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Capillary zone electrophoresis for separation and analysis of four diarylheptanoids and an α-tetralone derivative in the green walnut husks (*Juglans regia* L.)

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ABSTRACT

A fast capillary zone electrophoresis (CZE) method for the simultaneous determination of four cyclic diarylheptanoids (rhoiptelol, RH; juglanin A, JA; juglanin B, JB; juglanin C, JC) and an α-tetralone derivative (sclerone, SC) in the extract of the green walnut husks (*Juglans regia* L.) was developed. The optimized buffer was composed of 25 mM sodium tetraborate at pH 10.3. The applied voltage was 20 kV and the capillary temperature was kept constant at 20 °C. The detection wavelength was set at 220 nm using a photodiode array detection. The effects of several CE parameters, including pH value, buffer concentration, applied voltage and separation temperature on the separation were investigated systematically. Regression equations showed good linear relationships (correlation coefficients: 0.9996–0.9999) between the peak area of each compound (RH, JA, JB, JC and SC) and its concentration accordingly. The relative standard deviations (R.S.D.) of the migration time and peak area were less than 0.57 and 3.44% (intra-day), and 0.97 and 3.71% (inter-day), respectively. The contents of the five active compounds in the green walnut husks (*J. regia* L.) from different origins were determined with satisfactory repeatability and recovery.

1. Introduction

Walnuts, *Juglans regia* L. (*Juglandaceae*), are found throughout the world such as in the West Indies, Japan, China, Southern Asia from India and Turkey, in South Eastern Europe to the Carpathian Mountains of Poland, in the eastern and southern parts of the United States [1]. The seeds of *J. regia* L. (*Juglandaceae*) are a highly nutritious food and also used as a traditional remedy for treating cough, stomach ache and cancer in Asia and Europe [2]. Phenolic compounds are secondary metabolites, which occur in abundance in different parts of *J. regia* L., such as fresh walnut leaves, husks, inner root bark. Naphthoquinones and flavonoids are considered the major phenolic compounds, and the naphthoquinone juglone is of great interest due to its chemical reactivity [3]. In the shoots of *J. regia* L., the presence of flavanol myricitrin and glucoside of juglone were proved by past research [4]. Several diarylheptanoids and naphthoquinones from *Juglans* and other natural products have exhibited a broad range of potent biological activities, including anti-inflammatory, antihepatoxic, antifungal, antibacterial, anticancer, anti-androgen, NO donor effect, antioxidant and oxygen radical scavenging activities, and related effects [5–15]. In our previous study, four diarylheptanoids (rhoiptelol, RH; juglanin A, JA; juglanin B, JB; juglanin C, JC) and an α-tetralone derivative (sclerone, SC) were isolated from the extracts of the green walnut husks (*J. regia* L.) in our laboratory for the first time, and these compounds exhibited the cytotoxic activity in our previous investigations [16]. Their molecular structures are shown in Fig. 1. The purified compounds RH and SC were identified by comparing their MS, NMR data with literatures [17,18]. JA, JB and JC were all new diarylheptanoid derivatives that were isolated and identified by our group, and their structures were elucidated by various spectroscopic methods, including intensive 2D-NMR techniques, HR-ESI-MS and X-ray single-crystal diffraction analysis [19–21]. Based on the bioactivity of these compounds from the green walnut husks (*J. regia* L.), the identification and determination of these compounds will play an important role in the effectual and safe use of the green walnut husks for pharmaceutical purpose. Therefore, it is necessary to develop an analytical method to identify and determine these compounds. Up to now, there have been only a few reports on determination of linear diarylheptanoids, such as curcuminoids by high performance thin layer chromatography (HPTLC) with a diode

