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Rapid and simultaneous analysis of some bioactive components in *Eucommia ulmoides* by capillary electrophoresis

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Abstract

A micellar electrokinetic chromatography method was established for the qualitative and quantitative determination of three groups of bioactive components, iridoids, flavonoids and phenolic compounds, in *Eucommia ulmoides*. Of the eleven bioactive components being studied, ten were successfully separated in 50 m*M* boric acid buffer at pH 9.5, with 50 m*M* sodium dodecylsulfate and 4% 1-butanol, at a voltage of 20 kV, temperature of 20 °C and injection under high pressure at 138 kPa for 5 s in a fused-silica capillary with peak detection at 214 nm. A high reproducibility and good linearity was obtained. The relative standard deviations of the migration times in eight injections of the standards ranged from 0.64 to 1.88% and those of the corrected peak area ranged from 2.79 to 6.62%. A good linearity, with correlation coefficients in the range of 0.995–1.000, was obtained in the calibration curves of each standard from 1 to 50 ppm. The amount of these bioactive components in the bark and leaves of *Eucommia ulmoides* were determined.

Keywords: *Eucommia ulmoides*; Pharmaceutical analysis; Buffer composition; Plant materials; Iridoids; Flavonoids; Phenolic compounds

1. Introduction

Eucommia ulmoides Oliver, also called Du-zhong in Chinese, is one of the oldest tonic herbs in traditional Chinese Medicine [1]. The cortex of Du-zhong is used to strengthen tendons and bones, reinforce muscle, benefit liver and kidney, prevent miscarriage, as well as for the treatment of hypertension and as an anti-aging agent [1,2]. Previous studies have found that many bioactive components are present in *Eucommia ulmoides* including derivatives of iridoids, flavonoids and phenolic compounds [3–6].

To separate and analyze the bioactive components in traditional herbal medicine, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are generally used [4,7,8]. However, CE possesses advantages of high efficiency, high selectivity, high flexibility and ease of method development over other separation techniques [9]. Recently, because the method of validation of CE has been widely implemented, better separation results are reported in CE than in HPLC [10].

Micellar electrokinetic chromatography (MEKC)

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is a mode of CE that is capable of separating both charged and neutral analytes. Surfactant, such as sodium dodecylsulfate (SDS), at concentrations higher than its critical micelle concentration aggregates to form spherical micelles in buffer in MEKC. These micelles serve as a nonpolar pseudostationary phase within the capillary column that facilitates separation among both charged and neutral analytes [11].

Qualitative or quantitative analyses of single or individual groups of bioactive components in *Eucommia ulmoides* by either HPLC or CE have been reported. The separation and analysis of chlorogenic acid in bark and leaf of *Eucommia ulmoides* have been achieved by CE [4]. Another study using HPLC has determined the amount of protocatechuic acid in the leaf extract and cortex of *Eucommia ulmoides* [7]. However, to determine only one component at a time is less efficient and the determination of different groups of components simultaneously is rarely reported. In this experiment, all derivatives of iridoids, flavonoids and phenolic compounds in *Eucommia ulmoides* were simultaneously determined in both qualitative and quantitative point of view. The optimized pH conditions, buffer concentration, surfactant concentration and concentration of organic modifier in the separation buffer to provide a good separation method that can be used to analyze the different groups of bioactive components in *Eucommia ulmoides* at one time is described.

2. Experimental

2.1. Apparatus

MEKC was carried out on a capillary electrophoretic instrument, Beckman P/ACE 5000 (Beckman Instruments, Fullerton, CA, USA), equipped with a 57 cm (50 cm from the inlet to detector)×50 μm I.D. fused-silica capillary tube (Beckman Instruments). The instrument was connected to a personal computer, which controlled the P/ACE instrument. Data analysis was performed using P/ACE station software developed by Beckman.

2.2. Chemicals

All chemicals were of analytical grade. Purified aucubin, geniposide, geniposidic acid and pyrogallol were purchased from Wako (Osaka, Japan). Chlorogenic acid, protocatechuic acid, rutin, ferulic acid were purchased from Acros Organics (Geel, Belgium), while caffeic acid, quercetin and *p-trans*-coumaric acid were obtained from Sigma (St. Louis, MO, USA). These chemicals were used as reference materials for the analysis of components in the *Eucommia ulmoides* extract. SDS was obtained from Bio-Rad (Hong Kong) and boric acid used as buffer electrolyte was purchased from Sigma. Organic modifiers, ethanol, methanol and isobutanol were bought from Riedel-de Haën (Seelze, Germany), acetonitrile was bought from Lab-Scan (Bangkok, Thailand) and 1-butanol was purchased from Aldrich (Germany). Hydrochloric acid (analytic grade) was purchased from Riedel-de Haën and sodium hydroxide was obtained from Pharmacos (Southend on Sea, UK). Milli-Q deionised water was used throughout the study (Millipore, Bedford, MA, USA).

