Short communication

Preparative isolation and purification of polymethoxylated flavones from Tangerine peel using high-speed counter-current chromatography

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Abstract

Several polymethoxylated flavones including nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone were separated from Tangerine peel (Juhong in Chinese) by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of n-hexane–ethyl acetate–methanol–water (1:0.8:1:1, v/v). Then, 26 mg of nobiletin, 6 mg of 3,5,6,7,8,3′,4′-heptamethoxyflavone, 35 mg of tangeretin and 11 mg of 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone could be obtained from 150 mg crude extracts and their purities were 98.6%, 95.9%, 99.8% and 96.8%, respectively. All these constituents were identified by EI-MS and ^{1}H NMR.
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Keywords: Counter-current chromatography; Citrus reticulata Blanco; Preparative chromatography; Polymethoxylated flavones

1. Introduction

Tangerine peel (Juhong in Chinese), the dried ripe fruit peel of Citrus reticulata Blanco, is one of the most popular traditional medicinal herb which has been widely used in traditional Chinese medicine [1] and is officially listed in the Chinese Pharmacopoeia [2]. It has been used to regulate qi, normalizing the function of spleen and stomach, eliminate dampness, and resolve phlegm [1,2]. Recently, the studies have shown that polymethoxylated flavones (Fig. 1) in citrus exhibit anticancer effects against human cancer cell lines as well as anti-inflammatory and cardioprotective actions [3–9]. Unlike the glycosidic flavonoids, the polymethoxylated flavones are considerably less polar and assume planar structures. These features of the polymethoxylated flavones influence their biological properties, including their permeabilities to biological membranes, metabolic fates, and binding properties. These properties, in turn, play critical roles in influencing the molecules' modes of actions, which often differ from those exhibited by the glycosidic flavonoids [5,9].

Because of the importance of its biological properties, a large quantity of pure materials is urgently needed for further studies. However, the preparative separation and purification of polyhydroxylated flavones from other constituents of the plant by classical methods are tedious, requiring multiple chromatographic steps resulting lower recovery [10,11]. High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography technique that uses no solid support matrix. HSCCC eliminates the irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. This method has been successfully used for the preparative separation of natural products such as traditional Chinese medicinal herbs [12–18]. No report has been seen on the use of HSCCC for the isolation and purification of polymethoxylated flavones from plants. We herein report an efficient method for the preparative isolation and purification of polymethoxylated flavones by HSCCC.

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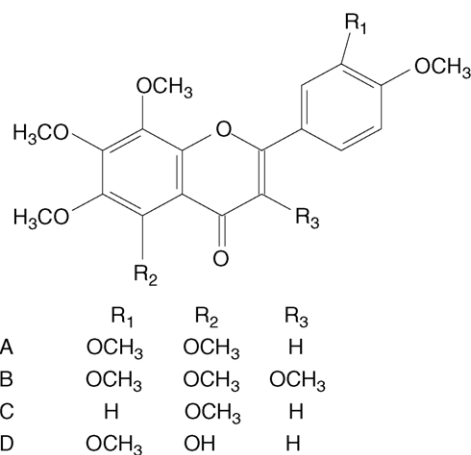E-mail address: wangx@keylab.net (X. Wang).

Fig. 1. Chemical structures of compounds A–D.

2. Experimental

2.1. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Tianjin Guangcheng Chemical Factory, Tianjin, China. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Tianjin Siyou Special Reagent Factory, Tianjin, China.

2.2. Apparatus

The preparative HSCCC instrument employed in the present study is a Model GS10A-2, with a miltilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a polytetrafluoroethylene multilayer coil of $110 \, m \times 1.6 \, mm$, I.D., with a total capacity of 230 ml. The β value of the preparative column varied from 0.5 at internal to 0.7 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The rotation speed is adjustable from 0 to 1000 rpm, and 800 rpm was used in the present studies. The system was also equipped with one NS-1007 constant flow pump, a Model 8823A-UV monitor operating at 254 nm, a Yakogawa 3057 recorder and a manual injection valve with a 15 ml sample loop.

The HPLC system used throughout this study consisted of a Waters 660 pump, a Waters 660 controller (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 10 µl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millenium[32] workstation (Waters, USA).

