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Short communication

Identification of major active constituents in the fingerprint of *Salvia miltiorrhiza Bunge* developed by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography was applied as a method to develop fingerprinting of *Salvia miltiorrhiza Bunge*, a popular traditional Chinese medicine, in our previous study. Important active constituents that were directly related to the therapy effect should be identified. Each effluent fraction and standard samples (cryptotanshinone, tanshinoneI and tanshinoneIIA) were analyzed by ultraviolet spectroscopy and liquid chromatography–mass spectroscopy. It was concluded from the UV-Vis spectrograms, retention times in LC analysis and mass spectrograms, that fractions 7, 8 and 11 were respectively cryptotanshinone (M_r 296), tanshinone I (M_r 276) and tanshinone IIA (M_r 294).

Keywords: *Salvia miltiorrhiza*; Counter-current chromatography; Fingerprinting; Pharmaceutical analysis; Cryptotanshinone; Tanshinones

1. Introduction

Fingerprinting is a method used to control quality of traditional Chinese medicine (TCM), which shows chemical information [1,2]. Chromatography is recommended by the Chinese Pharmacopoeia for fingerprinting [2,3]. Our previous studies showed for the first time that high-speed count-current chromatography (HSCCC) could be applied in fingerprint development of TCM [2].

S. miltiorrhiza Bunge has been used extensively for the treatment of coronary heart disease, hepatitis, chronic renal failure [4] and showed significant cytotoxicity against human tumor cell lines [5]. Cryptotanshinone, tanshinone I and tanshinone IIA are important active constituents in *S. miltiorrhiza Bunge*. Cryptotanshinone is usually used against inflammation, tanshinone I for therapy of angina pectoris and tanshinone IIA for improving blood circulation [6].

Separation and purification of *Salvia miltiorrhiza Bunge* by different types of counter-current chromatography have been reported by Tian and co-workers [7–10]. More components were prepared in HSCCC separation in our previous report [2].

Since fingerprint is applied as a method to control quality of TCM, important active constituents that are directly related to the therapy effect should be identified in fingerprint. In this study, three major active constituents were identified, so improving fingerprint of *S. miltiorrhiza Bunge*.

2. Materials and methods

2.1. Instruments and materials

HSCCC (TBE-300) is from Shenzhen Tauto Biotech, Shanghai, China, with three preparative coils connected in series (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 internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC systems are equipped with a Model S constant-flow

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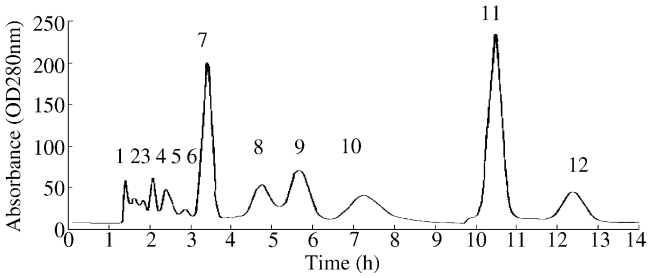


Fig. 1. Chromatogram of crude sample of *S. miltiorrhiza Bunge* from Hebei province by HSCCC separation. Conditions: column: multilayer coil of 2.6 mm i.d. tube with a total capacity of 300 mL; rotary speed: 900 rpm; stationary phase: the upper phase of solvent system A; mobile phase: 0–470 min, the lower phase of solvent system A and after 470 min, the lower phase of solvent system B; flow-rate: 2 mL/min; detection at 280 nm; sample size: 100 mg; retention of stationary phase: 78.8%.

pump, a Model 8823A UV monitor operating at 280 nm and a Model 3057 recorder.

Ethanol and *n*-hexane were analytical-grade chemicals from Atoz, Tianjin, China. Reverse osmosis water (18 MΩ, Milli-Q, Milipore, Bedford, MA, USA) was used for all solutions and dilutions. Acetonitrile and aqueous trifluoroacetic acid (TFA) were chromatographic-grade chemicals from Fisher Scientific, Leics., UK. Standard samples, including tanshinone I, tanshinone IIA and cryptotanshinone, were purchased from the State Food and Drug Administration (SFDA).

2.2. Solvent system for HSCCC

Solvent system A was: *n*-hexane–ethanol–water (10:5.5:4.5, v/v) and solvent system B was *n*-hexane–ethanol–water (10:7:3, v/v) [9].

