Comparison of Micellar Electrokinetic Capillary Chromatography and High Performance Liquid Chromatography on Fingerprint of *Cnidium monnieri*

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In our studies, micellar electrokinetic capillary chromatography (MEKC) was employed in fingerprint analysis of *Cnidium monnieri* for the first time. Average chromatography of 10 batches *Cnidium monnieri* from Jiangsu province, China, which have long been considered as the original and genuine herbal medicine, was first established as the characteristic fingerprint. Within 25 min the major effective components were separated by 18 mM borate, 12 mM phosphate and 50 mM SDS (pH 9.2) containing 20% methanol. The relative standard deviations of migration times and peak areas were less than 5%. As a new approach of fingerprint, MKCE was compared to the conventional approach—HPLC in our experiments. The fingerprint developed by HPLC comprised 8 peaks that were collected within 40 min. Relative standard deviation (RSD) values of retention times of corresponding peaks in HPLC analysis were very small (maximum 3% and average 0.9%). In conclusion, each two methods had its advantages and disadvantages. Furthermore, besides HPLC, MEKC as a feasible method, could be used in the development of fingerprint of *Cnidium monnieri*.

Key words micellar electrokinetic capillary chromatography; *Cnidium monnieri*; fingerprint; high performance liquid chromatography; capillary electrophoresis

Traditional Chinese Medicines (TCM) has been used to treat human disease in China for centuries. Because the content of each component varies significantly due to differences in geographic origin, climate condition, environment and other factors. It is therefore necessary to build up fingerprint chromatography on identifying and assessing the stability of the plants. Fingerprint is a kind of method to show chemical information of TCM with chromatograms, spectrograms and other graphs by chemical analytical techniques.[1—3] Fingerprint analysis has been introduced and accepted by WHO as a strategy for the assessment of herbal medicines.[4] And it is also required by the Drug Administration Bureau of China to standardize injections made from TCM and their raw materials.[5]

Cnidium monnieri (Chinese name Shechuangzi), the fruit of *Cnidium monnieri* (L.) CUSS., is a kind of herbal medicine commonly used in China for the treatment of kidney deficient and relieving rheumatism, chills and pains.[6] It also could delay aging, build up strength, enhance immune function and adjust sex hormone level.[7] The constituents in *C. monnieri* include osthole, umtatin, imperatorin, bergapten, isopimpinellin and xanthotoxin *et al.*[8] The quality control of TCM using fingerprint will play an important role in the efficacy, safety and therapeutic reproducibility of *C. monnieri*.

To check and prove the quality of TCM adequate analytical methods have to be applied to the quality control and quantitative analysis of the constituents.[9—12] HPLC, because of its advantages and popularization, has been regarded as the conventional way on fingerprint analysis of *C. monnieri* in our laboratory.[13] Recently, capillary electrophoresis (CE), as a more meaningful analysis technique for controlling the quality of herbal samples, has been attracting more and more people's attention, because it is suitable for a wider range of complex analytical problems.[14] High performance capillary electrophoresis (HPCE) is an automated analytical technique that separates species by applying voltage across buffer filled capillaries. It is generally used for the separation of charged compounds, which move at different speed depending on their size and charge. CE has the advantages of short analysis time, high separation efficiency and minimal consumption of the samples and solvents. In our study, we choose micellar electrokinetic capillary chromatography (MEKC) as the separation mode due to absence of charge of coumarins.[15—17] MEKC, an important mode of CE, was capable of separating charged or neutral compounds based on their relative affinities for the lipophilic interior and ionic exterior of a micellar pseudo-stationary phase. In our study, we introduce the MEKC in fingerprint analysis of *C. monnieri* for the first time. Throughout the study, we focused on the feasibility of this method by using HPCE in developing fingerprinting of *C. monnieri* and compared it with HPLC as a means of validating the CE assay.

Experimental

Materials and Reagents The *C. monieris* were collected from Jiangsu Province, China and identified by Professor Song Xuehua of China Pharmaceutical University. Isopimpinellin and bergapten were exacted by us and identified by UV, MS and ^{1}H-NMR. The purities of them are all-higher than 98.0%. Xanthotoxin came from Sigma-Aldrich (Steinhein, Germany). Imperatorin and osthole were provided by the National Institute for the control of Pharmaceutical and Biological Products (Beijing, China).

