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Capillary electrophoretic determination of the constituents of Artemisiae Capillaris Herba

Shuenn-Jyi Sheu*, Chum-Ling Chieh, Wu-Che Weng

Department of Chemistry, National Taiwan Normal University, 88, Sec. 4, Tingchow Road, Taipei 11718, Taiwan

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Abstract

Two capillary electrophoretic methods, a micellar electrokinetic electrophoretic (MEKC) one and a capillary zone electrophoretic (CZE) one, were developed for the separation of 12 constituents in Artemisiae Capillaris Herba. Detection at 254 nm with 20 m*M* sodium dodecyl sulfate and 20 m*M* sodium borate buffer (pH 9.82) in MEKC or with 25 m*M* sodium borate and 6.75 mg/ml 2,3,6-tri-*O*-methyl-β-cyclodextrin buffer in CZE was found to be the most suitable approach for this analysis. Within 42 min, the MEKC method could successfully separate 12 authentic constituents, whereof chlorogenic acid, however, appeared as a broad and split peak, and capillarisin and chlorogenic acid overlapped partially with other coexisting substances in crude extract of the herb. The CZE method could completely overcome these problems and was used to determine the amounts of capillarisin, chlorogenic acid, scopoletin and caffeic acid in the extract. The effect of buffers on the constituent separation and the validation of the two methods were discussed.

Keywords: *Artemisia capillaris*; Pharmaceutical analysis

1. Introduction

Artemisiae Capillaris Herba (capillaris) is the dried sprout of *Artemisia capillaris* Thumb. (Compositae), which is a commonly used Chinese herb drug indicated for removing fever and dispelling dampness [1]. A number of essential oils, coumarins, chromones, flavones and carboxylic acids in the herb have been isolated and identified, such as scopoletin (SP) [2–5], capillarisin (CP) [2–6], 6,7-dimethylesculetin (DE) [2,4,5], caffeic acid (CFA) [1], chlorogenic acid (CGA) [1], phenol (P) [7,8], cresols (C) [7,8], eugenol (EG) [7,8] and ethylphenols (EP) [7,8], etc. Among them, CP [9–20], DE [9–13,17–24], CFA [11–13,25,26] and CGA [25,26] were found to have specially biological activities. Several methods have been established for separating one or two of these constituents which are used as quality control markers for the crude drug of the herb. The methods include thin-layer chromatography (TLC) for CGA [27,28], supercritical fluid chromatography (SFC) for CP and DE [5] and high-performance liquid chromatography (HPLC) for CP and DE [29–31] as well as CGA [32,33]. Recently we have reported a reversed-phase (RP)-HPLC method for separating 10 constituents from the herb [34]. However, as our knowledge of the effective components of capillaris is still limited and efforts to develop

*Corresponding author. Tel.: +886-2-9350-749; fax: +886-2-9324-249.

E-mail address: chefvooz@scc.ntnu.edu.tw (S.-J. Sheu).

