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Supercritical fluid extraction of quinolizidine alkaloids from *Sophora flavescens* Ait. and purification by high-speed counter-current chromatography

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Abstract

Supercritical fluid extraction (SFE) was used to extract quinolizidine alkaloids from *Sophora flavescens* Ait. (Kushen). An orthogonal test $L_9(3)^4$ including pressure, temperature, flow rate of CO_2 and the amount of modifier was performed to get the optimal conditions. The process was then scaled up by 30 times with a preparative SFE system under 25 MPa, 50 °C and a flow rate of CO_2 (2 l/min) and the amount of modifier (0.04 ml/min). The crude extracts were separated and purified by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of chloroform–methanol–2.3×10^{-2} M NaH_2PO_4 (27.5:20:12.5, v/v), and the collected fractions were analyzed by high-performance liquid chromatography (HPLC). Three kinds of quinolizidine alkaloids were obtained, yielding 10.02 mg of matrine, 22.07 mg of oxysophocarpine and 79.93 mg of oxymatrine with purities of 95.6, 95.8, 99.6% in one-step separation, respectively.

Keywords: Supercritical fluid extraction; High-speed counter-current chromatography; Quinolizidine alkaloids; *Sophora flavescens* Ait

1. Introduction

The dried root of *Sophora flavescens* Ait. (Leguminosae), a typical traditional Chinese medicine, is commonly used for the treatment of viral hepatitis, cancer, viral myocarditis, gastrointestinal hemorrhage and skin diseases (such as colpitis, psoriasis and eczema) [1–3]. The principal bioactive constituents of *S. flavescens* Ait. are the major quinolizidine alkaloids matrine (MT) and oxymatrine (OMT), which were reported to exhibit sedative, depressant, anti-tumor, antipyretic, cardiotonic activities [4,5] and anti-hepatitis B virus (HBV) activity [6]. Oxysophocarpine (OSC), another alkaloid, obviously suppressed the biosynethesis of leukotrienes (LTC4 and LTB4) in dose-dependent manner [7]. OSC, MT and OMT (structure shown in Fig. 1) were also found in other plants of Leguminosae, such as *Sophora tonkinensis*, *Sophora subprostata* and *S. alopecuroides* [8,9].

Due to the high pharmacological activities, alkaloids from the root of *S. flavescens* Ait. has recently drawn great attention in natural medication researches. A large quantity of pure materials is urgently needed for further studies. Several methods such as high-performance liquid chromatography (HPLC), silica gel, polyamide column and thin-layer chromatography (TLC) have been applied to the separation and purification of matrine-type alkaloids in *S. flavescens* root. Undoubtedly, HPLC is the most widely used separation technique [10]. However, the conventional preparative separation and purification methods are tedious and time-consuming, requiring multiple chromatographic steps. As for HPLC, reconditioning of the column requires a long time and a large volume of organic solvent. Expensive columns and frequent changing of the columns for fear of loss of elution efficiency are also required. Hence, sensitive, rapid and specific methods for purification of quinolizidine alkaloids are of great interest.

As an alternative, supercritical fluid extraction (SFE) is a particularly suitable method for the research of natural materials. Carbon dioxide is an ideal solvent because it is non-toxic, non-explosive, readily available and easy to remove from extracted

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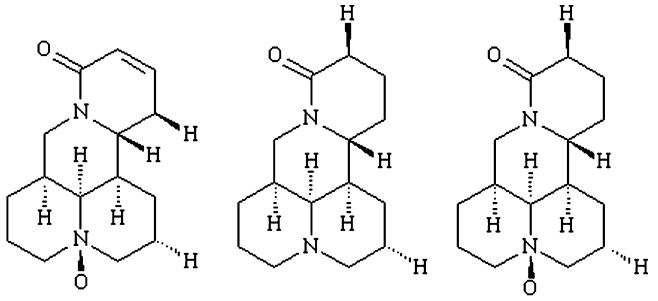

Fig. 1. Chemical structures of alkaloids from *Sophora flavescens* Ait.

products. SFE has the ability to use low temperatures, leading to less deterioration of the thermally labile components in the extract [11–16]. In addition, SFE using carbon dioxide ensures minimal alteration of the active ingredients, and the curative properties can be preserved.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography technique that uses no solid support matrix [17]. HSCCC eliminates the irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. This method has been successfully applied to the separation and purification of several natural products [18–22]. No reports on the use of SFE to extract and HSCCC to isolate matrine, oxysophocarpine and oxymatrine from natural plants have been found.

