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Reversed-phase retention characteristics of tocotrienol antioxidants☆

S.L. Abidi*

Food Quality and Safety Research, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA

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Abstract

Mixtures of α-, β-, γ- and δ-tocotrienols were separated by reversed-phase (RP) high-performance liquid chromatography (HPLC). Four sets of subcomponents (*cis*/*cis*-, *cis*/*trans*-, *trans*/*cis*- and *trans*/*trans*-geometrical isomers) were further resolved under various HPLC conditions. Retention characteristics of the β- and γ-isomers on octadecylsilica (ODS) were indistinguishable. However, complete separations of all sixteen tocotrienol components were achieved by RP-HPLC with a nonsilica-based octadecanoyl polyvinyl alcohol (ODPVA) column. In this system, β-tocotrienol peaks were found to interpose alternatively with those of γ-tocotrienols, which was attributable to apparent differences in intrinsic molecular polarity and hydrophobicity. HPLC with a pentafluorophenylsilica (PFPS) column led to partial separations of tocotrienols, of which two subclass species remained unresolved. Peak profiles obtained with PFPS columns of different packings were similar but not identical. Nonetheless, the component elution patterns on PFPS were markedly different from those on ODPVA. Furthermore, the use of a triacontylsilica column enabled separations of fifteen components and yielded an elution order parallel to that of an ODPVA column. In general, the elution sequence of tocotrienols appeared to depend largely on the type of organic modifier used, despite the subtle influence of stationary phases and the isomeric characteristics of alkenyl chains. While poorly resolved on silica, geometrical isomers were readily separated on the RP phases studied with acetonitrile–water as the preferred eluent. Factors affecting analyte retention and component resolution were delineated.

Keywords: Stationary phases, LC; Geometrical isomers; Tocotrienols; Antioxidants; Lipids

1. Introduction

Tocotrienols are natural lipid antioxidants occurring in plants (e.g. palm oil, coconut oil, rice bran oil and cereal grains) as a complex mixture of 2-methyl-6-chromanol-derived homologues and aromatic ring position isomers. Each compound has a three-terpene-unit side chain at the C-2 position containing C-3′, C-7′ and C-11′ olefinic bonds (Fig. 1). In view of their well-recognized nutritional values and health benefits [1,2], accurate analyses of these substances provide useful information on the antioxidant levels in vegetable oils. Distribution patterns of tocotrienol components are known to vary widely among different oilseeds [3–7] and have a direct bearing on the antioxidative properties of tocotrienol samples isolated from various natural origins. It has been speculated that tocotrienols may serve as potential

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*Tel.: +1-309-681-6390; fax: +1-309-681-6686.

E-mail address: abidis@ncaur1.ncaur.gov (S.L. Abidi)

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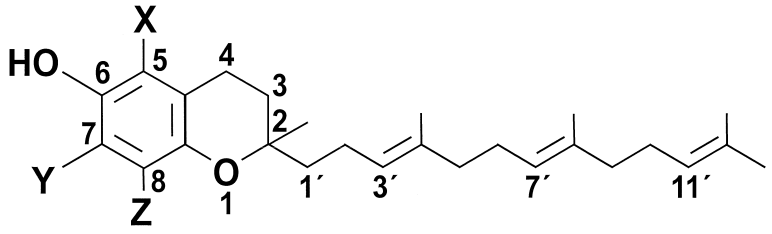

(A) X = CH_3, Y = CH_3, Z = CH_3
(B) X = CH_3, Y = H, Z = CH_3
(C) X = H, Y = CH_3, Z = CH_3
(D) X = H, Y = H, Z = CH_3

Fig. 1. Structures of investigated tocotrienols: (A) α-tocotrienol, (B) β-tocotrienol, (C) γ-tocotrienol and (D) δ-tocotrienol.

markers for the characterization and differentiation of vegetable oils [8,9].

