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Simultaneous Separation and Analysis of Five Compounds in *Cibotium barometz* by Micellar Electrokinetic Chromatography with Large-Volume Sample Stacking

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Abstract: A large volume sample stacking (LVSS) method in micellar electrokinetic chromatography (MEKC) with diode array detector was developed for the simultaneous separation and analysis of five compounds: protocatechuic acid, protocatechuic aldehyde, caffeic acid, syringetin and vanillin in *Cibotium barometz*. The electrophoretic separation was performed in a 10 mM sodium dodecyl sulfate (SDS) and 50 mM sodium borax-sodium dihydrogen phosphate system (pH = 8.5) with 10% methanol at a separation voltage of 30 kV after optimizing the typical parameters. The detection limits were from 32 pg to 65 pg, which were around 12–27 times lower than MEKC, and 500 times less than reported methods. Finally, the established method was validated to be applicable for the determination of protocatechuic acid and caffeic acid in *Cibotium barometz*. This proposed method is expected to facilitate the quality control of *Cibotium barometz*.

Keywords: capillary electrophoresis; *Cibotium barometz*; large volume sample stacking; micellar electrokinetic chromatography

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1. Introduction

As an important traditional Chinese medicine, *Cibotium barometz* is widely used to treat limb-ache, rheumatism, sciatica and osteoporosis [1]. It has the properties of being anti-inflammatory [2], reducing bone loss [3], increasing antioxidant activity [4,5] and promoting chondrocyte proliferation [6]. The pharmacological effects of herbs are often attributed to their chemical components. *Cibotium barometz* mainly contains protocatechuic acid [1,7] and protocatechuic aldehyde [1,7], in addition to caffeic acid [1,8], syringetin and vanillin [8]. Evidence shows that protocatechuic aldehyde, protocatechuic acid, caffeic acid and polysaccharides from rhizomes of *Cibotium barometz* had a significant proliferative effect on osteoblasts [9,10].

Up to now, some works on the main ingredients in *Cibotium barometz* have been reported. Protocatechuic acid and protocatechualdehyde were determined by HPLC [11,12]. The organic acids, alkaloids, small molecular aldehyde ketones and other substances [13–15] in *Cibotium barometz* were qualitied by UPLC/Q-TOF and GC-MS. However, in view of the fact that the physicochemical properties result from multiple components at multiple targets, the quantity of one or two components does not always reflect its quality. Thus, it is necessary to establish qualitative and quantitative methods for simultaneous analysis of more constituents in *Cibotium barometz*.

Based on the concepts of positive environmental impact and health, capillary electrophoresis (CE) has versatility and high separation power, which can satisfy the quantitative separation and identification of traditional Chinese medicine [16,17]. However, the sensitivity and selectivity of CE are limited due to the small injection volume (nL range) [18] and short optical path length, which hamper the determination of low-concentration components [19]. Fortunately, it can be solved by special electrophoresis patterns (e.g., capillary zone electrophoresis and electrokinetic chromatography) and on-line enrichment sample stacking techniques (e.g., large-volume sample stacking (LVSS), field-amplified sample injection and sweeping) [20].

In micellar electrokinetic chromatography (MEKC), analytes are separated by the differential partition between the pseudo-stationary phase and the surrounding electrolyte [21]. MEKC can also combine different stacking techniques to separate analytes simultaneously according to the properties of the analytes [22], and LVSS is one of the typical representatives [23].

In this work, LVSS method was combined with the MEKC to simultaneously determine five compounds in *cibotium baromet*, including protocatechuic acid, protocatechuic aldehyde, caffeic acid, syringetin and vanillin. Compared with traditional methods, this online approach can provide higher determination sensitivity and less use of organic solution. This environmentally friendly analysis method has great application potential in the quality control of *Cibotium barometz* and relevant crude plant extracts.

