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Separation and determination of coumarins in *Fructus cnidii* extracts by pressurized capillary electrochromatography using a packed column with a monolithic outlet frit

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ABSTRACT

The pressurized capillary electrochromatography (pCEC) was utilized for the separation and determination of coumarins in *Fructus cnidii* extracts from 12 different regions. After a thorough study of analytical parameters such as acetonitrile content of the mobile phase, the concentration and pH of the buffer, and the applied voltage, a methodology was proposed to separate and determine six coumarins of *F. cnidii* extracts in less than 15 min. The experiments were performed in an in-house packed column with a monolithic outlet frit under the optimal conditions: pH 4.0 ammonium acetate buffer at 10 mM containing 50% acetonitrile at −6 kV applied voltage. The calibration curves were linear in the range of 10.0–100.0 μg/mL for bergapten, 20.0–200.0 μg/mL for imperatorin, 5.0–400.0 μg/mL for osthole, 10.0–100.0 μg/mL for 2′-acetylangelicin, 10.0–200.0 μg/mL for oroselone, and 10.0–200.0 μg/mL for O-acetylcolumbianetin. The correlation coefficients were between 0.9967 and 0.9995. With this pCEC system, fingerprints of *F. cnidii* extracts were preliminarily established to distinguish three types of coumarins by characteristic peaks, and the quality of various sources of raw materials was evaluated by determining the contents of six coumarins.

1. Introduction

Fructus cnidii (Chinese name she chuang zi) is the dried ripe fruit of *Cnidium monnieri* (L.) Cusson, family Umbelliferae. As one of the most popular traditional Chinese medicinal herbs, it has been used for the treatments of impotence, frigidity and skin-related diseases such as suppurative dermatitis and pudendum itching [1,2]. Modern chemical and pharmacological studies have revealed the main bioactive components of *F. cnidii* are coumarins which mainly include simple coumarins (osthole), linear furocoumarins (bergapten and imperatorin) and angular furocoumarins (2′-acetylangelicin, oroselone, and O-acetylcolumbianetin) [3–8]. The chemical structures of six coumarins were shown in Fig. 1. Among six coumarins, bergapten, imperatorin and osthole are regarded as the biologically active principle constituents [9–11]. Bergapten possesses anti-inflammatory and analgesic activities [12], and imperatorin has the potential chemopreventive effects when administered in the diet [13], while osthole could prevent postmenopausal osteoporosis [14].

Several analytical methods for the quality control of *F. cnidii*, such as thin layer chromatography (TLC) [15], high performance liquid chromatography (HPLC) [16], high performance liquid chromatography–mass spectrometry (HPLC–MS) [7,17,18], high-speed counter-current chromatography (HSCCC) [6,19,20], gas chromatography (GC) [21], gas chromatography–mass spectrometry (GC–MS) [22] and capillary electrophoresis (CE) [10,23,24] had been established to determine the main active constituents, coumarins. However, how to realize the high resolution and rapid analysis of coumarin derivatives still represents a formidable challenge due to the very similar molecular structures.

Capillary electrochromatography (CEC), as a novel micro-column separation technology, couples the high efficiency of capillary electrophoresis with high selectivity of HPLC. The CEC analytes separation is usually achieved in capillaries containing packed stationary phases by an electroosmotic flow (EOF) generated by a high electric field. The usage of CEC method has been applied on many areas [25–27]. However, the intrinsic limitation of packed column such as bubble formation and “dry-out” problem encountered

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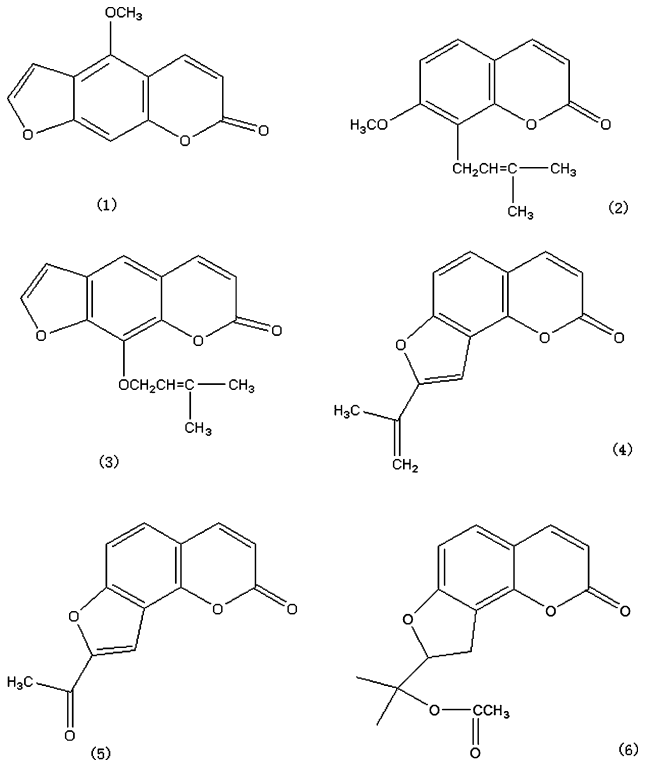

Fig. 1. Structures of the six coumarins: (1) begapten, (2) osthole, (3) imperatorin, (4) oroselone, (5) 2′-acetylangelicin, and (6) O-acetylcolumbianetin.

with "pure" CEC posted an obstacle to improve stability and reproducibility of experimental system [28,29]. By introducing applied pressure on electrochromatographic separation using HPLC pumps, pressurized capillary electrochromatography (pCEC) combines the benefits of EOF and the supplementary pressure to increase peak capacity, shorten analysis time, and to some extent, suppress the bubble formation [30]. Due to its good precision, high separation efficiency and resolution, pCEC has become an attractive separation tool for complex mixtures [31–35].

