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Review

# Chromatographic analysis of tocol-derived lipid antioxidants

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## Abstract

This paper provides a comprehensive overview of existing chromatographic methods for the analysis of tocol-derived lipid antioxidants in various sample matrices. After a brief introductory discussion on biological and nutritional aspects of the vitamin E active compounds, the review focuses on various techniques for the isolation, purification, chromatographic separation, and detection of tocopherols and tocotrienols. Compiled published normal-phase (NP) and reversed-phase (RP) high-performance liquid chromatographic (HPLC) methods demonstrate general trends and analytical variability and versatility of HPLC methodology. The relative merits of the two HPLC methods are assessed. NP and RP elution characteristics are delineated to aid in the identification of antioxidant components. Technical novelty of certain analytical procedures for non-food samples warrants their inclusion in this review in light of the potential applicability in food assays. © 2000 Published by Elsevier Science B.V.

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## 1. Introduction

Lipid antioxidants, tocopherols (T) and tocotrienols ( $T_3$ ) comprise a number of vitamin E active substances derived from a chromanol structure. These compounds are closely related homologues and isomers depending respectively on the number and position of methyl groups on the aromatic ring of the tocol backbone in tocopherols (Fig. 1). The unsaturated analogues of tocopherols are tocotrienols in which the carbon-2 triterpenyl side chain contains three double bonds at carbon-3', carbon-7', and carbon-11' positions. In addition, plastochromanol-8 (the octaterpenyl side chain analogue of  $\gamma$ -tocotrienol), dehydrotocopherols, and tocodienols occur in nature as tocopherol like substances in vegetable oils.

In 1964, Pennock and coworkers first reported the existence in nature of eight tocol-derived compounds,  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols of 2R,4'R,8'R-configuration along with the corresponding 2R-trans/trans-tocotrienols [1]. Synthetic materials from racemic precursors normally produce mixtures of diastereomers (Table 1). Due to the presence of three asymmetric carbon atoms at carbon-2, carbon 4', and

carbon 8', there are eight stereoisomers for each tocopherol molecule. Likewise, synthetic tocotrienols consist of an array of enantiomers (2R and 2S forms) and geometrical isomers (cis/cis-, cis/trans-, trans/cis-, and trans/trans-forms). As demonstrated in Table 1, a total of 64 stereoisomers are present in all racemic synthetic tocopherols and tocotrienols.

The vitamin E family of compounds offers multifaceted health benefits and is essential for mitochondrial electron-transport function in physiological systems. Owing to their lipid soluble antioxidative properties, these compounds inhibit lipid peroxidation processes of polyunsaturated fatty acids and other compounds in cell membranes [2]. Biological activities of the lipid antioxidants vary and depend largely on lipid solubility and standardization of bioassays. Nonetheless, it has been established that 2R4'R8'R- $\alpha$ -tocopherol possesses the highest activity among the various antioxidant structures. Other homologues and levorotatory stereoisomers including tocotrienols exhibit relatively weak vitamin E potency. Thus, the general trend of biological activity of some compounds has been reported as follows:  $\alpha T > \beta T > \alpha T_3 > \gamma T > \beta T_3 > \delta T$  [3].

The title lipid antioxidants have been shown [4] to exhibit an increasing order of relative autoxidative stabilities in methyl myristate:  $\alpha T = \alpha T_3 < \beta T_3 < \gamma T_3 < \delta T_3 < \gamma T < \delta T = \beta T$ , and in methyl linoleate:  $\alpha T < \alpha T_3 < \gamma T_3 < \beta T < \gamma T < \delta T$ . The same study has demonstrated different trends of photolytic stabilities in respective methyl myristate and methyl linoleate:  $\gamma T_3 < \alpha T_3 < \delta T < \alpha T < \gamma T < \beta T$ , and  $\alpha T < \alpha T_3 < \gamma T_3 < \beta T < \zeta T < \gamma T < \delta T$ . On the other hand, the relative stabilities of these compounds in frying oils are function of oil varieties: in soybean oil,  $\alpha T > \delta T > \beta T > \gamma T$ ; in corn oil,  $\alpha T > \gamma T > \delta T > \gamma T_3$ ; in palm oil,  $\alpha T > \delta T_3 > \alpha T_3 > \gamma T_3$  [5].

The structural complexity and the wide variation in antioxidative activity of the title compounds necessitate reliable analytical techniques for the isolation, separation, differentiation, and quantification of individual components in mixtures derived from various sample matrices. Availability of pure compounds facilitates structure–activity studies and furthers biomedical and pharmaceutical research on their health application. In particular, trace natural occurrence of certain precious members of the compounds in the series render it necessary to

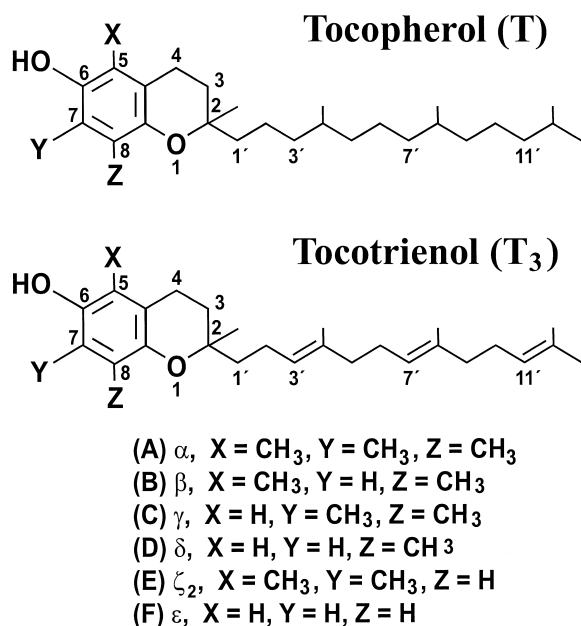


Fig. 1. Structures of tocopherols (T) and tocotrienols ( $T_3$ ).

Table 1  
Various forms of tocopherols and tocotrienols

Stereoisomer	Homologue <sup>a</sup>			δ
	α	Position isomer		
		β	γ	
Methyl substitution:				
Number	3	2	2	1
Position	C-5, 7, 8	C-5, 8	C-7, 8	C-8
Tocopherol:				
No. 1	2R4'R8'R	2R4'R8'R	2R4'R8'R	2R4'R8'R
No. 2	2R4'R8'S	2R4'R8'S	2R4'R8'S	2R4'R8'S
No. 3	2R4'S8'S	2R4'S8'S	2R4'S8'S	2R4'S8'S
No. 4	2R4'S8'R	2R4'S8'R	2R4'S8'R	2R4'S8'R
No. 5	2S4'S8'S	2S4'S8'S	2S4'S8'S	2S4'S8'S
No. 6	2S4'S8'R	2S4'S8'R	2S4'S8'R	2S4'S8'R
No. 7	2S4'R8'R	2S4'R8'R	2S4'R8'R	2S4'R8'R
No. 8	2S4'R8'S	2S4'R8'S	2S4'R8'S	2S4'R8'S
Tocotrienol:				
No. 1	2R-tr/tr	2R-tr/tr	2R-tr/tr	2R-tr/tr
No. 2	2R-tr/cis	2R-tr/cis	2R-tr/cis	2R-tr/cis
No. 3	2R-cis/tr	2R-cis/tr	2R-cis/tr	2R-cis/tr
No. 4	2R-cis/cis	2R-cis/cis	2R-cis/cis	2R-cis/cis
No. 5	2S-tr/tr	2S-tr/tr	2S-tr/tr	2S-tr/tr
No. 6	2S-tr/cis	2S-tr/cis	2S-tr/cis	2S-tr/cis
No. 7	2S-cis/tr	2S-cis/tr	2S-cis/tr	2S-cis/tr
No. 8	2S-cis/cis	2S-cis/cis	2S-cis/cis	2S-cis/cis

<sup>a</sup> C, carbon; Tr, trans.

recourse to sophisticated analytical procedures for sample enrichment, high-efficiency resolution, and sensitive detection of analytes at very low levels.

Tocopherols and tocotrienols occur in plants in variable abundance. Vegetable oils provide the best sources of these lipid antioxidants. Isolation and enrichment of the compounds from plant tissue matrices entails traditional solvent extraction or supercritical fluid extraction (SFE) of oilseeds followed by various chromatographic procedures. In comparison to solvent extraction, SFE is a sophisticated newer technique (see its discussion in the Other Chromatographic Techniques section) providing a convenient way to fasten up the extraction with reduced loss of tocol-derived compounds during extraction procedure. For characterization and quantification purposes, the crude isolate can subsequently be further purified and separated by a wide variety of chromatographic techniques: column chromatography, thin layer chromatography (TLC), normal-phase high-performance liquid chromatography

(HPLC), reversed-phase HPLC, and other chromatographic techniques. With the recent advancement in column technologies, complete separations of mixtures of synthetic lipid antioxidants (which contain more components than the natural compounds) can be achieved.

Based on the large numbers of publications available in the literature, most researchers have preferred normal-phase HPLC techniques as the methods of choice for their separations of lipid antioxidants because of the relatively easy separations of isomeric β-, and γ-tocopherols and tocotrienols. However, recent introduction of several reversed-phase stationary phases excluding octadecylsilica (ODS) phases enables the separation the β-, and γ-isomers of interest. The new reversed-phase column systems can eliminate the use of hazardous solvents. This paper present a critical review on chromatographic methods for the analysis of tocol-derived lipid antioxidants and their application in food analysis with emphasis on vegetable oils.

## 2. Isolation and enrichment procedures

Depending on sample matrices, isolation procedures vary among solid and liquid samples of animal or plant origins. Tocol-derived lipid antioxidants in tissues and oilseeds can generally be isolated by solvent extraction and saponification [6–8]. In a typical procedure, vegetable oilseeds (15 g) were homogenized (60 s) in a coffee bean grinder and extracted with absolute ethanol (100 ml) in a soxhlet thimble overnight on a steam bath. Water (100 ml), and light petroleum (50 ml) are added to the cooled extract and shaken (10 min) in a separator. Evaporation of the upper organic layer under reduced pressure leaves an oily residue. To the lipid residue (1 g), ascorbic acid (0.3 g) in absolute ethanol (4 ml) is added. The mixture is heated to boiling on a boiling water bath and immediately added with a concentrated solution of potassium hydroxide (1 ml). After refluxing for 15 min, water (20 ml) is added to the cooled reaction mixture and shaken three times with diethyl ether (25 ml). The ether extract is washed with several portions of water (25 ml) until neutral, dried over anhydrous sodium sulfate, and evaporated under nitrogen to give a crude antioxidant product devoid of esters. For faster hydrolysis at room temperature, the saponification reagent ethanolic potassium hydroxide can be substituted by tetraalkylammonium hydroxide in dimethyl sulfoxide [7,8].

