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Separation and determination of honokiol and magnolol in herbal medicines by flow injection-capillary electrophoresis

Abstract A simple, rapid, and accurate method for the separation and determination of honokiol and magnolol in *Magnolia officinalis* and related herbal medicines was developed by combination of flow injection (FI) and capillary zone electrophoresis (CZE). The analysis was carried out using an unmodified fused-silica capillary (50-μm I.D.; total length 7.5 cm; effective length 4.5 cm). A series of optimization steps afforded the following conditions: the sample solvent consisted of 150 mM NaOH and a running buffer composed of 10 mM sodium tetraborate/10 mM sodium dihydrogenphosphate (NaH$_2$PO$_4$) at pH 12 was applied for the separation of the analytes. The separation could be achieved within 5 min with a sample throughput rate of up to 28 h^{-1}. The repeatability (defined as the relative standard deviation, RSD) for honokiol and magnolol was 2.0% and 1.6% with peak area evaluation, 3.6% and 2.0% with peak height evaluation, and 2.0% and 1.4% with migration time evaluation, respectively. Regression equations revealed linear relationships ($r = 0.9991$–0.9998) between the peak area of each analyte and the concentration.

Keywords Flow injection-capillary electrophoresis ·
Honokiol · Magnolol · Herbal medicines

Introduction

Capillary electrophoresis (CE) has become a powerful analytical technique [1]. However, the discontinuous

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sample introduction mode (hydrodynamic and electrokinetic sample introduction) restricts the sample throughput and precision. The advantages of the combined FI-CE system have been demonstrated not only to produce an efficient and reliable mode of sample introduction with improved throughput and precision [2–8], but also to provide on-line sample pretreatment involving on-line column sorption [3], sample dialysis [4], gas diffusion [5], and reaction [7]. Additionally, the microchip with an H-channel design in this work presented a low-cost alternative for more basic studies on a microfluidic system [9–13].

Honokiol and magnolol are the two major phenolic components of the bark of *Magnolia officinalis* (Fig. 1). They have been reported to show many pharmacological activities including antiplatelet activity [14], anti-inflammatory effects [15], and antimicrobial activity [16]. The contents of honokiol and magnolol are key factors for quality control of herbal medicines. Therefore, a simple and rapid method to determine the two components is highly desired. Several methods have been reported for the determination of honokiol and magnolol, including thin-layer chromatography [17], high-performance liquid chromatography [18–20], micellar electrokinetic chromatography [21], and CZE [22–24]. However, few of these methods were entirely adequate because of poor accuracy, low resolution, or the requirement for tedious pretreatment. The purpose of this paper is to establish a simple, inexpensive, rapid, and continuous automated sampling FI-CE method to separate and determine honokiol and magnolol in herbal medicines.

Experimental

Chemicals and solutions

Honokiol and magnolol were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, China. The standards were used as received. The crude drug preparation of *Magnolia officinalis* was
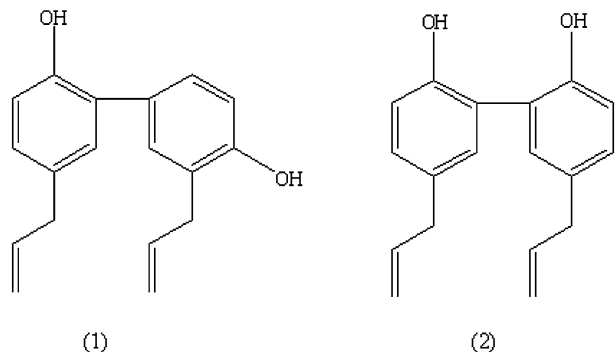
Fig. 1 Structures of honokiol (**1**) and magnolol (**2**)

purchased from a local drug store. The commercial herbal medicines Huoxiang Zhengqishui and Baoji pill were purchased from Sichuan Shuzhong Medicine Company (Guanghan, Sichuan, China) and Guangzhou Wanglaoji Medicine Company (Guangzhou, Guangdong, China), respectively. Sodium tetraborate was purchased from Jiangsu Taichang Second Chemical Factory (Taichang, Jiangsu, China), and sodium dihydrogenphosphate (NaH_2PO_4) was purchased from Beijing Chemical Factory (Beijing, China). All chemicals were of analytical reagent grade and were used as received. All solutions and buffers were made with distilled water.

