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Journal of Chromatography A, 782 (1997) 25–32

JOURNAL OF CHROMATOGRAPHY A

Reversed-phase high-performance liquid chromatographic separations of tocopherols[1]

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Received 10 February 1997; revised 18 April 1997; accepted 22 April 1997

Abstract

The retention behavior and separation characteristics of five natural products α-, β-, γ-, δ-, and ζ_2-tocopherols and their acetyl derivatives were studied by reversed-phase high-performance liquid chromatography (HPLC)–fluorescence detection. Investigated stationary phases included an octadecyl polyvinyl alcohol (ODPVA) column, several octadecylsilica (ODS) columns, and a pentafluorophenylsilica column. Mobile phases comprised methanol (or acetonitrile) and water at variable proportions. Separation factors for β- and γ-tocopherol isomers and capacity factors of the five tocopherols were determined under various conditions. The β- and γ-tocopherols were separable on ODPVA but not on ODS. However, the β–γ pair was resolved with the latter column only as their ester derivatives. HPLC with mobile phases containing alkanols with carbon atoms greater than 2 favored the separation of the β–γ pair on ODS and yielded results diametrically different but complementary with those obtained with the ODPVA phase. The combined effects of mobile phases, stationary phases and antioxidant structures on component separations were delineated. © 1997 Elsevier Science B.V.

Keywords: Antioxidants; Stationary phases, LC; Mobile phase composition; Tocopherols

1. Introduction

Tocopherols are important lipid antioxidants occurring ubiquitously in plants. They exist in nature as a complex mixture of 2-methyl-6-chromanol homologs and aromatic ring position isomers each having a three-terpene-unit side chain at the C-2-position (Fig. 1). Because of their closely related structures, chromatographic separations and isolation of individual tocopherol components have been of

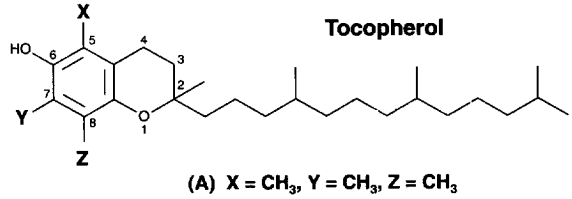

Fig. 1. Structures of investigated tocopherols. (A) α-Tocopherol, (B) β-tocopherol (C) γ-tocopherol, (D) δ-tocopherol, and (E) ζ_2-tocopherol.

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[1] Presented in part at the 20th International Symposium on High-performance Liquid Phase Separations, San Francisco, CA, USA, June 16–21, 1996.

PII S0021-9673(97)00471-8

interest to many investigators in various fields. Tocopherol contents of oilseeds serve as useful indicators of their antioxidative activities. Accurate analyses of these substances provide valuable information on the antioxidant levels in vegetable oils. The compositional distributions of the tocopherol components vary widely among different varieties of oilseeds [1,2].

There are four major tocopherols (α-, β-, γ-, and δ-) found in plants. These components of closely related structures have been successfully separated on silica-based columns by normal-phase high-performance liquid chromatography (HPLC) [3,4]. In spite of few difficulties associated with the normal-phase resolution of all tocopherol components, the use of undesirable toxic and volatile organic solvents and the requirement of long equilibration times have posed problems for reliable tocopherol assays. In addition, the nonaqueous mobile phases used in the normal-phase techniques are incompatible with interfaces used in electrochemical detection of tocopherols.

Numerous reports on the reversed-phase HPLC studies of tocopherols on octadecylsilica (ODS) have appeared in the literature [5–20]. However, none of these workers succeeded in the separation of β- and γ-tocopherols. Most recently, reversed-phase HPLC separations of all four compounds, α-, β-, γ-, and δ-tocopherols, have been described [21–23]. Of the published methods, the use of a pentafluorophenyl-bonded silica (PFPS) column in the reversed-phase HPLC system gave the most satisfactory separation of the β–γ pair [21]. We report here the results of our comprehensive reversed-phase HPLC study on the separation of the title compounds on an octadecyl polyvinyl alcohol (ODPVA) phase. Also, the first successful separations of tocopherol acetates on ODS are described in this paper.

