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Elution behavior of unsaponifiable lipids with various capillary electrochromatographic stationary phases

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Abstract

Capillary electrochromatographic (CEC) separations of unsaponifiable lipids, tocopherols (T), tocotrienols (T_3), and plant sterols were studied under various conditions. Investigated stationary phases include pentafluorophenylsilica (PFPS), triacontylsilica (TCS), and octadecylsilica (ODS) phases. A baseline separation of four sterols (ergosterol, lanosterol, sitosterol and stigmasterol) on ODS was achieved and their elution order was found to be dictated by side-chain structures. CEC of the tocol-derived compounds on PFPS in aqueous methanol yielded the most satisfactory results with complete resolution of all components eluting in the order $\delta T_3 > \beta T_3 > \gamma T_3 > \epsilon P > \alpha T_3 > \delta T > \zeta_2 T > \beta T > \gamma T > \alpha T$, while a reversal in elution of the $\epsilon T - \alpha T_3$ pair was observed in aqueous acetonitrile. CEC with a TCS phase in non-aqueous methanol led to a different elution pattern $\delta T_3 > \gamma T_3 > \beta T_3 > \alpha T_3 \epsilon T > \delta T > (\zeta_2 + \gamma)T > \beta T > \alpha T$, despite favorable resolution of the $(\gamma - \zeta_2)T$ pair along with the observation of inseparable $(\beta - \gamma)T$ and $(\beta - \gamma)T_3$ pairs in non-aqueous dimethylformamide. Non-aqueous acetonitrile mobile phases provided unique selectivity for the $(\gamma - \zeta_2)T$ pair and isomer separations on TCS. Variations in separation and retention factors of relevant antioxidant species with CEC variables were evaluated. Examples of CEC quantification of unsaponifiable fractions of rice bran oils and soybean oils are presented. Published by Elsevier Science B.V.

Keywords: Electrochromatography; Stationary phases, electrochromatography; Mobile phase composition; Vegetable oils; Lipids; Tocopherols; Tocotrienols; Sterols

1. Introduction

Lipid antioxidants, tocopherols (T) and tocotrienols (T_3), and sterols occur ubiquitously in oilseed plants as minor constituents. The tocol-derived antioxidants comprise closely related structures 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). Sterols are a class of compounds derived from 3-hydroxylated polycyclic isopentanoids possessing a 1,2-cyclopentanophenthrene structure (Fig. 2). Because of their important nutritional values/health benefits and structural similarity, chromatographic isolation and separation of these substances have been of much interest to lipid scientists. Evaluation of their compositional distribution in vegetable oils should provide valuable information on antioxidant/sterol levels which vary widely among different oil types [1]. Assessment of compositions of minor bioactive

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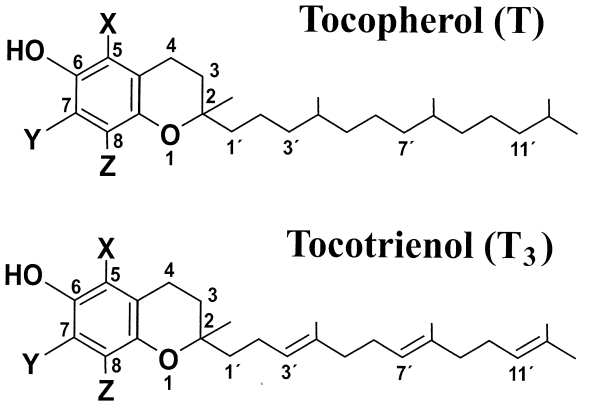

Fig. 1. Structures of tocopherols and tocotrienols.

oil chemicals constitutes an indispensable part of industrial quality control of the important agricultural products and provides a useful means for their correlation with frying oil stability.

Capillary electrochromatography (CEC) was first introduced in 1974 by Pretorius et al. [2] and is a fairly new technique in its infancy for the analysis of lipids and their antioxidants. CEC features high efficiency-, high resolution- and high speed micro-scale separations with minimal solvent consumption, and is a combination of capillary electrophoresis and high-performance liquid chromatography (HPLC). In comparison with electrophoretic techniques including CEC, the column efficiency in HPLC is low because of the need of an external pressure to pump analytes with the mobile phase through the column. In contrast, the mobile phase in CEC is driven by electroosmotic flow (EOF) to transport solutes through a packed capillary column. Thereby, the high column efficiency of CEC is its primary benefit derived from the flat flow profile of the EOF relative to the parabolic flow of HPLC.

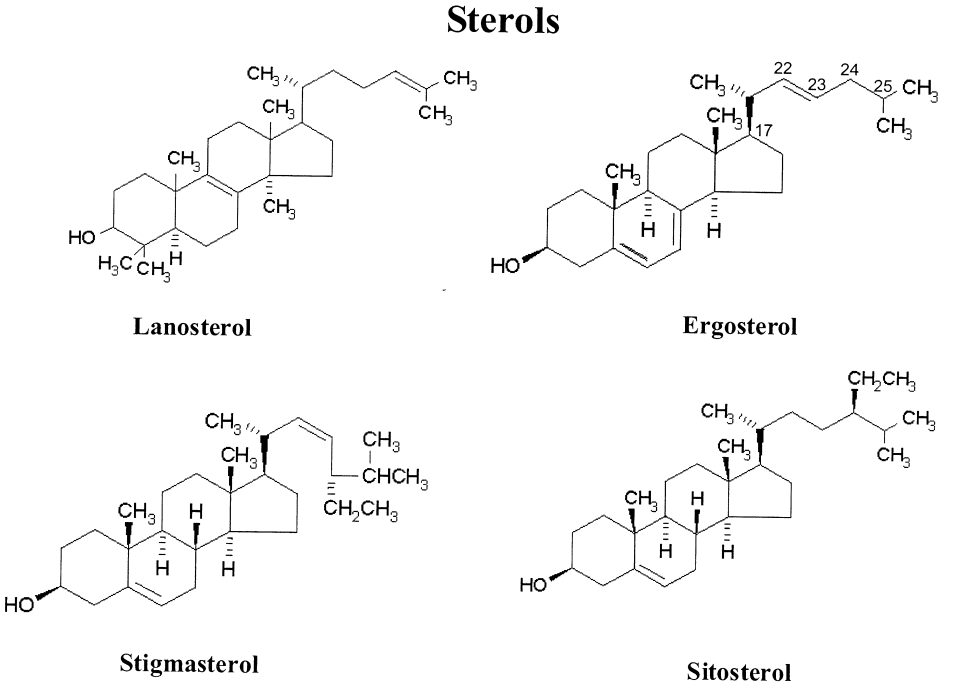

Fig. 2. Structures of investigated plant sterols.

