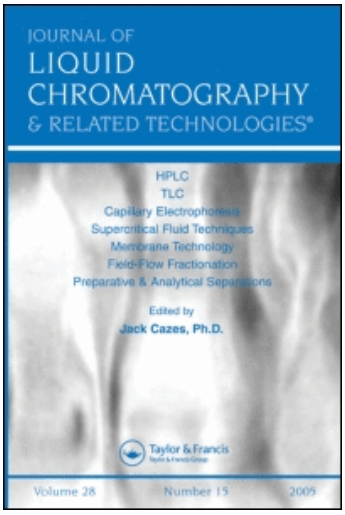

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of the Alkaloids in Coptis-Evodia Herb Couple by Capillary Electrophoresis

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To cite this Article Lee, Ming-Chung and Sheu, Shuenn-Jyi(1997) 'Determination of the Alkaloids in Coptis-Evodia Herb Couple by Capillary Electrophoresis', Journal of Liquid Chromatography & Related Technologies, 20: 1, 63 — 78
To link to this Article: DOI: 10.1080/10826079708010637
URL: http://dx.doi.org/10.1080/10826079708010637

J. LIQ. CHROM. & REL. TECHNOL., 20(1), 63–78 (1997)

DETERMINATION OF THE ALKALOIDS IN COPTIS-EVODIA HERB COUPLE BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

A method combining the techniques of micellar electrokinetic capillary chromatography (MECC) and capillary zone electrophoresis (CZE) has been developed to assay seventeen alkaloids in coptis-evodia herb couple. The MECC method, based on SDS, was applied to analyze three indolequinazoline alkaloids and six quinolone alkaloids in evodia within 30 minutes, and a CZE technique was used to determine eight quaternary alkaloids (dehydroevodiamine in evodia, and seven protoberberine alkaloids in coptis) within 25 minutes. The recovery efficiencies were 96.68-103.19% in MECC and 99.65-103.28% in CZE, with a relative standard deviation of 1.67-4.00% for MECC and 2.33-4.38% for CZE. The calibration curves exhibited good linearity over one order of magnitude of concentration, and their minimum detectable concentrations were approximately 15.78 to 47.33 μg/mL using a 0.75 μm inner diameter column. Contents of the seventeen alkaloids in a methanol-water crude extract of coptis-evodia herb couple could easily be determined by this method.

INTRODUCTION

Herbs simultaneously used together and in couples are the basic composition units of Chinese herbal formulas and have special clinical significance in Chinese medicine. They are much simpler than complicated formulas in composition, but retain the basic therapeutic features.[1] Coptis (Coptidis Rhizoma) possesses the effects of dispelling heat, drying dampness, purging fire and removing toxin, and evodia (Evodiae Fructus) has the actions of warming middle, dispelling cold, causing vitality to descend and controlling pain.[2] The combined use of these two herbs will enable the healing of hypochondric and costar pain, stomach ache, acid regurgitation, nausea and gastric upset.[1]

The pharmacologically active constituents of coptis are a number of protoberberine alkaloids such as coptisine (**1**), berberine (**3**), berberastine (**4**), epiberberine (**5**), columbamine (**6**), jatrorrhizine (**7**), palmatine (**8**),[3-7] and those of evodia are indolequinazoline alkaloids, dehydroevodiamine (**2**), evodiamine (**9**), rutaecarpine (**10**), carboxyevodiamine (**17**), and quinolone alkaloids, 1-methyl-2-nonyl-quinolone (**11**), 1-methyl-2-[(Z)-6-undecenyl]-4(1H)-quinolone (**12**), 1-methyl-2-undecyl-4(1H)quinolone (**13**), evocarpine (**14**), 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1H)quinolone (**15**) and dihydroevocarpine (**16**),[8-12] as shown in Figure 1. The former can be analyzed by a standard capillary zone electrophoretic (CZE) method,[13] and the latter have to be separated by both CZE and micellar electrokinetic capillary chromatographic (MECC) methods.[14] The two systems are distinctly different. In order to determine, simultaneously, the chemical constituents of the two herbs, this paper reports a facile and rapid CE analytical method that can be used to help assess not only the herb couple itself but also the quality of Chinese herbal preparations containing the herb couple mentioned herein.