2.3. Preparation of crude extract

The dried peel (500 g) of *C. reticulata Blanco* were shattered to powder (about 30 mesh) and extracted with 6000 ml of boiling light petroleum (boiling range 60–90 °C) four times. The extraction time was 3, 2, 1 and 1 h, respectively. Then the light petroleum solutions were combined and evaporated to about 200 ml by rotary vaporization at 40 °C under reduced pressure. The enriched liquid was frozen under −4 °C for 24 h. The deposit was separated and dried and 6 g of crude sample was obtained. It was stored in a refrigerator for subsequent HSCCC separation.

2.4. Selection of two-phase solvent system

A number of two-phase solvent systems were tested by changing the volume ratio of the solvent to obtained the optimum composition that gave suitable partition coefficient (K) values. The partition coefficient (K) values were determined according to the literature [18]. Two milliliters of each phase of the equilibrated two-phase solvent system was added to approximately 1 mg of a test sample placed in a 10 ml test tube. The test tube was caped, and was shaken vigorously for 1 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficients (K). The K value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

2.5. Preparation of two-phases solvent system and sample solution

The selected solvent system was thoroughly equilibrated in a separation funnel by repeatedly vigorously shaking at room temperature. The two phases were separated shortly prior to use. The aqueous phase was used as the mobile phase, while the organic phase was used as the stationary phase. The sample solution was prepared by dissolving the crude sample in the mixture solution of organic phase and aqueous phase (1:1, v/v) of the solvent system used for HSCCC separation.

2.6. Separation procedure

In the crude sample isolation and separation, the coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 1.5 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 15 ml sample solution containing 150 mg of the crude extract was injected through the injection value. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

2.7. HPLC analyses and identification of HSCCC peak fractions

The crude extract and each purified fraction from the preparative HSCCC separation were analyzed by HPLC with a Shim-pack VP-ODS column (250 mm × 4.6 mm, I.D.) at 270 nm and at a column temperature of 25 °C. The mobile phase, a solution of acetonitrile and water (50:50, v/v), was eluted at a flow rate of 1.0 ml/min. The effluent was monitored by a photodiode array detector.

The identification of HSCCC peak fractions was carried out, respectively, by MS on an Agilent 5973N mass spectrograph and by ^{1}H NMR spectra on a Varian 600 MHz NMR spectrometer.

3. Results and discussion

A successful separation of the target compounds using HSCCC requires a careful search for a suitable two-phase solvent system to provide an ideal range of partition coefficients for the applied material. Generally, the two-phase solvent system needs to satisfy the following requirements [18–20]: (1) for ensuring a satisfactory retention of the stationary phase, the settling time of the solvent system should be considerably shorter than 30 s; (2) for an efficient separation, the partition coefficient (K) value of the target compounds should be close to 1, and the separation factor between two components ($\alpha = K_2/K_1$, $K_2 > K_1$) should be greater than 1.5. In general, small K values usually result in a poor peak resolution, while large K values tend to produce excessive sample band broadening.

We have selected a two-phase solvent system composed of n-hexane, ethyl acetate, methanol and water because it provided a broad range of hydrophobicity by modifying the volume ratio of the four solvents. Table 1 shows that the n-hexane, ethyl acetate, methanol and water ratios ranging from 1:0.8:1:0.8, 1:1:1:1, 1:0.8:0.8:0.8 and 1:0.8:1:1 can be used to separate the samples. After trying all the above solvent systems, the ratio of 1:0.8:1:1 (v/v) was found to be suitable for the separation of polymethoxylated flavones from the crude extracts.

Fig. 2 shows the preparative HSCCC separation of 150 mg of the crude sample using the optimized solvent system. The retention of stationary phase is 59%. Based on the HPLC
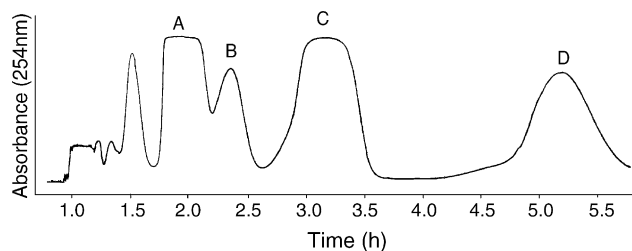

Fig. 2. HSCCC chromatogram of the crude extract. Conditions: Revolution speed: 800 rpm; solvent system: n-hexane–ethyl acetate–methanol–water (1:0.8:1:1, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; sample size: 150 mg; injection volume: 15 ml; detection wavelength: 254 nm; flow rate: 1.5 ml/min; retention of stationary phase: 59%.

analysis and the elution curve of the preparative HSCCC, all collected fractions were combined into different pooled fractions. Then, 26 mg compound A, 6 mg compound B, 35 mg compound C and 11 mg compound D were obtained, with the purity of 98.6%, 95.9%, 99.8% and 96.8%, respectively. The chromatograms of HPLC and UV spectra of these compounds were shown in Fig. 3.

