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Fingerprinting of *Salvia miltiorrhiza Bunge* by Thin-Layer Chromatography Scan Compared with High Speed Countercurrent Chromatography

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Abstract: The components of the traditional Chinese medicines (TCM) can be influenced by soil, climate, and growth stage. Fingerprinting is an important means for its quality control. Our previous studies showed that high speed countercurrent chromatography (HSCCC) was helpful in the development of TCM fingerprinting. Since the HSCCC method is new, it is necessary to compare it with more conventional ones, such as high performance liquid chromatography (HPLC), high performance capillary electrophoresis (HPCE), and thin-layer chromatography scan (TLCS). Comparisons with HPLC and HPCE were reported in our previous studies. In this study, HSCCC was compared with the thin-layer chromatography scan (TLCS). With TLC, a quick, convenient, and cost effective technique, 8 stable components were separated in common within 48 min, respectively, from 3 crude samples of *Salvia miltiorrhiza Bunge* from different growth locations. In HSCCC separation, 12 components were separated, respectively, with good correspondence and precision within 13 h. Both TLCS and HSCCC were effective in showing the whole concentration distribution of all kinds of constituents in different samples. HSCCC showed better performance in analysis of tanshinones, which produced a fingerprint which contained more chemical information than that of TLCS. It was further proven that HSCCC could be a feasible and cost effective method in the development of the fingerprint of TCM.

Keywords: Thin-layer chromatography scan (TLCS), High speed countercurrent chromatography (HSCCC), Fingerprint, *Salvia miltiorrhiza Bunge*, Tanshinones, Traditional Chinese Medicine (TCM)

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INTRODUCTION

Traditional Chinese medicines (TCM) have been used for clinical treatment for about two thousand years. Today, there are more than 500 species of medicinal plants recorded in the Chinese Pharmacopoeia.[1] The active components of TCM are influenced by its growing soil and climate, and the growth stage when harvested, which make quality control necessary for its application. Fingerprinting becomes a popular method in the quality control of TCM.[2]

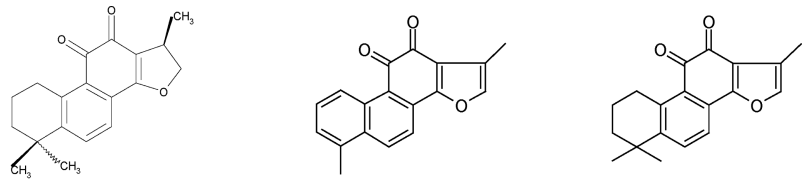
Chromatography, including thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), high performance capillary electrophoresis (HPCE), is recommended for the quality control of TCM in the Chinese Pharmacopoeia (2005 edition).[3] With the popular application of natural products, more and more techniques would be included in fingerprinting.

TLC is a quick, convenient, and cost effective technique widely used for pharmaceutical analyses. TLC has the special ability to assay many samples at the same time on a single plate.[4] TLC is recommended as an effective method for identification of plant derivatives by Chinese, American, and European Pharmacopoeias for the above reasons.[5] However, there are some limitations in this technology, for example, low revolution, accuracy, and repeatability compared with column analysis.[1]

High speed countercurrent chromatography (HSCCC) is a liquid-liquid partition chromatography without any solid matrix, which eliminates the irreversible adsorption of samples on solid support.[6,7] Its special structure makes it easy to analyze samples with high viscosity and it is easy to adsorb. It has been applied to develop a fingerprint of TCM in our study.[8] As a new method, it is, therefore, necessary to compare HSCCC with conventional approaches, for example HPLC, HPCE, and TLC, to evaluate its feasibility. Comparisons with HPLC and HPCE have been involved in our previous reports.[8,9]

Salvia miltiorrhiza Bunge, a popular traditional Chinese medicinal plant, has been used extensively for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrhea, and neuroasthenic insomnia.[10] *S. miltiorrhiza Bunge* also showed significant cytotoxicity against human tumor cell lines.[11] There are two kinds of major active constituents in *S. miltiorrhiza Bunge*, tanshinones and polyphenolics. Since it was impossible to separate these two kinds of compounds with the same condition, only tanshinones were involved in this study. Cryptotanshinone, tanshinone I and tanshinone II A (structures were shown in Figure 1) are major active components. Cryptotanshinone is usually used against inflammation, tanshinone I for therapy of angina pectoris, and tanshinone II A for improving blood circulation.

