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LIQUID COLUMN CHROMATOGRAPHY OF FREE STEROLS

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SUMMARY

Liquid column chromatography with an apolar sorbent–eluent system permits the separation of free sterols differing in the number and position of double bonds and in their alkyl side chains. The method is applicable to the isolation of minute quantities of sterols from a large excess of closely related sterols.

INTRODUCTION

In connection with a research problem on vitamin D in plants, we required a method for isolating minute quantities of radioactive sterols from a large excess of closely related sterols. Among the chromatographic methods available for the purpose, liquid column chromatography (LC) is doubtedly the most suitable[1]. It not only provides the largest load capacity, but also the greatest variety of sorption systems. However, application to plant sterols has given disappointing results, because naturally occurring mixtures contain many homologs and analogs of very similar physical properties. High-pressure liquid chromatography (HPLC) has been shown to resolve such mixtures effectively, provided they are in the form of acetates[2,3] or benzoates[2,4]. The HPLC of free sterols, which is more convenient as well as preferable for quantitative analyses and mass spectrometry, has so far been only partially successful[5]. We now wish to report the LC separation of free sterols in an apolar chromatographic system. The model compounds used in this work are listed in Table I.

EXPERIMENTAL*

The apparatus was assembled from commercially available components. The pump was of the dual-piston reciprocating type, Tracor Model 990 (Tracor, Austin, Texas, U.S.A.), and a variable-wavelength detector, Tracor Model 970, was set at 205

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

TABLE I

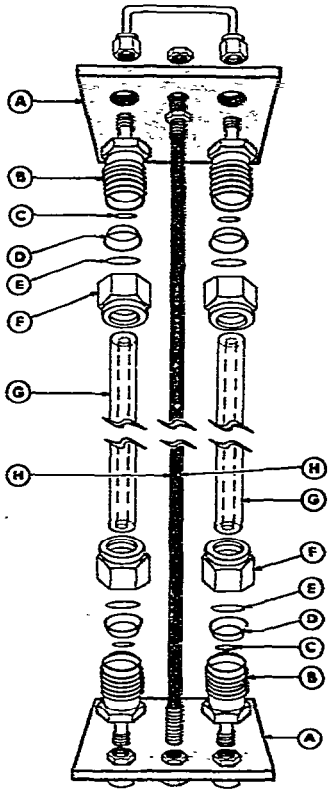
STEROLS USED AS MODEL COMPOUNDS

Trivial name	*IUPAC nomenclature*
Cholesterol	5-Cholesten-3β-ol
Lathosterol	5α-Cholest-7-en-3β-ol
7-Dehydrocholesterol	5,7-Cholestadien-3β-ol
Cholecalciferol	9,10-Seco-5,7,10(19)-cholestatrien-3β-ol
Campesterol	(24R)-24-Methyl-5-cholesten-3β-ol
Ergosterol	(24R)-24-Methyl-5,7,22(E)-cholestatrien-3β-ol
Ergocalciferol	9,10-Seco-5,7,10(19), 22(E)-ergostatetraen-3β-ol
Sitosterol	(24R)-24-Ethyl-5-cholesten-3β-ol
Stigmasterol	(24S)-24-Ethyl-5, 22(E)-cholestadien-3β-ol

nm. A single-channel recorder, Linear Model 355 (Linear Instruments, Irvine, Calif., U.S.A.), was attached to the output of the detector. The column was assembled from 1-ft. sections of Pyrex glass tubing, 3/8 in. O.D., 1/8 in. I.D., and filled with Bondapak C_{18}–Porasil B (Waters Assoc., Milford, Mass., U.S.A.).

For the packing of columns the following procedure was used (*cf.* Fig. 1). One end of the glass tube (G) was fitted with a 10-μm porosity end-fitting (B) (Waters Assoc.), into which a Viton O-ring (C) (National O-Rings-Federal-Mogul Corp., Detroit, Mich., U.S.A.) had been inserted. The end-fitting was fastened to the glass tube by means of a 3/8-in. brass nut (F), containing a 3/8-in. Nylon ferrule (D) (Crawford Fitting, Cleveland, Ohio, U.S.A.) which was backed by a 3/8-in. Buna O-ring (E) (National O-Rings-Federal-Mogul Corp.). After pressing the tube into the fitting, the brass nut was securely tightened. The fitting was then capped and the column was filled through a funnel by alternately pouring small portions of the sorbent into the tube and tapping its capped end against the table top. When a section was filled, it was capped with an assembly of fittings, as described above. The end-fitting of that assembly was filled with just enough sorbent to cover the O-ring, and the sorbent was wetted with isopropanol just before capping. Again, the fitting was pressed against the tube, and the brass nut was tightened. Pairs of columns prepared in this manner were then placed in the holder (A) shown in Fig. 1.

