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Development of a fingerprint of *Salvia miltiorrhiza Bunge* by high-performance liquid chromatography with a coulometric electrode array system

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Abstract

To standardize and control herbal medicines, a feasible approach and control system is necessary. In this paper, a high-performance liquid chromatography with a coulometric electrode array detector (HPLC-CEAD) system was applied to fingerprint *Salvia miltiorrhiza Bunge* (*S. miltiorrhiza Bunge*), a popular herbal medicine, for the first time. pH of mobile phase, working potentials and sample preparation were included in our research. Twenty-five common peaks were obtained from extracts of *S. miltiorrhiza Bunge* (Shandong province), more than that obtained in previous report. Fingerprints of *S. miltiorrhiza Bunge* from different locations were also studied. The content of main components varied in different samples. Overlapping ratio of peaks (ORP) in 10 batches of *S. miltiorrhiza Bunge* (Shandong province) was not less than 72.46%. In method validation, relative standard deviation (RSD) of relative retention times and relative peak areas were of not more than 3%. It was concluded that HPLC-CEAD system can be applied in fingerprinting herbal medicines.
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Keywords: HPLC; Coulometric electrode array; Fingerprint; *Salvia miltiorrhiza Bunge*

1. Introduction

Herbal medicines is a complete system of healing developed in China about 3000 years ago with very little change over centuries. However, owing to the fact that there are hundreds of complex active components in herbal medicines, it is impossible to identify all these substances and carry on quantitative analysis. Furthermore, active components are influenced by its growing soil and climate, and when it is harvested [1]. It is absolutely necessary to develop reliable analytic methods in order to control the quality of herbal medicines. Fingerprint technique was introduced and accepted by WHO as a powerful tool for identification and quality evaluation of multi-component herbal medicines [2].

Chromatography, ultraviolet spectroscopy (UV), infrared spectrometry (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS) are practically used in development of fingerprints [3]. Among them, chromatography, including thin-layer chromatography (TLC) [4], high-performance liquid chromatography (HPLC) [5–7] and gas chromatography (GC) [8], is recommended by Chinese Pharmacopoeia for fingerprinting. TLC is fast and easy to operate, while its precision is poor; GC is of high precision, but has limited application. High-performance liquid chromatography with a coulometric electrode array detector (HPLC-CEAD) is a new approach with high precision and selectivity based on redox activity of the analytes. It could separate and detect the co-eluting peaks caused by complicated components in herbal medicines by setting different potentials according to the tiny distinction on molecular structural formulas of the analytes. It has been successfully applied to analyze natural products [9].

Salvia miltiorrhiza Bunge (*S. miltiorrhiza Bunge*), a popular herbal medicine, has been commonly used for promoting blood circulation to remove blood stasis, clearing away heat, relieving vexation, nourishing blood, tranquilizing the mind,

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cooling the blood to relieve carbuncles, treating hemorrhages, menstrual disorders and miscarriages [10]. There are two kinds of major active constituents in *S. miltiorrhiza Bunge*, tanshiones and polyphenolics. Pharmacological experiments have demonstrated that tanshinones have the efficacy of resisting bacterium and tumour, as well as diminishing inflammation; salvianolic acids can effectively restrain lipid peroxidation of the brain, liver and kidney [11]. In addition, Danshensu and protocatechuic aldehyde also play an important role in the effect of *S. miltiorrhiza Bunge*.

Liu et al. [12] separated and detected 11 components from *S. miltiorrhiza Bunge* using HPLC-UV, a common method in fingerprinting. High-speed counter-current chromatography (HSCCC) [13] and non-aqueous capillary electrophoresis (NACE) [14] were also reported to develop fingerprint of *S. miltiorrhiza Bunge*. Although strict sample pretreatment was not necessary for them, HSCCC needed more than 10 h to separate and analyze 12 components from *S. miltiorrhiza Bunge* and NACE showed poor performance on fingerprinting of tanshinones. HPLC-CEAD has been reported to analyze phenols and flavonoid and develop its fingerprint [15], but no report about fingerprinting of *S. miltiorrhiza Bunge* has been found yet. In this paper, 25 components from *S. miltiorrhiza Bunge* (Shandong province) were separated and detected in two hours using HPLC-CEAD system, more than that obtained in the previous report. The results showed that HPLC-CEAD could offer more information and high precision in fingerprinting of herbal medicines.

