

Article

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A comparative study on analytical method of total alkaloids from cortex *Phellodendri amurens* by reversed phase high performance liquid chromatography (RP-HPLC) and pressurized capillary electrochromatography (pCEC)

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Abstract: A pressurized capillary electrochromatography (pCEC) method with post-column detection cell has been developed for the analysis of total alkaloids of cortex *Phellodendron amurense* Rupr., Rutaceae. The separation of total alkaloids (berberine, palmatine, oatrorrhizine, magnoflorine, phellodendrine, candicine, menisperine) was optimized by compositions of the mobile phase, ionic strength of buffers, pH value, and applied voltage. Separation of total alkaloids was achieved within 11 min by using a mobile phase of Na₂HPO₄-citric acid solution-acetonitrile (pH 4.00; 3 mM) (60:40, v/v) and applying a voltage of -10 kV. This method showed satisfactory retention times and peak shapes. Meanwhile, a reversed phase high performance liquid chromatography (RP-HPLC) has also been established for the separation of total alkaloids extracted from cortex *Phellodendri amurens*. Baseline separation of total alkaloids was achieved within 25 min by using a mobile phase of acetonitrile-0.1% phosphoric acid with 0.1 g sodium dodecanesulphonate per 100 mL (35:65, v/v). Compared to conventional RP-HPLC, pCEC led to higher column efficiency, less consumption of reagent, and shorter analysis time.

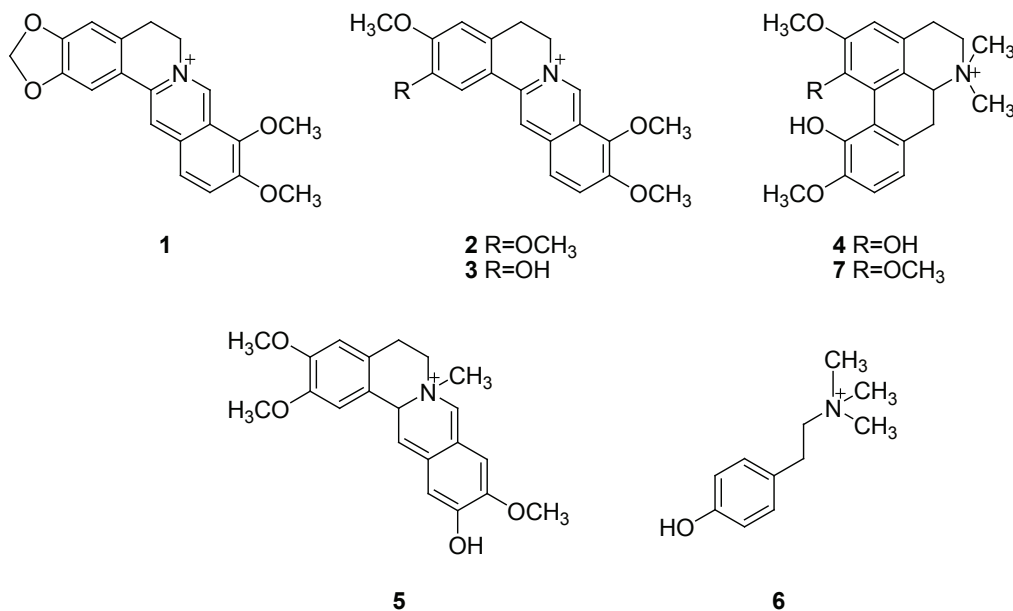
Introduction

Guanhuangbai (cortex *Phellodendri amurens*, the dried bark of *Phellodendron amurense* Rupr., Rutaceae) is one of the important traditional chinese medicines (TCM) (The State Pharmacopoeia Commission of People's Republic of China, 2005). It was commonly used in TCM to remove damp heat, quench fire, counteract toxicity, relieves consumptive fever and also was effective in curing dysentery, diarrhea and other syndromes. Alkaloids were reported to contribute to the biological activities of cortex *Phellodendri amurens* (CPA) (Birdsall & Kelly, 1997). Main alkaloids of CPA are berberine (1), palmatine (2), oatrorrhizine (3), magnoflorine (4), phellodendrine (5), candicine (6) and menisperine (7). Modern pharmacological research confirmed that alkaloids of CPA could be used for treating many diseases, such as inhibitions of original microbes, antiulcer, antiarrhythmia, antihypertension, anti-inflammation,

diuretic, relieving diarrhea, and so on. In order to control the quality of CPA comprehensively and effectively, the analytical method for total alkaloids of CPA is especially important.

