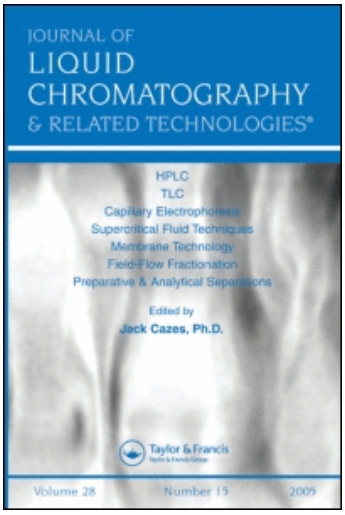

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Isolation of Hyperoside and Luteolin-Glucoside from *Agrimonia pilosa* Ledeb Using Stepwise Elution by High-Speed Countercurrent Chromatography

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Abstract: Preparative high-speed countercurrent chromatography was successfully used for isolation and purification of hyperoside and luteolin-glucoside from *Agrimonia pilosa* Ledeb. The separation was performed by stepwise elution with a pair of two-phase solvent systems composed of ethyl acetate-methanol-water at volume ratios of 50:1:50 and 5:1:5, which had been selected by analytical high-speed countercurrent chromatography (HSCCC). Using a preparative unit of the HSCCC centrifuge, about a 300 mg amount of the crude extract was separated, yielding 7.3 mg of hyperoside and 10.4 mg of luteolin-glucoside at a high purity of over 97%.

Keywords: Stepwise countercurrent chromatography, Isolation and purification, Hyperoside, Luteolin-glucoside, *Agrimonia pilosa* Ledeb

INTRODUCTION

Agrimonia pilosa Ledeb is a native plant that grows in many parts of China. It contains flavonoids and phenolic compounds. A pharmaceutical compound of

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Agrimonia pilosa Ledeb for treating or preventing hepatitis is reported. The compound has an inhibitory effect on HBV activity, as well as being effective in reducing HBV levels. Bioassays showed that flavonoid (hyperoside) effectively controls fungal pathogens in vitro, including *Alternaria alternate* and *Fusarium avenaceum*, although antifungal activity of this compound in the plant is limited.[1] Hyperoside (3,5,7,3′,4′-pentahydroxy-3-galactoside) also decreases blood pressure transiently.[2] The luteolin-7-O-D-glucoside (5,7,3′,4′-tetrahydroxy-7-glucoside) can reduce the hepatic total cholesterol content, which was elevated in mice fed the high fat diet alone.[3]

However, the separation of these active compounds from natural sources has always been difficult to achieve. High-speed countercurrent chromatography (HSCCC), being a support free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support,[4] and permits direct introduction of crude samples into the column, it has been successfully applied to isolate and purify a number of natural products.[5,6]

It is worthwhile to demonstrate the advantage of stepwise elution applied in the purification of natural products. One of target peaks with a large partition coefficient will be retained in the column for a long period of time. In this case the chromatographic condition should be changed to facilitate elution of the retained peaks without loss of peak resolution. In fact, a stepwise elution in countercurrent chromatography is an efficient way to achieve this goal. It has been used to separate the coumarins in our previous studies.[7] The present paper describes the successful preparative separation and purification of hyperoside and luteolin-glucoside from *Agrimonia pilosa* Ledeb by stepwise HSCCC.

EXPERIMENTAL

Apparatus

The analytical HSCCC instrument employed in the present study is a Model GS 20 analytical high-speed countercurrent chromatograph designed and constructed in 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 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 50 m long, 0.85 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 40 mL. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 to 2000 rpm, an optimum speed of

1800 rpm was used in the present studies. A manual sample injection valve with a 1.0 mL loop was used.

Preparative HSCCC was performed using a Model GS10A2 multilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a multilayer coil of 110 m long and 1.6 mm I.D. PTFE tubing with a total capacity of 230 mL. The β value of the preparative column ranged from 0.5 to 0.8.

The solvent was pumped into the column with a Model NS-1007 constant flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 nm. A manual sample injection valve with a 1.0 mL loop (for the analytical HSCCC) or a 10 mL loop (for the preparative HSCCC) (Tianjin High-New Science & Technology Company, Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to plot the chromatogram.) A rotary evaporator was also used.