2. Experimental

2.1. Instrumentation

The HPLC-CEAD system was an ESA chromatographic system (ESA, Chemsford, MA, USA) equipped with an ESA Model 582 solvent delivery module, a manual sample injector with a 20 μL loop (Rheodyne 7725i, CA, USA) and a 5600A 16 channels CoulArray detector. ESA software was used for data acquisition and processing. In addition, Sartorius BS 110S electronic balance (Beijing, China), WTW inolab level 1 pH meter (Weiheim, Germany) and Dupont Sorvall RC 5C plus centrifuge (Newtown, CT, USA) were used in this study.

2.2. Chemicals and reagents

Standards of Sodium Danshensu (I), protocatechuic acid (II), protocatechuic aldehyde (III), salvianolic acid B (IV), cryptotanshinone (V), tanshinone I (VI), and tanshinone IIA (VII) were bought from the Institute of Chinese Pharmaceutical and Biological Product Inspection (Beijing, China). Sodium acetate, glacial acetic acid and trifluoroacetic acid (TFA) were analytes from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were HPLC purity reagent from Fisher Science (NJ, USA). The water used in all experiments had a resistivity of 18.2 MΩ cm obtained from a Mill-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Standard solution preparation

Primary stock standard solutions of the seven compounds were prepared by dissolving them with methanol respectively (sodium Danshensu with 80% methanol) to get a concentration of 1 mg/mL and were stored at 4 °C for one week. Working mixed standard solutions were prepared daily by mixing and diluting the stock solutions with mobile phase.

2.4. Sample preparation

Dried root (0.5 g) of *S. miltiorrhiza Bunge* from three different locations (Shandong, Hebei and Jiangsu) was immersed in 200 mL 70% methanol overnight and extracted by thermal refluxing for 2 h, respectively. After, filtered, the extracts were centrifuged (10,000 × *g*, 10 min), and the supernatant was saved and filtered through 0.22 μm membrane. Then 20 μL of each sample was injected into the HPLC-CEAD system.

2.5. Chromatographic conditions

A ODS Hypersil (250 mm × 4.6 mm i.d., 5 μm, Hewlett-Packard, USA) column and a Hypersil guard column (Hypersil, 5 μm, Alltech Association, USA) was used in HPLC-CEAD system. The mobile phase was component A (50 mM sodium acetate-0.1% TFA, pH 2.7) and B (acetonitrile) in gradient mode as follows: 0–15 min, 6% B; 15–45 min, 6–20% B; 45–60 min, 20–53% B; 60–120 min, 53% B; the total flow rate was 0.8 mL/min. Electrode potentials were set at −600, 100, 250 and 500 mV, respectively. Injection volume was 20 μL and the analysis was carried out at room temperature.

3. Results and discussion

3.1. Selection of a suitable chromatographic condition

In this study, structures of main components in *S. miltiorrhiza Bunge* were given in Fig. 1. pH of mobile phase was considered because of phenolic hydroxyl groups and carboxylic groups in water-soluble compounds (I, II, III and IV). Its influence on them has been involved in our previous report [16]. In addition, in lipid-soluble components, interconversion between quinonyls and phenolic hydroxyl groups caused by their resonant structure is also affected by pH of mobile phase. In acidic solution, after quinonyls transform into hydroxyls group, the carbon that connects to these groups was electropositive, which increases its oxidizability. Influences of pH on retention time and peak area of lipid-soluble components were shown in Fig. 2a and b. The curves were gained by several repetitive experiments. From Fig. 2a and b, the retention time was the shortest for each compound and they could be separated well at pH 2.7. Considering our previous studies, pH 2.7 was finally chosen for further optimization.

Proper selection of applied electrode potentials was critical for accurate measurement. In this paper, working potentials of each detector cell were chosen through experimentation after other chromatographic conditions had been determined.