## 3. Thin-layer chromatography and column chromatography

Among the many chromatographic techniques available for the determination of lipid antioxidants, TLC and solid–liquid adsorption column chromatography methods employ the least expensive apparatus and instruments despite the lack of high quantitation precision. They are suitable for sample cleanup, purification [9,10], qualitative assays, and rough estimates of the antioxidants in assay samples.

### 3.1. Thin-layer chromatography

Tocopherols and tocotrienols in plant oils have been separated by either one-dimensional [11] or

two-dimensional TLC [12]. In a one-dimensional system, a silica gel plate spotted with an enriched antioxidant sample is developed three times in hexane–ethyl acetate (92.5:7.5) leading to the separation of six components:  $\alpha$ -,  $\beta$ -, and  $\delta$ -tocopherols along with  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocotrienols. The  $\gamma$ -tocopherol and  $\beta$ -tocotrienol remain inseparable under the conditions used. In a two-dimensional system, a crude antioxidant sample is applied onto a silica gel plate and developed in chloroform for the first dimension and in hexane–isopropyl ether (80:20) in the second dimension. Except for the unresolvable pair of  $\gamma$ -tocopherol and  $\beta$ -tocotrienol, all other six tocol-derived compounds can be separated by this method.

Visualization of TLC spots is normally carried out with a UV lamp on a silica gel plate impregnated with a fluorescence indicator, e.g. dichlorofluorescein. For quantitation, TLC spots are scrapped off the plate, treated with a solution (0.003 M) of 4,7-diphenyl-1,10-phenanthroline in absolute ethanol and determined by colorimetry [6]. Quantitation can also be achieved by densitometric scanning of the colored spots using a standard calibration curve. Crude materials of tocopherols and tocotrienols can be purified by preparative TLC using the one-dimensional technique described above. Streaks of the separated antioxidants on a preparative TLC plate are cut out, suspended in absolute ethanol–benzene (9:1), and filtered. Removal of solvent yields the individual antioxidants of improved purity. Recent applications of anticircular TLC [13] and unconventional starch/talc layers [14] to the analysis of plant lipids demonstrate potential analytical utility of the techniques in the purification and quantitation of lipid antioxidants.

### 3.2. Column chromatography

Crude plant extracts or oil hydrolysates of lipid antioxidants often contain undesirable impurities that interfere with the analytes of interest in subsequent quantitative analyses with high-precision chromatographic instruments. Utilization of liquid–solid adsorption in lieu of liquid–liquid partition can be advantageous for treatment of a saponification solution [7]. The column chromatographic procedure is simple and can overcome emulsion problems associ-

ated with the liquid–liquid extraction process. Thus, a hydrolysate in methanol–ethanol–butanol (4:3:1) is loaded on a Kieselgel column under atmospheric pressure and then eluted with isooctane to afford the investigated antioxidants. Generally, elution of the antioxidant compounds with appropriate solvents on a selected adsorption column removes interfering materials and recovers the compounds in satisfactory yields. A rapid sample cleanup procedure employs a short glass column packed with Kieselgel 60 followed by elution with ethyl acetate–hexane (5:95) [10]. Strongly activated adsorbents tend to cause analyte retention on the solid-phase. It has been shown that column chromatography with deactivated silica gel (15% water) or hydrated Florisil (20% water) in a light petroleum–ether (98:2) eluent system give quantitative recovery of some lipid antioxidants [15].

#### 4. Gas chromatography

Prior to the development of HPLC, lipid scientists have relied heavily on GC techniques for the accurate measurement of oil constituents including tocopherols and tocotrienols. Abreast with the pro-

gress in chromatographic column technologies, the column systems used in GC analyses of the title compounds have evolved from packed columns to capillary columns. Using the latter coated columns or chemically bonded columns, antioxidant sample assays can be achieved with high degrees of detection sensitivity and component resolution. In a typical analysis, a GC column is connected to a flame ionization detection (FID) system to monitor the column effluents or to a mass spectrometer for structural identification as well as single ion monitoring quantitation with improve detection sensitivity. As FID systems lack detection selectivity and specificity, GC–FID analyses are often preceded by TLC and column chromatography for cleanup and pretreatment of unsaponifiable samples. In addition, due to destruction of column effluents by FID, a splitter is needed to be inserted between the column outlet and a FID system to collect analyte peak components.

Table 2 summarizes some published methods pertaining to the analysis of tocopherols and tocotrienols in vegetable oils and related sample matrices. To volatilize the hydroxy-containing GC detectants, the compounds are more frequently analyzed as their ester [16,17] or trimethylsilyl (TMS) [18,19] deriva-

Table 2  
Gas chromatographic analysis of tocopherols and tocotrienols<sup>a</sup>

Method (detection)	Column	Elution order	Ref. (Matrices)
(1) FID	Packed, 8 ft.×4 mm, 6% SE-52 on Chromosorb W AW-DMCS	$\delta T \rightarrow (\beta + \gamma) T \rightarrow \alpha T$ (propionates)	[16] Vegetable oils
(2) FID	Packed, 6 ft.×5 mm, 3% SE-30 on Gaschrom Q	$\delta T \rightarrow (\beta + \gamma) T \rightarrow \alpha T$ (butyrates)	[17] Vegetable oils
(3) FID	Packed, 3 ft.×4 mm, 2% Silicon oil on Chromosorb W AW-DMCS	$\delta T \rightarrow (\beta + \gamma) T \rightarrow \delta T_3 \rightarrow \alpha T \rightarrow (\beta + \gamma) T_3 \rightarrow \alpha T_3$	[9] Palm oil
(4) FID	Capillary, 150 ft.×0.25 mm, Dexsil 400	$\rightarrow \zeta T \rightarrow \beta T_3 \rightarrow \gamma T_3 \rightarrow \alpha T \rightarrow \alpha_3$ (TMS ether)	[18] Fats & oils
(5) FID	Capillary, 90 ft.×0.25 mm, 0.25 $\mu$ m DB-5	$\delta T \rightarrow \beta T \rightarrow \gamma T$ (TMS ether)	[19] Oil distillate
(6) FID	Capillary, 45 ft.×0.32 mm, RTX-50	$\delta T \rightarrow \beta T \rightarrow \gamma T \rightarrow \delta T_3 \rightarrow \alpha T \text{-AC} \rightarrow \gamma T_3$	[20] Foods
(7) FID	Capillary, 30 ft.×0.53 mm, HP-1	$\alpha T \rightarrow \alpha \text{-AC}$	[22] Edible oils

<sup>a</sup> T, tocopherol; T<sub>3</sub>, tocotrienol; TMS, trimethylsilyl; AC, acetate.

tives. In view of the procedural simplicity and facile reaction duration, TMS derivatization methods are considered to be most suitable for analyzing the tocol-derived antioxidants. For example, in a septum-capped vial, a purified sample (200 mg) dissolved in dry pyridine (1 ml) is treated with a TMS reagent solution (2 ml) of trimethylchlorosilane–bis-(trimethylsilyl)trifluoroacetamide (1:99). The mixture is heated in a heating block at 55°C for 5 min. Upon cooling, the content of the vial is diluted to an exact volume with chloroform and the aliquots are analyzed by GC. Depending on sample matrices, the number of analyte species in samples, and specific application, GC analyses of lipid antioxidants can be run isothermally or by temperature programming to bring the peak of interest within reasonable retention times without peak overlapping.

A cursory perusal of the methods list in Table 2 reveals that the pairs of  $\beta$ -, and  $\gamma$ -positions isomers of tocopherols and tocotrienols are not resolved on packed GC columns [16,17,9] but are resolved on capillary columns [18–20]. On a capillary (150 ft.  $\times$  0.25 mm; 1 ft.=30.48 cm) Dexsil 400 column [18], TMS derivatives of all eight homologues/isomers of the title compounds have been separated emerging sequentially from the column in the following order:  $\delta T \rightarrow \beta T \rightarrow \gamma T \rightarrow \delta T_3 \rightarrow \zeta T \rightarrow \beta T_3 \rightarrow \gamma T_3 \rightarrow \alpha T \rightarrow \alpha T_3$ . For the analysis of underivatized tocopherols and tocotrienols, various capillary GC stationary phases such as RTX-50 [20], SE-30, or OV-17 [21] have been used. As outlined in method 7 [22] of Table 2, direct determination of vitamin E compounds along with other important chemicals in vegetable oils and fats has been accomplished by capillary GC–FID with continuous online removal of triglycerides via transesterification [22]. Generally, it is possible to detect individual lipid antioxidants in the low nanogram range by capillary GC–FID, which is about 20 times more sensitive than GC with a packed column. Further, additional advantages of capillary GC techniques over packed GC methods include reduction in analysis times and peak interferences, improvement in component resolution, and high thermal stability. Modern sophistication in the commercial manufacture of various capillary columns should provide convenient means for the accurate analysis of tocopherols and tocotrienols by capillary GC.

## 5. Normal-phase high-performance liquid chromatographic analysis

Since the emergence of the HPLC technology a few decades ago, the majority of investigators in lipid research have used HPLC methods for the analysis of lipid antioxidants. HPLC has outshone GC because less tedious sample cleanup is involved and milder column temperature conditions prevent the loss of labile analytes. By virtue of detection specificity and sample homogeneity, direct HPLC analysis of tocopherols and tocotrienols in vegetable oils can be carried out with little sample purification preventing from unwanted sample losses. On the other hands, samples of other complex matrices must go through TLC, column chromatography, or other chromatographic pretreatment to eliminate interfering endogenous matters. Hence, working HPLC procedures are much dictated by the degree of matrix complexity, the type and concentration of antioxidant components, routine or non-routine assays, and analytical or preparative scale separations.

