

Original Research Article

Development and Validation of Improved Method for Fingerprint Analysis of *Rhizoma Chuanxiong* by Capillary Zone Electrophoresis with Ultraviolet-Diode Array Detection

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Abstract

Purpose: To develop and validate an improved method by capillary zone electrophoresis with photodiode array detection for the fingerprint analysis of *Ligusticum chuanxiong* Hort. (*Rhizoma Chuanxiong*).

Methods: The optimum high performance capillary electrophoresis (HPCE) conditions were 30 mM borax containing 5 %v/v acetonitrile at pH 9.6, direct injection of water extract at 0.5 psi for 5s, 25kV running voltage at positive power, 20 °C capillary temperature and 295 nm detection. Between injections, capillary was rinsed using 0.1M sodium hydroxide solution for 4 min, with HPCE-grade water for 4 min and re-equilibrated with running buffer for 2 min at 20 psi, moreover, the running buffer was replaced after three consecutive injections.

Results: Baseline separation of most of the 17 detectable peaks in the water extract could be completed within 21 min. The relative standard deviations (RSD) of precision, reproducibility, and stability were < 2.79 % for retention time and 8.68 % for peak area of the main peaks.

Conclusion: The developed method is simple, fast, reliable and environment-friendly for the fingerprint analysis of *Rhizoma Chuanxiong* water extract.

Keywords: Capillary electrophoresis, *Rhizoma chuanxiong*, Water extract, Fingerprint analysis, Diode array

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INTRODUCTION

In Traditional Chinese Medicine practice, herbal medicines have usually been decocted in water for clinical application since thousands of years, more than 90% of Chinese herbal medicines contained in Chinese Pharmacopoeia (Volume I of 2010 edition) are taken orally after being decocted with water [1]. This indicates that direct analysis of water extracts of Chinese herbal medicines is a quality control method in accordance with their biological effects. High

performance capillary electrophoresis (HPCE) is well-known for analyzing directly water extracts, because the contaminated inner capillary wall can be easily regenerated by rinsing with acid or alkaline solution. The major drawback of HPCE in fingerprint analysis for Chinese herbal medicines is relatively low run-to-run reproducibility of separations caused by the change in the electroosmotic flow (EOF) due to a nonreproducible inner capillary wall [2]. Attempt to improve EOF reproducibility or overcome EOF's effects has been reported, including

adding markers to sample as aids, different standardization methods and capillary rinsing protocol [3-6].

Rhizoma Chuanxiong (*Ligusticum chuanxiong* Hort., RC) is a well-known Chinese herbal medicine for treatment of angina, cardiac arrhythmias by improving blood circulation and dispersing blood stasis [7]. The best-known habitat of RC is been acknowledged as Sichuan Province. More than 90% of RC materials used in clinical are from Sichuan province. In the reported papers about fingerprint analysis of RC, the analyzed samples were mostly prepared with organic solvent, and were analyzed using HPLC with organic reagent as mobile phases, these methods were also time-consuming, usually 40–120 minutes for an analysis period [8-10]. The aim of this work is to develop an improved fingerprint analysis method for *Rhizoma Chuanxiong* and to make a better quality control for it.

EXPERIMENTAL

Instruments

The capillary electrophoresis system with photodiode array detector (Beckman P/ACETM MDQ, Beckman Coulter, Inc. CA, USA) was used. Separations were performed in a 75 µm inner diameter (ID) fused-silica uncoated capillary with an effective length of 50 cm and a total length of 60 cm (Beckman Coulter, Inc. CA, USA). The Data were collected and processed using a 32 Karat™ operating system.

Materials and chemicals

Ten batches of *Rhizoma Chuanxiong* samples were collected from different towns in Sichuan province, China (Table 1). All samples were identified by associate professor Zhang Hongwei (Department of Medicinal Plants & Pharmacognosy, Southern Medical University,

Guangzhou, China) according to pharmacognostic standard documented in I Volume of 2010 Edition of China Pharmacopoeia. All samples were kept in a desiccator.

