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DETERMINATION OF STEROL AND TRITERPENE ALCOHOL ACETATES IN NATURAL PRODUCTS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A partial separation of nine sterol acetates and seven triterpene alcohol acetates by reversed-phase liquid chromatography is described. Good results are obtained using acetonitrile–water (90:10, v/v) as mobile phase with an UV detector at 205 nm. The variation in sterol sensitivity shows that this technique is not suitable for quantitative analyses. A combination of this technique for the fractionation of the natural sterol mixture, gas–liquid chromatography for quantitation and gas chromatography–mass spectrometry for identification is necessary for the determination of sterol compounds contained in natural products. An example of the separation, identification and quantitation of sterol acetates from sunflower seed oil is given.

INTRODUCTION

In the last decade the number of natural sterols known has increased significantly[1,2], specially sterols contained in marine algae and invertebrates[3–7]. The separation of groups of sterols from each other, *i.e.*, 4-desmethylsterols from 4-methylsterols and from triterpene alcohols, in complex natural mixtures has been achieved using column and thin-layer chromatography (TLC), sometimes using silver nitrate-impregnated adsorbents for the separation of unsaturated sterols from satu-

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rated ones. Knowledge of the structures of sterols has been increased by use of gas chromatography–mass spectrometry (GC–MS). GC is a very effective tool for separating sterols. The relationships between structure and GC retention data have been investigated by several authors. The relative retention times of free sterols[8–10], their trimethylsilyl ethers[8,11–13] and acetates using various liquid phases on conventional packed columns[14,15] have been reported, as have those for 168 acetate derivatives of sterols and triterpene alcohols on OV-1 and OV-17 glass capillary columns[16]. However, the GC method fails to distinguish all sterols[16] and various column systems have been used[15]. To distinguish some sterols, the use of a combination of chromatographic techniques is necessary. Until now, the separation within a class of sterol compounds has usually been achieved by argentation TLC of the acetate derivatives[17]. This separation is based on the number and the position of the double bonds in the sterol skeleton. Thus, homologous compounds obtained by increasing the chain length could not be separated. In addition, such methods are difficult, time-consuming and quantitative results are difficult to obtain.

Ten years ago, few attempts had been made to introduce high-performance liquid chromatography (HPLC) into the steroid field[18]. Although this technique has now been applied qualitatively to steroids[19–21], the HPLC of triterpenic alcohols has not been studied in detail[21–23]. Among the difficulties encountered in the HPLC analyses of these compounds, the more important ones are: detection, separation and identification.

In this paper, the utility of HPLC, GC and GC–MS in sterol analyses is considered in relation to the problems mentioned above. The aim was to develop a stable chromatographic method for the separation, identification and quantitation of sterols or triterpenic alcohols, using reversed-phase liquid chromatography (RPLC), GC and GC–MS. The capacity factors of various sterols and triterpenic alcohols were measured by RPLC using acetonitrile with various concentrations of water as eluent. Sensitivities and response factors were also established. This method was applied to a sample of sunflower seed oil.

EXPERIMENTAL

Reagents and standards

Spectrograde acetonitrile was purchased from E. Merck (Darmstadt, F.R.G.) and used without purification. Water was deionized, distilled from glass apparatus and then purifed on a RP-18 Lobar column (E. Merck). Hexane, diethyl ether, chloroform, carbon tetrachloride, dichloromethane, acetone, methanol, pyridine and acetic anhydride were of high purity and distilled before use. 7-Dehydrocholesterol, dihydrocholesterol, lanosterol with dihydrolanosterol were purchased from Sigma (St. Louis, MO, U.S.A.), cholesterol, ergosterol, stigmasterol, sitosterol with stigmasterol and campesterol from Fluka (Buchs, Switzerland), α- and β-amyrin from Pierce Chemical (Rockford, IL, U.S.A.), cycloartenol and 24-methylenecycloartanol from the unsaponifiable matter (UM) of rice bran oil, brassicasterol from the UM of rapeseed oil, isofucosterol from the UM of coconut oil and dammaradienol from Gum Damar (Sigma).

The saponification and extraction of the unsaponifiable lipids using hexane or diethyl ether was performed as described previously[24]. The UM (500 mg) was frac-

tionated on an alumina column (Brockmann grade II–III, 120 g)[25] using the following solvent mixtures: 200 ml hexane; 200 ml hexane–benzene (1:1, v/v); 200 ml hexane–benzene (1:4, v/v); 500 ml hexane–diethyl ether (1:1, v/v); 500 ml diethyl ether and 250 ml methanol. Fractions (20 ml) were collected and each subjected to silica gel TLC with chloroform–diethyl ether (9:1, v/v) as solvent.

The acetylation of standard compounds was performed using acetic anhydride–pyridine (1:1 v/v)[26]. The crude acetates were crystallized from acetone. Pure steryl acetates including brassicasterol, sitosterol, isofucosterol, dammaradienol, lanosterol, dihydrolanosterol, cycloartenol and cycloartanol were obtained by preparative RPLC using a Lobar RP-8 column (E. Merck) and acetonitrile as mobile phase.

