Analysis of rhubarb anthraquinones and bianthrones by microemulsion electrokinetic chromatography

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Abstract

In this work a method of microemulsion electrokinetic chromatography (MEEKC) has been developed for the analysis of nine anthraquinones and bianthrones in rhubarb. This study employed di-*n*-butyl tartrate as oil substance to make up the microemulsion. The composition of the microemulsion was 0.5% (w/w) di-*n*-butyl tartrate, 0.6% (w/w) SDS, 1.2% (w/w) 1-butanol and 97.7% (w/w) 10 mM sodium borate buffer, pH of the buffer being 9.2. Acetonitrile was added to the emulsion to improve the separation. The volume ratio between the emulsion solution and acetonitrile of an optimized separation was 70:30. With the optimized conditions all of the nine analytes were baseline-separated in peaks of good shapes within 20 min. After validation the method was used to analyze the components in a rhubarb sample. A solid-phase extraction procedure was employed. Five anthraquinones and two bianthrones had been detected in the sample and their amounts were determined. The method should be able to be used for the quantitative analysis of the main active components of rhubarb crude drugs.
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Keywords: Anthraquinones; Bianthrones; Rhubarb; Microemulsion electrokinetic chromatography

1. Introduction

Rhubarb is one of the oldest and best-known Chinese herbal medicines. It is used or recommended as a laxative, antiphlogistic and hemostatic in the treatment of obstipation, gastrointestinal indigestion, diarrhea, and jaundice [1–3]. This crude drug is not only officially listed in the Chinese Pharmacopoeia, but also appears in the British Pharmacopoeia and European Pharmacopoeia [4,5]. The major constituents of the rhubarb material (rhizome and root part) are anthraquinone and bianthrone derivatives. Until the 1950s, the free anthraquinones were considered to be the constituents producing the purgative action of rhubarb. Current evidence indicates that the major active principles are the bianthrone glycosides.

Due to its high resolution, short analysis time, and low sample and solvent consumption, capillary electrophoresis (CE) has become a powerful tool in natural product analysis. Microemulsion electrokinetic chromatography (MEEKC) is a separation mode of CE that shows similar potential as capillary electrochromatography (CEC) and micellar electrokinetic chromatography (MEKC), namely the possibility to separate both charged and neutral solutes simultaneously [6,7]. Microemulsions are solutions containing nanometer-sized droplets of an immiscible liquid dispersed in an aqueous buffer. The droplets are coated with a surfactant to reduce the surface tension between the two liquid layers. By the addition of a short-chain alcohol the surface tension of the droplet is further lowered. The oil-in-water microemulsion systems are similar to micelles in that they can solubilize hydrophobic compounds, but with a much larger capacity [8]. In addition, solutes are more easily able to penetrate the surface of the droplet than the surface of the micelle, which is much more rigid [9]. The solvating property of the microemulsion enables resolution

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of a wide range of solutes of differing hydrophobicity [10].

A microemulsion system is more complex than a micellar system as there are more operating variables such as type and concentration of the oil and co-surfactant to be treated. However, this might be favorable to separations because more choices are engendered for the manipulation of optimization. Octane is the oil most commonly used to form emulsions. However, there is a high surface tension between octane and water. Although a large amount of surfactant and co-surfactant are added to reduce the surface tension, such emulsion systems did not favor the use of relatively larger amounts of organic modifiers such as methanol and acetonitrile. In MEKC, organic solvents are widely used in micellar systems to improve the separation. In our previous experiments, a volume of 17% of acetonitrile was added to a micellar solution so as to obtain an adequate separation of nine anthraquinone and bianthrone compounds [11]. Microemulsions prepared with the oils of relatively low surface tension would allow the use of a larger amount of organic modifiers without breaking the emulsion system. These oils are the organics of much higher polarity than *n*-octane. Di-*n*-butyl tartrate, which is an ester compound, belongs to such oils. Aiken and Huie had used di-*n*-butyl tartrate as oil to separate a racemic mixture of ephedrine in an MEEKC experiment [12]. Mahuzier et al. had achieved a separation of four homologous parahydroxybenzoates with di-*n*-butyl tartrate as oil in a high-speed analysis by MEEKC [13]. Because of the structural similarities among the analytes to be separated in this work (for example, sennoside A and sennoside B are geometric isomers) di-*n*-butyl tartrate was investigated for its capability to separate the complex mixture of rhubarb via an MEEKC process.