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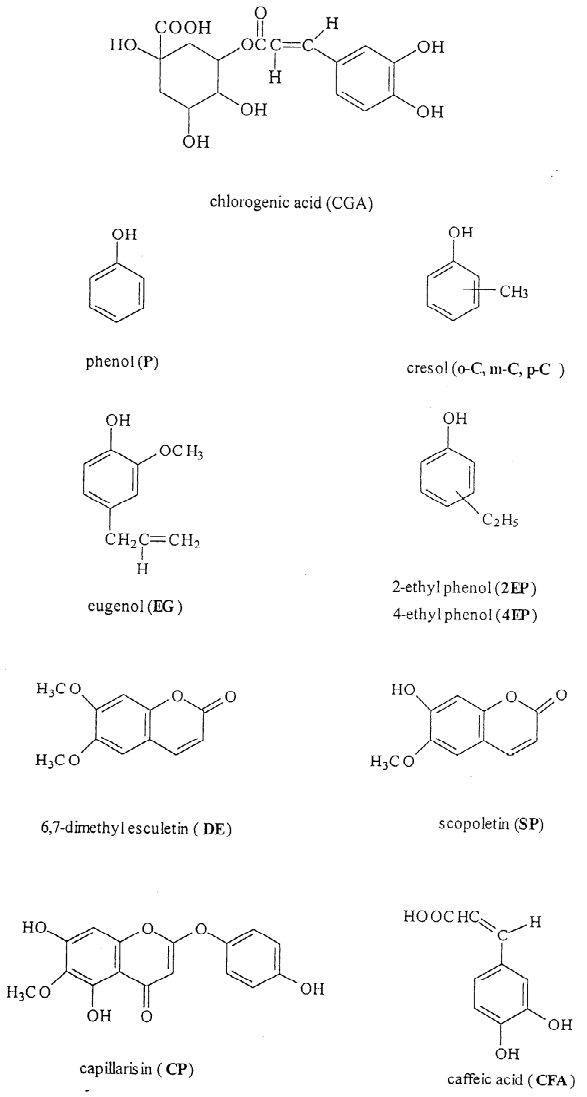

Fig. 1. The structures of capillaris constituents.

simpler and more rapid methods that can assay as many components as possible are therefore necessary. Here, we are going to describe two capillary electrophoretic (CE) methods, a micellar electrokinetic electrophoretic (MEKC) one and a cyclodextrin-capillary zone electrophoretic (CD-CZE) one, to separate from the herb a total of 12 bioactive constituents, namely CP, DE, SP, CFA, CGA, P, *o*-cresol (*o*-C), *m*-cresol (*m*-C), *p*-cresol (*p*-C), EG, 2-ethylphenol (2EP) and 4-ethylphenol (4EP) as shown in Fig. 1. The effects of pH value, borate and sodium dodecyl sulfate (SDS) concentrations in MEKC and borate and 2,3,6-tri-*O*-methyl-β-cyclodextrin (TM-β-CD) concentrations in CD-CZE on the migration times of the constituents were studied.

2. Experimental

2.1. Reagents and materials

Vanillic acid, CFA, CGA, SP, 2EP and 4EP were purchased from TCI (Tokyo, Japan), DEP from Yoneyama (Osaka, Japan), EG from Aldrich (Milwaukee, WI, USA), P, *m*-C, *p*-C, *o*-C, CP, sodium borate and TM-β-CD from Nacalai Tesque (Kyoto, Japan), benzyltriphenylphosphonium chloride from Merck (Darmstadt, Germany) and SDS from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were of LC grade (Fisons, Loughborough, UK). Deionized water was from a Milli-Q system (Millipore, Bedford, MA, USA). Artemisiae Capillaris Herba was purchased from the Chinese herb market in Taipei, Taiwan.

2.2. Preparation of Artemisiae Capillaris Herba extracts and standard solutions

A 4.0-g sample of pulverized Artemisiae Capillaris Herba was extracted with methanol (20 ml) for 30 min and then centrifuged at 1500 *g* for 5 min. The extraction was repeated twice. The extracts were combined and concentrated under reduced pressure at room temperature to 5.0 ml. After the addition of 1 ml of an internal standard solution ($I.S._1$, a 25 ml methanol solution containing 70.3 mg vanillic acid, 2.8 mg/ml; $I.S._2$, a 50 ml methanol solution containing 150.6 mg benzyltriphenylphosphonium chloride, 3.0 mg/ml), the Artemisiae Capillaris Herba extract was diluted to 10 ml with methanol, and then passed through a 0.45-μm filter. The filtrate was injected directly into the capillary electrophoresis system.