Column is the heart of pCEC and packed column is most usually utilized because it makes use of stationary phases such as octyldecyl silica (ODS) commonly employed in HPLC. Here the silica stationary phase is retained between two frits obtained by sintering the particles. This approach has usually brought the drawbacks such as bubble formation, fragility, band broadening, and poor reproducibility [36–39]. In order to improve the characteristics of the functional frits, several attempts of their preparation have been performed. Chen et al. [40] trapped silica particles within two monolithic frits obtained by UV photopolymerization, and this kind of frits possessed good permeability, stability and robustness. Rocco and Fanali [41] used packed columns with a monolithic inlet frit without pressure assistance and experimental results demonstrated that there were no significant differences in terms of efficiency and retention times when compared with column with both sintered frits. In the review of Mistry et al. [42], they mentioned μ-HPLC inlet frits were not required due to application of pressure. In this paper we propose the application of a new packed capillary column to pCEC experiments, which used a monolithic frit to displace the tradition sintered outlet frit and made the inlet of the column fritless. In the pCEC operation procedure the mobile phase flow entered the capillary column under constant pressure (about 13.8 MPa) controlled by a back-pressure regulator, and the charge stationary phases would not flow out of the column from the fritless inlet.

In our previous study, a special pCEC instrument has been developed and analysis of traditional Chinese medicines (TCM) by pCEC was first reported. Chen et al. [43] utilized successfully pCEC for the separation and the quantitative analysis of coumarin compounds from *Angelica dahurica* (*Fisch. ex Hoffm*) *Benth. et Hook. f* and Wang et al. [44] developed a quality evaluation method based on chromatographic fingerprint for *Fructus schisandrae* by pCEC. The present study focused on the development of a convenient and reproducible pCEC fingerprinting method to identify and compare the coumarins from *F. cnidii* in different regions using a packed column with a monolithic outlet frit, and distinguished three types of coumarins by characteristic peaks, and evaluated the quality of various sources of raw materials by determining the contents of the six coumarins.

2. Experimental

2.1. Materials and reagents

Capillary column (with two sintered frits) of 100 μm inner diameter (i.d.) × 375 μm outer diameter (o.d.) was obtained from Unimicro Technologies, the total length of the capillary used was 30 cm, of which 15 cm was packed with 3 μm spherical octadecylsilica (ODS) particles. Polyamide-clad fused-silica capillary tubing with 100 μm i.d. and 375 μm o.d. was purchased from Hebei Yongnian Optical Filter Factory (Hebei, PR China). The 3 μm ODS particles were purchased from Synchrom (Lafayette, IN, USA). 3-(Trimethoxysilyl) propyl methacrylate (98%, γ-MAPS), ethylene dimethacrylate (98%, EDMA), glycidyl methacrylate (97%, GMA), azobisisbutyronitrile (AIBN), dodecyl alcohol and cyclohexanol were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Reference compounds, bergapten, imperatorin, osthole were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Oroselone, O-acetylcolumbianetin and 2′-acetylangelicin were extracted and obtained from *F. cnidii* by preparative HPLC, and identified with NMR, IR, UV and MS (Table 1). The purity of oroselone, O-acetylcolumbianetin and 2′-acetylangelicin is 98.2%, 98.5% and 98.0%, respectively. Internal standard (IS) diazepam was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 99.9% ethanol of chemical grade, ammonium acetate and acetic acid were from China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, PR China). Twelve samples of *F. cnidii* were purchased from different provinces of China, labeled according to their regions (Table 2). Acetonitrile was of chromatographic grade (Merck, Darm-

Table 1
UV, MS and NMR data of three angular furocoumarins from *F. cnidii*.

Oroselone	UV λ_{max} (EtOH): 213, 274, 318; ESI-MS *m/z*: 227 $[M+H]^+$, 199 $[M+H-CO]^+$; ^1H NMR ($CDCl_3$) δppm: 2.14 (3H, s, $-C(CH_3)=CH_2$), 5.24, 5.83 (1H × 2, t, d, *J* = 0.74, 1.47 Hz, $-C(CH_3)=CH_2$), 6.36, 7.78 (1H × 2, d, *J* = 9.5 Hz, 3-H, 4-H), 7.31,7.36 (1H × 2, d, *J* = 8.5 Hz, 5-H, 6-H), 6.95 (1H, s, 3′-H)
O-acetylcolumbianetin	UV λ_{max} (EtOH): 210, 235, 252, 262, 318; ESI-MS *m/z*: 289 $[M+H]^+$, 229 $[M+H-60]^+$, 214, 187; ^1H NMR ($CDCl_3$) δppm: 1.51, 1.55 (3H × 2, s, $-C(CH_3)_2$), 1.98 (3H, s, $CH_3-C=O$), 3.31 (2H, d, *J* = 8.79 Hz, $-CH_2-CH-O-$), 5.14 (1H, t, *J* = 8.55 Hz, $-O-CH-CH_2-$), 6.18, 7.63 (1H × 2, d, *J* = 9.5 Hz, 3-H, 4-H), 7.27, 6.71 (1H × 2, d, J = 8 Hz, 5-H, 6-H)
2′-Acetylangelicin	UV λ_{max} (EtOH): 227, 250, 322; ESI-MS *m/z*: 229 $[M+H]^+$, 214 $[M+H-CH_3]^+$, 201 $[M+H-CO]^+$, 186 $[M+H-COCH_3]^+$; ^1H NMR ($CDCl_3$) δppm: 2.64 (3H, s, $-COCH_3$), 6.43, 7.82 (1H × 2, d, *J* = 9.5 Hz, 3-H, 4-H), 7.50, 7.55 (1H × 2, d, *J* = 8.5 Hz, 5-H, 6-H), 7.80 (1H, s, 3′-H)

Table 2
Sources of samples.