Borate Phosphate and sodium hydrate were purchased from Nanjing Chemical Reagent Factory (Nanjing, China). Sodium dodecyl sulfate (SDS) was obtained from Merck (Darmstadt, Germany). All of them were of analytical reagent grade. Solutions were prepared in deionised water. Methanol and acetonitrile, which were chromatographic grade, are from Merck (Darmstadt, Germany).

Capillary Electrophoresis All the experiments were carried out on a Hewlett Packard G1600A (Hewlett-Packard, U.S.A.) capillary electrophoresis instrument equipped with diode array detector, temperature-controlled equipment, a HP chemical station and an automatic injector. The applied voltage was held constant at 30 kV. Separation capillary was an untreated fused silica capillary with a total length of 50 cm and an effective length of 41.5 cm (50 μm, i.d.). The UV detector was operated at 245 nm. The temperature of the capillary cartridge was maintained at 20 °C. Before use, the buffer solutions were filtered through 0.22 μm films and degassed. Daily

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condition of the capillary was done by washing for 5 min with 1 N NaOH, 5 min with water and 5 min with buffer. Between the runs, the capillary was purged for 3 min with 1 N NaOH, 5 min with water and 3 min with buffer. After every 5 analyses the buffer was replaced. Samples were introduced from its anodic end by gas pressure for 5 s.

Sample Preparation for CE The fruits of *C. monnieri* were ground into fine powder. Then 3 g of the powder was weighted accurately and immersed with 20 ml methanol for half an hour in a conical flask. After ultrasonication for half an hour, it was cooled to room temperature and then filtered though filter paper. This extraction was repeated three times. The extracted solutions were combined and concentrated at 40 °C to 10 ml. The concentrated solution was diluted to 50 ml with aqueous phase of buffer (18 mM borate+12 mM phosphate+50 mM SDS, pH=9.2), filtered with 0.22 μm films as sample solution for future CE analysis.

HPLC Analysis The fruits of *C. monnieri* were ground into fine powder. Then 0.1 g of the powder was weighted accurately and immersed with 15 ml 70% ethanol for half an hour in a conical flask. After ultrasonication for 1 h and cool it to room temperature, 2 ml of the sample was diluted to 10 ml with 70% ethanol, filtered with 0.45 μm film as stock solution. The 20 μl of the sample solution was used for HPLC analysis.

The column used was an Alltech ODS column (4.6×250 mm, 5 μm). The mobile phase was solvent A (CH_3CN) and solvent B (0.1% acetic acid–water). The gradient mode was as follows: 0—15 min, 77% B; 15—30 min, 55% B; 30—40 min, 0% B. The flow-rate was 1.0 ml/min. The wavelengths are also in the gradient mode as follows: 0—26 min, 245 nm; 26—30 min, 325 nm; 30—40 min, 245 nm.

Results and Discussion

Fingerprint of *C. monnieri* by CE Due to absence of charge of the coumarins, in our study, we chose MEKC as the separation mode. In order to propose a specific and accurate way to analysis *C. monnieri* in CE analysis, it is crucial to find the best experimental conditions in which the components can be separated from each other. In our study, three factors were optimized: concentrations of borate, phosphate, pH values and concentration of SDS.

In the separation, the buffer concentration and the pH of the electrophoretic medium are two vital factors. In the separation of the *C. monnieri*, we applied five pH values, 8.0, 8.5, 9.0, 9.5, and 10.0. When the pH values were 8.0 and 8.5, the five common peaks were not eluted separately. When the pH were higher than 9.5, the migration times of last two coumarins were above 60 min. So we chose the pH values ranging from 9.0 to 9.5 for further study. And at last, it was found that pH 9.2 was the optimum.

Eight SDS concentrations 20, 25, 30, 35, 40, 45, 50 and 55, which had evident effect on resolution of peak fractions, were applied to separate crude samples. The last two analytes were overlapped and the resolution between peak 2 and peak 3 was lower than 1.0 with 20 mmol/l SDS. Although the separation of the second and the third analytes was highly improved with increasing the SDS concentration, the last two analytes were not eluted separately. Similar resolution, the concentration of 50 mmol/l was chosen for further optimization.