EXPERIMENTAL

Apparatus and Conditions

The analysis was carried out on a Waters (Milford, MA) Quanta 4000 capillary electrophoresis system, equipped with a UV detector set at 254 nm and a 90 cm x 75μm I.D. fused silica capillary tube (Polymicro Technologies, Phoenix, AZ) with the detection window placed 82.5 cm from the injection. The conditions were as follows: sampling time, 2 s, hydrostatic (injection volume, 3.4 nL); applied voltage, 25 kV (constant voltage, positive to negative polarity); and temperature, 24.5-25.0 °C. In CZE, the electrolyte was a buffer

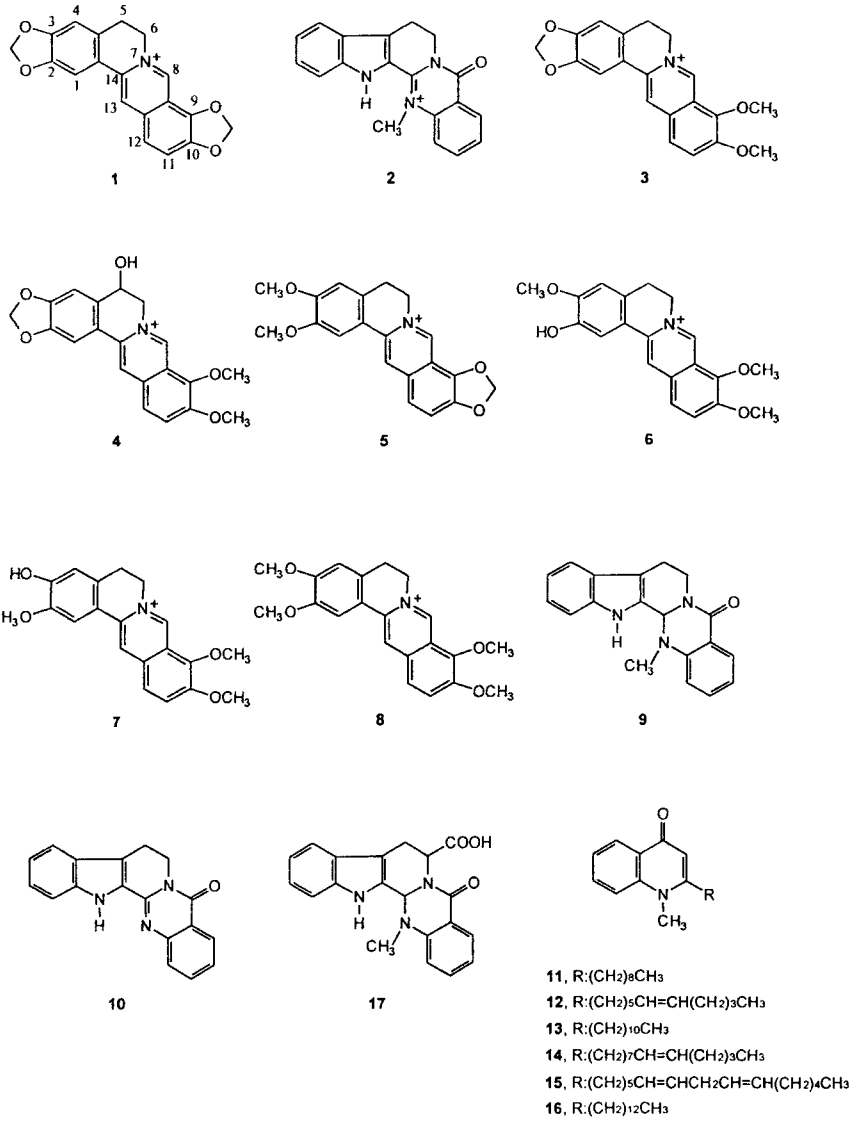

Figure 1. Structures of the seventeen alkaloids. **1**, coptisine; **2**, dehydroevodiamine; **3**, berberine; **4**, berberastine; **5**, epiberberine; **6**, columbamine; **7**, jatrorrhizine; **8**, palmatine; **9**, evodiamine; **10**, rutaecarpine; **11**, 1-methyl-2-nonyl-quinolone; **12**, 1-methyl-2-[(Z)-6-undecenyl]-4(1 H)-quinolone; **13**, 1-methyl-2-undecyl-4(1 H)-quinolone; **14**, evocarpine; **15**, 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1*H*)-quinolone; **16**, dihydroevocarpine; **17**, carboxyevodiamine.

solution of 50 mM maleic acid and 40 mM NaH_2PO_4 and acetonitrile (9:1); run time, 25 min. In MECC, the electrolyte was a buffer solution of 40 mM SDS, 20 mM NaH_2PO_4 and 9 mM $Na_2B_4O_7$ and acetonitrile (3:2); run time, 30 min. Before each run, the capillary was washed with 0.1 N NaOH for 5 min, with water for 5 min and then with buffer for 5 min.