Identification of HSCCC pure fractions was carried out by MS, ^{1}H NMR analysis as follows.

Compound A: Colorless needles (MeOH), $C_{21}H_{22}O_8$. EI-MS, m/z (%) 402 (M$^+$), 387 (M$^+$–CH$_3$, 100), 371 (M$^+$–OCH$_3$), 344, 326, 225, 197, 194, 179, 162, 153, 83. ^{1}H NMR (600 MHz, CDCl$_3$): 3.96 (3H, s, OCH$_3$), 3.97 (6H, s, 2 × OCH$_3$), 3.98 (3H, s, OCH$_3$), 4. 03(3H, s, OCH$_3$), 4.11 (3H, s, OCH$_3$), 6.65 (1H, s, H-3), 6.99 (1H, d, J = 8.4 Hz, H-5′), 7.42 (1H, d, J = 2.4 Hz, H-2′), 7.58 (1H, dd, J = 9.6, 2.4 Hz, H-6′). Comparing the above data with Refs. [11,21], the obtained product was identified as nobiletin [5,6,7,8,3′,4′-hexamethoxyflavone].

Compound B: Yellow needles (MeOH), $C_{22}H_{24}O_9$. EI-MS, m/z (%) 432 (M$^+$), 417 (M$^+$–CH$_3$, 100), 404 (M$^+$–CO), 402 (M$^+$–OCH$_3$), 401, 389, 386, 240, 225, 197, 165. ^{1}H NMR (600 MHz, CDCl$_3$) δ ppm: 3.89 (3H, s, OCH$_3$), 3.95 (3H, s, OCH$_3$), 3.97, (9H, m, 3 × OCH$_3$), 4.01(3H, s, OCH$_3$), 4.10 (3H, s, OCH$_3$), 7.01 (1H, d, J = 8.4 Hz, H-5′), 7.81 (1H,d, J = 2.4 Hz, H-2′), 7.83 (1H, dd, J = 9.0, 2.4 Hz, H-6′). Comparing the above data with Refs. [10,21], the obtained product was identified as 3,5,6,7,8,3′,4′-heptamethoxyflavone.