Amounts of 15.8 mg P, 19.2 mg *o*-C, 29.1 mg

m-C, 25.8 mg *p*-C, 20.3 mg 2EP, 14.4 mg 4EP, 21.2 mg EG, 23.7 mg SP, 18.8 mg CP, 15.5 mg DE, 30.5 mg CGA and 39.8 mg CFA were accurately weighed and added to methanol to make 50 ml exactly. The solution so made served as the standard stock solution. Aliquots of 0.1, 0.5, 1, 2, 3, 4, 5 and 7 ml of the standard stock solution were pipetted and each transfered to a 10-ml volumetric flask. To each flask were added 1 ml of the internal standard solution ($I.S._1$ for the MEKC method, $I.S._2$ for the CD-CZE method) and methanol to make exactly 10 ml. The solution in the various flasks represented the different concentrations of standard solution, which were used to plot a calibration curve by injecting each concentration of standard solution into the instrument, repeating the injection of each concentration three times and taking their average.

2.3. Apparatus and conditions

The analysis was carried out on a Waters Quanta 4000 CE system equipped with a UV detector set at 254 nm and a 75 μm I.D. fused-silica capillary tube (Polymicro, Phoenix, AZ, USA) 100 cm long with the detection window placed 92.4 cm from the injection for MEKC, and 80 cm long and 72.4 cm from the injection for CZE. The conditions were as follows: injection time, 2 s, hydrostatic (injection volume, 3.4 nl); applied voltage, 20 kV (constant voltage, positive to negative polarity); and temperature, 25–26°C. In MEKC, the electrolyte was a pH 9.82 (adjusted with 0.05 *M* NaOH) buffer solution of 20 m*M* sodium borate and 20 m*M* SDS; run time, 42 min. In CZE, the carrier was a 25 m*M* sodium borate solution with 6.75 mg/ml TM-β-CD; run time, 22 min.

At the beginning of experiment each day, the capillary was purged with 0.1 *M* NaOH for 5 min, followed by deionized water for 5 min and then with running buffer for 3 min. Between the runs, the capillary tube was flushed with 0.1 *M* NaOH for 3 min, followed by deionized water for 3 min and then with running buffer for 3 min. At the end of experiment each day, the capillary tube was purged with 0.1 *M* NaOH for 5 min, followed by deionized water for 10 min and then the two ends of the tube were immersed in deionized water.

3. Results and discussion

3.1. Analytical conditions

With the exception of DE, all the capillaris constituents possess phenolic hydroxyl groups and can be separated in a proper alkaline borate solution, in principle. However, preliminary experiments showed that only SP, CP, CGA and CFA could have a reasonable separation. DE, being a neutral molecule, was eluted out along with the electroosmotic flow (EOF). P, EG, *o*-C, *m*-C, *p*-C, 2EP and 4EP were all unseparable. As surfactants [SDS and sodium cholate (SC)] were added to the borate solution, SDS was found to have a stronger influence on the resolutions of the various constituents. After a series of trials, a pH 9.82 condition for MEKC was established as being capable of successful analysis of 12 authentic standards. However, as this condition was applied to the crude extract of Artemisiae Capillaris Herba, it was found that CGA and CP were subject to interferences from other unknown constituents of the herb. Besides, the CGA peak showed a severely forward leaning tendency. To solve these problems, we developed the CD-CZE method.

3.1.1. MEKC method

3.1.1.1. Effect of borate concentration

As the concentration of SDS was fixed at 20 m*M* and the borate concentration was varied among 10, 20, 30, 40 and 50 m*M*, it was then found that when the borate concentration was gradually increased, the zeta potential became diminished, and the migration times of the various constituents increased. Especially marked was the slowing down in the migration velocities of CGA and CFA. In addition, high electrolyte concentration caused the splitting and forward leaning of the CGA peak to become more serious, the individual peaks to become broader and so the theoretical plate numbers to become smaller. At concentrations of 20 and 30 m*M*, resolution was fairly good. At 20 m*M*, the analysis time was shorter and the theoretical plate number was higher. Hence, the 20 m*M* borate concentration was chosen.

