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Review

Chromatographic analysis of α-tocopherol and related compounds in various matrices

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Abstract

Tocopherols and tocotrienols (Vitamin E) are part of a group of "minor components" of main interest, present in the unsaponifiable fraction of many samples. Their importance in biological, metabolical and nutritional studies makes determination of tocopherols and related compounds of major interest. Present work critically reviews the different ways to perform sample pre-treatment and analysis of these compounds, related to the matrices, other analytes to be measured, sensitivity, and simplicity. The review includes well referenced tables that provide in-depth summaries of methodology for the chromatographic analysis of α-tocopherol and related compounds in foods, pharmaceuticals, plants, animal tissues and other matrices.

Keywords: Reviews; Tocopherols; Vitamins; Tocotrienols

Contents

1. Introduction

Development of analytical methods which work in a more rapid, simple and reliable manner than the existing ones is one of the objectives of analytical chemistry. The progress of performance features of analytical tools has been impressive in the last 10 years or less. Comprehensive reviews on vitamin E analysis have already been done [1–8]. Present

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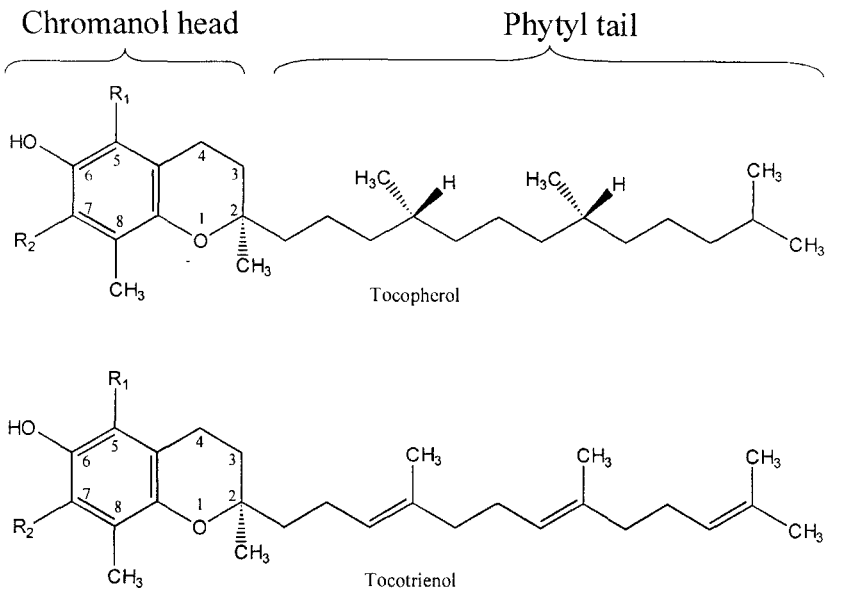
review focuses on discussing the contributions on the analysis of tocopherols and related substances mainly during the last decade, trying to give facts for those working in this area and trying to select an analytical method or to develop a new one.

Vitamin E is a term used to designate a family of related compounds (tocopherols and tocotrienols) which share a common structure, as shown in Fig. 1, with a chromanol head and a phytyl tail. They are named as a vitamin, because they cannot be synthesized by humans, and therefore must be obtained from diet. Vitamin E is found in fat products of vegetal origin, mainly oils. Each oil has different amounts of vitamin E, and the proportion of tocopherol or tocotrienol can be very different, too. The phytyl tail of tocopherols molecule has three quiral centers (carbons 2, 4′, and 8′), and organic synthesis from the reaction of trimethylhydroquinone with isophytol renders an enantiomeric equimolar mixture of the eight possible isomers, known as all *rac*-α-tocopherol. However in plants, specific enantiomeric synthesis occurs [9], and only RRR-forms



R_1	R_2	Compound
CH_3	CH_3	α
CH_3	H	β
H	CH_3	γ
H	H	δ

Fig. 1. Structure of tocopherols and tocotrienols.

are present for all the four possible tocopherols in samples from natural origin, with no extra vitamin E added.

Vitamin E is the natural most effective lipid soluble antioxidant [10,11], and the principal membrane antioxidant in mammalian cells, although antioxidant is not the only mechanism of action of this vitamin, because there are studies demonstrating activity of tocopherols or tocotrienols in cells and tissues that do not directly relate with antioxidant activity. Vitamin E decreases protein kinase C activity, and enhances both phospholipase A_2 and cyclooxigenase activity, and modulates several pathways involved in prevention of atherogenesis [12–14].

If oxidation of α-tocopherol occurs in the organism, a variety of compounds appears [15,16]. In case that such oxidation of tocopherol does not exceed 20%, the main oxidation product is α-tocopherolquinone (TQ), which could be reduced by cellular mechanisms to α-tocopherolhydroquinone (THQ) [17]. Other products of tocopherol reactions with free radicals, as 5,6-epoxy-α-tocopherolquinone (TQE1) and 2,3-epoxy-α-tocopherolquinone (TQE2) can also be formed [16].

A number of oxidative products of tocopherols have been detected in urine including 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman (α-CEHC) [18,19], metabolite of α-tocopherol, and similar metabolites of γ- and δ-tocopherol [20,21]. These compounds are derived from oxidation of the phytil side chain, while the chromanol structure remains intact indicating that α-CEHC is derived from α-tocopherol that has not reacted as an antioxidant and it may be used as an indicator of excess of α-tocopherol supply in the body. With such discovery it has been questioned wether "Simon metabolites" with an open ring system (α-tocopheronic acid and its lactone) [22] are a consequence of oxidation during sample preparation [23].

Different forms of α-tocopherol have different biological activity. These differences are not only due to their ability for free radical-quenching, but also to the specific affinity of α-tocopherol transfer protein (α-TTP), which is a protein that specifically incorporates α-tocopherol into lipoprotein particles during their assembly in the liver cells [24,25]. α-TTP may be responsible also for the different activities of the different stereoisomers of α-tocopherol. α-TTP has much higher affinity for the 2R forms, and thus synthetic α-tocopherol does not have the same biological activity as α-tocopherol from natural origin [26].

The biological activity of vitamin E in animals is defined by its influence on symptoms of deficiency, including neuropathy, fetal death, or myopathy (muscle disease), and is dependent upon distinct regulatory processes. RRR-α-tocopherol or d-α-tocopherol is a single stereoisomer and is derived from vegetable oils, primarily soybean, sunflower and corn oils. Synthetic vitamin E (all-*rac*-α-tocopherol or dl-α-tocopherol) is a mixture of eight stereoisomers in equal amounts. The other seven stereoisomers have different molecular configurations and lower biological activities that range from 21 to 90% of the activity of natural vitamin E based on rat assays. Vitamin E content is generally expressed by biological activity, using the scale of International Units (IU). By this system, 1 mg of d-α-tocopherol, biologically the most active of the naturally occurring forms of vitamin E, is equivalent to 1.49 IU vitamin E. The biological activity of 1 mg of dl-α-tocopheryl acetate, the synthesized form of vitamin E commonly used in food enrichment, is equivalent to 1 IU. Up until 1980, the recommended daily allowance (RDA) for vitamin E was expressed in IU. However, in 1980, the term tocopherol equivalents (TE) was used to express the RDA for vitamin E. One mg of d-α-tocopherol is equivalent to 1 TE. Other tocopherols and α-tocotrienol in the diet were assigned the following values: 1 mg β-tocopherol= 0.5 TE; 1 mg γ-tocopherol=0.1 TE, and 1 mg α-tocotrienol=0.3 TE.

Quantification of other tocopherols is important in some studies. γ-Tocopherol, in comparison to α-tocopherol, is more abundant in some diets, absorption in gut and intestines is the similar, its activity is 10–20% that of α-tocopherol, and its plasma levels are 5 to 10 times lower than those of α-tocopherol. In any case, γ-tocopherol or its metabolites may play a role in the protection of the body from damage by free radicals, because γ-tocopherol can be even more important than α-tocopherol in preventing pernicious effects of some specific radicals, as peroxynitrite and NO_x [27–29].

In addition, separation and characterization of

various forms of tocopherols are required to asses the impact of genetic modifications of oil seeds on their distribution.