CPA has been recorded by Chinese Pharmacopoeia (2005 edition), and the quantitative analytical method of berberine by RP-HPLC also has been recorded (The State Pharmacopoeia Commission of People's Republic of China, 2005). However, there is no report on analytical method for total alkaloids of CPA by HPLC. One of the objectives of this study was to establish an effective analytical method for total alkaloids of CPA by RP-HPLC.

A pressurized capillary electrochromatography (pCEC) is a relatively new microcolumn separation technique, which combines the advantages of HPLC and capillary electrochromatography (CE) (Jorgenson & Lukacs, 1981; Pretorius & Hopkins, 1974; Knox & Grant, 1987). By introducing high pressure on the electrochromatographic separation, pCEC combines



the benefit of an electroosmotic flow (EOF) which could increase peak capacity and shorten analysis time and the supplementary which could suppress bubble formation (Tsuda, 1988). A capillary column packing with spherical octadecylsilica (ODS) particles is usually used in pCEC, then, the chromatographic behavior of pCEC is in good agreement with that of RP-HPLC. Due to the advantages of pCEC, such as precision, high separation efficiency, and good resolution, pCEC has the potential to become a powerful separation tool for complex samples (Xie et al., 2005a; Liang et al., 2005b; Lim et al., 2000a). By now, there is no other report to analysis total alkaloids of CPA by pCEC. Accordingly, another aim of this study was to develop a pCEC method to analyze total alkaloids of CPA, which was compared with RP-HPLC method.

Material and Methods

Materials

Cortex Phellodendri amurens purchased from Huadong medicine Co., Ltd. (Liaoning, China) was identified by Professor Rusong Zhang of Zhejiang Chinese Medical University, and verified that the samples were indeed the dried bark of *Phellodendron amurense* Rupr., Rutaceae.

Instruments and reagents

Experiment was carried out by using a TrisepTM-2100 capillary electrochromatography system (Unimicro Technologies, USA) consisting of two solvent delivery modules, a UV/vis post-column cell

detector, a micro flow control module, a high voltage module and a solvent tray. High performance liquid chromatography system (Waters, USA) consisting of 1525 dual analytical pump, 717 automatic sampler and 2996 photodiode array detector. Digital pH meter was purchased from Shanghai Tianda instrument Co., Ltd. Methanol and acetonitrile used as mobile phase were of chromatographic grade (Merck, Ger). Water was purified by a Millipore Milli-Q purification system (Milford, MA, USA), and other reagents used were of analytical grade.

Sample preparation

An aliquot of 50 g CPA was accurately weighted and transferred into a round-bottom flask, then, it was extracted with 500 mL of 80% ethanol for 1 h. The extraction was repeated twice in the same way. The extracting solution was collected, evaporated by rotary vaporization to 150 mL. Hydrochloric acid was added to adjust pH to 2.0, after 1 h, the extracting solution was filtered, and the filtrate solution was adjusted pH to 10.0 with NaOH solution. Then, it was extracted with 150 mL chloroform for three times. The extracting solution was collected, and concentrated until dryness. 150 mL 1-butyl alcohol was also added to the filtrate solution to extract for three times. The extracting solution was collected, and concentrated to dryness. The extracts both from chloroform and 1-butyl alcohol were combined, for the future RP-HPLC and pCEC analysis. The extracts for RP-HPLC analysis were diluted fifty times with the mobile phase daily, and the extracts for pCEC analysis were diluted five times with the mobile phase daily. Furthermore, the

samples for RP-HPLC and pCEC analysis were filtered through 0.22 μm nylon filters after dilution.