3.1.1.2. Effect of SDS concentration

To investigate the effect of surfactant, amounts of 10, 20, 30, 40 and 50 m*M* SDS were added to 20 m*M* borate solution. Fig. 2 shows the influence of SDS at different concentrations on the migration times of the capillaris constituents. In the absence of SDS, with the exception of CGA and CFA which could be completely separated, and SP and CP which were partially separated, the other phenolic compounds and DE all moved out along with the EOF. As SDS was added, the EOF gradually retarded, the various constituents also varied in their migration times whereof DE was affected with the greatest retardation, the phenolic compounds retarded less, while compounds with carboxyl groups such as CGA and CFA were almost unaffected. Among the SDS concentrations used, 50 m*M* was found to be the best and 20 m*M* was next. In both cases, 10 constituents could be separated and *m*-C and *p*-Cl could be partly separated. (R_s values for this pair of isomers were 0.214 at 20 m*M* and 0.516 at 50 m*M*, respectively). Since a high SDS concentration would render a poor reproducibility in the migration times of CFA and EG, 20 m*M* was finally chosen.

3.1.1.3. Effect of pH value

Fixed concentrations of borate and SDS were used as mentioned above, while the pH values of the buffer were varied from 9.24 to 10.04 by adding suitable amounts of sodium hydroxide. The relation between the pH values and migration times are shown in Fig. 3. Fig. 3 shows that as the pH value increases the EOF retards and the migration times of all constituents increase markedly, especially for CGA and CFA which contain carboxyl groups and CP which contains three hydroxyl groups. It was noted that with the increase of pH value, the peak of CGA became smaller and broader, but at the same

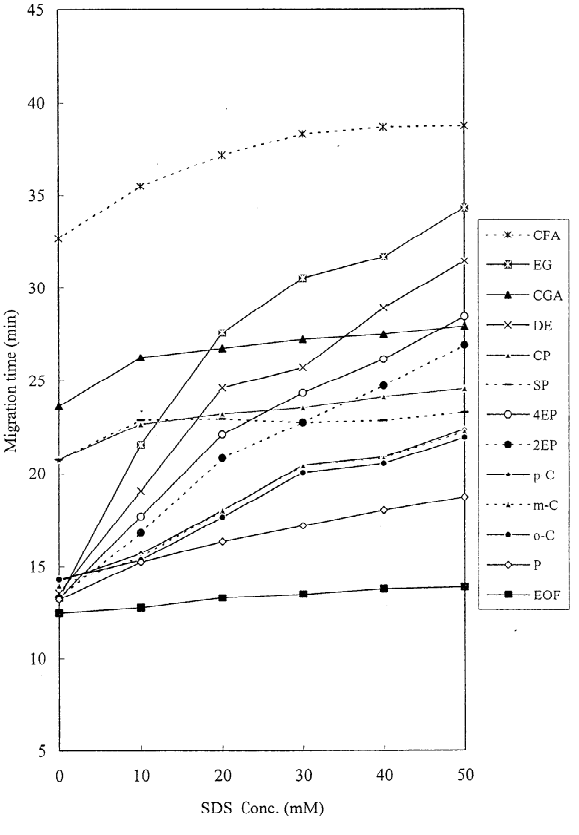

Fig. 2. Effect of SDS concentration on migration time.

