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Journal of Chromatography A, 1102 (2006) 44–50
JOURNAL OF CHROMATOGRAPHY A
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Efficient new method for extraction and isolation of three flavonoids from *Patrinia villosa* Juss. by supercritical fluid extraction and high-speed counter-current chromatography

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Received 18 July 2005; received in revised form 9 October 2005; accepted 17 October 2005
Available online 8 November 2005

Abstract

Supercritical fluid extraction (SFE) of orotinin, orotinin-5-methyl ether and licoagrochalcone B from *Patrinia villosa* was performed. The optimization of parameters including pressure, temperature, modifier and sample particle size on yield was carried out using an analytical-scale SFE system. The process was then scaled up by 100 times using a preparative SFE system under the optimized conditions of 25 MPa, 45 °C, a sample particle size 40–60 mesh and modified CO_2 with 20% methanol. The yield of the preparative SFE was 2.82% (crude extract I) and the combined yield of orotinin, orotinin-5-methyl ether and licoagrochalcone B was 0.82 mg/g of dry sample mass. Then the crude extract I was re-dissolved in methanol and methanol soluble fraction (crude extract II, 0.17%) was obtained, which was successfully isolated and separated by a preparative high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (5:6:6:6, v/v/v/v) by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 ml/min after 3 h. The target compounds isolated and purified by HSCCC were analyzed by high performance liquid chromatography. The separation produced total of 38.2 mg of orotinin at 99.2% purity, 19.8 mg of orotinin-5-methyl ether at 98.5% purity and 21.5 mg of licoagrochalcone B at 97.6% purity from 400 mg of the crude extract in a one-step separation. The recoveries of orotinin, orotinin-5-methyl ether and licoagrochalcone B were 91.1, 91.6 and 90.3%, respectively, and the chemical structure identification was carried out by UV, IR, MS, ^{1}H NMR and ^{13}C NMR.

Keywords: *Patrinia villosa*; Counter-current chromatography; SFE; Flavonoid

1. Introduction

Patrinia, a genus of about 20 species, is mainly distributed in central to east of Asia and northeast of North America, 10 of which growing in China. *Patrinia* species have been used as medicinal plants for more than 2000 years from *ShenNongBenCaoJing*, a famous ancient Chinese medicinal literary, and some of them still used in folk medicine as anti-virus and anti-bacteria [1,2], especially two species, *P. scabiosaefolia* Fisch and *P. villosa* (*BaiJiangCao* in Chinese).

With regard to the chemical constituents of this genus, we have found more research about *P. scabiosaefolia* Fisch [3], *P. scabra* [4] *and P. gibbosa* [5] than *P. villosa*. Except for some iridoids [6,7], we have isolated and separated two C-glycosylflavones (isovitexin and isoorientin) [8] and a peptide derivative aurentiamide acetate [9] from it. A literature search did not yield any more references to early report on study of chemicals from the medicinal herb *P. villosa*. So, further chemical research and discovery from *P. villosa* is warranted for exploitation new TCM products and pharmacological tests.

Natural products are often obtained by some conventional protocols of extraction and separation techniques, such as using organic solvents to extract and column chromatography including silica gel and high-performance liquid chromatography (HPLC) to isolate, in which organic solvents are unfriendly to our environment and the conventional separation methods are tedious, time consuming, needing multiple steps, and worse still the sample are adsorbed onto the stationary phase irreversibly.

Two new techniques, supercritical fluid extraction (SFE) and high-speed counter-current chromatography (HSCCC) are

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doi:10.1016/j.chroma.2005.10.045

widely used to extract and separate natural products from medicinal plants [10–16]. The former uses CO_2 instead of organic solvent and possesses unusual properties such as high compressibility, liquid-like density, high diffusivity, low viscosity and low surface tension. So, supercritical fluid shows a greater ability to diffuse into the ultrafine matrix than the conventional organic solvents, thus improving extraction yield of desired materials from complex matrices. The later, a support free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support [17], has an excellent sample recovery and permits directly introduction of crude samples into the column without more preparation. But there have no reports of using SFE to extract and HSCCC to isolate orotinin, orotinin-5-methyl ether and licoagrochalcone B from natural plants.

