notes on methodology

Improved separation of sterols by reversed-phase thin-layer chromatography

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SUMMARY Some closely related sterols have been separated with better resolution in a shorter period of time than has previously been reported. Separations were effected on the basis of carbon number and the number and location of double bonds through the use of paraffin-impregnated kieselguhr chromatoplates in the system (paraffin oil)/(acetone–water 4:1).

SUPPLEMENTARY KEY WORDS phytosterols · cholesterol precursors

THIN-LAYER chromatography (TLC) has been applied to the separation of sterols by a number of investigators (1–12). Closely related sterols like the phytosterols and the intermediates in the biosynthesis of cholesterol are difficult to separate by TLC on adsorbents and recourse to argentation TLC (2–10) and reversed-phase TLC (6, 10–12) has been necessary. We report here an adaptation of the method of Copius-Peereboom and Beekes (10–12) in which we use paraffin oil as the stationary phase and aqueous acetone, saturated with paraffin, as the mobile phase. Separation even of closely related sterols such as cholesterol and β-sitosterol was possible without the necessity of applying them to the chromatoplates as their acetates. The separation was better than has previously been reported with the acetates, and the time of development with this system is shorter (only 1 hr). Also, the use of anisaldehyde–sulfuric acid as the detecting reagent, a modification of a procedure first used for bile acids (13), usually allows as little as 2 μg of sterols to be detected.

Materials. Kieselguhr G (Merck, medium grain size: maximum 10 μ) was purchased from Brinkmann Instruments Inc., Westbury, L. I., N. Y. Paraffin oil, N. F., was purchased from Arthur H. Thomas Co., Philadelphia, Pa., and *p*-anisaldehyde from J. T. Baker Chemical Co., North Phillipsburg, N. J. Cholesterol was purchased from Fisher Scientific Company, Fair Lawn, N. J., and was recrystallized from methanol. Cholestan-3β-ol and 7-cholesten-3β-ol (lathosterol) were purchased from Steraloids, Inc., Pawling, N. Y. Desmosterol and commercial "β-sitosterol" (shown to be a mixture of campesterol and β-sitosterol, 2:3, by gas–liquid chromatography) were purchased from Calbiochem, Los Angeles, Calif. Pure β-sitosterol was prepared from stigmasterol by the method of Steele and Mosettig (14). Stigmasterol, 7-dehydrocholesterol, and ergosterol were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. 7-Dehydrocholesterol was purified by several recrystallizations from ethanol. Ergosterol was chromatographed on alumina and subsequently crystallized from ethanol. Fucosterol was extracted from *Fucus vesiculosus*. Commercial lanosterol (shown to be principally a mixture of 8,24-lanostadien-3β-ol and lanost-8-en-3β-ol, 1:1, by gas–liquid chromatography) and lanost-8-en-3β-ol were purchased from Mann Research Labs., Inc., New York. Spinasterol was obtained from the collection of the late Dr. Werner Bergmann through the courtesy of Dr. William Stokes. 24-Methylene cholesterol was prepared in this laboratory by Mr. Jerry W. Cannon according to established procedures (15,16). 24-Methylene dihydrolanosterol and 24-ethylene dihydrolanosterol were prepared in this laboratory by Dr. Joseph P. John and Dr. Karl H. Raab from lanosterol *via* 24-ketolanost-8-en-3β-yl acetate (17) and the appropriate Wittig reagents.

Preparation of Chromatoplates. About 300 ml of acetone–water 4:1 was shaken in a separatory funnel with 30 ml of paraffin oil. The mixture was kept for 18 hr at 24–25°C. The two layers were then separated. The paraffin oil layer (lower phase) was diluted with petroleum ether (bp 30–60°C) to a 5% solution; this solution was used for the impregnation procedure. The acetone–water mixture (100 ml, upper phase) was introduced into a chromatographic development tank (26 × 27 × 7 cm) that was lined on three sides with filter paper. The atmosphere in the tank was equilibrated at 24–25°C for 3 hr.

Glass plates, 20 × 20 cm, were coated with water–Kieselguhr G 2:1 according to the procedure of Stahl (18). The layers, 250 μ thick, were allowed to dry in air for 0.5 hr and then activated at 110°C for 1 hr. After cooling to room temperature, the layers were impregnated with the 5% paraffin solution by carefully introducing them into a tank filled with the solution (11). After 1 min the plates were removed, held upside down for a few seconds, then kept at room temperature on a horizontal surface for 30 min.

Development of Chromatoplates. The sterols (in about 2 μl of a 0.1% w/v solution in ethyl acetate) were applied to the plates by means of a microsyringe, and the chromatoplate was then developed. After the solvent front had ascended to a height of 15 cm (about 1 hr) the

Abbreviation: TLC, thin-layer chromatography.

plates were dried in air for 5 min, sprayed with a reagent consisting of 1.5 g of *p*-anisaldehyde and 1.5 ml of concentrated H_2SO_4 in 27 ml of 90% ethanol, and heated for 5–10 min at 110°C. The sterol spots were blue on a pale pink background. When more than 5 μg was used, any spots that were close together tended to merge. However, with thicker plates excellent separations, for example of lanosterol from 24,25-dihydrolanosterol, were achieved on a preparative scale. After elution from the plate the paraffin was removed by column chromatography.

