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Capillary electrochromatography of sterols and related steryl esters derived from vegetable oils

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Abstract

Capillary electrochromatographic (CEC) separations of plant sterols and related esters were evaluated under various conditions. Stationary phases included octadecylsilica (C_{18}) and triacontylsilica (C_{30}). Mobile phases comprised acetonitrile, tetrahydrofuran, and tris(hydroxymethyl) aminomethane buffers in aqueous or non-aqueous systems. Apart from notable differences in component resolution, both C_{18} and C_{30} phases had dramatic influence on the elution behavior of the title compounds. Generally, C_{18} had greater selectivity for most components with elution patterns in consistence with the hydrophobicity of side chain structures, while no predictable trend of analyte elution was observed in CEC with C_{30}. In the latter column systems, analyte separations appeared to be improved by conversion to benzoates or ferulates. Twenty-four-epimers of campesterol acetate and 7-campestenol acetate as well as the campesterol–stigmasterol pair were readily resolved by CEC with either phase. However, the cholesterol–stigmasterol pair was barely resolved and had an elution order opposite to that of their acetates or benzoates. Potential applicability of the CEC technique in the analysis of sterols and sterol ferulates in vegetable oil is demonstrated.

Keywords: Capillary electrochromatography; Elution pattern; Octadecylsilica; Phytosterol; Stationary phases; Steryl acetate; Steryl benzoate; Steryl ferulate; Triacontylsilica; Vegetable oil

1. Introduction

Sterols and steryl esters are widespread substances occurring in plants as bioactive minor constituents. They are a group of compounds derived from 3-hydroxylated polycyclic isopentanoids which have a 1,2-cyclopentanophenthrene structure (Figs. 1 and 2). The number of carbon atoms in a sterol structure varies from 27 to 30 and that of the side chain attached at the carbon-17 position can be equal to or greater than seven. Plant sterols (i.e. phytosterols) are important agricultural products for use in nutrition and pharmaceutical industries. Phytosterols and steryl esters containing unique structural moieties are potential antipolymerization and stabilizing agents for frying oils [1]. Hypocholesterolemic activities of some phytosterols derive from vegetable oils have been reported [2,3]. Evaluation of sterol content and composition in vegetable oils enables correlation between distribution patterns and oil properties including frying oil stability.

With the advent of the high-efficiency separation technology based on electrophoretic mobility and solute-stationary phase partitioning, numerous capillary electrochromatographic (CEC) studies with a wide variety of compounds in various sample matrices have been reported [4–19]. Some steroids and isomers have been separated by CEC [11,20,21]. In spite of the vast volume of published information on the gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) analysis of phytosterols [22], there are only sporadic reports available on the CEC separation of these compounds [23,24]. In general, for mixture analyses,

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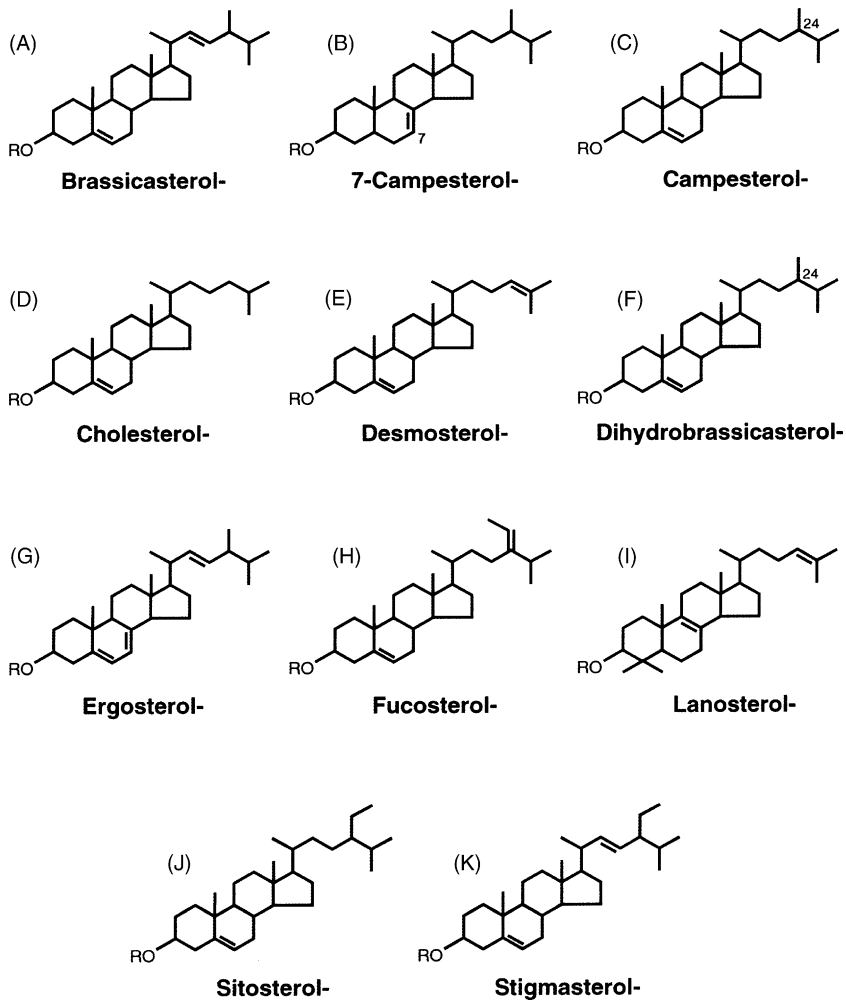

Fig. 1. Structures of investigated plant sterols (R = H), their acetates (R = CH_3CO), and benzoates (R = C_6H_5CO).

