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Capillary electrochromatographic evaluation of vitamin E-active oil constituents: tocopherols and tocotrienols

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Abstract

Separations of lipid antioxidants, tocopherols (T) and tocotrienols (T_3), on octylsilica (OS), octadecylsilica (ODS), phenylsilica, or silica were studied by capillary electrochromatography (CEC)–UV detection. The homologues and isomers of the vitamin E-active compounds were best separated with an OS column. CEC with an ODS column tended to yield broad peaks with poor resolution. Among the various mobile phases evaluated, [acetonitrile–methanol (64:36)]–[25 m*M* tris(hydroxymethyl)aminomethane, pH 8] (95:5) eluent systems produced the most satisfactory results. Under these conditions, a baseline separation of an 11-component mixture was obtained with elution order similar to that observed in reversed-phase HPLC: $\delta T_3 > (\gamma+\beta)T_3 > \alpha T_3 > \epsilon T > (\delta+\zeta_2)T > (\gamma+\beta)T > \alpha T > \alpha$T-acetate. CEC of the antioxidant acetates led to separations inferior to those of the parent compounds. Effects of CEC experimental variables (e.g., mobile phase solvents and buffers, stationary phases and electric field) on analyte separations were assessed in the context of resolution factors and retention factors. Published by Elsevier Science B.V.

Keywords: Mobile phase composition; Vitamins; Tocopherols; Tocotrienols

1. Introduction

Vitamin E-active substances, tocopherols and tocotrienols (derived from tocol), occur widely in oilseed plants. They are structurally related as a complex mixture of 2-methyl-6-chromanol homologues and aromatic ring position isomers with a three-terpene-unit side chain at the C-2 position (Fig. 1). Because of their antioxidative properties and close similarity in structure, chromatographic separations and isolation of these compounds have been of much interest to many interdisciplinary investigators. Compositions of tocopherols and tocotrienols in vegetable oils vary widely among different oil types [1]. Accurate analyses of them should provide valuable information on the antioxidant levels in vegetable oils. In addition, the nutritional values and health benefits of the title lipid antioxidants are well known. Compositional determinations constitute integral part of routine industrial processing of the important agricultural products.

Although chromatographic analysis of the tocol-derived lipid antioxidants has been extensively studied by normal-phase high-performance liquid chromatography (HPLC) [2,3] and reversed-phase HPLC [4–8], capillary electrochromatographic (CEC) separations of these compounds have not been investigated. Since the introduction of the CEC concept by Pretorius et al. [9] in 1974, applications of the relatively new technique for the analysis of

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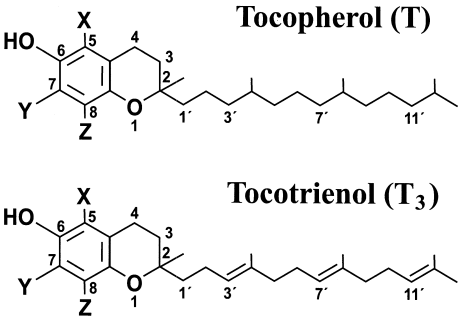

(A) α, X = CH_3, Y = CH_3, Z = CH_3
(B) β, X = CH_3, Y = H, Z = CH_3
(C) γ, X = H, Y = CH_3, Z = CH_3
(D) δ, X = H, Y = H, Z = CH_3
(E) ζ_2, X = CH_3, Y = CH_3, Z = H
(F) ε, X = H, Y = H, Z = H

Fig. 1. Structures of the investigated compounds.

neutral compounds [10–12,15,16], pharmaceuticals [13,14,17–19,23–25,28], food/biological samples [20,21,26,33,34], and environmental chemicals [11,12,22,27,29] have proven successful. In light of the demonstrated high-efficiency separations of CEC enabling high-speed micro analysis with minimal solvent consumption, the technique should be ideally suited for quality control assays of vitamin E-active compounds in various sample matrices. We report the results of a CEC study on the separation of the title compounds on alkyl-bonded silica phases.