Since its inception, CEC has met with success in the analysis of neutral compounds [3–7], pharmaceuticals [8–16], food/biological samples [17–21], and environmental chemicals [4,5,22–24]. Application of the technique in quality control assays of vitamin E active compounds in various sample matrices would be advantageous. Although chromatographic analysis of lipid antioxidants has been extensively studied by HPLC [25–32], CEC separations of these compounds have rarely been investigated. In continuation of our previous CEC study using octylsilica or octadecylsilica column [33], we report here the results of a CEC study on the separation of the title compounds on a polar reversed-phase stationary phase (pentafluorophenyl silica) [27,30–32] and on a long-chain alkylsilica phase (triacontylsilica) [20,31,32,34,35]. Cholesterol ester derivatives have been separated by CEC [38], but CEC of free sterols has not been described. The preliminary results of a CEC separation of selected sterols on octadecylsilica are included in this study.

2. Experimental

2.1. Chemicals and reagents

Rice bran oils were obtained from Riceland Foods (Stuttgart, AR, USA). Soybean oils were of Hardin cultivars provided by W.R. Fehr (Iowa State University, Ames, IA, USA). Taxane standards were obtained from Lipomed (Cambridge, MA, USA). Sterols (ergosterol, lanosterol, sitosterol and stigmasterol) and tocopherol standards including 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).

High-purity lanosterol was obtained from Sigma (St. Louis, MO, USA). Tocotrienol standards (α-, β-,γ- and δ-tocotrineols) were obtained from VWR Scientific (Batavia, IL, USA). All standard samples devoid of solvents and analytical samples were stored in amber vials at 30°C for protection from light.

Buffer reagents tris(hydroxymethyl)aminomethane (Tris) and lithium acetate were obtained from Sigma and Aldrich (Milwaukee, WI, USA), respectively. Dimethylformamide (DMF) was also obtained from Aldrich. Hydrochloric acid used for adjustment of Tris pH, HPLC-grade acetonitrile (MeCN), methanol (MeOH), and tetrahydrofuran (THF) were obtained from Fisher (Fair Lawn, NJ, 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 for tocol-derived compounds and 200 nm for sterols) and HP ChemStation software for system control. Mobile phases consisted of methanol, acetonitrile, DMF, THF or mixtures of these solvents. They were prepared by mixing individual buffer solutions with organic modifiers in variable proportions. Tris buffer solutions were prepared by titrating the desired amounts of the reagent in water with 30% hydrochloric acid to pH 8. In the optimization experiments with tocol-derived antioxidants on pentafluorophenylsilica, high percentages of MeCN (80–90%) and MeOH (90–95) in aqueous binary mobile phases were used. Otherwise, CEC with mobile phases beyond the specified ranges produced broad, irreproducible, ill-defined peaks of long retention times, presumably due to solubility problems of the hydrophobic analytes in water. Non-aqueous mobile phases were prepared by mixing organic solvents with buffer reagents. Since the lipid antioxidants are not stable in alkaline pH media, samples in mobile phases were injected immediately after preparation to minimize the contact time of analytes with alkaline eluents. There was no indication of the formation of any alkaline decomposition products of the title compounds analyzed under all CEC conditions employed. In all experiments, both inlet and outlet buffer vials in the CEC system were pressurized at 10 bar. Prior to CEC sample analyses, columns were equilibrated with fresh mobile phases.

Two fused-silica capillary columns (Unimicro Technologies, Pleasanton, CA, USA) custom-packed individually with 3-μm pentafluorophenylsilica (PFPS) (E S Industries, West Berlin, NJ, USA) and

5-μm triacontylsilica (TCS) (Phenomenex, Torrance, CA, USA) were used for the study of tocopherols and tocotrienols. The TCS packing material is of monomeric Develosil produced by Nomura Chemical Company (Seto, Japan). For CEC of sterols, a fused-silica capillary column packed in-house with Hewlett-Packard Hypersil 3-μm octadecylsilica (ODS) was used. The PFPS column and TCS column had dimensions of 25 cm×75 μm I.D. and 25 cm×100 μm I.D., respectively. The dimensions of the ODS column were 40 cm×100 μm I.D. In a typical CEC run for tocol compounds, samples dissolved in buffered mobile phase solutions (5–15 μg/μl) were injected electrokinetically onto the column at 10 kV for 2 s, while the CEC voltage and column temperature were maintained at 20 kV (the microamp (μA) currents ranging from 4 to 6 μA) and 25°C, respectively. Analytical samples of sterols were prepared by dissolving individual standards in acetonitrile–THF (1:1) at a concentration of 1 μg/μl while the respective CEC voltage, injection, and temperature were set at 25 kV (the microamp current was reading 9 μA), 15 kV/8 s, and 30°C. In optimization experiments, the CEC parameters were variable.

Individual chromatographic analyses were performed with three replicate injections. Retention factors (k') based on the average retention times (t) (relative standard deviations, RSD, 3.0–5.6%) were determined from the equation $k' = (t - t_0)/t_0$ where t and t_0 represent respective retention times of an analyte and the neutral marker, thiourea (i.e. EOF). Separation factors (α) were determined for adjacent tocopherol components as $\alpha = k'_{c+1}/k'_c$ where subscript ''c'' represents an analyte component.

2.3. Vegetable oil analyses

Prior to CEC analyses, all investigated vegetable oils were saponified following a published procedure [36] with some modification. A sample (2 g) of the oil was stirred with absolute ethanol (70 ml). To the mixture, a fresh solution (5 ml) of 5% (w/w) pyrogallol in absolute ethanol and a 76% (w/w) potassium hydroxide solution (20 ml) were sequentially added, while stirring slowly under a stream of nitrogen. Then, the reaction mixture was heated to 70°C in a water bath and continuously stirred at this temperature for 30 min. After cooling, the saponified matter was added with a solution (25 ml) of 2.5% (w/w) sodium chloride in water and extracted three times with 50-ml portions of hexane–ethyl acetate (85:15, v/v). Evaporation of solvents gave a residue which was dissolved in methanol (0.5 ml). The methanolic solution was filtered through a 0.45-μm polypropylene microfilter (Chrom Tech, Apple Valley, MN, USA) and diluted to exact volume. Aliquot samples were analyzed by CEC.

3. Results and discussion

3.1. Elution patterns of lipid antioxidants

Chromatographic separations of homologous compounds (Fig. 1) in the reversed-phase mode are straightforward and predictable based on hydrophobic interactions of the analyte solutes with alkyl-bonded silica phases. Analytes with the highest number of methyl substitution elute the last from a hydrocarbonaceous column. Thus, monomethyltocol (δ-T) elutes before dimethyltocols (β-, γ-, or ζ_2-T), followed by trimethyltocol (α-T). The same is true for the corresponding tocotrienols (T_3). By contrast, the separation of isomeric compounds (Fig. 1) in the tocopherol series (β-, γ- and ζ_2-T) and in the tocotrienol series (β- and γ-T_3) depends heavily on the stationary phases employed. As demonstrated in an earlier study [33], CEC of a mixture of the parent tocopherols and tocotrienols on octadecylsilica (ODS) or octylsilica (OS) yielded an elution order $\delta T_3 > (\gamma+\beta)T_3 > \alpha T_3 > \epsilon T > (\delta+\zeta_2)T > (\gamma+\beta)T > \alpha T$. In the present CEC study, the ten-component antioxidant mixture on pentafluorophenylsilica (PFPS) eluted in the sequence $\delta T_3 > \beta T_3 > \gamma T_3 > \epsilon T > \alpha T_3 > \delta T > \zeta_2 T > \beta T > \gamma T > \alpha T$ (Fig. 3A), whereas, with a triacontylsilica (TCS) column, they had elution order as follows $\delta T_3 > \gamma T_3 > \beta T_3 > \alpha T_3 > \epsilon T > \delta T > (\zeta_2+\gamma)\, T > \beta T > \alpha T$ (Fig. 3C). The elution patterns observed in CEC are very similar, if not identical, to those seen in reversed-phase HPLC [30,31] of these compounds (Fig. 3A vs. Fig. 4).