the separation power of CEC is greater than HPLC which, in turn, is much greater than supercritical fluid chromatography (SFC). The major disadvantage of GC is that it requires the use of thermally stable columns and chemical derivatization prior to sample analyses. However, it appears to have equal or greater ability to resolve complex mixtures than CEC. The latter technique tends to provide superior analyte selectivity and is ideally suitable for the analysis of thermally labile compounds. Analytical precision and sensitivity of the various techniques for sterol assays seems to follow the order GC > HPLC > SFC > CEC. The sensitivity order may varies depending on sterol structures (e.g. fluorescent labels) and detectors employed. While GC, HPLC, SFC, and CEC are individually meritorious for specific applications, GC–flame ionization detection (or mass spectrometry) is believed to be the method of choice for practical analyses of plant sterols in foods and vegetable oils. This paper presents the results of a CEC study on the separation of the title compounds on octadecylsilica (C_{18}) or triacontylsilica (C_{30}). Inclusion of the C_{30} phase in the study was plausible in light of its demonstrated selectivity for compounds with alkyl chain structures [8,24–28].

2. Experimental[1]

2.1. Chemicals and reagents

Sterol standards (purity 95–99%) were obtained from Matreya (Pleasant Gap, PA, USA). Lanosterol was obtained from Sigma (St. Louis, MO, USA). Most common steryl acetates were products of Sigma. Acetates of campestenol, desmosterol, and fucosterol were obtained from Research Plus (Manasquan, NJ, USA). Benzoates of brassicasterol and cholesterol were obtained from Steraloids (Newport, RI, USA). Sitosterol benzoate and stigmasterol benzoate were synthesized in house by treating the sterols (1 mg) with benzoyl chloride (10 μl) in pyridine (100 μl) at room temperature overnight. After addition of water (5 ml), extraction with methylene chloride (3 ml × 2 ml), removal of solvent, the benzoate products were purified by preparative thin layer

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

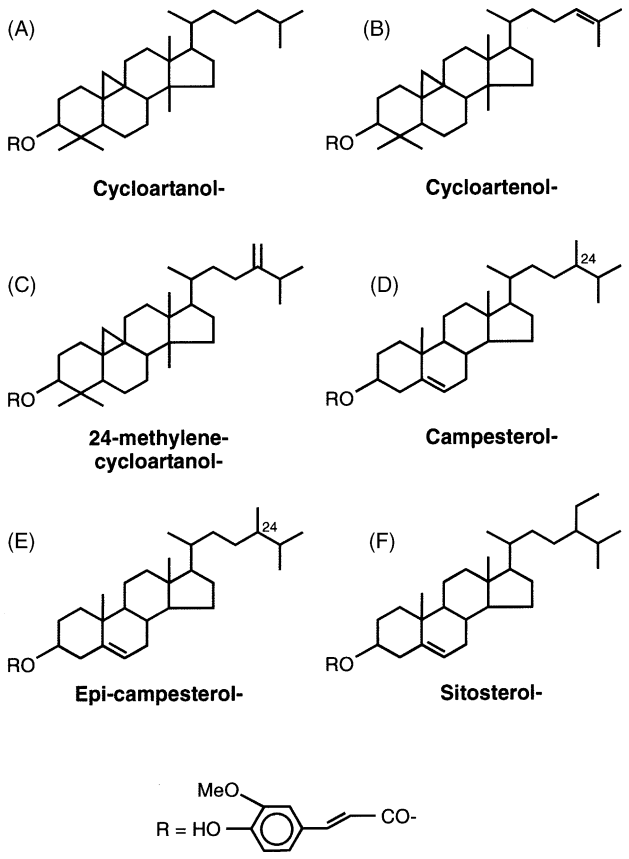

Fig. 2. Structures of γ-oryzanol (sterol ferulates).

chromatography [29]. γ-Oryzanol standards (purity 98–99%) were obtained either from CTC organics (Atlanta, GA, USA) or from Indofine Chem. Co. (Somerville, NJ, USA). All standard compounds and analytical samples were stored at −30 °C before use. Rice bran oils were obtained from Riceland Foods (Stuttgart, AR, USA). Soybean oils were obtained locally from grocery stores or provided by Professor W.R. Fehr (Iowa State University, Ames, IA, USA). Canola oils were obtained from InterMountain Canola Company (Cinnaminson, NJ, USA).

Buffer reagents tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma. Hydrochloric acid used for adjustment of Tris pH, acetonitrile, methanol, and tetrahydrofuran (THF) were obtained from Fisher (Fair Lawn, NJ, USA). Organic solvents utilized for CEC experiments were of HPLC grade. 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 photo diode array detector, and a HP ChemStation software for system control. The detector was set at 210 nm, 230 nm, and 330 nm for monitoring sterols, steryl benzoates, and steryl ferulates, respectively. Mobile phases consisted of methanol, acetonitrile, tetrahydrofuran in aqueous or non-aqueous Tris buffers. They were prepared by mixing individual buffer solutions with organic solvents in suitable proportions. Aqueous Tris buffer solutions were prepared by titrating the reagent in water with 30% hydrochloric acid to pH 8. Nonaqueous mobile phases containing no water were prepared by mixing organic solvents with Tris buffer in methanol–THF (1:1). Optimum compositions of organic modifiers in mobile phases for C_{18} and C_{30} columns were selected based on results obtained from initial mobile phase optimization experiments. These systems provided the best resolution and efficiency or selectivity as compared to any other systems attempted. All experiments were carried out with both inlet and outlet buffer vials in the CEC system pressurized at 10 bar. To ensure reproducible analysis results, columns were re-equilibrated with fresh mobile phases prior to CEC sample analyses.

