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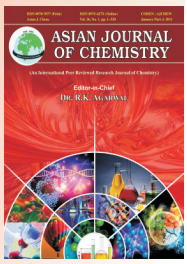

Rapid Separation and Determination of Five Phenolic Acids in *Eucommia ulmoides* Oliver by FASI-CZE

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A new method for simultaneous separation and determination of five active phenolic acids (chlorgenic acid, vanillic acid, caffeic acid, gallic acid and protocatechuic acid) in *Eucommia ulmoides* oliver (EuO) by field-amplified sample injection-capillary zone electrophoresis (FASI-CZE) has been proposed. The determination conditions, which contain pH in background solution, concentration of $Na_2B_4O_7$ and H_3BO_3, acetonitrile, injection time, injection voltage, separation voltage, were optimized. The operating buffer was composed of 40 mmol/L $Na_2B_4O_7$, 40 mmol/L H_3BO_3 and 10 % (by volume) acetonitrile at pH 9.5 with a capillary of 50 µm i.d. × 55 cm (35 cm to the detector window). In addition, it worked under a constant temperature of 25 °C. The UV detection wavelength was 215 nm. The applied voltage was -10 kV, the separation voltage was 20 kV and the injection time was 480 s. The linear ranges of the method were 77.6-1241.6 µg/L for chlorgenic acid, 2.25-36 µg/L for vanillic acid, 9.8-156.8 µg/L for caffeic acid, 11.4-182.4 µg/L for gallic acid and 7.35-117.6 µg/L for protocatechuic acid. The recoveries were 95-104, 93-107, 94-110, 98-107 and 94-105 %, respectively. Besides, the RSDs of the peak area were less than 4 % and detection limits (S/N = 3) were 9.21, 0.097, 0.350, 0.304 and 0.118 µg/L for chlorgenic acid, vanillic acid, caffeic acid, gallic acid and protocatechuic acid, respectively.

Keywords: Stacking, Field-amplified sample injection, Chlorgenic acid, Vanillic acid, Caffeic acid, Gallic acid.

INTRODUCTION

Eucommia ulmoides oliv. (EuO) also known as Duzhong, is a tall deciduous tree originating in China and the only plant in the Eucommia genus of the Eucommiaceae family. It's bark and leaf have been used in traditional Chinese medicine to treat kidney disease, lower back and knee pains and improve liver and spleen disorders[1,2]. *Eucommia ulmoides* oliver extracts have antioxidative, antihypertensive and antigastric ulcer effects[3,4]. According to some report, water extracts from *Eucommia ulmoides* oliver leaves have potent antimutagenic effects. *Eucommia ulmoides* oliver extracts have also been reported to be able to enhance collagen synthesis. It is generally assumed that natural substances in *Eucommia ulmoides* oliver give rise to these beneficial effects. The chemical compositions of *Eucommia ulmoides* oliver leaf contain phenolic acids such as chlorgenic acid (CGA), vanillic acid (VA), caffeic acid (CA), gallic acid (GA) and protocatechuic acid (PCA)[5]; iridoids, for example, eucommiol, geniposide and aucubin; and flavonoids, for instance, kaempferol and quercetin[1]. They have diverse biological functions such as antiinflammatory, anticarcinogenic, cardiovascular protective effects and so on. These functions might be associated with their antioxidative activity[6].

Capillary zone electrophoresis (CZE) is a useful alternative to HPLC. Though it can get a good separation result, it lacks sensitivity. In order to overcome this shortcoming, researchers have used capillary zone electrophoresis with different pre-concentration methods such as at-line solid phase extraction[7,8] and on-line solid phase extraction[9,10]. Field-amplified methods have been studied by several workers[11,12]. With these methods, a plug of low-conductivity sample solution (H_2O or CH_3CN) is introduced hydrodynamically or electrokinetically into a capillary, which is filled with a higher conductivity buffer. When high voltage is applied across the capillary, the sample ions experience higher field strength than the buffer ions, because of the difference in conductivity between the zones. This causes the sample ions to migrate quickly to the sample-buffer interface. Here, the field strength of the ions is lower. The sample ions slow down, when passing the boundary between the sample and the run buffer compartments and stack into a zone at the sample/separation buffer interface, which is much narrower than the original sample plug. Separation is then performed by reversing the polarity of the applied voltage (with polarity switching) or by suppressing the electroosmotic flow (EOF) (without polarity switching) using various strategies[8]. There are two primary modes of field-amplified method.