2. Experimental

2.1. Chemicals and reagents

Tocopherol standards, α-tocopherol (5,7,8-trimethyltocol), β- tocopherol (5,8-dimethyltocol), γ-tocopherol (7,8-dimethyltocol), δ- tocopherol (8-methyltocol), and ζ_2-tocopherol (5,7-dimethyltocol) (Fig. 1), were obtained from Matreya, Inc. (Pleasant Gap, PA, USA). The ester derivatives acetates, butyrates, benzoates, and pentafluorobenzoates were prepared by treating tocopherols (1 mg) with, respectively, acetic anhydride, butyric anhydride, benzoyl chloride, and pentafluorobenzoyl chloride in pyridine (2:1, v/v, 1 ml) at 110°C for 30 min. Each of the reaction mixtures was evaporated under nitrogen to leave a residue which was dissolved in a small amount of chloroform followed by solid-phase extraction with hexane using a Waters (Milford, MA, USA) SEP-PAK silica cartridge. After removal of hexane, the ester products were redissolved in methanol and diluted to exact volume for HPLC injections. Analytical samples were stored in amber vials at −30°C for protection from light.

HPLC solvents acetonitrile (ACN), dioxane, methanol (MEOH), and tetrahydrofuran (THF) were obtained from Fisher (Fair Lawn, NJ, USA). Isopropanol (ISP), and *tert.*-butyl alcohol (TBA) were high-purity products of Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC-quality water (H_2O) was obtained by purification of in-house distilled water through a Millipore (Bedford, MA, USA) Milli Q water purifier.

2.2. High-performance liquid chromatography

All HPLC experiments were performed on a Thermo Separation Products (San Jose, CA, USA) Model SP8700XR liquid chromatograph equipped with a Model 8500 dynamic mixer. The LC instrument was coupled to an Applied Biosystems (Foster City, CA, USA) Model 980 programmable fluorescence detector. The detector was set at an excitation wavelength of 298 nm and an emission wavelength of 345 nm for monitoring column effluents. Mobile phases employed variable proportions of methanol (or acetonitrile) and water. In some experiments, isopropanol, *tert.*-butyl alcohol and other aprotic solvents were also used as the organic modifiers. The mobile-phase eluents were degassed with a helium sparge, filtered through a 0.02-μm filter, and pumped at a flow-rate of 1 ml/min.

Several commercially prepacked columns each having a particle size of 5 μm, and dimensions of 250×4.6 mm I.D. (unless specified otherwise) were evaluated in this study. They include (1) YMC-ODS-

A (Wilmington, NC, USA), (2) Alltech Adsorbsphere C_{18} (Deerfield, IL, USA), (3) Beckman Ultrasphere ODS (a short column of 150 mm length was also used) (San Ramon, CA, USA), (4) Waters Novapak C_{18}, 4 μm (300×3.9 mm I.D.), (5) EM Separations Lichrosphere-RP, 3 μm (Gibbstown, NJ, USA), (6) Phenomenex Curosil-PFP (Torrance, CA, USA), and (7) Astec Asahipak ODP (Whippany, NJ, USA), an octadecyl polyvinyl alcohol (ODPVA) column.

Aliquots (2–5 μl) of analyte samples in methanol (70–100 μg/ml) were injected onto the column via a Rheodyne (Cotati, CA, USA) Model 7125 injector fitted with a 10-μl loop. Three replicate injections were made for each sample analysis. Average retention times (t) (coefficients of variation, 0.1–2%) were used for the determination of capacity factors (k'). The k' values were then calculated from the equation $k'=t-t_o/t_o$ where t and t_o represent retention times for an analyte and an unretained solute, respectively. Separation factors (α) were determined for adjacent tocopherol components as $\alpha=k'_{c+1}/k'_c$ where subscript 'c' represents an analyte component.

