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# STRATEGY FOR THE ANALYSIS OF STERYL ESTERS FROM PLANT AND ANIMAL TISSUES

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## SUMMARY

Methods are described for the analysis of intact steryl esters present in complex mixtures isolated from plant or animal tissues. A preliminary examination by analytical thin-layer chromatography (TLC) and capillary column gas chromatography–mass spectrometry (GC–MS) under electron impact (EI) ionisation reveals the complexity of the mixture and the nature of the steryl moieties. Preparative TLC is then utilised to separate the steryl esters into two broad groups, containing fatty acyl moieties of shorter ($C_2$–$C_8$) or longer chain length ($C_{10}$–$C_{22}$). The shorter-chain fatty acyl steryl esters are separated by adsorption high-performance liquid chromatography (HPLC) on a LiChrosorb Silica-60 column. The steryl esters with longer-chain fatty acyl moieties are analysed by reversed-phase HPLC on either an Ultrasphere ODS, 5-μm, or a S3 Spherisorb ODS, 3-μm, column. Steryl esters with unsaturated fatty acyl moieties are eluted with the shorter-chain fatty acyl steryl esters. The presence of the unsaturated fatty acyl esters can be monitored by analytical argentation TLC, which will also reveal the degree of unsaturation. The steryl esters are fractionated into the saturated, mono-, di-, tri- and polyene acyl types by preparative medium-pressure liquid chromatography on a column of 10% $AgNO_3$-silica gel. Each of these steryl ester types can then be resubmitted to reversed-phase HPLC or analysed by GC–MS on a short fused-silica capillary column with a bonded phase of the OV-1 type. GC–MS on a magnetic-sector instrument under negative-ion chemical ionisation conditions with ammonia as the reagent gas produces fragment ions for both the steryl and fatty acyl moieties, thus permitting identification of the individual intact steryl esters. These various methods are illustrated by analyses of the steryl ester mixtures obtained from human plasma, barley seedlings, palm oil and rape seed oil.

## INTRODUCTION

Steryl esters occur widely in nature. They have been reported in fungi, algae, plants, invertebrates and vertebrate animals[1]. In mammals, cholesteryl esters are major constituents of plasma low-density lipoproteins and they are an important transport form of cholesterol. The cholesteryl esters in some tissues are in a dynamic state[2] and play an integral role in cholesterol metabolism. An accumulation of cho-

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lesteryl esters is characteristic of some pathological conditions, such as atherosclerosis[3]. The biochemistry and functions of steryl esters in lower organisms and in plants are not well understood, although they may play a sterol storage role in some situations[4].

Complex mixtures of steryl esters can result from the conjugation of various steryl and fatty acyl moieties. The ability to identify and quantify the individual components of a steryl ester mixture could be a valuable asset for investigating such phenomena as steryl ester turnover, metabolism and function in various tissues. The steryl esters, although abundant in some particular situations, are more usually relatively minor constituents of the total lipid extracts of many plant and animal tissues. Hence, chromatographic purification procedures are a necessary first step in the characterisation procedure. The relatively low volatility of steryl esters has, until recently, tended to preclude the analysis of intact steryl esters by gas chromatography (GC). The most routine approach[3,5,6] to steryl ester identification has been saponification and subsequent separation and GC analysis of the released sterols and the fatty acids, the latter usually after conversion to the corresponding fatty acid methyl esters. The identification and proportions of the original steryl esters in the mixture are then inferred from the sterol and fatty acid analyses. However, this procedure can possibly result in misleading conclusions if the steryl esters have not been rigorously purified to remove other saponifiable fatty acyl ester impurities, such as wax esters and triglycerides. These compounds have similar chromatographic properties to steryl esters and they often occur in considerably larger amounts than the steryl esters in some tissues. Furthermore, when multicomponent mixtures of both sterols and fatty acids are obtained from a steryl ester mixture after saponification, there is no means of deducing which sterol was bound to which acid, and potentially valuable information is lost. To overcome these problems, attention has now turned to the development of methods for the unambiguous analysis of intact steryl esters by high-performance liquid chromatography (HPLC) and GC–mass spectrometry (GC–MS) techniques.

HPLC methods have been developed for the study of both plant and mammalian steryl esters. An early report was that of Duncan *et al.*[7] who employed reversed-phase HPLC to separate serum cholesteryl esters. Good separations of mixtures of cholesteryl esters were reported by Carroll and Rudel[6], who used three HPLC columns, connected in series, and a linear gradient elution program. Resolution was according to both carbon number and the degree of unsaturation in the fatty acyl moiety.

Reversed-phase HPLC has also been extended to the analysis of plant steryl esters by Billheimer *et al.*[8]. A wide selection of steryl esters were submitted to HPLC analysis, and the applicability of the technique was demonstrated by an analysis of corn oil steryl esters. Also, HPLC was demonstrated to be particularly useful for the analysis of radiolabelled steryl esters used in metabolic studies[9].

A problem with HPLC is the lack of resolution of different steryl esters[6,8]. This makes it difficult to assign unambiguous identifications of components in complex mixtures. This is particularly the case with steryl esters of plant origin, which may contain several different sterols, in contrast to the mammalian steryl esters, where cholesterol may be the major or only sterol component. Another major disadvantage of HPLC for the analysis of steryl esters lies in the relatively poor detection limits. The lack of a strong chromophore means that 10–50 $\mu$g of each component is generally required for analysis[6].

An alternative to HPLC which overcomes some of these difficulties is the analysis of steryl esters by GC and GC–MS. GC analysis on short, packed columns has been described[10], but recent reports have described the application of capillary columns coated with cross-bonded apolar and polar stationary phases[11,12]. The most notable merits of capillary column GC are superior resolution and sensitivity allowing detection of low nanogram amounts of steryl esters. A major advantage of capillary GC is that it can be readily interfaced to a mass spectrometer to provide further compelling evidence for individual compound identification[13–16]. The electron impact (EI) mass spectra of steryl esters do not display significant molecular ions or fatty acyl fragment ions[14,17,18]. However, chemical ionisation (CI) has been employed to obtain mass spectra displaying a molecular ion and/or fragment ions corresponding to the steryl and fatty acyl moieties[14–17,19,20]. Two reports describe the use of a quadrupole mass analyser, operating in the positive ion mode with methane[13] or ammonia[14] as reagent gas. In recent work, we have employed a double-focussing magnetic-sector GC–MS instrument, operating in the negative-ion scanning mode and using ammonia as the CI reagent gas to analyse steryl esters and to obtain compositional information on intact plasma cholesteryl esters[15,16].

In GC analysis of cholesteryl esters on apolar phases, which are the most suitable for GC–MS, the separations are largely by carbon number. However, resolution of steryl esters of the same carbon number but possessing differing degrees of unsaturation in the fatty acid moiety are less satisfactory. Consequently with multicomponent steryl ester mixtures there are often problems of unresolved peaks. Although careful examination of GC–MS data will usually permit some identifications, we have found the need for recourse to additional analytical techniques to obtain full compositional information on a complex mixture. We now describe protocols for the analysis of a steryl ester mixture which employ argentation chromatography, HPLC, GC and GC–MS.

## EXPERIMENTAL

### *Extraction of biological materials*

Human plasma (1–2 ml) was obtained by centrifugation of whole blood. This was extracted three times with 5 ml chloroform–methanol (2:1)[21] with vigorous mixing, followed by centrifugation to separate the aqueous and organic layers. The organic layers were removed by Pasteur pipette, combined, washed with water and dried over sodium sulphate. The solvent was evaporated to leave the total lipid extract.