2. Experimental[1]

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), ζ_2-tocopherol (5,7-dimethyltocol) and ϵ-tocopherol (tocol) (Fig. 1) were obtained from Matreya (Pleasant Gap, PA, USA). Tocotrienol standards were obtained from Merck (Vienna, Austria). The tocopherol acetate derivatives were prepared by treating tocopherols (1 mg) with acetic anhydride–pyridine (2:1, v/v, 1 ml) at 110°C for 30 min. Each reaction mixture was evaporated under nitrogen to leave a residue. Hexane (3 ml) was added to the residue and the resulting suspension was washed three times with a saturated solution (2 ml) of sodium chloride. After removal of hexane, the ester products were re-dissolved in methanol and diluted to exact volume for CEC evaluation. Analytical samples were stored in amber vials at −30°C for protection from light.

Buffer reagents tris(hydroxymethyl)aminomethane (Tris) and sodium phosphate monobasic were obtained from Sigma–Aldrich (St. Louis, MO, USA) and Fisher (Fairlawn, NJ, USA), respectively. Hydrochloric acid, phosphoric acid, HPLC-grade acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) were obtained from Fisher. Isopropanol (IPA) was an ultra-pure product of Aldrich (Milwaukee, WI, USA). Chromatography-quality water was obtained by purification of laboratory-distilled deionized water through a Millipore (Bedford, MA, USA) Milli-Q water purifier.

2.2. Capillary electrochromatography

CEC experiments were performed on a Hewlett-Packard HP^{3D} CE instrument (Wilmington, DE, USA) equipped with a diode array detector (290 nm) and a HP ChemStation software for system control. Mobile phases were prepared by mixing individual buffer solutions with organic modifiers. Weighed amounts of Tris and phosphate were titrated, respectively, with 30% hydrochloric acid to pH 8 and phosphoric acid to pH 2.60. In light of the instability of the vitamin E-active compounds in alkaline pH media, samples in mobile phases were injected immediately after preparation to minimize the contact time of analytes with alkaline eluents. Under the conditions employed in this study, no alkaline decomposition products of the tocopherols and tocotrienols were detected. Throughout analyses, both inlet and outlet buffer vials in the CEC system were pressurized at 12 bar. Prior to CEC runs, columns

[1]Names are necessary to report factually on available data; however, the US Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by the US Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

were equilibrated daily with fresh mobile phases. Three commercial CEC capillary columns (25 cm× 100 μm I.D.) packed with 3 μm alkyl- or aryl-bonded silica and one 3 μm silica column obtained from a different source were used in this study. These columns include (1) Hewlett-Packard CEC-Hypersil C_8, (2) Hewlett-Packard CEC-Hypersil C_{18}, (3) Hewlett-Packard CEC-Hypersil phenyl, (4) Unimicro CEC silica, 25 cm×75 μm I.D. (Unimicro Technologies, Pleasanton, CA, USA). Unless stated otherwise, samples dissolved in buffer solutions (0.5–20 μg/μl) were injected electrokinetically onto the column at 10 kV for 10 s, while the separation voltage and column temperature were maintained at 25 kV and 30°C, respectively.

Three replicate injections were made for each sample analysis. Average retention times (t_R) (relative standard deviations, RSDs, 2.5–5.3%) were used for the determination of retention factors (k'). The k' values were then calculated from the equation $k' = t_R - t_0/t_0$ where t_R and t_0 represent respective retention times of an analyte and the neutral marker, thiourea (i.e., CEC osmotic flow). Resolution 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

3.1. Comparison of CEC and HPLC separations

Homologues of the tocopherols (T) and tocotrienols (T_3) include one monomethyltocol (δ-tocopherol), three dimethyltocols (β-, γ- and ζ_2-tocopherols), one trimethyltocol (α-tocopherol), and their corresponding trialkenyl-side chain analogues (tocotrienols) (Fig. 1). The dimethylated compounds β-, γ- and ζ_2-tocopherols and β-, γ-tocotrienols are position isomers with respect to the aromatic ring of the tocol structure.

As observed in reversed-phase HPLC [7,8], CEC of a mixture of tocopherols and tocotrienols on octylsilica (OS), octadecylsilica (ODS), or phenylsilica in acetonitrile or methanol met with success in the separation of homologues, but failed to resolve the β- and γ-isomers. Conversion of the parent tocopherols to their acetates altered the HPLC differentiability of the isomeric β–γ pair on ODS and eventually achieved its separation. However, attempted CEC separations of the acetates on ODS were unsuccessful apparently due to poor selectivity of the ODS phase for the isomers of interest. It must be clarified that the ODS stationary phase used in the HPLC and CEC mode was not the same.

