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Separation and determination of three water-soluble compounds in *Salvia miltiorrhiza Bunge* and two related traditional medicinal preparations by flow injection-capillary electrophoresis

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Abstract

A sensitive and reproducible method was developed for the separation and determination of three water-soluble components—protocatechuic aldehyde (PAH), β-(3,4-dihydroxyphenyl) lactic acid (DSS) and protocatechuic acid (PA) in medicine plant *Salvia miltiorrhiza Bunge* and two related traditional medicinal preparations using flow injection-capillary electrophoresis system. This analysis was carried out by using an unmodified fused-silica capillary (28.4 cm × 75 μm i.d. × 375 μm o.d., 25 cm effective separation length) and direct ultraviolet detection at 214 nm, 7.0 kV applied voltage. With boric acid (200 mM) adjusted to pH 7.8 as a background electrolyte. The separation was achieved in 9 min. The sample throughput rate could reach up to 15 h^{-1}. Calibration curves showed good linearity with correlation coefficients (r) more than 0.9986. The repeatability (defined as R.S.D.) were 0.20%, 0.46%, 0.47% with migration time evaluation and 0.62%, 3.66%, 1.50% with peak area evaluation for PAH, DSS and PA, respectively. The limits of detection (S/N = 3) were 0.36 μg/mL, 0.84 μg/mL, and 0.73 μg/mL for PAH, DSS, and PA, respectively. The mean recoveries of PAH, DSS and PA were 103.2%, 98.1% and 100.5%, respectively. This method has been applied successfully to monitor these three components in *Salvia miltiorrhiza Bunge* and its two traditional medicinal preparations.

Keywords: Protocatechuic aldehyde; β-(3,4-Dihydroxyphenyl) lactic acid; Protocatechuic acid; Nicotinic acid; *Salvia miltiorrhiza Bunge*; Traditional medicinal preparation; Flow injection-capillary electrophoresis

1. Introduction

The root and rhizome of *Salvia miltiorrhiza Bunge* (Danshen in Chinese), a traditional herbal drug, is used for treatment of coronary heart disease, cerebrovascular disease, bone loss, hepatitis, hepatocirrhosis and chronic renal failure [1–3]. According to the pharmacological investigations, the active constituents of Danshen are divided into two groups: phenolic acids which are water soluble and tanshinones which are lipophilic [4]. In recent years, the water-soluble components of Danshen have attracted increasing attention because of their effectiveness in improving the renal function of rats with adenine-induced renal failure, as an antioxidant for the removal of free radicals and their potential in treating Alzheimer's disease [5]. β-(3,4-Dihydroxyphenyl) lactic acid or danshensu (DSS), protocatechuic acid (PA), protocatechuic aldehyde (PAH) have been identified as the main constitutes in water-soluble compounds [6]. Pharmacological experiments have demonstrated that DSS can protect cardiac muscle from lacking blood and oxygen, dilate isolated coronary artery, decrease the biosynthesis of cholesterol in cells and inhibits lipoprotein oxidation [7,8]. PAH can increase the flux of coronary [7], and it also has such biological activities as antiatherosclerosis, anti-phlegmonosis, anti-oxidation and protecting myocardial damage [3,9]. PA is the most effective in treating anticardium colic, improving flow in coronary arteries and inhibiting the aggregation of platelets caused by adenosine diphosphate [10], it also has antibacterial, anti-inflammatory, anti-oxidant and styptic activities [11]. Therefore, it is of great

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practical significance to establish an analytical method for determining them for the quality control of *Salvia miltiorrhiza Bunge* and its related traditional medicinal preparations containing Danshen.

Up to now, quantitative determination of water-soluble constituents in Danshen has been only focused on one or two compounds, which could not reflect the overall quality of Danshen. Therefore, a simple and rapid method to determine these three components simultaneously is highly desired for controlling drugs quantity validly. So far, several methods such as colorimetric method [12], ultraviolet spectrophotometric method [13], thin layer chromatography (TLC) [14], high-performance liquid chromatography (HPLC) [15–17], RP–HPLC [18,19], and high-performance capillary electrophoresis (HPCE) [20–23], MEKC [24] have been reported for the determination of water-soluble components in *Salvia miltiorrhiza Bunge* or in pharmaceutical preparations. Nevertheless, the first two methods were usually interfered by other compounds, TLC lacked quantitative precision, while HPLC appeared to be efficient and time-consuming; most of the CE methods only focused on one or two compounds of water-soluble constituents in Danshen. Although the literature 23 determined simultaneously the DSS, PAH and PA, it only analyzed a real sample and the peak of DSS occurred forfication.

Although CE has the advantages of high-resolution capability, high peak efficiencies and small sample volume, the injection methods of sample introduction (hydrodynamic and electrokinetic sample introduction) are discontinuous in nature, and sample throughput and precision are limited. Recent developments on the coupling of flow injection (FI) to CE demonstrated the potentials of achieving efficient continuous sampling frequencies for CE [25–29], and improved reproducibility, as compared to conventional sample introduction.