The optimized CE separation electrolyte was 18 mM borate, 12 mM phosphate and 50 mM SDS (pH 9.2). We also added 20% methanol as the organic modifier to induce the migration times and increase the resolution. The reason for

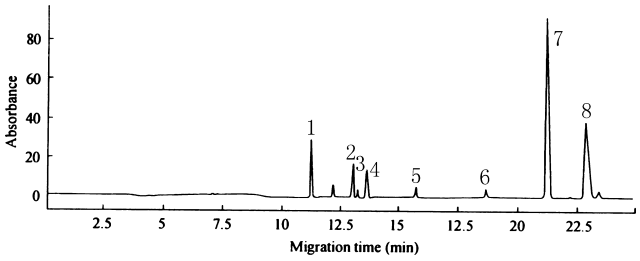

Fig. 1. Electropherograms of the Sample

Method: buffer: 18 mM borate, 12 mM phosphate and 50 mM SDS (pH 9.2) containing 20% methanol; applied voltage: 30 kV; injection time: 5 s; temperature: 20 °C; UV wavelength: 245 nm; capillary: 50 cm (effective length 41.5 cm)×50 μm I.D.

Table 1. Relative Migration Times of CE

Peak no.	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	Batch 9	Batch 10	RSD%
1	0.5315	0.5314	0.5282	0.5290	0.5309	0.5321	0.5328	0.5342	0.5355	0.5370	0.52
2	0.6139	0.6138	0.6110	0.6120	0.6139	0.6153	0.6159	0.6174	0.6191	0.6206	0.49
3	0.6249	0.6243	0.6222	0.6227	0.6250	0.6258	0.6262	0.6280	0.6303	0.6316	0.49
4	0.6464	0.6461	0.6435	0.6443	0.6463	0.6478	0.6483	0.6495	0.6509	0.6523	0.43
5	0.7428	0.7428	0.7412	0.7420	0.7441	0.7446	0.7453	0.7474	0.7485	0.7504	0.40
6	0.8779	0.8783	0.8778	0.8770	0.8776	0.8780	0.8786	0.8782	0.8787	0.8789	0.07
7	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0
8	1.0733	1.0733	1.0742	1.0735	1.0724	1.0722	1.0719	1.0709	1.0706	1.0696	0.14

Method: buffer: 18 mM borate, 12 mM phosphate and 50 mM SDS (pH 9.2) containing 20% methanol; applied voltage: 30 kV; injection time: 5 s; temperature: 20 °C; UV wavelength: 245 nm; capillary: 50 cm (effective length 41.5 cm)×50 μm I.D.

Table 2. Relative Peak Areas of CE

Peak no.	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	Batch 9	Batch 10	RSD%
1	0.1133	0.1187	0.1133	0.1168	0.1162	0.1160	0.1188	0.1155	0.1193	0.1051	3.59
2	0.0676	0.0704	0.0713	0.0711	0.0690	0.0737	0.0738	0.0686	0.0744	0.0635	4.77
3	0.0106	0.0111	0.0108	0.0099	0.0104	0.0104	0.0111	0.0100	0.0102	0.0109	4.15
4	0.0849	0.0850	0.0861	0.0930	0.0910	0.0880	0.0924	0.0880	0.0909	0.0863	3.44
5	0.0217	0.0229	0.0228	0.0230	0.0225	0.0217	0.0234	0.0218	0.0232	0.0204	4.12
6	0.0086	0.0095	0.0099	0.0095	0.0090	0.0097	0.0096	0.0089	0.0094	0.0087	4.76
7	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0
8	0.7105	0.7034	0.7045	0.7118	0.7034	0.7039	0.7066	0.7070	0.7093	0.6227	3.83

Method: buffer: 18 mM borate, 12 mM phosphate and 50 mM SDS (pH 9.2) containing 20% methanol; applied voltage: 30 kV; injection time: 5 s; temperature: 20 °C; UV wavelength: 245 nm; capillary: 50 cm (effective length 41.5 cm)×50 μm I.D.

