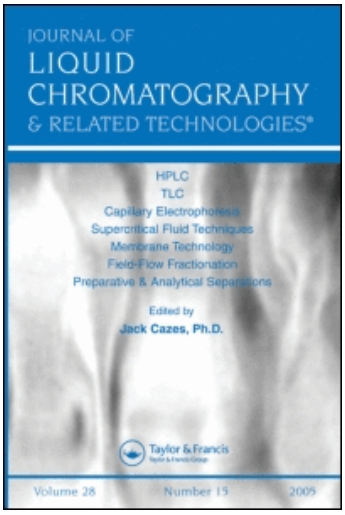

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PREPARATIVE SEPARATION AND PURIFICATION OF KAEMPFEROL, ISORHAMNETIN, AND QUERCETIN BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

High-speed countercurrent chromatography was used for the preparative separation and purification of kaempferol, isorhamnetin, and quercetin from leaf extracts of *Ginkgo biloba L.* and the commercial quercetin standard with a two-phase solvent system composed of chloroform-methanol-water (4:3:2, v/v/v).

HPLC analyses of the CCC fractions revealed that all three main flavone aglycones were over 99% pure. Their chemical structures were identified by mass spectrometric analysis.

INTRODUCTION

Extracts from the leaves of *Ginkgo biloba L.* are used as phytomedicines to increase peripheral and central blood flow. The ginkgo extracts contain active components, such as flavones and terpene lactones (Ginkgolides and bilobalide). They show pharmaceutical effects on vascular and cerebral metabolism and also inhibit platelet-activating factor.[1-3] While the ginkgo extract has been developed into an oral medicine, injection drug, nutrient, and health food, etc., the standard samples of three major flavonoids, i.e., isorhamnetin, kaempferol, and quercetin, are very expensive and not readily available in China. Consequently, some commercial quercetin standards, usually used in the quantitative analysis of total flavonoids in ginkgo extracts, are not pure and contain isorhamnetin, kaempferol, etc. as impurities.

The preparative separation and purification of flavonoids from plant materials by classical methods is tedious and usually requires multiple chromatographic steps on silica gel, polyamide, and sephadex column, etc. High-speed countercurrent chromatography (HSCCC), being a support-free liquid partition chromatography, eliminates complications such as irreversible adsorption onto the solid support, tailing of the solute peaks, etc.[4] and, therefore, is considered as a suitable alternative for the separation of flavonoids.[5-7]

The present paper describes the preparative separation and purification of three major flavone aglycones, i.e., isorhamnetin, kaempferol and quercetin by HSCCC.

EXPERIMENTAL

Apparatus

HSCCC was performed using a Model GS10A2 multilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a multilayer coil prepared from 1.6 mm ID, 110m long PTFE (polytetrafluoroethylene) tubing with a total capacity of 240 mL. The solvents were pumped with an NS1007 constant-flow pump (Beijing Institute of New Technology Application). Continuous monitoring of the effluent was provided with a Model 8823A-UV monitor (Beijing Institute of New Technology Application) operating at

254 nm. A manual sample injection valve with a 35 mL loop (Tianjin High-New Science & Technology Company, Tianjin, China) was used to load the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqin, China) was used to record the elution curve. A rotary evaporator used was Model RE-90 (Beijing Institute of New Technology Application). The HPLC equipment used was a Rainin Model SD-200 HPLC system (Rainin Instrument Company, Mack Road, Woburn, MA, USA).

Reagents

All organic solvents were of analytical grade and were purchased from Beijing Chemical Factory, Beijing, China. The commercial quercetin standard sample was purchased from Beijing Chemical Reagent Company, Beijing, China.

Preparation of Two-Phase Solvent System

Throughout the present study, a two-phase solvent system composed of chloroform-methanol-water at a volume ratio of 4:3:2 was used. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

Preparation of Sample and Sample Solutions

The extracts of the ginkgo leaves were prepared by using 70% aqueous acetone extraction. A 2.0g amount of the dried ginkgo extract was refluxed with 70 mL of methanol and 50 mL of 25% hydrochloric acid for 4 hours. After cooling, the reaction mixture was filtered through a glass filter (G3) covered with a filter paper (pore size 4.4 μm). The flavone aglycones were extracted from the solution with ethyl acetate for three times. The ethyl acetate extract was evaporated to dryness to yield crude ginkgo flavone aglycones.