Reagents and Materials

The alkaloids were isolated from Coptidis Rhizoma[3-7] and Evodiae Fructus[8-12] and their structures were elucidated on the basis of spectra such as IR, PMR, CMR and MS. The purities of these compounds were checked by HPLC. Maleic acid was purchased from Wako (Osaka, Japan), sodium dodecyl sulphate (SDS) and 18β-glycyrrhetinic acid from Sigma (St. Louis, MO), benzyltriethylammonium chloride from Merck (Darmstadt, Germany), sodium dihydrogenphosphate from Yoneyama (Osaka, Japan) and sodium borate from Kanto (Kyoto, Japan).

Acetonitrile and methanol were of LC grade (Fisons, Loughborough, England). Deionized water from a Milli-Q system (Millipore, Bedford, MA) was used to prepare all buffer and sample solutions. Coptidis Rhizoma and Evodiae Fructus were purchased from the Chinese herbal market in Taipei (Taiwan).

Preparation of Herb Couple and Chinese Herbal Preparation Extracts

A 1.0 g sample of the pulverized herb couple (containing 0.5 g Coptidis Rhizoma and 0.5 g Evodiae Fructus) was extracted with 70% methanol (6 mL) by stirring at room temperature for 30 min,, then centrifuging at 1500 g (Universal, Hettich Zentrifugen) for 5 min. Extraction was repeated three times. The extracts were combined and filtered through a No.1 filter paper. After adding a 5 mL aliquot of internal standard solution, IS_1, (20 mg of benzyltriethylammonium chloride in 1 mL of 70% methanol) for CZE, 2 mL aliquot of internal standard solution, IS_2, (2 mg of 18β-glycyrrhetinic acid in 1 mL of 70% methanol) for MECC, the extract was diluted to 25 mL with 70% methanol. This solution was passed through a 0.45 μm filter and the filtrate was then injected into the capillary electrophoresis system.

2.0 g of the constituent crude drugs of *Pien-tung-wan* (including 1.0 g Coptidis Rhizoma and 1.0 g Evodiae Fructus) was added with a twenty-fold mass of water (40 mL) and boiled mildly for 30 min until the volume becomes halved. After filtration while hot, to the filtrate was added suitable amounts of

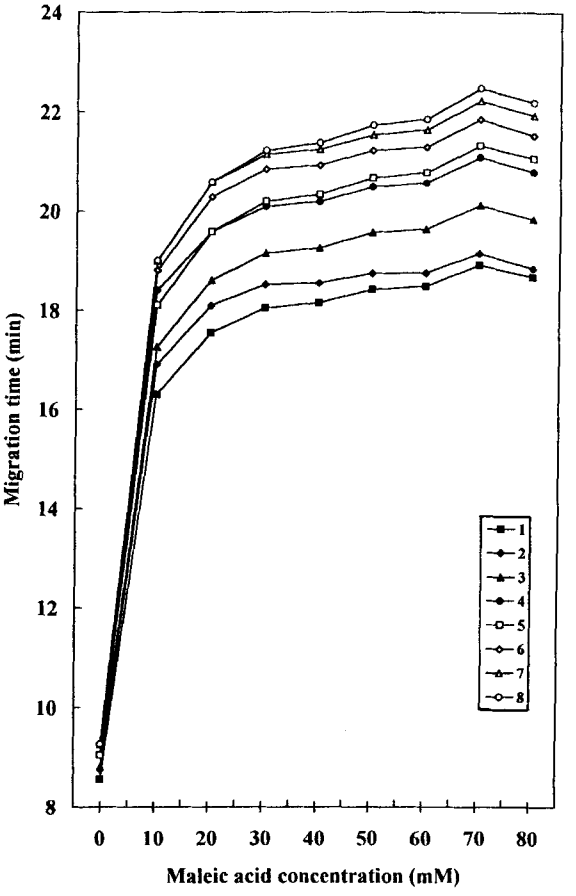

Figure 2. Effect of maleic acid concentration on migration time. All these experiments were conducted at a voltage of 25 kV across the 90 cm x 75 μm I.D. separating tube filled with phosphate buffers of different maleic acid concentrations containing 10% acetonitrile; temperature 24.5-25.0°C; detection wavelength, 254 nm. Symbols are the same as those in Fig. 1.

