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Preparative separation of nine flavonoids from *Pericarpium Citri Reticulatae* by preparative-HPLC and HSCCC

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ABSTRACT

An economical and systematized chromatographic separation process to isolate and purify flavonoids from *Pericarpium Citri Reticulatae* was developed. Solvent refluxing extraction, macroporous resin column, preparative HPLC and HSCCC (high-speed counter-current chromatography) were used for extraction, fraction, isolation, and purification of nine flavonoids including vicenin-2 (42 mg), hesperidin (108 mg), isosinensetin (15 mg), sinensetin (10 mg), tetramethyl-*O*-isoscutellarein (11 mg), nobiletin (121 mg), 3, 5, 6, 7, 8, 3′,4′-heptamethoxyflavone (34 mg), tangeretin (114 mg), and 5-demethylnobiletin (13 mg) from *Pericarpium Citri Reticulatae*, and their purities were all above 98%. All these constituents were identified by UV, MS, ^{1}H NMR, and ^{13}C NMR. The procedure and techniques described here can provide a useful reference for the preparative-scale isolation of some flavonoids in a fast, cost-effective, and environmentally-friendly method.

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Introduction

Pericarpium Citri Reticulatae (PCR, Chenpi in Chinese), the dried ripe pericarp of *Citrus reticulata Blanco* or its cultivars, is widely used in traditional Chinese medicine for its low toxicity and various pharmacological activities[1] and officially listed in the Chinese Pharmacopoeia.[2] Traditional Chinese medicine (TCM) theory indicates that it has the efficacy of strengthen spleen and liver, disperse stagnation, eliminate dampness, and resolve phlegm.[2] Modern pharmacological studies have shown that PCR exhibits significant anti-inflammatory,[3] antioxidant,[4–5] antibacterial,[6] and anticancer[7] functions. The major nonvolatile chemical compounds of this plant, which are responsible for the pharmacological activities are considered to be flavonoids. And the flavonoids mainly contain three types: flavone *C*-glycosides, flavone *O*-glycosides, and polymethoxyflavones. Among these, several compounds including vicenin-2, hesperidin, isosinensetin, sinensetin, tetramethyl-*O*-isoscutellarein, nobiletin, 3, 5, 6, 7, 8, 3′, 4′-heptamethoxyflavone (HMF), tangeretin and 5-demethylnobiletin are representative. A literature reported that vicenin-2 has the indirect gastrointestinal spasmolytic effect.[8] And hesperidin demonstrated anti-inflammatory, analgesic activities.[9] Since polymethoxyflavones have been reported to exhibit anti-inflammatory, anti-carcinogenic, anti-proliferative, and apoptotic effects, etc.,[10–13] it is urgently needed to acquire a large quantity of pure compounds for further pharmacological investigation and quality evaluation or standardization of PCR and its medical products that separation of these chemicals from raw materials is of critical importance.

Isolation and purification of flavonoids in PCR using silica gel column chromatography and HPLC has been reported.[14–16] Macroporous resins column and High-speed counter-current chromatography (HSCCC) have also been used in preparative separation of flavonoids from PCR.[17–21] Generally speaking, preparative high-performance liquid chromatography (prep-HPLC) is a method which can obtain the monomeric compound with high purity from crude materials, and it has also been industrially applied in separation for the isolation and purification of pharmaceuticals and other products.[22] However, the report for combining three techniques including Macroporous resins column, HSCCC, and prep-HPLC for the preparation of PCR of whole fraction was still infrequent.

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In our study, a method combining macroporous resins column which was used to fractioned the extract solution, HSCCC, and prep-HPLC was developed for isolation and purification of flavonoids from PCR systematically.

Experimental

Chemicals and reagents

Dried peels of *C. reticulata "Chachi"*, Guangchenpi, were bought from local TCM Company in Guangdong province of China. The species was verified by Prof. Shuyuan Li (Department of Chinese medicine of Guangdong Pharmaceutical University). The samples were ground into powder through a 50 mesh sieve. The powders were dried at 60°C until constant weight and were well blended before use.

HPLC grade acetonitrile and methanol from Merck (Darmstadt, Germany) were used. HPLC grade formic acid was purchased from ROE scientific INC. (Newcastle, USA). Water for HPLC analysis was purified by Milli-Q academic water purification system (Millipore, MA, USA). N-hexane, ethyl acetate, and methanol, which were analytical grade, were obtained from Shanghai Qiangshun Chemical Reagent Co., Ltd., China. The standards of hesperidin, nobiletin, and tangeretin were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Apparatus

Preparative HSCCC was carried out with a model TBE-300A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China). The apparatus equipped with a polytetrafluoroethylene three preparative coils (diameter of tube, 2.6 mm, total volume, 300 mL) and a 20 mL sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 at the internal terminal to 0.8 at the external terminal (β = r/R where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a model S constant-flow pump, a model UV-II detector operating at 280 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China).