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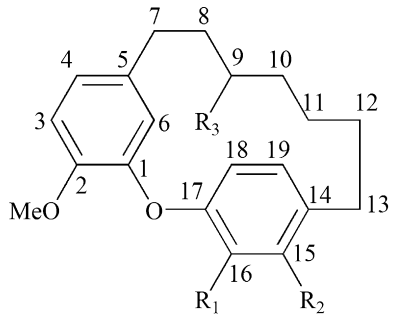
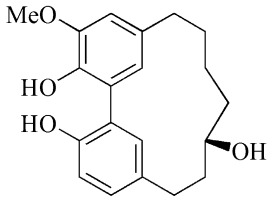


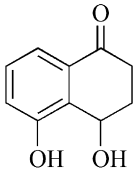
Rhoiptelol (RH): $R_1 = CH_3O$, $R_2 = OH$, $R_3 =$ 

Juglanin A (JA): $R_1 = CH_3O$, $R_2 = OH$, $R_3 = =O$

Juglanin C (JC): $R_1 = OH$, $R_2 = H$, $R_3 = =O$



Juglanin B (JB)



Sclerone (SC)

Fig. 1. Molecular structure of RH, JA, JC, JB and SC.

array detector (DAD) [22], curcumin, demethoxycurcumin, and bisdemethoxycurcumin in *Curcuma longa* L. by LC–ESI-MS/MS [23], five diarylheptanoids from *Alpinia officinarum* using HPLC/PAD and HPLC/electrochemical detection (ECD) [24], and a diarylheptanoid glycoside in *Alnus* plants and biological samples by CZE [25]. The determination of three considered diarylheptanoid JA, JB, RH and an α-tetralone derivative (regiolone, RE) in the green walnut husks (*J. regia* L.) by HPLC have been previously reported by our group [21]. To the best of our knowledge, there are few reports on the simultaneous separation and determination of cyclic diarylheptanoids and naphthoquinones in the plant samples by capillary electrophoresis (CE) method.

Capillary zone electrophoresis (CZE) has been applied widely owing to its minimal sample volume requirement, short analysis time and high separation efficiency. In this paper, we firstly developed a CZE method for the simultaneous determination of four cyclic diarylheptanoids RH, JA, JB, JC and an α-tetralone derivative SC in the green walnut husks (*J. regia* L.) from different origins of western China.

2. Experimental

2.1. Apparatus and conditions

A Beckman PACE/MDQ capillary electrophoresis instrument equipped with a photodiode array detection (Beckman Coulter, Fullerton, CA, USA) was used with a software of 32 Karat system and version 5.0 (Beckman Coulter, Fullerton, CA, USA) for data acquisition and evaluation. The Separation was carried out on an uncoated fused-silica capillary with a total length of 60 cm (50 cm effective length) × 75 μm i.d. (Yongnian Optical Fiber Factory, Hebei, China). The detection wavelength was set at 220 nm. The separation voltage was set at 20 kV and the temperature of the capillary was maintained at 20 °C. The optimal background electrolyte solution was 25 mM sodium tetraborate and the pH value was adjusted to 10.3 by titrating with 0.1 M NaOH. Before use, the capillary was rinsed with 1 M NaOH for 10 min, 0.1 M NaOH for 10 min, deionized water for 5 min, and then conditioned with running electrolyte for 5 min. Between runs the capillary was rinsed with running electrolyte only for 2 min. Samples were injected by applying a pressure of 0.5 psi for 5 s (1 psi = 6894.76 Pa).

An ultrasonic bath Model SB5200 (Branson, Shanghai China) was used to degas the buffer. A PB-10 pH meter (Beijing Sartorius Instrument & System Engineering Co., Ltd.) was used to adjust the borate buffer solutions to the desired pH value with 0.1 M NaOH or 0.1 M HCl.

2.2. Materials and reagents

The four samples of the green walnut husks (*J. regia* L.) were collected from different natural growth sites in western China, and immediately carried to the laboratory in cellulose bags. Voucher specimens, which were authenticated by Professor Zhi-gang Ma of Lanzhou University, China, were stored in the research center of natural products of this institution.

