirculation in the gastric mucosa (Fattori et al. 2016; Mozsik et al. 2005).

On the other hand, capsaicin in high concentrations harms the gastric mucosa, as it can cause inactivation of TRPV1 receptors, which blocks the cascade of reactions leading to the gastroprotective effect. Moreover, it can have much more serious consequences in the form of pathological changes in the liver or gastric mucosa (Arora et al. 2011; Fattori et al. 2016; Hayman and Kam 2008; Peng and Li 2010).

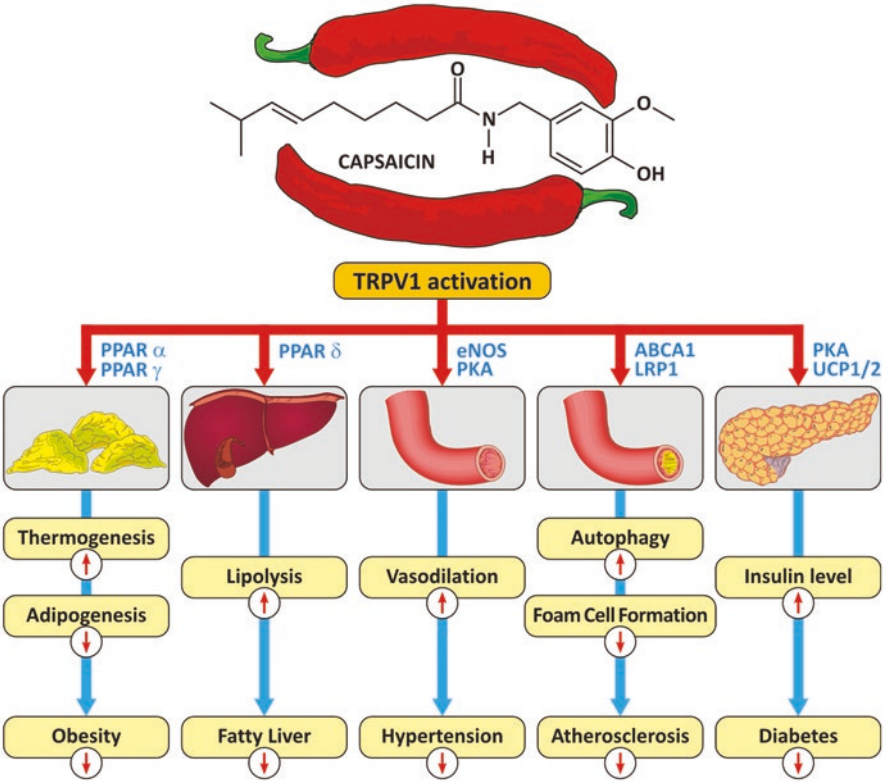


Fig. 8.5 The influence of capsaicin on cardiometabolic diseases and related target organ damage: *PPARα, PPARγ, PPARδ* peroxisome proliferation-activated receptors; *eNOS* endothelial nitric oxide synthase; *PKA* phosphorylation of protein kinase A; *ABCA1* ATP-binding cassette transporter; *LRP1* low-density lipoprotein receptor-related protein 1; *UCP1/2* uncoupling protein 1/2

Anticancerogenic Mechanisms of Capsaicin

Cancer cells acquire unique capabilities that most healthy cells do not possess. Cancer cells become resistant to growth-inhibitory signals, proliferate without dependence on growth-stimulatory factors, replicate without limits, escape apoptosis (the breakdown of cells), and acquire invasive and angiogenic (creation of new blood vessels) properties (Bley et al. 2012; Fattori et al. 2016).

The anticancer mechanism of capsaicin (Fig. 8.6) relies on the alteration of the expression of genes involved in

(a) activation of apoptosis,
(b) inhibition of angiogenesis,
(c) cell-growth arrest, and
(d) inhibition of metastasis (Arora et al. 2011; Clark and Lee 2016; Dou et al. 2011).

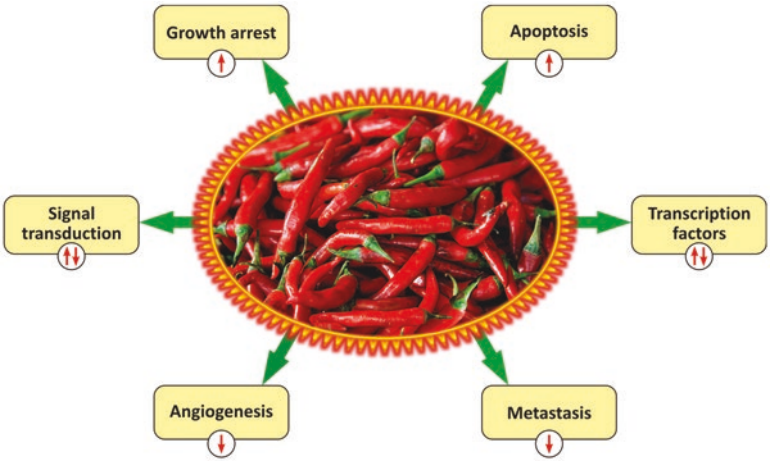


Fig. 8.6 The general scheme of anticancer mechanisms of capsaicin

Apoptosis is an essential barrier against cancer development and progression. The loss of this barrier is highly associated with malignancy. Many types of cancer cells disrupt apoptotic pathways and enhance anti-apoptotic ones.

The mechanism by which capsaicin activates apoptosis in cancer cells is not known in detail, but in this regard, it is believed that cancer cell apoptosis occurs as a result of inhibition of the last stage of cellular respiration in mitochondria. The impact of capsaicin on cellular respiration can be twofold. On the one hand, this substance may be an inhibitor of an electron transporting enzyme from nicotine adenine dinucleotide oxidoreductase (NADH) to ubiquinone occurring in the complex and the respiratory chain. On the other hand, capsaicin probably can also bind directly to coenzyme Q, which will change the direction of the electron flow and the excessive formation of reactive oxygen forms and, consequently, disperse the transmembrane potential in mitochondria (Chapa-Oliver and Mejía-Teniente 2016; Clark and Lee 2016; Surh 2002). The transmembrane potential is a part of the proton gradient arising during the flow of electrons through the respiratory chain and is fundamental for the functioning of mitochondria. Both formation of reactive oxygen species (ROS) and inhibition of electron transporting enzymes can result in oxidative stress, damage to the cell structure, and disruption of the functioning of mitochondria, and consequently support the development of the cancer cells (Archer and Jones 2002; Chapa-Oliver and Mejía-Teniente 2016; Clark and Lee 2016).

The interaction of capsaicin with the TRPV1 receptor and the associated migration of calcium ions cause another significant effect: there is a decrease in mesothelial mitochondrial potential. Such energetic changes can also lead to cell apoptosis. However, it is important that capsaicin activity in this area is limited only to the destruction of cancer cells, but does not occur in healthy cells.

Another mechanism of capsaicin interaction with cancer is associated with the process of angiogenesis, i.e., the formation of new blood vessels from the

endothelium. Angiogenesis is a key stage in cancer cell growth and metastasis (a complex process that occurs when cancer cells acquire the ability to invade the vasculature and migrate to distant organs). The proliferation of cancer-affected cells increases their oxygen demand. Over time, the primary network of blood vessels is unable to transport enough oxygen for cancer cells. So there is a need to shape new, additional blood vessels. Scientific studies have confirmed that capsaicin is an anti-angiogenic compound because it acts as an inhibitor of the angiogenic pathways induced by VEGF (vascular endothelial growth factor). This factor is the main regulator of the angiogenesis process not only in cancer cells but also in healthy ones. Hence, it is suggested that capsaicin may find use in the treatment of angiogenesis-dependent diseases (Bley et al. 2012; Clark and Lee 2016; Dou et al. 2011; Fattori et al. 2016; Talmadge and Fidler 2010).

The cell-growth arrest has gained much attention as a defense mechanism against cancer and target for cancer prevention. The cyclins, cyclin-dependent kinases (CDKs), and the CDK inhibitors are essential parts of the cell-cycle regulations. Scientific researches confirmed that capsaicin may stop the growth and division of cancer cells by targeting cell-cycle regulators (Kastan and Bartek 2004). Studies indicated that capsaicin exhibits anti-invasive and anti-migratory activity on metastasis through modulating signaling pathways involved in cell invasion and migration, and suppresses advanced stages of cancer (Clark and Lee 2016; Talmadge and Fidler 2010).

Reports indicated that capsaicin destroyed many different types of cancer cell lines, including pancreatic (Pramanik et al. 2011; Vendrely et al. 2017), colonic (Jin et al. 2014; Kim et al. 2007), prostatic (Mori et al. 2006; Ramos-Torres et al. 2016), liver (Lee et al. 2004a), esophageal (Mao et al. 2018; Wu et al. 2006), bladder (Lee et al. 2004b; Montani et al. 2015), skin (Hail and Lotan 2002), leukemia (Ito et al. 2004), lung (Athanasiou et al. 2007; Lau et al. 2014), and many others, leaving normal cells intact (Bley et al. 2012; Clark and Lee 2016; Fattori et al. 2016; Sarkar and Li 2009), mainly by activation of apoptosis as well as inhibition of angiogenesis.

Unfortunately, apart from many scientific reports on various anticancerogenic mechanisms of capsaicin, there are also studies partially confirming the carcinogenic or co-carcinogenic activity. Therefore, the carcinogenic potential of capsaicin requires further study (Bley et al. 2012; Surh and Lee 1996).

Extraction and Determination of Capsaicinoids

The first attempts to determine capsaicinoids in *Capsicum* peppers were carried out in the mid-twentieth century, and at that time, colorimetric photometry (North 1949), gas chromatography (GC; Hartman 1970), thin-layer chromatography (Pankar and Magar 1977; Spanyar and Blazovich 1969), spectrophotometry (Nikolaeva 1984), and micellar electrokinetic capillary chromatography (Laskaridou-Monnerville 1999) were used.

After 2000, the increased interest in capsaicinoids has led to the introduction of more sensitive, faster techniques used for their separation and determination (Ilie et al. 2019; Reyes-Escogido et al. 2011; Stoica et al. 2016). Nowadays, mainly gas chromatography (GC) with mass spectrometry (Peña-Alvarez et al. 2009; Wesołowska et al. 2011) and high-performance liquid chromatography (HPLC) with various detectors, such as fluorescence detector (Barbero et al. 2006, 2008a, b, 2014; Dang et al. 2018; Daood et al. 2015; Ha et al. 2010), diode array detector (Gonzalez-Zamora et al. 2015; Juangsamoot et al. 2012; Popelka et al. 2017; Sganzerla et al. 2014; Usman et al. 2014), and UV detector (Musfiroh et al. 2013; Othman et al. 2011; Perucka and Oleszek 2000; Ryu et al. 2017), as well as a mass spectrometer (Garces-Claver et al. 2006; Kozukue et al. 2005; Pavon-Perez et al. 2019; Reilly et al. 2001, 2002; Zhang et al. 2010), are most commonly used for the determination of capsaicinoids.

Various extraction methods of capsaicinoids from *Capsicum* fruits have been proposed during the past decades. When designing an extraction process, the first step is the selection of an appropriate solvent which can result in high extraction efficiency of capsaicinoids. Among all extractants of capsaicinoids, methanol (Barbero et al. 2008a; Daood et al. 2015; Juangsamoot et al. 2012; Kozukue et al. 2005; Pavón-Pérez et al. 2019; Sganzerla et al. 2014), ethanol (Barbero et al. 2006; Ha et al. 2010; Musfiroh et al. 2013; Othman et al. 2011), acetonitrile (Garces-Claver et al. 2006; Gonzalez-Zamora et al. 2015; Usman et al. 2014; Zhang et al. 2010), acetone (Ryu et al. 2017; Wesołowska et al. 2011), or mixtures of solvents (Perucka and Oleszek 2000; Popelka et al. 2017; Reilly et al. 2001) are the most commonly used. In addition to the solvent selection procedure, parameters such as temperature, time of extraction, and the amount of sample should also be considered.

The most commonly used extraction techniques include maceration (Kozukue et al. 2005; Musfiroh et al. 2013; Popelka et al. 2017), magnetic stirring extraction (MSE) (Juangsamoot et al. 2012), Soxhlet extraction (SOX) (Ha et al. 2010; Wesołowska et al. 2011), microwave-assisted extraction (MAE) (Barbero et al. 2006, 2008b), ultrasound-assisted extraction (UAE) (Barbero et al. 2008a, 2014; Daood et al. 2015; Gonzalez-Zamora et al. 2015; Popelka et al. 2017; Sganzerla et al. 2014), and solid-phase extraction (SPE) (Juangsamoot et al. 2012; Musfiroh et al. 2013), as well as solid-phase microextraction (SPME) (Peña-Alvarez et al. 2009). The conditions of extraction and determination of capsaicinoids published over the past 20 years are shown in Table 8.1.

Summary

Capsaicinoids, mainly capsaicin, are alkaloids naturally found in *Capsicum* fruit. Depending on the *Capsicum* species, they exhibit different degrees of pungency because of the diverse content of capsaicinoids and include numerous other ingredients that affect their antioxidant properties. *Capsicum* fruit is a popular spice with culinary and taste qualities. On the other hand, thanks to capsaicinoids, it is an

Table 8.1 The conditions of extraction and determination of capsaicinoids published over the past 20 years

Analytes	Matrices	Extraction conditions	Detection	Determination conditions	LOD (μg mL^{-1})	RSD (%)	References
CAP DHC	*Capsicum annuum* fruit	Extractant: Ac:PE (1:1, v:v), Washed out with H_2O Dried (Na_2SO_4)	HPLC-UV	Column: Eurospher 80 (C_{18}) ACN/H_2O: ACN (gradient) Flow rate: 1.0 mL min^{-1} - λ_{UV} = 280 nm	–	–	Perucka and Oleszek (2000)
CAP DHC NVA	Self-defense weapons (pepper spray)	Extractant: MeOH: n-BuCl (1:1, v:v) Dried (air, 40 °C) Dissolved in MeOH:H_2O (6:4, v:v)	LC-MS/MS	Column: MetaSil Basic (C_2–C_8) MeOH: 0.1% HCOOH (gradient) Flow rate: 0.25 mL min^{-1} Collision gas: argon Collision energy: −15 V	0.010	6.0 10.0 7.0	Reilly et al. (2001)
CAP DHC NVA	Blood, tissue	Extractant: n-BuCl Vortex (20 min, rt) Dried (air, 40 °C) Dissolved in MeOH:H_2O (7:3, v:v)	LC-MS/MS	Column: MetaSil Basic (C_2–C_8) MeOH: 0.1% HCOOH (gradient) Flow rate: 0.25 mL min^{-1} Collision gas: argon Collision energy: −15 V	0.0025	4.0 4.0 6.0	Reilly et al., (2002)
CAP DHC HC HDC NDC NVA	Fresh pepper, pepper-containing food	Maceration (MeOH) Centrifuged (10 min 2000 rpm)	LC-MS	Column: Zorbax Eclipse XDB-C18 ACN: 0.5% HCOOH (isocratic) 45:55 (v:v) Flow rate: 1.0 mL min^{-1} Capillary voltage 27 V	–	–	Kozukue et al. (2005)
CAP DHC HC HDC NDC	*Capsicum frutescens* and *Capsicum annuum* fruits	MAE (125 °C, 500 W) Extractant: EtOH Extraction time: 5 min	HPLC-FD	Column: Chromolith RP (C_{18}) 0.1% HCOOH in H_2O:0.1% HCOOH in MeOH (gradient) Flow rate: 6.0 mL min^{-1} λ_{ex} = 280 nm, λ_{em} = 310 nm	0.008 0.011 – – –	2.0 2.4 3.0 3.0 2.0	Barbero et al. (2006)

Analytes	Matrices	Extraction conditions	Detection	Determination conditions	LOD (μg mL^{-1})	RSD (%)	References
CAP DHC	*Capsicum* genotype	Extractant: ACN Shaken (rt, 1 h) Heated (65 °C, 1 h)	LC-ESI-TOF-MS	Column: Waters Symmetry (C_{18}) MeOH: H_2O (gradient) Flow rate: 0.9–1.8 mL min^{-1} Drying gas: N_2 (200 °C) Capillary voltage: 90 V	20[a] 4[a]	5.3 8.7	Garces-Claver et al. (2006)
CAP DHC HC HDC NDC	*Capsicum frutescens* fruits	UAE (50 °C, 360 W) Extractant: MeOH Extraction time: 10 min	HPLC-FD	Column: Chromolith RP (C_{18}) 0.1% HCOOH in H_2O:0.1% HCOOH in MeOH (gradient) Flow rate: 6.0 mL min^{-1} λ_{ex} = 278 nm, λ_{em} = 310 nm	0.008 0.011 – – –	1.7 1.8 2.4 1.8 2.0	Barbero et al. (2008a)
CAP DHC HC HDC NDC	Hot chili peppers cultivated in Spain	MAE (125 °C, 500 W) Extractant: pressurized fluids Extraction time: 5 min	HPLC-FD	Column: Chromolith RP (C_{18}) 0.1% HCOOH in H_2O:0.1% HCOOH in MeOH (gradient) Flow rate: 6.0 mL min^{-1} λ_{ex} = 278 nm, λ_{em} = 310 nm	0.008 0.011 – – –	<2.0	Barbero et al. (2008b)
CAP DHC	*Capsicum* genotype, pepper sauces	SPME (PDMS/DVB fiber) Extraction (pH = 7 50 °C, 30 min)	GC-MS	Column: Phenomenex ZB-5M (0.25 μm film) GC temp. 40–300 °C Ionization current: 350 μA ionization potential: 70 eV	– – – – –	9.9	Pena-Alvarez et al. (2009)
CAP DHC	Gochujang, chili oil, kimchi, snack	Extractant: EtOH Soxhlet (1 h, 90 °C)	UHPLC-FD	Column: LaChromUltra (C_{18}) ACN: 1% HCOOH (isocratic) (60:40, v:v) Flow rate: 0.6 mL min^{-1} λ_{ex} = 280 nm, λ_{em} = 325 nm	0.054[b] 0.053[b]	<5.2 <9.8	Ha et al. (2010)

(continued)

Table 8.1 (continued)

Analytes	Matrices	Extraction conditions	Detection	Determination conditions	LOD (μg mL^{-1})	RSD (%)	References
CAP DHC	Rat plasma	Extractant: EtOH Vortex 14,000 rpm, 5 min	HPLC-MS/MS	Column: Zorbax SB (C_{18}) ACN: 0.1% HCOOH (isocratic) (55:45, v:v) Flow rate: 0.2 mL min^{-1} Collision gas: Ar 10.0 L min^{-1}, Capillary voltage: 4.5 kV Collision energy: 23 eV (CAP), 15 eV (DHC)	–	6.0 5.7	Zhang et al. (2010)
CAP DHC	*Capsicum annuum* fruits	Extractant: EtOH Water bath (4 h, 80 °C)	HPLC-UV	Column: Betasil (C_{18}) ACN: H_2O (isocratic) (50:50, v:v) Flow rate 1.5 mL min^{-1} λ_{UV} = 222 nm	0.001[b] 0.001[b]	1.0 0.6	Othman et al. (2011)
CAP DHC	*Capsicum annuum* fruits	Extractant: Ac Soxhlet (reflux, 9 h)	GC-MS	Column: Agilent HP-5MS (30 m, 0.25 μm film) Flow rate: 2.0 mL min^{-1} (He) Injection 250 °C	– –	– –	Wesołowska et al. (2011)
CAP DHC	*Capsicum annuum* fruits	MSE (2 h, 60 °C) Extractant: MeOH SPE C_{18} cartridge (500 mg/4 cm^3)	HPLC-PDA	Column: Hypersil (C_{18}) MeOH:H_2O (isocratic) (70:30, v:v) Flow rate: 0.9 mL min^{-1} λ = 280 nm	0.5 1.0	1.0 4.0	Juangsamoot et al. (2012)
CAP	*Capsicum* annuum fruit	Maceration (EtOH, 24 h) SPE (HLB)	HPLC-UV	Column: Shim-Pack VP-ODS (C_{18}) ACN:2% CH_3COOH (isocratic) (60:40, v:v) Flow rate: 1.0 mL min^{-1} λ_{UV} = 280 nm	–	–	Musfiroh et al. (2013)

Analytes	Matrices	Extraction conditions	Detection	Determination conditions	LOD (μg mL^{-1})	RSD (%)	References
CAP DHC NDC	*Capsicum annuum* fruit, sweet paprika	UAE (US 20 min) Extractant: MeOH Shaken (15 min)	HPLC-FD	Column: RP-ODS-3 (C_{18}) ACN:0.01 M KH_2PO_4 (pH = 4) (isocratic) (55:45, v:v) Flow rate: 0.8 mL min^{-1} λ_{ex} = 280 nm, λ_{em} = 320 nm	0.004 0.002 0.003	2.3 2.1 1.8	Daood et al. (2015)
CAP DHC HC HDC NDC	Ripening of Cayenne pepper plant (*Capsicum annuum*)	UAE (50 °C, 360 W) Extractant: MeOH Extraction time: 15 min	HPLC-FD	Column: Chromolith RP (C_{18}) 0.1% HCOOH in H_2O:0.1% HCOOH in MeOH (gradient) Flow rate: 1.0 mL min^{-1} λ_{ex} = 278 nm, λ_{em} = 310 nm	– – – – –	– – – – –	Barbero et al. (2014)
CAP DHC	*Capsicum chinense* fruit	UAE Extractant: MeOH Extraction time: 10 min	UHPLC-DAD	Column: Hypersil Gold (C_{18}) ACN:H_2O (isocratic) (60:40, v:v) Flow rate: 0.5 mL min^{-1} λ = 280 nm	0.003 0.002	3.6 5.9	Sganzerla et al. (2014)
CAP DHC	Chili pepper genotypes	Extractant: ACN Water bath (4 h, 80 °C)	UFLC-PDA	Column: Purospher STAR RP (C_{18}) ACN:1% CH_3COOH (isocratic) (50:50, v:v) Flow rate: 1.2 mL min^{-1} λ = 280 nm	0.045[b] 0.151[b]	0.6 1.4	Usman et al. (2014)
CAP DHC NDC	Chiltepin pepper	UAE (65 °C, 20 min) Extractant: ACN	HPLC-DAD	Column: Purospher STAR RP (C_{18}) ACN:H_2O (isocratic) (50:50, v:v) λ = 202, 222, 280 nm	0.004 – –	0.1 0.1 –	Gonzalez-Zamora et al. (2015)

(continued)

Table 8.1 (continued)

Analytes	Matrices	Extraction conditions	Detection	Determination conditions	LOD (μg mL^{-1})	RSD (%)	References
Total capsaicinoids	*Capsicum annuum* fresh and dry fruits	Extractant: Ac Water bath (1 h, 50 °C)	HPLC-UV	Column: Hydrosphere S5 (C_{18}) ACN:1% CH_3COOH (isocratic) (40:60, v:v) Flow rate: 1.0 mL min^{-1} λ_{UV} = 280 nm	–	–	Ryu et al. (2017)
CAP DHC	*Capsicum annuum* fresh and dry fruits	UAE (30 min) Extractant: EtOH:H_2O (1:9, v:v) Maceration (4 h)	HPLC-DAD	Column: Ascentis RP-Amide ACN: 0.2% CH_3COOH (gradient) Flow rate: 0.5 mL min^{-1} λ = 254, 280 nm	0.001[b] 0.001[b]	– –	Popelka et al. (2017)
CAP DHC	Red pepper powder, red pepper paste	Extractant: MeOH Water bath (2 h, 80 °C)	HPLC-FD	Column: Zorbax Eclipse XDB (C_{18}) ACN:1% CH_3COOH (isocratic) (40:60, v:v) Flow rate: 0.8 mL min^{-1} λ_{ex} = 280 nm, λ_{em} = 325 nm	0.02–0.06[c] 0.02–1.38[c]	1.8 2.8	Dang et al. (2018)

Analytes	Matrices	Extraction conditions	Detection	Determination conditions	LOD (μg mL^{-1})	RSD (%)	References
CAP DHC NDC	*Capsicum annuum, Capsicum pendulum* fruits	Extractant: MeOH Shaken (4 h, 40 °C) Dried (N_2, 60 °C) Dissolved with MeOH:ACN:CH_3COOH (50:50:0.1, v:v:v)	LC-MS	Column: Zorbax Eclipse XDB (C_{18}) Eluent A: 0.1% CH_3COOH in H_2O Eluent B: MeOH:ACN:CH_3COOH (50:50:0.1, v:v:v) (gradient) Drying gas: N_2 (400 °C) 15.0 L min^{-1}, Capillary voltage: 4.5 kV	0.003 – –	1.5 1.0 –	Pavon-Perez et al. (2019)

CAP capsaicin, *DHC* dihydrocapsaicin, *NVA* nonivamide, *HC* homocapsaicin, *HDC* homodihydrocapsaicin, *NDC* nordihydrocapsaicin, *HPLC* high-performance liquid chromatography, *GC* gas chromatography, *UV* ultraviolet detector, *MS* mass spectrometer, *MS/MS* tandem mass spectrometer, *FD* fluorescence detector, *ESI* electrospray ionization, *TOF* time of flight, *PDAD* photodiode array detector, *DAD* diode array detector, *UFLC* ultra fast liquid chromatography, *MAE* microwave-assisted extraction, *UAE* ultrasound-assisted extraction, *SPME* solid-phase microextraction, *MSE* magnetic stirring extraction, *SPE* solid-phase extraction, *US* ultrasounds, *Ac* acetone, *PE* petroleum ether, *ACN* acetonitrile, *MeOH* methanol, *n-BuCl* n-butyl chloride, *EtOH* ethanol, *PDMS/DVB* polydimethylsiloxane/divinylbenzene, *HLB* hydrophilic lipophilic balance, *DCQ* 2,6-dichloroquinone-4-chloroimide

[a]pmol

[b]μg kg^{-1}

[c]mg kg^{-1}

interesting subject of scientific research, especially in terms of pro-health properties. It is worth noting that the mechanisms proposed by scientists confirm the analgesic effect of capsaicin or its effect on thermoregulation. Based on these mechanisms, the beneficial effect of capsaicinoids in the treatment of obesity, hypertension, diabetes, cardiovascular diseases, gastroprotective, or anticancerogenic activity was investigated and partially or completely confirmed.

However, especially in the case of the anticancerogenic activity of capsaicin, there are too many conflicting or incomplete studies to clearly confirm its activity in this regard. Despite numerous studies, knowledge regarding capsaicin is still not a sufficient foundation for its clinical application. High doses of capsaicin are toxic; therefore, the selection of an appropriate therapeutic dose may be a significant obstacle to the use of capsaicin as a potential pharmaceutical. As a result, the need to search for compounds with an analogous structure to capsaicin, but with slightly modified properties, is justified. This creates the possibility of obtaining compounds with properties desired for medical and pharmaceutical applications.

In anticipation of specific scientific evidence which will confirm the mechanisms of action of capsaicinoids in living organisms as well as their pro-health properties in the treatment of various diseases, it is worth introducing *Capsicum* fruits to the daily diet because, in addition to their taste, they possess many properties that can positively influence the human health.

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Chapter 9
Dietary Indoleamines: Bioavailability and Human Health

Akula Ramakrishna and Atanu Bhattacharjee

Abstract Indoleamines such as melatonin and serotonin have been found not only in mammalians but also in diverse living organisms, microorganisms, and plants. In the last decade of investigations, these molecules have been found in various edible plants and processed foods. Melatonin has been reported in edible fruits (grape, banana, strawberries, apple, pineapple, Kiwi fruit, tart cherries, etc.), vegetables (tomato, pepper, mushroom, cabbage, cauliflower, cucumber, carrot, beetroot, etc.), nuts and raw seeds (walnuts, sunflower, green cardamom, fenugreek, white and black mustard, etc.), juices and beverages (wines, green and roasted coffee beans, decoction brew). Also, the intake of these indoleamines-containing foods could significantly increase the melatonin/serotonin concentration in human serum, indicating these indoleamines could provide beneficial health effects. Studies have shown that melatonin has many bioactivities, such as antioxidant, anti-inflammatory, enhancing immunity, anticancer, improving circadian cycle, cardiovascular protecting, antidiabetic, anti-obese, antiaging, and neuroprotection. The nutritional implications of indoleamines as bioactive molecules are well documented and hence, it becomes one of the thrust areas of interest to explore their levels in various edible plants and food products. This chapter describes the occurrence of melatonin and serotonin in selected edible plants, fruit, vegetables, and processed foods and their bioavailability along with possible human health effects.

Introduction

Melatonin (MEL) and serotonin (SER) have been found in a wide variety of plants and plant products (Paredes 2009; Verde et al. 2019; Ramakrishna et al. 2011, 2012a, b; Ramakrishna and Soumya 2020; Ramakrishna and Roshchina 2019;

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Rayne 2010; Atanu and Dey 2018; Atanu et al. 2014, 2016). Various analytical methods such as immunoassay, high-performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS) have been used to measure MEL and SER levels in edible and medicinal plants (Garcia-Parrilla and Troncoso 2009; Fenga et al. 2014; Ramakrishna et al. 2012a; Ravishankar and Ramakrishna 2017; Soumya and Ramakrishna 2018). Studies have shown that the variation of MEL and SER contents in each plant also depends on the cultivator (Stürtza et al. 2011; Riga et al. 2014), part or age of the plant, ripeness, climate, radiation (Riga et al. 2014), and geographical and seasonal variations. Various fruits, vegetables, seeds, grains, herbs, and plant products have been shown to contain high levels of MEL and SER (Meng et al. 2017, 2018; Briguglio et al. 2018; Hano et al. 2017; Rayne 2010). Therefore, foods rich in MEL and SER are considered as nutraceuticals (Salehi et al. 2019; Briguglio et al. 2018; Gomes Domingos et al. 2017; Delgado et al. 2013; Iriti et al. 2006; Ferrari 2004; Sae-Teaw et al. 2013). Moreover, these indoleamines have been reported to improve brain functioning, mood regulation, and cardiovascular functioning (Pereira et al. 2020; Jiki et al. 2018).

Chemistry and Bioavailability of Indoleamines in Plants

Biosynthetic pathway of MEL from tryptophan has been reported in cultured *Hypericum perforatum* L cells (Murch et al. 2000). However, the first two steps of MEL biosynthesis from tryptophan in rice plants are reversed compared to that in animals (Park et al. 2012). In animals, SER is synthesized from tryptophan in two successive steps involving tryptophan hydroxylase (TPH; EC 1.14.16.4) and aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28) in which tryptophan hydroxylase acts as the rate-limiting enzyme (Veenstra-Vander Weele et al. 2000). In plants, SER is synthesized differently in that tryptophan is first catalyzed into tryptamine by tryptophan decarboxylase (TDC; EC 4.1.1.28), followed by the catalysis of tryptamine by tryptamine 5-hydroxylase (T5H) to form SER. However, in St. John's wort (*Hypericum perforatum*), SER synthesis has been reported to occur *via* 5-hydroxytryptophan akin to mammals (Murch et al. 2001; Ramakrishna et al. 2011).

Galano et al. (2013) reported an increase in plasmatic melatonin levels upon the consumption of MEL-enriched foods. When MEL is taken orally, it is absorbed from the gastrointestinal tract and largely metabolized in the liver. The residue is being excreted mainly as soluble metabolites (6-sulfatoxyMEL, aMT6-s) in the urine. Upon consumption of MEL-rich food, MEL enters blood and heads to target sites either directly or indirectly, through MEL receptors MT1 and MT2 (Dubocovich et al. 2003; Slominski et al. 2012). When 2-week old chickens were fed with a high MEL diet containing 3.5 ng g^{-1} MEL, a significant increase in plasma MEL levels from 20 pg mL^{-1} to 34 pg mL^{-1} after 1.5 h ($p < 0.01$) compared to controls (8 pg mL^{-1}) was observed. When rats were fed with walnuts (*Juglans regia* L.) containing MEL (3.5 ± 1.0 ng g^{-1}), an increase in serum MEL concentrations to 38.0 ± 4.3 pg mL^{-1} was found compared to control (11.5 ± 1.9 pg mL^{-1}). Moreover,

"Total antioxidant power" of the serum was increased for the walnut-fed animals (Reiter et al. 2005). The intake of lentil sprouts increased the concentration of MEL in the plasma of rats as well as the urinary levels of 6-sulfatoxymelatonin. Moreover, the plasmatic antioxidant capacity was augmented (Rebollo-Hernanz et al. 2020). The consumption of banana, orange, or pineapple juices significantly increased the serum MEL concentration (Sae-Teaw et al. 2013). Similarly, an increase in circulating MEL in human subjects after consuming coffee has been reported (Ursing et al. 2003). Individuals who consumed vegetables rich in MEL increased their urinary MEL metabolite excretion and also had an elevated level of MEL in the blood (Oba et al. 2008). Intake of MEL from tropical fruit altered urinary excretion of 6-sulfatoxymalatonin in healthy volunteers (Johns et al. 2013).

The Occurrence of Indoleamines in Food Products and Edible Plants

Both MEL (3.9 ± 0.2 μg 50 mL^{-1}) and serotonin (7.3 ± 0.6 μg 50 mL^{-1}) were detected in the coffee brew (Ramakrishna et al. 2012b). Da Silveira et al. (2007) reported that during the roasting of coffee, there is a significant reduction in the levels of amines. However, brewing the coffee reduced MEL to about half these levels. According to Lima and Gloria (1999), intake of four to five 50 mL cups of coffee beverage provides 24.5 μg tryptamine and 39 μg SER daily. The presence of indoleamines in roasted beans, which pass into coffee brew, might be useful for consumers. Various refined and extra-virgin Spanish olive oils were found to contain between 650 and 119 pg mL^{-1} MEL (de la Puerta et al. 2007). MEL was also determined in virgin argan oils (Venegas et al. 2011). Numerous wine cultivars (*Vitis vinifera*) have been reported to contain between 2.4 and 428 pg mL^{-1} (0.005–0.97 ng g^{-1}) MEL (Iriti et al. 2006; Iriti and Faoro 2006). Eight red wine varieties showed MEL level between 140 and 277 pg mL^{-1} (Rodriguez-Naranjo et al. 2011a). MEL content of 51.8–169.7 pg mL^{-1} was found in a wide variety of beer (Maldonado et al. 2009).

In animal foods, MEL concentrations were found higher in eggs and fish compared to meat (Tan et al. 2014). MEL was detected in the human breast milk and milk of animals (Tan et al. 2014; Karunanithi et al. 2014). MEL has been identified in the most popular beverages of the world such as coffee, tea, wine, and beer (Chen et al. 2003; Iriti 2009; Maldonado et al. 2009; Stege et al. 2010). MEL ranged 0.03–0.29 ng g^{-1} in the tested edible oils, among which refined linseed presented 0.29 ng g^{-1} and virgin soybean 0.19 ng g^{-1}. MEL in wine, thus, is believed to be derived from the grapes. MEL levels are higher in red wine than in white wine (Stege et al. 2010). MEL was found at 2.2 ± 0.14 ng g^{-1} in *Saccharomyces cerevisiae* yeast, which is widely used in the bread baking and alcohol industry (Tan et al. 2014; Rodriguez-Naranjo et al. 2011a). Alcoholic fermentation has also been shown to induce MEL synthesis in orange juice (Fernández-Pachón et al. 2014). MEL has

been found in popular alcoholic drinks like beer (0.09 ± 0.01 ng mL^{-1}) and wine (129.5 ± 3.5 ng mL^{-1}) (Garcia-Moreno et al. 2013; Rodriguez-Naranjo et al. 2011a; Stege et al. 2010). Besides the MEL originated from the main ingredients, it was also considered to be synthesized during the fermentation process due to the yeast growth (Rodriguez-Naranjo et al. 2011a). As for the other drinks, coffee usually contains a high concentration of MEL because coffee beans contained MEL at a very high level and even much higher in roasted beans (Ramakrishna et al. 2012b). MEL also existed in juices, cacao, and balsamic vinegar (Vitalini et al. 2013). However, it was not found in tea including green tea and black tea (Kocadagli et al. 2014). The occurrence of MEL in food products has been depicted in Table 9.1.

