Separation of steryl esters by reversed-phase liquid chromatography

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Abstract Thirty steryl esters composed of ten different fatty acids and 14 sterols as well as two wax esters were analyzed by reversed-phase liquid chromatography on a Zorbax ODS column at 205 nm using acetonitrile–isopropanol 60:40 (v/v) as a mobile phase. Contributions (σ values) of individual double bonds and alkyl groups in the steryl ester were determined from the relative retention values for steryl esters with and without the feature. A double bond and an alkyl group had a greater effect on the retention time of a steryl ester when present in the fatty acid rather than in the sterol moiety. The σ value can be used to obtain structural information about a steryl ester. Separation of a complex mixture of steryl esters found in corn oil was achieved using this technique.—**Billheimer, J. T., S. Avart, and B. Milani.** Separation of steryl esters by reversed-phase liquid chromatography. *J. Lipid Res.* 1983. **24:** 1646–1651.

Supplementary key words corn oil • wax esters • 24 alkyl sterols • desmosterol • lathosterol

Sterols are required by most eucaryotic organisms as an integral part of their cellular membranes. A diversity of sterols has been found in varying organisms and even within the same organism several sterols may predominate (1). Recently reverse-phase liquid chromatography (RPLC) was used to augment gas–liquid chromatography as a means to separate free sterols and to predict the structure of unknown sterols (2–4).

Besides free sterols, long chain fatty acid esters of sterols are also present in cells where they are thought to be a storage form for sterols. The structure of steryl esters is very complex since both the sterol and the fatty acid moiety are variable. The steryl ester fraction is typically studied following separation of the free sterols by silicic acid chromatography. Usually the steryl ester fraction is saponified and the individual components (sterols and fatty acids of the steryl esters) are analyzed. However, in addition to being time consuming, this procedure assumes that the individual sterols are equally likely to be esterified to the various fatty acids; also the fatty acid composition of the steryl esters may be in error due to various wax esters, which comigrate with the steryl esters upon silicic acid chromatography. Recently several reversed-phase liquid chromatography systems have been devised to separate cholesteryl esters according to their fatty acid moiety (5–8). These systems have primarily analyzed serum in which cholesterol is essentially the only sterol present. The purpose of this investigation is to study the RPLC separation of steryl esters differing in both sterol and fatty acid moiety and to determine the efficacy of this technique for the separation of complex steryl ester mixtures that are known to exist in plants and microorganisms.

MATERIALS AND METHODS

Oleic acid, oleoyl chloride, stearyl chloride, oleyl stearate, oleyl arachidate, and the cholesteryl esters of stearate, oleate, linoleate, linolenate, palmitate, and palmitoleate were obtained from Sigma Chemical Co. Ergosterol was purchased from ICN; desmosterol, sitosterol, campesterol, and cholestanol from Applied Science; lanosterol (dihydrolanosterol), lathosterol, 3-epicholesterol from Steraloids; and 7-dehydrocholesterol, 22-dehydrocholesterol, and brassicasterol from Research Plus. Fucosterol was a gift from Dr. W. R. Nes. Corn oil was a gift from Best Foods and purified pancreatic cholesterol ester hydrolase was the generous gift of Dr. Howard Brockman (Hormel Institute). Steryl stearates were synthesized according to the procedure of Pinter, Hamilton, and Muldrey (9). Unsaturated fatty acyl esters of the various sterols were synthesized either using acyl chloride according to the procedure of Teng, McGehee, and Smith (10) or enzymatically using pancreatic cholesterol ester hydrolase. In the latter case, 2 μmol of sterol and 4 μmol of fatty acid were dried on the bottom of a conical tube. Petroleum ether (8 μl) and a solution (0.45 ml) containing sodium taurocholate (8.9 mM) in 0.05 M phosphate, pH 6.0, were added. After sonication, 0.5 units of cholesterol ester hydrolase was added and the mixture was incubated

Abbreviations: SE, steryl esters; RPLC, reversed-phase liquid chromatography.

for 48 hr at 30°C. The reaction was terminated by the addition of 10 μl of 1% trichloroacetic acid and the lipids were extracted with 20 ml of chloroform–methanol 2:1 (v/v). The newly synthesized steryl esters, as well as the steryl ester fraction of corn oil, were isolated by column chromatography using acid-washed, heat-activated Silacar (Mallinkrodt) equilibrated in 1% diethyl ether in petroleum ether and eluted with the same solvent.