The aim of the present paper, therefore, was first to optimize the extraction conditions of three flavonoids including orotinin, orotinin-5-methyl ether and licoagrochalcone B (shown in Fig. 1) by an analytical-scale SFE system using an orthogonal test design. Then, the extraction was scaled up by 100 times using a preparative-scale SFE system. Subsequently, a preparative HSCCC was used to isolate and separate the targets from the crude extract by increasing the flow-rate of the mobile phase. To our best knowledge, this is the first report of discovering orotinin, orotinin-5-methyl ether and licoagrochalcone B from the plant of *P. genus*.

2. Experimental

2.1. Reagents

Carbon dioxide (CO_2, 99.95%) was obtained from Beijing Analytical Instrument Factory. All solvents and other chemicals including *n*-hexane, ethyl acetate, methanol and acetic acid were analytical grade and purchased from WuLian Chemical Factory, Shanghai, China. While acetonitrile used for HPLC was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions.

The *P. villosa* was purchased from a local drug store and identified by Doctor Luping Qin (Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, China).

2.2. Optimization of SFE extraction

A Suprex HA system (Hua An SFE Company, Nan Tong, Jiang Su Province, China) in the SFE mode was used for optimization the extraction conditions. In this study, extraction was accomplished with 100 ml volume extraction vessel. Nine extractions were carried out at temperature of 45, 55 and 65 °C, pressure of 15, 25 and 35 MPa, sample particle size of 10–20, 20–40, 40–60 mesh and two different concentrations of methanol (10 and 20%) were used as modifier. Table 1 shows the SFE experimental conditions for the extraction of orotinin, orotinin-5-methyl ether and licoagrochalcone B from *P. villosa*. After 1 h of static extraction (no liquid flow), the sample was subjected to dynamic extraction for 1 h by flowing liquid CO_2 at a rate of 0.4 ml/min. The extract was trapped into a collection vessel containing about 100 ml methanol, and the sample was then analyzed by HPLC.