In a typical CEC system, the six investigated tocopherol acetates eluted in the following order (Ac=acetate): εTAc>(δ+ζ_2)TAc>(γ+β)TAc> αTAc (not shown here), whereas a mixture of the parent tocopherols and tocotrienols had an elution order δT_3>(γ+β)T_3>αT_3>εT>(δ+ζ_2)T>(γ+β)T>αT>αTAc (Fig. 2).

The elution behavior of these compounds was predictable based on hydrophobic interactions of the analyte solutes with alkyl-bonded silica phases normally observed in reversed-phase HPLC. It was noteworthy that, under CEC conditions employed, the 5,7-dimethyl isomer (ζ_2-tocopherol) or its acetate was separated from the other two unresolved dimethyl isomers (β- and γ-tocopherols) or their acetates, but co-eluted with the corresponding δ compounds. In view of the coexistence of α-tocopherol acetate with the parent antioxidant compounds in oilseed plants, the acetate was included in the underivatized antioxidant test mixture for the study.

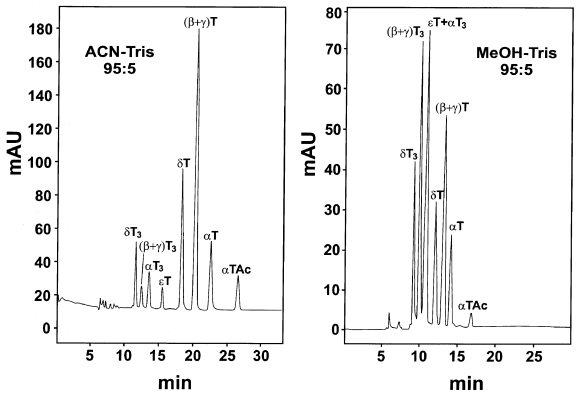

Fig. 2. Comparison of CEC separations obtained with acetonitrile (ACN) and methanol (MeOH). Conditions: column, octylsilica (OS); Tris, 25 m*M*; pH, 8; temperature, 30°C; voltage, 25 kV, pressure, 12 bar; injection, 10 kV/10 s.

3.2. Mobile phase solvent and stationary phase effect

Fig. 2 compares CEC separations obtained with two solvent systems acetonitrile and methanol. In both solvent systems, the antioxidants components on OS were well resolved except for the aforementioned position isomers. Notwithstanding longer retention times for component elution, CEC with an acetonitrile mobile phase provided a better selectivity for certain components than with methanol in which ϵ-tocopherol and α-tocotrienol remained as one unresolved peak (Fig. 2). Similar solvent effects were observed in experiments with ODS columns. Attempted uses of other organic solvents IPA and THF afforded no separation or inadequate speciation of the antioxidant complex.

Examination of CEC separations obtained with OS and ODS columns indicated that the latter proved to be much less efficient than the former when acetonitrile was used as the mobile phase. In the acetonitrile system, no baseline separation of peaks corresponding to δ-, (γ+β)-tocopherol, δ-, (γ+β)-, and α-tocotrienols on ODS was observed along with severe peak broadening in contrast to those obtained with an OS column shown in Fig. 2. Generally, the stationary effect was a function of mobile phases employed. With acetonitrile, the OS phase demonstrated peak characteristics superior to those obtained with ODS. Furthermore, CEC with the OS phase exhibited somewhat longer retention times of components than the ODS phase. An reversal of such a stationary phase effect on analyte retention times of later-eluting components was found in experiments with acetonitrile (i.e., under acetonitrile mobile phase conditions, more hydrophobic components on ODS had longer retention times than the same analyte species on OS).

The idea of using a phenylsilica column in the study was to enhance π–π interactions of aromatic moieties of solutes with those of the phenyl-bonded phase. Unfortunately, numerous exploratory experiments conducted under various CEC condition led to fruitless results. Peak resolution was very poor. A chromatogram of a 10-component mixture revealed only four partially resolved peaks eluting in the following manner: $(\delta T_3+\gamma T_3+\beta T_3+\alpha T_3>\epsilon T)>\delta T>(\gamma+\beta)T>\alpha T>\alpha TAc$. Evidently, the four tocotrienols were not separated at all. In another series of CEC attempts at separating tocopherols and tocotrienols on silica using aqueous buffers yielded no separation of any component. A study with a non-aqueous buffer system is in progress.

