THE ANALYSIS OF MIXTURES OF ANIMAL AND VEGETABLE FATS

V. SEPARATION OF STEROL ACETATES BY THIN-LAYER CHROMATOGRAPHY IN REVERSED-PHASE SYSTEMS AND ON SILICA GEL G–SILVER NITRATE LAYERS*

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INTRODUCTION

The separation of steroids and sterols by means of thin-layer chromatography (TLC) has recently received much attention. The analysis of steroids was reviewed among others by WALDI[6] in the handbook of STAHL *et al.* Many recent investigations were published in the "Thin-layer chromatography issue" of the Journal of Chromatography[7]. The TLC of the lipophilic cholesterol esters has also been studied intensively by many authors, *e.g.* VAN DAM[8], HEŘMÁNEK *et al.*[9], MAHADEVAN AND LUNDBERG[10], WEICKER[11], JATZKEWITZ AND MEHL[12], KAUFMANN *et al.*[13], MICHALEC *et al.*[14], and ZÖLLNER AND WOLFRAM[15].

However, only a few of the investigations deal with the group of sterols and the related provitamins D, vitamins D, and triterpenoid alcohols.

The TLC of steroids, usually also comprising some sterol types, on silica gel G layers was studied by BARBIER *et al.*[16], VAN DAM *et al.*[17], WALDI[6], JANECKE AND MAAS-GOEBELS[18], TSCHESCHE AND SNATZKE[18], BENNETT AND HEFTMANN[20], NORMAN AND DE LUCA[21], and COPIUS-PEEREBOOM (see ref. 5, p. 97), while HEŘMÁNEK *et al.*[9,22] and ČERNY *et al.*[23] have studied such separations on spread layers of aluminium oxide. In general, mono-unsaturated sterols like cholesterol, provitamins D (*e.g.* ergosterol), and vitamins D are separable, but closely related sterols like cholesterol, stigmasterol, and β-sitosterol were not resolved.

The double bond isomers cholesterol and Δ^7-cholestenol were separated by BENNETT AND HEFTMANN[20] in the solvent cyclohexane–ethyl acetate–water (600:400:1).

AVIGAN *et al.*[24] also studied the application of TLC systems to the separation of related sterols. On normal silica gel G layers related sterols like cholesterol, β-sitosterol, and desmosterol were not resolved. A slightly better fractionation of cholesterol, Δ^7-cholestenol, 7-dehydrocholesterol, and lanosterol was achieved by chromatog-

* For parts I, II, III and IV of this series, see refs. 1, 2, 3, 4. A comprehensive survey of our work on the analysis of sterols is given in the thesis of one of us (C.-P.), *viz. Chromatographic Sterol Analysis*, Pudoc, Wageningen, 1963, ref. 5.

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raphy on 40 cm plates in benzene–ethyl acetate (20:1) for 24–36 h. AVIGAN *et al.* also reported the separation of lanosterol acetate and 24-dihydrolanosterol acetate on silica gel + 5% $CaSO_4 \cdot {}^1/_2\ H_2O$ layers using benzene–ethyl acetate (20:1) as mobile phase.

The behaviour of sterols on thin layers of aluminium oxide and kieselguhr G was studied by COPIUS-PEEREBOOM[3]. Using the solvent mixture cyclohexane–ethyl acetate (99.5:0.5) a mixture of ergosterol, cholesterol, and vitamin D_2 was fractionated. Cholesterol and Δ^7-cholestenol were resolved in the solvent cyclohexane–ethyl acetate (99.9:0.1).

In the above "normal" TLC systems the best separation was accomplished by BENNETT AND HEFTMANN[20] by analysing the trifluoroacetates of some sterols. The trifluoroacetates of cholesterol, stigmasterol, and desmosterol were separated with a cyclohexane–heptane (1:1) mixture.

The various systems for the separation of sterol acetates investigated by the authors are discussed below. After the authors' own investigations were finalized, BENNETT AND HEFTMANN[25] reported another procedure for separating β-sitosterol acetate, cholesterol acetate, and stigmasterol acetate, by means of continuous development with hexane–ether (97:3) on Anasil B plates for 120 min.

THE REVERSED-PHASE SYSTEM UNDECANE/ACETIC ACID–ACETONITRILE (1:3)

While studying the analysis of mixtures of animal and vegetable fats, we were mainly interested in the separation of closely related sterols like cholesterol, β-sitosterol, stigmasterol, and other phytosterols. In the above "normal" systems a separation between mono-unsaturated sterols like cholesterol and β-sitosterol could not be accomplished. However, reversed-phase thin-layer chromatography of the sterol acetates proved to be very suitable for this purpose.

After testing several reversed-phase systems, we finally decided that the system undecane (b.p. 190–220°)/acetic acid–acetonitrile (1:3) gives the best separations in the group of sterol acetates. The procedure of this reversed-phase system is given in the experimental part. In this system cholesterol and closely related phytosterols, *e.g.* campesterol, stigmasterol, and β-sitosterol, and in addition the pair cholesterol–dihydrocholesterol are clearly separated[3,4,5]. Analogous separations of the latter pair and of other positional isomers have since been reported by CARGILL[26], who has employed systems like undecane/methanol or/methanol–ether (49:1).

In such reversed-phase systems, both in paper chromatography and in thin-layer chromatography, the sterols or their acetates are arranged in several bands (see Fig. 1). The sterol acetates belonging to a certain band have nearly the same R_s (s = cholesterol) value and constitute a so-called critical pair.

The introduction of a double bond in the molecule causes nearly the same increase in R_s value as shortening the carbon chain by one methylene or methyl group. Average ΔR_M values were calculated from the R_M values of the compounds given in Table I, *viz.* $\Delta R_M{}^{C=C} = -0.08$; $\Delta R_M{}^{CH_2} = +0.06$.