Analytical method for total alkaloids of CPA by RP-HPLC

Chromatographic conditions

A reversed-phase column (Kromasil ODS, 250 \times 4.6 mm, 5 μm) was used in this study. Acetonitrile-0.1% phosphate (35:65, 0.1% SDS) was used as a mobile phase. The flow-rate was 1.0 mL/min and the injection volume was 10 μL . The UV detection wavelength was set at 230 nm. The column temperature was maintained at 25 $^{\circ}\text{C}$.

Determination

A sample (10 μL) for RP-HPLC was injected into HPLC, analyzed sample according to the above conditions.

Analytical method for total alkaloids of CPA by pCEC

Chromatographic conditions

A 45 cm (packed to 20 cm) \times 100 μm I.D. reversed-phase column (EP-100-20/45-3-C18, Unimicro Technologies Inc.) was used. The pCEC mobile phase used for the experiment consisting of 3 mM, pH 4.00 Na_2HPO_4 -citric acid (60% v/v) solution and acetonitrile (ACN) (40% v/v). The flow-rate of the mobile phase was 0.1 mL/min; and the injection volume was 1 μL . The UV detection wavelength was set at 230 nm. pCEC experiments were carried out by applying a voltage of -10 kV at the two sides of the capillary. The capillary temperature was maintained at 25 $^{\circ}\text{C}$.

Determination

A sample (60 μL) (the volume of the sampling loop is 1 μL) for pCEC was injected into pCEC, analyzed sample according to the chromatographic conditions of the above.

Results and Discussion

Determination of the UV detection wavelength

Photodiode array detector (PAD) was used for the determination of UV detection wavelength for the analysis of total alkaloids of CPA. To determine the best wavelength, total alkaloids was separated by RP-HPLC firstly; then, each chromatogram peak was scanned from 210 nm to 400 nm by PAD. The results suggested that the wavelength at 230 nm led to highest intensities for most peaks. Thus, 230 nm was chosen as the best detection wavelength for RP-HPLC and pCEC.

Optimization of RP-HPLC conditions

Effects of ACN concentration

ACN concentration of the quantitative analytic method for berberine (**1**) recorded in Chinese Pharmacopoeia (2005 edition) is 50% (v/v). Under this condition, berberine could be separated. However, the resolution among other peaks is low. It is known that with the decrease of the content of organic phase, the reservation capability of the compounds will increase on the RP-column. To obtain the baseline separation among other alkaloids, the effect of ACN concentration was investigated. The result showed that other alkaloids also could be separated by using a mobile phase of ACN-0.1% phosphoric acid with 0.1g sodium dodecanesulphonate per 100 mL (35:65, v/v).

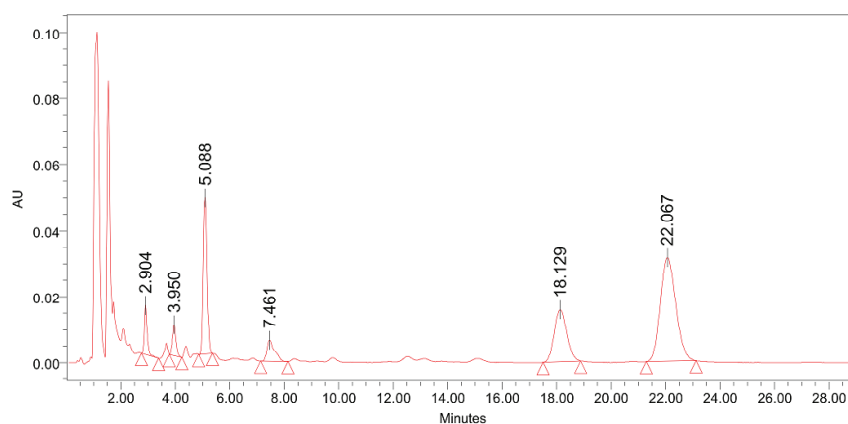


Figure 1. Chromatogram of the total alkaloids of cortex *Phellodendri amurens* by HPLC. Mobile phase: acetonitrile-0.1% phosphate (35:65, 0.1% SDS), flow rate: 1.0 mL/min, injection volume: 10 μL , detection wavelength: 230 nm, temperature: 25 $^{\circ}\text{C}$.