MEL has been discovered in widely consumed crops including corn, rice, wheat, barley, and oats (Wang et al. 2009; Hattori et al. 1995; Hernandez-Ruiz and Arnao 2008). And also reported in many edible fruits such as grape (Iriti et al. 2006; Mercolini et al. 2012), banana (Johns et al. 2013), strawberries (Hattori et al. 1995), tomatoes, and cherries (González-Gómez et al. 2009). Wang et al. (2009) reported MEL contents in 58 cultivars of corn and 25 of rice. Moreover, Setyaningsih et al. (2015) reported that pigmented rice contained higher MEL contents. MEL has been detected at quite high levels in fresh-frozen Montmorency tart cherries (13.5 ng g^{-1}) and Balaton tart cherries (22.4 ng g^{-1}) (*Prunus cerasus*) (Burkhardt, et al. 2001). However, MEL levels of only 0–0.22 ng g^{-1} fresh fruit have been reported in eight Spanish sweet cherry cultivars (*Prunus avium* L.) (González-Gómez et al. 2009), indicating that there can be great differences between individual species of fruit.

Table 9.1 Melatonin contents in processed food products

Food	Melatonin content	Reference
Chinese green tea	2.12 μg g^{-1} fw	Chen et al. (2003)
Olive oil	53–119 pg mL^{-1}	De la Puerta et al. (2007)
Spanish wines	50–80 pg mL^{-1}	Peyrot and Ducrocq (2008)
Beer	52–170 pg mL^{-1}	Garcia-Parrilla and Troncoso (2009)
Extra virgin olive oil	71–119 pg mL^{-1}	Paredes et al. (2009)
Coffee canephora decoction brew	3.0 ± 0.6 μg 50 mL^{-1}	Ramakrishna et al. (2012b)
Coffee arabica decoction brew	3.9 ± 0.25 μg 50 mL^{-1}	Ramakrishna et al. (2012b)
Chicken meat and skin	2.3 ± 0.23 ng g^{-1}	Tan et al. (2014)
Chicken liver and heart	1.1 ± 0.01 ng g^{-1}	Tan et al. (2014)
Lamb	1.6 ± 0.14 ng g^{-1}	Tan et al. (2014)
Beef	2.1 ± 0.13 ng g^{-1}	Tan et al. (2014)
Pork	2.5 ± 0.18 ng g^{-1}	Tan et al. (2014)
Salmon	3.7 ± 0.21 ng g^{-1}	Tan et al. (2014)
India human milk	15.92 ± 1.02 pg mL^{-1}	Karunanithi et al. (2014)
Cow milk	14.45 ± 0.12 pg mL^{-1}	Karunanithi et al. (2014)
Toned milk	18.41 ± 0.62 pg mL^{-1}	Karunanithi et al. (2014)
Orange juice	3.15–21.80 ng mL^{-1}	Fernandez-Pachon et al. (2014)
Pomegranate juice	8.78 ng mL^{-1}	Faveroa et al. (2017)

fw fresh weight, *dw* dry weight

The highest MEL content in fruits was reported in the range of 8.9–158.9 ng g^{-1} dry weight in the skin of grapes (*Vitis vinifera* L. cv. Malbec) (Boccalandro et al. 2011), 13.46 ± 1.10 ng g^{-1} fresh weight in tart cherries (*Prunus cerasus* L. cv. Balaton) (Burkhardt et al. 2001), and 11.26 ± 0.13 ng g^{-1} fresh weight in strawberry (*Fragaria ananassa* L. cv. Festival) (Sturtz et al. 2011). MEL was reported in many of common vegetables such as pepper (*Capsicum annuum* L. cv. F26) 11.9 ng g^{-1} fresh weight or 93.4 ng g^{-1} dry weight, tomato (*Solanum lycopersicum* L. cv. Optima) 14.77 ng g^{-1} fresh weight or 249.98 ng g^{-1} dry weight (Riga et al. 2014). Mushrooms also contain MEL 12,900 ± 770 ng g^{-1} dry weight in Basidiomycota (*Lactarius deliciosus*), 6800 ± 60 ng g^{-1} dry weight in Basidiomycota (*Boletus edulis*) (Kocadagli et al. 2014). However, it remains undetectable in potatoes and very low in beetroots (Badria 2002). The occurrence of MEL in edible plants has been depicted in Table 9.2.

Food sources rich in free tryptophan content can be considered as a good option for serotonin enhancement in the brain (Young 2007). Fruits, vegetables, and seeds are the major tissues in which serotonin occurs abundantly (Kema et al. 2000). SER occurs in pineapple, bananas and tomatoes, avocado, eggplant, and walnuts (Collier and Chesher 1956). Recent studies have shown that sweet cherries contain substantial amounts of MEL and SER, and may have several health benefits and would be of relevance in a healthy diet (Gonzalez-Gomez et al. 2009). Higher concentrations of SER in the peel (150,000 ng g^{-1}) of the banana compared to the pulp 24,000 ng g^{-1} were also found (Udenfriend et al. 1959). During ripening, concentrations of SER increased significantly in both the inner and outer banana peel. However, in French plantain (*Musa sapientum* var. *paradisica*) though very high serotonin concentrations were found during ripening (from 49,900 to 56,700 ng·g^{-1}) during ripening it decreased (12,000 ng·g^{-1}) (Foy and Parratt 1960). In Prata bananas (*M. acuminate* × *M. balbisiana*) during ripening, SER levels were between 15,000 and 20,000 ng·g^{-1} until the 14th day of storage (Waalkes et al. 1958). Bananas and walnuts are believed to be the major sources of dopamine and serotonin among various plant products (Badria 2002). MEL, SER, and total polyphenols were determined in four walnut cultivars (Tapia et al. 2013). The presence of SER in coffee beans might have a beneficial influence on consumer health (Casal et al. 2004). Even *Coffea* seed wax contains 4.1 mg g^{-1} SER (Kele and Ohmacht 1996), and also a Polish coffee called Astra contains 20–40 mg SER per 100 g (Strance 1993). Coffee (*Coffea canephora* and *Coffea arabica*) has been shown to contain significant amounts of MEL and SER. Both MEL and SER were detected in green coffee beans (5.8 ± 0.8 μg g^{-1} dry weight, 10.5 ± 0.6 μg g^{-1} dry weight) and also in roasted beans of *C. canephora* (8.0 ± 0.9 μg g^{-1} dry weight, 7.3 ± 0.5 μg g^{-1} dry weight), and MEL (3.0 ± 0.6 μg per 50 mL) and SER (4.0 ± 0.7 μg per 50 mL) were detected in the coffee brew. In *C. arabica*, serotonin was high in green beans (12.5 ± 0.8 μg g^{-1} dry weight) compared with roasted beans (8.7 ± 0.4 μg g^{-1} dry weight). The levels of MEL were higher (9.6 ± 0.8 μg g^{-1} dry weight) in roasted beans compared with green beans (6.8 ± 0.4 μg g^{-1} dry weight) (Ramakrishna et al. 2012b). The occurrence of serotonin in edible plants has been depicted in Table 9.3.

Table 9.2 Melatonin contents in edible plants

Plant/Food source	Melatonin content	Reference
Sweet corn	1.3 ± 0.28 ng g^{-1}	Tan et al. (2014)
Rice	1.50 ng g^{-1} fw	Badria (2002)
Wheat	124.7 ± 14.9 ng g^{-1} fw	Hernandez-Ruiz et al. (2005)
Barley	82.3 ± 6.0 ng g^{-1} fw	Hernandez-Ruiz et al. (2005)
Oats	90.6 ± 7.7 ng g^{-1} fw	Hernandez-Ruiz et al. (2005)
Pineapple	0.04 ng g^{-1} fw	Hattori et al. (1995)
Kiwi fruit	0.02 ng g^{-1} fw	Hattori et al. (1995)
Strawberry	11.26 ± 0.13 ng g^{-1} fw	Sturtz et al. (2011)
Banana	0.66 ng g^{-1} fw	Badria (2002)
Apple	5 ng g^{-1} fw	Lei et al. (2013)
Pomegranate	0.17 ng g^{-1} fw	Badria (2002)
Tart cherries	13.46 ± 1.10 ng g^{-1} fw	Badria (2002)
Grape (fresh)	3.9 ± 0.06 ng g^{-1}	Vitalini et al. (2011)
Cranberry	96 ± 26 μg g^{-1} dw	Brown et al. (2012)
Onion	0.30 ng g^{-1} fw	Badria (2002)
Garlic	0.59 ng g^{-1} fw	Badria (2002)
Cabbage	0.31 ng g^{-1} fw	Badria (2002)
Cauliflower	0.82 ng g^{-1} fw	Badria (2002)
Turnip	0.50 ng g^{-1} fw	Badria (2002)
Cucumber	0.59 ng g^{-1} fw	Badria (2002)
Carrot	0.49 ng g^{-1} fw	Badria (2002)
Radish	0.76 ng g^{-1} fw	Badria (2002)
Potato	nd	Badria (2002)
Ginger	1.42 ng g^{-1} fw	Badria (2002)
Beetroot	0.002 ng g^{-1}	Dubbels et al. (1995)
Purslane	19 ng g^{-1} ww	Simopoulos et al. (2005)
Indian spinach	0.04 ng g^{-1} fw	Hattori et al. (1995)
Pepper	11.9 ng g^{-1} fw 93.4 ng g^{-1} dw	Riga et al. (2014)
Tomato	14.77 ng g^{-1} fw 249.98 ng g^{-1} dw	Riga et al. (2014)
Ginger	0.58 ng g^{-1} fw	Hattori et al. (1995)
Mushroom	4300–6400 ng g^{-1} dw	Muszyńska et al. (2016)
Lentils	0.5 ng g^{-1} dw	Aguilera et al. (2015a)
Kidney beans	1.0 ng g^{-1} dw	Aguilera et al. (2015a)
Soybean	0.45 ± 0.03 ng g^{-1} dw	Zielin Ski et al. (2001)
Lupin (seed flour)	0.53 ± 0.04 ng g^{-1} dw	Hernandez-Ruiz et al. (2008)
Grape (seed)	10.04 ± 0.49 ng g^{-1}	Vitalini et al. (2011)
Black mustard	129 ng g^{-1} dw	Manchester et al. (2000)
White mustard	189 ng g^{-1} dw	Manchester et al. (2000)
Fenugreek	43 ng g^{-1} dw	Manchester et al. (2000)
Alfalfa	16 ng g^{-1} dw	Manchester et al. (2000)

(continued)

Table 9.2 (continued)

Plant/Food source	Melatonin content	Reference
Coriander	7 ng g^{-1} dw	Manchester et al. (2000)
Green cardamom	15 ng g^{-1} dw	Manchester et al. (2000)
Fennel	28 ng g^{-1} dw	Manchester et al. (2000)
Poppy	6 ng g^{-1} dw	Manchester et al. (2000)
Sunflower	29 ng g^{-1} dw	Manchester et al. (2000)
Flax	12 ng g^{-1} dw	Manchester et al. (2000)
Almond	39 ng g^{-1} dw	Manchester et al. (2000)
Mung bean	0.01 ± 0.0 ng g^{-1} dw	Aguilera et al. (2015a)
Broccoli	0.41 ± 0.04 ng g^{-1} dw	Aguilera et al. (2015a)
Walnuts	3.5 ± 1.0 ng g^{-1}	Reiter et al. (2005)
Groppello wines	5.2 ng mL^{-1}	Vitalini et al. (2013)
Coffee canephora green beans	5800 ± 800 ng g^{-1} dw	Ramakrishna et al. (2012b)
Coffee arabica green beans	6800 ± 400 ng g^{-1} dw	Ramakrishna et al. (2012b)
Coffee canephora roasted beans	8000 ± 900 ng g^{-1} dw	Ramakrishna et al. (2012b)
Coffee arabica roasted beans	9600 ± 800 ng g^{-1} dw	Ramakrishna et al. (2012b)
Green tea	nd	Kocadagli et al. (2014)
Black tea	nd	Kocadagli et al. (2014)

fw fresh weight, *dw* dry weight, *ww* wet weight, *nd* not detected

Table 9.3 Serotonin contents in edible plants

Common name	Serotonin content	Reference
Sweet cherry	2.8–37.6 ng 100 g^{-1} fw	González-Gómez et al. (2009)
Tomato	221.9 μg g^{-1} fw	Ly et al. (2008)
Cherry tomato	156.1 μg g^{-1} fw	Ly et al. (2008)
Hot pepper	17.9 μg g^{-1} fw	Ly et al. (2008)
Paprika	1.8 μg g^{-1} fw	Ly et al. (2008)
Strawberry	3.77 ± 0.66 μg g^{-1} fw	Ly et al. (2008)
Welsh onion	8 ± 0.8 μg g^{-1} fw	Ly et al. (2008)
Lettuce	3.3 ± 0.6 μg g^{-1} fw	Ly et al. (2008)
Chinese cabbage	110.9 ± 22.5 μg g^{-1} fw	Ly et al. (2008)
Chicory	8.5 ± 3.2 μg g^{-1} fw	Ly et al. (2008)
Spinach	34.4 ± 2.4 μg g^{-1} fw	Ly et al. (2008)
Pineapple	1.5 μg g^{-1} fw	West (1960)
Papaya	1.1–2.1 μg g^{-1} fw	Erspamer (1961)
Coffea canephora green beans	10.5 ± 0.6 μg g^{-1} dw	Ramakrishna et al. (2012b)
Coffea arabica green beans	12.5 ± 0.8 μg g^{-1} dw	Ramakrishna et al. (2012b)
Coffea canephora roasted beans	7.3 ± 0.5 μg g^{-1} dw	Ramakrishna et al. (2012b)
Coffea arabica roasted beans	8.7 ± 0.4 μg g^{-1} dw	Ramakrishna et al. (2012b)
Pineapple	1.5 μg g^{-1} fw	West (1960)
Runner bean (leaf)	0.6 μg g^{-1} fw	Applewhite (1973)
Pea	0.9–1 μg g^{-1} fw	Applewhite (1973)
Black walnut (seed)	180 μg g^{-1} fw	Grobe (1982)
Coffee	2.5 μg g^{-1} dw	Strance (1993)

fw fresh weight, *dw* dry weight

Analytical Methods for the Determination of Indoleamines

Several researchers reported the reliable analytical methodologies such as Microchip Electrophoresis, Capillary Electrophoresis, Liquid Chromatography, coupled to different detection strategies for the determination of MEL, its isomers, and precursors in different plant tissues and food samples (Gomez et al. 2017; Cao et al. 2006; Ramakrishna et al. 2012a). HPLC is a widely used technique for the determination of MEL, isocratic elution with a reversed-phase elution mode is the most commonly used. In this case, different detectors such as fluorescence (Hamase et al. 2004; Minami et al. 2009), electrochemical (Raynaud and Pevet 1991; Vieira et al. 1992), and UV (Ayano et al. 2007; Brömme et al. 2008) have been used. Current methods for the investigation of SER include thin-layer chromatography, radioimmunoassay, enzyme-linked immunosorbent assay, high-performance liquid chromatography with ultraviolet detection, and high-performance liquid chromatography with mass spectrometry (Mukherjee and Ramakrishna 2019).

Health Benefits of Dietary Indoleamines

Consumption of edible plant tissues that contain the SER and MEL will be useful, as their antioxidant activity is linked to their medicinal value for the treatment of human diseases (Yılmaz and Gökmen 2020; Reiter et al. 2005). Currently, it is believed that some of the health benefits from Mediterranean diets are derived from their phytochemicals including phenylpropanoids (or phenolic compounds), isoprenoids, alkaloids, and MEL (Iriti 2009). Phytomelatonin in walnuts is reported to contribute to the enhancement of indoles in offering protection against cardiovascular damage (Reiter et al. 2005). Hardman and Ion (2008) have reported the suppression of cancer growth by dietary walnut. Tryptophan represents a key element for brain functioning, because of its role as a precursor for the production of SER, which can have a positive impact on mood and cognition (Strasser et al. 2016). SER has been administered to boost its levels in the human brain in treating SER deficiency syndrome. Some of the extensive uses of SER include treating patients suffering from Parkinson-like symptoms (Bell and Janzen 1971) and controlling obesity (Cangiano et al. 1992). MEL exerts metabolic effects such as improved insulin resistance, antioxidant power, and circadian rhythm control. It was able to reduce amyloid-beta peptide (Aβ) deposits in the brains of Alzheimer's disease patients (Shen et al. 2002). MEL is also reported as having therapeutic effects on brain injuries (Traystman et al. 1991; Wakatsuki et al. 2001). Exogenous MEL was able to improve sleep, probably due to the chronobiotic role (Mayer et al. 1996). Moreover, MEL supplementation is helpful in hypertension, oxidative stress, and metabolic syndrome (Koziróg et al. 2010). MEL might contribute to the beneficial antitumoral action of coffee due to its known cancer-preventive effects (Bizzarri et al. 2013; Proietti et al. 2013; Wang et al. 2015).

Conclusion

MEL and SER were reported in many types of fruits, vegetables, and food products at widely varying levels depending on the cultivars, species, location, time of harvest, and ripening stage. Various reports have confirmed that consumption of fruits, vegetables, processed foods, and beverages containing MEL and SER can enhance plasma levels and have positive effects on human health. Various studies have reported that MEL and SER are having antioxidant, anti-inflammatory, enhancing immunity, anticancer, improving circadian cycle, antidiabetic, cardiovascular protecting, neuroprotection, anti-obese, and antiaging properties. However, dietary indoleamines intake and their impact on health benefits need to be further investigated, as there are no significant data available on bioavailability or clinical implications. Reports on foods riched in phytoserotonin, bioavailability, and health benefits are scanty and more studies are warranted.

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Chapter 10
Bioactive Peptide Analysis

Ilona Gałązka-Czarnecka and Grażyna Budryn

Abstract The introduction to this chapter describes the progress in discovering the prophylactic and therapeutic properties of bioactive peptides. Methods for analyzing bioactive peptides have been described in detail, including structure, composition, amino acid sequence determination, and quantitative analysis. Methods for designing active peptide sequences and methods for their synthesis have been presented. Examples of peptide bioactivity analysis using isothermal titration calorimetry have been also given, illustrating the energy effects associated with the interaction of bioactive peptides with body macromolecules.

Peptide Chemistry

In the nineteenth century, the German chemist Hermann Emil Fischer introduced the term "peptides" for a group of organic compounds having a characteristic peptide bond. In the twentieth century, food proteins began to be considered not only as a source of amino acids necessary for the proper functioning of the body but also as precursors of biologically active peptides. In 1950, Mellander was the first who introduced the name "biologically active peptides" for phosphopeptides – compounds derived from cow's milk casein (Mellander 1950). Through the 1960s and 1970s, a number of groups created different methodologies to produce small volatile peptide derivatives appropriate for gas chromatographic separation before introduction into a gas chromatograph coupled with a mass spectrometer (GC-MS) using electron impact ionization and fragmentation (Biemann et al. 1959; Biemann and Vetter 1960). In 1970, Burgus et al. (1970) used Edman sequencing, such as blocked N termini. Later, Hudson and Biemann (1976) employed this technique to completely sequence a small protein of unknown sequence by blocking N termini. Development of this technique included technical work on various combinations of mass analyzers in order to provide mixture analysis *via* the mass selection of precur-

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sor ions, fragmentation of the selected ion, and mass analysis of the resulting fragment ions what is now broadly thought of as tandem mass spectrometry (MS/MS) (Ma and Johnson 2012). In 1981, Michael Barber developed the fast atom bombardment method. It was the first method of non-gas phase analysis by which peptides in solution were analyzed and amino acid sequences were determined as a result of spontaneous fragmentation.

The greatest progress in peptide analysis and study of their activity occurred at the beginning of the twenty-first century. Inactive within the sequence of the parent protein, different short amino acid chains (2–20 amino acids or in some cases up to 40 amino acids) are food-derived bioactive peptides. These peptides can be released by gastrointestinal digestion or during food processing, for example, ripening, fermentation, cooking, and also by storage or in vitro hydrolysis by proteolytic enzymes (Aguilar-Toalá et al. 2017; Boutrou et al. 2015; Di Bernardini et al. 2011; Korhonen and Pihlanto 2006; Korhonen 2009). However, there are also bioactive peptides with longer chains, for example, lunasin soybean anti-tumor peptide, consisting of 43 amino acids (Shahidi and Zhong 2008), and the glycomacropeptide that contains 64 amino acid residues (Thomä-Wörringer et al. 2006). During the last years, the identification and characterization of bioactive peptides have become thorough research subjects. Food peptidomics can be seen as a subfield of the food proteomics concentrated on composition, interaction, and properties of peptides existing in a food matrix. Bioactive peptides are molecules of great interest that represent potent pharmacological tools that are often engaged in the development of new animal models of disease, pharmacophores for the design of new drugs, markers for substance abuse, or markers for disease (De Kock et al. 2001; Frank and Hargreaves 2003; Keramida et al. 2006; Sánchez-Rivera et al. 2014; Tamvakopoulos 2007). Techniques for determining, identifying, obtaining, and testing the activity of bioactive peptides are dynamically developing. This allows them to be used more effectively in medicine as compounds with a broad spectrum of activity. At the same time, the growing progress in the development of analytical tools used for the study of bioactive peptides, such as liquid chromatography (LC) assisted by mass spectrometry, computer and chemometric methods, allows designing the new drugs based on bioactive peptides as well as studying the molecular mechanisms of action and bioavailability of peptides in raw materials and food products.

The molecular structure of food peptides affects the mechanisms of absorption of these compounds, the quality of raw materials and food products, the psychophysical condition of consumers, and the prevention of diet-related diseases (Darewicz et al. 2008). Understanding of the relationship between the molecular structure of bioactive peptides and the biological activity of food (in which these compounds are present) often requires the use of very advanced analytical and bioinformatic methods in parallel. The determination of bioactive peptides is a complex issue, due to the diverse structure of these compounds, their properties, as well as the origin (endogenous and exogenous) and the specificity of the tested material (e.g., raw material, food, biological material, etc.). The study of bioactive peptides is facilitated by a broad knowledge of peptide biochemistry, their structure, physicochemical properties, peptidomics, and post-translational modifications (PTMs; i.e., the phenomenon of attachment or removal of various types of chemical particles from/

to a polypeptide chain, proteolytic degradation, or formation of covalent bonds within a protein molecule). Understanding the complexity of the phenomenon of protein modification in post-translational processes can help to understand many mechanisms at the cellular level, including cell regulation and carcinogenesis.

A subfield of the proteomic that concentrates on composition, interactions, and properties of peptides present in a food matrix is called peptidomics (Gagnaire et al. 2009). The definition refers also to research focused on the origin and dynamic changes of peptides liberated from parent food proteins obtained during processing and storage (Minkiewicz et al. 2008a). The methods used in proteomics and peptidomics depend on parameters of proteins such as the level of their expression and modification, and location within cells. Physical and chemical properties such as solubility, polarity, and chemical stability should be taken into account in bioactive peptide studies. In addition, it is also necessary to determine the stability of the biological matrix (the stability in biological fluids) because of peptides adsorption to surfaces, molecular weight, and ionization efficiency. These aspects should not be underestimated; otherwise, the risk of unreliable results is high. For example, due to the oxidation of methionine or tryptophan residues or disulfide bond cleavage, peptide alteration is possible (Tamvakopoulos 2007). Sample preparation is extremely important because the major concerns of proteomics are to avoid polypeptide loss or protein degradation and to guarantee the reproducibility of the data. Proteomic studies include one or more of the following aspects, including techniques: a high-resolution (HR) separation technique (two-dimensional gel electrophoresis, multidimensional liquid chromatography, nano-HPLC [high-pressure liquid chromatography], capillary electrophoresis [CE]) to separate proteins and receive a protein profile which can be then analyzed in databases; protein analysis, characterization, and identification, mainly by mass spectrometry (MS) or by western blotting; quantification of the peptide of interest by enzyme-linked immunosorbent assay (ELISA); the search for bioactive peptides for all of the protein sequences reported for the food protein source by databases, called virtual screening; and simulation of the docking between the receptor molecule and the peptide (Agyei et al. 2016; Bhat et al. 2015; Caron et al. 2016; Carrasco-Castilla et al. 2012). Proteomic research includes also the development of measurement tools used for identification of peptides and their protein precursors, optimization of hydrolysis for obtaining peptides with high activity, prediction of protein/peptide structure and bioactive peptide properties and quantitative structure–activity relationship (QSAR), modeling which provides the methodology to find mathematical expressions for such relationships useful for estimating activities of any related compound and for predicting structures of high activity (Carrasco-Castilla et al. 2012; Pripp et al. 2005). There are many interesting directions in peptide analysis, including methods for identifying, isolating, synthesizing, and sequencing biologically active peptides; including analytical, computer methods, as well as proteomic and peptidomic techniques used in vitro, in vivo, and in silico; including hydrolysis processes, separating, identifying, and determining the activity of the obtained protein fragments together with the analysis of the relationship between structure and biological function.

Identification and Quantification of Bioactive Peptides

The basic techniques for isolation and determination of peptides are various types of chromatography, especially high-pressure liquid chromatography (HPLC) used in analytical and preparative scales. HPLC techniques use various detectors to analyze bioactive peptides, for example, UV/VIS, photodiode, and fluorescence for qualitative and quantitative determinations (based on absorbance or fluorescence values) of analyzed compounds. Chromatographic techniques allow also to detect the adulteration of peptide-containing products of animal origin (meat and dairy products) and plant products, soy protein preparations with other proteins, for example, cow's milk proteins (El-Fattah et al. 2017; Fu et al. 2019). Chromatographic techniques and mass spectrometry that are used to analyze bioactive peptides are the main analytical tools, thanks to which this exciting field continues to evolve. Mass spectrometry can be used to perform basic proteomic studies for the quantification and identification of bioactive peptides. Mass spectrometry is an analytical technique in which the essence is to measure the mass to charge ratio (m/z) of ions in the gas phase. The mass spectrum shows the disintegration of particles of the analyzed substance into smaller fragments formed under the influence of various ionizing agents. In the MS technique, various ionization methods can be used to analyze bioactive peptides, including electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI). However, for mass analysis, one can use, for example, quadrupole analyzer (Q), ion trap (IT or QIT), and time of flight (TOF). Mass spectroscopy allows us to determine the molecular weight of the tested compound and the mass of fragments into which this compound breaks down. For many years, the review of the development of this technique in peptides and peptides of a given organism, food, and tissue has been described in review works (Carrasco-Castilla et al. 2012; Ferri et al. 2017; Mamone et al. 2009; Picariello et al. 2015). MS techniques are a fairly universal analytical tool because they can be used not only to identify peptides but also to identify products resulting from many complex reactions that these compounds undergo.

Over the last few years, there have been developed different methods of analysis of active biopeptides such as liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) with low-resolution (LR) quadrupole mass analyzers (Bougatef et al. 2010; Caron et al. 2016; Gomez-Ruiz et al. 2006; Guo et al. 2009) and high-resolution orbitrap system (Caron et al. 2016), or off-line systems including ESI-MS/MS used without the HPLC (Gomez-Ruiz et al. 2006) and fast atom bombardment (Saito et al. 2000). These tools help mass spectrometry experts to monitor up or down regulation of key proteins (often the signatures of human disease) and therefore play a significant role in the deciphering of complex biological problems (Silva-Sanchez et al. 2008). The methods using mass spectrometry allow the identification of enzymatic modifications of peptides (Li-Chan 2015; Lothrop et al. 2013) and are also effective for identifying products formed during other reactions (Hernández-Ledesma et al. 2004; Martin et al. 2008; Van Lancker et al. 2011). Over many years, liquid chromatography–mass spectrometry has been

widely applied to analyze intact milk proteins or the peptide sequences released from milk proteins in a wide variety of food products (e.g., fermented dairy products, hydrolysates from simulated gastrointestinal digestion of milk protein fractions, or hypoallergenic infant milk formulas) (Hernández-Ledesma et al. 2004; Weber et al. 2006; Martin et al. 2008; Catala-Clariana et al. 2010). Mass spectrometry was also combined for more complete analysis not only with high-performance liquid chromatography but also with capillary electrophoresis (CE) (Catala-Clariana et al. 2010; Gaspar et al. 2008; Gómez-Ruiz et al. 2007; Ibáñez et al. 2013; Kašička 2014; Miralles et al. 2001; Muller et al. 2008; Veledo et al. 2005).

In order to provide information about proteolytic profiles, mainly from milk and cheese ripening, CE has been applied (Albillos et al. 2005; Molina et al. 2000; Strickland et al. 2001). Moreover, an extensive review on the use of CE for the analysis of alterations in soybean proteins and peptides qualities, safety, functionality, and food processing has also been published (Saz and Marina 2007). Capillary electrophoresis–mass spectrometry (CE-MS) was a firmly established technique used to separate and characterize a diverse range of compounds, especially polar and charged molecules, such as peptides and proteins (Herrero et al. 2008; Staub et al. 2009). The main advantage of CE is the possibility of miniaturization of the equipment and low consumption of reagents, which is consistent with the philosophy of "green chemistry," recommending the search for analytical methods that are least harmful to the environment (Welch et al. 2010). The use of CE-MS was considered to be a good complement to LC-MS techniques because the mechanism of the electrophoretic separations based upon charge-to-mass ratios can be considered orthogonal to the partitioning mechanism of the conventionally used reversed-phase LC separations, which is mostly based on the hydrophobic character of the molecules (Catala-Clariana et al. 2010). The use of the CE-MS technique for peptide analysis employing almost all mass analyzers has been extensively described in the first decade of the twenty-first century (Catala-Clariana et al. 2010; Herrero et al. 2008; Staub et al. 2009). The online combination of CE and MS techniques was an attractive option, and they present some major benefits. Among them, they enhance sensitivity and enable the determination of co-migrating compounds with different mass to charge ratios (m/z) (Schappler et al. 2008). MS gives a great opportunity for the identification and confirmation of components in complex mixtures and may give some information about the structure of the separated compounds. For this reason, CE-MS coupling provides a powerful combination for performing rapid, efficient, and sensitive analysis (Schappler et al. 2008).

Other types of MS instruments used to identify peptides are the Q-TOF or TOF/TOF tandem spectrometers, ion trap (IT), orbitrap, and ion cyclotron resonance analyzer with Fourier-transform (FT-ICR), which are interesting tools in the analysis of the peptide/bioactive peptide sequence (Silve et al. 2013). Orthogonal-acceleration reflectron TOF (oa-TOF) mass spectrometers were regarded as a beneficial alternative for CE-MS. They provide high resolution and mass accuracy at a reasonable cost together with reduced bench space, user-friendly operation, high acquisition rates, and potentially unlimited mass acquisition range in a single run (Catala-Clariana et al. 2010). Analytical methods combined in various systems

include techniques where samples were first analyzed by CE-IT-MS, and the presence of some proposed bioactive peptides was confirmed using accurate molecular weight data obtained by CE-TOF-MS (Catala-Clariana et al. 2010). Almost all mass analyzers like Q, IT, TOF, and FT-ICR can be online coupled with CE, generally using ESI as the ionization source. The CE-ESI-TOF-MS method allows the highlighting of ca. 800 peptides. With a supplementary CE-MALDI-TOF/TOF-MS offline method, some of these biomarkers were found (Staub et al. 2009). However, MS peptide identification meets some technological challenges: peptide length, dynamic range, few copies of the peptide in a complex mixture, high mass accuracy, collision energy used for fragmentation, peptide retention time, and a computer-based validation. All mentioned points have to be taken into consideration and have been discussed thoroughly by Panchaud et al. (2012). The conclusion was that improvements in mass spectrometric systems still are needed to further extend the detailed analysis of the food peptidome (Carrasco-Castilla et al. 2012).

The primary structure (amino acid sequence) of a peptide is important from the activity point of view. It can be identified in an extensive chromatographic system containing diode array detector (DAD), for example, HPLC-DAD-ESI-MS, by comparison with the established peptide fragmentation fingerprint. Amino acid sequencing of peptides is often achieved through tandem MS or mass selection/mass separation experiments, and their biological activity can be foreseen (Gomez-Ruiz et al. 2006; Hernandez-Ledesma et al. 2005). Tandem mass spectrometry is a more precise method for identifying peptides and proteins. Tandem mass spectrometers have been and are applied in proteomic studies because they are used to determine the amino acid sequence of peptides, identify individual peptides, and even identify from several dozen to over a thousand proteins in a material. When using the MS/MS mode, the mass spectrometer is set to transmit only a narrow m/z range. The transmitted ions are then fragmented to acquire internal energy post-source decay (PSD) or, through collisions with gas molecules, collision-induced dissociation (CID). The sequence-specific fragments are then segregated according to their m/z values and detected (Carrasco-Castilla et al. 2012; Paulson et al. 2005). Also, MS can be used to determine the peptide mass fingerprinting in which an isolated protein is digested (often using trypsin), and the obtained mix of peptides is analyzed by a mass spectrometer, usually by MALDI-TOF, for example, the lunasin identification in four different amaranth seed varieties (Silva-Sanchez et al. 2008) and several soybean cultivars as well as the effect of environmental factors in the concentration of lunasin (Wang et al. 2008).

The use of MS/MS spectra for peptide sequencing is recommended in the case of bioactive food peptides, which are usually formed in the reaction of enzymatic hydrolysis of proteins (Ma and Johnson 2012; Picariello et al. 2013). Ma and Johnson (2012) described in detail how to use it in proteomics. Homology searches are particularly useful for the study of organisms whose genomes have not been sequenced. Caron et al. (2016) established the peptide map of each hemoglobin chain by low-resolution (LR) and high-resolution (HR) liquid chromatography (LC)-MS/MS approaches. LR technique (LC-LR-ESI-MS/MS) identifies without ambiguity 75 peptides, while the HR approach (nanoLC-HR-ESI-MS/MS) unam-

biguously identifies 971 unique peptides (innate or induced post-translational modifications included). The food peptidomic approach using the most performant separation methods and mass spectrometer with high-resolution capabilities appears as a promising source of information to evaluate the health potentiality of proteins, through their bioactive peptides (Caron et al. 2016). The enhanced peptide identification, which is the result of the valuable mass spectrometry development and the regular use of high-resolution techniques, supports the application of peptidomic approaches in the case of empirical bioactive peptide identification workflow (Sánchez-Rivera et al. 2014).

The applied detectors take advantage of the different properties of the peptides and can be used individually or in combination depending on the peptides themselves and the information one wants to obtain. Mass spectrometry is an analytical technique that uses gas ionization of analytes and the separation of ions obtained in the electric and magnetic fields. The separation of ions depends on the ratio of their mass value to the number of charges (m/z), and the identification of analytes is based on mass spectra. This technique is very useful in proteomics. Mass spectrometry can also be used in peptide sequential analysis by using MS/MS spectra. The MS/MS spectral analysis is currently considered to be a method that allows unambiguous identification of peptides and is a technique that allows the detection and location of changes occurring within the studied peptides. The identification of post-translational modifications (PTMs) due to the very complex nature of the results obtained during analyses without the use of necessary databases and computer programs would be impossible. Chromatographic or electrophoretic techniques combined with computer visualization methods are used for the proteomic study of proteins. The MALDI is a non-invasive technique of matrix-assisted laser desorption and ionization that serves as the ion source for mass spectrometers. The MALDI technique offers non-destructive evaporation (desorption) and ionization of both small and large biomolecules. It uses a matrix that mediates the transfer of energy to the test substance, which facilitates its ionization. Thanks to this, it is possible to analyze non-volatile, high molecular and polar substances. In the MALDI technique, an efficient and very precise laser-assisted energy transfer at the time of contact with the analyte causes the phenomenon of desorption of large ions and allows the measurement of the mass of the test compound with high accuracy and sensitivity of the order of picomoles. The analysis technique is fast, but the preparation of samples (plates) is quite tedious. The sample measurement does not exceed a few nanoseconds, so in order to achieve good performance and repeatability, the best homogenization of the sample is necessary. Therefore, in the MALDI technique, the method of sample preparation before measurement (matrix used, solvent, the concentration of solutions, and the method of mixing and applying) is of great importance. Getting a good separation is also a problem when the matrix is more complex. The MALDI technique can also be used to study the adulteration of peptides derived from the hydrolysis of a specific protein with other protein hydrolysates (Leopold et al. 2018).