The acetates of cholesterol (FC_{27}) from animal fats and of brassicasterol ($FC_{28}F$*) from rapeseed oil, therefore, have nearly same R_s value and thus belong

* This formula means a C_{28} sterol skeleton with one double bond in the nucleus (FC_{28}) and another double bond in the C_{17}-side chain of the molecule ($C_{28}F$).

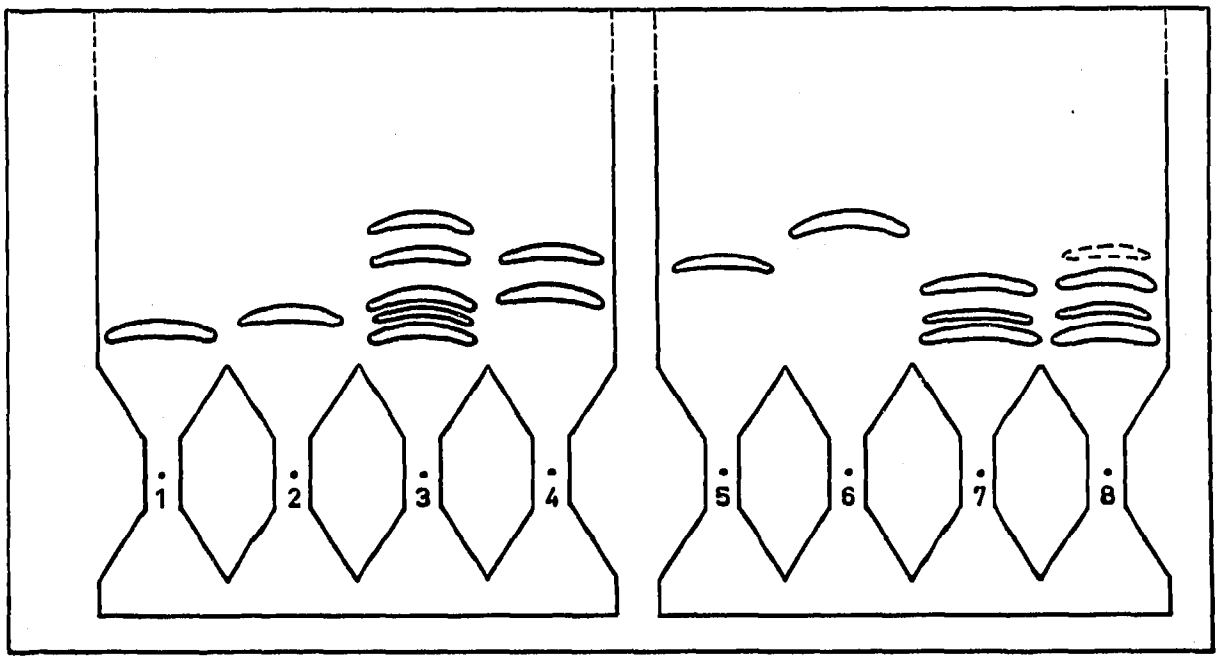

Fig. 1. Reversed-phase TLC of sterol acetates. Adsorbent: Kieselguhr G (Merck). System: undecane/acetic acid–acetonitrile (1:3). Time of run: 2 h. Detection: phosphomolybdic acid. Spot 1: 10 μg β-sitosterol acetates; spot 2: 10 μg stigmasterol acetate; spot 3: mixture of 1, 2, 4 and 6; spot 4: 20 μg of a mixture of cholesterol acetate–desmosterol acetate; spot 5: 10 μg ergosterol acetate; spot 6: 10 μg $\Delta^{9(11)}$-dehydroergosterol acetate; spot 7: 35 μg of phytosterol acetates from coconut fat; and spot 8: 35 μg of sterol acetates from *Asterina pectinifera*.

to the same critical pair. The equality of the migration rates of cholesterol, brassicasterol, and related $FC_{28}F$-sterols in this system hampers the suitability of our procedure, which was devised for the detection of animal fat in mixtures with vegetable fats. We were forced therefore to study the correlation between sterol structure and separability in more detail.

Naturally occurring complex sterol mixtures can be separated in this reversed-phase system and can be divided into six clearly separated bands, *viz.* critical pairs. The R_S values of some sterol acetates, classified according to these six critical pairs, are given in Table I.

The above arrangement of the sterol acetates into critical pairs is of course a rough classification. The position of the double bond in the nucleus and especially the occurrence of systems of double bonds will exert some influence upon the R_S value.

The R_S and also the R_M values are highly dependent on the experimental conditions of the applied reversed-phase system. A most unequivocal characterisation of the effect of introducing a double bond in a special position can be obtained by way of the so-called *carbon numbers* (*Nc*). This procedure has been applied by KAUFMANN AND MAKUS[27] to the separation of higher fatty acids and of triglycerides by reversed-phase systems of paper chromatography and of thin-layer chromatography. They introduced the so-called "papierchromatographische Wertzahl" (*pcW*), defined as the difference between the number of carbon atoms (n) and twice the number of double bonds (m) of the molecule, *viz.* $pcW = n - 2m$. However, such a procedure of calculating characteristic values, which are independent of the properties of the system, dates back to the so-called R_C values of DECKER[28].