Apparatus

The HPLC equipment consisted of an Altex pump Model 710 A (Altex, Berkeley, CA, U.S.A.) and a Pye Unicam Model LC UV spectrophotometer (Philips, Cambridge, U.K.). The cell used had a volume of 8 μl and a path length of 10 mm. Injection was carried out with a Rheodyne 70–10 valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with 10- or 50-μl loops. A Hibar pre-packed column (E. Merck) (250 × 4.6 mm I.D.) slurry packed with RP-8 LiChrosorb (5 μm) was used with various acetonitrile–water mixtures as moibile phase. A Girdel 30 gas chromatograph (Girdel, Suresnes, France) equipped with a flame ionization detector and a glass solid type injector was used for the analyses. A WCOT glass capillary column (30 m × 0.36 mm I.D.) coated with 0.3 μm OV-17 was operated isothermally at 260°C. The injection port and detector temperatures were 270 and 265°C respectively, and the inlet pressure of hydrogen used as carrier gas was 0.6 bar, split 55 ml min^{-1}. An ICR/1B Intersmat recorder integrator (Shimadzu, Kyoto, Japan) was used for the measurement of retention times and peak area determinations. The GC–MS equipment comprised a Girdel Ribermag R 10-10B apparatus coupled with a Sidar data computer. The chromatograph was fitted with the same injector and column as described above. Operating conditions: 250°C for column and 270°C for inlet; helium carrier gas at 0.5 bar; ion source, 150°C; ionizing voltage, 70 eV.

Procedure

In HPLC experiments each solvent was degassed separately by applying a mild vacuum while it was placed in a sonicator for 5 min, then filtered through a Millipore Fluoropore (PTFE) membrane filter of pore size 0.5 μm. The studies were carried out at ambient temperature (20–22°C) and the flow-rate was set at 1.0 ml min^{-1}. The solutions of sterol acetates, in pure acetonitrile, were filtered through a 0.5-μm Millipore filter before injection. Several standard mixtures were prepared, generally including cholesterol acetate, when the separation was possible, each sterol acetate or triterpenoid acetate being present in at least two standard solutions. Peak identification was done by two methods. The contents of the chromatographic peaks were collected, concentrated and analyzed by GC in order to compare the retention times with published literature values[16], and by GC–MS. In micropreparative liquid chromatography about 0.2 mg of the natural sterol acetate mixture, in pure acetonitrile, were injected on the same column using the 50-μl sample loop. At the outlet of the detector, the mobile phase including peak fractions of interest was collected in vials,

concentrated under a stream of nitrogen and dissolved in 100 μl of hexane, containing cholesterol acetate as internal standard, for further analyses. In GC and in GC–MS analyses, 2 μl of this hexane solution was spotted on to the needle of the solid type injector.

Retention data and precision studies

In RPLC experiments, the retention times and capacity factors, k', were determined from the positions of the peak maxima on the chromatogram, and the mobile phase hold-up time was obtained by injecting sucrose and measuring the retention time of the corresponding peak. Triplicate analyses were used to determine k'. The selectivity, α_1, of sterol acetates or triterpene alcohol acetates was measured relative to cholesterol acetate. In GC experiments, the retention times and capacity factors were calculated from the position of the peak maxima and the solvent hold-up time. The relative retention times, α_2, of sterol acetates or triterpene alcohol acetates were referenced to cholesterol acetate. The sensitivity of the method was determined at 200, 210 and 220 nm for dihydrocholesterol, cholesterol and ergosterol acetates. The sensitivity was defined as the smallest amount of sterol acetate that would give a peak height of twice the peak-to-peak noise level. Response factors determined at 200, 210 and 220 nm were referenced to cholesterol acetate.

Analysis of sunflower seed oil

Seeds of sunflower (*Helianthus annus*, Compositae) were purchased from a local grocery. Oil was extracted from crushed seeds with light petroleum (b.p. 40–60°C) by using a Soxhlet apparatus. The crude oil (1.0 g) was saponified and unsaponifiable material (9.6 mg) was recovered. The dried unsaponifiable material was dissolved in carbon tetrachloride (5%, w/v) and fractionated using TLC by depositing 200 μl on silica gel 50 F-254 (E. Merck) plate. The developing solvent was chloroform–diethyl ether (9:1, v/v). Cholesterol was used as standard for the identification of the sterol band (R_F 0.33–0.37) and a Rhodamine B spray for the detection at 366 nm. The zone corresponding to sterols was scraped off the plate and extracted with dichloromethane. The sterol fraction (4.2 mg) amounted to 44% of the unsaponifiable matter and 0.42% of the oil. Acetylation of this fraction was performed as described above and the composition was determined by GC. The sterol acetate mixture (0.2 mg, dissolved in pure acetonitrile) was separated by HPLC into five fractions: fraction 1 (α_1 *ca.* 0.7–1.04), fraction 2 (α_1 *ca.* 1.05–1.15), fraction 3 (α_1 *ca.* 1.16–1.25), fraction 4 (α_1 1.26–1.35) and fraction 5 (α_1 1.36–1.44), using acetonitrile–water (90:10, v/v) as mobile phase. The eluent was evaporated to dryness in a stream of nitrogen. For analysis by GC and GC–MS, hexane was added to yield a 5% solution. Ten sterol acetates were identified: cholesterol (2), 24ξ-methylcholest-5-en-3β-ol (probably campesterol, 3), 24ξ-ethylcholest-5-en-3β-ol (probably sitosterol, 4), 24ξ-methylcholesta-5,*E*-22-dien-3β-ol (probably brassicasterol, 6), 24ξ-ethylcholesta-5,*E*-22-dien-3β-ol (probably stigmasterol, 7), Δ5-avenasterol or isofucosterol (8), 24ξ-methyl-5α-cholest-7-en-3β-ol (probably Δ7-campestenol), 24ξ-ethyl-5α-cholest-7-en-3β-ol (probably Δ7-stigmastenol), 24*E*-ethylidene-5α-cholest-7-en-3β-ol (28-isoavenasterol) and 24*Z*-ethylidene-5α-cholest-7-en-3β-ol (Δ7-avenasterol).