There are also other synthetic esterified forms of α-tocopherol, obtained by esterifyng the hydroxyl group of the chromanol head with either acetate, nicotinate, succinate or phosphate. This ester molecules are more stable and less susceptible to oxidation than α-tocopherol. However, once ingested, the ester bond is easily split within the organism, and therefore these forms are used in medicine as sources of vitamin E for parenteral breeding, and are also used added to nutritional supplements, or to cosmetics (acetate). They are also used in the treatment of people with fat malabsorption, because of their higher water solubility (succinate, or synthetic vitamin E analogues derived from the non-phytil form of vitamin E, e.g. 2,2,5,7,8-pentamethyl-6-chromanol).

2. Vitamin E analysis

The first step for selecting the more adequate analytical method is to define the objective of the analysis and to know the sample matrix and complications related with it and with the analytes. The first question is then *which analytes*?, i.e., analyst should decide wether α-tocopherol determination is enough for the purpose of the experiment, or if some other tocopherols and tocotrienols, and/or analogues, derivatized forms, metabolites, etc. should be analysed, or even if structural information is also needed. The fact that other non-vitamin E compounds are to be measured is one of the critical points in every method, because this would conditionate every step of the analysis. In tissues, compounds co-analyzed with tocopherol are retinoids and carotenoids, coenzyme Q_{10} (ubiquinols and ubiquinones), and, in lesser extent, cholesterol, fatty acids an triacylglycerols. Tables 1–6 summarise the main characteristics of the methods currently in use. The different stages and outstanding contributions will be briefly discussed.

3. Sample treatment

Sample treatment is a critical step in the analytical process. Manipulation is time consuming, expensive and the main source of errors and, therefore, it should be kept at a minimum if possible. Nevertheless, the relative amount of tocopherols in the sample to be analyzed can be very low, and sometimes preconcentration and highly sensitive methods are required.

Except for vitamin E analysis in oils, which can be directly injected onto the HPLC system after dilution, vitamin E must be extracted from the sample matrix and concentrated in many cases. Vitamin E is not chemically bound to proteins, lipids or carbohydrates [12] and using harsh reagents and conditions to free it up (e.g. strong saponification) does not seem necessary and can destroy the vitamins. However, if adequate conditions are not used to release the vitamin from lipophylic millieu, recoveries will be poor, because vitamin E could be associated to other matrix components and must be freed up in the sample preparation steps.

Esters could need to be hydrolysed to the free form prior to the analysis and then total tocopherol content is measured, but in most cases, the same conditions could be applicable to the determination of free or acetate form (in normal-phase HPLC, in the same time of chromatography, and in reversed-phase HPLC, only some more min), as it was previously proposed [30].

Sample must be treated with some organic solvent, previous to or simultaneously with saponification or extraction process, in order to disrupt the structures where vitamin E can be associated to (membranes, lipoproteins, fat droplets . . .), to eliminate interferences from big molecules such as proteins or carbohydrates, that are non-soluble in organic phases, and to provide a medium in which analytes can be freely soluble. This organic solvent is almost always ethanol, but methanol is used too (see Tables 1–6). SDS (sodium dodecyl sulphate) as modifier for sample preparation previous to extraction was described by Burton et al. [31] and such an additive has been adopted by many researchers [32–39].

Sample treatment for vitamin E analysis often includes saponification, of the entire sample matrix, or of an isolated lipid fraction. Saponification prior to extraction is classically performed by heating with KOH, frequently in ethanol or methanol.

Following saponification, the unsaponifiable com-

Table 1
Summarized characteristics of the methods to analyze α-tocopherol in pharmaceuticals and medical food preparations

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Medical food	All-*rac*-α-tocopherol acetate, γ-tocopherol, δ-tocopherol	Extraction in 2-propanol and hexane–ethyl acetate BHT added	NP (LiChrosorb Si 60) HPLC–fluorescence	Isocratically 2-propanol–hexane (0.5:99.5, v/v)	Retinol palmitate can be measured changing mobile phase	[84]
Medical food	All-*rac*-α-tocopherol acetate	Matrix solid-phase dispersion in Bondesil C_{18}	NP (LiChrosorb Si 60) HPLC–fluorescence	Isocratically 2-propanol–hexane (0.5:99.5, v/v)	Retinol palmitate can be measured changing mobile phase	[72]
Multivitamin tablets	Retinol, retinol acetate, tocopherol succinate, γ-tocopherol, α-tocopherol acetate, α and β-carotene	Hexane extraction	Non-aqueous RP (Symmetry C_{18}) HPLC–UV	Step gradient Acetonitrile–0.25% ammonium acetate in methanol and 0.05% triethylamine in dichloromethane		[132]
Vitamin E-supplemented concentrates	α-Tocopherol acetate	Hexane extraction and/or enzymatic digestion	GC–FID	Isothermal 265°C	Colaborative assay Hexadecyl palmitate or dotriacontane as I.S.	[133]
Tablet preparations	Vitamin A, vitamin E and their esters acetate and palmitate	Supercritical fluid extraction (SFE) with CO_2	Non-aqueous RP (LiChrospher CH-8) HPLC–UV	Isocratic Methanol–acetonitrile (75:25, v/v)		[75]
Semisolid gelled preparations		Disolution in ethanol	RP C_{18} HPLC–UV	Methanol–water (93:7, v/v)	Retinol acetate as I.S.	[98]
Synthesized vitamin E	Two enantiomers of α-tocopherol and two structural isomers		MEKC–UV	7 m*M* borate, 14 m*M* phosphate, 15 m*M* SDS, 10 m*M* sodium cholate, 8% acetonitrile	Poor sensibility and recovery	[124]

ponents including vitamin E are extracted into the organic solvent, while fatty acid salts, glycerols and other potentially interfering substances remain in the alkaline aqueous phase. Several factors may interfere in the extraction of vitamin E from the saponification medium as evaluated by Ueda et al. [40]. Among these factors organic solvent, ethanol concentration and the levels of lipids used in the digest are included.

Nevertheless, the saponification is time consuming and complex. It has been mainly employed in food and samples of animal origin. De Leenheer et al. already in 1979 did prove that saponification was not needed, and results were not different from that of normal extraction [41]. Anyway, methods with or without saponification have been applied to the same type of sample by different scientists.

To overcome oxidation of fat-soluble vitamins

Table 2
Summarized characteristics of the methods used to analyze α-tocopherol in diverse samples

Source	Other analytes	Sample treatment	Technique/ detection	Conditions[a]	Comments	Ref.
Tobacco smoke		Collected in Fluoropore membrane and extracted with methanol	RP (Vydac 201TP104) HPLC–fluorescence	Methanol–water (88:12, v/v)		
Olive by-products	Four tocopherols	SFE with CO_2	GC–MS	Methylsilicone column from 230 to 275°C		[76]
Oil palm leaflet		Methanol–chloroform extraction	NP (Zorbax Sil) HPLC–fluorescence	Hexane–THF–methanol (97.25:2.5:0.25, v/v/v)		[134]
Palm-oil extract	Four tocotrienols and α-tocoenol	Dissolution in ethanol	RP C_{30} (laboratory-made)–UV–MS–NMR	Methanol Silver ions added prior to MS	Unambiguous structural assignement	[122]
Vegetable oil, soybean oil, deodorizer distillate, mixed tocopherol concentrate and pharmaceutical preparations	Four tocopherols	Dilution with 2-propanol or THF	RP (Taxsil, pentafluorophenyl) HPLC–UV	Methanol–water (92:8, v/v)	The four isomers separated in RP	[99]
Standards	Four tocopherols and 5,7-dimethyltocol		NP cyclodextrin-bonded silica (CDS)–fluorescence	Hexane or cyclohexane in combination with alcohol, ether or esters in binary and ternary mobile phases	Excellent separations in most cases. Comprehensive approach to explain the interactions of tocols and CDS	[135]

[a] THF=Tetrahydrofuran.

caused by saponification, some investigators have added antioxidants such as butylated hydroxytoluene (BHT) [42], ascorbic acid [32,43,44], pyrogallol [34,45], each one alone, or combinations [46,47].

