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Separation and identification of water-soluble salvianolic acids from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography and ESI-MS analysis

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Abstract

High-speed counter-current chromatography (HSCCC) technique in semi-preparative scale has been applied to separate and purify salvianolic acids from the water extract of Danshen, *Salvia miltiorrhiza* Bunge. High efficiency HSCCC separation was achieved on a two-phase solvent system composed of a mixture of *n*-hexane–ethyl acetate–water–methanol (1.5:5:5:1.5, v/v) by eluting the lower mobile phase at a flow-rate of 1.7 ml/min and a revolution of 850 rpm. A total of five well separated peaks were obtained in the HSCCC chromatogram, and their purities determined by HPLC-UV absorption. These peaks were characterized by UV–vis spectra and ESI-MS, and the data compared with the reference standards. Salvianolic acid B was positively identified as one of the major peaks. Three of the remaining four peaks were also tentatively identified as rosmarinic acid, lithospermic acid, and salvianolic acid E, an isomer of salvianolic acid B, all are members of the salvianolic acids group. In a typical run, tens of milligrams of samples can be separated with high efficiency to yield tens of milligrams of purified materials with over 98% purity. HSCCC thus provides a cost-effective alternative to preparative scale HPLC for the semi-preparative scale separation and purification of salvianolic acids in Danshen. With appropriate modifications, the technique should also be applicable to other herbs in general.

Keywords: *Salvia miltiorrhiza* Bunge; Danshen; Salvianolic acids; Salvianolic acid B; HSCCC

1. Introduction

The dried roots of *Salvia miltiorrhiza* Bunge, or Danshen in Chinese, is among the most popular herbs which has been reputed by ancient Chinese physicians to make the “sluggish” or “stuck” blood to flow more freely. It has been widely used to promote blood circulation, remove blood stasis, clear away heat, relieve vexation, nourish and cool the blood to relieve carbuncles [1]. The major active constituents of this herb include tanshinones, which belong to a group of lipid-soluble diterpenoids, and water-soluble polyphenolic compounds. Among these species, tanshinones have been studied extensively [2,3] and salvianolic acids have been attracting intense interest recently and many researchers have been investigating its pharmacological effects both in vivo and in vitro [4,5–7]. These results demonstrated that salvianolic acids possess various pharmacological effects including antioxidation, free radicals scavenging, and the protection of neural cells against injuries caused by anoxia, etc. Salvianolic acid B is the most abundant and bioactive member of the salvianolic acids in Danshen, and has been assigned as the marker species for Danshen in the 2005 edition of Chinese Pharmacopoeia [1]. The chemical structure of rosmarinic acid, lithospermic acid, salvianolic acid B and salvianolic acid E are shown in Fig. 1. In order to better understand the biochemical properties of salvianolic acids and to ascertain their clinical applications, there is an urgent need to develop efficient, preparative scale separation methods for their isolation and purification. Moreover, the preparation of these bioactive compounds or fractions is also in demand by the research and