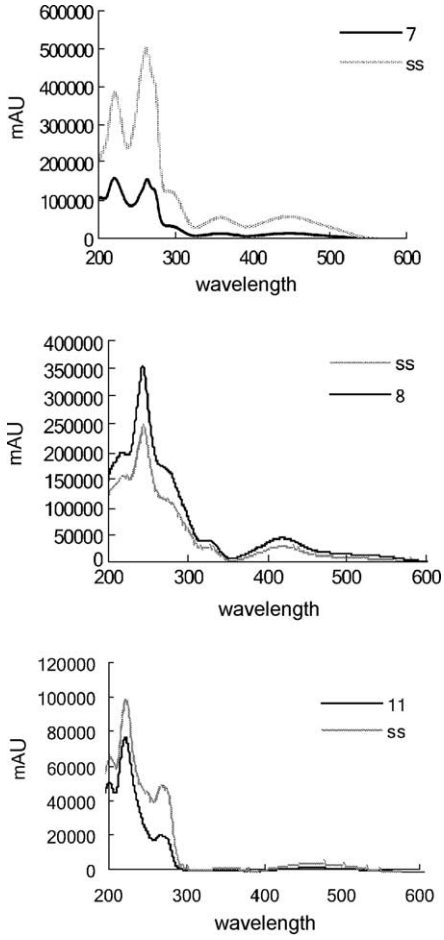
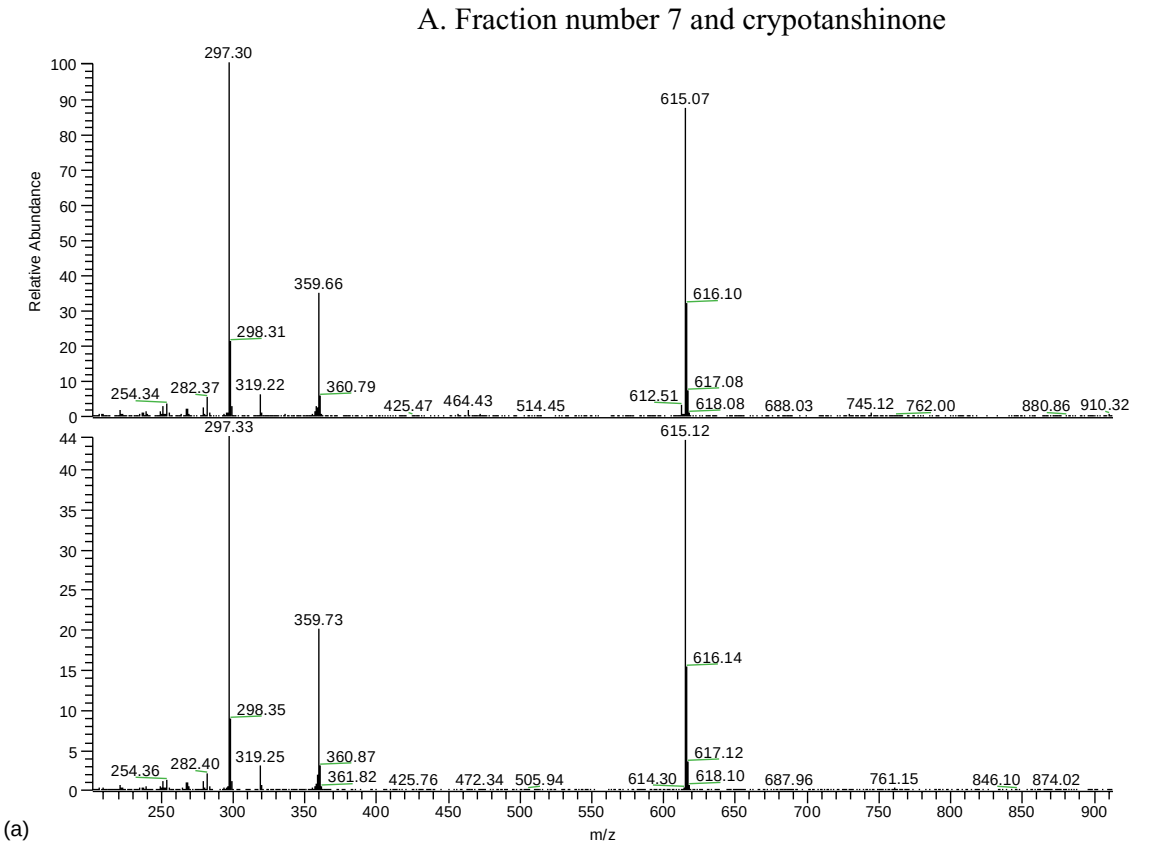


Fig. 2. Absorption spectra of effluent fractions and the corresponding standard samples. Condition: scanned at 600–200 nm, SS: standard sample.