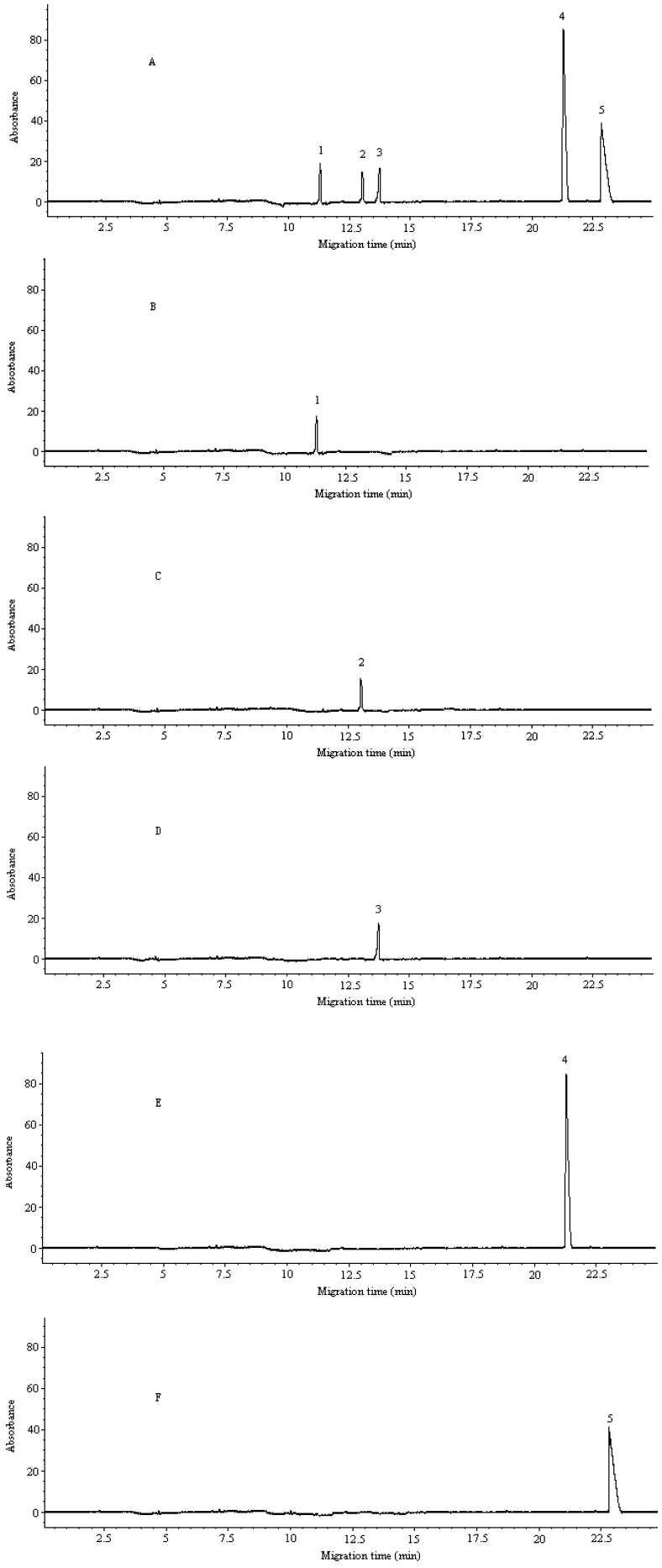

Fig. 2. Chromatograms of Five Standard Samples by CE Analysis

A mixture of the five standard samples; B standard sample of xanthotoxin; C standard sample of isopimpinellin; D standard sample of bergapten; E standard sample of imperatorin; F standard sample of osthole. Method: buffer: 18 mM borate, 12 mM phosphate and 50 mM SDS (pH 9.2) containing 20% methanol; applied voltage: 30 kV; injection time: 5 s; temperature: 20 °C; UV wavelength: 245 nm; capillary: 50 cm (effective length 41.5 cm)×50 μm I.D.

this is that the addition of methanol enhanced the mass transfer of analytes from the micellar to the aqueousphase. Prior to each sample injection, the capillary was rinsed with 1 N NaOH, consecutively, water and buffer for 5 min. Crude samples of *C. monnieri* from Jiangsu Province were prepared in accordance with method described Experimental.

There were 8 distinct and stable peaks respectively in ten batches of *C. monnieri* (Fig. 1). Relative standard deviation (RSD) of relative migration times of the corresponding peaks were calculated as labeled in the (Table 1). Eight peaks were defined as common peaks in the fingerprint.

Each peak area of peak 7 in the ten batches samples was set to 1, and other peak areas were expressed as relative values (Table 2). It was proven that CE could be an effective method in fingerprint.