Plant tissue was initially refluxed with methanol for 1 h. After decanting the methanol, the tissue was macerated and extracted twice by reflux (1 h) with chloroform–methanol (2:1). All solvent extracts were combined and evaporated to a small volume. Water (20 ml) was added, and the lipids were extracted into light petroleum (b.p. 40–60°C; three times 50 ml). The light petroleum extracts were combined, washed and dried over sodium sulphate, and the solvent was evaporated *in vacuo* to yield the total lipid. Alternatively, plant material can be extracted initially with acetone[22].

Palm oil and rape seed oil were obtained from a commercial source.

*Isolation of the steryl ester fraction from total lipid*

Steryl esters were isolated from large amounts of total lipid (100 mg to 2 g) by column chromatography on alumina (Brockmann, Grade III). For optimum resolution the column loading should be up to 10–20 mg lipid/g of alumina[22]. The column was eluted with petroleum ether to remove hydrocarbons, followed by light petroleum–diethyl ether (98:2) to give a fraction containing the steryl esters.

The steryl ester fraction, or small amounts of total lipid (up to 20 mg) were subjected to preparative TLC [silica gel; cyclohexane–diethyl ether (98:2); visualisation under UV after spraying with 0.005% berberine in ethanol][22]. The steryl esters gave an $R_F$ of *ca.* 0.5.

Steryl esters were also purified on straight-phase Sep-Pak cartridges (Waters Assoc., Harrow, U.K.) according to the method of Wang and Peter[23]. Total lipid (1–20 mg) was introduced onto the Sep-Pak cartridge as a solution in a minimum volume of light petroleum (b.p. 40–60°C). Elution with light petroleum–diethyl ether (98.5:1.5) gave the steryl esters.

The purity of the steryl ester fractions obtained by various methods was assessed by analytical TLC on 10 × 5 cm silica gel plates (Art. 5719, Merck, Darmstadt, F.R.G.), developed with cyclohexane–diethyl ether (98:2). Visualisation was by spraying with 20% sulphuric acid, followed by charing at 100°C. The steryl esters gave spots with a red colouration, $R_F$ *ca.* 0.5.

*HPLC*

Adsorption HPLC was employed to separate steryl esters bearing short-chain ($C_2$–$C_8$) fatty acyl moieties. A 25 cm × 4.6 mm LiChrosorb Silica 60 (10-μm) column was eluted, with hexane–methyl *tert.*-butyl ether (99.75:0.25) at a flow-rate of 2 ml/min. Solvent was delivered by a Kontron LC414-T pump (Kontron Analytical, St. Albans, U.K.). Detection was at 206 nm with an LKB 2238 Uvicord SII fixed-wavelength detector (LKB, Bromma, Sweden). The sample was introduced with a Rheodyne (Cotati, CA, U.S.A.) 7125 20-μl loop injector.

Reversed-phase HPLC was used to separate steryl esters bearing long chain ($C_{10}$–$C_{22}$) fatty acyl moieties. Two methods were used: (a) 25 cm × 4.6 mm Ultrasphere ODS 5-μm (Beckman) column, isocratic elution (2 ml/min) with acetonitrile–tetrahydrofuran–water (65:35:2); (b) 15 cm × 4.6 mm S3 ODS Spherisorb (3-μm) (Phase Separations, Queensferry, U.K.), isocratic elution (1 ml/min) with acetonitrile–tetrahydrofuran (65:35), containing up to 2% water. Solvent delivery was by a Beckman 110B pump and detection was at 206 nm as described above.

*Argentation chromatography*

Preparative TLC employed 2% $AgNO_3$-silica gel plates (0.5 mm), developed with hexane–toluene (70:30). Up to 10 mg of steryl ester mixture can be applied to a plate. After development, bands were detected by spraying with 0.005% berberine in ethanol and viewing under UV light; steryl esters with long chain ($C_8$–$C_{22}$) saturated fatty acyl groups had $R_F$ 0.75–0.80, monoenoic acyl groups had $R_F$ 0.65, dienoic acyl groups had $R_F$ 0.55, trienoic acyl group $R_F$ 0.35 and tetraenoic acyl group $R_F$ 0.20. Saturated, short chain ($C_2$–$C_6$) fatty acyl esters gave $R_F$ values in the range 0.45–0.7, depending upon chain length. Steryl esters were recovered by scraping off the bands and eluting them with diethyl ether.

For analytical TLC, 10 × 5 cm precoated silica gel plates (Merck) were immersed briefly in a 2% methanol solution of silver nitrate, then reactivated by heating (120°C, 2 min). The plates were developed with hexane–toluene (70:30), and spots were located by 20% sulphuric acid spray and heating (100°C).

Medium-pressure liquid chromatography on $AgNO_3$-silica gel was used to fractionate steryl ester mixtures. A 15 cm × 12 mm glass column (Omni, Anachem., Luton, U.K.) was dry-packed with *ca.* 4 g of 10% silver nitrate, dispersed on Kieselgel 60 (Merck) (230–400 mesh). The column was equilibrated by recycling with dichloromethane (2 ml/min for 2–4 h), and then 20 μl formic acid was injected into the column, followed after 5 min by a further 20-μl of formic acid (Dr. E. Hammond, personal communication). Once conditioning was completed, the column was used immediately. Steryl esters (10 mg) in dichloromethane were introduced via a Rheodyne 7125 injector. Solvent delivery was by an Altex 110A pump at 2 ml/min (Beckman Instruments, Berkeley, U.S.A.). Stepwise elution was performed with 15 ml dichloromethane, followed by 30 ml dichloromethane–methyl *tert.*-butyl ether (90:10) and finally with 15 ml dichloromethane–methyl *tert.*-butyl ether (50:50). Fractions (2 ml) were collected and monitored by analytical $AgNO_3$-silica gel TLC. Steryl esters bearing saturated fatty acyl groups were eluted in fractions 1–7, monoenoic esters in fractions 8–14, dienoic esters in fractions 15–18, trienoic esters in fractions 19–24, and tetraenoic esters in fractions 25–30.

### *GC*

GC was performed on a Becker 407 gas chromatograph, fitted with a SGE OCI-II on-column injector (S.G.E., Milton Keynes, U.K.). The column was either an 8 m × 0.22 mm flexible fused-silica BP-1 (0.25-μm film) capillary column or a 12 m × 0.22 mm BP-1 (0.1-μm film) (S.G.E.). Argon was the carrier gas; samples were injected at 50°C, followed by programming of the GC oven temperature from 50 to 330°C at 8 or 12°C/min.

### *GC–MS*

GC–MS analyses were performed on a Pye Unicam 204 GC instrument, interfaced to a VG 70-70H double-focussing magnetic-sector mass spectrometer (V.G. Analytical, Manchester, U.K.). Data acquisition and processing were on a Finnigan INCOS 2300 data system. The GC–MS interface was modified to allow heating to 350°C, as described elsewhere[15,16]. The GC columns and operating conditions were as described above, except that helium was used as the carrier gas. The mass spectrometer was operated in both positive EI and negative ion CI modes. Ammonia was used for the CI work, as described previously[15,16].