The high performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-20A system including two LC-20A solvent delivery units, an SPD-M20A UV-VIS photodiode array detector (PDA), a Model 7725 injection valve with a 20 μL loop, an SCL-20A system controller, and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan).

Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Methanol used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory (Tianjin, China). *Agrimonia pilosa* Ledeb was purchased from a local store (Tong Ren Tang Shop, Beijing, China). Hyperoside and luteolin-glucoside standards were purchased from National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China).

Preparation of Crude Extract of *Agrimonia pilosa* Ledeb

About 200 g of dried *Agrimonia pilosa* Ledeb was extracted (refluxed) for 3 h, three times, with 500 mL of petroleum ether (b.p.: 60–90°C), and then filtered by six-layer pledget. The residues were again extracted with 500 mL of 95% ethanol solution, centrifuged, and concentrated to dryness under reduced pressure, yielding 9.1 g of a crude sample, which contained hyperoside and luteolin-glucoside at purity of 3.1% and 4.3%, respectively, as determined by HPLC (Fig. 1).

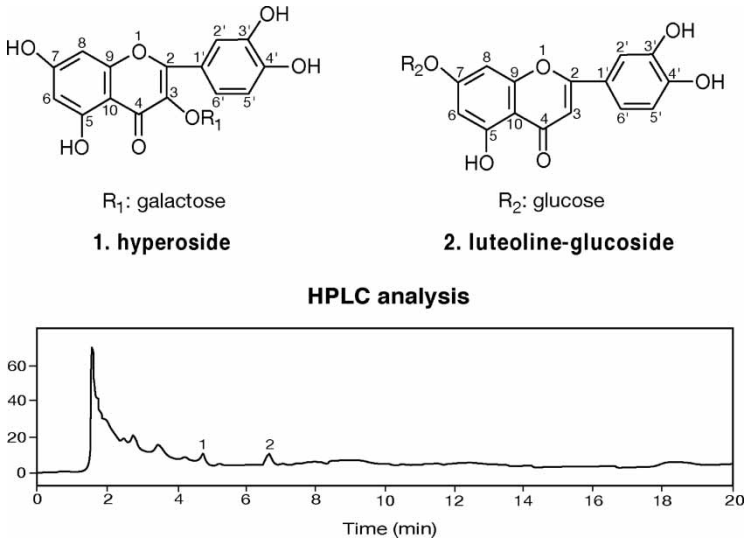

Figure 1. HPLC analyses of the crude extract of *Agrimonia pilosa* Ledeb. HPLC conditions: a Shimadzu ODS column (150 × 4.6 mm I.D.). Mobile phase:methanol: water (50:50, v/v), flow rate: 1.0 mL/min, monitored at 280 nm by a PAD detector. Peak 1: hyperoside, peak 2: luteolin-glucoside.

Preparation of Two-Phase Solvent System and Sample Solutions

The solvent system utilized in the present study was prepared by mixing ethyl acetate-methanol-water (50:1:50 or 5:1:5, v/v), and thoroughly equilibrating the mixture in a separatory funnel at room temperature, two phases being separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the lower phase at suitable concentrations according to the analytical or preparative purpose.

Separation Procedure

Analytical HSCCC was performed with a Model GS 20 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the inlet column at a flow rate of 1.0 mL/min, while the apparatus was run at a revolution speed of 1600 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (12 mg in 1 mL of lower phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 280 nm. Each peak fraction was collected according to the chromatogram.

Preparative HSCCC was performed with a Model GS10A2 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase as stationary phase. Then the lower phase of the solvent system 1 (volume ratio: 50:1:50) was pumped into the head end of the inlet column at a flow rate of 2 mL/min, while the apparatus was rotated at 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (300 mg in 10 mL of lower phase of solvent system 1) was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 280 nm. Each peak fraction was collected according to the chromatogram.

In the stepwise elution, after a certain time of elution using the first solvent system, the mobile phase was switched to the lower phase of the second solvent (volume ratio: 5:1:5). The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram.

