☘Rapid Separation of Free Sterols by Reversed-Phase High Performance Liquid Chromatography

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ABSTRACT

The separation of eight common, structurally closely related sterols on C8 and C18 reversed-phase columns with UV-detection at 206 nm is described. Good separation was obtained in less than 18 min on the C18 column using methanol-water as mobile phase and a column temperature of 30 C. Except for brassicasterol and cholesterol, the sterols also were readily separated on the C8 column. Applications of the method on sterols from natural sources are described.

INTRODUCTION

Because of their simplicity and wide area of application, reversed-phase columns with polar mobile phases are most used for separation by high performance liquid chromatography (HPLC) today. This mode has been applied for separation of both free sterols (1-4) and sterol derivatives (5,6). The relationship between molecular structure and the retention of sterols on such columns has been discussed by several authors (1,3,4), but no investigation of the influence of various mobile phases and different column temperatures on the separation of sterols has been reported. The previously reported chromatographic systems do not give satisfactory resolution of the most common sterols found in biological materials.

Nonpolar mobile phases also have been used for analysis of free sterols on reversed-phase columns, resulting in stronger retention of the more polar compounds. Good separation of sterols with 0.5% isopropanol in hexane as mobile phase has been reported, but the method implied long retention times (7). In a recent paper (8) reporting the separation of stigmasterol and cholesterol, using 0.1% isopropanol in hexane as eluent, it is claimed that the reversed-phase mode did not exhibit sufficient selectivity for separation of these two sterols.

The objective of this study has been to optimize the separation of eight common, free sterols, closely related in molecular structure, on C8 and C18 reversed-phase columns. The potential of the true reversed-phase mode (with polar mobile phase) for an efficient separation of sterols will be demonstrated.

EXPERIMENTAL

Chromatographic Equipment

The analyses were performed on a Spectra-Physics model 3500 B fitted with a Valco valve loop injector (10 μl), a Spectromonitor III UV-detector from Laboratory Data Control operated at 206 nm unless otherwise stated, and a Scintag SW 3120 Chart Recorder. The columns used were a Brownlee RP-8 (4.6 mm × 25 cm) and a Brownlee RP-18 (4.6 mm × 25 cm). Both columns were packed with 5 μm spherical particles and had about the same efficiency (number of theoretical plates). Separations were carried out at different temperatures using a homemade water jacket for column heating and with preheating of the mobile phases.

Chemicals

HPLC grade methanol, tetrahydrofuran and acetonitrile (grade S) were purchased from Rathburn (Walkerburn, Great Britain). Ethanol was obtained from A/S Vinmonopolet (Oslo, Norway). Water was distilled and filtered through a 0.45 μm Millipore filter before use. Brassicasterol, campesterol, β-sitosterol and stigmasterol were obtained from Supelco, Inc. (Bellefonte, Pennsylvania, USA). Ergosterol and desmosterol were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) and cholesterol from Koch-Light (Colnbrook, Great Britain). Fucosterol was a gift from T. Mortensen (Trondheim, Norway).

The sterol standards were purified by HPLC when necessary. Standard solutions were prepared by dissolving the reference substances in absolute ethanol at a concentration of approximately 1-2 mg/ml of each sterol (10-20 μg injected).

Preparation of Samples

About 1 g of rapeseed oil and mayonnaise, respectively, were hydrolyzed with 100 ml 0.8 M KOH in ethanol for about 30 min at 80 C. The unsaponifiable fractions were extracted with 2 × 100 ml ether. The combined extracts were washed with water, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residues were dissolved in absolute ethanol and filtered through a 1.0 μm Millipore filter before injection.

2.5 g of a dried sample of the brown alga *Fucus vesiculosus* were extracted with 2 × 90 ml $CHCl_3$. The combined extracts were concentrated to dryness and treated as above.