The development of research combining two-dimensional electrophoresis techniques with MALDI-TOF spectrometry has been observed for years. In these stud-

ies, mixtures of soluble proteins are initially separated by bidirectional electrophoresis based on the difference in pI (isoelectric point) and molecular weight values. Computer software for gel analysis is used to characterize peptides based on a comparison of the pI and molecular weight of the peptides tested and mixtures of standards. The spots that correspond to single peptides on the electropherogram are cut out of the gel in the next stage. As a result of the MALDI-TOF analysis, mass spectra are obtained, on which the signals are arranged according to the increase in the mass of the ion. Their intensity depends on the proportion and type of the obtained ions. The MALDI technique, due to its non-invasiveness and precision, has been used in biochemistry to quickly identify proteins isolated by gel electrophoresis. Using a MALDI-TOF mass spectrometer, hydrolysates obtained by the enzymatic reaction of peptides can be analyzed using proteolytic enzymes (e.g., trypsin or chymotrypsin). And the tool makes it possible to identify the relationship, structure, and mass confirmation (Ayala-Niño et al. 2019).

Proteomic techniques include those used to characterize proteins and generate protein maps (these are primarily two-dimensional electrophoresis and mass spectrometry as well as techniques for identifying proteins such as "protein fingerprint"/"peptide mass fingerprinting") and those used for studying protein functions and interactions between them, and in this case, for example, immunoaffinity-based methods are used. The "peptide mass fingerprinting" technique enables the identification of peptides using appropriate computer programming (SONAR PLUS or ProFound type). The programs are used to process peptide data (mass of obtained peptide fragments) and compare them with online databases (e.g., National Center for Biotechnology Information [NCBI] and OWL). It identifies the tested proteins due to the degree of coverage of the amino acid sequence, and additionally, the use of the PSD technique or its combination with chemically assisted fragmentation allows us to determine the amino acid sequence of peptides. Identification of peptide sequence by the Edman degradation procedure (labeling and cleavage of peptides from the N-terminal without disrupting the peptide bonds between other amino acid residues) using MALDI-TOF has been widely described (Catala-Clariana et al. 2010; Cheison et al. 2011; Jiang et al. 2010; Rejtar et al. 2004).

Proper separation of peptides is usually a necessary step before their actual identification. Almost all bioactive peptides obtained by enzymatic hydrolysis of food proteins can be isolated by reverse-phase high-performance liquid chromatography (RP-HPLC) (Agyei and Danquah 2011; Fekete et al. 2012; Picariello et al. 2013). There are many reports on the use of RP-HPLC for the isolation of peptides in the subject literature. To isolate the active peptide from the crude fraction, two reverse-phase HPLC purification cycles were used. Pheromone was isolated from the abdominal glands utilizing the reverse-phase gel chromatography and HPLC (Kikuyama et al. 2013). In other studies, 3 nmol of the final product was obtained, which was sufficient for subsequent amino acid analysis (Miyauchi and Goto 2013). The MS/MS in combination with RP-HPLC has been used in many studies to identify and quantify biologically active peptides. Boutrou et al. (2013) used this technique to analyze bioactive milk protein peptides obtained in the enzymatic process of the human gastrointestinal tract. The liquid chromatography column was used to

isolate peptides and proteins even on an industrial scale (Agyei and Danquah 2011). Electro-membrane filtration (EMF) has also been considered as a method for the isolation of highly charged biomolecules such as bioactive peptides. It combines conventional membrane filtration with electrophoresis, making it more selective than membrane filtration and cheaper than chromatography. Also, operating parameters such as membrane type, electric field strength, hydrolysate salinity, and hydrolysate concentration can be controlled to improve product transfer and separation (Agyei and Danquah 2011).

Experimental methods like X-ray crystallography, MS, and nuclear magnetic resonance (NMR) together with in silico methods such as QSAR, Robetta, PepLook, and PEPstr can be used to study the 3D structure of bioactive peptides from which functional properties can be inferred (Agyei and Danquah 2011). ELISA is the immunochemical technique used regularly for the detection and determination of food allergens and has been used for specific detection of very small quantities of peptides. This technique is more sensitive than HPLC and as specific as the radioimmune assay (Carrasco-Castilla et al. 2012; Wang and De Mejia 2005). Thanks to it, the protein allergens and some bioactive peptides, which exert a negative influence on organisms, might be identified. Despite the fact that peptides with a molecular mass over 1000 Da are usually not immunogenic, a length of 6–25 amino acids is usually suggested to produce an antibody (Carrasco-Castilla et al. 2012; Wang and De Mejia 2005).

Peptide Sequencing

An interesting direction of research is the study of peptide sequences. Such research can be carried out in several ways. The history of protein sequencing is inevitably associated with Peter Edman, who in the 1950s proposed to further cleavage the amino acids from the N-terminus of the protein by phenylisothiocyanate (PITC) and presented the mechanism of such a reaction (later called Edman degradation). Currently, one of the methods of peptide sequencing is the traditional but automated Edman degradation technique which uses a sequencer adapted for this purpose. The first sequencing apparatus was constructed in the 1950s at Saint Vincent's School of Medical Research in Melbourne, Australia. And the prototype automatic sequencer was finally completed in 1967. In contrast, Beckman launched the first commercial sequencer in 1969 based on Edman's design. In the following decades, the sequencers were improved, increasing their sensitivity by conducting sequencing reactions in the gas phase. Edman sequencing remains a useful method for determining the actual N-terminus of a protein or peptide (Chang et al. 2016).

Currently, most modern sequencers have many advantages: among others, they allow to receive high stability of the analysis, thanks to the system working in the field of microflows. This allows easy identification of phenylthiohydantoin-amino acids (PTH-amino acids) even from trace samples. Some devices are adapted to

analyze both liquid and immobilized samples on a polyvinylidene fluoride (PVDF) membrane. This method offers great possibilities for use in pharmacy.

Mass spectrometry has become a tool of great importance for researchers looking to sequence peptides. Peptide sequencing can be performed using the MALDI-TOF, MALDI-TOF/TOF, or LC-Q-TOF techniques. Depending on the purpose of the research, whether it is product quality control or advanced scientific work with peptides, the appropriate software package is used. One of the more known and advanced software dedicated to peptide analysis is PEAKS Studio. It is used to process the results from both MALDI-MS and LC-MS spectrometers of many manufacturers.

De novo sequencing is used to search for new, biologically active peptides. In this way, the protein proteolysis products are separated, for example, by size exclusion chromatography based on their size. The active fraction components are then identified by RP-HPLC-MS/MS. The last stage involves the synthesis of identified peptides and testing their activity (Lau et al. 2013, 2014). Additional information facilitating peptide identification by RP-HPLC in combination with MS can be obtained by using retention time prediction using software available on the Internet (Krokhin 2012). An alternative to de novo sequencing may be a research strategy called "hypothesis-driven proteomics" (Schmidt et al. 2009). The method is based on identifying and observing the formation, degradation, modification, or changes in the content of selected proteins or peptides that are markers of the occurrence or changes in the content of these proteins.

Proteins or peptides can be typed, among others, based on bioinformatics research (Sénéchal and Kussmann 2011). The strategy of imitating "hypothesis-driven proteomics" may include, for example, searching for protein chain fragments with potential biological activity, simulating in silico proteolysis, creating simulated MS/MS spectra, and searching for peptides in protein hydrolysates using the obtained data. In these peptides, one can search for fragments identical to previously known amino acid sequences stored in databases or predict the activity of protein fragments using programs such as Peptide Ranker.

In order to adapt the theoretical predictions to the analytical methods used in individual laboratories (the type of columns, mobile phase gradient, etc.), a strategy of calculating the expected retention time of the peptide set, measuring the retention time of the same peptides, and calculating the equation describing the relationship between the theoretical retention time calculated from using the available program and the real time of peptide retention can be used (Dziuba et al. 2011).

Peptide Synthesis

Bioactive peptides from food proteins can be produced by enzymatic hydrolysis (using proteolytic enzymes from plants or microbes), hydrolysis with digestive enzymes (simulated digestion in the digestive tract), or fermentation using starter cultures (Fig. 10.1). Some researchers also used a combination of these methods to

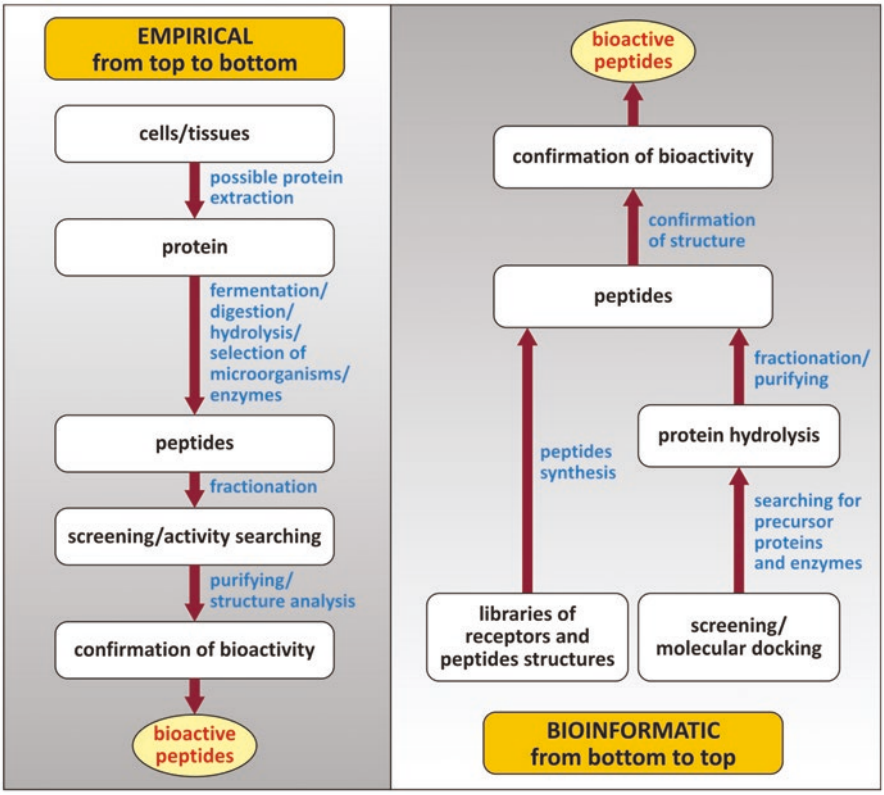

Fig. 10.1 Methods of bioactive peptides investigation

produce peptides with biological activity (Korhonen and Pihlanto 2006). What is more, bioactive peptides may be synthesized chemically because the number of these peptides occurring in nature is very low and the need of producing synthetic bioactive peptides is very high in commercial interest (Perez-Espitia et al. 2012).

Currently, interesting solutions for peptide synthesis can be used, which are peptide synthesizers, systems for both small- and large-scale synthesis of peptides, peptoids, peptide nucleic acids (PNA), and peptidomimetics. These devices are fully automated, semi-automatic, or manual, depending on the needs of peptide synthesis. The synthesis can be carried out using room temperature peptide synthesizers and microwave peptide synthesizers in both entry-level and high throughput configurations. Synthesizers allow the formation of single or multiple peptides in parallel. The synthesis of more peptides proceeds in a shorter time, resulting in the highest productivity. The use of microwave peptide synthesizers not only allows the synthesis of difficult peptide sequences that are problematic or even impossible to synthesize by conventional methods, but also allows for more efficient peptide production. Such solutions allow us to make complex peptide modifications like side-chain modification and elaboration, for example, attachment of a dye label, multiple

antigenic peptides, intramolecular cyclization, head-to-tail cyclization, side-chain to side-chain cyclization, and disulfide bridge formation. Advanced synthesizers allow us to control the course of peptide synthesis and to make changes in ongoing synthesis. These changes are made after pausing and checking the coupling without having to reprogram the synthesis (Banerjee et al. 2010).

Bioinformatics

The rapid development of bioinformatics has led to the rapid accumulation of a huge amount of biological data, stored in numerous databases. Bioinformatic tools enable simulation of protein hydrolysis and facilitate the identification of bioactive peptides (Barkia et al. 2010; Sánchez-Rivera et al. 2014). Algorithms enabling the analysis of mass spectrometric data in connection with information from bioinformatics databases are indispensable tools during proteomic research. This analysis is used to identify proteins based on molecular weights and amino acid sequences of the analyzed peptides. Currently, databases are an essential tool in the development of bioactive peptide science and extremely useful in defining the structure–function correlations of proteins and peptides, computer-aided structure simulations, searching for possible forerunners of bioactive peptides, the search of binary and multiple alignments between peptide sequences, foreseeing biological activity, proteolysis simulation, secondary structure prediction, calculation of physicochemical features, and protein–ligand docking. All the programs for peptides and proteins available on the Internet have been published and described in detail by Minkiewicz (Minkiewicz et al. 2008a, b, 2009).

The popular bioinformatics databases include Universal Protein Resource (UniProt Knowledgebase) protein sequence collections, National Center for Biotechnology Information (NCBI) protein sequence collections, and proteomic databases of the European Bioinformatics Institute (EBI). Information on the physicochemical properties of peptides or biological activities has been provided by chemometric analysis applied in science concerning food-derived biopeptides, and they can be predicted by using the peptide sequence identified by chromatographic, electrophoretic, or mass spectrometric complex profile (Sánchez-Rivera et al. 2014).

To predict the performance of bioactive peptides from food protein sources, QSAR and in silico bioinformatic-based method are often used (Gu et al. 2011). QSAR represents the method in molecular design. The method searches information relating the chemical structure to biological and other activities. The idea behind QSAR is that the biological activity is related to the structural variation of the compounds, and this correlation can be modeled as a function of molecular structure (Wu et al. 2006, 2008). In the food science, in silico (computer simulation) analysis may be used as a fast initial screening tool to search for high potential sources of bioactive peptides assisted by Food Eng Rev databases like BIOPEP, SwePep, PeptideCutter, POPS, NeuroPred BioPD, EROP-Moscow, and PepBank, although BIOPEP focuses mainly on peptides of food origin (Sánchez-Rivera et al.

2014). There are databases that can be applied in the selection of proteins/enzymes combinations, in silico hydrolysis, and also for the characterization of the peptides produced. These databases can be used for selecting proteins and enzyme(s) treatment which, after in silico digestion, may provide a peptide of pharmacological interest. It is expected that this approach will be especially beneficial to drug manufacturers since it has the ability to predict the possible bioactivity of a peptide beforehand, therefore making the approach more cost-effective and less labor-intensive (Agyei and Danquah 2011).

Analytical identification of proteins in proteomics is based on their initial proteolytic digestion into peptides, determination of the sequence of obtained peptides, and use of this sequence to identify proteins by searching relevant bioinformatics peptide sequence databases. The identification of peptides in proteomics is usually done by using MALDI-TOF-MS and ESI-MS/MS. Appropriate specialized algorithms are needed to effectively analyze information from mass spectrometry and bioinformatic databases. The PEAKS is a proteomics software program for MS/MS, which was designed for peptide sequencing, protein identification, and quantification. This tool has many advantages; it enables identification after translational modifications, analysis of samples before and after digestion, de novo sequencing and searching. It is one of the cheapest software in the world and one of the best rated by its users. In bioinformatics, in addition to using typical IT solutions related to computer-aided database searching, solutions based on statistical and chemometric methods are also used. Chemometrics is a field of extracting useful information from multidimensional measurement data, using the methods of statistics and mathematics. Bioinformatic-driven approaches have gradually gained importance through the wider application of in silico analysis, structure-activity relationship models, chemometrics, and peptide database management (Sánchez-Rivera et al. 2014). The use of chemometric and bioinformatics methods reduces costs and research time.

In Vitro Analysis of Peptides Bioactivity Using Isothermal Titration Calorimetry

Peptide bioactivity can be analyzed in a variety of ways. In these types of activities in which clearly separated and relatively simple mechanisms are involved, less complex methods can be used, showing the basic chemical relationships. For example, renin (aspartyl proteinase), which hydrolyzes angiotensinogen, a globulin containing 452 amino acids, is involved in regulating blood pressure. Prohormone decapeptide called angiotensin I is cleaved from angiotensinogen. It is activated by further hydrolysis, during which the dipeptide is cleaved and angiotensin II is formed, an active hormone that causes blood vessels to constrict and increases blood pressure. The enzyme peptidyl dipeptide hydrolase called angiotensin-converting enzyme (ACE) is involved in the conversion of angiotensin I to II. Peptides constituting ACE

inhibitors are the most numerous and best-known group of bioactive peptides derived from food (Hernández-Ladesma et al., 2007, Wanasundara et al., 2002). They bind to the enzyme by aligning the sequence in such a way that the hydrolysis of angiotensin I is reduced. These types of sequences are present in fish, cereal, and milk proteins. The antihypertensive activity of peptides is determined not only by the peptide sequence, enabling its interaction with ACE, but also by the affinity to the enzyme, including interaction energy. Therefore, the activity can be tested by isothermal titration calorimetry (ITC), which allows the assessment of inhibition of substrate hydrolysis in the presence of an inhibitor. The interaction energy alone can be assessed by the same method in only the enzyme and peptide system. For ACE activity evaluation, the spectrophotometric method of hippuryl-L-histidyl-L-leucine can be used comparatively. The cleavage product hippurate gives a yellow derivative, showing a maximum absorbance at 405 nm (Schnaith et al. 1994).

The mechanism that accompanies clot formation involves several stages. Clot formation is preceded by increased circulation of prothrombin, which is a zymogen, a glycoprotein containing 579 amino acids, which is an inactive form of thrombin. Prothrombin hydrolyzes to active thrombin, which is involved in the transformation of soluble fibrinogen into insoluble fibrin, from which the clot forms. Two factors are necessary for the conversion of prothrombin into thrombin: Xa, which is a serine protease, and Va, which is a stimulating protein. The Xa factor cleaves two prothrombin bonds in the presence of the Va factor. In this way, thrombin is produced, which is also a serine protease made up of 622 amino acids and consists of two resulting polypeptide chains connected by a disulfide bond. Thrombin hydrolyzes four fibrinogen bonds to release fibrin in the form of fibrinopeptides A and B. The resulting fibrinopeptides spontaneously form aggregates that transform into an insoluble clot. Collagen is a rich source of peptides that inhibit the formation of clots. In this case, ITC can also be used to test the activity of anticoagulant peptides by measuring heat changes in the peptide binding to the fibrinogen system. It is also possible to measure thrombin activity in an inhibitor and fibrinogen system, but all other hydrolysis accompanying interactions must be included. For comparison, using blood plasma, it is possible to determine the evolution of the fibrin intermediate, α-profibrin, from fibrinogen solutions by electrophoresis that enables separating insoluble fibrin from a fibrin intermediate soluble in a buffer. After electrophoresis, the band containing α-profibrin co-migrates with fibrinogen and is distinguished from it by immunoprobing the gel with an anti-fibrin antibody that reacts with fibrin or α-profibrin but not with fibrinogen (Siebenlist et al. 2005).

Opioid activity is measured in vitro by the dissociation constant of the opioid to opioid receptor complexes or by the maximal binding capacity of peptide to the opioid receptor by ITC. Since adenylate cyclase is sensitive to opioid concentration, due to opioid-induced changes in the mitogen-activated protein kinase signaling pathway, the opioid activity can also be measured by influencing adenylate cyclase activity. Another method is testing the antagonistic effect of opioid peptides on inhibition of muscular contraction (Siebenlist et al. 2005). The bioactivity of opioid peptides is not based on enzymatic reactions but on binding ligands to receptors. Opioid receptors are found in the central and peripheral nervous systems. They

come in four types, each of them in two subtypes. Opioid peptides are ligands of opioid receptors and, when attached, contribute to pain relief, sedation, dementia, respiratory depression, and reduction of some other activities. The opioid peptides are found in food mainly as fragments of gluten proteins, casein, and hemoglobin.

Antimicrobial activity is most often tested in viability tests of saprophytic or pathogenic microorganisms. The mechanism of action of these peptides mainly concerns the destabilization of the microbial cell wall, and the study of antibacterial activity can also be carried out in a peptidoglycan model, analyzing, for example, the energy effects of peptides interacting with elements of the bacterial cell wall (Dallas et al., 2013).

Peptide bioactivity measured in vitro does not always correspond to in vivo activity. This is due, inter alia, to the bioavailability of peptides. Although the peptide may be resistant to the action of gastrointestinal endopeptidases and may be easily absorbed into the blood plasma, it may later be digested by intracellular proteases before reaching the target system in which its activity is expected. Therefore, the bioactivity of the peptides should be finally verified in clinical trials.

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Chapter 11
Determination of Antioxidant Biomarkers in Biological Fluids

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Abstract The presence of various bioactive markers in the human body reflects its health condition, physical endurance, and also the intake of some valuable compounds from food. Analytical methods for their determination are becoming a useful tool for gaining information on the levels of these physiologically essential substances. Antioxidants that can prevent or slow the harmful action of free radicals belong to one of the most significant biomarkers. Reactive oxygen species/reactive nitrogen species produced under oxidative stress act together and cause damage to all cellular biomolecules. Therefore excessive levels of such reactive species pose a threat to human organisms contributing to inflammatory responses. For measurements of oxidative stress or damage indicators, both the reactive species are analyzed, and also different markers considered useful indexes of the level of the phenomenon are determined. Biological samples usually include whole blood derivatives (serum and plasma), urine, and saliva. The fluorescence methods are most commonly applied for the determination of oxygen radicals. At the same time, markers of cellular oxidative damage are most often tested in body fluids and tissue homogenates using enzyme-linked immunoassay kits, high-pressure liquid chromatography, or even gas chromatography, both combined with mass spectrometry. For the assay of protein carbonyls in biological matrices, the derivatization of the carbonyl group, usually with 2,4-dinitrophenylhydrazine, is performed and followed

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by the detection of various types, for example, using anti-DNP antibodies in immunoblotting.

Similarly, glutathione and antioxidant enzymes are determined by immunological assays and chromatographic and spectrophotometric techniques. For phenolic content quantification, different kinds of detection such as an electrochemical coulometric array, on-line connected photodiode-array and electro-array, chemical reaction detection techniques, mass spectrometry, and nuclear magnetic resonance can be used. Modern methods for assessing the levels of vitamins A, C, D, and E as well as total antioxidant capacity assays are also described.

Introduction

Most of the life processes lead directly or indirectly to the formation of oxidizing compounds. Some of these substances are transformed into radical species, molecules that have a deficit or surplus of electrons on the valence shell. It results in the appearance of very reactive agents, which cause damage to cell structures in the body. In the organism, the most sensitive structures to the effects of free radicals are nucleic acids (DNA and RNA as examples of polynucleotides) and low density lipoproteins (LDL) as well as proteins and carbohydrates. The impact of free radicals may contribute to the development of civilization diseases, including cancer or cardiovascular diseases. Also, during intensive physical activity, substances of acidic properties such as pyruvic and lactic acid are secreted into the bloodstream. They lower the pH value of blood, which in turn leads to the release of iron(III) ions (Fe^{3+}) associated with the transferrin and their excessive transformation into iron(II) ions (Fe^{2+}) (Cooper et al. 2002; Yamaji et al. 2004). These ions are involved in the processes responsible for the formation of free radicals in the body, which in the case of athletes, may adversely affect their endurance (Sjödin et al. 1990). It is also assumed that 5–10% of the oxygen involved in the oxidative phosphorylation process (respiratory chain) is converted into free radicals (Raha and Robinson 2000).

Small amounts of free radicals (reactive oxygen species (ROS)/reactive nitrogen species (RNS)) and other reactive species are found in the human body, but when the balance between formation of species and their neutralization is not maintained, the oxidative stress occurs. Oxidative stress in an organism caused by contamination, stress, and others factors can develop and progress various systemic disorders like Parkinson's disease, Alzheimer's disease, myocardial infarction, depression, diabetes, cancers and autism, and ADHD (attention-deficit/hyperactivity disorder) in children, among others (Fig. 11.1). The aging of the organism also results from oxidative stress, additionally connected with impaired antioxidant defense system (Goni and Hernández-Galiot 2019).

Both preventive and chain-breaking antioxidants are mainly divided into enzymatic and non-enzymatic systems, which represent prevalently cellular or non-cellular mechanisms of action (Fig. 11.2) (Ialongo 2017). To the first group belong

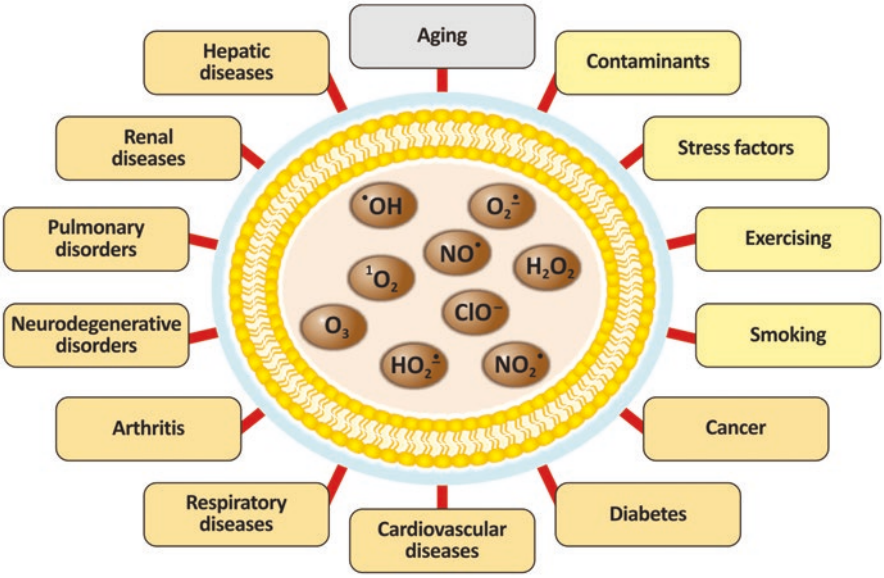

Fig. 11.1 Formation of reactive oxygen and nitrogen species in the cell by the exogenous and endogenous factors

the superoxide dismutases (SOD), the catalases (CAT), the peroxiredoxins (Prx), the thioredoxin reductase (TR), the reduced glutathione peroxidase (GPx), and the oxidized glutathione reductase (GR). The compounds of five major groups are the following:

(a) Endogenous low-molecular-weight antioxidants: free reduced glutathione (GSH), uric acid (UA), α-lipoic acid (ALA), coenzyme Q_{10} (CoQ_{10}), bilirubin, methionine, and cysteine,
(b) Exogenous low-molecular-weight antioxidants: ascorbic acid (vitamin C), the tocopherols (vitamin E), the carotenoids (e.g., β-carotene), and vitamin A,
(c) Proteins: albumin and cysteine-rich proteins,
(d) Polypeptidic antioxidants: thioredoxins, glutaredoxins, and sulfiredoxins,
(e) Metal-binding proteins: ceruloplasmin and metallothioneins are classes of the non-enzymatic system.

Exogenous and endogenous antioxidants interact with each other (e.g., urates →ascorbic acid → tocopherols) and inhibit the oxidative stress and the action of pro-oxidants (Ialongo 2017).

Plant food contains the compounds of high antioxidant activity. Proper intake of phenolics and antioxidant vitamins from food could help the antioxidant defense in the organism. Therefore, the measurement of phenolic compounds intake with dietary assessment (estimation of dietary intakes – questionnaire, diary, and 24-hour recall) and nutritional status are widely used (Jenab et al. 2009; Pico et al. 2019). However, these estimation methods are not sufficient and the specific biomarkers of

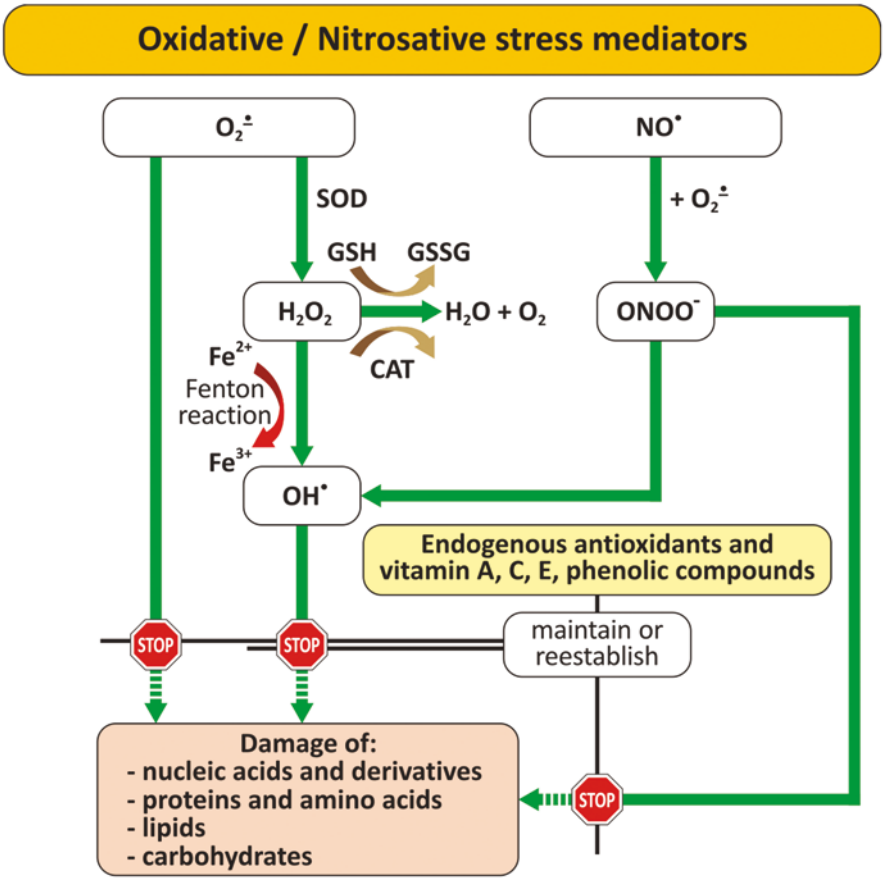

Fig. 11.2 The possible paths of reactive oxygen and nitrogen species in the organism. (SOD superoxide dismutase, GSH glutathione, GSSG glutathione disulfide, and CAT catalase)

oxidative damage/stress, as well as antioxidant status in the biological fluids, are becoming now more and more investigated for the medical and physiological research purposes (Frijhoff et al. 2015; Marrocco et al. 2017).

The World Health Organization (WHO) has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (WHO 2001). In the field of nutrition, the biomarkers are also used to reflect the intake, exposure, or status of some food or nutrient in the body. Also, the determination of the biomarkers in biological fluids in the future epidemiologic studies should be important to show the relations between dietary exposure, food composition, and risk of major chronic disorders such as cancer, cardiovascular diseases, or diabetes (Edmands et al. 2015; Stalmach et al. 2009).

Therefore, the biomarkers of oxidative stress or damage and antioxidant activities can be divided into the four following groups:

1. Reactive oxygen and nitrogen species
2. Markers of cellular structure oxidative damage
   (a) Oxidative damage of cell membrane (lipid fraction)
   - thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA),
   - 4-hydroxynonenal (4-HNE),
   - hexanal,
   - 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$).

   (b) Oxidative damage of nucleic acids and derivatives
   - 8-hydroxy-2′-deoxyguanosine (8-OHdG),
   - allantoin.

   (c) Oxidative damage of proteins and amino acids
   - protein carbonyl groups,
   - *o,o′*-dityrosine, *ortho*-tyrosine, *meta*-tyrosine,
   - homocysteine.
3. Glutathione and antioxidant enzymes.
   - glutathione (GSH),
   - glutathione peroxidase (GPx),
   - glutathione reductase (GR),
   - glutathione S-transferase (GST),
   - superoxide dismutase (SOD),
   - catalase (CAT).
4. Non-enzymatic markers – antioxidants and total antioxidant capacity (TAC) assays
   - phenolic compounds,
   - vitamins C, A, and E and in some cases vitamin D,
   - bilirubin,
   - ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay,
   - DPPH (2,2-diphenyl-1-picrylhydrazyl) assay,
   - FRAP (Ferric Reducing Ability of Plasma) assay,
   - ORAC (Oxygen Radical Absorbance Capacity) assay.

The evaluation of oxidative stress/damage remains a significant challenge for analysts. The biomarkers are mainly tested in the following biological samples: whole blood derivatives (serum and plasma), urine as well as in saliva, among others. Examples of the clinical or animal studies of the determination of antioxidant markers, also in the organism with oxidative stress/damage, are presented in Table 11.1.