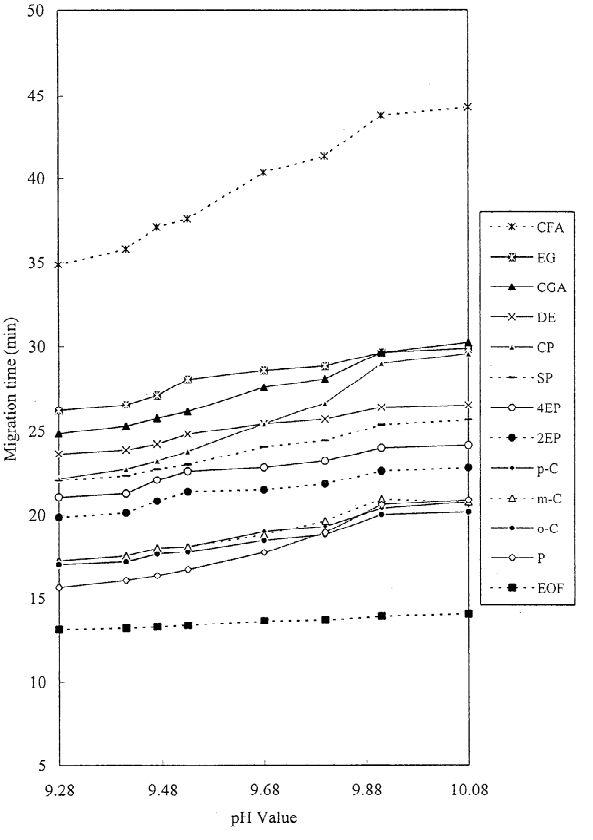

Fig. 3. Effect of pH on migration time.

time a number of peaks became separable. At pH 9.28, the resolution values for the pairs SP/CP and *m*-C/*p*-C were 0.52 and 0.00, respectively. As pH increased to 9.53, the values for the two pairs were 3.32 and 0.41, whereby SP/CP separated completely and *m*-C/*p*-C partially. As pH reached 9.82, a baseline-separated electropherogram was obtained and the values for the compounds were SP/CP 8.76, P/*o*-C 1.50, *o*-C/*m*-C 1.58, *m*-C/*p*-C 1.61 and CP/CGA 4.24, respectively. On further increase of pH, resolution decreased. So we chose a buffer solution at pH 9.82. Fig. 4A shows the separation of 12 authentic marker substances, using 20 m*M* borate and 20 m*M* SDS as the electrolyte at pH 9.82. The condition was found to be reproducible on different days.

3.1.2. CD-CZE method

Fig. 4A shows that the proposed MEKC method could separate the 12 marker substances though, it was not a perfect analytic condition in view of the facts that CGA had multiple forward leaned broad peaks and that the CP and CGA in the crude herb extract overlapped with unknown constituents. Therefore, we developed the following CD-CZE method with CP, CGA, SP and CFA as markers to solve the above problems.

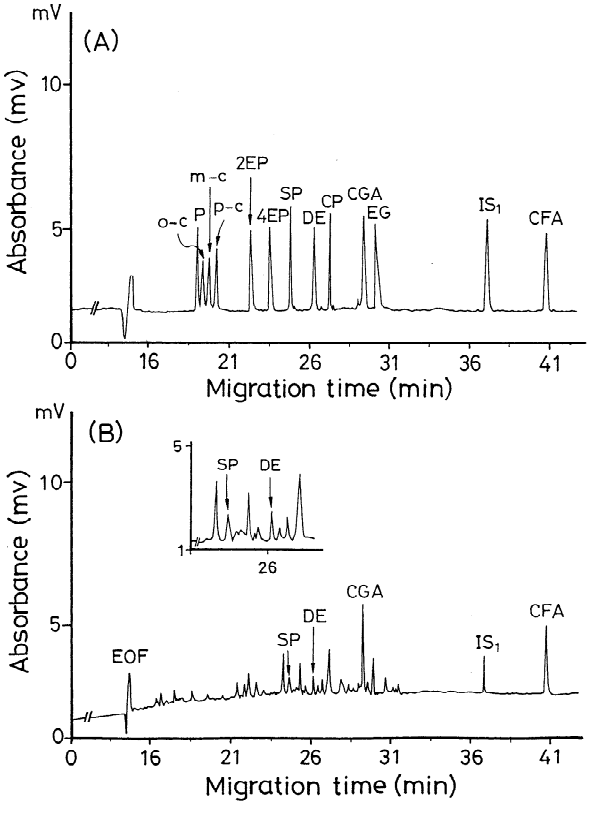

Fig. 4. The MEKC electropherograms of (A) the 12 authentic standards,(B) Artemisiae Capillaris Herba extract.