RESULTS AND DISCUSSION

Effect of Stationary Phase Chain Length

Two reversed-phase columns with C8 and C18 alkyl chains, respectively, were employed using mixtures of methanol and water as eluents (Table I). The retention of the sterols was, as expected, much stronger on the C18 column than on the C8 column. Therefore, a less polar mobile phase

TABLE I

Comparison of C18 and C8 Columns for Separation of Sterols

No.	Compound	Retention rel. to cholesterol[a]	
		C18[b]	C8[c]
1	Desmosterol	0.77	0.78
2	Ergosterol	0.83	0.83
3	Brassicasterol	0.92	0.99
4	Fucosterol	0.93	1.08
5	Cholesterol	1.00	1.00
6	Stigmasterol	1.04	1.21
7	Campesterol	1.08	1.16
8	Sitosterol	1.15	1.38

[a]Absolute retention times 18.1 min and 21.5 min on the C18- and C8 columns, respectively.

[b]Mobile phase methanol-water (99:1), flow-rate 1.2 ml/min and temp 22 C.

[c]Mobile phase methanol-water (85:15), flow-rate 1.2 ml/min and temp 22 C.

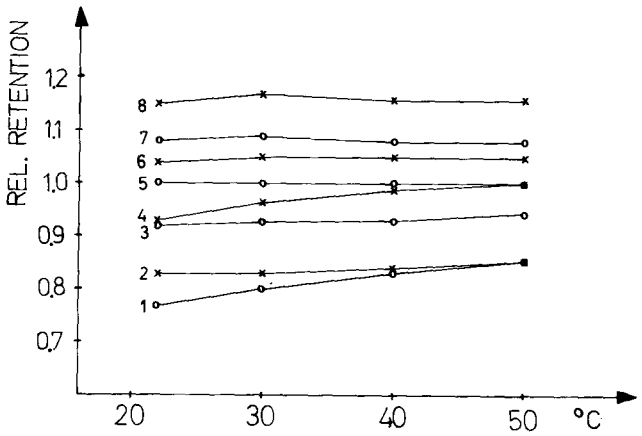

FIG. 1. Temperature effects on the separation of sterols. Column: Brownlee RP-18 with methanol-water (99:1) as eluent. Flow-rate, 1.2 ml/min. See Table I for identification of sterols.

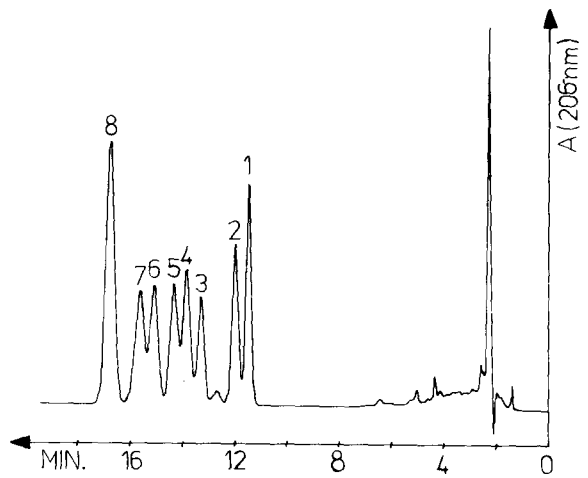

FIG. 2. Separation of sterols at 30 C on a Brownlee RP-18 column with methanol-water (99:1) as eluent. Flow-rate, 1.2 ml/min. See Table I for peak identification.

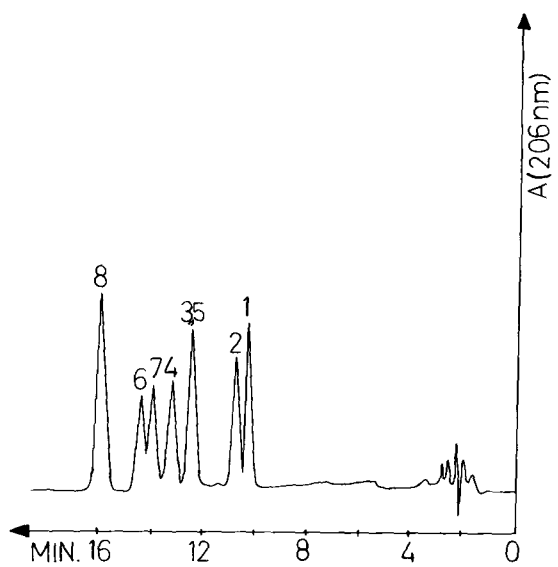

FIG. 3. Separation of sterols at 50 C on a Brownlee RP-8 column with methanol-water (85:15) as mobile phase. Flow-rate, 1.2 ml/min. See Table I for peak identification.

