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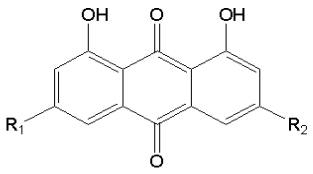
Simultaneous determination of anthraquinones in Rhubarb by pressurized liquid extraction and capillary zone electrophoresis

Rhubarb, a well-known Chinese herbal medicine, is also used in Europe and other places of the world. Anthraquinones derivatives are thought to be the major active components. A pressurized liquid extraction (PLE) and capillary zone electrophoresis (CZE) separation were developed for simultaneous determination of five anthraquinones including aloe-emodin, emodin, chrysophanol, physcion, and rhein in Rhubarb. The effects of the experimental variables on PLE and CZE have been optimized. The optimum conditions of PLE were: solvent, methanol; temperature, 140°C; particle size, 0.13–0.2 mm; static extraction time, 5 min; pressure, 1500 psi; and one extraction. The best separation of the five anthraquinones could be obtained using 50 mM borate buffer (pH 8.2) containing 25% isopropyl alcohol and 25% acetontrile as modifier, while the separation voltage was 25 kV and the temperature was at 20°C. The method developed is accurate, simple, and reproducible, and could be used for quality control of Rhubarb and its medical preparations.

Keywords: Anthraquinones / Capillary zone electrophoresis / Pressurized liquid extraction / Quantitative determination / Rhubarb
DOI 10.1002/elps.200400001

1 Introduction

Rhubarb, a well-known Chinese herbal medicine, has long been used in oriental preparations. It is also used in Europe and other places of the world [1, 2]. Pharmacological actions of Rhubarb include laxative, antibacterial, hemostatic, and antispasmodic effects [3, 4]. Anthraquinones derivatives, including emodin, aloe-emodin, rhein, physcion, and chrysophanol, are thought to be the major active components [5]. Thus, determination of the active components in Rhubarb is required for the evaluation of its quality and the control of dosage during clinical studies. It is commonly performed by using TLC [6, 7] and HPLC [8]. These methods suffer from low resolution or large consumption of organic solvent. High-performance capillary electrophoresis has become a powerful tool in natural product analysis [9–12], due to its high resolution, short analysis time, and low solvent and sample consumption. Several methods, such as micellar electrokinetic chromatography (MEKC) [13–19], capillary electrochromatography (CEC) [20], and capillary zone electrophoresis (CZE) [21], have been proposed for the analysis of anthraquinone derivatives in Rhubarb. In all of these works, simultaneous separation of emodin, aloe-emodin, physcion, chrysophanol, and rhein (Fig. 1) was not performed or achieved in CZE mode [21, 22]. We developed an efficient and reliable CZE method to analyze the five anthraquinones in Rhubarb. Pressurized liquid exraction (PLE, Dionex trade name ASE) was used for sample preparation.



	R1	R2
Chrysophanol	CH3	H
Emodin	CH3	OH
Physcion	CH3	OCH3
Aloe-emodin	H	CH2OH
Rhein	H	COOH

Figure 1. The structure of five anthraquinones.

2 Materials and methods

2.1 Instrumentation

All samples were extracted by a Dionex ASE 200 system (Dionex, Sunnyvale, CA, USA). All CE separations were performed on an Agilent CE instrument (Agilent Technol-

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Abbreviation: PLE, pressurized liquid extraction

ogies, Palo Alto, CA, USA), equipped with a diode-array detector and an Agilent ChemStation software. A fused-silica capillary (56 cm × 75 μm ID, 48 cm effective length) was used throughout this study.

2.2 Chemicals

Rhein, emodin, aloe-emodin, chrysophanol, physcion, and 4-methoxysalicyladehyde were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sodium tetraborate, acetonitrile, and isopropyl alcohol for liquid chromatography and sodium hydroxide was purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). Reagents not mentioned here were from standard sources. Rhubarb was obtained from Sichuan Province of China, which is identified as *Rheum officinale* Baill by Dr. Shaoping Li. The voucher specimen of Rhubarb was deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau SAR, China.