The sample solutions were prepared by dissolving the crude ginkgo flavone aglycones or the commercial quercetin standard sample each in equal volumes of the upper and lower phases.

HSCCC Separation Procedure

In each separation, the coiled column was first entirely filled with the upper aqueous stationary phase, and then the apparatus was rotated at 800 rpm while the

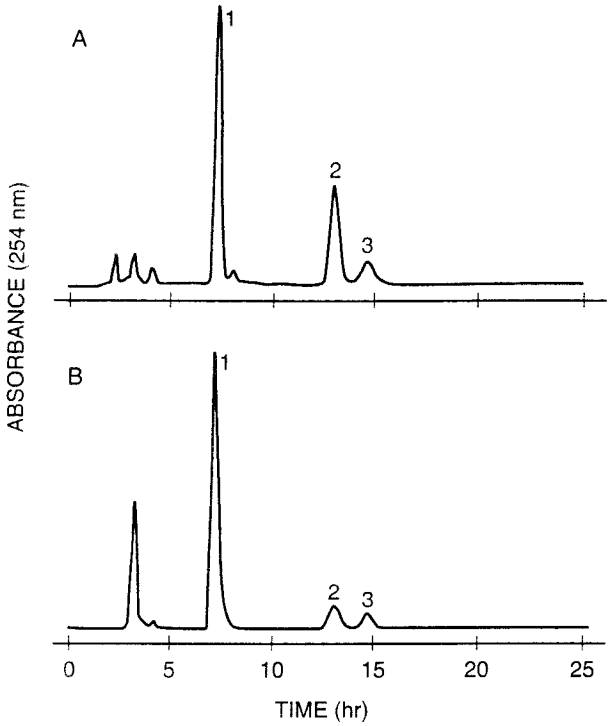

Figure 1. HPLC analyses of crude ginkgo flavone aglycones from the ginkgo extracts (A) and the commercial quercetin standard (B). Mobile phase: methanol-0.04% H_3PO_4 (50:50, v/v); flow-rate: 1.0 mL/min; detection: 254 nm. Peak 1: quercetin; Peak 2: kaempferol; Peak 3: isorhamnetin.

lower chloroform phase was pumped into the column at a flow-rate of 2.0 mL/min. After the mobile phase front emerged and two phases had established hydrodynamic equilibrium in the column, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm and collected in test tubes.

The crude ginkgo flavone aglycones, the commercial quercetin standard, and HSCCC peak fractions were analyzed by HPLC. The analysis were performed with a Microsorb-MV ODS column (150 x 4.6 mm ID, 5μm diameter) (Rainin Instrument Company, Mack Road, Woburn, MA, USA). The mobile phase, composed of methanol-0.04% H_3PO_4 (50:50, v/v), was isocratically eluted at a flow-rate of 1.0 mL/min and the effluent monitored at 254 nm. The CCC fractions corresponding to three main peaks were subjected to mass spectrometric analysis.

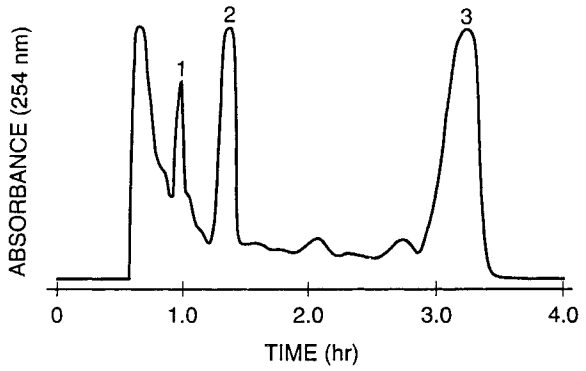

Figure 2. Chromatogram of the preparative isolation of the major flavonoids from the extract of *Ginkgo biloba* leaves by HSCCC. Solvent system: chloroform-methanol-water (4:3:2, v/v/v); mobile phase: lower phase; flow-rate: 2.0 mL/min; sample size: 200 mg; Peak 1: isorhamnetin; Peak 2: kaempferol; Peak 3: quercetin.