Separation and purification of *S. miltiorrhiza Bunge* by different types of countercurrent chromatography has been reported. G. L. Tian prepared four



Cryptotanshinone Tanshinone I Tanshinone IIA

Figure 1. Chemical structures of cryptotanshinone, tanshinone I and tanshinone IIA.

tanshinones by HSCCC[12] and eight tanshinones by multidimensional countercurrent chromatography.[13] H. B. Li separated eight tanshinones[14] and one water soluble phenolic compound by HSCCC.[15] Twelve components, more than other reports, were prepared by HSCCC in our study.[8]

Since TLCS is a traditional analytical method for medicinal plants, HSCCC, as a new method for fingerprinting, should be compared with it. In this study, TLCS was applied to develop fingerprinting of *S. miltiorrhiza Bunge* and the results were compared with those of HSCCC.

EXPERIMENTAL

Materials

Analytical grade ethanol, n-hexane, light petroleum (60–90°), and ethyl acetate were from Atoz Fine Chemicals Co. Ltd., Tianjin, China. All aqueous solutions were prepared with pure water produced by Milli-Q system (18MΩ, Milipore, Bedford, MA, USA). Reference materials of cryptotanshinone, tanshinone I and tanshinone II A were supplied by the State Food and Drug Administration of China (SFDA).

Thin-Layer Chromatography Scan (TLCS)

Silica gel 60 F254 plate, 10 × 20 cm (Qingdao, China), was used in the TLC experiment. Quantitative capillary for loading the sample was 5 μL (Alltech, IL, USA). Scanning procedure was carried out on CS9301 TLC scanner and software (Shimadzu, Japan). Optimized mobile phase was light petroleum (60–90°)-ethyl acetate (8 : 2, v/v).

Mobile phase was added to the chromatographic cylinder prior to use for presaturation. Each sample of 5 μL was loaded on the plate in parallel, and then the plate was put into the cylinder in a proper angle to develop the

sample. When the front of mobile phase was close to the upper fringe of plate, it was taken out and dried. Then, the plate was put into CS9301 TLC scanner and scanned at 280 nm with a linear mode. The sample loading dot was set to the origin and the migration length was calculated by the chromatograph and its software.

High Speed Countercurrent Chromatography (HSCCC)

The HSCCC (TBE-300) is from Tauto Biotech, Shanghai, China, with three preparative coils connected in series (diameter of 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 ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The HSCCC systems are equipped with a Model S constant flow pump, a Model 8823A UV monitor operating at 280 nm, and a Model 3057 recorder.

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).[8] Each mixture was equilibrated thoroughly in a separating funnel at room temperature. The upper phase and lower phase were separated before use.

Preparative separation was performed by 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 crude tanshinones was injected through the valve. The effluent was monitored with a UV-Vis detector at 280 nm and the peak fractions were collected respectively. After 470 min of elution, the mobile phase was changed to the lower phase of system B to the end.[8]

LC-MS Conditions

An Agilent 1100 system was applied for LC-MS with an Ultrasphere C18 column (150 × 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 mL/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 1,000 with full scan mode.

Crude Sample Preparation

The powdered dried roots (20 g) of *S. miltiorrhiza Bunge* from Hebei, Shandong and Jiangsu province were added to 50 mL n-hexane-ethanol (1 : 1, v/v). The mixture was shaken for 45 min before centrifugation at 10,000 g for 10 min, and the supernatant was saved. The above procedure was repeated once and the supernatants combined. The extract was diluted with water at a ratio of 1 : 2 and equilibrated for 2 h. The organic phase was separated and washed with 30% aqueous ethanol until the water phase was almost colorless. The organic extracts were dried by rotary vaporization at 40°C to yield the final crude samples. The crude sample (100 mg) was dissolved in 6 mL lower phase of solvent system A for HSCCC separation.[8] The crude sample, 10 mg, was dissolved in 1 mL mobile phase of TLCS analysis and 5 μL of each crude sample was loaded.