RESULTS AND DISCUSSION

Since all sterols and triterpene alcohols cannot be distinguish systematically by GC, we have tried to fractionate them using an HPLC procedure. The various fractions obtained could be used for the characterization and the quantitation of these compounds. The utilization of the acetate derivatives for the HPLC separation seems a convenient method for the determination of sterols and triterpene alcohols contained in natural oils and fats because the sensitivity is highly increased by the presence of an acetate group in the molecules. Furthermore, these acetate derivatives could be used directly, without further derivatization, for subsequent GC and GC–MS analyses. The nucleus and the side chain of the sterol acetates, 1–9 and triterpene alcohol acetates 10–16 investigated are given in Figs. 1 and 2 respectively. The systematic nomenclature of these compounds is given in Tables I and II.

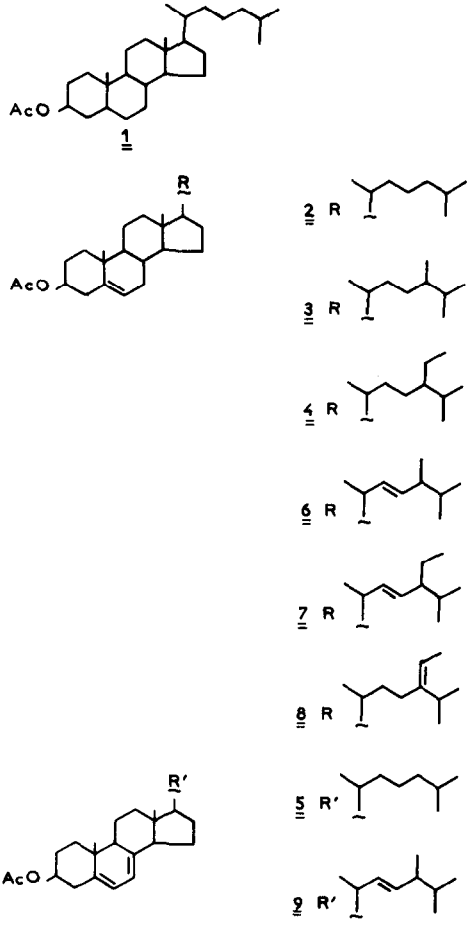

Fig. 1. Nucleus and side chain of the sterol acetates investigated. The numbers refer to Table I. Ac = CH_3CO–.

TABLE I

CAPACITY FACTORS AND RELATIVE RETENTIONS OBTAINED BY HPLC AND GC OF STEROL ACETATES

RP-HPLC on a LiChrosorb RP-8 column with acetonitrile–water (90:10, v/v) as eluent and detection at 210 nm. GC on a glass capillary column coated with OV-17, at 260°C. Formulae for sterol acetates as in Fig. 1.

Compound number	*Sterol*	*Nomenclature*	*Carbon number**	*Double bond number***	*k'****	α_1§	α_2§§
1	Dihydrocholesterol	5α-Cholestan-3β-ol	27	0	6.30	1.21	1.02
2	Cholesterol	Cholest-5-en-3β-ol	27	1	5.20	1.00	1.00
3	Campesterol	[24*R*]-24-Methylcholest-5-en-3β-ol	28	1	5.95	1.14	1.31
4	Sitosterol	[24*R*]-24-Ethylcholest-5-en-3β-ol	29	1	7.07	1.38	1.63
5	7-Dehydrocholesterol	Cholesta-5,7-dien-3β-ol	27	2	4.00	0.77	0.90
6	Brassicasterol	[24*R*]-24-Methylcholesta-5,*E*-22-dien-3β-ol	28	2	5.33	1.03	1.14
7	Stigmasterol	[24*S*]-24-Ethylcholesta-5,*E*-22-dien-3β-ol	29	2	6.33	1.22	1.43
8	Isofucosterol	24-Ethylcholesta-5,*Z*-24(28)-dien-3β-ol	29	2	5.35	1.03	1.81
9	Ergosterol	[24*R*]-24-Methylcholesta-5,7,*E*-22-trien-3β-ol	28	3	4.00	0.77	1.35

* Number of carbon atoms in the free sterol.

** Number of double bonds in the free sterol.

*** Capacity factor.

§ Selectivity in HPLC expressed as the ratio of the capacity factor of the sterol acetate to the capacity factor of cholesterol acetate.

§§ Relative retention in GC expressed as the ratio of the retention time of the sterol acetate to the retention time of cholesterol acetate.