RPLC was carried out on a Perkin Elmer Series 3-B Liquid Chromatograph using an LC-75 detector set at 205 nm. A 25 cm × 4.6-nm Zorbax ODS (C-18) column was used with a mobile phase composed of acetonitrile–isopropanol 60:40 (v/v) and a flow-rate of 2 ml/min at 60°C. Samples of steryl esters (0.5–1.0 mg/ml) were prepared in isopropanol. Using these conditions cholesteryl oleate had an average retention time of 12.8 min and a capacity factor [K′(4)] of 9.8. For comparative purposes and to minimize daily variances, the retention volumes of sample steryl esters are expressed relative to the retention volume of cholesteryl oleate (α_{co}) where α_{co} is equal to the K′ of sample steryl ester divided by the K′ of cholesteryl oleate. Cholesteryl oleate was either added as an internal standard or chromatographed immediately before the steryl ester of interest. Chromatography of all esters was carried out a minimum of three times and the α_{co} of individual compounds had a standard deviation of less than 5%.

RESULTS

The relative retentions (α_{co}) of the various steryl esters (SE) and wax esters employed in this study are shown in **Table 1.** The fastest eluting SE (cholesteryl linolenate: 16 ml, 8 min) was well separated from cholesterol, oleic acid, and triolein, which have retention volumes less than 10 ml (data not shown). The two wax esters studied (oleyl stearate and oleyl arachidate) had α_{co}'s similar to that of cholesteryl linolenate.

Reducing the percentage of isopropanol in the mobile phase from 40 to 30% resulted in a doubling of the retention times. This is probably due to the fact that iso-

TABLE 1. Relative retention (α_{co}) of esters on a Zorbax ODS column

Ester	α_{co}	Structure	
		Sterol Moiety[a]	Fatty Acid[b]
Oleyl stearate	0.52		C_{18}
Cholesteryl linolenate	0.58	Δ^5-3β-ol	$C_{18},\Delta^{9,12,15}$
Oleyl arachidate	0.66		C_{20},Δ^9
Cholesteryl palmitoleate	0.76	Δ^5-3β-ol	C_{16}
Cholesteryl linoleate	0.77	Δ^5-3β-ol	$C_{18},\Delta^{9,12}$
Ergosteryl oleate	0.79	$\Delta^{5,7,22}$-24β-methyl-3β-ol	C_{18},Δ^9
Desmosteryl oleate	0.81	$\Delta^{5,24}$-3β-ol	C_{18},Δ^9
7-Dehydrocholesteryl oleate	0.82	$\Delta^{5,7}$-3β-ol	C_{18},Δ^9
Stigmasteryl linoleate	0.83	$\Delta^{5,22}$-24α-ethyl-3β-ol	$C_{18},\Delta^{9,12}$
22-Dehydrocholesteryl oleate	0.84	$\Delta^{5,22}$-3β-ol	C_{18},Δ^9
Campesteryl linoleate	0.85	Δ^5-24α-methyl-3β-ol	$C_{18},\Delta^{9,12}$
Cholesteryl myristate	0.85	Δ^5-3β-ol	C_{14}
Sitosteryl linoleate	0.91	$\Delta^{5,22}$-24α-ethyl-3β-ol	$C_{18},\Delta^{9,12}$
Brassicasteryl oleate	0.95	$\Delta^{5,22}$-24β-methyl-3β-ol	C_{18},Δ^9
Fucosteryl oleate	0.95	$\Delta^{5,24(28)}$-24-ethylidene-3β-ol	C_{18},Δ^9
Lanosteryl oleate	0.95	$\Delta^{8,24}$-4,4,14-trimethyl-3β-ol	C_{18},Δ^9
Lathosteryl oleate	0.99	Δ^7-3β-ol	C_{18},Δ^9
Cholesteryl oleate	1.00	Δ^5-3β-ol	C_{18},Δ^9
Cholesteryl elaidate	1.03	Δ^5-3β-ol	C_{18},Δ^9 Trans
Stigmasteryl oleate	1.04	$\Delta^{5,22}$-24α-ethyl-3β-ol	C_{18},Δ^9
Desmosteryl stearate	1.04	$\Delta^{5,24}$-3β-ol	C_{18}
Cholesteryl palmitate	1.05	Δ^5-3β-ol	C_{16}
Campesteryl oleate	1.08	Δ^5-24α-methyl-3β-ol	C_{18},Δ^9
3-Epicholesteryl stearate	1.17	Δ^5-3α-ol	C_{18}
Cholestanyl oleate	1.17	3β-ol	C_{18},Δ^9
Sitosteryl oleate	1.18	$\Delta^{5,22}$-24-ethyl-3β-ol	C_{18},Δ^9
Cholesteryl heptadecanoate	1.19	Δ^5-3β-ol	C_{17}
Lanosteryl stearate	1.24	$\Delta^{8,24}$4,4,14-trimethyl-3β-ol	C_{18}
Cholesteryl stearate	1.30	Δ^5-3β-ol	C_{18}
Lathosteryl stearate	1.30	Δ^7-3β-ol	C_{18}
Campesteryl stearate	1.41	Δ^5-24α-methyl-3β-ol	C_{18}
Sitosteryl stearate	1.56	Δ^5-24α-ethyl-3β-ol	C_{18}
Cholesteryl arachidate	1.68	Δ^5-3β-ol	C_{20}