Stock standard solutions (400 µg mL^{-1}) of honokiol and magnolol were prepared in 150 mM NaOH. The standard solutions at various concentrations were prepared by appropriate dilution of the stock solution with 150 mM NaOH. The carrier solution, which also functioned as an electrophoretic buffer, was freshly prepared and typically consisted of 10 mM sodium tetraborate/10 mM NaH_2PO_4. The buffer was prepared daily from stock solution of 100 mM sodium tetraborate and 100 mM NaH_2PO_4, and then adjusted to the desired pH using either 2 M NaOH or 2 M HCl. The pH of the buffer was checked using a PHS-10A pH meter (Xiaoshan Science Instrumentation Factory, Zhejiang, China).

Magnolia officinalis powder (2 g) or Baoji pill (5 g) were extracted by ultrasonic treatment with ethanol (80 mL, respectively) for 1 h, and then extracted solutions were evaporated to dryness, 150 mM NaOH (144 mL or 20 mL, respectively) was added to dissolve the residues. Huoxiang Zhengqishui (2 bottles) was evaporated to dryness, and then 150 mM NaOH (60 mL) was added to dissolve the residue. All solutions were filtered through 0.45-µm syringe filters before use.

Apparatus

Experiments were conducted using an HPE-100 CE system with 12-kV maximum voltage (Bio-Rad, Hercules, CA) interfaced with a 486 personal computer utilizing the Chroma chromatography collection system (Bio-Rad) for integration and data collection. Uncoated fused silica capillaries (50-µm I.D. ×375-µm O.D.) were purchased from Yongnian Optical Fiber Factory (Handan, Hebei,

China). Detection was by monitoring UV absorbance at 254 nm.

A K-1000 flow injection analyzer (Hitachi, Japan) was equipped with a peristaltic pump, a 16-way injection valve, and a plunger pump. All components of the FI system were connected using 0.5-mm-I.D. poly(tetrafluoroethylene) (PTFE) tubing; this set up included a 33-cm-long transport line from the valve to the split-flow interface. A sample loop and two reagent loops were made from PTFE. The time period for the sample injection was selected through man/access mode.

The H-channel microchip (Fig. 2) was constructed using a planar plastic slide B (30 m × 80 mm × 2 mm) as the base plate integrated with a separation capillary (50-µm I.D. × 375-µm O.D. × 7.5-cm length, and 4.5-cm effective length) and two reservoirs. The two reservoirs were produced from Tygon tubing (T1) (3.0-mm I.D., 4.0-mm O.D., 7.0-cm length for the anodic reservoir and 11.0-cm length for the cathodic reservoir) which were first fixed sideways on the slide B with epoxy and then connected to the separation capillary whose both ends were inserted into the center of the Tygon tubing (T1) through holes punctured with a hypodermic stainless steel needle. Two platinum electrodes (the anode and cathode) were respectively inserted into the two reservoirs 30 mm above the capillary tip to avoid entrainment of electrolytically generated oxygen bubbles into the capillary. A 30-mm length of Tygon tubing (T2) was push-fitted into the anodic reservoir until 12 mm below the capillary tip, and this functioned as the carrier/sample (C/S) inlet. The lower section of cathodal reservoir (T1) was controlled by using a screw clamp except for filling or changing of the electrolyte solution, or for capillary cleaning.
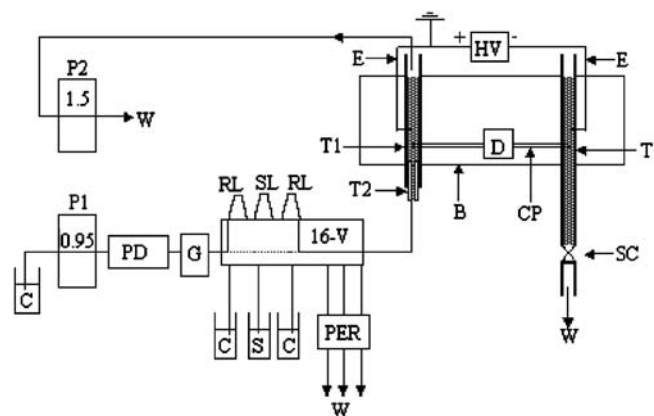