2. Materials and Methods

2.1. Chemicals and Materials

Protocatechuic acid, protocatechuic aldehyde, caffeic acid, syringetin and vanillin (the structures are shown in Table 1) were obtained from Yuanye Biotech (Shanghai, China) Co., Ltd. *Cibotium barometz* was obtained from Fujian Youxi Xianjin Pharmaceutical Co., Ltd. (Sanming, Fujian Province, China). All chemicals were analytical grade. High-purity water was prepared by a Milli-Q water purification system (Millipore, MA, USA).

2.2. Instruments

All CE separations were conducted on a Beckman PA800plus system (Beckman, Fullerton, CA, USA) equipped with DAD detector. The system was controlled by 32-Karat Software. Electrophoretic separation was carried out in a fused-silica capillary of 60.0 cm length (50.0 cm effective length) × 75 μm I.D. × 370 μm O.D. (Yongnian photoconductive Fiber Factory, Handan, Hebei, China). A PHS-3C meter (Shanghai Dapu Instrument Company, Shanghai, China) was used to measure the pH value of the running buffer. The ultrasonic cleaner was purchased from Kunshan Ultrasonic Instrument Co., Ltd. (Kunshan, Jiangsu Province, China).

2.3. Preparation of Solutions and Samples

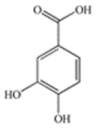
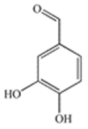
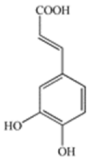
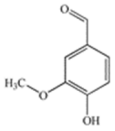
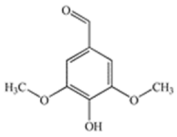
Stock solutions of each compound were prepared in anhydrous ethanol at 1 mg/mL, and diluted to the final concentration with water before using.

The dried *Cibotium barometz* was cut into pieces and finely ground, then 1.0 g *Cibotium barometz* powder was extracted with 6 mL ethanol in an ultrasonic bath for 0.5 h. This extraction process was repeated twice before filtering. Subsequently, the volume was fixed with ethanol to 25 mL and stored at 4 °C. Before use, it was diluted with water and filtered through a 0.22 μm membrane filter.

2.4. Electrophoretic Procedure

New capillaries were flushed with water, 1 M HCl, 1 M NaOH and running buffer alkali solutions [24]. To achieve better reproducibility, the capillaries were pretreated by flushing with 0.5 M NaOH for 10 min, water for 10 min every day. The capillaries were flushed with buffer for 5 min (20 psi) between injections.

Table 1. Five compounds in *Cibotium barometz*.

Representative Number	Analyte	Structure	pKa
1	protocatechuic acid		4.26
2	protocatechuic aldehyde		7.26 [25]
3	caffeic acid		4.58 [26]
4	syringetin		7.39
5	vanillin		7.80 [27]

MEKC injection conditions: 0.5 psi, 5 s. LVSS-MEKC injection conditions: 0.5 psi, 100 s, negative 30 kV, 0.5 min. The running buffer was 50 mM sodium borax-sodium dihydrogen phosphate (pH = 8.5) containing 10 mM sodium dodecyl sulfate and 10% methanol (Note: First borax was dissolved at the concentration of 50 mM, then the pH was adjusted to 8.5 with 50 mM sodium dihydrogen phosphate solution. Finally, sodium dodecyl sulfate was added to the concentration of 10 mM and mixed with methanol at the ratio of 10%.). The temperature of the capillary cartridge was set at 25 °C. Separations were carried out at a voltage of 30 kV and detected at 214 nm. Moreover, sample solution, standard solution and running buffer were all filtered through a syringe cellulose acetate filter (0.45 μm) prior to use.

3. Results and Discussion

3.1. Optimization of the Type and Concentration of Background Electrolytes (BGE)

Types of BGE, such as citrate buffer and borax buffer were tested first. The best reproducibility of five compounds was obtained in the borax buffer. Thus, the concentrations of borax buffer were further investigated. The best separation effect was achieved at 50 mM as shown in Figure 1, and this concentration was selected in the following experiments.