Tetramethylpyrazine hydrochloride (TMPH) and ferulaic acid (FA) were purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China (batch numbers were 0817-9803 and 0773-9910, respectively). Ultrapure water prepared with the Millipore Milli-Q SP water purification system (18.2MΩMillipore, Bedford, MA, USA) was used for all the buffer solutions and dilution process. All of the reagents, acetonitrile, methanol, ethanol, sodium tetraborate decahydrate (borax, Na₂B₄O₇·10H₂O), NaOH and HCl were all analytical-reagent grade and purchased from reagent company (Guangzhou, China).

Sample preparation

The samples were dried in sunshine, weighed (Table 1) and ground into powder. The powder of RC was exactly weighed (10.0 g) and put into a 250.0 ml of round-bottom flask, 100 ml water was added next. The mixture was shaken and then was extracted under reflux conditions for 60 min. The entire extraction procedure was repeated twice. The extracted solutions were combined, quantified to 200 ml with water and centrifuged at 2500 rpm at 4°C for 15 min, finally the supernatant as sample solutions was transferred into a new tube and stored for analysis at 4°C. Sample solutions were filtered with a 0.22 µm-membrane filter (Supelco Inc., Bellefonte, PA, USA) before injection.

Preparation of reference standard solutions

Tetramethylpyrazine hydrochloride solution (25 µg/ml) and 10µg/ml of ferulaic acid solution were prepared with 0.1M hydrochloric acid aqueous solution and methanol, respectively.

Table 1: The ten batches of *Rhizoma Chuanxiong* collected from different towns of Sichuan Province

Sample no.	Origin	Weight (g, approx.)
01	Mengyang town, Peng region	41
02	Aoping town, Peng region	35
03	Aoping town, Peng region	40
04	Xindu town, Xindu region	33
05	Xindu town, Xindu region	48
06	Shiyang town, Dujiangyan	50
07	Shiyang town, Dujiangyan	37
08	Zhuwa town, Dujiangyan	45
09	Daguan town, Dujiangyan	49
10	Daguan town, Dujiangyan	39

Optimization of CZE conditions

Effect of sodium tetraborate solution

The electro-osmotic flow can be modified by adjusting the concentration and ionic strength of buffer solution. The running voltage and capillary temperature were set at 25 kV and 20 °C, respectively. 20, 30, 40, 50 mM of sodium tetraborate solution were studied by the separation and appropriate migration time of composition peaks.

Effect of organic solvent modifier

Organic solvent can improve the separation performances and the selectivity of CZE by influencing the effective mobility of the analytes and the mobility of EOF [11]. Acetonitrile (10 %v/v) and methanol (10 % v/v) were added to 30 mM sodium tetraborate solution as additive, respectively. One of the organic solvents was chosen as a modifier.

Effect of buffer pH value

Buffer pH can affect the EOF rate and the degree of ionization of analytes. To obtain the baseline resolution of the complex ingredients in *Rhizoma Chuanxiong* (RC) water extract, its pH at 9.0, 9.3 and 9.6 in 30 mM sodium tetraborate solution containing 5 %v/v acetonitrile was evaluated. The optimum pH value was obtained by trickling into an appropriate amount of 0.1M NaOH solution.

Effect of capillary rinse procedures

The reproducibility of migration times and peak areas strongly depends on the capillary rinse procedures between each run in CZE system [12]. Capillary rinsing can make the system return to the initial running conditions, so the influence of the EOF variation is reduced [13-14]. The total rinsing time was set to 10.0 min in order to compare efficiency of rinsing methods under the same time consumption. To achieve maximal reproducibility of results, the buffer was renewed from the inlet and outlet reservoirs after three consecutive injections.

Validation of method

Precision and reproducibility were investigated by five successive analysis of the same No. 2 sample and five replicates of the No. 2 sample, respectively. The same solution of No.2 sample was analyzed at different time points (0, 4, 8, 16, 24 and 48h, between the samplings, the sample

was kept at 4°C) and evaluated for the stability test.

Fingerprint analysis

Data analysis was carried out by a professional software named Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004 A), recommended by the Chinese Pharmacopoeia Committee for evaluating similarities of different electropherograms by computing the correlation coefficient and/or cosine value of vectorial angel. In this study, all the results were treated by correlation coefficient [15]. The reference electropherogram of *Rhizoma Chuanxiong* was established by processing electropherograms of 10 batches of *Rhizoma Chuanxiong* from different regions in Sichuan Province.