The first separation of  $\beta$ -, and  $\gamma$ -tocopherols and that of all eight  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols and tocotrienols embarked in 1973 [89] and 1974 [90], respectively. Since then, normal-phase HPLC techniques have been extensively used for the analysis of the antioxidant mixtures in various sample matrices despite obvious shortcomings of long equilibration times inherent with the normal-phase systems and the employment of hazardous volatile organic solvents. In contrast to the inability of isomer separations by reversed-phase HPLC, complete normal-phase HPLC resolution of the title compounds has met with much success (Figs. 2 and 3). The demonstrated unusual selectivity of silica-based stationary phases for the differentiation of the aromatic ring position isomers of tocol-derived antioxidants has pivotal bearing on the proliferation of their applications in normal-phase HPLC of these compounds. As in any other chromatographic techniques, optimization of normal-phase HPLC experimental parameters (e.g. stationary phases, mobile phases, flow-rates, isocratic/gradient elution., etc.) is crucial for the accurate quantification of the lipid antioxidants of interest. Because of the ubiquitous occurrence of tocopherols and tocotrienols in certain vegetable oils

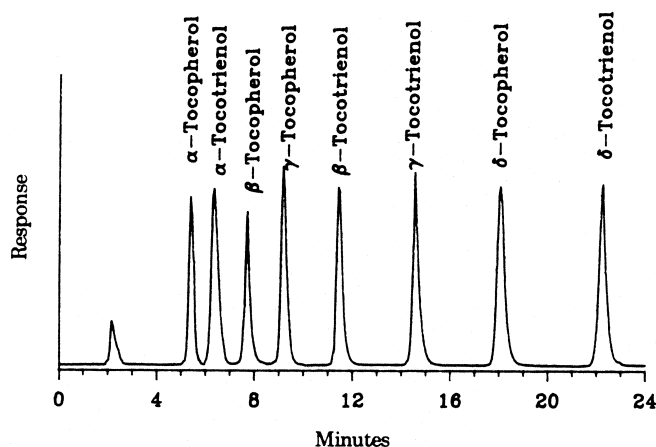


Fig. 2. Normal-phase HPLC–FL detection of tocopherols and tocotrienols (on silica) in a standard mixture (from [44] with permission).

such as rice bran oil and palm oil, separations of the tocol compounds in these oils have often been carried out in the normal-phase mode despite the absence of some antioxidant components [27,49].

### 5.1. Stationary phases

Table 3 summarizes selected published normal-phase HPLC methods for the analysis of tocopherols and tocotrienols. About 70% (a total of 19 methods) of the listed methods employed non-polar silica of diverse manufacturers' specifications and achieved

variable degrees of component separations. LiChrosorb Si 60 (5  $\mu\text{m}$ ) has been used by four different research groups [23,34,44,46] for the simultaneous determination of tocopherols and tocotrienols in assay samples. Each of the silica phases Zorbax Si (5  $\mu\text{m}$ ) [26,35], Ultrasphere Si (5  $\mu\text{m}$ ) [28,42], and Nucleosil Si (3,5  $\mu\text{m}$ ) [29,37] has been reported in two publications for the separation of antioxidant mixtures. Two reports [24,32] described the use of Spherisorb Si (5  $\mu\text{m}$ ) in the analysis of tocopherols. The rest of the methods in Table 3 singly employed RSIL(10  $\mu\text{m}$ ) [25], Polygosil 60 (5  $\mu\text{m}$ ) [31], naphthylethylsilica (5  $\mu\text{m}$ ) [33], Supelcosil LC-Si, (5  $\mu\text{m}$ ) [39], Hypersil Si (5  $\mu\text{m}$ ) [40], NovaPak Si, (4  $\mu\text{m}$ ) [43], Econosil Si,(10  $\mu\text{m}$ ) [45] for antioxidant assays.

As shown in Table 3, there are only eight publications in the literature describing the normal-phase HPLC separation of tocopherols and tocotrienols on polar silica-based phases where silica has been modified with polar moieties to form amino-, cyano-, cyclodextrin-, diol-, and nitro-bonded silica. Of these, five methods employed diol polar silica phases: LiChrospher 100 Diol (5  $\mu\text{m}$ ) [36,38], Chromega Diol (5  $\mu\text{m}$ ) [48], LiChrosorb Diol (5  $\mu\text{m}$ ) [49], supelcosil LC-Diol (5  $\mu\text{m}$ ) [50]. One publication [30] dealt with normal-phase HPLC separations of lipid antioxidants on Partisil PAC (5  $\mu\text{m}$ ) which contains both amino and cyano groups. Cyclodextrin-bonded silica known as Cyclobond I (5

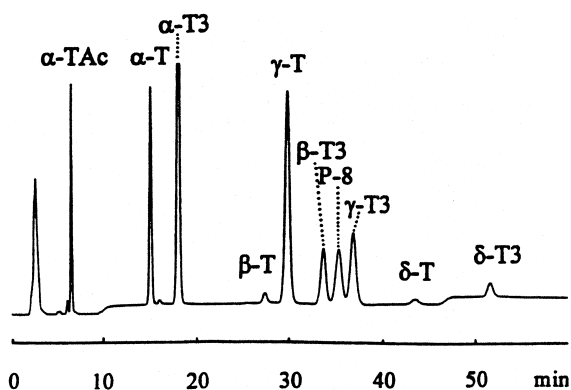


Fig. 3. Normal-phase HPLC–FL detection of tocopherols (T), tocotrienols ( $T_3$ ),  $\alpha$ -tocopherol acetate (TAC), and plastoquinone-8 (P-8) (on diol-silica) in a mixture of 2-*tert*-butyl-4-hydroxyanisole (BHA), linseed oil, and barley lipids (from [38] with permission).

Table 3  
Normal-phase HPLC analysis of tocopherols and tocotrienols<sup>a</sup>

Method (detection)	Stationary/mobile phase	Elution order (run time)	Ref. (Matrices)
(1) FL, 290 <sub>Ex</sub> 330 <sub>Em</sub>	LiChrosorb Si 60, 5 μm 250×3.2 mm HX-IP (99.8:0.2)	αT→αT <sub>3</sub> →βT→γT →βT <sub>3</sub> →γT <sub>3</sub> →δT	[23] Foods, tissues
(2)UV, 280 nm	Spherisorb Si, 5 μm 250×2 mm HX-IP (99.75:0.25)	αT→βT→γ→δT (15 mm)	[24]
(3) FL, 303 <sub>Ex</sub> 328 <sub>Em</sub>	RSIL, 10 μm 250×3.0 mm HX-ethyl acetate (97:3)	αT→αT <sub>3</sub> →βT→γT →βT <sub>3</sub> →γ <sub>3</sub> →δT→δT <sub>3</sub>	[25] Vegetable oils
(4) FL, 298 <sub>Ex</sub> 325 <sub>Em</sub>	Zorbax Sil, 5 μm 250×4.6 mm HX-THF-MeOH (97.25:2.5:0.25)	αT→αT <sub>3</sub> γT <sub>3</sub> →δT <sub>3</sub> (25 mm)	[26] Palm oils
(5) FL, 205 <sub>Ex</sub> 330 <sub>Em</sub>	Ultrasphere Si, 5 μm 250×4.6 mm HX-IP (98.8: 1.2)	αT→αT <sub>3</sub> →γT →γT <sub>3</sub> →δT (10 mm)	[28] Corn grain
(6) FL, 295 <sub>Ex</sub> 330 <sub>Em</sub>	Nucleosil Si, 3 μm 100×2.8 mm (2 in series) HX-IP (99.5:0.5)	αT→αT <sub>3</sub> →βT→γT βT <sub>3</sub> →γT <sub>3</sub> →δT	[29] Edible oils
(7) FL, 210 <sub>Ex</sub> 325 <sub>Em</sub>	Partisil PAC, 5 μm 250×4.6 mm HX-THF (94:6)	αT→αT <sub>1</sub> →αT <sub>3</sub> →βT→ γT→βT <sub>3</sub> →γT <sub>3</sub> →δT→δT <sub>3</sub> (20 mm)	[30] Seed oils
(8) FL, 296 <sub>Ex</sub> 320 <sub>Em</sub>	Polygosil 60, 5 μm 250×4.6 mm HX-PE (9:1)	αT→α <sub>3</sub> →βT→γT →δT (20 min)	[31] Seed oils
(9) UV 295 nm	Spherisorb Si, 3 μm 100×4.4 mm HX-IP (99.8:0.2)	αT→βT→γT→δT	[32]
(10) UV 295 nm	Naphthylethylsilica, 5 μm, 750×0.53 mm HX-HFIP (99.9:0.1)	αT→γT→T→δT (120 min)	[33]
(11) FL, 290 <sub>Ex</sub> 330 <sub>Em</sub>	LiChrosorb Si 60, 5 μm 250×4.6 mm HX-JP (99.5:0.5)	αT→αT <sub>3</sub> →βT→γT →γT <sub>3</sub> →δT→δT <sub>3</sub>	[34] Vegetable oils
(12) UV 295 nm	Zorbax Sil, 5 μm 250×4.6 mm HX-IP (99:1)	αT→αT <sub>3</sub> →βT→γT →γT <sub>3</sub> →δT→δT <sub>3</sub> (5 min)	[35] Palm oils
(13) FL, 295 <sub>Ex</sub> 330 <sub>Em</sub>	LiChrospher 100 Diol, S μm 250×4.0 mm HX-BME (96:4)	αT→αT <sub>3</sub> →βT→γT →βT <sub>3</sub> →γ <sub>3</sub> →δT→δT <sub>3</sub> (50 min)	[36] Seed oils, Cereals
(14) FL, 295 <sub>Ex</sub> 330 <sub>Em</sub>	Nucleosil 50 Si, 5 μm 250×4.0 mm HX-dioxane (95:5)	αT <sub>3</sub> →βT→γT <sub>3</sub> →δT <sub>3</sub> (20 mm)	[37] Stillingia oil
(15) FL, 295 <sub>Ex</sub> 330 <sub>Em</sub>	LiChrospher 100 Diol, 5 μm 250×4.0 mm HX→HX-BME (97:3) →HX-BME (95:5) (gradient elution)	αT→AC -αT→αT <sub>3</sub> →βT →βT <sub>3</sub> →γT <sub>3</sub> →δT→δT <sub>3</sub> (50 min)	[38] Foods
(16) FL, 290 E 330 <sub>Em</sub>	Supelcosil LC-Si, 5 μm 250×4.6 mm (i) IO-ethyl acetate (97.5:2.5) (ii) IO-ethyl acetate-ACA-DP (98.15:0.9:0.85:0.1)	αT→αT <sub>3</sub> →βT→γT -βT <sub>3</sub> →γT <sub>3</sub> →δT→δT <sub>3</sub> (i) (25 min) (ii) (15 min)	[39] Rice bran