The antioxidants have also been used even though no saponification was to be applied: BHT [32,34, 36,38,39,48–52], pyrogallol [53], ascorbic [37] or ascorbic and BHT [54] have been used. For determination of α-tocopherol, in most cases the addition of antioxidants has not been considered necessary, and most methods have been developed without antioxidants. However, if some other more labile compounds are to be analysed [47], special matrices are going to be analysed [30], or saponification is needed, then the presence of antioxidants in sample treatment may be important. Nevertheless, antioxidants do not ensure the accuracy: In fact, a recently described method uses BHT, pyrogallol and ascorbic, and recovery from samples was 86% [46]. In addition, Su et al. [55] tested stability of tocopherols and other analytes in human plasma during sample processing, and found tocopherol to be stable exposed to fluorescent light at room temperature for at least 4 h, and after 24 h a loss of only 4% was found, with no antioxidant present in the sample.

In our experience when analyzing vitamin E in

Table 3
Summarized characteristics of the methods used to analyze α-tocopherol in foods

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Vegetable oils, formulated preparations and biscuits	Tocopherols and tocotrienols	Extraction and silica Sep-Pak purification compared with saponification	RP (μ-Bondapak C_{18}) HPLC–UV and fluorescence	Methanol–water (95:5, v/v)	Significant differences with and without saponification were found	[74]
Seed oils	Tocopherols, tocomonoenols, tocotrienols and plastocromanol-8	Hexane dilution	Amino-cyano HPLC–fluorescence	Hexane–tetrahydrofuran (94:6, v/v)	LOD for α-tocopherol 1 ng	[136]
Vegetable oils	Four tocopherols	Hexane–methanol dilution	RP (ODS-2) HPLC–UV	Methanol–water (96:4, v/v)	α-Tocopherol acetate as I.S.	[100]
Virgin olive oil	Tocopherols, carotenoids and chlorophylls	Hexane–2-propanol dilution	NP-HPLC (LiChrospher-Si)–UV–Vis diode array	Gradient Hexane–2-propanol		[85]
Olive and hazelnut oil	Tocopherols and sterols	Saponification with pyrogallol added, extraction in cyclohexane, concentration, derivatization to *O*-trimethylsilyl ethers	GC–FID or MS	Temperature gradient from 230 to 294°C	Discriminant analysis that permits oil identification 5,7-Dimethyltocol as I.S.	[137]
Food oils	Four tocopherols	Hexane dilution	Non-aqueous RPC_{18} HPLC–fluorescence	Methanol–acetonitrile (50:50, v/v)	5,7-Dimethyltocol as I.S.	[115]
Vegetable oils	Four tocopherols and sterols	SPE with silica gel	GC–FID	Step gradient from 70 to 350°C	5,7-Dimethyltocol as I.S.	[73]
Vegetable oils	Three tocopherols	Continuous membrane extraction from oil samples disolved in Triton X-114	RP (OD-224 Brownlee Labs C_{18})–Electrochemical (coulometric) detection	2.5 m*M* acetic acid–sodium acetate in methanol–water (97:3, v/v)	Validated with a BCR Reference Material 2,2,5,7,8-Pentamethyl-6-chromanol as I.S.	[138]
Vegetable oils	Three tocopherols	Dilution with hexane and ethanol	RP (Tracer Extrasil ODS-2)–UV	Methanol–water (96:4, v/v)	Tocopherol acetate as I.S.	[100]
Vegetable oils	Three tocopherols and β-carotene	Saponification with ascorbic acid added, and extraction with hexane–ethyl acetate	RP (Tracer Extrasil ODS-2)–UV	Gradient with methanol–water–butanol		[100]
Vegetable oils	Four tocopherols	Saponification with pyrogallol added, extraction with light petroleum, concentration and dehydratation with sodium sulphate	ODS-silica gel column–fluorescence	CO_2 and methanol as modifier	Comparison with NP-HPLC Tocol as I.S.	[116]

Table 3. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Marine oils	Various tocopherols, triacylglycerols, free fatty acids, squalene, retinol, cholecalcipherol, cholesterol and wax esters	Dilution in heptane	Capillary supercritical fluid chromatography (SFC), non-polar column–FID	CO_2	Fingerprints of marine oils. Poor resolution for tocopherol	[139]
Edible oils and fats	Triacylglycerols and tocopherols	Dissolution	SFC cyanopropyl-phenyl-methylpolysiloxane capilary column–FID	CO_2	Characterization of oils. Poor resolution and sensitivity for tocopherols. Two injections needed	[126]
Soy-based infant formula	All-*rac*-α-tocopherol acetate, γ-tocopherol, δ-tocopherol	Extraction in 2-propanol and hexane–ethyl acetate BHT added	NP (LiChrosorb Si 60) HPLC–fluorescence	Isocratically 2-propanol–hexane (0.5:99.5, v/v)	Retinol palmitate can be measured changing mobile phase	[2]
Margarine and reduced fat products	Three tocopherols	Hexane extraction with anhydrous $MgSO_4$	NP (LiChrosorb Si 60) HPLC–fluorescence	Hexane–2-propanol (99.1:0.9, v/v)	LOD ranging from 23.2 to 1.98 μg/100 g	[86]
Margarine, infant foods and vegetables	Three tocopherols and tocotrienols	Saponification for 40 min at 80°C, extraction with hexane–ethylacetate, evaporation and dissolution in hexane–BHT	NP (LiChrosorb Diol) HPLC–fluorescence	Hexane–*tert*. butyl methyl-ether (94:6, v/v)	Some interferences. Recoveries ranging from 91 to 105% LOD=0.1 μg/ml	[42]
Margarines and margarine-like products		Saponification, extraction with light petroleum, evaporation and dissolution in hexane	RP (μ-Bondapak) HPLC–UV–Vis	Methanol–water (94:6, v/v)	Retinol and β-carotene measured in the same extract with different conditions or columns	[101]
Baby food composite Standard Reference Material 2383	Carotenoids, retinoids and tocopherols	Extraction followed by saponification as compared with saponification followed of extraction	RP (C_{18}) HPLC–UV–Vis		Comparison of results and uncertainties	[140]
Infant milk formulae	Retinol	Saponification at room temperature, subdued light and addition of ascorbic acid. Hexane–BHT extraction, evaporation and dissolution in methanol	RP (Trace Spherisorb ODS 2 C_{18}) HPLC–UV–Vis	Water–acetonitrile–methanol (4:1:95, v/v/v)		[43]

Table 3. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Milk powder	Vitamin A	SFE with CO_2 modified with 5% methanol followed of miniaturised saponification for ester hydrolisis	RP (LiChrospher RP-18) HPLC–UV–Vis	Methanol–water (96:4, v/v)	96 and 99% recoveries	[77]
Milk-based products	Vitamins A and E	Antioxidant addition, saponification, extraction, evaporation and redissolution in methanol	RP (Ultrasbase C_{18} HPLC–UV	Methanol–2-propanol (90:10, v/v)	AOAC official method of sample treatment but fully automated using a robotic station	[102]
Italian cheeses	Tocopherols, carotenes and retinol	Saponification, extraction with hexane–ethyl acetate, evaporation and dissolution in mobile phase	NP (Ultrasphere Si) HPLC–UV–Vis and fluorescence	Multilinear gradient with 2-Propanol–hexane	Data of cheeses and milks. Changes in column performance due to water accumulation	[141]
Dairy products with olive, basil or banana and cheese with tomato	Three tocopherols, total retinols, carotenes and several steroles	Saponification, extraction with hexane–ethyl acetate, evaporation and dissolution in methanol–dichloromethane (9:1)	RP (Supelco C_{18}) HPLC–UV and fluorescence	Methanol		[103]
Dairy products, foods and tissues	Tocopherols and α-tocopherol acetate	Saponification and extraction in the same test-tube	NP (silica) and RP (C_{18}) HPLC–fluorescence	Hexane–2-propanol (99:1, v/v) for NP methanol 100% for RP	Comparison of experimental and literarure data for different procedures	[87]
Diet and tissues	Vitamin E forms	Hexane extraction of the frozen sample. Saponification only employed when tocopherol esters were present	NP (Supelcosil LC-Diol)–fluorescence	Hexane–2-propanol (99:1, v/v)	5,7-Dimethyltocol	[88]
40 Food products	Three tocopherols	Saponification followed of hexane extraction	RP (Zorbax ODS) HPLC–fluorescence	Acetonitrile–methylene chloride–methanol (70:30:5, v/v/v)	Vitamin E data for food product important to the U.S. dietary	[142]
Key Foods in the U.S. Diet	Tocopherols and tocotrienols	Depending of the matrix: (i) Soxhlet extraction, (ii) saponification, (iii) Folch extraction	NP (LiChrosorb Si 60) HPLC–fluorescence	Hexane–2-propanol (99:1, v/v)	Tabulated data	[143]
Foods	Cholesterol, phytosterols and tocopherols	Saponification and extraction with hexane–diisopropylether in the same test-tube	NP (silica) and RP (C_{18}) HPLC–fluorescence and UV–Vis	Hexane–2-propanol (99.9:0.1, v/v) for NP, methanol 100% for RP		[89]

semisynthetic diets, to avoid the different oxidation processes that may occur both in aqueous and in organic phases and that can affect tocopherols is a critical point, and the same may be so in medical foods analysis. In fact, inorganic ions added to the diet as copper or iron can promote lipid oxidation