RESULTS

Optimization of HPCE conditions

Concentration increase of borax solution improved the separation of phenolic acid ingredients, simultaneously remarkably increased the migration time of ferulic acid. A better separation and appropriate migration time was achieved at 30 mM (Fig 1). Thus, 30 mM sodium tetraborate buffer was chosen as background electrolyte for subsequent experiments.

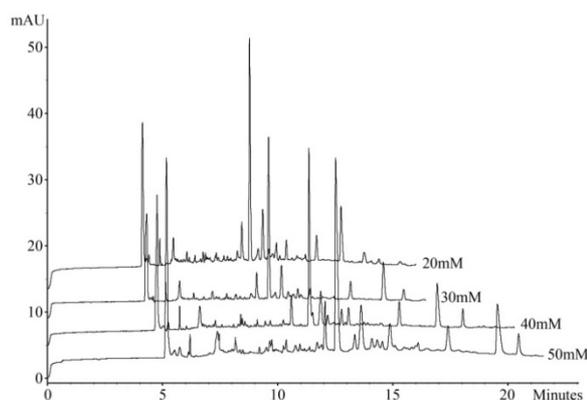


Figure 1: Effect of sodium tetraborate solution concentration on the separation of *Rhizoma Chuanxiong* water extract. **Conditions:** Running voltage, 25kV; capillary temperature, 20 °C; wavelength, 295nm; injection, 0.5psi for 5s

The separation performance and peak numbers with 10 % acetonitrile as modifier were better than tetraborate solution alone and than 10% methanol as modifier. Increasing the concentration of acetonitrile in buffer obviously improved the separation of FA with its neighbouring peaks (Fig 2). However, the

migration time of FA and its contiguous peaks became long notably. Based on the comprehensive analysis of separation performance, 5% acetonitrile was chosen for subsequent experiments.

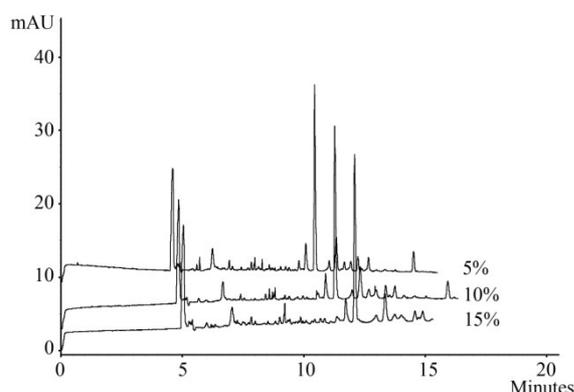


Figure 2: Effect of acetonitrile concentration in buffer on the separation of *Rhizoma Chuanxiong* water extract. **Conditions:** sodium tetraborate buffer, 30mM; running voltage, 25kV; capillary temperature 20 °C; wavelength, 295nm; injection, 0.5psi for 5s.

The buffer pH value had little influence on the migration time of TMPH, however increased clearly the migration time and improved the separation of FA and its neighbouring peaks when the pH was increased from 9.3 to 9.6, pH less than 9.3 the resolution of FA and its neighbouring peaks was decreased (Fig.3). Considering the higher pH increasing migration times, the best pH value of running buffer was 9.6.

Obviously, running voltage, capillary temperature and detection wavelength also have effect on migration time, separation and peak numbers of samples. A satisfactory fingerprint of CR water extract has been developed based on 25 kV running voltage, 20 °C capillary temperature and 295 nm detection wavelength. Therefore, further

optimization under these conditions was not continued.

The rinsing procedures and results are shown in Table 2. The RSD values in migration times and in peak areas were less than 3.17% and 6.25%, respectively, by rinsing with 0.1M NaOH for 4.0 min followed by rinsing with water for 4.0 min and lastly with BGE buffer for 2.0 min.