The Varian Prostar 1200L semi-preparative HPLC system (Aligent, USA) consisted of Prostar 210 pump, Prostar 330 PDA detector, a 500 sample loop, and the Varian star 6.42 workstation. The analytical HPLC system used throughout this study consisted of LC-10AT pump (Shimadzu, Japan), a SPD-10A UV-vis detector (Shimadzu, Japan), and a LC-solution workstation (version1.25, Shimadzu, Japan).

Extraction procedure

500 g dried crude powder of *C. reticulate "Chachi"* was immersed in 5 L of 50% methanol overnight. The mixture was extracted twice by refluxing extraction for 2 h at 80°C, then filtered and collected the combined filtrates. After condensing the aqueous extracts into approximate 500 mL, the solution was passed through an 800 g macroporous resin column which had been washed with 4 L 95% ethanol and then equilibrated with 8 L water. The column was serially eluted with 2.5 L water, 2.5 L 25% ethanol, 2.5 L 50% ethanol, and 2.5 L 95% ethanol. 25% ethanol, 50% ethanol, and 95% ethanol fraction were evaporated to dryness by rotary vaporization at 60□ under reduced pressure to yield 7.3 g (fraction A), 5.7 g (fraction B), and 4.5 g (fraction C) of the final residue, respectively.

Separation of fraction A and B

The separation of fraction A and B were performed by preparative high performance liquid chromatography (pre-HPLC). A lichrospher C_{18} preparative column (100 mm × 10 mm, 5 μm) (Hanbon, China) was used for the separation and isolation of the compounds in fraction A and B. The mobile phase for fraction A was composed of methonal(A)-water(B), using gradient elution of 0-10 min 20%-30% A, 10-30 min 30% A. The flow-rate was 4.5 mL/min and the effluent was monitored at 271 nm. For fraction B, the mobile phase consisted of acetonitrile-water (18:82, v/v) was isocratically at a flow rate of 4.7 mL/min and the detector was set at 280 nm. All injection volume was 300 μL. Peak fraction was collected according to the elution profile.

Separation of fraction C

The high-speed counter current chromatography (HSCCC) and pre-HPLC techniques were used for the separation and isolation of fraction C. The two-phase solvent system used in the present study was prepared by mixing n-hexane-ethyl acetate-methonal-water (1:0.8:0.8:0.9, v/v/v/v). After thoroughly equilibrating the mixtures in a separated funnel at room temperature, the two phases were separated shortly before use. And the organic upper phase was used as the stationary phase. The sample solution was

prepared by dissolving 4.0 g of fraction C in 100 mL of the mobile phase. For each separation, the coil column was first entirely filled with stationary phase. Then the apparatus was rotated at 800 rpm and the sample solution was injected into the HSCCC system through the PTFE sample loop with the mobile phase at a flow-rate of 1.5 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 5 mL sample solution was injected through the injection value. The effluent of the column was continuously monitored with a UV detector at 280 nm. Peak fractions were collected according to the elution profile. The temperature of the apparatus was set at 25°C. The HSCCC peaks yielded fractions I-VI. The fractions II and III containing two compounds were separated by pre-HPLC using Varian Prostar 1200L system equipped a lichrospher column (10 mm × 100 mm, 5 μm) eluted by acetonitrile(A)-water(B) using gradient elution of 0-35 min 25%-50% A.

HPLC analysis and identification of pre-HPLC and HSCCC peak fractions

The fractionated samples and each peak fraction obtained by HSCCC and pre-HPLC were analyzed by HPLC. The column used was a Hanbon Benatach C_{18} (250 mm × 4.6 mm, 5 μm) (Hanbon, China) with a pre-column equipped with the same stationary phase, the mobile phase was acetonitrile (A) and 0.2% aqueous formic acid (v/v) (B) with gradient elution of 5-10% A at 0-10 min, 10-30% A at 10-50 min, 30-45% A at 50-65 min, and 45-90% A at 65-85 min. The flow rate was 1.0 mL/min, and the effluent was monitored at 280 nm and the column temperature was set at 25°C.[23]

Electrospray ionization mass spectrometry (ESI-MS)

All ESI-MS experiments of the compounds obtained from our separation procedure were performed on a LCQ fleet ion trap multiple mass spectrometer (Thermo Finnigan, San Jose, CA, USA) in positive ion mode. Instrument parameters: electrospray voltage 4 kV; capillary temperature 350°C; capillary voltage 35 V; tube lens offset 75 V; sheath gas pressure 35 psi, auxiliary gas flow rate 8 arb (1 arb = 0.3 L/min).