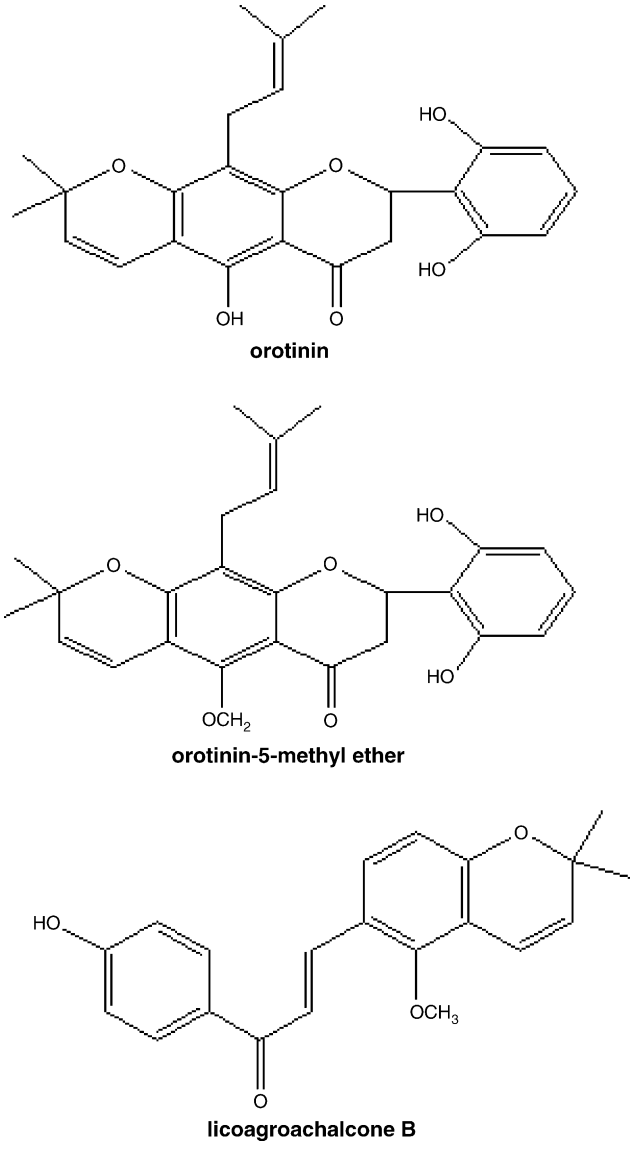

Fig. 1. The chemical structures of orotinin, orotinin-5-methyl ether and licoagrochalcone B.

2.3. Scaling-up SFE and preparation of the crude extract

After the SFE conditions were optimized, the extraction was scaled up by 100 times using a preparative system. Five kilograms amount of sample (40–60 mesh) was placed into an extraction vessel with a 1.0×10^4 ml capacity, and extracted statically for 1 h followed by another 5 h dynamically under the optimized conditions at 45 °C, 25 MPa. The flow-rate of carbon dioxide supercritical fluid was set at 40 kg/h, and the extract in supercritical fluid was depressed directly into a separate vessel. The SFE extract before methanol washing (crude extract I) was light yellow semi-solid and then re-dissolved in methanol, and

Table 1
L_9 $(3)^4$ orthogonal test design

Test no.	Factors							
	A: pressure (MPa)		B: temperature (°C)		C: particle size (mesh)		D: modifier (methanol %)[a]	
Matrix								
1	A_1	15	B_1	45	C_1	10–20	D_1	0
2	A_1	15	B_2	55	C_2	20–40	D_2	10
3	A_1	15	B_3	65	C_3	40–60	D_3	20
4	A_2	25	B_1	45	C_2	20–40	D_3	20
5	A_2	25	B_2	55	C_3	40–60	D_1	0
6	A_2	25	B_3	65	C_1	10–20	D_2	10
7	A_3	35	B_1	45	C_3	40–60	D_2	10
8	A_3	35	B_2	55	C_1	10–20	D_3	20
9	A_3	35	B_3	65	C_2	20–40	D_1	0

[a] Modifier (methanol %) = volume of added methanol (ml)/sample mass (g).

the methanol soluble fraction (crude extract II) was obtained and evaporated to dryness under reduced pressure at 60 °C, which was subjected to subsequent HSCCC isolation and separation.

2.4. Preparation of two-phase solvent system and sample solution

In the present paper, we selected several kinds of two-phase solvent systems. Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude extract II in the solvent mixture of lower phase and upper phase (1:1, v/v) of the solvent system for isolation because the sample was not easily dissolved in either phase.

2.5. HSCCC separation procedure

Preparative HSCCC was carried out with a Model TBE-300A high-speed counter-current chromatography system manufactured by Tauto Biotech Co., Shanghai, China. The apparatus equipped with a polytetrafluoroethylene three preparative coils (diameter of tube, 1.6 mm, total volume, 300 ml) and a 20 ml sample loop. The β value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta = r/R$, $R = 7.5$ cm, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the hold axis and central axis of the centrifuge). 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).

In each separation, the coil column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 900 rpm, while the lower phase (mobile phase) was pumped into the column at a flow-rate of 1.0 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, approximately 10 ml of the sample solution containing 400 mg of the crude extract II was injected into the head of the column through the injection valve. After 3 h, the flow-rate of the mobile phase was increased to 2.0 ml/min. The effluent of the column was continuously monitored with a UV–vis detector at 280 nm. Peak fractions were collected according to the elution profile.

