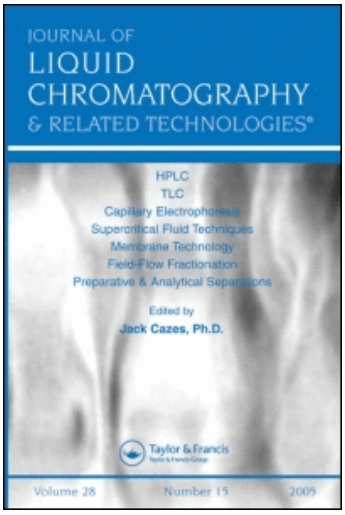

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PREPARATIVE SEPARATION OF β-SITOSTEROL BY HIGH SPEED COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

β-sitosterol was successfully separated and purified from both β-sitosterol standard (about 60% purity) and crude sterols using high-speed countercurrent chromatography (HSCCC) with a heptane–acetonitrile–ethyl acetate (5 : 5 : 1, v/v/v) solvent system. The purity of β-sitosterol product was improved to 97%, with the recovery being 50% for β-sitosterol standard and 45% for crude sterols, as indicated by HPLC analyses. Further mass spectrometric analyses confirmed the identity of the structures of the purified β-sitosterol products from crude sterols and β-sitosterol standard.

Key Words: Separation; Purification; Countercurrent chromatography; β-Sitosterol; Campesterol; Stigmasterol; Phytosterol

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INTRODUCTION

High-speed countercurrent chromatography (HSCCC) is a support-free liquid–liquid partition chromatography. As an important separation technique, it eliminates complications between solutes and solid supports, and has been used for preparative separation and purification for standards in recent years.[1,2]

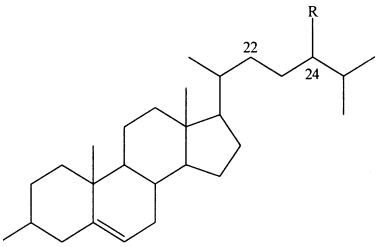
β-sitosterol, campesterol, and stigmasterol are important phytosterols and they are very similar in structures (Fig. 1). The sterols are usually separated by crystallization from plant oils. However, the method can just provide a mixture of the three sterols, or enrich stigmasterol only,[3] and β-sitosterol or campesterol can not be purified in this way. In the present study, we succeeded in applying HSCCC to the preparative separation of β-sitosterol from commercial β-sitosterol standard and crude sterols.

EXPERIMENTAL

Equipment and Materials

A Shimadzu series LC-10Avp HPLC apparatus (Kyoto, Japan) was used for quantitative analyses.

HSCCC was performed using a Model GS10A multilayer coil planet centrifuge (Beijing Institute of New Technology, Beijing, China). The total column (1.6 mm i.d.) volume was 234 mL. A manual injection valve with a 15 mL loop was used to introduce samples into the column (Tianjin High-New Science & Technology Company, Tianjin, China). The solvents were delivered with an NS-1007 pump (Beijing Institute of New Technology, Beijing, China). Column effluents were collected with a BSZ-100 fraction collector (Shanghai Huxi Instrument Plant, Shanghai, China).



Stigmasterol ($C_{29}H_{48}O$): R = C_2H_5, Δ^{22}
Campesterol $C_{28}H_{48}O$): R = CH_3
β-sitosterol $C_{29}H_{50}O$): R = C_2H_5

Figure 1. Structure of the three sterols.

The β-sitosterol standard and the stigmasterol standard were obtained from Sigma Chemical Co. (St. Lousis, MO, USA). The former contained campesterol (39.6%), stigmasterol (2.3%), and β-sitosterol (56.2%), and the latter contained stigmasterol (98.2%) and β-sitosterol (1.7%).[4] Crude sterols from soybeans were obtained from Beijing Fat and Oil Co. (Beijing, China).

Methanol used for HPLC was HPLC grade (Concord Tech Co. Ltd., China). Other solvents were analytical grade (Vas Lab Supplies Co. Ltd., China).

Preparation of Solvent System

Solvent system containing heptane, acetonitrile, and ethyl acetate (5 : 5 : 1, v/v/v) was used. The solvent system was thoroughly equilibrated in a separatory funnel, and the two phases were separated just before use.