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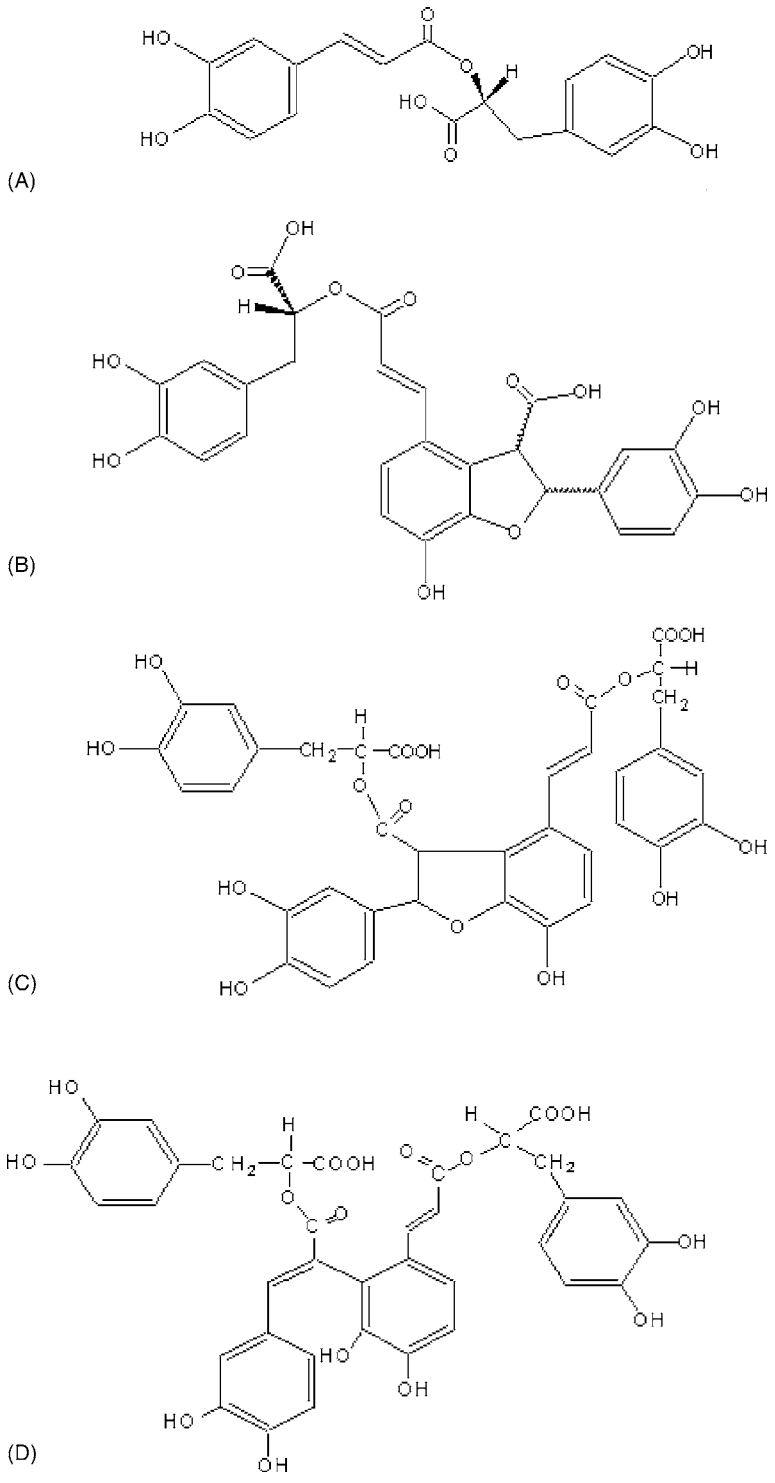

Fig. 1. The chemical structures of rosmarinic acid (A), lithospermic acid (B), salvianolic acid B (C) and salvianolic acid E (D).

industrial communities to serve as reference standards for quality control purposes.

High-speed counter-current chromatography (HSCCC) is a liquid–liquid chromatographic technique that is gaining increasing interest recently. It relies on the use of centrifugal force for the retention of the liquid stationary phase, which is continuously eluted by the counter-flowing mobile phase driven by external pumping [8]. Compared to the conventional liquid–liquid techniques, HSCCC is advantageous because of its shorter separation time, wider range of selection of solvent systems, and more quantitative material recovery. HSCCC has been used for the preparative separation of tanshinones, the lipid-soluble bioactive components in Danshen [9–13], as well as salvianolic acid B [14] and other herbal ingredient such as inflacoumarin A and licochalcone A in licorice [15]. However, only one component (salvianolic acid B) was separated and identified in the Ref. [14].

In this paper, the development of HSCCC method for the separation and purification of several salvianolic acids from the crude water extract of *S. miltiorrhiza* Bunge was described. These salvianoic acids were also characterized individually by ESI-MS and LC–MS techniques.

2. Experimental

2.1. Materials

Dried *S. miltiorrhiza* Bunge root, whose species was identified by Prof. Shouquan Lin (Institute of Medicinal Plants, Chinese Academy Medical Sciences), was provided by Danshen Base, New World Danshen Inc., Zhongjiang, Szechwan. Samples of *S. miltiorrhiza* Bunge roots were crushed into pieces by a disintegrator made in Hangzhou Chunjiang Pharmacy Machine Co. Ltd.

Acetonitrile is of chromatographically grade and trifluoroacetic acid is of protein sequencing grade. Both were purchased from Sigma Chemical Corporation. Water used as the mobile phase in HPLC was prepared with Millipore purifier (Millipore, USA) in our laboratory. All organic solvents used for HSCCC were of analytical grade and purchased from Shanghai chemical reagent corporation, Shanghai, China.

2.2. Preparation of crude salvianolic acids and sample solutions

Roots of *S. miltiorrhiza* Bunge (100 g) were extracted twice (2× 1 h), each time with 800 ml of water by sonication using a SK3200LH ultrasonic cleaning instrument (Shanghai Kudos Ultrasonic Instrument Co. Ltd., Shanghai, China). The extracts were combined and concentrated under reduced pressure with a Model SENCO R-201 rotary evaporator (Shanghai Shensheng Biotech Co. Ltd., Shanghai, China). The residue was purified by D101 macroporous resin (Xi'an Lanshen Technology Corporation, Xi'an China). The fraction obtained by ethanol–water (1:1) elution were concentrated under reduced pressure and freeze-dried with a Model FD-1 freezing drier (Beijing Boyikang Technology Corporation, Beijing, China) to get the crude salvianolic acids.