Three fused silica capillary columns (Unimicro Technologies, Pleasanton, CA, USA) were custom-packed individually with (1) 3 μm Hypersil octadecylsilica (C_{18}) (Hewlett-Packard) with specifications: 120 Å, 2.8 μmol/m^2, 170 m^2/g, 10% carbon loading, encapped, monomeric bonding, (2) 3 μm ProntoSil triacontylsilica [C_{30}-(I)] (Mac-Mod Analytical, Chadds Ford, PA, USA) with specifications: 200 Å, 200 m^2/g, 24% carbon loading, no endcapping, polymeric bonding, (3) 5 μm YMC carotenoid [C_{30}-(II)] (Waters, Milford, MA, USA) with specifications: 250 Å, 3.64 μmol/m^2, 200 m^2/g, 19.4% carbon loading, no endcapping, polymeric bonding. The dimension of the C_{18} and C_{30} columns were 40 cm × 100 μm i.d. and 25 cm × 75 μm i.d., respectively. The CEC columns were preconditioned by applying the potential in 5 kV increments for 15 min up to 20 kV and conditioned at 15 kV for 5 min between injections. The pressure (10 bar) was applied simultaneously with voltage to both sides. In a typical analysis, sterol samples dissolved in acetonitrile–THF (1:1) at concentrations of 1–5 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 and 25 °C, respectively. The CEC currents ranged 4–9 μA.

In order to evaluate analytical reproducibility, three replicate injections were made for each aliquot sample for obtaining average retention times (t). Retention factors (k') were determined from the equation $k' = t - t_o/t_o$ where t and t_o represent average retention times of an analyte and the neutral marker, thiourea (i.e. EOF), respectively. Separation factors (α) were determined for adjacent sterol components as $\alpha = k'_{c+1}/k'_c$ where subscript "c" represents an analyte component.

2.3. Oil sample analyses

Before analysis, samples of soybean oils or canola oils were purified using a published method [30] with some modification. An aliquot sample (100 mg) of the oil was stirred

overnight with 1 M potassium hydroxide in ethanol (20 ml). Water (20 ml) was added to the reaction mixture followed by extraction with three portions (40 ml) of diethylether. The combined ether extract was further treated with 0.5 M solution of potassium hydroxide in ethanol (20 ml) and washed with water until neutral. Removal of ether gave a residue which was streaked onto a silica gel (1 mm thickness) preparative thin layer plate (EM Sciences, Gibbstown, NJ, USA) which was developed with hexane–diethylether (7:3). The sterol bands were extracted with chloroform–diethylether (8:2) to give aliquot samples for CEC analyses.

Rice bran oils were purified as follows: an oil sample (0.5–5.0 g) was dissolved in 2–5 ml of hexane–ethyl acetate (9:1) and loaded onto a silica (30 g) column (45 cm × 2 cm i.d.). The column was eluted sequentially with 60-ml each of hexane–ethyl acetate (7:3) and hexane–ethyl acetate (1:1). Oryzanol fractions (15 ml) were monitored spectrophotometrically at 330 nm. They were combined and the solvents were evaporated to leave a residue suitable for the CEC analysis of steryl ferulates.

The percent composition of each oil sample was obtained by normalizing composition data processed by a HP ChemStation software interfaced with the CEC instrument. Percent areas were calculated by correcting to migration time as computed automatically by the computer ChemStation software. Using standards, calibration curves for each compound could be constructed for quantitation purposes.

3. Results and discussion

Since pK_a of hydroxyl groups of the investigated sterols (Fig. 1) is greater than 32, all plant sterol solutes were considered neutral species being separated by partitioning with no electrophoresis. On the other hand, oryzanol analytes (Fig. 2) were presumably separated by partitioning and electrophoresis due to weakly acidic phenolic hydroxyl groups (pK_a = 20). Throughout CEC experiments, an optimized temperature (25 °C) was used even though high temperature would have speeded analysis and possibly improved peak shape.

Structural modification of the 3-hydroxy group of sterols by derivatization to various steryl esters affects not only adsorption of sterol analytes on silica-based stationary phases but also their molecular polarity. It was logical to include steryl acetates, benzoates, and ferulates for studying structural factors affecting the CEC behavior of the title compounds (Figs. 1 and 2). Of the steryl esters evaluated, ferulates are the most important naturally occurring bioactive substances also known as oryzanols (Fig. 2). The sterol compounds are closely related homologues, isomers and epimers differing merely in side chain structures or in the number and position of double bonds. Their chromatographic behavior in either normal phase or reversed phase systems would be expected to be similar.