We herein optimized experiment parameters by an analytical-scale SFE system using an orthogonal test design. Then, the extraction was scaled up by 30 times by a prepared-scale SFE system. Subsequently, the crude extract was purified by HSCCC.

2. Experimental

2.1. Reagents and materials

Carbon dioxide (99.9% purity) was obtained from Daxing Gas Co., Beijing, China. All organic solvents used for HSCCC were of analytical grade and purchased from Guangcheng Chemical Factory, Tianjin, China. HPLC-grade acetonitrile was obtained from Tedia, USA. The roots of *S. flavescens* Ait. were purchased from a local drug store of Jinan, Shandong Province, in August 2006. Three quinolizidine alkaloids, matrine, oxysophocarpine and oxymatrine, were purchased from National Institute of the Control of Pharmaceutical and Biological Products, Beijing, China, and diluted to the desired concentration prior to use.

Compared with the voucher specimen collected from Bozhou, Anhui Province, the dried roots of *S. flavescens* were identified by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.2. Optimization of SFE extraction

A Spe-ed SFE system (Applied Separations, Allentown, PA, USA) fitted with a 10 ml stainless-steel extraction vessel was used for optimization of the extraction conditions with modifier adding by a WellChrom K-501 HPLC pump (Knauer, Berlin, Germany). A micro-metering valve was used as restrictor valve to control the flow rate of the supercritical CO_2 to the solvent collection. Extraction temperatures were monitored using a thermocouple and were found to be accurate to within ±1 K. The precision of the pressure measurement was ±1 Pa.

Table 1
Orthogonal experimental design

	A, pressure (MPa)	B, temperature (°C)	C, flow rate of CO_2 (l/min)	D, flow rate of modifier (ml/min)
1	20	45	1.0	0.02
2	25	50	1.5	0.03
3	30	55	2.0	0.04

To get more effective extraction, the roots of *S. flavescens* Ait. were shattered to powder (60–80 mesh) and dipped in 0.1 ml/l ammonia–ethanol at the ratio of 1:4 (v/v) for 24 h. An orthogonal test design $L_9(3)^4$ was employed where temperature, pressure, flow rate of CO_2 and the flow rate of 75% ethanol and 25% water as a modifier were considered to be the four major factors for effective extraction. Combinations of the three different levels of each factor are listed in Table 1. In each test, 5.500 g *S. flavescens* (60–80 mesh) was placed into the extraction vessel. After 0.5 h of static extraction (no liquid flow), the sample was subjected to dynamic extraction by flowing CO_2 at a set rate for 2 h.

2.3. Scaling-up SFE

Under the optimized SFE conditions determined above, the extraction was scaled up by 30-fold using a 1000 ml vessel. A 165 g amount of sample was extracted statically for 1 h and then dynamic extraction done for 3 h by flowing liquid CO_2 at a rate of 2 l/min; the extract was depressed directly into a collection vessel and stored in a refrigerator for subsequent HPLC analysis and HSCCC separation.

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

The selected solvent system, chloroform–methanol–2.3×10^{-2} M NaH_2PO_4 (27.5:20:12.5, v/v), was prepared by adding all the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated by shaking separately. After being thoroughly equilibrated, the upper phase and lower phase were separated and degassed by sonication for 45 min prior to use. The sample solution was prepared by dissolving the crude sample in 5 ml upper phase of the solvent system.

2.5. HSCCC separation

The extracted alkaloids from *S. flavescens* Ait. were separated by a TBE-300A high-speed counter-current chromatograph

(Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation column connected in series (I.D. of the tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop. The revolution radius was 5 cm, and the β values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal.

The upper phase of chloroform–methanol–2.3×10^{-2} M NaH_2PO_4 (27.5:20:12.5, v/v) was pumped into the multilayer-coiled column with a ÄKTA prime plus system (Amersham Bioscience, Piscataway, NJ, USA). After the column was totally filled with the two phases, only the lower phase was pumped at a flow-rate of 2.0 ml/min; and at the same time, the HSCCC apparatus was run at a revolution speed of 850 rpm. After hydrodynamic equilibrium was reached, 4.5 ml of the sample solution containing 175 mg of the crude extract was introduced into the column through the injection valve. All through the experiment the separation temperature was controlled at 25 °C by a Multi Temp III Thermostatic Circulator 230 VAC (GE Healthcare Life Sciences, Piscataway, NJ, USA).

