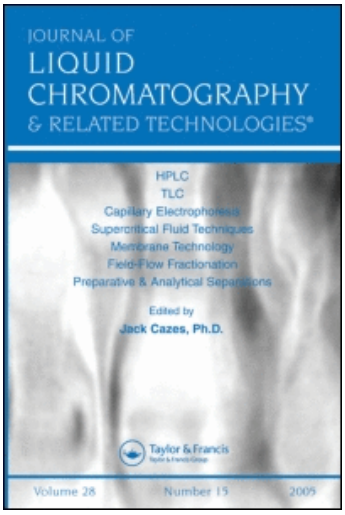

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Yi Yang[ab]; Tao Wu[ab]; Wu Xing Yang[a]; Haji Akber Aisa[a]; Tian You Zhang[ac]; Yoichiro Ito[d]

[a] Xinjiang Key Laboratory of Plant Resources and Natural Products Chemistry, Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Urumqi, P. R. China [b] Graduate University of the Chinese Academy of Sciences, Beijing, P. R. China [c] Beijing Institute of New Technology Application, Beijing, P. R., China [d] Bioseparation Technology, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

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Preparative Isolation and Purification of Four Flavonoids from *Flos Gossypii* by High-Speed Countercurrent Chromatography

Yi Yang,[1,2] Tao Wu,[1,2] Wu Xing Yang,[1] Haji Akber Aisa,[1] Tian You Zhang,[1,3] and Yoichiro Ito[4]

[1]Xinjiang Key Laboratory of Plant Resources and Natural Products Chemistry, Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Urumqi, P. R. China
[2]Graduate University of the Chinese Academy of Sciences, Beijing, P. R. China
[3]Beijing Institute of New Technology Application, Beijing, P. R. China
[4]Bioseparation Technology, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract: Following an initial cleanup step on the AB-8 macroporous resin, high-speed countercurrent chromatography (HSCCC) was successfully applied for the first time to the isolation and purification of four flavonoids from *Flos Gossypii*. HSCCC was performed with a two-phase solvent system composed of chloroform-methanol-isopropanol-water (5:5:1:3, v/v) adding 0.4% phosphoric acid in the aqueous stationary phase. The separation yielded quercetin (5.6 mg), quercetin-3′-O-D-glucoside (7.2 mg), quercetin-3-O-β-D-glucoside (16 mg), quercetin-7-O-β-D-glucoside (8.3 mg), from 100 mg of the crude extract in a one step separation. The structure identification was made by ^{1}H NMR, ^{13}C NMR.

Keywords: High-speed countercurrent chromatography, *Flos Gossypii*, Flavonoids

Correspondence: Dr. Yoichiro Ito, Bioseparation Technology, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bldg. 10, Room 8N230, 10 Center Dr., MSC 1762, Bethesda, MD 20892, USA. E-mail: itoy2@mail.nih.gov

INTRODUCTION

As a medicinal plant, *Flos gossypii* (Caomianhua, in Chinese) has been used as tranquilizations, detumescences, antipruritics, alleviating burn pain, and replenishing the heart and brain for a thousand years in Xinjiang Province in China. However, up to now few phytochemical studies on this plant have been described in the literature. The systematic phytochemical investigations recently carried out by our group revealed that *Flos gossypii* produces a rich source of secondary metabolites, such as flavonoids. The chemical structures of four such flavonoids were shown in Figure 1.

High-speed countercurrent chromatography (HSCCC), being a support-free liquid liquid partition chromatographic technique, eliminates the risk of irreversible adsorption of sample components that is often the case with conventional column chromatography with solid supports.[1] Consequently, HSCCC has been successfully applied to the isolation of various natural products,[2,3] especially for flavonoids.[4,5] This paper described the isolation of four flavonoids from *Flos Gossypii* by HSCCC.

EXPERIMENTAL

Apparatus

The analytical HSCCC instrument employed in this study is a Model GS 20A analytical high-speed countercurrent chromatograph designed and constructed at 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.