Table 3. Continued

Method (detection)	Stationary/mobile phase	Elution order (run time)	Ref. (Matrices)
(17) FL, 290 <sub>Ex</sub> 330 <sub>Em</sub>	Hypersil Si, 5 $\mu\text{m}$ 100 $\times$ 2.1 mm HP-IP (99.8:0.2)	$\alpha\text{T}\rightarrow\beta\text{T}\rightarrow\gamma\text{T}\rightarrow\delta\text{T}$ (10 min)	[40] Margarine
(18) FL, 298 <sub>Ex</sub> 345 <sub>Em</sub>	Cyclobond I, S $\mu\text{m}$ 250 $\times$ 4.6 mm Cyclohexane-IPE (95:5)	$\alpha\text{T}\rightarrow\zeta\text{T}\rightarrow\beta\text{T}\rightarrow\gamma\text{T}$ $\rightarrow\delta\text{T}$ (40 min)	[41]
(19) ELSD	Ultrasphere Si, 5 $\mu\text{m}$ 250 $\times$ 4.6 mm HX-IP (99.3:0.7)	$\zeta\text{T}\rightarrow\alpha\text{T}\rightarrow\beta\text{T}\rightarrow\gamma\text{T}$ $\rightarrow\delta\text{T}$ (15 min)	[42] Vegetable oils
(20) UV, 295 nm	NovaPak Si, 4 $\mu\text{m}$ 150 $\times$ 3.9 mm IO-ethyl acetate (97.5:2.5)	$\alpha\text{T}\rightarrow\alpha\text{T}_3\rightarrow\gamma\text{T}$ $\rightarrow\gamma\text{T}_3\rightarrow\delta\text{T}\rightarrow\delta\text{T}_3$ (10 min)	[43] Rice bran
(21) FL, 295 <sub>Ex</sub> 330 <sub>Em</sub>	LiChrosorb Si 60, 5 $\mu\text{m}$ 125 $\times$ 4.6 mm HX-dioxane (97:3)	$\alpha\text{T}\rightarrow\alpha\text{T}_3\rightarrow\beta\text{T}\rightarrow\gamma\text{T}$ $\rightarrow\beta\text{T}_3\rightarrow\gamma\text{T}_3\rightarrow\delta\text{T}\rightarrow\delta\text{T}_3$ (20 min)	[44] Tissues
(22) FL, 290 <sub>Ex</sub> 330 <sub>Em</sub>	Econosil Si, 10 $\mu\text{m}$ 250 $\times$ 10 mm HX-THF (gradient 0 $\rightarrow$ 15% THF)	(i) $\alpha\text{T}\rightarrow\beta\text{T}\rightarrow\gamma\text{T}\rightarrow\delta\text{T}$ (ii) $\alpha\text{T}_3\rightarrow\beta\text{T}_3\rightarrow\gamma\text{T}_3\rightarrow\delta\text{T}_3$ (i) (20 min) (ii) (25 min)	[45] Soybean oil, wheat bran
(23) FL, 290 <sub>Ex</sub> 330 <sub>Em</sub>	LiChrosorb Si 60, 5 $\mu\text{m}$ 250 $\times$ 4.0 mm HX-IP (99.7:0.3)	$\alpha\text{T}\rightarrow\alpha\text{T}_3\rightarrow\beta\text{T}\rightarrow\gamma\text{T}$ $\rightarrow\beta\text{T}_3\rightarrow\gamma\text{T}_3\rightarrow\delta\text{T}\rightarrow\delta\text{T}_3$ (25 min)	[46] Vegetable oils
(24) FL, 295 <sub>Ex</sub> 330 <sub>Em</sub>	Nucleosil 100-5 NO <sub>2</sub> , 5 $\mu\text{m}$ 250 $\times$ 4.0 mm HX $\rightarrow$ HX-BME (98:2) $\rightarrow$ HX-BME (98:2) $\rightarrow$ HX-BME (85:15) (gradient elution)	$\alpha\text{T}\rightarrow\text{AC}\rightarrow\alpha\text{T}\rightarrow\alpha\text{T}_3\rightarrow\beta\text{T}$ $\rightarrow\beta\text{T}_3\rightarrow\gamma\text{T}_3\rightarrow\delta\text{T}\rightarrow\delta\text{T}_3$ (50 min)	[47] Foods
(25) FL, 298 <sub>Ex</sub> 345 <sub>Em</sub>	Chromega Diol, 5 $\mu\text{m}$ 250 $\times$ 4.6 mm HX-IPE (95:5)	$\alpha\text{T}\rightarrow\zeta\text{T}\rightarrow\beta\text{T}\rightarrow\gamma\text{T}$ $\rightarrow\delta\text{T}$ (35 min)	[48]
(26) FL, 295 <sub>Ex</sub> 330 <sub>Em</sub>	LiChrosorb Diol, 5 $\mu\text{m}$ 250 $\times$ 4.0 mm HX-BME (94:6)	$\alpha\text{T}\rightarrow\alpha\text{T}_3\rightarrow\beta\text{T}\rightarrow\gamma\text{T}$ $\rightarrow\beta\text{T}_3\rightarrow\gamma\text{T}_3\rightarrow\delta\text{T}\rightarrow\delta\text{T}_3$ (40 min)	[49] Foods
(27) FL, 290 <sub>Ex</sub> 330 <sub>Em</sub>	Supelcosil LC-Diol, 5 $\mu\text{m}$ 250 $\times$ 4.6 mm HX-IP (99:1)	$\alpha\text{T}\rightarrow\alpha\text{T}_3\rightarrow\beta\text{T}\rightarrow\gamma\text{T}$ $\rightarrow\beta\text{T}_3\rightarrow\gamma\text{T}_3\rightarrow\delta\text{T}\rightarrow\delta\text{T}_3$ (20 min)	[50] Tissues, diet

<sup>a</sup> HX, hexane; THF, tetrahydrofuran; PE, dipropyl ether; HFIP, hexafluoroisopropanol; BME, t-butylmethyl ether; IO, isooctane; IPE, diisopropyl ether; IPE, diisopropyl ether; DP, 2,2-dimethoxypropane; HAC, acetic acid.

$\mu\text{m}$ ) has been useful for the separation of tocopherols [41]. Baseline separations of tocopherols and tocotrienols admixed with other food components have been achieved using a nitrosilica column [47].

In spite of the variation in the manufacture of silica-based columns, normal-phase HPLC with stationary phases of small particle size 3–5  $\mu\text{m}$  invariably allows efficient separations of antioxidant components with satisfactory peak symmetry. For

preparative HPLC (e.g. method 22 [45] in Table 3), a column packed with silica of 10  $\mu\text{m}$  suffices the separation of tocopherols and tocotrienols. In typical assays, standard column dimensions of 250 $\times$ 4.6 mm I.D. and 250 $\times$ 10 mm I.D. are employed for respective analytical and preparative separations of the compounds under consideration. Short columns (100–150 mm) with 3–4  $\mu\text{m}$  silica packings can have two columns linked in tandem (method 6) or used directly [32,40,43,44]. Method 17 [40] in Table

3 is an example of on-line HPLC–high resolution GC analysis of tocopherols on a short and small I.D. column (100×2.1 mm I.D.), which simplifies sample preparation and reduces analysis time and solvents.

### 5.2. Mobile phases

With a few exceptions, binary systems of a polar organic modifier in hexane are commonly used in normal-phase HPLC of tocol-derived lipid antioxidants. The organic modifiers cover a variety of alcohol, ether, and ester. Eleven of selected published methods (Table 3) employed hexane–isopropanol mobile phase systems in which the percentage of isopropanol varied from as low as 0.2% up to 1.2% [23,24,28,29,32,34,35,40,42,46,50]. Lowering the isopropanol content in hexane tends to increase separations of  $\beta$ – $\gamma$  pairs of tocopherols and tocotrienols. Table 3 also shows another set of eleven published methods using hexane (or cyclohexane)–ether binary systems as normal-phase HPLC mobile phases. The organic ether modifiers include linear ethers (dipropyl ether in method 8 [31]; t-butylmethyl ether in methods 13, 15, 24, and 26 [36,38,47,49]; diisopropyl ether in methods 18 and 25 [41,48]) and cyclic ethers (tetrahydrofuran in methods 4, 7, and 22 [26,30,45]; dioxane in methods 14 and 21 [37,44]). Usually the hexane–ether mobile phases contain fairly low proportions of ether modifiers somewhere in the 1.0–6.0% range, which is notably greater than hexane–alcohol solvent systems. Except for methods 15,22, and 24 [38,45,47], the HPLC experiments described above have been run under isocratic elution. In method 22 [45], individual components of tocopherols and tocotrienols were separated and isolated by preparative normal-phase HPLC under gradient elution of hexane–tetrahydrofuran, where a gradient of 0.0% to 15% of tetrahydrofuran was used. Gradient HPLC techniques can be applied to special cases to handle particular analyte species in complex sample matrices.

There are three groups of researchers [25,39,43] (Table 3) who have utilized hexane (or isooctane)–ethyl acetate mobile phases containing 2.5–3.0% of the ester modifier for the normal-phase HPLC separation of lipid antioxidants. It is noteworthy that straight- or branched-chain alcohols or ethers in mobile phases have subtle influence on the adsorp-

tion of analyte solutes on silica-based phases [41,48]. Generally, the presence of an aprotic oxygen-containing organic modifier of low polarity in a mobile phase leads to better separations of  $\beta$ – $\gamma$  pairs of antioxidants than that of a protic oxygen-containing solvent such as isopropanol. In other words, chromatographic peaks of the tocol-derived lipid antioxidants have tendency to be more equally dispersed among the components in the former solvent system. Strong interactions of the polar mobile phase solvents (e.g. alcohol or polar ether) have adverse effects on column selectivity for component separation. The published literature in Table 3 provides rather limited information on applications of ternary or quaternary solvent systems for the normal-phase HPLC analysis of the title compounds. A ternary mobile phase of hexane–tetrahydrofuran–methanol and a quaternary system of isooctane–ethyl acetate–acetic acid–dimethylpropane have been used in methods 3 [25] and 16 [39], respectively. Mobile phase effects of numerous ternary systems of hexane–isopropanol–ether (or ester) on the separation of the  $\beta$ -, and  $\gamma$ -tocopherols have been discussed previously [41]. In a unique case, a mixture of tocopherols has been separated on naphthylethylsilica and eluted with hexane–hexafluoroisopropanol (method 10 [33] in Table 3).