3.3. Mobile phase buffer effect

Several buffer solutions were tested for CEC optimization of experimental variables. The effect of both Tris and phosphate buffer concentrations on the CEC separation of the antioxidant components on OS (Fig. 3) paralleled to that noted in CEC runs with the ODS column. Thus, retention times of tocopherols and tocotrienols had the tendency to decrease with increasing buffer concentrations. The concentration effect was more prominent in the higher concentration range (25–50 m*M*). On the other hand, analyte separations were affected by changes in buffer pH values. An increase in a buffer pH brought about a decrease in retention times of the compounds under consideration. CEC with an ODS column often led to separations with poor resolution in cases where high buffer concentrations (~50 m*M*) were used. The results of the pH study also showed that component resolution appeared to improve with high buffer pH. The use of phosphate buffer at low pH offered few advantages over the Tris buffers in the resolution of position isomers. The optimal buffer pH conditions used in this study were 25 m*M* Tris buffer at pH 8.

Table 1 shows effects of buffers on retention factors (k') and resolution factors (α) of tocopherols and tocotrienols eluted through an ODS or OS column. Under CEC conditions (A) specified in the

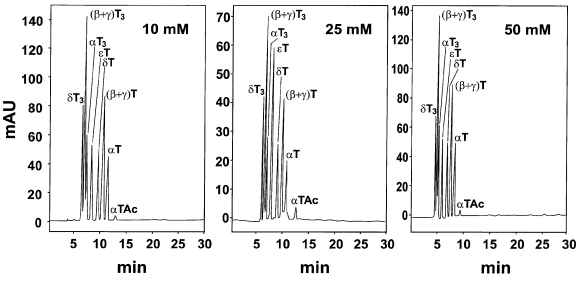

Fig. 3. Effect of Tris buffer concentration on CEC separations of tocopherols and tocotrienols. Conditions: [ACN–MeOH (64:36)]–Tris (95:5); other conditions as in Fig. 2.

Table 1
CEC separations of tocopherols and tocotrienols on ODS or OS under various buffer conditions[a]

Component	(A) pH value				(B) Tris concentration (m*M*)				(B′) Phosphate concentration (m*M*)	
	ODS				OS		ODS		OS	
	8.0 k' (α)	7.0 k' (α)	5.8 k' (α)	5.0 k' (α)	50 k' (α)	25 k' (α)	50 k' (α)	25 k' (α)	12 k' (α)	6.5 k' (α)
αTAc	7.27	8.21	8.52	9.56	2.60	3.23	2.64	4.29	6.96	9.40
$k'_{\alpha TAc}/k'_{\alpha T}$	(1.26)	(1.28)	(1.27)	(1.29)	(1.20)	(1.23)	(1.19)	(1.17)	(1.31)	(1.22)
αT	5.73	6.39	6.66	7.41	2.17	2.63	2.21	3.67	5.32	7.73
$k'_{\alpha T}/k'_{\beta T}$	(1.32)	(1.32)	(1.33)	(1.33)	(1.12)	(1.11)	(1.25)	(1.27)	(1.22)	(1.23)
βT	4.33	4.82	5.00	5.56	1.93	2.37	1.77	2.88	4.36	6.31
$k'_{\beta T}/k'_{\gamma T}$	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
γT	4.33	4.82	5.00	5.56	1.93	2.37	1.77	2.88	4.36	6.31
$k'_{\gamma T}/k'_{\delta T}$	(1.20)	(1.19)	(1.19)	(1.21)	(1.18)	(1.16)	(1.16)	(1.20)	(1.21)	(1.28)
δT	3.60	4.04	4.17	4.56	1.63	2.05	1.53	2.41	3.60	4.93
$k'_{\delta T}/k'_{\epsilon T}$	(1.38)	(1.39)	(1.39)	(1.39)	(1.28)	(1.24)	(1.35)	(1.32)	(1.36)	(1.35)
ϵT	2.60	2.89	3.00	3.26	1.27	1.65	1.14	1.83	2.65	3.65
$k'_{\epsilon T}/k'_{\alpha T_3}$	(1.29)	(1.26)	(1.26)	(1.27)	(1.26)	(1.22)	(1.30)	(1.26)	(1.25)	(1.35)
αT_3	2.01	2.28	2.38	2.56	1.01	1.35	0.88	1.45	2.12	2.71
$k'_{\alpha T_3}/k'_{\beta T_3}$	(1.25)	(1.23)	(1.23)	(1.23)	(1.15)	(1.12)	(1.19)	(1.22)	(1.19)	(1.24)
βT_3	1.60	1.84	1.92	2.07	0.88	1.21	0.74	1.19	1.78	2.19
$k'_{\beta T_3}/k'_{\gamma T_3}$	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
γT_3	1.60	1.84	1.92	2.07	0.88	1.21	0.74	1.19	1.78	2.19
$k'_{\gamma T_3}/k'_{\delta T_3}$	(1.22)	(1.23)	(1.23)	(1.24)	(1.16)	(1.14)	(1.17)	(1.19)	(1.00)	(1.20)
δT_3	1.31	1.49	1.56	1.66	0.76	1.06	0.63	1.00	1.78	1.83