Table 4
Summarized characteristics of the methods used to analyze α-tocopherol in animal diet

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Animal feedingstuffs		Saponification, extraction in light petroleum, evaporation and redisolution in hexane	NP (Partisil 5 Si) HPLC–fluorescence	Isooctane–2-propanol		[144]
Rodent feed	Vitamins A and E	Saponification with ascorbic acid added, hexane extraction	Normal-phase (LC-CN) HPLC–UV	Hexane–2-propanol–glacial acetic acid (99:1:0.02, v/v/v)		[145]
Animal feeds	Vitamins A, D, E and pro-vitamin D2	Acetone–chloroform extraction, evaporation, *n*-butanol redissolution	RP (Novapak C_{18}) HPLC–UV	Methanol	LOD 10 ng/g for the four vitamins. Various analytical conditions tested.	[68]
Aquatic organisms and feed	α-Tocopherol acetate and tocopherols	Extraction with methanol–BHT	RP (Hypersil ODS) HPLC–fluorescence and UV	Methanol–water (96:4, v/v)	LOQ 0.2 μg/ml in spiked samples Tocol as I.S.	[104]
Diets of animals	α-Tocopherol acetate	Addition of EDTA and BHT, hexane extraction, evaporation and dissolution in chloroform–methanol	RP (Nucleosil C_{18}) HPLC–fluorescence	Methanol–water (96:4, v/v)	Phenyldodecane as I.S.	[57]

and subsequent tocopherol oxidation [56]. This may be prevented by adding EDTA, plus an organic phase soluble antioxidant such as BHT [57].

Some of the most commonly used solvents for vitamin E extraction include the Folch extraction with chloroform–methanol (2:1), acetone, diethyl ether and Soxhlet extraction with a variety of solvents. Wet samples can be ground in the presence of anhydrous sodium or magnesium sulphate to facilitate extraction. The solvent must be capable of effectively penetrating tissues, and for this, extraction is usually achieved by strong vortexing or shaking. In our hands we have applied probe sonication for the extraction of tissues and diets with very good results [30,57,58]. It is however adviced that sonication should not be too intense, because samples warm up easily, and ultrasounds can produce free radicals [59]

Hexane, alone or with little amounts of more polar solvents as ethanol or ethyl acetate, or di-isopropylether (never more than 5%) is the most frequently used extractant. Nevertheless, its effectiveness may not be as high as that of other organic solvents [60,61] because there can be a problem if phases are very non-miscible, and probably hexane does not interact with all the molecules of analyte. Working with internal standard partially corrects this non-effectiveness, and recoveries are not affected, but in some cases sensitivity could be augmented if working with solvents like propanol [62], acetone [63–65], or other solvent mixtures [61]. In the other hand, solvents as acetone can have the problem of mixing with the aqueous phase. Other solvents as acetone can have the problem of mixing with the aqueous phase, wich would affect the unpredictable dilution of the sample, with its subsequent calculi mistakes.

If vitamins have been extracted with hexane or heptane, and chromatography is going to be performed by reversed-phase HPLC, solvent must be evaporated and replaced by other solvent more similar to mobile phase, or by the mobile phase itself. To avoid this evaporation–redisolution process, other organic solvents in which vitamins are soluble, eleiminate interferences and not interfere with separation of analytes in HPLC have been investigated [61,65,66]. Therefore, with these solvents, sample pretreatment is attained in one step

Table 5
Summarized characteristics of the methods used to analyze α-tocopherol in plant samples

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Spinach	Three tocopherols and α-Tocopherol acetate	Lyophilisation, extraction, evaporation and dissolution in methanol	RP (Spherisorb ODS) HPLC–UV	Methanol–water (95:5, v/v)	Only α-tocopherol detected in raw, frozen and canned spinach	[105]
Grain and seeds	Antioxidant capacity	Freezing, ground, extraction and incubation with reagent solution	Spectrophotometry of phosphomolybdenum complex		Compared with HPLC	[131]
Soybean	Four plant tocopherols	Only standards processed	NP (aminopropylsilica or diol-bonded silica) HPLC–fluorescence	Binary solvent systems comprising a hydrocarbon and an alcohol, an ether or an ester	Examination of structural effects on HPLC behaviour on different polar phases. 5,7-Dimethyltocol as I.S.	[94]
Soybean oil, wheat germ, wheat bran and rubber latex	Three tocopherols and four tocotrienols	Extraction of crude oil, saponification, crystallization and dissolution	Semi-preparative, NP (Alltech Econosil) HPLC–fluorescence and UV–Vis	Linear gradient with tetrahydrofurane and hexane	The purpose of the work is the isolation of highly purified tocopherols and tocotrienols as analytical standards	[146]
Brassica oleracea	Carotene and tocopherols	Saponification, extraction, evaporation and dissolution	RP (Prodigy ODS) HPLC–UV–Vis	Acetonitrile–methanol–THF (52:40:8, v/v/v)	Ascorbate extraction and analysis is also described	[147]
Brassica napus L. single seeds	Three tocopherols	Isooctane extraction	NP (LiChrospher 100 diol) HPLC–fluorescence	Isooctane–*tert.*-butylmethylether (94:6, v/v)	β-Tocopherol as I.S.	[148]
Rosmarinus officinalis and *Salvia officinalis*	Phenolic diterpenes and tocopherols	Liquid fat was extracted and purified in methanol	RP (Hypersil ODS) HPLC–electrochemical	Gradient Solvent A: methanol–water–2 mol citric acid–TEAH. Solvent B: methanol–2 mol citric acid–TEAH.		[149]
Fifteen plant species		Air-dried leaves grounded and extracted with hexane by percolation. Purification with column chromatography in silicagel	GC (capillary OV-17 column)–FID	Gradient of temperature from 200 to 320°C	Antioxidant activity was also evaluated. α-Tocopherol acetate as I.S.	[119]
Pumpkin seeds	Three tocopherols and four tocotrienols	Hexane extraction	NP (LiChrosorb Si 60) HPLC–fluorescence	Hexane–dioxane (96:4, v/v)		[150,151]

Table 5. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Oil plant seeds and corn products	Three tocopherols	Extraction and filtration	NP(μ-Bondapak NH_2) HPLC–UV	Hexane–2-propanol (98.7:1.3, v/v)		[152]
Xanthium strumarium	γ-tocopherol	Extraction with acetone, concentration, washing in separation funnel, alumina column chromatography and TLC for purification	GC (AW Chromosorb W glass column)–FID			[153]
Spinach thylakoid		Mixture with SDS and extraction with ethanol–*n*-heptane. Evaporation of the organic phase and dissolution in ethanol	RP (Supelcosil ODS) HPLC–UV	Methanol–water (98:2, v/v)		[106]
Oregano	Three tocopherols	Air-dried leaves extracted with hexane. Saponification and injection of the unsaponifiable fraction	NP (LiChrospher Si 60) HPLC–UV	Hexane–2-propanol (99:1, v/v)		[90]
Maize leaves		Extraction with acetone buffered with Tricine and ascorbate. Re-extraction with hexane, evaporation and dissolution in methanol	RP (Supelcosil LC 18) HPLC–amperometric	Methanol–acetonitrile–acetate buffer (50:40:0.75, v/v/v) followed by ethyl acetate for 1 min		[154]
Plant tissue		Saponification, hexane extraction, evaporation and dissolution in hexane	NP (Spherisorb 5S NH_2) HPLC–fluorescence	Hexane–2-propanol (99:1, v/v)	Small scale method	[91]
Plants	All major photosynthetic pigments, tocopherols, carotenoids and intermediates	Acetone extraction of frozen leaves	RP (Spherisorb ODS-1) HPLC–fluorescence and UV–Vis	Gradient Solvent A, acetonitrile–methanol–water (84:9:7, v/v/v). Solvent B, methanol ethyl acetate (68:32, v/v)	Identification of 20 components in the same run. Quantitative data not included	[155]
Vegetable extracts, vitamin drink and a commercial infant food	Carotenoids, tocopherols and tocopherol acetate	Dichloromethane extraction	RP (polymeric C_{30}) HPLC–UV–Vis and MS	Linear gradient acetone–water. Post-column derivatization with silver ions	LOD 300 fmol for β-carotene	[123]
Leaves of six edible plant species	Tocopherols and tocotrienols	Leaves dipped in hot water. Total lipids extracted with chloroform–methanol and pigment removed with activated charcoal	NP (Zorbax Sil) HPLC–fluorescence	Hexane–THF–methanol (97.35:2.5:0.15, v/v/v)		[156]