2.6. HPLC analysis and identification of HSCCC fractions

The analytical HPLC system used throughout this study consisted of 515 pump and 2487 detector (Waters), and a model N2000 workstation (Zhejiang University, Hangzhou, China). The crude sample and peak fractions obtained by HSCCC were analyzed by HPLC. The column used was a reversed-phase Lichrospher C_{18} (250 mm × 4.6 mm I.D., 5 μm) (Hanbang Science, Jiang Su Province, China) with a pre-column equipped with the same stationary phase, the mobile phase was CH_3CN–H_2O–HAC (60:40:2, v/v/v). The flow rate was 1.0 ml/min, and the effluent was monitored at 280 nm.

Identification of HSCCC fractions was carried out by UV (Cary-50), IR (Hitachi 275-50), MS (Finnigan MAT 711), ^{1}H NMR and ^{13}C NMR spectra (Varian Unity Inova–500).

3. Results and discussion

3.1. Optimization of the SFE conditions

The first step in the SFE is to optimize the operating conditions to obtain an efficient extraction of the target compounds and avoid the co-extraction of undesired compounds such as fatty acids and their esters.

Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of a SFE method. In fact, the fluid pressure, temperature, sample particle size and modifier are generally considered to be the most important factors. Optimization the suitable extraction conditions in SFE can be carried out step-by-step or by using an experimental design. In the present study, all selected factors were examined using an orthogonal L_9 $(3)^4$ test design.

The extract obtained from each test in SFE was quantitatively analyzed by HPLC for the contents of orotinin, orotinin-5-methyl ether and licoagrochalcone B. The results presented in Table 2 indicated that the maximum extraction yields of the crude extract I and crude extract II were 2.69 and 0.13%, and

Table 2
L_9 $(3)^4$ test result

Test no.	Parameters				Yield (%)[a]	Yield (%)[b]	Yield (μg/g)[c]		
	A	B	C	D			Compound 1	Compound 2	Compound 3
1	A_1	B_1	C_1	D_1	0.64	0.02	25.6	10.2	12.5
2	A_1	B_2	C_2	D_2	0.96	0.06	120.5	73.4	80.5
3	A_1	B_3	C_3	D_3	1.87	0.11	300.5	160.8	170.4
4	A_2	B_1	C_2	D_3	2.69	0.13	360.6	180.3	200.6
5	A_2	B_2	C_3	D_1	1.02	0.05	134.2	55.8	68.4
6	A_2	B_3	C_1	D_2	1.14	0.06	180.6	90.4	101.2
7	A_3	B_1	C_3	D_2	1.25	0.07	210.5	120.5	130.6
8	A_3	B_2	C_1	D_3	1.41	0.09	285.6	150.6	170.2
9	A_3	B_3	C_2	D_1	0.52	0.01	20.6	30.5	26.3

[a] Extraction yield (%) = (the amount of crude extract I/sample mass) × 100.
[b] Extraction yield (%) = (the amount of crude extract II/sample mass) × 100.
[c] Extraction yield (μg/g) = the amount of the target compounds/sample mass.

the maximum extraction yields of orotinin, orotinin-5-methyl ether and licoagrochalcone B were 360.6, 180.3 and 200.6 μg/g, respectively. In each test, the extraction yields of orotinin-5-methyl ether and licoagrochalcone B were lower than that of orotinin.

Extraction efficiencies at different sets of temperature, pressure, sample particle size and modifier were examined under L_9 $(3)^4$ test design. The results shown in Table 2 indicated that there are great yield differences among each set of SFE conditions. If the extraction yield of orotinin, orotinin-5-methyl ether and licoagrochalcone B was expressed as a control index, the results in Table 2 are transformed to Table 3 after orthogonal analysis.