TABLE I

R_s (s = CHOLESTEROL) VALUES OF STEROL ACETATES IN THE REVERSED-PHASE SYSTEM: UNDECANE/ACETIC ACID–ACETONITRILE (1:3)

Acetates of	*Shorthand designation*	*Band No.*	*Carbon number Nc*	*R_s value*
5α-Androstan-3β-ol	C_{19}		19.0	2.25
$\Delta^{9(11)}$-Dehydroergosterol	$3FC_{28}F$	6	22.7	1.45
Vitamin D_3	$3FC_{27}$	6	23.0	1.41
Dihydrovitamin D_2	$3FC_{28}$	5/6	23.6	1.34
Zymosterol	$FC_{27}F$	5	24.0	1.28
Vitamin D_2	$3FC_{28}F$	5	24.2	1.26
Desmosterol	$FC_{27}F$	5	24.35	1.24
3-Dehydrocholesterol	$2FC_{27}$	5	24.55	1.22
Ergosterol	$2FC_{28}F$	5	24.8	1.19
Epi-cholesterol	FC_{27}	5	24.8	1.19
7-Dehydrocholesterol	$2FC_{27}$	5	25.0	1.16
Ergosterol D ($\Delta^{7,9(11),22}$-ergostatrienol)	$2FC_{28}F$	5	25.4	1.11
Brassicasterol (7-dihydroergosterol)	$FC_{28}F$	4	25.8	1.07
5-Dihydroergosterol	$FC_{28}F$	4	25.8	1.07
22-Dihydroergosterol	$2FC_{28}$	4	25.4	1.07
Cholesterol	FC_{27}	4	≡ 26.0	≡ 1.00*
Lanosterol	$FC_{30}F$	4	26.0	1.00
Δ^{3}-Cholestenol	FC_{27}	4	26.0	1.00
Δ^{7}-Cholestenol	FC_{27}	4	26.5	0.99
Δ^{7}-Ergostenol	FC_{28}	3	27.0	0.92
Campesterol	FC_{28}	3	≡ 27.0	0.92
Stigmasterol	$FC_{29}F$	3	27.2	0.91
α-Spinasterol ($\Delta^{7,22}$-stigmastadienol)	$FC_{29}F$	3	27.2	0.91
Dihydrocholesterol	C_{27}	3	27.4	0.89
Agnosterol	$2FC_{30}F$	2/3	27.6	0.86
β-Sitosterol	FC_{29}	2	≡ 28.0	0.83
Dihydro-β-sitosterol (stigmastanol)	C_{29}	1	29.0	0.73

* The R_F value of cholesterol is about 0.28.

In the series of sterol acetates given in Table I the carbon number Nc is defined as $Nc = n - m$. Plotting the R_M values of the acetates of the mono-unsaturated sterols cholesterol ($Nc = 26$), campesterol ($Nc = 27$), and β-sitosterol ($Nc = 28$) against their Nc values, a linear correlation is found. The R_M values of the saturated compounds: 5α-androstan-3β-ol acetate (C19) and dihydro-β-sitosterol acetate (C29) also obey this relation. In the R_M–Nc graph through these 5 points the straight line $R_M = 0.609\ (Nc - 19) - 0.09$ can be drawn. The R_M values of all other sterol acetates are interpolated and the corresponding Nc values are calculated graphically (see Table I). The Nc values can be considered as characteristic values, dependent only on the structure of the sterol molecule.

The Nc value belonging to an unknown component of a naturally occurring sterol mixture already gives some indication as to the possible structure of that component.

By means of reversed-phase TLC several hitherto unknown sterols have been detected in special sterol mixtures[1,5]. In many phytosterol mixtures isolated from edible vegetable oils, *e.g.* coconut fat, we could detect the presence of so-called *third-*

band phytosterols with an R_s value of 1.07 and *Nc* value of about 25.8 (see Fig. 1, spot 7). The structure of these special phytosterols, therefore, was supposed tentatively to be isomeric to that of methylcholestadienol, $FC_{28}F$.[5]

In chromatographic analysis the sterol mixtures from animal fats also show some peculiarities. Thus, in the sterols from hardened whale oil a band of $R_s = 0.88$ due to dihydrocholesterol was detected. In some crude and refined whale and fish oils two other sterol bands were observed with R_s values of 1.22 ($Nc = 24.3$), and 1.38 ($Nc = 22.1$), belonging to the "ergosterol critical pair" and to the "vitamin D_3 critical pair", respectively*.

The R_s values of Δ^7-sterols like Δ^7-cholestenol and 5-dihydroergosterol are identical to those of the corresponding Δ^5-sterols, *viz.* cholesterol and brassicasterol. Mixtures of homologous and related Δ^7-sterols are similarly separated in this system. The Δ^7-sterol mixture isolated from the starfish *Asterina pectinifera*** by TOYAMA AND TAKAGI[29] is separated into three bands with a very faint fourth band at $R_s = 1.22$ (see Fig. 1, spots 8)[5].

In the structural analysis of such complex sterol mixtures reversed-phase thin-layer chromatography may thus give circumstantial evidence concerning the structure of unknown sterol components. In many instances, however, various theoretically conceivable structures, all belonging to the same critical pair *e.g.* $FC_{27}F$ (with all possible isomers), $2FC_{27}$, $2FC_{28}F$, should be taken into consideration.

A separation of sterols according to principally different rules of separability, *e.g.* according to the "degree of unsaturation" of the components therefore appeared to be necessary. For this purpose several possible systems were investigated. Finally, two systems enabling a separation according to the "degree of unsaturation" were established, *viz.* the so-called "bromine-system" and silver nitrate TLC.

THE "BROMINE-SYSTEM"

KAUFMANN *et al.*[30] have described a procedure of separating fatty acids or triglycerides, belonging to the same critical pair but having a different number of double bonds, by the mere addition of 0.5 % of bromine to the mobile phase. In this way compounds with a different degree of unsaturation were separated. We have applied the principle of these so-called "bromine-systems" to the separation of the before-mentioned critical-pair partners cholesterol acetate–brassicasterol acetate and stigmasterol acetate–campesterol acetate[23].

FABRO[31] has described a paper chromatographic separation of the mono- and di-unsaturated sterols cholesterol and desmosterol by way of their bromo derivatives. A 10 % bromine solution was spotted directly on to the sterol sample at its starting point on the paper. MICHALEC[32] described a paper chromatographic separation of cholesterol and dihydrocholesterol, using a benzene–bromine (100:0.5) mixture. CARGILL[26] devised an analogous procedure of separating this sterol pair by TLC. After spotting the sterol sample at the starting point some drops of bromine were spotted on the same place. The plate was then developed with a benzene–ethyl

* More details concerning the analysis of such natural sterol mixtures will be published in a further communication of this series.