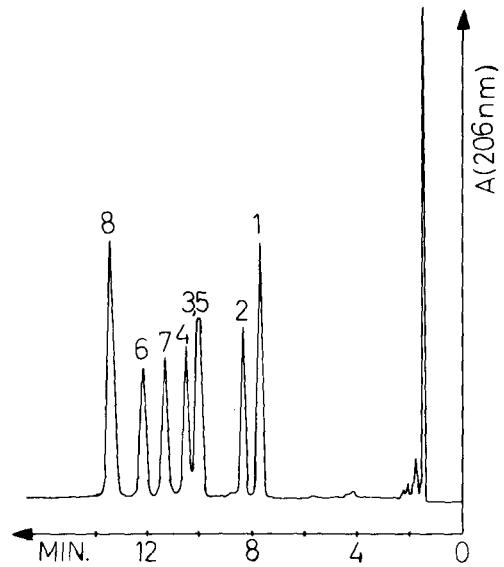

FIG. 4. Separation of sterols at 50 C on a Brownlee RP-8 column with acetonitrile-water (80:20) as eluent. Flow-rate, 1.6 ml/min. See Table I for peak identification.

was used to reduce retention times on the C18 column. In general, the elution was on both columns in the order of decreasing polarity of the sterols. However, the two columns exhibited marked differences in selectivity. On the C18 column, fucosterol eluted prior to cholesterol, and stigmasterol eluted prior to campesterol. The elution order of these sterols was reversed on the C8 column.

The difference in selectivity between brassicasterol and fucosterol on the C18 column was negligible, and they eluted as a single peak. The other sterols were well separated with a resolution (R_S) better than 0.85 (9). On the C8 column brassicasterol coeluted with cholesterol. The other sterols were nearly baseline separated, except stigmasterol and campesterol (R_S = 0.90).

Effect of Column Temperatures

The chromatographic behavior of the various sterols was somewhat differently affected by changing the temperature of the C18 column from 22 C to 50 C (Fig. 1). The retention of fucosterol and desmosterol increased relative to cholesterol at higher temperatures, while the other sterols showed only small variations. Owing to these changes fucosterol separated from brassicasterol at elevated temperatures, whereas the pairs fucosterol-cholesterol and desmosterol-ergosterol could not be separated above 40 C. The optimal temperature for separation on the C18 column appeared to be 30 C (Fig. 2).

Brassicasterol and cholesterol could not be separated on the C8 column by increasing the temperature. The selectivity of this column was only slightly affected by changes in the temperature. Analyses were performed at 50 C because column pressure and retention times were greatly reduced at high temperature (Fig. 3).

Choice of Mobile Phases

Figure 4 shows the separation achieved on the C8 column with acetonitrile-water as mobile phase. This solvent system gave the same elution order of the compounds as did the methanol-water system. The resolution of stigmasterol and