HPLC Analyses and Identification of HSCCC Peak Fractions

The crude extract of *Agrimonia pilosa* Ledeb and HSCCC peak fractions were each analyzed by HPLC. The analyses were performed with a Shimadzu ODS column (150 × 4.6 mm I.D.). The mobile phase composed of methanol:water (50:50, v/v) was isocratically eluted at a flow-rate of 1.0 mL/min and the effluent monitored at 280 nm by a PAD detector.

Identification of the target compounds (hyperoside and luteolin-glucoside) was based on MS, ^{1}H-NMR; and ^{13}C-NMR spectra.

RESULTS AND DISCUSSION

As shown in Fig. 1, the HPLC analysis of the crude extract of *Agrimonia pilosa* Ledeb shows several compounds where the purity of hyperoside and luteolin-glucoside in crude extract is 3.1% and 4.3%, respectively, based on HPLC external standard curve.

In order to achieve an efficient resolution of target compounds, a two phase solvent system composed of ethyl acetate-methanol-water was examined using analytical HSCCC by varying the mutual volume ratio, since this solvent system has been successfully applied to various samples with a moderate degree of polarity. The results are illustrated in Fig. 2 a–b.

As seen in Fig. 2a, the separation using solvent ratio (50:1:50) purified hyperoside (peak 1) from polar impurities while luteolin-glucoside is long retained in the column and is eluted after about 3-hour. On the other hand, the use of the solvent ratio (5:1:5) completely separated two target compounds in a short elution time, while hyperoside (peak 1) was

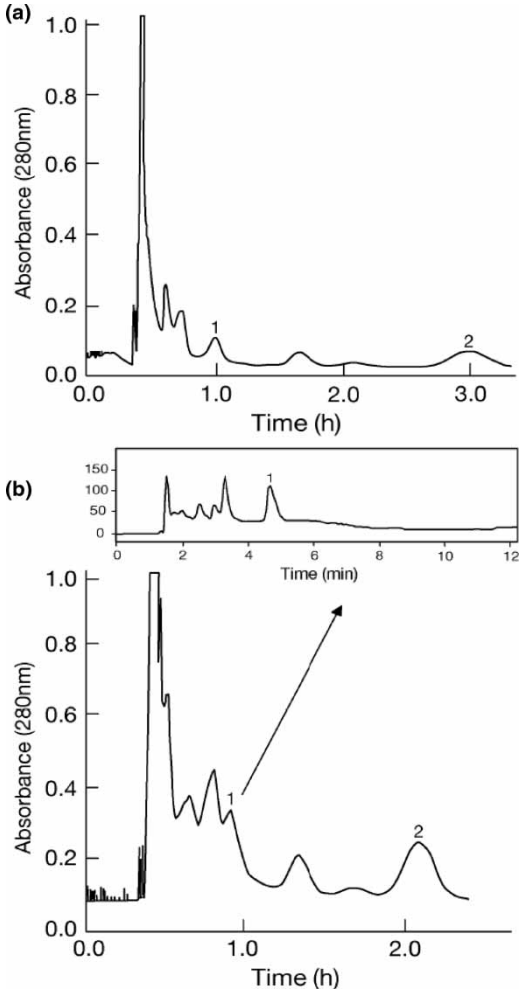

Figure 2. (a); Optimization of stepwise elution mode using analytical HSCCC for separation of the crude extract of *Agrimonia pilosa* Ledeb. Solvent system: ethyl acetate-methanol-water at volume ratios at 50:1:50 (solvent system 1) and 5:1:5 (solvent system 2) (b); stationary phase: organic phase; mobile phase: lower aqueous phase; flow-rate: 1.0 mL/min; revolution speed: 1600 rpm; sample: 12 mg dissolved in 1.0 mL lower phase. HPLC conditions: a Shimadzu ODS column (150 × 4.6 mm I.D.). Mobile phase: methanol: water (50:50, v/v), flow rate: 1.0 mL/min, monitored at 280 nm by a PAD detector. Peak 1: hyperoside, peak 2: luteolin-glucoside.