## RESULTS AND DISCUSSION

After preliminary purification of the steryl ester fraction from a total lipid by alumina column and/or preparative TLC, we have found it useful to subject the mixture to preliminary GC–MS analysis in the positive-ion EI mode. The GC profile gives an indication of the range of chain lengths of the fatty acyl moieties present in the esters (see Table I for GC retention times). GC–MS will also reveal the presence of other compounds, such as wax esters or triglycerides in the sample[13,18,24]. While

TABLE I

ANALYSIS OF STERYL ESTERS BY CAPILLARY COLUMN GC

The capillary column was a 12 m × 0.22 mm BP-1 (0.1-μm film thickness) temperature programmed from 150 to 350°C at 8°C/min. The standard used for calculation of the relative retention times (RRT) values is cholesteryl laurate.
Values in parentheses indicate the carbon number and number of double bonds in the fatty acyl residue.

| *Steryl ester* | *RRT* |
|---|---|
| Cholesteryl acetate (2:0) | 0.78 |
| Cholesteryl propionate (3:0) | 0.81 |
| Cholesteryl butyrate (4:0) | 0.83 |
| Cholesteryl caproate (6:0) | 0.88 |
| Cholesteryl caprylate (8:0) | 0.92 |
| Cholesteryl decylate (10:0) | 0.96 |
| Cholesteryl laurate (12:0) | 1.00 |
| Cholesteryl myristate (14:0) | 1.04 |
| Cholesteryl palmitate (16:0) | 1.07 |
| Cholesteryl stearate (18:0)* | 1.10 |
| Cholesteryl arachidate (20:0) | 1.13 |
| Cholesteryl oleate (18:1) | 1.10 |
| Cholesteryl linoleate (18:2) | 1.10 |
| Cholesteryl linolenate (18:3) | 1.10 |
| Cholesteryl arachidonate (20:4) | 1.12 |
| Campesteryl palmitate (16:0) | 1.11 |
| Campesteryl stearate (18:0) | 1.15 |
| Stigmasteryl palmitate (16:0) | 1.12 |
| Stigmasteryl stearate (18:0) | 1.14 |
| Sitosteryl palmitate (16:0) | 1.13 |
| Sitosteryl stearate (18:0) | 1.17 |

* Cholesteryl stearate has Kováts' retention index of 5000 (co-elutes with *n*-pentacontane).

operation in the EI mode will not give significant molecular ions or fragment ions characteristic of the fatty acyl moiety, it does provide an ion for the steryl moiety, derived by loss of the fatty acyl group ($[M-RCO_2H]^+$)[14,17,18]. For esters of $\Delta^5$-sterols, the spectrum produced is very similar to that of the corresponding steryl acetate. This permits a tentative identification of the sterols in the mixture by the data system searching against a suitable library of steryl acetate spectra. Mass chromatograms can also be produced to reveal the distribution of particular sterols in the mixture. This is illustrated in Fig. 1, which shows an analysis of palm oil steryl esters by GC–MS. Mass chromatograms are presented for the ions representative of the sitosteryl (*m/z* 396), stigmasteryl (*m/z* 394) and campesteryl (*m/z* 382) moieties. These show that all three sterols occur in two esterified forms. The ones of shorter retention time are the palmitates, while those of longer retention time are the stearates.

Analytical TLC on small silica gel plates will quickly provide some information on chain length of the fatty acyl moieties in the steryl ester mixture. Steryl esters with a $C_2$–$C_8$ fatty acyl unit will separate from those with longer-chain fatty acyl moieties (Tables II). The presence and approximate proportions of saturated, monoenoic, dienoic, trienoic and tetraenoic fatty acyl steryl esters can be rapidly monitored by analytical argentation TLC of a small sample of the material (Table II).

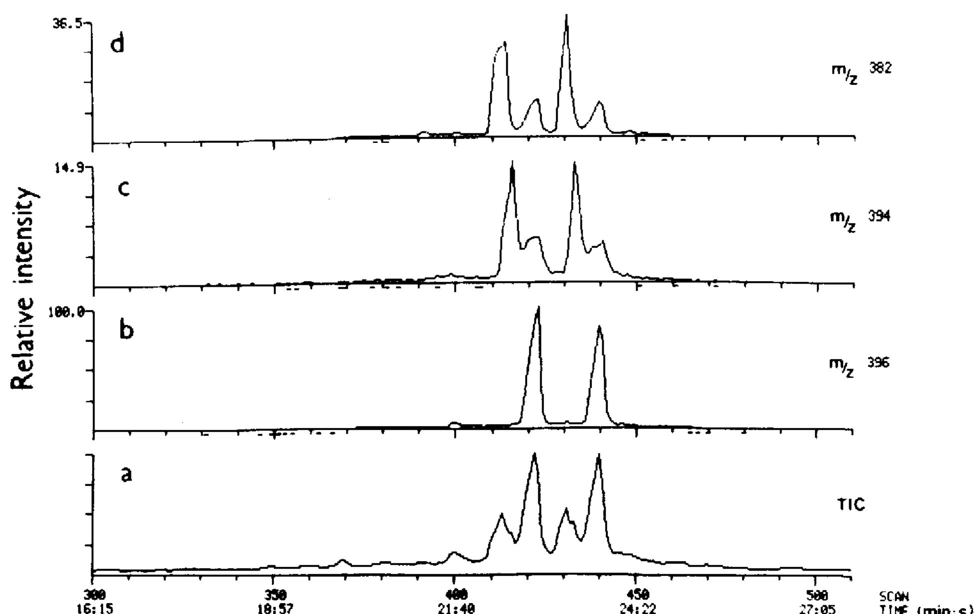


Fig. 1. Analysis of the steryl esters isolated from palm oil by GC–MS operating in the EI mode. (a) Total-ion chromatogram; (b) mass chromatogram for the ion *m/z* 396 ($[M-RCO_2H]^+$), typical of sitosteryl esters; (c) mass chromatogram for the ion *m/z* 394, typical of stigmasteryl esters; (d) mass chromatogram for the ion *m/z* 382, typical of campesteryl esters.

TABLE II

ANALYTICAL TLC OF STERYL ESTERS

Solvents: toluene–hexane (3:2) for silica gel and hexane–toluene (7:3) for $AgNO_3$-silica gel.

| *Steryl ester* | $R_F$ | |
|---|---|---|
| | *Silica gel* | *$AgNO_3$-silica gel* |
| Cholesteryl acetate (2:0) | 0.42 | 0.45 |
| Cholesteryl propionate (3:0) | 0.51 | – |
| Cholesteryl butyrate (4:0) | 0.54 | – |
| Cholesteryl caproate (6:0) | 0.62 | 0.70 |
| Cholesteryl caprylate (8:0) | 0.69 | 0.75 |
| Cholesteryl decylate (10:0) | 0.72 | 0.77 |
| Cholesteryl laurate (12:0) | 0.75 | 0.79 |
| Cholesteryl myristate (14:0) | 0.75 | 0.80 |
| Cholesteryl palmitate (16:0) | 0.76 | 0.80 |
| Cholesteryl stearate (18:0) | 0.77 | 0.80 |
| Cholesteryl oleate (18:1) | 0.75 | 0.65 |
| Cholesteryl linoleate (18:2) | 0.70 | 0.55 |
| Cholesteryl linolenate (18:3) | 0.67 | 0.35 |
| Cholesteryl arachidonate (20:4) | 0.66 | 0.20 |

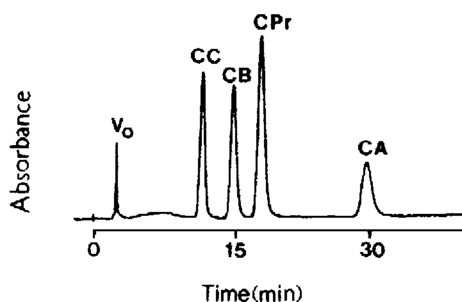


Fig. 2. Separation of short-chain fatty acyl esters of cholesterol by HPLC on a LiChrosorb Silica-60 column. Operating conditions are described in Experimental. Peak identifications: $V_0$ = void volume, CC = cholesteryl caproate, CB = cholesteryl butyrate, CPr = cholesteryl propionate, CA = cholesteryl acetate.