Reference Material Preparation

Reference materials of tanshinone I, tanshinone IIA, 2.5 mg/mL, and 5 mg/mL reference material of cryptotanshinone were prepared with mobile phase of TLCS analysis.

Calculation of Relative Amounts of Components in HSCCC and TLCS Analysis

It was difficult to use the area percent of components to compare the content of the same component in different samples in HSCCC and TLCS analysis. Relative amounts of components were calculated for easy comparison. Cryptotanshinone, whose peak was in the middle part of the densitograms, was a major active compound in *S. miltiorrhiza Bunge* and it was suitable to be a reference peak to calculate relative amounts of the components. In each sample, the peak area percent of cryptotanshinone was set to 1 and area percents of other peaks were divided by that of cryptotanshinone.

RESULTS AND DISCUSSION

Fingerprinting of Tanshinones in *S. miltiorrhiza Bunge* by TLCS

Several mobile phases were tried according to the literature:[2] chloroform-n-hexane (10 : 1), chloroform-methanol-light petroleum (60–90°) (10 : 1 : 10), light petroleum (60–90°)-ethyl acetate (19 : 1, 8 : 2, 8 : 3, 2 : 1), n-hexane-ethanol-water (10 : 5.5 : 4.5, upper phase), n-hexane-ethanol-water (10 : 5.5 : 4.5, lower phase).

The crude sample couldn't be separated and the retention factor (R_f) was close to 1 with chloroform-methanol-light petroleum (60–90°) (10 : 1 : 10) and ethyl acetate-ethanol-water (10 : 5.5 : 4.5, lower phase). Also, crude sample couldn't be separated and its R_f was less than 0.2 with chloroform-n-hexane (10 : 1). Four or five visible spots came out with n-hexane-ethanol-water (10 : 5.5 : 4.5, upper phase) or light petroleum (60-90°)-ethyl acetate (2 : 1), however, their retention factors were not in the suitable range and the resolution was not good. The separation became a little better with light petroleum (60–90°)-ethyl acetate (19 : 1) and (8 : 3). Finally, seven visible spots developed with light petroleum (60–90°)-ethyl acetate (8 : 2), with good resolution and suitable retention factors in the range of 0.15-0.7, which showed better performance than other mobile phases.

Three crude samples and three reference materials were developed on the same plate with light petroleum (60–90°)-ethyl acetate (8 : 2). Several visible spots came out from the three crude samples, respectively. Only one spot was developed from each reference material with suitable R_f. After drying, the plate was scanned at 280 nm, because the major absorption peaks of the important active components were in the range of 280 nm. Scanned data of samples were translated into densitograms by the scanner and its software as shown in Figure 2.

Since precision of TLCS was not that good as HPLC, 3% was set to the limit of relative standard deviation (RSD) of migration length for estimation of the correspondence of peaks according to the literature.[16] There were eight peaks in common in the three crude samples and their RSDs of migration lengths were in the range of 0.47–2.57% as shown in Table 1. It was concluded that the corresponding peaks were the same constituent.

The densitograms evidently showed that the contents of corresponding components in the three crude samples were different. For easy quantitative comparison, relative peak area was calculated. Each peak area of peak no. 3, as a reference peak, in the three crude samples was set to 1 and other peak areas were divided by it as shown in Table 2. For example, relative peak areas of peak no. 7 in the three crude samples were 1.03, 1.57, and 1.24, respectively, which confirmed that location had a great effect on the quality of TCM.