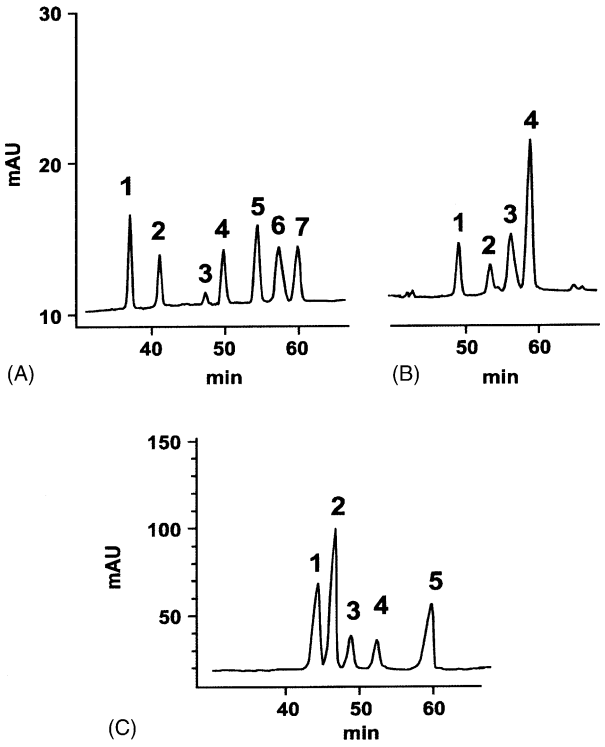

Fig. 3. CEC separations of (A) sterol standards, (B) purified canola oil sample spiked with stigmasterol, and (C) steryl benzoates on octadecylsilica. Conditions: Tris buffer, 25 mM/pH 8; temperature, 25 °C; voltage, 20 kV pressure, 10 bar; injection, 10 kV/2 s. Mobile phases, acetonitrile–tetrahydrofuran–25 mM Tris buffer in water (60:35:5). Detection, UV, (A) 210 nm, (B) 210 nm, (C) 230 nm. Current, 7.5 μÅ. Peaks in (A): (1) lanosterol, (2) ergosterol, (3) dihydrolanosterol, (4) brassicasterol, (5) stigmasterol, (6) campesterol, (7) sitosterol. Peaks in (B): (1) brassicasterol, (2) stigmasterol, (3) campesterol, (4) sitosterol. Peaks in (C): benzoates of (1) brassicasterol, (2) cholesterol, (3) stigmaterol, (4) campesterol, (5) sitosterol.

While no results were obtained with a silica column, CEC with bonded silica phases led to variable degrees of separations of free sterols and ester derivatives. In a typical CEC experiment with a C_{18} column in an aqueous mobile phase system, analytes with a longer alkyl- or alkenyl chain having a higher total number of carbon atoms eluted later from the hydrocarbonaceous column (Fig. 3A). Thus, campesterol (with saturated side chain) and brassicasterol (with unsaturated side chain) emerged from the column before their respective homologues sitosterol and stigmasterol. On the other hand, compounds with a higher number of double bond eluted earlier from the column. For example, ergosterol with three double bonds had a shorter retention time than brassicasterol which contains two double bonds. An anomaly existed in the case of lanosterol (containing two double bonds) which eluted before ergosterol despite the latter has three double bonds. The seemingly unusual elution order suggested that the influence of the 4,4-dimethyl group on separation processes was apparently far less significant than the total number of carbon atoms and the position of double bond at side chains. The presence of the terminal isobutenyl group in

lanosterol contributed to its diminished hydrophobicity in relation to ergosterol. The observed retention order lanosterol < ergosterol < dihydrolanosterol < brassicasterol < stigmasterol < campesterol < sitosterol (Fig. 3A) was in consistence with reversed-phase solvophobic partition principles. The baseline separation of stigmasterol from campesterol was noteworthy as the pair has not been resolved by HPLC at an ambient temperature.

Under the same conditions as above, CEC of a purified canola oil sample fortified with stigmasterol gave well-separated sterol components which were identified in the order of elution as brassicasterol (13.3%), stigmasterol (6.90%), campesterol (25.9%), and sitosterol (53.9%) (Fig. 3B). Careful examination of structures of campesterol and stigmasterol aided in understanding why the pair has been indistinguishable by HPLC. The coexistence of an extra methylene and a double bond at the side chain of stigmaterol might counterbalance differences among polarity, hydrophobicity, and volatility of the compound. By virtue of its higher resolution efficiency in comparison to HPLC, CEC differentiation of campesterol and stigmasterol resulted in elution of the latter preceding the former in accord with analyte hydrophobicity. The less hydrophobic stigmasterol was presumably due to the polar 22–23 double bond disregarding the 24-ethyl group. Interestingly, in GC where molecular volatility and polarity play important roles in separation processes, campesterol elutes ahead of stigmasterol in reversal of the elution sequence observed in CEC (Fig. 3A and B).

For the purpose of eliminating undesirable solute adsorption on a stationary phase due to the hydroxy group in a sterol molecule, derivatization techniques have been commonly adopted by chromatographers for the GC analysis of sterols. Often cholesterol benzoate has been used as reference compound for HPLC assays. CEC of a sample of selected sterol benzoates on C_{18} yielded a elution pattern (Fig. 3C) similar to the corresponding free sterols (Fig. 3A). As expected, cholesterol benzoate eluted prior to its 24-methyl analogue, campesterol. The elution sequence for the brassicaterol–cholesterol pair was analogous to that for the campesterol–stigmasterol pair explainable in terms of the same rationale discussed earlier for the latter pair. Benzoylation was often advantageous as demonstrated in the case of the separation of benzoates of cholesterol and stigmasterol at retention times of 46.6 min and 48.7 min, respectively (Fig. 3C). The corresponding free sterols were only partially resolved with a α-value of 1.02 (Table 1) had a reversed elution sequence: stigmasterol → cholesterol. The retention data in Table 1 clearly indicate that, in an aqueous mobile phase system (B), all three free sterols (cholesterol, campesterol, and sitosterol) containing saturated side chains eluted as a group from a C_{18} column after those with unsaturated side chains.

Throughout the study, selection of sterol compounds for CEC evaluation was far from systematic and much limited by their availability. CEC of several sterol acetates on C_{18} resulted in a partially separation of some of the components (Fig. 4A). Similar to the situation in benzoates, cholesterol aceteate eluted before stigmasterol acetate sequentially followed by campesterol acetate and sitosterol acetate. Acetylation of sterols appeared to have an adverse effect on the C_{18} separation of the last pair. Although the pair of acetates was not as well resolved as the benzoates, the free sterols of the pair cannot be separated by HPLC as mentioned