CE Analysis for Major Peaks Identification In *C. monnieri*, xanthotoxin, isopimpinellin, bergapten, imperatorin and osthole were major active constituents and needed to be identified in the fingerprint. Each standard sample and the mixture of the five standard samples were separated respectively, on CE under the same conditions (Fig. 2). Migration times of standard sample and the corresponding peaks in crude samples were very similar. It could be inferred that peak 1 was xanthotoxin, peak 2 was isopimpinellin, peak 4 was bergapten, peak 7 was imperatorin, and peak 8 was osthole.

Fingerprint of *C. monnieri* by HPLC HPLC being conventionally applied in analysis and separation of natural products is recommended by Chinese Pharmacopoeia in development of the fingerprint. As a comparison to MEKC, HPLC was used to develop fingerprint of *C. monnieri* in our studies as described in Fig. 3.

In the ten batches of samples, there are 8 common peaks eluted in 40 min by HPLC. RSD values of the relative retention times of the corresponding peaks in HPLC analysis were very small (maximum 3% and average 0.9%) as shown in Table 4. Each peak area of peak 5 in the ten batches samples was set to 1, and other peak areas were expressed as relative values (Table 5). Total peak area of non-common peak was less than 10%, which met the standards.

Comparisons of CE and HPLC Both CE and HPLC could separate and detect the components of the *C. monnieri*, which are important to fingerprint. However, because of the different principles of the two methods, there're some advantages and disadvantages between them. CE is an automated

Table 3. Migration Times of Standard Samples in CE Analysis

Standard sample	Migration time (min)			RSD (%)
	1	2	3	
Xanthotoxin	11.33	11.30	11.45	0.70
Isopimpinellin	13.01	13.06	13.23	0.88
Bergapten	13.78	13.75	13.92	0.66
Imperatorin	21.32	21.28	21.43	0.36
Osthole	22.88	22.85	22.94	0.20

1: analysis of only one standard sample, 2: analysis of mixture of standard samples, 3: analysis of the corresponding peak in crude sample, Method: buffer: 18 mM borate, 12 mM phosphate and 50 mM SDS (pH 9.2) containing 20% methanol; applied voltage: 30 kV; injection time: 5 s; temperature: 20 °C; UV wavelength: 245 nm; capillary: 50 cm (effective length 41.5 cm)×50 μm I.D.

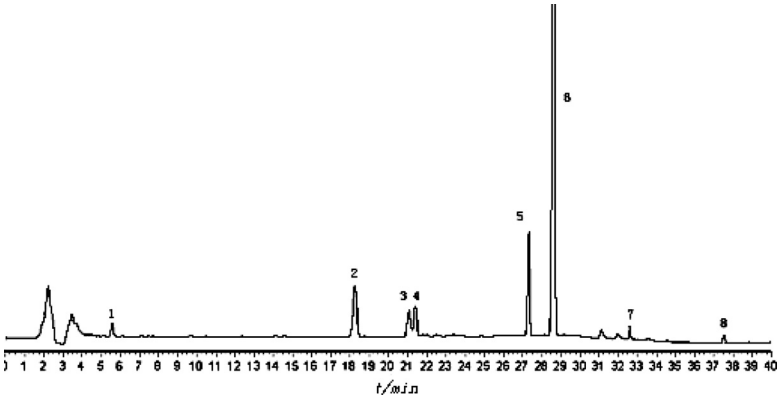

Fig. 3. Chromatograms of Sample by HPLC Analysis

Method: an Alltech ODS column (4.6×250 mm, 5 μm); mobile phase: solvent A (CH_3CN) and solvent B (0.1% acetic acid–water); the gradient mode: 0—15 min, 77% B; 15—30 min, 55% B; 30—40 min, 0% B; the flow-rate was 1.0 ml/min. The wavelengths' gradient mode: 0—26 min, 245 nm; 26—30 min, 325 nm; 30—40 min, 245 nm.

Table 4. Relative Retention Times of HPLC

Peak no.	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	Batch 9	Batch 10	RSD%
1	0.2040	0.2094	0.2263	0.2072	0.2054	0.2072	0.2057	0.2094	0.2063	0.2077	3.04
2	0.6679	0.6700	0.6929	0.6699	0.6708	0.6693	0.6686	0.6700	0.6708	0.6699	1.10
3	0.7713	0.7747	0.7903	0.7745	0.7751	0.7738	0.7717	0.7747	0.7751	0.7744	0.69
4	0.7833	0.7863	0.8008	0.7862	0.7869	0.7856	0.7837	0.7863	0.7869	0.7862	0.63
5	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0
6	1.0472	1.0465	1.0450	1.0464	1.0465	1.0464	1.0472	1.0465	1.0465	1.0466	0.06
7	1.1934	1.1912	1.1826	1.1916	1.1919	1.1910	1.1931	1.1912	1.1919	1.1917	0.26
8	1.3113	1.3711	1.3579	1.3720	1.3721	1.3703	1.3726	1.3711	1.3721	1.3722	1.40