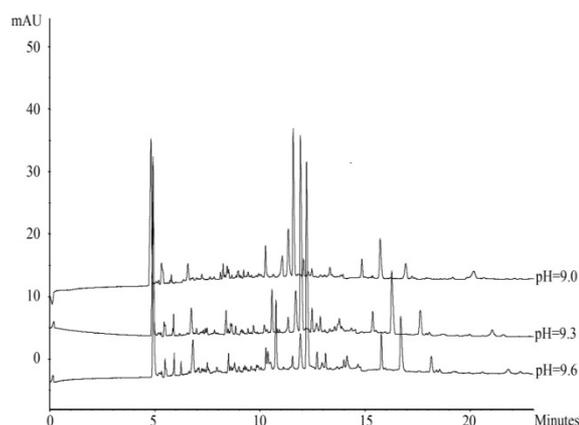


Figure 3: Effect of pH on the separation of *Rhizoma Chuanxiong* water extract. **Conditions:** sodium tetraborate buffer, 30mM containing 5 % acetonitrile; running voltage, 25 kV; capillary temperature, 20 °C; wavelength, 295nm; injection, 0.5psi for 5s.

This improved method for fingerprint analysis of *Rhizoma Chuanxiong* was as follows: 30 mM borax containing 5 %v/v acetonitrile at pH 9.6 as buffer solution, samples were injected at the anodic end at 0.5 psi for 5s, 25 kV at positive power as running voltage, capillary temperature was 20°C and 295 nm as detection wavelength. In addition, between each injection, rinsing the capillary with 0.1M sodium hydroxide solution for 4 min, with HPCE-grade water for 4 min and re-equilibrated with running buffer for 2 min at 20 psi, moreover, the running buffer was replaced

Table 2: Relative standard deviation of migration times and peak areas of characteristic ingredients in *Rhizoma Chuanxiong* water extracts after different capillary rinsing procedures

Procedure of preconditioning		Relative standard deviation (% , n = 5)						
		Peak 1	Peak 4	Peak10	Peak 11	Peak 12	Peak15	Peak16
Rinsing with distilled water (8 min), followed by BGE (2 min) between each run	Migration	1.09	1.47	4.59	4.78	5.06	6.39	8.25
	Peak area	3.76	4.60	6.72	6.38	7.33	9.67	12.21
Rinsing with 0.1M HCl (4 min), distilled water (4 min), followed by BGE (2 min) between each run	Migration	1.13	1.39	4.48	4.69	5.13	8.23	10.98
	Peak area	4.22	3.52	3.29	7.02	10.36	13.04	10.69
Rinsing with 0.1M NaOH (4 min), distilled water (4 min), followed by BGE (2 min) between each run	Migration	0.89	1.08	1.73	1.97	2.08	2.11	2.49
	Peak area	2.41	3.06	2.94	4.03	3.04	3.01	3.25

after three consecutive injections. Based on their UV spectra, migration time and standard addition, peak-1 and peak-11 were identified as TMP and FA (two main compounds in RC), respectively. The representative electropherogram of 2-sample was showed in Figure 4.

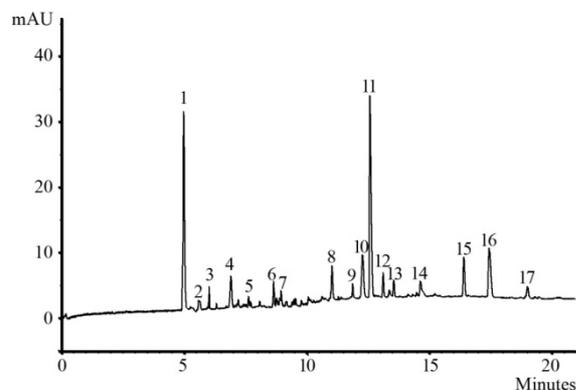


Figure 4: Representative electropherogram of the water extract of *Rhizoma Chuanxiong* (2-sample). Peak-1 = tetramethylpyrazine; Peak-11 = ferulic acid.

Validation results

As shown in Table 3, the RSD of migration times and peak areas of characteristic peaks in the precision test, the reproducibility test and the stability test were < 2.79 and < 8.68 %, respectively. The results indicate that the conditions of fingerprint analysis were satisfactory.

Fingerprint analysis

The electropherograms of 10 batches of RC samples from different regions in Sichun province had good similarity and a reference standard electropherogram was constructed (Fig.5), It indicated there is no obvious difference in the quality, the similarity values are listed in Table 4. These results are identical to the reports about *Rhizoma Chuanxiong* [8-9].