The sample solutions were prepared by dissolving the crude salvianolic acids in the upper phase of the HSCCC solvent system (see next section) at the suitable concentration range of 30–40 mg/ml.

2.3. Selection and preparation of two-phase solvent system

The chromatographic process in HSCCC is based on the partition of a solute between the two liquids that are used as the mobile and stationary phases, respectively. Successful separation requires a suitable choice of the two-phase solvent system, which provides an ideal partition coefficient (K). A series of experiments were performed to determine the partition coefficient in different solvent systems. The values of the partition coefficients were determined by HPLC. In brief, about 20 ml of the pre-equilibrated two-phase solvent system was mixed with 10 mg crude salvianolic acids sample. The mixture was thoroughly mixed by vibration for 1 min and the mixture was allowed to separate into two phases by standing for 5 min. Then, an aliquot of each phase was evaporated to dryness under vacuum. Finally, the residue was diluted with water and analyzed by HPLC. The K-value was expressed as the peak area of salvianolic acids in the upper phase relative to those of the lower phase.

Two solvent systems finally utilized in HSCCC separation and purification were prepared by first mixing a solvent mixture of *n*-hexane, ethyl acetate, water, and methanol. The volume ratios of the four solvents in the order listed were 1.5:5:5:1.5. The mixture was thoroughly equilibrated and allowed to separate into two phases in a separator funnel at 25 °C in a self-designed thermostat shortly before use. The two separated phases each contain the above four solvents but in different volume ratios. The heavier phase at the bottom is used as the mobile phase in HSCCC and was enriched in water and methanol, while the upper lighter phase used as the stationary phase is enriched in organic solvents of *n*-hexane and ethyl acetate.

2.4. High-speed counter-current chromatography (HSCCC)

2.4.1. Instrumentation

HSCCC was performed with a Model TBE-300A HSCCC system manufactured by Tauto Biotech Co. Ltd., Shanghai, China. The multi-layer coil planet centrifuge (CPC) was prepared by winding 1.8 mm i.d. PTFE tubing coaxially onto the column holder with a total capacity of 350 ml. The β-value varied from 0.42 at the internal terminal to 0.63 at the external terminal. $\beta = r/R$, where r is the distant from the coil to the holder shaft and R is the revolution radius or the distant between the holder axis and central axis of the centrifuge. The rotation speed is adjustable from 500 to 1000 rpm; and 750–900 rpm was used in the present study. Different from ordinary HSCCC systems, the system used in the present study was equipped with a thermostatic jacket. The jacket can keep the CPC at constant temperature with the aim to eliminate the harmful effect of temperature variation on separation efficiency.

The solvent was pumped into the column with a Model S1007 constant-flow pump (Beijing Shengyitong Technology Development Co. Ltd., Beijing, China). The effluent was detected on-line at 254 nm with a Model 8823 A UV detector (Beijing Institute of New Technology Application). A manual sample injection valve with a 20 ml loop was used to introduce the sample into the column. A Model 3057 portable recorder (Sichuan Instrument Factory, Chongqing, China) was used to record the chromatogram. In addition, the data were also displayed and analyzed simultaneously on a Model Sepu3000 chromatographic data station provided by Hangzhou Puhui Scientific Technology Co. Ltd.

2.4.2. HSCCC separation procedure

The multi-layer coiled column was first filled completely with the upper solvent phase (stationary phase). Then, the lower phase (mobile phase) was pumped into the head of the column at a flow-rate of 1.7 ml/min while the column was rotating at 850 rpm.

After the hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected through the sample injection valve. The column effluent was monitored with a UV detector at 254 nm as stated earlier, and the individual peak fractions were collected manually, guided by the chromatographic elution profile displayed on the recorder.

2.5. HPLC analysis

Five peak fractions were collected from HSCCC separation and analyzed by HPLC. The analyses were performed with a HPLC instrument (Agilent1100, USA). The column (Kromasil KR100-5C_{18}, 150 mm × 4.6 mm i.d., Dalian Elite Analytical Instruments Co. Ltd.) was eluted isocratically with a mixture of acetonitrile and water (volume ratio 1:3) with 0.05% trifluoroacetic acid modifier at a total flow-rate of 0.8 ml/min. Elution was monitored at 290 nm using a diode array detector (DAD). The purities of the collected peak fractions were determined by HPLC based on the peak area of the target species normalized to the sum of all observed peaks.

2.6. Identification of the separated peaks

The eluted peak IV (see Fig. 2 given later) and the standard of salvianolic acid B were each dissolved in CH_3OH and scanned by a Model DU-7400 UV–vis spectrophotometer (Beckman, USA) at 600–200 nm.