Nuclear magnetic resonance (NMR) analysis

^{1}H and ^{13}C NMR spectra were recorded in DMSO-d6 on a Bruker Avance 500 II (Karlsruhe, Germany) with 500 MHz for ^{1}H measurements and 125 MHz for ^{13}C measurements, respectively.

Results and discussion

Optimization of HPLC method

The crude extract solution and three fractionated parts used for further pre-HPLC or HSCCC isolation were analyzed by HPLC-UV method. To obtain LC chromatograms with good resolution of adjacent peaks within a reasonably short analysis time, different mobile phase compositions, different flow rates, detection wavelength, and column temperature were screened. It was found that the mobile phase was composed of acetonitrile and 0.2% aqueous formic acid, and the flow rate, column temperature, and detection wavelength were set at 1.0 mL/min, 25°C and 280 nm, which were most suitable for our analysis.[23] Under the conditions mentioned above, a satisfactory separation of the target compounds was obtained, and the HPLC chromatograms of the crude extract and three fractions is shown in Fig.1, which mainly contained nine peaks, and peaks 1, 2, 3, 4, 5, 6, 7, 8, and 9 correspond to vicenin-2, hesperidin, isosinensetin, sinensetin, tetramethyl-*O*-isoscutellarein, nobiletin, 3, 5, 6, 7, 8, 3′, 4′-heptamethoxyflavone, tangeretin, and 5-demethylnobiletin. And the structure of nine compounds was shown in Fig. 2.

Preparative HPLC separation of fraction A and B

In order to obtain the targeted compounds with high purity from fraction A and B, the pre-HPLC technique was choose for separation in place of HSCCC method. The pre-HPLC chromatograms of fraction A and B were shown in Fig.3. And The HPLC chromatograms of peaks 1 (vicenin-2) and 2 (hesperidin) were shown in Fig. 4. Finally, 42 mg vicenin-2 and 108 mg hesperidin were obtained with the purity of 98.9% and 99.4%, respectively.

HSCCC and pre-HPLC isolation and separation for fraction C

Preparative isolation of fraction C by HSCCC employed a two-phase solvent system composed of n-hexane-ethyl acetate-methonal-water (1:0.8:0.8:0.9, v/v/v/v) yielded six fractions (Fig. 4). Then, 15 mg of peak I, 36 mg of peak II, 121 mg of peak III, 34 mg of peak IV, 114 mg of peak V, and 13 mg of peak VI were obtained from 4.0 g of fraction C by HSCCC separation. As shown in Fig. 5, the HPLC analysis of each HSCCC fraction revealed that five pure compounds could be obtained from fraction C. And the purities of peak I (isosinensetin), peak III (nobiletin), peak IV