For the first one, which is called large-volume sample stacking (LVSS)[13-15], the sample is introduced into the capillary hydrodynamically. For the second one, which is called field-amplified sample injection (FASI)[16-19], the sample is introduced into the capillary electrokinetically.

In order to further improve the sensitivity of capillary zone electrophoresis, here we present a field-amplified sample injection-capillary zone electrphoresis (FASI-CZE) method. And the basic schematic diagrams are illustrated in Fig. 1. At the beginning, capillary was filled with redistilled water. Then we injected the sample solution into capillary by electrokinetic-injected method (negatively). Meanwhile, sample matrix was gradually moved to the injection end (inlet) and exited from the capillary. When the electric current increased suddenly and reached 95 % of the value corresponding to the normal capillary zone electrophoresis conditions, the program of electrokinetic injection was finished. Finally, a positive polarity was applied and the separation and determination of the sample was completed. In this paper, the mentioned method was applied to the analysis of chlorgenic acid, vanillic acid, caffeic acid, gallic acid and protocatechuic acid in the *Eucommia ulmoides* oliver with a satisfying result.

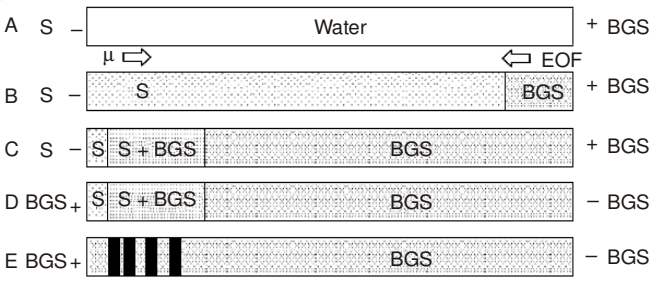


Fig. 1. Schematic diagram of the FASI-CZE

EXPERIMENTAL

All analyses mentioned above were carried out on an ACS2000 capillary electrophoresis system (Beijing Cailu Instrumental Co., Beijing, China). The apparatus mainly consisted of a power supply (up to constant voltage 30kV), a HW-2000 chromatography workstation and a UV-visible detector (double light beams, λ = 190-740 nm, set at 215 nm). A fused-silica capillary (Factory of Yongnian Optical Fiber, Hebei, China) was also used, with total length 55 cm, effective length 35 cm, I.D.50 μm. The runs were carried out under 25 °C cooling air. A UV-2102PC UV-visible spectrophotometer from UNICO instruments Co., Ltd. was used. DL-60D ultrasonic bath from Letter to Shanghai Instrument Co., Ltd. was used to degas solutions. Analytical balance from Austrian House (Shanghai) Co., Ltd. was used. The pH of the solutions was measured by employing a model EF20 Laboratory pH meter Mettler-Toledo Instruments (Shanghai).

A commercially available microwave oven (Shanghai Xintuo Microwave Decomposition and Testing Technology Co. Ltd, PR China), equipped with a magnetron of 2450 MHz with a nominal maximum power of 800 W, was used for microwave-assisted extraction. A one-meter-long condensation pipe was connected to the sample cartridge vessel, which was located at microwave irradiation zone. The whole system was operated under atmospheric pressure. The schematic diagram of UMAE apparatus is illustrated in Fig. 2.

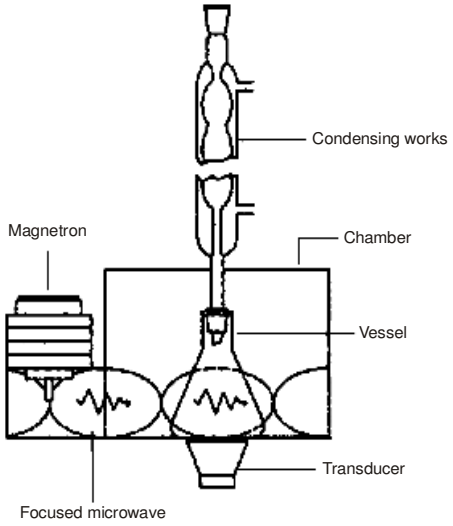


Fig. 2. Schematic diagram of UMAE apparatus

Chlorgenic acid, vanillic acid, caffeic acid, gallic acid and protocatechuic acid were of analytical standard purity, which were purchased from the Beijing Medicine Inspection Institute, China. Fig. 3 shows the chemical structures and pK values of these compounds. Dried barks of *Eucommia ulmoides* oliver were bought from the Traditional Chinese Medicine Co. Ltd (Hunan, PR China). Sodium borate, boric acid, sodium hydroxide and sodium dodecyl sulfate (SDS) were of analytical grade. Acetonitrile and methanol were LC grade solvents. All solutions and samples were filtrated and degassed before analysis by capillary electrophoresis.