Table 1
CEC of free sterols on various stationary phases

Free sterol	Capacity factor (k')		Free sterol	Capacity factor (k')	
	C_{18}			C_{30}-(I), A	C_{30}-(II), A
	A	B			
Lanosterol	1.20	2.88	Lanosterol	2.06	1.47
α	1.60	1.15	α	1.11	1.09
Ergosterol	1.92	3.30	Ergosterol	2.29	1.60
α	1.18	1.27	α	1.04	1.06
Brassicasterol	2.27	4.18	Brassicasterol	2.39	1.69
α	1.07	1.11	α	1.05	1.03
Stigmasterol	2.42	4.64	Campesterol	2.50	1.74
α	1.08	1.02	α	1.00	1.00
Cholesterol	[a]	4.73	Sitosterol	2.50	1.74
α	1.03	1.04	α	1.06	1.14
Campesterol	2.61	4.94	Stigmasterol	2.66	1.99
α	1.00	1.05	α	1.00	1.00
Sitosterol	2.61	5.20	Cholesterol	2.66	1.99
α	1.03				
Cholesterol	2.69	[a]			

A, Acetonitrile–tetrahydrofuran–25 mM Tris in [methanol–tetrahydrofuran (1:1)] (60:35:5) and B, acetonitrile–tetrahydrofuran–25 mM Tris in water (60:35:5). C_{30}-(I) and C_{30}-(II) were obtained from different manufacturers (see Section 2). α: separation factor.

[a] Elution of cholesterol was dependent of the mobile phase (A or B) used.

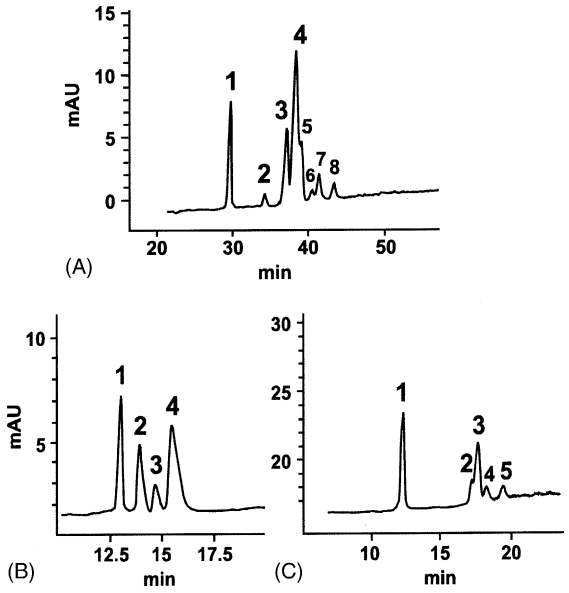

Fig. 4. CEC separations of (A) sterol acetates on octadecylsilica and (B) free sterols, and (C) steryl acetates on triacontylsilica [C_{30}-(I)]. Mobile phases: (A) same as in Fig. 3; (B and C) acetonitrile–tetrahydrofuran–25 mM Tris buffer in [methanol–tetrahydrofuran (1:1)] (60:35:5). Detection, UV, (A–C) 210 nm. Current, 4.0 μÅ. Peaks in (A): acetates of (1) desmosterol, (2) fucosterol, (3) cholesterol, (4) 7-campestenol + stigmasterol, (5) campesterol, (6) epi-7-campestenol, (7) epi-campesterol, (8) sitosterol. Peaks in (B): (1) lanosterol, (2) ergosterol, (3) sitosterol + campesterol, (4) cholesterol + stigmasterol. Peaks in (C): acetates of (1) desmosterol + fucosterol, (2) campesterol + sitosterol, (3) cholesterol + 7-campestenol, (4) epi-campesterol + stigmasterol, (5) epi-7-campestenol.

earlier. Depending on the commercial sources, some standard samples of campesterol acetate and 7-campestenol acetate contain 24-epimers (discussed later) which appeared on the chromatogram as minor peaks 6 and 7 shown in Fig. 4A. In general, under the same CEC conditions, separations of certain steryl acetates with the C_{18} phase seemed to be inferior to those of the corresponding free sterols or steryl benzoates (Fig. 4A versus Fig. 3A and C).

In view of previous successful CEC separations of vitamin E-active antioxidants with a C_{30} phase [24], CEC experiments were conducted with this phase to evaluate the elution behavior of selected mixtures of sterols and steryl esters under conditions similar to those described in preceding paragraphs except for the use of only organic solvents in the mobile phase. With C_{30} as the stationary phase, it was imperative to employ a non-aqueous mobile phase in order to avoid problems associated with solute precipitation and appearance of ill-defined broad CEC peaks with unreasonably long retention times. CEC of a six-component sterol mixture on C_{30}-(I) produced four adequately separated peaks two of which contained co-eluting components as shown in Fig. 4B. Under the conditions specified in the figure, this column showed no selectivity for individual components of pairs of campesterol–sitosterol and cholesterol–stigmasterol both with an α-value of 1.00 (Table 1). It was apparent that the observed elution order lanosterol → ergosterol → sitosterol → cholesterol was independent on the stationary phase (C_{30} or C_{18}) employed as long as the non-aqueous mobile phase was used in the CEC experiment (Fig. 4B and Table 1, mobile phase A). However, stigmaterol was more strongly retained by C_{30}-(I) than sitosterol, in contrast to the elution sequence stigmasterol → sitosterol observed in CEC with C_{18} (Fig. 3 and Table 1). Generally, the elution of sterols on C_{30} was rather erratic and difficult to predict based on hydrophobic interactions between analyte solutes and a stationary phase.