2.3. Procedure

2.3.1. Optimization of conditions

The capillary electrophoretic equipment was operated in normal mode with the cathode at the detector end. Prior to running any standard or sample solutions through the system, the capillary was conditioned with 0.1 *M* NaOH for 15 min followed by Milli-Q water for 2 min and finally the running buffer for 15 min. In between each injection, the capillary was flushed again with 0.1 *M* NaOH, Milli-Q water and the running buffer in use. Each of these solutions was rinsed through for 2 min in order to regenerate and equilibrate the capillary. The operating conditions for the system were set at a voltage of 20 kV, temperature of 20 °C and injection under high pressure at 138 kPa for 5 s with UV–visible detection at wavelength 214 nm.

A mixed standard solution was prepared of 50 ppm of each standard. The separation of standard mixture was first performed by optimizing the pH of the buffer. Buffer solutions of 50 m*M* boric acid and 50 m*M* SDS at pH 7.5, 8.0, 8.5, 9.0 and 9.5 were tested. The effect of buffer concentration was then determined. Boric acid at concentrations of 30, 50, 70 and 90 m*M* with 50 m*M* SDS at pH 9.5 were investigated. The next factor was the surfactant

concentration; varying SDS concentrations of 25, 50, 75 and 100 m*M*, with 50 m*M* boric acid at pH 9.5 were studied. For a further optimization, different organic modifiers, methanol, ethanol, isobutanol, 1-butanol and acetonitrile were tested. After selection of the appropriate organic modifier, 3, 4, 5 and 10% solutions of that organic modifier were investigated. Spiking of each standard was performed for peak identification.

2.3.2. Method validation

After an optimum operating condition for the mixed standard solution had been established, validation of the method was performed. The mixed standard solution was injected eight times and the repeatability in terms of the relative standard deviation (RSD) of migration time and corrected peak area was determined. Five different concentrations, 1, 5, 10, 20 and 50 ppm, were analyzed in triplicate and the corrected peak area of each concentration was used to produce a calibration curve. The linearity of each calibration curve was studied. The recovery of the method was determined by spiking 5 ppm of each standard into the sample. This process was repeated three times.

2.4. Sample preparation

Bark and leaves of *Eucommia ulmoides* were homogenized and 10 g of each sample was refluxed with 150 ml methanol for 4 h. The extract was filtered through a 0.2-μm cellulose filter and then dried by speed vacuum. The dried extract was stored at −20 °C. Prior to injection, 0.2 g of bark and 0.1 g of leaves were dissolved in 50 ml methanol.

3. Results and discussion

3.1. Method development

Optimization of the MEKC separation conditions for the simultaneously qualitative and quantitative analysis of iridiods, flavonoids and phenolic compounds in *Eucommia ulmoides* (structures shown in Fig. 1) involved the variation of buffer pH, buffer concentration, surfactant concentration and organic solvent content.

3.1.1. Optimization of pH

Variation of pH from 7.5 to 9.5 in 50 m*M* boric acid and 50 m*M* SDS shows a significant effect on the separation of the bioactive components in *Eucommia ulmoides* (Fig. 2). An alkaline buffer system was tested because of phenolic nature of flavonoids and phenolic compounds [12]. The results revealed that the migration time increased as the pH of the running buffer increased and the standard peaks are best resolved at pH 9.5. The observation is similar to a previous study for the separation of rutin and quercetin by CE. When pH of the running buffer increased, the migration time increased and the resolution improved as a result of the dissociation of hydroxyl group in analytes [13]. However, aucubin could not be resolved from the solvent peak and geniposide overlapped completely with geniposidic acid under any pH condition tested. Buffer pH higher than 9.5 was not tested because except iridoid compounds, phenolic compounds and flavonoids have already been separated. Therefore, pH 9.5 was chosen as the optimum.