This paper presented a sensitive and reproducible method for the simultaneous determination of compounds PAH, DSS, PA in *Salvia miltiorrhiza Bunge* and its related traditional medicinal preparations using FI–CE system. And compared the R.S.D. of migration time and peak area between without IS way (normal way) and internal standard (IS) method. In addition, the extraction solvents and extraction time have been investigated to find the simplest and most efficient sample preparation method for Danshen products.

2. Materials and methods

2.1. Chemicals

Standards of sodium danshensu, PA, PAH and nicotinic acid (IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their structures were shown in Fig. 1. The crude drug of *Salvia miltiorrhiza Bunge* and two commercial traditional medicinal preparations containing Danshen such as fufang danshen tablet (FDT), xiangdan injection (XDI), were purchased from local drug stores (Lanzhou, China). Boric acid and NaOH was supplied by Tianjin Chemical Graduate School (Tianjin, China). Methanol was supplied by Tianjin Secondary Chemical Factory (Tianjin, China). All the reagents used were of analytical grade and were used as received, and distilled water was used throughout the study.

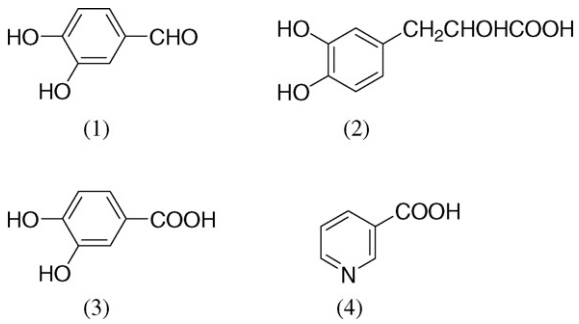

Fig. 1. Structures of (1) PAH, (2) DSS, (3) PA and (4) IS.

2.2. Apparatus

A model HPE-100 CE system with 12 kV maximum voltage (BioRad, Hercules, CA, USA) was used for the separations, which was connected to a 486 PC. Data collection was achieved by a Chroma chromatography collection system (BioRad). Uncoated fused-silica capillaries of 75 μm i.d., 375 μm o.d. and 28.4 cm length (25 cm effective length) were purchased from Yongnian Optical Fiber Factory (Baoding, China). UV detection was carried out at 214 nm.

A K-1000 Flow Injection Analyzer (Hitachi, Japan) was used throughout for transporting background electrolyte (BGE, buffer/carrier) and samples. It was composed of a double plunger pump, a 16-way auto-switching valve with a sample loop and two reagent loops and a peristaltic pump used for delivery of sample solution to sample loops. A 33 cm length polytetrafluoroethylene (PTFE) tubing (0.5 mm i.d.) from the valve to the split-flow interface was used as transport line. The time period for the injecting sample was defined by man/access mode. The manifold of FI–CE was shown in Fig. 2. The detailed description of the H-channel microchip has been given elsewhere [30].

The pH values were determined by a PHS-10A pH meter (Xiaoshan Instrumental Factory, Zhejiang, China).

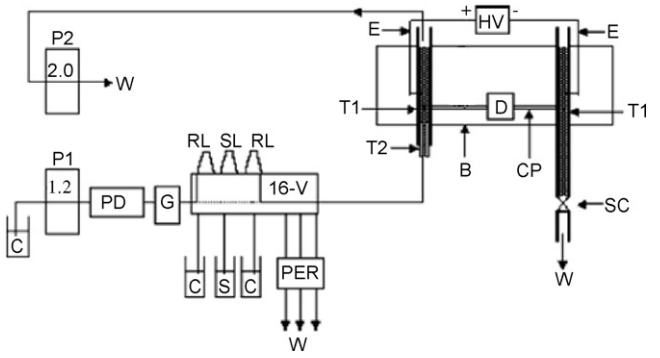

Fig. 2. Manifold for the FI–CE (not to scale). C, carrier solution; S, sample; P1 and P2, pumps; PD, pressure damper; G, pressure gauge; SL, sampling loop; RL, reagent loop; 16-V, 16-way valve; PER, peristaltic pump; B, planar plastic base; T1, Tygon tubing; T2, Tygon tubing (1.2 mL/min); CP, separation capillary column; E, platinum electrode; W, waste; C/S, carrier/sample; HV, high voltage; D, detector; SC, screw clamp.

2.3. Procedures

2.3.1. Reagents preparation

Stock standard solutions (1000 μg/mL) of sodium danshensu, PA, PAH and IS were prepared in 70% (v/v) aqueous methanol and stored in refrigerator at 4 °C, and diluted to required concentration with distilled water. Buffer solutions were prepared by diluting 0.5 M boric acid stock solutions with distilled water, and then adjusted with 2 M NaOH to the required pH. All solutions were filtered through a 0.45 μm syringe filter before use.