contaminated with unknown impurities as shown in Fig. 2b. These results suggest that the combined use of these two solvents in stepwise elution would provide an excellent purification of these two target compounds. This strategy was successfully demonstrated in Fig. 3, where the crude extract

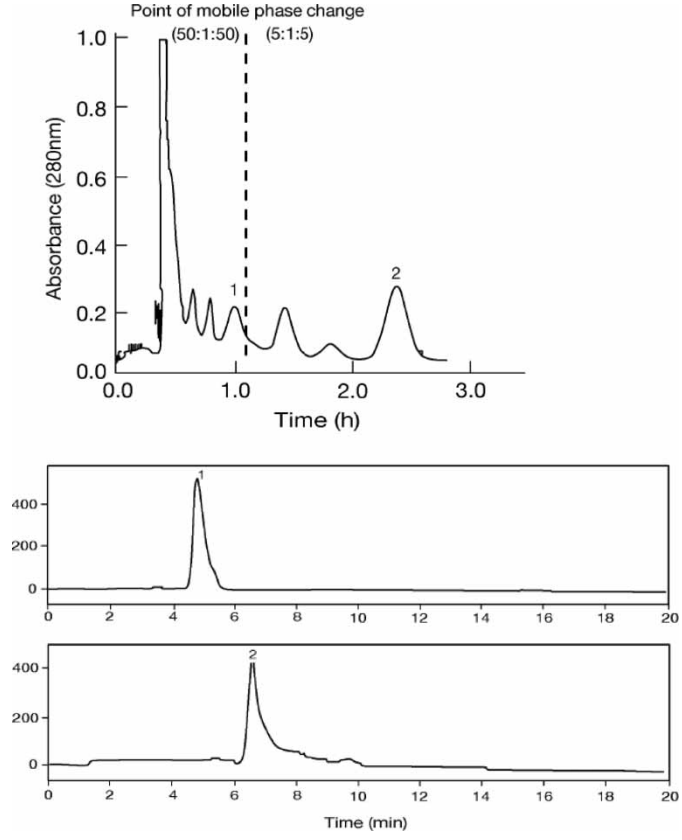

Figure 3. Analytical HSCCC separation of the crude extract of *Agrimonia pilosa* Ledeb using stepwise elution with solvent systems 1 and 2. Solvent system 1: ethyl acetate-methanol-water (50:1:50, v/v), solvent system 2: ethyl acetate-methanol-water (5:1:5, v/v), stationary phase: upper organic phase of solvent system 1, mobile phase: 63 mL lower aqueous phase of solvent system 1 and 100 mL lower aqueous phase of solvent system 2; flow rate: 1.0 mL/min; revolution speed: 1600 rpm, sample: 12 mg dissolved in 1 mL lower phase of solvent system 1. HPLC conditions: a Shimadzu ODS column (150 × 4.6 mm I.D.). Mobile phase: methanol: water (50:50, v/v), flow rate:1.0 mL/min, monitored at 280 nm by a PAD detector. Peak 1: hyperoside, peak 2: luteolin-glucoside.

was first eluted with the solvent system with a volume ratio at 50:1:50 until the polar impurities were eluted out, followed by the elution with the second solvent system with a volume ratio at 5:1:5. In this way, these two components were purified in 2.5 hours as shown in Fig. 3.

This stepwise elution was applied for the preparative separation of 300 mg of the crude extract of *Agrimonia pilosa* Ledeb. As shown in Fig. 4, the separation was started with the solvent system 1 (50:1:50) and, after

most of the polar impurities were eluted (2 hours and 45 minutes), the mobile phase was switched to the lower phase of solvent system 2 (5:1:5). Then, hyperoside (peak 1) and luteolin-glucoside (peak 2) were well resolved and eluted in less than 9 hours. This separation yielded 7.3 mg of hyperoside and 10.4 mg of luteolin-glucoside at a high purity of over 97% based on HPLC analysis.