3.2. Optimization of the pH of BGE

The separation of CE was based on the differences in the charge-to-mass ratio of the analytes and the electroosmotic flow (EOF). Based on their pKa value (4.26–7.80), the effect of buffer pH in the range of 7.5–9.0 on the resolution of separation and intensity of analyte peaks was investigated. As shown in Figure 2, the best separation was obtained at the pH of 8.5. Thus, pH 8.5 was selected in the following experiments.

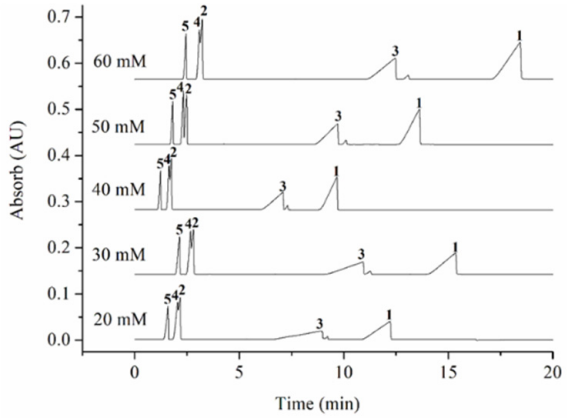


Figure 1. Electropherograms of five compounds at different buffer concentrations. Running buffer: sodium borax-sodium dihydrogen phosphate system. Separation voltage: 25 kV. Wavelength: 214 nm. The concentrations of analytes: 50 μg/mL. Inject: (0.5 psi, 10 s). The compounds represented by the numbers 1–5 are listed in Table 1.

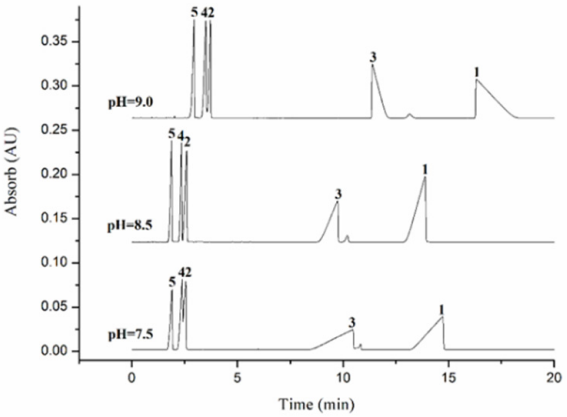


Figure 2. Electropherograms of five compounds at different pH values of buffer. Running buffer: 50 mM sodium borax-sodium dihydrogen phosphate system. Other conditions are the same as Figure 1.

3.3. Optimization of MEKC Conditions

In order to increase the solubility of analytes, micelles were further introduced in this work. SDS concentrations in this study were ranged from 10 mM to 30 mM (slightly higher than the critical micelle concentration value of 7.4 mM [16,28,29]). The best peak area and peak-to-peak resolution of the five compounds were obtained at 10 mM SDS, so 10 mM SDS [30] was added to the buffer to improve the solubility of samples in the buffer.

In addition, since nonaqueous media can increase the solubility of hydrophobic compounds and also improve the selectivity [17], organic solvents are widely used in MEKC analysis. Thus, several types of organic solvents, such as methanol, acetonitrile and ethanol, were added to the buffer to investigate their influence on the separation effect. As shown in Figure 3, when a constant concentration of 10% methanol was maintained in buffer, none of the analytes precipitated, and a stable current was obtained in the process of electrophoretic separation [19].

3.4. Optimization of LVSS Stacking Time and Injection Time

In order to eliminate the interference of sample matrix, the stacking time was varied in the range of 0.3–1.0 min at negative 30 kV (as shown in Figure 4). Long sample stacking time would lead to the loss of analytes (Figure 4A,B), but insufficient stacking time would lead to incomplete elimination of sample matrix (Figure 4D), so 0.5 min was chosen for the further study (Figure 4C).

