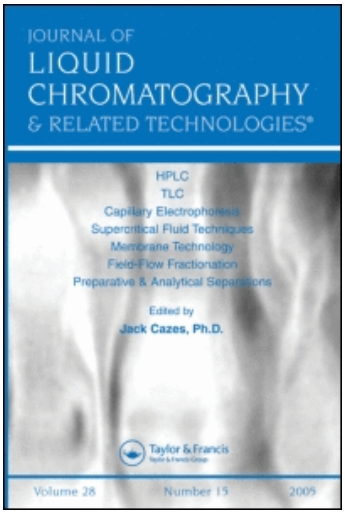

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PREPARATIVE SEPARATION OF FRUIT EXTRACT OF *SILYBUM MARIANUM* USING A HIGH-SPEED COUNTERCURRENT CHROMATOGRAPH WITH SCALE-UP COLUMNS

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ABSTRACT

A crude extract (12 g) of fruits of *Silybum marianum* was separated with a two-phase solvent system composed of water/methanol/ethyl acetate/*n*-hexane (4 : 3 : 4 : 1, v/v/v/v) by high speed countercurrent chromatography. Using a set of three large multilayer coil separation columns with a total capacity of 2460 mL, the separation yielded 1.37 g of silycristin at 93.1% purity, 3.47 g of silybin at 95.7% purity, and 0.93 g of isosilybin at 89.7% purity.

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INTRODUCTION

Silymarin, an antihepatotoxic substance isolated from fruits of *Silybum marianum*, has been subjected to pharmacological, biochemical, and chemical studies.[1–6] Silymarin was first considered as a single compound with the structure of 7-chromanol-3-methyl-taxifolin,[7] but later studies with more advanced analytical methods have shown that it is a mixture of four components, i.e., silybin, isosilybin, silydianin, and silycristin.[8,9] Preparation of pure standards of each component is desired for further scientific studies on these compounds.

The present paper describes preparative separations of three major components from a crude extract of silymarin by high-speed countercurrent chromatography (HSCCC).

EXPERIMENTAL

A triplet coil planet centrifuge countercurrent chromatograph was designed and manufactured in our institute (Institute of Food and Biological Engineering, Hangzhou University of Commerce, Hangzhou, China). Each multilayer coil separation column was made by winding 42 m long PTFE tubing (5.0 mm i.d. and 6.0 mm o.d.) onto a column holder hub (22 cm long, 7 cm o.d.) making five coiled layers. A set of three columns were connected in series to bring a total capacity of 2460 mL.

The stationary phase was introduced into this separation column by pressurized nitrogen gas through a special solvent container and the mobile phase was pumped using a Waters 510 pump (Millipore, Milford, MA, USA). A Model 8823A-UV Monitor (Beijing Institute of New Technology Application Beijing, China) was used to continuously monitor the effluent at 254 nm, and a Model S-100 recorder (Shanghai Analytical Instrument Factory, Shanghai, China) was used for recording the chromatogram. The effluent was collected with a Model BS 100 fraction collector (Shanghai Puxi Instrument Factory, Shanghai, China).

Reagents

Organic solvents including *n*-hexane, methanol, and ethyl acetate used for HSCCC were of analytical grade. Methanol used for HPLC analysis was an HPLC grade reagent. Silybin, isosilybin, silydiain, and silycristin standards were provided by Panjin JiangYuan Biological Products Co. Ltd, Pangjin, Liaoning, China.

Preparation of Two-Phase Solvent System

The HSCC separation was performed with a two-phase solvent system composed of water/methanol/ethyl acetate/*n*-hexane (4 : 3 : 4 : 1, v/v/v/v). After thoroughly equilibrating the solvent mixture in a separatory funnel at room temperature, two phases were separated shortly before use. The upper organic phase was used as the stationary phase, and the lower aqueous phase as the mobile phase.

Preparation of Sample Solution

A 1.0 kg amount of powdered dry fruits of *S. marianum* was extracted with 5 L of methanol three times. The extracts were combined and evaporated into dryness in vacuum. Then, the part of impurity in the extactum was removed using *n*-hexane and chloroform, successively. Finally, the residue was extracted with ethyl acetate three times. The extracts were combined and evaporated in vacuum and lyophilized to yield 103 g of a crude sample for HSCCC separation. The sample solution was prepared by dissolving 12 g of the above in 30 mL each of upper and lower phases of the solvent system used for separation.

Separation Procedure

The multilayer coil separation columns were first entirely filled with the upper organic phase (the stationary phase). Then the apparatus was rotated at 650 rpm, followed by sample injection through a injection loop (12 m of 2.6 mm i.d. Teflon tube). Then the lower aqueous phase (mobile phase) was pumped into the column at a flow rate of 5 mL/min. The effluent was monitored at 288 nm and collected with a fraction collector according to the chromatogram.