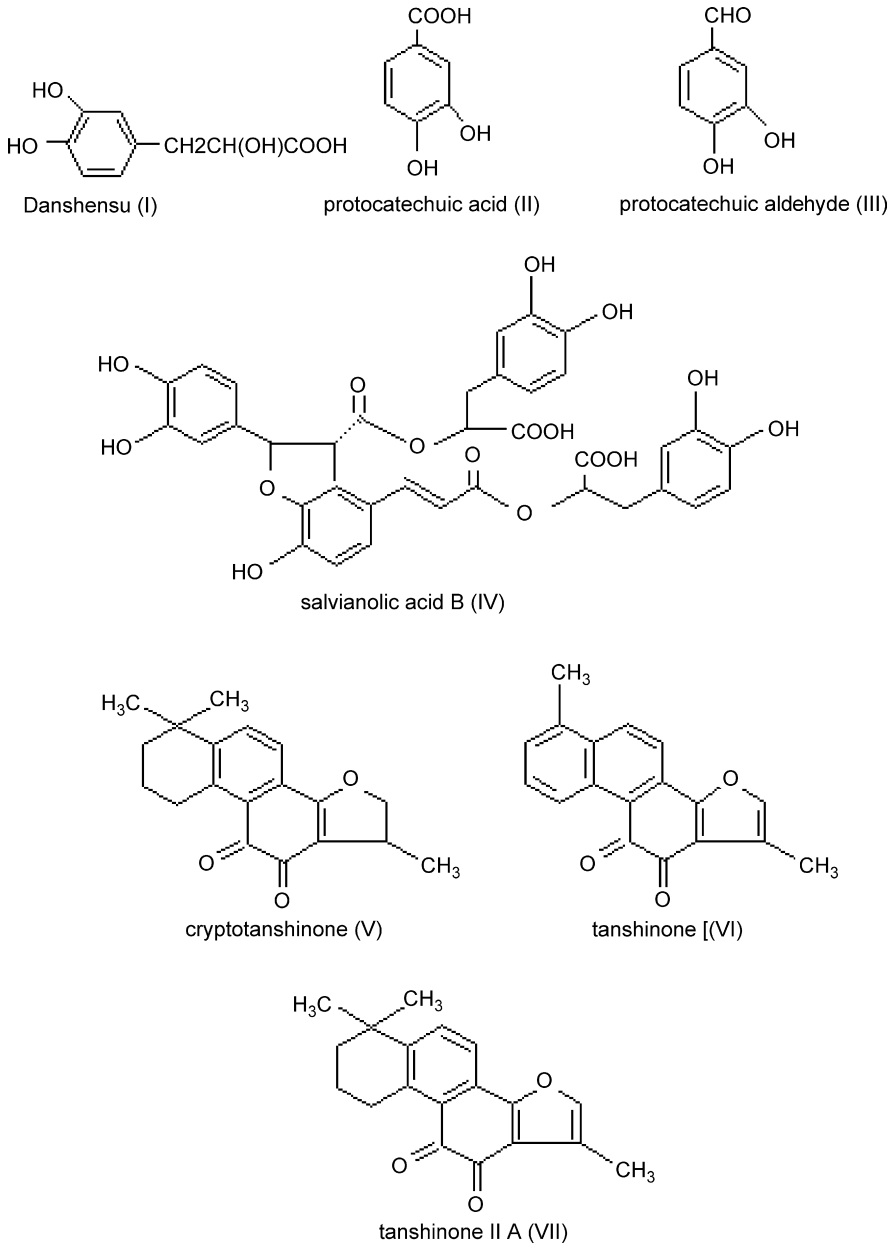

Fig. 1. Structures of the main components in *S. miltiorrhiza Bunge*.

Because phenolic hydroxyls groups and quinonyl groups could be oxidized and reduced, respectively, two electrodes were selected so that they could be detected completely. In the first channel, potential was set at −300 mV and decreased by 50 mV each time until it was −700 mV and the second channel was kept at −700 mV invariably; in the third channel, potential was set at 50 mV and increased by 50 mV each time until 700 mV; whereas while the fourth channel was constantly kept at 700 mV. Relationships between the peak area (quantity of electricity) in the first (for the lipid-soluble compounds) and third channel (for the water-soluble compounds) and potentials (voltage) were shown in Fig. 2c and d, respectively. From Fig. 2c and d, we can see that I and IV can be oxidized below 50 mV while II and III at 100 mV. Their peak areas reached a plateau at 500, 250, 300 and 300 mV, respectively, where no increase in peak area occurred when the potential was heightened. V, VI and VII could be reduced at −350 mV and their peak areas reached a plateau at −600 mV. Although greater response could be obtained at higher potential, the background current or noise was also increased. The ratio of a signal to noise was 3:1, and then potentials of −600, 100, 250 and 500 mV were selected to provide adequate sensitivity for fingerprinting of *S. miltiorrhiza Bunge*.