3.1.2.1. Effect of borate concentration

As different amounts of sodium borate ranging from 5, 10, 15, 20, 25 to 30 m*M* were each added to a TM-β-CD solution of fixed concentration (6.75 mg/ml), this range (5~30 m*M*) shows very good separatory effect for the authentic standards of SP, CP, CFA and CGA. However, for the crude herb extract, it was 25 m*M* that afforded the best resolution. The resolution values for the constituents with unknown neighboring compounds were as follows: at 20 m*M*, CP 1.16, SP 2.15, CGA 1.88; at 25 m*M*, CP 1.51, SP 2.63, CGA 2.27; at 30 m*M*, CP 1.66, SP 1.63, CGA 2.04. Therefore, we chose the 25 m*M* borate solution.

3.1.2.2. Effect of TM-β-CD concentration

The carrier was prepared, using the buffer solution containing 25 m*M* borate and different concentrations of TM-β-CD, namely 0, 1.0, 2.5, 5.0, 6.75, 7.5, 10 and 12.5 mg/ml. The TM-β-CD concentration could markedly affect either the migration time or resolution of a number of constituents. For example, the migration time of CP was 10.5 min in the absence of TM-β-CD, but 8.1 min at 1 mg/ml. The other compounds did not noticeably change with the addition of TM-β-CD though, their theoretical plate numbers and peak symmetry increased enormously. As the TM-β-CD concentration exceeded 5 mg/ml, the forward leaning condition of the CGA peak disappeared and instead a highly symmetrical peak was obtained. This was especially true for the crude herb extract for which the addition of TM-β-CD was found to be able to improve very markedly the resolution between the marker constituents and the unknown substances. Table 1 shows the CP, SP, CGA and CFA of the herb with their theoretical plate numbers, tailing factors and resolutions in various TM-β-CD buffer solutions. Experiments showed that addition of 6.75 mg/ml TM-β-CD into 25 m*M*

Table 1
Effects of TM-β-CD on the theoretical plate numbers (N), tailing factors (T) and resolutions (R_s) of constituents in a capillaris crude extract

Concentration of TM-β-CD (mg/ml)	Parameter	CP	SP	CGA	CFA
0	$N \cdot 10^6$	1.35	2.44	1.35	1.23
	T	0.23	0.63	0.24	3.50
	R_s	0.11	0.11	0.22	11.90
2.50	$N \cdot 10^6$	2.72	2.02	1.46	1.30
	T	1.04	0.67	0.53	2.20
	R_s	0.78	0.34	0.67	12.10
5.00	$N \cdot 10^6$	5.53	2.49	1.67	1.32
	T	0.90	0.85	0.84	2.25
	R_s	1.12	0.96	1.34	13.80
6.75	$N \cdot 10^6$	5.78	2.71	1.83	1.39
	T	0.95	1.01	0.94	1.17
	R_s	1.51	2.63	2.27	14.10
10.00	$N \cdot 10^6$	5.70	2.18	1.38	1.28
	T	0.98	0.73	0.95	1.26
	R_s	1.35	1.50	2.45	15.10

borate solution had the best separatory effect and also showed reproducible on different days. Fig. 5A presents an electropherogram showing the separation of four authentic marker substances.

3.2. Method validation

The best resolution was obtained with a pH 9.82 buffer containing 20 m*M* sodium borate and 20 m*M* SDS in MEKC, and an electrolyte containing 25 m*M* sodium borate and 6.75 mg/ml TM-β-CD in CZE.

In the MEKC method using vanillic acid as an internal standard, the reproducibility [by the relative standard deviation (RSD) in both pure standards and plant extract components] was, in terms of peak-area ratio, 0.35–1.72% (intra-day, $n=6$) and 0.68–2.51% (inter-day, $n=6$), and, in terms of migration time, 0.25–2.49% (intra-day, $n=6$) and 1.02–3.04% (inter-day, $n=6$).