The Electrospray MS experiments were performed using Model 1100 MSD pneumatically assisted electrospray octopole–quadrupole mass spectrometer (Agilent, USA). The eluted peak IV and the standard of salvianolic acid B were each dissolved in water and injected at a rate of 200 μl/min. Lens voltages was optimized in neutral ion mode by turning on the respective ions of interest. A spray voltage of 4.5 kV was employed and the collision induced dissociation (CID) voltage was set to 70 V. The temperature of the heated transfer capillary was 325 °C. The mass spectrometer was scanned from *m*/*z* 100 to 1000 in full scan mode, and the mass spectra were acquired with a step size of 0.15 μm.

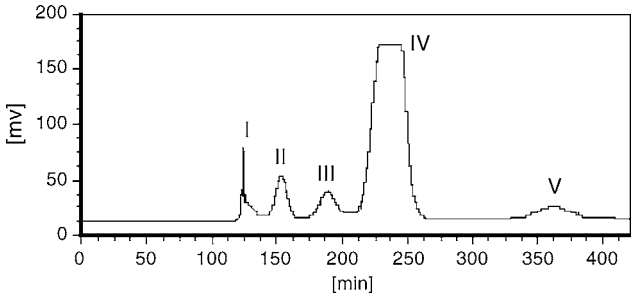

Fig. 2. Chromatogram of the crude salvianolic acids by HSCCC. Solvent system: hexane–ethyl acetate–water–methanol (1.5:5:5:1.5, v/v); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 1.7 ml/min, revolution speed: 850 rpm; temperature: 25 °C; sample size: 80 mg dissolved in 2 ml upper phase; detection: 254 nm.

An Agilent Model 1100 LC–MS system was also used for compound identification. The chromatographic conditions are identical to those described in Section 2.5 and the outlet of flow cell was connected to a splitting valve, from which a flow of 200 μl/min was diverted to the electro spray ion source via a short length of fused silica tubing.

3. Results and discussion

3.1. Selection of solvent system

To be effective in HSCCC separation, the samples need to have a favorable partition coefficient in the two-phase solvent system. The partition coefficient is defined as the mass concentration ratio of the solute in the stationary phase versus those in the mobile phase. For chromatographic processes such as HPLC, CE and TLC, the optimal partition coefficient should be within the range of 0.67–1.50 [16–18]. A series of experiments were performed to determine the optimum two-phase solvent system for HSCCC separation. The crude salvianolic acids extract, which was known to contain all seven salvianolic acids including salvianolic acid B (see Fig. 3A), was used as the testing material. The measured *K*-values for

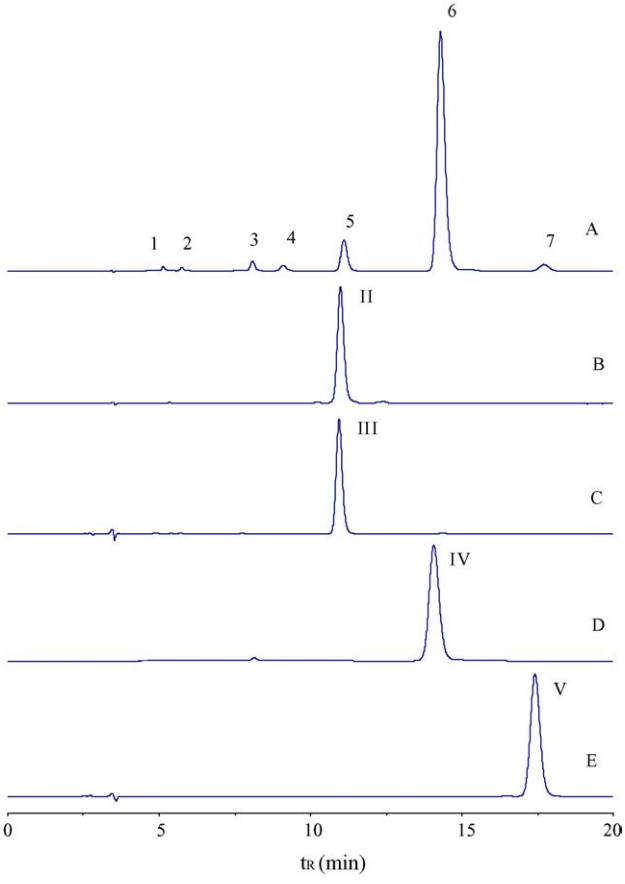

Fig. 3. HPLC chromatograms of the crude salvianolic acids (A), purified peak II (B), purified peak III (C), purified peak IV (D) and purified peak V (E), column: Kromasil KR100-5C18 (150 mm × 4.6 mm) at room temperature; elution: acetonitrile–water (added 0.05% trifluoroacetic); flow-rate: 0.8 ml/min; detection at 290 nm.