3. Results and discussion

Structures of the investigated five tocopherols are depicted in Fig. 1. These structurally similar, naturally occurring substances include a monomethyltocol (δ-tocopherol), three dimethyltocols (β-, γ-, and ζ_2- tocopherols) and a trimethyltocol (α-tocopherol). Separations of the dimethyltocol trio β-, γ-, and ζ_2-tocopherols have been of tantalizing challenges to analysts, as the compounds differ merely in the position of the two methyls on the aromatic ring of the tocol structure. The minimal differences in their molecular polarity and hydrophobicity appeared to be governed by the location of the methyls in relation to that of the 6-hydroxy group, as demonstrated in previous normal-phase HPLC studies [3,4]. The reversed-phase HPLC retention behavior of the β- and γ-tocopherols on a conventional ODS phase in methanol (or acetonitrile) mobile phases has been indistinguishable. Modification of the tocopherol structures would alter the differentiability of the isomeric β–γ pair on ODS. Hence, reversed-phase HPLC separations of tocopherol acetates were evaluated along with the parent compounds.

In a recent reversed-phase HPLC study of carotenoids [24], Sander et al. developed a stationary phase based on three desirable column properties: enhanced shape recognition, high absolute retention, and silanol activity. Although tocopherols are structurally similar to the polar carotenoids with both cyclic and hydrocarbon side chain moieties, their position isomers resulting from aromatic ring substitution (Fig. 1) are significantly different from the carotenoidal compounds. Therefore, in addition to the bonded-phase parameters identified by Sander's group, separations of tocopherols appeared to be governed by dipole or hydrogen-bonding interactions and others of steric origins [3,4]. Thus, the two polar PFPS and ODPVA phases employed in this study favored the separation of the position isomers, β- and γ-tocopherols, while all the investigated ODS columns failed to resolve the isomeric pair. On the other hand, the corresponding acetate isomers were separated on ODS presumably due to an increase in hydrophobicity of the isomeric solutes.

Table 1 shows stationary effects of various ODS packings on the capacity factors (k') of five tocopherols and on the separation factors (α) of β- and γ-tocopherols. With a mobile phase of isopropanol (ISP)–water (67:33), the β–γ pair was resolved (separation factor, $\alpha=k'\gamma/k'\beta>1.00$) on all of the investigated silica-based columns of diversified specifications. In a previous HPLC study [22], Satomura et al. overstated that the observed separation of β- and γ-tocopherols was attributed to the particular slurry packing technique employed in the manufacture of a YMC-ODS column. As demonstrated in Table 1, HPLC with all eight ODS phases including the YMC-ODS column led to variable degrees of separations of the β- and γ-isomers with α values ranging from 1.04 to 1.12. A small percentage of acetic acid in the mobile phase somewhat enhanced the separation of the pair of interest. Evidently, when eluted with ISP in water, the pair of tocopherols was also resolved on the PFPS phase with selectivity comparable to the ODS phases under study. On the other hand, the isomeric β–γ tocopherol pair was not resolved ($\alpha=1.00$) on ODPVA in the same mobile phase system as for the silica-based phases (Table 1).

Table 1
Reversed-phase HPLC separations of tocopherols on various stationary phases with a mobile phase of isopropanol (ISP)–water (67:33) at a flow-rate of 0.3 ml/min

Column[a]	Capacity factor, k'					
	Tocopherol					
	δ	ζ_2	β	$k'\gamma/k'\beta$	γ	α
Asahipak ODP	6.18	7.73	8.02	1.00	8.02	9.80
Prodigy ODS	13.6	15.5	16.7	1.04	17.3	20.3
Adsorbsphere	6.43	7.48	7.89	1.07	8.43	10.0
Beckman-250	9.29	10.9	11.5	1.07	12.3	14.5
Beckman-150	8.55	10.0	10.7	1.07	11.4	13.6
Beckman-150-A	6.14	7.29	7.57	1.10	8.29	9.95
Novapak-C_{18}	7.33	8.43	8.52	1.12	9.57	11.2
Lichrosphere-RP	9.52	11.0	11.8	1.04	12.3	14.8
Curosil PFP	5.40	6.45	6.45	1.06	6.85	8.15
YMC-ODS-A	10.4	11.9	12.8	1.05	13.4	15.8

[a] Beckman-250, Ultrasphere ODS, 250×4.6 mm I.D.; Beckman-150-A, Ultrasphere ODS, 150×4.6 mm I.D.; mobile phase contained 0.25% acetic acid. For detailed column specifications, see Section 2.