2.3 Procedures

A running buffer composed of 50 mM borate with 25% acetonitrile and 25% isopropyl alcohol was adjusted to pH 8.2 with 0.1 M hydrochloric acid. The solution was filtered through a 0.25 μm Econofilter (Agilent Technologies) before it was transferred to the inlet/outlet vials. The anthraquinones derivatives were first dissolved in the buffer at ~0.1 mg/mL as a stock solution and then diluted with the buffer to the desired concentration. All solutions were found stable when stored at 4°C for two months. Rhubarb powder (0.5 g) was mixed with diatomaceous earth in a proportion of 1:1 and placed into an 11 mL stainless steel extraction cell. The sample was extracted by PLE, which was performed on a Dionex ASE 200 system under the optimized condition. The extract was transferred into a volumetric flask and adjusted to the desired volume with the buffer, so as to make the five analyte concentrations in the linear range based on the preliminary determination. Before sample injection, the capillary was rinsed with 1 M sodium hydroxide and running buffer for 10 min, respectively. Twenty-five kV voltage was applied over the capillary. No pair of running vials (inlet and outlet) was used for more than a total of 2 h running time. Pressure injection was 50 mbar for 6 s, and the detection was performed at 230 nm. The running time was 50 min at 20°C.

3 Results and discussion

3.1 Optimization of PLE

The PLE procedure was optimized and the parameters including the type of solvent, particle size, temperature, static extraction time, and pressure were studied by using a univariate approach. Five analytes, including rhein, emodin, aloe-emodin, chrysophanol, and physcion, were used as markers for evaluation of extraction efficiency. The influences of solvent, particle size, temperature, static extraction time, and pressure on the PLE are shown in Fig. 2. The extraction efficiency for PLE was determined by performing consecutive pressurized liquid extractions on the same sample under the optimized conditions, until no investigated compounds were detected by the analysis. It was calculated based on the total amount of individual investigated components. The recoveries at one-time extraction obtained for every analyte were higher than 99.6% (RSD $< 3\%$, $n = 3$). The optimum conditions of the PLE method were: solvent, methanol; temperature, 140°C; particle size, 0.13–0.2 mm; static extraction time, 5 min; pressure, 1500 psi; one extraction.

3.2 Optimization of the separation of anthraquinones

For optimization of the running condition, CZE was performed by using a mixture of five anthraquinone derivatives: emodin, aloe-emodin, rhein, physcion, and chrysophanol. Borate was chosen as buffer component according to the literature [21, 22] and our primary test with *e.g.*, phosphate, Tris-phosphoric acid, Tris-HCl, and carbonate was performed). It was found that the separation of aloe-emodin and chrysophanol was poor under the initial condition of 50 mM borate solution (pH 8.5) with 10% acetonitrile as organic modifier. Therefore, the effect of the individual parameters was determined mainly for the resolution of aloe-emodin and chrysophanol.

Of all parameters, pH plays a significant role. A slight change of the pH can remarkably affect the resolution. The effect of pH on the resolution of aloe-emodin and chrysophanol is shown in Fig. 3A. A pH of 8.2 was found to be optimum. The concentration of borate (40–60 mM) was changed to optimize the running condition. The better separation could be obtained at 50 mM borate (Fig. 3B). The influence of an organic modifier, acetonitrile and isopropyl alcohol, on the CE separation of aloe-emodin and chrysophanol was also investigated. The best separation could be achieved using 50 mM borate (pH 8.2) containing 25% isopropyl alcohol and 25% acetontrile as modifier (Fig. 3C). The resolution of aloe-emodin and