2. Experimental

2.1. Anthraquinones and bianthrones

Aloe-emodin, emodin and rhein were purchased from Sigma (St. Louis, MO, USA). Chrysophanol was purchased from Fluka (Buchs, Switzerland). Physcion, sennidin A and sennidin B were purchased from Extrasynthese (Genay, France). Sennoside A and sennoside B were provided by Leiras Oy (Helsinki, Finland). Anthraquinone-2-carboxylic acid was purchased from Aldrich (Milwaukee, WI, USA).

2.2. Chemicals and SPE material

n-Octane, 1-butanol, acetic acid, sodium hydrogen carbonate and tetrahydrofuran were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was purchased from Sigma. Di-*n*-butyl tartrate was purchased from Aldrich. Methanol (MeOH) and acetonitrile (ACN) were purchased from Mallinkrodt (Paris, KY, USA). Iso-

propanol was purchased from Alps (Taipei, Taiwan). All of the above reagents and solvents were of analytical or chromatographic grade.

Oasis HLB SPE cartridges were purchased from Waters (Milford, MA, USA). The SPE procedure was performed on a Waters extraction manifold system.

2.3. Instrument

MEEKC separations were carried out on a CE system consisting of a Lauer Labs (Emmen, The Netherlands) Prince programmable injector and a 30 kV high-voltage supply, connected to a Dynamax (Rainin, Emeryville, CA, USA) UV-C absorbance detector. A fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA) was used. The electropherograms were recorded using an EZChrom (Scientific Software, San Ramon, CA, USA) chromatographic data system.

2.4. Electrophoretic conditions

Separations were performed on a 74 cm (60 cm effective length) × 50 μm i.d. fused-silica capillary. The new capillary was pre-conditioned prior to use by flushing successively with 1.0 M sodium hydroxide for 10 min, 0.2 M sodium hydroxide for 10 min, de-ionized water for 10 min, and running buffer for 10 min. At the beginning of each experiment, the capillary was washed with 0.2 M sodium hydroxide for 10 min followed by running buffer for 10 min.

Operating conditions were listed as follows: voltage, 25 kV; injection time, 4.8 s (hydrodynamic, 0.725 p.s.i. (50 mbar); injected volume about 5.0 nl); detection wavelength, 270 nm; temperature, 30 °C.

2.5. Preparation of the microemulsions

Microemulsion solution A was prepared by weighing *n*-octane (0.81%, w/w), 1-butanol (6.61%, w/w), SDS (3.31%, w/w), and the sodium tetraborate aqueous buffer (10 mM, pH 9.2) (89.27%, w/w) to an appropriate volumetric flask. Microemulsion solution B was prepared by weighing di-*n*-butyl tartrate (0.5%, w/w), 1-butanol (1.2%, w/w), SDS (0.6%, w/w), and sodium tetraborate aqueous buffer (10 mM, pH 9.2) (97.7%, w/w) to an appropriate volumetric flask. Both solutions were sonicated for 30 min to form optically transparent microemulsions. Various volumes of ACN were added to microemulsion solution B.

2.6. Standard preparation

Standard working solutions were prepared by dissolving the appropriate amount of anthraquinones and bianthrones in 70% ACN in water (v/v). Concentrations of these compounds were as follows: aloe-emodin and emodin, 0.1480 mM; chrysophanol, 0.1967 mM; physcion, 0.0879 mM; rhein,

0.0704 mM; sennoside A and sennoside B, 0.0695 mM; sennidin A and sennidin B, 0.1115 mM. Anthraquinone-2-carboxylic acid (0.1190 mM) was used as internal standard for quantitative analysis.

2.7. Sample preparation

2.7.1. Extraction of sample

Rhubarb (*Rheum officinale* Baill., Polygonaceae) sample powder (0.2 g) was ultrasonically extracted with 70% MeOH (5 ml) for 20 min and followed by centrifugation at $1500 \times g$ for 5 min. This procedure was repeated three times. The extracts were combined together and filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA). One millilitre of this solution was dried by vacuum and the residue was dissolved in 2.4 ml of 70% ACN for injection.

