THE ANALYSIS OF MIXTURES OF ANIMAL AND VEGETABLE FATS

III. SEPARATION OF SOME STEROLS AND STEROL ACETATES BY THIN-LAYER CHROMATOGRAPHY*

J. W. COPIUS PEEREBOOM AND HENNY W. BEEKES

*Government Dairy Station, Leiden (The Netherlands)***

(Received April 12th, 1962)

Small amounts of animal and vegetable fats in mutual mixtures can be detected by chromatographic analysis of their sterols. A separation of cholesterol (abbreviated formula FC27) and phytosterols, such as β-sitosterol (FC29), stigmasterol (FC29F), and campesterol (FC28), has been accomplished by reversed-phase paper chromatography in the system paraffin oil/acetic acid–water (84:16)[1]. The migration rates of these sterols as well as of several others and the relationship between structure and R_F value were studied[2]. However, a more general application of this sterol analysis to the routine procedures of biochemistry and fat chemistry is hampered by the time-consuming character of reversed-phase paper chromatography, the times of accommodation and of elution being 16 h and 44 h respectively.

At present thin-layer chromatography (TLC) is being applied to the analysis of several groups of lipoid substances (for reviews see, *e.g.*, ref.[3]). Separations in the group of sterols and related triterpenoid alcohols by TLC on layers of silicic acid were already mentioned by JANECKE[4] (cholesterol–vitamin D_3), and TSCHESCHE[5] (*e.g.* lanosterol–β-amyrin).

We have found that separations within this group of sterols and triterpenoid alcohols can also be achieved on layers of "kieselgur G" (Merck). The relative R_S values of some sterols are given in Table I.

Although some sterols that differ in the type and number of double bonds are clearly separated (*e.g.* cholesterol–ergosterol, and lanosterol–cholesterol), the separation of cholesterol from the phytosterols could not be achieved. The structural differences between these related sterols, which only consist in the presence of one or two methyl groups or a double bond in the side chain, are too small to make separations by methods based on adsorption chromatography possible.

Recently, the technique of reversed-phase TLC has been applied by KAUFMANN *et al.*[6] to the analysis of several lipoid substances, such as fatty acids, cholesterol esters, fatty alcohols, diglycerides, and even of closely related mixed triglycerides, such as palmitodiolein and tripalmitin. For the greater part the principles and results of this technique are analogous to those of reversed-phase paper chromatography, but the advantages are a tenfold reduction in the time of analysis, smaller, less diffuse spots, which permit better separations, and the possibility of spraying with

* For Parts I and II of this series, see refs. [1] and [2].

** Rijkszuivelstation, Vreewijkstraat 12b, Leiden, The Netherlands.

TABLE I

R_S[a] VALUES OF SOME STEROLS ON KIESELGUR G

Mobile phase: cyclohexane–ethyl acetate (99.5:0.5). Temperature: 23°. Detection: phosphomolybdic acid. Spotted amounts: 0.6 μg. Time of run: 2–3 h.

Compound	R_S	*Compound*	R_S
Cholesterol	≡ 1.0	7-Dehydrocholesterol	0.93
β-Sitosterol	1.00	Zymosterol	1.02
Stigmasterol	1.00	Dihydrolanosterol	1.38
Dihydrocholesterol	0.93	Lanosterol	1.37
Ergosterol	0.89	Agnosterol	1.35
Vitamin D_2	1.11	Dihydroagnosterol	1.34

[a] S = cholesterol.

more agressive colour reagents. We have applied the techniques described by KAUFMANN *et al.* to the analysis of some sterols and sterol acetates.

Chromatoplates of kieselgur G (Merck) are impregnated with undecane* by dipping them into a 10% solution of this hydrocarbon in petroleum ether. After drying in the air, hexagonal holes are brushed out of the kieselgur layer, leaving four chromatograms modelled on the MATTHIAS technique (Fig. 1). Quantities of about