Fig. 2 Schematic of the FI-CE manifold (not to scale). *C* carrier solution, *S* sample, *P1* and *P2* pumps, *PD* pressure damper, *G* pressure gauge, *SL* sampling loop, *RL* reagent loop, *16-V* 16-way valve, *PER* peristaltic pump, *B* planar plastic base, *T1* Teflon tubing, *T2* Tygon tubing, *CP* separation capillary column, *E* platinum electrode, *W* waste, *C/S* carrier/sample, *HV* high voltage, *D* detector, *SC* screw clamp

Procedure

Figure 2 shows a schematic of the FI-CE system. During the sampling loading stage (Fig. 3a), samples and buffer solutions were drawn by a peristaltic pump to fill the sample loop (SL) and reagent loops (both of 20-μL volume) of the injector valve (V) in the fill position, respectively. The buffer solutions were drawn by a plunger pump at a constant flow rate through split-flow interface. When sample and reagent loops were filled, the loading stage was finished and the peristaltic pump was stopped. In the injecting stage, the injector was automatically changed to the inject position, as shown in Fig. 3b. The buffer solution flows through the sample and reagent loops. The sample solution was sandwiched by the buffer solution and transported through the connecting conduit into the Tygon tubing (T2), where the flow was split and a fraction of the sample zone injected by the FI system was introduced into the separation capillary by electrokinetic means for 8 s. After the injection stage was finished, the injector was automatically changed to the loading position and prepared for the next injection. A series of samples were injected

Fig. 3 Schematic of the 16-way auto-switching valve: **a** loading stage, **b** injecting stage