2.7.2. SPE procedure

Ten millilitre of the 70% methanolic extract from Section 2.7.1 was rotavaporated to remove the solvent and the residue was dissolved in a 25% MeOH (20 ml). An Oasis HLB SPE cartridge (capacity 1 ml; 30 mg) was conditioned with 1 ml of MeOH and then 1 ml of water. After conditioning, 1 ml of the above 25% MeOH solution was loaded onto the cartridge. The glycosidic bianthrones (including sennoside A and sennoside B) were collected in the tubes as fraction A during the elution with 1 ml of 35% MeOH in 20 mM NaHCO$_3$. The cartridge was then washed successively with 1 ml of 20 mM acetic acid and 1 ml of 65% MeOH. Afterwards, the non-glycosidic anthraquinones (including aloe-emodin, chrysophanol, emodin, physcion and rhein) were collected in the tubes as fraction B during the elution with 1 ml of 95% ACN. The vacuum pressure was kept at 2 mmHg during the loading step. In other steps, it was kept at 5 mmHg. Fractions A and B were rotavaporated. Internal standard (anthraquinone-2-carboxylic acid dissolved in 70% ACN) was added to the residue of fraction A and 70% ACN was added to make the volume to 1.5 ml. This was termed sample solution A. The same internal standard was added to the residue of fraction B and 70% ACN was added to make the volume to 0.35 ml. This was termed sample solution B.

2.8. Calculation of effective mobility

The effective mobilities of the analytes were calculated according to the following equation [14]:

$$\mu_{\text{eff}} = \left(\frac{1}{t_0} - \frac{1}{t_R} \right) \frac{lL}{V}$$

where μ_{eff} is the effective mobility of the analyte, t_0 the migration time of the electroosmotic flow, t_R the migration time of the analyte, l the capillary length to the detector, L the total length of the capillary, V the applied voltage.
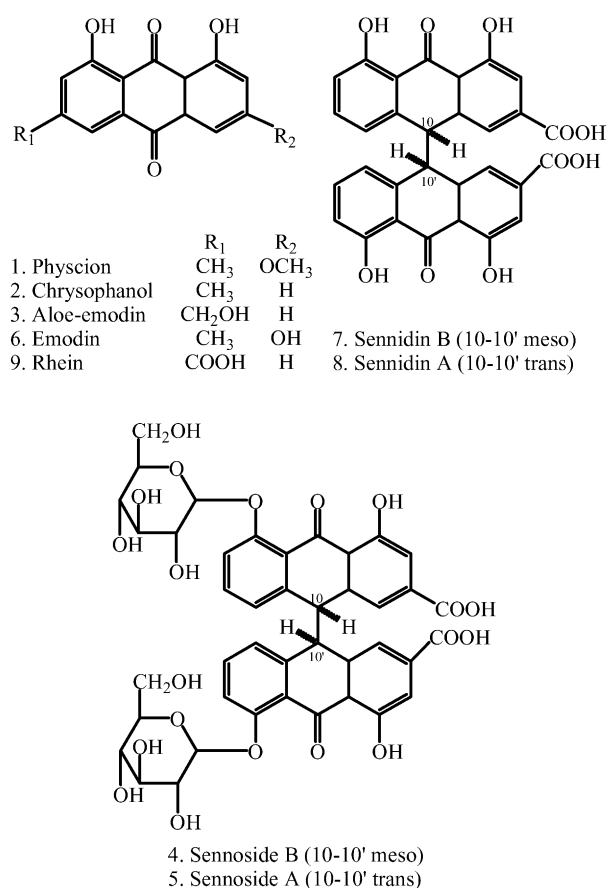

	R$_1$	R$_2$
1. Physcion	CH$_3$	OCH$_3$
2. Chrysophanol	CH$_3$	H
3. Aloe-emodin	CH$_2$OH	H
6. Emodin	CH$_3$	OH
9. Rhein	COOH	H

7. Sennidin B (10-10' meso)
8. Sennidin A (10-10' trans)

4. Sennoside B (10-10' meso)
5. Sennoside A (10-10' trans)

Fig. 1. Structures of the anthraquinones and bianthrones analyzed.

3. Results and discussion

3.1. Method development

The structures of the nine rhubarb anthraquinones and bianthrones analyzed in this work are shown in Fig. 1. Among these analytes aloe-emodin, chrysophanol, emodin, physcion and rhein are free anthraquinones ('free' means 'not binding with sugars'); sennidin A and sennidin B are free bianthrones; while sennoside A and sennoside B are bianthrone glycosides (each binding with two glucoses). Sennidin A and sennidin B are geometric isomers. They are approximately as twice in molecular weight as the free anthraquinones. Sennoside A and sennoside B are sennidin A and sennidin B bonded with two glucoses, respectively. They are also geometric isomers and much more polar than sennidin A and sennidin B. The above nine compounds therefore cover a wide range of lipophilicity and molecular size. Because the solvating property of the microemulsions enables resolution of a wide range of solutes of differing hydrophobicities as stated before [10], MEEKC was therefore applied for the separation of the complex mixture of the analytes.