IS_1 and IS_2, and the resultant solution was diluted with methanol to give a 70% methanol solution. This solution was passed through a 0.45 μm filter and the filtrate was then injected into the capillary electrophoresis system. Similarly, a standard decoction of *Kan-lu-san* (including 2.0 g Coptidis Rhizoma and 1.0 g Evodiae Fructus) was also prepared in the same procedure.

RESULTS AND DISCUSSION

Analytical Conditions

The quaternary alkaloids in Coptidis Rhizoma have been well separated by a CZE technique with the use of acetate as counter ion.[13] However, a similar process was found to fail in the analysis of coptis-evodia herb couple. Under this condition (0.1 M CH_3COONa and methanol in the ratio of 85:15), the peak of dehydroevodiamine not only overlapped with berberine, but also exhibited serious tailing. The alkaloids in Evodiae Fructus were analyzed by two modes, CZE and MECC.[14] Following the evodia CZE condition (40 mM NaH_2PO_4 and acetonitrile in the ratio of 9:1), all evodia alkaloids were eluted along with EOF except for dehydroevodiamine (**2**) which was overlapped with coptis alkaloids; however, using the MECC condition (40 mM SDS, 20 mM NaH_2PO_4, 9 mM $Na_2B_4O_7$ and 40% CH_3CN), the evodia alkaloids (except **2**) in the herb couple were separated as well as those obtained with evodia crude drug itself, without interference. The migration times of those compounds were: **9**, 17.15 min; **10**, 17.47 min; **11**, 18.19 min; **12**, 18.35 min; **13**, 19.10 min; **14**, 19.39 min; **15**, 19.64 min; **16**, 20.30 min; **17**, 20.65 min.

In order to separate the quaternary alkaloids (**1-8**) in coptis-evodia herb couple, a new CZE method was developed with the addition of a dicarboxylic acid to the phosphate buffer in evodia's CZE system. Acetic acid and four dicarboxylic acids, tartaric acid, oxalic acid, succinic acid and maleic acid, had ever been used in this series of trials. From the experiments, maleic acid was found to be the best. Acetonitrile was added to the carrier as organic modifier to achieve a better resolved electropherogram. Other organic solvents such as methanol, ethanol and isopropanol had also been tried as substitutes for acetonitrile, but a much broader peak was obtained.

Effect of Maleic Acid Concentration

Nine electrolyte systems containing 40 mM NaH_2PO_4 and 10% CH_3CN at different maleic acid concentrations, ranging from 0 to 80 mM were used to study the effect of maleic acid concentration on the separability. The results obtained are shown in Figure 2, where the migration times are plotted against maleic acid concentrations. In Figure 2, the migration times of all quaternary alkaloids increased rapidly from 0 to 10 mM maleic acid, showing that there should be a strong interaction between a carboxylic group and a positive nitrogen atom. After that, the migration times were found to increase gradually with the increasing maleic acid concentrations. Usually, the migration times of