** We gratefully acknowledge the generous gift of the *Asterina pectinifera* sterol sample from Prof. TOYAMA.

acetate (2:1) mixture, by which means the spots of cholesterol and dihydrocholesterol were separated.

The R_S values of some sterol acetates in our "bromine-system", *viz.* undecane/acetic acid–acetonitrile (1:3) + 0.5 % of bromine are given in Table II. The identity of the bands of the dibromo derivatives which are made visible by spraying with antimony (III) chloride, was verified by a specific reaction with cadmium chloride[5].

The separability in this system is partly due to a normal reversed-phase separation of mono-unsaturated FC_{29}-, FC_{28}- and FC_{27}-sterol acetates, and partly to an enhanced migration of the more polar di-unsaturated sterols.

Because of both effects the migration rates of stigmasterol acetate and cholesterol acetate in this system are nearly equal. Likewise, the migration rate of brassicasterol acetate, present in rapeseed oil, is enhanced by a ΔR_S value of +0.06 to an R_S of 1.13, as compared with the "normal" reversed-phase system (see Fig. 2 and ref. 5, Fig. 34).

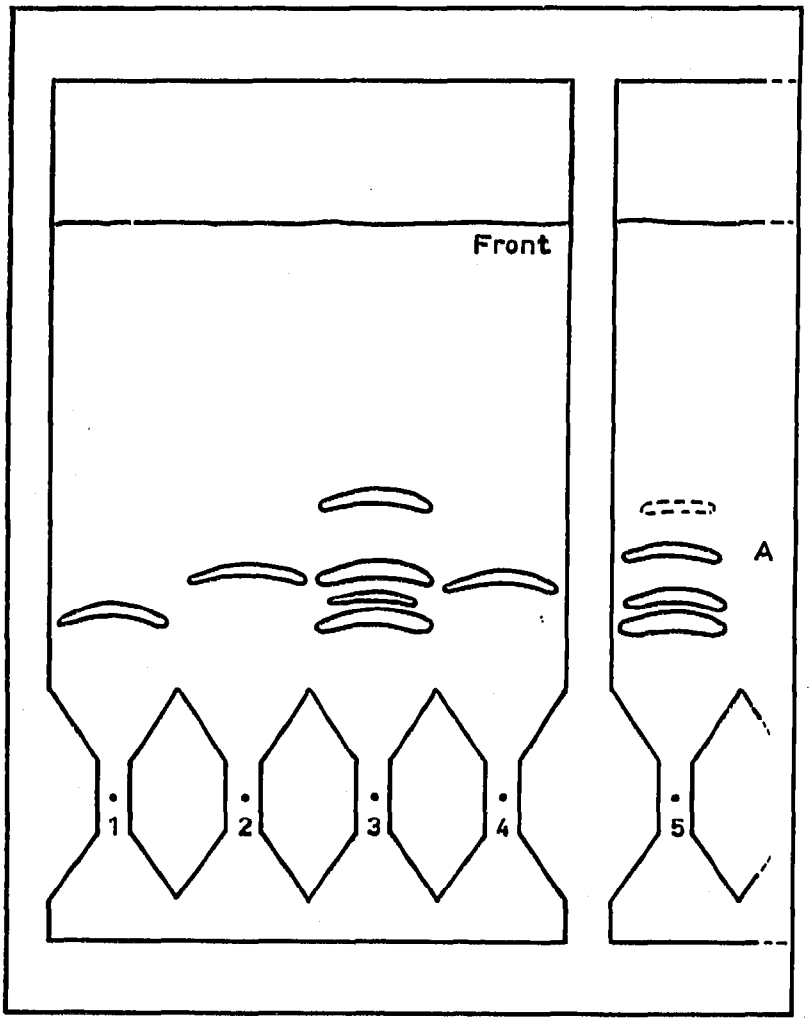

Fig. 2. TLC of sterol acetates in the "bromine-system". Adsorbent: Kieselguhr G (Merck). System: undecane/acetic acid–acetonitrile (1:3) + 0.5 % of bromine. Time of run: 2 h. Detection: heating, 50 % antimony(III) chloride. Spot 1: 40 μg β-sitosterol acetate; spot 2: 20 μg stigmasterol acetate; spot 3: 80 μg of phytosterol acetates from coconut fat; spot 4: 20 μg cholesterol acetate; and spot 5: 80 μg of phytosterol acetates from rapeseed oil (A = brassicasterol).

In the reversed-phase system in the previous section the R_s value of the third-band phytosterols from vegetable oils is equal to that of brassicasterol. In the bromine-system the third-band phytosterols, however, have a somewhat higher R_s value *viz.* 1.40 as compared to that of brassicasterol. In this way the three critical-pair partners cholesterol–brassicasterol–third-band phytosterols are clearly separated. In the bromine-systems compounds having a higher polarity, *e.g.* a higher degree of

TABLE II

R_s VALUES OF STEROL ACETATES IN THE SO-CALLED BROMINE-SYSTEM: UNDECANE/ACETIC ACID–ACETONITRILE (1:3) + 0.5% OF BROMINE

Acetates	*Shorthand designation*	R_s *value*
Ergosterol	$2FC_{28}F$	front
7-Dehydrocholesterol	$2FC_{27}$	front
Third-band phytosterols from coconut fat	"$FC_{28}F$"?	1.40
Brassicasterol	$FC_{28}F$	1.13
Stigmasterol	$FC_{29}F$	1.06
Cholesterol	FC_{27}	≡ 1.00
Δ^7-Ergostenol	FC_{28}	front
Campesterol	FC_{28}	0.89
Dihydrocholesterol	C_{27}	0.85
Lanosterol	$FC_{30}F$	front
β-Sitosterol	FC_{29}	0.82

unsaturation, display a higher migration rate. The tentative $FC_{28}F$-structure for the third-band phytosterols should therefore have a higher polarity than that of brassicasterol with a double bond at 24 (28) or 25 (26) etc.