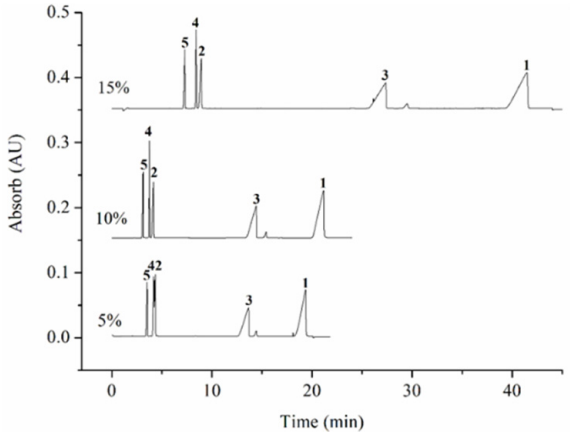


Figure 3. Electropherograms of five compounds at different concentration of methanol in buffer. Running buffer: 10 mM sodium dodecyl sulfate and 50 mM sodium borax-sodium dihydrogen phosphate system (pH = 8.5). Separation voltage: 30 kV. Other conditions are the same as Figure 2.

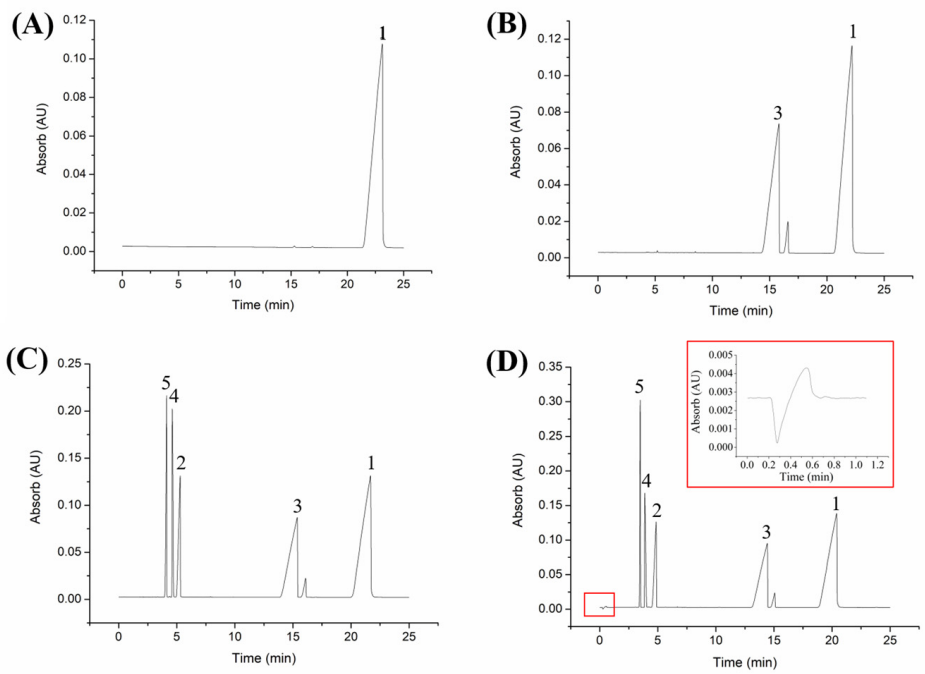


Figure 4. Electropherograms of five compounds at the sample stacking time of 1.0 min (**A**), 0.75 min (**B**), 0.5 min (**C**) and 0.3 min (**D**). LVSS-MEKC inject: 0.5 psi, 130 s, negative 30 kV. Running buffer: 50 mM sodium borax-sodium dihydrogen phosphate (pH = 8.5) containing 10 mM sodium dodecyl sulfate and 10% methanol. Other conditions are the same as Figure 3.

Increasing the injection volume is an efficient way to attain the highest sensitivity in peak area. Herein, the injection time was varied between 80 s and 180 s at 0.5 psi (as shown in Figure 5). It was found that both of the peak area and peak height of the analytes increased with the injection time from 80 s to 100 s. However, when the time was longer than 100 s, there was almost no change in peak efficiency and peak areas. Based on the above results, the optimum time occurred at 100 s.