CEC of β- and γ-T dimethyltocol isomers and their unsaturated side-chain analogues on either PFPS or TCS resulted in variable degrees of com-

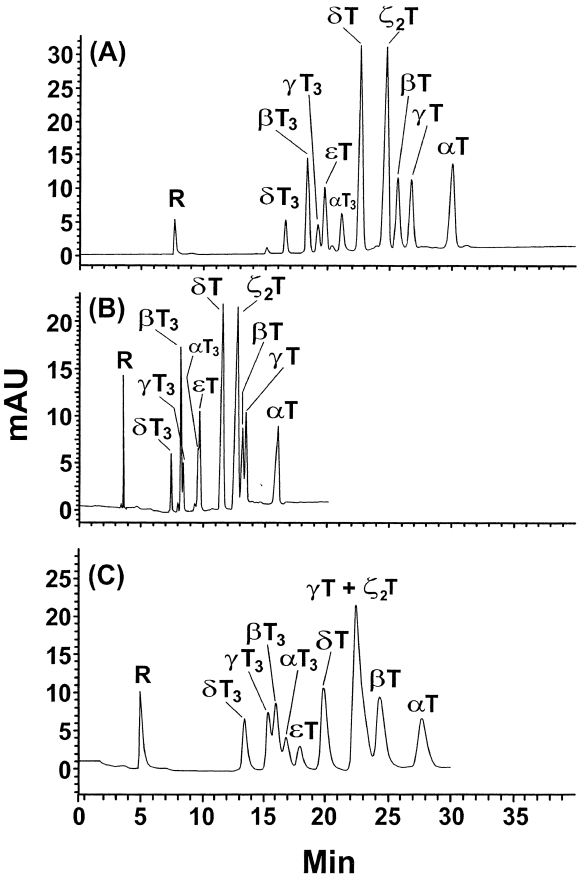

Fig. 3. CEC separations of lipid antioxidants on pentafluorophenylsilica (A,B) and on triacontylsilica (C). Conditions: Tris buffer, 25 m*M*, pH 8; temperature, 25°C (A,B), 20°C (C); voltage, 20 kV (A,B), 25 kV (C); pressure, 10 bar; injection, 10 kV/2 s. Mobile phases: (A) methanol–water/Tris buffer (92:8), (B) acetonitrile–water/Tris buffer (80:20), (C) methanol + Tris buffer. R, thiourea.

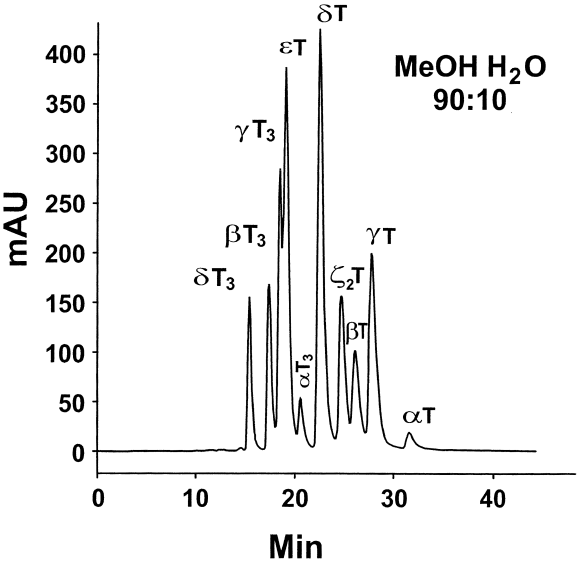

Fig. 4. HPLC separations of tocopherols and tocotrienols on pentafluorophenylsilica.

ponent resolution despite their inseparability on a short-chain alkylsilica phase [33] (e.g. OS or ODS). The unusual selectivity of the polar PFPS reversed-phase column for β-, γ- and ζ_2-T was noteworthy as it facilitated differentiation of the isomers by combined effects of analyte hydrophobicity and molecular polarity. Thus, in the PFPS stationary phase system, γT appeared to be more hydrophobic than βT, which seemed to be less polar than ζ_2T. Likewise, the same trend of differentiability in hydrophobicity between β- and γ-T_3 was observed (Fig. 3A). However, when CEC experiments were carried out with a TCS column, the elution order of the β- and γ-antioxidant isomers was reversed and the γ–ζ_2T pair was not resolved (Fig. 3C).

Careful examination of Fig. 3A revealed that εT emerged from the polar PFPS column before αT_3, contradicting the common elution patterns of the T_3 group preceding the T group observed in CEC with a short chain non-polar alkylsilica phase, OS, or ODS [33]. Increasing the number of alkyl carbon atoms of the alkylsilica phase such as TCS retained the normal elution order with the εT component eluting after αT_3 (Fig. 3C), explicable in terms of simple hydrophobic interactions of the non-polar TCS phase with the analyte solutes in reversed-phase separation processes. The anomaly of the elution order $\epsilon T > \alpha T_3$ on PFPS occurred only in methanol–Tris mobile phases. Replacing methanol with acetonitrile tended to shift the elution order to give $\alpha T_3 > \epsilon T$ (Fig. 3B) normally observed in CEC of these compounds with non-polar alkyl-bonded silica phases. The results indicated that CEC mobile phases and stationary phases had dramatic effects on the elution behavior of the lipid antioxidant evaluated. Evidently, elution patterns obtained with polar reversed-phase station-

ary phases were notably affected by both hydrophobic and polar properties of analyte structures.

3.2. Mobile phase effect in pentafluorophenylsilica systems

Fig. 5 shows the effect of methanol composition in mobile phases on CEC separations of lipid antioxidants on PFPS. A small decrease in the content of methanol in methanol–Tris/water rendered a significant increase in component resolution. When the percent composition of methanol was changed from 95 to 92%, the only partially resolved components 3 (γT_3) and 4 (ϵT) were adequately separated with a total analysis time of ~30 min to complete the CEC run. At 90% methanol in the mobile phase, a very well-resolved base-line separation of all-ten component mixture was obtained at the expense of a rather long analysis time of over 80 min with the last component, peak 10, appearing at 85 min (not shown due to the graphic limitation of the time scale used in Fig. 5). Therefore, a mobile phase consisting of methanol–25 m*M* Tris/water (92:8) was chosen as optimized conditions for CEC assays of standards and oil samples.