### 5.3. Elution characteristics

In general, the reported analysis times for the lipid antioxidants to elute through a normal-phase column are variable and take about 5–120 min (Table 3). Under identical experimental conditions, short HPLC runs are inversely related to component resolution. Therefore, it is a good general practice to conduct sample assays under optimal conditions to achieve rapid separations with maximal analyte resolution. As to elution characteristics, it has been well established that elution of tocopherols and tocotrienols in the normal-phase mode has the following order:  $\alpha T \rightarrow \alpha T_3 \rightarrow \beta T \rightarrow \gamma T \rightarrow \beta T_3 \rightarrow \gamma T_3 \rightarrow \delta T \rightarrow \delta T_3$  (Figs. 2 and 3), which is consistent with all but one (method 10 [33]) of the methods given in Table 3. In method 10 [33], an anomalous elution order  $\alpha T \rightarrow \gamma T \rightarrow \beta T \rightarrow \delta T$  was observed when an unusual nonpolar arylalkylsilica stationary phase was used under normal-phase conditions.

To understand the normal-phase HPLC behavior of the closely related isomeric compounds, it is worthwhile to study the relation between structures and elution characteristics of the three closely related dimethyltocols  $\zeta$ -,  $\beta$ -, and  $\gamma$ -tocopherols. In a normal-phase HPLC system, differential adsorptive interactions between analyte solutes and a silica-based stationary phase during chromatographic processes result in the separation of compounds of interest. Examination of the methyl substitution patterns of antioxidant structures (Fig. 1) shows that each of the respective  $\beta$ -, and  $\gamma$ -compounds has a 5-methyl- and 7-methyl groups ortho to the polar 6-hydroxy group that plays an important role in normal-phase HPLC partition processes. Therefore, the sufficient polarity difference between the  $\beta$ -, and  $\gamma$ -isomers of tocopherols and tocotrienols appears to be attributable to the observed separation of these isomers. On the other hand, both of the 5-, and 7-dimethyl groups in  $\zeta$ -tocopherol are flanked by the 6-hydroxy group rendering the latter less accessible for interactions with the stationary phase. Accordingly, the observed elution order  $\zeta T \rightarrow \beta T \rightarrow \gamma T$  [41,48] (Table 3) parallels with increasing polarity of the dimethyl-compounds. A reversal in the normal elution order  $\beta T \rightarrow \gamma T$  has been found in method 10 [33] (Table 3) where a micro naphthylethylsilica column was used.

## 6. Reversed-phase high-performance liquid chromatographic analysis

Although  $\beta$ -, and  $\gamma$ - isomers of tocopherols and tocotrienols have not been resolved on traditional octadecylsilica (ODS) columns with acetonitrile (or methanol)–water mobile phases (Fig. 4), reversed-phase HPLC techniques have been widely used in the analysis of lipid antioxidants in cases where one component of an isomer pair is absent in samples and the need for the isomer separation is unimportant in specific research projects. Some practical advantages offered by reversed-phase are easy equilibration of mobile phases, reproducible chromatographic peak characteristics, compatible with highly sensitive electrochemical detection, low volatility of mobile phase solvents, and good selectivity for geometrical isomers of tocotrienols. In non-routine applications, the  $\beta$ – $\gamma$  pairs of the antioxidant position isomers can be resolved on ODS provided mobile phases of an aqueous alcohol with carbon number  $\geq 2$  (e.g. ethanol) must be used. In these systems, the analytes elute from the column at unusually long retention times due to high column pressure and low eluent flow-rates. Most recently, several investigators have reported the separation of  $\beta$ -, and  $\gamma$ - isomers of tocopherols and tocotrienols on new stationary phases encompassing pentafluorophenylsilica [63],

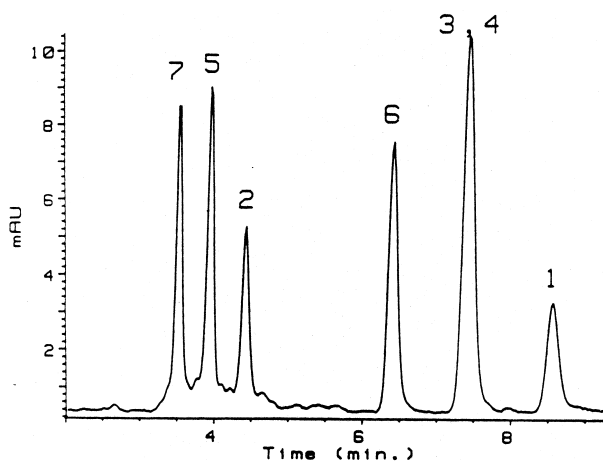


Fig. 4. Reversed-phase HPLC–UV detection of tocopherols (T), tocotrienols ( $T_3$ ) (on ODS) in a standard mixture. 1,  $\alpha T$ ; 2,  $\alpha T_3$ ; 3,  $\beta T$ ; 4,  $\gamma T$ ; 5,  $\gamma T_3$ ; 6,  $\delta T$ ; 7,  $\delta T_3$  (from [35] with permission).

long-chain alkylsilica [67,70], and nonsilica-based octadecanoyl polyvinyl alcohol [65,69]. Also, it has been demonstrated recently that esterification of the 6-hydroxy group enables separations of the  $\beta$ - $\gamma$  pairs on ODS. The future outlook for high efficiency reversed-phase resolution of the the position isomers is auspicious and the task for the isomer separation will continuously impose a formidable challenge to many lipid scientists.

### 6.1. Stationary phases

Table 4 summarizes selected reversed-phase HPLC methods for the analysis of tocol-derived lipid antioxidants. From the table, it is clearly indicated that traditional ODS methods overwhelmingly outnumber those employing other stationary phases. An Ultrasphere ODS phase has been used in four methods [55,57,44,71] given in Table 4. Also shown in the table, there are two methods each for Spherisorb ODS- [56,46], Hypersil ODS- [61,72], and YMCPack ODS- [58,65] phases used in the antioxidant separation. The remainder (11 methods) of the listed ODS phases each appears only once in Table 4. As stated earlier,  $\beta$ -, and  $\gamma$ -tocopherols and tocotrienols have not been separated on ODS under standard mobile phase conditions (aqueous acetonitrile or methanol). However, with an ODS column, resolution of these position isomers can be achieved under elution with isopropanol–water eluents [58]. Also, their ester derivatives can be resolved on this phase under regular HPLC conditions [65]. A weak separation of  $\beta$ -, and  $\gamma$ -tocopherols was observed in method 8 [33], where a long polymeric ODS column was used and eluted with a non-aqueous mobile phase.

In recent years, new stationary phases other than ODS have found valuable applications in antioxidant analysis. In 1994, a group of researchers reported the first separation of position isomers of tocopherols with a non-traditional stationary phase, pentafluorophenylsilica (PFPS) [63] (method 16 in Table 4). A few years later, another group utilized a nonsilica-based column, octadecanoyl polyvinyl alcohol (ODPVA) for obtaining isomer separations of tocopherols [65] (method 19 in Table 4) (Fig. 5) and tocotrienols [69] (method 24 in Table 4) (Fig. 6).

Most recently, a long chain alkyl-bonded silica phase, triacontylsilica ( $C_{30}$ -silica), has been successfully applied to the separation of isomers and homologues of tocopherols [67] and tocotrienol [70] (Fig. 7) as well as geometrical isomers of the latter [69]. Among the three types of unconventional phases described, the PFPS phase offers the best baseline resolution and appears to be best suited for the routine reversed-phase analysis of the title compounds. For selection of reversed-phase column specifications, a standard size of 250 $\times$ 4.6 mm column packed with a stationary phase of 3–5  $\mu$ m particle size has been chosen by many chromatographers in the field. Other specific considerations concerning reversed-phase HPLC column selection are similar to those discussed earlier on normal-phase HPLC.

### 6.2. Mobile phases

As gathered from Table 4, classic mobile phases for isocratic reversed-phase HPLC of tocopherols and tocotrienols consist of aqueous acetonitrile (methanol) or a combination of these solvents [51,52,54,56,63,46,65,69,72]. In many cases, investigators have used non-aqueous mobile phases for their assays [53,55,33,35,59,60,62,44,64,70,73]. A variety of organic modifiers (alcohol, dichloromethane, ether, ester, and hexane) can be added to either aqueous or non-aqueous solvent systems to meet optimal separation requirements for specific analytical applications [55,57,33,35,59–62,44,66,68,71,73]. Mobile phases for HPLC–electrochemical detection (ED) contain various electrolytes such as sodium perchlorate [53,56,62,46], lithium perchlorate [55,71,73] or tetraethylammonium hydroxide [57]. With regard to the choice of reversed-phase elution modes, isocratic elution methods (23 methods in Table 4) have been more frequently used for the antioxidant determinations than gradient elution procedures [60,61,67,68,71] outlined in methods 12, 13, 22, 23, and 26 of Table 4 because of the methodological convenience of the former. HPLC of complex antioxidant samples containing a wide range of endogenous analytes usually call for gradient elution to cover all structural types within reasonable run times.