[a] Based on changes to 5α-cholestane.
[b] All double bonds are *cis* unless otherwise stated.

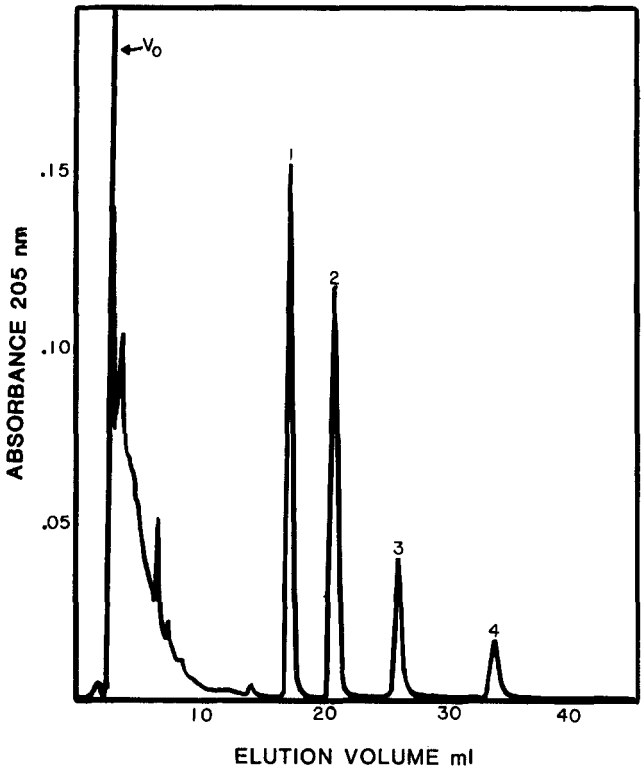

Fig. 1. Separation of cholesteryl esters by RPLC. Elution profile of cholesteryl esters (10 μg each) on a Zorbax ODS column. Peak identification, V_0, void volume, (1) cholesteryl linolenate, (2) cholesteryl linoleate, (3) cholesteryl oleate, (4) cholesteryl stearate.

propanol but not acetonitrile is capable of hydrogen bonding with the SE. Increasing the percentage of isopropanol was avoided because of its high viscosity.

The presence of increased unsaturation in the fatty acid moiety decreased the retention time of the SE (**Fig. 1**). The contribution (σ) of an individual feature to the mobility of a compound can be determined by the following equation (4).

$$\sigma = \frac{\alpha_{co} \text{ SE with feature}}{\alpha_{co} \text{ SE without feature}}$$

As is illustrated in **Table 2,** a value of $\sigma = 0.77$ is obtained for the presence of a *cis* double bond in the fatty acid portion of the SE. This value does not appear to be affected (in the SE studied) by the presence of additional unsaturation in the fatty acid, position of the double bond, or the sterol present. Cholesteryl elaidate which contains a *trans* double bond has a slightly higher value, $\sigma = 0.79$.