2.3.2. Sample preparation

The dried roots of *Salvia miltiorrhiza Bunge* from Gansu province (China) were crushed to powder, 5 g of the dried powder was accurately weighed and marinated in 25 mL 70% (v/v) aqueous methanol at room temperature for 24 h and then extracted in an ultrasonic bath.

The sugar coats of 60 tablets FDT were washed off with water and the tablets were dried followed by being grinded into fine powder. Then 3 g of the dried powder was accurately weighed and marinated in 25 mL 70% (v/v) aqueous methanol at room temperature for 24 h and then extracted in an ultrasonic bath. The supernatant was diluted with distilled water appropriately so that the final concentration was within the working range.

Without any pretreatment, 2 mL (a potion) XDI was diluted directly to 10 mL with distilled water and used for an analysis. All solutions were filtered through 0.45 μm syringe filters before use. The sample solutions were stored in the dark.

2.3.3. FI procedure

The manifold of the FI system was shown schematically in Fig. 3. During the sampling charging, with the valve in the "load" position (charge stage, Fig. 3A), sample solution was pumped by the peristaltic pump to fill the middle sample loop of the 16-way auto-switching valve, running buffer plug was pumped by the peristaltic pump to fill the first loop and the third loop of the 16-way auto-switching valve. Simultaneously, the carrier solution was pumped by the double plunger pump through the split-interface. When the charge stage was finished, peristaltic pump was stopped and the valve was switched automatically to the "inject" position. In the injection stage (Fig. 3B), the buffer solution flowed through the sample and reagent loops. The sample solution in the middle loop of the 16-way auto-switching valve was sandwiched by the buffer solution and transported through the connecting conduit into the split-flow interface and a fraction of the sample zone was introduced into the separation capillary by the electrokinetic injection. After the injection sequence, the valve returned to the load position, and the next cycle began. The charging time (CT) and injecting time (IT) were set by manual acting. A series of samples were injected continuously without interrupting the voltage (7.0 kV).

2.3.4. CE procedure

Prior to the first use, the new capillary was conditioned with distilled water for 10 min, 0.1 M NaOH for 10 min and distilled water for 10 min, followed by the running buffer for 10 min from the capillary outlet reservoir using a water-circulating vacuum pump. At the beginning of each working day, the capillary was flushed sequentially with distilled water for 5 min, 0.1 M NaOH for 5 min and distilled water for 5 min, followed by the running buffer for 5 min. Moreover, between runs, the capillary was washed with distilled water (2 min), 0.1 M NaOH (3 min), distilled water (2 min) and equilibrated with the fresh BGE (3 min) to ensure the good reproducibility.

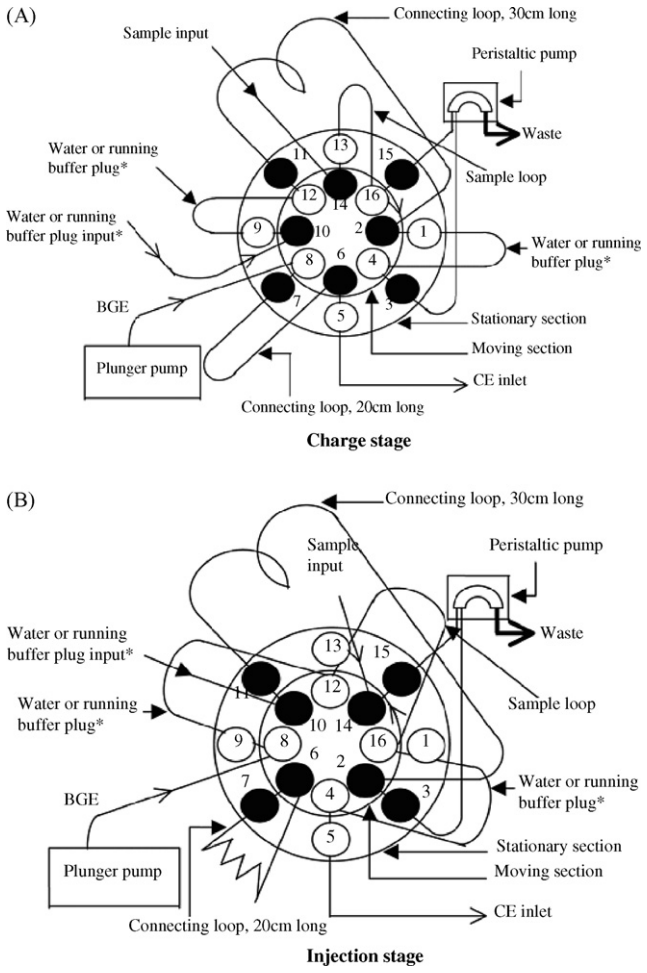

Fig. 3. Schematic diagram of two stages of an FI system with a 16-way switching valve. (A) Charge stage, (B) injection stage.