The modifier was found to be the most important determinant of the yield. The extraction yields of orotinin, orotinin-5-methyl ether and licoagrochalcone B significantly increased as the concentration of the modifier increased (shown in Fig. 2). Pressure, temperature and sample particle size have no significant influence on the yield of orotinin, orotinin-5-methyl ether and licoagrochalcone B, and the 25 MPa of pressure, 45 °C of temperature and 40–60 mesh of sample particle size seem favorable for the extraction of the compounds (shown in Fig. 2 and Table 3). High temperature, large sample particle size and no modifier were not satisfactory. Moderate pressure was favorable to our aim. These results indicated that the optimal conditions for extraction of orotinin, orotinin-5-methyl ether and licoagrochalcone B by SFE were 25 MPa of pressure, 45 °C of temperature, 40–60 mesh of sample particle size and modified with 20% methanol.

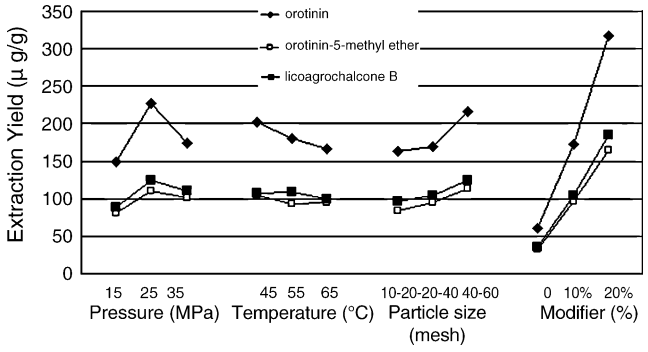

Fig. 2. Effects of pressure, temperature, sample particle size and modifier on yield of orotinin, orotinin-5-methyl ether and licoagrochalcone B.

3.2. Preparative-scale SFE

Under the optimal conditions, 5 kg of the powder was extracted by SFE and 141 g (extraction yield 2.82%) crude

Table 3
Analysis of L9 (3) 4 test results

	Compound 1 (μg/g)				Compound 2 (μg/g)				Compound 3 (μg/g)			
	A	B	C	D	A	B	C	D	A	B	C	D
K_1	446.6[a]	606.6	491.8	180.4	244.4	314.0	254.2	96.5	265.4	321.7	289.9	107.2
K_2	680.3	540.3	506.6	516.6	329.5	280.8	285.2	288.3	375.2	325.1	312.4	312.3
K_3	521.7	501.7	650.2	951.6	304.6	283.7	339.1	493.7	333.1	299.9	371.4	554.2
k_1	148.9[b]	202.2	163.9	60.1	81.5	104.7	84.7	32.2	88.5	107.2	96.6	35.7
k_2	226.8	180.1	168.9	172.2	109.8	93.6	95.1	96.1	125.1	108.4	104.1	104.1
k_3	173.9	167.2	216.7	317.2	101.5	94.6	113.0	164.6	111.0	100.0	123.8	184.7
R	77.9[c]	35.0	52.8	257.1	28.3	11.1	28.3	132.4	36.6	8.4	27.2	149.0
Optimal level	A_2	B_1	C_3	D_3	A_2	B_1	C_3	D_3	A_2	B_1	C_3	D_3

[a] $K_i^A = \sum$ the amount of target compounds at A_i.
[b] $k_i^A = K_i^A/3$.
[c] $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