The composition of microemulsion most commonly used in MEEKC was *n*-octane 0.81% (w/w), SDS 3.31% (w/w) and 1-butanol 6.61% (w/w), as proposed by Watarai [15].
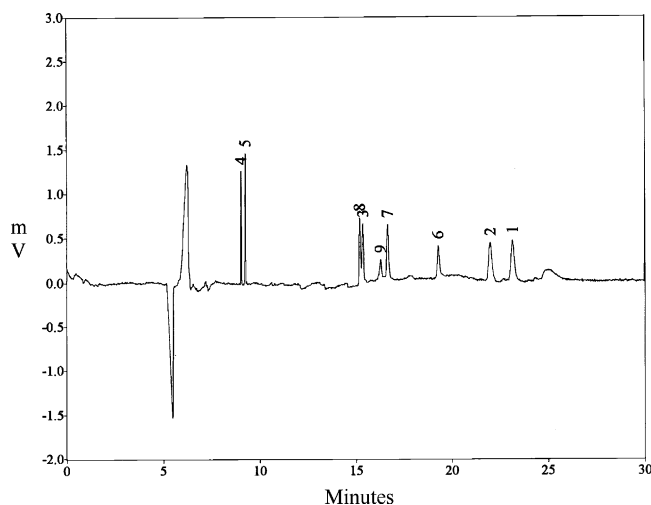
Fig. 2. Electropherogram of the anthraquinone and bianthrone analytes. Fused-silica capillary 74 cm × 50 μm i.d., 60 cm detection length; 0.81% (w/w) n-octane, 3.31% (w/w) SDS, 6.61% (w/w) 1-butanol and 89.27% (w/w) 10 mM $Na_2B_4O_7$ buffer, pH 9.2; 25 kV; 30 °C; 270 nm; injection, 0.725 p.s.i. (50 mbar), 4.8 s; sample dissolved in 70% ACN.

Using this kind of microemulsion the electropherogram obtained for the nine analytes is shown in Fig. 2. In this figure some of the peaks are not well separated and the baseline is not sufficiently flat. Besides, the peak heights of the analytes are too low with respect to their concentrations injected. Among the nine tested analytes, physcion and chrysophanol (compounds 1 and 2) are the most lipophilic. They are readily solubilized in the oil drops and thus retained much more than the other compounds. In contrast, sennosides A and B (compounds 4 and 5) are most hydrophilic and thus migrate far in front of the other analytes.

Long-chained aliphatic alkanes such as n-octane and n-heptane have a much stronger surface tension when mixed with the aqueous buffers. A large amount of surfactant is required to diminish the surface tension so that the microemulsion drops could be formed. These microemulsion systems possess a high stability and a good reproducibility. However, the amount of organic solvent that could be added to the buffer is relatively limited for them. In our experiments, the maximum amount of addition of MeOH and ACN to the microemulsion system n-octane 0.81% (w/w), SDS 3.31% (w/w) and 1-butanol 6.61% (w/w) was about 12 and 6% (v/v), respectively. MeOH and ACN were added in various amounts within these limits but little improvement in separation of the analytes was observed.

Aiken and Huie used di-n-butyl tartrate as oil in a microemulsion system to separate enantiomers of ephedrine [12]. Hu and co-workers employed ethyl acetate as oil in another microemulsion system to separate anthraquinone compounds [16]. Unlike the long-chained aliphatic alkanes, di-n-butyl tartrate and ethyl acetate might be characterized as the oils having much less surface tension, which allows the organic modifiers to be added in a much larger amount to a microemulsion system. In this experiment the liquid
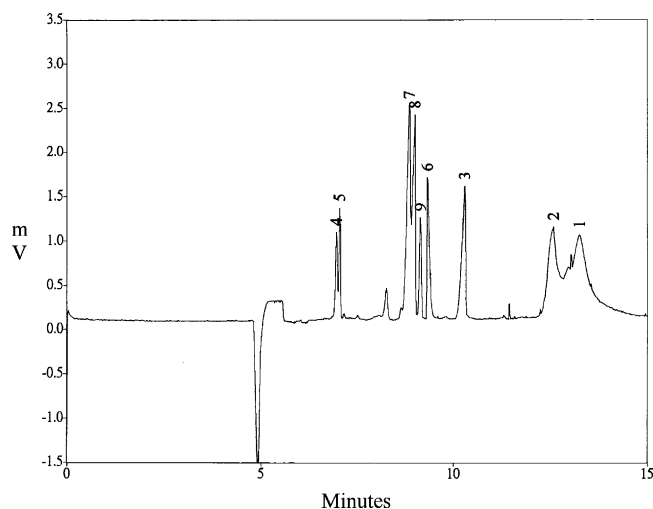

