



Optimization of micellar electrokinetic capillary chromatography method using central composite design for the analysis of components in Yangwei granule*

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Received Apr. 14, 2010; Revision accepted May 31, 2010; Crosschecked Jan. 26, 2011

Abstract: Central composite design (CCD), together with multiple linear regression, was successfully used to optimize the electrophoretic buffer system of micellar electrokinetic capillary chromatography (MEKC) for the determination of albiflorin, paeoniflorin, liquiritin, and glycyrrhizic acid in the traditional Chinese medicine (TCM) prescription, Yangwei granule. Concentrations of sodium deoxycholate (SDC) and borate, and proportions of ammonia, acetonitrile, and methanol were optimized. The total resolutions of peaks between the analytes and their adjacent peaks in real samples were integrated into the evaluation index of separation efficiency. The optimum electrophoretic buffer contained 80 mmol/L SDC, 20 mmol/L borate, 5% (v/v) methanol, 0.5% (v/v) ammonia, and 5% (v/v) acetonitrile. The correlation coefficients (R^2) between the peak areas and the corresponding concentrations of analytes were greater than 0.9956. The limits of detection (LODs) ($S/N=3$) of the analytes were 0.97–4.00 $\mu\text{g/ml}$. The results indicate the superiority of CCD in optimizing the separation conditions of complex samples such as TCM prescriptions.

Key words: Micellar electrokinetic capillary chromatography, Traditional Chinese medicine, Central composite design, Yangwei granule

doi:10.1631/jzus.B1000135

Document code: A

CLC number: R917

1 Introduction

Yangwei granule, a traditional Chinese medicine (TCM) prescription, is composed of eight herbs, including Radix Astragali, Radix Codonopsis, Radix Paeoniae Alba, Radix et Rhizoma Glycyrrhizae, Pericarpium Citri Reticulatae, Rhizoma Cyperi, Fructus Mume, and Rhizoma Dioscoreae. It is commonly used in China in the treatment of stomach ache and chronic atrophic gastritis (Wang *et al.*, 2006). Albiflorin and paeoniflorin (Fig. 1) are two of the active components in Radix Paeoniae Alba, and

liquiritin and glycyrrhizic acid (Fig. 1) are two of the active constituents in Radix et Rhizoma Glycyrrhizae (Gu *et al.*, 2006; Feng *et al.*, 2008). Thus, determination of these active components in Yangwei granule is required for its quality evaluation.

In the evaluation of TCM quality, high-performance liquid chromatography (HPLC) is commonly used as a powerful analytical technique. Wang *et al.* (2006) determined albiflorin, paeoniflorin, benzoylalbiflorin, and gallic acid in Yangwei granule by HPLC in more than 40 min. Capillary electrophoresis (CE) has become a powerful technique in natural product analysis, as well as TCM analysis, because of its high efficiency, short analysis time, and low solvent and sample consumptions. As an important mode of CE, micellar electrokinetic capillary chromatography (MEKC) has great advantages in the analysis of TCM. It has been used for analyses of

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* Project supported by the National Science and Technology Major Project of Ministry of Science and Technology of China (No. 2009ZX09313-036) and the Program for New Century Excellent Talents in University of Ministry of Education of China (No. NCET-06-0515)

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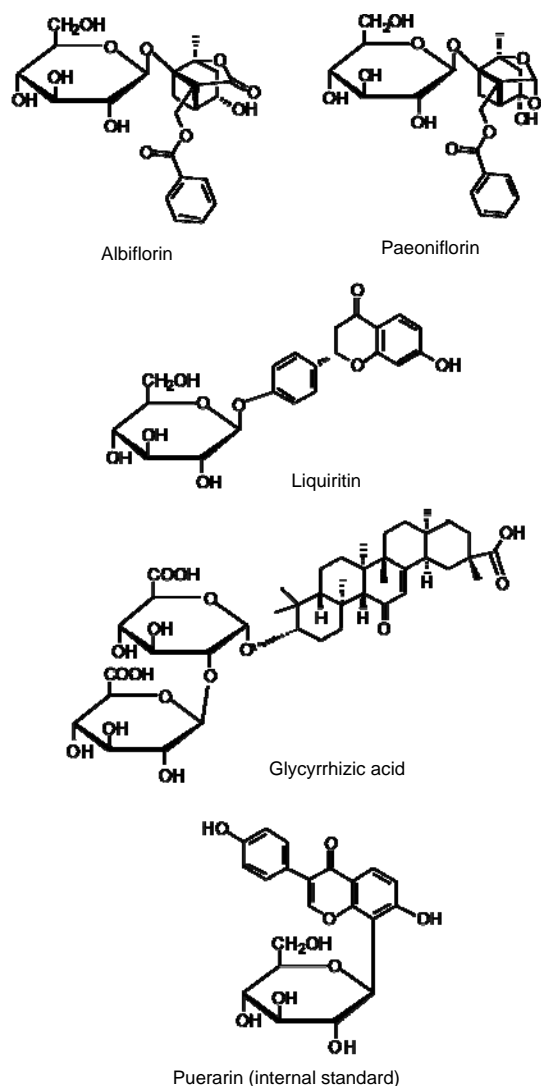