Table 5. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Oat (*Avena sativa* L.) extracts		Evaporation of extracts and dissolution in hexane	NP (μPorosil) HPLC–fluorescence	Hexane–2-propanol (99.5:0.5, v/v)	Correlation of antioxidant capacity and phenolic and tocol antioxidants	[92]
Rice Bran	Two novel tocotrienols plus the known four tocopherols and tocotrienols	Hexane extraction, evaporation and re-dissolution	NP (silica) HPLC–fluorescence	Hexane–2-propanol (99.8:0.2, v/v)	Structural data and purification system for the two novel tocotrienols	[93]
Rosmarinus officinalis L. and *Melissa officinalis* L.		Methanol extraction, evaporation and re-dissolution in acetonitrile	RP (Hypersil ODS) HPLC–UV	Acetonitrile–water (citric acid 2 *M*) (98:2, v/v)	Abietane diterpenes analysis is also described	[157,158]

only, just by adding this solvent to the sample. Extraction has also been done with butanol–ethyl acetate, with direct injection of an aliquot of the upper layer [67].

In addition, Qian et al. [68] have tested several conditions that may affect the analysis, including sample particle size, extraction solvents, ratio of solvent to sample, extraction approach, extraction time, N_2 protection, prepurification, etc. Results have been evaluated compared to the AOAC method [69].

Nowadays, sample preparation methods are considered the central point in methods development and several techniques have been developed as alternative to liquid–liquid extraction. Although recently starch-talk layers have been applied to the analysis of plant lipids and have demonstrated to be of potential utility in the purification and quantitation of lipid antioxidants [70], TLC and column chromatography, the oldest types of chromatography, are currently applied to sample clean up [54,71]

The use of solid-phase extraction has proven to be an efficient technique for simplifying sample clean-up prior to HPLC analysis [34,72–74].

The use of supercritical fluids to perform extractions is a relatively new technology with a large potential for future. Behind the recent expansion in use are environmental concerns, such as the need to eliminate organic solvents and to find alternatives for incineration. Some laboratories [75–77] are extracting fat soluble vitamins from foods or tablet preparations using carbon dioxide in the form of a supercritical fluid. This technique, called supercritical fluid extraction (SFE), is rapid and generates little or no hazardous reagent and solvent waste.

4. Gas chromatography

GC-flame ionization detection (FID) is a technique that preceeded HPLC. Methods for determining vitamin E with this technique are known since the early 1970s [78,79] but nowadays it has been used at a lesser extension. The GC analysis of tocopherols normally implies warm saponification, TLC separation, and formation of the trimethylsilyl derivatives prior to the chromatographic injection. Recently Lechner et al. [73] isolated the silyl derivatives by solid-phase extraction (SPE) and quantified them by GC–FID. Derivatizations has been done by heating a purified extract with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) [73,80], *N,O*-(bistrimethylsilyl)trifluoroacetamide (BTSFA)–trimethylchlorosilane (TMCS) (10:1, v/v) [33] and with TriSil Z-reagent (trimethylsiylimidazole–pyridine) [81].

The main goal of GC is the coupling to MS for the identification of metabolites. It has been applied to fractions collected from HPLC [80], for structural elucidation of the compunds [32]. If deuterated compounds are available, identification of estereoisomers is possible too [11]. GC–MS has been

Table 6
Summarized characteristics of the methods used to analyze α-tocopherol in animal tissues

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Human plasma and lipoproteins	Free and esterified cholesterols, triacylglycerols	Ethanol precipitation. Hexane extraction–evaporation. Redissolve in ethanol.	RP (Hitachi Gel 3057 ODS) HPLC–UV–FL	Acetonitrile–2-propanol (75:25)	Cholesteryl benzoate or heptadecanoate as I.S. Sample can be as little as 5 μl	[159]
Rat plasma, liver, lung, mamma	5 retinoids, 8 carotenoids, α-tocopherol acetate	Plasma: add NaCl. Tissues: homogenize with ascorbic, EDTA, acetic. Methanol (BHT) precipitation. Chloroform extraction–evaporation	RP (Nucleosil C_{18} or Hypersil ODS) HPLC–UV	Nucleosil: acetonitrile–ammonium acetate (70:30) Hypersil: acetonitrile–methylene chloride–methanol–water (70:10:15:5)	Methods developed for retinoids and carotenoids analysis. α-tocopherol can be measured in both conditions. Ethanol for precipitation and hexane for extraction are also tested. LOD=60 ng/ml	[48]
Liver of farm animals	Retinol, β-, γ-, δ-tocopherol and α-tocotrienol	Add ascorbic, methanol precipitation, saponification, hexane:toluene (1:1) extraction	NP (LiChrosorb Si 60). HPLC–FL	Hexane (3% 1,4-dioxane)	Recovery 92–100%	[95]
Human red blood cells	α-Tocopherol quinone	Saponification (antioxidant), hexane extraction–evaporation	RP (Ultrasphere ODS) HPLC–ED	Methanol–acetonitrile–phosphate buffer (83:12:5)	Tocol as I.S. Recoveries 80–100% depending on antioxidant. LOQ=50 pg BHT, ascorbic, and pyrogallol (and combinations)	[47]
Human plasma, lung, colon, breast, skin	Retinol lutein, cryptoxanthin, lycopene, α- and β-carotene	Enzymatic digestion (collagenase) with ascorbic, homogenization, ethanol precipitation, hexane extraction–evaporation	RP (Ultracarb ODS) HPLC–UV	Acetonitrile–THF–methanol–1% ammonium acetate (65:25:6:4)	α-Tocopherol nicotinate as I.S. Different homogenization procedures, saponification, or SDS are tested as well as two other columns	[160]
Human plasma (premature newborns)	Retinol, retinol palmitate	Ethanol precipitation. Hexane extraction–evaporation	RP (Superspher RP_{18}). HPLC–UV	Gradient: (A) Acetonitrile–water (90:10) (B) Ethyl acetate–2-propanol (90:10)	Tocol of own synthesis as I.S. Recovery=93% LOD=1.3 μg/ml	[117]
Plasma	(γ+β)-Tocopherol	*n*-Butanol–ethyl acetate–acetonitrile (1:1:1) extraction	RP (Superspher RP_{18}). HPLC–ED	Methanol–ethanol (10:90), 2.5 m*M* perchloric acid, 7.5 m*M* sodium perchlorate	δ-Tocopherol as I.S. LOD=60 pg (3 ng/ml plasma)	[61]
Human serum, rat serum, brain, lung, heart, liver and kidney	Oxidized and reduced coenzyme Q and homologes	Ethanol precipitation, hexane extraction–evaporation.	RP (Capcell Pak C_8). HPLC–Postcolumn Pt reduction–ED	Ethanol–methanol–water (82:8:10), 0.05 *M* sodium perchlorate	8-Menaquinone as I.S.	[112]