3. Results and discussion

3.1. Optimization of the separation conditions

To achieve a low limit of detection (LOD) and satisfactory separation, the optimization of separation conditions was of primary importance. In this work, the separation conditions were optimized by a univariate approach taking the peak areas, migration time and resolution as the principal figures of merit. To obtain the electropherograms with the good separation, buffer pH, the concentration of boric acid and methanol, applied voltage, flow rate, CT and IT were investigated, respectively. The concentration of PAH, DSS and PA in optimization studies were 100 μg/mL, 50 μg/mL and 100 μg/mL, respectively. The peak sequence of the three compounds was PAH, DSS and PA. The identity of the recorded peaks was confirmed by independent

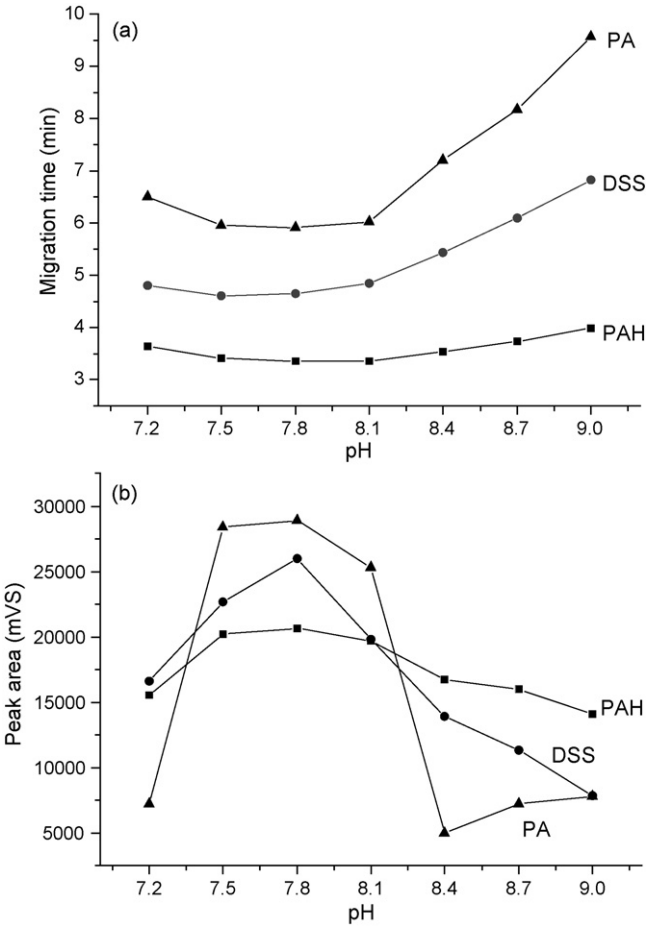

Fig. 4. Effect of pH on migration time and peak area. CE conditions: uncoated separation capillary, 75 μm i.d. × 375 μm o.d. × 28.4 cm length (25 cm effective length); voltage, 7.0 kV; detection wavelength, 214 nm. Conditions of K-1000 FIA: man/access mode; CT, 2 s; IT, 7 s; sample volume, 20 μL; carrier flow-rate, 1.2 mL/min frequency of injecting sample, 4 min; separation temperature, room temperature; buffer, 200 mM boric acid–2.5% (v/v) methanol; sample, 100 μg/mL PAH, 50 μg/mL DSS and 100 μg/mL PA.

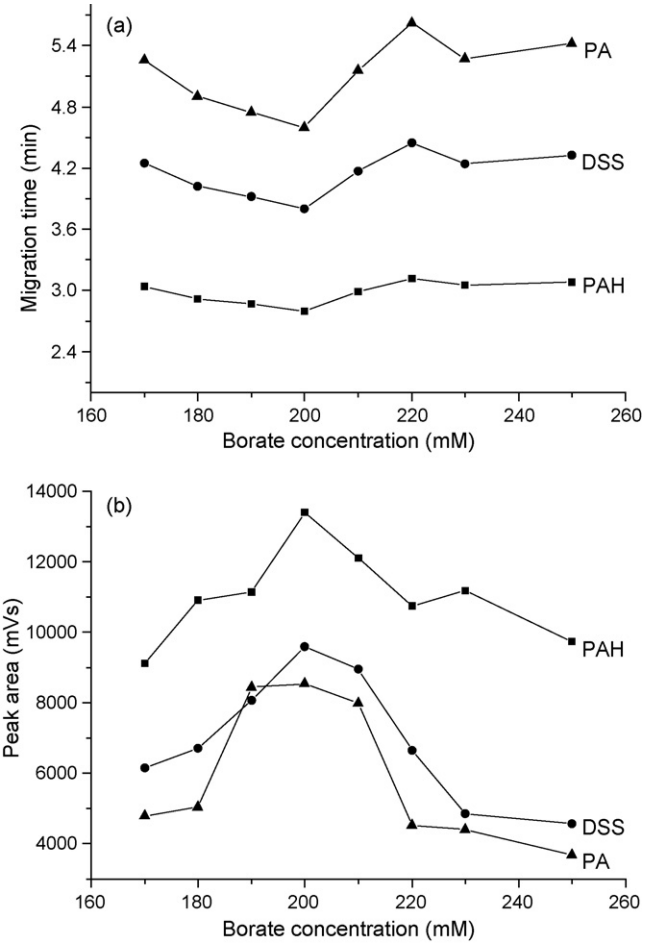

Fig. 5. The effect of boric acid concentration on the separation. Experimental conditions: pH 7.8; the other experimental conditions were as in Fig. 4.

injection of the pure compounds. The sequence of the peaks was invariable when the conditions were changed.