Preparation of Sample and Sample Solutions

The crude sterol, which had been recrystallized with ethanol to eliminate impurities, stigmasterol standard, and β-sitosterol standard were dried before use in a vacuum drying chamber at 50°, 100 kPa (vacuum).

The sample solutions were prepared by dissolving a given amount of the dried sterols in equal volume of the upper and lower phases.

HSCCC Separation Procedure

The ascending mode applied in the separation, the column being filled with the lower phase, and the sample solution was injected through the injection valve. Then the column was rotated at 800 rpm while upper phase was delivered into the column with a flow-rate of 0.5 mL/min. After hydrodynamic equilibrium had been established, the effluent was collected in test tubes and would be analyzed by HPLC to monitor the sterols.

HPLC Analyses

For the quantitative analyses of β-sitosterol, campesterol, and stigmasterol,[5] a Shimadzu CLC-ODS column (150 × 6.0 mm i.d.) (Kyoto, Japan) was employed at 40°. Methanol was used as the mobile phase with a flow-rate of 1 mL/min. The sterols were detected at 204 nm.

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Measurement of Partition Coefficients

A mixture of β-sitosterol standard, and stigmasterol standard with a mass ratio of 20 : 1, was prepared to measure the partition coefficients of the three phytosterols with the classical "shake-flask" method.[6] At room temperature, about 0.2 mg of the mixture was gently shaken with 1 mL of each phase of the equilibrated two-phase solvent system in a caped graduated centrifuge tube. After settling for 2 h, the two phases were separated. The concentrations of the three sterols in both phases were quantitatively analyzed by HPLC, and partition coefficient of each sterol was calculated from the concentration of the upper phase divided by that of the lower phase.

RESULTS AND DISCUSSION

Selection of Solvent Systems

The polarities of the sterols are weak, and the polarity of β-sitosterol is weaker than campesterol and stigmasterol, which can be judged from their structures directly. The polarity differences may result in difference of partition coefficients between β-sitosterol and the other two sterols, and gives the probability for separation by HSCCC. As the polarities of aqueous solvent systems are strong for the sterols in HSCCC separation, non-aqueous solvent systems should be selected.[7] Partition coefficients of the three sterols in six non-aqueous solvent systems were determined for searching proper solvent systems (Table 1).

As can be seen from Table 1, in all solvent system observed, the partition coefficients of the sterols were very similar, especially for stigmasterol and campesterol, their separation factors (K_2/K_1) nearly equaled to 1 in the six solvent systems. The partition coefficients of β-sitosterol were a little different with the other two sterols as anticipated. Among these solvent systems, heptane–acetonitrile–ethyl acetate (5 : 5 : 1, v/v/v) gave the most prominent difference of partition coefficients between β-sitosterol and the other two sterols. Therefore, it should be the best solvent system for HSCCC separation in these solvent systems. Considering the large K values of the three sterols in this system, we selected ascending mode, i.e., lower stationary phase and upper mobile phase, or the sample bands would be broadened by excessive mixing.

Preparative Separation by HSCCC with Heptane–Acetonitrile–Ethyl Acetate (5 : 5 : 1, v/v/v)

8 mg of β-sitosterol standard was separated by HSCCC, and the mass concentration distribution of the three sterols in the effluent was shown in Fig. 2.

Table 1. Partition Coefficients and Separation Factors of the Three Sterols in Six Non-Aqueous Solvent System (at 25°C)

Solvent System	Strigmasterol (K_1)	Campesterol (K_2)	β-Sitosterol (K_3)	α		
				K_2/K_1	K_3/K_1	K_3/K_2
1	1.23	1.22	1.29	0.99	1.05	1.06
2	4.68	4.56	6.17	0.97	1.32	1.35
3	3.10	3.24	3.26	1.05	1.05	1.01
4	0.94	0.94	0.98	1.00	1.04	1.04
5	2.89	2.83	3.11	0.98	1.08	1.10
6	1.89	1.86	2.08	0.98	1.10	1.12

1. Heptane–methanol (1 : 1, v/v).
2. Heptane–acetonitrile–ethyl acetate (5 : 5 : 1, v/v/v).
3. Heptane–acetonitrile–ethyl acetate (10 : 10 : 3, v/v/v).
4. Hexane–methanol (1 : 1, v/v).
5. Hexane–aetonitrile–methylene dichloride (5 : 5 : 1, v/v/v).
6. Hexane–acetonitrile–methylene dichloride (5 : 5 : 2, v/v/v).