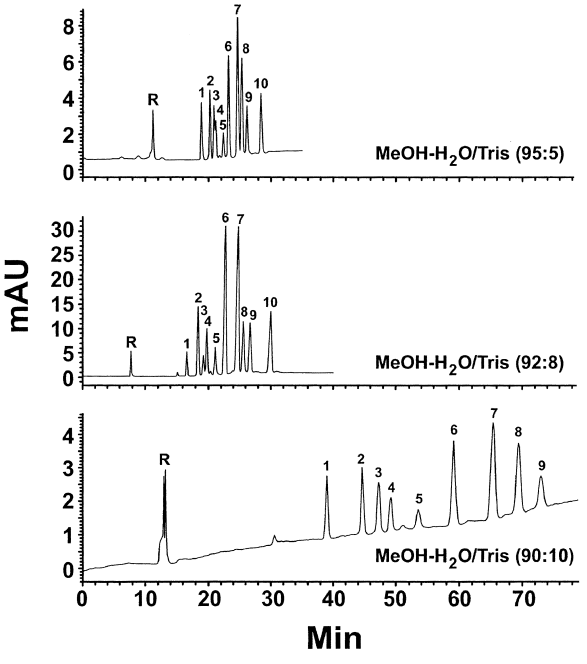

Fig. 5. Effect of water content on CEC separations of lipid antioxidants on pentafluorophenylsilica. Conditions: same as in Fig. 3A. Mobile phases, variable ratios of methanol–water/Tris buffer. R, thiourea. Peaks: 1, δT_3; 2, βT_3; 3, γT_3; 4, ϵT; 5, αT_3; 6, δT; 7, $\zeta_2 T$; 8, βT; 9, γT; 10, αT. The missing peak 10 on the bottom chromatogram eluted at 85 min.

Table 1 compiles retention factors (k') and separation factors (α) of tocopherols and tocotrienols separated on PFPS under various organic modifier conditions. CEC with an optimal composition of 92% methanol as modifier as described earlier yielded k' and α values of the ten components ranging from 1.16 to 2.90 and from 1.05 to 1.20, respectively. The lowest α value, 1.05, belonged to the hard-to-resolve γT_3–ϵT pair, which was, however, poorly dispersed ($\alpha = 1.02$) under elution with a non-optimal composition of 95% methanol.

Evidently, the separation factors were adversely affected by increasing the percentage of methanol in mobile phases. Such detrimental effects of high concentrations of the mobile phase organic modifier on separation factors were also manifested in cases where acetonitrile was used in the mobile phases (Table 1). Thus, increasing the percentage of acetonitrile in mobile phases of acetonitrile–25 m*M* Tris/water from 80 to 90% somewhat reduced the α values of the weakly resolved pairs (β–γ)T and (β–γ)T_3 to 1.02 and 1.00, respectively. In other words, at 90% acetonitrile in the mobile phase specified, the latter (β–γ)T_3 pair was inseparable. Conversely, when the composition of acetonitrile was elevated from 80 to 90%, the α value of the ϵT–αT_3 pair (please note the reversed order of k' values of these components (MeCN vs. MeOH) shown in Table 1) increased from 1.01 to 1.06, a unique phenomenon observed in reversed-phase CEC with acetonitrile alone as organic modifier. The opposite effects of the two organic modifiers acetonitrile and methanol on the separation of components ϵT and αT_3 were reflected in the finding that they co-eluted in all tested mobile phases containing mixed organic modifiers of acetonitrile–methanol (64:36) (Table 1).

3.3. Mobile phase effect in triacontylsilica systems

Initial attempts to separate mixtures of tocopherols

Table 1
CEC of tocopherols and tocotrienols on pentafluorophenylsilica under various solvent conditions

Component	% MeOH			% MeCN in MeOH[a]			Component	% MeCN		
	90	92	95	85	90	95		80	85	90
	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)		k' (α)	k' (α)	k' (α)
αT	5.23	2.90	1.54	5.57	2.18	0.94	αT	3.44	2.97	1.71
$k'_{\alpha T}/k'_{\gamma T}$	(1.13)	(1.17)	(1.15)	(1.23)	(1.20)	(1.16)	$k'_{\alpha T}/k'_{\gamma T}$	(1.26)	(1.26)	(1.22)
γT	4.63	2.47	1.34	4.52	1.82	0.81	γT	2.72	2.36	1.40
$k'_{\gamma T}/k'_{\beta T}$	(1.06)	(1.06)	(1.06)	(1.03)	(1.05)	(1.04)	$k'_{\gamma T}/k'_{\beta T}$	(1.03)	(1.02)	(1.02)
βT	4.35	2.32	1.26	4.33	1.74	0.78	βT	2.63	2.31	1.37
$k'_{\beta T}/k'_{\zeta T}$	(1.08)	(1.05)	(1.04)	(1.06)	(1.03)	(1.04)	$k'_{\beta T}/k'_{\zeta T}$	(1.03)	(1.04)	(1.05)
ζ_2T	4.04	2.22	1.21	4.10	1.69	0.75	$\sim_2$T	2.56	2.22	1.31
$k'_{\zeta T}/k'_{\delta T}$	(1.14)	(1.14)	(1.13)	(l.14)	(l.13)	(l.12)	$k'_{\zeta T}/k'_{\delta T}$	(1.15)	(l.16)	(1.12)
δT	3.55	1.95	1.07	3.60	1.49	0.67	δT	2.22	1.92	1.17
$k'_{\delta T}/k'_{\alpha T3}$	(1.14)	(1.12)	(1.07)	(1.34)	(1.26)	(1.20)	$k'_{\delta T}/k'_{\epsilon T}$	(1.31)	(1.31)	(1.29)
αT_3	3.12	1.74	1.00	2.69	1.18	0.56	ϵT	1.69	1.47	0.91
$k'_{\alpha T3}/k'_{\epsilon T}$	(1.12)	(1.11)	(1.12)	(1.00)	(1.00)	(1.00)	$k'_{\epsilon T}/k'_{\alpha T3}$	(1.01)	(1.01)	(1.06)
ϵT	2.79	1.57	0.89	2.69	1.18	0.56	αT_3	1.68	1.46	0.86
$k'_{\epsilon T}/k'_{\gamma T3}$	(1.06)	(1.05)	(1.02)	(1.24)	(1.22)	(1.27)	$k'_{\alpha T3}/k'_{\gamma T3}$	(1.26)	(1.25)	(1.25)
γT_3	2.64	1.49	0.87	2.17	0.97	0.44	γT_3	1.33	1.17	0.69
$k'_{\gamma T3}/k'_{\beta T3}$	(1.08)	(1.07)	(1.09)	(1.05)	(1.02)	(1.00)	$k'_{\gamma T3}/k'_{\beta T3}$	(1.04)	(1.03)	(1.00)
βT_3	2.44	1.39	0.80	2.07	0.95	0.44	βT_3	1.28	1.14	0.69
$k'_{\beta T3}/k'_{\delta T3}$	(1.22)	(1.20)	(1.18)	(1.22)	(1.20)	(1.13)	$k'_{\beta T3}/k'_{\delta T3}$	(1.21)	(1.21)	(1.21)
δT_3	2.00	1.16	0.68	1.69	0.79	0.39	δT_3	1.06	0.94	0.57

[a] % [MeCN–MeOH (64:36)]. All mobile phases contained water with 25 m*M* Tris buffer.

and tocotrienols with triacontylsilica (TCS) with mobile phases containing water were not successful. The CEC peaks were either too broad or had elution times too long to be analytically useful. Therefore, non-aqueous mobile phases of Tris in methanol were used to study effects of various CEC variables on retention times (t) and separation factors, α values, of ten-component mixtures on the TCS phase (Table 2). Based on our previous experience with an OS or ODS column where an optimal buffer concentration range was determined [33], two different concentrations, 25 and 50 m*M*, of Tris buffer were used. As demonstrated in the retention data in Table 2, CEC under otherwise identical conditions invariably led to decreases in analyte retention times when the buffer concentration was increased from 25 to 50 m*M* at temperature ≥25°C and voltage ≥25 kV. Apparently, the influence of buffer concentrations on component retention times as well as k' values (not shown) became negligible as the column temperature and CEC voltage were lowered to 20°C and 20 kV, respectively.