Sterols with a system of conjugated double bonds and sterols devoid of the normal $\Delta^{5(6)}$ double bond, *e.g.* Δ^7-sterols, lanosterol, and zymosterol, are completely decomposed in the bromine-system. Sterol structures with two or more conjugated double bonds like ergosterol, 7-dehydrocholesterol, vitamin D_3 etc. are not likely to be attributed to these third-band phytosterols.

By combining the R_s value of an unknown sterol in the normal reversed-phase and in this bromine-system respectively, ample circumstantial evidence concerning its structure can be obtained.

Δ^7-Sterol mixtures *e.g.* from *Asterina pectinifera* are similarly decomposed and only show blue spots near the solvent front, caused by decomposition products. Both Δ^7-sterols and "conjugated sterols" cannot be analysed in this system. The possibility of analysing sterols with "silver nitrate TLC", which is known to separate compounds mainly according to their degree of unsaturation without any decomposition effects, therefore had to be investigated.

SEPARATION OF STEROLS AND STEROL ACETATES ON SILVER NITRATE PLATES

In gas–liquid chromatography the admixture of silver salts to stationary phases like benzyl cyanide or glycols in order to enable a good resolution of paraffins and olefins is a well-known procedure.

The application of a silver nitrate coated adsorbent to the column chromatographic fractionation of lipids was described by DE VRIES[33,34]. The procedure was soon extended to thin-layer chromatography. MORRIS[35] and DE VRIES[36] succeeded in separating the methyl esters of oleic acid, linoleic acid, and linolenic acid, which differ in the number of double bonds. Furthermore, *cis–trans* isomers like methyl oleate and methyl elaidate and their corresponding epoxy and hydroxy esters were separated.

The fractionation of synthetic mixtures of triglycerides and of natural oils and fats on silver nitrate plates, using mixtures like carbon tetrachloride–chloroform–ethanol, was described by BARRET *et al.*[37,38].

Phosphatide mixtures *e.g.* from eggs were resolved on silica gel G–silver nitrate layers by KAUFMANN *et al.*[39] with the solvent mixture chloroform–ether–acetic acid (97.0:2.3:0.5).

Some poly-unsaturated olefins like humulene, caryophyllene, and thujopsene were separated on silica gel–silver nitrate plates by GUPTA AND SUKH DEV[40]. A fractionation of aldehyde 2,4-dinitrophenylhydrazones into "unsaturation classes" is described by URBACH[41], using aluminium oxide–silver nitrate plates, and by BADINGS AND WASSINK[42], employing kieselguhr G–silver nitrate chromatoplates. Since naturally occurring sterol mixtures are composed of saturated, mono-, di-, and poly-unsaturated sterols, it is to be expected that silver nitrate adsorbent layers will become a powerful tool in sterol and steroid analysis.

The analysis of the saturated dihydrocholesterol and the mono-unsaturated Δ^5-cholesterol in mutual mixtures incurs many difficulties, but because of their different degree of unsaturation dihydrocholesterol and cholesterol were readily separated on silica gel G–silver nitrate plates by DE VRIES, using chloroform or chloroform–acetic acid mixtures.

We have previously reported a clear resolution of these sterols in our reversed-phase system[5]. In this way small amounts of dihydrocholesterol (down to 5 %) were detected in an excess of cholesterol.

AVIGAN *et al.*[24] have briefly described the separation of cholestenols (Δ^5 and Δ^7), desmosterol, and 7-dehydrocholesterol on silica gel layers impregnated with $AgNO_3$ by spraying with a saturated $AgNO_3$ solution. A mixture benzene–ethyl acetate (5:1) was used as mobile phase. Cholesterol esters were fractionated by MORRIS[43] on silica gel G–silver nitrate plates with the solvent mixture ether–hexane (1:4), according to the unsaturation of the fatty acid chain.

In the present investigation we have studied the behaviour of several sterols and sterol acetates on silica gel G–silver nitrate layers more thoroughly.

Separation of sterols

We have followed the procedure of preparing silica gel G–silver nitrate chromatoplates described by DE VRIES[36] (see also KAUFMANN *et al.*[39]). 13 g of silver nitrate (Merck) were dissolved in 60 ml of water and added to 30 g of silica gel G (Merck); chromatoplates of 20 × 20 cm were then prepared from this mixture. After drying (for 2 h at 20° and then for 2 h at 110°) and spotting with 110–150 μg of sterols, the plates were developed in the solvent mixture chloroform–ether–acetic acid (97:2.3:0.5), according to KAUFMANN *et al.*[39].

After the development procedure the plates were sprayed with a 0.2 % ethanolic

solution of dibromofluorescein and viewed under U.V. radiation of 365 mμ. The sterol spots then showed a bright fluorescence. More details of the procedure are given in the experimental part.