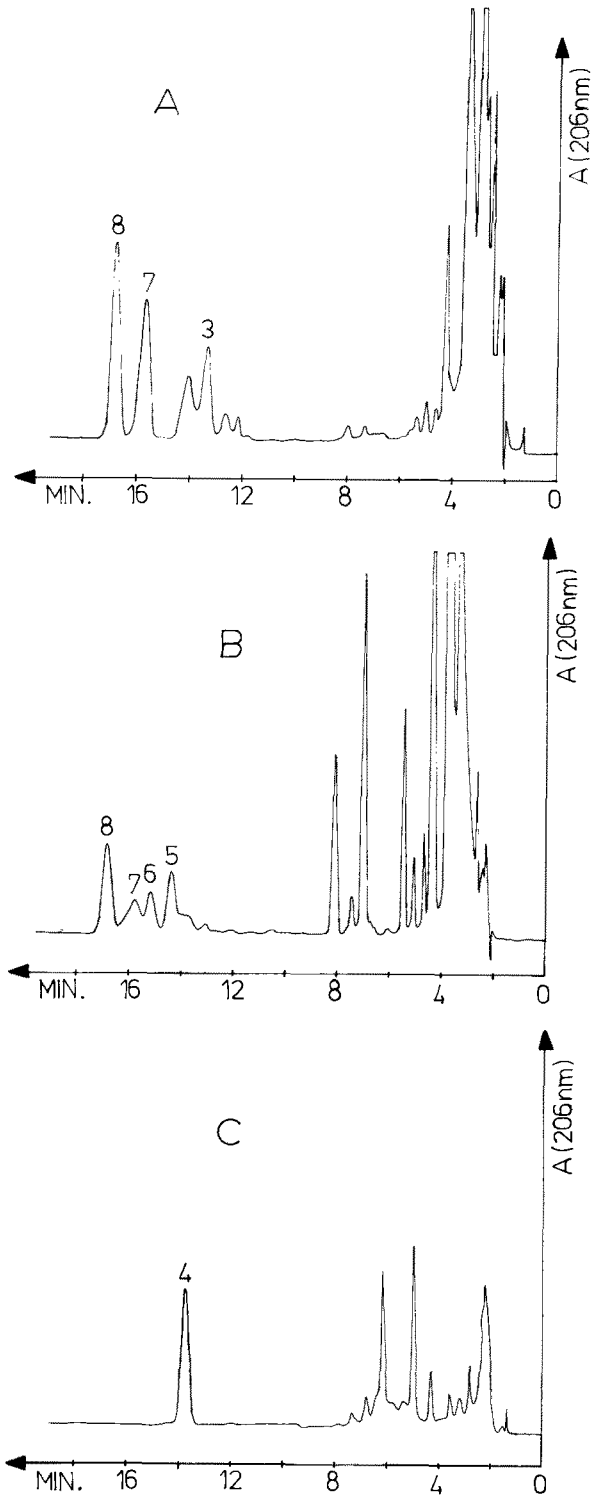

FIG. 5. Separation of sterols in A. rapeseed oil; B. mayonnaise, and C. the brown alga *Fucus vesiculosus*. Chromatographic conditions as in Fig. 2. See Table I for peak identification.

campesterol was improved, but brassicasterol still could not be separated from cholesterol. Use of acetonitrile significantly reduced the column pressure, allowing higher flow rates and, consequently, shorter retention times. Analyses also were performed on the C8 column with binary mixtures of tetrahydrofuran-water (UV-detection at 210 nm) and ethanol-water. Neither of these mobile phases effected separation of brassicasterol and cholesterol. Furthermore, separation of the other compounds was poorer than with methanol-water or acetonitrile-water mixture.

On the C18 column methanol-water mixtures were superior to acetonitrile-water mixtures. The latter solvent system (0-5% water in acetonitrile) revealed strong retention accompanied by peak tailing.

Applications

Samples of rapeseed oil, mayonnaise and the brown alga *Fucus vesiculosus* were analyzed in order to demonstrate the applicability of the reversed-phase system for determination of sterols. The chromatograms are shown in Figure 5. Although there are several unidentified peaks in the chromatograms, the sterols were well separated from the other compounds. The results are in good agreement with previous works using gas chromatography for analysis of sterols in rapeseed oil and mayonnaise (10,11).

The present method requires no sample derivatization or extra clean up except ether extraction of the unsaponifiable matter. Thus, it is a simple and rapid method for analysis of free sterols. By using a UV-detector at 206 nm, amounts as small as 0.4 μg (1 nmol) of cholesterol could be detected easily.

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