A   Loading stage

B   Injecting stage
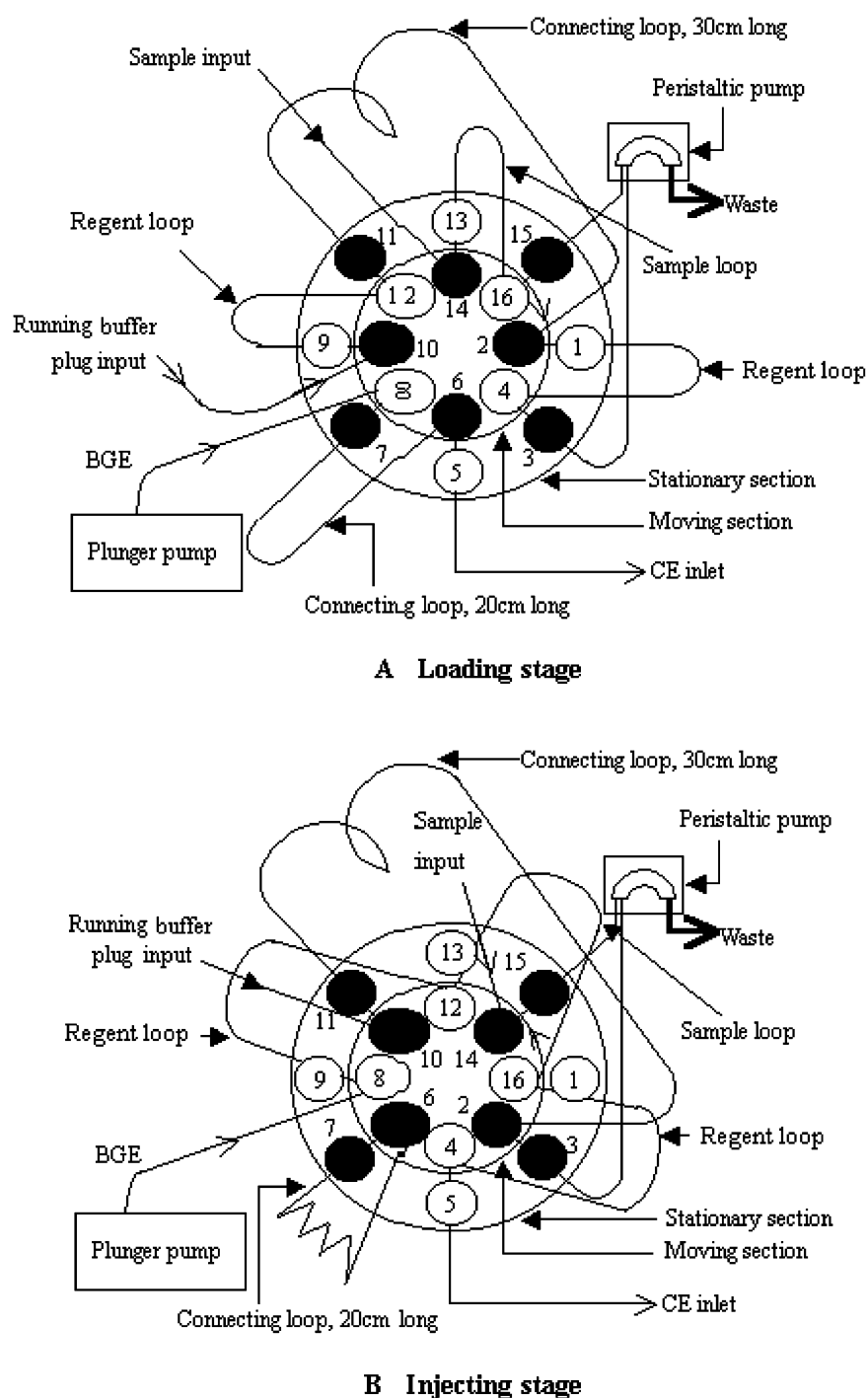
continuously without interrupting the high voltage (1.2 kV). For CE operations, an unmodified fused-silica capillary was used for all analyses. At the beginning of each working day, the capillary was flushed sequentially with distilled water (5 min), 100 mM NaOH (5 min), and distilled water (5 min), followed by running buffer (5 min) from the capillary outlet reservoir using a water-circulating vacuum pump. The CE instrument was simultaneously warmed up until a stable baseline was achieved. Moreover, the capillary was rinsed for 2 min with distilled water, 2 min with 100 mM NaOH, 2 min with distilled water, and then 3 min with fresh buffer by manual handling after each run, prior to the next injection. The column was left filled with distilled water overnight.

Results and discussion

To achieve low a limit of detection (LOD) and satisfactory separation, the optimizations of sample medium and separation conditions were of primary importance. In this work, the FI and CE parameters were optimized by a univariate approach taking the peak areas and resolution as the principal figures of merit. The identity of the recorded peaks was confirmed by independent injection of the pure compounds.

NaOH as the medium of sample solution

The sample medium plays an important role in CE. In this study, we dissolved honokiol and magnolol in NaOH solution. The concentration of NaOH has an important effect on the resolution and sensitivity of analytes: the use of NaOH solution not only turns them into anions, but also get rid of insoluble matter. To optimize the method, the concentrations of NaOH were varied from 20 to 500 mM using 10 mM sodium tetraborate/10 mM NaH_2PO_4 buffer (pH 12) and 1.2-kV applied voltage. The results indicated that the resolution of the analytes increased with increasing concentration of NaOH up to 150 mM, and then decreased at higher concentration, but the peak area increased throughout. Additionally, the peak shape (height/width) of the analytes became poor with increasing NaOH. As we know, if the concentration of NaOH was too high, it would damage the silica surface of the capillary inner wall, thus reducing the lifetime of capillary. On considering the resolution of these analytes, peak area, peak shape, and lifetime of capillary, 150 mM NaOH was preferred in further studies.