Fig. 1 Structures of the analytes and internal standard

albiflorin, paeoniflorin, benzoylalbiflorin, paeonol, and oxypaeoniflorin in *Radix Paeoniae Alba* (Wu and Sheu, 1996). As many factors have effects on the separation of MEKC and there are interactions between some factors, it is usually difficult to rapidly achieve the optimal separation efficiency by step-by-step procedure, i.e., by changing one factor at a time while keeping the others constant (Lin, 2006). Thus, the selection of a suitable method to optimize the separation conditions is important. There are many approaches available for the optimization of separation conditions, such as experimental design coupled with response surface method, multivariate analysis, or artificial neural network method (Ben Hamed *et al.*, 2006; Lin, 2006; Shaban *et al.*, 2006; Hanrahan

et al., 2008; Liu *et al.*, 2009). Central composite design (CCD), one of experimental design methods, has been widely used to optimize the separation conditions of CE, owing to its simple procedure, fewer experimental runs, and its advantage in the full evaluation on interactions of factors (Lin, 2006; Liu *et al.*, 2006; Yu *et al.*, 2006a; 2006b; 2006c; 2007; Liu *et al.*, 2008). Although several papers using CCD to optimize the separation conditions of CE have been published, many problems still exist. First, in many papers, the optimum separation conditions were obtained from the response surface (Servais *et al.*, 2004). From the CCD results, regression equations reflecting the relationship between the responses (Y) and the factors (X) are obtained. Response surface is just the visualization of the equation. From this point of view, the optimum separation conditions are actually obtained from the equations, but not the response surface. Second, many authors used standard solutions in experimental designs to obtain an optimum separation, but this optimum condition may not separate the target peak from the adjacent peaks in complex real samples such as TCM prescriptions (Liu *et al.*, 2008).

In the present work, a simple, accurate and reproducible MEKC method was developed for the determination of albiflorin, paeoniflorin, liquiritin, and glycyrrhizic acid in Yangwei granule. Although an HPLC method had been established for the quality control of Yangwei granule by Wang *et al.* (2006), the HPLC method just determined four components of *Radix Paeoniae Alba* (Wang *et al.*, 2006). Here, the established MEKC method could determine components of both *Radix Paeoniae Alba* and *Radix et Rhizoma Glycyrrhizae*.

2 Materials and methods

2.1 Chemicals

Albiflorin and paeoniflorin were isolated from *Radix Paeoniae Alba* in our lab. The structures of these isolated standards were confirmed on the basis of spectra data such as mass spectrometry (MS) and nuclear magnetic resonance (NMR), and their purities checked by HPLC were over 98%. Liquiritin, glycyrrhizic acid, and puerarin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China).

Sodium deoxycholate (SDC), disodium tetraborate decahydrate, and ammonia were of analytical grade; acetonitrile and methanol were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli-Q Plus system (Millipore, Molsheim, France). Yangwei granule and eight herbs, including Radix Paeoniae Alba, Radix et Rhizoma Glycyrrhizae, Pericarpium Citri Reticulatae, Radix Codonopsis, Rhizoma Dioscoreae, Fructus Mume, Rhizoma Cyperi, and Radix Astragali, were provided by Chiatai Qingchunbao Pharmaceutical Co., Ltd., Hangzhou, China.