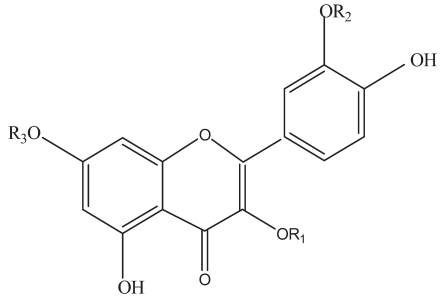

Figure 1. Chemical structures of target compounds from *Flos Gossypii* by HSCCC. Quercetin: $R_1 = H$ $R_2 = H$ $R_3 = H$; Quercetin-3-O-β-D-glucoside: $R_1 = Glc$ $R_2 = H$ $R_3 = H$; Quercetin-3′-O-β-D-glucoside: $R_1 = H$ $R_2 = Glc$ $R_3 = H$; Quercetin-7-O-β-D-glucoside: $R_1 = H$ $R_2 = H$ $R_3 = Glc$.

The multiplayer coil separation column was prepared by winding 50 m × 0.85 mm I.D. PTFE (polytetrafluoroethylene) tubing 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.

The preparative HSCCC instrument holds a Model GS10A2 multiplayer coil of 110 m × 1.6 mm I.D. with a total capacity of 230 mL. The β value of this preparative column ranged from 0.5 to 0.8. The revolution speed of the apparatus could be regulated with a speed controller in a range between 0 to 1000 rpm. An optimum speed of 800 rpm was used in this study.

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) at 254 nm. A manual sample injection valve with a 2.0 mL loop (for the analytical HSCCC) or a 20 mL loop (for the preparative HSCCC) (Tianjin High New Science Technology, 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 draw the chromatogram.

The high performance liquid chromatography equipment used was a DIONEX system (DIONEX, USA) including a P680 pump, an ASI-100 Automated sample injector, a TCC-100 temperature controlled column compartment, a UVD170U detector. The analysis was carried out with a Luna reversed phase C18 column (4.6 mm I.D. × 250 mm, 5 μm; Phenomenex, USA). Evaluation and quantification were made on a Chromeleon WorkStation.

Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Tianjing Chemical Factory (Tianjing, China). Methanol used for HPLC was of HPLC grade and purchased from Fisher Scientific Company (USA).

Preparation of Crude Sample

Flos gossypii purchased from a local store in China was powdered and extracted with aqueous ethanol in reflux for three times. All the extracts

were combined and evaporated under reduced pressure at 60ºC, and the residue was dissolved in water, which was loaded into a glass column with AB-8 macroporous resin. The column was first eluted with water until the eluate becomes colorless, followed by stepwise elution with 50% and 70% aqueous ethanol to elute out the target compound. Fractions of 50% and 70% aqueous ethanol were combined, evaporated to dryness, and used for HSCCC separation.

Preparation of Two-Phase Solvent System

The two-phase solvent system used was composed of chloroform-methanol-isopropanol-water (5:5:1:3, v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. The upper phase was used as the stationary phase after being acidified with phosphoric acid at 0.4%.

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

HSCCC Separation

The analytical HSCCC separation was performed with a Model GS 20A HSCCC instrument as follows: the multiplayer coiled column was first entirely filled with the stationary phase. The lower phase was then pumped into the head end of the column at a flow rate of 1.0 mL/min, while the apparatus was run at a revolution speed of 1800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (10 mg in 2 mL of the acidified upper aqueous phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram.

The preparative HSCCC was similarly performed with a Model GS-10A2 HSCCC instrument as follows: the multiplayer coiled column was first entirely filled with the upper aqueous stationary phase. The lower organic phase was then pumped into the head end of the column at a flow rate of 2.0 mL/min, while the apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium was established in the column, the sample solution (100 mg in 20 mL of the upper aqueous phase) was injected through the sample port. The effluent from the outlet of the column was monitored with a UV detector at 254 nm. Peak fractions were manually collected according to the chromatogram.

HPLC Analysis and Identification of Crude Sample and Peak Fractions from HSCCC

The crude extract of *Flos gossypii* and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a C_{18} column (4.6 mm I.D. × 250 mm, 5 μm) at a column temperature of 35ºC. The mobile phase was a linear gradient of 0.4% phosphate acid (A) and methanol (B) as follows: A-B (75:25, v/v) to A-B (65:35, v/v) in 15 min, then to A-B (60:40, v/v) in 5 min, then to A-B (37:63, v/v) in 14 min, then to A-B (37:63, v/v) in 6 min. The flow rate was 1.0 mL min^{-1} and the effluent was monitored at 360 nm by a UV detector.