In the CZE method using benzyltriphenylphosphonium chloride as the internal standard, the RSD was, in terms of peak-area ratio, 0.32–1.54% (intra-day, $n=6$) and 0.18–1.50% (inter-day $n=6$), and, in terms of migration time, 1.25–1.59% (intra-day, $n=6$) and 0.76–1.56% (inter-day, $n=6$),as shown in Table 2.

Calibration curves were plotted by correlating the

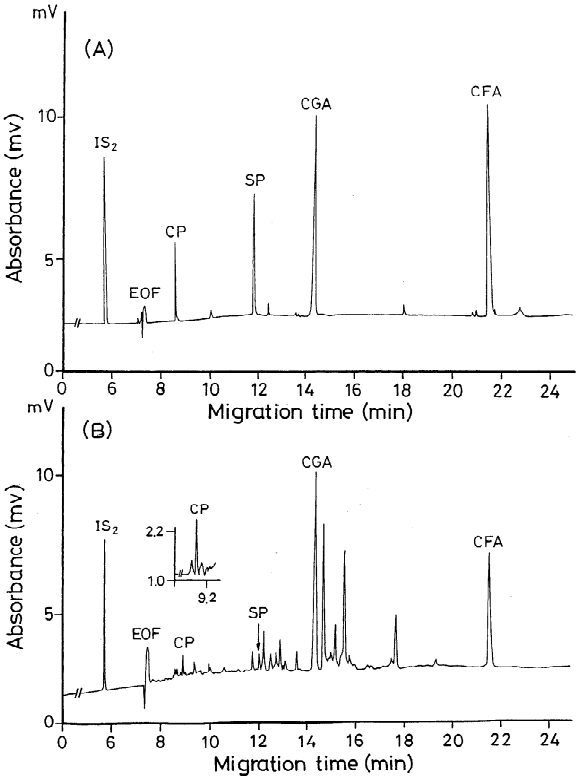

Fig. 5. The CD-CZE electropherograms of (A) authentic standards, (B) Artemisiae Capillaris Herba extract.

Table 2
Reproducibility of peak-area ratios (*A*) and migration times (*t*) of the constituents in Artemisiae Capillaris Herba

Constituent	CZE				MEKC			
	Intra-day (RSD, %)		Inter-day (RSD, %)		Intra-day (RSD, %)		Inter-day (RSD, %)	
	A	*t*	*A*	*t*	*A*	*t*	*A*	*t*
P					1.58	0.25	1.42	1.99
o-C					1.08	0.87	2.23	3.04
m-C					0.57	0.75	1.99	1.02
p-C					0.59	0.66	1.74	1.07
2EP					0.78	1.13	1.18	2.06
4EP					0.62	1.21	1.14	2.04
DE					1.50	1.05	1.12	2.56
EG					1.13	1.12	2.06	2.18
SP	0.32	1.25	0.18	0.90	1.72	1.57	1.33	1.81
CP	0.44	1.59	0.33	0.76	0.35	1.46	0.68	1.84
CGA	1.54	1.25	1.50	0.93	1.62	2.02	2.51	1.77
CFA	0.84	1.40	0.70	1.56	1.69	2.49	2.51	1.78

peak-area ratios with the corresponding concentrations. The regression lines linear in the concentration range studied are shown in Table 3, where the detection limits ($S/N=3$) for the constituents were 1.1–68.0 ng (0.32–20.00 μg/ml) in MEKC and 0.3–4.2 ng (0.27–1.24 μg/ml) in CZE, and the correlation coefficients were all higher than 0.999.