Influence of separation conditions

Influence of the buffer pH on separation

In electrophoretic separations of ionizable compounds, pH plays an important role as it determines the extent of ionization of each individual analyte; hence, it is important

to manipulate the pH of the buffer in optimizing the separation conditions. Honokiol and magnolol (Fig. 1) are weak acids due to the presence of phenolic hydroxyl group. The mobility of the two analytes is influenced by the degree of dissociation, which is dependent on their pK_a values and pH of the electrolyte solution. In this study, the influence of buffer pH on the FI-CE separation of honokiol and magnolol was investigated in the pH range 6.0–12.2 with 10 mM sodium tetraborate/10 mM NaH_2PO_4 solution and 1.2-kV applied voltage. Figure 4 shows the effect of buffer pH on the resolution and migration time. The results indicate that the resolution of the two analytes increased with increasing pH up to pH 9, and then decreased at higher pH; however, the resolution increased dramatically when the pH was higher than 11. In the pH range 6–11, the peak sequence of the two compounds in CZE was honokiol and then magnolol. When the pH is low, magnolol is more easily ionized than honokiol; however, when the pH is higher than 11, the reverse happens. Increasing pH resulted in a reversal of the migration order of honokiol and magnolol. This could be explained by the pK_a values of magnolol (pK_{a1}=6.94±0.11, pK_{a2}=10.38±0.28) and honokiol (pK_{a1}=9.82±0.32, pK_{a2}=10.35±0.34) [25]. In addition to resolution of the solutes, the retention time, column efficiency, and sensitivity all tend to change with the changing pH. When the pH was increased from 6 to 12.2, there was an increase in the migration time from 2.45 to 11.34 min; the peak area also increased throughout this change. With concurrent consideration of peak area, resolution, and migration time, pH 12 was therefore preferred for further studies.
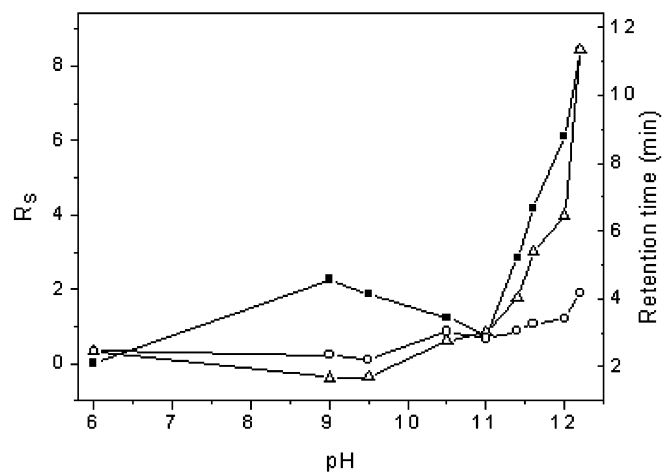

Fig. 4 Influence of buffer pH on the separation of the peaks. Conditions: 50-μm I.D. × 375-μm O.D. × 7.5-cm length (4.5-cm effective length), uncoated; buffer 10 mM sodium tetraborate/10 mM NaH_2PO_4 (pH 12); sample solution NaOH (100 mM), voltage 1.0 kV; detection wavelength 254 nm; sample volume 20 μL; carrier flow rate 0.90 mL min^{-1}; sample: 100 μg mL^{-1} honokiol and magnolol; (△, magnolol; ○, honokiol; ▪, resolution of honokiol and magnolol)

Table 1 Analytical performance of the FI-CE in a pharmaceutical preparation testing system ($n=8$)

Sample	Magnolol	Honokiol
LOD (S/N=3) (μg mL^{-1})	0.64	0.86
Peak area RSD (%)	1.6	2.0
Peak height RSD (%)	2.0	3.6
Migration time RSD (%)	1.4	2.0
Regression equation[a]	$y=49.91x-22.03$	$y=60.11x-28.71$
r	0.9998	0.9991
Linear range (μg mL^{-1})	5–300	5–300

[a]y=peak area; x=standard concentration (μg mL^{-1})

Influence of separation voltage

The effect of the separation voltage was investigated in the range 0.8 to 1.5 kV under the conditions selected above. As may be expected, under such conditions, with increasing separation voltage, the migration time was shortened; however, a decrease of resolution was also apparent, probably owing to an increase in the electroosmotic flow. A higher voltage was necessary for rapid analysis, which could reduce molecular diffusion in the mobile phase and band spread. At lower voltage, because the migration of compound became slow, the band would also be broadened due to diffusion effects [26]. With concurrent consideration of peak shape, sample throughput, and resolution, the separation voltage finally chosen was 1.2 kV.