Table 4  
Reversed-phase HPLC analysis of tocopherols and tocotrienols<sup>a</sup>

Method (detection)	Stationary/mobile phase	Elution order (run time, min)	Ref.
(1) FL, 295 <sub>Ex</sub> 330 <sub>Em</sub>	Vydac C <sub>8</sub> , 10 μm 250×3.2 mm MeOH–water (95:5) +acetic acid	δT→(β+γ)T→αT	[51] Foods
(2) FL, 296 <sub>Ex</sub> 330 <sub>Em</sub>	μBondapak C <sub>18</sub> , 10 μm 300×3.9 mm MeOH–water (95:5)	(γ+β)T <sub>3</sub> →αT <sub>3</sub> →δT→(β+γ)T→αT (10 min)	[52] Feeds
(3) ED, +0.8 V	Yanapak ODS-T C <sub>18</sub> , 5 μm 250×4.0 mm MeOH+50 mM NaClO <sub>4</sub>	δT→(β+γ)T→αT	[53] Feeds
(4) UV, 300 nm	Spheri-5 RP-18, 5 μm 100×2.1 mm MeOH–water (95:5)	δT→(β+γ)T→αT	[54] Vegetable oils
(5) ED, +0.8 V	Ultrasphere ODS, 5 μm 250×4.6 mm MeOH–EtOH (1:9)+20 mM LiClO <sub>4</sub>	δT→γT→αT (10 min)	[55] Tissues
(6) ED, +0.6 V	Spherisorb ODS II, 3 μm 150×4.6 mm MeOH–water (96:4)+NaClO <sub>4</sub>	δT→γT→αT (15 min)	[56] Tissues
(7) ED, +0.3 V	Ultrasphere ODS, 5 μm 150×4.6 mm IP–ACN–water–TEA–acetic acid (60:20:19.4:0.5:0.1)	δT→γT→αT (50 min)	[57] Tissues
(8) UV, 295 nm	Polymeric C <sub>18</sub> , 5 μm 750×0.53 mm ACN–HX (91.5:8.5)	δT→βT→γT→αT (130 min)	[33]
(9) UV, 295 nm	Zorbax ODS, 5 μm 250×4.6 mm ACN–MeOH–CH <sub>2</sub> Cl <sub>2</sub> (60:35:5)	δT <sub>3</sub> →γT <sub>3</sub> →αT <sub>3</sub> →δT→(β+γ)T→αT (10 min)	[35] Palm oil
(10) FL, 298 <sub>Ex</sub> 325 <sub>Em</sub>	YMCPack-A-ODS, 5 μm 150×4.6 mm IP–water (65:35)	δT→γT→βT→αT (50 min)	[58] Tissues
(11) UV, 300 nm	Resolve C <sub>18</sub> , 5 μm 300×3.9 mm ACN–CH <sub>2</sub> Cl <sub>2</sub> –MeOH–octanol (90:15:10:0.1)	γT→αT (10 min)	[59] Tissues
(12) FL, 295 <sub>Ex</sub> 335 <sub>Em</sub>	Bakerbond C <sub>18</sub> , 5 μm 250×4.6 mm ACN–MeOH+ ammonium acetate–ethyl acetate (gradient elution)	εT→δT→γT→αT (10 min)	[60] Foods
(13) FL, 290 <sub>Ex</sub> 330 <sub>Em</sub>	Hypersil ODS, 5 μm 200×2.1 mm ACN–MeOH–IP–water (45:45:5:5) →ACN–MeOH–IP (50:45:5) (gradient elution)	δT <sub>3</sub> →(β+γ)T <sub>3</sub> →αT <sub>3</sub> →δT→(β+γ)T→αT (15 min)	[61] Rice bran oil
(14) ED, 0.35 V	Superspher 100RP-18, 4 μm 250×4 mm MeOH–EtOH (1:9)+ 2.5 mM HClO <sub>4</sub> +7.5 mM NaClO <sub>4</sub>	δT→(β+γ)T→αT (15 min)	[62] Tissues
(15) FL, 298 <sub>Ex</sub> 328 <sub>Em</sub>	Ultrasphere ODS, 5 μm 250×4.6 mm ACN–THF–MeOH– 1% ammonium acetate (684:220:68:28)	δT <sub>3</sub> →(β+γ)T <sub>3</sub> →αT <sub>3</sub> →δT→(β+γ)T→αT (22 min)	[44] Tissues

Table 4. Continued

Method (detection)	Stationary/mobile phase	Elution order (run time, min)	Ref.
(16) UV, 290 nm	Taxsil PFP, 5 $\mu$ m MeOH–water (92:8)	$\delta$ T $\rightarrow$ $\beta$ T $\rightarrow$ $\gamma$ T $\rightarrow$ $\alpha$ T (20 min)	[63] Vegetable oils
(17) PDA, 200 nm $\rightarrow$ 800 nm	C <sub>18</sub> Vydac201TP54, 5 $\mu$ m 250 $\times$ 4.6 mm MeOH–ACN (9:1)	$\delta$ T $\rightarrow$ $\alpha$ T (10 min)	[64] Tissues
(18) ED, +0.6 V	Spherisorb ODS, 5 $\mu$ m 250 $\times$ 4.5 mm MeOH–water (90:10)+NaClO <sub>4</sub>	( $\beta$ + $\gamma$ )T <sub>3</sub> $\rightarrow$ ( $\beta$ + $\gamma$ )T $\rightarrow$ $\alpha$ T (10 min)	[46] Vegetable oils
(19) FL, 298 <sub>Ex</sub> 345 <sub>Em</sub>	Asahipak ODP, 5 $\mu$ m 250 $\times$ 4.6 mm ACN–water (85:15)	$\delta$ T $\rightarrow$ $\zeta$ T $\rightarrow$ $\beta$ T $\rightarrow$ $\gamma$ T $\rightarrow$ $\alpha$ T (130 min)	[65]
(20) FL, 298 <sub>Ex</sub> 345 <sub>Em</sub>	YMCPack-ODS-A, 5 $\mu$ m 250 $\times$ 4.6 mm MeOH–H <sub>2</sub> (95:5)	$\delta$ T-AC $\rightarrow$ $\zeta$ T-AC $\rightarrow$ $\beta$ T -AC $\rightarrow$ $\gamma$ T-AC $\rightarrow$ $\alpha$ T-AC (60 min)	[65]
(21) PDA, 200 nm $\rightarrow$ 800 nm	Suplex pKb-100, 5 $\mu$ m 250 $\times$ 4.6 mm MeOH–BME–water (80:20:5)	$\alpha$ T-AC $\rightarrow$ $\gamma$ T $\rightarrow$ $\alpha$ T (20 min)	[66] Tissues
(22) UV, 295 nm MS	YMCPack-C30, 3 $\mu$ m 250 $\times$ 4.6 mm Acetone–water+AgClO <sub>4</sub> (90:10) $\rightarrow$ (100:0) (gradient elution)	$\delta$ T $\rightarrow$ $\gamma$ T $\rightarrow$ $\beta$ T $\rightarrow$ $\alpha$ T $\rightarrow$ $\alpha$ T-AC (10 min)	[67] Foods
(23) PDA, 200 nm $\rightarrow$ 800 nm	Microsorb MV-C <sub>18</sub> , 3 $\mu$ m 100 $\times$ 4.6 mm A:MeOH–water (3:1) +ammonium acetate B:MeOH–CH <sub>2</sub> Cl <sub>2</sub> (4:1) A $\rightarrow$ B $\rightarrow$ B (gradient elution)	$\gamma$ T $\rightarrow$ $\alpha$ T (25 min)	[68] Tissues
(24) FL, 290 <sub>Ex</sub> 330 <sub>Em</sub>	Asahipak ODP, 5 $\mu$ m 250 $\times$ 4.6 mm ACN–water (70:30)	$\delta_1$ T <sub>3</sub> $\rightarrow$ $\delta_2$ T <sub>3</sub> $\rightarrow$ $\delta_3$ T <sub>3</sub> $\rightarrow$ $\delta_4$ T <sub>3</sub> $\rightarrow$ $\beta_1$ T <sub>3</sub> $\rightarrow$ $\gamma_1$ T <sub>3</sub> $\rightarrow$ $\beta_2$ T <sub>3</sub> $\rightarrow$ $\gamma_2$ T <sub>3</sub> $\rightarrow$ $\beta_3$ T <sub>3</sub> $\rightarrow$ $\gamma_3$ T <sub>3</sub> $\rightarrow$ $\beta_4$ T <sub>3</sub> $\rightarrow$ $\gamma_4$ T <sub>3</sub> $\rightarrow$ $\alpha_1$ T <sub>3</sub> $\rightarrow$ $\alpha_2$ T <sub>3</sub> $\rightarrow$ $\alpha_3$ T <sub>3</sub> $\rightarrow$ $\alpha_4$ T <sub>3</sub> (40 min)	[69]
(25) UV, 295 NMR. MS	YMCPack-C30, 3 $\mu$ m 250 $\times$ 4.6 mm MeOH	$\delta$ T <sub>3</sub> $\rightarrow$ $\gamma$ T <sub>3</sub> $\rightarrow$ $\beta$ T <sub>3</sub> $\rightarrow$ $\alpha$ T <sub>3</sub> $\rightarrow$ $\alpha$ T <sub>1</sub> $\rightarrow$ $\alpha$ T (40 min)	[70] Palm oil
(26) ED, 0.5 V	Ultrasphere ODS, 5 $\mu$ m 250 $\times$ 4.6 mm MeOH–water–EtOH+0.2%LiClO <sub>4</sub> (gradient elution)	$\gamma$ T <sub>3</sub> $\rightarrow$ $\alpha$ T <sub>3</sub> $\rightarrow$ $\gamma$ T $\rightarrow$ $\alpha$ T (20 min)	[71] Tissues
(27) FL, 296 <sub>Ex</sub> 340 <sub>Em</sub>	Hypersil ODS, 5 $\mu$ m 150 $\times$ 4.6 mm MeOH–water (96:4)	$\epsilon$ T $\rightarrow$ $\delta$ T $\rightarrow$ $\gamma$ T $\rightarrow$ $\alpha$ T (15 min)	[72] Tissues
(28) ED, +0.6 V	SuperPac PeP-S RP <sub>C<sub>2</sub>/C<sub>18</sub></sub> , 5 $\mu$ m 250 $\times$ 4.6 mm MeOH–EtOH–IP (88:24:10) +13 mM LiClO <sub>4</sub>	$\gamma$ T <sub>3</sub> $\rightarrow$ $\gamma$ T $\rightarrow$ $\alpha$ T (10 min)	[73] Tissues

<sup>a</sup> IP, isopropanol; ACN, acetonitrile; TEA, tetraethyl ammonium hydroxide; HX, hexane; THF, tetrahydrofuran; BME, t-butylmethyl ether; MeOH, methanol; EtOH, ethanol.