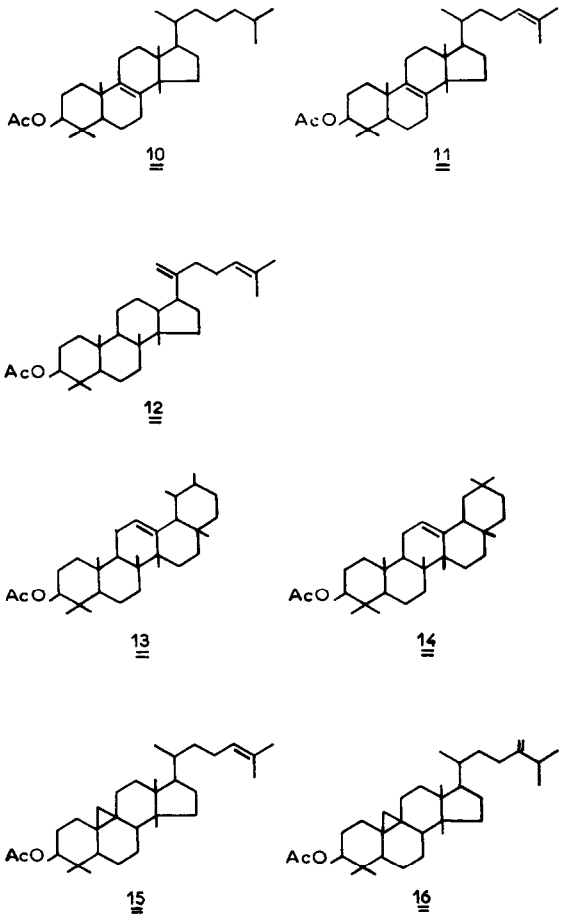

Fig. 2. Structure of the triterpene alcohol acetates investigated. The numbers refer to Table II. Ac = CH_3CO–.

Eluent efficiency

Several authors have separated sterols in the form of their acetate or benzoate derivatives: Rees *et al.*[27] used a μBondapak C_{18} column and various mixtures of methanol, chloroform and water as eluent; Thowsen[28] employed a μPorasil column and hexane–benzene (9:1, v/v) as eluent. The separation of free sterols and triterpene alcohols by RPLC has also been achieved using methanol[22], acetonitrile–methanol[21] and methanol–water[23]. Since improved separations have been achieved in our laboratory with other lipid systems using RPLC and acetonitrile–water as eluent[29,30], we attempted to use this solvent mixture for the peak resolution of sterol acetates and triterpene alcohol acetates. We chose to use a LiChrosorb RP-8 column and a UV detector. Fig. 3 shows the chromatograms of the acetates of dihydrolanosterol (10), dammaradienol (12), α- and β-amyrins (13 and 14) and 24-methylenecycloartanol (16), using various acetonitrile–water eluent mixtures. Compounds 11, 15 and 16 were not well separated in pure acetonitrile.

TABLE II

CAPACITY FACTORS AND RELATIVE RETENTIONS OBTAINED BY HPLC AND GC OF TRITERPENE ALCOHOL ACETATES

Chromatographic details as in Table I. Formulae for triterpene alcohol acetates as in Fig. 2.

Compound number	*Triterpene alcohol*	*Nomenclature*	*Carbon number**	*Double bond number***	*k'*	α_1***	α_2§
10	Dihydrolanosterol	5α-Lanost-8-en-3β-ol	30	1	7.36	1.42	1.30
11	Lanosterol	5α-Lanosta-8,24-dien-3β-ol	30	2	5.55	1.07	1.57
12	Dammaradienol	5α-Dammara-20,24-dien-3β-ol	30	2	4.37	0.84	1.64
13	α-Amyrin	5α-Urs-12-en-3β-ol	30	1	5.90	1.13	1.84
14	β-Amyrin	5α-Olean-12-en-3β-ol	30	1	5.70	1.11	1.65
15	Cycloartenol	9β,19-Cyclo-5α-lanost-24-en-3β-ol	30	1	5.60	1.08	1.86
16	24-Methylenecycloartanol	24-Methylene-9β,19-cyclo-5α-lanostan-3β-ol	31	1	6.44	1.24	2.07

* Number of carbon atoms of the free triterpene alcohol.

** Number of double bonds of the free triterpene alcohol.

*** Selectivity in HPLC expressed as the ratio of the capacity factor of the triterpene alcohol acetate to that of cholesterol acetate.

§ Relative retention in GC expressed as the ratio of the retention time of the triterpene alcohol acetate to that of cholesterol acetate.