Preparation of standard and sample solutions: The standard stock solutions of chlorgenic acid (388 mg/L), vanillic acid (360 mg/L), caffeic acid (392 mg/L), gallic acid (456 mg/L) and protocatechuic acid (588 mg/L) were prepared by dissolving weighed amount of the five standards in volumetric flask with 25 mL methanol, respectively. Calibration standard was prepared by diluting calculated volume of the stock solution with redistilled water.

Recently, microwave-assisted extraction (MAE) has received more and more attention as a potential alternative to traditional solid-liquid extraction methods, mainly due to considerable saving in processing time and solvent consumption[20-22]. Microwave-assisted extraction was being used for the extraction of a variety of organic compounds: Polybrominated diphenylethers (PBDEs)[23], phenols and organochlorine pesticides[24,25] and hydrocarbons[26]. Dried barks of *Eucommia ulmoides* oliver were finely powdered in a mortar. An appropriate amount about 3 g of *Eucommia ulmoides* oliver powder was placed into the cartridge vessel, by adding appropriate solvent which was about 80 mL redistilled water. With ice water running through the condensation pipe, the sample was treated under microwave irradiation in an intermittent way, *i.e.*, irradiation-cooling-irradiation. The power of the microwave was 50 % (with maximum power 800 W). The irradiation time was kept for 100 s. Then, the resulted solution was cooled off and then centrifuged at 2500 rpm for 10 min. After being filtrated from a 0.45 μm

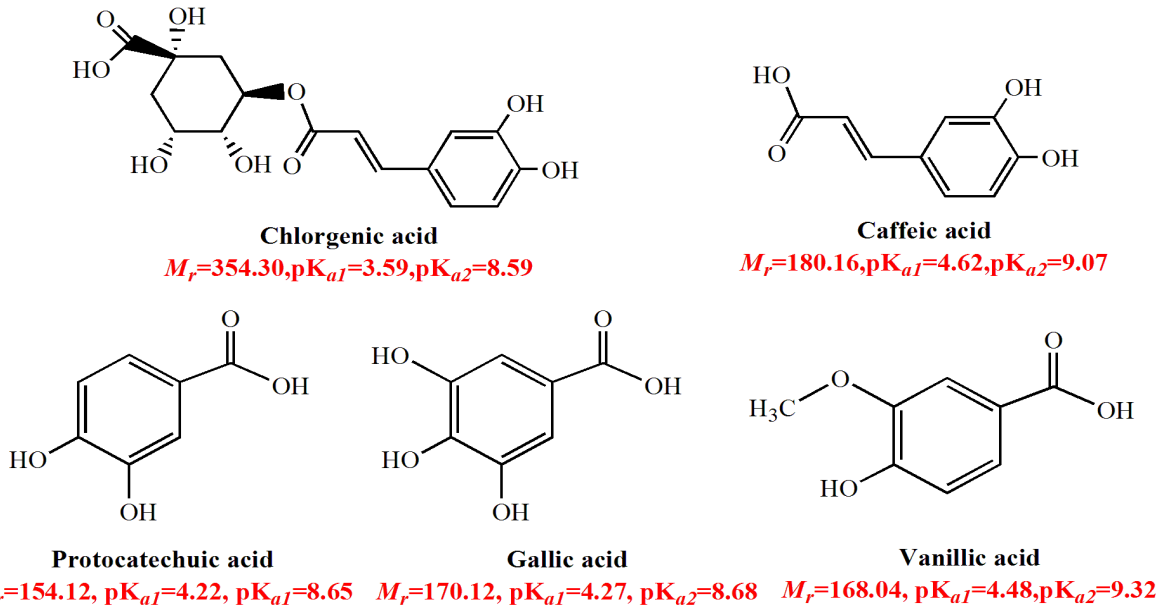


Fig. 3. Chemical structures and pK values of the analyzed phenolic acids

microporous membrane and degassed in an ultrasonic bath, the solutions were used in capillary electrophoresis analysis. All the solutions were stored in a refrigerator at 4 °C.