Each pair of columns was tested for the ability to separate cholesterol and stigmasterol (*cf.* Fig. 2). The eluent was a 0.5% solution of isopropanol in *n*-hexane ("distilled in glass" quality, Burdick and Jackson, Muskegon, Mich., U.S.A.). It was continuously recycled at a flow-rate of 1 ml/min. Samples were applied directly to the column by the stop-flow method. After stopping the pump and removing the 1/16-in. cap at the end-fitting, the excess solvent was aspirated. For the test shown in Fig. 2, a sample, containing 75 μg stigmasterol and 100 μg cholesterol in 5 μl of *n*-hexane–isopropanol (1:1) was injected into the fitting. The latter was connected to the pump which was then restarted. The quality of each pair of columns was thus tested and pairs giving over 90% separation of cholesterol and stigmasterol were assembled to form longer columns. Sixteen-foot columns used in this study were operated at pressures of ca. 200 p.s.i., well below the rupture point of the glass (1600 p.s.i.). The identity of the separated sterols was confirmed by collecting the column effluent and analyzing it by gas–liquid chromatography (GLC) according to the method of Grunwald[6].



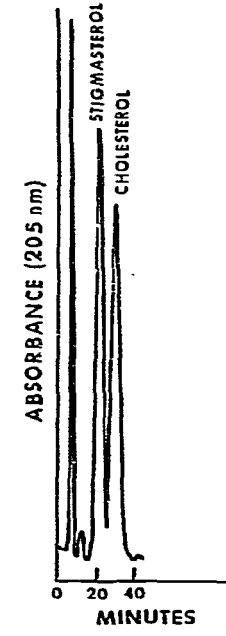


Fig. 1. Dual column assembly (exploded view). A = Aluminum holder, 1 in. × 2 in. × 1/8 in., with two 2 3/8-in. holes and one 1/8-in. hole; B = end-fitting containing a 10-μm porosity steel disk; C = Viton O-ring; D = Nylon ferrule; E = Buna O-ring; F = brass nut; G = glass tube; H = threaded brass rod.

Fig. 2. Quality test. Separation of 25 μg stigmasterol and 30 μg cholesterol. Column, 2 ft. × 1/8 in. I.D.; Bondapak C_{18}–Porasil B, 37–75 μm; eluent, 0.5% isopropanol in *n*-hexane; flow-rate, 1.0 ml/min at 120 p.s.i.

RESULTS AND DISCUSSION

A mixture of C_{27} sterols involved in the biosynthesis of vitamin D_3 was separated by LC, as shown in Fig. 3. A mixture of 18.7 μg cholecalciferol, 25.2 μg cholesterol, 16.1 μg lathosterol, and 28.9 μg of 7-dehydrocholesterol in 10 ml of *n*-hexane–isopropanol (1:1) was applied to a 16-ft. column of Bondapak C_{18}–Porasil B and eluted at a rate of 0.4 ml/min with 0.5% isopropanol in *n*-hexane over a period of 12 h. Under these conditions, the photo-isomers vitamin D_3 (cholecalciferol) and provitamin D_3 (7-dehydrocholesterol) were eluted *ca.* 8 h apart. The difference in elution time between the double-bond isomers cholesterol and lathosterol was *ca.* 100 min, and cholesterol was eluted *ca.* 2.5 h before its dehydrogenation product, 7-dehydrocholesterol.

Under the same conditions (Fig. 4), a sample containing 99 μg ergocalciferol, 248 μg sitosterol, 257 μg stigmasterol, 257 μg campesterol, 260 μg cholesterol, and 373 μg ergosterol gave a difference in elution time between the photoisomers vitamin D_2

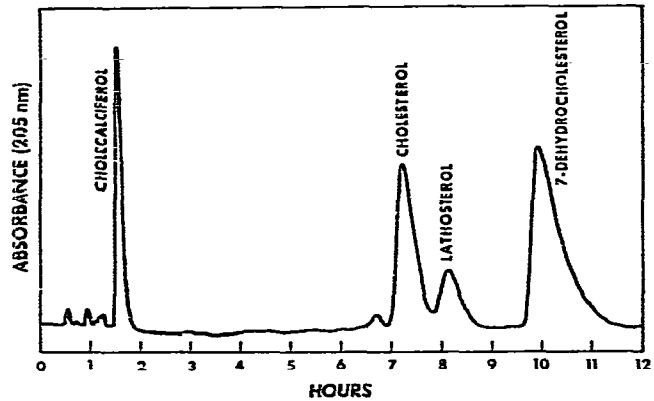


Fig. 3. Separation of C_{27} sterols by LC. Column 16 ft. × 1/8 in. I.D. Bondapak C_{18}–Porasil B, 37–75 μm; eluent, 0.5% isopropanol in *n*-hexane; flow-rate, 0.4 ml/min at 190 p.s.i.