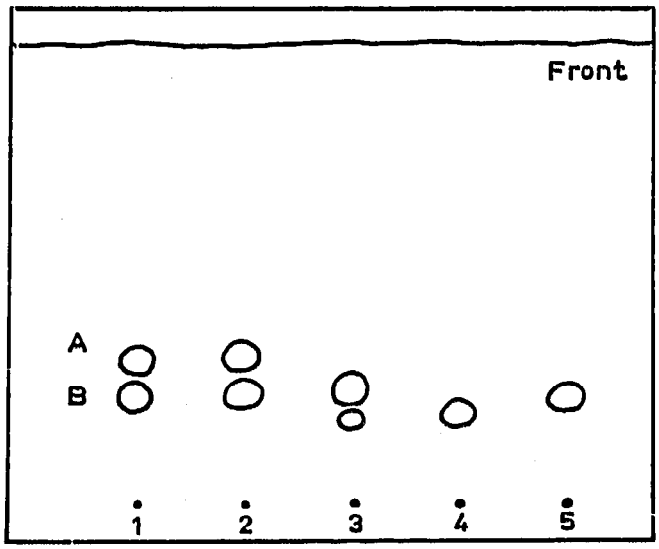

Fig. 3. TLC separation of sterols. Adsorbent: silica gel G–silver nitrate. Solvent: chloroform–ether–acetic acid (97:2.3:0.5). Time of run: 1.5–2 h. Detection: dibromofluorescein. Spot 1: 150 μg of a 1:1 mixture of dihydrocholesterol (A) and cholesterol (B); spot 2: 150 μg of a 1:1 mixture of Δ^7-cholestenol and cholesterol (B); spot 3: third band phytosterols isolated from coconut fat; spot 4: 80 μg ergosterol D; and spot 5: 80 μg stigmasterol.

The R_s values of several sterols in this system (A) are given in Table III. Besides separating dihydrocholesterol and cholesterol, this system enables the separation of several other pairs of closely related sterols *e.g.* cholesterol (FC_{27})–Δ^7-cholestenol (FC_{27}), cholesterol (FC_{27})–lanosterol ($FC_{30}F$), vitamin D_2 ($3FC_{28}F$)–dihydrovitamin D_2 ($3FC_{28}$), cholesterol (FC_{27})–"third-band phytosterols" ($FC_{28}F$), and 5-dihydroergosterol ($FC_{28}F$)–stigmasterol ($FC_{29}F$). Some of these sterol separations are shown in Fig. 3.

The R_s values of Table III and the chromatoplate of Fig. 3 indicate that in this system mono- and many di-unsaturated sterols have the same migration rate. Cholesterol, β-sitosterol, and stigmasterol show identical R_s values. Similarly, sterols having one double bond in the $\Delta^{7(8)}$ position like Δ^7-cholestenol, Δ^7-ergostenol, and 5-dihydroergosterol cannot be separated in this system.

Remarkably, these Δ^7-sterols form a critical pair with dihydrocholesterol and not with the corresponding Δ^5-sterol *viz.* cholesterol. The possibility of *separating Δ^5- and Δ^7-sterols* in this system is therefore of practical importance.

The triterpenoid alcohols lanosterol, agnosterol, and their 24-dihydro compounds show quite a high migration rate ($R_s \simeq 1.70$). Ergosterol D, *viz.* $\Delta^{7,9(11),22}$-ergostatrien-3β-ol ($2FC_{28}F$), with two conjugated double bonds has an R_s value of 0.83.

Sterols and steroids having a system of *three* conjugated double bonds like vitamins D_2 ($3FC_{28}F$) and $\Delta^{9(11)}$-dehydroergosterol ($3FC_{28}F$) have quite low migration rates *viz.* 0.64 and 0.69, respectively.

On account of their migration rates in the normal reversed-phase system and in the "bromine-system", the third-band phytosterols of *e.g.* coconut fat were ten-

TABLE III

SEPARATION OF STEROLS AND STEROL ACETATES ON SILICA GEL G–SILVER NITRATE LAYERS

System A: chloroform–ether–acetic acid (97:2.3:0.5); system B: chloroform–light petroleum (b.p. 60–80°)–acetic acid (25:75:0.5).
Spotted amount: 50–100 μg.
Time of run: 1–2 h.
Detection: 0.2% dibromofluorescein.

Compound	*Shorthand designation*	*R_s value of sterols in A*	*R_s value of sterol acetates in B*
Agnosterol	$2FC_{30}F$	1.68	0.40
24-Dihydroagnosterol	$2FC_{30}$	1.61	
Lanosterol	$FC_{30}F$	1.70	0.78
Dihydro-β-sitosterol	C_{29}	1.14	1.30
Dihydrocholesterol	C_{27}	1.14	1.25
Cholesterol	FC_{27}	≡ 1.00	≡ 1.00
β-Sitosterol	FC_{29}	1.00	1.00
Δ^7-Cholestenol	FC_{27}	1.17	1.14
Δ^7-Ergostenol	FC_{28}	1.22	1.21
5-Dihydroergosterol	$FC_{28}F$	1.13	0.88
Stigmasterol	$FC_{29}F$	0.98	0.87
Desmosterol	$FC_{27}F$	0.88	—
Vitamin D_2	$3FC_{28}F$	0.64	
Dihydrovitamin D_2	$3FC_{28}$	0.47	
Ergosterol D ($\Delta^{7,9(11),22}$-ergostatrienol	$2FC_{28}F$	0.83	
$\Delta^{9(11)}$-Dehydroergosterol	$3FC_{28}F$	0.69	
Brassicasterol	$FC_{28}F$	0.98(?)	0.68(?)
"Third-band phytosterols"	"$FC_{28}F$"?	0.87	0.33
Ergosterol	$2FC_{28}F$	0.44	0.35 *
7-Dehydrocholesterol	$2FC_{27}$	0.44	0.43 *

* After two developments; spotted amount 30 μg.

tatively supposed to have an $FC_{28}F$-sterol structure, slightly more polar than that of brassicasterol. The third-band phytosterols of coconut fat were isolated on a preparative scale from ten chromatoplates. After removing the contaminating undecane and after saponification of the sterol acetates, the residue was spotted on a silica gel G–silver nitrate plate. After the development procedure two spots appeared under U.V. radiation, a minor spot at $R_s = 1.05$ and a major one at $R_s = 0.87$. The latter spot has about the same migration rate as some poly-unsaturated sterols *e.g.* ergosterol D.

This experiment confirmed the conclusion, which was already drawn from the results in the bromine-system, that the polarity of the third-band phytosterols is somewhat higher than that of the isomerous brassicasterol.