Reference compounds of RH, JA, JB, JC, and SC, were extracted, isolated and purified from the green walnut husks (*J. regia* L.) in our laboratory. The purities of these compounds were determined to be more than 95% by normalization of the peak areas detected by HPLC–DAD. The reference compounds were accurately weighted and then dissolved with aqueous methanol (50%, v/v) to produce stock standard solutions, which were diluted to appropriate concentration with aqueous methanol (50%, v/v) for the construction of calibration curves.

All chemicals were purchased from Beijing Chemical Reagents Plant (Beijing, China) and were analytical grade. Deionized water was used throughout. All solutions and samples were filtered through a 0.45 μm syringe filter before use.

2.3. Sample preparation

The samples were washed, cut and air-dried then finely milled to powder (particles within 82–26 mesh were chosen). A 1.0 g sample of the pretreated powder was accurately weighed and added into a 50 ml flask together with 20 ml aqueous methanol (80%, v/v), then extracted in an ultrasonic bath at the frequency of 40 kHz for 30 min at 25 °C, and repeated two times. The two extracts were combined and filtered through a filter paper, and then concentrated under reduced pressure. The residue was dissolved and fixed with aqueous methanol (80%, v/v) in a final volume of 10 ml. After being deposited still for 2 h, the 0.6 ml of supernatant fluid was accurately aspirated and fixed with water in a final volume of 1 ml. Then, the sample was filtered through a 0.45 μm syringe filter, and injected directly after degasification by ultrasonication.

3. Results and discussion

3.1. Choice of buffer system

At the pH 10.0, different buffer systems, including Tris ($C_4H_{11}NO_3$)–H_3PO_4 (20 mM), KH_2PO_4–NaOH (20 mM), H_3BO_3–NaOH (50 mM) and $Na_2B_4O_7$–NaOH (25 mM), were studied, respectively. As a result, buffer of boric acid (H_3BO_3) and sodium tetraborate ($Na_2B_4O_7$) were selected for their ability to form the complexation with hydroxyl group of diarylheptanoids and α-tetralone derivative at the pH 10.0. It was found that the pH value of buffer was unstable using boric acid than sodium

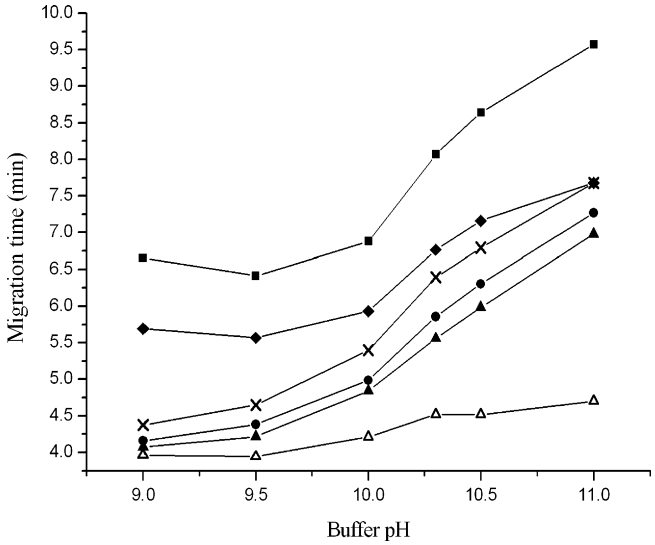


Fig. 2. Effect of buffer pH on the migration time of the five analytes. (▲) RH; (●) JA; (×) JC; (♦) JB; (■) SC; (△) EOF. Separation conditions: 25 mM borate; applied voltage, 25 kV; temperature, 25 °C; detection wavelength, 220 nm; the pH values are as indicated in the figure.