In CEC experiments with a TCS phase (Table 2) at constant buffer concentrations and voltages, the retention times of the antioxidant components tended to decrease with increasing temperature from 20 to 30°C, because mobile phase eluent viscosity decreased at a high temperature leading to an increase in EOF. Thus, at a Tris concentration of 50 m*M* and a voltage of 20 kV, the changes in retention times of the respective last- and earliest-eluting components α-T and δ-T_3 were 34.7→29.9 min and 16.8→14.8 min, as the temperature increased from 20 to 30°C (Table 2). Analogously, the accelerated EOF at high voltage also accounted for decreases in analyte retention times observed in CEC with variable voltages of 20–30 kV, while keeping the buffer concentration and temperature at constant values. For example, at a Tris concentration of 25 m*M* and a temperature of 25°C, the retention times of the last-

Table 2
CEC of tocopherols and tocotrienols on triacontylsilica in non-aqueous methanol–Tris buffer under various CEC conditions[a]

	Tris conc.													
	25 m*M*								50 m*M*					
	20°C		25°C		30°C		20 kV		20°C	25°C		30°C		20 kV
	20 kV	30 kV	25 kV	30 kV	25 kV	30 kV	25°C	30°C	20 kV	25 kV	30 kV	25 kV	30 kV	30°C
	t (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)	*t* (α)
αT	34.7	22.7	26.2	21.3	24.5	20.1	32.6	30.8	34.7	25.5	21.1	23.4	19.3	29.9
	(1.17)	(1.18)	(1.18)	(1.18)	(1.19)	(1.19)	(1.19)	(1.18)	(1.17)	(1.18)	(1.19)	(1.18)	(1.18)	(1.19)
βT	30.5	19.9	23.0	18.6	21.3	17.5	28.4	26.9	30.5	22.3	18.4	20.5	16.9	26.0
	(1.11)	(1.11)	(1.06)	(1.09)	(1.07)	(1.08)	(1.09)	(1.08)	(1.07)	(1.09)	(1.09)	(1.08)	(1.08)	(1.07)
γT	28.1	18.4	22.0	17.4	20.1	16.5	26.6	25.3	29.0	20.8	17.2	19.4	16.0	24.6
	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)	(1.04)	(1.00)	(1.00)	(1.00)	(1.00)	(1.00)
C_2T	28.1	18.4	22.0	17.4	20.1	16.5	26.6	25.3	28.2	20.8	17.2	19.4	16.0	24.6
	(1.18)	(1.16)	(1.22)	(1.18)	(1.18)	(1.18)	(1.18)	(1.18)	(1.18)	(1.17)	(1.18)	(1.18)	(1.20)	(1.18)
δT	24.8	16.3	18.9	15.3	17.7	14.6	23.5	22.3	24.9	18.4	15.2	17.1	14.1	21.7
	(1.15)	(1.15)	(1.15)	(1.15)	(1.17)	(1.17)	(1.16)	(1.16)	(1.16)	(1.17)	(1.16)	(1.17)	(1.17)	(1.17)
εT	22.4	14.7	17.1	13.8	15.8	13.0	21.1	20.0	22.4	16.4	13.6	15.3	12.6	19.4
	(1.09)	(1.10)	(1.09)	(1.08)	(1.05)	(1.05)	(1.08)	(1.07)	(1.09)	(1.07)	(1.07)	(1.06)	(1.06)	(1.06)
αT_3	21.0	13.8	16.1	13.1	15.2	12.5	20.0	19.1	21.1	15.6	12.9	14.7	12.1	18.6
	(1.07)	(1.08)	(1.10)	(1.11)	(1.13)	(1.12)	(1.10)	(1.11)	(1.08)	(1.09)	(1.11)	(1.12)	(1.12)	(1.12)
βT_3	20.0	13.1	15.1	12.2	14.0	11.6	18.7	17.7	20.0	14.7	12.1	13.6	11.2	17.2
	(1.06)	(1.06)	(1.04)	(1.04)	(1.03)	(1.04)	(1.04)	(1.03)	(1.06)	(1.04)	(1.03)	(1.00)	(1.00)	(1.02)
γT_3	19.3	12.6	14.7	11.9	13.7	11.3	18.2	17.3	19.3	14.3	11.8	13.6	11.2	16.8
	(1.23)	(1.23)	(1.24)	(1.23)	(1.21)	(1.22)	(1.23)	(1.22)	(1.23)	(1.23)	(1.24)	(1.26)	(1.26)	(1.23)
δT_3	16.8	11.0	12.8	10.4	12.1	9.93	15.9	15.2	16.8	12.5	10.3	11.7	9.67	14.8

[a] *t*, retention time. All mobile phases contained specified concentrations of Tris buffer in methanol.

and earliest-eluting components α-T and δ-T_3 decreased from 26.2 to 21.3 min and from 12.8 to 10.4 min, respectively, as the voltage increased from 25 to 30 kV (Table 2). In general, the CEC data in Table 2 establish inverse relationships between analyte retention times and changes in the concentration, temperature, and voltage parameters.

For most lipid antioxidant adjacent pairs having α values in the 1.05–1.26 range, increasing either the voltage up to 30 kV or the temperature up to 30°C, appeared to render no adverse effect on component resolution under conditions specified (Table 2). However, in CEC experiments with a Tris buffer concentration of 50 m*M*, the α values of the (β–γ)T_3 pair reduced from 1.06 to 1.00 as the CEC variables changed from 20 kV and 20°C to 30 kV and 30°C, respectively. This isomeric pair remained unresolved ($\alpha = 1.00$) at 50 m*M* buffer, 25 kV and 30°C. In most experiments conducted in this work, CEC conditions of 25 m*M* Tris buffer, 25 kV voltage and 30°C temperature were used for achieving optimal separations within reasonable analysis times. The apparent inferior column efficiency of TCS relative to PFPS (Fig. 3C vs. Fig. 3A) was partly due to the difference in the respective particle size of 5 and 3 μm. As compared to PFPS, the lower selectivity of TCS might partly stem from the use of the monomeric TCS instead of a polymeric material which is most successfully used for the separation of isomeric species. At the time of conducting this study, the only available TCS packing material in the market was 5-μm monomeric Develosil (see Experimental section) and numerous attempts at obtaining bulk packing materials of other specifications including polymeric TCS packings were unsuccessful. Inspection of CEC data in Tables 1 and 2 showed that under optimal conditions, all three dimethyltocol isomers, β-, γ- and ζ_2T, were separ-

able on a PFPS phase with α values ranging from 1.08 to 1.02 (Table 1), but only two of them, β- and γ-T, were resolved ($\alpha = 1.06–1.11$) with the (γ–ζ_2)T pair remaining inseparable ($\alpha = 1.00$) on a TCS phase (Table 2). The failure of the long-chain alkylsilica phase TCS to distinguish the (γ–ζ_2)T isomers was rather unanticipated and unparalleled to their ready resolution with a short-chain alkylsilica OS or ODS phase [33].