In the bromine-system, only those sterols having a $\Delta^{5(6)}$ double band can be detected while all other sterol types are decomposed. Therefore, we may assume that one of the double bonds of the third-band phytosterols should be at $\Delta^{5(6)}$, while the special position of the other one may cause the relatively high polarity of the molecule[5]. In this respect we tentatively might consider a double bond situated nearer to the terminal alkyl groups *e.g.* $\Delta^{24(28)}$ ($\Delta^{5,24(28)}$-ergostadienol), $\Delta^{24(25)}$ (desmosterol) or $\Delta^{25(26)}$. Such positions are known to cause longer retention times in the gas chromato-

graphic analysis of fatty acids (ACKMAN AND BURGHER[44]) and sterols (RECOURT AND BEERTHUIS[45]). Since we did not succeed in resolving mono- and di-unsaturated sterols like cholesterol and stigmasterol in this system, it was supposed[46] that the free hydroxyl group exerts too great an influence on the adsorption of the sterols. It seemed worthwhile to study the separation of the less polar sterol acetates.

Separation of sterol acetates

After testing various solvent mixtures the best results for fractionating mixtures of sterol acetates on silica gel G–silver nitrate layers were obtained when using the mobile phase B: chloroform–light petroleum (b.p. 60–80°)–acetic acid (25:75:0.5). In this system still better separations of the groups of saturated, mono-, di-, and polyunsaturated sterols may be accomplished. The R_s values of a number of sterols in this system *viz.* B are given in Table III.

Although the acetates of cholesterol and stigmasterol have slightly different migration rates in this system their separation is only possible under favourable conditions *e.g.* at a spotted amount of only 30 μg of the mixture (see Table III).

The triterpenoid alcohols, lanosterol and agnosterol were clearly separated, analogous to the investigations of DEN BOER[46].

In contrast to the results of system A, in system B the spot of Δ^7-cholestenol does not coincide with that of dihydrocholesterol, but has an R_s value intermediate to those of dihydrocholesterol and cholesterol. Under favourable conditions it is possible to resolve the spots of Δ^7-cholestenol and Δ^7-ergostenol.

This system enables a clear separation between the acetates of Δ^7-cholestenol (or Δ^7-ergostenol) and the di-unsaturated 5-dihydroergosterol, whereas the corresponding sterols have identical R_s values in system A. The sterol mixture from the starfish *Asterina pectinifera*, which according to TOYAMA contains Δ^7-cholestenol,

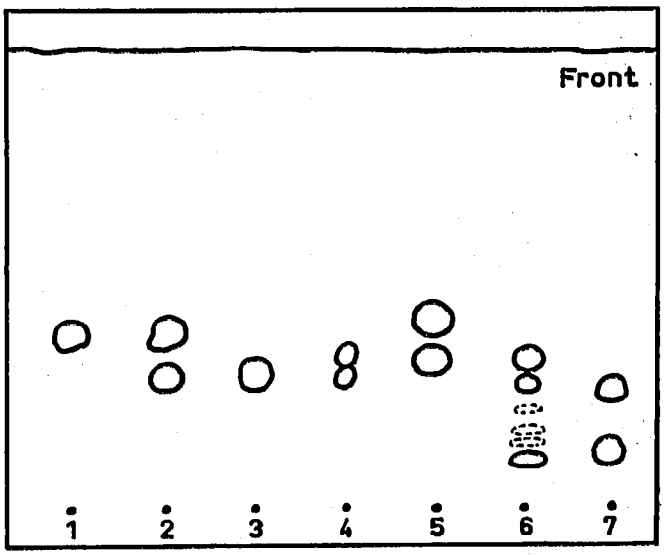

Fig. 4. TLC separation of sterol acetates. Adsorbent: silica gel G–silver nitrate. Solvent: chloroform–light petroleum (b.p. 60–80°)–acetic acid (25:75:0.5). Time of run: 1–2 h. Detection: dibromofluorescein. Spot 1: 100 μg Δ^7-cholestenol acetate; spot 2: 150 μg of the acetates of *Asterina pectinifera* sterols; spot 3: 100 μg 5-dihydroergosterol; spot 4: 30 μg of a 1:1 mixture of cholesterol acetate and stigmasterol acetate; spot 5: 200 μg of a 1:1 mixture of dihydrocholesterol acetate and cholesterol acetate; spot 6: 60 μg of the phytosterol acetates from coconut fat; and spot 7: 100 μg of a 1:1 mixture of lanosterol acetate and agnosterol acetate.

$\Delta^{7,22}$-C_{28} sterol, α-spinasterol and Δ^{7}-stigmastenol, was thus fractioned into two major spots at R_S values of 0.88 and 1.08. These spots are most likely attributed to the acetates of Δ^{7}-cholestenol and 5-dihydroergosterol (see Fig. 4, spot 2). Furthermore, two minor spots at lower R_S values *viz.* 0.48 and 0.29 were visible when spotting 200 μg of the sterol acetate mixtures. Most probably, these minor spots are due to "polar" sterols, causing the fourth band at $R_S = 1.22$ in the normal reversed-phase system (see Fig. 1, spot 8).

In spite of their low migration rates the acetates of the critical-pair partners 7-dehydrocholesterol and ergosterol are separable in system B. In general, in this system the acetates of "polar" sterols like these provitamins D and triterpenoid alcohols, *e.g.* agnosterol, have quite low migration rates.

Using system B the phytosterol mixtures isolated from various edible oils and fats can be fractionated into several distinct spots. An amount of 60 μg of the acetates of the phytosterols isolated from coconut fat is fractionated in this way into two clearly separated spots at $R_S = 1.00$ and $R_S = 0.88$, caused by β-sitosterol and stigmasterol acetate, respectively. Furthermore, three weak fluorescent spots are visible at R_S values of 0.68, 0.49 and 0.44, respectively, and a relatively large spot at $R_S = 0.33$, due to the "third-band phytosterols" (see Fig. 4, spot 6).