Sample no.	Regions (province)	Sample no.	Regions (province)
1	Xinyi (Jiangsu)	7	Sheshan (Shanghai)
2	Xuzhou (Jiangsu)	8	Shanghe (Shangdong)
3	Jurong (Jiangsu)	9	Dali (Shangxi)
4	Jiaxing (Zhejiang)	10	Zhengding (Hebei)
5	Lin'an (Zhejiang)	11	Wafangdian (Liaoning)
6	Xiamen (Fujian)	12	Zhaodong (Heilongjiang)

stadt, Germany). Pure water prepared by Milli-Q System (Millipore, Bedford, MA, USA) was used for all buffer and mobile phase solutions.

2.2. Instrumentation

pCEC was performed on reconstructed pCEC system which comprised a pump (Waters, USA), CE system (Bio-Rad, USA, containing a high-voltage power supply and a variable wavelength UV–vis detector), a microfluid manipulation module with a 2 μL injector (Unimicro Technologies, Inc.) and a data acquisition module, as our previous study [43,44]. A packed capillary column with the inlet connected with a four-way valve and the outlet connected with electrolytic bath contained an uncoated fused-silica section to be a detection flow-cell.

2.3. Column preparation

2.3.1. Surface pretreatment of the capillary

In order to improve the stability of the monolithic section, the inner wall of a 100 μm i.d. and 375 μm o.d. capillary was treated according to the following procedure. The capillary (about 40 cm) was flushed by a syringe pump with 0.1 M sodium hydroxide (12 h), H_2O (1.5 h), 0.1 M HCl (6 h), H_2O (1.5 h), methanol (1 h), and stream of nitrogen (3.5 h). Then, 50% γ-MAPS dissolved in methanol was drawn into the capillary and left overnight at room temperature. Next day, the capillary was washed with methanol (15 min) and dried with a stream of nitrogen (1 h).

2.3.2. Fabrication of outlet monolithic section

The preparation of monolithic section is similar to that reported by Ye et al. [45]. The polymerization mixture consists of the GMA as functionalizable monomer, EDMA as crosslinker, cyclohexanol and dodecyl alcohol as pore-forming solvents and AIBN as thermal initiator. Their composition in the mixture was summarized as follows: 0.3 mL GMA, 4 mg AIBN, 0.1 mL EDMA, 1.1 mL cyclohexanol, and 0.1 mL dodecyl alcohol were mixed together and stirred for 5 min in an ice bath. Then the pretreated capillary was immediately filled to about a 20 cm segment with polymerization mixture by a syringe pump and sealed with a silicon rubber and put into a thermostating bath at 50 °C for 12 h. Once the polymerization was completed, the outlet monolithic section of capillary was washed with methanol for 2 h, distilled water for 2 h to eliminate all the residuals of polymerization solution using a HPLC pump.

2.3.3. Column packing and precondition

The capillary column employed in this study had an i.d. of 100 μm and an o.d. of 375 μm, which was packed with a 4 mM phosphate (pH 6.5) methanol suspension of 3 μm spherical octadecylsilica (ODS) (90%, w/w) and 1 μm spherical silica particles (10%, w/w), in the open inlet end for about 20 cm length using the electrokinetic packing method described previously [43,44,46]. The column was then inspected carefully under a microscope (at magnifications of 10–40×) to verify the packing density and the structural appearance of the outlet monolithic section of capillary. Two millimeter monolithic segment after the interface of packed part and monolithic section was used to be an outlet frit. We chose an alternative way to prepare the detection window in monolithic section that has been reported by Ericson et al. [47]. The detection window was prepared by burning off 1–2 mm of the polyimide coating after the outlet frit with the aid of flame while pumping cold water continuously through the column by approximately 20.7 MPa. The heat from flame breaks covalent bonds at the inner wall and creates a small water-filled gap. The detection window prepared in this way was found to have about the same UV transmission as a window in empty capillary. The resulting column was preconditioned with the mobile phase by pressurizing the column inlet to approximately 20.7 MPa with a HPLC pump for 5 h prior to its use. At last, the capillary was cut for suitable length, 15 cm for the packed end and 15 cm for the monolithic end (in our reconstructed pCEC system, the length from detection window to electrolytic bath was 15 cm).

2.4. Sample preparation

Sample preparation was performed using ultrasonic extraction associated with soaking under the optimized conditions published by our group [7]. In brief, about 1 g dry *F. cnidii* samples was ground into powder and then soaked at room temperature for 24 h away from light, following ultrasonical extraction with 95% ethanol (20 mL) for 1 h at room temperature (25 °C). After centrifugation at 3500 rpm for 10 min (twice), the supernatant was filtered through a 0.45 μm membrane filter, and then mixed with 100 μg/mL IS methanol solution and mobile phase in the proportion of 1:1:2. All samples were degassed with ultrasonication for 10 min before analysis.