[a] (A)=[Acetonitrile–methanol (64:36)]–Tris, pH *X* (90:10), where *X* represents a pH value; for pH 5, an acetate buffer was used. Other parameters: 25 m*M* Tris; temperature, 30°C; voltage, 25 kV; pressure, 12 bar; injection, 10 kV/10 s. (B)=[Acetonitrile–methanol (64:36)]–*X* m*M* Tris (95:5), where *X* represents a buffer concentration; other conditions as in (A). (B′)=Acetonitrile–*X* m*M* phosphate (90:10); pH 2.6; other conditions as in (A).

table, k' values increased systematically as pH values reduced from 8.0 to 5.0 apparently independent of the type of buffers used, because the pH–k' trend was unaltered by employment of the acetate buffer. It must be pointed out that, in the pH study, all but pH 5 (acetate) solutions were of Tris buffer systems. The influence of pH changes on α values was generally slight. The observed α values were between 1.00 and 1.39 for the β–γ unresolved pairs and the best separated δ–ϵ-tocopherol components, respectively.

The effects of buffer concentrations on k' values resembled those of pH values. Within the Tris buffer concentration range of 10 to 50 m*M* evaluated under CEC conditions (B) (Table 1), lower k' values of the antioxidant components were invariably obtained by using higher concentrations of buffer solutions.

Comparisons of the Tris buffer concentration data obtained with OS and ODS columns (Table 1) revealed that some degrees of variations in α values of the corresponding adjacent pairs occurred as a result of the change in the buffer concentration. However, in a given stationary phase system, the concentration effects on α values were relatively small. The α values in these cases ranged from 1.00 to 1.35.

CEC experiments were also carried out with phosphate buffers (pH 2.6) to see whether the low-pH buffer system would separate the pairs of antioxidant components that were not resolved with Tris buffers. The results are summarized in Table 1. Under conditions (B′) specified in the table, k' values increased with decreasing concentrations of phosphate buffers. Mostly, a reduction in the phosphate concentration tended to give relatively high α values except for the two last eluting components α-tocopherol and its acetate. The attempted resolution of β–γ pairs (α=1.00) with the low pH buffers

in the concentration range 6.5–12 m*M* failed to materialize. It was of note that additional unresolved early-eluting components γ–δ-tocotrienols ($\alpha = 1.00$) were found in CEC with the phosphate buffer at 12 m*M*. At a lower buffer concentration of 6.5, the compounds were ultimately well resolved having an α value of 1.20 [(B′), Table 1]. The α values obtained in the phosphate study ranged from 1.00 to 1.35.

3.4. CEC voltage and temperature effect

The results of this study illustrated that increases in CEC voltage from 15 to 30 kV caused decreases in analyte retention times on OS because of the accelerated electroosmotic velocity in the CEC system. Likewise, the electroosmotic velocity increased with decreasing viscosity at a high temperature, retention times of the antioxidant components on OS had inverse relationships with temperature changes. Moreover, increasing either voltage up to 30 kV or temperature to 30°C appeared to render no adverse effect on component resolution under conditions specified. In most experiments conducted in this work, the 25 kV voltage and 30°C temperature were used for achieving optimal separations.