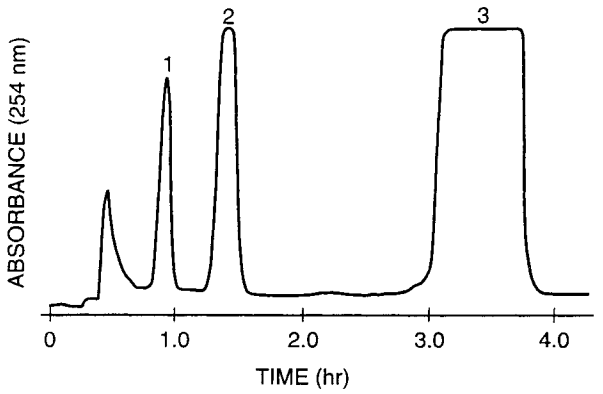

Figure 3. Chromatogram of the preparative purification of the quercetin from the commercial standard quercetin by HSCCC. For experimental conditions see Fig. 2 caption.

RESULTS AND DISCUSSION

HPLC analysis of the crude ginkgo extracts (Fig. 1A) showed that it contained three main flavone aglycones, i.e., quercetin, kaempferol and isorhamnetin (identified by MS analyses), and some other impurities. HPLC analysis of the commercial quercetin standard (Fig. 1B) showed that it also contained a small amount of kaempferol and isorhamnetin in addition to a large amount of polar

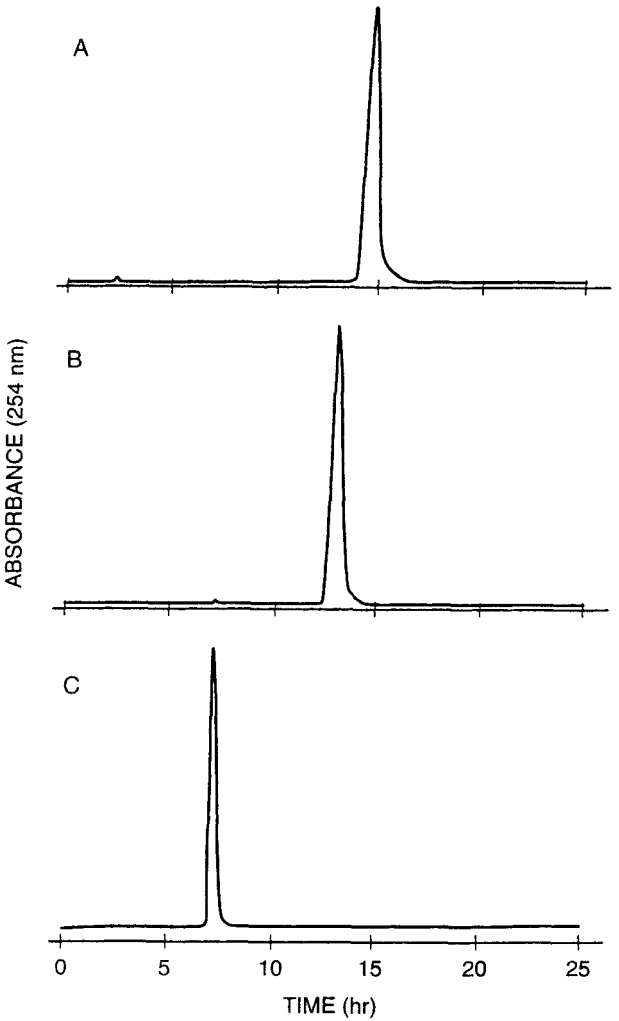

Figure 4. HPLC analyses of major HSCCC peak fractions (Figs. 2 and 3). For experimental conditions, see Fig. 1 caption.

impurity of unknown nature. A 200 mg amount of the crude flavone aglycones from the ginkgo extracts was separated by HSCCC (Fig. 2). After rechromatography of each peak by HSCCC, the purity of the three flavone aglycones was more than 99% according to the results of HPLC analysis (Fig. 4).

A 200 mg amount of the commercial quercetin standard was also separated by HSCCC (Fig. 3). Although the sample volume was as large as 33 mL in this separation, a baseline separation between peaks 1 and 2 was achieved. This indicates that HSCCC has a very large sample loading capacity and it will be useful for the separation and purification of crude and semi-pure natural products.

The chemical structures of the three flavone aglycones from the CCC fractions were identified by mass spectrometric analyses which indicated that peaks 1 to 3 correspond to isorhamnetin, kaempferol, and quercetin, respectively.

In conclusion, we have described a method suitable for the preparative separation and purification of isorhamnetin, kaempferol, and quercetin from the crude extracts of ginkgo leaves and semi-pure commercial quercetin standard. The high degree of purity achieved in our study makes it possible to use the recovered flavone aglycone as ginkgo standards.

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