Analysis of total alkaloids of CPA by RP-HPLC

Total alkaloids of CPA were separated under the above chromatographic conditions, as shown in Figure 1.

Optimization of pCEC conditions

Effect of organic phase of the mobile phase

Water was used as the aqueous phase and either methanol or ACN was used as the organic phase respectively to optimize the separation of total alkaloids of CPA. The results displayed that using ACN as the organic phase was better than that of methanol with lower column pressure and better separation efficiency, as illustrated in Figure 2. 40% ACN could lead to better separation, due to weaker eluting power, 70% methanol could result in better separation. However, separation efficiency of 70% methanol was not as good as 40% ACN. Thus, ACN was selected as the optimum organic phase for the further experiment.

Effect of buffers of the mobile phase

In order to study the separation of total alkaloids of CPA, the buffer solution which consisting of 5 mM Na_2HPO_4 -citric acid (pH 4.00) was added to the mobile phase. Compared with ACN-water system, the addition of the buffer solution shortened the retention times of the main alkaloids and improved the efficiency of the column.

Effect of pH value of the mobile phase

Na_2HPO_4 -citric acid buffer system has a wide pH range from 2.2 to 8.0. To study the effect of pH value on the separation, the mobile phase containing 5 mM Na_2HPO_4 -citric acid at different pH values ranging from 4.00 to 7.00 (4.00, 5.00, 6.00, 7.00) was investigated. As shown in Figure 3, pH value influenced the separation significantly, and total alkaloids had a better separation with a low pH value. With the increasing of pH, the retention times of the main peaks and the resolution among total alkaloids decreased. On consideration of both analysis time and resolution, pH 4.00 was chosen as the optimum value, at which total alkaloids could achieve a better separation.

Effect of buffer concentration

The change of buffer concentration will affect the double layer on the silica surface and finally influence the EOF of the pCEC system via changing the migration and separation of ions (VanOrman et al., 1990; Desiderio & Fanali, 2000b). To improve the separation of total alkaloids, the effect of buffer concentrations (3 mM, 5 mM, 7 mM and 9 mM, pH 4.00, Na_2HPO_4 -citric acid solution-ACN) of the mobile phase was studied in this experiment. As shown in Figure 4, low concentration of buffer resulted in longer retention times and better resolution. In addition, high concentrations of buffer led to the risk of bubble formation when the column was performed electricity. Therefore, the buffer concentration was set at 3 mM for the further study.