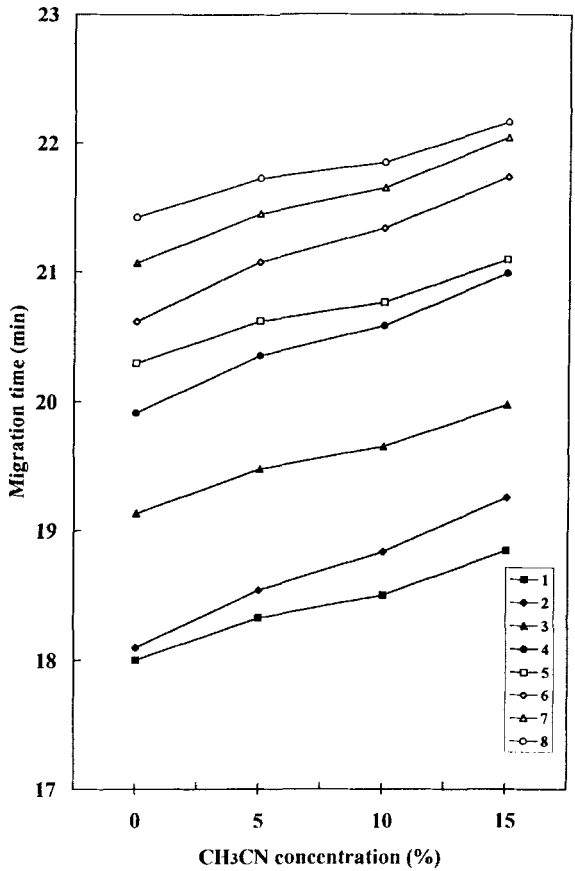

Figure 3. Effect of acetonitrile concentration on migration time. The carriers were 50 mM maleic acid-40 mM NaH_2PO_4 solutions containing 0-15% acetonitrile. Other conditions are the same as those in Fig. 2.

quaternary alkaloids in Coptidis Rhizoma were influenced much more by maleic acid concentration than in Evodiae Fructus. From the results, concentrations at 50 and 60 mM were found to be the best conditions for the separation of these eight alkaloids. However, 60 mM maleic acid gave more baseline noise and, therefore, 50 mM was chosen.

Effect of Acetonitrile Concentration

Four electrolyte systems, containing 50 mM maleic acid and 40 mM NaH_2PO_4 at different acetonitrile concentrations ranging from 0 to 15%, were

used to study the effect of the acetonitrile concentration on the resolution. The results obtained are given in Figure 3. As the acetonitrile concentration increased, the migration times of all quaternary alkaloids became longer, but the resolution of these compounds did vary. When acetonitrile was absent, the resolution value (Rs) between 7 and **8** was 1.19; however, that of **1** and **2** was only 0.50. At 5%, 10% and 15% acetonitrile, the Rs values between **1** and **2** were 1.24, 1.38 and 2.82, respectively, but were 1.35, 0.83 and 0.64 between **7** and **8**. From the calculated results, the best resolution for all the compounds was at 10% acetonitrile. Meanwhile, the addition of acetonitrile to the buffer could also result in a sharper peak and smoother baseline, which came from the reduction of interaction between the silanol group on the capillary wall and the quaternary alkaloids.

Effect of Phosphate Concentration

Six electrolyte systems, at different phosphate concentrations (10-60 mM sodium dihydrogenphosphate), were used to study the effect of phosphate concentration on the resolution. The number of theoretical plates and the resolution values between **1** and **2** and between **7** and **8,** obtained at different phosphate concentrations are listed in Table 1.

Data in Table 1 show that the numbers of theoretical plates for these eight alkaloids, especially those of **1**, **2** and **6**, varied markedly with the change of phosphate concentration of the buffer, and ideal plate numbers could be successfully achieved when the concentrations were higher than 30 mM. However, influence on resolution by changing the phosphate concentration was found to be reverse between **1-2** and **7-8**.

As the phosphate concentration was increased, the Rs values on **1-2** were increased, but that on **7-8** were decreased. In order to assay all the quaternary alkaloids simultaneously, a concentration of 40 mM was therefore selected.

From the above results, a buffer solution consisting of 50 mM maleic acid and 40 mM NaH_2PO_4 and acetonitrile (9:1) were chosen. Figure 4 is an electropherogram showing the separation of a mixture of the quaternary alkaloids with the following migration times: **1**, 19.39 min; **2**, 19.70 min; **3**, 20.69 min; **4**, 21.55 min; **5**, 21.70 min; **6**, 22.33 min; **7**, 22.71 min; **8**, 22.95 min.

Cyclodextrins (CD's) had also been added to the electrolyte in an attempt to get more improvement on resolution, but this approach failed. However, some information about the relationship between the structures of alkaloids and

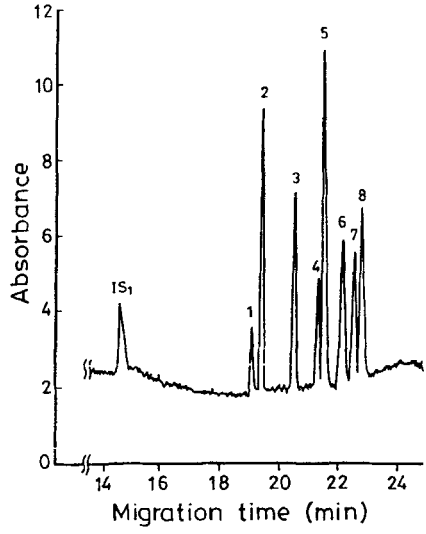

Figure 4. Capillary electropherogram of a mixture of eight quaternary alkaloids in CZE. IS_1 = benzyltriethylammonium chloride; other symbols are the same as those in Fig. 1.