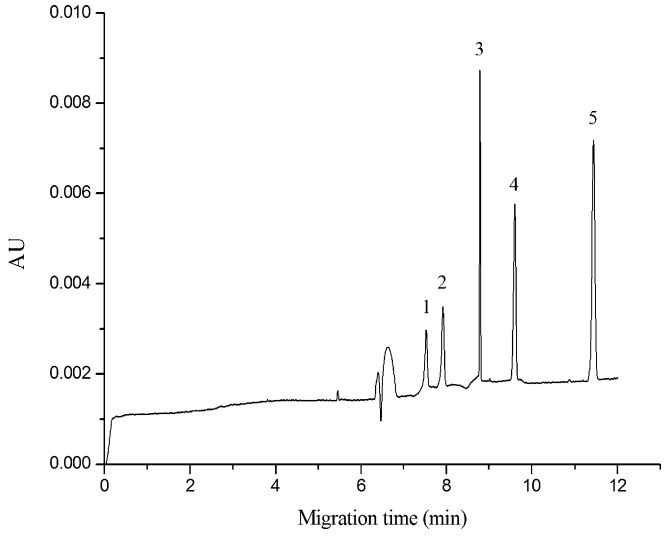


Fig. 3. Electropherogram of the standard mixtures. (1) RH; (2) JA; (3) JC; (4) JB; (5) SC. Separation conditions: 25 mM borate; pH, 10.3; applied voltage, 20 kV; temperature, 20 °C; detection wavelength, 220 nm; sampling size, 0.5 psi for 5 s.

tetraborate in the further experiment, so sodium tetraborate was chosen as buffer system.

3.2. Effect of buffer pH

The pH value of buffer is a very important parameter for its effect on zeta potential, electroosmotic flow (EOF), as well as the overall charge of all the analytes, which can affect the migration time and the separation of the analytes. At the pH lower than 9.0, RH and JA would co-migrate with EOF. Therefore, a series of buffers using the different pH value ranging from 9.0 to 11.0 were investigated, with the buffer concentration of 25 mM sodium tetraborate at applied voltage of 25 kV and capillary temperature of 25 °C. Fig. 2 shows the influence of buffer pH on separation from 9.0 to 11.0. It can be found that with increasing the buffer pH value, the migration time increased. It is possible that diarylheptanoids and α-tetralone derivative as phenol compounds are weak acids, and the phenolic group of all the analytes will be constantly ionized to form negative charges in higher pH values. The migration of the analytes towards negative electrode was slackened for having more negative charges, so the migration time increased. The effect to SC was more obvious than other analytes, which is possibly because the small molecular mass of SC (resulting in slower migration). Although all the reference compounds could be baseline separated at pH 9.5, SC was poorly separated from other unknown compounds in the real sample. The baseline of electropherogram was poorer at pH 10.5 than pH 10.3, which could be caused by the evident increase of joule heat. JB and JC would migrate together at pH 11.0. Considering the analysis time, sensitivity and resolution, the pH 10.3 was finally selected for further experiments.

3.3. Effect of borate concentration

To verify the effect of buffer concentration on migration behavior, the running buffer consisting of sodium tetraborate with different concentration (10, 15, 20, 25 and 30 mM) at pH 10.3, applied voltage of 25 kV and capillary temperature of 25 °C, was investigated. The result revealed that the migration time of RH, JA, JB, JC and SC increased with the increase of the concentration of running buffer. It is as a result of the decreased EOF, and this effect is directly related to the decrease of the zeta potential at the capillary wall–solution interface. Additionally, with the increase of buffer concentration, the ability to form complexations between sodium tetraborate and the analytes became stronger. Considering the joule heats, analysis time, sensitivity and resolution of real samples, 25 mM sodium tetraborate was selected as optimum concentration.

3.4. Effect of applied voltage and separation temperature

The effect of applied voltage of 10, 15, 20, 25 and 30 kV on the separation was tested under above conditions at 25 °C. The result indicated that higher voltage can bring higher separation efficiency and shorter analysis time, but reduce the peak areas of all analytes. And the joule heats existent in the buffer system also increased when increasing the applied voltage. Considering the analysis time, joule heats, sensitivity and separation of real samples, the voltage of 20 kV was finally used. Subsequently, the effect of capillary temperature at 20, 25, 30 and 35 °C on separation was studied. In view of better resolution for the analysis of the herbal samples and proper migration time, the capillary temperature of 20 °C was chosen as separation temperature. Under the optimum separation conditions (an electrolyte containing 25 mM sodium tetraborate at pH 10.3, the applied voltage of 20 kV, and the capillary temperature at 20 °C), the analysis of standard solution was completed within 12 min as exhibited in Fig. 3.