3.5. Effect of aqueous buffer content and organic modifier composition

Much like in reversed-phase HPLC, increasing the percentage of aqueous buffer solutions in CEC mobile phases favored component separations at the expense of increased retention times. Table 2 compiles CEC data showing the effects of mobile phase [acetonitrile–methanol (64:36)] compositions on retention and separation characteristic of tocopherols and tocotrienols on ODS. Examination of chromatograms (not shown here) indicated that, with the exception of the β- and γ-position isomers, a near baseline separation of the 10-component mixture was obtained with a mobile phase of [acetonitrile–methanol (64:36)]–Tris/water (90:10) [CEC conditions (C), Table 2)] despite the 40 min long run time. On

Table 2
CEC separations of tocopherols and tocotrienols on ODS under various solvent conditions[a]

Component	(C) % Buffer solution			(D) % Acetonitrile			
	5.0, k' (α)	7.5, k' (α)	10, k' (α)	0.0, k' (α)	50, k' (α)	64, k' (α)	100, k' (α)
αTAc	4.08	5.11	8.52	2.42	3.15	4.29	13.8
$k'_{\alpha TAC}/k'_{\alpha T}$	(1.20)	(1.23)	(1.27)	(1.42)	(1.24)	(1.16)	(1.36)
αT	3.40	4.15	6.66	1.70	2.54	3.67	10.1
$k'_{\alpha T}/k'_{\beta T}$	(1.26)	(1.28)	(1.33)	(1.24)	(1.25)	(1.27)	(1.18)
βT	2.68	3.22	5.00	1.37	2.02	2.88	8.52
$k'_{\beta T}/k'_{\gamma T}$	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
γT	2.68	3.22	5.00	1.37	2.02	2.88	8.52
$k'_{\gamma T}/k'_{\delta T}$	(1.19)	(1.19)	(1.19)	(1.19)	(1.17)	(1.19)	(1.09)
δT	2.24	2.70	4.17	1.15	1.72	2.42	7.78
$k'_{\delta T}/k'_{\epsilon T}$	(1.31)	(1.33)	(1.39)	(1.27)	(1.33)	(1.32)	(1.22)
ϵT	1.70	2.03	3.00	0.90	1.29	1.83	6.35
$k'_{\epsilon T}/k'_{\alpha T_3}$	(1.22)	(1.26)	(1.26)	(1.00)	(1.22)	(1.26)	(1.21)
αT_3	1.39	1.60	2.38	0.90	1.05	1.45	5.22
$k'_{\alpha T_3}/k'_{\beta T_3}$	(1.21)	(1.22)	(1.23)	(1.21)	(1.25)	(1.21)	(1.13)
βT_3	1.15	1.31	1.92	0.74	0.87	1.19	4.61
$k'_{\beta T_3}/k'_{\gamma T_3}$	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
γT_3	1.15	1.31	1.92	0.74	0.87	1.19	4.61
$k'_{\gamma T_3}/k'_{\delta T_3}$	(1.20)	(1.20)	(1.23)	(1.19)	(1.19)	(1.19)	(1.05)
δT_3	0.96	1.09	1.56	0.62	0.73	1.00	4.39

[a] (C)=[Acetonitrile–methanol (64:36)]–Tris, pH 5.8 ($100-X$:X), where X represents a % buffer solution. Other conditions as in (A). (D)=[Acetonitrile–methanol (X:$100-X$)]–Tris, pH 8 (95:5), where X represents % acetonitrile. Other conditions as in (A).

the other hand, a CEC analysis with [acetonitrile–methanol (64:36)]–Tris/water (95:5) was accomplished in less than 15 min [CEC conditions (C), Table 2)].