Table 1

Theoretical Plates and Resolutions of the Alkaloids at Different Phosphate Concentrations

	No. of Theoretical Plates ($X10^{-5}$)								Resol'n (Rs)	
Conc'n (mM)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1-2**	**7-8**
10	1.30	0.83	0.27	0.93	0.75	0.16	0.91	0.63	0.80	1.02
20	1.17	1.20	0.44	1.51	1.12	1.06	1.56	0.70	0.92	1.09
30	0.68	0.75	0.38	1.45	1.08	1.02	1.52	0.91	0.96	0.99
40	1.00	1.39	0.44	1.48	1.14	0.83	1.10	0.82	1.38	0.83
50	1.17	1.25	0.41	1.66	1.12	0.82	1.63	0.95	1.61	0.75
60	1.45	1.39	0.49	1.58	1.41	0.95	1.54	1.00	1.93	0.70

the size of cyclodextrins was obtained. There was almost no influence on all compounds when α-CD was added, but a marked change on the migration times of **1**, **3**, **4**, **6** and **7** was observed when β- and γ-CD were used.

Table 2

Detection Limits and Reproducibility of Migration Time (Mt) and Peak Area Ratio (Ar, Peak Area/IS Area) (n=6) of the Alkaloids

Compound	Detection Limit ng (μg/mL)	Intra-Day RSD, %		Inter-Day RSD, %	
		Mt	Ar	Mt	Ar
1	0.06 (17.43)	0.73	2.33	1.96	3.59
2	0.11 (31.40)	0.77	3.10	1.34	3.17
3	0.06 (16.77)	0.67	3.24	1.68	3.76
4	0.05 (15~97)	0.86	2.89	1.46	4.38
5	0.06 (16.77)	0.87	3.71	1.52	3.71
6	0.06 (16.46)	0.91	2.33	1.53	3.31
7	0.06 (16.46)	0.95	2.77	1.55	3.28
8	0.05 (15.78)	0.95	2.75	1.62	3.07
9	0.13 (39.50)	0.69	2.07	1.52	2.48
10	0.16 (47.33)	0.69	1.67	1.53	2.00
11	0.12 (35.85)	0.75	2.60	1.51	3.46
12	0.12 (35.88)	0.79	2.92	1.51	3.68
13	0.12 (35.65)	0.83	3.33	1.46	4.00
14	0.12 (35.25)	0.89	2.39	1.46	2.68
15	0.12 (35.91)	0.91	3.53	1.47	3.88
16	0.12 (35.85)	0.92	2.69	1.41	2.93
17	0.06 (16.50)	0.66	3.57	1.36	3.78

Compound **1-8** were measured by CZE and **9-17** were determined by MECC.

Obviously, the alkaloids with a highly hydrophilic group on their 2,3-positions, and a dialkoxy group on their 9,10-positions, and the CD with a large-size cavity are the paramount factors for forming a reasonable complex.

METHOD VALIDATION

Precision

The reproducibility (relative standard deviation) of the proposed method, on the basis of peak-area ratios for six replicate injections, was 1.67-4.00% in

Table 3

Data of Linear Ranges and Correlation Coefficients (r) of the Alkaloids

Compound	Linear Range (mg/mL)	Slope	Intercept	r
1	0.016-0.492	14.024	0.434	0.9954
2	0.076-1.216	3.750	0.086	0.9993
3	0.067-2.138	13.495	0.417	0.9950
4	0.015-0.116	13.221	0.147	0.9962
5	0.022-0.350	13.763	0.149	0.9963
6	0.012-0.198	15.790	0.171	0.9962
7	0.012-0.197	13.508	0.147	0.9963
8	0.023-0.720	12.948	0.140	0.9960
9	0.089-0.890	2.611	0.353	0.9999
10	0.085-0.850	2.180	0.102	0.9999
11	0.013-0.130	3.803	0.006	0.9990
12	0.011-0.110	3.485	0.005	0.9985
13	0.017-0.170	3.463	0.005	0.9982
14	0.071-0.710	3.197	0.005	0.9987
15	0.016-0.160	2.969	0.004	0.9991
16	0.021-0.210	3.178	0.005	0.9989
17	0.041-0.410	3.567	0.064	0.9997

Compound 1-8 were measured by CZE and 9-17 were determined by MECC.