For decades, the development of a suitable analytical technique for the isolation and separation of closely related tocotrienols has been a challenge for chromatographers. Complete speciation of the antioxidant complex is of special interest to lipid scientists for pursuing structure–activity studies. Tocotrienol components have been analyzed by gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). Most GLC techniques suffer from some limitations of incomplete resolution and extensive sample clean-up. Normal-phase HPLC separations of the major α-, β-, γ- and δ-tocotrienol components are fairly straightforward and have met with moderate success [10]. Unfortunately, existing normal-phase HPLC methods fail to further resolve geometrical isomeric subcomponents [11], require variable long equilibration times [12] between analyses, and entail the use of toxic and volatile organic solvents. Moreover, nonaqueous mobile phases are not compatible with electrochemical detection [13,14]. The majority of the normal-phase technical problems can be overcome by using a reversed-phase procedure.

Because of the inability of reversed-phase methodology to effect resolution of β- and γ-isomers with a conventional octadecylsilica column, publications on reversed-phase (RP) HPLC analyses of tocotrienols are scarce. Unlike the popular normal-phase method, the RP-HPLC technique has found few applications in oil assays [14–16]. In light of our recent successful separation of β- and γ-tocopherols, the saturated analogues of tocotrienols, with unconventional reversed-phase columns [17], a similar RP-HPLC approach was extended to the separation of tocotrienol mixtures containing a total of sixteen components.

2. Experimental

2.1. Chemical and reagents

Tocotrienol standards, α-, β-, γ- and δ-tocotrienols, were synthetic materials obtained from Merck (Wien, Austria). Natural tocotrienols were either gifts from Professor C.C. Akoh (University of Georgia, Athens, GA, USA) or isolated from palm oils by preparative HPLC. The four tocopherol standards were obtained from Matreya, (Pleasant Gap, PA, USA). The standard samples were stored at −30°C. The HPLC solvents acetonitrile, isopropanol and methanol were obtained from Fisher (Fair Lawn, NJ, USA). HPLC-grade water was obtained by passing in-house distilled water through a Millipore (Bedford, MA, USA) Milli-Q water purifier.

2.2. High-performance liquid chromatography

HPLC experiments were carried out with a

Thermo Separation Products Model SP8700XR liquid chromatograph coupled to a Model 8500 dynamic mixer. An Applied Biosystems Model 980 programmable fluorescence detector was interfaced to the LC system.

Column effluents were monitored at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. For mobile phases, various combinations of acetonitrile, isopropanol or methanol in water were pumped at flow-rates of 0.3–1 ml/min. The mobile-phase solvents were degassed with a helium sparge and filtered through a 0.02-μm filter prior to entering a reversed-phase column.

Samples (1–3 μl) of standard solutions in mobile phase solvents (50–70 μg/ml) were injected onto a reversed-phase column through a Rheodyne (Cotati, CA, USA) Model 7125 injector housed with a 10-μl loop. Average retentions (t) were mean values of three replicate injections with RSDs of 0.1–1.0%. Capacity factors (k') were determined based on the equation $k=(t/t_0-1)$, where t and t_0 are the respective retention times of an analyte and an unretained solute.

A number of commercial RP-HPLC columns (5 μm, 250×4.6 mm I.D.) were evaluated: (1) YMC-ODS-A (Wilmington, NC, USA), (2) Waters NovaPak C_{18}, 4 μm (300×3.9 mm I.D.; Milford, MA, USA), (3) Phenomenex Curosil-PFP (Torrance, CA, USA), (4) MetaChem Taxsil, 3 μm (Torrance), (5) Astec AsahiPak ODP (Whippany, NJ, USA), an octadecanoyl poly(vinyl alcohol) phase, and (6) YMC-C30, a triacontylsilica phase.