The effluent from the tail end of the column was continuously monitored with ÄKTA prime plus system at 254 nm, and the chromatogram was recorded. Each peak fraction was collected manually according to the obtained chromatogram, and each collection was treated by vacuum freeze-dry and dissolved by methanol for analysis by HPLC.

2.6. HPLC analysis and identification of HSCCC fractions

The crude extract and each purified fraction from the preparative HSCCC separation were analyzed by Agilent 1100 HPLC system including a G1311A QuatPump, a G1315B photodiode array detector, a G1332A degasser and Agilent HPLC workstation, with a Zorbax NH_2 column (250 mm × 4.6 mm I.D., 5 μm) at 220 nm and at a column temperature of 40 °C. The mobile phase, a solution of acetonitrile:ethanol:H_3PO_4 (pH 2) = (80:10:10, v/v), was eluted at a flow rate of 1.0 ml/min. Identification of the target compounds (matrine, oxysophocarpine, oxymatrine) was based on comparison with their standard samples. The identification of HSCCC peak fractions was carried out, respectively, by MS on an Agilent 5973N mass spectrometer and by ^{1}H NMR and ^{13}C NMR spectra on a Bruker Avance 400 MHz NMR spectrometer.

3. Results and discussion

3.1. Optimization of SFE conditions

The products obtained from each $L_9(3)^4$ test of the analytical SFE were quantitatively analyzed, and the results are shown in Table 2. The maximum extraction yields of matrine, oxysophocarpine and oxymatrine were 0.501, 1.315 and 3.945%, respectively. Extraction efficiencies at different sets of temperature, pressure, flow rate of CO_2 and flow rate of modifier were examined under $L_9(3)^4$ test design. The results shown in Table 2 indicate that there are great yield differences among each set of SFE conditions as a control index. Table 3 shows the results of orthogonal analysis. The flow rate of modifier was found to be the most important determinant of the yield. Pressure, temperature and the flow rate of CO_2 have significant influence on the yields. The optimal conditions for extraction of matrine, oxysophocarpine and oxymatrine by SFE were 25 MPa of pressure, 50 °C of temperature, 2.0 l/min of flow rate of CO_2 and 0.04 ml/min of flow rate of 75% ethanol as a modifier.

3.2. Preparative-scale SFE

Under the above optimized SFE conditions, 165 g of *S. flavescens* sample was extracted, yielding 0.52% matrine, 1.343% oxysophocarpine and 4.062% oxymatrine, respectively. The combined yield was approximately 59.25 mg/g of dry seeds. HPLC analysis in Fig. 2 shows that the total 12.90 g of SFE extract contained 6.65% matrine, 17.18% oxysophocarpine and 51.95% oxymatrine.

3.3. HSCCC purification and HPLC identification

Fig. 3 shows the preparative HSCCC separation of 175 mg of the crude sample using the optimized solvent system. The retention of the stationary phase was 75.6%, and the separation time was about 150 min in each separation run. Based on the HPLC analysis and the elution curve of the preparative HSCCC, all collected fractions were combined into different pooled fractions. Then, three kinds of compounds were obtained, yielding 10.02 mg of A, 22.07 mg of B and 79.93 mg of C with the purity of 95.6, 95.8, 99.6% in one-step separation, respectively. The

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

Test no.	A	B	C	D	Yield (%)[a]		
					Martine	Oxysophocarpine	Oxymatrine
1	1	1	1	1	0.389	0.676	2.031
2	1	2	2	2	0.426	0.930	2.789
3	1	3	3	3	0.493	1.200	3.596
4	2	1	2	3	0.501	1.315	3.945
5	2	2	3	1	0.459	1.220	3.657
6	2	3	1	2	0.418	0.971	2.914
7	3	1	3	2	0.433	1.159	3.478
8	3	2	1	3	0.487	1.264	3.792
9	3	3	2	1	0.443	1.054	3.160

[a] Extraction yield (%) = (the amount of martine, oxysophocarpine or oxymartine in extract/sample mass) × 100.