Table 11.1 Clinical or animal studies on the determination of antioxidant markers

Experimental system	Tested compounds/ material/food	Antioxidant marker/s	Reference
Clinical studies			
A randomized clinical trial (healthy older adults)	LBP-standardized *L. barbarum* fruit juice	SOD, GPx, and MDA compared to pre-intervention and placebo	Amagase et al. (2009)
Antidepressant drug – patients with oxidative stress (total 132 patients in every five groups)	Vitamin C, α-lipoic acid, Vitamin C + α-lipoic acid	Vitamin C status, TAC (ABTS test), plasma cholinesterase levels compared to placebo	Kadar Ali and Raja (2019)
20 patients with autism spectrum disorder (ASD) (6–22 years old) and 12 controls (5–21 years old)	No special diet or vitamin – normal diet (TAC for ASD group was higher)	Urinary TAC – higher in controls Urinary levels of 8-OHdG and plasma SOD – not changed	Yui et al. (2017)
Chronic hemolytic anemic children (60 patients: 6–18 years old) and 30 healthy children	No special diet or vitamin supplementation	Serum levels of vitamin E and selenium – higher in control patients	Hamdy et al. (2015)
24 healthy men (20–22 years old) before and after 7, 14, 21, and 28 days (two charcoal-broiled beefburgers per day)	Diet with PAH	CAT, GPx, SOD, TBARS in erythrocytes; Plasma vitamin A, C and E	Elhassaneen (2004)
68 patients with acute ischemic stroke and 41 healthy controls	No special diet	Plasma concentrations of α and β-carotene are lower in patients with acute ischemic stroke than in healthy controls and negatively correlated with hs-CRP level and neurologic deficits	Chang et al. (2005)
14 healthy volunteers (11 men and 3 women) (0, 4, and 8 weeks)	Hydroxytyrosol supplementation	Serum TAC, MDA, vitamin A, D, & E – not changed; Serum vitamin C level higher after supplementation	Lopez-Huertas and Fonolla (2017)
25 Behcet's disease patients (13 men and 12 women) and 25 controls (6 weeks)	Vitamin E supplementation	Plasma vitamins A, E and β-carotene CAT, GPx, and MDA HNE-lipid adducts GSSG	Kokcam and Naziroglu (2002)
50 PCOS patients and 14 healthy controls	No special diet	Vitamin C B complex (eight vitamins)	Szczuko et al. (2020)

(continued)

Table 11.1 (continued)

Experimental system	Tested compounds/ material/food	Antioxidant marker/s	Reference
10 younger (18–40 years old) and 10 older (55–70 years old) healthy adults	Nitrate, vitamin C, and in combination	Vitamin C, nitrate, nitrite, c-GMP, tetrahydrobiopterin (BH4) and 3-NT	Ashor et al. (2020)
67 patients with vascular parkinsonism and 39 controls	No special diet	Vitamins A, C, E – not changed	Paraskevas et al. (2003)
Meta-analysis of 12 randomized controlled trials, the effects on mental health, biomarkers of inflammation and oxidative stress in patients with psychiatric disorders were assessed	Probiotic supplementation	CRP, IL-10, and MDA; TAC, NO, GSH – not changed	Amirani et al. (2020)
Meta-analysis of 23 trials (dietary anthocyanins more useful for unhealthy subjects)	Dietary anthocyanins	MDA, ox-LDL, isoprostane, TAC level, SOD, and GPx activities	Fallah et al. (2020)
Meta-analysis of randomized controlled trials patients with psychiatric disorders	Vitamin D supplementation	GSH, TAC, and CRP levels	Jamilian et al. (2019)
7 randomized controlled trials with 317 participants	Garlic supplementation	TAC and MDA	Moosavian et al. (2020)
Meta-analysis of randomized controlled trials in the pathophysiology of cystic fibrosis	No special diet	PC, 8-iso-prostaglandin F2α, MDA, vitamins A and E and β-carotene in plasma or serum	Causer et al. (2020)
Alzheimer's disease and brain aging: a meta-analysis	No special diet	MDA (slight difference); 4-hydroxynonenal, 8-hydroxyguanine, PC level, antioxidant enzymes activities, vitamin C, and α-tocopherol, all not changed	Zabel et al. (2018)
112 patients with chronic schizophrenia (18–65 years old)	Clozapine or risperidone or perphenazine	SOD, GSH – higher in patients with clozapine treatment, other antipsychotic agents not; protein carbonyl and MDA level not changed	Hendouei et al. (2018)

(continued)

Table 11.1 (continued)

Experimental system	Tested compounds/ material/food	Antioxidant marker/s	Reference
13 (8 male and 5 female) patients with ankylosing spondylitis and 13 (8 male and 5 female) controls	No special diet	Vitamins A, C, E, and β-carotene in plasma, GSH and GPx values in erythrocyte -lower in patients and TBARS higher	Naziroglu et al. (2011)
Systemic review of markers in depressive disorders	No special diet, vitamins C, A, or retinol palmitate	8-OHdG, 3-NT, PC, F2-ISO, 4-HNE, 8-iso-prostaglandin, MDA, SOD, CAT, GPx, and vitamins A and C	Barbosa et al. (2020)
10 healthy adults (0 and after 2, 4,5, 6, and 8 hours)	α-tocopherol and tocotrienol fractions	MDA-only FRAP, TEAC-ABTS, SOD, GSH – not changed	Fairus et al. (2019)
83 patients with T2DM 81 controls	No special diet	SOD, CAT, vitamin C, TAC, Serum NO, ROS, and MDA; GSH not changed	Mandal et al. (2019)
30 young patients with acute myocardial infarction	Folic acid and folic acid + vitamin E	Homocysteine, serum TAC [Cu(II) – Cu(I)]	Assanelli et al. (2004)
29 chronic lymphocytic leukemia patients in stage A, 21 patients in stages B and C, and 31 healthy volunteers	No special diet	CAT, MDA; GPx, H_2O_2 – not changed	Zelen et al. (2010)
28 cement workers and 30 volunteers	No special diet	MDA, GSH, vitamin C and E, total bilirubin; SOD and GPx in erythrocytes	Aydin et al. (2004)
116 lactating women during 3 months	No special diet	Serum vitamin A and E – lower during lactation	da Silva et al. (2019)
Animal studies			
Hypercholesterolemic Wistar rats (6 weeks)	High-fat diet with mulberry leaves extract in the diet	Plasma FRAP, total phenolics, and TBARS levels compared to control	Jeszka-Skowron et al. (2017)
Diabetic Wistar rats (4 weeks)	High-fat diet with mulberry leaves extract in the diet	Plasma FRAP, total phenolics, TBARS levels compared to control	Jeszka-Skowron et al. (2014)
Diabetic hindlimb ischemia rats (6 weeks)	Supplementation of simvastatin, vitamins E, and C, simvastatin + vitamin E, and simvastatin + vitamin C	Nitrite in plasma and tissue, MDA in plasma, SOD, CAT, and GSH in tissue	El-Azab et al. (2012)

(continued)

Table 11.1 (continued)

Experimental system	Tested compounds/ material/food	Antioxidant marker/s	Reference
Mice treated with mixture of heavy metals (Pb, Hg, Cd, and Cu) (7 weeks)	Heavy metals and vitamin E Heavy metals Vitamin E	Plasma urea Plasma uric acid Kidney SOD and GSH Testis SOD and GSH	Al-Attar (2011)
Cadmium induced lipid peroxidation in Wistar rats	CoQ_{10}, vitamin E, and olive oil	Tissue SOD, CAT, GPx, GR and GST, vitamins C and E	Ognjanović et al. (2010)
Hypercholesterolemic hamsters (10 weeks)	Vitamin E compared with black tea	ox-LDL LDL-α-Tocopherol LDL-γ- Tocopherol	Nicolosi et al. (1999)
Hepatocellular carcinoma Wistar rats (4 weeks)	Ginger extract	MDA and GSH	Vipin et al. (2017)
Parkinson's disease in rats (3 weeks)	Edaravone-caffeine combination	3-NT and MDA	Bandookwala et al. (2019)
Acute and chronic immobilization stress in swiss albino mice	*Celastrus paniculatus* seed oil (Jyothismati oil)	GPx, GST, and GR in brain tissues	Lekha et al. (2010)

Control group – without antioxidant material/vitamins and/or healthy
3-NT 3-nitrotyrosine, *4-HNE* 4-hydroxynonenal, *8-OHdG* 8-oxo-2′-deoxyguanosine, *CAT* catalase, *c-GMP* cyclic guanosine monophosphate, *CoQ_{10}* coenzyme Q10, *F2-ISO* F2-Isoprostanes, *FRAP* Ferric Reducing Ability of Plasma, *GR* glutathione reductase, *GSH* reduced glutathione, *GPx* glutathione peroxidase, *GSSG* glutathione disulfide, *GST* glutathione *S*-transferase, *IL-10* interleukin 10, *MDA* malondialdehyde, *PAH* polycyclic aromatic hydrocarbons, *PC* protein carbonyls, *T2DM* diabetes type 2, *TAC* total antioxidant capacity, and *TBARS* thiobarbituric acid reactive substances

Qualitative and Quantitative Analysis of Free Radicals and Other Reactive Species

The main free radicals and other reactive species in the organism are the following:

- hydrogen peroxide H_2O_2
- singlet oxygen $^1O_2$
- ozone O_3
- hydroperoxyl radical $HO_2^{\bullet -}$
- superoxide anion radical $O_2^{\bullet -}$
- hydroxyl radical $OH^{\bullet}$
- nitric oxide radical $NO^{\bullet}$
- nitric dioxide radical $NO_2^{\bullet}$
- hypochlorite anion ClO^-

Free radicals have in vivo a short half-life of 10^{-6} s ($O_2^{\bullet}$) to 10^{-9} s ($OH^{\bullet}$). Most free radicals have specific, usually enzymatic neutralization mechanisms. Hydroxyl radical ($OH^{\bullet}$) has the shortest life span and it is the most reactive species among

radicals. In its utilization, the body uses antioxidants derived from food, that is antioxidant vitamins or/and polyphenolic compounds. On the other hand, free radicals and other reactive species are a part of the human body defense system against microbes.

Hydrogen Peroxide (H_2O_2)

Amplex Red reagent, a colorless and non-fluorescent derivative of resorufin, can be oxidized by H_2O_2, in the presence of horseradish peroxidase (HRPO), producing a highly fluorescent product. H_2O_2 production is calculated by measuring the specific fluorescence of oxidized Amplex Red molecule (extinction = 350 nm, emission = 399 nm) according to a modification of the method of Zhou et al. (1997).

The buffered phenol red solution (PRS) contains: 140 mmol L^{-1} NaCl, 10 mmol L^{-1} potassium phosphate buffer, pH 7.0, 5.5 mmol L^{-1} dextrose, 0.28 mmol L^{-1} (0.1 g L^{-1}) phenol red, and 8.5 U mL^{-1} (50 μg mL^{-1}) of HRPO. PRS and HRPO were added to the buffer just before the start of the experiment. To 1 mL of PRS, 10 μL of H_2O_2 solution was added, to result in final concentrations of H_2O_2 from 0.1 to 100 pmol L^{-1}. The tubes were incubated for 5 min at room temperature (25 °C) and brought to pH 12.5 by the addition of 10 μL (1 mol L^{-1}) NaOH. The absorbance was read at 610 nm in a spectrophotometer against a blank sample containing 1 mL PRS, 10 μL H_2O_2, and 10 μL (1 mol L^{-1}) NaOH. There was a linear relationship between absorbance at 610 nm and H_2O_2 concentration in the 1–60 μmol L^{-1} range, corresponding to 1–60 nmol H_2O_2 per mL (Pick and Keisari 1980).

Superoxide ($O_2^{\bullet-}$)

For superoxide anion radical ($O_2^{\bullet-}$) determination of a novel fluorescence method has recently been developed (Han et al. 2017). The researchers have studied CdTe quantum dots (QDs) modified by L-cysteine Schiff base and characterized them with the use of Fourier transform infrared (FT-IR), thermal gravimetric analysis (TGA), ultraviolet-visible (UV-Vis) and fluorescent methods. X-ray powder diffraction pattern (XRD) and transmission electron microscope (TEM) were applied to confirm the particle size of CdTe QDs (3.1 nm) in the presence of β-cysteamine. The fluorescence intensity of CdTe QDs decreased with a modified Schiff base at 305 nm and enhanced at 610 nm with the addition of $O_2^{\bullet-}$. The method was characterized by the high sensitivity fluorescence for detecting the concentration of $O_2^{\bullet-}$ with high selectivity toward $O_2^{\bullet-}$.

Hydroxyl Radical ($OH^{\bullet}$)

The principle of the method (Korotkova et al. 2011) is based on the selective reaction of the hydroxyl radical with non-fluorescent terephthalic acid (TA), resulting in the formation of a fluorescent compound 2-hydroxy-terephthalic acid (TA-OH).

For the initial detection of fluorescence, a reaction solution (10 mL) is prepared. The following solutions are successively added: 2 mL of terephthalate solution (0.01 mol L^{-1}); 30 μL of standard Mohr salt solution (0.002 mol L^{-1}); 0.03 μL of standard hydrogen peroxide solution (0.001 mol L^{-1}); and 2 mL of phosphate buffer (0.025 mol L^{-1} equimolar mixture of Na_2PO_4 and KH_2PO_4 and 2% KCl; pH = 6.86). The remaining volume is made up with distilled water (V = 6.4 mL). The mixture is incubated at room temperature for 6 min.

The concentration of hydroxyl radicals in blood serum is determined by mixing 2 mL of standard TA solution (0.01 mol L^{-1}), 2 mL of phosphate buffer (0.025 mol L^{-1}; pH = 6.86), and 0.1 mL of serum. Distilled water is added to make up to a volume of a total 10 mL. TA-OH fluorescence is read at 327 nm. The intensity of light emission is directly proportional to the concentration of hydroxyl radicals in the biological material. The serum concentration of hydroxyl radicals in healthy people is from 270 to 410 μmol L^{-1}.

Nitric Oxide ($NO^{\bullet}$)

There are at least two methods for the determination of nitric oxide. One of them is the electrochemical analysis in which the electrode reacts by changing the voltage in contact with $NO^{\bullet}$. The difficulty of the method is the short half-life of this free radical in body fluids and tissues (Nagano and Yoshimura 2002). It is possible to bind $NO^{\bullet}$ using iron-dithiocarbamate complexes, whose product (mono-nitrosyl-iron complex) is measured by electron paramagnetic resonance (EPR) (Vanin et al. 2002). Total nitric oxide, as well as nitrate/nitrite, may be determined using enzyme-linked immunosorbent assay (ELISA) kit in serum, plasma, cell culture supernates, and urine. The reference values of these parameters in serum and urine are 13–97 and 369–2684 μmol L^{-1}, respectively.

Markers of Structural Oxidative Damage

Determination of TBARS and MDA

The direct effects of reactive oxygen species are damage to lipid cellular structures and lipid damage in blood. The LDL fraction is very sensitive to those compounds (Steinberg and Chait 1998). The impact of free radicals of aerobic origin on these

structures leads to the formation of a low-molecular compound – malondialdehyde (MDA), the concentration of which is directly proportional to the quantity of ROS in the organism and indirectly determines the body exposure to them (Kanter et al. 1993). Under the influence of peroxidation, the four-carbon segments of higher fatty acids are then disconnected. They are precursors of the produced adducts with thiobarbituric acid (TBARS), such as MDA, 4-HNE (4-hydroxynonenal), hexanal, and others (Janero 1990; Suarez-Pinzon et al. 1996; Zhou et al. 2005). The determination of MDA concentration with the use of the high-pressure liquid chromatography (HPLC) method, similarly to TBARS (determined by the spectrophotometric method), can be considered as an indicator of oxidative stress, that is the degree of body exposure to free radicals.

The concentration of TBARS is also modulated by physical activity. These changes depend on the individual variety of athletes, the intensity and duration of the activity, and the method of regeneration, as well as on blood sampling to post-effort analysis. A higher level of MDA is observed in smokers (Lykkesfeldt et al. 2004), in the conditions diagnosed as asthma (Hanta et al. 2003), cancer (Carbonneau et al. 1991), cirrhosis (Wasowicz et al. 1993), and diabetes (Arshad et al. 1991; Wasowicz et al. 1993), as well as in people on dialysis (Carbonneau et al. 1991; Wasowicz et al. 1993).

TBARS measurement is based on the methodology proposed by Buege and Aust (1978). Plasma (50 μL) was mixed with 50 μL of 0.01% butylated hydroxytoluene, 300 μL of 20% acetic acid, and 300 μL of 0.8% TBA and placed in 2 mL polypropylene screw-cap microcentrifuge tubes. After shaking, the tubes were placed into a water bath for 60 min at a temperature of 100 °C. Subsequently, all samples were brought to room temperature, vigorously shaken, and centrifuged for 10 min at 4000 g at 4 °C. The supernatant (200 μL) was pipetted onto an enzyme-linked immunoassay (ELISA) plate. Measurements were performed at 532 nm with a multimode microplate reader. The standard curve is created from stoichiometrically diluted 1,1,3,3-tetramethoxypropane (TMP) solutions. TBARS concentration is expressed in μmol L^{-1} of plasma. The modification of this method is proposed by Ohkawa et al. (1979).

8-Iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) and 8-Hydroxy-2-deoxyguanosine (8-OHdG)

8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is an isoform of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). It is characterized by higher stability than MDA in blood plasma (Roberts and Morrow 2000). 8-iso-$PGF_{2\alpha}$ is formed as a result of non-enzymatic free radical peroxidation of arachidonic acid and is excreted by the kidneys. Therefore, their concentration in the urine may be much higher than in the blood.

The serum or urine determination of 8-OHdG is an indicator of oxidative DNA damage and a measure of mutagenicity or carcinogenicity. The body exposure to

harmful agents, such as cigarette smoke, smog, mutagenic substances, as well as ultraviolet and ionizing radiation, increases the concentration of 8-OHdG. Usually, the cells have abilities that allow 8-OHdG to be converted back to deoxyguanosine. Sometimes such change does not occur, and 8-OHdG appears in the DNA chain, which can lead to carcinogenesis (Yasui et al. 2014). Furthermore, the presence of 8-OHdG may cause defective gene expression or induce subsequent mutations alone (Valavanidis et al. 2013).

Currently, both substances are most often determined in body fluids (blood and urine) and tissue homogenates using commercially available ELISA kits (Table 11.2). However, greater detection accuracy is possible due to liquid (LC) and gas chromatography (GC), especially with the use of mass spectrometers (Milne et al. 2007).

Allantoin

Allantoin, a heterocyclic compound formed in most mammals, except for the higher primates and man, by the oxidation of uric acid and by the catabolism of purine is also taken into account in the characterization of free radicals (Young et al. 1944). In the physiological conditions, this compound is not determined in blood because in the human body the enzyme, which converts the uric acid to allantoin, is not produced (Hellsten et al. 1997). Only the oxidation of uric acid by free radicals leads to the appearance of this substance in the organism. Therefore, allantoin is a marker of the intensity of free radical reactions in the body. It is sometimes considered to be a more sensitive indicator of these reactions because due to its production under both aerobic and anaerobic conditions, the concentration of this compound in the blood increases faster than that of TBARS (Mikami et al. 2000).

It has been revealed that allantoin possesses the features of a substance accelerating wound healing (Robinson 1935), regeneration of skin (Ho et al. 2006), and nerves (Loots et al. 1979). Moreover, it has anti-inflammatory properties and may alleviate the negative impact of pro-inflammatory factors, which are cytokines, secreted, among others, by leukocytes during physical activity.

Allantoin is determined in 250 μL of deproteinized serum, following the methodology developed by Grootveld and Halliwell (1987). After that, the samples are centrifuged (4000 g, 10 min, 4 °C), 250 μL of the supernatant is transferred to a

Table 11.2 Biochemical parameters of lipids and DNA degradation by ROS and other reactive species

Biochemical parameter	Reference values	References
8-iso-prostaglandin $F_{2\alpha}$ [pg mL^{-1}]	31.8±5.5	Bielecki et al. (2012)
8-hydroxy-2′-deoxyguanosine [ng mL^{-1}]	0.3–5.9 0.4–8.0	El-Zein et al. (2010) and Hamurcu et al. (2010) Kono et al. (2006) and Pan et al. (2007)

glass test tube, and 250 μL of 0.12 M NaOH is added. Next, the samples are boiled for 20 min in a water bath at 100 °C and mixed with 250 μL of 1 M HCl and 50 μL of 2,4-dinitrophenol and warmed up again for 5 min to the same temperature. The mobile phase consists of trisodium citrate and sodium acetate (pH 4.75). The measurements are performed using HPLC with UV detection at 360 nm. The standard curve is created from subsequent dilutions of the allantoin solution. Allantoin concentration is expressed in mmol L^{-1} of serum.

Oxidative Damage of Proteins and Amino Acids

Protein oxidation is both a part of cellular regulatory mechanisms and a consequence of damage to tissues as proteins are major targets for radicals, two-electron oxidants, and glycation reactions. Protein oxidation is believed to play a crucial role in the pathophysiology of diseases (atherosclerosis, neurodegenerative disorders, diabetes, pulmonary fibrosis, end-stage renal disease) and the aging process (Vivekanandan-Giri et al. 2011; Weber et al. 2015).

Oxidative damage to proteins may lead to the modification of sulfur-containing aromatic and aliphatic amino acids. Various reactive oxygen species present in blood, tissues, and cells generate protein carbonyl groups, which are used as a marker of global protein oxidation. Protein carbonyls are an irreversible and relatively stable form of protein modification. As a result of the impact of different oxidants on amino acids, both protein-bound and released carbonyl groups are produced.

The standard methods for determining protein carbonyls in biological samples (plasma, cellular extracts, erythrocytes, or isolated proteins) are based on the derivatization of the carbonyl group, most often with 2,4-dinitrophenylhydrazine (2,4-DNPH) to obtain the stable 2,4-dinitrophenylhydrazone (2,4-DNP) of an absorption maximum at 370 nm (Purdel et al. 2014). Derivatization is usually followed by a detection either by spectrophotometric methods, by an HPLC-based technique or using anti-DNP antibodies in immunoblotting or ELISA (Weber et al. 2015).

Levine et al. (1990) described methods relying on the modification of the carbonyl group (1) to alcohol with tritiated borohydride; (2) with 2,4-DNPH to form the 2,4-DNP; (3) with fluorescein thiosemicarbazide to form the thiosemicarbazone; and (4) with fluorescein amine to form a Schiff base followed by reduction to the secondary amine with cyanoborohydride. To avoid contamination of nucleic acids, they should be precipitated with streptomycin sulfate. If utilizing the borotritide or 2,4-DNPH methods, the protein should be concentrated by precipitation with trichloroacetic acid. According to Levine, the most sensitive procedure for carbonyl determination in purified proteins, homopolymers, extracts of cells and tissues is the method with tritiated borohydride, suitable for the assay of total protein content not exceeding 1 mg. This method requires less sample than that with

2,4-DNPH (Levine et al. 1990). However, nowadays, the most widespread procedure for the analysis of protein carbonyls is their derivatization with 2,4-DNPH, followed by various techniques of detection (Weber et al. 2015).

One of them can be the Western blot immunoassay. Shacter and co-workers (1994) evaluated the susceptibility of the major plasma proteins to oxidative modification based on immunoblotting. As protein oxidation leads to the formation of carbonyl groups (aldehydes and ketones) on some amino acids, these were derivatized with 2,4-DNPH, separated by sodium dodecyl sulfate-gel electrophoresis, and analyzed for carbonyl content by immunoassay with anti-DNP antibodies. This approach is very useful for measuring protein carbonyls as markers of oxidative modification in biological samples. The scientists concluded that 1 pmol of protein-associated carbonyls could be detected, and plasma fibrinogen turned out to be more susceptible to oxidative modification compared to the other major plasma proteins, albumin, immunoglobulins, and transferrin (Shacter et al. 1994).

Buss and co-workers (1997) carried out an ELISA and a colorimetric analysis for measuring protein carbonyls in plasma and lung aspirate samples to assess the oxidative injury. Protein samples were subjected to reaction with DNP and adsorbed to wells of an ELISA plate. After probing with a commercial biotinylated anti-DNP antibody, the reaction with streptavidin-linked horseradish peroxidase was performed to obtain a product for quantification. In the colorimetric assay, a proper amount of protein in phosphate buffer saline was subjected to reaction with DNP in 2 mol L^{-1} hydrochloric acid for 45 min with occasional mixing. The trichloroacid precipitates were washed three times with the solution ethanol/ethyl acetate (1:1). Pellets were sonicated, finally dissolved in 6 mol L^{-1} guanidine hydrochloride, 0.5 mol L^{-1} potassium phosphate, and the absorbance at 375 nm resulting from the presence of carbonyl groups was colorimetrically assessed. A blank with the protein was subjected to reaction with HCl without DNP and its absorbance was subtracted after adjusting for the protein loss that occurred with this method. Protein concentrations were determined for final samples (for albumin) by diluting and measuring absorbance at 280 nm or, for biological samples, by the simple colorimetric assay called BioRad (Buss et al. 1997).

The scientists compared immunohistochemistry and dihydroethidium fluorescence with mass spectroscopy in studies on amino acid oxidation markers, which served as molecular fingerprints of specific oxidative pathways. The firstly mentioned technique, despite its high sensitivity, appeared nonspecific and semiquantitative. In contrast, mass spectrometry (MS), in combination with GC, turned out to be a powerful, highly sensitive, and specific method that should be recommended for the determination of specific markers. MS detection enables identification of a target biomolecule based on its unique fragmentation pattern. The quantification is performed by adding a stable, isotopically labeled internal standard, identical to the target analyte except for the heavy isotope, which is introduced to the mixture. Due to specific ionization processes, such as electron capture negative-ion chemical ionization, even low femtomole levels of biomarkers can be determined. Additionally, the researchers confirmed the hypothesis that unique oxidants are generated in regions vulnerable to diabetic damage, and the role of antioxidant therapies seems

to be of great importance in preventing microvascular and macrovascular disease in diabetic patients (Vivekanadan-Giri et al. 2008).

Agarwal and Sohal (1995) separated protein fractions based on their molecular weight by gel filtration, and for determination of carbonyl proteins, they developed the HPLC method with diode array detection (at 370 nm). They noted that the gel filtration HPLC is more sensitive for the quantitation of oxidized purified proteins than electrophoresis but proteins of similar molecular weights cannot be separated entirely.

The Spanish scientists presented a very interesting approach with fluorescent hydrazides to the detection of carbonylated proteins (Tamarit et al. 2012). They carried out derivatization with fluorescent Bodipy-hydrazide of low molecular weight and no net charge. This enabled by two-dimensional gel electrophoresis. Bodipy means boron dipyrromethene and its analogs, whereas fluorescent Bodipy-hydrazide is a green-fluorescent dye used for the detection of carbonyl groups. Their reaction results in forming a Schiff base that can be later stabilized by reduction. Derivatization with Bodipy-hydrazide allowed easy matching of the spots of target proteins and those obtained by general fluorescent protein staining methods. Due to the cyanine hydrazide derivatization (Cy3-Hz and Cy5-Hz) analysis of two samples in the same gel was possible.

Measurement of Tyrosine Derivatives

Reactive oxygen species cause damage to proteins and amino acids. One of the structures particularly sensitive to the effects of free radicals are cross-linked two tyrosine molecules. They are present in many proteins (elastin and collagen) of vertebrates, including humans, and are sensitive to hydrogen peroxide (H_2O_2), resulting in proteolysis. Heinecke (2002) has shown that the product of the oxidative conversion of dityrosine to *o,o′*-dityrosine is 100 times higher in LDL fraction in patients with atherosclerotic symptoms compared to healthy people. Also, the hydroxyl radical increases the concentration of the modified forms of tyrosine, namely *ortho*-tyrosine and *meta*-tyrosine. These substances appear to be useful markers of protein and amino acid damage by free radicals. The concentration of these compounds is determined using a GC-MS method with isotope dilution. Research material may be: LDL isolated from blood serum (Leeuwenburgh et al. 1997) and urine (Bhattacharjee et al. 2001). Orhan et al. (2005) showed that the isotope dilution reversed-phase liquid chromatography-atmospheric pressure chemical ionization-ion-trap-tandem mass spectrometry (LC-APCI-MS/MS) with a triple quadrupole instrument is 2.5 times more sensitive than the ion-trap instrument. The concentration of *o,o′*-dityrosine in the urine of smokers is on average 0.08 ± 0.01 μmol L^{-1} or calculated as 10.1 ± 0.4 μmol mL^{-1} of creatinine.

Pennathur et al. (2001) identified amino acid oxidation markers and utilized highly sensitive and specific GC-MS in *Cynomolgus* monkeys. The animals were hyperglycemic due to streptozotocin (STZ) induced diabetes. Samples from seven

controls and eight diabetic monkeys were analyzed. *Ortho*-tyrosine, *meta*-tyrosine, and *o,o′*-dityrosine levels were higher in aortic proteins from diabetic monkeys than in those from control animals, whereas 3-nitrotyrosine levels remained unchanged. These proportions of oxidized amino acids can lead to the conclusion that a hydroxyl radical-like oxidant promotes aortic damage in hyperglycemic animals. The scientists also searched for the relationship between the level of glycemic control (measured as serum glycated hemoglobin) and levels of amino acid oxidation products in aortic tissue in control and diabetic *Cynomolgus* monkeys. They found a strong correlation between levels of both *ortho*-tyrosine and *meta*-tyrosine and glycated hemoglobin.

Homocysteine

Homocysteine is a product of methionine demethylation. The increased concentration of this amino acid is directly related to cardiovascular diseases (lifestyle diseases).

The homocysteine concentration is determined in serum or plasma using commercially available kits. About 80% of circulating homocysteine in the blood is bound to proteins. To determine the total concentration, the disulfide bridges must be reduced to release free homocysteine. Due to a specific enzyme cutting the homocysteine molecule, an intermediate product is created, which in combination with the appropriate reagent, leads to the formation of a stable fluorophore emitting far-red spectrum light (extinction = 625 nm, emission = 708 nm). The test is not influenced by the physiological concentrations of other biological thiols (such as glutathione, cysteine, and methionine) and it can be quickly adjusted (Kar et al. 2019). Also, the LC-MS method can be used to determine the homocysteine level in blood samples, even in 30 μL of a sample (Oosterink et al. 2015).

The concentration of homocysteine is usually higher in men than in women samples. Also, elevated levels were observed for the elderly people and those with a deficiency of B vitamins (B_6, B_9, B_{12}) (Oosterink et al. 2015). Reference values for this compound: 5–15 μmol L^{-1} (Maron and Loscalzo 2009).

Glutathione and Antioxidant Enzymes

Glutathione Analysis

Glutathione is a tripeptide built of three amino acids: glutamic acid, cysteine, and glycine. A unique role is played by cysteine, containing a thiol group (-SH), which has strong reducing properties. Therefore, glutathione has a high antioxidant potential, acting as a so-called free radical scavenger. This compound and antioxidant

enzymes arise in significant amounts during physical exercise, especially along with endurance characteristics.

Glutathione removes from erythrocytes hydrogen peroxide, which is a natural product formed during its metabolism. H_2O_2 significantly reduces the survival of red blood cells (erythrocytes) from 90 to 120 days and increases the rate of oxidation of hemoglobin to methemoglobin (metHgb). MetHgb loses the ability for oxygen transport, which reduces the physical performance of the athlete. NADPH (nicotinamide adenine dinucleotide phosphate – reduced form), which is formed in the pentose phosphate pathway, causes a reduction of glutathione, that is its re-activation (Fig. 11.3). Glutathione is also able to neutralize singlet oxygen ($^1O_2$) and other electrophile molecules (Lafleur et al. 1994).

Both glutathione and antioxidant enzymes are currently determined by ELISA. Most often, antioxidant enzyme activity is determined in erythrocyte hemolysate. The method is based on the isolation of erythrocytes by washing the red blood cells three times with saline in a 1:1 ratio. After each addition of physiological saline and thorough (gently) mixing, the sample is centrifuged, and the supernatant removed. Red blood cells are then frozen to induce hemolysis. GSH concentration is calculated per 1 g of hemoglobin. The concentration of hemoglobin is measured using a hematological analyzer.

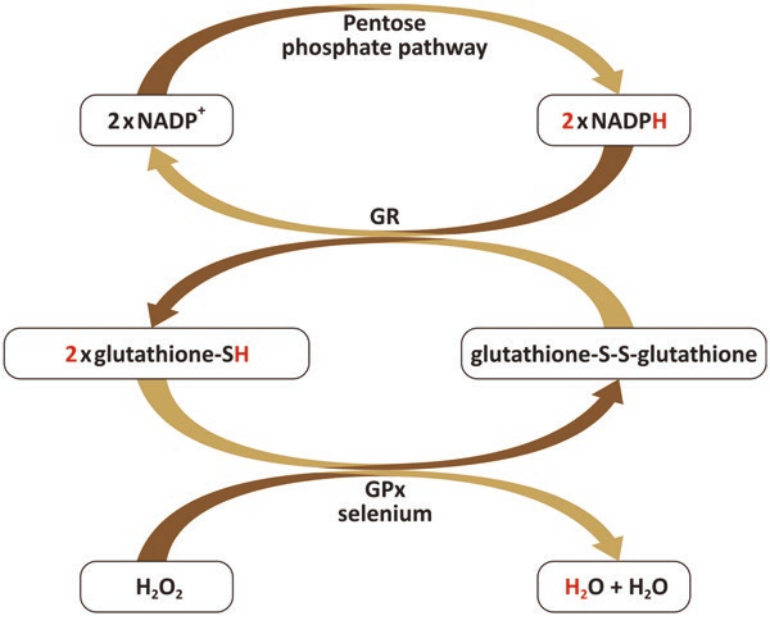

Fig. 11.3 Neutralization of hydrogen peroxide (H_2O_2) to the neutral molecule of water (H_2O) by reduced glutathione (glutathione-SH), which has previously been reduced by NADPH formed in the pentose phosphate pathway. The hydrogen atom, disconnected from NADPH, reduces glutathione, which in turn transfers it to H_2O_2 and transforms it into two water-neutral molecules

The concentration of the reduced and oxidized forms of glutathione in blood hemolysate presented in the literature:

- GSH – 8342 ± 769 nmol per gram Hb; glutathione disulfide (GSSG) – 23.3 ± 5.06 nmol per gram Hb (Rossi et al. 2002),
- GSH – 8460 ± 1750 nmol per gram Hb; GSSG – 13.2 ± 4.0 nmol per gram Hb (Khazim et al. 2013).

Also, HPLC and spectrophotometric techniques are used to determine glutathione and glutathione disulfide concentrations in blood and other tissues. Giustarini et al. (2013) have compared these two methods. The GSH recycling assay is a specific method with minimal or without interference from other thiols and disulfides for the determination of total GSH, and the limit of detection is 0.1 μmol L^{-1} in most tissues. GSH measurement was carried out using HPLC with UV detection at 265 nm in supernatants obtained from NEM-treated (N-ethylmaleimide) blood after acidification. For the preparation of human or animal blood samples, tripotassium ethylenediaminetetraacetic (EDTA) acid is needed and 100 μL of NEM per mL of blood.

Antioxidant enzymes neutralize two toxic reactive oxygen species, that is the superoxide radical and hydrogen peroxide to a water molecule.

Superoxide dismutase in humans (SOD; E.C. 1.15.1.1) is an enzyme that has a coenzyme in the form of a copper and zinc metal atom (SOD-1 and SOD-3) or manganese (SOD-2). This enzyme is involved in the utilization of the superoxide radical ($O_2^{\bullet -}$). In this way, it protects intracellular structures from oxidation and the generation of other free radicals, for example, nitric oxide ($NO^{\bullet}$). Liver cells show the highest SOD activity.

$$Cu^{2+} - SOD + O_2^- \rightarrow Cu^+ - SOD + O_2$$

$$Cu^+ - SOD + O_2^- + 2H^+ \rightarrow Cu^{2+} - SOD + H_2O_2$$

SOD triggers the production of hydrogen peroxide, which in turn is deactivated by the catalase enzyme (CAT; EC 1.11.1.6). It is an enzyme that contains iron (Fe^{3+}) in its structure. Its high activity is observed in the cytosol, peroxisomes, and erythrocytes.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Hydrogen peroxide (H_2O_2) is neutralized by another enzyme – glutathione peroxidase (GPx; EC 1.11.1.9). Also, it can reduce lipid radicals to alcohols. GPx has eight isoenzymes that contain a selenium atom (Fig. 11.4).

$$2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$$

Currently, antioxidant enzymes are determined using commercial 96-well plate kits basing on the ELISA method. As mentioned earlier, erythrocyte hemolysate is most often analyzed, although it is possible to determine the analytes in blood serum

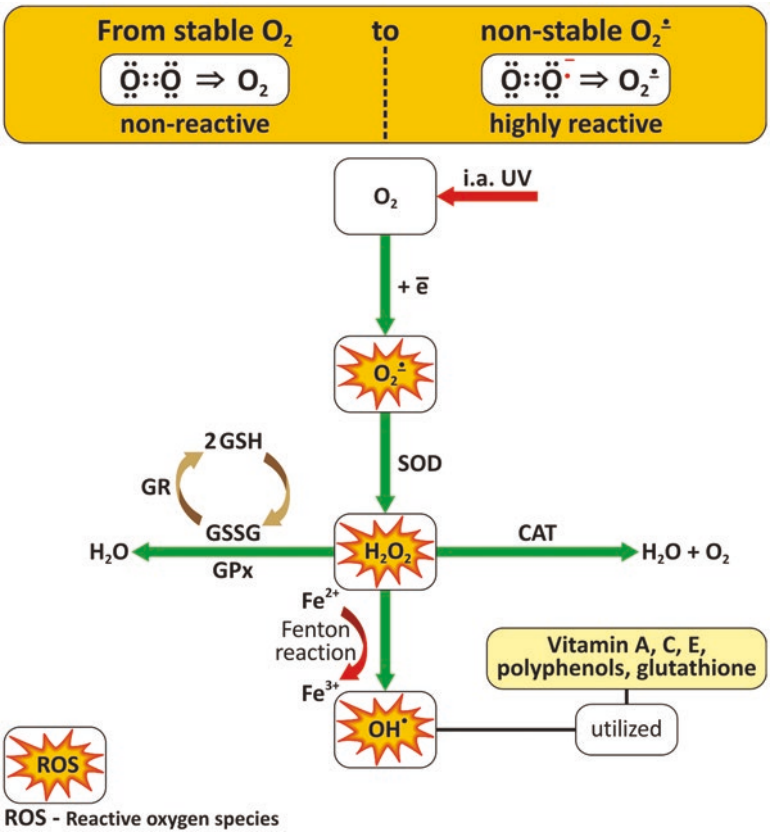

Fig. 11.4 The relationship between antioxidant enzymes in the utilization of free radicals. (SOD superoxide dismutase, GSH glutathione, GSSG glutathione disulfide, CAT catalase, GPx glutathione peroxidase, and GR reduced glutathione)

Table 11.3 The activity of antioxidant enzymes in human erythrocytes and plasma

	Enzyme activity	
Enzyme	Erythrocytes (U 10^{10} mL^{-1})	Plasma (U L^{-1})
Glutathione peroxidase	7.8–10.6	0.4
Glutathione reductase	2.7–3.7	0.03
Superoxide dismutase	550–800	5–20
Catalase	3800–5400	46–52

or tissue biopsies, for example, liver. Weydert and Cullen (2010) also demonstrated the method for determining the above mentioned enzymes in tissues and cell cultures using active gels. It takes 24–48 hours to carry out this type of determination. The activity of antioxidant enzymes in human erythrocytes and plasma can be found in Table 11.3.

Non-enzymatic Markers of Antioxidant Capacity Assessment and Antioxidants

Total Phenolic Compounds and Determination of Phenolic Compounds and Antioxidant Vitamins with the Use of Modern Techniques

Phenolic compounds (mainly flavonoids and phenolic acids) are the group of over 8000 plant origin substances. They also belong to natural plant dyes, the role of which is to protect against UV radiation, insects, and parasites. Their concentration in blood is an effect of proper nutrition and dietary supply of these compounds (Wiseman 1999). It has been revealed that a higher concentration of phenolic compounds in the blood protects the organism from civilization diseases, especially atherosclerosis and some cancers. Particularly symptomatic are the results of studies carried out in France, where the habit of drinking red wine for meals, despite a high-fat diet, leads to a lower number of deaths caused by cardiovascular disorders compared to other populations of European countries. Therefore, the phenomenon is named the French Paradox (Ferrières 2004).

Phenolic compounds possess potent antioxidant properties, often of greater strength than vitamins C, A, or E. The proper levels of these substances in human organisms indirectly protect antioxidant vitamins from oxidation. Phenolics, especially from fruit and vegetables, turned out to modulate the cellular concentration of natural antioxidants, as well as glutathione synthesized in the organism through gene regulation (Moskaug et al. 2005).