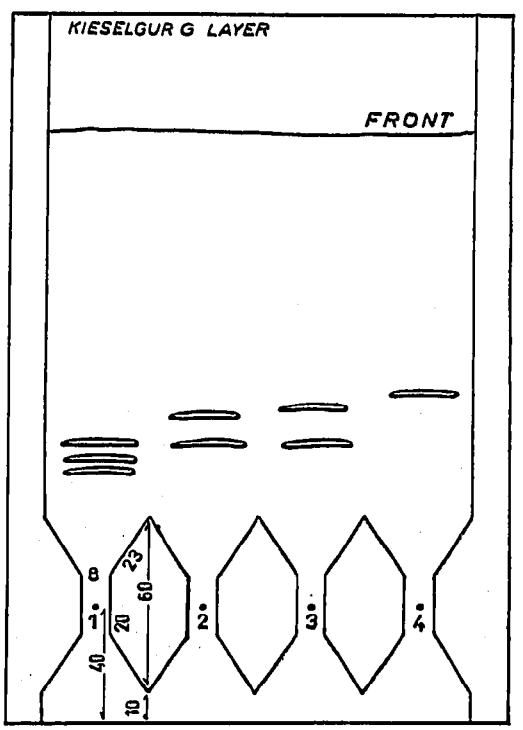

Fig. 1. Separation of some sterol acetates by reversed-phase TLC. Adsorbent: kieselgur G (Merck). Stationary phase: undecane stand. Mobile phase: acetic acid–water (92:8). Time of run: 5 h. Length of run: 20 cm. Detection: phosphomolybdic acid. Temperature: 23°. Measurements in mm. Spot 1 = 5 μg of a cholesterol acetate–soya bean oil phytosterol acetate 4:6 mixture. Spot 2 = Cholesterol acetate–epicholesterol acetate 1:1 mixture. Spot 3 = Ergosterol acetate–cholesterol acetate 1:1 mixture. Spot 4 = 7-Dehydrocholesterol acetate.

* Available from J. HALTERMANN, Hamburg.

5 μg of the sterols and sterol acetates are spotted, and finally the chromatoplate is developed for 5–6 h with acetic acid–water, 90:10 or 92:8 mixtures respectively. After drying, the plate is sprayed with a phosphomolybdic acid solution or with other sensitive colour reagents. The small, clearly discernible blue-green bands of ergosterol, cholesterol, stigmasterol, β-sitosterol, agnosterol and their respective acetates are completely separated, the results being for the greater part analogous to those obtained in the separation of sterols by reversed-phase paper chromatography (Fig. 1).

Preliminary experiments stressed the importance of mutual saturation of the mobile and stationary phases at a temperature of 22–24°. If this condition has not been fulfilled, dissolutions occur caused by solving of the stationary phase, or by variations in temperature. In that case a second front line above the starting points appears on the chromatoplate. The reproducibility of the degree of impregnation is far less than with reversed-phase paper chromatography; consequently there is a greater variation in the R_F values. The relative R_S values (S = cholesterol) in both systems, which prove to be fairly reproducible, are given in Table II.

TABLE II

R_S[a] VALUES OF SOME STEROLS AND STEROL ACETATES OBTAINED IN REVERSED-PHASE TLC
Systems: (A) undecane/acetic acid–water (90:10); (B) undecane/acetic acid–water (92:8).

Compound	*Abbr. formula*	*R_S values of sterols in system A*	*R_S values of sterol acetates in system B*
Cholesterol	FC27	≡1.0	≡1.0
Stigmasterol	FC29F	0.93	0.91
β-Sitosterol	FC29	0.86	0.83
Brassicasterol	FC28F	1.00	1.00
Ergosterol	2FC28F	1.16	1.22
7-Dehydrocholesterol	2FC27	1.12	1.26
Lanosterol	FC30F	0.84	0.97
Dihydrocholesterol	C27	0.90	0.89
Epicholesterol	FC27	0.90	1.16
Agnosterol	2FC30F	0.76	0.86

[a] S = cholesterol.

By applying other stationary and mobile phases, *e.g.* silicone oil, paraffin oil, etc., with acetic acid, propionic acid, monochloroacetic acid etc. water mixtures, similar results can be obtained; an example is the separation of sterol acetates in the system tetradecane* (b.p. 240–250°)/acetic acid–water (96:4).

The results obtained in the separation of sterols and sterol acetates are, on the whole, analogous. A difference in behaviour is shown, for instance, by the pair cholesterol–epicholesterol; the former has the highest R_F value in system A, whereas the migration rate of epicholesterol acetate in system B appears to be higher than that of cholesterol acetate. In the analysis of mixtures of vegetable and animal fats, reversed-phase TLC of the sterol acetates offers some practical advantages; the melting point of these sterol acetate mixtures is used in the phytosterol acetate test of BÖMER and gives already some indication about the composition of the fat mixture.