3. Results and discussion

As shown in Fig. 1, structures of tocotrienol homologues can be classified by the number of the aromatic ring methyl group: (1) δ-tocotrienol with one methyl group, (2) β- and γ-tocotrienols with two methyl groups, (3) α-tocotrienol with three methyl groups. The two dimethylated compounds in class (2) differ in structures merely by the position of the methyls. This isomeric pair can be separated by adsorptive differentiation in the normal-phase mode but cannot be differentiated by hydrophobic interaction in the reversed-phase mode. On the other hand, due to definitive differences in polarity and hydrophobicity, separations of the homologues in (1) and (3) classes can easily be achieved by normal-phase- or RP-HPLC.

As each of the four investigated (α-, β-, γ- and δ-) tocotrienol classes contains four subcomponents attributed to geometrical isomerization of alkenyl moieties (*cis*/*cis*, *cis*/*trans*, *trans*/*cis* and *trans*/*trans*), there are sixteen chemical entities in a tocotrienol mixture. The geometrical isomers were preferably separated by RP-HPLC because initial attempted normal-phase HPLC experiments yielded ill-defined broad subcomponent bands with no baseline separation. Solvophobic partition of analyte solutes with alkyl-bonded phases appeared to discriminate more effectively among geometrical isomers than normal-phase adsorption on silica.

Table 1 compares retention data obtained with two ODS columns of different specifications. It is clearly

Table 1
Retention characteristics of tocotrienols on different octadecylsilica phases with a mobile phase of acetonitrile–water (90:10, v/v) at a flow-rate of 1 ml/min

Stationary phase	Tocotrienol class	Capacity factor, k' Geometrical isomer			
		1	2	3	4
YMC-Pak-ODS	δ	7.76	8.19	8.47	8.89
	β	10.2	10.7	11.0	11.7
	γ	10.2	10.7	11.0	11.7
	α	13.6	14.3	14.8	15.6
NovaPak C_{18}	δ	5.03	5.19	5.51	5.83
	β	6.62	6.94	7.25	7.73
	γ	6.62	6.94	7.25	7.73
	α	9.00	9.32	9.79	10.4

demonstrated that the employment of a highly retentive column (% carbon loading: YMC-Pak column> NovaPak column) offered no advantages for the resolution of the β-γ isomers despite the observed clean separation of the four geometrical subcomponents of either isomer. Hence, regardless of the lack of uniformity in manufactural packings, HPLC of tocotrienols on ODS gave only twelve component peaks, leaving β1–γ1-, β2–γ2-, β3–γ3- and β4–γ4-pairs unresolved (Fig. 2 and Table 1). In the absence of any additional experimental evidence, the retention sequence of geometrical isomers was tentatively identified: *cis*/*cis* (peak 1) <*cis*/*trans* (peak 2) <*trans*/*cis* (peak 3) <*trans*/*trans* (peak 4), based on a traditional polarity rationale (Fig. 2). It was of note that the magnitude of isomer retention increased in the same fashion as peak intensity. Thus, the *cis*/*cis* component had the weakest peak intensity, while the most intense peak corresponded to the *trans*/*trans* component.

Fig. 3 shows the first successful RP-HPLC separation of tocotrienols on a nonsilica-based octadecanoyl polyvinyl alcohol (ODPVA) phase. At a slow flow-rate of 0.5 ml/min, all sixteen components were separated (Fig. 3A). Elution of the column with a mobile phase at a higher flow-rate (e.g. 1 ml/min) adversely affected the separation of γ1- and α1-tocotrienols, which appeared as hump shoulders of adjacent β2- and γ4-components, respectively (Fig. 3B). The two sets of individual β–γ component peaks (a total of eight components) were found to interpose with one another. Generally, separations of tocotrienol geometrical isomers on ODPVA were much more efficient with acetonitrile mobile phases than with those of methanol. Table 2 shows the effect of the water content in mobile phases of acetonitrile–water on the separation of tocotrienol components on ODPVA. Examination of retention data in the table indicated that it required a minimum of 25% water in the acetonitrile mobile phase to achieve adequate component resolution. With 12.5% water in acetonitrile, the α1–γ4 and β2–γ1 pairs were not resolved with k' values of 4.36 and 5.39, respectively. The latter pair remained unresolved