In all experiments where ISP mobile phases were used (Table 1 and Table 2), it was necessary to set the flow-rate at 0.3 ml/min because of pressure limitations. Comparisons of the retention data (Table 1) obtained with three Beckman columns indicated that effects of column lengths on k' values of tocopherols seemed to be less profound than those of modified mobile phases or column types. As shown in Table 1, switching Beckman columns from a 250-mm column to a 150-mm column led to a small decrease in the k' values of δ-tocopherol from 9.29 to 8.55. However, when the above HPLC experiments were repeated with the exception of using a mobile phase modified with acetic acid, the k' values were reduced from 9.29 to 6.14. In general, tocopherols were found to be retained more strongly on Prodigy ODS, Beckman ODS, Lichrospher-RP, and YMC-ODS-A columns than others of the same dimensions (Table 1).

To explore the possibility of enhancing the α value of the β- and γ-tocopherols by incorporating various organic modifiers into a mobile phase of

Table 2
Effects of mobile phase modifiers on capacity factors, k', of tocopherols on ODS (YMC—ODS—A) (flow-rate=0.3 ml/min)

Mobile phase	Capacity factor, k'					
	Tocopherol					
	δ	ζ_2	β	$k'\gamma/k'\beta$	γ	α
ISP—MEOH—H_2O (67:10:23)	6.16	7.05	7.56	1.05	7.95	9.22
ISP—MEOH—H_2O (60:20:20)	7.86	8.92	9.73	1.05	10.2	12.0
ISP—MEOH—H_2O (64:15:21)	7.22	8.20	8.92	1.05	9.34	10.9
ISP—THF—H_2O (64:15:21)	2.39	2.86	2.86	1.04	2.98	3.36
ISP—THF—H_2O (71:5:24)	3.87	4.51	4.68	1.04	4.89	5.57
ISP—DIOX—H_2O (71:5:24)	4.68	5.36	5.70	1.04	5.95	6.88
ISP—DIOX—H_2O (67:10:23)	4.47	5.15	5.44	1.04	5.65	6.63
ISP—ACN—H_2O (67:10:23)	6.33	7.01	7.73	1.03	7.95	9.34
ISP—ACN—H_2O (71:5:24)	5.63	6.33	6.84	1.05	7.18	8.33
ISP—H_2O (75:25)	4.94	5.66	5.95	1.05	6.25	7.18
ISP—H_2O (67:33)	10.4	11.9	12.8	1.05	13.4	15.8
TBA—H_2O (75:25)	4.21	4.72	4.85	1.06	5.15	5.78
TBA—H_2O (67:33)	6.84	7.78	8.11	1.06	8.62	9.85

ACN, acetonitrile; DIOX, dioxane; ISP, isopropanol; THF, tetrahydrofuran; TBA, tertiary butyl alcohol.

ISP-water (75:25), effects of mobile-phase solvents on the reversed-phase HPLC behavior of tocopherols on ODS were studied. In general, no significant improvement in the separation of the β- and γ-isomers was observed in most experiments with the selected ternary solvent systems, as shown in Table 2. The k' values of the analyte components tended to decrease with the presence of additional strong modifiers such as tetrahydrofuran and dioxane in the ISP–water mobile phase. The α values of the β–γ pair were only slightly affected by these strong solvents. Addition of a high percentage (greater than 10%) of acetonitrile to the mobile phase appeared to have an adverse effect on the resolution of the pair of interest. It was noteworthy that replacing ISP with *tert.*-butyl alcohol in binary solvent systems (Table 2) lowered the k' values of analytes while maintaining a similar degree of selectivity for the β- and γ-tocopherols. An analogous solvent effect stemming from low concentrations of hexanol has been reported previously [25].