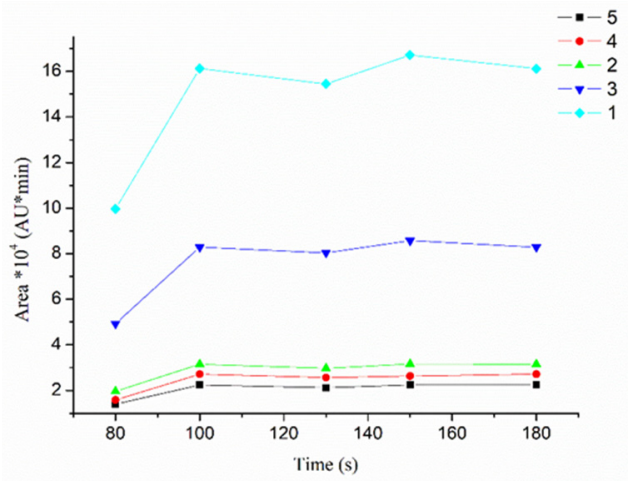


Figure 5. Effect of injection time on peak area of five compounds. LVSS-MEKC inject: 0.5 psi, negative 30 kV, 0.5 min. The concentrations of analytes: 0.5 μg/mL. Other conditions are the same as Figure 4.

3.5. The Principles of This LVSS-MEKC Method

The on-line sample enrichment technique combines the principles of large volume [22], and the focusing mechanism is shown in Figure 6. The fused silica capillary was filled with BGE, and then a large volume of a sample with low electric conductivity was injected hydrodynamically (Figure 6A). The inlet and outlet vials were then replaced by vials containing the BGE, and a reverse polarity voltage was applied. The negatively charged sodium dodecyl sulfate (SDS) micelles started to focus the analytes rapidly to an outlet because of the high electric field strength of the sample matrix. At the same time, the sample matrix was pumped out from the inlet driven by electroosmotic flow (EOF) (Figure 6B). Once the SDS micelles reached the boundary between the sample matrix and BGE at the outlet, they slowed down because of the reduced electric field strength in BGE. Finally, a narrow sample zone was formed (Figure 6C). Combined with Figure 4, it can be inferred that the analytes were arranged in order of pKa values, which may be due to the interaction between the analytes and SDS (Figure 6B,C). After the sample matrix was completely removed from the capillary by the EOF, the inlet and outlet vials were replaced by vials containing the same new BGE. Then a normal polarity voltage was applied, the analytes moved toward the detector and started to separate (Figure 6D). Finally, the negatively charged SDS micelles and the analytes migrated to the detector end (outlet end) according to the value of pKa (Figure 6E).

3.6. Stacking Efficiency

The stacking efficiency was investigated by calculating the ratio of peak areas ($A^{LVSS\text{-}MEKC}/A^{MEKC}$). Compared with the hydrodynamic injection, the LVSS gave a stacking efficiency of about 24, 24, 27, 12 and 14 folds for vanillin, syringetin, protocatechuic aldehyde, caffeic acid and protocatechuic acid, respectively (see Figure 7).

3.7. Method Validation

Standard mixture solution of the five analytes with a concentration of 0.5 μg/mL was analyzed three times in a day and for three consecutive days to determine the intraday and interday repeatability under the optimum condition. The results were given in Table 2. The relative standard deviations (RSDs) for analyte peak area response of the five analytes were 0.6–2.0% (intra-day, n = 5), and 2.4–4.9% (inter-day, n = 5), respectively.