2.2 Instrumentation and electrophoretic procedure

All MEKC separations were performed on an HP^{3D} CE system (Agilent Technologies, Waldbronn, Germany), equipped with a diode array detector (DAD) and an Agilent ChemStation software. A fused-silica capillary (Φ 60 cm \times 75 μ m, 51.5 cm effective length; Yongnian Photoconductive Fiber Factory, Hebei, China) was used throughout this experiment.

An electrophoretic buffer containing 80 mmol/L SDC, 20 mmol/L borate, 5% (v/v) acetonitrile, 5% (v/v) methanol, and 0.5% (v/v) ammonia was prepared for the electrophoretic analysis. The new capillary was pre-conditioned by flushing with acetonitrile, 0.1 mol/L hydrochloric acid, deionized water, 1 mol/L NaOH, deionized water, and electrophoretic buffer for 10, 10, 5, 10, 5, and 10 min, respectively. Each day, the capillary was first conditioned by flushing with deionized water, 0.1 mol/L NaOH, deionized water, and electrophoretic buffer for 2, 10, 5, and 10 min, respectively. To obtain good reproducibility, the capillary was flushed with deionized water, 0.1 mol/L NaOH, deionized water, and electrophoretic buffer for 1, 1, 1, and 3 min, respectively, between every run. Each pair of running vials (inlet and outlet) was used for no more than four runs. The positive voltage was 20 kV and the capillary temperature was 25 °C. The sample was injected under 5 kPa pressure for 5 s and the detection wavelength was 254 nm. The running time was 25 min.

2.3 Preparation of standard solution

Albiflorin and paeoniflorin were dissolved in deionized water at 6.03 and 10.24 mg/ml, respectively, as stock solutions. Liquiritin and glycyrrhizic acid were dissolved in methanol at 6.07 and 6.16 mg/ml,

respectively, as stock solutions. Puerarin (internal standard) was dissolved in deionized water at 0.093 mg/ml as a stock solution. The standard stock solutions were diluted to the desired concentration with 25% (v/v) methanol aqueous solution. Then the mixture solutions of standards were mixed with puerarin solution in the ratio of 4:1 (v/v) before use.

2.4 Sample preparation

Yangwei granule was ground to powder, then 0.5 g powder was mixed with 5 ml 25% (v/v) methanol aqueous solution and ultrasonic extraction was performed at room temperature for 15 min. After centrifugation at 13 000 r/min for 15 min, the supernatant was mixed with puerarin solution (internal standard) in the ratio of 4:1 (v/v) for the electrophoretic analysis.

2.5 Statistical software package

The statistical software packages for experimental design and result analysis were Minitab system release version 14 for windows.

3 Results and discussion

3.1 Optimization of capillary electrophoresis conditions

3.1.1 Preliminary investigation

As albiflorin and paeoniflorin have similar structures, the separation of the two components was difficult. Many electrophoretic buffer systems, including borate, phosphate, and borate-phosphate containing sodium dodecyl sulfate (SDS) or SDC, had been tested. It was found that surfactant had decisive effects on the separation of albiflorin and paeoniflorin. Only the electrophoretic buffer containing SDC allowed baseline separation of albiflorin and paeoniflorin, while the electrophoretic buffer containing SDS cannot separate them at all. Therefore, borate-phosphate containing SDC electrophoretic buffer was selected. Furthermore, it was found that the separation was not good when the electrophoretic buffer contained only acetonitrile or methanol. Thus, acetonitrile and methanol were both added into electrophoretic buffer as the organic modifiers to improve separation and make the peak shape better. Ammonia and NaOH solutions were both attempted to adjust pH

of electrophoretic buffer. The electrophoretic buffer adjusted with ammonia could separate the four analytes well, whereas the buffer adjusted with NaOH could not separate glycyrrhizic acid from adjacent peaks. Therefore, ammonia was selected to adjust the pH of electrophoretic buffer.

Preliminary experiments were carried out to study the effects of factors on the separation. The factors included concentrations of SDC and borate, proportions of ammonia, acetonitrile, and methanol, temperature, and voltage. Out of the seven factors, the former five factors had pronounced effects on the separation. Then CCD experiments were designed with the five factors and five levels (Table 1).