Table 3
Analysis of $L_9(3)^4$ test results

	Matrine yield (%)				Oxysophocarpine yield (%)				Oxymatrine yield (%)			
	A	B	C	D	A	B	C	D	A	B	C	D
K_1	1.308[a]	1.323	1.308[a]	1.323	2.806	3.150	2.911	2.950	8.416	9.454	8.737	8.848
K_2	1.378	1.372	1.378	1.372	3.506	3.414	3.299	3.060	10.516	10.238	9.894	9.181
K_3	1.363	1.354	1.363	1.354	3.477	3.225	3.579	3.779	10.430	9.670	10.731	11.333
k_1	0.436[b]	0.441	0.436[b]	0.441	0.935	1.050	0.970	0.983	2.805	3.151	2.912	2.949
k_2	0.459	0.457	0.459	0.457	1.169	1.138	1.100	1.020	3.505	3.413	3.298	3.037
k_3	0.454	0.429	0.454	0.429	1.159	1.075	1.193	1.260	3.477	3.220	3.577	3.778
R	0.023[c]	0.028	0.023[c]	0.028	0.234	0.088	0.223	0.277	0.700	0.262	0.665	0.829
Optimal level	A_2	B_2	C_3	D_3	A_2	B_2	C_3	D_3	A_2	B_2	C_3	D_3

[a] $K_i^A = \sum$ extraction yield at A_i.
[b] $k_i^A = \frac{K_i^A}{3}$.
[c] $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

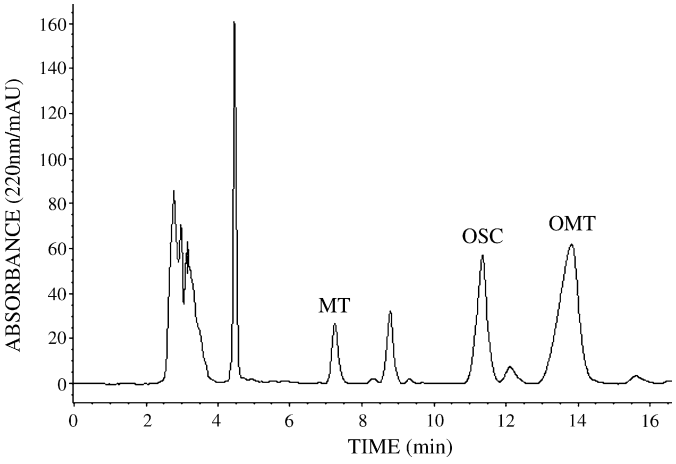

Fig. 2. HPLC chromatography of the extract from preparative SFE. Sample: ethanol solution of preparative SFE extraction without any further treatment. Column: Zorbax NH_2 column (250 mm × 4.6 mm I.D., 5 μm); mobile phase: acetonitrile:ethanol:H_3PO_4 (pH 2) = (80:10:10, v/v); flow rate: 1.0 ml/min; detection wavelength: 220 nm.

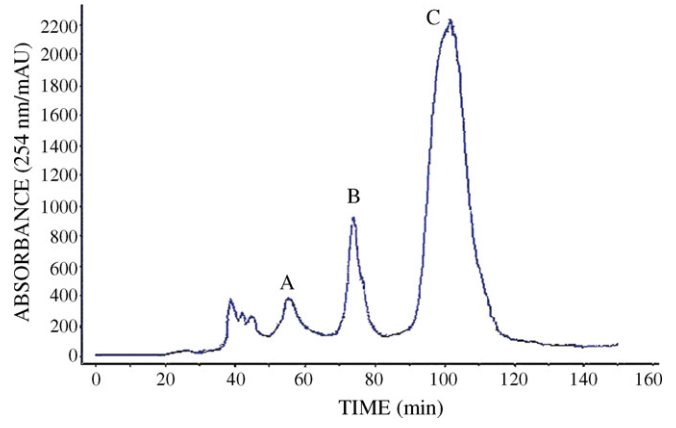

Fig. 3. HSCCC chromatogram of crude extract from *Sophora flavescens* Ait. Two-phase solvent system: chloroform–methanol–2.3 × 10^{-2} M NaH_2PO_4 (27.5:20:12.5, v/v); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 2.0 ml/min; revolution speed: 850 rpm; detection wavelength: 254 nm; sample size: 175 mg; injection volume: 4.5 ml; retention of stationary phase: 75.6%.

chromatograms of HPLC and UV spectra of these compounds are shown in Fig. 4.

The structural identification of matrine, oxysophocarpine and oxymatrine was carried out by electron impact ionization mass spectrometry (EI-MS), ^{1}H NMR and ^{13}C NMR spectra as follows.