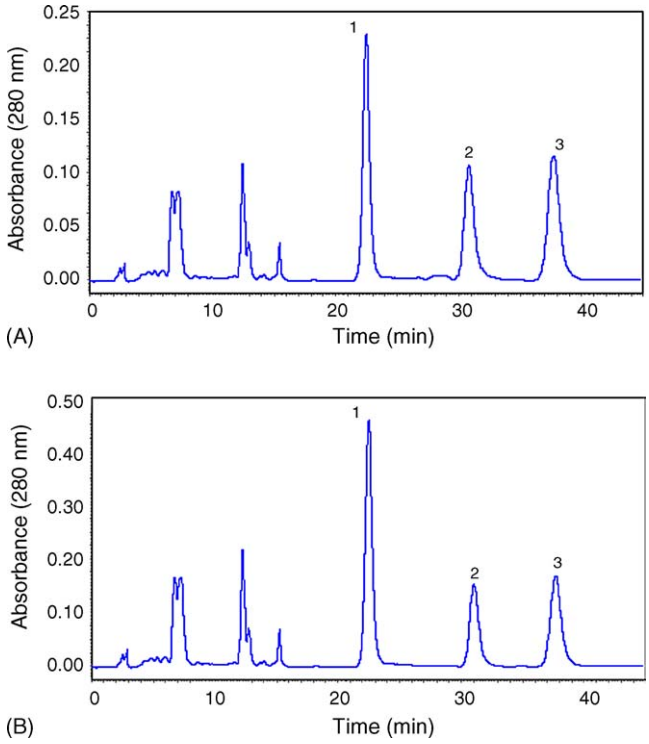

Fig. 3. (A) HPLC chromatogram of the crude extract I from preparative SFE; (B) HPLC chromatogram of the crude extract II from preparative SFE. Column: reversed-phase Lichrospher C_{18} (250 mm × 4.6 mm I.D., 5 μm); mobile phase: CH_3CN—H_2O—HAC (60:40:2, v/v/v); flow rate: 1.0 ml/min; UV wavelength: 280 nm; column temperature: 25 °C; 1: orotinin; 2: orotinin-5-methyl ether; 3: licoagrochalcone B.

extract I was obtained, which contained 1.40% orotinin, 0.72% orotinin-5-methyl ether and 0.78% licoagrochalcone B (Fig. 3A). Then the crude extract I was re-dissolved in methanol and 8.5 g (extraction yield 0.17%) methanol soluble fraction (crude extract II) was obtained, in which orotinin, orotinin-5-methyl ether and licoagrochalcone B were present at 20.2, 10.3 and 11.3%, respectively, according to the HPLC analysis (Fig. 3B). The recoveries of orotinin, orotinin-5-methyl ether and licoagrochalcone B in the step of changing the crude extract I into the crude extract II were 87.0, 86.3 and 87.3%, respectively. The crude extract II was then subjected to purification by HSCCC.

3.3. Selection HSCCC separation conditions

In HSCCC, the selection of two-phase solvent system is the most important for successful separation, and is also the most difficult step; it is estimated that about 90% of the entire work in HSCCC is spent on that. If only one component requires to be isolated from others, the standard HSCCC method, which uses a constant flow-rate of the mobile phase, could be used. In order to isolate more different compounds, stepwise elution or stepwise increasing the flow-rate of the mobile phase might be adopted [18–20]. First, HSCCC experiment was carried out with a two-phase solvent system composed of *n*-hexane–methanol at a volume ratio of 1:1 (v/v). However, it was difficult to purify the target compounds from the crude extract II, because the time they were retained in the column was short. Subsequently, a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at a volume ratio of 1:1:3:3 (v/v/v/v) was tested. Although the peak resolution was improved, and orotinin could be isolated from others, it was difficult to separate orotinin-5-methyl ether and licoagrochalcone B. Then, a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 5:6:6:6 (v/v/v/v) was selected and the lower phase was used as the mobile phase at the flow rate of 1.0 ml/min. Although orotinin and orotinin-5-methyl ether were purified and separated, licoagrochalcone B was retained in the column for a long time (10 h) and more mobile phase was required. While the flow-rate of the mobile phase was increased to 2.0 ml/min, orotinin-5-methyl ether and licoagrochalcone B were obtained from other constituents, but orotinin could not be separated. Finally, the method with stepwise increasing the flow-rate of the mobile phase was attempted with this two-phase solvent system. That is, the flow-rate of the mobile phase was kept at 1.0 ml/min before 3 h, and then increased to 2.0 ml/min after 180 min.

At the same time, the influence of the separation temperature and the revolution speed was also investigated. The temperature has significant effect on *K* values, the retention of stationary phase and the mutual solvency of the two-phase. After tested at 15, 20, 25, 30, 35 and 40 °C, it can be seen that good result can be obtained when the separation temperature was controlled at 30 °C. The revolution speed has a great influence to the retention of the stationary phase, high rotary speed can increase the retention of the stationary phase. In our experiment, the revolution speed was set at 900 rpm.