As demonstrated in Table 2 under experiments (C), both k' and α values of tocopherols and tocotrienols were markedly affected by the compositional change in the mobile phases discussed in the preceding paragraph. Without exception, the k' values (e.g., 4.08→8.52 for α-tocopherol acetate) increased substantially when the percentage of the aqueous buffer solutions was raised merely from 5 to 10%. The majority of the α values increased with an increase in the percentage of buffer solutions, excluding those of the unresolved β–γ pairs (α=1.00) and the γ–δ-tocopherols. The latter pair had a constant α value of 1.19 over the composition range analyzed. A mixture of the investigated compounds assayed under CEC conditions (C) (Table 2) had α values ranging 1.00–1.39 for the adjacent components.

Since mixing acetonitrile and methanol complements benefits of fast analyte elution with methanol and superior component selectivity with acetonitrile, it was pragmatic to optimize the composition of the two organic modifiers in CEC mobile phases for achieving CEC assays within desirable retention times. Inspection of CEC data indicated that elution times of the various antioxidant species in pure organic modifiers appeared to be somewhat longer than those in mixtures of these solvents. When portions of acetonitrile were gradually added to methanol, the unresolved peak of ε-tocopherol and α-tocotrienol in methanol alone began to split leading to complete separation of the two components on OS in 100% acetonitrile (Fig. 2). With the ODS column used in this study, the early-eluting components including those two just described were partially resolved in an acetonitrile–methanol mixture.

To explore the effects of organic solvent compositions on the separation of the title antioxidant mixture separated with an ODS column, chromatographic characteristics k' and α values were determined under variable organic solvent conditions [(D), Table 2]. CEC of the compounds with an increased amount of acetonitrile in the acetonitrile–methanol organic modifier consistently yielded analyte components having increased k' values. At 100% acetonitrile in the absence of methanol, the high k' values were too long to be analytically acceptable for routine assays. With a mobile phase void of acetonitrile (i.e., 100% methanol), three pairs (β–γ-tocopherols, β–γ-tocotrienols, and ε-tocopherol+α-tocotrienol) of the 10-component mixture remained unresolved (α=1.00, Table 2). It was necessary to use at least 50% but no more than 64% acetonitrile in the mixed organic modifier to adequately separate ε-tocopherol from α-tocotrienol both having relatively low k' values. Under the conditions specified in (D) of Table 2, the observed α values of the pair of interest ranged from 1.00 to 1.26.

3.6. Calibration study

Linear correlation between peak areas and analyte concentrations (0.5–20 μg/μl) was established for each of the antioxidants examined. While the linear regression coefficients (R^2) were fairly constant averaging 0.9984, the abscissa and slope values obtained from regression analysis were variable particularly among the homologues and saturated/unsaturated side chain analogues (e.g., α-tocopherol vs. α-tocotrienol).

3.7. Other stationary phases of interest

In our earlier publications [7,8,30] on the separation of vitamin E-active compounds, the use of a pentafluorophenylsilica (PFP) phase [4] or triacontylsilica (C_{30}) phase [31,32] for superior component separations have been documented. In consideration of common and popular uses of ODS columns in reversed-phase HPLC assays of antioxidants in vegetable oils, an OS or ODS column was chosen in this study to compare the CEC results with HPLC data in similar short-chain alkylsilica column systems. Moreover, at the outset of this work, the only commercially packed bonded phases available in our laboratory were those of the Hewlett-Packard materials listed in the Experimental section. CEC columns packed with PFP and C_{30} were not commercially available then. However, since we launched a second phase of CEC work about 6 months ago using these superior phases for antioxidant separations, we have

custom packed PFP and C_{30} CEC columns and have made significant progress.

4. Conclusions

In conclusion, this study represents the first CEC separations of mixtures of vitamin E-active tocopherols and tocotrienols. CEC elution behavior of the compounds is explicable in terms of a hydrophobic partition rational in parallel with reversed-phase HPLC. Of the three bonded-silica phases evaluated, OS appears to exhibit the most satisfactory separation and peak characteristics. Based on the inseparability of components under conditions employed, CEC with a silica column offers limited uses in the analysis of the compounds, in contrast to the complete resolution of all the investigated antioxidant components by normal-phase HPLC. Optimization of CEC conditions coupled with calibration data enables speedy sample analyses with adequate component resolution and enhanced detection sensitivity. The CEC method developed can serve as a viable alternative to existing reversed-phase HPLC method and can be used in the routine analysis of tocopherols and tocotrienols in oil samples (e.g., rice bran oil and palm oils).

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