Considering that the coumarins are often photosensitive compounds, especially C-8 of the molecular structure of imperatorin and osthole has a unsaturation side chain, it is easily degenerated by oxidization [48]. So, during the experiment it is important to conserve the extracts away from light.

2.5. pCEC procedures

Standard stock solutions were prepared in methanol to give a final concentration of 1 mg/mL for bergapten and 2′-acetylangelicin, 2 mg/mL for imperatorin, oroselone, O-acetylcolumbianetin, and 4 mg/mL for osthole. A further dilution was performed by diluting stock solution with mobile phase to give a desired concentration. The buffer containing ammonium acetate was adjusted to the desired pH with either acetic acid or sodium hydroxide, then the mixture of the buffer and a certain ratio of acetonitrile was used as the mobile phase. All the buffer solutions were filtered through 0.45 μm membrane filters and degassed by ultrasonication for approximately 5 min before being used as the mobile phase and transferred to the outlet vial. A negative voltage was added on the column outlet and the column inlet was grounded. Pressure was applied to the column inlet during the separation. Flow rate of the pump was set at 50 μL/min. The wavelength of the UV–vis detector was set at 320 nm. The backpressured regulator was set at 10.3 MPa. Both the pump flow and the sample in the injection loop were split, and therefore the actual flow rate in the capillary column was estimated to be 100 nL/min and the actual injection volume of sample was about 4 nL at the split ratio 1:500.

2.6. Method validation

The suitability of the optimized method for the analysis of bergapten, imperatorin and osthole was evaluated with validation studies including linearity, limit of detection and quantification, repeatability, accuracy, robustness and the stability of the solution. Linearity was demonstrated from

10.0 μg/mL to 100.0 μg/mL (bergapten), 20.0 μg/mL to 200.0 μg/mL (imperatorin), 5.0 μg/mL to 400.0 μg/ml (osthole), 10.0 μg/mL to 100.0 μg/mL (2′-acetylangelicin), 10.0 μg/mL to 200.0 μg/mL (oroselone), 10.0 μg/mL to 200.0 μg/mL (O-acetylcolumbianetin), respectively. For each calibration curve, at least five different concentrations were used. The linear regression analysis of six ingredients was obtained by the ratios of the peak area of each analyte to the peak area of the IS versus the concentration of the analytes. Three injections of each standard solution were performed. The limit of detection (LOD), defined as signal-to-noise ratio (S/N) of 3, and the limit of quantification (LOQ) was defined as the S/N of 10. The precision of the method was represented by measurement of intra- and inter-day variability. And the intra- and inter-day precision was determined by the RSD obtained on one day and on three different days at three concentration levels. Recovery test was used to evaluate the accuracy of this method, which was investigated by standard addition method studied by spiking the sample of *F. cnidii* with the standard solution at three different concentrations and triplicate experiments were performed at each concentration level. The robustness of the method experimental conditions was remained unaffected by small, but deliberate changes in electrochromatographic conditions. The stability was tested within the standard solution of three different concentrations that were stored at room temperature, away from light and analyzed every 2 h within 12 h.

3. Results and discussion

3.1. Analytical conditions by pCEC

3.1.1. Effect of acetonitrile concentration

The organic modifier in the mobile phase is an important parameter in the pCEC system. In order to examine the effect of organic modifier on the separation of six coumarins by pCEC on the novel packed column, different contents of acetonitrile, in the range of 40–55% (v/v), were added to a 10 mM ammonium acetate buffer (pH 5.0), while keeping an applied voltage of −6 kV. As can be seen from Fig. 2, the $\log k^*$ ($k^* = (t_R - t_0)/t_0$) of all analytes almost linearly decreased with the increasing concentration of acetonitrile in mobile phase ($r > 0.9963$). This indicated the retention action was mainly controlled by the hydrophobic interactions between the analytes and stationary phase and the neutral coumarins were separated primarily on the basis of differential partitioning into the alky-bonded phase. In the experiment, good separation can be obtained using a mobile phase containing lower concentration of organic solvent. Considering the analysis time and resolution, 50% (v/v) acetonitrile was used to further optimize separation conditions for the coumarins of *F. cnidii*.

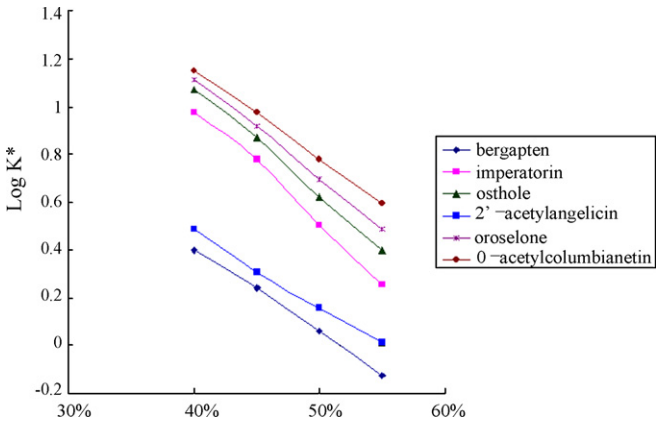

Fig. 2. Effect of acetonitrile content on the retention of six coumarins. Experimental conditions: mobile phase, 10 mM ammonium acetate buffer (pH 5.0); applied voltage: −6 kV; injection: 40 nL; backpressure: 10.3 MPa; flow rate: 0.05 mL/min; and detection wavelength: 320 nm.