For sample preparation, thermal refluxing extraction was used in this paper. 10, 30, 50, 70 and 90% (v/v) methanol were put on trial as extract solvent and kept refluxing for 1, 1.5, 2 and 2.5 h for each respectively. Experimental results revealed that proportion of methanol and refluxing time had significant effects on total number of peaks and peak areas. Refluxing for two hours under a certain proportion of methanol obtained the optimal results, and there was no obvious difference between

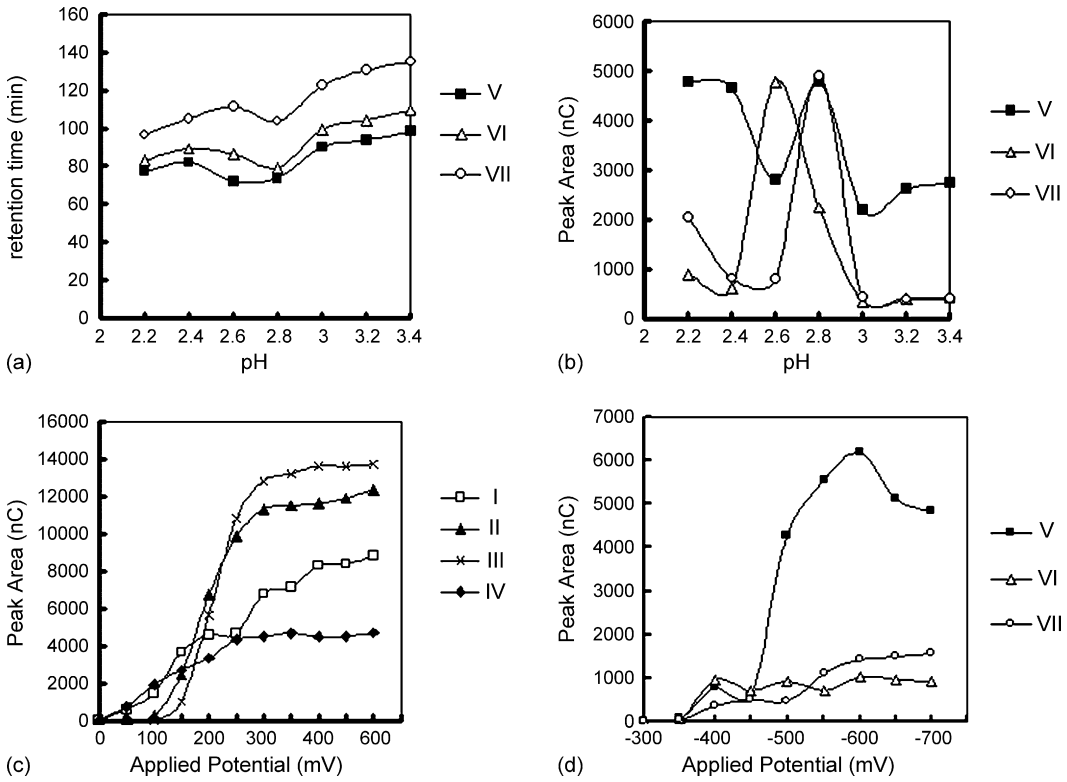

Fig. 2. Effect of pH of mobile phase on: (a) retention time of V, VI and VII; (b) peak area of these three compounds; and effect of applied potential on (c) I, II, III and IV (the applied potentials were positive due to the reducibility of these four compounds); (d) V, VI and VII (the applied potentials were negative due to the oxidizability of these three compounds).

refluxing for 2 and 2.5 h. Ten percent methanol extracted more water-soluble compounds whereas 90% extracted more lipid-soluble compounds, but both of them could not provide complete information for fingerprinting of *S. miltiorrhiza Bunge*. The number of peaks was nearly the same when extracted by 50 and 70% methanol; however, peak area was greater in the latter. Consequently, 70% methanol was chosen as extract solvent and refluxing time was kept at 2 h in order to offer more accurate information for the fingerprint.