The identity of the third-band phytosterol spot was deduced among other things from the study of the acetates of the phytosterols from almond oil, which only exhibit a β-sitosterol band in the undecane/acetic acid–acetonitrile (1:3) system. Besides a β-sitosterol and a "third" band at $R_S = 1.00$, on silica gel G–silver nitrate plates only one other band (at $R_S = 0.33$) is shown, thus identifying itself with the third-band phytosterols in the reversed-phase system.

EXPERIMENTAL

Reversed-phase system

A volume of about 400 ml of the solvent mixture acetic acid–acetonitrile (1:3) is saturated with 18 ml of undecane* by shaking both phases in a separatory funnel. After a settling time of 16 h at 22–23° both layers are separated. The undecane is diluted with light petroleum (b.p. 40–60°) to a 10 % solution; the latter is used for the impregnation procedure.

The acetic acid–acetonitrile mixture is introduced into a small chromatographic vessel of about 19 × 30 × 7 cm, which is lined with filter paper on all sides to ensure complete saturation. The vessel is equilibrated at 22–23° for 24 h.

Glass plates of 14 × 24 cm are coated with a kieselguhr G (Merck)–water (1:2)-mixture, according to the procedure of STAHL. The 0.24–0.28 mm layers are activated by heating at 100° for 15 min. After cooling to room temperature, the layers are impregnated with the 10 % undecane solution. The chromatoplate is taken between thumb and forefinger of both hands (wearing rubber gloves) and is dipped carefully into a shallow tray containing this solution. Care must be taken that the layer is not damaged. After the impregnation procedure, lasting only a few seconds, the plate is held upside down for some ten seconds to remove the excess of undecane solution. The plate is then stored at room temperature on a horizontal surface for a standard

* Undecane standardized, b.p. 190–220°, $n_D^{20} = 1.4307$, available from J. HALTERMANN, Hamburg.

evaporation period. The duration of this period depends on the thickness of the layer, dimensions of the plate, temperature of the room etc. In our case a period of 80 min was selected, after which time 0.19 g of undecane was left on the plate *viz.* 0.04 g/g of kieselguhr G*.

During this evaporation period hexagonal** pieces (of 20 × 23 mm) are removed from the layer with a brush, using an appropriate template. In this way 4 chromatograms are modelled. The distance of the centre of the 8 mm wide "bridges" to the bottom of the plate is 40 mm. At the end of the evaporation period the sterol acetate solution (preferably 15 mm^3 of a 0.2 % ethereal solution) is spotted at that centre by means of a micro pipette.

The chromatoplate is then developed in the chromatographic vessel. When the solvent front has travelled 20 cm in some 1.5 h, the development is discontinued and the plate is dried in air for 2–4 h and afterwards for 45 min at 100°.

The plate is sprayed with a 20 % ethanolic solution of phosphomolybdic acid (Merck) and heated for 5–10 min at 100° till the bands are coloured to the maximum intensity. After each development procedure, the solvent mixture must be discarded and a freshly prepared mixture should be used.

"Bromine-system"

In this bromine-system nearly the same procedure applies. The temperature during development should preferably be 18–20°. Prior to the development procedure 0.5 % of bromine is added *within* the chromatographic vessel. The developed chromatoplate is dried in air for 2 h and then for some 10 min at 100°. Generally, during the heating bright blue bands appear. The bands of brassicasterol and of the FC_{28}F-sterols are coloured faintly. To increase the colour intensity the warm plate is afterwards sprayed with a 50 % solution of antimony(III) chloride in acetic acid or with an acetic anhydride–sulfuric acid 50 % (1:2) mixture. The colour of the bands is now intensified, especially those of brassicasterol and FC_{28}F-sterols, which attain a violet shade. The bands of the other sterols are coloured bright blue.

"Silver nitrate layers"

13 g of silver nitrate (Merck) is dissolved in 60 ml of water and 30 g of silica gel G (Merck) is admixed. The mixture is spread upon 4 chromatoplates of 20 × 20 cm. The plates are dried in the dark for 2 h and then activated for 1 h at 110°. After spotting 100–200 μg of the ethereal solutions of the sterols or their acetates, the plate is developed with either the solvent mixture A, chloroform–ether–acetic acid (97:2.3:0.5), or B, chloroform–light petroleum–acetic acid (25:75:0.5). The chromatographic vessel is lined with filter paper which is saturated with the solvent mixture and equilibrated for some 16 h. After the development procedure, the chromatoplate is dried in air and then sprayed with a 0.2 % solution of dibromofluorescein in 96 % ethanol. The plate is then viewed under U.V. radiation of 365 mμ and the fluorescent spots are marked with a pencil.

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* On the common 20 × 20 cm "Desaga" plates with layers of about 0.18 mm, a pentagonal figure with its base at 10 mm distance from the bottom of the plate is modelled. In this case an evaporation period of 60 min in air is employed (degree of impregnation 0.08 g/g). Length of run 17 cm.

** Pentagonal figures may also be used.

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SUMMARY

The analysis of sterols by means of thin-layer chromatography (TLC) is investigated. Previous experiments of the authors on the reversed-phase system undecane/acetic acid–acetonitrile (1:3) are amplified. For identification purposes the sterols are characterised by their so-called carbon numbers.

By adding bromine to the mobile phase several critical pairs of sterols were resolved. The properties of this "bromine system" are discussed.

On silica gel G–silver nitrate layers the sterols or their acetates are separated according to their degree of unsaturation. The procedures for this $AgNO_3$ TLC, which enable a clear separation of many sterols, are described in detail. The above chromatographic analyses can be applied to the identification of unknown sterols isolated from natural sources, *e.g.* coconut fat and *Asterina pectinifera*.

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