Fig. 3. Electropherogram of the anthraquinone and bianthrone analytes. 0.5% (w/w) di-n-butyl tartrate, 0.6% (w/w) SDS, 1.2% (w/w) 1-butanol and 97.7% (w/w) 10 mM $Na_2B_4O_7$ buffer, pH 9.2. Other conditions are as described in Fig. 2.

di-n-butyl tartrate was chosen as the oil to make up the microemulsion. In the beginning the microemulsion system composed of 0.5% (w/w) di-n-butyl tartrate, 0.6% (w/w) SDS, 1.2% (w/w) 1-butanol and 97.7% (w/w) 10 mM sodium tetraborate was tested, according to that used by Aiken and Huie [12]. This microemulsion system was stable for weeks even months. The electropherogram obtained is shown in Fig. 3. In this Figure, the nine analytes were separated notwithstanding some overlapping of peaks. However, the peak shapes of compounds 1 and 2 were unacceptable. Under such circumstances various volumes (0–40%) of MeOH, isopropanol, tetrahydrofuran and ACN were added to the
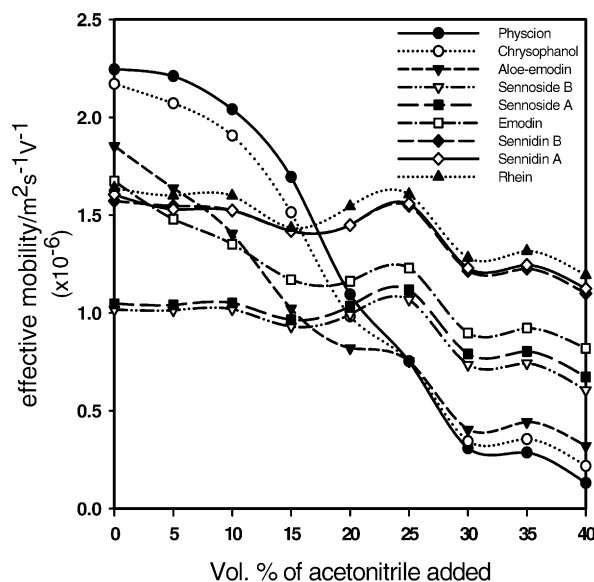

Fig. 4. Mobility changes of the anthraquinones and bianthrones with acetonitrile %. 0.5% (w/w) dibutyl-L-tartrate, 0.6% (w/w) SDS, 1.2% (w/w) 1-butanol and 97.7% (w/w) 10 mM $Na_2B_4O_7$ buffer, pH 9.2, 0–40% (v/v) ACN. Other conditions are as described in Fig. 2.
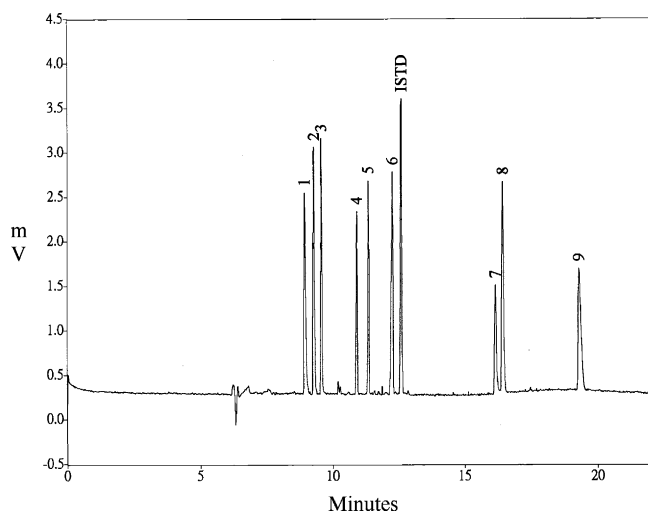
Fig. 5. Electropherogram of the anthraquinone and bianthrone analytes obtained with optimum MEEKC separation conditions. 0.5% (w/w) di-*n*-butyl tartrate, 0.6% (w/w) SDS, 1.2% (w/w) 1-butanol and 97.7% (w/w) 10 mM $Na_2B_4O_7$ buffer, pH 9.2, 30% (v/v) ACN. Other conditions are as described in Fig. 2.