3.2. Chromatograms of S. miltiorrhiza Bunge by HPLC-CEAD

S. miltiorrhiza Bunge from Shandong, Hebei and Jiangsu province was studied and compared under the same experimentation conditions. Their chromatograms are shown in Fig. 3. From Fig. 3, responses in the first channel (−600 mV) were produced by lipid-soluble compounds which were reduced at negative potential. Then the reduced products were partly oxidized at positive potential, so at the corresponding position in the second channel, there were also responses.

From Fig. 3a, 25 distinct common peak fractions were eluted and detected (samples from Shandong) within 2 h using HPLC-CEAD, more than that in previous report. They were separated well from each other under the chosen experimentation conditions. Retention times of peak 16 and 17 were 70.1 and 70.2 min, respectively, and that of peak 20 and 21 were 79.7 and 79.9 min, respectively. The differences of their retention times were so tiny that it was difficult to separate and detect them just by their UV absorption character. However, this problem could be solved using HPLC-CEAD according to redox character of these compounds which were analyzed in this paper. That indicated the advantage of our method. From Fig. 3a, retention times of water-soluble compounds were shorter owing to the ionization of phenolic hydroxyls groups. Quinones are more hydrophobic, therefore they elute later.

From Fig. 3b and c, there were 24 and 23 common peaks in chromatograms of samples from Hebei and Jiangsu, which were less than that from Shandong. Overlapping peaks existing in these chromatograms were also separated by HPLC-CEAD. The content of main compounds varied in different samples as shown in Fig. 3b and c, which confirmed that location and climate had a great impact on the quality of herbal medicines. This shows that a feasible control system is necessary for herbal medicines.

3.3. Analysis of chromatogram

Peaks were identified using standards. Chromatogram of standards was shown in Fig. 4. The highest peak in the chromatogram is peak 10, IV, which was used as internal standard for method validation. According to Fig. 4, peak 1, 2, 3, 10, 22, 23 and 25 in Fig. 3a were I, II, III, IV, V, VI and VII, respectively. However, HPLC fingerprint determination is not a method for quantitative analysis. Parameters evaluation and validation aspects are different from general assaying methods. Authentication and identification of an herbal medicine can be accurately