HPLC Analysis

The HPLC system was composed of two Waters HPLC 510 pumps, a manual injector, a Waters 486 UV detector, an ODS column (5 μm, 250 × 4.6 mm, Beckmann, Germany), and a chromatogram processor (Elite, Dalian, China). High performance liquid chromatography analyses were performed with a linear gradient elution, 30% to 50% of methanol in water from 0 to 25 min at room temperature.

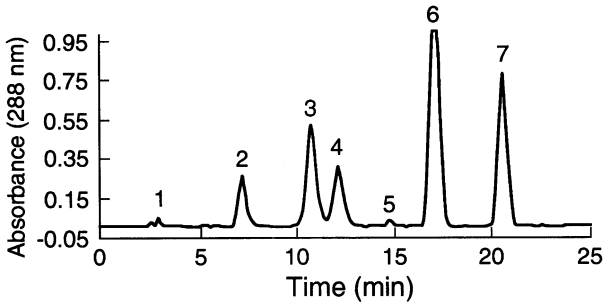

Figure 1. HPLC analysis of the crude extract of *S. marianum*. Peak 3: silycristin, peak 4: silydianin, peak 6: silybin, peak 7: isosilybin.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC analysis of the extract of *S. marianum.* Peaks 3, 4, 7, and 8 were silycristin, silydiain, silybin, and isosilybin, respectively; each consisting of 11.6%, 7.9%, 28.1%, and 7.7% of the dry extract. Figure 2 is the HSCCC chromatogram of 12 g of the crude extract of *S. marianum.* Four peaks were obtained from the separation, while the yellow components were eluted out before the peak 1. There were also yellow components retained in the stationary phase, which was collected from the column after completing the separation. The fractions corresponding to these four peaks were collected and evaporated in vacuum to remove the organic solvents, then lyophilized to yield 1.37 g of light yellow component 1, 2.01 g of light yellow component 2, 3.47 g of white component 3, and 0.93 g of white component 4, respectively. By HPLC analyses, components 1, 3, and 4 were each identified as silycristin at a purity of 93.1% (Fig. 3a), silybin at a purity of 95.7% (Fig. 3b), and isosilybin at a purity of

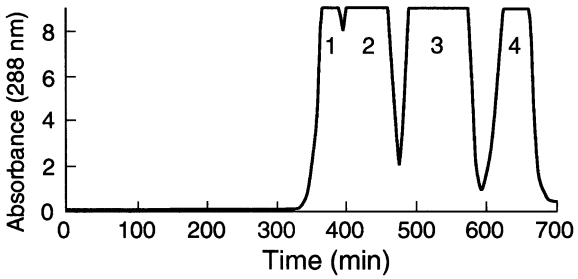

Figure 2. HSCCC separation of 12 g of the crude extract of *S. marianum*.

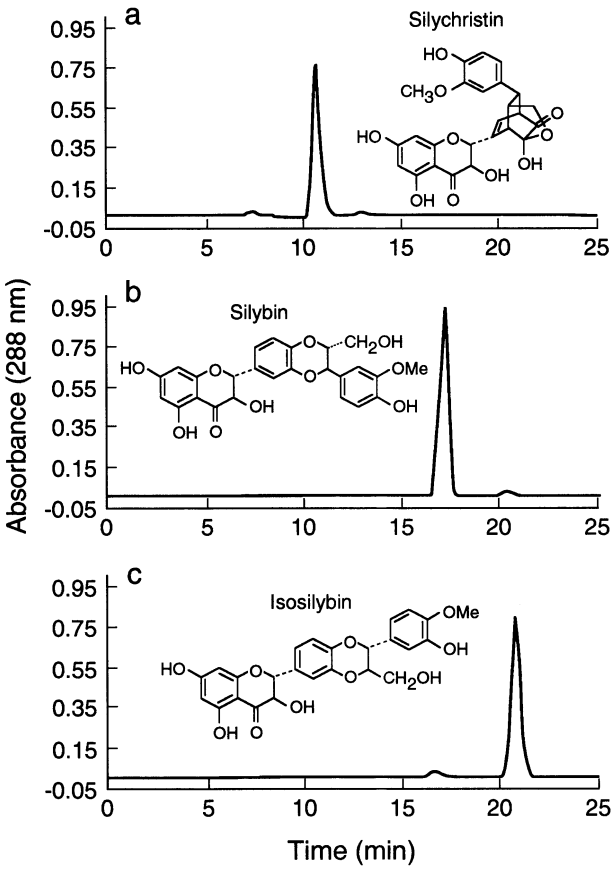

Figure 3. HPLC analyses of the components obtained from the HSCCC separation. (a) component 1, (b) component 3, (c) component 4.

89.7% (Fig. 3c). Component 2 was found to be a mixture of two major components including silydianin.

The above results demonstrate that three major components in silymarin were successfully purified by HSCCC using a large preparative column.

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