buffer. Results showed that ACN gave the largest change in selectivity. Migration orders of physcion, chrysophanol and aloe-emodin (compounds 1, 2 and 3, respectively) change greatly following the addition of ACN (Fig. 4). Among the nine tested analytes, physcion, chrysophanol and aloe-emodin are the three compounds with larger lipophilicities. Without ACN in the buffer, these three compounds showed the largest affinity for the oil drops. When ACN was added to the buffer these three compounds started entering into the aqueous phase. As the ACN percentage is greater than 23%, they migrate as fast as to overtake the other six compounds. This phenomenon has also been observed in the previous experiments carried out with MEKC [11]. Emodin (compound 6) behaved in a somewhat similar way; in the absence of ACN it migrated behind sennidin B, sennidin A and rhein (compounds 7–9); however, following the addition of a little ACN (below 5%) it moved in front of them.

According to the result shown in Fig. 4, the optimum conditions for separation of the nine compounds was determined. With these conditions the electropherogram obtained is shown in Fig. 5. In this figure, the nine compounds are well separated from one another. The first three peaks are due to physcion, chrysophanol and aloe-emodin, respectively. In a

Table 2
Recoveries of the anthraquinones and bianthrones ($n = 3$)

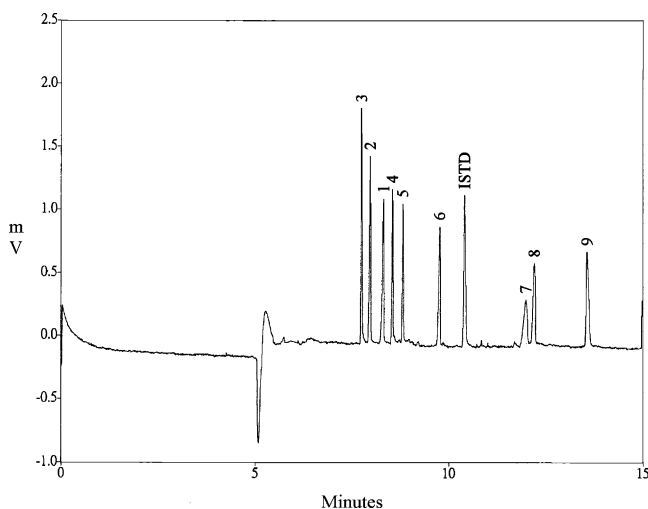| Compounds	Conc. added (μg/ml)	Conc. found (μg/ml)	Recoveries (%)
Physcion	5	4.99	99.81 ± 3.98
Chrysophanol	5	5.03	100.61 ± 6.72
Aloe-emodin	5	5.23	104.68 ± 4.32
Sennoside B	5	5.22	104.37 ± 5.43
Sennoside A	10	10.14	101.41 ± 7.61
Emodin	5	4.79	95.83 ± 5.55
Rhein	5	4.89	97.83 ± 10.75

Fig. 6. Electropherogram of the anthraquinone and bianthrone analytes obtained with optimum MEKC separation conditions. Fused-silica capillary 72 cm × 50 μm i.d., 60 cm detection length; 15 mM $Na_2B_4O_7$/15 mM NaH_2PO_4, 30 mM sodium deoxycholic acid, pH 8.6 and ACN 17% (v/v); 28 kV; 30 °C; 270 nm; injection, 50 mbar, 4.8 s; sample dissolved in 70% ACN.

sharp contrast with the electropherogram in Fig. 2, these three peaks are much narrower and highly symmetrical.

3.2. Method validation

Run-to-run repeatability ($n = 8$) and day-to-day reproducibility ($n = 3$) of the method in terms of migration times were within 1.28 and 2.14% relative standard deviation (R.S.D.), respectively. Run-to-run repeatability and day-to-day reproducibility of peak-area ratios (with respect to internal standard, anthraquinone-2-carcoxylic acid) were within

Table 1
Linear relationships between peak-area ratios and concentrations (μg/ml), and limit of detection (LOD) for the anthraquinones and bianthrones

Compounds	Linear range (μg/ml)	Intercept	Slope	r[a]	LOD (μg/ml)
Physcion	5–20	1.23×10^{-2}	1.94×10^{-2}	0.9996	0.42
Chrysophanol	5–40	0.83×10^{-3}	1.24×10^{-2}	0.9948	0.48
Aloe-emodin	5–40	0.59×10^{-3}	1.49×10^{-2}	0.9997	0.50
Sennoside B	5–60	4.73×10^{-3}	0.78×10^{-2}	0.9998	0.96
Sennoside A	10–80	5.94×10^{-3}	0.83×10^{-2}	0.9984	0.73
Emodin	5–40	-5.98×10^{-3}	2.12×10^{-2}	0.9998	0.52
Rhein	10–40	-7.39×10^{-3}	2.67×10^{-2}	0.9968	0.72

[a] Correlation coefficient.