3.1.2 Optimization of the separation condition by central composite design

Experimental design for CCD was shown in Table 1. The total experimental design contained 32 experiments, which included six additional experiments at the centre point to estimate the repeatability of operation. The experiments were performed in random order to avoid systematic error. Yangwei granule extract was used as the sample during the CCD experiments and the total resolutions (R_s) between peaks of albiflorin, paeoniflorin, liquiritin, and glycyrrhizic acid, and their adjacent peaks were chosen as the evaluation indices or the responses.

Table 1 Optimization method parameters for central composite design and response results for resolutions

Run	SDC (mmol/L)	Borate (mmol/L)	Methanol (% v/v)	Ammonia (% v/v)	Acetonitrile (% v/v)	R_s
1	50	12.5	12.5	0.75	7.5	2.58
2	50	17.5	7.5	0.75	7.5	3.79
3	50	12.5	7.5	0.75	12.5	2.32
4	60	15.0	10.0	0.50	10.0	3.01
5	70	17.5	7.5	1.25	7.5	6.23
6	70	12.5	7.5	1.25	12.5	3.73
7	60	12.5	12.5	1.25	12.5	6.91
8	70	17.5	12.5	0.75	7.5	5.30
9	80	15.0	10.0	1.00	10.0	3.64
10	60	15.0	10.0	1.50	10.0	5.84
11	60	15.0	15.0	1.00	10.0	2.76
12	60	15.0	10.0	1.00	5.0	3.58
13	50	17.5	12.5	0.75	12.5	1.88
14	50	17.5	12.5	1.25	7.5	3.18
15	50	17.5	7.5	1.25	12.5	7.99
16	60	15.0	10.0	1.00	15.0	3.48
17	70	17.5	12.5	1.25	12.5	3.78
18	40	15.0	10.0	1.00	10.0	5.07
19	70	12.5	7.5	0.75	7.5	4.27
20	70	12.5	12.5	1.25	7.5	4.82
21	60	10.0	10.0	1.00	10.0	6.15
22	50	12.5	7.5	1.25	7.5	6.78
23	70	17.5	7.5	0.75	12.5	3.93
24	70	12.5	12.5	0.75	12.5	5.04
25	60	20.0	10.0	1.00	10.0	6.43
26	60	15.0	5.0	1.00	10.0	4.70
27-32	60	15.0	10.0	1.00	10.0	3.82

SDC: sodium deoxycholate; R_s : resolution

The results of CCD experiments were analyzed and the corresponding analysis of variance (ANOVA) was performed to choose factors for establishing a multiple linear regression model. The ANOVA results were shown in Table 2. The factors which had a P value less than 0.05 were considered having significant effects on the separation efficiency and were chosen to establish the regression equation related to the evaluation index of separation efficiency. Though the P values of SDC, methanol, and acetonitrile were greater than 0.05, the interactions of these factors had significant effects on the separation efficiency. Therefore, they were included in the regression model.

The experimental results were fitted to a multiple linear regression model described as follows:

$$Rs=f(A, B, C, D, E) \\ =-18.6828+0.3468A-1.8370B+0.9368C+ \\ 31.2633D+1.0103E+0.0948B^2+0.0178AC- \\ 0.3567AD-0.0173AE-0.1005BC-0.6530CD.$$

The coefficient (r) of multiple linear model was 0.9247, which demonstrated that the multiple linear model was good. In the equation, A and B were the concentration of SDC and borate, respectively, and C , D , and E were the proportions of methanol, ammonia, and acetonitrile, respectively.

By using the Minitab program, the optimized condition was obtained when the Rs had the maximum value ($Rs=9.87$). The optimized CE condition was: 80 mmol/L SDC, 20 mmol/L borate, 5% (v/v) methanol, 0.5% (v/v) ammonia, and 5% (v/v) acetonitrile. Keeping three factors fixed at their optimum, the response surface plots (Fig. 2) were made, which showed significant interactions between the other two factors. The concentrations of SDC and borate had positive effects on Rs . On the contrary, the proportions of methanol and acetonitrile had negative effects on Rs .