Table 3 shows the influence of non-aqueous methanol, acetonitrile, and dimethylformamide (DMF) [37] mobile phases (B, C and D in Table 3) containing lithium acetate on the separation of the lipid antioxidants on a TCS phase. All CEC data in this table were obtained with a TCS column non-identical with the one used in Table 2. The data obtained with a mobile phase of non-aqueous methanol–Tris buffer in the same new TCS phase system (A in Table 3) is included in the table for comparison. In mobile phase systems containing high percentages of DMF (Table 3, mobile phases C and D), an elution order $\delta T_3 > (\gamma+\beta)T_3 > \alpha T_3 > \epsilon T > \delta T > \zeta_2 T > (\gamma+\beta)T > \alpha T$ was observed. Such an elution order is reminiscent of that observed in CEC with OS or ODS [33]. Obviously, CEC with TCS in DMF offered little selectivity for the β- and γ-isomers of tocopherols and tocotrienols, even though the separation of the (γ–ζ_2)T pair was unexpectedly favored. As noted earlier, the latter isomeric pair was not differentiable in CEC with non-aqueous methanol (Table 2). It appears that TCS in DMF system provides no advantages over OS or ODS for assays where individual isomer measurements are critical. Retention factors k' values (Table 3) of the components of interest decreased sharply by changing the non-aqueous solvent from methanol ($k' = 1.25–3.32$) to DMF ($k' = 0.46–1.48$). Increases in the content of methanol or acetonitrile in the DMF mobile phase systems led to higher analyte k' values and had variable degrees of effects on the separation factors,

Table 3
CEC of tocopherols and tocotrienols on triacontylsilica in different non-aqueous solvent systems[a]

Component	A	B (% MeCN)			C (% DMF)		D (*x*% DMF)		
		0	50	99	99	95	90	50	10
	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)
αT	3.32	3.62	5.02	8.66	1.48	1.60	1.87	3.06	6.80
$k'_{\alpha T}/k'_{\beta T}$	(1.19)	(1.19)	(1.20)	(1.23)	(1.17)	(1.19)	(1.19)	(1.24)	(1.20)
βT	2.79	3.05	4.17	7.05	1.27	1.35	1.57	2.46	5.69
$k'_{\beta T}/k'_{\gamma T}$	(1.04)	(1.06)	(1.05)	(1.04)	(1.00)	(1.00)	(1.00)	(1.00)	(1.06)
γT	2.62	2.87	3.97	6.75	1.27	1.35	1.57	2.46	5.36
$k'_{\gamma T}/k'_{\zeta T}$	(1.00)	(1.03)	(1.06)	(1.07)	(1.08)	(1.05)	(1.06)	(1.06)	(1.07)
ζ_2T	2.62	2.80	3.76	6.29	1.18	1.28	1.48	2.32	5.02
$k'_{\zeta T}/k'_{\delta T}$	(1.22)	(1.19)	(1.14)	(1.12)	(1.10)	(1.11)	(1.11)	(1.12)	(1.12)
δT	2.14	2.36	3.29	5.60	1.07	1.15	1.33	2.08	4.48
$k'_{\delta T}/k'_{\epsilon T}$	(1.16)	(1.16)	(1.28)	(1.29)	(1.20)	(1.25)	(1.21)	(1.24)	(1.28)
ϵT	1.85	2.03	2.58	4.35	0.89	0.95	1.10	1.68	3.50
$k'_{\epsilon T}/k'_{\alpha T3}$	(1.06)	(1.07)	(1.17)	(1.24)	(1.51)	(1.44)	(1.45)	(1.39)	(1.30)
αT_3	1.74	1.89	2.20	3.50	0.59	0.66	0.76	1.21	2.70
$k'_{\alpha T3}/k'_{\beta T3}$	(1.09)	(1.10)	(1.18)	(1.17)	(1.13)	(1.16)	(1.15)	(1.19)	(1.18)
βT_3	1.59	1.72	1.86	2.98	0.52	0.57	0.66	1.02	2.29
$k'_{\beta T3}/k'_{\gamma T3}$	(1.05)	(1.04)	(1.03)	(1.03)	(1.00)	(1.00)	(1.00)	(1.00)	(1.05)
γT_3	1.52	1.65	1.80	2.89	0.52	0.57	0.66	1.02	2.19
$k'_{\gamma T3}/k'_{\delta T3}$	(1.25)	(1.23)	(1.21)	(1.18)	(1.13)	(1.16)	(1.18)	(1.19)	(1.20)
δT_3	1.25	1.34	1.49	2.44	0.46	0.49	0.56	0.86	1.82

[a] The data were obtained with a different triacontylsilica column non-identical with the one used for data in Table 2. A, MeOH–25 m*M* Tris buffer. B, % MeCN in MeOH containing 2.5 m*M* lithium acetate. C, % DMF in MeOH containing 2.5 m*M* lithium acetate. D, *x*% DMF in [DMF–MeCN (*x*:100 − *x*]–MeOH (99:1) containing 2.5 m*M* lithium acetate.

α values, of various analyte pairs (Table 3, mobile phases C and D). In addition, k' values increased with increasing percent composition of acetonitrile in methanol (Table 3, mobile phases B).

Because of the insolubility of Tris and lithium acetate buffer reagents in non-aqueous acetonitrile, the elution behavior of the lipid antioxidants on TCS was not studied with the non-aqueous acetonitrile–Tris buffer. As alternatives, non-aqueous acetonitrile–thium acetate mobile phases [37] containing minimal 1% methanol were used for studying their elution behavior (Table 3, mobile phases B). Except for the slight improvement in the separation of the $(\gamma-\zeta_2)$T pair ($\alpha=1.03$), CEC with methanol/lithium acetate yielded results very similar to those obtained with methanol–Tris buffer (Table 3, B (0% MeCN) vs. A). Resolution of this pair was enhanced by increasing the amount of acetonitrile in methanol (Table 3, mobile phases B). On the other hand, when the amount of acetonitrile in DMF was raised to 90%, some degrees of separations of the $(\beta-\gamma)$T and $(\beta-\gamma)T_3$ pairs were observed with α values of 1.06 and 1.05, respectively (Table 3, mobile phases D). However, the two pairs were not resolved at lower concentrations (10 and 50% of acetonitrile in DMF, $\alpha=1.00$).