Table 1
Identification of major active constituents by ultraviolet absorption peak, retention time and molecular mass

Absorption peak	Group 1				Group 2				Group 3			
	No. 7	SS	Reference	R.S.D. (%)	No. 8	SS	Reference	R.S.D. (%)	No. 11	SS	Reference	R.S.D. (%)
1	219.2	219	219	0.05	246.2	245	245	0.28	223.9	224	224	0.02
2	264.4	264	263	0.32	269.3	270.2	270	0.24	252.7	253	250	0.66
3	270.9	270.9	271	0.06	324	325.5	325	0.24	269.9	270	268	0.42
4	296.5	294.5	290	1.13	424.8	422.9	420	0.57	356.7	357	352	0.79
5	362.6	360	357	0.78					468.2	467	455	1.58
t_R in LC analysis (min)	30.79	30.79		0	30.91	30.99		0.18	34.69	34.65		0.08
Molecular mass	296.30	296.33	296.35	0.01	276.27	276.27	276.28	0	294.30	294.19	294.33	0.03

SS: The corresponding standard sample, reference: properties reported in literature. Conditions: an Agilent 1100 system was used for LC–MS with Ultrasphere C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Shimadzu) at a flow rate of 1.0 mL/min. The mobile phase was solvent A (0.1% aqueous trifluoroacetic acid, TFA) and solvent B (0.1% TFA + acetonitrile) in the gradient mode as follows: 0–5 min, 0% B; 5–25 min, 0–70% B; 25–40 min, 70% B; 40-50 min, 80% B, 51–60 min, 70% B, 61–70 min, 0% B. The flow-rate was 1.0 mL/min [8]. A UV6000LP photodiode array detector (Finnigan MAT) was used to monitor continuously at 280 nm. The outlet of the flow cell was connected to a splitting valve and a flow of 100 μL/min was achieved and induced to the electrospray ion source via a short length of fused silica tubing. ESI-MS was performed on a Finnigan LCQ DecaXP ion trap mass spectrometry (Thermo Finnigan). A spray voltage of 4.5 kV was employed and the temperature of heated transfer capillary was set to 275 °C. The mass spectrometer was scanned from *m/z* 100 to 1000 in the full scan mode.