Analogous to the results obtained with C_{18}, CEC of steryl acetates on C_{30} showed no significant improvement over the parent free sterols in component resolution. CEC of a nine-component mixture produced five barely resolved peaks four of which had two unresolved components (Fig. 4C). The last peak of the chromatogram was assigned as the epimer of 7-campestenol. Similar to the elution characteristics of the underivatized compounds, the more hydrophobic sitosterol acetate emerged from the C_{30}-(I) column earlier than stigmasterol (Fig. 4C). It was noteworthy that the separation of the acetates of campesterol–stigmasterol pair with the C_{30}-(I) phase was notably better than the C_{18} phase (Fig. 4C versus Fig. 4A). Nonetheless, C_{30} failed to separate desmosterol acetate from fucosterol acetate ($\alpha = 1.00$, Table 2) despite the excellent separations ($\alpha = 1.15$–1.24, Table 2) obtained with the C_{18} phase in aqueous or non-aqueous mobile phase. The inability of C_{30} to recognize the shape difference [31] between the two olefinic side chain structures could be accounted for the co-elution of the pair from the column.

In nature, 24-epimers coexist with plant sterols as minor constituents. Low-grade commercial samples of both campesterol acetate and 7-campestenol acetate contained their 24-epimers as impurities. HPLC of the epimeric pairs of free sterols requires subambient temperature to effect separations [32]. GC separations of trimethylsilyl ether derivatives of sterol epimers have been reported previously [33]. The first CEC separations of 24-epimers of campesterol acetate and 7-campestenol acetate on C_{18} or C_{30}-(I) at ambient temperature are shown in Fig. 5. The C_{18} column having longer packed column length was more efficient than C_{30}-(I). The observed sharper peaks in Fig. 5C and 5D [obtained with C_3-(I)] in comparison to the corresponding Fig. 5A and B (obtained with C_{18}) were attributed to the use of respective non-aqueous- and aqueous mobile phases in the two different column systems. Also, the migration times of the analyte components on C_{30}-(I) (shorter column length) were significantly shorter under the non-aqueous mobile phase conditions than those on C_{18} (longer column length) with the aqueous mobile phase. Each epimeric pair was efficiently resolved with either stationary phase to give epimeric components. Thus, a commercial sample of 7-campestenol acetate (24*R*/α) had about 10% of its epimer, 7-ergostenol acetate (24*S*/β), as impurity (Fig. 5A and C), whereas a sample of campesterol acetate (24*R*/α) contained about 35% of its epimer, dihydrobrassicasterol acetate (24*S*/β) (Fig. 5B and D). Inspection of the elution sequence indicated that the

Table 2
CEC of sterol acetates on various stationary phases

Sterol acetate	Capacity factor (k')		Sterol acetate	Capacity factor (k')	
	C_{18}			C_{30}-(I), A	C_{30}-(II), A
	A	B			
Desmosterol	0.52	1.99	Desmosterol	2.09	1.91
α	1.15	1.24	α	1.00	1.00
Fucosterol	0.60	2.46	Fucosterol	2.09	1.91
α	1.22	1.12	α	1.62	1.70
Cholesterol	0.73	2.75	Campesterol	3.38	3.24
α	1.07	1.04	α	1.00	1.00
Stigmasterol	0.78	2.86	Sitosterol	3.38	3.24
α	1.00	1.00	α	1.04	1.04
7-Campestenol	0.78	2.86	7-Campestenol	3.50	3.37
α	1.00	1.03	α	1.00	1.00
Campesterol	0.78	2.95	Cholesterol	3.50	3.37
α	1.00	1.05	α	1.04	1.06
Epi-7-campestenol	0.78	3.10	Stigmasterol	3.65	3.59
α	1.00	1.03	α	1.00	1.00
Epi-campesterol	0.78	3.19	Epi-campesterol	3.65	3.59
α	1.00	1.07	α	1.32	1.32
Sitosterol	0.78	3.42	Epi-7-campestenol	4.81	4.73

A, Acetonitrile–tetrahydrofuran–25 mM Tris in [methanol–tetrahydrofuran (1:1)] (60:35:5) and B, acetonitrile–tetrahydrofuran–25 mM Tris in water (60:35:5). C_{30}-(I) and C_{30}-(II) were obtained from different manufacturers (see Section 2). α: separation factor.

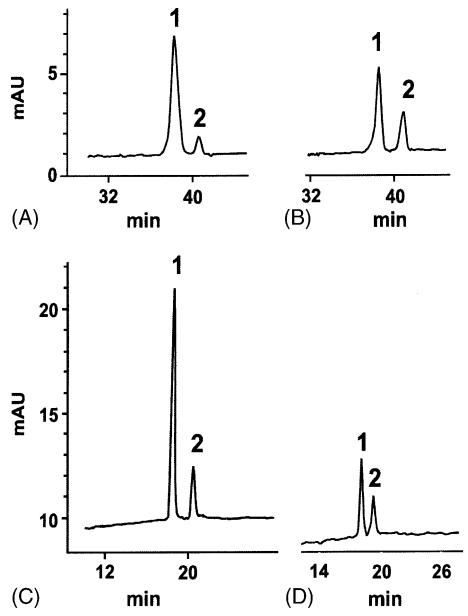

Fig. 5. CEC separations of epimers of campesteryl acetate and 7-campestenyl acetate on (A and B) octadecylsilica and (C and D) triacontylsilica [C_{30}-(I)]. Mobile phases: (A and B) same as in Fig. 3; (C and D) same as in Fig. 4. Detection, UV, (A–D) 210 nm. Current, 4.3 μÅ. Peaks in (A): acetates of (1) 7-campestenol and (2) epi-7-campestenol. Peaks in (B): acetates of (1) campesterol and (2) epi-campesterol. Peaks in (C): acetates of (1) 7-campestenol and (2) epi-7-campestenol. Peaks in (D): acetates of (1) campesterol and (2) epi-campesterol.

minor components with 24*S*/β configuration were more hydrophobic or less polar than their 24*R*/α-epimers in agreement with HPLC and GC results [32,33].