Table 2 Results from the ANOVA of factors contributing to the mathematical model

Effect	P	Effect	P
Constant	0.927	$A \times B$	0.286
A: SDC	0.698	$A \times C$	0.027
B: borate	0.036	$A \times D$	0.000
C: methanol	0.498	$A \times E$	0.031
D: ammonia	0.010	$B \times C$	0.004
E: acetonitrile	0.584	$B \times D$	0.544
$A \times A$	0.383	$B \times E$	0.869
$B \times B$	0.001	$C \times D$	0.039
$C \times C$	0.768	$C \times E$	0.112
$D \times D$	0.318	$D \times E$	0.164
$E \times E$	0.504		

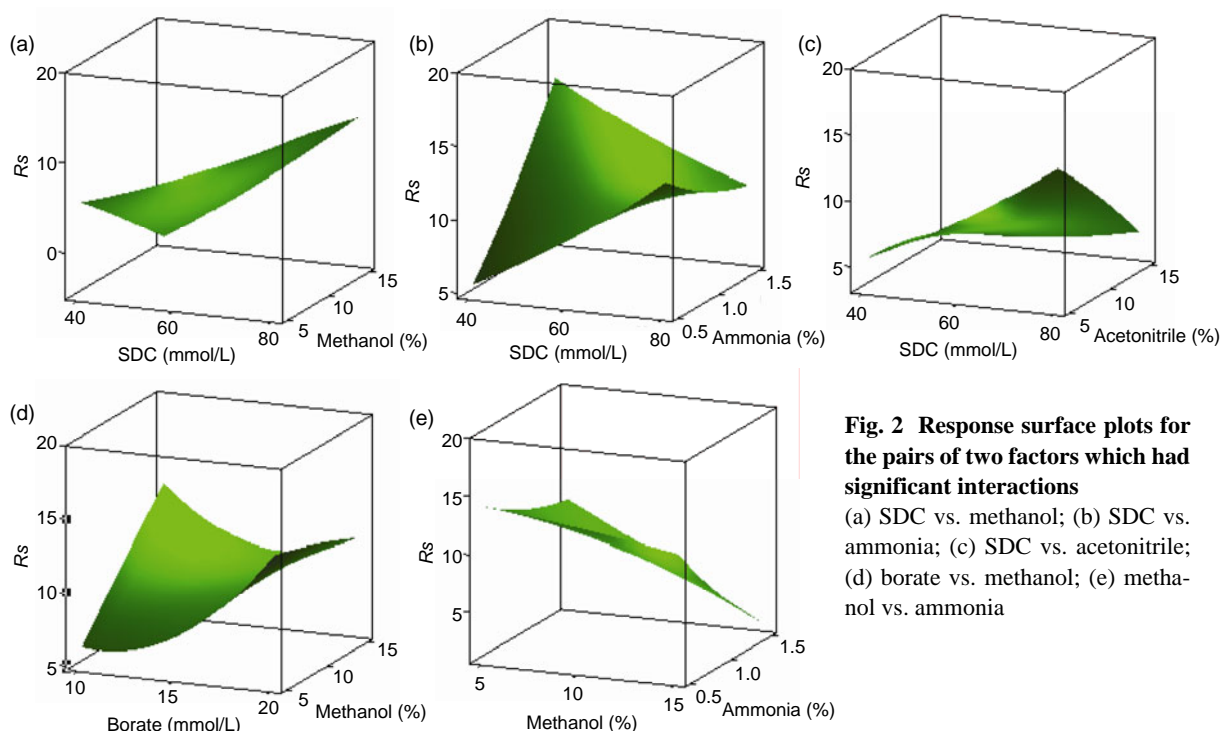


Fig. 2 Response surface plots for the pairs of two factors which had significant interactions

(a) SDC vs. methanol; (b) SDC vs. ammonia; (c) SDC vs. acetonitrile; (d) borate vs. methanol; (e) methanol vs. ammonia

As shown in Fig. 2b, there was a rather significant interaction between SDC and ammonia. When the SDC concentration was 40 mmol/L, R_s increased significantly with the increasing of ammonia concentration; however, it decreased slightly with the increasing of ammonia concentration when the SDC concentration was 80 mmol/L.

As shown in Table 1, it was apparent that the optimum condition was neither the one at the 15th run that had the biggest R_s in CCD experiments, nor the one at the centre point that had the best separation efficiency obtained by use of the step-by-step procedure. The electropherograms of Yangwei granule extract under the optimum condition predicted by the established equation, the condition with the biggest R_s in CCD runs, and the condition at the centre point in CCD runs are shown in Fig. 3. It was clear that the optimized condition provided better separation and shorter analysis time.