3.1.2. Optimization of electrolyte concentration

The effect of boric acid concentration ranging from 30 to 90 m*M* with 50 m*M* SDS at pH 9.5 on the separation of the three groups of components was investigated and the results are shown in Fig. 3. The electrophoretic mobilities of the compounds were greatly influenced by the boric acid concentration. Migration time and resolution increased as the concentration of boric acid increased. However, the separation between quercetin and *p*-coumaric acid decreased at concentrations higher than 50 m*M*. The decrease in electrophoretic mobilities is due to the formation of a complex between the hydroxyl groups on the polyphenol ring and borate which gives negatively charged complexes. The bulky size of the complex also facilitates its partitioning into the micellar phase, which leads to longer migration times [14]. At concentrations lower than 50 m*M* (such as 30 m*M*), the pyrogallol and chlorogenic acid pair partially overlapped. Also, aucubin could not be resolved from the solvent peak and the geniposide–geniposidic acid pair was not separated at any buffer concentration tested. Thus, boric acid at 50 m*M* was chosen as the optimum concentration for separation.

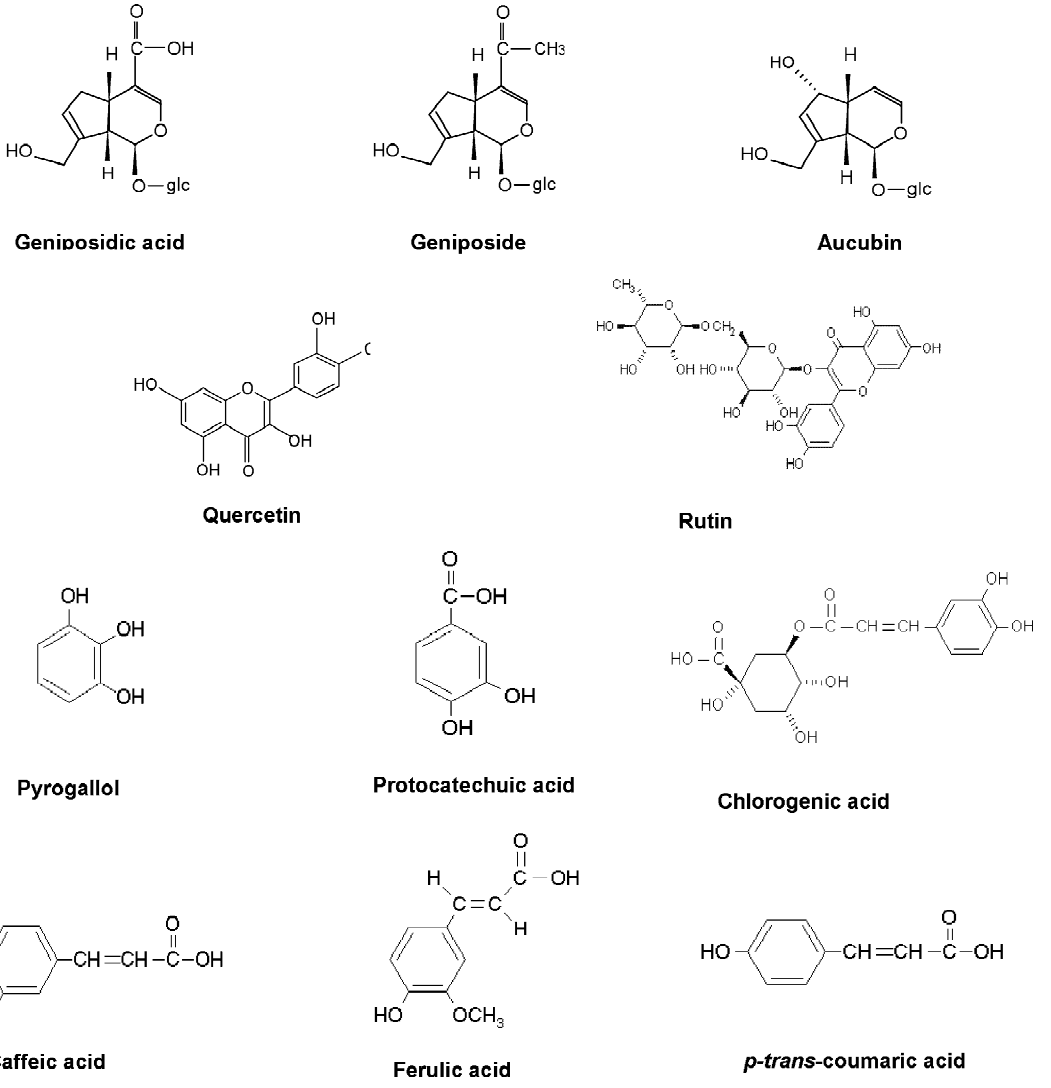

Fig. 1. Chemical structure of iridoids, flavonoids and phenolic compounds.