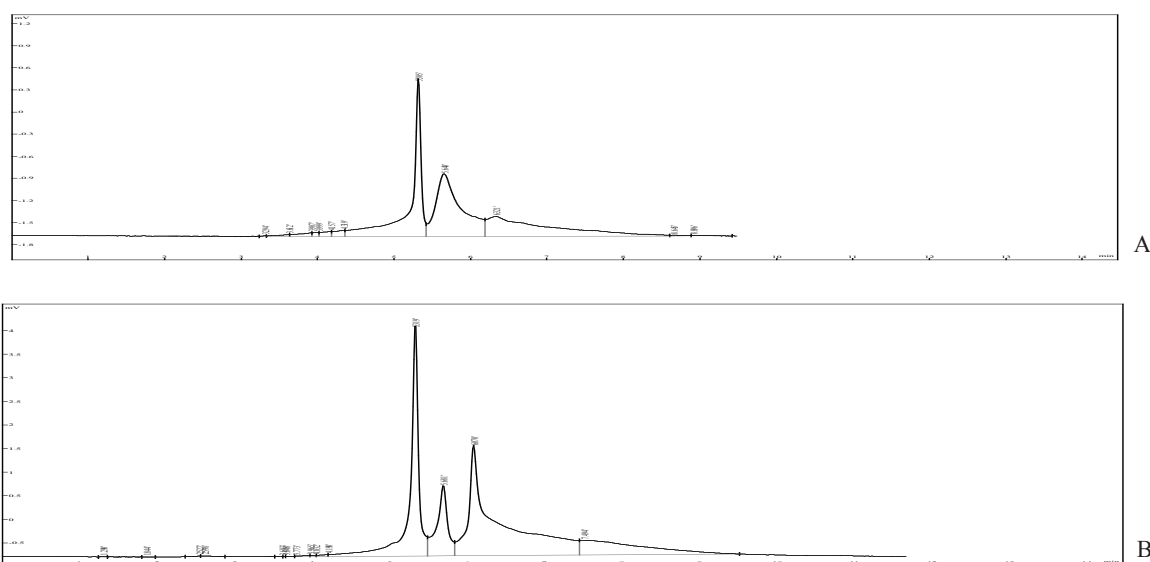


Figure 2. Electrochromatogram of the total alkaloids of cortex *Phellodendri amurens* with different organic phase as the mobile phase. A: 70% methanol; B: 40% acetonitrile; flow rate: 0.1 mL/min; injection volume: 1 μL ; detection wavelength: 230 nm; temperature: 25 $^{\circ}\text{C}$.