Chromatography condition: The operating background electrolyte (BGE) was composed of 40 mmol/L $Na_2B_4O_7$, 40 mmol/L H_3BO_3 and 10 % (by volume) ACN at pH 9.5 with a constant temperature of 25 °C. The UV detection wavelength was 215 nm. The separation voltage was 20 kV.

Capillary zone electrophoresis: Separation voltage was 20 kV with hydrostatically (16 cm) and injection time was 60 s. FASI-CZE: The samples were injected by electromigration for 480 s (at -10 kV).

Rinsing procedure: New capillaries were conditioned by rinsing with sodium hydroxide solution (1 mol/L) for 60 min, followed by separate rinses with redistilled water and background electrolyte for 15 min, respectively. Finally, the capillaries were equilibrated for 30 min at 20 kV using background electrolyte. To obtain reproducible results from the first run on, two complete runs with buffer injection were performed before a measurement sequence was started. In order to ensure the repeatability of the migration time, the capillaries were rinsed between the runs with sodium hydroxide (1 mol/L) and water (each for 5 min) followed by background electrolyte equilibration for 5 min using 20 kV.

RESULTS AND DISCUSSION

Effect of organic solvents: Acetonitrile and alcohols have several other practical advantages such as improving dissolution of many organic compounds and removal of proteins. Organic solvents added into background electrolyte will have influence on analysis time and sometimes even on resolution of the analytes. There is a decrease of electro-osmotic velocity with addition of the organic solvent, because it leads to changes in viscosity[27] and dielectric constant of background electrolyte that affect both electro-osmotic flow of the capillary and the electrophoretic mobilities of the analytes. Both acetonitrile and methanol were tested with respect to the quality of separation and total analysis time. With adding methanol into background electrolyte, we found that the peaks of caffeic acid and gallic acid were non-Gaussian and resolution and peak shapes were not satisfactory. Better results (better resolution of analytes without longer analysis time) were achieved when using background electrolyte consisting of 20 mmol/L $Na_2B_4O_7$, 40 mmol/L H_3BO_3 and different concentration of acetonitrile. Figs. 4 and 5 show that with addition of acetonitrile, the peak area and the analysis time increases but the theoretical plate number decreases. The optimum trade-off between the peak area and the theoretical plate number was achieved with 10 % (v/v) acetonitrile.

Effect of borate concentration: Borate buffer is often used in capillary electrophoresis analysis of naturally occurring polyphenols due to its possibility to form anionic complexes with compounds possessing vicinal -OH groups[28]. The effect of borate concentration was investigated by using 10, 20, 30, 40 and 50 mmol/L sodium tetraborate in the background electrolyte [containing 40 mmol/L boric acid and 10 % (v/v) acetonitrile]. Figs. 5 and 6 show that with the increase of sodium tetraborate concentration, the peak area was increased while the theoretical plate number decreases. Besides, the analysis time was prolonged. When sodium tetraborate concentrations less than 30 mmol/L, the peaks of caffeic acid and vanillic acid merged. Experimental data showed no detectable decreasing trend of the theoretical plate numbers with increasing sodium tetraborate concentration 30 mmol/L to 40 mmol/L, but the peak area increased. However, when the concentration of sodium tetraborate was above 50 mmol/L, the baseline noise was increased. Hence, 40 mmol/L sodium tetraborate was chosen as the optimum concentration.

Optimization of separation voltage: Under the electrophoretic conditions, different separation voltages were tested. Optimal conditions were as follows. Fused silica capillary effective length 35 cm (55 cm total length) I.D. 50 µm, background electrolyte consisting of 40 mmol/L $Na_2B_4O_7$, 40 mmol/L H_3BO_3 and 10 % (by volume) CH_3CN at pH 9.5, detection at