2.21 and 4.01% (R.S.D.), respectively. These were tested at the lowest concentration level of each calibration curve, i.e. physcion, chrysophanol, aloe-emodin, emodin and sennoside B at 5 μg/ml, sennoside A and rhein at 10 μg/ml. Linearity was assessed by preparing five different concentrations of standard solutions and measuring the relative responses (with respect to internal standard) at each concentration level. The ranges of linearity and the regression lines along with the limits of detection (LOD, S/N = 3) are listed in Table 1. The accuracy of the method was determined by adding a suitable amount of standard to sample solutions A and B and the recoveries were calculated. The recoveries are listed in Table 2 and they are found within 96–105%.

3.3. Comparison with MEKC

The electropherogram shown in Fig. 6 was obtained in a previous experiment carried out with an MEKC system for the separation of the same nine analytes [11]. The micellar solution was made with a 30 mM sodium deoxycholate (SDC) in a 30 mM borate/phosphate buffer (pH 8.6). Acetonitrile was added to the solution in 17%. Comparison of Figs. 5 and 6 shows that the plate number and peak shape of compound 7 in MEKC separation is well below that in MEEKC separation. Results of reproducibility and accuracy were similar between the two separations.

3.4. Determination of anthraquinones and bianthrones in the rhubarb sample

Optimized separation conditions were used to analyze the crude extract from the rhizome part of the plant *Rheum officinale* Baill. (Section 2.7.1). All the nine analytes had been detected except the two non-glycosidic bianthrones, sennidin A and sennidin B. However, co-migration of other constituents of the extract with the analytes, especially for compounds 1, 2 and 9 (physcion, chrysophanol and rhein) occurred (Fig. 7a). On account of the accuracy in quantification, these
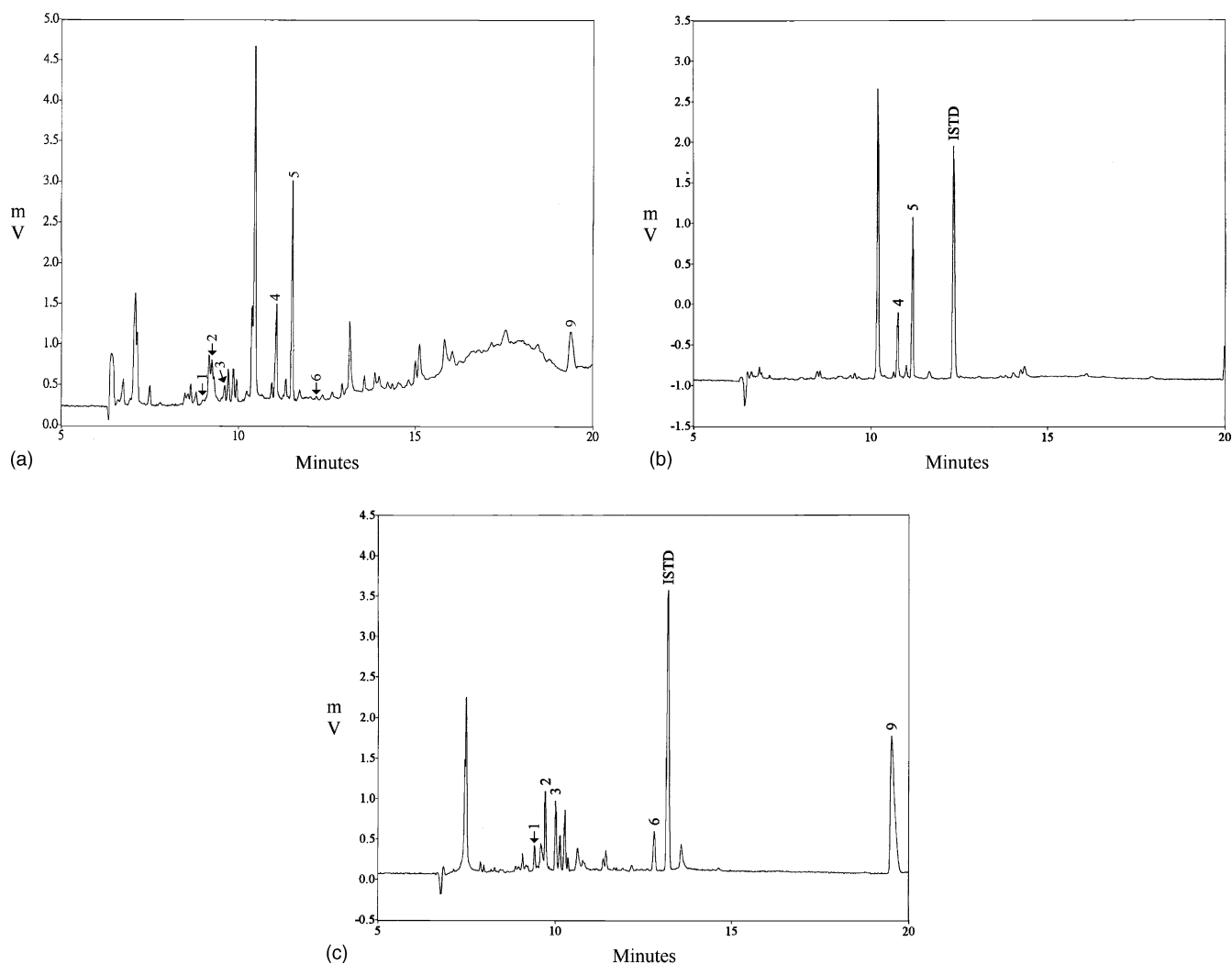