3.1.3. Optimization of surfactant concentration

Fig. 4 shows the effect of SDS concentration ranging from 25 to 100 m*M* in 50 m*M* boric acid at pH 9.5 on the separation of analytes. The electrophoretic mobilities of all standards decreased to different extents with increasing surfactant concentration. This is because an increase in the SDS concentration leads to increases in the apparent capacity factor. The partitioning of solutes towards the SDS pseudostationary phase is enhanced at high micellar concentration [15]. At 100 m*M* SDS concentration, peaks of pyrogallol and ferulic acid merged together. At 75 m*M* SDS, quercetin and *p*-coumaric acid partially overlapped. The resolution of the pyrogallol–ferulic acid and the quercetin–*p*-coumaric acid pairs were greatly improved by decreasing the SDS concentration to 50 m*M*. However, none of the conditions could resolve aucubin from

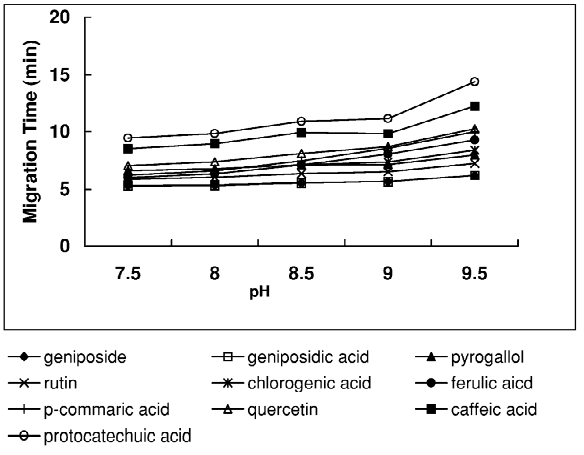

Fig. 2. Effect of pH on the separation of standards.

the solvent peak or the geniposide–geniposidic acid pair. Therefore, 50 m*M* SDS was added to the buffer for the best separation.

3.1.4. Optimization of buffer with organic modifiers

The effect of 5% methanol, ethanol, 1-butanol, isobutanol and acetonitrile in buffer with 50 m*M* boric acid, 50 m*M* SDS at pH 9.5 on the separation of the standard peaks was studied. Addition of organic modifiers to the buffer system altered the partition coefficient of the solutes and slowed down the electroosmotic flow by increasing the viscosity of the buffer system, thus improving resolution [16].

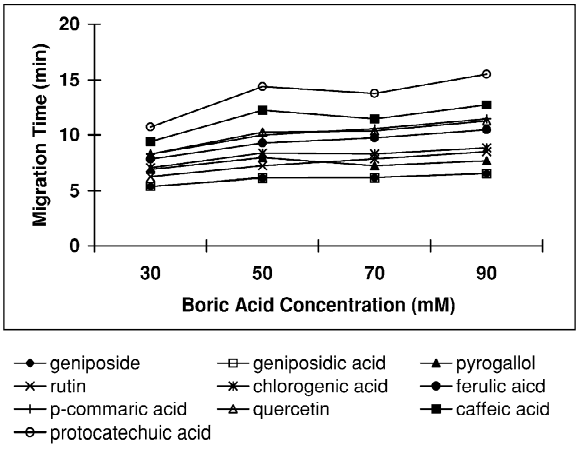

Fig. 3. Effect of electrolyte concentration on the separation of standards.

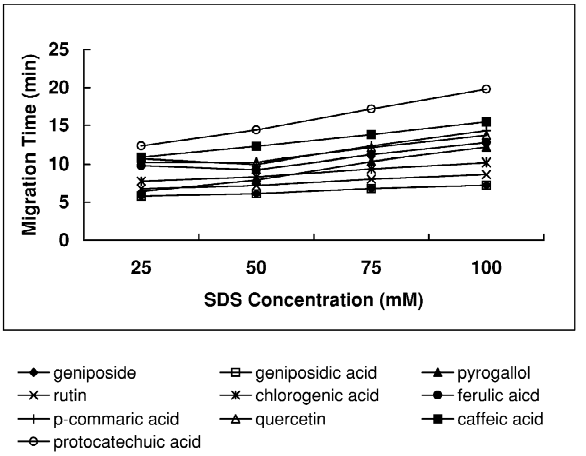

Fig. 4. Effect of SDS concentration on the separation of standards.