3.1.1. Optimization of the pH of BGE

In CZE, the pH value of the running buffer is a key strategy in optimizing the separation conditions. It influences electroosmotic flow, resolution and sensitivity. In this study, the effect of buffer pH on the peak area and migration times in the pH range of 7.2–9.0 with 200 mM boric acid solution–2.5% methanol, and 7.0 kV applied voltage was investigated (Fig. 4). Relationships between pH and retention time, pH and peak area were shown in Fig. 4a and b. From Fig. 4a, retention time of PAH, DSS, PA had a trend of decreasing and followed by increasing tardily. The three analytes can be separated in the pH range of 7.2–9.0. However, when pH is 7.2, 8.7 and 9.0, the peaks of DSS and PA were relatively wide. From Fig. 4, the peak area of PAH, DSS, PA had a trend of increasing and followed by decreasing with the changing pH. Simultaneously, with the pH increasing, considerable Joule heating from the larger current would be also produced. A good separation condition should satisfy the need that the sensitivity was higher within a short analysis time as far as possible and the peaks were symmetrical and synchronously avoiding the generation of excessive Joule heating. So pH 7.8 was chosen finally.

3.1.2. Effect of BGE concentration

Buffer concentration has obvious influence on the separation because it could influence the electroosmotic flow (EOF) and the viscosity of the electrolyte. In order to achieve the optimum resolution of the test mixtures, the effect of the boric acid concentration at pH 7.8 on these separations was studied in the range of 170–250 mM, 2.5% methanol, and 7.0 kV applied voltage (Fig. 5a and b). When boric acid concentration was lower than 190 mM, the figures of three analytes became broad, and when the concentration was higher than 200 mM, the migration times of the analytes were increased and peaks areas decreased. For the sake of better resolution and shorter analysis time, 200 mM boric acid concentration was adopted for the method.

3.1.3. Influence of methanol concentration in buffer on separation

Organic solvents in the buffer could improve selectivity, change electroosmotic velocity, and thus would expand the migration window [31]. The system without methanol as a

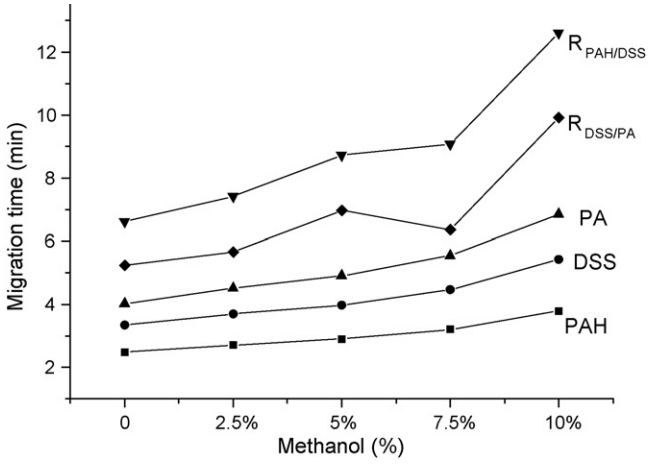

Fig. 6. Influence of methanol concentration in buffer on migration time. 200 mM boric acid (pH 7.8); other conditions and symbols were as in Fig. 4.

modifier, the analytes and impurities in real sample were not separated completely. When the methanol was added in the running buffer, there was an increase in the migration time due to the decrease in EOF, and the resolutions of the three analytes increased and the peaks area decreased. The effect of the addition of methanol to the buffer electrolyte on the separation of the analytes was studied in the range of 0–10% (v/v) (Fig. 6). Although further addition of methanol gave better resolution, peak figures were worse and sensitivity decreased. Therefore, 2.5% methanol was chosen for further studies.

3.1.4. Effect of BGE flow-rate

With fixed sample injection volumes, the carrier flow rate determined the length of the sample plug entering the capillary and therefore the time available for electrokinetic split-sampling into the capillary [32]. The effects of the BGE flow-rate of K-1000 FIA in the range 0.6–1.6 mL/min were studied (Fig. 7). A lower flow-rate ensured the better sensitivity and used fewer reagents, but deteriorated the resolution and theory plate number. Considering different factors, a flow-rate of 1.20 mL/min was selected as optimum.