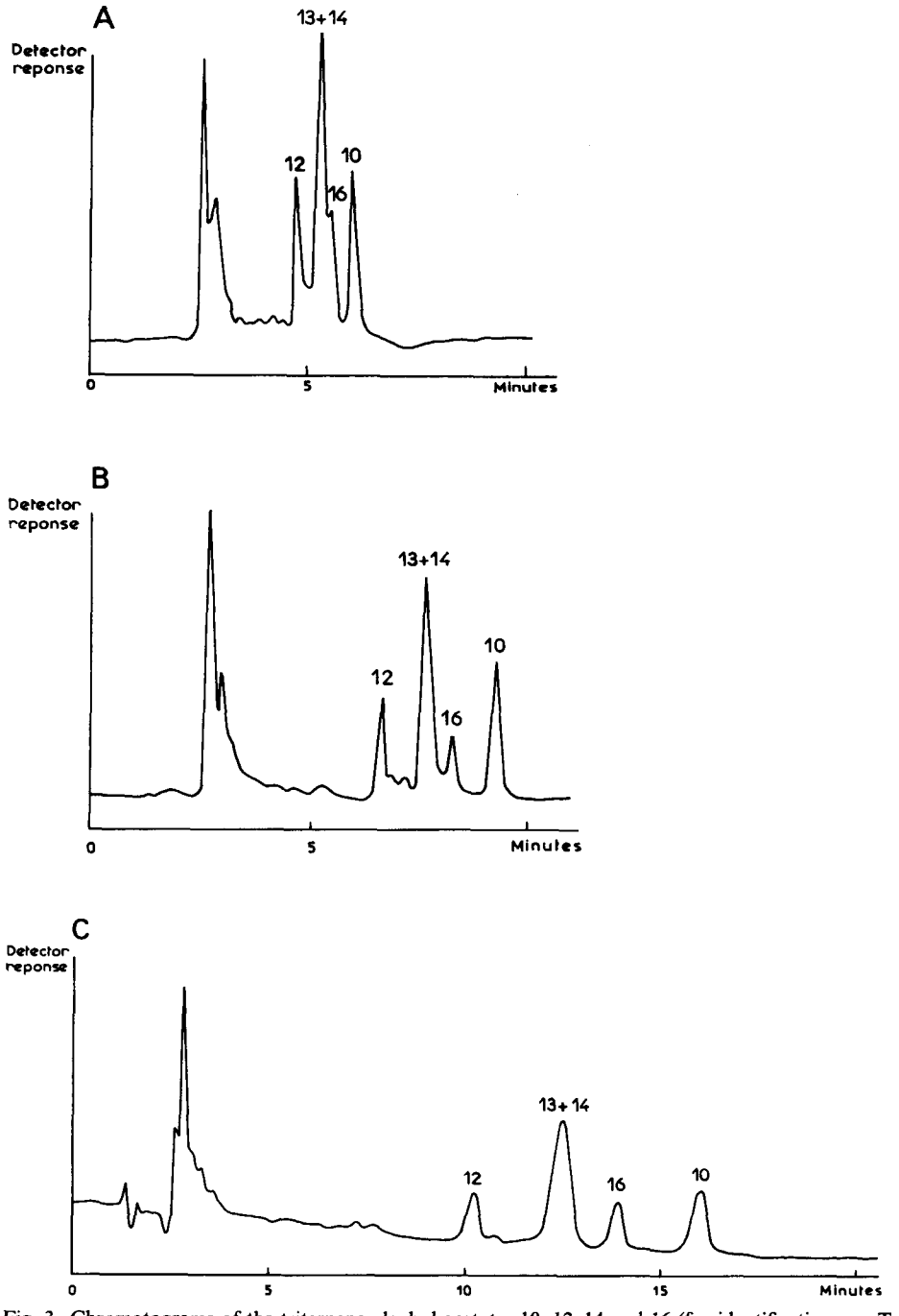

Fig. 3. Chromatograms of the triterpene alcohol acetates 10, 12–14 and 16 (for identification, see Table II). Chromatographic conditions: column, 250 × 4.6 mm I.D., LiChrosorb RP-8 (5 μm) at 20–22°C; flow-rate 1.0 ml min^{-1}; sample volume 10 μl; UV detection at 210 nm. Mobile phases: A, pure acetonitrile; B, acetonitrile–water (95:5, v/v); C, acetonitrile–water (90:10, v/v).

An addition of 5% water in the mobile phase enhanced the separation of the triterpenol acetates. The separation of compounds 11 + 15 from 16 was complete when 10% water was added. When the water content was raised to 15% the separation was better but the solubility of the steroid acetates in the mobile phase became too low and retention times were considerably increased (up to 30 min for dihydrolanosterol, 10). For these reasons, the water content in the eluent mixture was kept at 10% for all the following studies. The separation of five sterol acetates (cholesterol, 2; campesterol, 3; sitosterol, 4; stigmasterol, 7 and ergosterol, 9) is shown in Fig. 4.

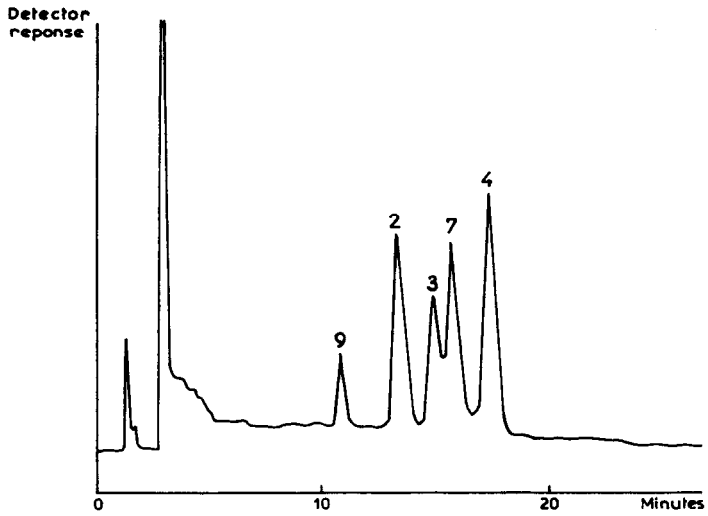

Fig. 4. Chromatogram of five sterol acetates 2–4, 7 and 9 (for identification see Table I). Chromatographic conditions as in Fig. 3C.

