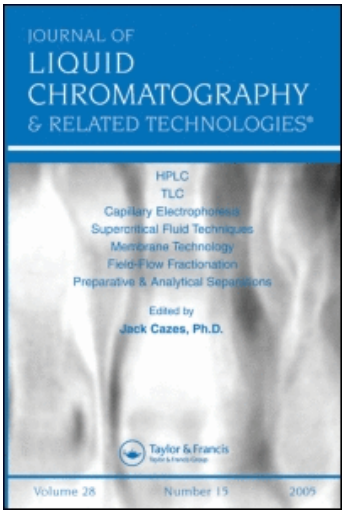


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# VERSATILE TWO-PHASE SOLVENT SYSTEM FOR FLAVONOID PREFRACTIONATION BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

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## ABSTRACT

High-speed countercurrent chromatography was used for the prefractionation of flavonoids from the crude extracts of Pericarpium citri reticulatae, Radix puerariae, Radix glycyrrhizae, Radix scutellariae, and Flos genkwa. All separations were performed only with a two-phase system composed of $CHCl_3 : CH_3OH : H_2O = 4 : x : 2$. The x was changed for each sample between $2.5 \sim 4.5$. The fractionated components were identified by thin-layer chromatography, which confirmed this solvent system was versatile and very useful for prefractionation of flavonoids.

## INTRODUCTION

High-speed countercurrent chromatography (HSCCC) performs liquid-liquid partitioning using no solid support. Elimination of the solid support

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provides various advantages over the conventional chromatographic methods (1). When the mobile phase is pumped through the sample, components are partitioned between the mobile and stationary phase and separated on the basis of differences in their partition coefficients. HSCCC is very suitable for separations of active components from the traditional Chinese medicinal herbs and other natural products.

The selection of a solvent system is the most important step in performing HSCCC. Selecting a solvent system for HSCCC means simultaneously choosing the column and the eluent. In the past, there were many successful studies for the separation of various components such as alkaloids, flavonoids, lignans, tannins, terpenes, saponins etc. (2). But those methods have been not used widely by the researchers in phytochemistry or pharmacy, because the selection of a two-phase solvent system is difficult for them. It is very useful to develop a fast and simple selection method with a two-phase solvent system as a general prefractionation step, which is able to separate a broad range of compounds. We have studied two versatile two-phase solvent systems for separation of alkaloids (3) and anthraquinones (4), respectively. In this paper, an extensive search for a HSCCC solvent system for the prefractionation of flavonoids from the crude plant extracts is described.

The polarity of flavonoid is of wide range. In general, they were extracted from medicinal herbs with methanol or ethanol (5). This fact shows that the solubility of flavonoid is good in methanol. The $CHCl_3/CH_3OH/H_2O$ solvent system contains methanol, provides reasonably short settling time, and has been used widely by HSCCC (6). Pericarpium citri reticulatae, Radix puerariae, Radix glycyrrhizae, Radix scutellariae, and Flos genkwa are Chinese traditional medicinal herbs. Their main active components are flavonoids (5). The crude extracts were resolved by HSCCC.



## EXPERIMENTAL

### Apparatus

The present studies were performed with a multilayer coil planet centrifuge constructed at the Beijing Institute of New Technology Application, Beijing, China. The apparatus holds a pair of column holders, symmetrically, on the rotary frame, at a distance of 8 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 1.6 mm i.d. polytetrafluoroethylene (PTFE) tube directly onto the holder hub to form multiple coiled layers with a total capacity of 260 mL. The system was equipped with a metering pump (Model NS-1007, Beijing Institute of New Technology Application, China), a UV

detector (Model 8823A-UV, Beijing Institute of New Technology Application, China), a recorder, and a sample injection valve.

## Reagents

All organic solvents and chemical reagents are analytical-reagent grade (Beijing Chemical Factory, China). Silica gel plates were from Qingdao Ocean Chemical Factory (China).

Pericarpium citri reticulatae, Radix puerariae, Radix glycyrrhizae, Radix scutellariae, and Flos genkwa were purchased from the Kunming medical market of China.

## Extraction of Crude Flavonoids

The preparations of samples of crude flavonoids from the herbs were extracted with ethanol in normal temperature. Then, the ethanol solutions were evaporated to obtain the crude flavonoids (5).

## Preparation of Two-Phase Solvent System and Sample Solution

The two-phase solvent system was composed of chloroform : methanol : water (4 : x : 2, v/v/v) in which the x was changed for each sample between 2.5 ~ 4.5. Each solvent mixture was thoroughly equilibrated in a separating funnel at room temperature and the two phases were separated before use.

The sample solutions were prepared by dissolving 20 mg crude flavonoid extract in 2 mL of the above phase mixture consisting of equal volumes of each phase.