Fig. 7. Electropherograms obtained from (a) crude extract, (b) sample solution A and (c) sample solution B of a rhubarb crude drug (*R. officinale* Baill.). Separation conditions are as described in Fig. 5.

Table 3
Contents of anthraquinones and bianthraones in a rhubarb sample (*R. officinale* Baill.) (*n* = 3)

Compounds	Content (mg/g)
Physcion	0.14 ± 0.01
Chrysophanol	0.77 ± 0.04
Aloe-emodin	0.65 ± 0.01
Sennoside B	5.87 ± 0.14
Sennoside A	13.68 ± 0.09
Emodin	0.35 ± 0.01
Rhein	2.36 ± 0.02

interfering species must be removed or separated from the analytes.

SPE procedure was applied for this purpose (Section 2.7.2). Because of the differences in lipophilicity between the glycosidic bianthrones and free (non-glycosidic) anthraquinones, two fractions were collected from the SPE treatment of the crude extract. Fraction A was obtained by eluting the cartridge with alkaline bicarbonate to dissolve sennosides A and B that carried two carboxyl groups. Before the last eluting step with 95% ACN to obtain fraction B, some interfering substances must be removed. This was accomplished by two washing steps, the first using an aqueous acid (20 mM acetic acid) to bring the material back to neutral, the second using a 65% MeOH to remove the interfering substances. Fraction A and B, as described in Section 2.7.2, were taken to determine the content of the respective anthraquinone and bianthrone analytes in a powdered sample of rhubarb (*Rheum officinale* Baill.). The electropherograms acquired from the two fractions are shown in Fig. 7b and c. It is observed that fraction A contains only the glycosidic bianthrones–sennosides A and B; while the five non-glycosidic free anthraquinones reside in fraction B. Recoveries of the compounds for the SPE procedure were tested and they were all greater than 97.5% except rhein (82.3%). The content of these analytes in the sample is listed in Table 3. The two sennosides, sennoside A and sennoside B, and rhein are the main components of the tested compounds, especially sennoside A.

4. Conclusion

An efficient and reliable MEEKC method for separating the main cathartic principles of rhubarb has been developed in this work. To reduce the surface tension between the oil drops and water, a kind of oil having much less lipophilicity than *n*-octane, i.e. di-*n*-butyl tartrate, was used. With this oil-substance organic solvent could be added in a larger amount to the buffer for the optimization of separation. Acetonitrile of 30% was found to give an optimum separation. Using the optimized conditions nine anthraquinones and bianthrones were completely separated with good peak shapes. Their amounts in a rhubarb sample were determined after an SPE treatment. Like the MEKC method previously developed in this laboratory [12], this work also provides a method suitable for the analysis of rhubarb crude drugs in their quality control as well as in their evaluations.

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