With this preliminary GC and TLC information regarding the composition of the steryl ester mixture, a strategy can be designed for the subsequent more detailed analysis of the steryl ester mixture.

If the presence of steryl esters with short-chain ($C_2$–$C_8$) fatty acyl moieties is indicated by the above procedures, these compounds can be readily separated from the long-chain ($C_{10}$–$C_{20}$) steryl esters by preparative TLC on silica gel (Table II). Application of appropriate steryl ester markers is useful to locate precisely the bands of interest. Elution of a broad band in the $R_F$ range 0.40–0.69 provides a mixture of steryl esters with acyl groups in the $C_2$–$C_8$ range, although caution in subsequent analysis is required, since this material could also include some long-chain polyunsaturated steryl esters, such as the arachidonate (Table II). Elution of the band of higher $R_F$ at 0.70–0.80 will provide the long-chain ($C_{10}$ to $< C_{22}$) steryl esters which tend to be unresolved.

The short-chain steryl esters can be analysed by GC (Table I) or by HPLC on an adsorption column. For the HPLC separation, we employ a Lichrosorb Silica-60 column (Fig. 2, Table III) and elute with hexane–methyl *tert.*-butyl ether (99.75:0.25).

TABLE III

ANALYSIS OF STERYL ESTERS WITH SHORT-CHAIN FATTY ACYL MOIETIES BY HPLC ON A LICHROSORB SILICA-60 COLUMN

The operating conditions are described in Experimental. $\alpha_{CD}$: relative to cholesteryl decylate, retention time 6.25 min.

| *Steryl ester* | *Relative retention* ($\alpha_{CD}$) |
|---|---|
| Cholesteryl decylate (10:0) | 1.00 |
| Cholesteryl caprylate (8:0) | 1.20 |
| Cholesteryl caproate (6:0) | 1.36 |
| Cholesteryl butyrate (4:0) | 1.84 |
| Cholesteryl propionate (3:0) | 2.28 |
| Cholesteryl acetate (2:0) | 4.00 |

TABLE IV

SEPARATION OF CHOLESTERYL ESTERS BY REVERSED-PHASE HPLC

Ultrasphere ODS (5-$\mu$m) column (25 cm × 4.6 mm), eluted with acetonitrile–tetrahydrofuran–water (65:35:2) at 2 ml/min. $\alpha_{CP}$: relative to cholesteryl palmitate, retention time 25 min.

| *Steryl ester* | *Relative retention ($\alpha_{CP}$)* |
|---|---|
| Cholesteryl decylate (10:0) | 0.40 |
| Cholesteryl laurate (12:0) | 0.56 |
| Cholesteryl myristate (14:0) | 0.74 |
| Cholesteryl palmitate (16:0) | 1.00 |
| Cholesteryl stearate (18:0) | 1.32 |
| Cholesteryl oleate (18:1) | 1.04 |
| Cholesteryl linoleate (18:2) | 0.76 |
| Cholesteryl linolenate (18:3) | 0.54 |

Cholesteryl esters with fatty acyl moieties longer than $C_{10}$ are not resolved by this system. It can be used as an alternative to preparative TLC for the recovery of the mixture of longer chain steryl esters from a lipid sample for subsequent analysis by other means.

For the HPLC analysis of the long-chain steryl esters, a reversed-phase system is required, as reported by other workers[6–9]. We have utilised two reversed-phase columns. Table IV presents the results achieved with some cholesteryl esters on an Ultrasphere ODS column. The separations are comparable to those reported by Carroll and Rudel[6] and Billheimer *et al.*[8], who used Zorbax ODS columns. An analysis time of 30–40 min is required under the conditions described to ensure elution of the longer-chain ($C_{18}$–$C_{22}$) steryl esters from either an Ultrasphere (Table IV) or Zorbax[6,8] column. We have achieved a considerably more rapid analysis (10–12 min) using a short S3 Spherisorb ODS (3-$\mu$m) column. Good resolution is obtained (Fig. 3, Table V), and this system has the merit of better economy in the use of solvent.

Although the reversed-phase HPLC systems described here and elsewhere[6,8] give good resolution of cholesteryl ester mixtures containing only saturated fatty acyl moieties, some problems of unresolved peaks arise with the introduction of one or more double bonds into the fatty acyl group. Steryl esters with unsaturated fatty acyl groups are eluted faster than those with the corresponding saturated fatty acyl unit[6,8]. This can result in the unsaturated compound having a retention time very similar to that of a shorter chain ester. For example, the pairs cholesteryl oleate and cholesteryl palmitate, cholesteryl linoleate and cholesteryl myristate and cholesteryl linolenate and cholesteryl laurate are rather difficult to distinguish by reversed-phase HPLC on the Ultrasphere ODS column (Table IV). Some separation of these particular "critical pairs" can be obtained on the Spherisorb ODS column (Table V), but in this case other compounds overlap with them, and when structural changes in the steryl moiety are also introduced (Table V), such as would occur in a plant steryl ester mixture, the problem of unambiguous identification becomes considerably more difficult. In such cases, a separation of the steryl ester mixture into fractions of simpler composition is desirable and will facilitate the later HPLC and GC analyses. When the

TABLE V

SEPARATION OF STERYL ESTERS BY REVERSED-PHASE HPLC

Column: Spherisorb ODS (3-$\mu$m), 15 cm × 4.6 mm; eluent: acetonitrile–tetrahydrofuran–water (65:35:1.5); flow-rate: 1 ml/min. $\alpha_{CP}$: relative to cholesteryl palmitate, retention time 6 min. $C_{27}$, $C_{28}$ and $C_{29}$ indicate the number of carbons in the sterol; cholesterol, campesterol and sitosterol are $\Delta^5$-sterols, stigmasterol is a $\Delta^{5,22}$-sterol; 8:0 etc. indicates the numbers of carbons and double bonds in the fatty acid.