Table 6. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Human plasma	γ-Tocopherol, lycopene, β-carotene, ubiquinol, ubiqinone	Ethanol precipitation, hexane extraction–evaporation.	(Two methods) RP. HPLC–ED	Method 1: Methanol–water (96:4), 20 μ*M* lithium perchlorate. Method 2: Methanol–ethanol–2-propanol (22.5:73.6:3.9), 20 m*M* lithium perchlorate		[107]
Rat plasma, red blood cells, liver, brain, adipose tissue, adrenal glands	(*RRR*-, *RRS*-, *RSR*-, *RSS*-), (*SSR*-, *SSS*-) *SRS*-, *SRR*-α-tocopherol, *RRR*-β-, γ-, δ-Tocopherol (all in acetate form)	NaCl 1%, ethanol (pyrogallol) precipitation, saponification, NaCl 1%, hexane (10% ethyl acetate) extraction–evaporation, acetylation: pyridine+acetic anhydride, NaCl 1%, hexane extraction–evaporation	Chiral phase (Chiralpak OP (+)). HPLC–UV	Methanol–water (96:4)	Recoveries between 90 and 100% 2,2,5,7,8-Pentamethyl-6-hydroxychroman as I.S.	[45]
Human plasma (neonates) and erythrocytes		Ethanol (pyrogallol,) precipitation, hexane extraction–evaporation	RP (Spherisorb ODS). HPLC–UV	Methanol	Tocopherol acetate as I.S.	[53]
Plasma and tissues	γ-, δ-, β-Tocopherol (γ-, and β- not resolved in RP), γ-, δ-, β-tocotrienol	Add ascorbic, methanol precipitation, saponification, hexane–toluene (1:1) extraction	(Two methods) NP (LiChrosorb Si60) and RP (Ultrasphere ODS). HPLC–FL	(1) Hexane (3% 1,4-dioxane) (2) Acetonitrile–tetrahydrofran–methanol–ammonium acetate 1% (68.4:22:6.8:2.8)		[96]
Rat plasma and liver, brain, adipose tissue	*RRS*-, *RSR*-, *RSS*-, *SSS*-, *SSR*-, *SRS*-, *SRR*-α-Tocopherol	SDS mixed, methanol (BHT) precipitated, heptane (BHT) extraction, dried with sodium sulphate, evaporation. Purification of tocopherols by repeated HPLC. Alternative: homogenize in methanol (ascorbic), saponification, purification in Extrelut column with isooctane, evaporation, purification in Kieselgel Si60 column with hexane (2% ethyl acetate), evaporation. Purification of tocopherols by single HPLC. Methylation of purified tocopherol.	(Two methods) (1) Chiral phase. (Chiralcel OD) HPLC–UV (2) Capillary GC (GC Silar 10)	(1) Hexane (2) Isothermal 165°C		[32]

Table 6. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Human plasma (neonates)	Ubiquinol-10, ubiquinone-10, γ-tocopherol, β-cryptoxanthin, β-carotene	Ethanol (BHT) precipitation, hexane extraction–evaporation	RP (SuperPac Pep-S RP C_2/C_{18}) HPLC–ED	Methanol–Ethanol–2-propanol (88:24:10), 13.4 m*M* lithium perchlorate	I.S.: γ-tocotrienol, ubiquinone-7, ubiquinol-9, ubiqinone-9, ethyl β-apo-8′.carotenoate	[113]
Pork adipose tissue and muscle		Saponification (ascorbic with or without KI), ethanol precipitation, hexane–toluene (1:1, BHT) extraction–evaporation	NP (LiChrosorb Si60). HPLC–FL	Hexane–ethyl acetate (95:5)		[44]
Rat liver microsomes	TQE1, TQE2, TQ, THQ	Add SDS, ethanol, hexane extraction–evaporation. Dissolve in ethanol–HCl (1:1). Hexane extraction–evaporation. Treat with BSTFA–TMCS (10:1)	GC–MS (DB-5 ms column)	Programmed temperature	$[^2H_6]$TH, $[^2H_6]$-TQE1, $[^2H_6]$-TQE2, $[^2H_6]$-TQ, $[^2H_3]$-THQ as I.S.	[33]
Mice brain, heart, kidney, liver and skin	γ-Tocopherol, α-, and γ-tocotrienol, ubiquinol-9 and -10, ubiquinone-9 and -10	Skin: homogenized in buffer (NaCl, EDTA, Na_3PO_4) (BHT), SDS added, ethanol precipitation, hexane extraction–evaporation. Other tissues: homogenate with SDS, ethanol precipitation, heptane extraction	RP (Ultrasphere ODS). HPLC–UV–ED	Gradient: (A) Methanol–water (80:20), 0.2% $LiClO_4$ (B) Ethanol, 0.2% $LiClO_4$	LOD: 0.1 pmol	[35]
Aquatic organisms: *Artemia*, rotifers, turbot, sea bass larvae, shrimp postlarvae	γ-, δ-Tocopherol	Alternatively: (a) Homogenize in methanol (BHT). SPE cleanup with hexane–2-propanol (97:3) (b) Homogenize in methanol (BHT), saponification (pyrogallol), light petroleum–diisopropyl ether (3:1) extraction–evaporation (c) Homogenize in methanol (BHT), hexane extraction–evaporation (d) Homogenize in methanol–SDS (BHT). Hexane extraction–evaporation (e) Homogenize in hexane–methanol (2:1). Hexane reextraction–evaporation.	RP (Hypersil ODS). HPLC–FL	Methanol–water (96:4)	Tocol as I.S. in every method	[34]

Table 6. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Human plasma, lipoproteins		(a) NaCl, ethanol precipitation (BHT), hexane or ethyl acetate extraction–evaporation (b) Dilution with methanol–ethanol (8:1, BHT, δ-tocopherol as I.S.)	RP (Novapak C_{18}) HPLC–FL	Methanol	δ-Tocopherol as I.S.	[49]
Mice brain, heart, kidney, liver and skin	γ-Tocopherol, α-, and γ-tocotrienol, ubiquinol-9 and -10, ubiquinone-9 and -10	Skin: homogenized in buffer (NaCl, EDTA, Na_3PO_4) (BHT), SDS added, ethanol precipitation, hexane extraction–evaporation. Other tissues: homogenate with SDS, ethanol precipitation, heptane extraction	RP (Ultrasphere ODS). HPLC–UV–ED	Gradient: (A) Methanol–water (80:20), 0.2% $LiClO_4$ (B) Ethanol, 0.2% $LiClO_4$	LOD: 0.1 pmol	[35]
Human plasma, erythrocite membranes	α-Tocopherol quinone, (γ+β)-, δ-tocopherol	Reaction with sodium hydrogensulphite, ethanol precipitation, hexane extraction–evaporation	RP (MC MEDICAL C_{18}). HPLC–ED	Methanol–water (96:4), 40 m*M* $NaClO_4$.	LOD: 50 pg, recovery=98.3±1.2% (plasma), 91.5±2.85 (erythrocite membrane)	[108]
Human plasma	Retinol, all-*trans*-α-carotene, all-*trans*-β-carotene, 9-, 13- and 15-*cis*-β-carotene	Ethanol precipitation, hexane extraction–evaporation, redissolve in ethanol (tocopherol acetate as 2nd I.S.)	RP (Suplex pKb-100) HPLC–UV	Methanol–methyl *tert.*-butyl ether–water	Retinol acetate as I.S.	[110]
Piglets heart and liver	β-, γ- and δ-tocopherol, α-, β-, γ- and δ-tocotrienol	Ethanol precipitation, sonication, hexane extraction–evaporation	NP (Supelcosil LC-Diol). HPLC–FL	Hexane–2-propanol (99:1)	Recovery >95%	[88]
Human plasma (preterm infants)	Retinol, γ- and δ-tocopherol	Ethanol precipitation (BHT), hexane (BHT) extraction–evaporation	RP (LiChrospher 100, RP_{18}) HPLC–UV	Acetonitrile–THF–methanol–ammonium acetate 1% (68.4:22:6.8:2.8)	LOQ=2.370 μ*M*. Tocol as I.S. but only for chromatography. Recovery: 96±7%	[50]
Serum	Retinol, lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, lycopenes, α- and β-carotene (9 more carotenoids and γ-tocopherol separated but not identificated-quantitated)	Water–ethanol precipitation, hexane extraction–evaporation	RP (Adsorbosphere C_{18}, two columns in serie). HPLC–UV	Gradient: (A) Acetonitrile–methanol (60:40), 0.05% acetic acid (B) Acetonitrile–methanol–dichloromethane (45.6:30.4:24), 0.04%	Tocol and echinenone as internal standard. Recoveries >92%. LOQ: 2 ng.	[111]