Capacity factors

The effect of the properties of the mobile phase in RPLC has often been studied. The logarithm of the capacity factor is approximately a linear function of the solvent composition expressed as the percentage (v/v) of water[31,32]. We observed the same relationship for sterol acetates (Fig. 5) and triterpene alcohol acetates (Fig. 6). However, deviations were found for triterpene alcohol acetates at small percentages (< 5%) of water, as shown in Fig. 6. These results have been explained in terms of a dual retention mechanism[33]. The logarithm of the capacity factors of the sterol acetates given in Table I are plotted *versus* the number of carbons of the free sterols in Fig. 7. Since in RPLC the retention is largely based on the sorption of the hydrophobic part of the molecule from a polar solvent to an apolar sorbent, the order of elution should be according to the chain length. In the case of homologous series, such as fatty acids, the variation of log k' *versus* the number of carbon atoms in the chain is linear[29,34]. For the sterols 1–9 investigated in this study, the increase in the number of carbon atoms (one or two) occurs at the same position of the sterol side chain, *i.e.*, at the C24 position. $\Delta 5$ sterols, 2–4, with a homologous side chain, lie on a straight line and the diunsaturated sterols $\Delta 5,22$ (6 and 7) lie on a line parallel to that for the monounsaturated ones (Fig. 7). The introduction of a double bond at

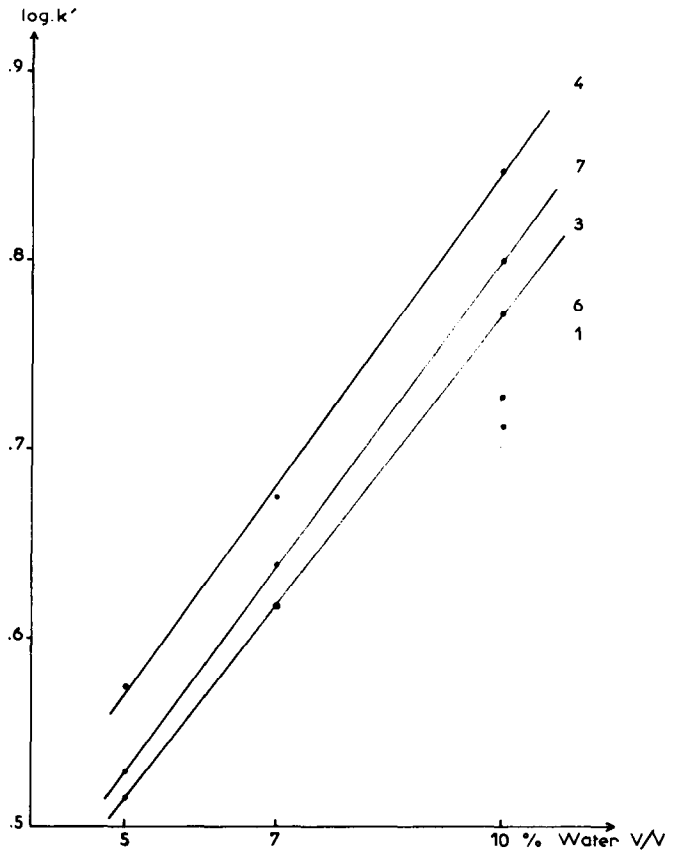

Fig. 5. Variation of the capacity factors, k', of sterol acetates with the concentration of water in the mobile phase. Solvent: acetonitrile. Column: LiChrosorb RP-8 (5 μm). Flow-rate: 1.0 ml min^{-1}. Detection: UV, 210 nm. Compound identification as in Table I.

position C24 (28) has a larger effect than at C22, as seen for compound 8 *versus* 6 and 7. This effect is of the same order as those observed when a $\Delta 7$ double bond is conjugated with a $\Delta 5$ double bond. Thus, in RPLC using acetonitrile–water (90:10, v/v) as eluent we obtained the following order of elution for sterol acetates: $\Delta 5,7,22$; $\Delta 5,7$ and $\Delta 5,24$ (28); $\Delta 5,22$; $\Delta 5$ and $\Delta 0$. These results are similar to those observed for RPLC on a Zorbax C8 column using acetonitrile–methanol (95:5, v/v) as mobile phase[21].

Sensitivity and response factors

The sensitivity in chromatographic analyses depends upon numerous factors. The peak height depends upon the column efficiency, capacity factor, cell pathlength and absorbance at a particular wavelength. The baseline noise on the other hand depends mainly upon the stability of the detector. Using the same chromatographic conditions, the sensitivity was determined for three sterol acetates (1, 2 and 9) at three wavelengths (200, 210 and 220 nm). The results are given in Table III. The best