| *Steryl ester* | *Relative retention ($\alpha_{CP}$)* |
|---|---|
| Cholesteryl caprylate ($C_{27}$, 8:0) | 0.45 |
| Cholesteryl linolenate ($C_{27}$, 18:3) | 0.54 |
| Cholesteryl decylate ($C_{27}$, 10:0) | 0.58 |
| Cholesteryl arachidonate ($C_{27}$, 20:4) | 0.59 |
| Cholesteryl laurate ($C_{27}$, 12:0) | 0.67 |
| Cholesteryl palmitoleate ($C_{27}$, 16:1) | 0.67 |
| Cholesteryl linoleate ($C_{27}$, 18:2) | 0.68 |
| Campesteryl linolenate ($C_{28}$, 18:3) | 0.73 |
| Stigmasteryl linolenate ($C_{29}$, 18:3) | 0.73 |
| Campesteryl linoleate ($C_{28}$, 18:2) | 0.75 |
| Sitosteryl linolenate ($C_{29}$, 18:3) | 0.79 |
| Sitosteryl linoleate ($C_{29}$, 18:2) | 0.81 |
| Cholesteryl myristate ($C_{27}$, 14:0) | 0.83 |
| Campesteryl myristate ($C_{28}$, 14:0) | 0.85 |
| Cholesteryl oleate ($C_{27}$, 18:1) | 0.87 |
| Campesteryl oleate ($C_{28}$, 18:1) | 0.88 |
| Stigmasteryl myristate ($C_{29}$, 14:0) | 0.89 |
| Sitosteryl myristate ($C_{29}$, 14:0) | 0.90 |
| Stigmasteryl oleate ($C_{29}$, 18:1) | 0.90 |
| Cholesteryl palmitate ($C_{27}$, 16:0) | 1.00 |
| Sitosteryl oleate ($C_{29}$, 18:1) | 1.00 |
| Campesteryl palmitate ($C_{28}$, 16:0) | 1.04 |
| Stigmasteryl palmitate ($C_{29}$, 16:0) | 1.08 |
| Sitosteryl palmitate ($C_{29}$, 16:0) | 1.10 |
| Cholesteryl stearate ($C_{27}$, 18:0) | 1.21 |
| Campesteryl stearate ($C_{28}$, 18:0) | 1.27 |
| Stigmasteryl stearate ($C_{29}$, 18:0) | 1.32 |
| Sitosteryl stearate ($C_{29}$, 18:0) | 1.36 |
| Cholesteryl arachidate ($C_{27}$, 20:0) | 1.46 |
| Campesteryl arachidate ($C_{28}$, 20:0) | 1.52 |
| Stigmasteryl arachidate ($C_{29}$, 20:0) | 1.58 |
| Sitosteryl arachidate ($C_{29}$, 20:0) | 1.62 |

preliminary investigations reveal the presence of steryl esters with unsaturated fatty acyl moieties, separation by argentation chromatography[3,22,25–27] can be employed to yield fractions containing esters with saturated, monoenoic, dienoic, trienoic and polyenoic fatty acyl constituents. Preparative TLC on $AgNO_3$-silica gel is effective for this purpose, but we have experienced rather poor recoveries of eluted steryl esters. Therefore, we have explored as an alternative the use of medium-pressure liquid chromatography (MPLC) on a column of 10% $AgNO_3$-silica gel. The column was prepared as described in the Experimental part and eluted stepwise with di-

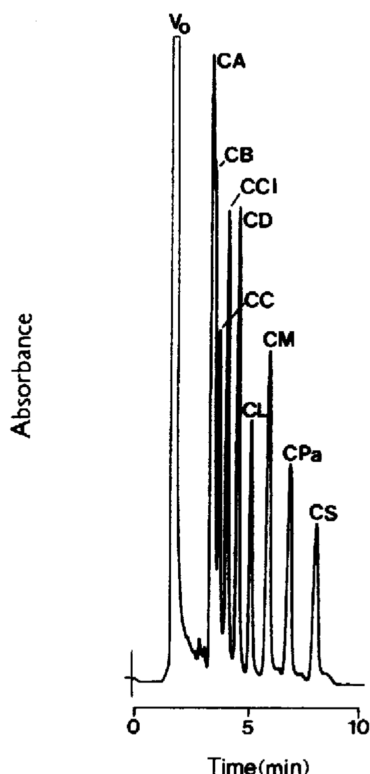


Fig. 3. Separation of cholesteryl esters with saturated fatty acyl moieties by reversed-phase HPLC on a S3 Spherisorb ODS (3-μm) column. Operating conditions are as described in Experimental. Peak identifications: $V_0$ = void volume, CA = cholesteryl acetate, CB = cholesteryl butyrate, CC = cholesteryl caproate, CCl = cholesteryl caprylate, CD = cholesteryl decylate, CL = cholesteryl laurate, CM = cholesteryl myristate, CPa = cholesteryl palmitate, CS = cholesteryl stearate.

chloromethane and dichloromethane–methyl *tert.*-butyl ether mixtures. The eluents were monitored by analytical $AgNO_3$-silica gel TLC. A clean separation of steryl esters with saturated, monoenic, dienoic, trienoic and polyenoic fatty acyl groups was achieved as indicated in Experimental. However, it should be noted that these separations were achieved using cholesteryl esters. With more complex mixtures, the esters of sterols with additional unsaturation in the steryl moiety may be more strongly retarded by the $AgNO_3$-silica gel column and separation will not then result solely on the basis of the degree of unsaturation of the fatty acyl moiety. This problem is currently being investigated in our laboratory using suitable phytosteryl esters with additional double bonds in the side chain. Recoveries of various steryl esters from argentation TLC and MPLC were checked by adding tritium-labelled cholesteryl palmitate, cholesteryl oleate, cholesteryl linoleate and cholesteryl linolenate. With all four types of cholesteryl esters the recoveries were significantly better in MPLC than in preparative TLC (Table VI).

The effectiveness of an argentation chromatography step for the analysis of a cholesteryl ester mixture is illustrated by the results obtained for an examination of

TABLE VI

RECOVERIES OF TRITIUM-LABELLED STERYL ESTERS FROM PREPARATIVE $AgNO_3$-SILICA GEL MPLC AND TLC

TLC and MPLC were performed as described in Experimental. Tritium-labelled cholesteryl esters (20 000–25 000 dpm, Sp. act. 44 Ci/mmole) were separated and the radioactivity recovered in the appropriate fractions was determined by liquid scintillation counting.

| *Compound* | *Recovery (%)* | |
|---|---|---|
| | *TLC* | *HPLC* |
| [1,2-$^3H_2$]Cholesteryl palmitate | 70 | 100 |
| [1,2-$^3H_2$]Cholesteryl oleate | 65 | 94 |
| [1,2-$^3H_2$]Cholesteryl linoleate | 80 | 90 |
| [1,2-$^3H_2$]Cholesteryl linolenate | 75 | 84 |

human plasma cholesteryl esters (Fig. 4). We have described elsewhere[16] the analysis of human plasma cholesteryl esters by GC–MS with negative-ion CI. Reversed-phase HPLC on Spherisorb ODS now also showed the presence of components with reten-