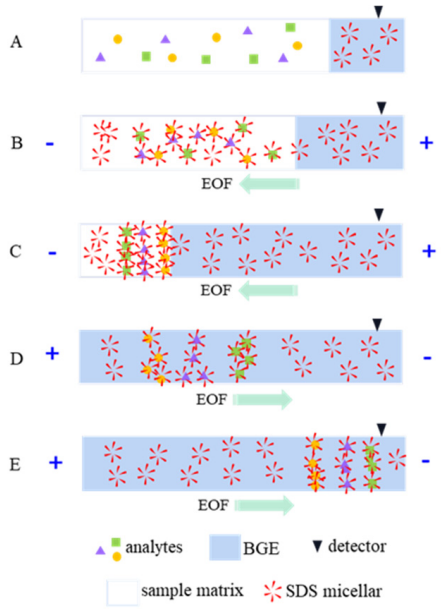


Figure 6. Schematic presentation of this LVSS-MEKC method. (**A**) A large volume of a sample is injected hydrodynamically. (**B**) SDS micelles transported together with the analytes migrate rapidly toward the outlet end. (**C**) The analytes were enriched and arranged in order of pKa values. (**D**) The sample matrix was pumped out from the inlet and the analytes started to separate. (**E**) The analytes migrated to the detector according to the value of pKa.

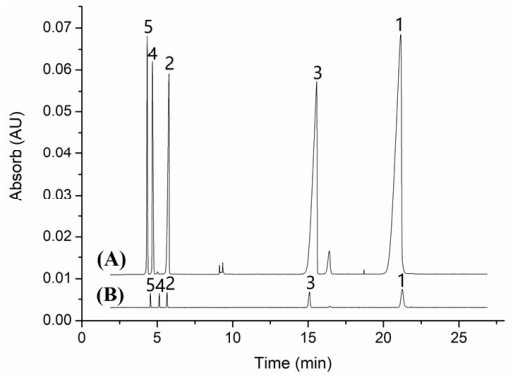


Figure 7. Electropherograms of five compounds by (A) LVSS-MEKC and (B) normal MEKC. MEKC inject: 0.5 psi, 5 s; LVSS-MEKC inject: 0.5 psi, 100 s, negative 30 kV, 0.5 min. The concentrations of analytes: 5.0 μg/mL. Other conditions are the same as Figure 5.

Table 2. Intraday and interday repeatability of the peak area for all analytes (n = 3) by using LVSS-MEKC method.

Analyte	Content (μg/mL)	Intra-Day Peak Area RSD (%)	Inter-Day Peak Area (%)
protocatechuic acid	0.5	0.68	5.47
protocatechuic aldehyde	0.5	0.47	3.50
caffeic acid	0.5	0.94	5.07
syringetin	0.5	0.47	2.05
vanillin	0.5	0.48	2.71

Furthermore, the linearity, linear range and detection limits (LODs) of this method were evaluated, and the results were presented in Table 3. The calibration curves exhibited excellent linearity over the concentration range of 0.25–10 μg/mL for all the five compounds. Three times the area signal-to-noise ratio (S/N = 3.0) was defined as LOD [31]. Under the optimum condition of online-MEKC method, the injection amount of each

compound was calculated by CE Expert software, so the detection limits were from 32 pg to 65 pg, which were around 500 times the level of conventional methods.

Table 3. Analytical performance of LVSS-MEKC for five compounds.

Analyte	Calibration Curve	Determination Coefficient (R^2)	Linearity Range(μg/mL)	Detection Limit(μg)	
				This Work	Reference
protocatechuic acid	y = 48,389x + 1603.2	0.999	0.25–10	6.5×10^{-5}	0.032 [11], 0.0124 [12]
protocatechuic aldehyde	y = 52,313x + 6216.2	0.9927	0.25–10	6.5×10^{-5}	0.0144 [11], 0.0102 [12]
caffeic acid	y = 61,388x + 11,869	0.9985	0.25–10	6.5×10^{-5}	ND [1]
syringetin	y = 163,658x − 38,019	0.9961	0.25–10	3.3×10^{-5}	ND [1]
vanillin	y = 293,371x + 51,259	0.9993	0.25–10	3.3×10^{-5}	ND [1]

[1] ND stands for not detect.