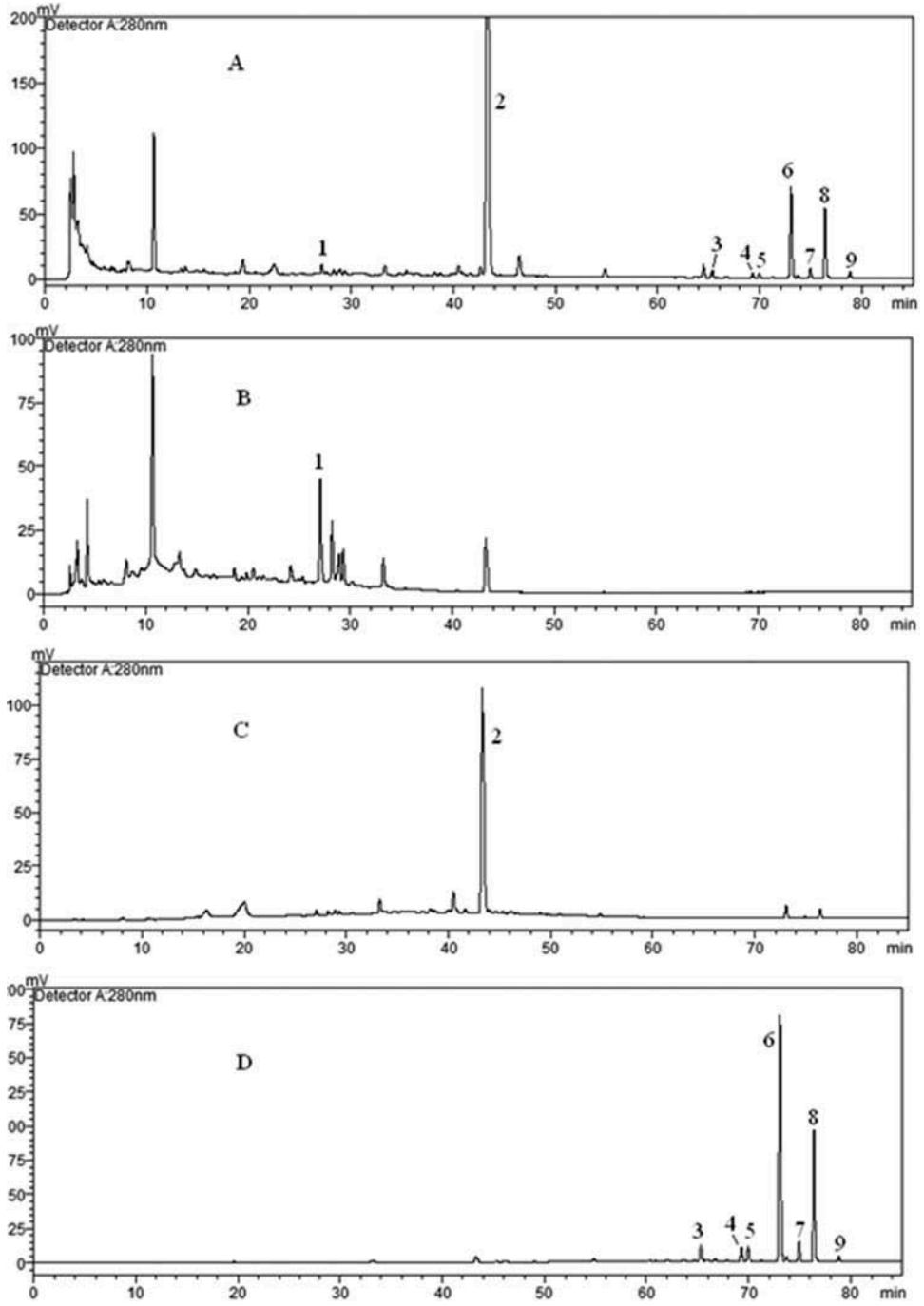

Figure 1. The HPLC-UV chromatograms of the crude extract (A), fraction A (B), fraction B (C) and fraction C (D). Peaks 1, 2, 3, 4, 5, 6, 7, 8 and 9 correspond to vicenin-2, hesperidin, isosinensetin, sinensetin, tetramethyl-O-isoscutellarein, nobiletin, HMF (3, 5, 6, 7, 8, 3′, 4′-heptamethoxyflavone), tangeretin and 5-demethylnobiletin.

(HMF), peak V (tangeretin), and peak VI (5-demethylnobiletin) were 98.8%, 98.4%, 99.2%, 98.1%, and 98.3%, respectively.

In order to obtain sinensetin and tetramethyl-*O*-isoscutellarein with high purity, preparative HPLC was used to further purify peak II. The effluent of target compounds (peaks VII and VIII) were collected and analyzed by HPLC (Figs. 6i and j). And 10 mg sinensetin and 11 mg tetramethyl-*O*-isoscutellarein were totally obtained, with the purity of 99.1% and 99.3%, respectively.

Identification of nine isolated citrus flavonoids

The obtained pure compounds were identified by UV, MS, NMR and compared with the standards. The MS and

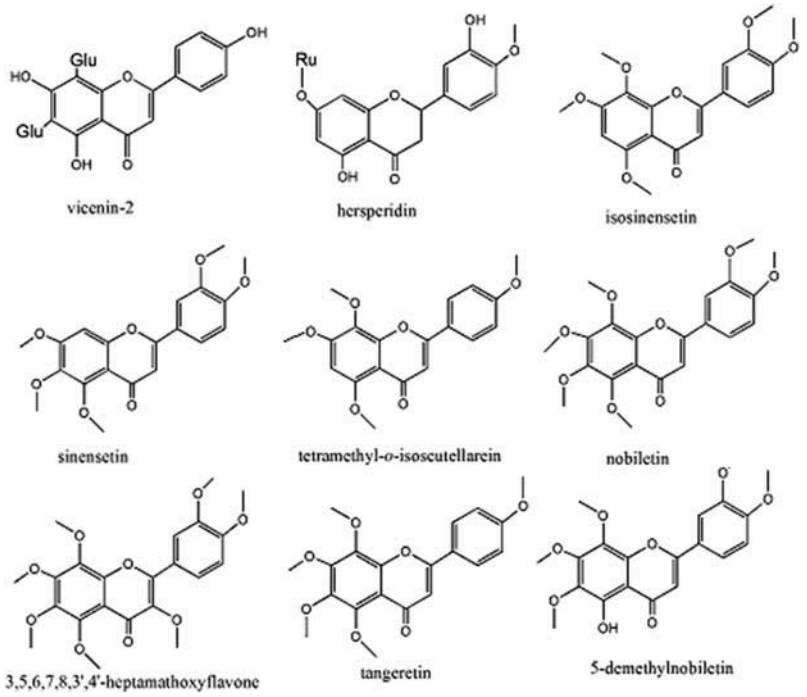

Figure 2. The chemical structures of nine compounds in *Pericarpium Citri Reticulatae*.