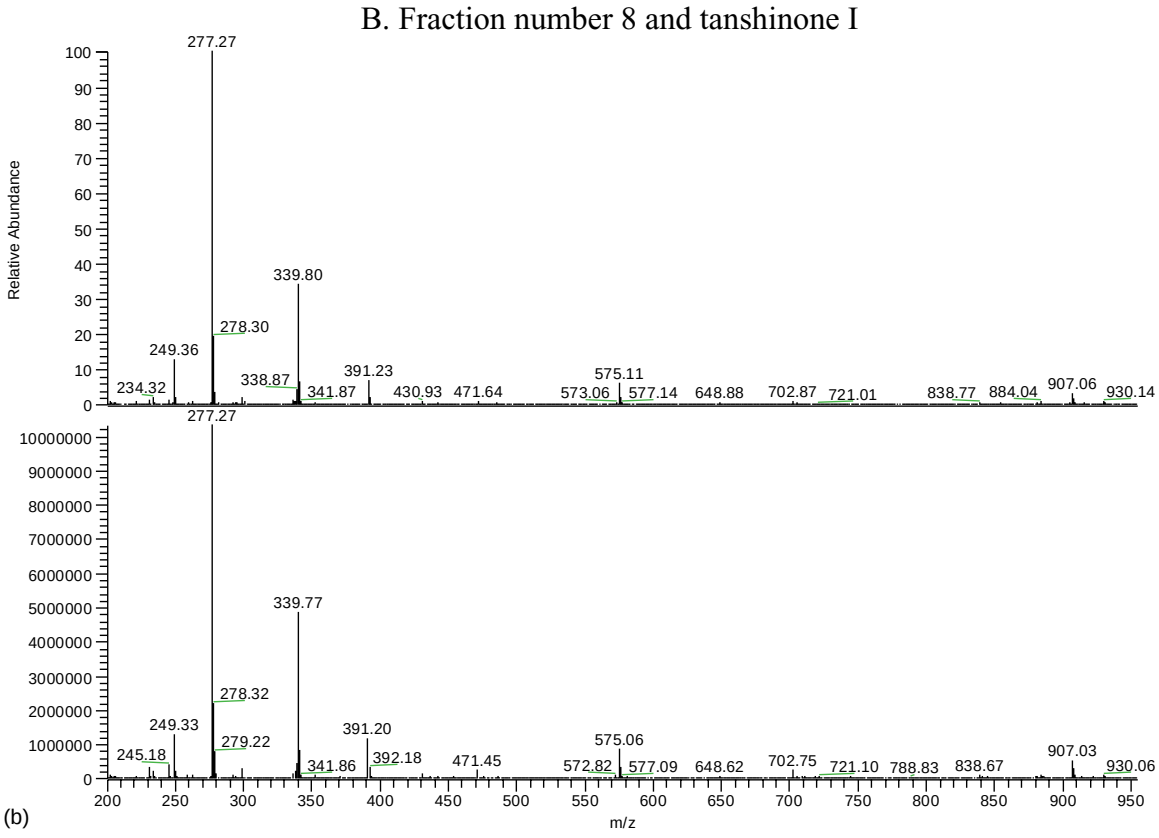


Fig. 3. Mass spectra of effluent fractions and the corresponding standard samples.

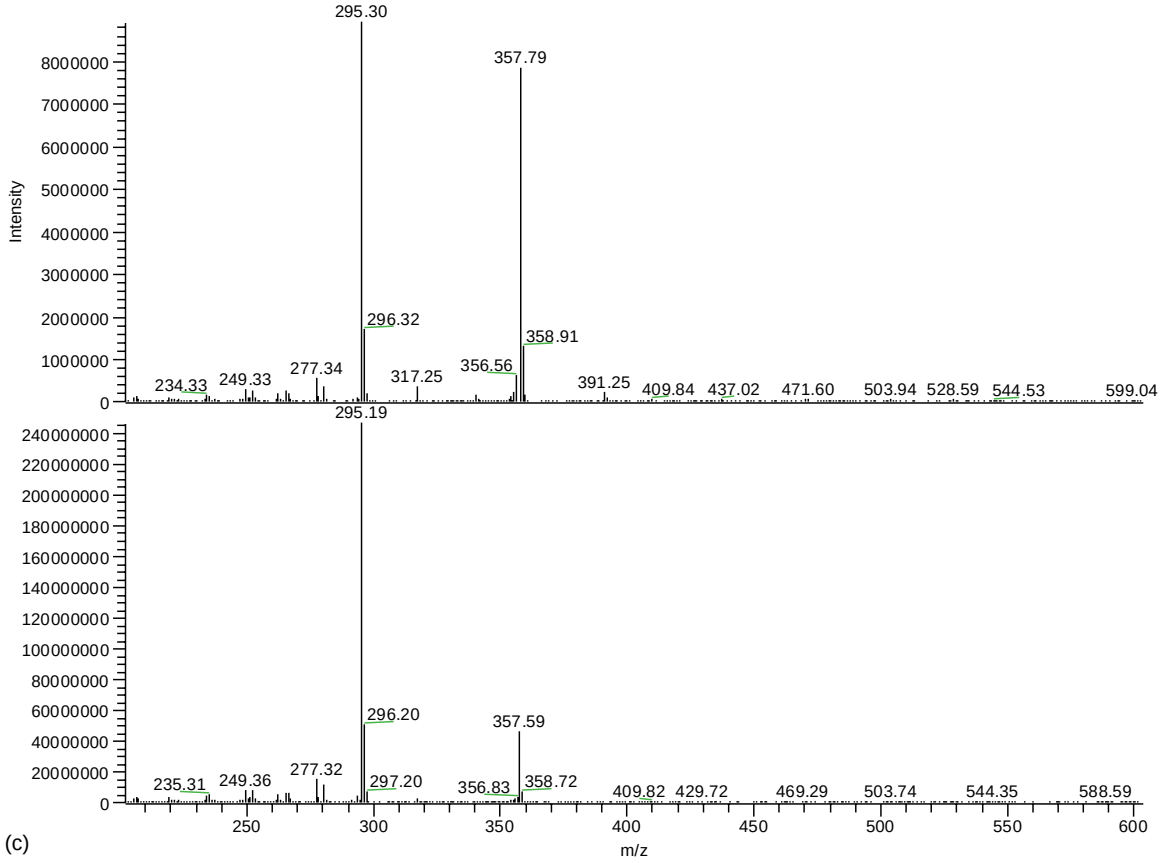


Fig. 3. (*Continued*).

