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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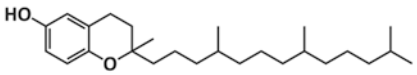
M. Jeszka-Skowron et al. (eds.), *Analytical Methods in the Determination of Bioactive Compounds and Elements in Food*, Food Bioactive Ingredients,
https://doi.org/10.1007/978-3-030-61879-7_6

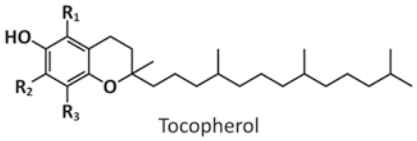
analytical point of view. For this reason, the preparation of samples for analysis is based on different methodologies for various foodstuffs.

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

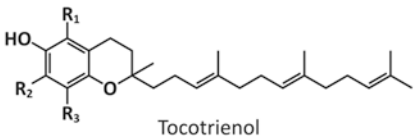


Tocol
2-methyl-2-(4`,8`,12`-trimethyltridecyl)chroman-6-ol



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

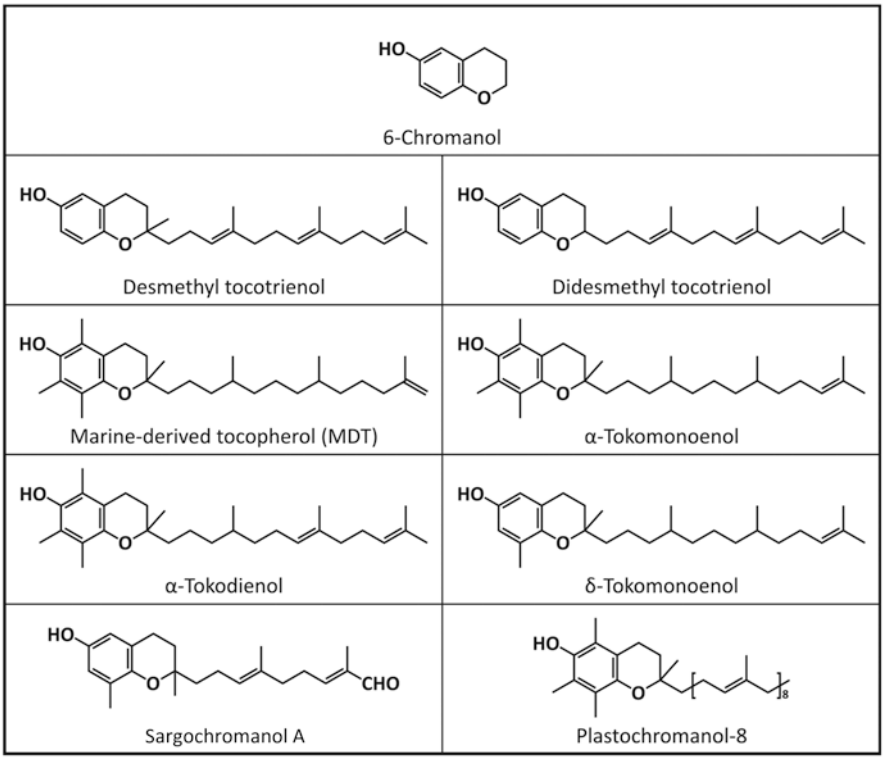


Fig. 6.2 Structures of other tocols

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

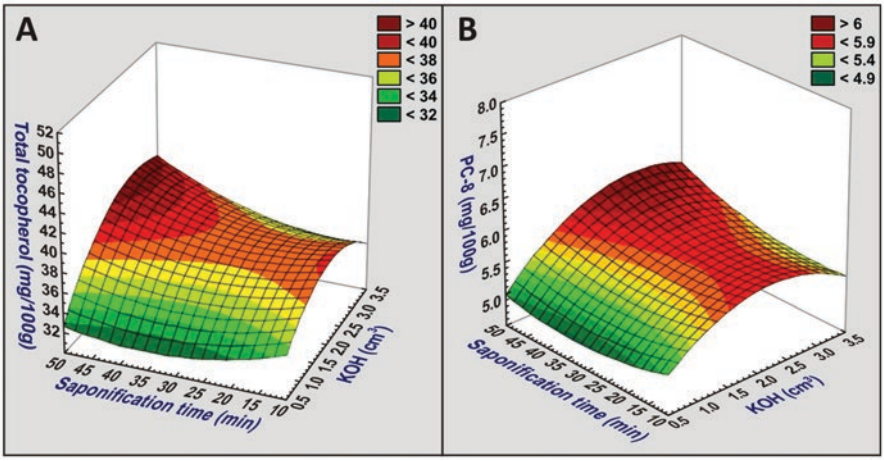


Fig. 6.3 Effect of 60% KOH concertation during saponification and saponification time on tocopherol and PC-8 contents (sample – seed of rape)

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

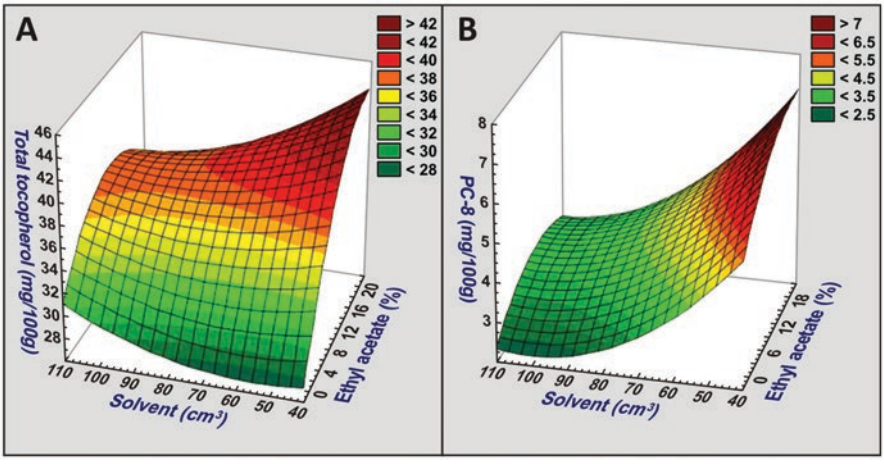


Fig. 6.4 Effect of the amount of solvent (*n*-hexane) and % share of ethyl acetate in the extraction mixture on the tocopherol and PC-8 contents (sample – seed of rape)

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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