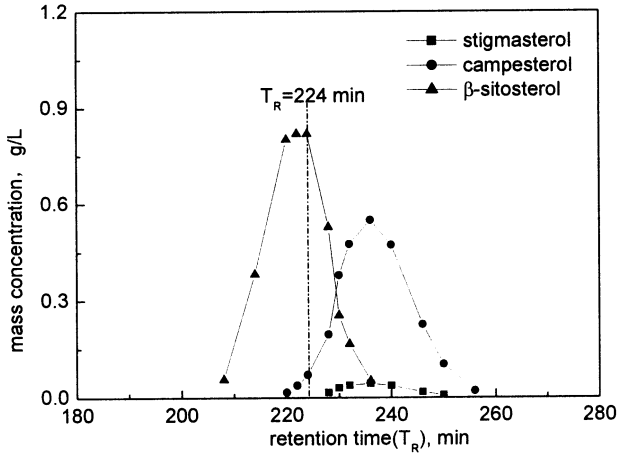

Figure 2. Mass concentration distribution of the three sterols in the effluent from HSCCC. Solvent system: heptane–acetonitrile–ethyl acetate (5 : 5 : 1, v/v/v); mobile phase: upper phase; flow-rate: 0.3 mL/min; sample size: 8 mg (β-sitosterol standard); fraction volume: 0.6 mL.

As can be seen in Fig. 2, peaks of campesterol and stigmasterol were overlapped completely as anticipated, and although the flow-rate of the mobile phase was quite slow and the sample size was small enough, peak resolution between β-sitosterol and the other two sterols was less efficient, which resulted from the small retention volumes of the sterols and the small separation factors between them.

Separations of different flow-rates and sample sizes were also performed to search suitable conditions for preparative separation, and satisfied results were

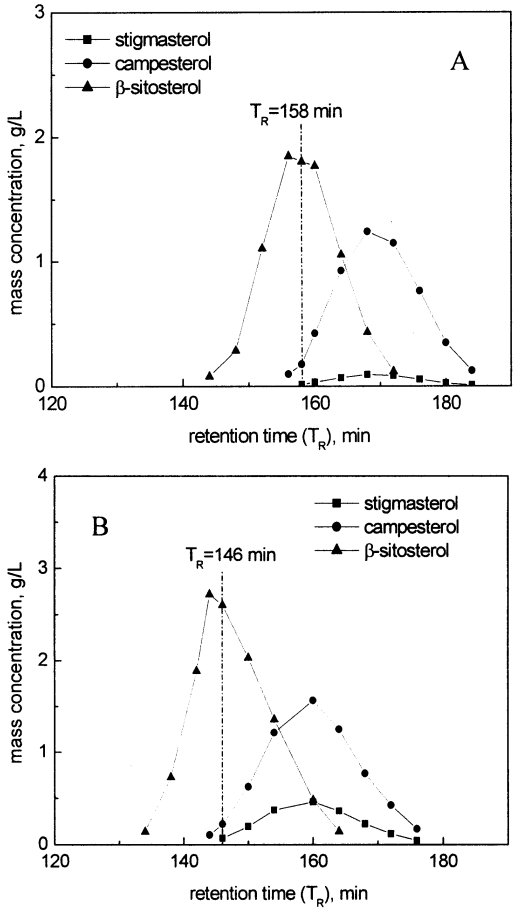

Figure 3. Mass concentration distribution of the three sterols in the effluent from HSCCC. Solvent system: heptane–acetonitrile–ethyl acetate (5 : 5 : 1, v/v/v); mobile phase: upper phase; flow-rate 0.5 mL/min; fraction volume: 1 mL sample size: 24 mg (β-sitosterol standard) (A); 45 mg (crude sterol) (B).

obtained under the conditions of flow-rate at 0.5 mL/min and sample size around 25 mg for β-sitosterol standard, or around 50 mg for crude sterols, as indicated in Fig. 3A and Fig. 3B.