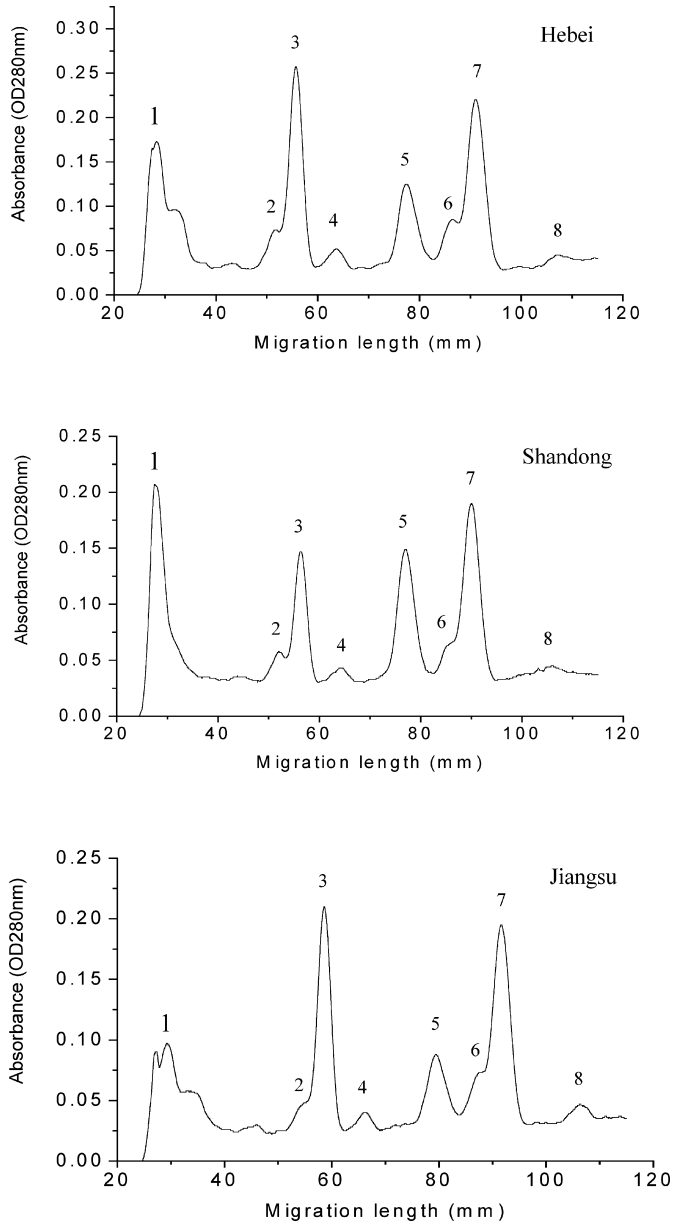


Figure 2. Chromatograms of crude samples of *Salvia miltiorrhiza Bunge* from 3 different provinces by TLCS. Conditions: TLC plate: silica gel 60 F254 plate, 10×20 cm; volume of quantitative capillary for loading sample: 5 μL; mobile phase: light petroleum (60–90°)-ethyl acetate (8 : 2, v/v); wavelength of scanning: 280 nm; mode of scanning: linear. Peak 3: cryptotanshinone, peak 5: tanshinone I, peak 7: tanshinone IIA.

Table 1. Migration lengths of peaks in TLCS analysis

	Migration length (mm)			
Peak no.	Hebei	Shandong	Jiangsu	RSD (%)
1	28.88	28.24	28.28	1.27
2	52.65	52.96	55.46	0.47
3	56.10	56.74	58.90	2.57
4	64.84	65.15	67.34	2.07
5	78.15	77.51	80.30	1.55
6	87.34	86.40	88.28	1.08
7	91.57	90.57	92.05	0.82
8	108.28	107.03	107.34	0.54

Conditions: TLC plate: silica gel 60 F254 plate, 10×20 cm; volume of quantitative capillary for loading sample: 5 μL; mobile phase: light petroleum (60–90°)-ethyl acetate (8 : 2, v/v); wavelength of scanning: 280 nm; mode of scanning: linear.