Table 1
The *K*-values (partition coefficient) of salvianolic acid B in different two-phase solvent systems used in HSCCC

Solvent system	*K*-value
Ethyl acetate–water (1:1, v/v)	48
n-Hexane–water (1:1, v/v)	0.03
Chloroform–ethyl acetate–water (1:3:3, v/v)	0.64
n-Hexane–ethyl acetate–water (1:5:5, v/v)	15
Chloroform–ethyl acetate–water–acetic acid (1.8:3:3:1, v/v)	2.47
Chloroform–ethyl acetate–water–acetic acid (1.8:3:3:0.6, v/v)	2.64
Chloroform–ethyl acetate–water–acetic acid (1.6:3:3:0.6, v/v)	2.54
Chloroform–ethyl acetate–water–acetic acid (1.5:3:3:0.5, v/v)	1.33
n-Hexane–ethyl acetate–water–methanol (1.5:5:5:0.5, v/v)	4.52
n-Hexane–ethyl acetate–water–methanol (1.5:5:5:1, v/v)	2.86
n-Hexane–ethyl acetate–water–methanol (1.5:5:5:1.2, v/v)	2.11
n-Hexane–ethyl acetate–water–methanol (1.5:5:5:1.5, v/v)	1.43

The partition coefficient is defined as the mass concentration ratio of the solute in the stationary phase vs. the mobile phase. In our systems, the stationary phase is the upper, aqueous rich phase in the chloroform containing systems. In all other systems, the situation is reversed, where the stationary phase is the organic-rich (hexane or ethyl acetate) phase at the top.

salvianolic acid B in different solvent systems are summarized in Table 1. Based on the criteria of having *K*-value in the range of 0.5–2.0, two systems selected for further evaluation were *n*-hexane–ethyl acetate–water–methanol (1.5:5:5:1.5, v/v) and chloroform–ethyl acetate–water–acetic acid (1.5:3:3:0.5, v/v).

Table 2 lists the distributions of the seven salvianolic acids in the two quaternary-solvent systems of: (1) *n*-hexane–ethyl acetate–water–methanol (1.5:5:5:1.5, v/v) and (2) chloroform-ethyl acetate–water–acetic acid (1.5:3:3:0.5, v/v). In system (1), the solvent mixtures exist as two phases with the aqueous-rich phase at the bottom and the organic-rich phase on the top. In system (2), the solvent mixtures exist as two phases with the organic-rich phase at the bottom and the aqueous-rich phase on the top. The results indicated that the partition coefficient of a majority of salvianolic acids vary within the range of 0.5–2. The values of individual salvianolic acids are significantly different from each other salvianolic acids. The results thus suggest that both these two solvent systems are suitable for the separation of salvianolic acids. The performance of the two systems was further compared in terms of the stability of the stationary phase under the operating conditions (800 rpm revolution speed, 2.0 ml/min flow-rate, and 25 °C). The upper phase was used as the stationary phase while the lower phase was used as the mobile phase in all runs. System (1) was found to give better retention of the stationary phase, i.e., 53.6% versus 38.4% for solvent systems (2). The retention of more stationary phase material in the separation channel resulted in better separation and was therefore used for all later HSCCC runs. In system (2), the stripping of excessive solvent from the stationary phase resulted in poor separation and also suffered from run to run irreproducibility problems.

Table 2
Partition coefficient of seven salvianolic acid compounds in two solvent systems

	Peaks						
	1	2	3	4	5	6	7
Solvent systems (1)	0.43	11.3	1.37	3.72	0.64	1.43	1.9
Solvent systems (2)	0.43	1.22	1.5	2.49	0.59	1.33	3.59

Note: solvent systems (1) *n*-hexane–ethyl acetate–water–methanol (1.5:5:5:1.5, v/v); solvent systems (2) chloroform–ethyl acetate–water–acetic acid (1.5:3:3:0.5, v/v).

3.2. Optimization of operational parameters

Previous study in our laboratory showed that temperature, flow-rate, revolution speed and sample size, etc. could all affected to a different extent the separation performance of HSCCC [15]. The highest separation efficiency and best resolution were achieved within the temperature range of 20–30 °C, and thus 25 °C was selected as the CPC temperature. Within the flow-rate range of 1.5–2.5 ml/min, the flow-rate greatly affects the overall run time, and to a certain extent also affects resolution. In order to shorten separation time while still maintaining adequate resolution to meet different analytical objectives, 1.7 ml/min was used in preliminary separation experiments. Typical run time for sample separation was 7 h.