Table 6. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Human platelets, culture endothelial cells		Methanol–chloroform extraction–evaporation. TLC with chloroform–methanol–water–triethylamine (35:30:7:35) (ascorbic and BHT). Spot R_F=0.86–0.89 to silica gel G60 column, hexane–*tert.*-butyl methyl ether (92.5:7.5) elution–evaporation.	RP (LiChrosorb RP_{18}). HPLC–FL or UV or light-scattering detection	Methanol–water (98:2)	α-Tocopherol acetate or δ-tocopherol as I.S. Objective of sample preparation: from the same sample obtain three extracts to measure cholesterol, tocopherol and phospholipid fatty acids	[54]
Rat liver, placenta, brain	Retinol, Retinol palmitate	Hexane extraction by sonication, evaporation	RP (Nucleosil C_{18}). HPLC–UV–FL	Methanol–water (96.5:3.5)	Vitamin K_1 as I.S.	[58]
Human plasma	Retinol, lycopene, lutein, α- and β-carotene	Ethanol precipitation (ascorbic), hexane extraction–evaporation	RP (Nucleosil ODS). HPLC–UV	Methanol–acetonitrile–THF (75:20:59), 0.01% ascorbic acid	α-Tocopherol acetate and retinol acetate as I.S. Echinenone for carotenoids, but "not commercially available". Recovery=96%, LOD= 2.5 μ*M*	[120]
Rat liver, liposome suspension	α-Tocopherol hydroquinone, α-tocopherol quinone, γ-tocopherol, α- and β-tocotrienol, ubiquinol-9 and -10, ubiquinone-9 and -10	NaCl, SDS, ethanol (BHT) precipitation, hexane extraction–evaporation	RP (LiChrosorb RP_{18}). HPLC–Zn post column reduction–ED	Methanol–water (98:2), 5 m*M* $ZnCl_2$, 2.5 m*M* sodium acetate, 2.5 m*M* acetic acid.	Recovery >95%, LOD=0.3 pmol	[36]
Rat adipose tissue and mammary gland		Homogenize in ethanol–water, hexane extraction–evaporation	RP (Nucleosil C_{18}). HPLC–FL	Methanol–water (96.5:3.5)	Recovery >95%, Vitamin K_1 as I.S.	[30]
Rat liver, cell culture	α-Tocopherol oxybutiric acid	Homogenize in SDS (ascorbic acid) and ethanol with sonication. Hexane extraction–evaporation	RP (Spherisorb C_{18}). HPLC–FL	Methanol 96%	δ-Tocopherol as I.S. LOD: 3 pmol	[37]
Human serum (newborns)	Retinol	Ethanol precipitation, hexane extraction–evaporation	RP (NovaPak C_{18}). HPLC–FL–UV	Methanol–water (95:5)	Retinol acetate and α-tocopherol acetate as I.S. LOQ=11.6 μ*M*, Recovery=103%	[109]
Human serum and liver, rat serum, liver, intestines, spleen, brain, heart, skeletal muscle, kidney and lung	Retinol and 11 retinoids, γ-tocopherol, lycopene, β-carotene	2-Propanol–dichloromethane (2:1) (BHT) precipitation–extraction (+acetic acid)	RP (Microsorb-MV). HPLC–UV	Gradient: (A) Methanol:water (3:1), 10 m*M* ammonium acetate; (B) Methanol–dichloromethane (4:1)	Retinol acetate as I.S.	[38]

Table 6. Continued

Source	Other analytes	Sample treatment	Technique/ detection	Conditions	Comments	Ref.
Mice brain, heart, kidney, liver, skin	γ-Tocopherol, α-, and γ-tocotrienol, ubiquinol-9 and -10, ubiquinone-9 and -10	Homogenize in buffer (phosphate-buffered saline) (BHT), SDS added, ethanol precipitation, hexane extraction–evaporation	RP (Ultrasphere C_{18}) HPLC–UV–ED	Gradient: (A) Methanol–water (80:20), 0.2% $LiClO_4$ (B) Ethanol, 0.2% $LiClO_4$	Recoveries >90%, LOD 0.1 pmol	[51]
Plasma or serum	12 carotenoids, (γ+β)-tocopherol	Water dilution, ethanol precipitation, hexane extraction–evaporation	RP (Primesphere C_{18}). HPLC–UV–FL	Acetonitrile–THF–methanol–1% ammonium acetate (68.4:22.0:6.8:2.8)	LOD=20 μg/l	[161]
Human serum	Retinol, γ-tocopherol	Ethanol precipitation. Butanol–ethyl acetate (1:1) extraction, Na_2SO_4 dried.	RP. (Supelcosil LC-18). HPLC–FL	Program: 90–100–90% A (A) Acetonitrile–butanol (95:5) (B) Water	α-Tocopherol acetate as I.S. LOD=0.35 μ*M* Recovery=97%	[67]
Human stratum corneum	Cholesterol	Ethanol/SDS (BHT) homogenize, hexane extraction–evaporation	RP. (Ultrasphere ODS). HPLC–ED	Methanol–ethanol (1:9), 20 m*M* $LiClO_4$		[39]
Human plasma		Acetonitrile–THF (3:2) precipitation–extraction	RP (Crestpak C_{18}). HPLC–UV	Methanol (6% THF)	Recovery 81–97% (low concentrations give poor recoveries)	[66]
Human plasma	Retinol, β-carotene	Ethanol–water (2:1) precipitation, hexane (BHT) extraction–evaporation	RP (LiChrospher RP_{18}) HPLC–UV	Acetonitrile–THF–methanol–1% ammonium acetate (68.4:22.0:6.8:2.8)	LOD: 0.7 μ*M*, recovery=97%	[52]
Rat plasma, heart, lung, liver, kidney	α-Tocopherol quinone, (β+γ)-tocopherol	Homogenize in Tris buffer (tissues), ethanol precipitation (BHT, ascorbic acid, pyrogallol). Saponification, hexane–ethyl acetate (9:1) extraction–evaporation	RP (Neo-pack ODS). HPLC–ED	Methanol 95%, 50 mm Na ClO_4	Recovery=86.%, LOD=0.25 pmol	[46]

used by Liebler and coworkers for determination of vitamin E and its oxidation products [33,82].

5. High performance liquid chromatography

HPLC separations of tocopherols provides a fast, simple, sensitive, selective and more robust technique than GC [83]. HPLC separations of tocopherols are performed on both normal- and reversed-phase columns.

6. Normal-phase high-performance liquid chromatography

Normal-phase systems show elution of homologues in order of increasing polarity with separation

based on methyl substituents on the chromanol moiety. The normal-phase columns provide separation of all isomers, while β and γ-tocopherols are not easily separated in conventional reversed-phase columns. Besides their ability to separate β- and γ-isomers, normal-phase separations present also the advantages of operating with organic solvents allowing a high solubility for lipids and of to tolerating high loads of lipids which are easy to wash-out by non-polar solvents. Normal-phase HPLC is very suitable for the direct analysis of oils and fats.

Silica columns with binary isocratic or gradient mobile phases of hexane–2-propanol have been widely applied [2,72,84–93]. Abidi and Mounts [94] studied and interpreted resolution provided by aminopropylsilica and diol-bonded silica with binary solvent systems comprising a hydrocarbon and an alcohol, an ether or an ester. Recently six silica, three amino and one diol columns were tested for the separation of vitamin E compounds in a prepared mixture containing oat extracts and palm oil as matix [83]. The best separation were obtained on three silica columns and two amino columns using 5% dioxane in hexane as mobile phase, as well as on a diol column using 4% *tert.*-butyl methyl ether in hexane as mobile phase. Anyway, poor reproducibility, prolonged equilibration times, low stability and the employment of hazardous volatile organic solvents have long been discussed. For α-tocopherol analysis in plasma and tissues, normal-phase has fallen in disuse, because most of the times, data about other tocopherols are not relevant, and simplicity is a goal for reversed-phase HPLC. Nevertheless, normal-phase HPLC is still succesfully used [88,95,96].