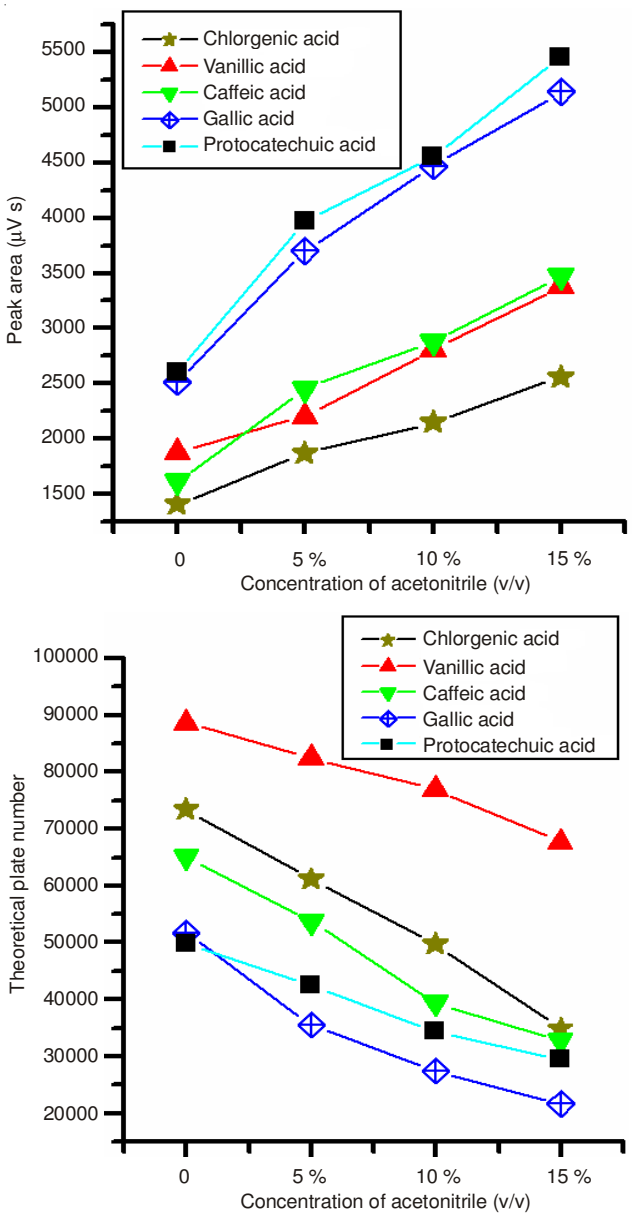
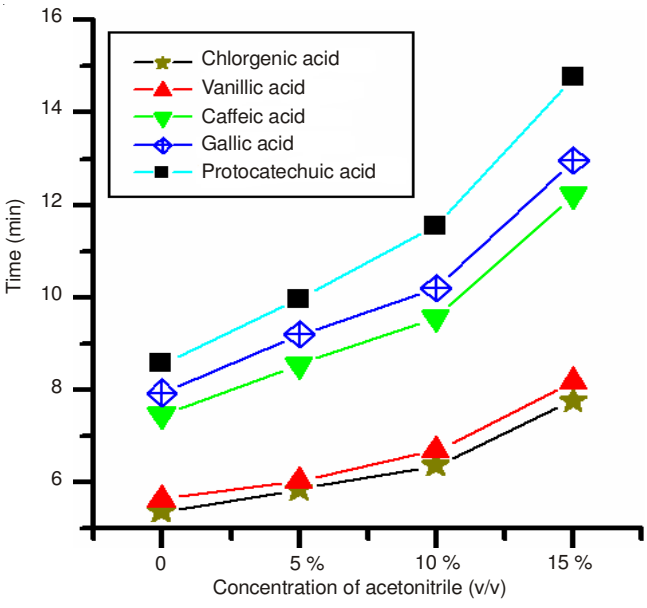


Fig. 4. Effect of concentration of acetonitrile on peak area and theoretical plate number



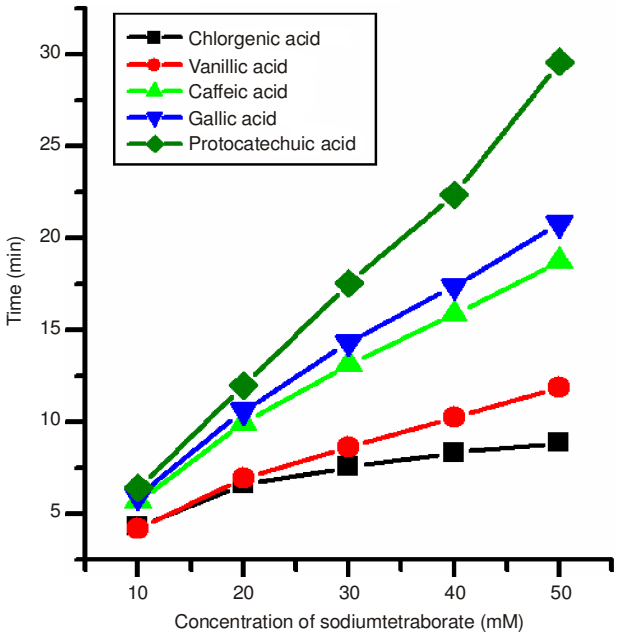


Fig. 5. Effect of concentration of acetonitrile and sodium tetraborate on migration time