Fig. 2 presents two examples of the reversed-phase separation of five tocopherol components on ODPVA. This is the first inclusive study of the separation of β- and γ-tocopherols on the nonsilica-based column with mobile phases of acetonitrile (or methanol) and water. For a comparison purpose, a typical separation of the tocopherols on PFPS is also shown in Fig. 2. Examination of the chromatograms in the figure clearly indicated that the analyte solutes were more strongly retained by the ODPVA phase than the PFPS phase (Fig. 2B vs. Fig. 2C). This observation was further corroborated with the capacity factor (k') data summarized in Table 3. The results from Table 3 also show that the α values $k'\zeta_2/k'\delta$, $k'\beta/k'\zeta_2$, $k'\gamma/k'\beta$, and $k'\alpha/k'\gamma$ for the corresponding ζ_2–δ, β–ζ_2, γ–β, and α–γ adjacent pairs separated with the ODPVA and PFPS phases are fairly close in magnitude (1.05–1.23 for ODPVA vs. 1.04–1.20 for PFPS). It was of note that HPLC with these two phases of diametrically different materials yielded the same order of component elution: $k'\delta < k'\zeta_2 < k'\beta < k'\gamma < k'\alpha$.

Results of HPLC optimization experiments showed that ODPVA column packings containing hydroxy functionality appeared to be more efficient in acetonitrile than in methanol. With the ODPVA phase, underivatized tocopherols were separated in either solvent system. By contrast, the corresponding acetylated tocopherol derivatives were not completely resolved on ODPVA with mobile phases containing relatively high percentages of organic modifiers (i.e. methanol or acetonitrile). Surprisingly, with mobile phases of methanol and water, β- and γ-tocopherol acetates were separated on ODPVA, while δ- and ζ_2-tocopherol acetates remained unresolved.

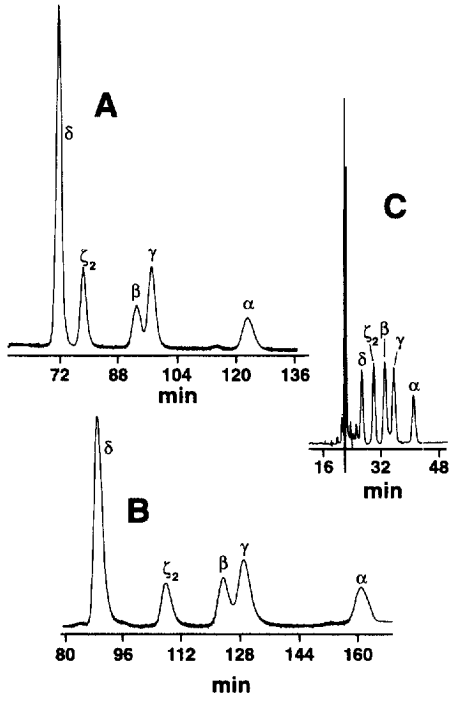

Fig. 2. Reversed-phase HPLC separations of tocopherols. Columns: (A,B) an ODPVA phase (Asahipak ODP); (C) a PFPS (Curosil-PFPS) phase. Mobile phases: (A) ACN–H_2O (85:15); (B,C) MEOH–H_2O (87.5:12.5).

It was of importance to note that the five tocopherol acetate derivatives were readily separated by eluting various proportions of aqueous methanol through an ODS column with which the underivatized parent β- and γ-tocopherols have not been separated in identical HPLC systems. Under similar mobile phase conditions, base-line separations of the acetylated tocopherols on PFPS were also obtained. However, different separation patterns were manifested in HPLC with the ODS and PFPS phases. With acetonitrile mobile phases, neither of the δ–ζ_2 and β–γ pairs could be resolved on ODS.

Inspection of the HPLC data in Table 4 indicated

Table 3
Comparisons of reversed-phase HPLC separations of tocopherols on octadecyl poly(vinyl alcohol) phase (ODPVA) and pentafluorophenyl-bonded silica (PFPS)

Tocopherol	Capacity factor, k'			
	ODPVA		PFPS	
	ACN—H_2O	MEOH—H_2O	ACN—H_2O	MEOH—H_2O
δ	8.95	11.3	3.33	6.50
$k'\zeta_2/k'\delta$	1.06	1.21	1.10	1.13
ζ_2	9.49	13.7	3.66	7.33
$k'\beta/k'\zeta_2$	1.20	1.13	1.11	1.09
β	11.4	15.5	4.08	8.00
$k'\gamma/k'\beta$	1.05	1.05	1.04	1.06
γ	12.0	16.2	4.25	8.50
$k'\alpha/k'\gamma$	1.23	1.23	1.20	1.16
α	14.8	19.9	5.08	9.83