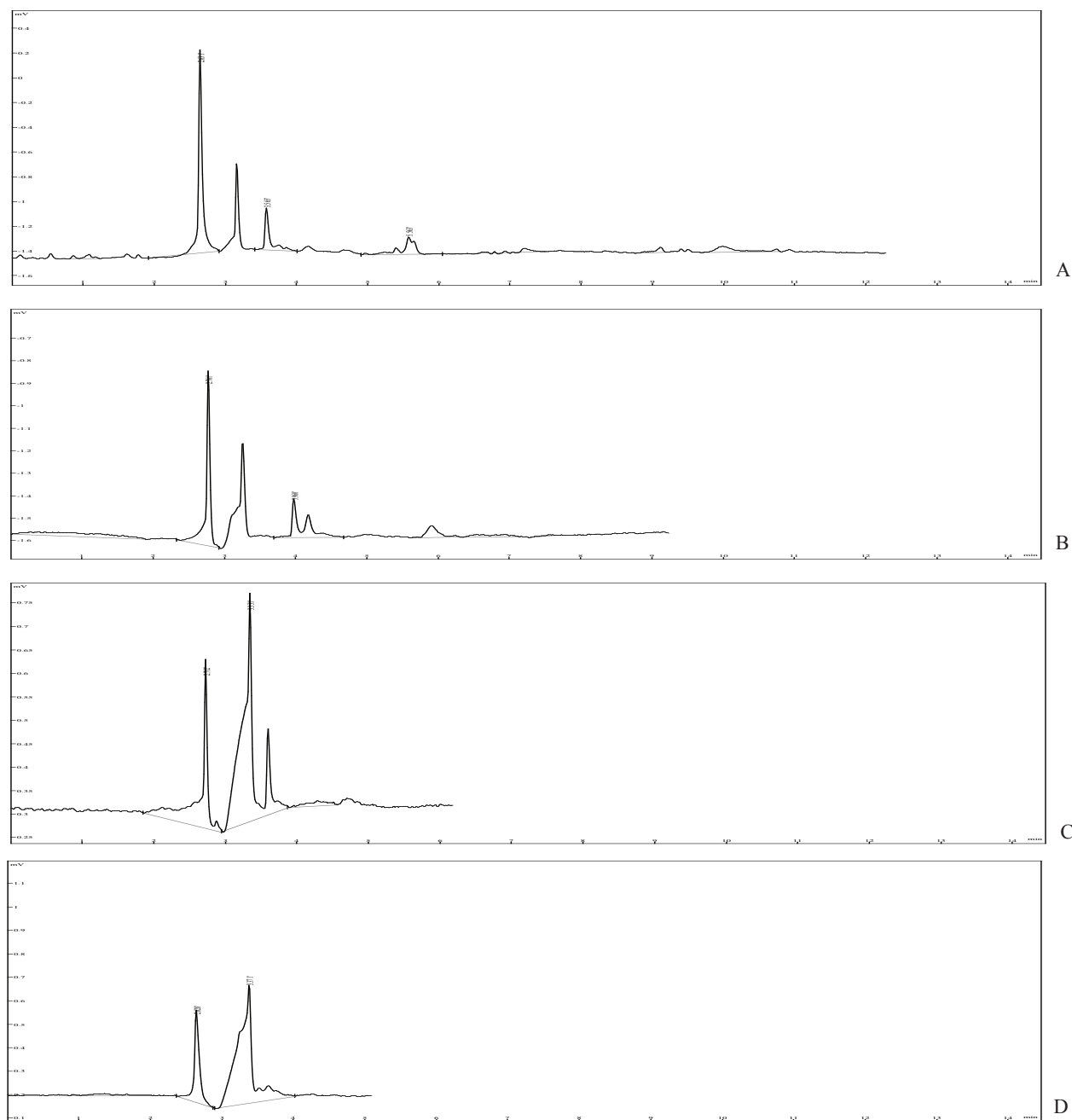


Figure 3. Electrochromatogram of the total alkaloids of cortex *Phellodendri amurens* with different pH value of mobile phase. Mobile phase: 5 mM, Na_2HPO_4 -citric acid solution (60% v/v) and acetonitrile (40% v/v). A: pH 4.00; B: pH 5.00; C: pH 6.00 and D: pH 7.00. Flow rate: 0.1 mL/min; injection volume: 1 μL , detection wavelength: 230 nm, temperature: 25 °C.

Effect of applied voltage

The effect of the applied voltage on the resolution and retention times of total alkaloids was investigated in a mobile phase consisting of 40% (v/v) ACN, and 60% (v/v) 3 mM Na_2HPO_4 -citric acid solution (pH 4.00). The applied voltage was varied from

-4 to -14 kV. Results showed that applying voltages led to less retention times, symmetrical peaks and better resolution as compared to without applying voltage. Moreover, with the increasing of the voltage applied, the analysis time was reduced significantly. However, there was a higher electric current and a risk of bubble formation coupled with increasing voltage. Considering

all, a voltage of -10 kV was chosen as the optimal value for this study.

Effect of ACN concentration

The organic modifier in the mobile phase is an important parameter in pCEC. In order to examine the effect of organic modifier on the separation of total alkaloids by pCEC, different contents of ACN from 30 to 80% (v/v) were added to a 3 mM Na_2HPO_4 -citric acid solution (pH 4.00). The results showed that the

retention times and the resolution of these analyses decreased with the increasing content of ACN in the mobile phase, which may be attributed to the change of EOF and chromatographic partitioning. At the same time, it was found that the column pressure and the systematic current increased gradually with decreasing the content of ACN in the mobile phase. The results showed that the total alkaloids had a better separation when the concentration of ACN was 40% (v/v) which was used for the further studies.