HSCCC fraction A in Fig. 4: EI-MS (*m*/*z*): 248[M]$^+$, 247[M-H]$^+$, 219, 205, 192, 177, 150, 137, 96, 55; ^{1}H NMR($CDCl_3$) δ: 4.40(1H, dd, J = 12.7, 4.3 Hz, He-17), 3.82(1H, dt, J = 10.1, 7.7 Hz, H-11), 3.05(1H, t, J = 12.7 Hz, Ha-17), 2.80(2H, m, H-10, He-2), 2.43(1H, m, He-14), 2.25(1H, m, Ha-14), 2.09(3H, m, H-6, H-10, Ha-2), 1.94(4H, m, Ha-3, H-5, H-12, He-4), 1.87(1H, m, Ha-9), 1.50–1.85(9H, m, H-13 × 2, H-8 × 2, H-3, He-9, H-7, H-4, Ha-12); ^{13}C NMR($CDCl_3$) δ: 57.3(C-2), 21.2(C-3), 27.2(C-4), 35.4(C-5), 63.8(C-6), 41.5(C-7), 26.5(C-8), 20.8(C-9), 57.2(C-10), 53.2(C-11), 27.8(C-12), 19.0(C-13), 32.9(C-14), 169.5(C-15), 43.2(C-17).

HSCCC fraction B in Fig. 4: EI-MS (*m*/*z*): 262[M]$^+$, 261[M-H]$^+$, 246[M-O]$^+$, 245[M-OH]$^+$, 217, 203, 177, 150, 138, 112, 96; ^{1}H NMR($CDCl_3$) δ: 6.85(1H, m, H-13), 5.98(1H, d, J = 9.6 Hz, H-14), 4.55(1H, m, H-11), 4.07(1H, dd, J = 12.6, 4.2 Hz, He-17), 3.85(1H, t, J = 12.6 Hz, Ha-17), 3.51(3H, m, H-6, He-10, 2), 3.36(2H, m, Ha-2,10), 2.86(1H, m, He-12), 2.45(1H, m, Ha-3), 2.36(1H, m, Ha-9), 2.20(2H, m, H-5, Ha-12), 1.70–2.00(5H, m, He-4, 3, 9, Ha-4,8); ^{13}C NMR($CDCl_3$) δ: 69.1(C-2), 17.1(C-3), 26.2(C-4), 33.5(C-5), 66.9(C-6), 40.6(C-7), 24.9(C-8), 17.1(C-9), 69.3(C-10), 51.5(C-11), 28.8(C-12), 137.0(C-13), 125.0(C-14), 166.3(C-15), 42.6(C-17).

HSCCC fraction C in Fig. 4: EI-MS (*m*/*z*): 264[M]$^+$, 247[M-OH]$^+$, 205[M-OH-ethylketone]$^+$, 150, 148, 96; ^{1}H NMR($CDCl_3$) δ: 4.50(1H, dd, J = 12.5 Hz, H-17β), 4.10(1H, t, J = 12.5 Hz, H-17α), 3.20(5H, m, H2-10, H2-2, H-6), 2.60(2H, m, H-3, Ha-9), 2.45(1H, m, He-14), 2.25(2H, m, Ha-14, He-12), 2.05(1H, m, He-8), 1.50–1.90(9H, m, H-5, H-13, H-4, H-3, He-9, H-8, H-4, Ha-13, H-7), 1.30(1H, m, Ha-12); ^{13}C NMR($CDCl_3$) δ: 68.7(C-2), 17.2(C-3), 25.9(C-4), 34.4(C-5), 67.1(C-6), 42.5(C-7), 24.5(C-8), 17.1(C-9), 68.1(C-10), 53.1(C-11), 28.5(C-12), 18.8(C-13), 32.9(C-14), 170.0(C-15), 41.6(C-17).