Under the above optimized separation conditions, the isolation of the target compounds was achieved with good resolution and the retention of the stationary phase was satisfactory (52%), and the purification was listed approximately 450 min (HSCCC chromatogram is shown in Fig. 4). After orotinin, orotinin-5-methyl ether and licoagrochalcone B were eluted out, in order

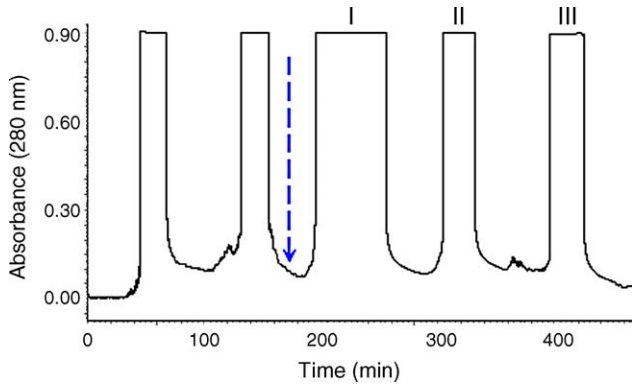

Fig. 4. HSCCC chromatogram of the crude extract II from *P. villosa*. Solvent system: *n*-hexane–ethyl acetate–methanol–water (5:6:6:6, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate, 0–3 h, 1.0 ml/min and 3–8 h, 2.0 ml/min; detection wavelength: 280 nm; sample size: 400 mg; retention of stationary phase: 52%; separation temperature: 30 °C; revolution speed: 900 rpm; sample loop: 20 ml. The arrow indicates the flow-rate of the mobile phase was increased stepwise from 1.0 to 2.0 ml/min after 3 h.

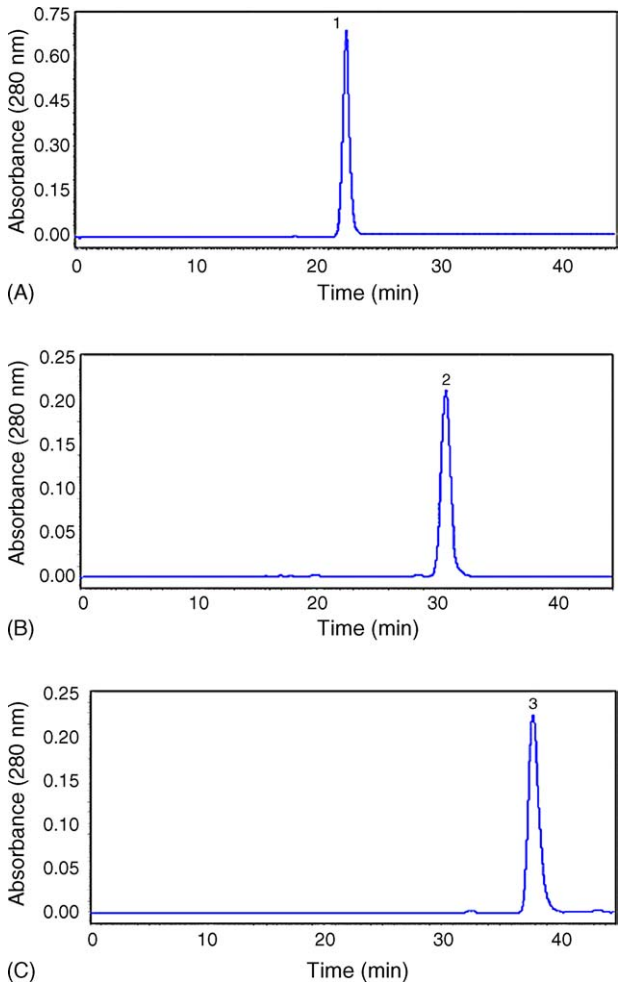

Fig. 5. HPLC chromatograms of orotinin, orotinin-5-methyl ether and licoagrochalcone B purified from *P. villosa* by HSCCC. HPLC conditions and the peaks were the same as showed in Fig. 2. (A) fraction I purified by HSCCC; (B) fraction II purified by HSCCC; (C) fraction III purified by HSCCC.