3.4. Capillary electrochromatography of sterols

Plant sterols are important unsaponifiable lipids in vegetable oils and have been traditionally analyzed by gas chromatography [39]. With the exception of a recent publication on cholesterol esters [38], CEC separations of the free sterols derived from plants have remained obscure. After initial unsuccessful attempts at using a 30-cm capillary column for CEC of test sterols, a mixture of lanosterol, ergosterol, stigmasterol and sitosterol was completely resolved with a 40-cm long column packed with ODS (Fig. 6). Examination of the electrochromatogram shown in the figure indicated that the elution sequence appeared to be dictated by the structures of the C_{17}-side-chains (Fig. 2). The elution of lanosterol ahead of ergosterol might be due to the presence of a terminal 24,25-double bond contributing to the diminished hydrophobicity of the former in relation to the latter. The two later-eluting sterols noted from the elution order lanosterol>ergosterol>stigma-

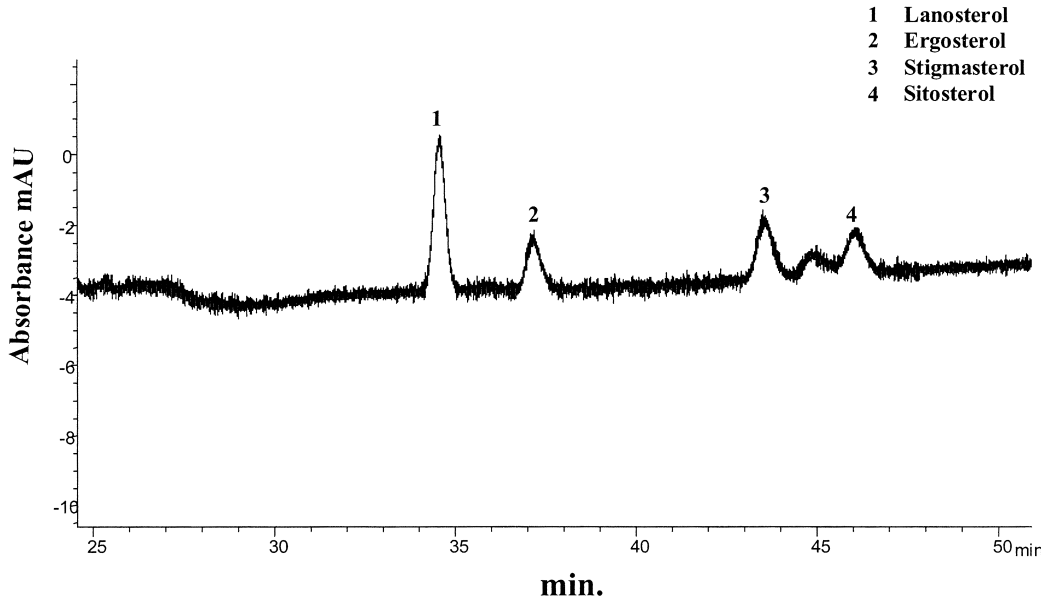

Fig. 6. CEC separation of selected plant sterols on octadecylsilica. Conditions: Tris buffer, 25 m*M*, pH 8; temperature, 30°C; voltage, 25 kV; pressure, 12 bar; injection, 15 kV/8 s. Mobile phase, acetonitrile–tetrahydrofuran–water/Tris buffer (60:35:5).

sterol>sitosterol emerged from the ODS column in a sequence consistent with hydrophobicity of the side-chain moieties of the sterols analyzed. Additional CEC optimization experiments with different mobile phases and stationary phases are in progress.

3.5. Analysis of oil samples and taxane standards

Since the CEC instrument had a built-in photodiode array detector system, all test vegetable oils required sample clean-up prior to the quantification of unsaponifiable lipids. To this end, oil samples were subjected to several purification procedures: supercritical fluid fractionation, solid-phase extraction, and saponification. After elaborate trial experiments, the saponification technique was found to be the most satisfactory. PFPS-CEC electrochromatograms of a rice bran oil (Fig. 7A) and soybean oil (Fig. 7B) showed adequate separations of analyte peaks with few interferences from endogenous substances present in oil samples. The shift in retention times observed for δ-, γ- and α-T in Fig. 7B in relation to those in Fig. 7A was probably derived from combined effects of sample matrices and fluctuations in CEC systems run months apart. Consequently, peak identification in all oil sample assays required spiking with standards. Linear calibration plots were constructed from peak areas obtained with a PFPS phase and analyte concentrations (1.0–30 μg/μl) for each of the antioxidants examined. Although linear regression coefficients (R^2) were constant, averaging 0.9981, the abscissa and slope values obtained from regression analysis varied with homologues and side-chain structures.

PFPS has been developed for use in the HPLC analysis of taxane compounds partially represented by 10-deacetylbaccatin III, baccatin III, 10-deacetyltaxol, cephalomannine and paclitaxel. They are naturally occurring diterpenoid substance with polycyclic (five, eight and six member) fused ring systems found in yew trees and have been proven to be effective breast cancer drugs. In view of their vital importance in chemotherapeutic programs, a sample of taxane standard mixture containing the five analogous compounds described above was chosen to study the feasibility of high speed separation by CEC. Under the isocratic conditions employed, CEC of the five-component mixture of taxane standards on PFPS produced very well separated peaks with excellent peak symmetry in less than 15 min (Fig. 8). Optimization of CEC mobile phases should further reduce analysis times.

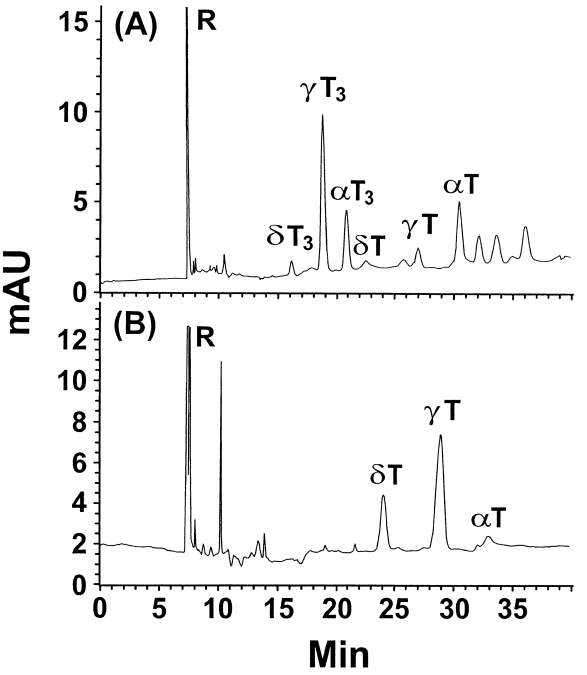

Fig. 7. CEC of crude rice bran oil (A) and crude soybean oil (B) on pentafluorophenylsilica. Conditions: same as in Fig. 3A. R, thiourea.