Identification of Major Active Constituents in TLCS Analysis

Cryptotanshinone, tanshinone I, and tanshinone IIA were major active constituents in *S. miltiorrhiza Bunge* and needed to be identified in the fingerprint. Three reference materials were separated on the same plate with three crude samples. Scanned data were translated into densitograms as shown in Figure 3. Each RSD of the reference material and its corresponding peak in the crude sample was less than 3% as shown in Table 3. It could be inferred

Table 2. Relative contents of components of three crude samples in TLCS analysis

	Relative content		
Peak no.	Hebei	Shandong	Jiangsu
1	0.71	1.86	0.22
2	0.29	0.32	0.24
3	1	1	1
4	0.30	0.48	0.29
5	0.74	1.31	0.65
6	0.35	0.41	0.33
7	1.03	1.57	1.24
8	0.32	0.72	0.35

Conditions: TLC plate: silica gel 60 F254 plate, 10×20 cm; volume of quantitative capillary for loading sample: 5 μL; mobile phase: light petroleum (60–90°)-ethyl acetate (8 : 2, v/v); wavelength of scanning: 280 nm; mode of scanning: linear.

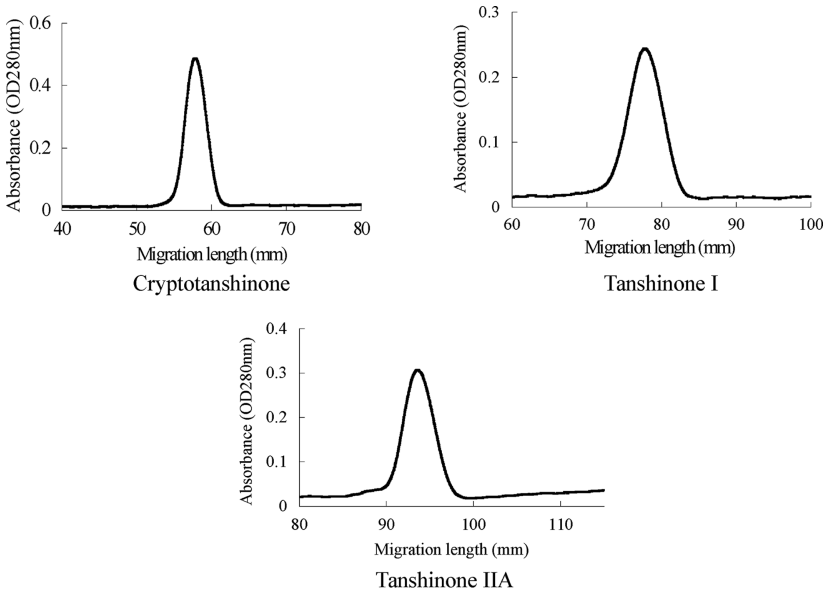


Figure 3. Chromatograms of three standard samples in TLCS analysis. Conditions: TLC plate: silica gel 60 F254 plate, 10 × 20 cm; volume of quantitative capillary for loading sample: 5 μL; mobile phase: light petroleum (60–90°)-ethyl acetate (8 : 2, v/v); wavelength of scanning: 280 nm; mode of scanning: linear.

that peak no. 3 was cryptotanshinone (Mw 296), no. 5 was tanshinone I (Mw 276) and no. 7 was tanshinone II A (Mw 294).

Comparisons of TLCS and HSCCC

An optimized step wise elution strategy was performed on HSCCC for better resolution:[8] 0–470 min, in solvent system A; then in solvent system B. The

Table 3. Identification of major active components in TLCS analysis

	Migration length (mm)		
Components	1	2	RSD (%)
Crypototanshinone	58.22	56.10	2.61
Tanshinone I	78.40	78.15	0.22
Tanshinone IIA	94.13	91.57	1.95

Conditions: TLC plate: silica gel 60 F254 plate, 10 × 20 cm; volume of quantitative capillary for loading sample: 5 μL; mobile phase: light petroleum (60–90°)-ethyl acetate (8 : 2, v/v); wavelength of scanning: 280 nm; mode of scanning: linear.