Since steryl ferulates (oryzanols) are important ester derivatives of phytosterols present as bioactive minor constituents in rice bran oil, they were included in this study to evaluate the effect of feruloylation on CEC separations (Table 4) of sterol compounds. In consideration of the improved C_{18} separation of cholesterol benzoate from stigmasterol benzoate ($\alpha = 1.06$, Table 3) in comparison to the corresponding free sterols ($\alpha = 1.02$, Table 1), steryl ferulates, which contain an aryl group (Fig. 2), would be suitable natural compounds for CEC evaluation. Several oryzanol samples isolated from various rice bran oils were analyzed by CEC with the C_{30}-(I) column to give chromatograms of nearly identical major component peaks (Fig. 6A) but with variable compositions (Table 5). It must be pointed out that as the C_{30} packed columns (17 cm) were shorter than the C_{18} packed columns (32 cm), field strength (V/cm) was higher in C_{30} than in C_{18}. Elution patterns obtained with the C_{30}-(I) phase and the C_{18} phase (Fig. 6B) were distinctly different. With the exception of its olefinic analogues classified collectively as 4,4-dimethylsterols (Fig. 2, structures B and C), cycloartanol ferulate ($k' = 3.66$, Table 4) eluted from the C_{30}-(I) column before 4,4-desmethylsterol ferulates (Fig. 2, structures D–F), the k' values of which ranged 4.07–4.68 (Table 4). However, owing to its highly hydrophobic nature, cycloartanol was strongly retained by the C_{18} phase and eluted after all the major unsaturated streol ferulates found in the

Table 3
CEC of sterol benzoates on various stationary phases

Sterol benzoate	Capacity factor (k')		Sterol benzoate	Capacity factor (k')	
	C_{18}			C_{30}-(I), A	C_{30}-(II), A
	A	B			
Brassicasterol	0.94	3.26	Brassicasterol	8.75	5.42
α	1.14	1.07	α	1.04	1.04
Cholesterol	1.07	3.48	Campesterol	9.07	5.65
α	1.05	1.06	α	1.06	1.03
Stigmasterol	1.12	3.68	Sitosterol	9.58	5.82
α	1.03	1.10	α	1.04	1.07
Campesterol	1.15	4.03	Cholesterol	9.94	6.20
α	1.03	1.17	α	1.04	1.06
Sitosterol	1.19	4.73	Stigmasterol	10.3	6.57

A, Acetonitrile–tetrahydrofuran–25 mM Tris in [methanol–tetrahydrofuran (1:1)] (60:35:5) and B, acetonitrile–tetrahydrofuran–25 mM Tris in water (60:35:5). C_{30}-(I) and C_{30}-(II) were obtained from different manufacturers (see Section 2). α: separation factor.

sample (Fig. 6B). Under conditions employed, the separation of epi-campesterol ferulate as a singly component on C_{30} was unique because it co-eluted with sitosterol ferulate on C_{18}. In addition, the facile separation of the campesterol ferulate–sitosterol ferulate pair on C_{30} signified the beneficial effect of the feruloylation on component resolution in light of the observed inseparability of the corresponding free sterols ($\alpha = 1.00$, Table 1) and acetates ($\alpha = 1.00$, Table 2) on this phase.

Tables 1–4 summarize effects of mobile phases and stationary phases on capacity factors (k') and separation factors (α). Regardless of the column used, cholesterol had the highest k' value among the compounds evaluated in a non-aqueous mobile phase (A) but eluted with an intermediate k' value in an aqueous mobile phase (B) (Table 1). No analogy of the abnormal retention behavior of cholesterol was found in CEC of its steryl esters (Tables 2–4). With the C_{18} phase, addition of water to a mobile phase generally resulted in an increase in k' values and a decrease in the number of co-eluting sterol species with $\alpha = 1.00$ (Tables 1–3). Such an effect was conspicuous in the case of steryl acetates (Table 2). With mobile phase (B), the best separations of free sterols and steryl benzoates on C_{18} had α values ranging 1.02–1.27 (Table 1) and 1.06–1.17 (Table 3), respectively. On the other hand, the best separation of steryl ferulates on C_{30}-(I) had α values ranging 1.04–1.38 (Table 4). With the exclusion of steryl benzoates and ferulates where the sterol components were either well resolved or partially separated, the number of unresolved pairs ($\alpha = 1.00$) on C_{30} was higher than C_{18} (Tables 1 and 2).

Although C_{30}-(I) and C_{30}-(II) were obtained from different commercial sources, the column specifications were similar and their CEC results were comparable with the former

Table 4
CEC of sterol ferulates in commercial oryzanol samples on various stationary phases

Oryzanol component	Capacity factor (k')[a]	Oryzanol component	Capacity factor (k')[a]	
	C_{18}, B		C_{30}-(I), A	C_{30}-(II), A
Cycloartenol	1.85	Cycloartenol	2.38	2.15
α	1.06	α	1.11	1.11
24-Methylenecycloartanol	1.96	24-Methylenecycloartanol	2.65	2.39
α	1.08	α	1.38	1.50
Campesterol	2.12	Cycloartanol	3.66	3.58
α	1.04	α	1.11	1.09
Epi-campesterol	2.20	Campesterol	4.07	3.90
α	1.00	α	1.04	1.05
Sitosterol	2.20	Epi-campesterol	4.24	4.10
α	1.09	α	1.10	1.07
Cycloartanol	2.40	Sitosterol	4.68	4.38
α	1.04			
Sitostanol	2.50			

A, Acetonitrile–tetrahydrofuran–25 mM Tris in [methanol–tetrahydrofuran (1:1)] (60:35:5) and B, acetonitrile–tetrahydrofuran–25 mM Tris in water (60:35:5). C_{30}-(I) and C_{30}-(II) were obtained from different manufacturers (see Section 2). α: separation factor.