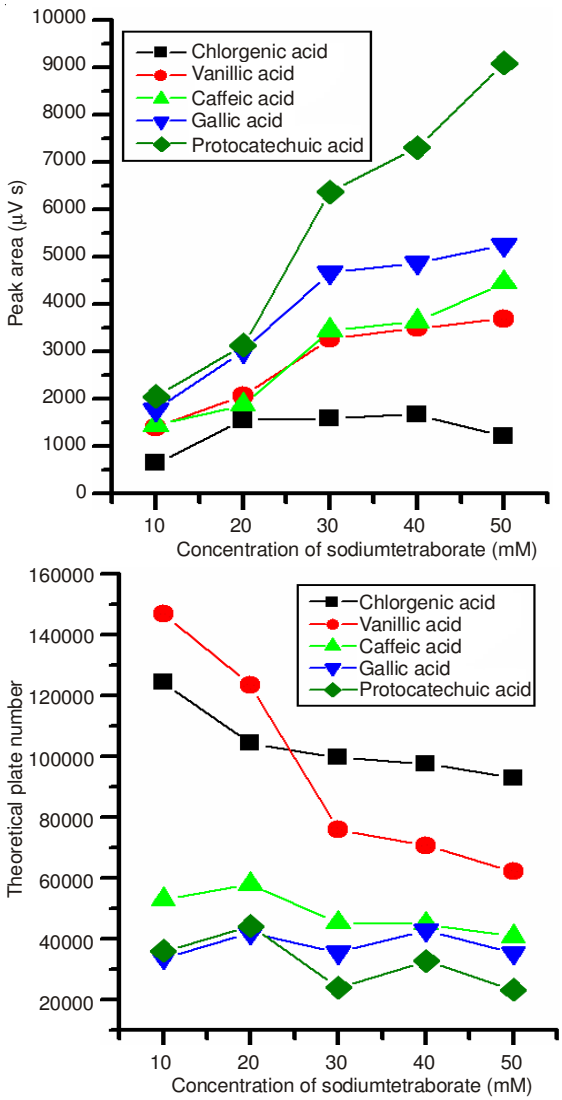


Fig. 6. Effect of sodium tetraborate concentration on peak area and theoretical plate number

215 nm, hydrostatical (16 cm) injection for 60 s. When the separation voltage fluctuated in the range of 15-25 kV, the bigger the separation voltage was, the shorter the migration time was. But the resolution of the analytes decreased. In order to achieve better theoretical plate number and column efficiency, 20 kV was selected as the separation voltage in this paper.

Optimization of injection time and electrokinetic injection voltage: Capillary zone electrophoresis was performed by injection of solution of sample using hydrodynamic mode with system raising the sample vial to a level 16 cm higher than that the electrolyte and with different injection time. The peak area significantly increased as the injection time increased from 40 to 120 s, but the theoretical plate number decreased. Injection time was chosen as 60 s, because all target compounds got a separation with better resolution and higher sensitivity. FASI-CZE was performed as follows: Firstly, the whole column was filled with water. Secondly, sample stacking was performed in the electrokinetic injection mode with negative voltage. Time of polarity was optimized through monitoring the magnitude of electric current in the capillary zone electrophoresis system during the preconcentration step. Only after the low conductivity matrix (water) was gradually removed electrokinetically did the electric current increase suddenly and the program of electrokinetic injection was finished. Then, the polarity was switched and the positive voltage was used for separations. The stacking voltage was optimized and was changed from -8 to -16 kV. We found the lower the stacking voltage was, the longer the migration time was. But when we used higher voltage, the peak area decreased. As a result, -10 kV was chosen as the optimized stacking voltage. The injection time was studied. Once the redistilled water was introduced into the capillary, a voltage of -10 kV was applied for sample injection. While the solvent (water) was flushed out of the capillary in the injection end, the current increased until it reached 95 % of the value corresponding to the normal capillary zone electrophoresis conditions, which took about 480 s. At this point, the capillary zone electrophoresis sepa-ration was started by applying 20 kV voltage.