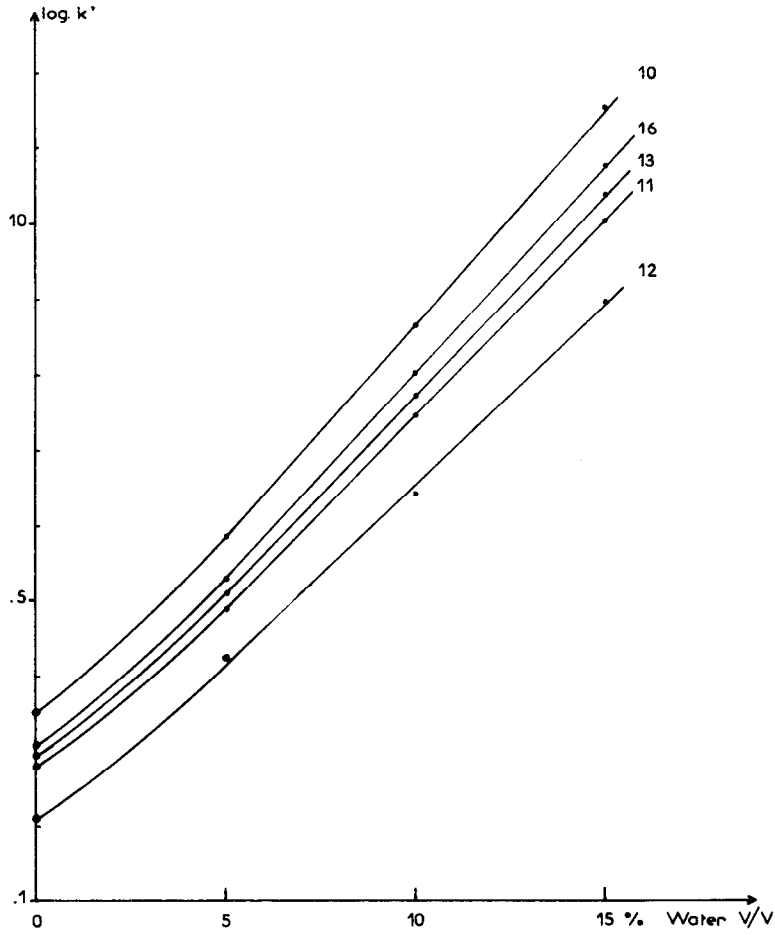

Fig. 6. Variation of the capacity factors, k', of triterpene alcohol acetates. Chromatographic conditions as in Fig. 5. Compound identification as in Table II.

sensitivity was observed at 200–210 nm for unsaturated compounds 2 and 9, ranging from 3.4 to 6.5 ng. In the case of dihydrocholesterol (1), the sensitivity was poor (288 and 330 ng at 200 and 210 nm respectively). The relative response factors (RRFs), expressed as the ratio of the response factor of the compound under examination to the response factor of cholesterol acetate, were determined for compounds 1, 2 and 9 at three wavelengths (Table III). At 200 and 210 nm the RRFs of compounds 1 and 2 were in the same range, but very small for compound 9. These results show that RPLC alone would not be a convenient method for analysis of sterols and triterpene alcohol acetates. For the determination of a natural mixture of sterols and for triterpene alcohols, the use of GC after fractionation by RPLC is necessary. The identification of the acetate derivatives can be achieved by GC–MS.

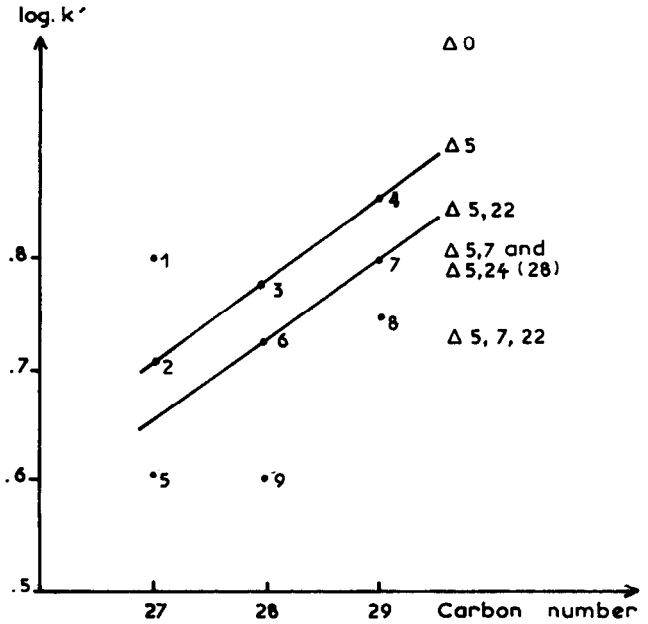

Fig. 7. Variation of the capacity factors, k', of sterol acetates with the number of carbons in the free sterols. Chromatographic conditions and compound identification as in Table I.

Analysis of the sterol fraction of sunflower seed oil

RPLC–GC–MS was applied to the sterol fraction of an edible oil sample. Sunflower seed oil, which is known to contain various sterols[34–37], was investigated. Table IV shows the composition of the sterol fraction of this oil, determined as the acetate derivatives by GC on an OV-17 glass capillary column. The direct GC analysis indicates that only four sterols have a relative concentration higher than 9%. Thus, the identification of the minor sterols using direct GC–MS without previous fractionation is generally unsuitable. The acetates of the sterol fraction were separated into five fractions using an RP-8 column, acetonitrile–water (90:10, v/v) as mobile

TABLE III

SENSITIVITY AND RELATIVE RESPONSE FACTORS OF SOME STEROL ACETATES AT VARIOUS WAVELENGTHS

Determined using a LiChrosorb RP-8 column and acetonitrile–water (90:10, v/v) as eluent. RRF = Relative response factor expressed as the ratio of the response factor of the sterol acetate to the response factor of cholesterol acetate. For nomenclature, see Table I.