An increase in the length of the alkyl chain of the fatty acid increases the relative retention of the SE, $\sigma = 1.13$ (**Table 3**).

The effect on retention time of unsaturation and alkyl groups in the sterol moiety is shown in Table 3 and **Table 4** and **Fig. 2.** The σ values for double bonds in the sterol vary much more (0.81–0.91) than observed for those in the fatty acid; as shown by the higher σ value, double bonds decrease the retention time to a lesser extent than unsaturation in the fatty acid ($\sigma = 0.77$). The closer the σ value is to unity, the less effect a feature has on retention time. This allows one to separate two SE that have the same degree of unsaturation but differ in the occurrence of the double bond in the sterol or fatty acid moiety [e.g., 22-dehydrocholesteryl oleate (α_{co} 0.84) and cholesteryl linoleate ($\alpha_{co} = 0.77$)]. A Δ^{24} double bond has the greatest effect on the elution of the SE, possibly due to its greater ability to interact with the solvent than a nuclear double bond. Similarly, the effect of a Δ^{22} double bond is a special case and is reduced by alkyl substituents at C-24 which may sterically hinder solvent-SE interaction (Table 4).

The addition of alkyl groups to the sterol lengthens

TABLE 2. Contribution of unsaturation in the C_{18} fatty acid series on the retention time of steryl esters

Double Bond	Steryl Ester		σ Value
	With Double Bond	Without Double Bond	
Δ^{9}*cis*	Campesteryl oleate	Campesteryl stearate	0.77
	Cholesteryl oleate	Cholesteryl stearate	0.77
	Desmosteryl oleate	Demosteryl stearate	0.78
	Lanosteryl oleate	Lanosteryl stearate	0.77
	Lathosteryl oleate	Lathosteryl stearate	0.76
	Sitosteryl oleate	Sitosteryl stearate	0.76
Δ^{9}*trans*	Cholesteryl elaidate	Cholesteryl stearate	0.79
Δ^{12}*cis*	Campesteryl linoleate	Campesteryl oleate	0.79
	Cholesteryl linoleate	Cholesteryl oleate	0.77
	Sitosteryl linoleate	Sitosteryl oleate	0.77
	Stigmasteryl linoleate	Stigmasteryl oleate	0.79
Δ^{15}*cis*	Cholesteryl linolenate	Cholesteryl linoleate	0.75

TABLE 3. Contribution of additional alkyl groups in fatty acid or sterol on the retention time of steryl esters

Steryl Esters Compared	σ Value/CH_2[a]
Cholesteryl myristate and palmitate	1.11
Cholesteryl palmitate and stearate	1.11
Cholesteryl palmitoleate and oleate	1.14
Cholesteryl heptadecanate and palmitate	1.13
Cholesteryl aracidate and stearate	1.14
Cholesteryl and campesteryl oleate	1.08
Campesteryl and sitosteryl oleate	1.08
Cholesteryl and campesteryl stearate	1.08
Campesteryl and sitosteryl stearate	1.10
Desmosteryl and lanosteryl oleate	1.06
22-Dehydrocholesteryl and stigmasteryl oleate	1.11
22-Dehydrocholesteryl and brassicasteryl oleate	1.13

[a] When the compounds differed by more than one methyl group, each methyl group was considered to be of equivalent contribution.

the retention time of the SE and the σ value is similar for substitution at C-4 or C-24 (Table 3). An exception is observed when a Δ^{22} double bond is present, then as seen above, C-24 alkyl groups also increase the α_{co} by steric hindrance. As observed with the presence of unsaturation, additional methyl groups in the sterol have less of an effect than similar substitution in the fatty acid (average σ = 1.07 and 1.13, respectively).

The α_{co} of a known SE can be used along with the σ values to determine the α_{co} of a particular SE for which one does not have a standard. For example the α_{co} of ergosteryl oleate can be determined by multiplying the α_{co} of cholesteryl stearate (1.30) by the average σ values for the Δ^7 bond (0.83), the Δ^{22} bond with alkyl group at C-24 (0.89), 24-methyl (1.08), and the Δ^9 bond of the fatty acid (0.77). The calculated α_{co} for ergosteryl oleate (0.80) is in close agreement with that obtained from an ergosteryl oleate standard (0.79).