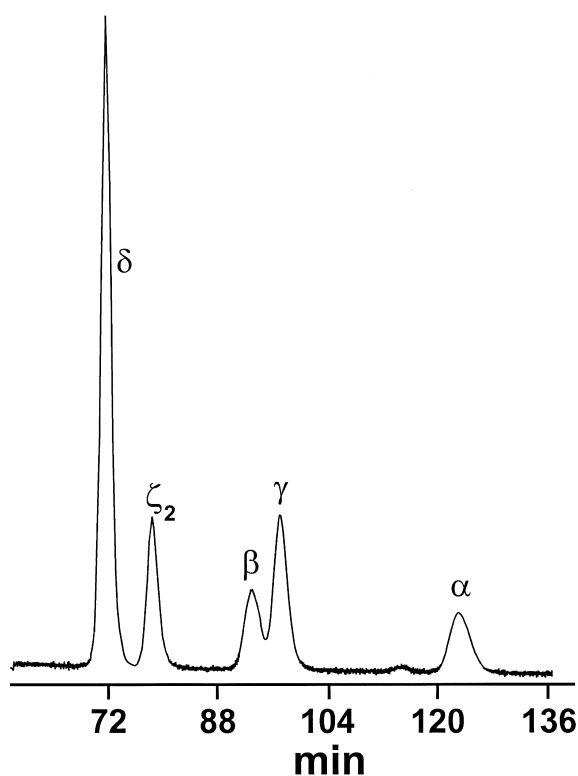


Fig. 5. Reversed-phase HPLC–FL detection of tocopherols (on ODPVA) in a standard mixture (from [65] with permission).

### 6.3. Elution characteristics

Table 4 contains published information on the reversed-phase HPLC elution order of tocopherols and tocotrienols analyzed under various experimental conditions. In a traditional ODS system, the elution of the antioxidant components in aqueous acetonitrile(methanol) is in order of increasing analyte

hydrophobicity:  $\delta T_3 \rightarrow (\beta + \gamma) T_3 \rightarrow \alpha T_3 \rightarrow \delta T \rightarrow (\beta + \gamma) T_6 \rightarrow \alpha T$  where the  $\beta$ -, and  $\gamma$ -species co-elute (Fig. 4). Apparently, tocopherols as a group are consistently more hydrophobic than members of the tocotrienol group and all the unsaturated  $T_3$  components elute through an ODS column before the saturated  $T$ -components. This elution pattern is markedly different from that observed in normal-phase elution  $\alpha T \rightarrow \alpha T_3 \rightarrow \beta T_6 \gamma T \rightarrow \beta T_3 \gamma T_3 \delta T \rightarrow \delta T_3$  in which the saturated  $\alpha$ -, and  $\delta$ -tocopherols are adjacently followed by the corresponding unsaturated analogues presumably due to small polarity differences between tocopherols and tocotrienols of the same homologous types ( $\alpha T$  vs.  $\alpha T_3$ ;  $\delta T$  vs.  $\delta T_3$ ).

Under reversed-phase HPLC conditions, injection of a sample of a four-component tocopherol mixture onto either of the two unconventional polar pentafluorophenylsilica (PFPS)- or octadecanoyl polyvinyl alcohol (ODPVA) phase (Fig. 5) leads to sequential elution of  $\delta T$ ,  $\beta T$ ,  $\gamma T$ , followed by  $\alpha T$  as shown in methods 16 [63] and 19 [65] of Table 4. With a non-aqueous mobile phase of hexane in acetonitrile, HPLC of tocopherols on a polymeric  $C_{18}$  column [33] (method 8 in Table 4) also produces the same elution order as on PFPS or ODPVA columns. However, subjecting the tocopherol sample to reversed-phase HPLC on a triacontylsilica column [67], a long-chain alkyl variant of alkylsilica, results in differential elution of the  $\beta$ -, and  $\gamma$ -tocopherols on the highly hydrocarbonaceous  $C_{30}$  phase:  $\delta T_6 \gamma T_6 \beta T_6 \alpha T$ , which is in order directly opposite to normal-phase elution of the same sample. In an exceptional situation employing a mobile phase of aqueous isopropanol, the  $\beta$ – $\gamma$  pair of tocopherols has been resolved on ODS [58] and the elution order of

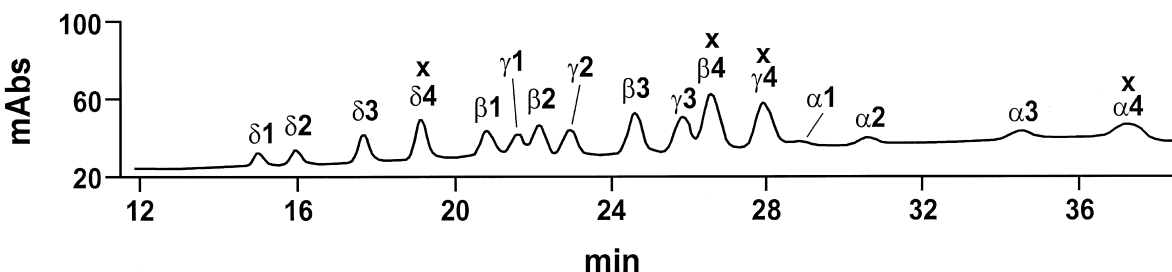


Fig. 6. Reversed-phase HPLC–FL detection of tocotrienols (on ODPVA) in a standard mixture. 1, *cis/cis*; 2, *cis/trans*; 3, *trans/cis*; 4, *trans/trans*. Peaks with 'x' correspond to natural compounds (from [69] with permission).

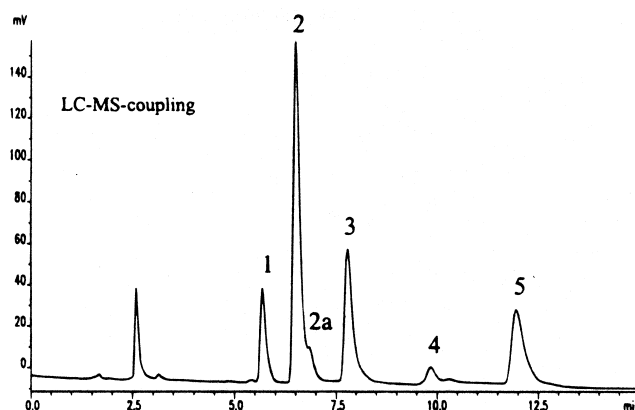


Fig. 7. Reversed-phase HPLC–UV detection of tocopherols and tocotrienols (on triacontylsilica) in a palm oil extract. 1,  $\delta T_3$ ; 2,  $\gamma T_3$ ; 2a,  $\beta T_3$ ; 3,  $\alpha T_3$ ; 4,  $\alpha T_1$ ; 5,  $\alpha T$  (from [70] with permission).

the four tocopherol components of interest is identical to that obtained with the  $C_{30}$ -silica phase described [67]. Further, as mentioned earlier, the first complete reversed-phase HPLC separation of tocopherol ester derivatives including  $\beta$ -, and  $\gamma$ -isomers with an ODS column has been reported [65]. Replacing the hydroxy group with an ester function group appears to enhance hydrophobic differentiation of the  $\beta$ - and  $\gamma$ -tocopherols on ODS phases. Under aqueous mobile phase conditions, the elution pattern of tocopherol acetates (AC) ( $\delta T$ -AC  $\rightarrow$   $\zeta T$ -AC  $\rightarrow$   $\beta T$ -AC  $\rightarrow$   $\gamma T$ -AC  $\rightarrow$   $\alpha T$ -AC) on ODS (method 20 in Table 4) [65] resembles that of parent tocopherols on ODPVA.

Comparisons of ODPVA reversed-phase HPLC elution characteristics [65] (method 19, Table 4) of three dimethyltocols ( $\zeta$ -,  $\beta$ -, and  $\gamma$ -tocopherols) with their silica-normal-phase HPLC elution behavior [41,48] (methods 18 and 25, Table 3) show the same order of elution in the two antithetical HPLC modes:  $\zeta T \rightarrow \beta T \rightarrow \gamma T$ . Conceivably, hindered interactions between the hydrophobic moiety at the 6-position (adjacent to 5- and 7-methyls) of the  $\zeta$ -tocopherol and a stationary phase might result in weak retention relative to the  $\beta$ - and  $\gamma$ -isomers.

In connection with another study on lipid anti-oxidants, the author succeeded in the total reversed-phase separation of a 16-component mixture of synthetic tocotrienols on ODPVA or triacontylsilica ( $C_{30}$ -silica) in aqueous acetonitrile systems and observed an elution order  $\delta_1 T_3 \rightarrow \delta_2 T_3 \rightarrow$

$\delta_3 T_3 \rightarrow \delta_4 T_3 \rightarrow \beta_1 T_3 \rightarrow \gamma_1 T_3 \rightarrow \beta_2 T_3 \rightarrow \gamma_2 T_3 \rightarrow \beta_3 T_3 \rightarrow \gamma_3 T_3 \rightarrow \beta_{4-3} \rightarrow \gamma_4 T_3 \rightarrow \alpha_1 T_3 \rightarrow \alpha_2 T_3 \rightarrow \alpha_3 T_3 \rightarrow \alpha_4 T_3$  for either column (method 24, Table 4) [69]. With a pentafluorophenylsilica phase in an aqueous methanol system, the mixture was partially resolved into 15 tocotrienol components with two remaining unresolved. The separated analytes exhibited elution characteristics ( $\beta_1 T_3 \rightarrow \beta_2 T_3 \rightarrow \gamma_1 T_3 \rightarrow \gamma_2 T_3 \rightarrow \beta_3 T_3 \rightarrow \beta_4 T_3 \rightarrow \gamma_3 T_{3-3} \rightarrow \gamma_4 T_3$ ) of the  $\beta$ -, and  $\gamma$ -components distinctly different from those obtained with ODPVA, while the  $\delta$ -, and  $\alpha$ -tocotrienol components maintained identical elution order for both columns. Hence, the elution order of the eight  $\beta$ -, and  $\gamma$ -tocotrienols was found very sensitive to variation in HPLC stationary phase and mobile phase conditions.