3.1.2. Effect of concentration and pH of the buffer

The change of buffer concentration will affect the double layer on the silica surface and finally change the EOF of the pCEC system [49]. In our experiment the effect of the concentration of buffer on the retention of six coumarins was investigated using 5 mM, 10 mM and 15 mM at pH 5.0 containing 50% acetonitrile. The result showed that the resolution and retention time of the analytes slightly increased with the increase of buffer concentration from 5 mM to 15 mM. However, when the concentration of the buffer solution was over 10 mM, we found that the increase of ionic strength caused a high background current that leaded to high frequency of bubble formation. So, a relative optimal buffer concentration was selected at 10 mM in the mobile phase.

In pCEC, the pH of mobile phase is an important factor to maintain EOF, because change of pH determines the number of dissociated residual silanol groups on the surface of the capillary wall and the packing particles, and further affects the surface charge density and zeta potential. The chemical skeleton in the six coumarins of *F. cnidii* contains fused benzene and α-pyrone rings and it indicates that they are hardly charged both in the condition of acidity or alkalinity. Therefore, the effect of pH is almost based on changing the speed of EOF. The retention times of the coumarins were approximately on the same level when changing pH from 3.0 to 5.0 (3.0, 4.0, 4.5 and 5.0). However, the increase of the buffer pH leaded to a slightly loss of resolution. To think of the separation and the retention time together so we selected pH 4.0 in the mobile phase.

3.1.3. Effect of the applied voltage

The increase of the applied voltage enhanced the EOF and decreased the retention time of the neutral compounds. The effect of the applied voltage on the retention time and the separation of the analytes of *F. cnidii* sample from Lin'an (Zhengjiang Province) were determined in a mobile phase containing 50% (v/v) acetonitrile, 10 mM ammonium acetate buffer (pH 4.0) at constant pressure. The applied voltage was varied from −2 kV to −10 kV. A higher voltage reduced the retention times due to an increase of EOF, but such high applied voltage makes the pCEC system unstable due to Joule heating caused by high background current. Therefore, we chose −6 kV to be the optimal voltage.

3.1.4. Selection of internal standard

In order to increase the repeatability and accuracy of assay, internal standard (IS) is usually necessary for the pCEC system. Our principal criterion for choosing IS was UV absorption and elution time. Other coumarins such as, oxypeucedanin hydrate, psoralen and isopsoralen were tried as potential IS. The retention time of the above coumarins was too short under experimental conditions , therefore some compounds, acyclovir, acebutolol hydrochloride, diazepam and so on with relatively strong UV absorption were compared in our experiment. Diazepam was selected as the IS, since it presented appropriate retention time and were well resolved from the other target analytes with a baseline separation.

3.1.5. Optimized separation

After optimization selection of analytical conditions, pCEC separation of six coumarins has been performed in 10 mM ammonium acetate buffer (pH 4.0)/acetonitrile (50/50, v/v), applied voltage −6 kV, flow rate 0.05 mL/min. The backpressured regulator was set at 10.3 MPa. The standard chromatogram was shown in Fig. 3.

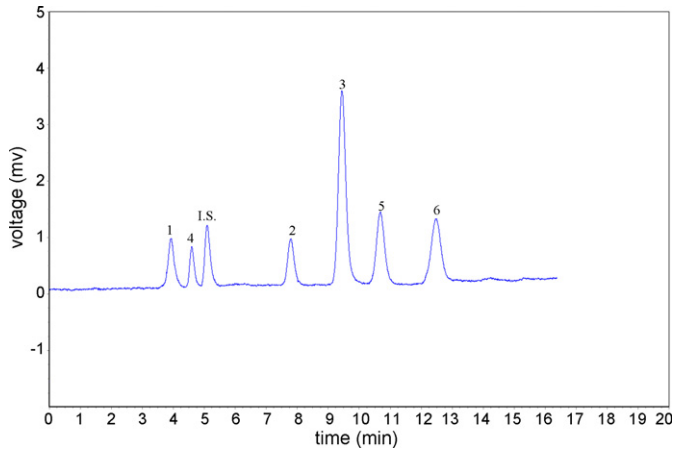

Fig. 3. Standard pressurized capillary electrochromatogram of six coumarins. 10 mM ammonium acetate buffer (pH 4.0)/acetonitrile (50/50, v/v), injection: 40 nL, applied voltage: −6 kV, backpressure: 10.3 MPa, flow rate: 0.05 mL/min, detection wavelength: 320 nm and standard sample: six standard coumarins solution. Peaks: (1) bergapten, (2) imperatorin, (3) osthole, (4) 2′-acetylangelicin, (5) oroselone, and (6) O-acetylcolumbianetin.

Table 3
Comparison of monolithic outlet frit and sinterized frits ($n = 10$).

Coumarins	Monolithic outlet frit				Sinterized frits			
	t_R	RSD	N/m	RSD	t_R	RSD	N/m	RSD
Bergapten	3.96	1.12	20,728	2.15	3.87	1.42	20,563	2.77
Imperatorin	5.27	1.33	52,356	1.31	5.48	1.53	50,328	2.01
Osthole	9.65	0.85	62,580	1.18	10.13	1.37	60,659	1.23

Sample was from Linan (Zhengjiang), experimental conditions, see Fig. 3.