* Available from J. HALTERMANN, Hamburg.

The critical pair cholesterol–brassicasterol, both of which have the same R_F values in these reversed-phase systems, was separated by adding 0.5 % Br_2 to the mobile phase according to the technique of KAUFMANN *et al.*[6]. The band of brominated brassicasterol acetate then moves ahead of the cholesterol acetate dibromide with a relative R_S value of about 1.19. The detection was accomplished by means of a chlorosulphonic acid–acetic acid, 1:2 mixture[5], blue to purple bands being obtained.

Because of this differentiation of cholesterol and all the phytosterols, reversed-phase TLC was applied with success to the analysis of mixtures of animal and vegetable fats.

Further experiments are in progress.

Experimental procedure

Glass plates of 14 × 24 cm were coated with a mixture of kieselgur G (Merck)–water (1:2) according to the technique of STAHL. After heating for 1/2 h at 115° the resulting 0.2 mm layer was impregnated with undecane* by dipping the chromatoplate carefully into a 10% solution of undecane (b.p. 190–220°) in petroleum ether (b.p. 40–60°). Care must be taken that the layer is not damaged by unnecessary handling. The chromatoplate is held 1 min with the bottom side upwards and is then stored 1 h at room temperature to evaporate the petroleum ether. An amount of approx. 0.50 or 0.30 g undecane is left on the plate (about 0.13 g or about 0.09 g undecane per g kieselgur respectively). After placing an appropriate mould on the chromatoplate hexagonal holes are scratched out of the layer with a brush. The sterol or sterol acetate solution (5 mm^3 of a 0.1 % ethereal solution) is spotted in the centre of the 8 mm wide "bridges". The chromatoplate is developed with the acetic acid–water, 90:10 or 92:8 mixture by the ascending technique at a temperature of 23°. The 10 % undecane solution and the mobile phase are mutually saturated at the same temperature the day before. The saturated acetic acid–water layer is introduced into a chromatographic vessel of 19 × 7 × 30 cm, supplied with filter paper at the sides to ensure complete saturation. When the solvent front has travelled 20 cm in 5–6 h at a temperature of 23–25° the development is interrupted, and the plate is dried about 3 h in the air and 45 min at 90°. After spraying with a 20 % ethanolic solution of phosphomolybdic acid (Merck), the chromatoplate is heated about 5–10 min at 90°. Blue-green bands appear on a light green background.

ACKNOWLEDGEMENTS

The authors express their thanks to Ir. J. B. Roos and to Dr. J. G. VAN GINKEL, Director of the Government Dairy Station.

SUMMARY

The behaviour of several sterols and related compounds on chromatoplates prepared from kieselgur G has been studied. Using a cyclohexane–ethyl acetate mixture (99.5:0.5), the separation of some sterols, *e.g.* ergosterol–cholesterol, cholesterol–lanosterol, cholesterol–vitamin D_2 has been achieved. In the reversed-phase system: undecane/acetic acid–water several sterols or their corresponding acetates can be

* Availabe from J. HALTERMANN, Hamburg.

fractionated. In this way cholesterol acetate and the acetates of the major phytosterols, *viz.* β-sitosterol and stigmasterol, are clearly separated, thus enabling the analysis of mixtures of vegetable and animal fats. By adding bromine to the mobile phase a differentiation of the critical pair of sterols cholesterol–brassicasterol has been accomplished.

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NOTE ADDED IN PROOF

Further experiments showed that in addition to the acetic acid–water mixtures the same separation of sterols etc. can be accomplished applying an acetic acid - acetonitrile 1:3 solvent mixture. The time of development with this undecane/acetic acid–acetonitrile 1:3 system is considerably less than with the acetic acid–water systems, *viz.* only 1 1/2–2 h.

The R_S values given in Table II indicate that a fractionation of cholesterol and dihydrocholesterol in these systems is possible. Spotting 50–80 μg of sterol acetates we were able to detect even small amounts of dihydrocholesterol acetate (down to 5 %) in an excess of cholesterol acetate.