The RPLC system was used to analyze the SE fraction isolated from corn oil by silicic acid chromatography (**Fig. 3**). The major sterol of corn oil is sitosterol with lesser amounts of campesterol and stigmasterol also being present (11). Corn oil is composed primarily of the unsaturated fatty acids, linoleic acid and oleic acid (12). The three major sterols of corn, sitosterol, campesterol, and stigmasterol, were all found to be esterified; the linoleate esters were predominant with lesser quantities of the oleate esters.

DISCUSSION

RPLC has been shown to be a useful tool in the separation and identification of various cholesteryl esters and its application has been extended in this study to the separation and identification of SE differing in either the sterol or fatty acid moiety. Of special usefulness is the fact that changes in the structure of the fatty acid have a more pronounced effect on the retention time of a SE than similar changes in the sterol moiety; this allows for the separation of various molecular weight isomers (e.g., cholesteryl oleate and desmosteryl stearate). While not capable of separating all possible SE (in practice a difference between two SE in α_{co} of about 0.04 is necessary for two individual peaks), RPLC can be augmented by other analytical methods to identify individual components. For example, cholesteryl palmitoleate and cholesteryl linoleate which have similar α_{co}'s can be distinguished by analysis of the fatty acid constituents after saponification. Similarly, RPLC of SE is not meant to replace but to compliment existing analytical techniques.

The use of RPLC of SE allows an investigator to do the following. *1*) Study the distribution of radiolabeled

TABLE 4. Contribution of unsaturation in the sterol on retention time of steryl esters

Double Bond	Steryl Ester		σ Value
	With Double Bond	Without Double Bond	
Δ^5	Cholesteryl oleate	Cholestanyl oleate	0.85
Δ^7	Lathosteryl oleate	Cholestanyl oleate	0.85
	7-Dehydrocholesteryl oleate	Cholesteryl oleate	0.82
	Ergosteryl oleate	Brassicasteryl oleate	0.83
Δ^{22}	22-Dehydrocholesteryl oleate	Cholesteryl oleate	0.84
Δ^{22} plus alkyl group at C-24	Stigmasteryl oleate	Sitosteryl oleate[a]	0.88
	Stigmasteryl linoleate	Sitosteryl linoleate[a]	0.91
	Brassicasteryl oleate	Campesteryl oleate[a,b]	0.88
$\Delta^{24(25)}$	Desmosteryl oleate	Cholesteryl oleate	0.81
$\Delta^{24(28)}$	Fucosteryl oleate	Sitosteryl oleate	0.81

[a] Interaction of alkylation at C-24 with Δ^{22} makes Δ^{22} less polar.
[b] Stereochemistry at C-24 was ignored.

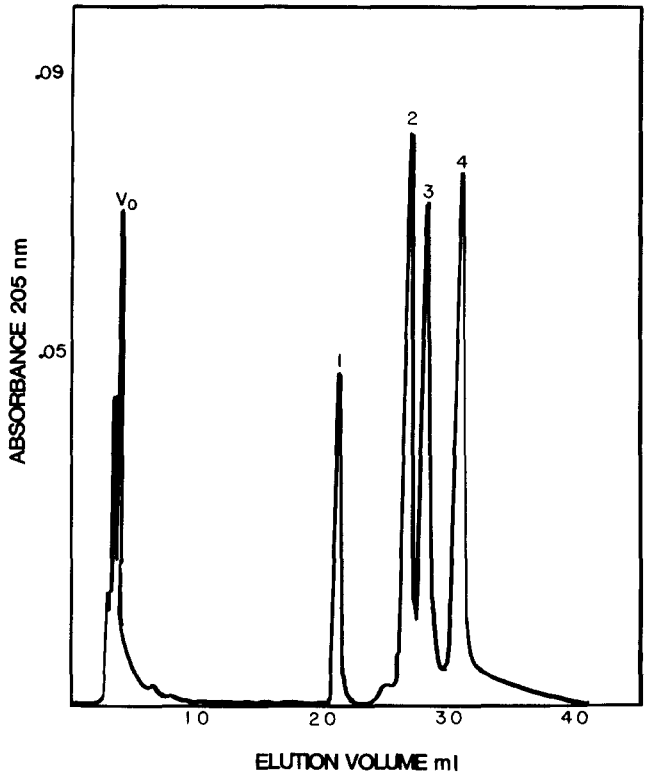

Fig. 2. Separation of steryl oleates by RPLC. Elution profile of steryl oleates on a Zorbax ODS column. Peak identification, V_0, void volume, (1) ergosteryl oleate, (2) cholesteryl oleate, (3) stigmasteryl oleate, (4) sitosteryl oleate.