Results. The R_s (s = cholesterol), R_f, and R_m [log $(1/R_f - 1)$] values of the sterols used are given in Table 1. The R_s and R_f values listed indicate that better separations were obtained than those reported by Copius-Peereboom and Beekes (10–12) for corresponding sterol acetates. An example of the separation is given in Fig. 1. The decrease in polarity with increase in chain length is revealed by the series cholesterol, campesterol, and β-sistosterol as well as by the series lanosterol, 24-methylene dihydrolanosterol, and 24-ethylidene dihydrolanosterol. The plots (Figs. 2 and 3) of R_m/(carbon number) for the two series gave approximately straight lines. The effect of introduction of double bonds in the molecule is observed by four sets of compounds, namely (*a*) the C_{27} series: cholestan-3β-ol, cholesterol, lathosterol, 7-dehydrocholesterol, and desmosterol; (*b*) the C_{28} series: compesterol, 24-methylenecholesterol, and ergosterol; (*c*) the C_{29} series: β-sitosterol, stigmasterol, spinasterol, and fucosterol; and (*d*) the lanosterol series: lanosterol and lanost-8-en-3β-ol. Plots (Figs. 4 and 5) of (R_m values)/(number of double bonds) in the C_{27} and C_{28} series show an almost linear relation in each series.

TABLE 1 R_s (s = CHOLESTEROL), R_f, AND R_m VALUES* OF STEROLS IN THE REVERSED-PHASE SYSTEM: (PARAFFIN OIL)/(ACETONE–WATER 4:1)

Sterol	R_s	R_f	R_m*
5,7,22-Ergostatrien-3β-ol (ergosterol)	1.26	0.65	−0.269
5,24-Cholestadien-3β-ol (desmosterol)	1.24	0.64	−0.25
5,7-Cholestadien-3β-ol (7-dehydro cholesterol)	1.22	0.63	−0.23
24-Methylene-5-cholestene-3β-ol	1.12	0.58	−0.14
5,24(28)-Stigmastadien-3βol (fucosterol)	1.01	0.53	−0.052
5-Cholesten-3β-ol (cholesterol)	1.00	0.52	−0.035
7-Cholesten-3β-ol (lathosterol)	0.99	0.51	−0.017
7,22-Stigmastadien-3β-ol (spinasterol)	0.94	0.49	0.017
5,22-Stigmastadien-3β-ol (stigmasterol)	0.92	0.48	0.035
5-Ergosten-3β-ol (campesterol)	0.90	0.47	0.052
5-Stigmasten-3β-ol (β-sitosterol)	0.78	0.41	0.158
8,24-Lanostadien-3β-ol (lanosterol)	0.68	0.35	0.269
Cholestan-3β-ol	0.66	0.34	0.288
24-Methylene-8-lanosten-3β-ol	0.58	0.30	0.368
24-Ethylidine-8-lanosten-3β-ol	0.47	0.25	0.477
8-Lanosten-3β-ol (dihydrolanosterol)	0.47	0.25	0.477

* R_m = log $(1/R_f - 1)$.

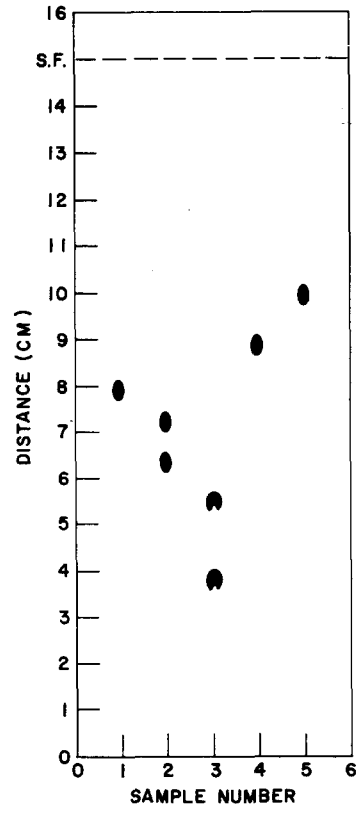

FIG. 1. Chromatogram of *1*, cholesterol; *2*, campesterol and β-sitosterol; *3*, lanosterol and 24,25-dihydrolanosterol; *4*, 24-methylene cholesterol; and *5*, ergosterol. Movement of the compounds was, respectively (in cm), 7.80, 7.05, 6.15, 5.25, 3.75, 8.70, and 9.75. S.F., solvent front.