3.2 Validation of the method

3.2.1 Linearity and limit of detection

The linearity of every analyte was calculated by plotting the relative peak area (Y) (the ratio of the analyte to internal standard) versus the concentration (X) of the analyte. The linearity results and limits of detection (LODs) of analytes are shown in Table 3, which indicates good correlations between the peak area ratios of analytes and their concentrations and higher sensitivity.

3.2.2 Precision and accuracy

The injection precision, intra-day precision, and inter-day precision were expressed as relative standard deviations (RSDs) of both relative peak areas and migration times. The injection precision was conducted by continuously injecting the same sample for six times. The injection precisions of the analytes were below 1.2% and 3.1% for the relative migration time and the relative peak area, respectively.

The intra-day precision was detected through analyzing six parallel samples extracted by the same procedures in a day, while the inter-day precision was performed by analyzing six parallel samples extracted everyday over 6 d. The intra-day precisions of the analytes were 0.95%–1.30% and 1.6%–4.3% for the relative migration time and the contents of analytes, respectively. The inter-day precisions of the analytes were 1.0%–2.4% and 4.0%–4.9% for the relative migration time and the contents of analytes, respectively.

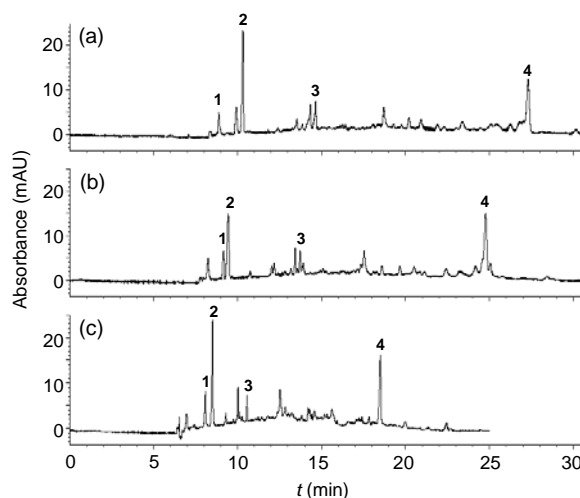


Fig. 3 Electropherograms of Yangwei granule extract with different buffer systems

(a) Buffer systems at the centre point: 60 mmol/L SDC, 15 mmol/L borate, 10% (v/v) methanol, 1% (v/v) ammonia, and 10% (v/v) acetonitrile; (b) buffer systems which had the biggest R_s in the CCD test: 50 mmol/L SDC, 17.5 mmol/L borate, 7.5% (v/v) methanol, 1.25% (v/v) ammonia, and 12.5% (v/v) acetonitrile; (c) the optimum buffer systems predicted by the established mathematical model obtained from CCD results: 80 mmol/L SDC, 20 mmol/L borate, 5% (v/v) methanol, 0.5% (v/v) ammonia, and 5% (v/v) acetonitrile. The other electrophoresis conditions were: capillary, Φ 60 cm \times 75 μ m, 51.5 cm effective length; positive voltage, 20 kV; temperature, 25 $^{\circ}$ C; pressure injection, 5 kPa \times 5 s; detection wavelength, 254 nm. Symbols: **1**, albiflorin; **2**, paeoniflorin; **3**, liquiritin; **4**, glycyrrhizic acid

Table 3 Results of linearity and LODs of analytes

Analyte	Regression equation	Linear range (μ g/ml)	R^2	LOD (μ g/ml)
Albiflorin	$Y=0.0025X-0.0009$	$9.04-4.52\times 10^2$	0.9993	2.71
Paeoniflorin	$Y=0.0025X+0.0168$	$9.11-9.11\times 10^2$	0.9998	2.73
Liquiritin	$Y=0.0070X+0.0230$	$9.10-4.55\times 10^2$	0.9990	0.97
Glycyrrhizic acid	$Y=0.0071X+0.0633$	$9.24-4.62\times 10^2$	0.9956	4.00

Y : relative peak area; X : concentration; R^2 : correlation coefficient; LOD: limit of detection ($S/N=3$)

The recovery experiments were performed by adding accurately an amount of the four standards into 0.25 g powder of Yangwei granule, and then the sample was extracted according to the procedure of sample preparation. The average recoveries for albiflorin, paeoniflorin, liquiritin, and glycyrrhizic acid were 96.5%–104.0% with the RSD values below 4.4%.