MECC and 2.33-4.38% in CZE, and the variation of the migration time of each peak was less than 2% (n=6). The detection limits (S/N=3) for the quaternary alkaloids were 0.05-0.16 ng (15.78-47.33 μg/mL, column inner diameter 75 μm). Detailed data for R. S. D. and detection limits are shown in Table 2.

Linearity

Calibration graphs for the seventeen alkaloids were obtained over a range of one order of magnitude of concentration. Results of the regression analyses and the correlation coefficients (r) are shown in Table 3. The results showed good linear relationships between peak-area ratios (y) and concentration (x, mg/mL).

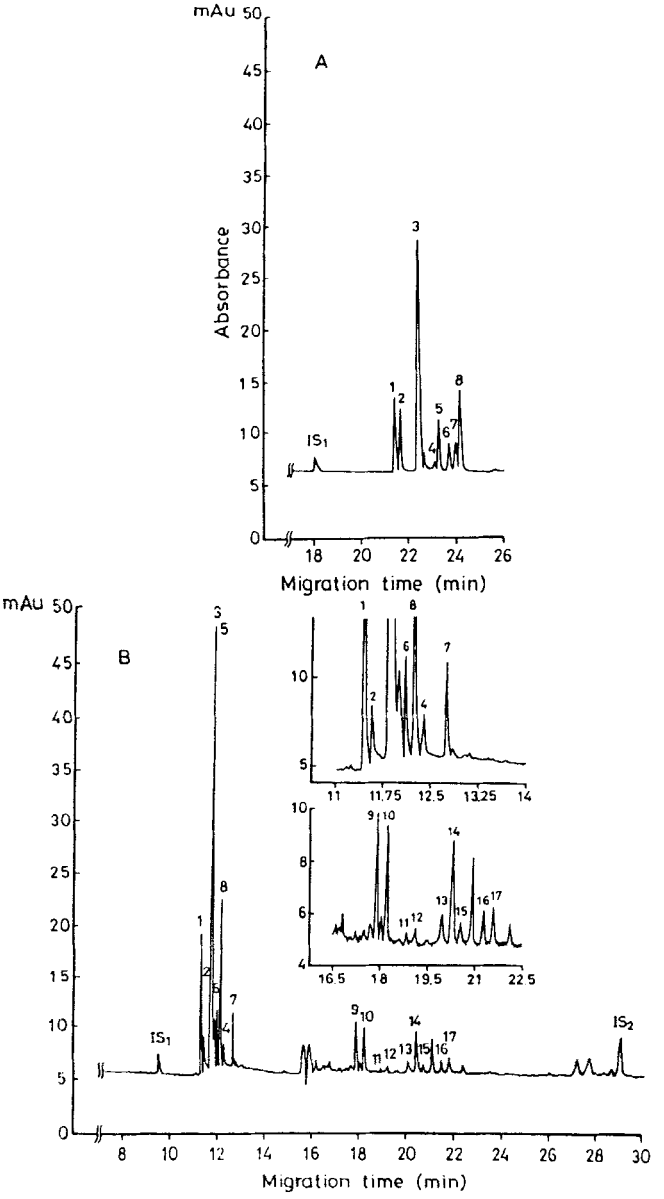

Figure 5. Capillary electropherograms of the coptis-evodia herb couple in (A) CZE and (B) MECC. IS_2 = 18β-glycyrrhetinic acid and other peaks as in Fig. 4.

Accuracy

Suitable amounts (0.01-10.45 mg) of the seventeen alkaloids were added to a sample of coptis-evodia herb couple of known alkaloid content and the mixture was analysed using the proposed procedure.

The recoveries of the alkaloids were 96.68-103.19% in MECC, and 99.65-103.28% in CZE.