A methanol solution containing suitable amounts of authentic standards (2 ml solution containing 0.6 mg P, 0.7 mg *o*-C, 1.1 mg *m*-C, 1.0 mg *p*-C, 0.8 mg 2EP, 0.5 mg 4EP, 0.8 mg EG, 1.0 mg SP, 0.7 mg CP, 0.5 mg DE, 1.3 mg CGA and 15.6 mg CFA) was added to a capillaris sample of known contents and the mixture was analyzed, using the proposed methods. The recoveries of the constituents were 94.4–101.3% as shown in Table 3. Owing to the fact that analyte–matrix interactions are always existing in the medicinal plant extract, there may have somewhat

Table 3
Data of linear ranges, regression equations, detection limits and recoveries of capillaris constituents

Constituent	Linear range (μg/ml)	Slope	Intercept ($\cdot 10^{-1}$)	Detection limit		Recovery (%)
				ng	μg/ml	
P[a]	3.0–178	36.7	0.34	31.4	9.2	96.8
o-C[a]	3.6–255	27.3	0.04	38.8	11.4	97.2
m-C[a]	5.4–241	5.6	0.02	36.0	10.6	101.0
p-C[a]	5.2–310	5.8	0.15	34.3	10.1	101.0
2EP[a]	20.3–284	2.7	0.09	43.2	12.7	95.1
4EP[a]	13.4–161	3.2	0.07	45.2	13.3	95.4
DE[a]	2.7–135	17.3	0.11	68.0	20.0	96.2
EG[a]	4.1–283	42.7	0.44	1.1	0.3	98.1
CP[a]	12.7–235	37.3	0.22	3.8	1.1	96.8
CP[b]	17.8–107	7.9	0.94	0.9	0.3	96.4
SP[a]	22.7–337	27.3	2.31	21.0	6.2	98.4
SP[b]	12.4–173	5.1	0.16	2.0	0.6	98.6
CGA[a]	32.7–435	57.3	3.12	17.5	5.2	95.5
CGA[b]	65.0–455	26.1	7.75	4.2	1.2	94.4
CFA[a]	77.9–545	0.7	0.34	10.1	3.2	95.0
CFA[b]	77.9–545	12.2	3.63	3.0	0.9	101.0

[a] Analyzed by the MEKC method.
[b] Analyzed by the CZE method.

differences between the measured values and the true recoveries.

The peaks in the electropherograms of the plant extract were identified by comparing their migration times with those of the authentic standards, by spiking the extract with a single standard compound in the subsequent run and also by checking the results obtained from dual wavelength calculations.

3.3. Determination of the contents of Artemisiae Capillaris Herba

A methanol extract of Artemisiae Capillaris Herba was analyzed with the proposed methods and was found that the contents of P, *o*-C, *m*-C, *p*-C, 2EP and 4EP were all lower than the detection limits, and the other constituents could be determined by either MEKC (for DE, SP, EG and CFA) or CZE (for CP and CGA) method. Figs. 4B and 5B show their electropherograms. By substituting the peak-area ratios of the individual peaks for y in the equations listed in Table 3, the contents of the individual constituents in Artemisiae Capillaris Herba were obtained as (mean, $n=6$): DE, 0.035; SP, 0.037; EG, 0.068; CFA,0.225; CP, 0.034; CGA, 0.926 mg/g.

In conclusion, by optimizing the pH value and the SDS, borate and cyclodextrin concentrations, it was possible to separate the 12 constituents in a crude extract of Artemisiae Capillaries Herba by joint use of the proposed MEKC and CZE methods. The MEKC method could separate more constituents of the herb as well as the isomers of cresol and ethylphenol, but it could not be used to assay the CGA and CP in the herbs' crude extract. The former shortcoming was due to the presence of a split, broadened and forward leaning peak, and the latter case was due to the methods' incapability to get rid of interferences from the other constituents of the herb. As for the CZE method, it could overcome those problems, took shorter analysis time and had lower detection limits. Thus, the proposed methods can serve as suitable tools for the quality assurance work in the production of commercial Chinese herb drugs and herbal preparations.

Acknowledgements

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