NMR data for several compounds were as follows. The UV and MS spectrum (shown in Figs. 4 and 5) of nine compounds are in agreement with the literature.[18, 24–28] And the retention times and MS data of compounds 2, 6, and 8 were the same as the standards (shown in Figs. 4 b/c, 6 c/d, and 6 f/g), thus compounds 2, 6, and 8 were identified to be hesperidin, nobiletin, and tangeretin, repectively.

Compound 1: $C_{27}H_{30}O_{15}$, ESI-MS: 595$[M+H]^+$, 617$[M+Na]^+$; 593$[M-H]^{-\cdot}$ ^{1}H NMR(500 MHz, DMSO-d6): δ6.9(1H, s, H-3), 7.9(2H, d, J=8.8Hz, H-2′, H-6′), 6.8 (2H, d, J = 8.2Hz, H-3′, H-5′), 5.6(1H, d, J = 7.8Hz, H-1″), 5.0(1H, d, J = 8.3Hz, H-1‴);^{13}C NMR(125MHz, DMSO-d6):δ164.2(C-2), 103.5(C-3), 180.6(C-4), 161.2 (C-5), 108.1(C-6), 158.9(C-7), 105.1(C-8), 152.1(C-9), 103.5(C-10), 124.1(C-1′), 128.9(C-2′),116.1(C-3′), 160.7 (C-4′), 115.8(C-5′), 129.1(C-6′), 75.4(C-1″), 70.4(C-2″), 77.1(C-3″), 71.1(C-4″), 83.4(C-5″), 60.9(C-6″), 73.5(C-1‴), 70.9(C-2‴), 78.1(C-3‴), 70.2(C-4‴), 81.5(C-5‴), 59.6(C-6‴). Compound 1 was identified as apigenin 6,

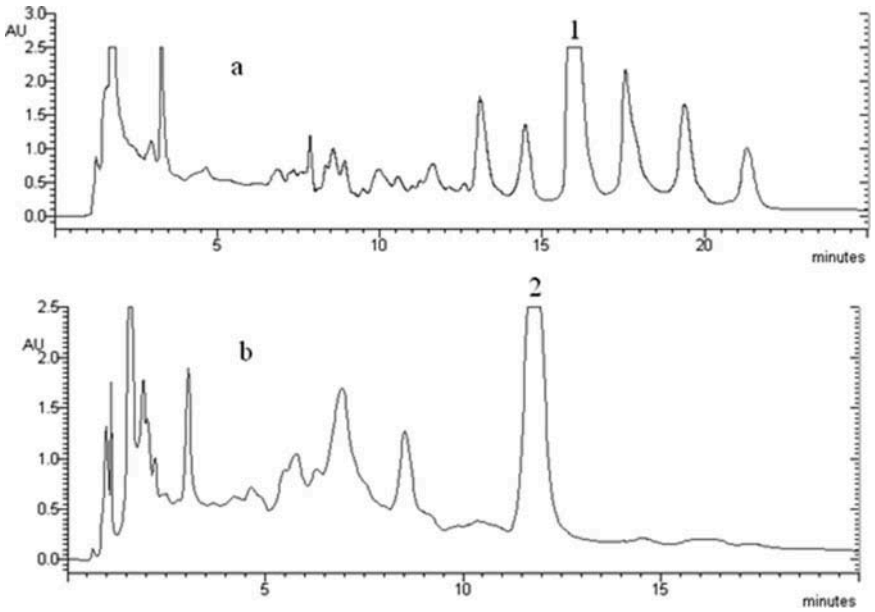

Figure 3. Pre-HPLC chromatograms of fraction A (a) and B (b). Peak 1: vicenin-2 Peak 2: hesperidin.