HSCCC system was performed at a speed of 900 rpm and at a flow rate of 2 mL/min. Retention of the stationary phase was 78.8%, which assured the resolution of separation. Twelve distinct peak fractions were eluted from the three crude samples within 13 hours (figure was shown in reference [8]). It was proven that the corresponding peaks in the three crude samples were the same constituents.[8] The average of RSDs of retention time was 2.7% as referred in our previous report.[8]

Each peak area of peak no. 7, as reference peak, in the three crude samples was set to 1 and relative peak areas were calculated (table was shown in reference [8]). It was very easy to compare different samples by these relative values. For example, relative peak areas of peak no. 11 in the three crude samples were 2.05, 3.78, and 3.17 respectively, which showed great difference in samples from different locations (Table 4).

Table 4. Comparisons of TLCS and HSCCC

Content of comparison	TLCS	HSCCC
Scale	Analytical	Semi-preparative
Volume of separation column or plate	10 × 20 cm	300 mL
Temperature	Non-controlled	Non-controlled
Pretreatment of sample	Load directly	Load directly
Mass of sample	mg	mg ~ g
Volume of sample	5 μL	2 mL
Common peaks	8	12
Non-common peaks	<10%	0
Run time	48 min	13 h
Sequence of elution peaks	CPTT, T I and T IIA	CPTT, T I and T IIA
Relative content	T I/CPTT = 0.74,1.31, 0.65 T IIA/CPTT = 1.03, 1.57, 1.24	T I/CPTT = 0.27, 1.56, 0.59 T IIA/CPTT = 2.05, 3.77, 3.17
Average of RSD of l_M or t_R (%)	1.30	2.7
Cost of apparatus	US$20,000	US$12,000

l_M: Migration length; t_R: retention time.

Cryptotanshinone: CPTT; tanshinone IIA: T IIA; tanshinone I: T I.

Conditions of TLCS: TLC plate: silica gel 60 F254 plate, 10 × 20 cm; volume of quantitative capillary for loading sample: 5 μL; mobile phase: light petroleum (60–90°)-ethyl acetate (8 : 2, v/v); wavelength of scanning: 280 nm; mode of scanning: linear.

Conditions of HSCCC: 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%.

Analyses were performed on liquid chromatography-mass spectrometry (LC-MS). It was concluded from ultraviolet absorption spectra, retention times in LC analysis, and mass-spectrograms, that fractions 7, 8, and 11 were cryptotanshinone, tanshinone I, and tanshinone II A, respectively (see reference [17]).

Both TLCS and HSCCC could display the whole concentration distribution of components, which was the most important character of fingerprinting. Differences of TCM from different locations could be shown directly by TLCS and HSCCC qualitatively (by retention time) and quantitatively (by peak area). Moreover, strict sample pretreatment was not necessary for both methods, which made their operations easier than HPLC.

The fingerprint developed by HSCCC included twelve peaks, which contained more chemical information than by TLCS. And, there was no noncommon peak in the HSCCC fingerprint. The precision of TLCS was a little better than HSCCC. The average RSD of elution time in HSCCC separation was 2.7% and the average RSD of migration length in TLCS separation was 1.3%. However, both of them were acceptable for fingerprinting. HSCCC was used in almost the same way for sample loading and on-line detection as HPLC, which could be more stable than TLCS.

CONCLUSION

There are complicated constituents in TCM leading to difficult quality control. Since each method has its own advantages and disadvantages, it is necessary to develop more approaches in fingerprinting of TCM. Both HSCCC and TLCS were effective in showing the concentration distribution of components in different samples with acceptable precision, which meant that they were both effective methods in fingerprinting. HSCCC showed better performance on fingerprinting of tanshinones, which contained more chemical information than TLCS. It was further proven, that HSCCC could be a feasible and cost effective method for development of fingerprinting of TCM, based on the comparison with TLCS, HPLC, and NACE.

HSCCC, as a new method in fingerprinting, should be further studied in the following aspects: repeatability, detection limit, range of linearity, application range, and other factors.

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