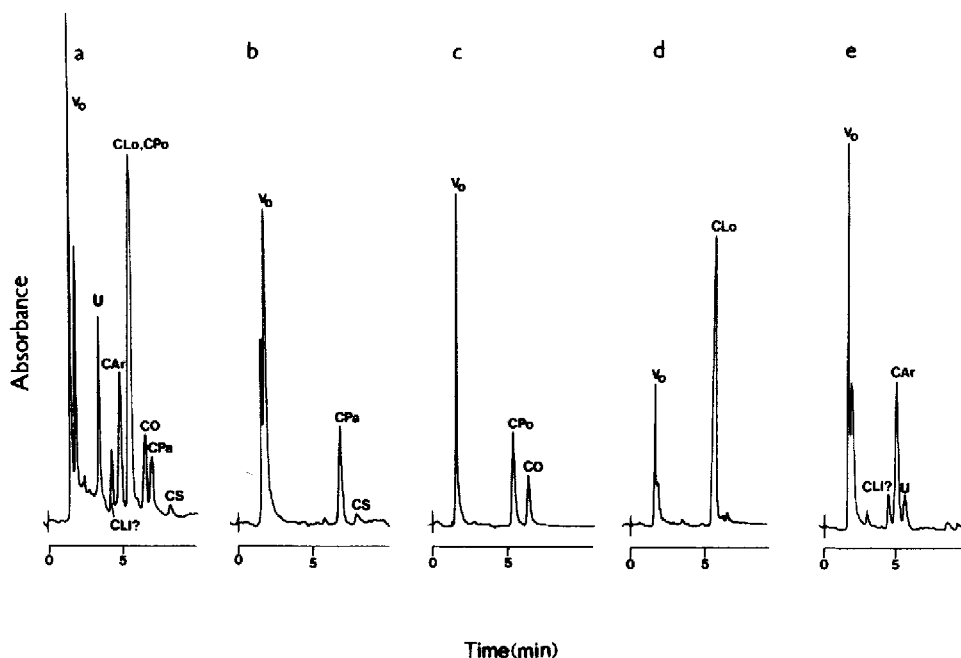


Fig. 4. Analysis of human plasma cholesteryl esters by reversed-phase HPLC on a S3 Spherisorb ODS column. (a) Total cholesteryl esters, (b) saturated fatty acyl esters, (c) monoenoic fatty acyl esters, (d) dienoic fatty acyl esters, and (e) polyenoic fatty acyl esters. The various cholesteryl ester fractions (b–e) were obtained by argentation chromatography of the total cholesteryl ester. Peak identifications: $V_0$ = void volume, U = unidentified, CAr = cholesteryl arachidonate, CPo = cholesteryl palmitoleate, CLo = cholesteryl linoleate, CO = cholesteryl oleate, CPa = cholesteryl palmitate, CS = cholesteryl stearate, CLI? = possibly cholesteryl linolenate.

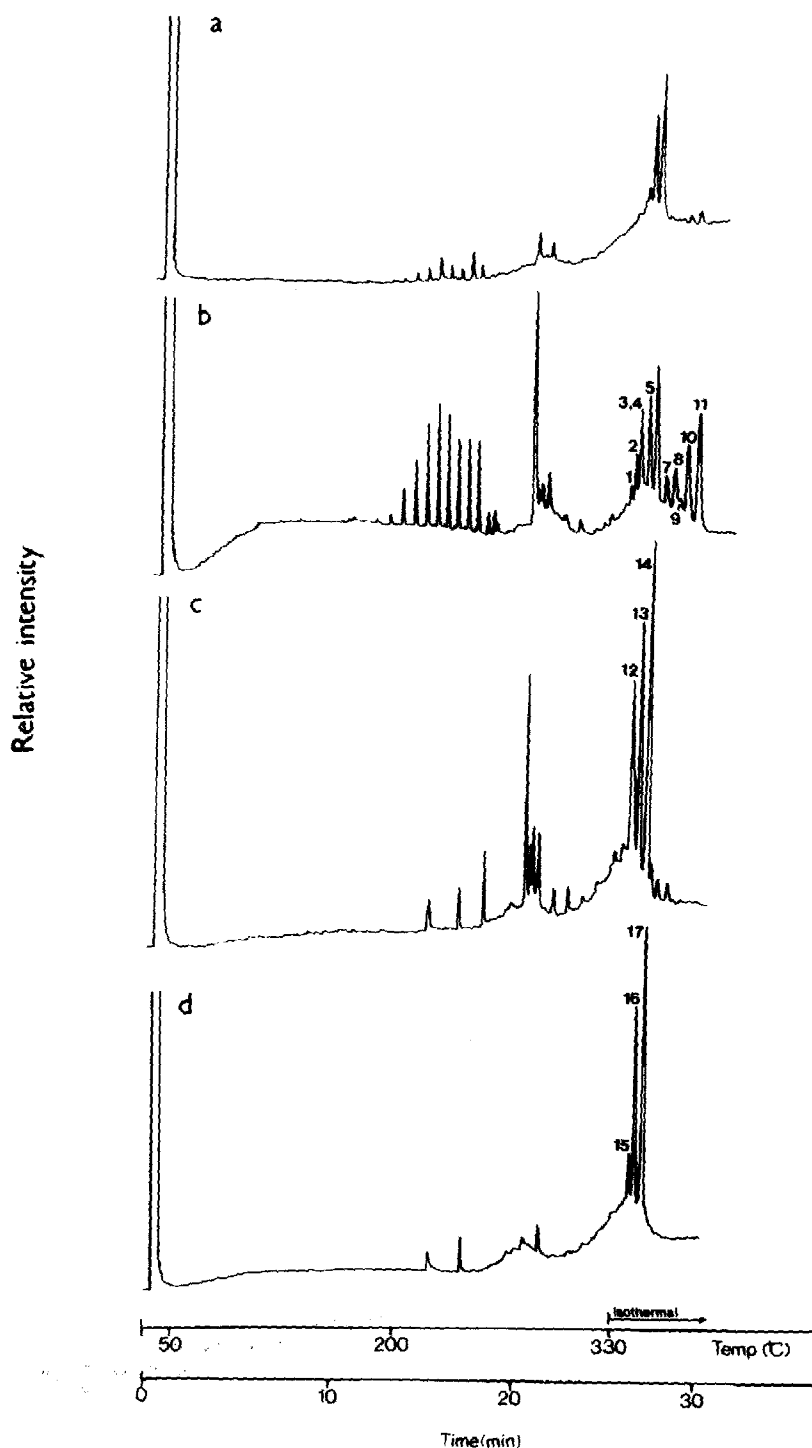


Fig. 5. Analysis of the steryl esters isolated from rape seed oil by GC. The total steryl esters (a) were separated into the saturated (b), monoenoic (c) and dienoic (d) fractions by argentation chromatography prior to GC analysis. GC operating conditions were as described in Experimental. For peak identifications see Table VII.

tion values in accord with the known cholesteryl ester constituents of human plasma[6,16,23,28]. Fractionation of the steryl ester mixture by argentation chromatography gave the saturated, monoenoic, dienoic and polyenoic fractions, which are shown by reversed-phase HPLC to contain the various cholesteryl esters previously identified in human plasma by either saponification and GC of the fatty acid methyl esters[23] or GC–MS of the intact steryl esters[16]. In particular, it is possible to recognise the presence of both cholesteryl palmitoleate and cholesteryl linoleate which are unresolved in the original total steryl ester analysis (Fig. 4).

It should be noted that, when using HPLC with UV detection for quantitative purposes, it is necessary to prepare a series of calibration curves for individual steryl esters, since the UV absorption is dependent upon both carbon number and degree of unsaturation in the fatty acyl moiety[6]. The HPLC methods of analysis are relatively insensitive, requiring 10–50 $\mu$g of material[6]. They are ideally suited for (a) preparative purposes to obtain individual compounds for further characterisation and (b) the determination during metabolic studies of the radioactive labelling of individual steryl esters[9] by an on-line radioactivity monitor or by sample collection and radioassay.