## HSCCC Procedure

In each separation, the multilayer, coiled column was entirely filled with the upper phase. This was followed by injection of a 2.0 mL sample solution containing 20 mg crude flavonoids. Then, the apparatus was rotated at 800 rpm while the lower phase was pumped into the head of the column at a flow of $2.0\,mL \cdot min^{-1}$. When the run of apparatus was 120 min, the centrifuge run was stopped while pumping was continued for 65 min to fractionate polar components retained in the column at a flow of $4\,mL \cdot min^{-1}$. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Fractions of the top peaks were collected according to the chromatograms.

### TLC Analysis

All fractions from HSCCC were spotted on silica gel plates and developed in saturated normal chambers (saturation time 30 min). The visual detection was done under UV 254 nm and UV 366 nm, then by spraying 1% $AlCl_3$ ethanol solution.

## RESULTS AND DISCUSSION

The polarity range of flavonoids is wide. The solubility of flavonoids is good in methanol. As the $CHCl_3/CH_3OH/H_2O$ solvent system contains methanol which can be dissolved in chloroform and water, and provides nearly equal volumes of the upper and lower phase with reasonably short settling times, the two-phase solvent system composed of $CHCl_3 : CH_3OH : H_2O = 4 : x : 2$ was selected for separations of flavonoids from five medical herbs. The x was changed from 2.5 to 4.5 in a different sample. Changing the ratio of methanol in the solvent system, the selectivity of upper and lower phase is changed.

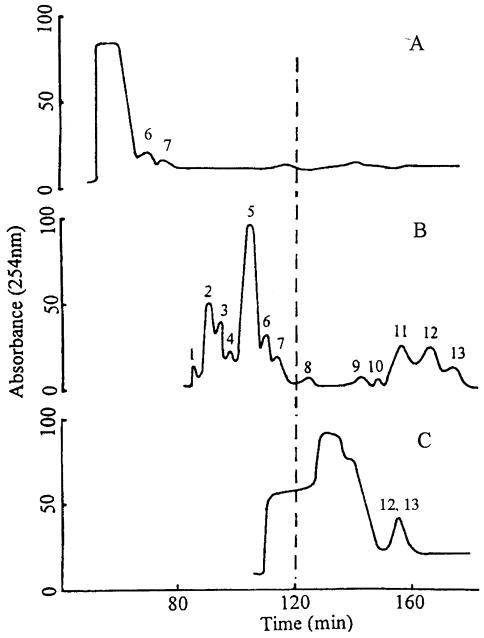


***Figure 1.*** Chromatograms of crude flavonoids extract from Pericarpium citri reticulatae obtained by HSCCC. Solvent system: $CHCl_3 : CH_3OH : H_2O = 4 : x : 2$, in which the x was 3 (A), 4 (B), and 4.5 (C).

Figure 1 shows three chromatograms of crude flavonoids extracted from Pericarpium citri reticulatae obtained by HSCCC. When the solvent system was 4 : 4:2, the separation was best. The summits of each peak were collected. The analyses were made on three silica gel G TLC plates developed with ethyl acetate-methanol (4.7 : 0.3, v/v), ethyl acetate-methanol (2.3 : 0.2, v/v), and ethyl acetate-methanol (4 : 1, v/v), respectively. Each peak gave a positive color reaction on the TLC. Peaks 1, 7, 8, 9, 12, 13 were single flavonoids. Peaks 3, 4, 5, 6, 7, 11 had two monochromatic spots. Peaks 2 and 10 had three monochromatic spots.

Figure 2 shows the chromatograms of crude flavonoids extracted from Radix puerariae. The best separation was obtained by using solvent system 4 : 2.9 : 2. Flavonoids were identified by TLC using three solvent mixtures composed of chloroform-methanol (4.9 : 0.1, v/v), chloroform-methanol (5 : 1, v/v), and chloroform-methanol (4 : 1, v/v). The summits of peaks 1 to 8 produced