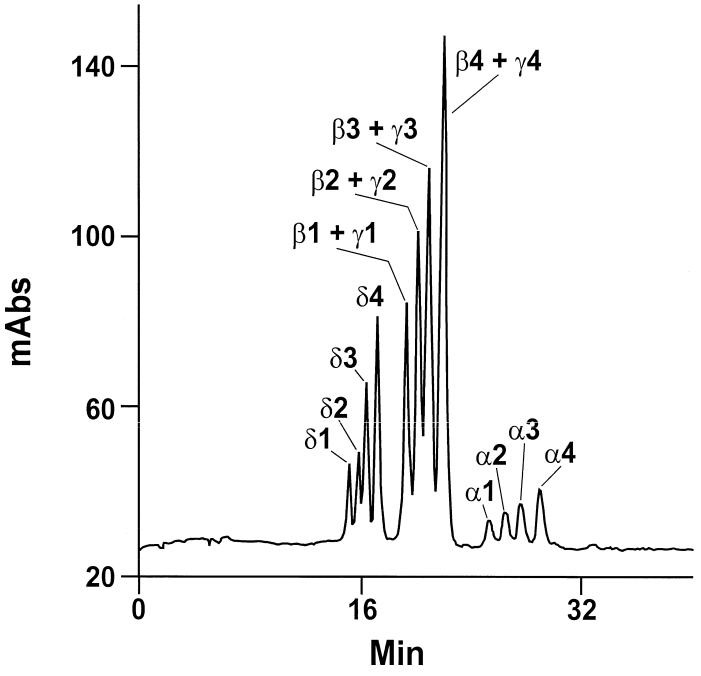

Fig. 2. Reversed-phase HPLC separations of synthetic tocotrienols on octadecylsilica. Column, NovaPak C_{18}. Mobile phase, acetonitrile–water (90:10, v/v). Flow-rate, 1 ml/min. Detector, fluorescence. Amounts injected: α, 53 ng; β, 90 ng; γ, 67 ng; δ, 50 ng. Component designations: (1) *cis*/*cis*, (2) *cis*/*trans*, (3) *trans*/*cis* and (4) *trans*/*trans*.