The structural identification of hyperoside and luteolin-glucoside was carried out by MS, ^{1}H-NMR, and ^{13}C-NMR Spectra as follows: EI-MS: m/z 464 M^{+}, 302 (100), 274, 217, 153, 137; it showed the molecular ion at m/z 464, which is in agreement with the molecular formula $C_{21}H_{20}O_{12}$ of hyperoside.[8] The EI-MS: m/z 448, 258, 153, 134 showed the molecular ion at m/z 448, which is in agreement with the molecular formula $C_{21}H_{20}O_{11}$ of luteolin-glucoside.[9,10]

Hyperoside: ^{1}H-NMR (500 MHz, DMSO) δ ppm: 5.351(1H, H-1″), 6.191(1H, H–6), 6.402(1H, H-8), 6.811(1H, H-5′), 7.533(1H, H-2′), 7.632(1H, H-6′). ^{13}C-NMR (500 MHz, DMSO) δ ppm: 156.305(C-2), 133.502(C-3), 177.411(C-4), 161.210(C-5), 98.702(C-6), 164.102(C-7), 93.604(C-8), 156.302(C-9), 104.104(C-10), 121.101(C-1′), 115.212(C-2′), 144.810(C-3′), 148.403(C-4′), 116.003(C-5′), 121.903(C-6′), 101.905(C-1′), 71.306(C-2′), 73.311(C-3′), 68.012(C-4′), 75.906(C-5′), 60.211(C-6′).[11,12]

Luteolin-glucoside: ^{1}H-NMR (500 MHz, DMSO) δ ppm: 3.301–3.812 (H-2′, H-3′, H-4′, H-5′, H-6′ of glucoside), 5.023(1H, H-1′), 6.401(1H, H-3), 6.582(1H, H-6), 6.752(1H, H-8), 6.851(1H, H-5′), 7.364(2H, H-2′, H-6′), 12.872 (1H, 5-OH). ^{13}C-NMR (500 MHz, DMSO) δ ppm: 147.905(C-2), 103.102(C-3), 176.111(C-4), 160.510(C-5), 99.102(C-6), 162.802(C-7),

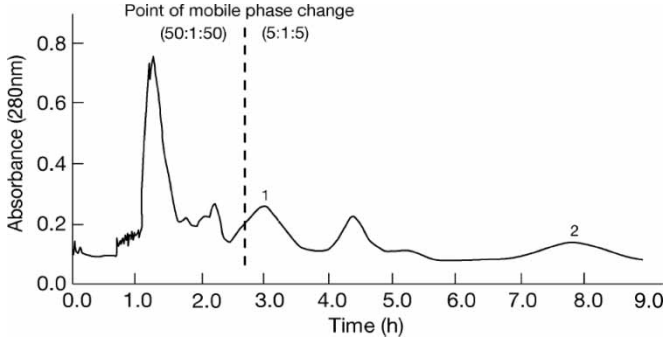

Figure 4. Preparative HSCCC separation of the crude extract of *Agrimonia pilosa* Ledeb using stepwise elution with solvent systems 1 and 2. Solvent system 1: ethyl acetate-methanol-water (50:1:50, v/v), solvent system 2: ethyl acetate-methanol-water (5:1:5, v/v), stationary phase: upper organic phase of solvent system 1, mobile phase: 330 mL lower aqueous phase of solvent system 1 and 750 mL lower aqueous phase of solvent system 2; flow rate: 2.0 mL/min; revolution speed: 800 rpm, sample: 300 mg dissolved in 10 mL lower phase of solvent system 1.

94.634(C-8), 156.002(C-9), 105.014(C-10), 121.701(C-1′), 129.612(C-2′), 145.510(C-3′), 159.405(C-4′), 115.603(C-5′), 129.603(C-6′), 100.505(C-1′), 78.406(C-2′), 76.711(C-3′), 70.112(C-4′), 77.286(C-5′), 61.201(C-6′). The results were similar to those in Reference [11,13].

The results of our studies demonstrated that stepwise countercurrent chromatography is a useful method for the preparative separation of hyperoside and luteolin-glucoside from a crude extract of *Agrimonia pilosa* Ledeb. It represents a method of choice especially for separation of natural products, which contains compounds of a broad spectrum of hydrophobicity.

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