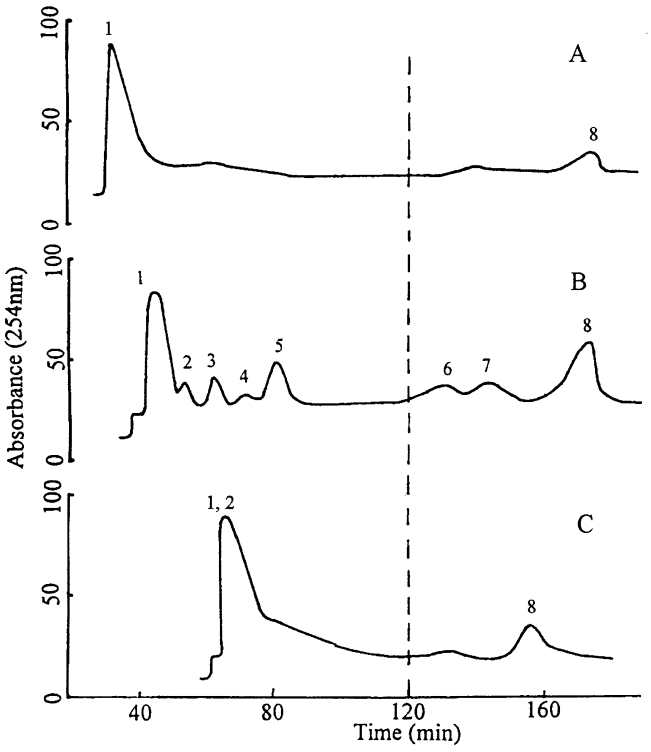


***Figure 2.*** Chromatograms of crude flavonoids extract from Radix puerariae obtained by HSCCC. Solvent system: $CHCl_3 : CH_3OH : H_2O = 4 : x : 2$, in which the x was 2.5 (A), 2.9 (B), and 3.5 (C).

a positive color reaction on the TLC. Peaks 3, 6 were essentially pure. Peaks 2, 4, 5, 8 gave two single monochromatic spots. Peaks 1, 7 contained three compounds.

The chromatograms of crude flavonoids extracted from Radix glycyrrhizae were showed in Figure 3. The best separation was solvent system 4 : 4 : 2. Three different solvent systems were used for the TLC analyses: ethyl acetate-benzene (5 : 1, v/v), ethyl acetate-benzene (4 : 1, v/v), and ethyl acetate-methanol (2.4 : 0.1, v/v). Each summit corresponding to peaks 2, 3, 9, 10 were identical with a single flavonoid. Other peaks produced two positive monochromatic spots on the TLC.

The crude flavonoids extracted from Radix scutellariae also were separated by HSCCC (Figure 4). The chromatogram of 4 : 3.8 : 2 solvent system was better than 4 : 3 : 2 or 4 : 3.2 : 2. The summits of each peak were collected. The TLC was

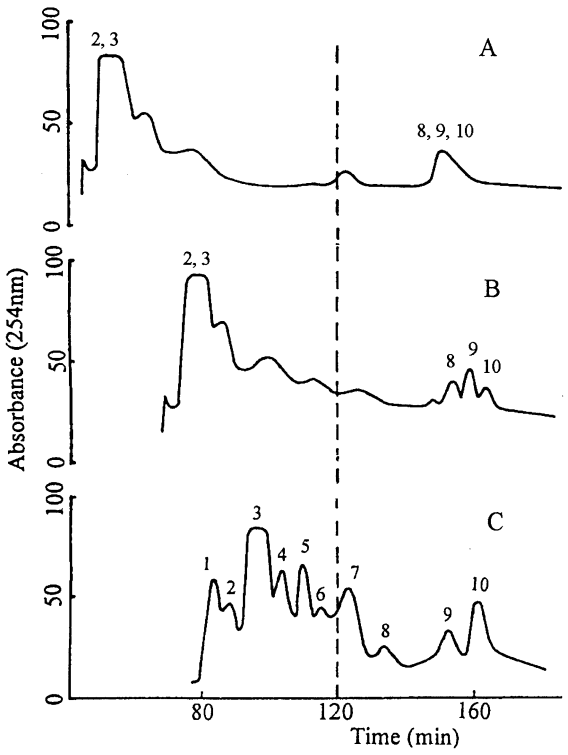


***Figure 3.*** Chromatograms of crude flavonoids extract from Radix glycyrrhizae obtained by HSCCC. Solvent system: $CHCl_3 : CH_3OH : H_2O = 4 : x : 2$, in which the x was 3.5 (A), 3.8 (B), and 4 (C).