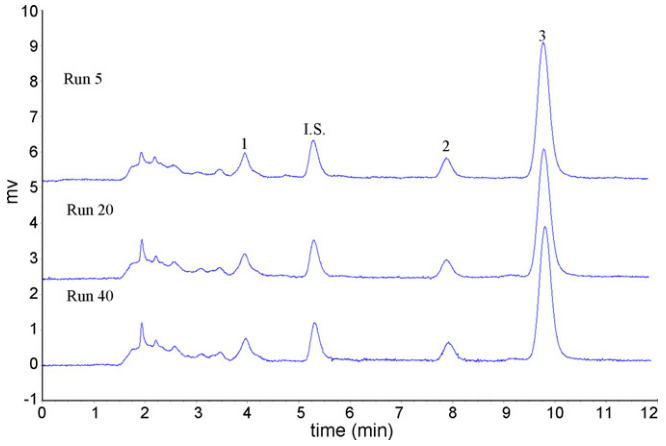

Fig. 4. Pressurized capillary electrochromatograms of run 5, 20 and 40. Samples were from Lin'an (Zhengjiang Province). Peaks: (1) bergapten, (2) imperation, and (3) osthole. pCEC conditions can be seen in Fig. 3.

Table 4
Column-to-column reproducibility for retention time (t_R), retention factor (k) and peak efficiency (N).

Coumarins	Reproducibility (RSD%, 3 columns, $n = 12$)		
	t_R	k	N
Bergapten	4.53	5.87	6.03
Imperatorin	4.15	4.93	5.41
Osthole	3.78	4.58	4.78

Experimental conditions: capillary column packed with C-18, 3 μm particles, with monolithic outlet frit; other experimental conditions, see Fig. 3. Sample of *F. cnidii* was from Lin'an (Zhengjiang).

3.2. Performance of the column with outlet monolithic frit

Table 3 was the data we obtained by the novel column with outlet monolithic frit, which was compared with a traditional column with both sintered frits. The novel columns showed the same features offered by capillary columns with both sintered frits in term of retention time and efficiency. In pressure electrochromatograms (Fig. 4) of runs 5, 20, and 40, there was almost no variation in retention times of the analytes of *F. cnidii* sample from Lin'an (Zhengjiang Province) with the novel column and the electrochromatogram behavior demonstrated that the monolithic porous polymer frit was strongly bound to the inner surface of the capillary wall, which could withstand applied pressure. The integrity of the monolithic outlet frit remained unchanged, and the column was assumed to be useful for a much longer period of time. Reproducibility was calculated after running experiments in three packed capillaries where the inlet was fritless and the outlet was obtained with the same polymerization mixture and conditions. Quite satisfactory results were achieved with RSD <4.53% for retention times while the reproducibility of efficiency was in the range 3.78–6.03% (see Table 4).

Comparing the results with the column with both sintered frits, we observed that the novel columns are more resistant since the inlet and the outlet are both protected by polyimide layer. The high permeability of the monolithic frit allows good packing and optimal flow through the packed part essential for a high-quality monolithic frit, moreover the simple and reproducible procedure for fabricating frit makes the novel one better.

3.3. Method validation results

3.3.1. Linear range, LOD and LOQ

At least five concentrations of the solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak area ratios versus the concentrations of coumarins. Correlation coefficients of all the calibration curves were found to be higher than 0.9967. The regression data obtained showed a good linear relationship (Table 5). Using a signal-to-noise ratio of 3 and 10, respectively, the LODs and LOQs for the six coumarins were less than 5.0 μg/mL and 20.0 μg/mL, which showed a high sensitivity under these experimental conditions (Table 5).

Table 5
Linear regression data, LOD and LOQ of the investigated analytes.

Coumarins	Linear regression data				
	Calibration curve	Linear range (μg/mL)	r^2 ($n = 3$)	LOD (μg/mL)	LOQ (μg/mL)
Bergapten	$y = 0.0019x - 0.0790$	10.0–100.0	0.9967	2.5	10.0
Imperatorin	$y = 0.0096x - 0.0587$	20.0–200.0	0.9995	5.0	20.0
Osthole	$y = 0.0264x - 0.0387$	5.0–400.0	0.9990	1.0	5.0
2′-Acetylangelicin	$y = 0.0112x - 0.0513$	10.0–100.0	0.9984	2.5	10.0
Oroselone	$y = 0.0184x - 0.0425$	10.0–200.0	0.9991	2.5	10.0
O-acetylcoumbianetin	$y = 0.0159x - 0.0476$	10.0–200.0	0.9993	2.5	10.0

Table 6
Intra- and inter-day precision of the method ($n = 3$).