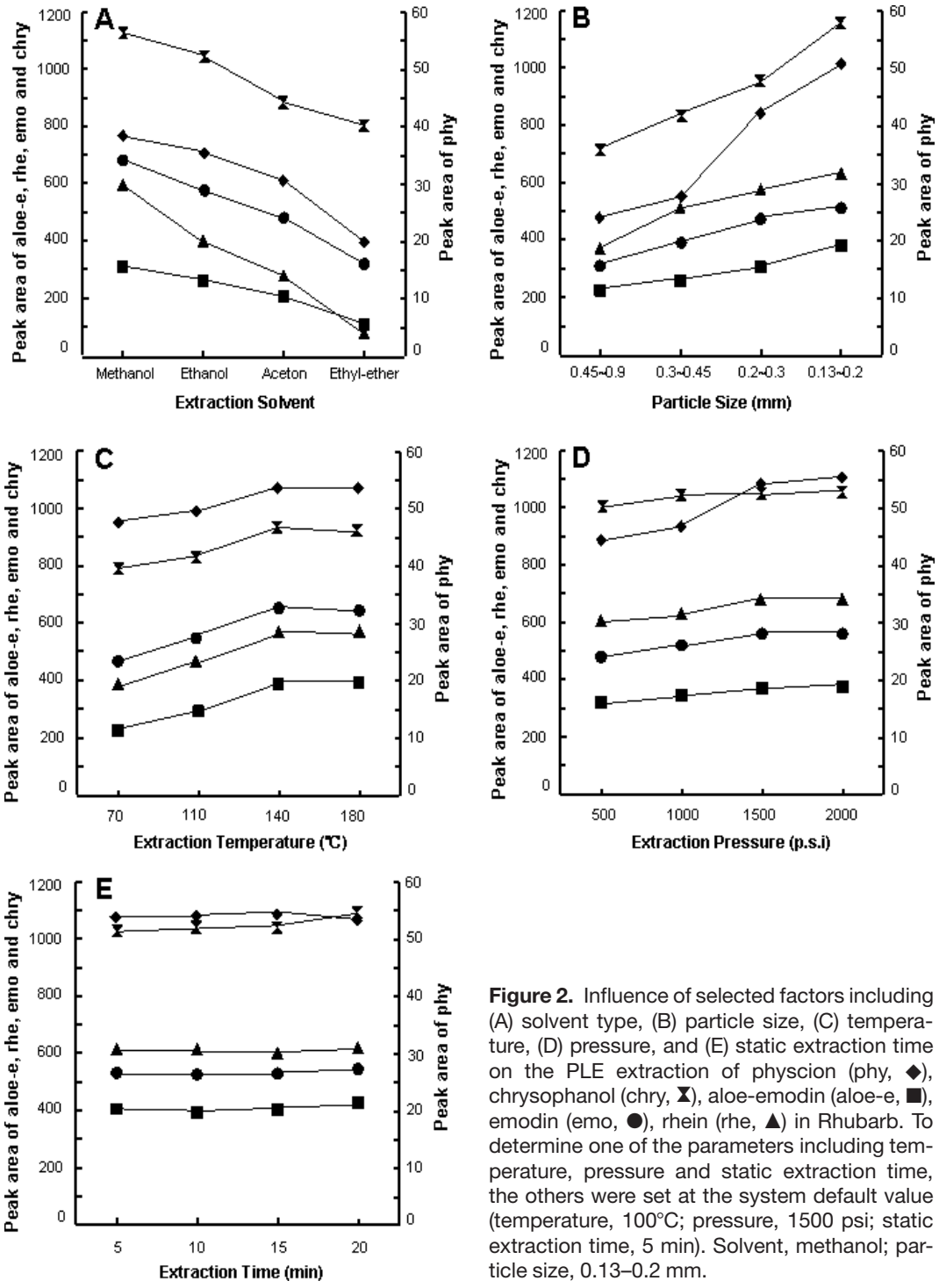

Figure 2. Influence of selected factors including (A) solvent type, (B) particle size, (C) temperature, (D) pressure, and (E) static extraction time on the PLE extraction of physcion (phy, ◆), chrysophanol (chry, ⧗), aloe-emodin (aloe-e, ■), emodin (emo, ●), rhein (rhe, ▲) in Rhubarb. To determine one of the parameters including temperature, pressure and static extraction time, the others were set at the system default value (temperature, 100°C; pressure, 1500 psi; static extraction time, 5 min). Solvent, methanol; particle size, 0.13–0.2 mm.

chrysophanol, however, did not obviously change with different temperatures or voltages (data not shown). Therefore, 25 kV and 20°C were chosen as separation conditions, considering the electro-osmotic flow being proportional to the voltage.