2.3. HSCCC separation

Preparative separation was performed using a stepwise elution with solvent systems A and B in sequence. First, the coiled column was filled with the upper phase of solvent system A. Then, the apparatus was rotated at 900 rpm, and at the same time, the lower phase of solvent system A was pumped through the column at a flow-rate of 2.0 mL/min. After the mobile phase emerged in the effluent, and hydrodynamic equilibrium was established in the column, 6 mL of the sample solution containing 100 mg of the crude tanshinones was injected through the valve. The effluent was monitored with a UV-Vis detector at 280 nm and the peak fractions collected separately. After 470 min of elution, the mobile phase was changed to the lower phase of system B to the end [2].

2.4. LC–MS conditions

An Agilent 1100 system was applied for LC–MS with Ultrasphere C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Shimadzu, Tokyo, Japan) at a flow rate of 1.0 mL/min. The mobile phase was solvent A (0.1% aqueous trifluoroacetic acid, TFA) and solvent B (0.1% TFA + acetonitrile) in the gradient mode as follows: 0–5 min, 0% B; 5–25 min, 0–70% B; 25–40 min, 70% B; 40–50 min, 80% B, 51–60 min, 70% B; 61–70 min, 0% B. The flow-rate was 1.0 mL/min [11]. A UV6000LP photodiode array detector (Finnigan MAT, San Jose, MA, USA) was used to monitor continuously at 280 nm. The outlet of the flow cell was connected to a splitting valve and a flow of 100 μL/min was achieved and induced to the electrospray ion source via a short length of fused silica tubing. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Finnigan LCQ DecaXP ion trap mass spectrometry (Thermo Finnigan, San Jose, CA, USA). A spray voltage of 4.5 kV was employed and the temperature of heated transfer capillary was set to 275 °C. The mass spectrometer was scanned from *m*/*z* 100 to 1000 with full scan mode.

2.5. Ultraviolet absorption spectrum condition

Each peak fraction and standard sample were scanned by a UV6000 LP photodiode array detector at 600–200 nm.

3. Results and discussion

3.1. Separation of S. miltiorrhiza Bunge by HSCCC

The crude sample from Hebei province was chosen for identification in this study (Fig. 1). Twelve distinct peak fractions were eluted respectively within 13 h.

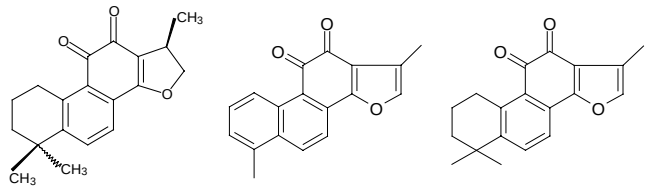


Fig. 4. Chemical structures of the three constituents.

Cryptotanshinone, tanshinone I and tanshinone IIA are important active constituents of *S. miltiorrhiza Bunge*., which need to be marked in the fingerprint according to the relative national standard. Three standard samples were available. The correspondence between standard sample and effluent fraction was studied by LC–MS (and ultraviolet spectra).

3.2. Analysis of the correspondence between effluent fractions and standard samples

Each effluent fraction and standard samples were scanned by a UV-Vis spectrophotometer at 600–200 nm. Absorption spectra of cryptotanshinone, tanshinoneI and tanshinoneIIA were similar to those of fractions 7, 8 and 11 respectively (Fig. 2). Absorption peaks in ultraviolet spectrums of fractions 7, 8 and 11 were compared to those of standard samples and properties of the corresponding constituents as reported in literature [9] (Table 1). The average of R.S.D.s of the absorption peaks was 0.51%, which showed very good correspondence.

The t_R (retention time) values of cryptotanshinone, tanshinone I and tanshinone IIA were respectively similar to those of fractions 7, 8 and 11 with satisfied R.S.D.s (maximum 0.018%, minimum 0%) as shown in Table 1.

Mass spectrograms of fractions 7, 8, 11 were almost identical to those of cryptotanshinone, tanshinone I and tanshinone IIA (Fig. 3). The molecular masses of fractions 7, 8 and 11 were further compared to those of standard samples and reported properties [12] as shown in Table 1. The average of R.S.D.s of molecular masses was 0.012%, very good accuracy.

It was concluded from the ultraviolet absorption spectra, retention times in LC analysis and mass-spectrograms, that fractions 7, 8 and 11 were respectively cryptotanshinone (M_r 296), tanshinone I (M_r 276) and tanshinone IIA (M_r 294). Their chemical structures were listed in Fig. 4.

3.3. Discussion

As a new method to develop fingerprint of TCM, HSCCC should be further compared to other conventional analytical means, such as high-performance capillary electrophoresis (HPCE) and thin-layer chromatography (TLC), to study its feasibility and explore the special advantages of this method.

Acknowledgements

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