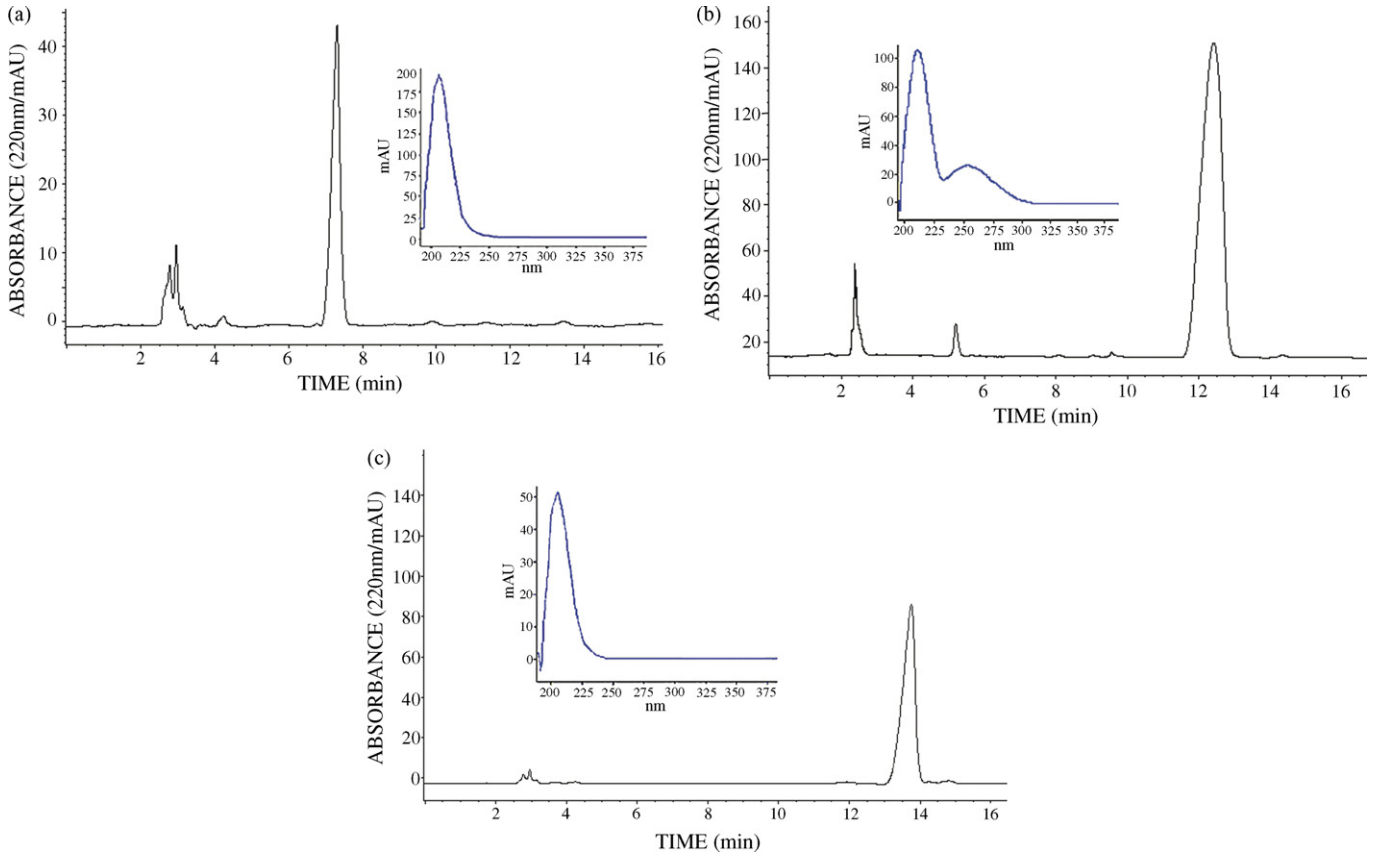

Fig. 4. HPLC analyses and UV spectrum of matrine, oxysophocarpine and oxymatrine purified from *Sophora flavescens* Ait. with HSCCC. HPLC conditions were the same as shown in Fig. 2. (a) Fraction A purified by HSCCC; (b) fraction B purified by HSCCC; (c) fraction C purified by HSCCC.

Compared with the data given in refs. [23,24], peak A–C in Fig. 4 corresponded to matrine, oxysophocarpine and oxymatrine, respectively.

4. Conclusion

Under optimal SFE conditions, 25 MPa, 50 °C and a flow rate of CO_2 (2 l/min) and the amount of modifier (0.04 ml/min), from a crude extract, matrine, oxysophocarpine and oxymatrine were obtained with greater than 95% purity by HSCCC with a two-phase solvent system composed of chloroform–methanol–2.3×10^{-2} M NaH_2PO_4 (27.5:20:12.5, v/v) in one step. In conclusion, SFE and HSCCC were successfully used for the extraction, separation and purification of three quinolizidine alkaloids including matrine, oxysophocarpine and oxymatrine from the traditional Chinese herb *S. flavescens* Ait.

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