During physical exercises, there may be an increase in the production of free radicals, particularly of aerobic origin (ROS), which results in oxidative stress. Polyphenols are therefore considered to be one of the essential exogenous substances that protect the body from the negative effects of this condition in sports practice. It is known from the literature that high concentrations of antioxidants in the body prevent damage and premature muscle fatigue. However, a clear interpretation of this issue is contrary to research results, which indicate an increase in the total antioxidant potential during a long-term exercise – marathon (Liu et al. 1999) or half-marathon (Child et al. 1998), while moderate exercises have not led to its changes so far (Karolkiewicz et al. 2002).

For these compounds, various methods were used (for the determination of total phenolics – spectrophotometric method, and high-performance liquid chromatography (HPLC), or LC-MS/MS where the selectivity was needed). In the routine work, the method should be easy, repeatable, and when it is possible inexpensive. Phenolics are mainly detected in UV/VIS and UV-fluorescence regions. Besides, electrochemical coulometric array detection, on-line connected photodiode-array, and electroarray detection, chemical reaction detection techniques, mass spectrometric, and nuclear magnetic resonance (NMR) detection are used. It is known that phenol compounds with the intrinsic existence of conjugated double and aromatic bonds

exhibit a higher or lower absorption in the UV or UV/VIS region. For benzoic acids, the maximum is in the 200–290 nm range, excluding gentisic acid, which extends the absorbance to 355 nm. The cinnamate carbon framework derivatives, because of the additional conjugation, show a wide-ranging absorbance band from 270 to 360 nm. In the case of flavonoid aglycones, they possess at least one aromatic ring and, consequently, efficiently absorb UV light. There are two maxima, and the first is found in the 240–285 nm range due to the A-ring. The second maximum in the range: 300–550 nm is attributed to the substitution pattern and conjugation of the C-ring (Mabry et al. 1970).

Determination of phenolic compounds (also named as total polyphenols) in plasma (after preparation) is based on a method developed by Singleton and Rossi (1965), which uses the ability to oxidize phenolic groups by the Folin-Ciocalteu reagent. The resulting substances are complexed to form a blue compound. The color of the solution is measured using a spectrophotometer or a multimode microplate reader at $\lambda = 765$ nm. The standard curve is created from standard solutions of gallic acid. Total polyphenols concentration is expressed as gallic acid equivalent in g L^{-1} of plasma.

Some substances present in biological material (blood, urine, and saliva) may affect the polyphenol concentration obtained by the Folin-Ciocalteu method. To eliminate protein interference, mainly aromatic amino acids (tyrosine and tryptophan) (Sánchez-Rangel et al. 2013), deproteinization of the sample should be performed by protein precipitation, for example, with methanol. For this purpose, the serum is mixed with 80% methanol (1:1) and then centrifuged for 5 min at 14,000 g to separate proteins from a residual fluid (Lee et al. 2017). Also, the effect of uric acid can be eliminated from the sample, especially in people consuming products with a high content of polyphenols, for example, apple juice. Uric acid reacts with the Folin-Ciocalteu reagent, overstating the result of polyphenols content in the blood (Godycki-Cwirko et al. 2010).

When determining the concentration of polyphenols in urine, compounds dissolved in water should be removed (Singleton and Rossi 1965). For this purpose, the urine is acidified and then applied to the active cartridge for the extraction of biological material. The resulting filtrate containing polyphenols is eluted with methanol and formic acid solution (Roura et al. 2006).

The extraction and isolation methods of phenolics start with the protein precipitation with acetonitrile (Miniati 2007). In the chlorogenic acid analysis, liquid-liquid extraction (LLE) for preparing the blood and/or urine samples was also used (Han et al. 2020; Zhang et al. 2010). An internal standard (puerarin or tinidazole) was added with hydrochloric acid, methanol, and ethyl acetate. After vortexing and centrifugation, the clear supernatant was separated and evaporated to dryness under a gentle stream of nitrogen at room temperature. Then it was reconstituted with 100 μL of 0.5% formic acid in 50% methanol/water (with centrifugation 12,000 rpm in 5 min). Finally, an aliquot of 2 μL was injected into the UPLC-MS/MS system.

Another novel method was developed to analyze the markers of green coffee bean extract consumption (rich in chlorogenic acids) in the urine of healthy volunteers (Peron et al. 2018). Urine samples were centrifuged at 13,000 rpm for 10 min

and then 1 μL was directly injected into the UPLC-QTOF with C18 stationary phase. For better separation of the polar and smaller molecules, the researchers used LC-ESI-MS and C-3 stationary phase. They monitored the metabolites related to polyphenol administration such as hippuric acid, benzoic acid derivatives, dihydroferulic and dihydrosinapic acid sulfate, as well as carnitine derivatives and dicarboxylic acids. Stalmach et al. (2009) revealed that the usage of LC-PDA-MS^n to identify and quantify total 21 metabolites circulating in the bloodstream and being excreted in urine after the acute consumption of coffee by healthy human subjects was efficient to show the bioavailability and metabolic fate of dietary phenolics and their nutritional value. Other markers of coffee and other product consumption with the techniques of analysis can be found in Table 11.4.

Yang et al. (2019) proposed a method for the determination of 22 urinary polyphenol biomarkers with the use of LC-ESI-MS/MS. The urine samples were extracted and purified with Plexa PCX (polymeric cation-exchange resin) solid extraction cartridges, and 10 μL of the reconstituted solution was injected into LC with the QTRAP tandem mass spectrometer (triple quadrupole linear ion trap spectrometer). In another study of Yang et al. (2013), plasma with internal standard (ginsenoside Rc) and acetic acid was vortexed and centrifuged at 12,000 g for 5 min at 4 °C, and then the solution was applied to the SPE (solid phase extraction) cartridge (SEP-PAK C_{18}, 100 mg). The 5 μL of residues after methanol eluate evaporation was injected into LC-MS. The chlorogenic acid, kaempferol-7-*O*-β-d-glucoside, and ilexgenin A were determined after oral administration of *Ilex hainanensis* leaves extract in normal and non-alcoholic fatty liver disease (NAFLD) rats.

It was also revealed that after oral administration of the *Flos Lonicera Japonica* extract in rats, 68 CGA (chlorogenic acids) metabolites were found (Wang et al. 2018). UHPLC-LTQ-Orbitrap (LTQ – linear trap quadrupole) MS was used to identify the major-to-trace in vivo metabolites of CGAs in the urine and plasma samples of Sprague–Dawley rats. This method showed the profile of CGA metabolites, which can be useful in understanding the in vivo metabolic fate, effective forms, both pharmacological and toxic actions of CGA.

For the biological samples, the proper quality control has been used (Yang et al. 2013; Yang et al. 2019). Apart from linearity, plasma calibration curves, precision and accuracy, recoveries, the stability of samples have also been investigated at different concentrations.

Vitamin C Analysis

Vitamin C (L-ascorbic acid) is a water-soluble vitamin present mainly in fruit and vegetables. This vitamin is not absorbed in the human organism, and therefore it should be ingested with the diet. As an antioxidant, it protects neurons from oxidative damage also by restoring the reduced form of vitamin E (Grunewald 1993; Niki 1991). The ascorbic acid is essential for collagen formation, necessary as a cofactor in the biosynthesis of catecholamines, amino acids, and peptide hormones, in the

Table 11.4 Determination of selected markers of chlorogenic acids and other phenolic acids from food consumption in healthy adults plasma and/or urine samples

Analytes (after food consumption)	Samples of healthy adults (amount)	Sample pretreatment and extraction	Stationary phase and mobile phase	Technique	Reference
Caffeic, ferulic, and chlorogenic acids (after 100 g prune consumption)	Human plasma, urine (3)	Incubated in sulfatase and glucuronidase; acidified with H_3PO_4, extracted with methylene chloride, vortexed, centrifuged. (urine) mixed with sodium acetate buffer (pH 5.5) and $CaCl_2$ solution; incubated in a manner similar to that of the plasma samples; acidified with 6 mol L^{-1} HCl; extracted with ethyl acetate, vortexed, and centrifuged	Hamilton PRP-1 column/ A: 1% acetic acid in water; B: ACN; gradient: 20% B, 4 min; 50% B, 6 min, 100% B, 15 min	LC-ESI-MS	Cremin et al. (2001)
3-,4-, and 5-CQA, 3-diCQA, and caffeic, ferulic, isoferulic, and *p*-coumaric acids in plasma; 4-CQA, 5-CQA, and sinapic, *p*-hydroxybenzoic, gallic, vanillic, dihydrocaffeic, caffeic, ferulic, isoferulic, and *p*-coumaric acids in urine (after hydroalcoholic decaffeinated green coffee extract from *C. canephora* cv. Pierre beans 170 mg of CGA)	Human plasma, urine (10)	Plasma and urine aliquots + HCl	Magic C30 HPLC column (5 μm, 150 × 20 mm, 100 Å)/aqueous solution of 0.3% formic acid, and methanol	HPLC–DAD (confirmation by LC-MS)	Farah et al. (2008)

Analytes (after food consumption)	Samples of healthy adults (amount)	Sample pretreatment and extraction	Stationary phase and mobile phase	Technique	Reference
Chlorogenic acids and metabolites Six CGA (3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) and 16 other phenolic compounds: caffeic, vanillic, ferulic, isoferulic, *p*-coumaric, gallic, 4-hydroxybenzoic, dihydrocaffeic, syringic, sinapic, 2,4-dihydroxybenzoic, hippuric, 3,4-dihydroxyphenylacetic, 3-(4′-hydroxyphenyl)propionic, trans-3-hydroxycinnamic, and benzoic acids (after consumption of water, coffee, or coffee with milk)	Human urine (5)	Helix pomatia (Sigma-Aldrich) extract containing β-glucuronidase and sulfatase activities for deconjugation of glucuronic acid conjugates and sulfated forms	C18 Kromasil guard column and column/a gradient with methanol and 0.3% formic acid	HPLC–DAD (confirmation by LC-MS)	Duarte and Farah (2011)

(continued)

Table 11.4 (continued)

Analytes (after food consumption)	Samples of healthy adults (amount)	Sample pretreatment and extraction	Stationary phase and mobile phase	Technique	Reference
3-Caffeoylquinic acid lactone-O-sulfate 4-Caffeoylquinic acid lactone-O-sulfate Caffeic acid-3′-O-sulfate 3-Feruloylquinic acid 4-Feruloylquinic acid 5-Feruloylquinic acid 5-Caffeoylquinic acid Ferulic acid-4′-O-sulfate Dihydrocaffeic acid Dihydrocaffeic acid-3′-O-sulfate Dihydroferulic acid Dihydroferulic acid-4′-O-sulfate (after instant coffee consumption)	Human plasma (11)	Plasma (450 μL), acidified with 13.5 μL of 50% aqueous formic acid, was added drop-wise to an 1125 μL of acetonitrile to which had been added 50 μL of 10% (m/v) ascorbic acid containing 0.5 μmol L^{-1} EDTA, (and 1 μg of sinapic acid as an internal standard). Samples were vortexed for 30 s every 2 min over a 10 min and centrifuged at 1500 g for 20 min at 4 °C. The supernatant was decanted and the pellet re-extracted with 1125 μL of methanol and after centrifugation the two supernatants were combined and reduced to dryness under a stream of nitrogen at 35 °C. The dried samples were then re-suspended in 250 μL of 0.1% aqueous formic acid containing 10% methanol, and centrifuged at 4 °C for 20 min at 16,110 g in a 0.2 μm filter	SYNERGI POLAR-RP (4 μm, 4.6 mm × 250 mm)/5–16% gradient of acetonitrile in 0.5% aqueous acetic acid	LC-PDA-MS^n	Stalmach et al. (2014)

Analytes (after food consumption)	Samples of healthy adults (amount)	Sample pretreatment and extraction	Stationary phase and mobile phase	Technique	Reference
Chlorogenic acids and other metabolites: caffeic acid, ferulic acid, isoferulic acid, gallic acid, dihydrocaffeic acid, vanillic acid, benzoic acid, *p*-hydroxybenzoic acid, syringic acid, sinapic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, trans-3-hydroxycinnamic acid, 3-(4-hydroxyphenyl) propionic acid and 2,4-dihydroxybenzoic acid (after consumption of soy, coffee, and beverages)	Human urine (6)	Helix pomatia (Sigma-Aldrich) extract containing β-glucuronidase and sulfatase activities for deconjugation of glucuronic acid conjugates and sulfated forms. The pH 5.0 and in water bath at 37 °C for 2 hours. Than acidified with HCl and methanol aqueous solution (40%, v:v) was added with subsequent vortexing. Eppendorf tube with a cellulose filter (Microcon YM-10, Millipore) and centrifuged at 17,000 g, for 30 min, at 4 °C	C18 Kromasil column (5 μm, 4.6 mm × 250 mm)/ gradient with 0.3% formic acid solution and methanol	HPLC–DAD (confirmation by LC-MS)	Felberg et al. (2015)
Benzoic acid, hippuric acid, 4-hydroxybenzoic acid, quinic acid and dihydrocaffeic acid sulfate (after 400 mg of dried green coffee bean extract consumption [GCBE, 9% w/w CGA and 0.5% w/w caffeine] each day for a month)	Human urine (10)	Urine was centrifuged at 5000 rpm for 10 min	Zorbax Rapid Resolution High Definition (RRHD) SB-C18 column (1.8 μm, 2.1 mm × 50 mm)/solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 0.1% formic acid)	UPLC-QTOF (LC-ESI-MS with C-3 stationary phase)	Peron et al. (2018)

prevention of bleeding and for reduction of toxic effects of most xenobiotics (Daud et al. 2016; Uchendu et al. 2012). There is some evidence that this vitamin may be a useful biomarker in overall health and nutritional status, especially in regular measurements (Chung et al. 2001; Szeto et al. 2004). The levels of ascorbic acid in serum or plasma are affected by dietary intake, age, and gender as well as circadian rhythm, and therefore leukocyte and lymphocyte vitamin C levels are also analyzed (Emadi-Konjin et al. 2005). Nowadays, this biomarker shows the antioxidant activity in cancer prevention, also antioxidant/anti-inflammatory, antihypertensive properties, and lipid-lowering effects in cardiovascular diseases and antioxidant activities in cataract and ocular diseases (Daud et al. 2016; Szeto et al. 2004). It also possesses antioxidant activity in critically ill patients with sepsis. However, in high doses, it demonstrates prooxidant activities, the ability to generate reactive oxygen species, cytotoxicity, also in stimulating apoptotic way of the tumor cells (Carr and Frei 1999). The study based on the European Prospective Investigation into Cancer and Nutrition (EPIC) showed that higher concentration of vitamin C in plasma, but not the level of dietary vitamin C intake assessed by country-specific questionnaires, is linked to decreasing risk of gastric cancer (Jenab et al. 2006, 2009). It has been lately revealed that ascorbic acid in proper concentration and as supportive intervention could be used in oncologic care (Klimant et al. 2018).

After a recent systematic review of literature, researchers have found that cancer risk prevention consequent to the reduction of oxidative damage has been observed in some cases at low doses of retinol palmitate – a derivative of vitamin A and/or vitamin C in contrary to its higher doses (de Carvalho Melo-Cavalcante et al. 2019).

In terms of the determination of vitamin C due to its susceptibility to the environmental conditions, sample collection and stabilization have to be adequately performed. There are protocols that include the precipitation of proteins, usually with meta-phosphoric or trichloroacetic acid, depending on the method used (Salminen and Alfthan 2008; Vuilleumier and Keck 1989). Chung et al. (2001) proposed heparin as an anticoagulant apart from meta-phosphoric acid and then ascorbic acid, which is stable for up to 10 days at −70 °C.

The best option for the determination of ascorbic acid in plasma is HPLC with ultraviolet or electrochemical detection and mass spectrometry as well (Emadi-Konjin et al. 2005; Ross 1994). For lymphocyte separation and lymphocyte vitamin C extractions, these processes should be completed no later than 3 hours after blood drawing. The whole lymphocyte preparation/extraction process is prepared on ice, the lysate is protected from light and then the lymphocyte vitamin C extracts are stored at −80 °C before HPLC analysis (Emadi-Konjin et al. 2005).

The problem in the ascorbic acid analysis in serum consists mainly in DHAA (dehydroascorbic acid) reconversion to ascorbic acid, and it may lead to incorrect results (Margolis and Duewer 1996). In HPLC-UV methods, dithioerythritol (DTE) and 1,4-dithiothreitol (DTT) are used to determine total ascorbic acid in samples, but they are not used in clinical settings due to the unknown contribution of DHAA in patients samples (Card 2019). Therefore, the dual-wavelength UV detector can be employed (Washko et al. 1992). The injection volume should be 20–50 μL, and the samples in the autosampler should be protected from light and refrigerated to

4 °C. Typical C18 columns, 5 μm, and 250 × 4.6 mm, with methanol or acetonitrile in acidic pH, are used.

The FRASC method is the modification of the ferric reducing antioxidant power (FRAP) assay, which allows determining ascorbic acid at the same time as the assay (Benzie and Strain 1996; Benzie and Strain 1999). This method has also been validated and compared with the reference HPLC method (Chung et al. 2001). The addition of enzyme – ascorbate oxidase – is necessary.

Another example of methods used to quantify ascorbic acid in plasma is its oxidation with Cu^{2+} to a dehydroascorbic acid that reacts with acidic 2,4-dinitrophenylhydrazine to form a red bis-hydrazone. This color compound is measured spectrophotometrically with the use of a 2,4-dinitrophenylhydrazine method. There are modern spectrophotometry methods used with microvolume amounts at 265 nm (Witmer et al. 2016). In the spectrophotometric techniques, which are relatively cheaper, the results could be higher than in HPLC procedures because of lower selectivity. The normal levels of vitamins A, C, D, and E can be found in Table 11.5.

Simultaneous Determination of vitamin A, Carotenoids, Vitamin D, and Vitamin E

Vitamin A refers mainly to retinol in the organism but retinal, retinoic acid, and retinyl acetate are also known as retinoids (Blomhoff and Blomhoff 2006). The role of this vitamin in the organism is to participate in vision and many different aspects of mammalian physiology, including embryonic development, growth and development, immunity, and the maintenance of epithelial barriers (Dawson 2000). The retinol in the blood is linked to the specific retinol-binding protein (RBP), important for the determination of this vitamin. Its provitamins are specific carotenoids also considered as potent antioxidants in food and then in the human organism. In the case of vitamin E, α-tocopherol is another lipid-soluble antioxidant that is found in all cellular membranes and protects cells from lipid peroxidation (Sies and Stahl 1995). What is essential, the chain-breaking antioxidant prevents the chain initiation and propagation of free radical reaction and lipid peroxidation in cellular membranes. It also influences the cellular response to oxidative stress through the modulation of the signal-transduction pathway (Azzi et al. 1992; Kamal-Eldin and

Table 11.5 Normal levels of selected vitamins

Vitamin level	Plasma/serum	Leukocyte
Vitamin A	>70 μmol L^{-1}	
Vitamin C	23–85 μmol L^{-1} (5–15 mg L^{-1})	8.73 ± 4.13 μg per 10^8 cells
Vitamin D	50–75 nmol L^{-1}	
Vitamin E	>16.2 μmol L^{-1}	

Appelqvist 1996). Nevertheless, high doses (greater than or equal to 400 IU daily) of vitamin E supplements may increase the mortality (Miller et al. 2005).

It is worth mentioning that healthy humans have no detectable quantities of retinol in urine samples and it can be determined in patients with kidney pathologies and diabetes; a higher level of vitamin E in poorly controlled diabetic children was revealed, and it could also be a marker of oxidative stress (Gavrilov et al. 2006, 2012). What is more, the increased levels of lipid-soluble antioxidant vitamins in plasma could be beneficial to treat childhood obesity (Guerendiain et al. 2017).

The preparation of biological fluid samples is the most crucial step in analyzing fat-soluble vitamins and antioxidants. The blood can be extracted and left to spontaneous coagulation, centrifuged at 3000 g for 10 min (Rodríguez-Delgado et al. 2002). The previous methods were not "green," and chemicals were also expensive (Albahrani et al. 2016; Guerendiain et al. 2017) with the use of hexane and dichloromethane in methanol to prepare the samples. Lazzarino et al. (2017) have described the single-step, efficient, simple, rapid, and low-cost method to extract the following fat-soluble vitamins and antioxidants: all-trans-retinoic acid, all-trans-retinol, vitamin D: 25-hydroxycholecalciferol, carotenoids: astaxanthin, lutein, zeaxanthin, trans-β-apo-8′-carotenal, β-cryptoxanthin, phylloquinone, lycopene, α-carotene, β-carotene, vitamin E: α-tocopherol, γ-tocopherol, and coenzyme Q_{10} in biological fluids (serum, plasma, seminal plasma, seminal fluid, urine, cerebrospinal fluid, saliva, and synovial fluid). 250 μL of serum or seminal plasma sample was mixed with 500 μL of acetonitrile for 60 s, incubated at 37 °C for 1 hour in a water bath under agitation (extraction of lipid-soluble compounds); centrifuged at 20,690 g for 15 min at 4 °C to precipitate proteins. During every procedure, samples must be protected from light to avoid degradation of photo-sensitive compounds. Also, the SPE method for 100 μL of the sample has been recently developed (Wills et al. 2019).

The determination method of retinol and α-tocopherol should be fast, selective, and economical in routine use. HPLC is usually used to determine these vitamins (and also other compounds such as carotenoids) in few minutes with methanol on the C18 columns (Guerendiain et al. 2017; Khan et al. 2010; Rodríguez-Delgado et al. 2002) as well as methanol and acetonitrile (Lazzarino et al. 2017). Fluorometric, electrochemical, and MS detectors are also used. Detection of the vitamins is carried out at 292 nm for α-tocopherol and 325 nm for retinol. Standards are dissolved in a mixture of hexane–dichloromethane or dichloromethane and methanol with the addition of butylated hydroxytoluene (BHT) to protect vitamins from oxidation processes.

In 2004 the automated HPLC method not only for vitamins A and E but also for vitamin D (24,25-$(OH)_2$ Vitamin D_3 and 25-(OH) Vitamin D_3) determination was described (Quesada et al. 2004).

The validation of methods is also necessary. Mata-Granados et al. (2008) have used standard reference material (SRM 968c) which provides certified concentration values for all-trans retinol (vitamin A), α-tocopherol (vitamin E) and reference concentration values for 25-hydroxyvitamin D_3. They have also found a low level of vitamin D and a high level of vitamin A in the serum of healthy adults, which

reflects a problem for the public health of chronic pathologies. The high concentration of vitamin E in the Spanish population may be used as a cardiovascular risk marker.

Bilirubin

Bilirubin is the product of the degradation of heme, the hemoglobin component in erythrocytes. Bilirubin concentration indicates indirectly the quality of liver function, which is responsible for its metabolizing. Some people, mostly men, have increased bilirubin levels. In the disorder, called Gilbert's syndrome, there is an impaired conjugation of this metabolite in the liver. Gilbert's syndrome is most often asymptomatic, and yellowing of the skin or conjunctival layers of the eye can be observed during the consumption of large amounts of alcohol, fatigue, or intense exercise. The study conducted by Floreani et al. (1993) has not indicated any influence of elevated blood bilirubin concentration on sports performance. Due to the weak solubility of bilirubin in water, it is bound in blood with glucuronate or possibly albumin. It protects water spaces as well as cell membranes. It is also responsible for the protection from the peroxidation of unsaturated fatty acids, including linoleic and linolenic acids (Stocker et al. 1987). As an antioxidant, it also participates in singlet oxygen ($^1O_2$) neutralization.

Due to the light sensitivity of bilirubin, blood samples should not be exposed to sunlight. This may result in false under-estimation. In serum, bilirubin bound (conjugated) can be calorimetrically determined with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde; DMAB). Preparation of the reagent is the following: 1 g of DMAB is dissolved in 50 mL of 95% ethanol and 50 mL of concentrated HCl. The absorbance of samples is measured at 650 nm wavelength (Suzuki 1997). After the addition of methanol to the serum sample, free bilirubin is bound so that the concentration of total bilirubin can be determined. The concentration of free bilirubin can be estimated by calculating the difference between total and bound bilirubin.

In the whole heparinized blood, it is possible to determine the concentration of bilirubin by reading absorption at two wavelengths, 455 and 575 nm. This method can be used, among others, in gasometric analyzers (Grohmann et al. 2006).

Bilirubin present in the blood occurs in four fractions (α, β, γ, and δ). The concentration of individual fractions is determined by using HPLC (Table 11.6).

Table 11.6 The analysis of bilirubin fractions

Fraction	Bilirubin form	Wavelength (nm)
α (Bα or Bu)	Unconjugated	459
β (BMG)	Monoglucuronide	422
γ (BDG)	Diglucuronide	422
δ (Bδ)	Albumin-bound bilirubin	433

Also, Adachi et al. (1988) divided bilirubin present in the blood serum of people with hyperbilirubinemia into five fractions, additionally secreting the β′ fraction. The authors fractionated serum bilirubin by newly developed HPLC method.

A healthy person's urine does not contain bilirubin (below limit of detection, <LOD).

Total Antioxidant Capacity (TAC)

The antioxidant capacity is the term defined as "the moles of oxidants neutralized by one liter of body fluids; the ability of chemical compounds to prevent or delay the oxidation of various substances, such as lipids, proteins or DNA, necessary for the proper functioning of the body" (Lettieri-Barbato et al. 2013; Serafini et al. 2006). Delayed oxidation or antioxidation occurs in living organisms (plants and animals), but also in food products.

Plasma non-enzymatic antioxidants include endogenous substances (e.g., uric acid, bilirubin, and thiols) and nutritional/exogenous substances (e.g., tocopherols, ascorbic acid, carotenoids, and phenolic compounds) which cannot be determined directly because this is an activity of all compounds found in the biological fluid (Lettieri-Barbato et al. 2013; Serafini et al. 2006). TAC concerns "the cumulative action of all the antioxidants present in the matrix (plasma, saliva, food extracts, tissues, etc.), providing an integrated parameter rather than the simple sum of measurable antioxidants and giving an insight into the assessment of the antioxidant network" (Serafini et al. 2006). This value consists of the sum of concentrations of vitamins C, E, and A, trace elements, for example, selenium, proteins with thiol groups (–SH), bilirubin, metal ions affecting the antioxidant potential (Fe^{2+} and Cu^{2+}), and compounds of plant origin including polyphenols (Rice-Evans and Miller 1994). The in vivo antioxidant level has been found essential for a health condition, therefore measuring antioxidant capacity in biological fluids can enable the prediction of not only nutritional health condition but also the diseases. It is worth mentioning that the TAC parameter does not reflect the full capacity of the organism to deal with oxidative stress but it helps to compare the results of different biological samples (from patients, healthy subjects, etc.) (Bartosz 2010).

Some methods have been incorporated from food to biological fluids and/or vice versa, especially ABTS, DPPH, and FRAP assays are commonly used to measure antioxidant activity of food and beverages (Cano and Arnao 2018; Jeszka-Skowron and Zgoła-Grześkowiak 2017). These easy and fast spectrophotometric methods are based on the radical-scavenging activity (stable radical scavenger) and redox potential of antioxidants. Among other methods, ORAC (Oxygen Radical Absorbance Capacity) is a procedure based on the hydrogen atom transfer (HAT) in contrast to the single-electron transfer SET-based methods (ABTS, DPPH, or FRAP) (Ialongo 2017).

The preparation of plasma could involve isolation from ethylenediaminetetraacetic acid-treated blood samples by centrifugation at 3000 g for 20 min at 4 °C (Fairus

et al. 2019). Plasma samples can also be diluted with methanol and centrifuged for 5 min at 14,000 g to remove precipitated proteins (Lee et al. 2017).

Trolox equivalent antioxidant capacity (TEAC) assay with the use of stable cation radical ABTS was firstly reported by Miller et al. (1993). Trolox is a structural hydrosoluble analog of vitamin E (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and it is used as a standard for calibration curve not only in ABTS but also in DPPH or FRAP assays. Other standards, such as vitamin C, uric acid, or glutathione, are also used (Bartosz 2010; Janaszewska and Bartosz 2002; Lee et al. 2017). Reference values of TBARS, allantoin, total polyphenols, bilirubin, and antioxidant status parameters in human blood are presented in Table 11.7.

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) Assay

A blue/green $ABTS^{\bullet+}$ chromophore is generated in the chemical reaction between ABTS and potassium persulfate. This compound possesses absorption maxima at wavelengths 645 nm, 734 nm, and 815 nm. When it is mixed with antioxidants, the pre-formed radical cation is reduced (Re et al. 1999). The reduction of the radical cation $ABTS^{\bullet+}$ (green-blue color) to the decolored form takes several seconds except for GSH (hours) (Janaszewska and Bartosz 2002).

To form the ABTS radical cation ($ABTS^{\bullet+}$) it is necessary to mix 7 mmol L^{-1} ABTS with 2.45 mmol L^{-1} potassium persulfate at a ratio of 2:1. Then the stock solution is incubated in the dark for 12–16 hours at room temperature before storage at 2 °C. The TEAC working reagent is prepared by mixing 1 mL of $ABTS^{\bullet+}$ and 19 mL of phosphate-buffered saline stock solution. A 200 μL volume of the reagent

Table 11.7 Reference values of TBARS, allantoin, total polyphenols, bilirubin, and antioxidant status parameters in blood

Biochemical parameter	Reference values	Unit
TBARS	1.0–5.0	μmol L^{-1}
Allantoin	3.1–36.4	μmol L^{-1}
Total polyphenols	2.8–4.0	g L^{-1}
Bilirubin	5.1–17.1	μmol L^{-1}
ABTS	1300–1600	μmol L^{-1}
DPPH[a]	7.9–27.1	%
FRAP	400–1600	μmol L^{-1}
ORAC[a]	1500–3100	μmol L^{-1}

ABTS 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), *DPPH* 2,2-diphenyl-1-picrylhydrazyl, *TBARS* thiobarbituric acid reactive substances, *FRAP* ferric reducing ability of plasma, *ORAC* Oxygen Radical Absorbance Capacity
[a]Deproteinized serum

is continuously mixed with 20 μL of the sample, incubated at 37 °C, and after 6 min measured at 734 nm. The TEAC values of plasma samples are expressed as mmol L^{-1} of Trolox equivalent (TE) (Fairus et al. 2019; Re et al. 1999). The assay has a precision lower than 3% (Erel 2004; Lee et al. 2017).

DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

DPPH antioxidant status of plasma is measured by the method described by Brand-Williams et al. (1995) with the use of a stable free radical – DPPH. This compound possesses the spare electron over the whole molecule (molecule does not dimerize) with the delocalization in deep violet color. The absorption band in methanol/ethanol solution is centered at 515–520 nm. After the reaction of the DPPH solution with the antioxidants (substances that can donate a hydrogen atom), the reduced form of DPPH remains in the form of the picryl group and changes the color to pale yellow.

Fresh DPPH radical solution is usually prepared by dissolving 1 mol L^{-1} of DPPH in 200 mL of methanol. 5 μL of plasma is subjected to reaction with DPPH working solution (245 μL) for 30 min in the dark and the absorbance is measured at 517 nm (Lee et al. 2017). The TEAC values of plasma samples are expressed as mmol L^{-1} of TE.

FRAP Assay

The determination of FRAP is based on the methodology elaborated by Benzie and Strain (1996) and modified by Janaszewska and Bartosz (2002). Plasma (10 μL) is mixed with 30 μL of deionized water on an ELISA plate. Then, 300 μL of the reagent (37 °C), consisting of 250 μL of 300 mmol L^{-1} acetate buffer (pH 3.6), 25 μL of 10 mmol L^{-1} 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol L^{-1} HCl, and 25 μL of 20 mmol L^{-1} $FeCl_3 \times 6H_2O$ solutions, is added to all wells and mixed carefully. After 6 min of incubation, the color of the solutions is read on a multimode microplate reader at 593 nm. The standard curve is determined with a stoichiometrically diluted solution of iron(II) sulfate(VI) ($FeSO_4 \times 7H_2O$). FRAP is expressed in μmol L^{-1} of plasma.

ORAC Assay

The ORAC assay aims to measure the antioxidant capacity of plasma by the determination of the oxidative degradation of a fluorescent molecule (fluorescein), which has been previously mixed and heated with AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride). Plasma samples react with the ORAC working solution (150 μL). After adding AAPH for peroxyl radical generation, the fluorescence is measured for 80 min to determine the area under the curve (AUC) at 485 nm as excitation and 520 nm as emission. TAC is expressed as mg L^{-1} of TE (Cao et al. 1993; Huang et al. 2002; Lee et al. 2017).

Conclusions

There is a growing need to find markers of oxidative stress in the organism. In the literature reviews, researchers found out that damage to lipids in samples of adults was lower (level of oxidized proteins in plasma), and superoxide anion levels in neutrophils were also lower than in samples of the elderly individuals (Belenguer-Varea et al. 2019). They also observed that antioxidant biomarkers such as superoxide dismutase were lower and glutathione reductase activities were higher, as well as higher levels of vitamins A and E, lower level of coenzyme Q_{10} in comparison to elderly controls. Another meta-analysis on patients with cystic fibrosis revealed some markers of antioxidant status and oxidative stress such as protein carbonyl groups (DNPH assay), total F2-isoprostane, 8-iso-prostaglandin $F_{2\alpha}$, MDA, vitamins A and E, β-carotene (Causer et al. 2020). Whalley et al. (2003) have concluded that the general factors believed to raise the risk of vascular disease such as higher homocysteine levels, and also lower vitamin C levels can increase the likelihood of brain shrinkage or brain cell loss. Moreover, metabolomics can identify the biomarkers of dietary patterns and the influence of nutrition on breast cancer risk (Noh et al. 2017; Playdon et al. 2017a, b). It is also essential that there should be more research on biomarker levels in patients because it is necessary to use them for medical purposes.

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Chapter 12
Speciation Analysis of Food Products

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Abstract Elements in the food products are rarely found in free form or as individual ions. The quantity and quality of element forms (species) carry extremely important information for understanding its bioavailability for living organisms, as well as its transport cycles in the environment. Therefore, identification and determination of inorganic species and organometallic compounds have become a very important aspect of modern analytical chemistry. This chapter provides a short description of the speciation forms of selected elements (mercury, arsenic, and selenium) occurring in food products. Their properties and impact on human health are presented depending on the chemical form. In the chapter, the main emphasis is placed on issues related to analytical procedures used in speciation analysis of food products. The next stages of these procedures (sampling, storage of samples, preparation of samples for the analysis, as well as the separation of analytes and final detection) are discussed, with emphasis on stages that differ from the techniques for determination of the total elements' contents. In the separation and determination stages, the presentation of chromatographic and non-chromatographic approaches as well as hyphenated techniques are provided. The crucial role of spectrometric techniques as the primary detection tools in the determination of elemental species in the food products is also discussed in detail.

Introduction

The studies of the trace elements effect on living organisms have been of interest to researchers from various fields of science for many years. Particular interest in the chemical forms in which an element occurs in the system was initiated by dramatic cases of environmental contamination with extremely toxic substances. The groundbreaking incident took place in Japan, where a "Minamata disease" was diagnosed in the inhabitants of Minamata Bay. The case of poisoning with methylmercury

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species has been described in many papers, especially in the area of biologically active chemical substances (Ekino et al. 2007; Eto 2000; Harada 1995). A lot of research has been conducted since then, and the results of the studies have shown that knowing only the simple elemental composition is not sufficient. Hence the interest increased in individual chemical forms of the element present in the examined object. This issue is closely related to speciation analysis, which allows the determination of the variety of chemical forms present in a sample. Often these specific forms of a particular element or its compounds are referred to as "species" (Sanz-Medel and Fernández-Sanchez 2005; Templeton et al. 2000). They can be defined by isotopic composition, electronic or oxidation state, and/or complex or molecular structure (Sanz-Medel and Fernández-Sanchez 2005). According to the definitions given by IUPAC, "speciation (of an element)" is "the distribution of an element amongst defined chemical species in a system" (IUPAC 1997; Sanz-Medel and Fernández-Sanchez 2005; Templeton et al. 2000). Whereas for the analytical chemistry purpose, "speciation analysis is analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample" (IUPAC 1997; Sanz-Medel and Fernández-Sanchez 2005; Templeton et al. 2000).