Within the rotary speed range of 700–900 rpm, the rotary speed has a slightly affect on the overall run time and resolution. At speed below 700 rpm, the stationary phase does not adequately retain the components. The best resolution and separation times were achievable with the rotary speed of 850 rpm. The resolution of neighboring peaks was calculated using adjacent peaks III (lithospermic acid) and IV (salvianolic acid B) in the HSCCC chromatograms as shown in Fig. 2. Resolution was calculated by equation $R = 2(t_{R_2} - t_{R_1})/(w_1 + w_2)$, in which t_R is the retention time of the compound standard and w is the peak width. At 850 rpm revolution speed, the resolution (R) of the two neighboring peaks III and IV is 1.97, which is better than the run with revolution speed of 800 rpm ($R = 1.28$).

The resolution was found to increase slightly with increasing rotary speed. On the other hand, high rotary speed runs the risk of stripping away the stationary phase material through the emulsification of the solvent system. A compromise of 850 rpm was therefore selected for operation.

Sample size also affects the separation resolution. When sample was over 80 mg, the purities of the separated peak IV in Fig. 2 reduced noticeably. Thus, the sample size is set to be no more than 80 mg.

For real sample runs, the crude salvianolic acids can be dissolved in either the upper or the lower phase of the solvent system. Sample solubility is higher in the upper organic phase than those in the lower phase. However, if the sample was dissolved in the upper phase or a mixture of upper and lower phases (1:1, v/v), loss of the stationary phase could occur, resulting in baseline drifting problems because of the upset of hydrodynamic balance in CPC. On the other hand, if lower phases were used for sample dissolution, higher volume of solvent would be needed because of its lower solubility towards the sample. The injection of large sample size would then degrade the resolution of separation. Results indicated that resolution is a more important factor in maintaining the peak purities than the drifting baseline. Thus, the upper phase was chosen as the sample solvent to dissolve the crude salvianolic acids sample.

3.3. Purity analyses by HPLC

Fig. 3A shows the HPLC chromatogram of the crude salvianolic acids. It can be seen that the crude salvianolic acids fractions consisted of seven peaks among which peak 6 (salvianolic acid B) was the major one. The peaks I–V in Fig. 2 were individually collected and their dry weights obtained after solvent evaporation. The yield distribution of the separation is given in Table 3. The purities of each of the collected fractions were checked by HPLC, and quantified by its peak area at 290 nm absorption relative to the sum of all observed peak areas. The calculated purity is therefore only UV purity rather than mass purity. Peak I consists of several peaks in the HPLC chromatogram. Since there was no major peak, and the overall contents of them were very low, no further study was therefore carried out. The purity of peak IV (peak 6 in Fig. 3A) estimated by HPLC analyses is 98.6% (Fig. 3D). Peaks II, III both appeared as peak 5 and peak V appeared as peak 7 in the HPLC chromatogram (Fig. 3A).

Table 3
Salvianolic acids isolated from the crude water extract of *Salvia miltiorrhiza* by HSCCC

Peaks	Isolated amount (mg)	Yield (%)	Purity (%)
I	5.4	6.8	No major peak
II	1.9	2.4	98
III	1.2	1.5	98
IV	63.4	79.2	>98
V	0.8	1.0	98

The purities of the three peaks increased to 98% (Fig. 3B, C, and E) after repeating HSCCC separation of the isolated peaks from the first run under the same HSCCC conditions.

3.4. Identification of the separated peaks

The separated fraction of peak IV was positively identified as salvianolic acid B by comparing to the standard using HPLC