Comparison of Fig. 3A and Fig. 3B with Fig. 2, overlapped regions between peaks of β-sitosterol and the other two sterols appeared to be broadened more, due to the higher flow-rate and larger sample size. However, less separation time was needed and more purified β-sitosterol was obtained. In Fig. 2 and Fig. 3, the dash-dot lines signified the retention time of the last fraction for β-sitosterol collection. By collecting all the fractions before the signified time in Fig. 3A and Fig. 3B, we obtained purified β-sitosterol products, and their chromatograms are in Fig. 4 and Fig. 5 compared with respective samples before separation. HPLC analyses showed they were about 97% pure with the approximate recovery being 50% and 45%, respectively. The molecular structures of the purified β-sitosterol from the crude sterol and β-sitosterol standard were identified by mass spectrometry, and the results showed that their structures are identical.

During the whole separation, once the hydrodynamic equilibrium was established, carryover of stationary phase was not notable after successive injections (3 times), and just about 100 mL of mobile phase was needed for one total separation, which showed that the solvent system is suitable for the separation.

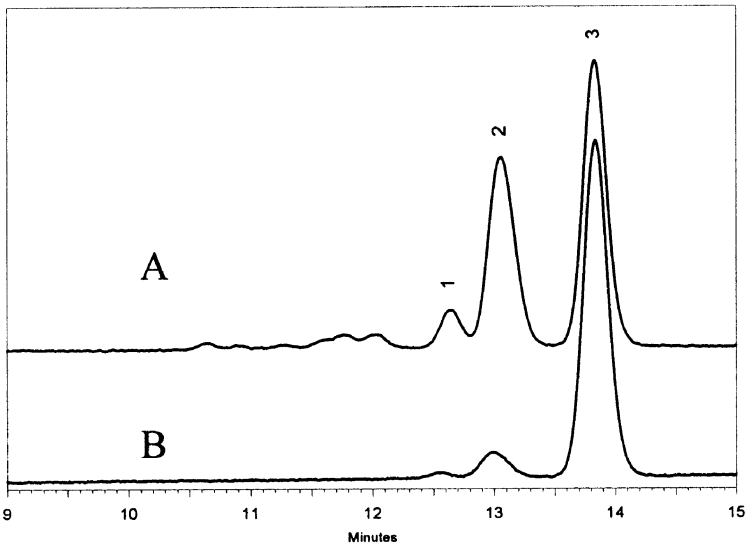

Figure 4. HPLC Chromatograms of β-sitosterol standard (A) and purified β-sitosterol product (Fig. 3A) (B). Peak identities: 1 = stigmasterol; 2 = campesterol; 3 = β-sitosterol.

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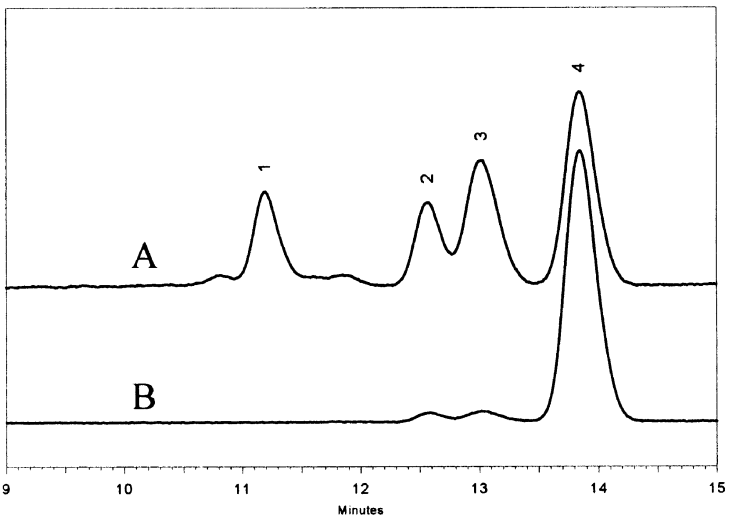

Figure 5. HPLC Chromatograms of crude sterols (A), and purified β-sitosterol product (Fig. 3B) (B). Peak identities: 1 = unknown component; 2 = stigmasterol; 3 = campesterol; 4 = β-sitosterol.

CONCLUSION

In the present study, we separated and purified β-sitosterol from both β-sitosterol standard and the crude sterol, though just partial separation was achieved. It has been known that high purity standards are usually very expensive, and hence, it is believed that HSCCC will be used widely in preparative separation in the future for more standards as a fast, efficient, and economical separation technique.

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