The presence of different species of elements in the food products is connected not only with the toxicity but also with bioaccessibility and bioavailability (Moreda-Piñeiro et al. 2011; Schumann and Elsenhans 2002). Bioaccessibility has been defined as the fraction of a compound that is released from the gastrointestinal matrix in the lumen of the gastrointestinal tract and thus made available for intestinal absorption (Gropper and Smith 2013b). While bioavailability is defined as the rate and extent to which a compound present in food is absorbed, retained, or metabolized normally (Gropper and Smith 2013a; Moreda-Piñeiro et al. 2011; Schumann and Elsenhans 2002).

Bioavailability of minerals and trace elements is one of the main parameters for assessing the nutritional value of food in general. It depends heavily on the chemical form of the target nutrient, and therefore, chemical speciation analysis or at least fractionation analysis is an extremely important task in food analysis. Bioavailability also depends on the interaction of the target compounds with other food ingredients. To study such interactions, e.g., complexing reactions and transformation of species, a chemical analysis must provide data on chemical compounds in their presence (Gropper and Smith 2013a; Moreda-Piñeiro et al. 2011; Schumann and Elsenhans 2002).

Information obtained as the result of the chemical speciation analysis is not only essential for assessing the nutritional value or health risk but is also a useful tool in the evaluation of food quality. Additionally, risk assessment of contaminants (e.g., toxic species) is another crucial task in food analysis. Since it is known that the toxicity of many trace elements depends largely on their speciation form, only the determination of the individual species allows for assessment of the health risks associated with the presence of such potentially toxic compounds (Armenta and de la Guardia 2015; Gropper and Smith 2013b).

In the first years of the speciation analysis, development researchers focused primarily on elements that were classified as toxic. The obtained results allowed for

the study of the impact of individual chemical forms of the element on living organisms. Currently, speciation analysis deals with a much larger group of ultratrace elements and their compounds. The obtained data allow for understanding their role in living organisms, circulation in nature, as well as in the context of the identification of substances necessary for the proper functioning of living organisms (Apostoli et al. 2006; Cornelis et al. 2005).

An extremely important step in speciation analysis is the selection of the appropriate measurement method, preceded by proper sample collection, storage, and preparation of the tested objects (samples). The entire analytical procedure should be optimized to obtain reliable results providing data on the chemical composition of the original material. Performing speciation analysis is a huge challenge for an analyst, primarily due to the need to adapt the analytical procedure to a given problem. The challenge is not only the expected specificity and sensitivity of the detection system but the use of the procedure, especially at the stage of sample preparation, which does not affect the original chemical equilibrium in the sample. For this reason, careful attention is paid to the proper selection of analytical procedures, which includes both sample preparation and measurement of the content of a species of interest. Most speciation studies are conducted using different extraction, leaching, and derivatization approaches followed by the use of hyphened techniques. The combination of selective techniques for separation of species (most often chromatographic techniques) with sensitive and specific detection techniques (atomic spectrometry and mass spectrometry techniques are most commonly used) allows advanced speciation analysis (Gonzalvez et al. 2009; Khouzam et al. 2012).

Mercury Species in Food Products

The study of mercury speciation was the first major analytical challenge in the aspect of environmental research. Although the toxic effects of mercury have been known since ancient times, only modern analytical procedures have allowed for a better understanding of mercury biochemical changes and their impact on living organisms (Bjørklunda et al. 2017).

The adverse effects of organic mercury species on human health attracted wide attention in the late 1950s. It was strictly associated with methylmercury poisoning and discovery of the neurological diseases among the inhabitants of Minamata Bay in Japan. In this area, the chemical industry producing acetaldehyde and vinyl products was located and was finally recognized as a source of pollution. In the production processes, catalytic inorganic mercury compounds were used and were accidentally discharged with process wastewater to nearby fisheries (Ekino et al. 2007; Eto 2000; Harada 1995; Lambertsson 2005). After transformation into methylmercury, it was accumulated in the trophic water level, thus people highly dependent on a diet rich in fish were exposed to significant amounts of the compound (Eto 2000). Other serious cases of mercury poisoning occurred in Iraq in the early 1970s. The poisoning occurred among farmers whose wheat grain had been dressed with

the fungicide (ethylmercury-p-toluene sulfonanilide). The number of hospital-admitted cases exceeded 5500, and the deaths reached 280 (Bakir et al. 1973; Damluji and Tikriti 1972).

The term "inorganic mercury" includes elemental metallic mercury (Hg0) and its two oxidation states, the mercurous (Hg–Hg(II)) and mercuric (Hg(II)) cations (Clarkson et al. 2007; Magos and Clarkson 2006; Mutter et al. 2006). Water solubility, gastrointestinal absorption, and oral toxicity decrease in the following order: $Hg(NO_3)$>$HgCl_2$>$HgSO_4$>Hg_2Cl_2>HgO. Inorganic mercury in the human body causes kidney damage, neurotoxicity, and cardiovascular disorders (Clemensa et al. 2012).

From mercuric mercury derive all the organic forms. "Organic mercury" is defined as those compounds in which divalent mercury is covalently bound to one or two carbon atoms. Bonding to phenyl or short-chained alkyl substituents yields, for example, dimethylmercury or phenylmercury, and of particular environmental deep concern – methylmercury. Toxicologically there are two distinct classes of organic mercury species, those that rapidly degrade to inorganic mercury in the body such as phenyl mercury and those that are relatively stable (Clarkson and Magos 2006; Clarkson et al. 2007; Magos and Clarkson 2006). In Table 12.1, selected inorganic and organic mercury compounds present in environmental and food samples are summarized.

Metallic mercury occurs as a component of the earth's crust (IARC 1993). It is found in the form of the sulfide, as cinnabar ore (mercury(II) sulfide, HgS), which has an average mercury content of 0.1–4%. The major source of atmospheric mercury is suggested to be the degassing of the earth's crust and the oceans (Berlin et al. 1986; WHO 1990). Other sources of this element in the environment are, for example, volcanoes and activity of people as the production of nonferrous metals (steelworks), cement, corrosive soda, as well as garbage disposal (Ferreira et al. 2015). Additionally, plant foods may also be contaminated with mercury due to the use of phosphate fertilizers in agriculture processing (de Jesus et al. 2013).

Methylmercury compounds are formed in aquatic and terrestrial environments from the methylation of metallic mercury and mercuric mercury. Methylation is likely to occur in the presence of bacteria in sea or lake sediments. The methylmercury compounds formed are accumulated by aquatic organisms, and dimethylmercury vapor is formed by degradation and released into the air. Dimethylmercury can be decomposed in the atmosphere by acidic rainwater to monomethylmercury species and thus reenter the aquatic environment (Berlin et al. 1986). The quantitative aspects of these cycles depend on many factors, and the local methylmercury compounds pollution can be increased considerably by anthropogenic sources (Clarkson and Magos 2006; WHO 1990).

From the atmosphere, mercury is removed mainly by precipitation. The chemical species of mercury in water is mainly ionic mercury(II). Concentrations of mercury in surface water are very low. In soil, the most common form of mercury is the bivalent ion. Concentrations measured in soils are generally less than 1 ppm (mg kg^{-1}) and strictly depends on human activities in the area. Methylation of mercury has been demonstrated in soil and is influenced by humidity, temperature, and

Table 12.1 Selected species of mercury, arsenic, and selenium commonly detected in environmental and food samples

Element	Species	Abbreviation	Formula
Hg	Inorganic bivalent mercury	Hg(II)	Hg^{2+}
	Methylmercury	MeHg ($MeHg^+$)	CH_3Hg^+
	Dimethylmercury	DMeHg	$(CH_3)_2Hg$
	Ethylmercury	EtHg	$CH_3CH_2Hg^+$
	Diethylmercury	DEtHg	$(CH_3CH_2)_2Hg$
	Phenylmercury	PhHg	$C_6H_5Hg^+$
As	Arsenite	As(III)	As^{3+}
	Arsenate	As(V)	As^{5+}
	Monomethylarsonic acid	MMA(V)	$CH_3AsO(OH)_2$
	Dimethylarsinic acid	DMA(V)	$(CH_3)_2AsO(OH)$
	Trimethylarsinic oxide	TMAO	$(CH_3)_3AsO$
	Arsenobetaine	AsB	$(CH_3)_3As^+CH_2COO^-$
	Arsenocholine	AsC	$(CH_3)_3As^+(CH_2)_2OH$
	Triethylarsine	Et_3As	$(CH_3CH_2)_3As$
	Triphenylarsine	Ph_3As	$(C_6H_5)_3$ As
Se	Selenite	Se(IV)	H_2SeO_3
	Selenate	Se(VI)	H_2SeO_4
	Selenomethionine	SeMet	$CH_3SeCH_2CH_2CH(NH_2)COOH$
	Selenocysteine	SeCys	$HSeCH_2CH(NH_2)COOH$
	Se-methylselenocysteine	Se-MetSeCys (Se-MetCys)	$CH_3SeCH_2CH(NH_2)COOH$
	Selenoethionine	SeEt	$CH_3CH_2SeCH_2CH_2CH(NH_2)COOH$
	Selenocystine	$SeCys_2$	$HOOCCH(NH_2)CH_2Se\text{-}SeCH_2CH(NH_2)COOH$

the mercury concentration of the soil (Simon et al. 1990). The accumulation of mercury in plants increases with increasing soil concentration. Soil type has a considerable influence on this process; the high content of organic matter decreases the uptake. Generally, the highest concentrations of mercury are found at the roots, but translocation to other organs (e.g., leaves) also occurs. In contrast to higher plants, mosses take up mercury from the atmosphere (Berlin et al. 1986; IARC 1993; WHO 1990).

Environmental contamination with mercury leads to a critical concentration effect in animals that occupy higher positions in the food chain (large fish and fish-eating sea fowl) (Simon et al. 1990). The factors that determine the methylmercury concentration in fish are the mercury content in the water and bottom sediments, the pH and redox potential of the water, and the type and size of fish (Berlin et al. 1986). The concentrations of mercury in most foods are generally in the range μg kg^{-1} (Clemensa et al. 2012; Hanna et al. 2015; Lambertsson 2005; WHO 1990).

The methyl- and ethylmercury compounds have quite similar chemical properties and are often referred to as the "short-chain alkyl mercurials." After the absorption of these substances into the human body, the central nervous system is most at risk. The ethyl- species differ from the methyl- ones in a more rapid conversion into inorganic mercury in the body and cause kidney damage. On the other hand, methylmercury appears to exclusively damage the central nervous system, at least in primates (Berlin et al. 1986; Clarkson and Magos 2006). The high mobility of methylmercury in the body is due to the formation of a complex with the amino acid cysteine (Berlin et al. 1986; Clarkson and Magos 2006; Clarkson et al. 2007). The compound is transported out of liver cells into bile as a complex with reduced glutathione using glutathione carriers (Ballatori et al. 1995). It may be that these two processes, entry into the cell as the cysteine complex and exit *via* the glutathione pathway, are sufficient to explain the mobility in the body (Clarkson and Magos 2006; Clarkson et al. 2007).

The phenyl- and methoxyethylmercury compounds are rapidly converted to inorganic mercury so that their toxic effects are similar to those of mercuric mercury compounds. However, they are more efficiently absorbed into the body than inorganic mercury (Berlin et al. 1986; Clarkson and Magos 2006; Clarkson et al. 2007). Additionally, mercury species are volatile, thus inhalation of their vapor has a detrimental effect on nervous and digestive processes, immune systems, as well as the lungs and kidneys.

Methylmercury in the diet is efficiently absorbed in the gastrointestinal tract and then transferred to the bloodstream due to its affinity to thiol groups. From the bloodstream, it distributes to all tissues readily crossing the blood-brain and placental barriers (Fig. 12.1). It is avidly accumulated into scalp hair. After a single meal of fish, methylmercury absorption and disposition is complete within 3 days (Clarkson et al. 2007; Kershaw et al. 1980). After oral administration, the central nervous system is organic mercury's main target organ, especially during fetal development. Toxic effects are changes in sensory functions (sight, hearing), motor coordination, memory, attention, and learning (Clemensa et al. 2012; NRC 2000). As a safeguard for human health, maximum permitted levels of Hg in fish (0.50 or 1 mg kg^{-1} essentially for predatory fish) and crustaceans (0.50 mg kg^{-1}) were set by Commission Regulation (EC) No 629/2008 to limit dietary exposure of consumers (Commission Regulation 2008). The World Health Organization (WHO) recommends the maximum methylmercury intake as 0.1–2.0 mg kg^{-1} of body weight per week for numerous national diets (FAO/WHO 2004).

Analytical techniques and methodology for mercury species determination in food samples with relevant literature references are presented in Table 12.4.

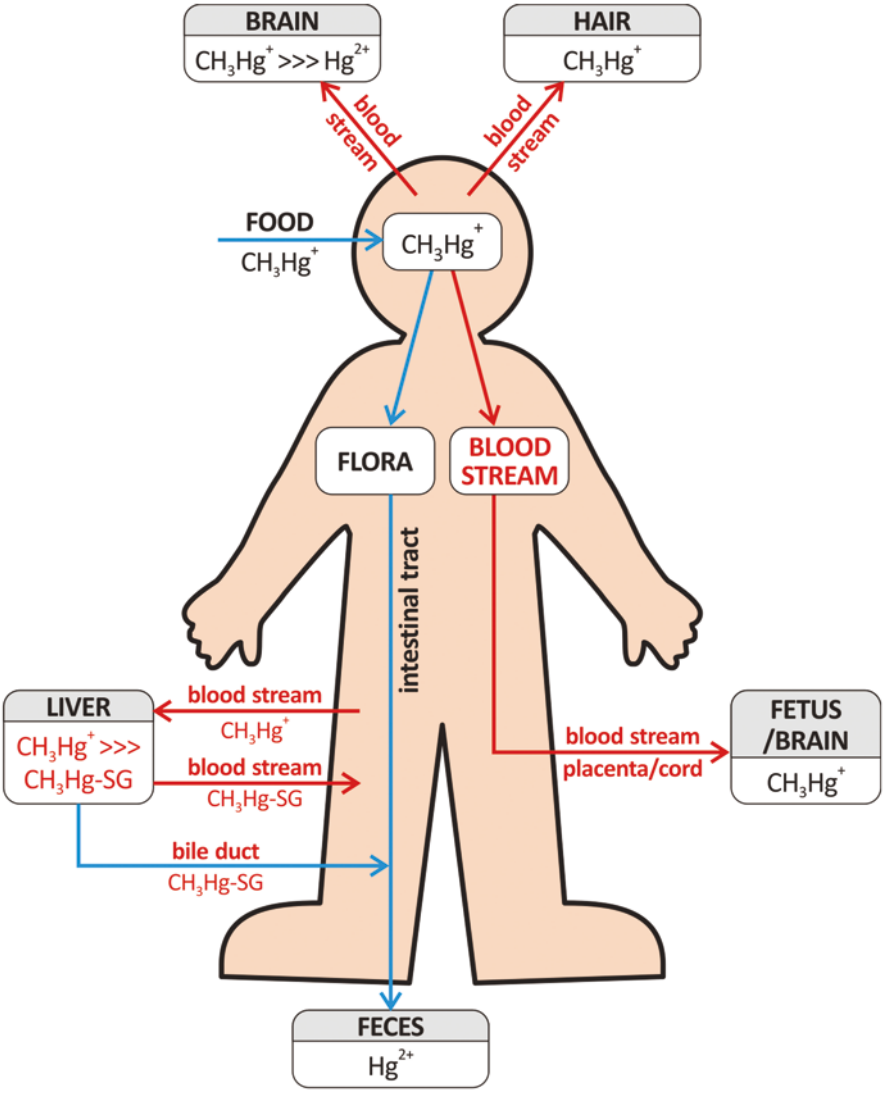

Fig. 12.1 A schematic representation of the disposition and metabolism of methylmercury (CH_3Hg^+) in the human body (Clarkson et al. 2007): methylmercury combines with reduced glutathione to form a complex (CH_3Hg-SG) that is secreted into bile. After hydrolysis of glutathione to its constituent amino acids, the methylmercury-cysteine complex is released. It is reabsorbed partly in the gallbladder into the bloodstream, and in part secreted into the intestinal tract along with any unhydrolyzed glutathione complex. Once in the intestinal tract, it is in part reabsorbed into the portal circulation as the cysteine complex and in part demethylated by intestinal microflora (Hg^{2+} in the feces)

Sources of Arsenic Food Contamination

Among chemical contaminants of food, arsenic is an element characterized by a very large difference in the toxicity of particular species (Armenta and de la Guardia 2015). The element is found in nature in the form of sulfides (As_2S_3 orpiment, As_4S_4 realgar), arsenosulfides (FeAsS arsenopyrite), and arsenide ($FeAs_2$ loellingite). Most heavy metal sulfide ores (e.g., nickel, copper, tin, lead, cobalt, gold) are contaminated with arsenic. The main sources of air and soil pollution with this element are the mining industry of coal and liquid fuels as well as mining and metallurgy of nonferrous metals. Other sources that are associated with human activities include the production of glass, electronics, ammunition, batteries, feed additives, plant and wood protection products, and medicines. The use of contaminated phosphorus fertilizers and sewage sludge to fertilize farmlands can directly contribute to the accumulation of arsenic in food (Armenta and de la Guardia 2015; IARC 1993, 2012).

The element is present in compounds at various degrees of oxidation (As(-III), As(0), As(III), and As(V)) depending on the oxidation-reduction conditions. In the strongly reducing environment, the As(III) form dominates, which after oxidation changes to As(V). There are many arsenic compounds found in water, foods, and environmental samples. Some of them are presented in Table 12.1 (Armenta and de la Guardia 2015; FAO/WHO 2011a). Examples of the determination of arsenic species in food products are described in detail in Table 12.5. Except for biochemical and toxicological considerations of specific arsenic species, the valency of MMA (monomethylarsonic acid) and DMA (dimethylarsinic acid) is usually not presented. In this chapter, the terms MMA and DMA are used as cited in the original references (Table 12.5). The determination of MMA(III) and DMA(III) has become possible recently. However, if MMA and DMA are determined in foods, they have been measured as the pentavalent form. For biological samples analysis, it is assumed that MMA and DMA refer to total [MMA(III) + MMA(V)] and total [DMA(III) + DMA(V)], respectively (FAO/WHO 2011a).

The following three major groups of arsenic compounds can be distinguished due to biological and toxicological perspectives: inorganic arsenic compounds, organic arsenic compounds, and arsine gas. Among inorganic arsenic compounds, arsenic trioxide, sodium arsenite, and arsenic trichloride are the most common trivalent compounds, and arsenic pentoxide, arsenic acid, and arsenates (e.g., lead arsenate and calcium arsenate) are the most common pentavalent compounds. Common organic arsenic compounds include methylarsonic acid, dimethylarsinic acid (cacodylic acid), and arsenobetaine (Armenta and de la Guardia 2015; WHO 2000).

Arsenic is a common component of water. Its natural content in water is about 10 μg L^{-1}. Water pollution with the element may be related to the presence of industrial and municipal sewage, precipitation of atmospheric dust, and as a result of leaching of soils containing residues of plant protection products (IARC 2012).

Forms in which arsenic is present in soils are approximately 60% arsenates and 20% sulfides and sulfosalts; the remaining 20% includes arsenides, arsenites,

oxides, silicates, and elemental arsenic (IARC 2012; WHO 2001). These forms are generally weathered to the inorganic water-soluble species, arsenate (As(V)) and arsenite (As(III)), with arsenate dominating under oxidized conditions and arsenite under reduced conditions (Cullen and Reimer 1989). Under both aerobic and anaerobic conditions, microorganisms can transform inorganic arsenic into organic forms such as MMA, DMA, and volatile TMA (trimethylarsine). TMA in the air is then rapidly converted into water-soluble species, As(V) and TMAO (trimethylarsine oxide) (Turpeinen et al. 2002). These compounds can also be degraded by microflora. In certain materials, organic arsenic compounds naturally build up to high concentrations (IARC 2012; Mandal and Suzuki 2002; Smedley and Kinniburgh 2002).

Arsenic compounds have been used by people for thousands of years. In the past, arsenic trioxide (As_2O_3, arsenic(III) oxide) was used not only as a poison but also for the preservation of leather and wood as well as in the production of paint sand glass. Arsenic compounds have also found their application in medicine, due to their antiseptic, antipyretic, antispasmodic, sedative, and tonic properties. Hippocrates used arsenic disulfide and trisulfide to treat skin ulcers. In Chinese medicine, on the other hand, arsenic trioxide was used to treat syphilis, psoriasis, and joint diseases (FAO/WHO 2011a).

Nowadays the International Agency for Research on Cancer (IARC) has classified "arsenic and inorganic arsenic compounds" as carcinogenic to humans (group 1), i.e., with proven carcinogenic effects for people (IARC 2012). Arsenic, and especially its inorganic compounds, have genotoxic and neurotoxic (hearing disorders) properties, may contribute to cardiovascular diseases, peripheral vascular disorders, anemia, reproductive system disorders, may also be the cause of diabetes (FAO/WHO 2011b), and causes lung, skin, bladder, prostate, kidney, and liver cancer (Hopenhayn-Rich et al. 1998).

Arsenic compounds enter the human body primarily through the digestive tract. It is also possible to be absorbed through the respiratory system and to penetrate the skin, especially in the case of people working in mines, nonferrous metal smelters, and agriculture (FAO/WHO 2011a; WHO 2001). Inorganic compounds are absorbed in approximately 95%, organic compounds in 75–85%, while insoluble compounds are absorbed to a much lesser extent. Inorganic arsenic from vegetables is absorbed in approximately 50% due to its occurrence in combinations with polysaccharides. Absorption of arsenic by the respiratory system is about 60% of total exposure (Cui et al. 2008; Mandal and Suzuki 2002).

Arsenic accumulates primarily in the bones, liver, kidneys, and hair, nails, skin, and gastrointestinal epithelium. The content of the element in human hair is a good indicator of the population's exposure to arsenic (Hindmarsh 2002). Prolonged intake of arsenic with contaminated water can lead to the so-called "Blackfoot disease" due to cardiovascular impairment, which can cause ischemia and putrefaction in the feet and hands (Mukherjee et al. 2005). Cancer can also be a consequence of chronic poisoning associated with the consumption of water containing large amounts of arsenic (Hall 2002; Hopenhayn-Rich et al. 1998). Arsenic is an element that has a large toxicity difference depending on the degree of oxidation and its chemical form. Apart from the dose and time of exposure, the chemical form of this

element determines its absorption and bioavailability. For example, pentavalent arsenic has less toxicity than trivalent arsenic. Arsenic organic compounds are also less toxic and they are less reactive and excreted faster. The most toxic is arsenic trihydride (AsH_3, arsane), classified as an extremely hazardous substance and called "the king of poisons," which arises naturally as a product of many chemical reactions (Mandal and Suzuki 2002; WHO 2001).

For humans, food products are the main source of arsenic. Low levels of inorganic and organic arsenic species have been measured in most foods (typical concentrations are less than 0.25 mg kg^{-1}) (FAO/WHO 2011a, b; WHO 2001). In general, seafood contains less toxic organic arsenic compounds (including arsenocholine, arsenobetaine, and arsenic sugars). Factors influencing the total concentration of arsenic in food include, besides growing conditions (e.g., soil type, water, use of arsenic-containing pesticides), food type (e.g., seafood *versus* meat or dairy) and food-processing techniques. For example, the arsenic content in rice may be several times higher than in other cereals. It was also found that depending on the area of cultivation, there are noticeable differences in the content of various forms of arsenic, e.g., rice from Asia contains much higher amounts of inorganic arsenic than rice from the USA (FAO/WHO 2011b; Mandal and Suzuki 2002).

In Table 12.2, the ranges of total arsenic concentrations in selected food products are summarized (FAO/WHO 2011a, b). The highest concentrations of arsenic have been found in seafood, followed by mushrooms and fungi, meats, fruit, rice, and vegetables. Inorganic arsenic is the predominant form found in meats, poultry, dairy products, and cereal, and organic arsenic (e.g., arsenobetaine) predominates in seafood, fruit, and vegetables (IARC 2012; WHO 2000, 2001). Regional differences are seen in the daily intake of total arsenic through food and are mainly attributable to variations in the quantity of seafood consumed. For example, the daily dietary intake of total arsenic in Japan is higher than that in Europe and the USA (IARC 2012; WHO 2000).

Analytical techniques and methodology for arsenic species determination in food samples with relevant literature references are presented in Table 12.5.

Selenium in Food Products

Selenium is a trace element that was first isolated by Jacob J. Berzelius in 1817. Initially, it was thought, because of the properties, that it was tellurium. However, it was quickly found that although similar to tellurium, it has several new, unique properties (Trofast 2011).

The main organic forms found in nature are selenomethionine (SeMet) and selenocysteine (SeCys), and among inorganic forms, there are selenate(VI) (SeO_4^{2-}), selenite(IV) (SeO_3^{2-}), selenide (Se^{2-}), and elemental selenium (Se(0)). The distribution of the valence states depends on microbial activity, solution pH, and redox conditions. Selenium in the (-II) oxidation state exists mainly as hydrogen selenide (HSe^-) and as a number of metallic selenides. Heavy metal selenides

Table 12.2 Summary of selected data on total arsenic concentrations in food products (FAO/WHO 2011a, b)

Food product	Total arsenic concentration[a], range (mg kg^{-1})
Dairy products and analogs	
Milk and milk powder	0.001–0.15
Milk products	0.010–0.35
Fats and oils	0.003–0.18
Meat and meat products	
Meat	0.004–0.78
Offal	0.009–0.45
Meat products	0.003–3.25
Eggs and egg products	0.003–0.04
Confectionery products	0.002–1.13
Sweeteners	0.003–0.26
Bakery wares	0.002–0.25
Beverages	
Alcoholic beverages (except rice-distilled spirits)	0.001–0.05[b]
Rice-distilled spirits	0.050–1.64[b]
Nonalcoholic beverages	0.001–0.26[b]
Vegetables/fruits/nuts/seaweed	
Fruits	0.005–2.20
Vegetables (except mushrooms and fungi)	0.001–1.27
Mushrooms and fungi	0.011–5.79
Nuts and oilseeds	0.005–0.88
Dried seaweeds	0.114–236
Cereals and cereal products	
Cereals (except rice)	0.007–0.43
Rice	0.002–1.83
Breakfast cereals	0.017–0.27
Pasta	0.003–0.18
Fish and fish products	
Marine fish	0.10–62
Shellfish	0.090–66
Freshwater fish	0.060–4.72
Baby food products	0.001–4.66

[a]Detailed literature sources listed in the reference (FAO/WHO 2011a)
[b]Expressed as mg L^{-1}

are the most insoluble forms of the element. H_2Se is a toxic gas at room temperature and is thermodynamically unstable in aqueous solutions. Elemental selenium exists as several allotrophic forms and is very stable and highly water-insoluble (Martens 2003; White 2016). Alkaline soils release more selenium than acidic soils. Additionally, in an alkaline environment, selenite(IV) is oxidized to easily

soluble selenates(VI), which are easily assimilated by plants (Mehdi et al. 2013). The organic form of selenium, selenomethionine is assimilated faster by plants than selenocysteine (Kikkert and Berkelaar 2013; Navarro-Alarcon and Cabrera-Vique 2008). The most important selenium species found in environmental and food samples are summarized in Table 12.1.

Industrial and agricultural activity has hastened the release of selenium compounds from geological sources, making them available to fish and wildlife in aquatic and terrestrial ecosystems around the globe. In recent years, the results of many investigations on pollution with selenium conclude that element exhibits its toxicity in animals primarily through the food chain (Hamilton 2004; Navarro-Alarcon and Cabrera-Vique 2008). Selenium compounds are also widely used in glass manufacture, electronic applications, as well as in the production of inorganic pigments, rubbers, ceramics, and plastics (Akl et al. 2006; Navarro-Alarcon and Cabrera-Vique 2008).

In the aquatic environment, agricultural drain water, sewage sludge, fly ash from coal-fired power plants, as well as mining of phosphates and metal ores are the main sources of selenium contamination (Hamilton 2004). Fish can take up the element from water, plants, or by eating other marine species. Accumulation of selenium in marine animals from dietary sources (phytoplankton and zooplankton) is more important than that accumulated directly from the water (Akl et al. 2006; Navarro-Alarcon and Cabrera-Vique 2008).

Descriptions of the toxicity of selenium ingested with food can be found in notes from the thirteenth century (Reid et al. 2004). Marco Polo during his travels found selenium-rich plants in the mountainous region of western China that, eaten by animals, caused degradation of their hooves. Similar observations were made in Nebraska and other western areas of the USA in the late nineteenth and early twentieth centuries (Holben and Smith 1999). The interest in the biological role of this element developed in the 1950s, when it was discovered that selenium toxicity causes muscular dystrophy in breeding animals, and when selenium and vitamin E deficiencies have been shown to cause acute liver necrosis in rats (Foster and Sumar 1995).

The year 1973 was significant for understanding the biochemical role of selenium. It was discovered that selenium is an integral component of the active center of the glutathione peroxidase enzyme (Rotruck et al. 1973). Since then, several other functional selenoproteins have been identified that contain selenocysteine as the form of selenium. These groundbreaking discoveries, as well as the identification of many selenium proteins and selenoenzymes, have become an impulse for the development of research on the physiological role of selenium, nutritional problems associated with the need for this element, clinical symptoms of selenium deficiency or excess, and the role of the element deficit in the etiology and pathological states (Burk and Hill 1993; Holben and Smith 1999).

Selenium bioavailability is affected by its chemical form. In general, organic compounds of the element are more bioavailable than inorganic species (Combs Jr 2001; Navarro-Alarcon and Cabrera-Vique 2008; Thomson 2004). The parameters such as total protein content, fat, and the presence of heavy metals are also important. The element can interact with several other trace elements, and these interac-

tions can be additive, antagonistic, or synergistic. In some cases, they reverse the interaction, i.e., antagonism changed to synergism (Akl et al. 2006; Hamilton 2004). Selenium is recognized to decrease mercury toxicity when both elements are absorbed together (Cabañero et al. 2007).

In addition to bioavailability, possible toxicity is the most important issue affecting the number of consumed foods containing selenium (Pedrero and Madrid 2009). The beneficial effects of the element on human health are strongly dependent on its concentration. The concentration range in which selenium is considered toxic or essential is very limited. It has been estimated that the ingestion of foods with a selenium content above 1 mg kg^{-1} may cause toxic effects, meanwhile, a concentration below 0.1 mg kg^{-1} leads to deficiency (Pedrero and Madrid 2009).

The selenium level ingested through the diet of local products is related to the selenium concentration in the soil of the area. Most plants cannot accumulate large amounts of the element (concentrations rarely exceed 100 μg g^{-1}, dry weight). However, various plant species such as garlic (*Allium sativum*), Indian mustard (*Brassica juncea*), canola (*Brassica napus*), and some mushrooms have been recognized as selenium accumulators. They can take up large amounts of the element (above 1000 mg Se kg^{-1}) without exhibiting any negative effects (Dumont et al. 2006; White 2016). This is mainly due to the reduction of the intracellular selenium concentration of SeCys and SeMet which are normally incorporated into proteins. When consumed in appropriate amounts, these foods can be a significant food source of the element (Dumont et al. 2006).

An extremely important phenomenon is that food processing such as cooking (boiling, baking, or grilling) could decrease selenium food content by volatilization (Dumont et al. 2006; Navarro-Alarcon and Cabrera-Vique 2008). Therefore, some authors postulate that the content of trace elements in food based only on food tables is insufficient information. The losses during food processing and preparation, variation due to seasonal changes or geographical location, as well as food habits should also be taken into account (Navarro-Alarcon and Cabrera-Vique 2008). Data on selenium content in different foods are collected in Table 12.3 (Food Data Central 2019).

Analytical techniques and methodology for selenium species determination in food samples with relevant literature references are presented in Table 12.6.

Procedures Used in Speciation Analysis of Food Samples

Methods of speciation analysis differ in several key stages from the corresponding analytical procedures for the determination of total metal content (Cornelis et al. 2005; Gonzalvez et al. 2009; Khouzam et al. 2012; Ruzik 2012). The following chapter is a short presentation of analytical procedures used in the determination of selected species of mercury, arsenic, and selenium. The main methods of these procedures are discussed, highlighting the role of spectrometric techniques as the primary detection tools in the determination of elemental species in the food products.

Table 12.3 Selected food sources of selenium (Food Data Central 2019)

Food product	Serving size	Selenium content (μg) per serving
Grains and cereals		
Bread, white	1 slice	6
Rice, brown, long-grain, cooked	1 cup	19
Corn flakes	1 cup	2
Oatmeal, unenriched, cooked with water	1 cup	13
Puffed wheat ready-to-eat cereal	1 cup	15
Meats and meat alternatives		
Ham, roasted	3 ounces	42
Shrimp, canned	3 ounces	40
Macaroni, enriched, cooked	1 cup	37
Turkey, boneless roasted	3 ounces	31
Beefsteak, bottom round, roasted	3 ounces	33
Beef, liver, pan-fried	3 ounces	28
Chicken, light meat, roasted	3 ounces	22
Tuna, yellowfin, cooked, dry heat	3 ounces	92
Halibut, cooked, dry heat	3 ounces	47
Sardines, canned in oil, drained solids with bone	3 ounces	45
Egg, hard-boiled	1 large	15
Brazil nuts	6–8 nuts	544
Dairy products		
Milk, 1% fat	1 cup	8
Cottage cheese, 1% milk fat	1 cup	20
Yogurt, plain, low fat	1 cup	8
Vegetables		
Green peas, frozen, boiled	1 cup	2
Bananas, sliced	1 cup	2
Potato, baked, flesh and skin	1 potato	1
Peaches, canned in water, solids, and liquids	1 cup	1
Spinach, frozen, boiled	1 cup	11

The separation techniques (mainly high-performance liquid chromatography (HPLC) and gas chromatography (GC)) coupled with atomic spectrometric techniques (inductively coupled plasma atomic emission spectrometry (ICP-AES) or mass spectrometry (ICP-MS), electrothermal (graphite furnace) atomic absorption spectrometry (ETAAS or GFAAS)) in speciation analysis of food products are presented in the form of a tabular compilation (Tables 12.4, 12.5, and 12.6). The most common approaches to speciation analysis of food samples are schematically presented in Fig. 12.2.