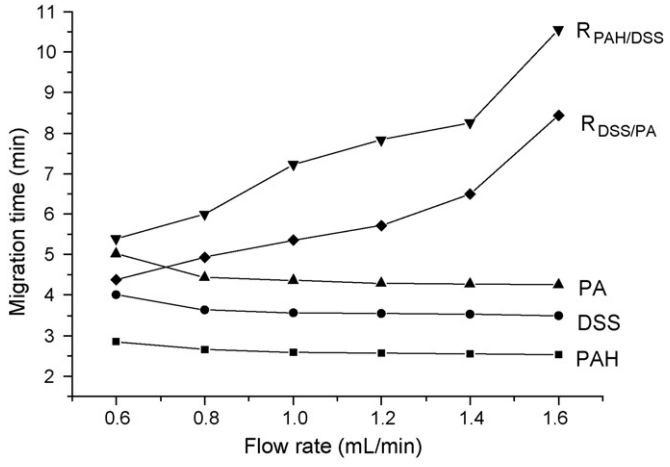

Fig. 7. Effect of flow rate on migration time. 200 mM boric acid–2.5% (v/v) methanol (pH 7.8); other conditions and symbols were as in Fig. 4.

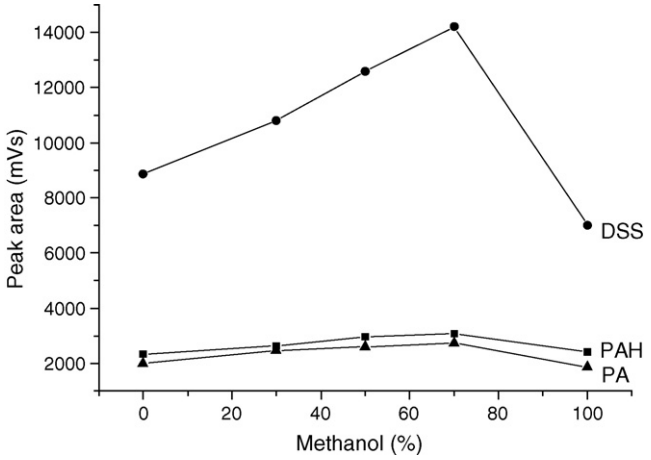

Fig. 8. Extraction efficiency of different concentrations of methanol.

In addition, we studied the effects of applied voltage in the range of 4.5–8.0 kV, CT in the range of 1–5 s and IT in the range of 1–9 s. The optimum separation condition was: 200 mM boric acid–2.5% (v/v) methanol buffer (pH 7.8), 7.0 kV voltage, 214 nm UV detection, 1.2 mL/min flow rate, 20 μL sample volume, CT 2 s and IT 7 s.

3.2. Optimization of sample extraction conditions

In order to compare the effect of extraction solvent on the product quantification so as to determine the most efficient extraction procedure applicable for detecting the above markers from Danshen products. Variables involved in the procedure such as solvent concentration and extraction time were optimized. Five grams of the dried powder of *Salvia miltiorrhiza Bunge* was accurately weighed and 25 mL methanol, 70% (v/v) methanol, 50% methanol, 30% methanol and water were employed as extraction solvents and were extracted respectively in an ultrasonic bath for1 h. Pure water could not extract the PA completely while DSS and PAH could not be efficiently extracted by pure methanol. From the extraction efficiency of the different solvents (see Fig. 8), it is clear that, when 70% methanol was employed, the peak areas of the three analytes reached the highest values. Therefore, 70% methanol was selected as the extraction solvent, which was consistent with the literature 3.

Then the optimal extraction time was investigated. Three grams of the dried powder of FDT was extracted with 25 ml 70% methanol for 20, 30, 40, 50 and 60 min, respectively. The peak areas of the marker constituents obtained by different extraction times were shown in Fig. 9. It could be seen from Fig. 9 that when the sample was extracted for 50 min, the peak areas of the constituents reached the highest values. So 50 min was selected as the optimal extraction time.

From above experiment, the most suitable extraction method was that the analytes were extracted by 25 mL 70% methanol for 50 min.

3.3. Performance of the combined FI–CE

Calibration graphs were obtained by injecting standard solutions at seven different concentrations. Each point on the

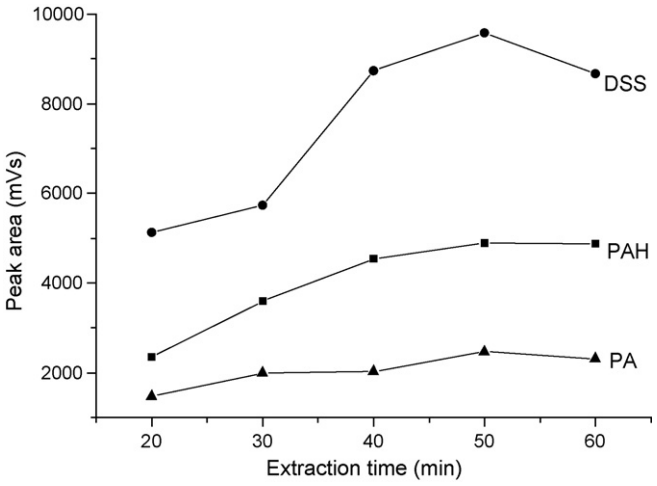

Fig. 9. Extraction efficiency of different extraction time.