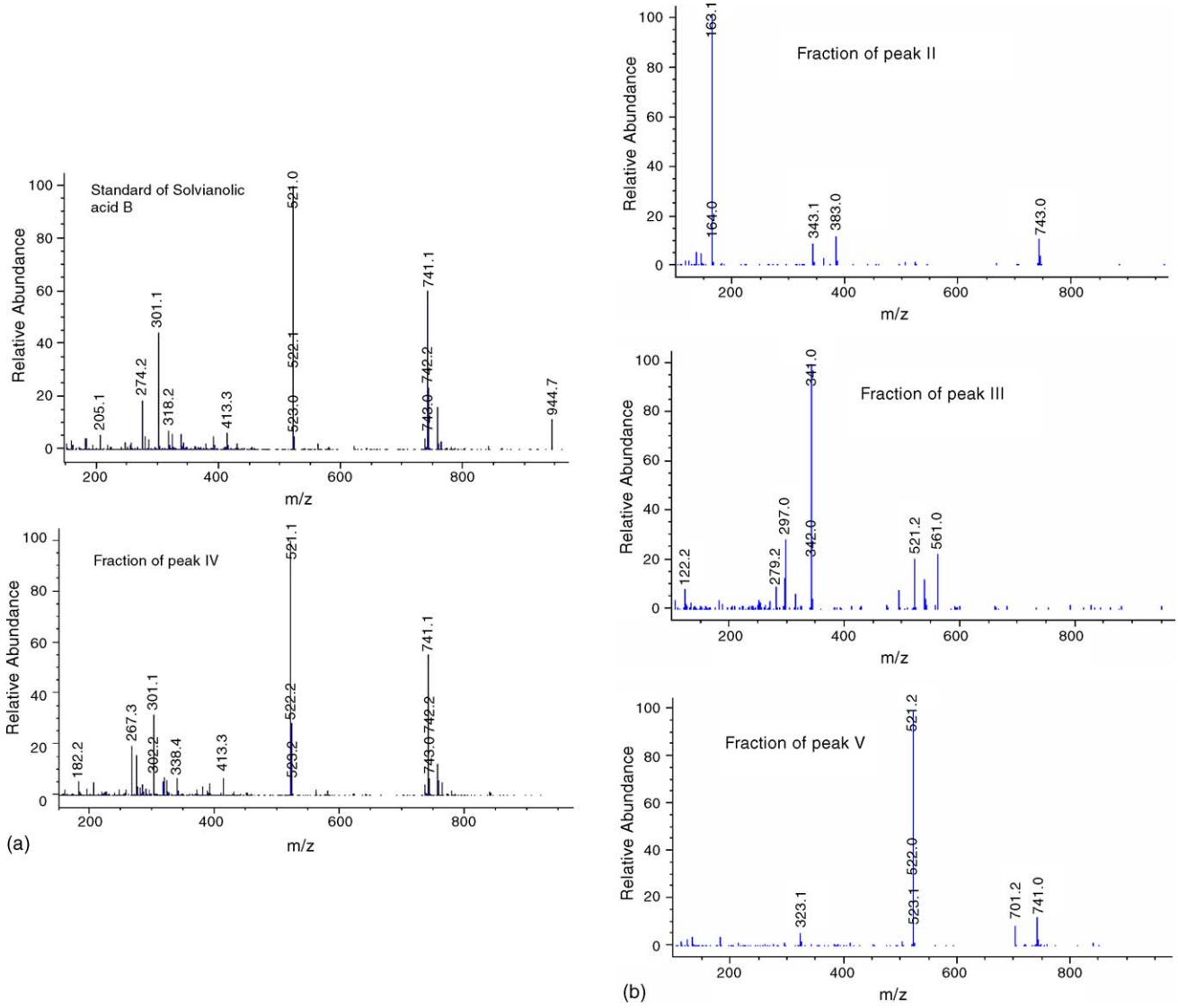

Fig. 4. Mass spectra of salvianolic acid B standard and HSCCC separated fractions. (A) Standard of salvianolic acid B and fraction of peak IV and (B) fraction of peaks II, III and V.

Table 4
Identification of the elution fraction of peak IV by ultraviolet absorption peak, LC retention time and molecular mass

Absorption peak	Peak IV	SS	Reference
1	203	203	203
2	256	255	253
3	289	288	288
4	312	310	308
5	331	330	330
t_R in LC analysis (min)	14.72	14.72	
Molecular mass	718.10	718.00	718.59

SS: standard of salvianolic acid B, reference: reported in literature. Conditions: A 1100 MSD pneumatically assisted electrospray octopole–quadrupole mass spectrometer (Agilent, USA) was used for electrospray MS. A spray voltage of 4.5 kV was employed and the collision induced dissociation (CID) voltage was set to 70 V. The temperature of heated transfer capillary was 325 °C. The mass spectrometer was scanned from *m/z* 100 to 1000 with full scan mode and the mass spectra were acquired with a step size of 0.15 μm.

retentions time, and UV–vis spectrophotometric and ESI-MS analyses. The t_R (retention time) value of peak IV was found to be the same as those of salvianolic acid B standard. The spectral properties of peak IV also agree well with the salvianolic acid B standard and the literature [19] values. In MS analyses, the observed major ions observed from peak IV included ions of *m/z* 741.1 (M + Na), 757.1 (M + K) and 521.1 ($M - C_9H_{10}O_5^+$) with a molecular mass of 718 (Fig. 4A). The molecular spectrogram of peak IV is in excellent agreement with those of salvianolic acid B standard and the reported properties [19]. The UV–vis and ESI-MS results along with HPLC retention time data are outlined in Table 4.