ODPVA, Asahipak-ODP; PFPS, Curosil-PFPS. Mobile phases employed ACN (or MEOH)—H_2O (9:1). For detailed column specifications, see Section 2.

that α values of adjacent components of tocopherol acetates were dramatically influenced by the type of mobile phases and stationary phases employed. Regardless of the type of columns used, the methanol–water (9:1) eluent system proved to be the mobile phase of choice for the separation of β- and γ-tocopherol acetates (α=1.04–1.07). With acetonitrile–water (9:1) as mobile phase, the pair of isomers was not resolved on either ODPVA or PFPS phase (α=1.00).

Representative reversed-phase chromatograms showing separations of two homologs of tocopherol derivatives on selected stationary phases are shown in Fig. 3. To assess the effect of increasing hydrophobicity (carbon chain length) in the acyloxy group of tocopherol esters, the HPLC behavior of a sample of tocopherol butyrates (the number of carbon atoms=4) was compared with that of the corresponding acetates (carbon atoms=2). Although the tocopherols having longer acyloxy chain had longer retention times than the lower members of the homologous family, differences in the selectivity for β- and γ-tocopherols were very small (Fig. 3B vs. Fig. 3C). It was apparent that HPLC with the ODS phase led to a better separation of the ζ_2--β pair than with the PFPS phase (Fig. 3B and Fig. 3C vs Fig.

Table 4
Comparisons of reversed-phase HPLC separations of tocopherol acetates on ODPVA, PFPS, and ODS

Tocopherol acetate	Capacity factor, k'					
	ODPVA		PFPS		ODS-A	ODS-B
	ACN—H_2O	MEOH—H_2O	ACN—H_2O	MEOH—H_2O	MEOH—H_2O	MEOH—H_2O
δ	8.75	16.1	4.83	9.67	29.3	43.9
$k'\zeta_2/k'\delta$	1.07	1.00	1.14	1.10	1.05	1.05
ζ_2	9.34	16.1	5.50	10.6	30.9	46.2
$k'\beta/k'\zeta_2$	1.16	1.12	1.00	1.04	1.13	1.13
β	10.8	18.0	5.50	11.0	35.0	52.3
$k'\gamma/k'\beta$	1.00	1.07	1.00	1.05	1.05	1.04
γ	10.8	19.2	5.50	11.5	36.6	54.3
$k'\alpha/k'\gamma$	1.20	1.16	1.15	1.20	1.24	1.24
α	13.0	22.2	6.33	13.8	45.5	67.4

ODPVA, Asahipak-ODP; PFPS, Curosil-PFPS; ODS-A, NovaPak; ODS-B, YMC-ODS-A. Mobile phases employed acetonitrile (or methanol)–H_2O (9:1). ACN, acetonitrile. For detailed column specifications, see Section 2.

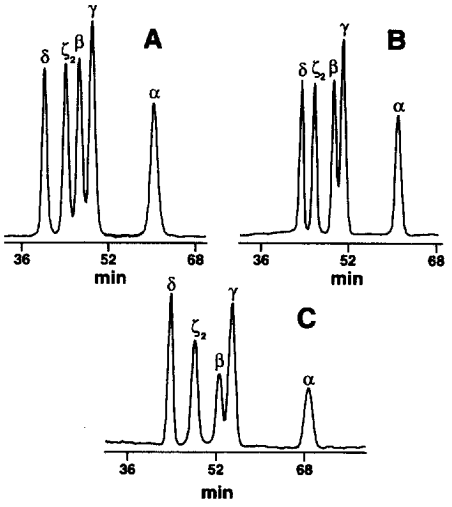

Fig. 3. Reversed-phase HPLC separations of acyloxy tocopherol derivatives: (A,B) acetates; (C) butyrate. Columns: (A) a PFPS phase (Curosil-PFPS); (B,C) an ODS phase (YMC-ODS). Mobile phases: (A) MEOH–H_2O (87.5:12.5); (B,C) MEOH–H_2O (95:5).