Sterol acetate	*Sensitivity (ng) at λ (nm)*			*RRF at λ (nm)*		
	200	*210*	*220*	*200*	*210*	*220*
1 Dihydrocholesterol	288	330	431	0.95	0.99	3.43
2 Cholesterol	3.8	6.5	45.4	1.00	1.00	1.00
9 Ergosterol	3.4	5.5	10.9	0.015	0.023	0.104

TABLE IV

ANALYSIS (%) OF THE STEROL FRACTIONS OF SUNFLOWER SEED OIL

Determined as sterol acetates from the unsaponifiable matter of the seed oil, after fractionation by TLC. For nomenclature, see Experimental and Table I.

Sterol	α_2*	*Direct GC***	*Fractions****				
			1	*2*	*3*	*4*	*5*
Cholesterol	1.00	1.1	15.6	1.0			
Brassicasterol	1.14	0.4	11.2				
Campesterol	1.31	9.2	2.6	83.2	12.7		
Stigmasterol	1.43	9.1		7.3	78.3	8.4	
Δ7-Campestenol	1.55	0.9	10.3	3.1			
Sitosterol	1.63	60.5				28.8	91.8
Δ5-Avenasterol	1.81	1.8	18.9	2.1			
Δ7-Stigmastenol	1.94	12.4			7.4	59.6	6.7
28-Isoavenasterol	2.04	0.3	5.5				
Δ7-Avenasterol	2.15	2.8	29.6	2.1			
Unknowns		1.6	6.3§	1.2	1.6	3.2§§	1.5

* Relative retention time of the sterol acetate on an OV-17 WCOT glass capillary column (cholesterol acetate 1.00) determined at 260°C.

** Without previous fractionation by HPLC.

*** The five fractions were obtained by HPLC on a LiChrosorb RP-8 column with acetonitrile–water (90:10, v/v) as eluent.

§ Among the other minor sterols, 24-methylenecholest-5-en-3β-ol (α_2 = 1.35) and 24*E*-ethylidenecholest-5-en-3β-ol (fucosterol, α_2 = 1.72) were tentatively identified.

§§ This fraction contained 1.5% of a sterol which was tentatively identified to 24ξ-ethyl-5α-cholest-7,*E*-22-dien-3β-ol (spinasterol, α_2 = 1.70).

phase and detection at 210 nm. Each fraction was subjected to GC–MS in order to identify the components and the composition was determined by GC (Table IV). The determination of the configuration at C-24 of 24-methyl- and 24-ethylsterols is possible using ^{1}H NMR[38–40] and ^{13}C NMR[41,42] spectroscopies, or by GC[43]. In this study, the absolute configuration of the C-24 alkylsterols was not examined. Although the 24α-alkylsterols are usually present in terrestrial plants[2], the co-occurrence of the two epimers of 24-ethyl-5α-cholest-7-en-3β-ol (Δ7-stigmastenol), *i.e.*, 22-dihydrospinasterol (24α-epimer) and 22-dihydrochondrillasterol (24β-epimer), has been demonstrated in the roots of *Tricosanthes japonica*[41]. The names of the sterols given in Table IV are trivial names generally found in the literature. In fraction 1, the identification was based upon MS of six sterol acetates (cholesterol, brassicasterol, Δ7-campestenol, Δ5-avenasterol, 28-isoavenasterol and Δ7-avenasterol). Fraction 2 gave campesterol acetate (83%) accompanied by small amounts of the acetates of cholesterol, stigmasterol, Δ7-campestenol, Δ5- and Δ7-avenasterols. In fraction 3, stigmasterol acetate was prominent (78%). Fraction 4 afforded a mixture of three acetates of which Δ7-stigmastenol acetate (60%) was the major one. Fraction 5 also contained Δ7-stigmastenol acetate (7%) but the prominent component was sitosterol acetate. The identification of these ten sterols and their relative composition in sunflower seed oil is in agreement with previous results[34–37]. Some other sterols, not previously found in sunflower seed oil, were also tentatively identified in trace

amounts using α_1 and α_2: 24-methylenecholest-5-en-3β-ol, fucosterol and spinasterol, which have been identified in higher plants[17].

CONCLUSIONS

A sensitive technique for the identification and the determination of sterols and triterpene alcohols in natural fats as their acetates using RPLC, prior to GC–MS analysis, has been developed. This method has certain advantages. (i) Low amounts of sterol acetates per injection are required with an UV detector since the sensitivity is greater than for the free sterols. (ii) Several sets of sterol acetates having the same or similar relative GC retention time may be separated. (iii) The fractions obtained are enriched in minor sterols which can more easily be identified using GC–MS. (iv) Repeated fractionation using this method may give pure sterol acetates for subsequent NMR analyses. (v) Quantitation of sterol acetates is possible using GC without further derivatization since large variations in sensitivity of the components are observed in RPLC experiments with an UV detector. (vi) Analyses can be carried out rapidly, with good precision and less than 100 mg of oil are necessary for the determination and the identification of the sterols.

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