Coumarins	Concentration (μg/mL)	Intra-assay RSD (%)		Inter-assay RSD (%)	
		Retention time	Peak area	Retention time	Peak area
Bergapten	10.0	2.04	3.75	2.94	7.86
	40.0	1.84	3.54	2.64	6.48
	100.0	1.98	3.32	3.15	5.98
Imperatorin	20.0	1.62	2.84	2.48	5.13
	60.0	1.64	2.46	2.32	4.98
	200.0	1.73	2.21	2.53	4.53
Osthole	10.0	1.78	2.02	2.42	3.68
	100.0	1.96	1.98	2.14	3.87
	300.0	2.05	1.83	2.65	3.42
2′-Acetyangelicin	10.0	1.85	2.54	3.45	6.74
	40.0	1.64	2.13	2.65	5.88
	100.0	2.41	1.64	2.15	4.62
Oroselone	20.0	1.42	2.25	2.91	5.23
	60.0	1.69	2.03	2.46	5.02
	200.0	1.45	1.97	2.15	4.67
O-acetylcolumbianetin	20.0	1.57	2.98	2.46	4.46
	60.0	1.98	2.46	2.14	4.62
	200.0	2.25	2.12	2.23	4.51

3.3.2. Precision, accuracy and stability

Under optimized conditions, the intra-day precision and inter-day precision were studied by analyzing three concentrations of standard solutions. Each assay was repeated three times every day and three times every day on three different days. Variations were expressed by the RSD of retention times and peak area ratios (the analytes/IS) which were less than 2.41% and 3.75% for intra-day, and 3.45% and 7.86% for inter-day, respectively (Table 6). The results showed good reproducibility of the developed method. The stability was tested at the same concentration levels of those in intra- and inter-day precision study and the analytes were found to be rather stable within 12 h.

The accuracy of the method was determined by recovery studies using the standard addition method. Three different concentrations of six coumarins were added to approximately 1 g of the sample 5 (Lin'an, Zhengjiang), and then extracted and analyzed using the method mentioned above. The total amount of each analyte was calculated from the corresponding calibration curve. The recoveries were counted by the formula: recovery (%) = (amount found-original amount)/amount spiked × 100%, and RSD (%) = (SD/mean) × 100%. The recoveries of the investigated analytes were in the range from 95.32% to 105.3% with RSDs less than 4.71%. The results were given in Table 7. Considering the results, the method was deemed to be accurate.

3.3.3. Robustness

No significant effect was observed on system suitability parameters such as capacity factor, resolution and theoretical plates of respective components, when small, deliberate changes were made to electrochromatographic conditions. The results showed that the method was considered robust and reliable.

3.4. Analysis of F. cnidii from different regions

Twelve samples of raw materials collected from different regions of China were analyzed with the pCEC condition and pCEC chromatograms were shown in Fig. 5. After careful analysis of the fingerprint profiles of the collected samples, six peaks with reason-

Table 7
Recovery of coumarins determined by standard addition method ($n = 3$).

Coumarins	Original (mg)	Added (mg)	Found (mg)	Recovery (%)	Mean recovery (%)	RSD (%)
Bergapten	3.24	2.56	5.74	97.66	98.26	2.74
		3.25	6.43	98.15		
		3.94	7.14	102.5		
Imperatorin	5.26	4.16	9.38	99.04	98.94	2.54
		5.25	10.32	96.38		
		6.34	11.69	101.4		
Osthole	14.78	11.89	26.47	99.24	97.67	1.32
		14.62	29.11	98.02		
		17.64	31.83	96.66		
2′-Acetyangelicin	ND	4.12	4.32	104.9	102.3	4.71
		5.22	5.05	96.74		
		6.04	6.36	105.3		
Oroselone	ND	4.06	3.87	95.32	96.95	1.50
		5.32	5.22	98.12		
		6.18	6.02	97.41		
O-acetylcolumbianetin	ND	4.14	4.32	104.3	101.6	4.22
		5.06	4.87	96.25		
		6.17	6.33	102.6		

Recovery (%) = (amount found − original amount)/amount spiked × 100%. RSD (%) = (SD/mean) × 100%. Sample of *F. cnidii* was from Lin'an (Zhengjiang). ND: not detected

Table 8
Contents of the coumarins in *F. cnidii* ($n = 3$).

Sample no.	Contents (mg/g)					
	Bergapten ±SD	Imperatorin ±SD	Osthole ±SD	2′-Acetylangelicin ±SD	Oroselone ±SD	O-acetylcolumbianetin ±SD
1	2.84 ± 0.08	5.76 ± 0.14	12.14 ± 0.24	ND	ND	ND
2	3.41 ± 0.06	7.16 ± 0.22	16.10 ± 0.52	ND	ND	ND
3	3.25 ± 0.03	6.53 ± 0.21	16.31 ± 0.54	ND	ND	ND
4	3.38 ± 0.09	5.34 ± 0.16	16.21 ± 0.38	ND	ND	ND
5	3.24 ± 0.06	5.26 ± 0.15	14.78 ± 0.31	ND	ND	ND
6	3.12 ± 0.11	6.44 ± 0.18	16.41 ± 0.51	ND	ND	ND
7	3.12 ± 0.09	5.64 ± 0.21	16.32 ± 0.49	ND	ND	ND
8	2.94 ± 0.04	7.25 ± 0.25	15.63 ± 0.48	ND	ND	ND
9	1.29 ± 0.05	3.84 ± 0.11	ND	4.41 ± 0.16	6.45 ± 0.21	7.86 ± 0.24
10	1.10 ± 0.04	4.17 ± 0.16	ND	4.07 ± 0.11	8.26 ± 0.25	7.27 ± 0.21
11	ND	2.95 ± 0.11	ND	6.13 ± 0.15	4.01 ± 0.11	3.89 ± 0.14
12	ND	ND	ND	ND	3.88 ± 0.09	4.31 ± 0.10

Samples 1–12 as Table 2. ND: not detected.

able heights and good resolution were selected as characteristic peaks for identification of the raw materials. Bergapten (peak 1, 3.97 min), imperatorin (peak 2, 7.82 min), osthole (peak 3, 9.42 min), 2′-acetylangelicin (peak 4, 4.62 min), oroselone (peak 5, 10.68 min), and O-acetylcolumbianetin (peak 6, 12.50 min) were characterized by using the standard addition method. These coumarins could be divided into three kinds of coumarins which were simple coumarins (osthole), linear furocoumarins (bergapten and imperatorin) and angular furocoumarins (2′-acetylangelicin, oroselone and O-acetylcolumbianetin).