GC is a considerably more sensitive analytical procedure than HPLC for the investigation of steryl esters[11–18]. Moreover, GC has a major advantage that it is easily interfaced to a mass spectrometer, thus providing the opportunity for more certain compound identification[13–16,18]. We have evaluated several fused-silica capillary columns with various bonded phases for their application to GC and GC–MS analysis of intact steryl esters[15,16]. Because of the relative involatility of steryl esters, a short column is required with good thermal stability for temperature programming up to about 350°C. We have found that the most suitable columns for our purposes are 8–12 m × 0.22 mm flexible fused-silica capillaries with a 0.1–0.25-$\mu$m film thickness of an OV-1-equivalent bonded phase (see Experimental). Best results are obtained[15,16] with on-column injection of the sample, followed by oven programming from 50 to 330°C. The GC separations obtained for a range of cholesteryl and plant steryl esters are presented in Table I. As with HPLC the overlapping of compounds is a problem which can render difficult the GC analysis of a complex steryl ester mixture. The prior fractionation of the steryl ester by argentation chromatography is again advantageous, as illustrated by the analysis of rape seed steryl esters (Fig. 5). The identities of the individual steryl esters were established by GC–MS, which is the final step in the sequence of analytical procedures required for intact steryl ester characterisation.

GC–MS of steryl esters has been described by other investigators who employed a quadrupole instrument operating in the positive-ion CI mode with methane or ammonia as the reagent gas[13,14]. Our GC–MS work has been performed on a magnetic-sector instrument in the negative-ion CI mode with ammonia as the reagent gas and operated under the conditions described elsewhere[15,16]. Under these conditions, a molecular ion is not observed, but instead, characteristic ions are seen for the steryl moiety ($[M-RCO_2H]^-$) and the fatty acyl moiety ($[RCO_2]^-$) and ($[RCO_2-18]^-$), from which the structure of the original steryl ester can be readily deduced. This is illustrated by the mass spectra for two of the steryl esters present in the rape seed oil (Fig. 6). In the mass spectrum of campesteryl palmitate the ion representing the campesteryl moiety is seen at $m/z$ 381, while the ions at $m/z$ 255 and

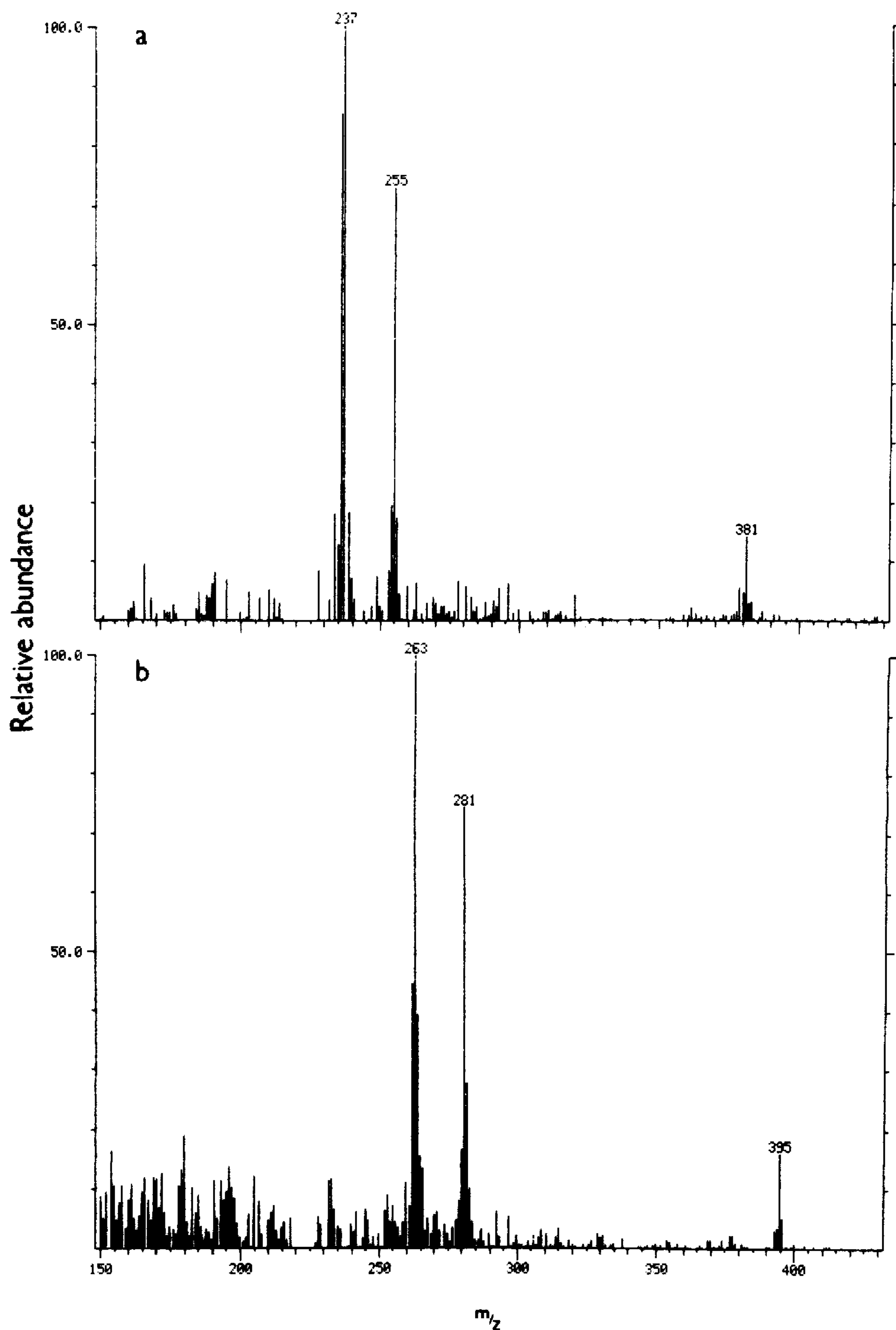


Fig. 6. Mass spectra of (a) campesteryl palmitate and (b) sitosteryl oleate present in rape seed oil steryl esters. The mass spectra were obtained by the GC–MS negative ion CI procedure described in Experimental.

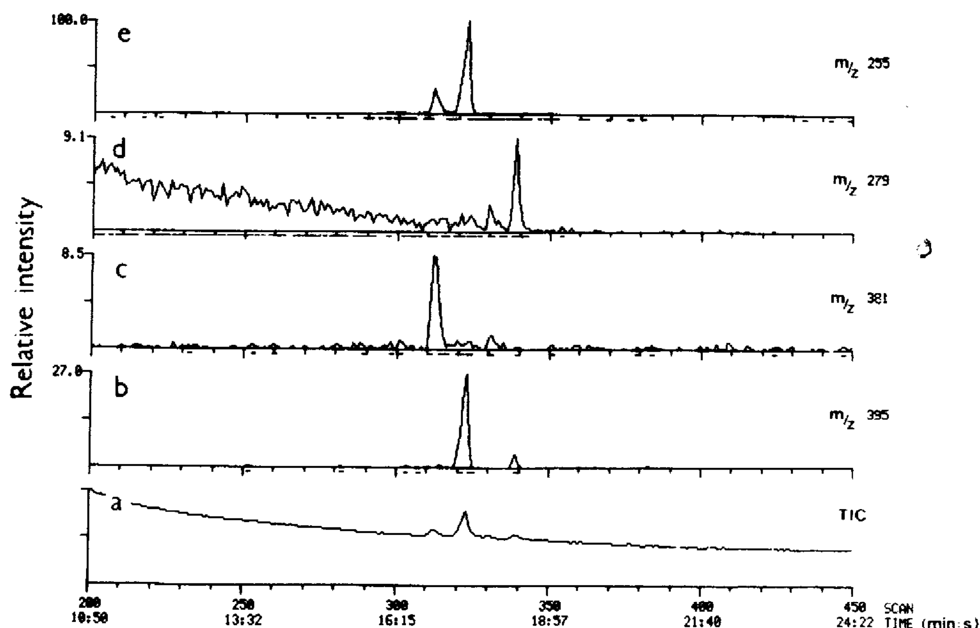


Fig. 7. Analysis of the steryl esters from barley seedlings by GC–MS with ammonia CI. (a) Total ion chromatogram; (b) mass chromatogram of the sitosteryl ion ($m/z$ 395); (c) mass chromatogram of the campesteryl ion ($m/z$ 381); (d) mass chromatogram of linoleate ion ($m/z$ 279); (e) mass chromatogram of the palmitate ion ($m/z$ 255).