Identification of the HSCCC peak fractions was carried out by ^{1}H NMR and ^{13}C NMR.

RESULTS AND DISCUSSION

The crude extract of *Flos gossypii* was first analyzed by HPLC. The result indicated that it contained several flavonoids, including quercetin (retention time 33.26 min), quercetin-3′-O-β-D-glucoside (retention time 30.12 min), quercetin-3-O-β-D-glucoside (retention time 23.78 min), quercetin-7-O-β-D-glucoside (retention time 19.53 min), and some other flavonoids, as shown in Figure 2A.

During the selection of the two-phase solvent system for HSCCC, a solvent system composed of chloroform-methanol-isopropanol-water (10:10:1:5, v/v) was first applied for the analytical separation of the crude extractions. In this separation, all major flavonoids components were eluted, but with poor resolution. Further studies were carried out using a similiar solvent system composed of chloroform, methanol, isopropanol, dilute phosphoric acid, or dilute acetic acid. The polarity of this system could be adjusted by varying the amount of each component. If the amount of chloroform was increased and the amounts of methanol and isopropanol were reduced, the polarity of the organic phase would decrease, and vice versa. The result indicated that the volume ratio of 5:5:1:3 with 0.4% phosphate acid in the stationary phase could separate the major flavonoids well.

Figure 3 shows the chromatogram obtained from 100 mg of the crude extracts from *Flos gossypii* by preparative HSCCC using a two-phase solvent system composed of chloroform-methanol-isopropanol-water (5:5:1:3, v/v), with 0.4% phosphoric acid added to the stationary phase. We cut these peaks, and collected fractions I, II, III, and IV (see Figure 3), and then washed each fraction with water to remove the acid, finally yielding 4 crystallized flavonoids. After drying, fraction I (shadowed portion) yielded 5.6 mg of quercetin, fraction II yielded 7.2 mg of quercetin-3′-O-β-D-glucoside, fraction III of this separation yielded 16 mg of quercetin-3-O-β-D-glucoside, and fraction IV yielded 8.3 mg of quercetin-7-O-β-D-