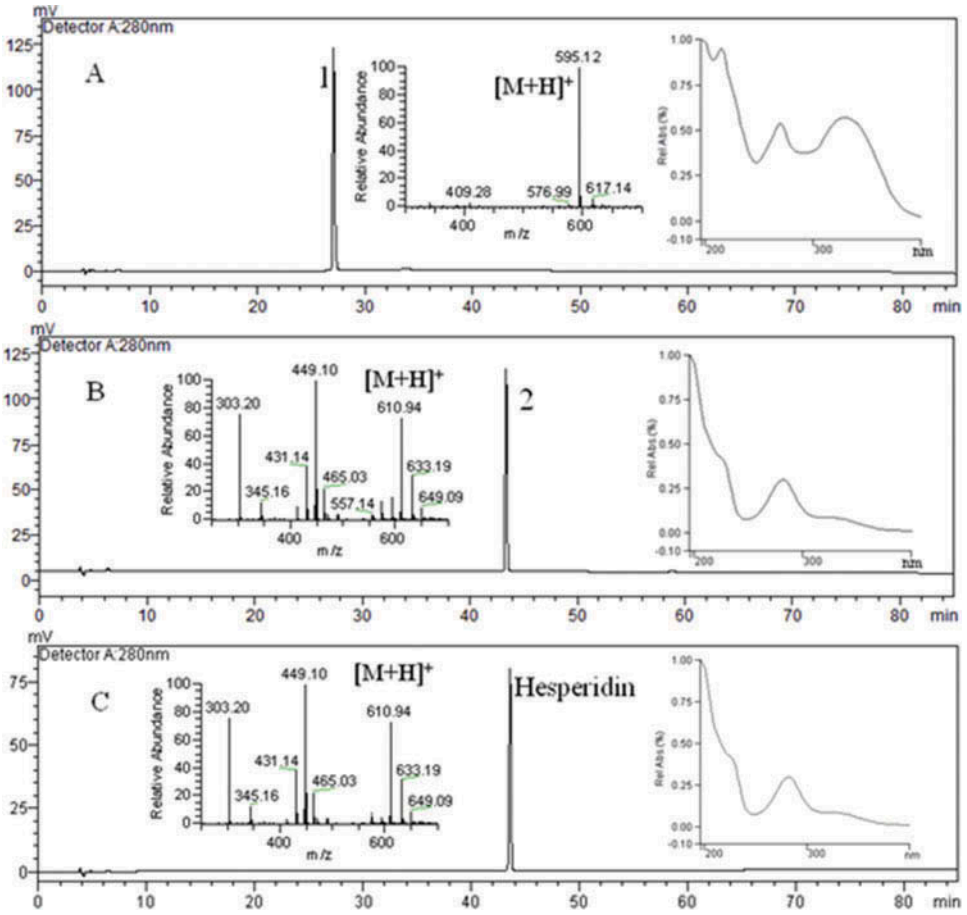

Figure 4. (A) HPLC chromatogram and UV, MS spectrum of compound 1 isolated by preparative HPLC (B) HPLC chromatogram and UV, MS spectrum of compound 2 isolated by preparative HPLC (C) HPLC chromatogram of the standard hesperidin.

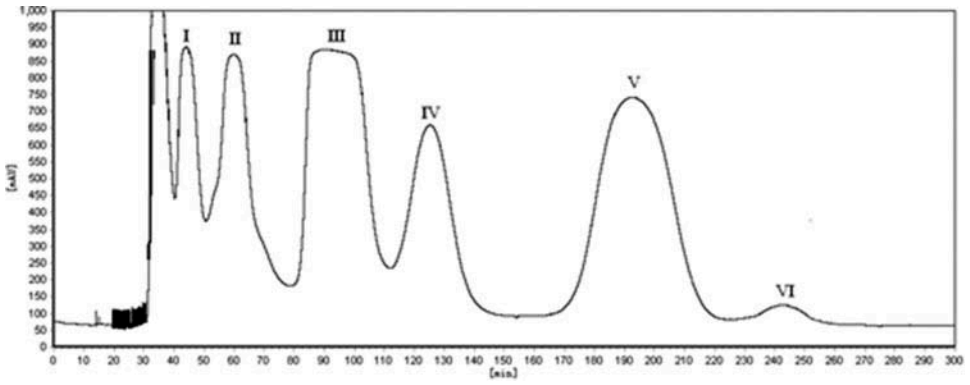

Figure 5. HSCCC chromatogram of fraction C.

8-di-C-glucoside (vicenin-2) from these spectral data and physical properties.[25, 26]

Compound 3: $C_{20}H_{20}O_7$. ESI-MS: 373$[M+H]^+$, 395$[M+Na]^+$, 767$[2M+Na]^+$; ^1H NMR(500MHz, DMSO-d6) δ: 6.8(1H, s, H-3), 6.6 (1H, s, H-6), 7.5(1H, s, H-2′), 7.1 (1H, d, J=7.8Hz, H-5′), 7.5(1H, d, J = 8.1Hz, H-6′), 3.8, 3.8, 3.7, 3.7, 3.8(15H, s, 5×OMe); ^{13}C NMR(125MHz, DMSO-d6) δ:158.9 (C-2), 105.7(C-3), 176.1(C-4), 154.3 (C-5), 104.1(C-6), 157.0(C-7), 135.6(C-8), 152.4 (C-9), 110.5(C-10), 122.9(C-1′), 107.8(C-2′), 147.5(C-3′), 150.1 (C-4′), 110.9(C-5′), 117.8 (C-6′), 60.2, 54.3, 57.1, 53.5, 54.1(5×OMe). Compound 3 was identified as 5, 7, 8, 3′, 4′-pentamethoxyflavone (isosinensetin) from these spectral data and physical properties.[24, 28]

Compound 4: $C_{20}H_{20}O_7$. ESI-MS: 373$[M+H]+$, 395$[M+Na]+$, 767$[2M+Na]+$; ^1H NMR(500MHz, DMSO-d6)