calibration graph corresponded to the mean value obtained from three independent peak area measurements. The corresponding regression equations, as well as other characteristic parameters for the determination of PAH, DSS and PA are listed in Table 1. The LODs (S/N = 3) and LOQs (S/N = 10) were also given. The peak areas were employed for quantification. The reproducibility of FI–CE was evaluated under optimized conditions using a standard solution containing 100 μg/mL PAH, 100 μg/mL PA and 50 μg/mL DSS. The analytes were repeatedly injected into the CE system every 4 min. The electropherogram of three continuous sampling was shown in Fig. 10(a). The precision test was carried out by the intra-day and inter-day variability. In order to compare the repeatability between the normal way and IS methods, 100 μg/mL nicotinic acid as IS was spiked into the standard solutions. The repeatability was expressed as the R.S.D. values of the migration time and peak areas obtained from five injections (given in Table 2).

Table 1
Analytical performance of FI–CE in three components analysis ($n = 5$)

	PAH	DSS	PA
LOD (S/N = 3) (μg/mL)	0.36	0.84	0.73
LOQ (S/N = 10) (μg/mL)	1.21	2.81	2.45
Regression equation[a]			
a	31	320	344.5
b	125.59	67.79	72.38
Correlation coefficient	0.9999	0.9986	0.9989
Linear range(μg/mL)	6.2–400	12.5–800	12.5–800

[a] $y = a + bx$; y, peak area; x, standard concentration (μg/mL).

3.4. Application

The established method has been applied to the determination of the *Salvia miltiorrhiza Bunge*, FDT and XDI. Quantitative