3.3 Linearity of the investigated anthraquinones

The five anthraquinones proved to exhibit good linearity in the measured concentration range using 4-methoxysalicyladehyde (0.1 mg/mL) as an internal standard

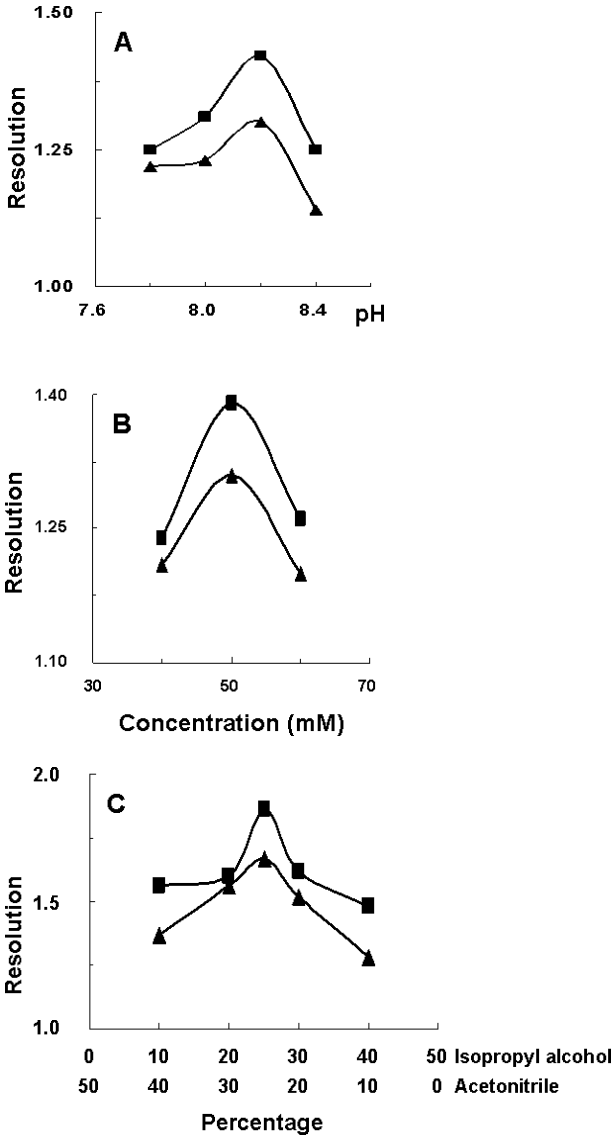

Figure 3. Effects of (A) buffer pH, (B) concentration, and (C) organic modifier on the resolution of (■) chrysophanol and (▲) aloe-emodin. Conditions: fused-silica capillary (56 cm × 75 μm ID, 48 cm effective length), pressure injection, 50 mbar for 6 s; (A) borate buffer concentration, 50 mM; (B) buffer pH 8.2; (C) 50 mM borate buffer (pH 8.2) with different ratios of acetonitrile and isopropyl alcohol as organic modifier; UV detection at 230 nm. Voltage, 25 kV applied over the capillary at 20°C.

(Table 1). The lowest concentrations of linearity for physcion, chrysophanol, aloe-emodin, emodin, and rhein was 1.5, 1.7, 1.6, 1.9, and 1.4 μg/mL, respectively. The limit of detection (LOD) was defined as the signal-to-noise ratio of 3. The LOD values were 0.5 μg/mL in the tested compounds, indicating the sensitivity and performance of the signal-to-noise ratio in our calibrated system. The amount of analytes in Rhubarb was determined within the linear range. The short-term (1 day) repeatability (RSD < 0.9%) as well as the long-term (4 days) repeatability (RSD < 1.2%) of the migration time were calculated on ten runs. The migration time was relatively stable. The area repeatability was also calibrated. Both short-term and long-term repeatability (RSD) of the five investigated components were less than 3.7%. Thus, the quantitation of the analytes was well performed within a few days after sample extraction. In order to determine the recovery, a known amount of the five anthraquinones was added into an accurately weighed Rhubarb sample. The mixture was extracted and analyzed using the mentioned method above. The reproducibility (n = 3) was also determined. The recovery of the tested components was in the range of 96.3%–97.5%, and the RSD of the reproducibility for the five analytes was in the range of 1.6%–2.2%, where n = 3 (Table 1).