[a] Samples analyzed with C_{18} and C_{30} were from different sources.

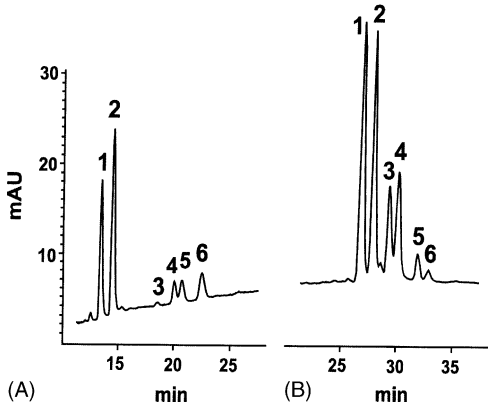

Fig. 6. CEC separations of a purified sample of γ-oryzanol (steryl ferulates) in rice bran oil. Columns (A) triacontylsilica [C_{30}-(I)]; (B) octadecylsilica. Mobile phases: (A) same as in Fig. 4 and (B) same as in Fig. 3. Detection, UV, 330 nm. Current (A) 5.1 μÅ, (B) 8.0 μÅ. Peaks in (A): ferulates of (1) cycloartenol, (2) 24-methylenecycloartanol, (3) cycloartanol, (4) campesterol, (5) epi-campesterol, (6) sitosterol. Peaks in (B): ferulates of (1) cycloartenol, (2) 24-methylenecycloartanol, (3) campesterol, (4) epi-campesterol + sitosterol, (5) cycloartanol, (6) sitostanol.

somewhat more retentive (higher k' values of analyte components) than the latter owing to the higher percent carbon loading (24% versus 19.4%) of C_{30}-(I). While these bonded phases C_{30}-(I) and C_{30}-(II) were normally prepared by reaction of triacontyltrichlorosilane with silica followed by polymerization in the presence of water as initiator, the exact proprietary structures and polymeric bonding chemistry have remained undisclosed to column users. In light of the close similarity in CEC separations of the title compounds on these phases including their current profiles, differences in structures and polymeric bonding chemistry of C_{30}-(I) and C_{30}-(II) would be expected to be small. At the onset of this study, our experimental design was to use a monomeric and a polymeric C_{30} phases for comparative CEC study. C_{30}-(I) was initially thought to be monomeric bonding (based on supplier's information) until its CEC results showed close similarity to those of the polymeric C_{30}-(II) phase. At the final stage of this study, the manufacturer of C_{30}-(I) confirmed the column bonding to be polymeric.

To demonstrated the analytical applicability of the CEC technique, compositions of major sterol compounds in soybean oils, canola oils and rice bran oils were determined (Table 5). Before CEC quantification, test oils were subjected to cleanup procedures for obtaining sufficiently purified samples. CEC chromatograms of the aliquot samples showed adequate separations of analyte peaks with few interferences from sample matrices. Two examples are given in Figs. 3B and 6A. Analyte components were identified by peak matching with standards. Linear calibration lines were obtained by plotting peak areas against concentrations (1.0–25 μg/μl) standards. Abscissa and slope values from linear regression analysis varied with sterol structures. Linear regression coefficients (R^2) averaged 0.9985 maintaining fairly constant values.

Table 5
Composition of sterol compounds in selected vegetable oils

Component	Composition (%)			
	CEC			Other method[a]
	Sample no.			Sample no. i
	i	ii	iii	
Soybean oil (free sterol)				
Brassicasterol	nd	nd	nd	nd
Stigmasterol	19.5	21.5	25.2	20.0
Campesterol	27.2	22.0	22.2	26.9
Sitosterol	53.3	56.5	52.6	53.1
Canola oil (free sterol)				
Brassicasterol	10.8	14.3	13.3	10.3
Stigmasterol	nd	nd	nd	nd
Campesterol	26.9	30.0	28.9	27.1
Sitosterol	62.3	55.8	57.7	62.6
Rice bran oil (steryl ferulate)				
Cycloartenol	34.2	28.9	37.9	33.9
24-Methylene cycloartanol	44.0	42.4	31.8	44.0
Cycloartanol	1.22	1.51	9.90	1.30
Campesterol	4.92	8.15	3.78	11.8
Epi-campesterol	6.71	7.14	7.58	[b]
Sitosterol	8.95	11.9	9.04	9.00

Composition data are normalized values. Soybean and canola oils were analyzed by CEC with a C_{18} column in mobile phase B: acetonitrile–tetrahydrofuran–25 mM Tris in water (60:35:5). nd: none detected. Rice bran oils were analyzed by CEC with C_{30}-(I) column in mobile phase A: acetonitrile–tetrahydrofuran–25 mM Tris in [methanol–tetrahydrofuran (1:1)] (60:35:5).

[a] Soybean and canola oils were analyzed by GC; rice bran oil was analyzed by HPLC.

[b] Epi-campesterol and campesterol were not resolved by HPLC.

In conclusion, this is the first comprehensive report on CEC separations of selected sterols and steryl esters occurring ubiquitously in oilseed plants. C_{18}-CEC with an aqueous mobile phase led to elution order predictable in terms of analyte hydrophobicity at sterol side chains. C_{30}-CEC in non-aqueous mobile phase system provides no conclusive results. Hence, elution patterns of the title compounds depend largely on the stationary phases (C_{18} and C_{30}) employed. CEC with either phase facilitates separations of not only pairs of 24-epimers but also the campesterol–stigmasterol pair which has been very difficult to resolve by HPLC. Analysis of phytosterols in vegetable oils can be carried out by CEC preferably with a C_{18} column in an aqueous mobile phase, whereas oryzanols in rice bran oils can best be analyzed by CEC with a C_{30} column in a non-aqueous mobile phase.

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