Resolution was enhanced as the chain length of the alcohol increased. The resolution of the pyrogallol–geniposidic acid and the quercetin–*p*-coumaric acid pairs was worsened by the addition of methanol and ethanol. Addition of acetonitrile, on the other hand, promoted the merging of the peaks of pyrogallol, geniposidic acid and geniposide. Likewise, the resolution between quercetin and *p*-coumaric acid decreased. Although aucubin was not resolved from the solvent peak, isobutanol and 1-butanol did improve the resolution and successfully separated geniposide from geniposidic acid. Hence, 1-butanol was chosen as the organic modifier as a better resolution of the geniposidic acid and pyrogallol peaks was obtained. Next, the optimum concentration of 1-butanol was determined. Fig. 5 shows the effect of 1-butanol ranging from 3 to 10% on the separation efficiency of the standard peaks. Similar separation efficiency was obtained at 3 and 4%. Therefore, 4% 1-butanol was added to the buffer to further increase the resolution of the bioactive components in *Eucommia ulmoides*.

3.2. Method validation

3.2.1. Precision

The reproducibility of migration time and peak area is critical for the quantitative analysis of CE. Migration time precision is significant for peak identification and assessment for the stability of the

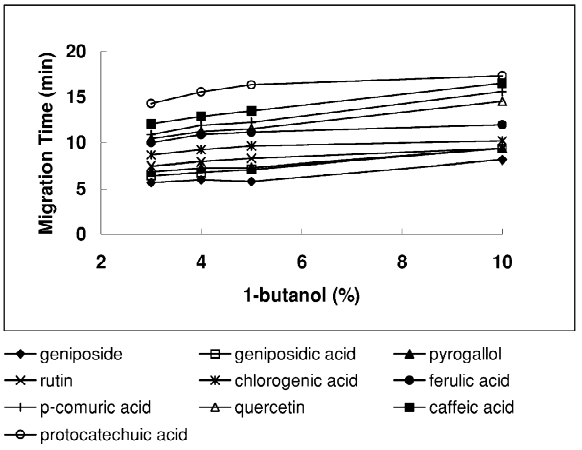

Fig. 5. Effect of concentration of 1-butanol on the separation of standards.

analytical method while peak area precision is significant for the quantitative assay [17]. Results in Table 1 shows the RSDs of peak area and migration time of the standards. The RSDs of migration time ranged from 0.64 to 1.88% show good reproducibility and indicate excellent control of electroosmotic flow in the whole experiment [18,19]. Although the RSDs of the corrected peak areas ranged from 2.79 to 6.62%, it is regarded as good reproducibility.

3.2.2. Linearity

Calibration of each bioactive component was performed by analyzing the corrected peak areas of each standard ranged from 1 to 50 ppm. Five concentrations were used for each calibration curve and triplicate analyses were carried out at each concentration. Correlation coefficients of the calibration curves were in the range of 0.993–1.000 as shown in Table 1. As the results implied good linearity at the defined concentration range, results of samples within this range are reliable for quantitative analysis.

3.3. Sample analysis and recovery

With the above optimization and validation, quantification of the amount of bioactive components in both bark and leaves of *Eucommia ulmoides* became possible. The electrokinetic chromatograms of standards, bark and leaves of *Eucommia ulomides* are shown in Fig. 6. Peak identification was carried out by spiking the sample with standards. Geniposide, geniposidic acid, rutin, chlorogenic acid and protocatechuic acid were found in the leaves of *Eucommia ulmoides* while geniposide, geniposidic acid, rutin and protocatechuic acid were present in bark. The amounts of these bioactive components found in leaves and bark of *Eucommia ulmoides* are shown in Table 2.

In order to ensure analytes are not lost during the analytical procedure, the recoveries were determined.