7. Reversed-phase high-performance liquid chromatography

Reversed-phase systems show separation based on the saturation of the phytyl side chain. The more saturated isomers are retained longer. C_{18} reversed-phase systems do not completely resolve β- and γ-tocopherols, which may however be separated by a polymeric column [97], nowadays commercially available. Nevertheless, when β- and γ-tocopherols separation is not a critical point, C_{18} reversed-phase systems are preferred, because equilibration times are shorter, and better reproducibility is achieved. Isocratically methanol with small amounts of water or other solvents is the most frequently employed mobile phase [34,36,37,43,49,54,57,58,66,68,74,75, 77,98–109], and is enough for tocopherol quantification. If other analytes are to be measured, more complicated mobile phases, gradients, etc., need to be developed [35,38,48,51,110,111]. Moreover, if electrochemical detector is used, mobile phase must be modified with an electrolite, usually perchlorate salts [35,39,46,61,107,108,112–114]. Zinc chloride has also been used in methods that reduce analytes after separation, before detection in oxidative mode [36].

Due to the fact that reference materials with certified reference values for the analyte of interest are nor commercially available, results of two isocratic reversed-phase separation were tested by method comparison for serum samples versus an isotope dilution–gas chromatography–mass spectrometry (ID–GC–MS) method [81]. It was made to access the inaccuracy of the HPLC methods. Both HPLC methods were evaluated as precise and accurate enough for clinical use.

8. Internal standard

Recoveries can be a major problem in vitamin E analysis. Internal standards are desirable when analyte loss can occur during sample preparation steps prior to analysis. If a known quantity of standard is added to the unknown prior to any manipulations, the ratio of standard to analyte remains constant because the same fraction of each is lost in any operation. Chemical and physical properties of analyte and internal standard must be as similar as possible to come true this affirmation. 5,7-Dimethyltocol [73,88,94,115], and tocol are among the employed internal standards that better fulfil these characteristics. Tocol (the desmethylated-chromanol form of tocopherols) is one of the internal sandards most used, historically, but currently is not easily found in commercial brands [88,116], and only as a generous gift or synthesized in the same laboratory [117] is currently used.

β-Tocopherol [118], δ-tocopherol [38,49,54,61]

and γ-tocotrienol [113,114] have been used as internal standards, though they can be present in some samples. α-Tocopherol acetate widely used [53,54,67,100,100,109,119,120] is a non-natural form of vitamin E, but can be present in manipulated oils and foods, in pharmaceutical formulae, and in tissues of people receiving parenteral or intravenous α-tocopherol acetate as part of diet or medical treatment, and therefore precautions must be taken prior to its use to confirm its absence. Moreover, it has no electrochemical or fluorescent properties.

There are other compounds, described in Tables 1–6, employed as internal standards when other analytes are quantificated, because internal standard with similar properties to all the analytes must be chosen, and many times interferences coming from the matrices make the election difficult. Franke et al. have reported a list of synthetic carotenoids (and the way to synthesize them) for plasma micronutrient analysis [121].

9. Detection

The four usual HPLC detection methods: Electrochemical detection (ED), fluorescence (FL), UV absorbance detection and light-scattering detection have been employed for determining tocopherols and related substances with sensitivities decreasing in the mentioned order.

HPLC–ED is the most sensitive and specific detection method of tocopherols by virtue of their low oxidative potentials. Moreover, as the process is reversible, oxidation products can also be measured, which is very interesting when studying tocopherols role in oxidative stress [13,16] Quinones do not fluoresce, therefore oxidation products cannot be determined with this detection method and UV detection has not enough sensitivity in some cases. Both amperometric [35,51,107] and coulometric [47,61,107,108,113,114] detection have been employed. ED is most suitable for RP-HPLC because the electrolytes needed are miscible with aqueous mobile phases.

FD as compared with ED leads to a 10-fold loss in sensitivity, but it depends on the composition of the mobile phase, which greatly affects both fluorescence yield and conductivity in ED. Nevertheless FD it is the top choice for many researchers because it is easier to operate than ED and compatible with normal-phase mobile phases. Both detection methods permit the employment of smaller sample amounts when it is important.

HPLC–UV poses a loss in sensitivity of around 10^3-fold, but UV is the most common detection method and its sensitivity is enough in many cases for α-tocopherol measurement. Moreover, in the diode array mode it permits to obtain the spectrum and it is a way of peak identification and purity test.

Evaporative light-scattering detection, widely used in lipid analysis due to the ability to detect compounds with or without chromophore, shows poor sensitivity and selectivity.

Coupling HPLC or GC with mass spectrometry (MS) or nuclear magnetic resonance (NMR) also provides a powerful means for structural elucidation [122]. Nonpolar substances cannot easily be ionized by standard electrospray ionization (ESI). The ionization of carotenoids and tocopherols can be greatly enhanced by the addition of silver ions, the procedure has been named coordination ion spray (CIS) [123].

10. Other methods

In spite of the immense analytical potential of capillary electrochromatography, no reference have been found showing a good resolution for tocopherols, although Abidi [6] remarks that they are working in it with preliminary good results. Two enantiomers and two structural isomers of synthetic tocopherol have been separated by micellar electrokinetic chromatography (MEKC) [124], nevertheless, sensitivity will be always poorer than in HPLC because of the smaller amount of sample injected.

Supercritical fluid extraction (SFE) and chromatography (SFC) are ideally suited for medium to non polar labile compounds analysis and several works have been published with one of them or both techniques. Nevertheless, nowadays SFE has progressed rapidly and many applications have continued arising, but SFC has not followed the prospective rhythm, probably due to the complexity and cost of the instrumental system. Yarita et al. used a laboratory-built SFC system for the determination of

tocopherols [116], whereas capillary SFC coupled to MS has been used for the determination of the four tocopherols and other fat-soluble vitamins [125,126]. Recently, Buskov et al. [127] have developed a method for the determination of α-tocopherol acetate, α-, β-, γ- and δ-tocopherol by packed column SFC using UV detection. The method has been applied to the determination of tocopherols in plant oils extracted by SFE.

Chiral separations of tocopherols and tocotrienols provide pure individual stereoisomers for nutrition, metabolism and pharmaceutical studies [32,45]. Several reports suggest differences among the bioavailability and biopotency of α-tocopherol isomers [128,129] and the relevance of the configuration of the α-tocopherol molecule on biodiscrimaination is under study.

In 1990, Vecchi et al. reported a separation method of all-*rac*-α-tocopheryl acetate isomers combining HPLC and capillary GC [130]. With this method, the eight stereoisomres were separated into two groups of 2R and 2S-configurations by HPLC, and each of the groups was determined by GC. Subsequently, these time-consuming procedures were improved and simplified by omitting the acetylation of α-tocopherol and by applying a commercially available HPLC chiral phase [32]. The new method consisted of extraction of α-tocopherol from biological samples, purification by semipreparative HPLC, conversion to α-tocopherol methylesters, separation by HPLC into five peaks and GC analysis of one of the peaks formed by four isomers.

Ueda et al. [45] developed a chiral HPLC method to separate the eight stereoisomers of unlabelled α-tocopheryl acetate into four peaks. The first peak consisted of RRR, RRS, RSS and RSR; the second peak consisted of SSS and SSR; the third peak was SRR and the fouth peak was SRS. Afterwards, the method was newly revised and applied [129]. During sample treatment plasma and lipoprotein samples were acetylated because the analysed standards are in that form.

Between other methods it is interesting to quote a spectrophotometric method [131] newly published that permits simultaneous determination of total antioxidant activity in plan extracts based in phosphomolybdenum complex formation. The phosphomolybdenum method, in combination with hexane monophasic extraction, has also been adapted for the specific determination of vitamin E in seeds. Other works related to α-tocopherol analysis [132–161] have been included in Tables 1–4 and they do not deserve further comments than those included in the tables.

Finally, it is interesting to mention that in the USA there is a Micronutrients Measurement Quality Assurance Program for these analytes run by the National Institute of Standards and Technology (NIST) and in the UK an equivalent scheme is run. With these programs the quality of tocopherol measurement in the laboratory can be evaluated.

As general comments we could say that GC–MS is the selected tool for tocopherol metabolites identification and quantification, but GC is not adequate for routine tocopherol analysis mainly due to sample pre-treatment, which requires more steps than other methods. Normal-phase HPLC is the selected tool for fat matrices and when β and γ isomers need to be separated. The more general technique is reversed-phase HPLC, which nowadays resolves most of the analytical problems related to tocopherols analysis in a more rapid and simple way. Supercritical fluids are mainly applied to clean fractionated extractions from foods, while SFC is not a generaly available tool. Finaly, developing techniques, such as capillary electrophoresis, need further development in this area to be able of competing with established techniques.

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