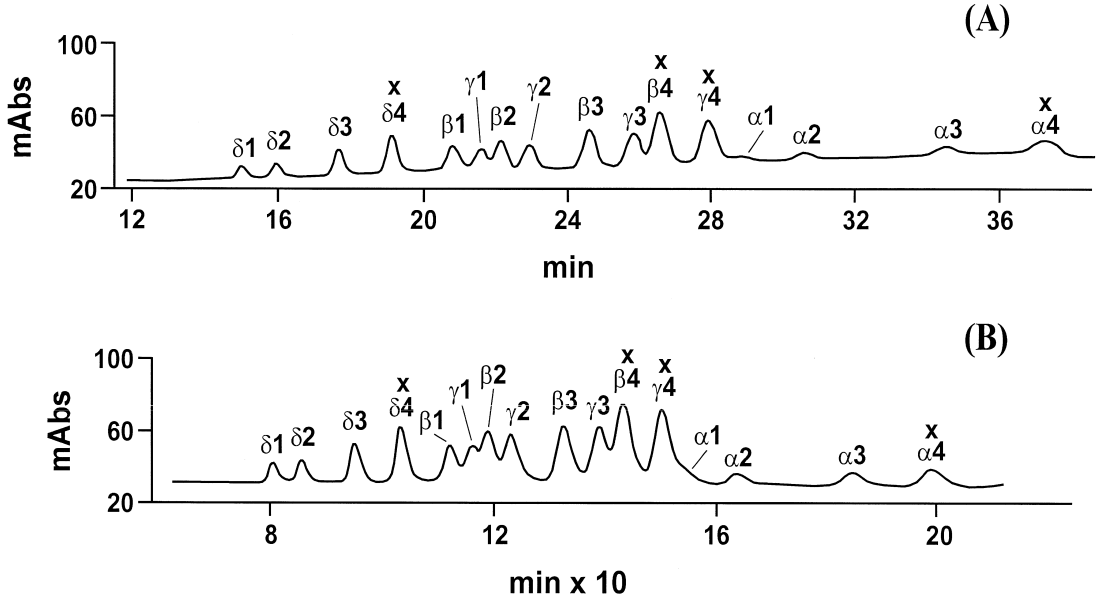

Fig. 3. Reversed-phase HPLC separations of synthetic tocotrienols on octadecanoyl polyvinyl alcohol. Column, AsahiPak ODP. Mobile phase, acetonitrile–water (70:30, v/v). Flow-rates, (A) 0.5 ml/min and (B) 1 ml/min. Detector, fluorescence. For amounts injected and component designations, see Fig. 2. Peaks with the label 'x' correspond to natural compounds.

even after the water content was increased to 20% (Table 2).

Fig. 4 compares RP-HPLC separations of tocotrienols on two different pentafluorophenylsilica (PFPS) phases, namely Taxsil [18] and Curosil-PFP. Under the conditions employed, HPLC with either Taxsil or Curosil columns led to the partial separation of fifteen components. With the Taxsil column, γ4- and α1-components were not separated (Fig. 4A), whereas β3- and γ2-tocotrienols were the

Table 2
Retention characteristics of tocotrienols on an octadecyl polyvinyl alcohol phase with mobile phases of acetonitrile–water at a flow-rate of 1 ml/min

Mobile phase (v/v)	Tocotrienol class	Capacity factor, k' Geometrical isomer			
		1	2	3	4
Acetonitrile–water (87.5:12.5)	δ	3.14	3.39	3.68	3.97
	β	4.17	4.36	4.75	5.09
	γ	4.36	4.56	5.09	5.39
	α	5.39	5.73	6.31	6.80
Acetonitrile–water (80:20)	δ	6.90	7.29	8.02	8.70
	β	9.39	9.92	10.9	11.8
	γ	9.92	10.2	11.4	12.4
	α	12.7	13.4	14.9	16.0
Acetonitrile–water (75:25)	δ	11.3	12.1	13.3	14.4
	β	15.7	16.2	18.9	19.8
	γ	16.6	17.2	18.8	20.8
	α	21.3	22.8	25.6	27.6