237 represent the $[RCO_2]^-$ and $[RCO_2 - 18]^-$ ions, respectively for the palmitate moiety. Similarly, in the mass spectrum of sitosteryl oleate the sitosteryl ion is seen at $m/z$ 395 while the typical oleate ions are at $m/z$ 281 and 263.

A useful technique for the identification of steryl esters in a mixture is to use the MS data system to generate mass chromatograms for ions typical of the steryl and fatty acyl moieties which are of interest. This is demonstrated in Fig. 7 for the steryl esters extracted for barley seedlings. Examination of the mass chromatograms permits the easy recognition of campesteryl and sitosteryl palmitate and campesteryl and sitosteryl linoleate.

The steryl esters identified in the rape seed oil are listed in Table VII. Of particular interest is the recognition of the series of eicosenoate (20:1) and brassidate (22:1) esters, which, although containing one double bond in the fatty acyl moiety, were nevertheless eluted from the argentation column with the saturated steryl ester fractions. Whilst this indicates that some refinement of the argentation procedure may be required, it also illustrates the power of the GC–MS procedure for compound identification. A further notable point is the presence of a considerable amount of wax esters in the rape seed oil steryl ester fraction. These accompanied the "saturated" compounds on argentation chromatography (Fig. 5) but were readily recognised by GC–MS. When using the saponification and fatty acid methyl ester GC analysis procedure, these wax esters would have made a contribution to the fatty acids and, hence, confused the characterisation of the steryl esters.

A strategy for the analysis of a complex steryl ester mixture, based upon the

TABLE VII

IDENTIFICATION OF THE STERYL ESTERS PRESENT IN RAPE SEED OIL BY GC–MS WITH NEGATIVE-ION CI

The steryl esters were separated by argentation chromatography and submitted to GC–MS analysis employing the conditions described in Experimental.

| *Compound* | *GC peak* | *Characteristic ions (m/z)* | | |
|---|---|---|---|---|
| | | *Sterol* | *Fatty acid* | |
| | | $[M-RCO_2H-H]^-$ | $RCO_2^-$ | $[RCO_2-18]^-$ |
| *"Saturated" fraction* | | | | |
| Brassicasteryl palmitate | 1 | 379 | 255 | 237 |
| Campesteryl palmitate | 2 | 381 | 255 | 237 |
| Sitosteryl palmitate | 3 | 395 | 255 | 237 |
| Cholesteryl stearate | 4 | 367 | 283 | 265 |
| Campesteryl stearate | 5 | 381 | 283 | 265 |
| Sitosteryl stearate | 6 | 395 | 283 | 265 |
| Campesteryl eicosenoate | 7 | 381 | 309 | 291 |
| Sitosteryl eicosenoate | 8 | 395 | 309 | 291 |
| Brassicasteryl brassidate | 9 | 379 | 337 | 319 |
| Campesteryl brassidate | 10 | 381 | 337 | 319 |
| Sitosteryl brassidate | 11 | 395 | 337 | 319 |
| *Monoenoic fraction* | | | | |
| Brassicasteryl oleate | 12 | 379 | 281 | 263 |
| Campesteryl oleate | 13 | 381 | 281 | 263 |
| Sitosteryl oleate | 14 | 395 | 281 | 263 |
| *Dienoic fraction* | | | | |
| Brassicasteryl linoleate | 15 | 379 | 279 | 261 |
| Campesteryl linoleate | 16 | 381 | 279 | 261 |
| Sitosteryl linoleate | 17 | 395 | 279 | 261 |

various chromatographic methods described in this paper, is outlined in Scheme 1. The application of the procedures for steryl ester analysis described may prove useful for studies on steryl ester metabolism in various organisms. The GC–MS analysis of steryl ester mixtures should permit the recognition of subtle changes in composition and quantities of the component steryl esters. Studies on steryl ester metabolism with radioactively labelled steryl esters could benefit from the use of preparative reversed-phase HPLC. We are now evaluating the use of deuterium labelling, in both the steryl and fatty acyl moieties, in conjunction with GC–MS negative-ion CI analysis, in order to develop a very sensitive technique for metabolic studies. Deuterium-labelled steryl esters could also prove useful as an added internal standard for the quantitation of steryl esters.

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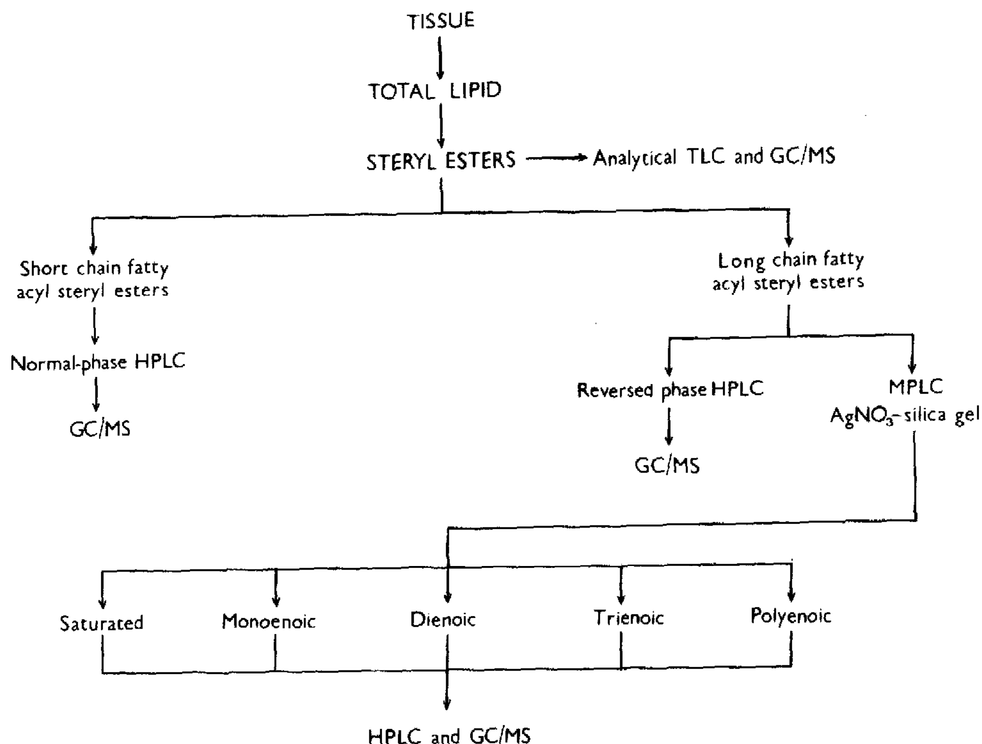


Scheme 1. An outline for the chromatographic procedures for separating and identifying a complex steryl ester mixture.

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