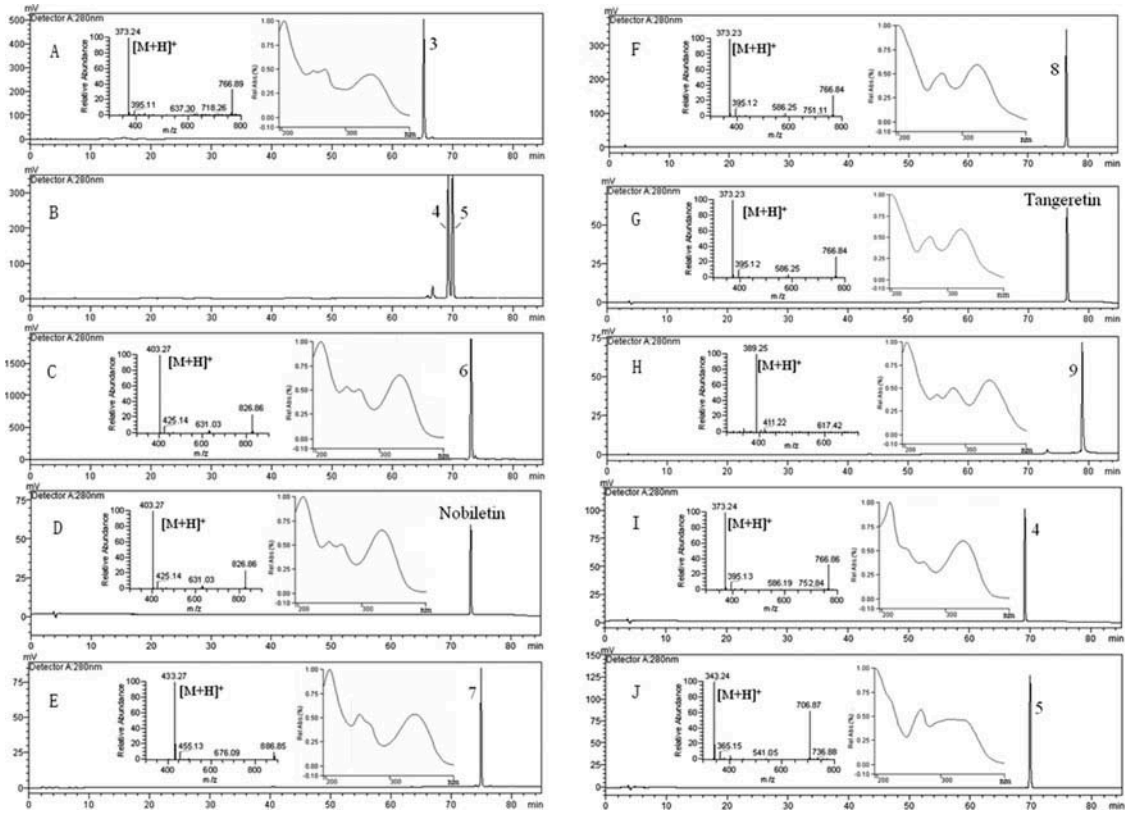

Figure 6. (A) HPLC chromatogram and UV, MS spectrum of HSCCC peak I; (B) HPLC chromatogram of HSCCC peak II; (C) HPLC chromatogram and UV, MS spectrum of HSCCC peak III; (D) HPLC chromatogram of the standard nobiletin; (E) HPLC chromatogram and UV, MS spectrum of HSCCC peak IV. Peaks 3, 4, 5, 6 and 7 correspond to isosinensetin, sinensetin, tetramethyl-O-isoscutellarein, nobiletin and HMF. (F) HPLC chromatogram and UV, MS spectrum of HSCCC peak V; (G) HPLC chromatogram the standard tangeretin; (G) HPLC chromatogram and UV, MS spectrum of HSCCC peak VI; (I) HPLC chromatogram and UV, MS spectrum of HSCCC-pre-HPLC peak VII; (J) HPLC chromatogram and UV, MS spectrum of HSCCC-pre-HPLC peak VIII. Peaks 4, 5, 8 and 9 correspond to sinensetin, tetramethyl-O-isoscutellarein, tangeretin and 5-demethylnobiletin.