Table 3: Results of precision, reproducibility and stability with relative standard deviation

Peak no.	RSD of migration time (%)			RSD of peak area (%)		
	Precision	Reproducibility	Stability	Precision	Reproducibility	Stability
1	0.51	0.43	1.23	2.29	2.49	3.10
4	0.76	0.82	1.37	2.64	3.14	3.53
10	1.09	2.10	2.13	3.12	4.52	3.69
11	1.22	2.14	2.28	4.17	5.68	6.22
12	1.28	2.42	2.36	3.36	6.24	5.75
15	1.79	2.51	2.69	3.26	8.11	7.02
16	2.11	2.77	2.79	4.01	7.47	8.68

Table 4: The similarity values of 10 samples

Correlation coefficient	Sample no.									
	1	2	3	4	5	6	7	8	9	10
Median	0.997	1.000	0.996	0.998	0.995	0.998	0.997	0.999	0.994	0.991
Average	0.998	1.000	0.997	0.999	0.996	0.998	0.998	0.999	0.996	0.995

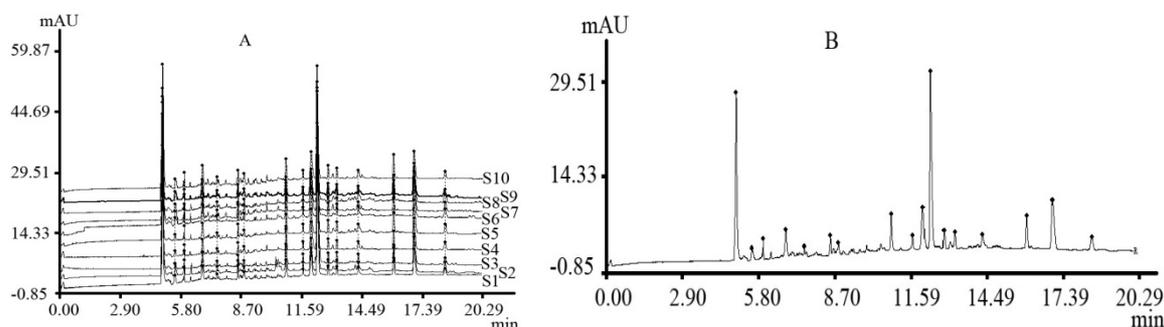


Figure 5: A = the overlay HPCE fingerprints of 10 *Rhizoma Chuanxiong* water extract by SES software; B = the reference standard fingerprint of *Rhizoma Chuanxiong* water extract by SES software. **Key:** S1 = Mengyang town, Peng region; S2 = Aoping town, Peng region; S3 = Aoping town, Peng region; S4 = Xindu town, Xindu region; S5 = Xindu town, Xindu region; S6 = Shiyang town, Dujiangyan; S7 = Shiyang town, Dujiangyan; S8 = Zhuwa town, Dujiangyan; S9 = Dagan town, Dujiangyan; S10 = Dagan town, Dujiangyan

DISCUSSION

Against HPLC, CZE presents four outstanding advantages: (a) the CZE separation is faster, (b) it has low requirement for sample pretreatment, (c) reagents consumption is low, and (d) capillaries are less expensive than HPLC columns. In this paper, the baseline separation of most of seventeen detectable peaks in *Rhizoma Chuanxiong* water extract was completed within 21 min, and 5 % acetonitrile alone was used as organic solvent modifier in the buffer.

The mean cost of a 75 cm capillary made in China was less than US\$15.00, which is much cheaper than HPLC column (usually more than \$330). Also, since HPCE capillary can tolerate high pressure rinsing procedures, CE capillary can be easily regenerated. Electroosmosis not only affects efficiency but also resolution, migration time and detector performance in HPCE analysis. The relative standard deviation for retention time and peak area of the main peaks demonstrate that a relative stable electroosmosis was obtained by optimizing the rinsing procedure.

CONCLUSION

Appropriate capillary rinsing is a simple method that is helpful for developing fingerprints of multi-component samples without adding markers/internal standards. This method is simple, fast, reliable and environment-friendly using capillary zone electrophoresis with photodiode array detection for the fingerprint analysis of *Rhizoma Chuanxiong* water extract.

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