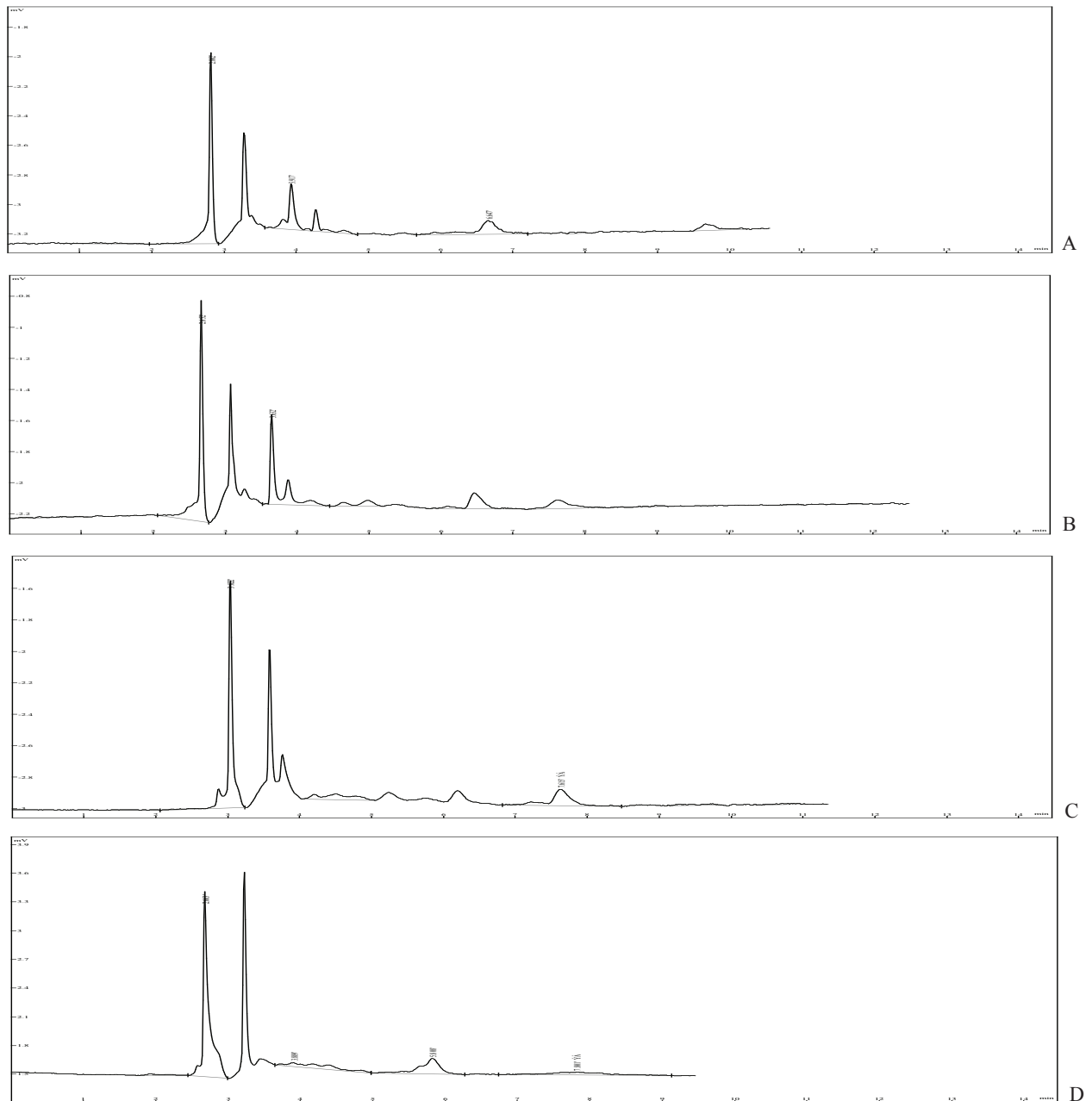


Figure 4. Electrochromatogram of the total alkaloids of cortex *Phellodendri amurens* with different concentrations of buffer in the mobile phase. Mobile phase: Na_2HPO_4 -citric acid solution (60% v/v) and acetonitrile (40% v/v) at pH 4.00. A: 3 mM; B: 5 mM; C: 7 mM and D: 9 mM; Flow rate: 0.1 mL/min; injection volume: 1 μL , detection wavelength: 230 nm, temperature: 25 $^\circ\text{C}$.

Analysis of the total alkaloids by pCEC

Under the optimal conditions, which are 40% (v/v) ACN and 60% 3 mM Na₂HPO₄-citric acid (pH 4.00) solution and applied voltage of -10 kV, total alkaloids of CPA can be well separated as shown in Figure 5.

Method validation

Precision of standard solutions

The repeatability of intra and interday was obtained by analyzing the retention time and the peak area variations of six injections of berberine (**1**) standard solutions (0.1668 mg/mL). The intraday RSD of the retention time was below 1.58 and RSD of the peak area below 3.26, interday RSD of the retention time below 2.87 and RSD of the peak area below 4.32.