(ergocalciferol) and provitamin D_2 (ergosterol) of *ca.* 7 h. Campesterol was separated from its dehydrogenation product, ergosterol, by *ca.* 170 min, whereas sitosterol was eluted *ca.* 25 min before its dehydrogenated analog, stigmasterol. Comparing homologous sterols, the 5-mono-unsaturated sterols are eluted at least 1 h apart in the order sitosterol (C_{29}), campesterol (C_{28}), cholesterol (C_{27}).

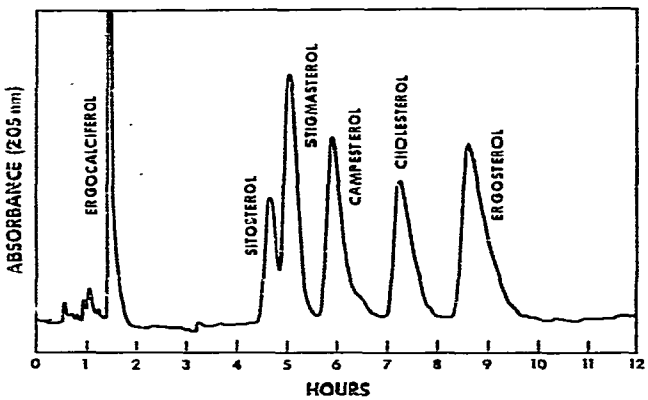


Fig. 4. Separation of C_{27}, C_{28}, and C_{29} sterols by LC. For conditions see Fig. 3.

The isolation of cholesterol from a *ca.* 500-fold excess of other plant sterols is illustrated by Fig. 5. A 10-mg sample of commercial sitosterol was mixed with 18 μg of cholesterol and chromatographed under the same conditions as before. The sitosterol preparation contained at least four major components, two of which were identified as sitosterol and campesterol by GLC. Just before cholesterol was eluted, the sensitivity of the detector was increased 100-fold to reveal the cholesterol peak.

The results show that difficult separations of sterols can be accomplished by LC, provided that an apolar sorbent–eluent system is selected. Bondapak C_{18} has previously been used as carrier of the apolar phase in reversed-phased partition chromatography of sterol esters[2]. In our method, it serves as the stationary phase of a

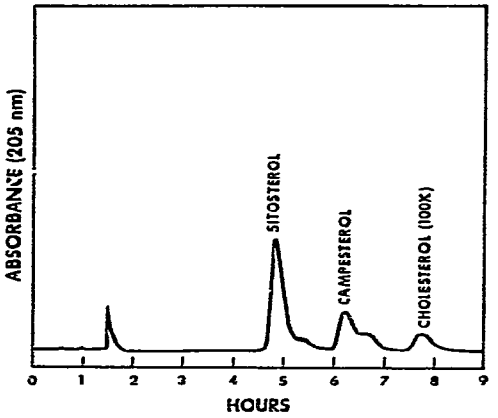


Fig. 5. Isolation of cholesterol from a *ca.* 500-fold excess of other plant sterols. For conditions see Fig. 3. Sensitivity increased 100-fold for cholesterol detection.

normal chromatographic sorbent–eluent system. Thus, the elution sequences are the reverse of those reported by Rees *et al.*[2]. By sacrificing speed, we have succeeded in separating such pairs as sitosterol and stigmasterol, which were previously only separable in the form of esters on Anasil B[7] or by argentation chromatography[1,3,4]. Rees *et al.*[2] separated the benzoates of sitosterol and stigmasterol by HPLC, but in that instance stigmasteryl benzoate was inseparable from campesteryl benzoate.

The use of short glass columns, which we can very satisfactorily pack ourselves, has allowed us to experiment with a variety of sorbents. Glass is a very desirable column material, because it is perfectly smooth and inert. Moreover, the high load capacity of these columns makes our method suitable for preparative applications in biochemical research. Further applications of this method are in progress.

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