Influence of FI sample carrier flow rate

With fixed sample injection volumes, the carrier flow rate determined the residence time of the sample zone within the flow-through reservoir and therefore the time available for electrokinetic split-sampling into the capillary [27]. The effects of FI carrier flow rate in the range 0.85–1.52 mL min^{-1} were studied using a 20-μL sample volume with 10 mM sodium tetraborate/10 mM NaH$_2$PO$_4$ solution
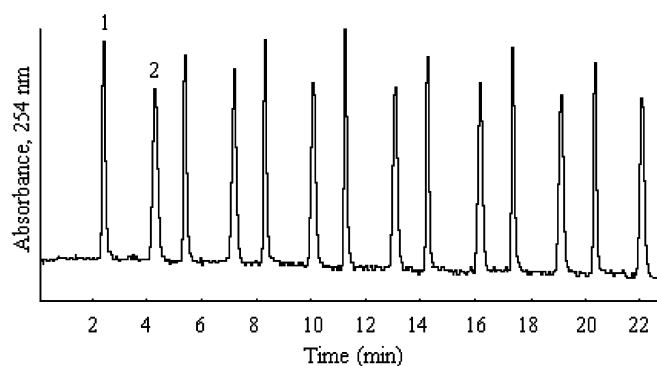
Fig. 5 Recordings of seven consecutive injections of a standard mixture of honokiol and magnolol (*1* magnolol, *2* honokiol). Dissolution condition: 150 mM NaOH. Separation condition: buffer 10 mM sodium tetraborate/10 mM NaH$_2$PO$_4$ (pH 12), 1.2-kV voltage, 20-μL sample volume, 254-nm UV detection, buffer flow rate 0.95 mL min^{-1}

(pH 12) and 1.2-kV applied voltage. With increasing flow rate, the migration time was slightly shortened and the resolution between the two analytes increased; however, the peak area was decreased drastically and a higher flow rate would consume more buffer solution. On considering the resolution of these analytes, peak area, and consumption of buffer solution, 0.95 mL min^{-1} was finally chosen as the flow rate of buffer solution.

Final optimization

The final optimized conditions were: (1) dissolution conditions: 150 mM NaOH solution, (2) separation conditions: 10 mM sodium tetraborate/10 mM NaH$_2$PO$_4$ buffer (pH 12), 1.2-kV voltage, 20-μL sample volume,
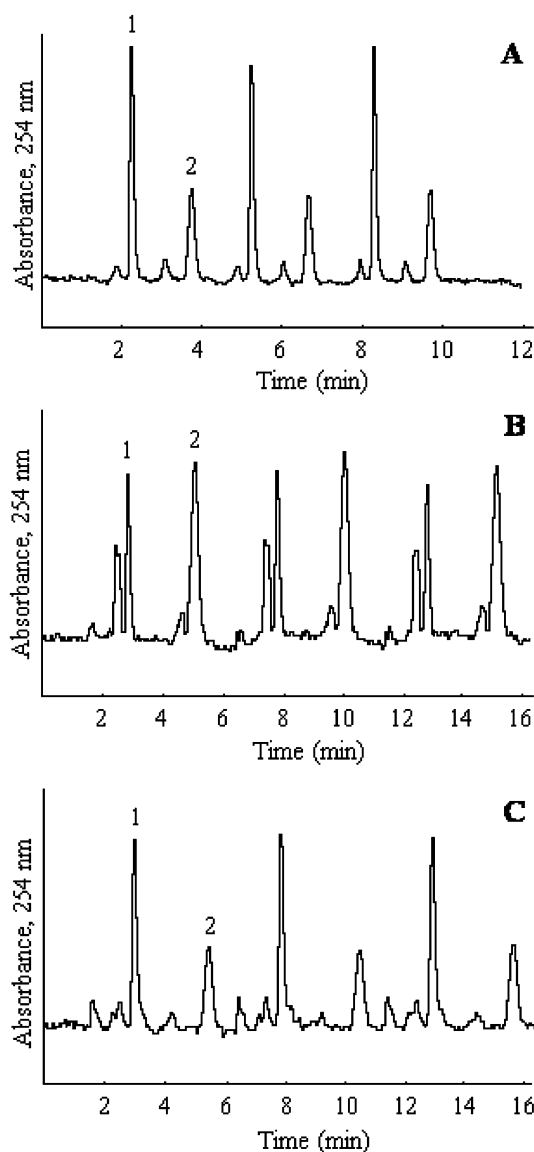
Fig. 6 Electropherograms of herbal drug and related herbal preparations: **a** *Magnolia officinalis*, **b** Huoxiang Zhengqishui, **c** Baoji pill. Other conditions as in Fig. 5