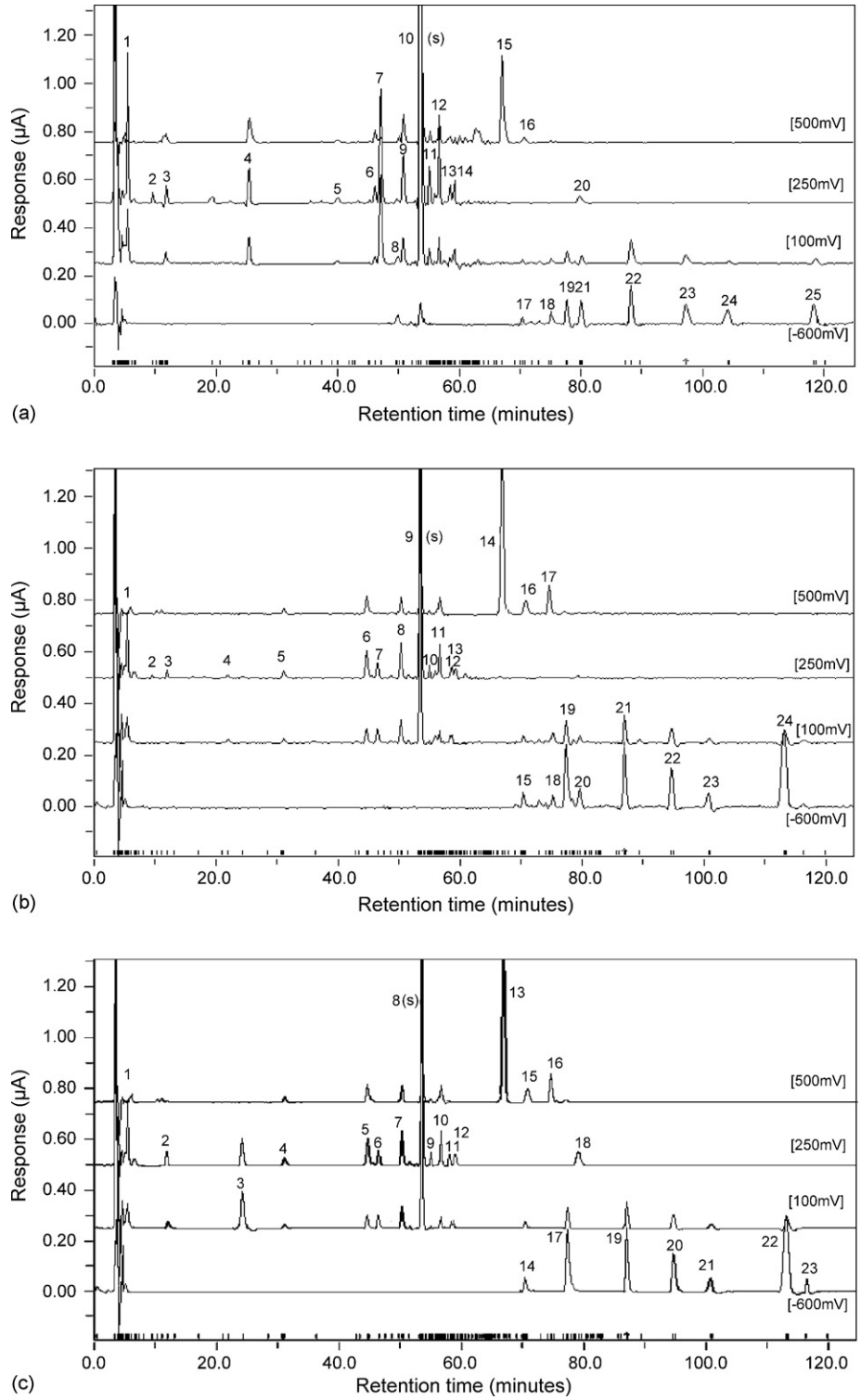

Fig. 3. HPLC chromatograms of: (a) sample from Shandong province: Peak 1, Danshensu (I); 2, protocatechuic acid (II); 3, protocatechuic aldehyde (III); 10, salvianolic acid B (IV); 22, cryptotanshinone (V); 23, tanshinone I (VI); 25, tanshinone IIA (VII); (b) sample from Hebei province: Peak 1, 2, 3, 9, 22, 23 and 24 were those corresponding compounds above, respectively; (c) sample from Jiangsu province: Peak 1, 2, 8, 19, 20 and 22 were I, III, IV, V, VI and VII, respectively (II could not be detected).

carried out using chromatographic fingerprints, even if batches or concentrations are not the same in different samples of this drug. Considering these characteristics of fingerprints, relative retention time (the ratio between retention time of sample constituents to that of internal standard) and relative peak area (the ratio between peak area of sample constituents to that of internal standard) of main peaks (>1% of total peak area) in 10 batches of samples were used to evaluate the quality of fingerprint. The results were shown in Table 1. From Table 1, relative standard deviation (RSD) of relative retention time and relative peak area

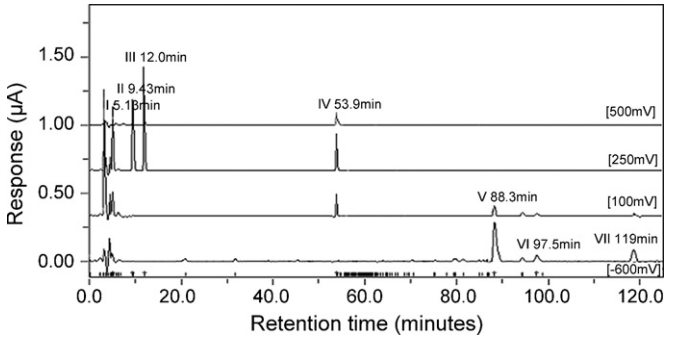

Fig. 4. HPLC chromatogram of standards of I, II, III, IV, V, VI and VII.

for 10 batches of *S. miltiorrhiza Bunge* sample from Shandong were not exceeding 0.99 and 2.94%, respectively. Mean ratio of uncommon peak area was 4.325%.

In addition, another two parameters were introduced in this paper: ten biggest peaks (TBP) and overlapping ratio of peaks (ORP). TBP are ten peaks with higher content compared with other peaks in chromatogram and they are arranged in the order from high to low. It could describe the differences between two compared samples in content. From Fig. 3a, TBP in 10 batches of *S. miltiorrhiza Bunge* sample from Shandong were peaks 10, 7, 4, 15, 1, 22, 9, 12, 11 and 13, respectively.