Based on pCEC chromatographic patterns in terms of peak composition and retention time, samples 1–12 were divided into four groups. The pCEC chromatograms of samples 1–8 were similar, which contained one simple furocoumarin (osthole) and two linear furocoumarins (bergapten and imperatorin). In sample 9, there were one simple furocoumarin (osthole), two linear furocoumarins (bergapten and imperatorin) and three angular furocoumarins (2′-acetylangelicin, oroselone and O-acetylcolumbianetin). Samples 10 and 11 contained both two linear furocoumarins (bergapten and imperatorin) and three angular furocoumarins (2′-acetylangelicin, oroselone and O-acetylcolumbianetin), and sample 12 only contained two angular furocoumarins (oroselone and O-acetylcolumbianetin) as the main components.

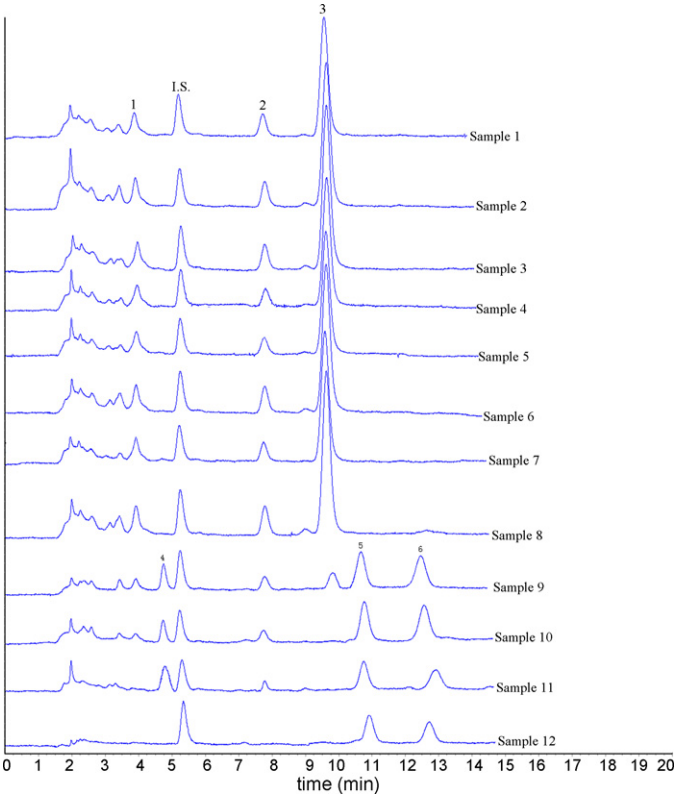

Fig. 5. Pressurized capillary electrochromatograms of coumarins from different regions of China. Samples 1–12 (see Table 2). pCEC conditions can be seen in Fig. 3. Peaks: (1) bergapten, (2) imperatorin, (3) osthole, (4) 2′-cetylangelicin, (5) oroselone, and (6) O-acetylcolumbianetin.

3.5. Quantitation of the six coumarins in F. cnidii

The quantitative analysis was performed by means of the internal standard methods. Contents (% w/w) of analytes in different samples are summarized in (Table 8). Data of quantitative analyses were expressed as mean ± deviation.

The quantitative analysis results showed that the content ranges (w/w, mg/g) of the six coumarin constituents in the raw materials from different regions of China were 0–3.38 (bergapten), 0–7.25 (imperatorin), 0–16.41 (osthole), 0–6.13 (2′-acetylangelicin), 0–8.26 (oroselone) and 0–7.86 (O-acetylcolumbianetin), respectively. Large variations in the contents of these constituents were found. From the results obtained, the content of osthole was the highest among the selected constituents in samples 1–8, but not detected in samples 9–12. Compared with the qualified content limit of *F. cnidii* stating in the Chinese Pharmacopoeia (2005 edition, 10 mg/g of osthole), four unqualified samples in the total twelve samples were found. However, these four samples contained angular furocoumarins which did not appear in samples 1–8. The large variation of coumarin composition and content in the different samples may be attributed to the geographical source, cultivation, harvest, storage, and processing of the herb, etc. Among these factors, different geographical sources play an important role in the concentrations of the selected constituents in *F. cnidii*. Samples 1–8 have the distribution in semitropical evergreen broadleaf forest zone such as Jiangsu, Zhejiang, etc.; samples 9–11 have the distribution in warm temperate deciduous broadleaf forest zone such as Heibei, Shangxi, etc.; sample 12 distributed in conifer broadleaf mix forest zone [4]. Since bergapten, imperatorin and osthole were considered as the main bioactive coumarins in *F. cnidii*, the results suggested that samples 1–8 might have stronger pharmacological effects than other samples.

4. Conclusions

We used the home-packing column with a monolithic outlet frit to qualitative and quantitative the *F. cnidii* from 12 different regions of China. And the method with high separation efficiency

and resolution, and acceptable repeatability indicates that it is a powerful analytical technique with much potential to the analysis of herbal extracts. The novel column showed the same features offered by capillary columns with both sintered frits in term of retention time and efficiency.

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