Table 12.4 Determination of mercury species[a] in selected food samples

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
Hg, organoHg	Fish, squid, sea urchins	Total Hg: 0.1% cysteine, 5 mol L^{-1} NaOH, 80 °C, 1–2 h. Fat removal with methyl isobutyl ketone and hexane. OrganoHg: As for total Hg then HBr/$CuCl_2$/toluene added to extract organoHg. Toluene layer removed and organoHg back-extracted into a mixture of 0.2% cysteine and 2% sodium acetate.	CV-AAS	–	Yoshimoto et al. (2016)
$MeHg^+$, Hg^+	Rice	Extraction with 6 mol L^{-1} HCl for 45 min, then overnight shaking. After two extractions, extract pH was adjusted to the value of 6.7. Next, 1 mL of solution was extracted and diluted to 4 mL with previously prepared mobile phase and filtered using a 0.22 mm microporous membrane.	HPLC-ICP-MS	CH_3Hg^+: 0.2 μg L^{-1} Hg^+: 0.3 μg L^{-1}	Xu et al. (2016)
OrganoHg	Sushi	Selected microwave-assisted extraction with toluene in an acid solution (30% HCl). After extraction, the organic phase was stabilized in a 2.5% L-cysteine solution.	AAS	3.8 μg kg^{-1}	de Paiva et al. (2016)
$MeHg^+$, Hg^+	Zebrafish tissues	Digestion with tetramethylammonium hydroxide in an analytical microwave system.	GC-ICP-MS	–	Gentès et al. (2015)
OrganoHg	Raw and cooked fish	Extraction with 18% KBr in 5% H_2SO_4, transfer into toluene and back-extraction into aqueous sodium thiosulfate solution.	AAS	–	Mieiro et al. (2016)
Total Hg, Hg^{2+}, and $MeHg^{2+}$	Dogfish liver	Ultrasound-assisted dispersive micro solid-phase extraction (USA DMSPE), with the use of TiO_2 nanoparticles as the adsorbent. Total Hg was determined in samples after microwave-assisted digestion. Hg^{2+} species were determined in samples after ultrasonic solubilization in the presence of tetramethylammonium hydroxide.	CV-AAS	Hg^{2+}: 0.004 μg L^{-1}	Krawczyk and Stanisz (2016)

(continued)

Table 12.4 (continued)

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
Total Hg, $MeHg^+$, Hg^+	Fresh and canned fish	Total Hg contents were determined after sample mineralization in an open system using a mixture of HNO_3, $HClO_4$, and H_2SO_4. Before the determination of CH_3Hg^+, the sample was weighed into a micro-diffusion cell containing cysteine-impregnated paper and after addition of NaCl solution, saturated solution of $K_4[Fe(CN)_6]$ and H_2SO_4 the mixture was left in an oven at a temperature of 70 °C overnight, where volatilization of CH_3Hg^+ from the samples onto the cysteine-impregnated paper took place. A mixture of KBr and H_2SO_4 saturated with $CuSO_4$ was added to the cysteine-impregnated paper. CH_3Hg^+ was extracted from the cysteine-impregnated paper into 0.5 mL of toluene.	Total Hg was determined using CV-AAS. CH_3Hg^+ was determined using gas chromatography-electron capture detection.	Total Hg: 0.1–0.2 ng g^{-1} CH_3Hg^+: 0.2 ng g^{-1}	Miklavčič et al. (2011)
Hg^+, $MeHg^+$, and $EtHg^+$	Dogfish muscles	Chromatographic separation with the reversed-phase mode was used to separate Hg species as their 2-mercaptoethanol complexes. The mobile phase consisted of an ammonium acetate buffer to assure a neutral environment and acetonitrile as an organic modifier.	CV-AFS	Hg^+: 0.11 µg L^{-1} $MeHg^+$: 0.03 µg L^{-1} $EtHg^+$: 0.09 µg L^{-1}	Guzmán-Mar et al. (2011)
Hg^+, $MeHg^+$	Seafood	Separation of inorganic mercury (Hg^{2+}) and $MeHg^+$ was accomplished on a Hamilton PRP X-200 polymer-based exchange column with a mobile phase of acetonitrile, L-cysteine and pyridine and formic acid at pH 2.4 within 7 min. Once separated, both species are reduced by formic acid in the mobile phase under UV radiation to convert Hg^0 on-line, which is subsequently swept (by argon carrier gas) to the detector.	Photo-induced CV-AFS	Hg^+: 0.1 µg L^{-1} $MeHg^+$: 0.08 µg L^{-1}	Liu (2010)

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
Hg^{2+}, $MeHg^{+}$	Edible mushrooms	Conventional extraction, ultrasound (US), and microwave (MW) radiation were evaluated for Hg species extraction using water, hydrochloric acid, or L-cysteine solutions as extraction media. The best results were obtained by using 1.0% (m/v) L-cysteine and US amplitude set at 10%.	CV-ICP-MS	Hg^{2+}: 0.41 ng g^{-1} $MeHg^{+}$: 0.35 ng g^{-1}	Pilz et al. (2011)
Hg^{2+}, $MeHg^{+}$	Seafood, fruits and vegetables	The ultrasound-assisted acid leaching method was used for sample preparation.	Hg^{2+} was determined using AFS. $MeHg^{+}$ was determined using UV-AFS.	Hg^{2+}: 0.015 mg L^{-1} $MeHg^{+}$: 0.081 mg L^{-1}	Hu et al. (2018)
Hg^{2+}, MeHg	Fish	Two-step hollow fiber liquid-phase microextraction (two-step HF-LPME) procedures with two/three phases.	ETAAS	Hg^{2+}: 0.14 μg L^{-1} MeHg: 0.06 μg L^{-1}	Thongsaw et al. (2019)

[a]The most frequently determined mercury species are total and inorganic mercury as well as two organic forms (methylmercury and ethylmercury)
[b]In some cases, separation method is presented

Table 12.5 Determination of arsenic species[a] in selected food samples

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
Inorganic As	Fish	The samples were solubilized in the presence of 1 mol L^{-1} HCl using a microwave digestion system. Inorganic As was determined as As(III). As(V) was pre-reduced to As(III). The prereduction was performed employing a solution containing 5% w/w KI + 5% w/w ascorbic acid.	FI-HG-AAS	5 μg kg^{-1}	Oliveira et al. (2016a)
As(III), inorganic As and total As	Rice	All solid samples were decomposed by enzymatic treatment with microwave assistance. After microwave-assisted extraction, the supernatant was filtered and stored at 4 °C before analysis. Water samples were collected in polyethylene bottles, filtered, and stored at 4 °C.	Electrolytic HG-AFS	As(III): 0.25 μg L^{-1} inorganic As: 0.22 μg L^{-1} Total As: 0.10 μg L^{-1}	Yang et al. (2016)
As(III), As(V), MMA, and DMA	Rice	Ultrasound-assisted extraction with 5 mM malonic acid at pH 5.6 and a temperature of 80 °C for 120 min was performed before the speciation of arsenic.	ICP-MS for total arsenic, HPLC-ICP-MS for arsenic species	As(III): 0.03 μg kg^{-1} As(V): 0.02 μg kg^{-1} DMA: 0.02 μg kg^{-1} MMA: 0.02 μg kg^{-1} Total As: 0.039 μg kg^{-1}	Choi et al. (2016)
As(III), As(V), MMA, and DMA	Seafood, rice and rice products	Total arsenic was determined after microwave-assisted digestion. Arsenic compounds were determined after ultrasound-assisted extraction with methanol. The separation was performed using an anion-exchange narrow-bore Nucleosil 100 SB column and 12 mM ammonium dihydrogen phosphate of pH 5.2 as a mobile phase.	HPLC-ICP-MS	As(III): 0.3 μg L^{-1} As(V): 0.4 μg L^{-1} DMA: 0.3 μg L^{-1} MMA: 0.4 μg L^{-1}	Terol et al. (2016)
As(III), As(V), and DMA	Rice	Extraction was performed using 2 M trifluoroacetic acid for 6 h at 100 °C. Chromatographic separation was performed using columns: Waters IC-Pak Anion HR, mobile phase 10 mM $(NH_4)_2CO_3$, pH 10; Dionex AS7 & AG7, mobile phase 12.5 mM HNO_3, pH 1.8, Hamilton PRP-X100, mobile phase 10 mM $NH_4H_2PO_4$ and 10 mM NH_4NO_3, pH 6.3.	HPLC-ICP-MS	As(III): 0.10 μg L^{-1} As(V): 0.10 μg L^{-1} DMA: 0.13 μg L^{-1}	Heitkemper et al. (2001)

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
As(III), As(V), MMA, and DMA	Rice	Total arsenic was determined after microwave-assisted digestion. Extraction was performed using different solvents (water, methanol, and tetramethylammonium hydroxide), as well as enzymatic hydrolysis. Chromatographic separation was performed using the PRP-X100 anion exchange column, mobile phase 10 mM $HPO_4^{2-}/H_2PO_4^-$, pH 6.0.	HPLC-HG-AAS	As(III): 0.015 μg L^{-1} As(V): 0.06 μg L^{-1} MMA: 0.06 μg L^{-1} DMA: 0.06 μg L^{-1}	Sanz et al. (2005)
As(III), As(V), MMA, and DMA	Rice	Total arsenic was determined after microwave-assisted digestion. Microwave extraction was performed using 10 mL of 1% HNO_3 and 1% H_2O_2 (5 min 50 °C, 5 min 75 °C, 30 min 95 °C).	HG-ICP-MS or HPLC-ICP-MS	–	Musil et al. (2014)
As(III), As(V), MMA, DMA, and AsB	Carrots	Total arsenic was determined after microwave-assisted digestion. Solid-phase extraction was used for preconcentration/separation of different species of As.	Determination of total As by ICP-MS and As speciation by HPLC-ICP-MS	As(III): 0.15 μg L^{-1} As(V): 0.11 μg L^{-1} MMA: 0.13 μg L^{-1} DMA: 0.24 μg L^{-1} AsB: 0.14 μg L^{-1}	Vela et al. (2001)
As(III), As(V), MMA and DMA	Apple	Ultrasound-assisted extractions with mixture methanol/water as well as a two-step procedure using initial overnight treatment with amylase enzyme followed by sonication with a 60/40 (v/v) acetonitrile/water solvent mixture were used.	HPLC-ICP-MS	As(III): 0.089 μg L^{-1} As(V): 0.19 μg L^{-1} MMA: 0.063 μg L^{-1} DMA: 0.034 μg L^{-1}	B'Hymer and Caruso (2002)
As(III), As(V), MMA, and DMA	Bean, rice, and hot pepper	Total arsenic was determined after microwave-assisted digestion. Species of arsenic were determined after microwave-assisted extraction with 0.3 M H_3PO_4.	Determination of total As by HG-AFS and As speciation by HPLC-HG-AFS	As(III): 1.5 μg L^{-1} As(V): 1.8 μg L^{-1} MMA: 2.1 μg L^{-1} DMA: 2.4 μg L^{-1}	Bohari et al. (2002)

(continued)

Table 12.5 (continued)

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
As(III), As(V), MMA, DMA and AsB	Fish	Enzymatic extraction using pronase E/lipase mixture assisted by microwave energy was used.	IC-ICP-MS	As(III): 15 μg L^{-1} As(V): 17 μg L^{-1} MMA: 14 μg L^{-1} DMA: 16 μg L^{-1} AsB: 22 μg L^{-1}	Hinojosa-Reyes et al. (2009)
Et_3As and Ph_3As	Canned cod liver tissue	Solid-phase extraction (SPE) using a silica gel column and ethyl acetate/methanol as eluent was used.	GC-ICP-MS	Et_3As: 0.00005 μg L^{-1} Ph_3As: 0.00013 μg L^{-1}	Arroyo-Abad et al. (2010)

FI flow injection

[a]Arsenic speciation procedures based on the determination of inorganic (As(III) and As(V)) as well as organic forms (MMA, DMA, AsB, Et_3As, or Ph_3As) of this element

[b]In some cases, separation method is presented

Table 12.6 Determination of selenium species[a] in selected food samples

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
Seleno-amino acids	Rice and yeast	Magnetic solid-phase extraction (MSPE) was combined with hollow fiber liquid-liquid-liquid microextraction (HF-LLLME). Magnetic nanoparticles were modified with graphene oxide.	HPLC-ICP-MS	0.0075–0.013 mg L^{-1}	Guo et al. (2016)
Se(IV) and Se(VI)	Black tea, milk powder, mushroom, soybean, bamboo shoots, energy drink, bottled water, carbonated drink, and mineral water	Magnetic effervescent tablet-assisted ionic liquid dispersive liquid-liquid microextraction (MEA-IL-DLLME) was used for the separation of different forms of selenium.	GFAAS	0.021 μg L^{-1}	Wang et al. (2016)
SeMet and SeCys	Fish	SeMet was determined in samples after enzymatic hydrolysis. SeCys was determined after derivatization using iodoacetamide. This step was performed to form a stable carboxymethylated complex before enzymatic hydrolysis.	HPLC-ICP-MS	–	Jagtap et al. (2016)
Selenium species	Animal tissues	Microwave-assisted digestion with concentrated nitric acid	HPLC-ICP-MS	–	Maher et al. (2016)
Se(IV), Se(VI), SeMet, SeCys, and SeOMet	Beef	Total selenium was determined after microwave-assisted digestion with concentrated nitric acid and 30% v/v hydrogen peroxide. Speciation analysis was performed after extraction. During extraction, a mix of the enzymes protease type XIV, proteinase K, and pancreatin was used. Extraction was performed at pH 7.5. Incubation in a buffer containing calcium chloride, sodium phosphate, and ammonium citrate was performed at 37 °C for 24 h with a combination of sonication.	HPLC-ICP-MS	–	Oliveira et al. (2016b)

(continued)

Table 12.6 (continued)

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
Se(IV) and SeMet	Biofortified yeast	The sample was hydrolyzed with methanesulfonic acid. Chromatographic separation was performed with a mobile phase containing 0.08% v/v heptafluorobutyric acid and methanol (92:8).	HPLC-HG-MP-AES	Se(IV): 59 μg L^{-1} SeMet: 0.52 mg L^{-1}	Yañez Barrientos et al. (2016)
SeCys, Se-MetSeCys, and SeMet	Extra virgin olive oils	Protein isolation from olive oils was performed using hexane/acetone precipitation. SEC analysis was applied to determine Se distribution within the fraction. To achieve seleno-amino acids determinations, fractions from SEC were treated for protein hydrolysis assisted by microwave.	LC-ICP-MS	SeCys: 0.17[c] μg kg^{-1} Se-MetSeCys: 0.20[c] μg kg^{-1} SeMet: 0.09[c] μg kg^{-1}	Torres et al. (2016)
Selenium species	Sunflower oil	Selol was dissolved in isopropanol. The polar selenium species were recovered from the mixture using an extraction method consisting of partitioning the species between hexane and 90% (v/v) methanol. Chromatographic separation was required before selenium species ESI MS characterization. The HPLC method was optimized using ICP-MS.	HPLC-ICP-MS/ESI-MS	5 μg L^{-1}	Bierla et al. (2016)
Total Se, soluble Se content, protein Se content, Se-MetSeCys	Mung bean sprouts	Total selenium was determined after microwave-assisted digestion with concentrated nitric acid. Soluble Se contents were determined in samples after extraction with NaOH, centrifugation, and microwave digestion with nitric acid. Protein Se contents were determined in samples after extraction with NaOH, centrifugation, adjustment to the pH of the isoelectric point, and microwave-assisted digestion with nitric acid. Se-MetSeCys was determined after isolation of the protein precipitate.	GFAAS was used to determine the total Se content, soluble Se content and protein Se content. Se-MetSeCys was determined by HPLC-MS.	Total Se: 1.2 μg L^{-1} Se-MetSeCys: 0.25 μg L^{-1}	Tie et al. (2016)

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
Se(IV), Se(VI), SeMet, SeCys, and glutathione peroxidase (GPx)	Roots and shoots of chickpea plants	Total selenium was determined after digestion with concentrated nitric acid. Soluble Se species were extracted by enzymatic hydrolysis. HPLC system was connected to an anion exchange pre-column and an analytical column for species separation. Size exclusion chromatography was used as the primary separation step to prepare mass-range-characterized SEC fractions for a 2-D approach for species identification.	Determination of total Se by ICP-AES and Se speciation by HPLC-ICP-DRC-MS	–	Lyubenova et al. (2015)
SeMet	Cape gooseberry	Extraction with sodium dodecyl sulfate (SDS) at a pH of 7.4 was used. Size exclusion chromatography was used as the separation step.	ICP-MS	–	Wojcieszek and Ruzik (2016)
Se(IV), Se(VI), and total Se	Milk, beer, red wine, tap water, and bottled water	Coprecipitation of Se(IV) using magnesium hydroxide. Total Se was determined after microwave-assisted reduction of Se(VI) to Se(IV) using concentrated HCl.	GFAAS	0.030 μg L^{-1}	Tuzen et al. (2007)
Se(IV), Se(VI), SeMet, and Se-MetSeCys	Wine, beer, yeast, and garlic	The complete separation was attained within 12 min using a C_8 column and a gradient performed with a mobile phase containing 0.1% (v/v) [C_6mim]Cl at pH 6.0.	RP-HPLC-HG-AFS	Se(IV): 0.92 μg L^{-1} Se(VI): 0.86 μg L^{-1} SeMet: 1.41 μg L^{-1} Se-MetSeCys: 1.19 μg L^{-1}	Castro Grijalba et al. (2017)
Se(IV), Se(VI), and total Se	Ice tea, soda, mineral water, beer, milk, red wine, mixed fruit juice, date, apple, orange, grapefruit, egg, and honey	Se(IV) and Se(VI) were determined after separation and preconcentration using ultrasound-assisted ionic liquid dispersive liquid-liquid microextraction. Total Se was determined after microwave-assisted reduction of Se(VI) to Se(IV) using concentrated HCl.	GFAAS	Se(IV): 150 ng L^{-1} Se(VI): 12 ng L^{-1}	Tuzen and Pekiner (2015)

(continued)

Table 12.6 (continued)

Analyte species	Matrix	Sample preparation[b]	Determination technique[b]	Limit of detection	Reference
Inorganic and organic Se	Rice	Sequential room-temperature ultrasound-assisted extraction with water and cyclohexane. Total Se was determined after sample digestion using HNO_3 and $HClO_4$.	GFAAS	Total Se: 0.08 μg L^{-1}	Sun et al. (2013)

SeOMet selenomethionine-Se-oxide, *MP* microwave plasma, *ESI* electrospray ionization, *DRC* dynamic reaction cell, *RP* reversed-phase

[a]Mainly inorganic forms Se(IV) and Se(VI) as well as total selenium and organic species such as selenomethionine and selenocysteine were determined

[b]In some cases, separation method is presented

[c]Limit of quantification

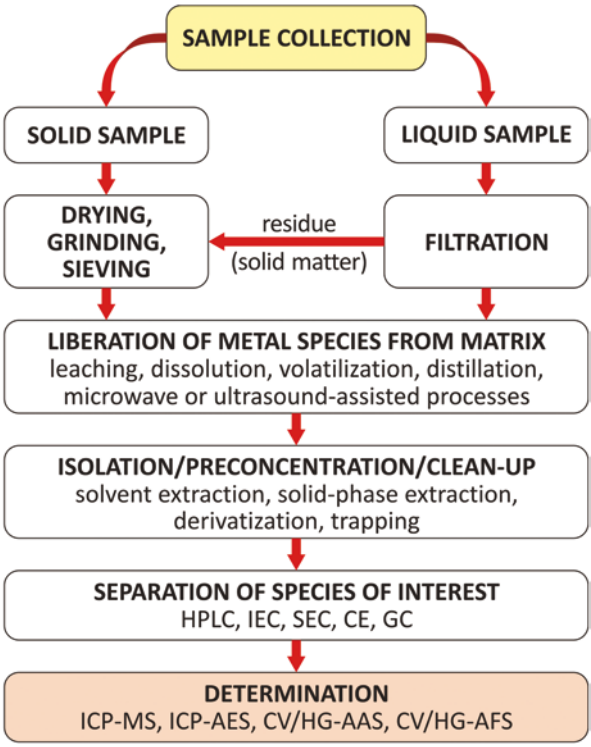

Fig. 12.2 General scheme of speciation analysis of food samples (the diagram does not include the sampling and sample storage stages). HPLC high-performance liquid chromatography, IEC ion-exchange chromatography, SEC size exclusion chromatography, CE capillary electrophoresis, GC gas chromatography, ICP -MS inductively coupled plasma mass spectrometry, AES atomic emission spectrometry, CV-AAS cold vapor atomic absorption spectrometry, HG hydride generation, AFS atomic fluorescence spectrometry

Sample Collection and Storage

The major problem arising in speciation analysis is to preserve the integrity of the tested object and the species of interest in the course of sample collection, storage, and preparation steps (Sadee et al. 2015; Gonzalvez et al. 2009). During these steps, difficulties may result from: unrepresentative samples collection, losses at the time of sampling and preparation steps, contamination, interconversion between species, inefficient sample preparation (e.g., extraction/isolation of the analyte), as well as effects from the sample containers and reagents used. Thus, procedures that result in a modification of equilibrium or a transformation of the different species must be avoided (Sadee et al. 2015; Gonzalvez et al. 2009). Additionally, the integrity of the elemental species throughout the analysis is highly dependent upon the nature of the species and sample matrix components may also affect the stability of the tested material (Apostoli et al. 2006). Microorganisms can participate in a range of element transformations including a change in valence (i.e., oxidation/reduction) or chemical form (i.e., solid, liquid, and gas). Many microorganisms (bacteria, fungi, and yeast) have the ability to biomethylation (e.g., arsenic) and as a result, volatile (e.g., methylarsines) and nonvolatile (e.g., monomethylarsonic acid and dimethylar-

sinic acid) species can be formed (Bentley and Chasteen 2002). The volatile ones may be lost during the collection and storage of the samples (Pizarro et al. 2003).

Biological samples should be kept at low temperatures as bacteria can degrade the integrity of the material. Too high storage temperature or drying procedure may cause major changes in equilibrium by converting fractions relevant to trace elements into highly unstable and reactive forms (Muñoz Olivas and Cámara 2003). For the stabilization of samples particularly, freeze-drying or lyophilization can be conducted, which tend to reduce analyte loss and species transformations (Apostoli et al. 2006). It is generally recommended storing biological objects at a temperature below 7 °C but only for a few days, and deep-freezing for longer periods. It is also important to be aware that elemental species in powders may also suffer from a lack of stability due to residual humidity (Apostoli et al. 2006). For many food samples, the long-term preservation of species can be guaranteed only when they are kept in the dark and at very low temperatures. To prevent microbiological activity over many years, the samples should be kept at below 130 °C. The other approach is to remove all the residual water, when temperatures of only 20 °C may be acceptable. Thus, it is necessary to know the stability of each elemental species in its particular matrix (Emons 2003). When no data are available from reliable studies, the effect of sampling, storage, and preparation conditions on the stability of the species in the sample should be studied (Apostoli et al. 2006).

Many species are thermodynamically unstable and therefore the simple act of sampling and storing of the sample may alter them (Apostoli et al. 2006). Hence only keeping the elemental species unaffected by the procedure both in composition and in concentration will enable obtaining reliable analytical information (Emons 2003).

Sample Preparation

The possible risk of species interconversion to other compounds can be minimized using an appropriate sample preparation procedure (Bouyssiere et al. 2003; Muñoz Olivas and Cámara 2003). Therefore, sample preparation is the most important and problematic step in the speciation analytical procedure. Foods, like other biological materials, are characterized by complex matrices, and direct analysis is difficult or often impossible. In many cases, it is necessary to extract species and isolate them from the material. The preparation of food samples depends on their nature: whether they are solid or liquid, on the concentration of fats as well as on the nature of analytes, the methodology used, and the final determination technique (Ruzik 2012).

Preparation of the sample for speciation analysis requires various unit processes, including mineralization or digestion of the sample, dissolution or leaching, removing or masking interfering substances, analyte extraction/isolation, analyte preconcentration, extract purification (clean-up) and derivatization of analytes. These processes can be carried out in a separable manner; however, there is a strong tendency to combine, at least some of them, into one operation (Khouzam et al.

2012). This approach reduces sample preparation time, the possibility of analytes loss by eliminating the transfer of solutions, and the risk of sample contamination, which is particularly important in the case of trace analysis. Figure 12.2 shows a general scheme of speciation analysis of food samples. This diagram was prepared based on a number of original publications (see Tables 12.4, 12.5, and 12.6) and reviews (Apostoli et al. 2006; Khouzam et al. 2012; Muñoz Olivas and Cámara 2003; Szpunar et al. 2004; Werner et al. 2018; Yu et al. 2019) regarding both methods of sample preparation for speciation analysis and determination of individual species in samples of various types. It should be noted that this is a very generalized scheme. Not all of the presented operations occur in every procedure, other work patterns than those presented, are also possible. Detailed examples of analytical procedures used in sample preparation before the determination of mercury, arsenic, and selenium species are provided in Tables 12.4, 12.5, and 12.6.

Separation of Analytes

Separation of analytes is an essential stage that distinguishes methodologies in the field of speciation analysis from the methods of total elements determination. Methods traditionally used for the identification and determination of trace elements do not allow for recognition between organic, inorganic, and other forms (Ruzik 2012).

For this purpose, a wide range of separation techniques, but primarily selected chromatographic techniques, are used (high-performance liquid chromatography (HPLC), ion-exchange chromatography (IEC), size exclusion chromatography (SEC), capillary electrophoresis (CE), and gas chromatography (GC)).

Liquid chromatography (LC) techniques are irreplaceable when high molecular weight, nonvolatile, or thermally unstable compounds need to be separated. This does not mean that the use of LC is limited only to this group of compounds; in some cases, liquid chromatography enables the analysis of samples without prior derivatization of analytes (Michalke 2002a, b).

Unfortunately, the limitation of the method using LC separation may be associated with the physical state of the sample that is required for the technique. This is because most detectors used in speciation analysis require analytes to be introduced in the form of vapor or aerosol. This entails the need to spray the eluent flowing out of the LC column, which may cause losses of analytes (Michalke 2002a, b). Additionally, many stationary phases used in LC as well as buffers or organic modifiers can change the nature of native species. Chelating eluents may cause the recomplexation of free or labile-bound metal species. The mobile phases can also cause severe alterations in the species (e.g., buffers can stabilize biomolecules but may also alter species equilibrium through complexing tendencies or can be a source of contamination) (Michalke 2003a; Ruzik 2012).

Ion exchange chromatography is used to separate free ions and easily ionizable species. IEC is a commonly used technique in speciation analyses because metallic species of interest frequently occur in the ionized form (Ackley and Caruso 2003).

Size exclusion chromatography is a commonly used separation technique for complexes with bioligands, where the complexes are separated based on the retention mechanism sufficient for the molecular weight of the analyte. Elements in food are complexed with various bioligands, and their bioaccessibility and bioavailability depend on the form of this complex. Therefore, understanding which element is complexed with each ligand is an important issue of speciation analysis. SEC techniques can only be recommended for fractionation of the element compounds, and they only provide information about groups of compounds (Ackley and Caruso 2003; Ruzik 2012). Additionally, the technique is used in elemental speciation analysis to study such issues as metalloproteins (Mason et al. 1990) and metabolites of metal-containing drugs (Matz et al. 1989). It may also be used to separate species of interest from interfering low molecular weight components of the sample matrix (e.g., interfering low molecular weight) (Ackley and Caruso 2003).

The application of capillary electrophoresis in speciation studies is wide. It may be used as a primary separation mechanism or as a secondary separation technique after, e.g., HPLC, in a second dimension for identification. The technique uses the separation principle of differences in the electrically driven mobility of charged analytes, similar to conventional electrophoresis. Using an electric field along an open tube column with a low inner diameter at high voltage (typically between 20 and 30 kV) enables relatively short analysis time and very high separation efficiency (Michalke 2003b).

Gas chromatography undoubtedly occupies an important place in the application among separation techniques for the needs of elemental speciation analysis. It is used in the determination of compounds that have appropriate volatility and thermal stability under the conditions of separation or can be carried out in this form. They mainly include alkyl and aryl metal derivatives of mercury, arsenic, and selenium (Bouyssiere et al. 2002; Lobinski and Adams 1997). The need for derivatization is related to the fact that analytes occurring in real samples are in most cases ionic compounds and also polar with very low thermal stability. In general, GC is inadequate to separate compounds of this nature. In the case of speciation analysis, this problem was solved by using various analytical derivatization approaches. As a result, nonpolar compounds with sufficient thermal stability are obtained, that are highly suitable for separation by GC (Bouyssiere et al. 2002; García Alonso and Ruiz Encinar 2003; Lobinski and Adams 1997).

The benefits of using gas chromatography in speciation analysis are primarily due to the ease of combining (hyphening) this technique with various systems for the determination and identification of the species. In contrast to LC, GC enables the introduction of the entire volume of the sample into the detection system (García Alonso and Ruiz Encinar 2003).

The Detection Systems

The final stage of the speciation analysis is the determination and (optional) identification of analytes. For this purpose, several spectroscopic techniques find the wide application, mainly atomic absorption spectrometry (AAS) and emission spectrometric techniques (inductively coupled plasma atomic emission spectrometry (ICP AES) and mass spectrometry (ICP-MS)).

ICP is a sensitive and selective detection system used in both the determination of organometallic compounds, as well as the total content of individual metals (Bouyssiere et al. 2002; Lobinski and Adams 1997; Vanhaecke 2003). ICP cannot be used for elemental speciation as a single technique, as all molecules introduced into the high-temperature ion source (an Ar ICP) are broken down into atoms, which are subsequently ionized. Therefore, elemental species of interest can be separated from one another before their introduction into the plasma by using selected separation techniques (HPLC, IEC, SEC, CE, and GC). In a hyphened system constructed this way, ICP can be used as a highly sensitive and specific on-line multielement detector (Vanhaecke 2003).

In addition, spectroscopic techniques (especially AAS) can be combined with the generation of volatile forms of selected elements. In the case of mercury, the technique of cold vapor generation (CV) is used, and in the case of other elements such as arsenic and selenium, the technique of volatile hydrides generation (HG) is often proposed (Ritsema et al. 1995; Tsalev 2000).

Due to lower sensitivity, the use of atomic absorption spectrometry is limited to combinations of GC-AAS and LC-AAS (Zhang and Zhang 2003). For some metals, there are specific detectors with high sensitivity, e.g., atomic fluorescence detector (AFS), successfully used in the determination of mercury, arsenic, and selenium compounds.

AAS with flame atomization (FAAS) has been successfully coupled to gas and liquid chromatography as the eluent from GC/LC can be easily introduced into the flame atomizer. The interface between chromatography and FAAS is very simple. However, FAAS could not provide the necessary sensitivity for element speciation analysis in most cases. Electrothermal atomic absorption spectrometry (ETAAS) combines high sensitivity with an extensive range of elements suitable for detection. Unfortunately, the sequential nature of the drying and pyrolysis steps before atomization makes it difficult to couple onto the continuous flow of the chromatography effluent (Zhang and Zhang 2003).

Detailed examples of detection systems used in the determination of mercury, arsenic, and selenium species are provided in Tables 12.4, 12.5, and 12.6.

Summary

Investigating the total content of elements is not enough to fully determine the effects – both positive and negative – that their presence may cause in food products. However, it is necessary to study the quantity and quality of species in which the element occurs in a tested material. The field of analytical chemistry dealing with this challenge is chemical speciation analysis. A fundamental tool for speciation analysis has become the combination of chromatographic separation techniques, with atomic spectrometry. The first step of the procedure ensures that the elemental species of interest leaves the column unaccompanied by other compounds and spectrometry enables sensitive and specific detection of the target elements. All the data received from the separation, determination, and identification of elemental species are important for the characterization of unknown compounds in food products. Additionally, recent impressive progress toward higher resolution in separation techniques and higher sensitivity in mass spectrometry for trace elemental species in complex matrices allows new frontiers to be crossed.

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Chapter 13
Two Sides of Selenium: Occurrence and Determination of Selenium Forms in Food and Environmental Samples Using Analytical Methods

Joanna Zembrzuska, Bożena Karbowska, and Iwona Gołębiewska

Abstract Historically, selenium has been considered a toxic element, suggesting its elimination from the diet. However, it has been recently discovered that it is a crucial trace element for human physiology, playing an important role in the metabolism, production of hormones, and functionality of the immune system. Among others, selenium has been found to exhibit protective effects in the etiology of cancer that are related to its activity against oxidative stress on cell membranes as well as the stabilizing effect on DNA and enhancing the cellular immune responses. Selenoproteins are involved in human metabolism, and so their deficiency causes degeneration of tissues and organs, resulting in an increased risk of various degenerative diseases. Bioavailability of selenium differs depending on the form it is supplied in, organic species being the ones that are absorbed to the highest extent in the human intestine. Therefore, a preferred supplementation method is through dietary routes, including the selenium-rich foods obtained through soil fertilization, livestock fodder fortification, or utilizing functional foods. This warrants an efficient analytical methodology for determining the selenium content and its chemical forms in the food products. The total, often trace amount of selenium, is usually determined by radiometric, electroanalytical, and spectroscopic methods. However, determining the speciations of selenium is an equally important task. Most analytical methods developed for selenium speciation analysis in food products focus on the determination of selenates(IV), selenates(VI), and selenoproteins. Among all the techniques used for selenium speciation analysis, liquid chromatography com-

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bined with mass spectrometry and excitation in inductively coupled plasma is undoubtedly characterized by the greatest analytical capabilities and the widest application.

Introduction

Selenium (Se) has been the object of scientific interest for many years. Although it was considered a toxic element by scientists for a long time, clinical studies conducted with this element have elucidated important information regarding its positive impact on human health. As a result, selenium was included as a trace element necessary for the proper functioning of the human body. Undoubtedly, it deserves to be called one of the most interesting elements necessary for maintaining health. Among other micronutrients, it is distinguished by a small difference between the toxic and therapeutic dose. Both an excess and a deficiency of selenium in the diet can be a cause of diseases. Ongoing studies are conducted in many research groups around the world, to obtain scientific confirmation regarding the effectiveness of chemical forms of selenium in preventing the formation and development of malignant tumors. Despite many scientific reports on this topic, the exact scope of this element's activity is still unknown.

Selenium: Characteristic of the Element

Selenium (Se) with an atomic mass of 78.96 u belongs to the group of chalcogens (the 16th group of the periodic table) and therefore has chemical properties similar to sulfur. Selenium speciation is a complex issue. It occurs in nature at oxidation levels ranging from −II, −I, 0, and + IV to +VI, in both inorganic and organic forms. It exists in solid, liquid, and gas phases, in the form of six stable isotopes, among which the isotope with a mass equal to 80 U is the most common (49.61%). Selenium is found in six allotropic forms, three amorphous forms, and three annular crystalline forms. Elemental selenium is characterized by a melting point of 494 K and a boiling point of 958 K (Tinggi 2003). The basic forms of selenium occurring in the environment and the human body are presented in Table 13.1.

Water-soluble selenite and selenate can be reduced to insoluble elemental selenium by nonspecific reduction using sulfates (Lenz et al. 2008; Tucker et al. 1998) or nitrates (Sabaty et al. 2001). Selenium oxyanions can be reduced to elemental selenium aerobically or microaerobically by various bacterial strains (Hunter 2007; Hunter and Kuykendall 2006). Formation of elemental selenium is desirable since the resulting forms are less soluble and thus less bioavailable (Combs et al. 1996; Fernández-Martínez and Charlet 2009). Insoluble elemental selenium can be converted into a mobile form by reoxidation to soluble oxyanions (mainly selenite)

Table 13.1 Common selenium compounds (Sentkowska 2019)

Name	Chemical formula
Selenium(IV) oxide	SeO_2
Selenate(IV) acid	H_2SeO_3
Selenate(VI) acid	H_2SeO_4
Sodium selenite(IV)	Na_2SeO_3
Sodium selenite(VI)	Na_2SeO_4
Hydrogen selenide	H_2Se
Diethyl selenide	CH_3-CH_2-Se-CH_2-CH_3
Dimethyl selenide	CH_3-Se-CH_3
Dimethyl diselenide	CH_3-Se-Se-CH_3
Dimethyl selenium sulfide	CH_3-Se-S-CH_3
Trimethyl selenium ion	$(CH_3)_3Se^+$
Dimethyl selenoxide	$(CH_3)_2SeO$
Selenocysteine	H-Se-CH_2-CH(NH_2)-COOH
Selenomethionine	CH_3-Se-$(CH_2)_2$-CH(NH_2)-COOH
Selenocystine	HOOC-(NH_2)CH-CH_2-Se-Se-CH_2-CH(NH_2)-COOH
Selenocystamine	NH_2-CH_2-CH_2-Se-Se-CH_2-CH_2-NH_2
Se-methylselenocysteine	CH_3-Se-CH_2-CH(NH_2)-COOH
Selenohomocysteine	H-Se-CH_2-CH_2-CH(NH_2)-COOH
Selenocystathionine	HOOC-CH(NH_2)-CH_2-Se-$(CH_2)_2$-CH(NH_2)-COOH

under aerobic conditions (Dowdle and Oremland 1998; Losi and Frankenberger Jr 1998; Sarathchandra and Watkinson 1981). Solubilization of elemental selenium can alternate based on a reduction to dissolved selenide (Herbel et al. 2003), which easily reacts with metal cations and forms strong metal selenium deposits (Seby et al. 2001).

Selenium is an essential element in organisms and is involved in intracellular redox homeostasis and thyroid hormone metabolism (USHHS 2003), similar to selenoproteins. Historically, glutathione peroxidase was the first identified enzyme containing selenium which protects the cell from oxidative damage (Flohe et al. 1973; Rotruck et al. 1973). Later, selenium was identified in different varieties of the selenium proteins, including at least 25 selenoproteins in humans (Papp 2007), but the function of many compounds belonging to this group remains unknown.

The Discovery of Selenium (the Origin of Selenium)

The medieval physician Paracelsus, considered the father of toxicology, once said, "All things are poison and nothing is without poison, only the dose permits something not to be poisonous" (Krieger 2001). Selenium is a particularly good example of this saying, as most living organisms require small amounts of selenium in food to stay healthy, while higher intake can cause illness and even death. Selenium was

discovered in 1817 by the Swedish chemist Jöns Jacob Berzelius and its name comes from the Greek goddess of the moon "Selene." Since the discovery of selenium, for the next hundred years there have been literature publications dealing mainly with inorganic selenium compounds found in nature in the form of minerals. In 1957, there was a breakthrough in terms of investigating the properties of the element, in which the relationship between selenium and the health of animal organisms was demonstrated (Hefnawy and Tortora-Perez 2010; Lopez et al. 2010; Rayman 2000; Tinggi 2003; Vinceti et al. 2020).