ORP indicated the correlation of two comparative samples according to their respective total number of peaks and common peaks. This calculation method was based on a certain sample

Table 1
RSD for relative retention times and relative peak areas of common peaks in 10 batches of *S. miltiorrhiza Bunge* sample from Shandong

Peak no.	Relative retention time		Relative peak area	
	Mean	RSD%	Mean	RSD%
1	0.102	0.65	0.175	1.23
2	0.178	0.99	0.016	2.88
3	0.224	0.83	0.072	1.67
4	0.483	0.37	0.287	0.46
5	0.750	0.29	0.021	2.25
6	0.863	0.22	0.054	2.23
7	0.883	0.24	0.404	0.38
8	0.929	0.28	0.015	2.94
9	0.947	0.25	0.156	1.07
10(s)	1.000	0.00	1.000	0.00
11	1.027	0.22	0.105	1.82
12	1.057	0.23	0.155	1.14
13	1.094	0.20	0.096	1.56
14	1.104	0.17	0.046	2.51
15	1.247	0.19	0.255	0.80
16	1.307	0.13	0.013	2.75
17	1.312	0.12	0.012	2.89
18	1.358	0.09	0.016	2.79
19	1.398	0.12	0.037	2.55
20	1.447	0.15	0.019	2.47
21	1.492	0.10	0.034	2.71
22	1.642	0.10	0.165	0.93
23	1.814	0.07	0.047	2.47
24	1.947	0.12	0.045	2.52
25	2.208	0.05	0.057	2.26

Table 2
Overlap ratios of peaks (ORP) in 10 batches of *Salvia miltiorrhiza Bunge* sample from Shandong

Batch	Number of common peaks	Total number of peaks	ORP (%)
1		34	72.46
2		31	75.76
3		28	79.37
4		33	73.53
5(s)	25	35	–
6		29	78.13
7		30	76.92
8		27	80.65
9		32	74.63
10		30	76.92

(reference sample) and the expression was defined as follows:

$$\gamma\,(\%) = \frac{2 \times \lambda}{\alpha + \beta} \times 100\% \quad (1)$$

where γ means the overlapping ratio; λ means the number of common peaks in two samples; α and β represent the number of total peaks in reference sample and compared sample, respectively. λ, α and β are determined under the same chromatographic conditions. Generally speaking, α and β are usually different numbers and they are influenced by growing soil, climate and when harvested. In this paper, ORP in 10 batches of sample from Shandong were calculated with batch 5 as the reference. The results were showed in Table 2. From Table 2, ORP of each batch was no less than 72.46%. ORP can be used further in compare of diversity between different samples, which verify the remarkable differences between varied samples in the simple view of mathematics.

3.4. Methodology validation

3.4.1. Precision test

Injection precision was assessed by repetitive injection of the same sample solution for six times in a day. The results were shown in Table 3. From Table 3, RSD of relative retention time and relative peak area were not exceeding 0.76 and 2.98%, respectively.

3.4.2. Repeatability test

Repeatability was determined by analyzing six independently prepared samples of *S. miltiorrhiza Bunge* using the same method. The results were shown in Table 4. From Table 4, RSD of relative retention time and relative peak area were no more than 1.99 and 2.98%, respectively.

3.4.3. Sample stability test

Sample stability test was determined with one sample at regular intervals of 3 h in 24 h. During this period, the solution was stored at 4 °C. The results were shown in Table 5. From Table 5, RSD of relative retention time and relative peak area found were below 1.13 and 2.98%, respectively. The similarity of these results indicated that the sample remained stable in 24 h.

Table 3
Results of precision test on HPLC fingerprint of *S. miltiorrhiza Bunge* from Shandong ($n = 6$)

Peak no.	Relative retention time		Relative peak area	
	Mean	RSD%	Mean	RSD%
1	0.102	0.76	0.178	1.05
2	0.180	0.31	0.017	2.44
3	0.224	0.37	0.071	2.00
4	0.483	0.11	0.286	0.51
5	0.751	0.16	0.021	2.67
6	0.865	0.16	0.054	2.73
7	0.882	0.16	0.402	0.58
8	0.927	0.20	0.014	2.87
9	0.947	0.28	0.157	0.90
10(s)	1.000	0.00	1.000	0.00
11	1.029	0.18	0.106	1.33
12	1.058	0.17	0.154	0.89
13	1.093	0.17	0.098	0.91
14	1.103	0.21	0.047	2.48
15	1.247	0.19	0.254	0.68
16	1.308	0.17	0.013	2.98
17	1.314	0.20	0.011	0.00
18	1.358	0.18	0.015	2.70
19	1.399	0.10	0.038	2.35
20	1.447	0.21	0.018	2.83
21	1.492	0.16	0.034	2.43
22	1.643	0.14	0.167	0.80
23	1.814	0.12	0.048	2.21
24	1.946	0.18	0.046	2.55
25	2.207	0.10	0.057	1.86