3.5. Optimization of sample preparation

An ultrasonic bath at room temperature was commonly used in the extraction of herbal samples, it is convenient and faster than reflux and soxhlet extraction. In our study, the ultrasonic bath extraction was chosen as preferred method. To obtain optimal extraction efficiency, the parameters, such as extraction solvents, extraction time and extraction times were investigated. Firstly, pure methanol, 80% and 50% (v/v) aqueous methanol were employed as extraction solutions, respectively. The results showed that the contents of all the analytes were higher using 80% aqueous methanol as extraction solution, compared with those extracted by pure methanol or 50% aqueous methanol. The effects of different extraction time (30, 45, and 60 min) on extraction efficiency were then

Table 1
Regression equations, linear range, correlation coefficient, LOD and LOQ of the analytes

Analyte	Regression equation[a]	Linear range (μg/ml)	Correlation coefficient	LOD (μg/ml)[b]	LOQ (μg/ml)[b]
RH	$y = 738.12x - 700.04$	1.00–128	0.9999	0.50	0.83
JA	$y = 784.57x - 803.17$	1.35–172	0.9999	0.68	1.12
JB	$y = 529.24x - 1204.6$	3.57–457	0.9999	1.79	2.97
JC	$y = 834.34x - 319.21$	1.46–186	0.9999	0.49	0.81
SC	$y = 757.9x + 3228.5$	3.69–472	0.9996	1.11	1.84

[a] y and x stand for the peak area and the concentration (μg/ml) of the analytes, respectively.
[b] The LOD was defined as the concentration where the signal-to-noise ratio is 3 and the LOQ was defined as the concentration where the signal-to-noise ratio is 5.

Table 2
Recoveries of the five analytes

Analyte	Original amount (mg)	Added amount (mg)	Found amount (mg)	Recovery (%)	R.S.D. (%)[a]
RH	0.064	0.128	0.192	97.7	2.74
		0.064	0.128	96.9	1.13
JA	0.285	0.172	0.457	108.1	2.50
		0.086	0.371	102.3	2.59
JB	0.886	0.457	1.343	93.4	1.40
		0.228	1.114	94.7	0.35
JC	0.117	0.186	0.303	94.1	1.21
		0.093	0.210	94.6	2.93
SC	0.222	0.472	0.694	94.5	1.13
		0.236	0.458	97.9	1.95

[a] $n = 3$.

Table 3
Contents of five analytes in the green walnut husks (*J. regia* L.) collected from different origins

Origin	Content (mean ± S.D., mg/g of dry sample, $n = 3$)[a]				
	RH	JA	JB	JC	SC
Baoji, Shanxi province	0.064 ± 0.001	0.285 ± 0.001	0.886 ± 0.009	0.117 ± 0.003	0.222 ± 0.006
Ziyang, Sichuan province	0.040 ± 0.001	0.070 ± 0.002	0.778 ± 0.032	0.139 ± 0.001	0.200 ± 0.006
Zhangye, Gansu province	0.041 ± 0.001	0.120 ± 0.002	0.806 ± 0.005	0.060 ± 0.002	ND
Xinjiang province	0.042 ± 0.002	0.092 ± 0.003	1.212 ± 0.018	ND	0.278 ± 0.008

[a] "ND" means not to be detected.

tested, and the result showed that the extraction time of 30 min was enough. Finally, the extraction times (once, twice, and three times) was observed. The result showed that the extraction repeated two times was adequate. As a result, the extraction for the samples with aqueous methanol (80%, v/v) for 30 min and repetition for two times was confirmed. The result of the recovery test also demonstrated that the extraction method was appropriate for the analysis. Additionally, owing to the solubility of the sample and the stability of the running buffer, the standard and herbal samples were all prepared to aqueous methanol (50%, v/v) before introducing into the electrophoretic capillary.