As pointed out at the beginning of the text, each natural tocotrienol has the trans/trans olefinic geometry at the side chain and corresponds to peak component 4 of a homologue or an isomer in a synthetic mixture (Fig. 6). These natural tocotrienols elute through the ODPVA column in the same fashion as tocopherols:  $\delta_4 T_3 \rightarrow \beta T_3 \rightarrow \gamma_4 T_3 \rightarrow \alpha_4 T_3$ . Very recently, samples of tocotrienols and related compounds have been analyzed by reversed-phase HPLC–MS with a triacontylsilica ( $C_{30}$ -silica) column [70] (method 25, Table 4) (Fig. 7). In the analysis, mobile phases employed methanol in the absence of water. The compounds assayed emerge from the column in the following sequence:  $\delta T_3 \rightarrow \gamma T_3 \rightarrow \beta T_3 \rightarrow \alpha T_3 \rightarrow \alpha T_1 \rightarrow \alpha T$ . This elution order differs from that obtained with an ODPVA



column as a result of the acute susceptibility of  $\beta$ -, and  $\gamma$ -tocotrienols to mobile phase- and stationary phase effects derived from interplay of polarity and hydrophobicity among analyte solutes, stationary phases, and mobile phases. For general application, the first example of separating all eight components of natural lipid antioxidants in the reversed-phase mode can be found in method 24 (Table 4) [69]. In the method, a standard mixture of tocopherols and tocotrienols was completely resolved into eight components which elute through a ODPVA column in order of increasing solvophobicity:  $\delta T_3 \rightarrow \beta T_3 \rightarrow \gamma T_3 \rightarrow \alpha T_3 \rightarrow \delta T \rightarrow \beta \rightarrow \gamma T \rightarrow \alpha T$ . The order is not exactly the reversal of that normally found in normal-phase HPLC of the antioxidants ( $\alpha T \rightarrow \alpha T_3 \rightarrow \beta T \rightarrow \gamma T \rightarrow \beta T_3 \rightarrow \gamma T_3 \rightarrow \delta T \rightarrow \delta T_3$ ).

## 7. Detection techniques

There are several commercial HPLC detectors (Tables 3 and 4) available in the market for evaporative light-scattering detection (ELSD), UV absorbance detection, fluorescence (FL) detection, and electrochemical detection (ED) of tocol-derived lipid antioxidants present in HPLC column effluents. Of these, ED is known to provide the highest sensitivity and has proven useful for the trace analysis of the antioxidants by reversed-phase HPLC–ED. In recent years, ELSD methodology has been widely utilized in lipid analysis. In a comparative study on detector sensitivity of two detection systems, a group of scientists demonstrated that detection sensitivity of an ELSD system was by far inferior to that of a FL detector (method 19, Table 3) [42]. Accordingly, the observed detection sensitivity of various detectors appear to fall in the following trend: EC > FL > UV > ELSD. Irrespective of the general acceptance of ELSD for routine lipid analyses, investigators opt to use other detection methods (i.e. ED, FL, or UV) for antioxidant determinations because of the poor sensitivity and selectivity inherent with ELSD.

A survey of the published methods compiled in Table 3 indicates that the FL detection technique (21 out of 27 methods) has been the top choice for many investigators pursuing normal-phase HPLC assays of the title antioxidants. This prevailing inclination

toward the use of FL detection is ascribed to its increased selectivity, sensitivity, and specificity in comparison to UV detection. On the other hand, the number of applications utilizing ED, and FL and UV detection in conjunction with reversed-phase HPLC of tocopherols and tocotrienols are nearly equal among the published methods shown in Table 4. Thus, ED, and FL and UV detection techniques have been taken up in eight, ten, and ten methods, respectively. Three of the UV methods in Table 4 employ photodiode array (PDA) detection modes [64,66,68] as given in methods 17, 21, and 23 of the table.

By virtue of their low oxidative potentials, tocol-derived antioxidants can easily be analyzed by reversed-phase HPLC–ED, which is the most sensitive and specific detection method known to date for the tocol compounds. Using either an amperometric or a coulometric detector, the compounds of interest can be detected with detection sensitivity in the neighborhood of low picograms. As mentioned earlier, ED is most suitable for reversed-phase HPLC work because the required electrolytes (e.g. perchlorates or acetates) at 5–50 mM concentrations are normally miscible with the aqueous mobile phase eluents. The insolubility of the electrolytes in normal-phase eluents preclude the practical applicability of ED in the normal-phase HPLC analysis of the lipid antioxidants. In rare cases, HPLC–ED analysis can be carried out by mixing an electrolyte solution with column effluents under normal-phase conditions.

Switching detection methods from ED to FL detection of lipid antioxidants often leads to a 10–20-fold loss in sensitivity, while a sensitivity loss of as high as three to four orders of magnitude (i.e.  $10^3$ – $10^4$ -fold) can be brought about by a change in the method from HPLC–ED to HPLC–UV detection. Vegetable oil samples containing major tocopherols and tocotrienols at moderate levels are frequently analyzed by HPLC–UV detection at a single wavelength detector setting. In some instances, detection of the antioxidants in multi-component samples containing other valuable compounds mandates the use of variable-wavelength detection systems to facilitate measurement of each compound at  $\lambda_{\max}$  for achieving maximal detection sensitivity and selectivity. An example of such multiple scanning ab-

sorbance detection is the PDA method mentioned earlier for tocopherol analyses [64,66,68].

As an integral part of antioxidant assays entailing complex sample matrices, analysts must identify and characterize with certainty each component including trace analytes of interest in the sample mixture. To this end, interfacing HPLC with mass spectrometer (MS) [67,70] (methods 22 and 25, Table 4) or nuclear magnetic resonance spectrometry [70] (method 25, Table 4) not only allows simultaneous separations and quantitation but also provides a powerful means for structural elucidation of new antioxidants as well as confirmation of known antioxidant structures. Tocopherols and tocotrienols in crude palm oil extracts have been analyzed by HPLC–coordination ion spray MS through the addition of silver ions to form the adducts (method 25, Table 4) [70]. Generally, in most MS experiments, interferences from sample matrices must be eliminated by sample purification or column switching at the LC–MS interface. MS detection in the single-ion monitoring mode and tandem techniques (HPLC–MS–MS) [88] can afford antioxidant assays with high sensitivity and specificity.

## 8. Other chromatographic techniques

In the past few years, a new breed of chromatographic technology, capillary electrochromatography (CEC), has progressed rapidly and received a great deal of interest from the separation science community. The CEC technique features high-efficiency, high-resolution, and high-speed separations and poses immense analytical potential for the determination of tocol-derived lipid antioxidants which possess no net charge. A preliminary study [74] conducted at the author's laboratory shows that, except for the  $\beta$ - and  $\gamma$ -isomers, tocopherols and tocotrienols can be separated by CEC with a  $C_8$ -silica column and a mobile phase of acetonitrile–tris buffer, 25 mM (95:5). Under these conditions, the antioxidant mixture elute in the same manner as observed in reversed-phase HPLC:  $\delta T_3 \rightarrow \beta T_3 + \gamma T_3 \rightarrow \alpha T_3 \rightarrow \delta T \rightarrow \beta T + \gamma T \rightarrow \alpha T$ . Further evaluation of the CEC behavior of the antioxidant compounds and CEC optimization experiments with various mobile phase buffers and stationary phases are in progress.

Lately, supercritical fluid chromatography (SFC), a bridge between GC and HPLC, has gained popularity in food industries on account of the employment of inert, low-temperature supercritical carbon dioxide as mobile phase eluent. The technique is ideally suited for the analysis of labile tocopherols and tocotrienols in food including vegetable oils. Coupling supercritical fluid extraction (SFE) with SFC allows sample extraction, preconcentration, chromatographic quantitation and preparative fractionation in a single operation. Food materials have been subjected to SFE, preparative SFC, and SFE–SFC to enrich and isolate individual tocopherols and tocotrienols [75–78]. With an ODS column,  $\beta$ - and  $\gamma$ -tocopherols in vegetable oils have been separated by SFC under elution with carbon dioxide containing 0.5% methanol as modifier [79]. Capillary SFC methods for the analysis of tocopherols and other compounds in edible oils and fish oils have been reported [80,81]. Using a microelectrochemical detector, a group of scientists was able to determine tocopherols at nanogram levels in vegetable oil samples by packed capillary column SFC [82]. The silica capillary was packed with 5  $\mu$ m ODS and eluted with a mobile phase of carbon dioxide modified with 8% methanol.

The observed differences in biopotency of tocopherol stereoisomers mentioned earlier have potential implications for human nutrition. Chiral separations of synthetic tocopherols and tocotrienols would provide pure individual stereoisomers for nutrition, metabolism, and pharmaceutical studies. Four (all-*rac*)- $\alpha$ -tocopherol acetate racemates (2R4'R8'S/2S4'S8'R, 2R4'R8'R/2S4'S8'S, 2R4'S8'R/2S4'R8'S, and 2R4'S8'S/2S4'R8'R) in production batch samples have been converted to their methyl ether via lithium aluminum hydride reduction prior to methylation with dimethyl sulfate, and analyzed by capillary GC–FID [83]. All eight stereoisomers of (all-*rac*)- $\alpha$ -tocopherol can be separated as their ethyl ether derivatives by consecutive chiral phase HPLC and GC, whereby two groups of 2R- and 2S-configurations are initially resolved by HPLC followed by GC separations of each of the four isomers in the 2R- and 2S-groups [84]. Chiral-phase HPLC of (all-*rac*)- $\alpha$ -tocopherol acetates on a commercially packed Chiralpak OP(+) column produces four peaks. The first peak consists of four unresolved stereoisomers (2R4'R8'R, 2R4'S8'R,

2R4'R8'S, and 2R4'S8'S), whereas there are two unresolved components (2S4'S8'S and 2S4'S8'R) in the second peak. The third peak is due to 2S4'R8'R- $\alpha$ -tocopherol acetate, the antipodal isomer of the natural 2R4'R8'R-component [85]. A commercial Chiralcel OD-H column has been connected in series with Sumichiral OA4100 column to separate natural tocopherol from its enantiomer [86]. A most recent study [87] showed that structures of homologous and isomeric tocopherols, tocotrienols, and their acetates had dramatic influence on the chiral phase HPLC retention behavior and separation factors of antipodal pairs.

## 9. Nomenclature

ACN	Acetonitrile
BME	tert.-Butyl methyl ether
CEC	Capillary electrochromatography
IPE	Diisopropyl ether
PE	Dipropyl ether
ED	Electrochemical detection
ELSD	Evaporative light scattering detection
FL	Fluorescence
FID	Flame ionization detection
HAc	Acetic acid
HFIP	Hexafluoroisopropanol
HX	Hexane
IO	Isooctane
IP	Isopropanol
MS	Mass spectrometry
ODPVA	Octadecanoyl polyvinyl alcohol
ODS	Octadecylsilica
PFPS	Pentafluorophenylsilica
PDA	Photodiode array
SFC	Supercritical fluid chromatography
TEA	tetraethyl ammonium hydroxide
THF	Tetrahydrofuran
T-AC	Tocopherol acetate
T <sub>3</sub>	Tocotrienol
TMS	Trimethylsilyl

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