Table 4
Results of stability test on HPLC fingerprint of *S. miltiorrhiza Bunge* from Shandong ($n = 6$)

Peak no.	Relative retention time		Relative peak area	
	Mean	RSD%	Mean	RSD%
1	0.103	1.13	0.177	1.22
2	0.182	0.90	0.017	2.44
3	0.226	0.94	0.071	2.52
4	0.486	0.59	0.288	1.11
5	0.754	0.35	0.021	2.67
6	0.865	0.44	0.054	2.73
7	0.880	0.60	0.407	0.99
8	0.923	0.46	0.014	2.87
9	0.943	0.43	0.158	0.93
10(s)	1.000	0.00	1.000	0.00
11	1.025	0.40	0.108	1.37
12	1.056	0.45	0.154	2.45
13	1.092	0.28	0.098	2.63
14	1.107	0.36	0.048	2.95
15	1.245	0.32	0.254	1.48
16	1.308	0.34	0.013	2.98
17	1.316	0.25	0.011	0.00
18	1.359	0.26	0.015	2.70
19	1.400	0.17	0.039	2.72
20	1.449	0.27	0.018	2.83
21	1.494	0.22	0.034	2.63
22	1.644	0.22	0.167	2.00
23	1.816	0.19	0.048	2.43
24	1.947	0.18	0.046	2.53
25	2.210	0.12	0.057	2.48

$n = 6$: determine the same sample solution at 0, 3rd, 6th, 9th, 12th, 24th hour after it was prepared, respectively.

Table 5
Results of repeatability test on HPLC fingerprint of *S. miltiorrhiza Bunge* from Shandong ($n = 6$)

Peak no.	Relative retention time		Relative peak area	
	Mean	RSD%	Mean	RSD%
1	0.103	1.99	0.177	1.80
2	0.180	1.38	0.017	2.44
3	0.223	1.28	0.072	2.98
4	0.483	0.77	0.288	1.01
5	0.752	0.59	0.021	2.67
6	0.864	0.79	0.053	1.69
7	0.882	0.46	0.402	0.88
8	0.929	0.40	0.014	2.87
9	0.949	0.53	0.156	1.93
10(s)	1.000	0.00	1.000	0.00
11	1.026	0.44	0.105	1.40
12	1.056	0.46	0.155	1.95
13	1.094	0.36	0.097	2.44
14	1.106	0.34	0.047	2.50
15	1.248	0.27	0.253	1.10
16	1.310	0.35	0.013	2.98
17	1.314	0.31	1.000	0.00
18	1.358	0.30	0.015	2.70
19	1.399	0.25	0.039	2.72
20	1.448	0.22	0.018	2.83
21	1.490	0.27	0.034	2.63
22	1.641	0.23	0.167	1.07
23	1.816	0.21	0.048	3.00
24	1.946	0.19	0.048	2.21
25	2.208	0.18	0.057	2.72

4. Conclusions

In this paper, the process of development, optimization and validation of fingerprint of *S. miltiorrhiza Bunge* using HPLC-CEAD was introduced in detail. Selecting a suitable chromatographic system, screening the optimal pH of mobile phase, working potentials and preparation method were studied in this paper. In addition, analyzing chromatograms and validating this method were also included in our research. Under optimized analysis conditions, 25, 24 and 23 common peaks of *S. miltiorrhiza Bunge* from Shandong, Hebei and Jiangsu province were obtained, respectively, which made the separation and detection quality of the fingerprint improved. The results of method validation, based on RSD of relative retention times and relative peak areas, were acceptable. Finally, an integrated and feasible new HPLC fingerprint approach was developed using HPLC-CEAD system and it can not only be utilized for *S. miltiorrhiza Bunge* but also for other herbal medicines to identify and assess their quality.

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