Table 2 Results for the determination of the two components in sample extracts (*n*=4)

Sample	Ingredient	Content	Concentration spiked (μg mL^{-1})	Concentration found (μg mL^{-1})	Recovery (%)	Average (%)	RSD (%)
Magnolia officinalis	Magnolol	12.2 mg/g	50	53.2	106	102	3.9
			100	101.7	102		
			125	122.0	98		
	Honokiol	6.2 mg/g	50	47.2	94	95	3.8
			100	92.2	92		
			125	123.7	99		
Huoxiang Zhengqishui	Magnolol	2.9 mg/bottle	25	25.7	103	104	4.4
			40	43.7	109		
			50	50.1	100		
	Honokiol	4.8 mg/bottle	25	22.4	90	98	7.4
			40	41.6	104		
			50	49.8	100		
Baoji pill	Magnolol	0.59 mg/g	40	38.0	95	101	6.4
			50	54.2	108		
			75	76.1	101		
	Honokiol	0.33 mg/g	40	38.8	97	105	6.7
			50	55.1	110		
			75	81.3	108		

254-nm UV detection, 0.95 mL min^{-1} flow rate of buffer solution.

Performance of the combined FI-CE

The linearity was measured by plotting the peak area (*y*) of each analyte versus the corresponding concentration (*x*). Calibration graphs were obtained by injecting standard solutions at eight different concentrations (5.0, 10.0, 25.0, 50.0, 100.0, 150.0, 250.0, and 300.0 μg mL^{-1}) in turn and a good linearity was obtained. As the solute concentrations increased (>300 μg mL^{-1}), the plot eventually deviates from linearity. The most dilute concentration of the analyte that produces a measurable peak is considered its low-limit. The RSD of the peak area for four replicate runs was <2.0% for the analytes, and the peak areas were employed for quantification. The corresponding regression equations and other characteristic parameters for the determination of honokiol and magnolol are listed in Table 1. The LOD was calculated as the peak height at a signal-to-noise ratio of 3 (S/N=3). The proposed method allowed the determination of honokiol and magnolol at low levels.

The repeatability of the FI-CE method was evaluated under optimized conditions by seven consecutive injections of a standard solution containing 150 μg mL^{-1} honokiol and magnolol. The repeatability (RSD) of peak height, peak area, and migration time for the analytes are summarized in Table 1. The electropherograms obtained are shown in Fig. 5. The sampling frequency achievable per hour could be estimated according to Fang's equation [27], and, in this work, it was calculated to be 28 h^{-1}.

Application

Quantitative analysis was performed under the optimum conditions obtained from the experiments described above. The method was applied to the analysis of honokiol and magnolol in *Magnolia officinalis* and related herbal medicines. Typical electropherograms of the samples are shown in Fig. 6. The peaks were identified by the standard addition methods. The accuracy of the methods and the potential matrix effects were established by analyzing spiked samples. The results are presented in Table 2.

Conclusions

The coupling of the FI system with CE equipment has successfully been used to analyze honokiol and magnolol in *Magnolia officinalis* and related herbal preparations for the first time. The use of NaOH as sample solvent improves sensitivity and resolution. Furthermore, the capillary used in the above methods was relatively short (total length 7.5 cm) which made the analysis time relatively short. The results indicate that FI provides reproducible and reliable sample introduction for CE. This sample introduction scheme has several compelling advantages over methods described in the literature [21–24], including ease of operation and automation and enhanced sampling frequencies (standard solution 28 h^{-1}, real samples 13–20 h^{-1}).

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