Validation of method

Linearity: Under the optimized buffer condition, capillary zone electrophoresis and FASI-CZE were studied and validated. These validated data are given in Tables 1 and 2. The repeatability of the results expressed as the relative standard deviation can be considered satisfactory since the RSD values of peak areas did not exceed 4 %. When the method of capillary zone electrophoresis was used, the LODs based on three times of the signal-to-noise (S/N = 3) were to be 218 μg/L for chlorgenic acid, 8 μg/L for vanillic acid, 45 μg/L for caffeic acid, 22 μg/L for gallic acid and 13 μg/L for protocatechuic acid. While the method of FASI-CZE was used, the LODs (S/N = 3) were to be 9.210 μg/L for chlorgenic acid, 0.097 μg/L for vanillic acid, 0.350 μg/L for caffeic acid, 0.304 μg/L for gallic acid and 0.118 μg/L for protocatechuic acid. LODs of FASI-CZE method were 24, 82, 129, 72 and 110 times lower than that obtained by capillary zone electrophoresis, respectively. The electropherograms of a standard mixture of the five organic acids are shown as Fig. 7.

TABLE-1
LINEARITY, REGRESSION EQUATION, LODs BY CAPILLARY ZONE ELECTROPHORESIS

Compound	Linear range (mg/L)	Regression equation	Correlation coefficient	LOD (μg/L)	RSD (%) (n=5)
Chlorogenic acid	2.91-46.56	y=37.07x -12.30	0.9999	218	2.7
Vanillic acid	0.23-3.60	y=832.87x-16.22	0.9999	8	2.3
Caffeic acid	0.98-15.68	y=200.27 x-66.65	0.9997	45	3.0
Gallic acid	1.14-18.24	y=291.35 x-286.04	0.9998	22	2.1
Protocatechuic acid	0.735-11.76	y= 526.11x-48.435	0.9990	13	2.3

TABLE-2
LINEARITY, REGRESSION EQUATION, LODS BY FASI-CZE

Compound	Linear range (μg/L)	Regression equation	Correlation coefficient	LOD (μg/L)	RSD (%) (n=5)
Chlorogenic acid	77.60-1241.60	y=1.14x+28.50	0.9995	9.210	3.5
Vanillic acid	2.25-36.00	y=98.59x-58.67	0.9996	0.097	3.2
Caffeic acid	9.80-156.80	y=24.82x+166.58	0.9991	0.350	2.9
Gallic acid	11.40-182.40	y=34.55x-19.83	0.9997	0.304	3.5
Protocatechuic acid	7.35-117.60	y=61.12x+274.63	0.9988	0.118	3.7

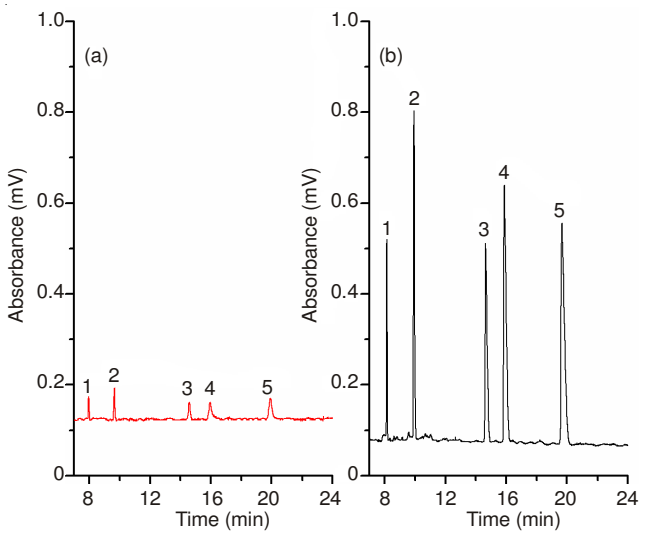


Fig. 7. Electropherogram of the five organic acids when use capillary zone electrophoresis (a) and FASI-CZE (b): [A] capillary zone electrophoresis; [B] FASI-CZE; background electrolyte: 40 mmol/L $Na_2B_4O_7$ + 40 mmol/L H_3BO_3 (pH 9.5) + 10 % (v/v) CH_3CN; injection voltage: 10 kV; separetion voltage: 20 kV; injection time: [A] 60 s; [B] 480s; concentration of five phenolic acids in standards: [A] 5695, 450, 1960, 2280 and 1470 μg/L; [B] 1241.6, 36, 156.8, 182.4 and 117.6 μg/L; (1) Chlorgenic acid; (2) Vanillic acid; (3) Caffeic acid; (4) Gallic acid; (5) Protocatechuic acid