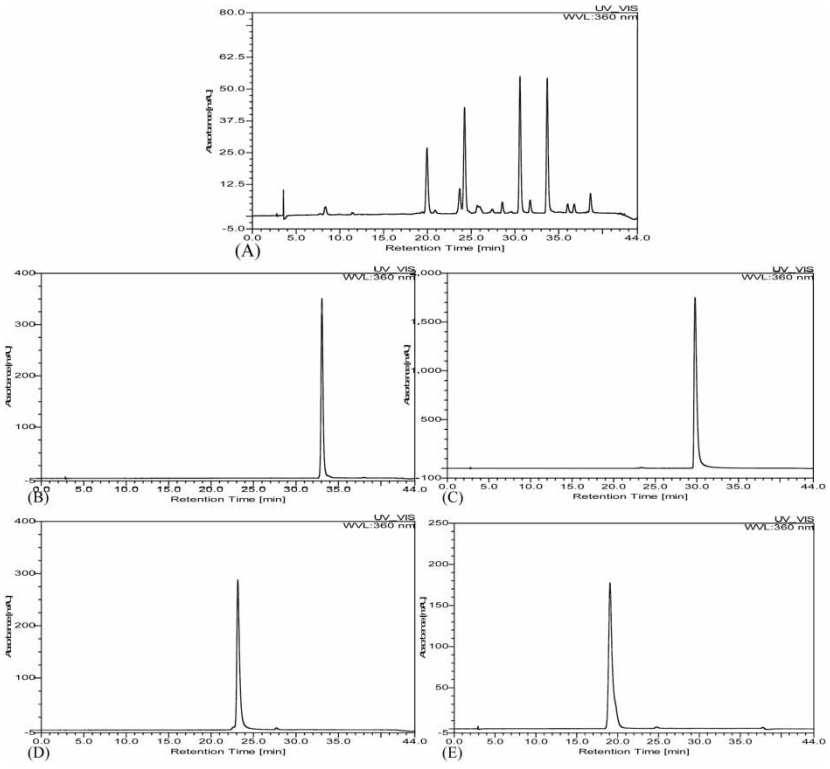

Figure 2. HPLC analysis of the crude sample and the HSCCC fractions. Separation column: a C_{18} column (4.6 mm I.D. × 250 mm, 5 μm); column temperature: 35ºC; detection wavelength: 360 nm; The mobile phase: a linear gradient of 0.4% phosphoric acid (A) and methanol (B) that follows: A-B (75:25, v/v) to A-B (65:35, v/v) in 15 min, then to A-B (60:40, v/v) in 5 min, then to A-B (37:63, v/v) in 14 min, then to A-B (37:63, v/v) in 6 min.; flow rate: 1.0 mL min^{-1}. (A) Crude sample; (B) Quercetin; (C) Quercetin-3′-O-β-D-glucoside; (D) quercetin-3-O-β-D-glucoside; (E) Quercetin-7-O-β-D-glucoside.

glucoside. Each fraction was analyzed by HPLC analysis, as shown in Figures 2B, C, D, and E.

The structural identification of the fraction was carried out by ^{1}H NMR and ^{13}C NMR as follows:

Quercetin (shadow I), ^{1}H NMR spectrum (600 MHz, DMSO, δ, ppm, J/Hz): 6.19 (1H, d, J = 1.8, H-6), 6.41 (1H, d, J = 2.4, H-8), 6.88 (1H, d, J = 8.4, H-5′), 7.55 (1H, q, J = 8.4, J = 2.4, H-6′), 7.68 (1H, d, J = 2.4, H-2′), 9.32 (1H, s, 4′-OH), 9.39 (1H, s, 3-OH), 9.61 (1H, s, 3′-OH), 10.79 (1H, s, 7-OH), 12.50 (1H, s, 5-OH); ^{13}C NMR: 93.55 (C-8), 98.38 (C-6), 103.21 (C-10), 115.80 (C-2′), 116.39 (C-5′), 120.17 (C-6′), 122.15 (C-1′),

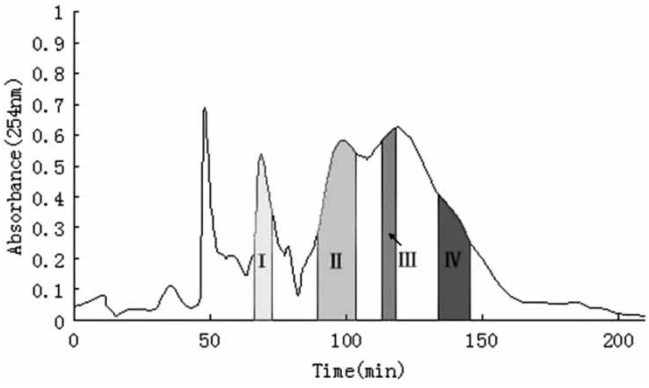

Figure 3. HSCCC chromatogram of the crude extract from *Flos Gossypii* after cleaning-up by AB-8 macroporous resin. Solvent system: chloroform-methanol-isopropanol-water (5:5:1:3, v/v); stationary phase: upper aqueous phase with 0.4% phosphate acid; mobile phase: lower organic phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm; sample size: 100 mg; detection.

135.95 (C-3), 145.26 (C-3′), 146.99 (C-2), 147.91 (C-4′), 156.32 (C-9), 160.92 (C-5), 164.09 (C-7), 176.05 (C-4).[6–8,10]