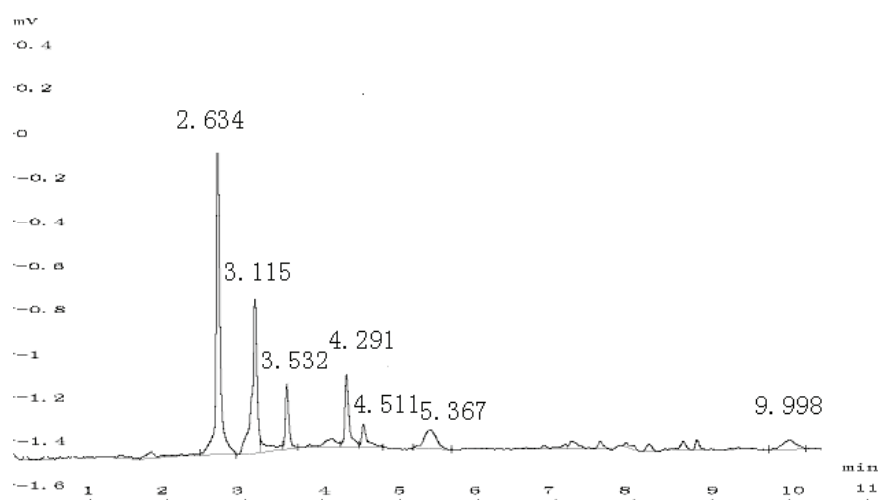


Figure 5. Electrochromatogram obtained from the analysis of total alkaloids from cortex *Phellodendri amurens* with optimized condition. Mobile phase: 3 mM Na₂HPO₄-citric acid solution (60% v/v) and acetonitrile (40% v/v) at pH 4.00; flow rate: 0.1 mL/min; voltage: -10 kV; injection volume: 1 µL, detection wavelength: 230 nm, temperature: 25 °C.

Table 1. Precision of sample (n = 3).

	Intraday		Interday	
	RSD for time (%)	RSD for area (%)	RSD for time (%)	RSD for area (%)
Peak 1	1.32	3.54	2.69	4.78
Peak 2	1.75	3.97	2.24	5.21
Peak 3	1.21	3.24	2.76	4.64
Peak 4	1.35	3.36	2.47	4.82

Precision and recovery of samples

To assess the repeatability of the extraction technique, sample was extracted and injected for pCEC analysis. Triplicate performances were made to evaluate the intraday variations. The RSD of the retention time and peak area of four alkaloids in the three extracts of sample were determined. The interday variations of the retention time and peak area of four alkaloids in the daily extract of sample were also determined for consecutive three days. The precision of sample is shown in Table 1.

The total alkaloids of CPA could be separated by pCEC as well as by RP-HPLC. The analysis was accomplished in less than 11 min by pCEC whereas it took 24 min to obtain the separation by RP-HPLC. Therefore, the efficiency of pCEC separation is better than that of RP-HPLC. The flow rate of mobile phase by RP-HPLC was ten times more than that by pCEC.

Table 2. Compared with RP-HPLC and pCEC for total alkaloids separating.

	pCEC		RP-HPLC	
	Retention time (min)	Theoretical plate number	Retention time (min)	Theoretical plate number
Peak 1	2.634	12539	2.904	3917
Peak 2	3.115	12410	3.950	3786
Peak 3	3.532	29325	5.088	5808
Peak 4	4.291	27558	7.461	3674
Peak 5	4.511	19935	18.129	7247
Peak 6	5.367	6461	22.067	7086
Peak 7	9.998	15380		

So, the reagent consumption by pCEC was less than that by RP-HPLC. From the aspect of the separation efficiency, seven chromatogram peaks were obtained by pCEC while six peaks were obtained by RP-HPLC. Accordingly, the efficiency of pCEC separation was superior to RP-HPLC. The column efficiencies by pCEC were also better than that by RP-HPLC. The retention times and theoretical plate number of main peaks were shown in Table 2.

Conclusion

Under the optimal conditions of 40% (v/v) ACN in 3 mM Na₂HPO₄-citric acid solution at pH 4.0 and applied voltage of -10 kV, the total alkaloids of CPA was well separated by pCEC, which was better than by RP-HPLC obviously. This study demonstrated that pCEC was a valuable analytical technique coupled with RP-HPLC, moreover, the results indicated that this method was adequate, valid, and applicable. Based on this, further studies will aim at establishing the quantitative analytic method for the main alkaloids of CPA by pCEC, in the meanwhile, the chemical fingerprint for total alkaloids of CPA by pCEC should also be studied subsequently to control the quality of CPA comprehensively and effectively.

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