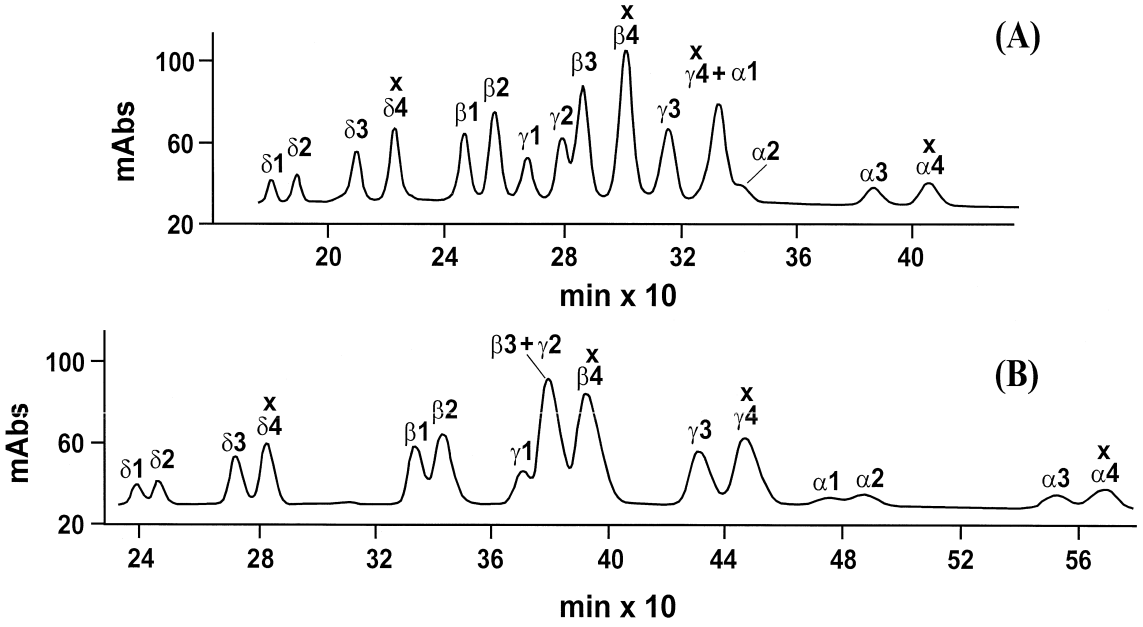

Fig. 4. Reversed-phase HPLC separations of synthetic tocotrienols on pentafluorophenylsilica. Column, (A) Taxsil and (B) Curosil-PFP. Mobile phase, methanol–water (75:25, v/v). Flow-rate, 0.5 ml/min. Detector, fluorescence. For amounts injected and component designations, see Fig. 2. Peaks with the label 'x' correspond to natural compounds.

unresolved components on the Curosil-PFP column (Fig. 4B). Although differences in column particle size (Taxsil, 3 μm vs. Curosil-PFP, 5 μm) of the two columns from different manufacturers are small, the observed column selectivity for specific tocotrienol components was noteworthy. Elution patterns for both PFPS phases were similar (Fig. 4A vs. Fig. 4B), but were different from those obtained with an ODPVA column (Fig. 4 vs. Fig. 3). Thus, the elution order of β–γ tocotrienols on PFPS (a silica-based phase) in methanol and water was found to be as follows: β1>β2>γ1>γ2>β3>β4>γ3>γ4 (Fig. 4), while a distinctly different pattern of elution was obtained with a nonsilica-based ODPVA phase in acetonitrile and water: β1>γ1>β2>γ2>β3>γ3>β4>γ4 (Fig. 3). Hence, the elution order of these components was found to be very sensitive to variation in the HPLC conditions.

Table 3 compiles some of the reversed-phase retention data obtained with a PFPS column. It is clearly demonstrated that the type and composition of mobile-phase solvents had notable influence on the retention characteristics of tocotrienol components. In comparison with acetonitrile mobile phases, HPLC with methanol–water systems produced better peak characteristics and methanol proved to be the solvent of choice for the separation of tocotrienol homologues and position isomers. However, acetonitrile was the preferred solvent for the speciation of geometrical isomers. A combination of the two organic modifiers (i.e. an acetonitrile–methanol–water ternary solvent system) gave a more satisfactory separation with reasonable analyte retention times than the binary solvent system of acetonitrile or methanol in water. When one half of the methanol in the methanol–water (77:23) mobile phase was replaced with acetonitrile, the k' values of all of the tocotrienol components were drastically lowered, to nearly one third of the original values, e.g. k'(α4), 58.2→19.1 (Table 3). Replacing methanol in the methanol–acetonitrile–water mobile phase (37.5:37.5:25) with acetonitrile resulted in approximately 50% reduction in k' values, e.g. k'(α4), 25.7→10.2, but the β1–β2, γ1–γ2, β3–β4 and γ3–γ4 pairs were not separated unless the water content in acetonitrile was raised from 25 to 35% (Table 3).