3.6. Linearity, LOD, LOQ and reproducibility

The linear relationships between the concentrations of the five compounds and the peak areas accordingly, are shown in Table 1. Seven calibration levels were used, and each calibration level was replicated for three times. The linear ranges, the limits of detection (LODs) (S/N = 3) and the limits of quantitation (LOQs) (S/N = 5) for the five analytes are also listed. The correlation coefficients (r) are all more than 0.9996. The method was validated for reproducibility of the migration time and the peak area of the standards. The relative standard deviation (R.S.D.) of the migration time and the peak area of each peak for six repeated injections were 0.33–0.57 and 2.28–3.44% (intra-day), and 0.49–0.97 and 2.13–3.71% (inter-day), respectively.

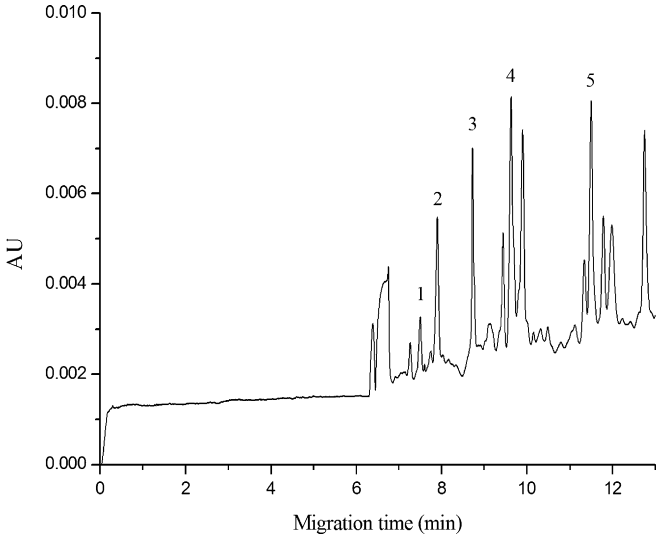


Fig. 4. Electropherogram of the extract sample of the green walnut husks (*J. regia* L.) (Baoji, Shanxi province of China). (1) RH; (2) JA; (3) JC; (4) JB; (5) SC. Separation conditions as in Fig. 3.

3.7. Recovery and application

Recovery experiments were also conducted to evaluate the accuracy and precision of the method. Accurate amounts of RH, JA, JB, JC and SC with different concentrations were added to the accurately weighed samples of *J. regia* L. before extraction. The samples were prepared under the selected optimal conditions as in Section 3.5, and the results are shown in Table 2. It indicated that this method is accurate, sensitive, reproducible and useful for the analysis of diarylheptanoids and α-tetralone derivative in herbal medicines.

Four samples of the green walnut husks (*J. regia* L.) were collected from different origins of western China. The contents of RH, JA, JB, JC and SC were simultaneously determined using the proposed method. Peaks were identified by comparing the migration time and spiking the standard RH, JA, JB, JC and SC to the sample solution. The analytical results are summarized in Table 3. The results showed that the difference of the contents of RH and JB was not much within these samples. Fig. 4 illustrates the typical electropherogram of 80% methanol extract of *J. regia* L. from Baoji, Shanxi province of China.

4. Conclusion

In this paper, a simple, accurate and sensitive method has been firstly developed for the simultaneous determination of diarylheptanoids and α-tetralone derivative by CZE. As stated in the beginning, the compound RH, JA, JB, JC and SC exhibited the cytotoxic activity in our previous investigation, which has the potential to be developed as a new drug, so an efficient analytical method is very important and required to study the content of these compounds in herbal samples. The developed method was successfully applied to determine the five compounds in *J. regia* L. This work shows that CZE is a suitable and powerful technique to analyze of diarylheptanoids and α-tetralone derivative in the complex extract of the medicinal plants.

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