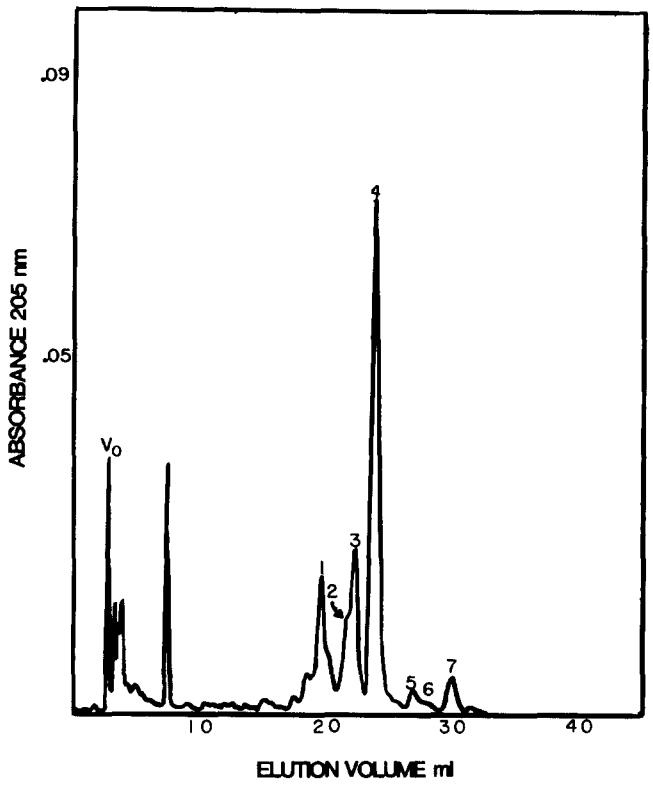

Fig. 3. Separation of the steryl esters of corn oil by RPLC. A total of 20 μg of steryl ester fraction was chromatographed on a Zorbax ODS column using the conditions described in Materials and Methods. Peak identification, V_0, void volume, (1) unknown (α_{co} = 0.73), (2) stigmasteryl linoleate (α_{co} = 0.82), (3) campesterol linoleate (α_{co} = 0.84), (4) sitosteryl linoleate (α_{co} = 0.90), (5) stigmasteryl oleate (α_{co} = 1.04), (6) campesteryl oleate (α_{co} = 1.08), (7) sitosteryl oleate (α_{co} = 1.16).

precursors in various SE. This method was used to distinguish the esterification of desmosterol from that of cholesterol by acyl CoA:cholesterol acyl transferase using labeled oleoyl CoA (13). *2*) Identify an unknown SE by comparison of its α_{co} with that of a standard SE. *3*) Make certain judgements on the structure of an unknown SE from only μg amounts from its α_{co} and the contribution (σ values) of various groups to the retention time. *4*) Analyze SE obtained from natural sources intact, i.e., prior to saponification. This may allow one to identify contamination by other lipid components such as wax esters (assuming the presence of a chromophor or use of a refractive index detector). It also would allow one to verify whether individual sterols are equally likely to be esterified to various fatty acids. *5*) Quantitate the amount of SE present. Because of the difference in absorptivity of the various SE at 205 nm (Fig. 1) which is dependent primarily on the number of double bonds present in the fatty acid, this requires a standard curve for each SE (5). In addition this RPLC system should be useful in analysis of wax esters.

The authors would like to thank Ms. Janet Coogan for her technical assistance. This work was supported in part by a grant from the American Heart Association (Southeastern Pennsylvania Chapter) #1009.

Manuscript received 17 June 1983.

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