δ:6.8 (1H, s, H-3), 6.7 (1H, s, H-8), 7.3(1H, s, H-2′), 7.0 (1H, d, J = 8.0Hz, H-5′), 7.4(1H, d, J = 8.0 Hz, H-6′), 3.7, 3.8, 3.8, 4.0, 3.7 (15H, m, 5×OMe); ^{13}C NMR (125MHz, DMSO-d6) δ:158.7 (C-2), 105.9 (C-3), 171.5 (C-4), 145.2 (C-5), 145.5 (C-6), 150.5(C-7), 111.4 (C-8), 149.1 (C-9), 116.1(C-10), 120.7(C-1′), 106.2(C-2′), 147.1(C-3′), 152.3(C-4′), 110.2 (C-5′), 118.1 (C-6′), 60.5, 59.8, 59.2, 58.8, 56.9, 56.1(5×OMe). Compound 4 was identified as 5, 6, 7, 3′, 4′-pentamethoxyflavone (sinensetin) from these spectral data and physical properties.[25]

Compound 5: $C_{19}H_{18}O_6$. ESI-MS: 343[M+H]+, 365[M+Na]+, 737[2M+Na]+; ^{1}H NMR (500MHz, DMSO-d6) δ:6.6(1H, s, H-3), 6.7 (1H, s, H-6), 7.2 (2H, d, J = 7.9 Hz, H-3′, 5′), 8.1 (2H, d, J = 8.2 Hz, H-2′, 6′), 3.8, 3.8, 3.7, 4.1 (12H, m, 4×OMe); ^{13}C NMR(125MHz, DMSO-d6) δ:159.2(C-2), 107.1(C-3), 176.3 (C-4), 153.1(C-5), 102.9(C-6), 154.8(C-7), 124.1(C-8), 148.7 (C-9), 108.1(C-10), 129.1(C-1′), 126.4(C-2′), 118.1(C-3′), 155.3(C-4′), 117.2(C-5′), 127.1 (C-6′), 63.8, 58.1, 57.1, 56.2(4×OMe). Compound 5 was identified as 5, 7, 8, 4′-tetramethoxyflavone (tetramethyl-*O*-isoscutellarein) from these spectral data and physical properties.[25]

Compound 7: $C_{22}H_{24}O_9$. ESI-MS: 433[M+H]+, 455[M+Na]+, 887[2M+Na]+; ^{1}H NMR (500MHz, DMSO-d6) δ: 7.3 (1H, d, J = 2.1Hz, H-2′), 6.7(1H, d, J = 8.1Hz, H-5′), 7.8(1H, dd, J = 2.1, 8.1 Hz, H-6′), 3.9, 3.8, 3.9, 4.0, 3.9, 3.8, 4.1(21H, s, 7×OMe); ^{13}C NMR(125MHz, DMSO-d6) δ:150.9 (C-2), 141.5 (C-3), 172.8(C-4), 147.5(C-5), 145.4(C-6), 149.9(C-7), 136.5(C-8), 144.7 (C-9), 118.5(C-10), 117.8(C-1′), 108.7(C-2′), 152.4(C-3′), 151.1(C-4′), 109.4 (C-5′), 118.7(C-6′), 63.6, 62.7, 62.5, 62.1, 61.0, 57.7, 57.4(7×OMe). Compound 7 was identified as 3, 5, 6, 7, 8, 3′, 4′-heptamethoxyflavone from these spectral data and physical properties.[18, 28]

Compound 9: $C_{20}H_{20}O_8$. ESI-MS: 389[M+H]$^+$, 411[M+Na]$^{+;}$ ^{1}H NMR(500MHz, DMSO-d6) δ:6.6(1H, s, H-3), 12.7(1H, s, 5-OH), 7.2 (1H, d, J = 1.9Hz, H-2′),

7.1(1H, d, J=8.7Hz, H-5′), 7.6(1H, dd, J = 2.2, 8.8 Hz, H-6′), 4.1, 4.0, 4.0, 3.8, 3.9(15H, s, 5×OMe); ^{13}C NMR (125MHz, DMSO-d6) δ:165.8(C-2), 103.5(C-3), 182.9 (C-4), 149.2(C-5), 137.1(C-6), 153.4(C-7), 132.7(C-8), 145.8(C-9), 107.3(C-10), 122.9(C-1′), 108.2(C-2′), 149.9 (C-3′), 153.9(C-4′), 111.1(C-5′), 119.8 (C-6′), 63.1, 61.4, 61.3, 57.1, 56.2(5×OMe). Compound 9 was identified as 5-hydroxy-6, 7, 8, 3′, 4′-pentamethoxyflavone (5-demethylnobiletin) from these spectral data and physical properties.[14, 28]

Conclusions

Nine flavonoids including vicenin-2, hesperidin, isosinensetin, sinensetin, tetramethyl-*O*-isoscutellarein, nobiletin, HMF, tangeretin, and 5-demethylnobiletin were successfully separated from *Pericarpium Citri Reticulatae* by preparative HPLC and HSCCC, and their chemical structures were identified by ESI-MS and ^{1}H and ^{13}C NMR. This procedure provides an effective method for the isolation and purification of bioactive components from *Pericarpium Citri Reticulatae* and our results prove that HSCCC incorporating pre-HPLC is a powerful technique for separating natural chemicals from medicinal plants.

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