Method: an Alltech ODS column (4.6×250 mm, 5 μm); mobile phase: solvent A (CH_3CN) and solvent B (0.1% acetic acid–water); the gradient mode: 0—15 min, 77% B; 15—30 min, 55% B; 30—40 min, 0% B; the flow-rate was 1.0 ml/min. The wavelengths' gradient mode: 0—26 min, 245 nm; 26—30 min, 325 nm; 30—40 min, 245 nm.

Table 5. Relative Peak Areas of HPLC

Peak no.	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	Batch 9	Batch 10	RSD%
1	0.1428	0.1419	0.1379	0.1448	0.1399	0.1427	0.1393	0.1434	0.1424	0.1448	1.62
2	0.9399	0.9128	0.9476	0.9187	0.9335	0.9564	0.9162	0.9345	0.9399	0.9128	1.65
3	0.4093	0.4357	0.4031	0.4338	0.4378	0.4168	0.4468	0.4598	0.4000	0.4172	4.63
4	0.4298	0.4468	0.4103	0.4533	0.4311	0.4416	0.4220	0.4791	0.4311	0.4335	4.32
5	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0
6	8.9601	8.5798	7.4534	8.5551	8.4213	8.5678	8.7994	8.5647	8.4213	8.4949	4.69
7	0.0944	0.0972	0.0825	0.0973	0.0951	0.0968	0.0970	0.0977	0.0951	0.0959	4.75
8	0.0721	0.0692	0.0663	0.0684	0.0666	0.0674	0.0706	0.0688	0.0665	0.0669	2.83

Method: an Alltech ODS column (4.6×250 mm, 5 μm); mobile phase: solvent A (CH_3CN) and solvent B (0.1% acetic acid–water); the gradient mode: 0—15 min, 77% B; 15—30 min, 55% B; 30—40 min, 0% B; the flow-rate was 1.0 ml/min. The wavelengths' gradient mode: 0—26 min, 245 nm; 26—30 min, 325 nm; 30—40 min, 245 nm.

Table 6. Comparison of Two Methods

	CE	HPLC
Separation column	41.5 cm (50 μm, i.d.)	4.6×250 mm
Temperature	20 °C	40 °C
Run time (min)	25	40
RSD of t_R (%)	0.31	0.76
Non-common peak area	<10%	<10%

analytical technique that separated species by applying voltage across buffer filled capillaries. When the voltage is applied, different migration speeds make the components separated. HPLC is a liquid–liquid partition chromatography. Separation on HPLC is depending on the partition coefficient of different components in the two phases. The principle differences led to different separation performance, retention time and relative peak area.

Commonness and difference of the two methods were shown in Table 6. In our study, at first, we found the amounts of the sample injected, were very small in the CE, and therefore the higher concentration of the sample was demanded. While in HPLC, we could detect the tiny concentration of the sample because of the higher injection amounts. Secondly, in CE analysis, the detection would be interfered by solvents. So at the last step we chose the buffer as solvents to obtain an excellent chromatography. While in HPLC analysis, the solvent peaks would not have effect on the basic line. Thirdly, CE analysis has the advantage in building up the fingerprint of TCM: As we all know, there are many impurities in TCM (such as chlorophyll), which will stain chromatography columns. The column of CE is hollow for us to wash easily. On the other side, the column of HPLC is difficult to wash so as to cause the high column pressure. As a result, the life of column is short and the cost will increase.

Conclusion

Our researches show, HPLC had some advantages in sensitivity, while CE can achieve high efficiency, low cost per analysis, rapidness and a minimum use of organic solvents. For example, isopimpinellin and bergapten were baseline separated by the CE method, while the resolution of the two coumarins in *C. monnieri* was only about 0.9 by previously published HPLC method.[8,13,18] Although CE show worse sensitivity than HPLC, there're no demands for the low limit of quantization and the entire datum were relative values in fingerprint.

In a word, CE with some special advantages is suitable for the developing of fingerprint.

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