4. Conclusions

In conclusion, this study represents the first application of packed PFPS, TCS and ODS phases in CEC separations of mixtures of important unsaponifiable lipids found in vegetable oils and plants. CEC elution patterns of the compounds resemble those of reversed-phase HPLC and are influenced dramatically by stationary and mobile phases employed. Further, CEC provides much better analyte resolution than HPLC. CEC with a PFPS column allows

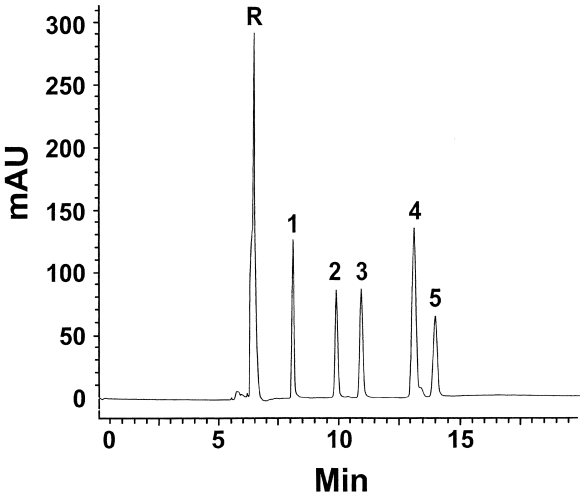

Fig. 8. CEC of taxane-related structures on pentafluorophenylsilica. Conditions: same as in Fig. 3A. Mobile phase, acetonitrile–water/Tris buffer (60:40). Peaks: 1, 10-deacetylbaccatin III; 2, baccatin III; 3, 10-deacetyltaxol; 4, cephalomannine; 5, paclitaxel. R, thiourea.

complete separations of all test components with excellent peak characteristics, whereas all but one pair of antioxidant analytes were separated on TCS with lower efficiency than PFPS. Due to high hydrophobicity of TCS, non-aqueous solvents are required in mobile phases for achieving optimal separations. Replacing methanol or acetonitrile with DMF in CEC of the lipid antioxidants on TCS loses the selectivity for the β- and γ-isomers of tocopherols and tocotrienols. In other words, TCS-CEC in DMF is similar to OS (or ODS)-CEC in any solvent where separations of these isomers are hardly possible. The CEC method developed can be used in the analysis of tocopherols and tocotrienols in various oil samples.

5. Disclaimer

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.

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References

[1] T.L. Mounts, S.L. Abidi, K.A. Rennick, J. Am. Oil Chem. Soc. 73 (5) (1996) 581.
[2] V. Pretorius, B.J. Hopkins, J.D. Shielie, J. Chromatogr. 99 (1974) 23.
[3] N.W. Smith, M.B. Evans, Chromatographia 38 (1994) 649.
[4] H. Rebscher, U. Pyell, Chromatographia 38 (1994) 737.
[5] C. Yan, R. Dadoo, H. Zhao, R. Zare, Anal. Chem. 67 (1995) 2026.
[6] R. Dadoo, C. Yan, R. Zare, D.S. Anex, D.J. Rakestraw, G.A. Hax, LC–GC Int. 15 March (1997) 164.
[7] M. Taylor, P. Teale, S.A. Westwood, Anal. Chem. 69 (1997) 2554.
[8] N.W. Smith, M.B. Evans, Chromatographia 41 (1995) 197.
[9] M. Eurby, C.M. Johnson, K.D. Bartle, P. Meyers, S. Roulin, Anal. Commun. 33 (1996) 403.
[10] J.H. Miyawa, M.S. Alasandro, C.M. Riley, J. Chromatogr. A 769 (1997) 145.
[11] M.M. Robson, M.G. Cikalo, P. Meyers, M.R. Eurby, K.D. Bartle, J. Microcol. Sep. 9 (1997) 357.
[12] M.R. Eurby, D. Gilligan, C.M. Johnson, S.C.P. Roulin, P. Meyers, K.D. Bartle, J. Microcol. Sep. 9 (1997) 372.
[13] J. Wang, D.E. Schaufelberger, N.C. Guzman, J. Chromatogr. Sci. 36 (1998) 155.
[14] J.H. Miyama, D.K. Lloyd, M.S. Alasandro, J. High Resolut. Chromatogr. 21 (1998) 161.
[15] I.S. Lurie, R.P. Meyers, T.S. Conver, Anal. Chem. 70 (1998) 3255.
[16] J. Reily, M. Saeed, J. Chromatogr. A 829 (1998) 175.
[17] P. Sandra, A. Dermaux, V. Ferraz, M.M. Dittmann, G. Rozung, J. Microcol. Sep. 9 (1997) 409.
[18] J. Ding, P. Vouros, Anal. Chem. 69 (1997) 379.
[19] J. Ding, P. Vouros, Am. Lab. (Boston) 30 June (1998) 15.
[20] L.C. Sander, M. Pursch, B. Marker, S.A. Wise, Anal. Chem. 71 (16) (1999) 3477.
[21] L. Roed, B. Lundanes, T. Greibrokk, J. Microcol. Sep. 11 (1999) 421.
[22] V. Lopez-Avila, J. Benedicto, C. Yan, J. High Resolut. Chromatogr. 20 (1997) 615.
[23] C.G. Bailey, C. Yan, Anal. Chem. 70 (1998) 3275.
[24] E. Dabek-Zlotorzynska, E.P.C. Lai, J. Chromatogr. A 853 (1999) 487.

[25] S.L. Abidi, T.L. Mounts, J. Liq. Chromatogr. 19 (1996) 509, and references cited therein.
[26] S.L. Abidi, T.L. Mounts, J. Chromatogr. A 670 (1994) 67.
[27] S.L. Richheimer, M.C. Kent, M.W. Bernart, J. Chromatogr. A 677 (1994) 75.
[28] Y. Satomura, M. Kimura, Y. Itokawa, J. Chromatogr. 625 (1992) 372.
[29] W.T. Wahyuni, K. Juno, J. Chromatogr. 448 (1988) 398.
[30] S.L. Abidi, T.L. Mounts, J. Chromatogr. A 782 (1997) 25.
[31] S.L. Abidi, J. Chromatogr. A 844 (1999) 67.
[32] S.L. Abidi, J. Chromatogr. A 881 (2000) 197.
[33] S.L. Abidi, K.A. Rennick, J. Chromatogr. A 913 (2001) 379.
[34] S. Strohschein, M. Pursch, D. Lubda, K. Albert, Anal. Chem. 70 (1) (1998) 13.
[35] S. Strohschein, C. Rentel, T. Lacker, B. Bayer, K. Albert, Anal. Chem. 71 (1999) 1780.
[36] E. Gimeno, E. Calero, A.I. Castellote, R.M. Lamuela-Raventos, M.C. de la Torre, M.C. Lopez-Sabater, J. Chromatogr. A 881 (2000) 255.
[37] L. Roed, E. Lundanes, T. Greibrokk, Electrophoresis 20 (1999) 2373.
[38] S. Thiam, S.A. Shamsi, C.W. Henry III, J.W. Robinson, I.M. Warner, Anal. Chem. 72 (2000) 2541.
[39] S.L. Abidi, J. Chromatogr. A 935 (2001) 173.