Inspection of the HPLC data in Table 3 also revealed that the elution order of the four sets of

Table 3
Retention characteristics of tocotrienols on a pentafluorophenylsilica phase (Taxsil) with various mobile phases at a flow-rate of 0.5 ml/min

Mobile phase (v/v)	Tocotrienol class	Capacity factor, k' Geometrical isomer			
		1	2	3	4
Methanol–water (77:23)	δ	26.2	27.1	30.1	31.9
	β	35.3	37.2	41.3	43.4
	γ	39.1	40.7	45.4	48.0
	α	48.0	49.4	55.6	58.2
Methanol–acetonitrile–water (38.5:38.5:23)	δ	9.94	10.3	10.9	11.5
	β	12.8	13.2	14.0	14.7
	γ	13.5	13.9	14.9	15.7
	α	16.6	17.0	18.2	19.1
Methanol–acetonitrile–water (37.5:37.5:25)	δ	12.8	13.2	14.0	14.8
	β	16.7	17.2	18.5	19.3
	γ	17.9	18.3	19.8	20.9
	α	22.0	22.7	24.5	25.7
Acetonitrile–water (75:25)	δ	5.60	5.60	5.94	5.94
	β	7.12	7.12	7.57	7.57
	γ	7.49	7.49	7.98	7.98
	α	9.45	9.45	10.2	10.2
Acetonitrile–water (65:35)	δ	14.1	14.5	15.0	15.8
	β	18.5	19.2	19.9	21.0
	γ	19.7	20.3	21.3	22.5
	α	26.0	26.8	28.1	29.7
Isopropanol–water (55:45)[a]	δ	8.25	8.65	9.13	9.62
	β	10.3	10.8	11.5	12.1
	γ	10.8	11.3	12.3	13.0
	α	13.1	13.6	14.9	15.6

[a] Flow-rate=0.3 ml/min.

tocotrienol geometrical isomers on PFPS was solvent-dependent. An experiment with a mobile phase of either isopropanol–water or methanol–acetonitrile–water yielded the same elution pattern (β1>β2 >γ1>γ2>β3>β4>γ3>γ4) as that with methanol–water, which was discussed earlier. An anomaly to the general elution trend was noticed in the case of the acetonitrile–water (65:35, v/v) eluent, where the compounds eluted in the following manner: β1 >β2>γ1>β3>γ2>β4>γ3>γ4 (Table 3). This elution sequence appears to be the hybrid of those shown in Figs. 3 and 4.

Fig. 5 shows RP-HPLC separations of tocotrienols on a triacontylsilica phase (TCS). This is the first demonstration of using a long-chain alkyl-bonded silica phase to achieve separations of the title compounds in the reversed-phase mode. The results are of particular significance in light of the inseparability of the β–γ tocotrienol pair on a relatively shorter-chain alkyl-bonded silica (e.g. ODS, Table 1 and Fig. 2). Analogous to the recently reported separation of carotenoids on TCS [19], the unique column properties of high absolute retention, augmented shape recognition and modest silanol activity were the pivotal factors contributing to the favorable separations. As illustrated in Fig. 5, the HPLC elution pattern resembles that obtained with ODPVA (Fig. 3). HPLC with acetonitrile–water mobile phases used in both column systems (TCS vs. ODPVA) might lead to the same sequence of analyte elution. With the exception of the γ1-component, which emerged as a shoulder of the β2-isomer,