3.2.3 Specificity

The specificity experiment was conducted by analyzing the negative samples using the optimized condition. The two negative samples were the extracts of all the seven medical materials except *Radix Paeoniae Alba* or *Radix et Rhizoma Glycyrrhizae*, and were prepared according to the preparation technology of the Yangwei granule. The electropherograms of the negative samples were shown in Fig. 4. Obviously, there was no interference peak at the position of target peak, which indicates excellent specificity of the MEKC method.

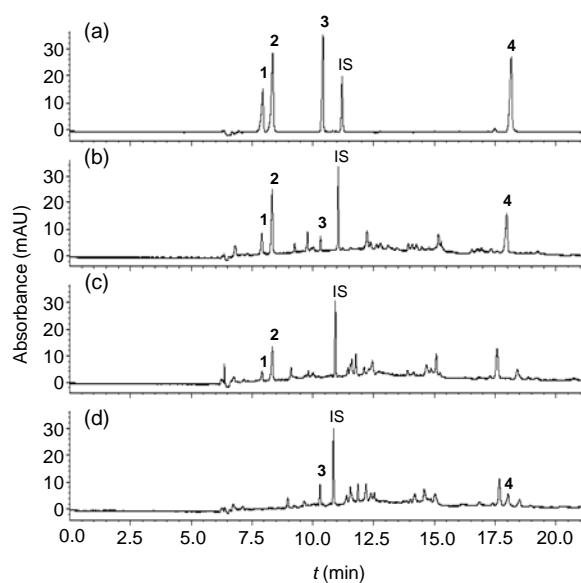


Fig. 4 Electropherograms of specificity experiments with the optimum buffer systems

(a) The standard solution; (b) Yangwei granule extract; (c) The negative sample extract missing *Radix et Rhizoma Glycyrrhizae*; (d) The negative sample extract missing *Radix Paeoniae Alba*. Electrophoresis conditions: 80 mmol/L SDC, 20 mmol/L borate, 5% (v/v) methanol, 0.5% (v/v) ammonia, and 5% (v/v) acetonitrile; The other electrophoresis conditions were: capillary, Φ 60 cm \times 75 μ m, 51.5 cm effective length; positive voltage, 20 kV; temperature, 25 $^{\circ}$ C; pressure injection, 5 kPa \times 5 s; detection wavelength, 254 nm. Symbols: 1: albiflorin; 2: paeoniflorin; 3: liquiritin; 4: glycyrrhizic acid; IS: puerarin (internal standard)

3.3 Analysis of Yangwei granule

The developed MEKC method was applied to the analysis of active components in Yangwei granule. The peaks of components in Yangwei granule extract were identified by comparing the migration time and ultraviolet (UV) spectra of the peaks with those of the standards and by spiking with standard solutions. The known components of the eight herbs were tested but only the four components of *Radix Paeoniae Alba* and *Radix et Rhizoma Glycyrrhizae* were detected in Yangwei granule as shown in Fig. 4. The components in Yangwei granule ($n=6$) were (2.50 \pm 0.04) mg/g for albiflorin, (7.26 \pm 0.31) mg/g for paeoniflorin, (0.38 \pm 0.01) mg/g for liquiritin, and (2.65 \pm 0.09) mg/g for glycyrrhizic acid. The main active components of the other six herbs, such as astragaloside IV, astragaloside I, astragaloside II, and isoastragaloside II in *Radix Astragali*, naringin, naringenin, hesperidin, and hesperetin in *Pericarpium Citri Reticulatae*, allantoin and diosgenin in *Rhizoma Dioscoreae*, ursolic acid and oleanolic acid in *Fructus Mume*, apigenin, ferulic acid, and vanillic acid in *Radix Codonopsis*, and physcione in *Rhizoma Cyperi*, were not detected in Yangwei granule.

4 Conclusions

In this study, CCD was successfully used to optimize MEKC conditions for the analyses of four components in Yangwei granule. The evaluation index of separation efficiency that considered the total resolutions of peaks between the analytes and their adjacent peaks in the real sample was used to predict the optimal separation condition. The results indicate that the method developed was accurate, simple, and reproducible. This study reveals that CCD is an effective tool in optimizing the separation conditions of complex samples such as TCM prescriptions.

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