Table 1
Partition coefficients and separation factors (a) of these components

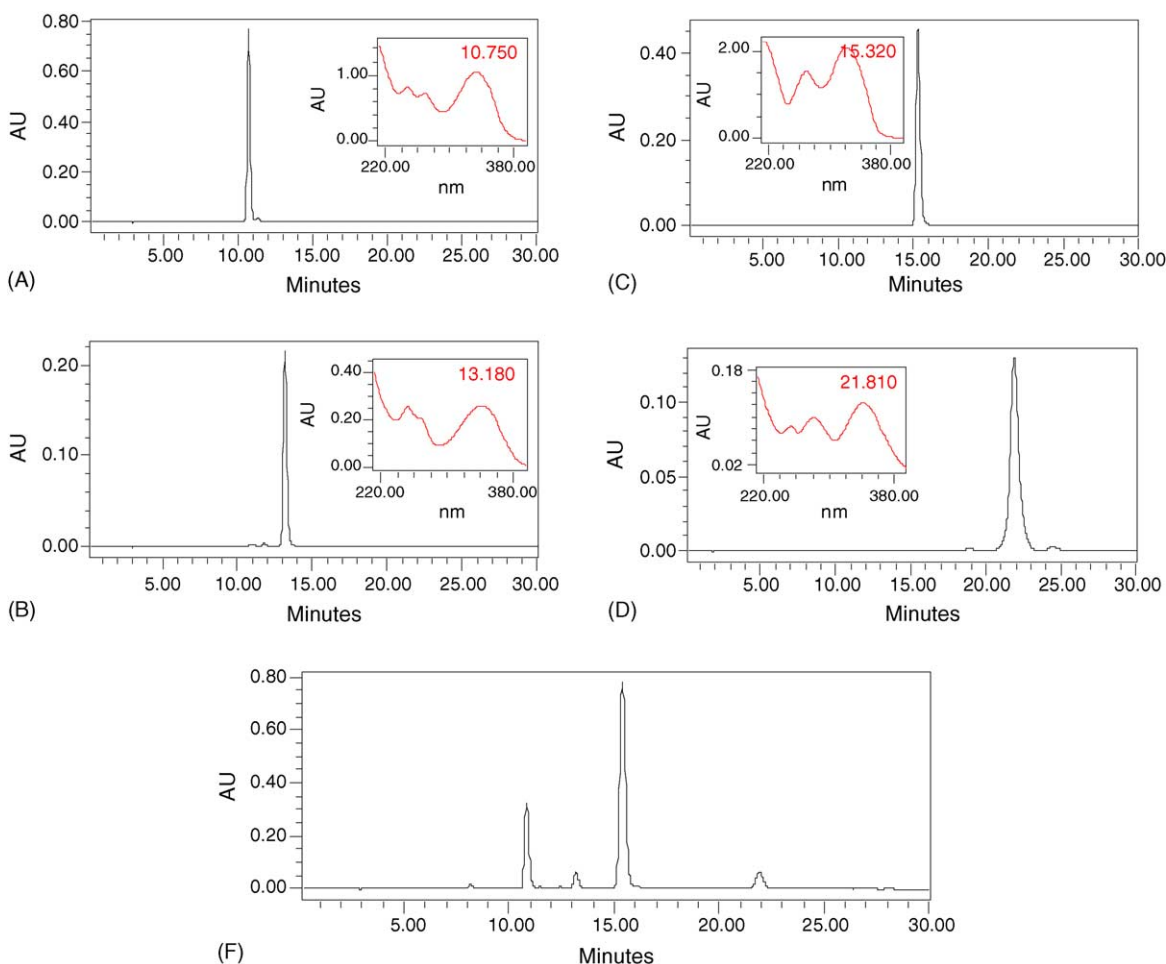| Solvent systems (hexane–ethyl acetate–methanol–water)	Peak no.						
	A	(a)	B	(a)	C	(a)	D
1:1:1:1	0.65	(1.49)	0.97	(1.65)	1.60	(2.30)	3.68
1:0.8:1:1.2	1.10	(1.45)	1.60	(1.63)	2.61	(2.16)	5.64
1:0.8:1:0.8	0.33	(1.42)	0.47	(1.83)	0.86	(2.12)	1.82
1:0.8:0.8:0.8	0.57	(1.49)	0.85	(1.49)	1.27	(1.69)	2.15
1:0.8:1:1	0.48	(1.54)	0.74	(1.78)	1.32	(2.42)	3.20

Fig. 3. HPLC chromatograms and UV spectrum of compounds A–D and HPLC chromatogram of the crude extract (F).

Compound C: Colorless needles (MeOH), $C_{20}H_{20}O_7$. EI-MS, m/z (%) 372 (M$^+$), 357 (M$^+$–CH_3 100), 341 (M$^+$–OCH_3), 329, 314, 296, 225, 197, 182, 167, 153, 132, 117, 83. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 3.89 (6H, s, 2 × OCH_3), 3.96 (3H, s, OCH_3), 4.02 (3H, s, OCH_3), 4. 11 (3H, s, OCH_3). 6.64 (1H, s, H-3), 7.03 (2H, d, J = 9.6 Hz, H-3′,5′), 7.90 (2H, d, J = 8.4 Hz, H-2′,6′). Comparing the above data with Refs. [11,21], the obtained product was identified as tangeretin [5,6,7,8,4′-pentamethoxyflavone].

Compound D: Yellow needles (MeOH), $C_{20}H_{20}O_8$. EI-MS, m/z (%) 388 (M$^+$), 373 (M$^+$–CH_3, 100), 358 (M$^+$–CH_3), 343 (M$^+$–CH_3–OCH_3), 327, 259, 211, 194, 183, 165, 163, 148, 147, 127, 69. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 3.96 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 3.98, (6H, m, 2 × OCH_3), 4.12 (3H, s, OCH_3), 6.62 (1H, s, H-3), 7.00 (1H, d, J = 2.4 Hz, H-5′), 7.42 (1H, d, J = 1.8 Hz, H-2′), 7.58 (1H, dd, J = 10.4 Hz, 1.8 Hz, H-6′). Comparing the above data with Refs. [11,21], the obtained product was identified as 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone.

In conclusion, an HSCCC method for the preparative separation and purification of nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3′,4′-

pentamethoxyflavone from Tangerine peel was developed with a two-phase solvent system comprised of hexane–ethyl acetate–methanol–water (1:0.8:1:1, v/v). The present study demonstrates that HSCCC is a valuable method in separating and purifying bioactive components from natural products.

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