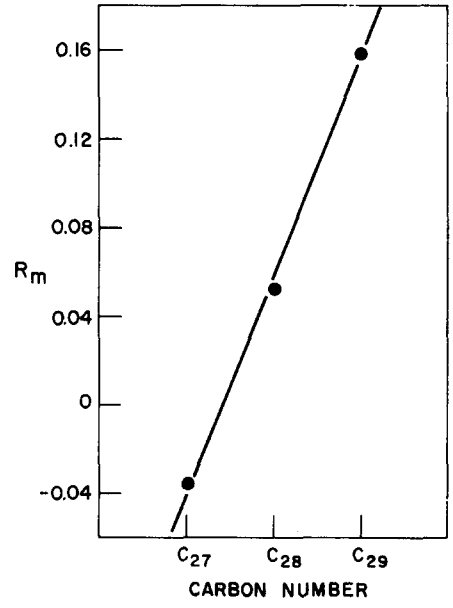

FIG. 2. Relationship of R_m to carbon number in the Δ^5-series (cholesterol, campesterol, and β-sitosterol).

Cholestanol and lathosterol, which have previously been difficult to separate (5, 7), are readily resolved by this technique. Also, a rapid indication of the structures of components of naturally occurring sterol mixtures may be obtained.

An illustration of the utility of this technique is the elucidation of a sterol mixture isolated from the blue-green alga, *Phormidium luridum*. After chromatography the mixture showed an intensely colored spot at R_s 0.78 diffusing as a faint band to about R_s 1.0. By an extrapolation of the data in Table 1 for R_m/(number of double bonds) in the C_{27} series, a sterol with three double bonds should have had an R_m value of 0.475 (R_s 1.44). In the C_{29} series the graph of R_m/(number of double bonds), when extrapolated, gave an R_m value of −0.15 (R_s 1.12). The data in Table 1 together with these extrapolations show that R_s values for common naturally occurring sterols possessing from one to three double bonds should range from 0.99 to 1.44 in the C_{27} series, from 0.90 to 1.26 in the C_{28} series (24-substituted), and from 0.78 to 1.12 in the C_{29} series (24-substituted). The R_s value (0.78) of the principal component in the mixture of sterols isolated from the algae indicates that the predominant sterol is a C_{29} compound with one double bond. The tailing to R_s 1.0 showed the presence of lesser amounts of other sterols. This conclusion was confirmed (19) by mass spectral, gas–liquid chromatographic, and other spectrophotometric data, which revealed that the mixture was composed principally of 7-stigmasten-3β-ol and smaller amounts of 5-stigmasten-3β-ol and other C_{29} sterols with two and three double bonds.

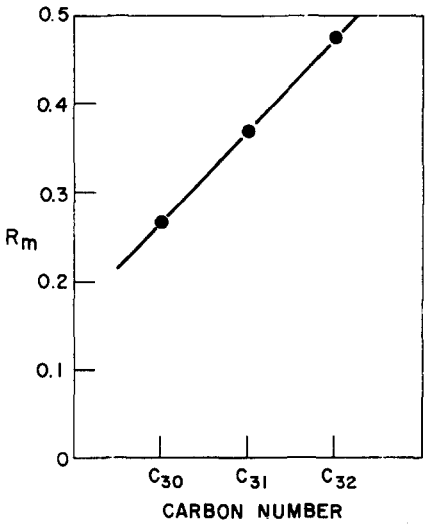

FIG. 3. Relationship of R_m to carbon number in the 4,4′,14-trimethyl series (lanosterol, 24-methylene-24,25-dihydrolanosterol, and 24-ethylidene-24,25-dihydrolanosterol).

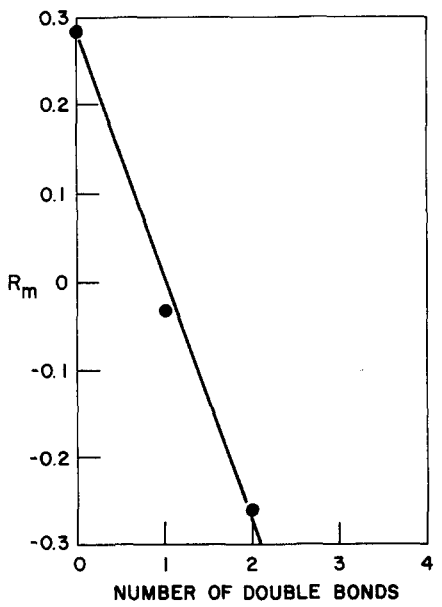

FIG. 4. Relationship of R_m to number of double bonds in the C_{27} series (cholestanol, cholesterol, and 7-dehydrocholesterol).

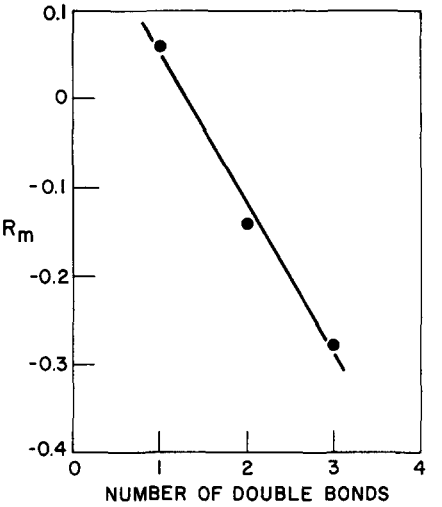

FIG. 5. Relationship of R_m to number of double bonds in the C_{28} series (campesterol, 24-methylene cholesterol, and ergosterol).

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