Related sensitivity enhancement (SE) of FASS-CZE is calculated with the formula: $SE = S_{FASI\text{-}CZE}/S_{CZE} \times K$, where $S_{FASI\text{-}CZE}$ is the peak area of the target chemical when we use the method of FASI-CZE, S_{CZE} is the peak area of the target chemical, while we use the method of capillary zone electrophoresis and K is the dilution factor). The sensitivity enhancement of chlorgenic acid, vanillic acid, caffeic acid, gallic acid and protocatechuic acid is shown as follows: $SE_{CGA} = 1326/202 \times 4.6 = 30$, $SE_{VA} = 3520/360 \times 12.5 = 122$, $SE_{CA} = 4012/347 \times 12.5 = 145$, $SE_{GA} = 6255/399 \times 12.5 = 196$, $SE_{PCA} = 7445/633 \times 12.5 = 147$. The related sensitivity enhancement is different

from compounds due to molecular charge property, molecular weight and structure.

Analysis of samples and recoveries: The recoveries of chlorgenic acid, vanillic acid, caffeic acid, gallic acid and protocatechuic acid from the extracts of *Eucommia ulmoides* oliver samples were determined by the method of standard addition. For each compound, three concentration levels were tested. Each sample was analyzed in triplicate. The recovery values were obtained by comparing the results from samples and fortified samples. As a result, the recoveries were satisfactory between 93-110 % in all tests. The results are listed in Table-3. This new method was utilized to separate and determine the five organic acids in the extracted sample of *Eucommia ulmoides* oliver. After analyzing the extracted samples, we found the results were as good as those obtained from pure standard substances without any interference. The electrophoretograms were shown in Fig. 8.

TABLE-3
RESULTS OF RECOVERY TEST

Compound	Added (μg/L)	Found (μg/L)	Recovery (%)	RSD (%) (n=5)
Chlorogenic acid	155.2	147.4	95	3.8
	310.4	322.6	104	
	620.8	616.8	99	
Vanillic acid	4.5	4.2	93	2.2
	9.0	9.6	107	
	18.0	17.2	96	
Caffeic acid	19.6	21.5	110	3.5
	39.2	36.9	94	
	78.4	75.8	97	
Gallic acid	22.8	24.5	107	2.7
	45.6	46.9	103	
	91.2	89.3	98	
Protocatechuic acid	14.7	15.4	105	3.3
	29.4	27.7	94	
	58.8	56.9	97	

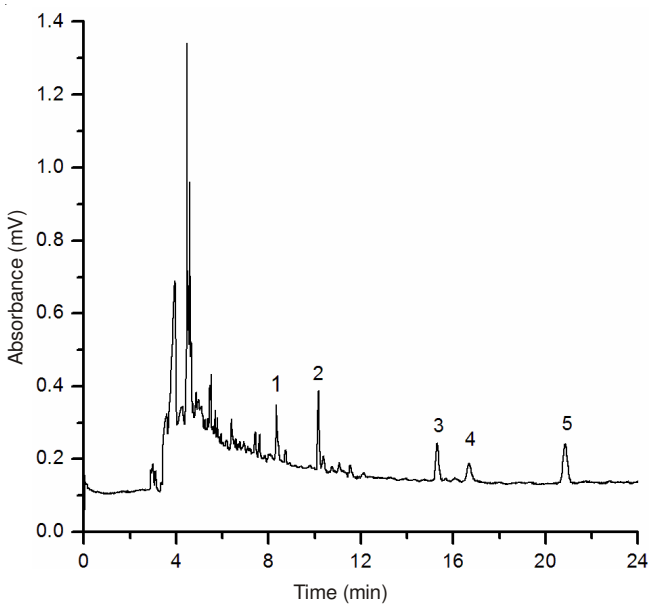


Fig. 8. Electropherogram of samples; (1) Chlorgenic acid; (2) Vanillic acid; (3) Caffeic acid; (4) Gallic acid; (5) Protocatechuic acid

Conclusion

The proposed method is simple while the sensitivity has been significantly improved without modification in the commercial capillary electrophoresis instrument. The separation takes place in less than 22 min. The linearity range, limits of detection and quantification, precision and accuracy were processed to determine the suitability of the method. The confirmed results were obtained. It has been demonstrated that the five phenolic acids could be analyzed in *Eucommia ulmoides* oliver easily and the LOD of the five phenolic acids is 24-110 times lower than that obtained without FASI.

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