The analysis of peaks II, III, V with LC–MS showed that the mass spectrogram of peak II was almost identical to the literature [20] values of rosmarinic acid (Fig. 4B) with *m/z* of 383.1 (M + Na), 343.1 ($M - H_2O^+$), and 163.1 ($M - C_9H_{10}O_5^+$), while the mass spectrogram of peak III was almost identical to the literature [20] values of lithospermic acid (Fig. 4B), with its *m/z* values of 561.2 (M + Na), 521 ($M - H_2O^+$), and 341.1 ($M - C_9H_{10}O_5^+$). The mass spectrogram of peak V is similar to those of salvianolic acid B, with *m/z* ions including 741.0 (M + Na), 701.0 ($M - H_2O^+$), and 521.1 ($M - C_9H_{10}O_5^+$). However, their retention times in both HPLC and HSCCC are noticeably different. Peak V is likely salvianolic acid E, an isomer of salvianolic acid B, judging from LC–MS results (Fig. 4B) and in comparison with literature data [4]. The LC–MS results are summarized in Table 5. MS analyses suggest that peaks II–V are all different members of the salvianolic acids group with the common pattern of losing $C_9H_{10}O_5^+$ ion in the mass spectrogram.

Table 5
Identification of the elution fraction of peak II, III, V by molecular mass

	Peaks		
	II	III	V
Molecular mass	360.3	538.2	718.0
Reference	360.1	538.5	718.0

4. Conclusion

HSCCC technique has been developed and successfully applied to the separation and purification of four kinds of salvianolic acids in crude salvianolic acids extract. The results demonstrate the effectiveness of HSCCC as a semi-preparative separation technique for the isolation and purification of water-soluble ingredients of salvianolic acids from *S. miltiorrhiza* Bunge. A total of five major peaks were observed in the HSCCC chromatogram. The peaks were individually collected and their identities studied by UV–vis and ESI-MS analysis. One of the peaks was positively identified as salvianolic acid B. The other four peaks were also tentatively identified as members of the alvianolic acids group based on MS fragmentation patterns. However, further confirmation is needed for these peaks because of the lack of standards.

In a typical run, tens of milligrams of samples can be separated with high efficiency to yield tens of milligrams of purified materials with over 98% purity. HSCCC thus provides a cost-effective alternative to preparative scale HPLC for the semi-preparative scale separation and purification of bioactive components in herbal extracts. In a previous study in our laboratory, HSCCC has been successfully developed for the preparative separation of bioactive flavonoids inflacoumarin A and licochalcone A from the crude extract of *Glycyrrhiza inflata* Bat. The current study further demonstrates the wide applicability of the technique in the separation and purification of bioactive components from herbal extracts with diverse chemical compositions and properties.

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References

[1] ChP. vol. I (2005) 52–53.
[2] M. Gu, G.F. Zhang, Z.G. Su, F. Ouyang, J. Chromatogr. A 1041 (2004) 239–243.
[3] Z.H. Shi, J.T. He, W.B. Chang, Talanta 64 (2004) 401–407.
[4] J.L. Zhang, M. Cui, Y. He, H.L. Yu, D.A. Guo, J. Pharm. Biomed. Anal. 36 (2005) 1029–1035.
[5] G.H. Du, J.T. Zhang, Bas. Med. Sci. Clin. 20 (2000) 394–398.
[6] S.J. Zhao, G.Y. Zhang, D. Liu, Trad. Chin. Herb. Drugs 35 (2004) 341–344.
[7] S.X. Peng, S.X. Zhao, Q.J. Liao, Developments in Chemistry of Drugs, CM Press, Beijing, 2000, pp. 210–211.
[8] D.S. Dai, Y.M. Wang, G.A. Luo, J. Chin. Anal. Chem. 29 (2001) 586.
[9] G.L. Tian, Y.B. Zhang, T.Y. Zhang, J. Chromatogr. A 904 (2000) 107–111.
[10] H.B. Li, F. Chen, J. Chromatogr. A 925 (2001) 109–114.
[11] G.L. Tian, Y.B. Zhang, T.Y. Zhang, J. Chromatogr. A 945 (2002) 281–285.
[12] M. GU, F. Ouyang, Z.G. Su, J. Chin. Biotechnol. 19 (2003) 740–744.

[13] M. GU, F. Ouyang, Z.G. Su, J. Chromatogr. A 1022 (2004) 139–144.
[14] H.B. Li, J.P. Lai, Y. Jiang, J. Chromatogr. A 943 (2002) 235–239.
[15] Q.E. Wang, Frank Sen-Chun Lee, X.R. Wang, J. Chromatogr. A 1048 (2004) 51–57.
[16] W.D. Conway, Modern Counter-Current Chromatography, 1995, pp. 78–86.
[17] L.Q. Tan, S.M. Zhang, Q.Y. Ou, J. Chin. Anal. Chem. 24 (1996) 1448–1451.
[18] L.Q. Tan, Z.Q. Ruan, Q.Y. Ou, J. Chin. Anal. Chem. 25 (1997) 515–518.
[19] H.F. Chen, Wordbook on Plant Active Components of Plants, I, CM Press, Beijing, 2001, pp. 863–864.
[20] C.X. Zhou, H.W. Luo, Z.W. Danyu, J. Chin. Pharm. Univ. 30 (6) (1999) 411–416.