Table 1
Precision and linearity of CE method of different standards[a]

Compound	RSD (%)		Regression equation [c]	r^2 [d]
	Migration time[b]	Peak area[b]		
Geniposide	0.64	5.84	$y = -6.846 + 3.65x$	0.996
Geniposidic acid	0.84	6.62	$y = 0.047 + 8.48x$	0.995
Pygrogallol	0.93	5.11	$y = 0.919 + 91.708x$	1.000
Rutin	0.91	4.05	$y = 0.399 + 41.564x$	1.000
Chlorogenic acid	1.00	2.86	$y = 0.419 + 22.766x$	1.000
Ferulic acid	1.71	3.30	$y = -1.346 + 55.793x$	1.000
p-Coumaric acid	1.38	3.08	$y = -0.879 + 56.987x$	1.000
Quercetin	1.88	3.03	$y = -0.047 + 63.334x$	0.999
Caffeic acid	1.27	2.79	$y = -0.693 + 68.459x$	1.000
Protocatechuic acid	1.73	3.57	$y = -0.583 + 102.5x$	1.000

[a] Conditions: 50 m*M* boric acid, 50 m*M* SDS, pH 9.5 and 20 kV with an effective length of 50 cm of capillary.
[b] Standard concentration is 50 ppm, $n = 8$.
[c] Concentrations of each compound were in the range of 1–50 ppm.
[d] r, correlation coefficient, $n = 3$.

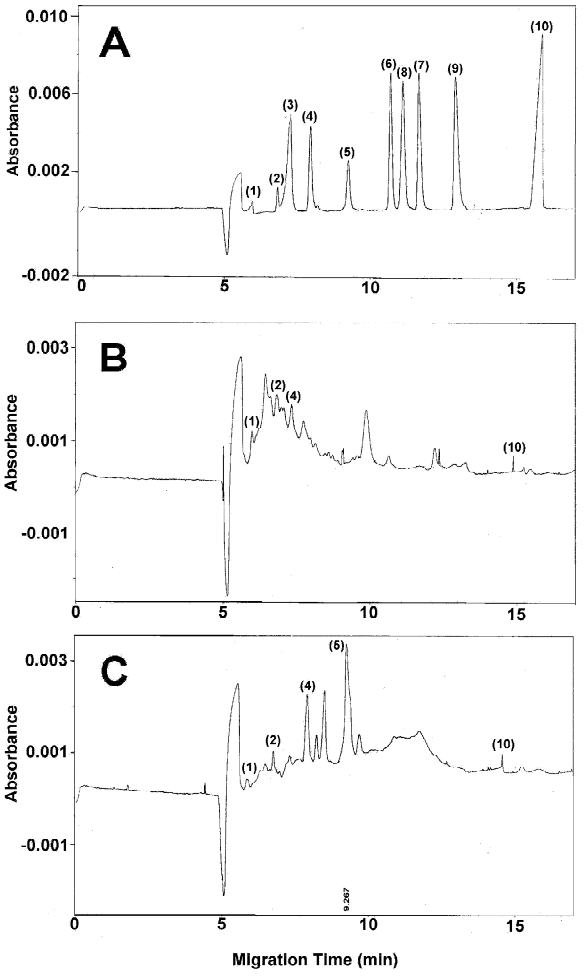

Fig. 6. Electrokinetic chromatogram of *Eucommia ulmoides*. (A) Standards; (B) bark; (C) leaves. Peaks: 1=geniposide; 2=geniposidic acid; 3=pyrogallol; 4=rutin; 5=chlorogenic acid; 6=ferulic acid; 7=*p*-coumaric acid; 8=quercetin; 9=caffeic acid; 10=protocatechuic acid.

Table 2
Quantity and recovery of compound in *Eucommia ulmoides* bark and leaves by CE ($n=3$)

Sample	Bark (mg/g)	Leaves (mg/g)	Recovery (%)
Geniposide	0.58	0.81	98
Geniposidic acid	0.94	2.24	99
Rutin	0.21	2.01	98
Chlorogenic acid	NF[a]	8.6	102
Protocatechuic acid	0.04	0.11	102

[a] NF, not found.

The sample was spiked with 5 ppm of each standard. The corrected peak area of each standard in the sample after spiking was compared with the sample alone plus the amount spiked into it. Three replicate analyses were carried out. The recovery of the compounds varied from 98 to 102% (Table 2). This result indicates that the method is reliable for the analysis of biochemical compounds.

4. Conclusions

In conclusion, a qualitative and quantitative analytical method for the simultaneous determination of three different groups of bioactive components in *Eucommia ulmoides* has been successfully established in this study. The method is simple, efficient, sensitive, accurate and reliable. It can be widely applied for the quantification in *Eucommia ulmoides* as well as other traditional Chinese medicine in the future in order to have a better understanding of the complex composition of traditional Chinese medicines.

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