3.8. Analysis of Sample and Recoveries of Spiked Sample

The proposed LVSS-MEKC method was applied to analyze the five compounds in *Cibotium barometz* under the optimum conditions above. Figure 8 shows the chromatograms of the sample of *Cibotium barometz* injected for 100 s (Figure 8 left) and standard spiked sample (Figure 8 right) under the same conditions. Finally, protocatechuic acid and caffeic acid were detected in *Cibotium barometz* (Table 4). The other three compounds were not quantified because baseline isolation was not achieved and the content of syringetin may be lower than its LOD. The contents of protocatechuic acid and caffeic acid found in the herb were 0.11 mg/g and 0.04 mg/g, respectively. Our results were consistent with the previous studies [11,12], while the content of protocatechuic acid was 0.11 mg/g (0.01%), which was lower than 0.02% of dry products in the Chinese pharmacopoeia (2020 edition) [32]. This may be attributed to the differences in the origin, storage conditions and time of the medicinal materials [12]. The recovery rates were 83.2–97.4% for protocatechuic acid and 81.5~95.4% for caffeic acid (Table 5).

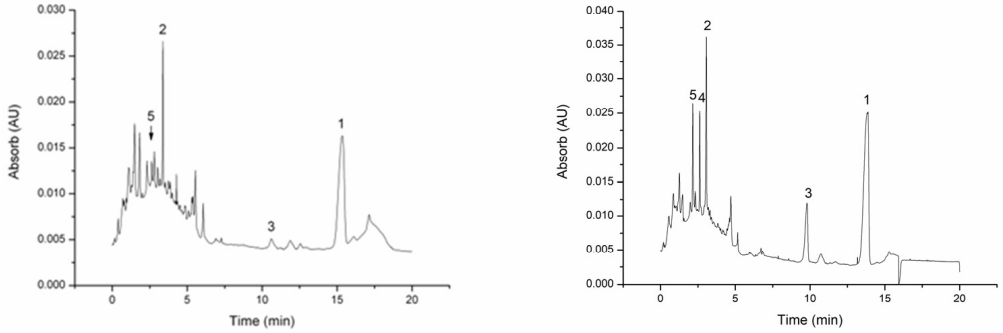


Figure 8. Electropherograms of *Cibotium barometz* nonspiked (left) and spiked (right). Running buffer: 10 mM sodium dodecyl sulfate and 50 mM sodium borax-sodium dihydrogen phosphate −10% methanol system (pH = 8.5). Separation voltage: 30 kV. Wavelength: 214 nm. LVSS-MEKC inject: 0.5 psi, 100 s, negative 30 kV, 0. 5 min. The extract was diluted 5 times with water. The compounds represented by the numbers 1–5 are listed in Table 1.

Table 4. Determination of protocatechuic acid and caffeic acid with LVSS-MEKC in *Cibotium barometz*.

Number	Protocatechuic Acid (mg/g)	Caffeic Acid (mg/g)
1	0.11	0.04
2	0.12	0.04
3	0.11	0.04
average	0.11	0.04
RSD (%)	3.81	3.39

Table 5. Recoveries (%) of protocatechuic acid and caffeic acid spiked at different levels using LVSS-MEKC in *Cibotium barometz*.

Analyte	Spiked (μg/mL)	Recovery (%)	RSD (%)
protocatechuic acid	0.5	97.4	1.1
	1.0	96.4	2.7
	1.5	83.2	3.1
caffeic acid	0.5	95.4	3.2
	0.7	81.5	4.1
	1.0	84.4	4.8

4. Conclusions

A simple micellar electrokinetic chromatography with large-volume sample stacking method for the analysis of five compounds in *Cibotium barometz* sample was reported in this work. Under the optimized conditions, obvious enrichment efficiency from 12 to 27 folds was confirmed and the detection limits were around 500 times less than conventional methods. In addition, satisfactory recovery and repeatability were obtained. This method was successfully applied to determine protocatechuic acid and caffeic acid in *Cibotium barometz*. Good recoveries at three spiked concentrations between 81.5% and 97.4% and the relative standard deviations (RSDs, n = 3) between 1.1% and 4.8% were obtained for protocatechuic acid and caffeic acid. In conclusion, the developed LVSS-MEKC method was simple, sensitive, low-cost, more environmentally acceptable and can be used for simultaneous qualitative analysis of five compounds and quantitative analysis of two compounds in *Cibotium barometz*.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors have declared no conflict of interest.

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