3.4 Analysis of Rhubarb

The content of the five anthraquinones contained in Rhubarb was determined by using the developed method of CZE. Peaks were identified by three means: (i) by comparing the migration times of the unknown peaks with those of the standards eluted under the same conditions, (ii) by comparing the UV spectra with those of the standards under the same conditions, and (iii) by spiking Rhubarb with stock standard solutions of anthraquinones. The resolution of the anthraquinone peaks was distinct in Rhubarb (Fig. 4). By using the calibrated elec-

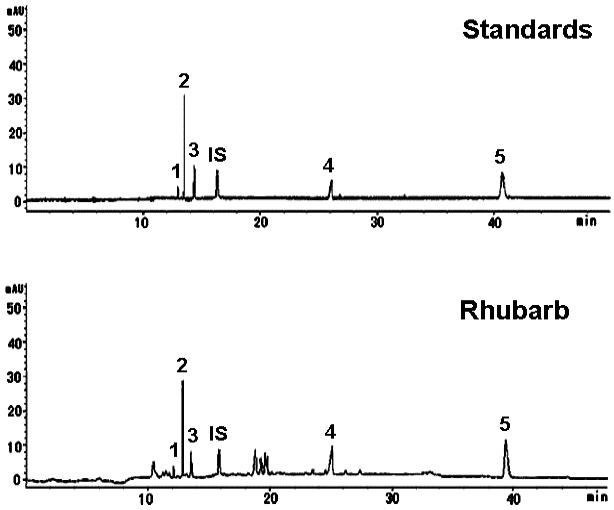

Figure 4. CZE profiles of Rhubarb. Conditions: fused-silica capillary (56 cm × 75 μm ID, 48 cm effective length), pressure injection, 50 mbar for 6 s; running buffer, 50 mM borate buffer (pH 8.2) with 25% acetonitrile and 25% isopropyl alcohol as organic modifier; voltage, 25 kV; temperature, 25°C; detection at 230 nm. Standards and Rhubarb are shown. 1, physcion; 2, chrysophanol; 3, aloe-emodin; 4, emodin; 5, rhein; IS, 4-methoxysalicyladehyde.

Table 1. Linear regression data and recoveries (%) of the investigated compounds from Rhubarb

Analyte	Linear regression data				Recovery (%)	Reproducibility RSD (%)
	Linear range (μg/mL)	Slope	Intercept	r^2 (n = 6)		
Physcion	1.5–60.0	0.56	0.31	0.9984	96.7	2.1
Chrysophanol	1.7–66.0	0.68	0.51	0.9989	97.1	1.9
Aloe-emodin	1.6–64.5	6.83	0.06	0.9982	97.5	1.7
Emodin	1.9–77.4.	18.2	−1.49	0.9994	97.4	1.6
Rhein	1.4–56.4	11.1	−0.51	0.9983	96.3	2.2

r^2, squares of correlation coefficients for the standard curves; percentage of relative standard deviation (RSD) for three replicates

trophoresis system, Rhubarb was analyzed. The results showed that the contents of physcion, chrysophanol, aloe-emodin, emodin, and rhein were 0.54, 5.56, 2.08, 2.74, and 4.33 mg/g, respectively, which is in accordance with the previous report [15].

The developed CZE method can simultaneously separate the five anthraquinones which are frequently used as markers for the quality control of Rhubarb [15, 16, 19, 21]. This could not be achieved in a previous study [22]. In addition, CZE is the most frequent and simplest separation mode from all modes of capillary electrophoresis, which makes the optimization of conditions easy. On the other hand, CZE uses an uncoated capillary column that requires less maintenance. CE also has several advantages over HPLC, particularly in terms of its high efficiency and small volume requirements [23], thus enabling the analysis of a number of sophisticated biological and clinical samples, even if the amounts are limited [24]. In addition, the time spent on HPLC conditioning and regenerating is saved. Moreover, CE is more environment-friendly.

4 Concluding remarks

In conclusion, a PLE and CZE separation of five anthraquinones was described. High extraction efficiency and automation were achieved by using PLE. The separation was performed without the use of a surfactant as pseudostationary phase. The fused-silica capillary was not contaminated by crude drugs considering the complex components in Chinese herbal medicines. The method developed is accurate, simple, and reproducible, and can be used for quality control of Rhubarb and its medical preparations.

We are grateful to Sandy Lao and Leon Lai from our laboratory for their expert technical assistance.

Received September 6, 2004

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