to save solvents and time, the remaining compounds in the column were removed by forcing out the stationary phase with pressurized nitrogen gas instead of eluting them with the mobile phase because the stationary phase was not to be reused. Fig. 4 shows the preparative HSCCC isolation of 400 mg of crude extract II using the solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 5:6:6:6 (v/v/v/v) by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 ml/min after 3 h. This separation yielded 38.2 mg of orotinin at 99.2% purity, 19.8 mg of orotinin-5-methyl ether at 98.5% purity and 21.5 mg of licoagrochalcone B at 97.6% purity according to HPLC analysis (HPLC chromatograms are shown in Fig. 5). The recoveries of orotinin, orotinin-5-methyl ether and licoagrochalcone B were 91.1, 91.6 and 90.3%, respectively. The overall recoveries of orotinin, orotinin-5-methyl ether and licoagrochalcone B in the present paper were 79.3, 79.0 and 78.8%, respectively, which were calculated by multiplication of the recoveries in the step of changing the crude extract I into the crude extract II and the recoveries in HSCCC step and lower than that of only in HSCCC step.

3.4. Chemical structure identification

The chemical structure identification of orotinin, orotinin-5-methyl ether and licoagrochalcone B was carried out by UV, IR, MS, ^{1}H NMR and ^{13}C NMR spectra as follows.

Fraction I: yellow powder. UVλ_{max}^{MeOH} : 273, 310 nm IR (KBr) υ_{max} cm^{-1}: 3425, 1630, 1600, 1458, 1378, 1140, 880, 785. ESI-MS (*m*/*z*): 421 $[M-H]^-$. These spectra data, ^{1}H NMR and ^{13}C NMR data are in agreement with orotinin in the literature [21].

Fraction II: light yellow solid. UVλ_{max}^{MeOH} : 264 nm. IR (KBr) υ_{max} cm^{-1}: 3450, 1670, 1660, 1600, 1461, 1380. ESI-MS (*m*/*z*): 435 $[M-H]^-$. These spectra data, ^{1}H NMR and ^{13}C NMR data are in agreement with orotinin-5-methyl ether in the literature [21].

Fraction III: yellow powder. UVλ_{max}^{MeOH} : 290, 350 nm. IR (KBr) υ_{max} cm^{-1}: 3430, 1630, 1590, 1560. ESI-MS (*m*/*z*): 335 $[M-H]^-$. These spectra data, ^{1}H NMR and ^{13}C NMR data are in agreement with licoagrochalcone B in the literature [22].

4. Conclusion

Three flavonoids including orotinin, orotinin-5-methyl ether and licoagrochalcone B from the traditional Chinese medicine *P. villosa* were extracted, separated and purified by SFE and HSCCC. Under optimal conditions i.e., a pressure of 25 MPa, a temperature of 45 °C, a sample particle size of 40–60 mesh and modifier of 20% methanol, the extraction yields of orotinin, orotinin-5-methyl ether and licoagrochalcone B were 1.40, 0.72 and 0.78% in crude extract I, and increased to 20.2, 10.3 and 11.3% in crude extract II, respectively. At last, high purities of orotinin, orotinin-5-methyl ether and licoagrochalcone B were obtained by HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 5:6:6:6 (v/v/v/v) by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 ml/min after 3 h. The results of the present paper demonstrated that SFE and HSCCC are very useful techniques for the extraction, isolation and purification of orotinin, orotinin-5-methyl ether and licoagrochalcone B from *P. villosa*.

Acknowledgement

Financial support from Ministry of Science and Technology of China (863 project) is gratefully acknowledged.

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