Separation of 1, 4, 6, 7 and 8 with MECC; an Alternative Route

Although compounds **1-8** had better to be analyzed by CZE method, Figure 5-B shows clearly that **1**, **4**, **6**, **7** and **8** could also be determined by MECC technique. After comparing the electropherograms in Figure 5-A and 5-B, it was found that MECC provided not only sharper peaks but also shorter migration times than that of CZE. In order to investigate the differences obtained from CZE and MECC systems, the following data were calculated. The Rs values of **1-2, 6-8** and **4-8** were 2.10, 2.09 and 2.00, and the numbers of theoretical plate of **1**, **4**, **6**, **7** and **8** were 541945, 166641, 627312, 317957 and 285733, respectively. The detection limits were 0.10 ng (30.36 μg/mL) for **1**, 0.08 ng (23.96 μg/mL) for **4**, 0.08 ng (23.01 μg/mL) for **6**, 0.08 ng (23.25 μg/mL) for **7**, 0.08 ng (24.18 μg/mL) for **8**. Regression equations and correlation coefficients were calculated as follows: **1**, y=15.695x-0.175 (r=0.9995); **4**, y=14.060x-0.035 (r=0.9996); **6**, y=1 4.773x-0.038 (r=0.9993); **7**, y=14.491x-0.035 (r=0.9995); **8**, y=13.894x-0.034 (r=0.9995).

The recoveries were 98.52-103.58%. These data indicate that MECC method could give higher theoretical plate numbers and Rs values, but has lower detection limits than that of CZE. As a result, CZE was able to analyze all the quaternary alkaloids **1-8** simultaneously, and MECC was suitable for the determination of **1**, **4**, **6**, **7**, **8** and **9-17** in a single run.

Determination of the Alkaloids in Herb Couple and Chinese Herbal Preparations

When the test solution of the herb couple was analysed by CE under the selected conditions, the electropherograrns shown in Figure 5-A and B were obtained. The peaks were identified by comparison of the migration times and by spiking the mixture with a single alkaloid in a subsequent run. By substituting the peak-area ratios of the individual peaks for y in the above

Table 4

Contents (mg/g Crude Drug) of the Alkaloids in the Herb Couple and the Chinese Herbal Preparations (Mean±SD; n=3)

Compound	Herb Couple	*Pien-tung-wan*	*Kan-lu-san*
1	7.43±0.14	4.69±0.27	5.32±0.36
2	17.34±1.00	7.59±0.23	6.35±0.12
3	32.47±0.80	12.17±0.35	17.11±0.39
4	1.24±0.11	1.32±0.02	1.07±0.02
5	4.89±0.27	2.58±0.22	3.11±0.17
6	2.77±0.18	1.86±0.19	1.89±0.22
7	2.76±0.17	2.00±0.22	1.89±0.06
8	10.07±0.52	4.21±0.26	5.93±0.20
9	6.56±0.05	-	-
10	10.15±0.21	-	-
11	0.43 ±0.03	-	-
12	0.77±0.07	-	-
13	1.33 ±0.03	-	-
14	4.96±0.24	-	-
15	1.29±0.14	-	-
16	1.19±0.08	-	-
17	2.38±0.08	-	-

-: The content was below detection limit. Compound **1-8** were measured by CZE and **9-17** were determined by MECC.

equations, the contents of the individual alkaloids in the coptis-evodia herb couple were calculated. Alkaloid contents in the herb couple containing Chinese herbal preparations such as *Pien-tung-wan* and *Kan-lu-san* could also be determined by the same procedure.

The results are given in Table 4. Data in Table 4 show that there are only the water-soluble components, quaternary alkaloids, present in the preparation decoction. Those alkaloids (**9-17**) that were water insoluble or practically insoluble were absent or existed in the amount below detection limits.

This work has successfully demonstrated that, by optimizing parameters such as maleic acid, phosphate and acetonitrile concentration of the electrophoretic media, high resolution separation of a complicated mixture can

easily be achieved. Furthermore, the results obtained indicate that the CZE method proposed for the separation and determination of quaternary alkaloids may be conveniently used for quality assurance of commercially available samples of some Chinese herbal preparations that contain coptis and evodia and also for quality control in pharmaceutical factories.

ACKNOWLEDGEMENT

Financial support from the National Science Council, Republic of China, is gratefully acknowledged (NSC 85-2113-M-003-008).

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Received April 14, 1996
Accepted May 28, 1996
Manuscript 4141