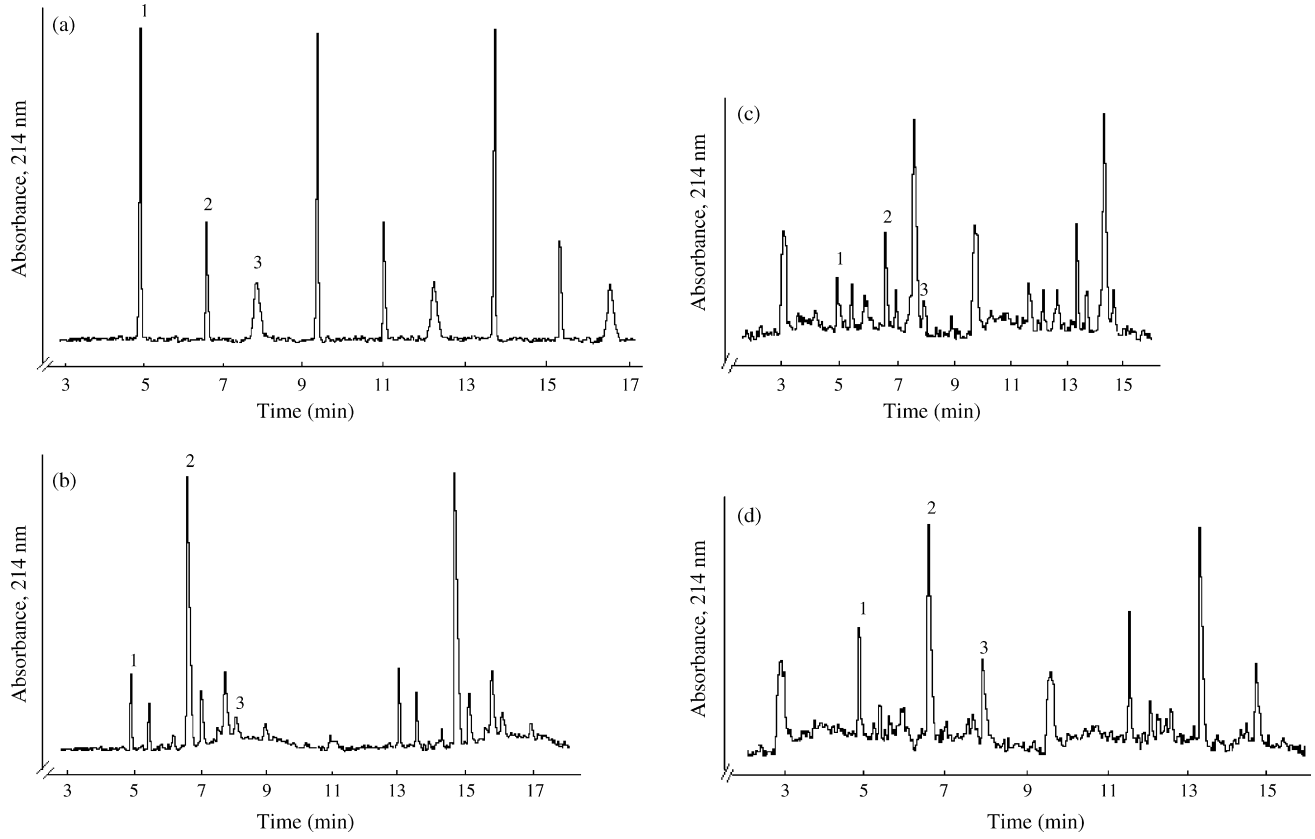

Fig. 10. Electrochromatograms of the standard mixture solution and the real samples: (a) standard mixture solution, concentration, 100 μg/mL PAH, 50 μg/mL DSS and 100 μg/mL PA (b) XDI (c) FDT (d) *Salvia miltiorrhiza Bunge* Peaks: 1 = PAH, 2 = DSS, 3 = PA. Separation condition: buffer, 200 mM boric acid–2.5% (v/v) methanol (pH 7.8); capillary, 75 μm i.d. × 375 μm o.d. × 28.4 cm length (25 cm effective length); applied voltage, 7.0 kV; sample volume, 20 μL; UV detection, 214 nm; flow rate, 1.2 mL/min.

Table 2
Compare of the sensitivity between current and previous studies (μg/mL)

Component	Current method (FI-CZE)	HPLC-DAD[a] [15]	CZE-AD[b] [20]	MEKC-UV [24]
PAH	0.36	0.03	0.10	1.11
PA	0.73	0.04	0.25	–[c]
DSS	0.84	0.16	–	0.236

[a] Diode-array detector.
[b] Amperometric detection.
[c] Not reported.

Table 3
Compare of the R.S.D. (%) between normal way and IS method ($n = 5$)

		PAH		DSS		PA	
		Normal way	IS method	Normal way	IS method	Normal way	IS method
Peak area	Intra-day	0.62	0.19	3.66	1.84	1.50	1.19
	Inter-day	5.53	0.67	11.62	5.81	1.65	1.48
Migration time	Intra-day	0.20	0.22	0.46	0.21	0.47	0.29
	Inter-day	0.57	0.46	0.87	0.58	1.56	0.036

analysis was performed under the optimum conditions obtained from the experiments described above. The method was applied to the analysis of the commercial medicines containing these analytes. The contents of the analytes found in different kinds of medicines are given in Table 3. The typical electropherograms of *Salvia miltiorrhiza Bunge*, FDT and XDI which were obtained by two continuous sampling were shown in Fig. 10(b–d). The peaks were identified by the standard addition methods. Accuracy of the methods and the potential matrix effects were established by analyzing samples. The recovery test was carried as follows: three different quantities (low, medium and high) of standard solutions were added into sample solutions. The

Table 4
Results for the determination of the three components in sample extracts ($n = 4$)

Sample	Ingredient	Content	Concentration spiked (μg/mL)	Concentration found (μg/mL)	Recovery (%)	Average (%)	R.S.D. (%)
S.[a]	PAH	0.29 mg/g	40	41.9	104.8	103.0	4.81
			80	77.9	97.4		
			120	128.2	106.8		
	DSS	4.81 mg/g	50	49.1	98.2	97.0	2.92
			100	93.8	93.8		
			150	148.6	99.1		
	PA	0.52 mg/g	40	39.8	99.5	99.4	3.82
			80	76.5	95.6		
			120	123.8	103.2		
FDT	PAH	0.87 mg/g	50	52.4	104.8	101.7	3.0
			100	101.5	101.5		
			150	148	98.7		
	DSS	4.13 mg/g	25	25.7	102.8	99.2	3.34
			50	49.2	98.4		
			75	72.2	96.3		
	PA	0.36 mg/g	50	49.0	98.0	102.5	5.73
			100	100.3	100.3		
			150	163.7	109.1		
XDI	PAH	0.54 mg/dose	33.2	35.4	106.6	104.8	6.42
			66.4	73.4	110.5		
			99.6	97	97.4		
	DSS	7.35 mg/dose	31.7	33.6	106	98.1	7.31
			63.4	59.4	93.7		
			95.1	90.0	94.6		
	PA	0.44 mg/dose	33.2	33.9	102.1	99.7	2.11
			66.4	65.6	98.8		
			99.6	97.8	98.2		

[a] S., *Salvia miltiorrhiza Bunge*.

quantity of each analyte was subsequently obtained from the corresponding calibration curve. The recovery of the three analytes ranged from 97.0 to 104.8% (see Table 3). From the results of precision test and recovery test, it was known that the method manifested good precision and accuracy (Table 4).

4. Concluding remarks

The coupling of an FI system with CE equipment has been successfully used to analyze the PAH, DSS and PA in the samples of roots of *Salvia miltiorrhiza Bunge* and two traditional medicinal preparations for the first time. Excellent separation efficiency, high sampling frequencies and good repeatability were achieved, using boric acid electrophoretic buffer modified with methanol. The disadvantage of this technique was the big waste of buffer solutions because of the using of FI. The result indicated that the proposed FI–CE system was suitable for the determination of *Salvia miltiorrhiza Bunge* and its related traditional medicinal preparations. In the paper, we compared the R.S.D. of migration time and peak area between normal way and IS method, the result indicated that the IS method can obviously improve the reproducibility.

Acknowledgement

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