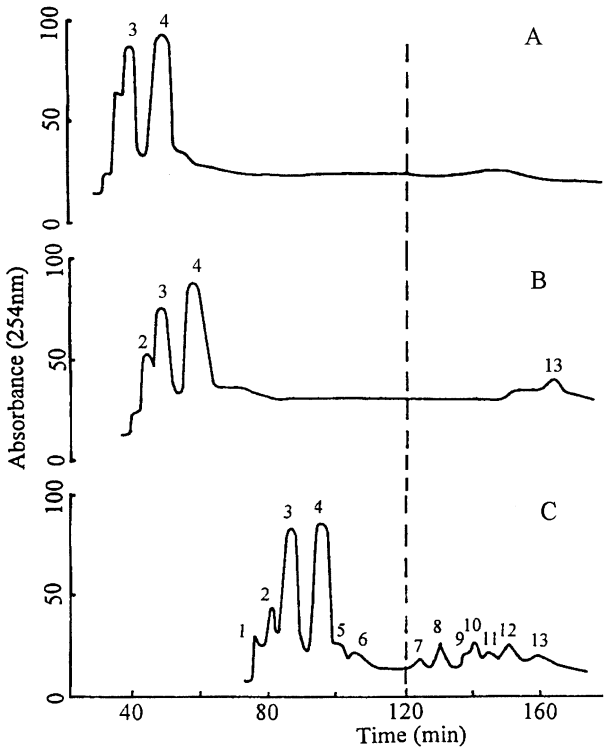


***Figure 4.*** Chromatograms of crude flavonoids extract from Radix scutellariae obtained by HSCCC. Solvent system: $CHCl_3 : CH_3OH : H_2O = 4 : x : 2$, in which the x was 3 (A), 3.2 (B), and 3.8 (C).

developed with either chloroform-methanol (4.7 : 0.3, v/v), chloroform-methanol (4 : 1, v/v) (80 : 3 : 1, v/v/v), or chloroform-methanol (3 : 1, v/v) (C). All of them produced one to three positive monochromatic spots. Peaks 2, 4, 5, 6, 8, 11 contained one flavonoid and peaks 1, 7, 9, 6, 10, 12, 13 gave two monochromatic spots.

Figure 5 shows the chromatogram of crude flavonoids extracted from Flos genkwa. The separation was best when the solvent system was 4 : 3.6 : 2. The TLC was developed with chloroform-methanol (2.4 : 0.1, v/v), chloroform-methanol (3 : 1, v/v), and chloroform-methanol (7 : 3, v/v), respectively. The summits corresponding to all peaks also gave the one to three positive monochromatic spots on the TLC with the color reagent. Peak 6 was essentially pure. Peaks 2, 3, 4, 5, 7, 9, 10 contained two compounds.

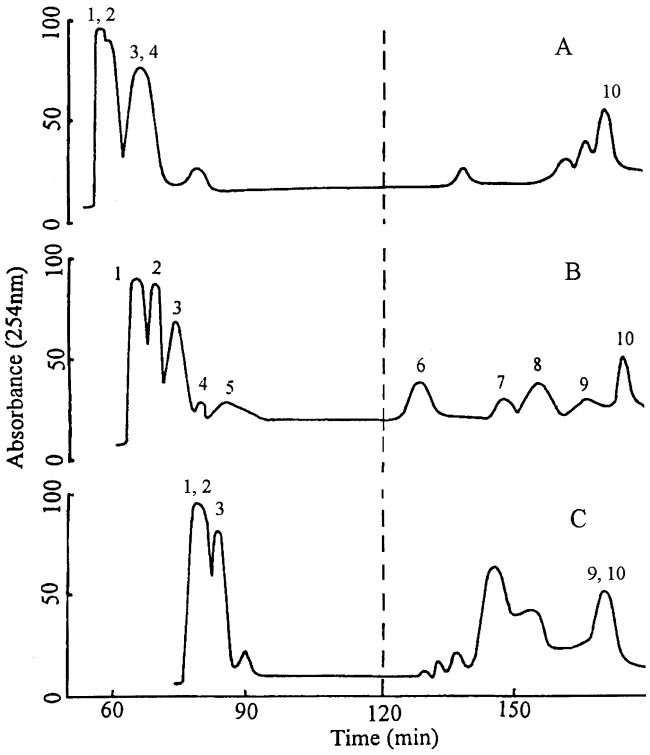


*Figure 5.* Chromatograms of crude flavonoids extract from Flos genkwa obtained by HSCCC. Solvent system: $CHCl_3$:$CH_3OH$:$H_2O = 4 : x : 2$, in which the x was 3.4 (A), 3.6 (B), and 3.8 (C).

Besides the above investigation, there are some other articles published on the separation of flavonoids by $CHCl_3/CH_3OH/H_2O$ solvent system (2,6–9).

## CONCLUSIONS

From the above comprehensive studies, we know that $CHCl_3/CH_3OH/H_2O$ was an excellent solvent system for HSCCC. The polarity of flavonoids is of a wide range. Most of them have good solubility in methanol. Controlling the rate of methanol may simultaneously change the selectivity of upper phase and lower phase in the two-phase solvent system. Therefore, $CHCl_3 : CH_3OH : H_2O = 4 : x : 2$, in which x is changed for each sample between $2.5 \sim 4.5$, is a versatile

high speed countercurrent chromatography solvent system for efficient prefractionation of flavonoids from crude plant extracts.

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