Quercetin-3′-O-β-D-glucoside (shadow II), ^{1}H NMR spectrum (600 MHz, DMSO, δ, ppm, J/Hz): 3.17–5.09 (sugar protons), 5.15 (1H, d, J = 4.2, H-1″), 6.19 (1H, d, J = 1.8, H-6), 6.49 (1H, d, J = 1.8, H-8), 6.98 (1H, d, J = 8.4, H-5′), 7.85 (1H, q, J = 8.4, J = 2.4, H-6′), 7.96 (1H, d, J = 1.8, H-2′), 9.22 (1H, s, 4′-OH), 9.42 (2H, s, 3-OH), 10.67 (1H, s, 7-OH), 12.46 (1H, s, 5-OH); ^{13}C NMR: 60.76 (C-6″), 69.73 (C-4″), 73.49 (C-2″), 76.14 (C-3″), 77.42 (C-5″), 93.90 (C-8), 98.44 (C-6), 102.59 (C-1″), 103.22 (C-10), 115.93 (C-2′), 116.20 (C-5′), 122.42 (C-6′), 123.77 (C-1′), 136.20 (C-3), 145.49 (C-3′), 146.43 (C-2), 148.98 (C-4′), 156.39 (C-9), 160.84 (C-5), 164.22 (C-7), 176.13 (C-4).[9,10]

Quercetin-3-O-β-D-glucoside (shadow III), ^{1}H NMR spectrum (600 MHz, DMSO, δ, ppm, J/Hz): 3.07–5.29 (sugar protons), 5.47 (1H, d, J = 7.2, H-1″), 6.20 (1H, d, J = 2.4, H-6), 6.40 (1H, d, J = 1.8, H-8), 6.84 (1H, d, J = 8.4, H-5′), 7.58 (1H, d, J = 2.4, H-6′), 7.56 (1H, br.s, H-2′), 9.24 (1H, s, 4′-OH), 9.74 (1H, s, 3′-OH), 10.86 (1H, s, 7-OH), 12.65 (1H, s, 5-OH); ^{13}C NMR: 61.97 (C-6″), 70.13 (C-4″), 74.29 (C-2″), 76.69 (C-3″), 77.81 (C-5″), 91.02 (C-8), 98.85 (C-6), 101.01 (C-1″), 104.18 (C-10), 115.40 (C-2′), 116.39 (C-5′), 121.37 (C-1′), 121.82 (C-6′), 133.50 (C-3), 145.02 (C-3′), 148.66 (C-4′), 156.36 (C-9), 156.52 (C-2), 161.45 (C-5), 164.32 (C-7), 177.65 (C-4).[10]

Quercetin-7-O-β-D-glucoside (shadow IV), ^{1}H NMR spectrum (400 MHz, DMSO, δ, ppm, J/Hz): 3.20–3.72 (sugar protons), 5.07 (1H, d, J = 7.2, H-1″), 6.43 (1H, d, J = 2, H-6), 6.78 (1H, d, J = 2, H-8), 6.94 (1H, d, J = 8.4, H-5′), 7.58 (1H, d, J = 8.4, J = 2, H-6′), 7.72 (1H, d, J = 2,

H-2′), 9.39 (1H, s, 4′-OH), 9.55 (1H, s, 3-OH), 9.76 (1H, s, 3′-OH), 12.49 (1H, s, 5-OH); ^{13}C NMR: 61.11 (C-6″), 70.04 (C-4″), 73.60 (C-2″), 76.78 (C-3″), 77.69 (C-5″), 94.99 (C-8), 99.35 (C-6), 100.47 (C-1″), 105.23 (C-10), 115.87 (C-2′), 116.16 (C-5′), 120.76 (C-6′), 122.44 (C-1′), 136.59 (C-3), 145.50 (C-3′), 148.08 (C-2), 148.31 (C-4′), 156.38 (C-9), 160.70 (C-5), 163.28 (C-7), 176.51 (C-4).[10]

CONCLUSION

We obtained four target flavonoids from the crude extract of *Flos gossypii* by HSCCC, collecting the corresponding portions of the peak. Though the yield is not very high, we got four compounds directly from a very complex system. Compared with other methods, HSCCC is an advantageous tactic in natural product separation. In the purification of natural products, it is a good choice to combine HSCCC and other isolation technology. The crude extract is separated with HSCCC first, and then the fractions are purified with other methods; PTLC, column chromatography, including silica gel, Sephadex LH 20, and so on.

The present study clearly indicates that HSCCC is a valuable method in separating, purifying and identifying bioactive components from Chinese herbal medicinal products.

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