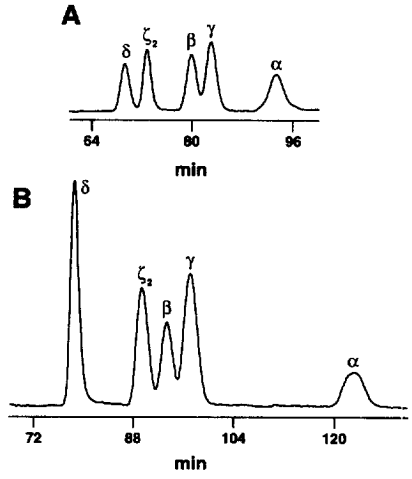

Fig. 4. Reversed-phase HPLC separations of aryloxy tocopherol derivatives: (A) benzoates; (B) pentafluorobenzoates. Column: an ODS phase (Prodigy ODS-2). Mobile phase: MEOH–H_2O (95:5).

3A). In other words, the elution patterns of the three dimethyltocols (ζ_2-, β-, and γ-tocopherols) on respective PFPS and ODS phases resemble those observed in normal-phase HPLC with ether and alcohol in hexane binary solvent systems [3,4].

Examination of HPLC profiles of tocopherols and their derivatives revealed that the elution order ($k'\zeta_2 < k'\beta < k'\gamma$) of the three dimethyltocols in reversed-phase HPLC unexpectedly maintained the same elution sequence as in normal-phase HPLC [3,4]. Conceivably, hindered interactions between the polar functionality at the 6-position (flanked by the 5- and 7-methyls) of the ζ_2-tocopherol and the polar sites of a stationary phase might result in weak retention in relation to the β- and γ-isomers. For the δ- and α-homologs, a reversal in the elution order was observed as usual in the two antithetical modes of HPLC separations.

Fig. 4 compares reversed-phase HPLC chromatograms of tocopherol benzoates and pentafluorobenzoates both obtained with an identical ODS column. Interestingly, the pentafluoro analogs appeared to be more strongly retained by the ODS phase than the benzoates (Fig. 4B vs. Fig. 4A). Comparisons of chromatograms in Fig. 3 and Fig. 4 showed that the peak profile of the pentafluoro derivatives corresponded well with that of the acetates on a PFPS phase (Fig. 4B vs. Fig. 3A). Likewise, the separation pattern of the tocopherol benzoates on ODS were parallel to those of their acetates and butyrates on ODS (Fig. 4A vs. Fig. 3B and Fig. 3C). As illustrated in Fig. 4, the set of three dimethyltocol pentafluorobenzoates (ζ_2-, β-, and γ-isomers) (Fig. 4B) is conspicuously dispersed more evenly and closer to one another than the corresponding set of the benzoate counterparts (Fig. 4A). The latter pattern of separation is presumably due to the apparent ortho effect [3,4] of the two methyls of the ζ_2-component on the comparative retention behavior of the three dimethyltocols. Further, it was definitely advantageous to replace the 6-hydroxy groups with either acyloxy or aryloxy groups of tocopherols in order to enhance hydrophobic differentiation of the β- and γ-tocopherols on ODS phases.

In conclusion, the results of this study represent the first methodical report on the successful HPLC reversed-phase separation of the five tocopherols on a nonsilica-based column. Chemical derivatization of the title compounds enabled reversed-phase resolution of the tocopherol complex as their derivatives on ODS with which the underivatized parent β–γ pair cannot be resolved. Optimization of reversed-phase HPLC variables with respect to mobile phases, stationary phases, and analyte structures led to adequate separations of all components of interest.

The combined effects of the HPLC parameters had significant bearing on the retention behavior of tocopherol structures. The reversed-phase HPLC methods developed can be applied to the analysis of tocopherols with electrochemical detection or other techniques that require aqueous mobile phases. In addition, the reversed-phase techniques facilitate analyses of trace amounts of later-eluting tocopherol components observed in the normal-phase mode. The reversed-phase HPLC solvent systems are more environmentally acceptable than those used in normal-phase HPLC.

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