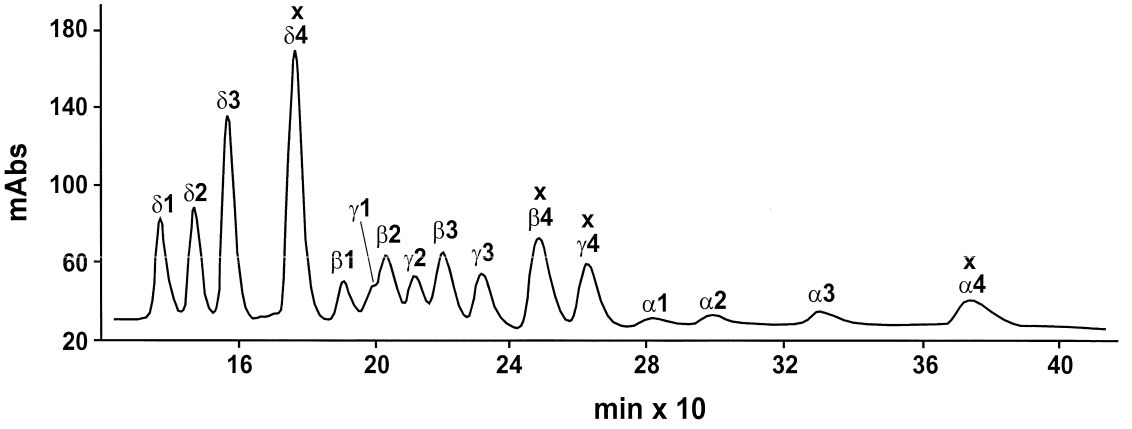

Fig. 5. Reversed-phase HPLC separations of synthetic tocotrienols on triacontylsilica. Column, YMC-C30. Mobile phase, acetonitrile–water (72.5:27.5, v/v). Flow-rate, 0.5 ml/min. Detector, fluorescence. Amounts injected: δ, 96 ng; others same as in Fig. 2. For component designations, see footnote to Fig. 2. Peaks with the label 'x' correspond to natural compounds.

fifteen tocotrienol peaks were adequately separated on this TCS column.

Three of the various types of stationary phases evaluated in this study are silica-based columns (ODS, PFPS and TCS). The HPLC results suggested that the presence of either polar groups (e.g. the PFPS phase) or highly retentive hydrocarbonaceous groups (e.g. the TCS phase) in silica packings appeared to be a prerequisite for differentiating β- and γ-tocotrienols in the reversed-phase mode. In HPLC with an ODS- or a phenyl silica phase (results not shown) devoid of such groups, the required polar dipole or hydrogen-bonding interactions [20–22] and high degrees of specific hydrophobic interactions [19] with analyte species were apparently truant in the chromatographic processes. Separations of the isomeric tocotrienols on the nonsilica-based ODPVA phase were effective by virtue of the desired solvophobic interactions involving both of its polar and nonpolar sites.

For each geometrical isomer, it was observed that in both normal-phase- and RP-HPLC, β-tocotrienol consistently emerged first followed by the γ-isomer: β (1, 2, 3 or 4)>γ (1, 2, 3 or 4). This retention behavior might reflect the interplay of molecular polarity and hydrophobicity. Nonetheless, in addition to mobile phase- and stationary phase factors affecting the elution order, peak interposition for β- and γ-isomers derived from adjacent geometrical isomers might be due partially to the steric and geometrical influence of the alkenyl side chains on the retention of individual tocotrienol isomers.

To demonstrate the general applicability of the RP-HPLC methods in the vegetable oil analysis, a sample of crude palm oil was analyzed with an ODPVA column, yielding a chromatogram (not shown) showing the complete resolution of both tocopherol (T) and tocotrienol (T3) antioxidants. The natural T3-antioxidants were found to correspond to the *trans*/*trans*-geometrical isomer peaks labeled with ''x's'' on the chromatograms in Figs. 3–5. A standard mixture of T and natural-T3 had the following retention sequence: δ4-T3 <β4-T3 <γ4-T3 <α4-T3 <δ-T <β-T <γ-T <α-T. The sample of palm oil showed the presence of five well-separated components δ4-T3, β4-T3, γ4-T3, α4-T3 and α-T.

In summary, tocotrienols derived from dimethyltocol cannot be separated with any of the ODS columns evaluated. Accordingly, RP-HPLC of a mixture containing the β- and γ-isomers yielded four geometrical isomers with identical retention times. The results of this study represent the first report on the complete reversed-phase separation of sixteen tocotrienols on a nonsilica-based polymer octadecanoyl polyvinyl alcohol column. Baseline separations of all components of interest can be achieved by optimization of HPLC variables including solvent composition, flow-rate and column efficiency. Partial separations (fifteen components) of tocotrienols can be achieved by using non-conven-

tional silica-based reversed-phase columns such as pentafluorophenylsilica or triacontylsilica phases. Application of the methods developed enables the analysis of tocotrienols by RP-HPLC–electrochemical detection or other techniques that require aqueous mobile phases.

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