Selenium in Soils

Selenium is present in the soil at all basic oxidation levels: −II, 0, IV, and VI, which depends on humidity, free oxygen concentration, and pH, as well as the redox potential. Apart from the geochemical properties, the behavior of selenium compounds in the soil is also determined by the multivalence of the element. The transformation of inorganic selenium into organic forms is a very important process that occurs in soil. The dominant reaction in this environment is the biomethylation carried out in plants, fungi, bacteria, and other microorganisms. The volatile alkyl derivatives resulting from this process – dimethyl selenide $(CH_3)_2Se$ and dimethyl diselenide $(CH_3)_2Se_2$ – significantly impact the geochemical circulation of this element in nature. Selenates(VI) in the form of SeO_4^{2-}, which occur in an alkaline environment, constitute a thermodynamically stable group of compounds. They are well soluble in water, which results in easier leaching from the soil, transport to groundwater, and uptake by plants. Selenates(IV) occur under poorly oxidative conditions and can be reduced to the Se^0 elemental form. Selenides (Se^{2-}) present in the acidic environment are poorly mobile and difficult to access for plants. The organic matter associated with mud and clay fractions significantly affects selenium content, further influencing its mobility and bioavailability in the soil (Pezzarossa and Petruzzelli 2001; Tam et al. 1995; Wang and Gao 2001).

It is estimated that selenium is present in the earth's crust in the amount of 0.05–0.5 mg Se·kg^{-1} (Amoatey and Baawain 2019; Lemly 2004), with total selenium concentrations in rocks (mainly in sandstone, quartzite, and limestone) constituting 40% of the earth's crust (Wang and Gao 2001). It is reported that the global content of selenium in most soils (classified as poor in selenium) is equal to 0.4 mg Se·kg^{-1}, while in seleniferous soils (rich in selenium) this content may reach up to 1200 mg Se·kg^{-1}. Such selenium-rich soils occur in the USA, Canada, Columbia, Great Britain, China, and Russia (Fordyce 2005).

The average selenium content in low selenium soil in the Keshan area in China is equal to 0.121 mg·kg^{-1}, with a range of 0.059–0.19 mg·kg^{-1} (Sun et al. 1985; Guilherme et al. 2017). Other low values have also been reported globally: 0.198 mg·kg^{-1} and 0.23 mg·g^{-1} in former Yugoslavia (Jović 1998; Maksimovic et al. 1992) and less than 0.1 mg·kg^{-1} in New Zealand, Hungary, and Finland (Westermarck et al. 1977). The low selenium content in the soil in the Al-Kharj region (Saudi

Arabia) can be attributed to the nature of the soil in the study area, which is well-drained, with a predominance of limestone and clays with a sandy top layer (Al-Sahel Mphil et al. 2009).

Selenium is bound to natural sulfides, such as pyrite, chalcopyrite, and sphalerite (Wiberg et al. 2001). However, selenium ore deposits have no economic significance (Butterman and Brown 2004). Selenium is also present in high sulfur carbons. Although the world average is equal to only 1.6 and 1.0 mg Se kg^{-1} (hard and brown coals, respectively) (Yudovich and Ketris 2006), in the USA, Russia, and China there are regions of increased selenium content in carbons up to 43 mg Se kg^{-1}. Furthermore, black slate and volcanic tuff may contain high concentrations of 22 and 32 mg kg^{-1} of selenium in the Daba region, China (Kunli et al. 2004).

Selenium in Surface Waters

Selenium occurs in natural waters in the following forms: H_2SeO_3, $HSeO_3^-$, SeO_3^{2-}, $HSeO_4^-$, or SeO_4^{2-}. Its content ranges from 0.1 to 60 ng·L^{-1} for ocean waters and from 0.1 to 400 μg·L^{-1} for surface and groundwaters. According to the World Health Organization (WHO), the acceptable content of Se in drinking water is 10 μg·L^{-1} (Barceloux 1999). In natural water, this element is found in trace amounts as a result of weathering of minerals, soil erosion, and volcanic activity. Its levels vary from region to region but are usually below 10 ng·mL^{-1}. Surface waters can absorb selenium from the atmosphere by dry and wet deposition, from adjacent waters that may contain selenium, from surface runoff, and from subsurface drainage. In the study regarding direct discharges from an oil refinery in the San Francisco Bay, the average concentration of selenium in wastewater was equal to 0.067 mg·L^{-1} with a range of 0.0066–0.156 mg·L^{-1} (Barceloux 1999; Cutter 1989). Approximately 50–76% of total selenium in the wastewater occurred in the form of selenate(IV) (Cutter, 1989).

The most oxidized forms of selenate(VI) and selenate(IV) found in surface waters exhibit high bioavailability and a tendency for bioaccumulation (Dungan and Frankenberger Jr. 1999).

Selenium in Plants

Although selenium is not considered as an essential plant nutrient, more selenium species have been identified in plants than in animals, e.g., inorganic compounds such as selenate Se(VI) and selenite Se(IV), as well as organic forms such as selenomethionine (SeMet), selenocysteine (SeCys), Se-methyl-selenocysteine (Se-methyl-SeCys), γ-glutamyl-Se-methyl-selenocysteine (γ-glutamyl-Se-methyl-SeCys), and selenoproteins. Some plants also volatilize selenium as dimethyl selenide or dimethyl diselenide (Aureli et al. 2012; Ogra et al. 2007; Torres et al. 2016).

The concentration of selenium in plants depends on the region and soils used to cultivate the plants. Plants can also absorb volatile selenium from the atmosphere. In species sensitive to selenium, the concentration threshold of this metalloid in shoot tissues ranges from 2 mg kg^{-1} dry mass (d.m.) in rice to 330 $mg \cdot kg^{-1}$ d.m. in white clover. Selenium hyperaccumulators can tolerate concentrations exceeding even 4000 $mg \cdot kg^{-1}$ d.m. without any signs of adverse effects on their growth. Such hyperaccumulators include, among others, plants belonging to the *Compositae*, *Leguminosae*, *Cruiferae*, and *Allium* families (Wierzbicka et al. 2007; Ellis et al. 2003; Terry et al. 2000).

The majority of crops and grasses usually contains less than 25 $mg \cdot kg^{-1}$ d.m. of selenium and does not tend to accumulate this element in tissues in concentrations exceeding 100 $mg \cdot kg^{-1}$ d.m. even during growth on soils with a high content of this element.

The content of selenium in cereals and cereal products is usually in the range of 4–267 $\mu g \cdot kg^{-1}$ d.m. Potatoes contain approximately 0.43 $mg \cdot kg^{-1}$ d.m., tomatoes and onions 0.03 $mg \cdot kg^{-1}$ d.m, carrots approximately 0.40 $mg \cdot kg^{-1}$ d.m., broccoli approximately 1.0 $mg \cdot kg^{-1}$ d.m., cauliflower 0.44 $mg \cdot kg^{-1}$ d.m., cabbage 0.72 $mg \cdot kg^{-1}$ d.m., and lettuce 0.36 $mg \cdot kg^{-1}$ d.m. In case of legumes, the content ranges as follows: soybean 435 $\mu g \cdot kg^{-1}$ d.m, peas 1345 $\mu g \cdot kg^{-1}$ d.m, and beans 938 $\mu g \cdot kg^{-1}$ d.m. Fruits (e.g., pears, apples, and plums) contain up to several $\mu g \cdot kg^{-1}$ d.m.

Various types of nuts were also analyzed for selenium content; lower levels were found in cashews (*Anacardium occidentale* – 0.27 $mg \cdot kg^{-1}$ d.m.), walnuts (*Juglans regia* – 0.03 $mg \cdot kg^{-1}$ d.m.), hazelnuts (*Corylus avellana* – 0.02 $mg \cdot kg^{-1}$ d.m.), peanuts (*Arachis hypogea* – 0.04 $mg \cdot kg^{-1}$ d.m.), pecans (*Carya pecan* – $mg \cdot kg^{-1}$ d.m.), and macadamia nuts (*Macadamia whelanii* – 0.07 $mg \cdot kg^{-1}$ d.m) (Barclay et al. 1995; Ihnat 1989; Pennington et al. 1995).

It is worth emphasizing that the differences in selenium content are not only species-based but also population-based. They depend on the area where a given plant species grows.

Selenium in Fish and Bird Organisms

In the aquatic environment, selenium is a particular threat to wildlife. Bioaccumulation (increased concentration in the body compared to the surrounding environment) and biomagnification (increasing concentration due to chain transfer) increase the risk of toxic selenium forms and are a threat to the wildlife. As a result, selenium concentration in the tissues of lower invertebrates or fish can reach levels up to 2000 times higher than the selenium concentration in water (Wu 2004). It has been shown that adverse effects on fish can occur at a selenium concentration in water of 5 $\mu g \cdot L^{-1}$, (Frankenberger et al. 2004; Hamilton 2004). The most thoroughly studied case of selenium contamination in wildlife took place at the National Wildlife Refuge Kesterson Reservoir, California, USA (Hamilton 2004; Presser and Luoma

2007), where selenium-rich subsurface drainage water entered the ponds in the wildlife reserve. In some acid leachates, concentrations were as high as 4200 μg $Se \cdot L^{-1}$ (Kharaka et al. 1996; Stefaniak et al. 2018). Contamination with selenium caused the death of many fish and waterbird populations. Also, developmental abnormalities were found in nesting birds in 20% of nests, whereas more than 40% contained one or more dead embryos (Ohlendorf 2002). Selenium caused deformation and reduced the survival of various fish species, e.g., bluefin larvae (*Lepomis macrochiros*).

Impact of Selenium on Human Health

Selenium in the Human Diet

Selenium has been reported to play an important role in human and animal nutrition as one of the antioxidant microelements. However, awareness regarding the importance of this element is scarce. The sources of selenium in food are often characterized with high protein content and include Brazilian nuts (up to 6.86 $\mu g \cdot g^{-1}$), milk products (up to 0.55 $\mu g \cdot g^{-1}$), or beef (up to 0.47 $\mu g \cdot g^{-1}$) (Fairweather-Tait et al. 2010; Ip et al. 2000; Smrkolj et al. 2005). Garlic and onions contain up to 0.50 $\mu g \cdot g^{-1}$ of selenium, making them a good source of this element without the high protein content. Selenium is present in these vegetables in the form of γ-glutamyl-Se-methylselenocysteine or Se-methylselenocysteine (Finley 2005).

Selenium plays an important role in human physiology. It participates in the metabolism, production of hormones, and functionality of the immune system. It is involved in cell growth and in modulating the action of transcription factors and cell signaling systems. It has been reported that appropriate selenium intake prevents diabetes, infertility, cancer, and cardiovascular diseases (Hendrickx et al. 2013). The functioning of the endocrine system and healthy inflammatory responses depend on the correct supply of this element (Ruseva et al. 2013). Furthermore, selenium exhibits protective activity against the toxic effects of metals such as lead, cadmium, arsenic, mercury, and some organic compounds (Rosen and Liu 2009).

Selenium is crucial for the correct functioning of the thyroid gland (along with iodine), as it is involved in the deiodination of thyroxine (T4) to triiodothyronine (T3) through the selenoprotein enzyme – iodothyronine deiodinase. Thus, selenium deficiency results in impaired iodine removal and, in turn, dysfunctions of the thyroid gland (Rosen and Liu 2009). Also, this element is also one of the neurotransmitters crucial for the correct functioning of the central nervous system (Lipinski 2015; Rayman 2012).

Selenoprotein P (SEPP1) is involved in the protection of the organism against free radicals and the damage they cause. Furthermore, SEPP1 is a heavy metal chelator, which forms nontoxic selenium–metal complexes (Pappa et al. 2006; Rayman 2012). Other proteins have also been identified to take part in important biological

processes: selenoprotein W is involved in muscle metabolism (Holben and Smith 1999), selenoprotein S in control of redox balance in cells, and selenoprotein R in probable antioxidant function (Brozmanová et al. 2010; Dokoupilová et al. 2007; Papp et al. 2007; Rosen and Liu 2009).

Selenium is also crucial for immune system regulation (Ruseva et al. 2013). It stimulates the immune system to increase the production of antibodies (e.g., IgG and IgM) and increases the activity of T cells and macrophages (Drutel et al. 2013). It acts in synergy with vitamin E, contributing to the limitation of the aging process and aiding cell regeneration. This microelement has been linked to inhibition of the progression of HIV infection into AIDS (Kamwesiga et al. 2011). Selenium also exhibits antibacterial and antiviral properties and alleviates the course of disease in patients infected with hepatitis, including hepatitis A (HAV) and hepatitis E (HEV) (Szucik et al. 2014), in addition to its protective properties against hepatitis B and C (Rayman 2012).

When supplied in the diet, approximately 85–95% of selenium quantity in food is absorbed in the intestine. However, the bioavailability of selenium differs depending on the form it is supplied in. Organic selenium compounds are absorbed with the extent of 90–95%, while inorganic compounds are only accessible at 10%. Immediately after entering into the bloodstream, selenium is bound by red blood cells, albumins, and globulins of the blood serum and is further transported to the tissues. It can also penetrate the placenta. The highest amounts of this element are found in the skeletal muscles, liver, renal cortex, pancreas, thyroid gland, pituitary gland, and testis, but selenium also accumulates in hair and nails (Kieliszek and Błażejak 2016).

As selenium absorption depends on the form in which it is being consumed, the best way of supplying the correct amount is through a proper diet. Enrichment of food with compounds containing selenium may be conducted through fertilization of soils with selenium compounds in order to obtain plants enriched with this element or through the enrichment of fodder with selenium compounds to obtain, e.g., selenium-rich eggs. Enrichment of soil or fodder represents an indirect method of selenium supplementation. Soil fertilization with selenium compounds is referred to as “biofortification” and presents one of the most efficient methods of resolving the societal issue of selenium deficiency. In contrast, a direct method of dietary supplementation with selenium is based on the intake of dietary supplements which constitute a source of this element (Kieliszek and Błażejak 2013; Mehdi et al. 2013; Ogawa-Wong et al. 2016; Ramos et al. 2010).

As the significance of the concept of functional foods is currently increasing, the use of microorganisms for selenium accumulation has been explored (Kieliszek et al. 2016). Through this method, it is possible to introduce increased amounts of selenium into grain products such as baker’s goods, produced using sourdough with the addition of bacteria and yeasts enriched with selenium (Stabnikova et al. 2008). Selenium yeasts are effective and safe sources of selenium in its most bioavailable form (selenomethionine), and its absorption is enhanced by vitamins present in the yeast biomass (mainly vitamins B and E) (McSheehy et al. 2006). The accumulation and retention of selenium originating from selenium yeast by the human organism

are estimated at between 75% and 90% (Dumont et al. 2006b; Gaikwad and Rajurkar 2016).

Currently, WHO recommends selenium intake of 70 μg day^{-1}, whereas in most European countries the dietary intake ranges between 30 and 50 μg day^{-1} (Kieliszek and Błażejak 2013). Selenium toxicity is estimated to start at an intake level of 400 μg day^{-1}. Consuming high doses of selenium can result in adverse health effects such as hair loss, diarrhea, and emesis (Fordyce 2007).

Protective Properties of Selenium Against Cancer Development

The anticancer properties of selenium are mainly linked to its antioxidant activity. In addition to the free radical scavenging function of selenium, the significant impact of this element on the cytotoxic activity of natural killer (NK) cells can be highlighted in the activity against tumor development (Rayman 2012). Clinical studies have shown that selenium may also protect against the occurrence of prostate, lung, and colorectal cancers (Brozmanová et al. 2010). In turn, decreased amounts of selenium in blood plasma can result in becoming more prone to cell damage and thus to the occurrence of certain cancer diseases. When comparing the individuals suffering from lung, prostate, liver, and stomach cancers to healthy individuals, it has been found that the level of selenium content in plasma of the cancer patients was decreased in comparison to healthy individuals (Wasowicz et al. 2003). Furthermore, a correlation between geographical differences of selenium content in the soil, the element consumption in the diet, and mortality related to cancer of various organs has been reported (Jönsson-Videsäter et al. 2004).

The presence of selenium as a selenocysteine residual in the four active centers of the GSH-Px (glutathione peroxidase enzyme), as one of the principal antioxidant systems in the organism, was identified, which suggests that a deficiency of this element would result in an impairment of the GSH-Px activity. The main action of this enzyme is to catalyze the reduction of the organic and inorganic hydroperoxides produced during the oxidative stress of phospholipids in the membrane and metabolic oxidation of the xenobiotics (Tato Rocha et al. 1994). Efficient removal of the free radicals maintains the integrity of membranes, therefore reducing the risk of cancer and slowing the aging process (Chan et al. 1998; Juhasze-Toth and Csapo 2018). Tissues with high metabolic activity, such as liver, heart, diaphragm, and striated muscle, are highly vulnerable to oxidative stress. This fact explains the selenium deficiency in cases of hemolysis, hepatic necrosis, or impairment of the immune and inflammatory function (Tato Rocha et al. 1994). Therefore, this element has high importance for the prevention of the cellular injury associated with these diseases, because an impairment in the GSH-Px activity cannot be compensated with other non-Se-dependent antioxidant systems.

Based on current research, selenium compounds such as methylselenocysteine (MeSeCys) and γ-glutamyl derivatives were identified as agents exhibiting the highest anticancer activity (Szucik et al. 2014). Se-methylselenocysteine is an active

compound detected in selenium yeast cells. However, it is known that the anticancer effect of methylselenocysteine depends on the expression of β-lyase. It has been observed that MeSeCys supplementation can significantly reduce the incidence of metastasis and tumors in the lungs can result in the reduction of tumor size in mice. Supplementation with a mixture of soy proteins containing high selenium amounts has demonstrated similar results (Chen et al. 2013).

As already mentioned, the protective effects of selenium in the etiology of cancer diseases are related to its activity against oxidative stress on cell membranes as well as the stabilizing effect on DNA and enhancing the cellular immune responses (Kieliszek and Błażejak 2013; Stabnikova et al. 2008). It has also been found that selenium inhibits tumor cell proliferation via the effect exerted on the expression of p53 tumor suppressor gene and Bcl-2 apoptosis suppressor gene (Zablocka and Biernat 2010). However, the anticarcinogenic effect of selenium depends on the chemical form of the element administered, its dosage, and type of agent which induces the development of cancer (Gromadzińska et al. 2008; Venza et al. 2015; Zachara 2015). Although administered selenium compounds exhibit different protective effects, they are metabolized to a final product (methylselenol) which exerts the most potent anticarcinogenic effect (Lavu et al. 2016; Zeng et al. 2009).

According to the Nutritional Prevention of Cancer indications, a dose of 200 μg Se·day^{-1} in the form of selenium yeasts decreases the risk of stomach, colon, rectal, prostate, and lung cancers (Hoffmann and Berry 2008; Lavu et al. 2016). Some reports have been carried out regarding the selenium-enriched broccoli, which was effective in inhibiting the formation of colon tumors (Gong et al. 2012).

Kenfield et al. (2015) have reported that supplementing selenium at doses higher than 140 μg·day^{-1} may increase the risk of death in metastatic prostate cancer patients, which warrants caution in administering the course of treatment for this group (Kenfield et al. 2015). In contrast, the studies presented by Heras et al. (2011) have shown that selenium supplementation at 200 μg·g^{-1} prevents the occurrence of high-grade tumors. The 15 kDa selenoprotein (Sep15) and TrxR1 (thioredoxin reductase 1) proteins are of particular importance (Heras et al. 2011).

Selenium Deficiency

Selenium deficiency is a socially important dietary aspect as it has been linked to some of civilization disease prevention, and a diet lacking in trace amounts of this microelement can cause a long list of health issues. This is especially true for individuals with dietary difficulties, such as phenylketonuria (Alves et al. 2012). As selenoproteins are involved in human metabolism, their deficiency causes degeneration of tissues and organs (Pedrero and Madrid 2009). Most health issues are related to joint and muscle tissues (including cardiovascular and reproductive degeneration), but can also affect the nervous system (Kryczyk and Zagrodzki 2013). Examples of selenium deficiency-related conditions include the Keshan disease (dilated cardiomyopathy) or Kashin–Beck disease (endemic osteoarthropathy)

(Pedrero and Madrid 2009). The Kashin–Beck disease can cause rheumatoid arthritis and growth disorders as well as bone and cartilage damage, leading to necrosis. Other conditions caused by selenium deficiency can include increased risk of asthma (related to impaired activity of glutathione peroxidase) or inducing AIDS progression. Selenium deficiency has also been positively correlated with sudden infant death syndrome (SIDS) (Navarro-Alarcon and López-Martınez 2000; Patelski and Dziekonska 2012; Vinceti et al. 2018).

Toxicity of Selenium

Adverse effects of consuming excessive amounts of selenium have been noticed in the first half of the twentieth century (Khanal and Knight 2010). It has been reported that inorganic forms of selenium have higher toxicity compared to the organic forms (Thiry et al. 2012).

Acute excessive consumption of selenium compounds can lead to various symptoms from the digestive system (including diarrhea and vomiting) and also neurological disorders (Fordyce 2007; Navarro-Alarcon and López-Martınez 2000; Vinceti et al. 2018). Selenosis, chronic selenium overdosing, manifests as hair loss, infertility, digestive and nervous system issues, and thyroid and liver dysfunctions. Certain hematological abnormalities have also been associated with selenium toxicity. Severe selenium toxicity level has been established at 2 μg per g of serum (Khanal and Knight 2010; Li et al. 2012). The toxicity mechanism has been attributed to the DNA damage by the free radicals generated when selenium is overdosed, which corresponds to impaired functions of produced proteins (Letavayová et al. 2008). Symptoms of selenosis have been observed in individuals consuming over 850 ug·day^{-1} (Mistry et al. 2012), and in patients supplementing this microelement at a dose of 600 μg·day^{-1} to aid rheumatoid arthritis, improvements in their treatment were observed with no signs of selenosis (Rayman 2004).

Methods for Determining Selenium Forms in Biological Materials

Speciation analytics is currently one of the most rapidly growing branches of analytical chemistry. It is of high importance because the proper functioning of living organisms is affected by the form in which the element occurs and not its total content. Therefore, it plays an important role in clinical analysis, toxicity determination, quality control of pharmaceuticals and food products, health risk assessment, and study of biochemical cycles of chemical compounds.

Interest in selenium speciation is constantly increasing due to the properties of selenium compounds that can be both toxic and necessary for the proper function-

ing of the human body (Cuderman and Stibilj 2008). Understanding the chemical forms of selenium in plants seems particularly important because of the possibility of using them, after prior enrichment, as a source of dietary supplementation with this element. Selenium-enriched yeast, garlic, onion as well as nuts and mushrooms are used to study selenium speciation.

In the case of speciation analysis, it is important to know the total content of an element and its chemical forms. This approach forces the use of separate procedures that provide an answer regarding the total content of Se and its chemical forms. These procedures differ in terms of sample preparation as well as in the selection of the analytical technique for the determination of the analyte. If the determination of the total selenium content is the goal, it is important to prepare the sample in a form in which the determination is possible. For solid samples, mineralization of the samples is necessary, which leads to its complete dissolution. If the determination of the chemical forms during sample preparation is intended, the employed methods should not affect the chemical forms of the tested element. The extraction procedures are most commonly used for this purpose. The procedure for preparing the material for the Se speciation is shown in Fig. 13.1.

Preparation of a Sample of Plant Material for Speciation Analysis

Mineralization – Determination of the Total Se Content

Determination of the total content of an element in the tested material requires its full mineralization. Food samples are wet mineralized in a closed system, most often in a mixture of HNO_3 and H_2O_2 (Denovics et al. 2002; Hsieh and Jiang 2013; Krawczyk-Coda 2019).

Extraction – Determination of Selenium Chemical Forms

Three extraction procedures are most often used for the preparation of samples in which the chemical forms of selenium are determined: extraction with water (or other solvents), enzymatic hydrolysis, and sequential extraction (Zembrzuska et al. 2014).

Water extraction is a frequently used technique for separating selenium compounds from food, usually with ultrasound-assisted hot water (Gosetti et al. 2007). This technique can be used to separate inorganic forms of selenium and water-soluble organic forms of this element (mainly selenoproteins). Water extraction is not a very efficient technique as only 10% of selenium can be isolated (Wróbel et al. 2004).

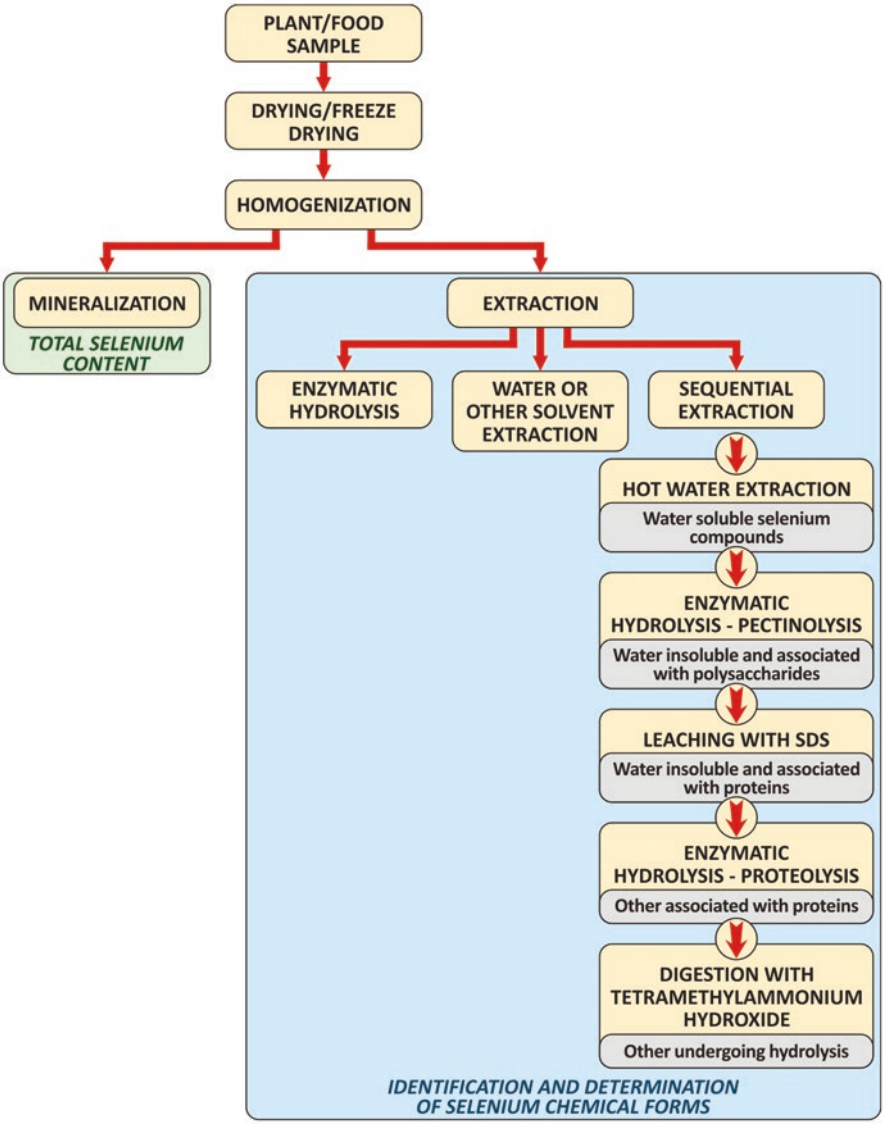

Fig. 13.1 The procedure for preparing the material for the Se speciation

Very often, the extraction of selenium compounds from biological materials is carried out with the use of a surfactant – sodium dodecyl sulfate (SDS), which denatures proteins and is used to elute selenium proteins from animal tissues (Wang et al. 2009; Wróbel et al. 2004).

Aside from water, many solutions can be used in the classic extraction of selenium compounds, such as hydrochloric acid (Cuderman et al. 2010; Montes-Bayon

et al. 2002), tetramethylammonium hydroxide, and methanesulfonic acid (Wang et al. 2009).

In case when selenium is incorporated into protein structures, it is necessary to use enzymatic extraction to isolate selenium compounds, which is based on enzymatic hydrolysis using proteolytic enzymes (Encinar et al. 2003; Vale et al. 2010). Proper selection of enzymes, pH of the hydrolyzed solution, and temperature is important for successful enzymatic extraction (Wang et al. 2009). The specificity of the enzyme plays an important role. Several specific and nonspecific enzymes can be used. Protein-cleaving protease is one of the most commonly used nonspecific enzymes (Casiot et al. 1999; Zou et al. 2018). Goenaga-Infante et al. (2008) used proteases and lipases to determine selenomethionine in pharmaceutical yeast tablets. The authors employed the catalysis for the hydrolysis of ester bonds in water-insoluble lipid substrates. Protease XIV and proteinase K are the enzymes most commonly used to extract selenium from plants, which were used to extract selenium compounds from edible fungi (Stefánka et al. 2001, Denovics et al. 2002). Proteinase K, aminopeptidase, and carboxypeptidase Y have been successfully used to extract selenium compounds from yeast (Wang et al. 2009).

The complementary separation of five basic fractions of selenium compounds from biological materials is possible through the use of sequential extraction. This method, which included five stages, was proposed by Encinar et al. (2003) to isolate the chemical forms of selenium present in yeast. The fractionation resulted in the isolation of selenium compounds: (1) water-soluble (hot water extraction), (2) water-insoluble and associated with polysaccharides (enzymatic hydrolysis – pectinolysis), (3) water-insoluble and associated with proteins (leaching with SDS), (4) other, associated with proteins (enzymatic hydrolysis – proteolysis), and (5) other, undergoing hydrolysis (digestion with tetramethylammonium hydroxide).

Total Selenium Determination

The total, often trace amount of selenium is usually determined by radiometric, electroanalytical, and spectroscopic methods. The most commonly used radiometric method is neutron activation analysis (NAA). This is one of the most sensitive methods of elemental analysis. In this technique, the sample is irradiated in the first stage in a reactor, followed by gamma radiation measurement. The advantages of this technique include no mineralization stage of the sample and no need for sample sacrifice. NAA has been used to determine the total selenium content of dietary supplements (Zembrzuska et al. 2014). Voltammetry is the most commonly used electroanalytical method. There are reports regarding the use of electrochemical methods to determine trace amounts of selenium, e.g., in mushrooms (Piech et al. 2014); however, these methods are very susceptible to matrix effects. Hence, their use in the study of complex environmental samples is very limited. The last group of methods used to determine the total amount of selenium includes spectroscopic methods, which rely on molecular spectrometry and atomic spectrometry. Molecular

spectrometry is based on the relationship between absorbance and the concentration of a colored substance in a solution. A suitable reagent is added to the sample to form colored complexes with the test element. The formation of colored complexes, e.g., with 2,3-diaminonaphthalene, is often used for the determination of selenium. Selenium in feed and cereal mixtures was determined using this method (Ramachandran and Kumar 1996). Due to the selectivity of molecular spectrometry, spectrophotometric methods can be used to selectively determine the content of Se(IV) in the presence of Se(VI). Molecular spectrometry methods are commonly used for the determination of selenium, although their sensitivity is often too low to carry out trace testing.

The following atomic spectrometry methods are widely used for trace amounts of selenium: flame atomic absorption spectrometry (FAAS), electrothermal atomic absorption spectrometry (ETAAS) (Méndez et al. 2002), high-resolution continuum source graphite furnace atomic absorption spectrometry (HR-CS GFAAS) (Krawczyk-Coda 2019), atomic fluorescence spectrometry (AFS) (Stefánka et al. 2001), optical emission spectrometry (OES), and mass spectrometry (MS). In spectroscopic techniques, the atomization process occurs under the influence of high flame temperature, electrothermal atomizer, or plasma, most often inductively coupled plasma (ICP). In most cases, the sample is introduced into the atomizer as a solution. Selenium can also be initially separated from the solution by a hydride generation (HG) and introduced into the atomizer in this form with a carrier gas (Tuzen et al. 2007).

The most common and also the most sensitive analytical technique is inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS allows for the determination of very low elemental contents, but in the case of selenium, the possibility of interference should also be taken into account (Gawor et al. 2020; Wysocka et al. 2003).

Determination of Chemical Forms of Selenium

Most analytical methods developed for selenium speciation in food products focus on the determination of selenates(IV), selenates(VI), and selenoproteins (Połeć-Pawlak et al. 2005). Selenium compounds are mainly separated by chromatographic methods (Yu et al. 2019). The research uses various variants of gas and liquid chromatography as well as electrophoretic methods. The selection of the separation technique primarily depends on the chemical and physical properties of the substances present in the sample.

Gas chromatography (GC) is mainly used for the separation of volatile alkyl selenides (Uden et al. 1998) and selenoproteins after the derivatization process (Pelaez et al. 2000). Gas chromatography–mass spectrometry (GC-MS) was used to identify selenium forms in yeast (Iscioglu and Henden 2004). Electron capture detector (ECD) and flame ionization detector (FID) are most often used for the detection of GC-separated forms of selenium.

Nevertheless, selenium compounds are separated by liquid chromatography rather than gas chromatography. The quantitative and qualitative determination of selenium forms described in the literature is mainly based on combined techniques. Among them, high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC-ICP-MS) is the most popular (Auger et al. 2004; Encinar et al. 2003; Gao et al. 2018; Gawor et al. 2020; Lipiec et al. 2010; Tsopelas et al. 2005; Wróbel et al. 2004). Due to the different forms of selenium present in the tested samples, chromatography with different separation mechanisms is used: size-exclusion (Acosta et al. 2018; Moreno et al. 2004; Pyrzyńska and Sentkowska 2019), anion or cation exchange (Cai et al. 1995; Chassaigne et al. 2002), reversed-phase (Do et al. 2001; Gao et al. 2018; Hsieh and Jiang 2013; Tsopelas et al. 2005; Zembrzuska et al. 2014), and hydrophilic interaction (Sentkowska and Pyrzynska 2018).

In addition to HPLC-ICP-MS technique, high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) is increasingly used to identify and quantify selenium compounds (Dumont et al. 2005; Dumont et al. 2006a; Gawor et al. 2020; Gosetti et al. 2007; Vu et al. 2018; Zembrzuska et al. 2014). The application of this technique allows to identify masses of apparent molecular ions of selenium compounds by searching in the mass spectrum of signal groups corresponding to the isotopic composition of selenium, confirm the structure of the determined compounds based on pseudomolecular ion fragmentation, and quantify them using the MRM mode (multiple reaction monitoring). HPLC-ESI-MS/MS was used to determine the chemical forms of selenium in dietary supplements (Dumont et al. 2005; Gosetti et al. 2007; Infante et al. 2005; Zembrzuska et al. 2014) as well as in onions (Sentkowska and Pyrzyńska 2018), yeast (Bierła et al. 2018), and brazil nuts (Dumont et al. 2006a).

The chemical forms of selenium separated on a column are sometimes subjected to atomic absorption spectrometry detection. In this case, the column fractions are first collected, and then the element content is tested. ETAAS (Do et al. 2001) or Hydride Generation Atomic Absorption Spectroscopy (HGAAS) (Marchante-Gayón et al. 1996) are the most frequently used detectors in such cases, due to the low detection limit of selenium and the low volume of solution needed for the determination.

In addition to chromatographic techniques, selenium compounds are also separated by electrophoretic techniques, primarily capillary electrophoresis (CE). The main advantages of this technique include low sample volume, low reagent consumption, short analysis time, and high separation efficiency. The combination of this technique with ICP-MS results in its increasing use for the study of selenium speciation (Kannamkumarath et al. 2002). Moinicou et al. (2002) used this technique to determine chemical forms in yeast. In addition to CE, isotachophoresis (ITP) is another electromigration technique used to determine the selenium (Grass et al. 2002). It was used to determine SeMet and selenocysteine in beer (Zembrzuska and Matusiewicz 2010).

Among all the techniques used for selenium speciation analysis, liquid chromatography combined with mass spectrometry and excitation in inductively coupled

plasma is undoubtedly characterized by the greatest analytical capabilities and the widest application. This technique is characterized by very high sensitivity and allows simultaneous multi-element determinations at the $ng \cdot L^{-1}$. The used analytical procedures provide detailed information regarding the chemical forms of selenium in the samples. Unfortunately, there are no procedures for testing selenium speciation which can be used routinely and widely. Speciation testing requires special treatment for each sample.

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