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Sensitivity improvement on detection of Coptidis alkaloids by sweeping in capillary electrophoresis

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Abstract

A simple and rapid sweeping method for the online improvement of detection sensitivity of the main alkaloids of Coptidis Rhizoma has been developed in this work. Optimum separation conditions were found as follows: electrophoretic running solution comprising 100 mM phosphoric acid, 15 mM sodium dodecyl sulfate (SDS) and 10% (v/v) tetrahydrofurane with pH 1.82; running voltage of −25 kV; sample matrix composed of 50 mM phosphoric acid and sample injection at 1000 mbar for 60 s (sample injection volume ca. 2.75 μl). With this sweeping method, the concentration limits of detection of berberine, coptisine and palmatine were found to be 2.5 ppb (ng/ml), which was about 500 times lower than those from conventional sample injections. Baseline separation was achieved for the main alkaloids within 15 min. After validation, the developed method was applied to determine the quantity of berberine, coptisine and palmatine in a Coptidis Rhizoma sample. The method should be able to be used in identification and quantitative evaluation of the crude drugs requiring only a minor amount of sample.

Keywords: Sweeping; Capillary electrophoresis; Coptidis Rhizoma alkaloids

1. Introduction

Coptidis Rhizoma, the dry rhizome of *Coptis chinensis* Franch (or related plants with the same genus), is one of the most well-known and widely used herbs in traditional Chinese medicine. It is indicated as a bitter-tasting gastric and intestinal regulative [1]. A number of quaternary protoberberine alkaloids have been isolated from the rhizomes of *Coptis* species, among which three alkaloids—berberine, coptisine and palmatine—were found in a greater amount and berberine is the most abundant [2].

Capillary electrophoresis (CE) has many of its advantages in natural product analysis owing to its high separation power, rapid analysis time, and low expenditure of reagents. However, the low concentration sensitivity of CE associated with UV detection has long been a problem due to its small injection volume and short optical path-length for detection. In general, the UV detectability of CE is about two orders worse than that encountered in high-performance liquid chromatography (HPLC) [3]. To improve the concentration sensitivity of CE in many fields involving trace analysis, the online approach to the concentration of samples was proposed. This approach is done by manipulating the composition of the samples and background solutions together with simple injection procedures without change of present commercial instrumentation. Examples of online approach to sample concentration are field-amplified sample stacking (FASS) and sweeping. FASS is connected to the change of electrophoretic velocity of sample molecules due to uneven distribution of electric field across the concentration boundary between the sample zone and the background solution zone [4]. Sweeping is the picking and accumulation of analyte molecules by the pseudostationary phase that penetrates

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the sample zone during application of voltage [5,6]. This technique generally uses very low pH buffers so that an environment of nearly zero electroosmotic flow (EOF) is generated. Unlike the FASS, in sweeping the analytes are prepared in a matrix with the same conductivity as the background solution (BGS). Besides, the analytes can be either neutral or charged molecules. The sweeping technique is used in micellar eletrokinetic chromatography (MEKC) and the injected length of an analyte can be narrowed by a factor of $(1 + k)$, where k is the retention factor. A concentration of over 5000-fold had been achieved with this technique [5]. To date, although not a few applications of sweeping for the online focusing of drugs or metabolites present in biological samples have been reported [7–14], quite rare studies concerning the improved detection of natural products by sweeping could be found in the literature. Durham et al. used the sweeping technique to examine phyllanthusols A and B, the two active constituents, in the extracts of roots of *Phyllanthus acidus* [15]. The technique involved application of a negative potential to the inlet end of the capillary and very much longer than conventional injection times. According to the authors, this sweeping technique in the application appeared superior to capillary zone electrophoresis (CZE) for the determination of the active constituents. It allowed increased sensitivity of detection of the desired analytes and high sample loading which enabled detection of additional components of the extract matrix. Sheu and co-workers separated successfully five saikosaponins in the crude extract of Bupleuri Radix by the sweeping method [16]. They found that the sweeping method gave better result than conventional MEKC method with a higher separation efficiency, lower detection limit and ability to detect five saikosaponins. Chiang and Sheu developed a sweeping method for the separation of very dilute solutions of ephedra alkaloids [17]. Under an optimized injection time, they found that the more diluted a solution is, the greater the sweeping effect will be.

Liu and Sheu developed a separation of eight quaternary alkaloids in Coptidis Rhizoma by capillary electrophoresis [18]. However, the limits of detection of the individual alkaloids had not been mentioned. It is necessary to consider the online concentration techniques to elevate the sensitivity of detection. In a previous work, the application of FASS techniques to the detection of the alkaloids of Coptidis Rhizoma was presented [19]. With that technique, a stacking efficiency of about 200-fold, with respect to the conventional sample injection, was obtained. The limits of detection were lowered with approximately the same magnitude. Although the sweeping technique proposed by Quirino and Terabe was initially used for concentration of neutral molecules [5], it could be applied equally well to ionic analytes as long as they have great affinities toward the pesudostationary phase [6]. Because several 100-fold increases (400–500-fold) in peak heights of some chargeable hydrophobic analytes were reported in the literature [6], it was envisioned here that a suitable sweeping method with higher detection sensitivity than the previous FASS method could be developed for determining the main alkaloids in dilute solutions of extract of Coptidis Rhizoma.

2. Experimental

2.1. Chemicals and material

Berberine chloride and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). Coptisine chloride and palmatine chloride were purchased from Wako (Osaka, Japan). Ethidium bromide was purchased from Fluka (Buchs, Switzerland). Sodium tetraborate, phosphoric acid and tetrahydrofuran (THF) of chromatographic grade were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile of chromatographic grade were purchased from J.T. Baker (Phillipsburg, NJ, USA). De-ionized water was obtained from Barnsted/thermolyne water purification system (Dubuque, IO, USA). Coptidis Rhizoma was purchased from the Chinese herbal market in Taipei (Taiwan).

2.2. Instrument

CE experiments were carried out on a 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. For conductivity measurements, a Radiometer CDM 83 conductivity meter (Copenhagen, Denmark) calibrated with a 0.05% (w/w) NaCl solution to a value of 1015 μS/cm (at 25 °C) was used.

2.3. Electrophoretic conditions

CE experiments were performed on a 72 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 methanol for 3 min, 0.2 M sodium hydroxide for 3 min followed by running buffer for 3 min. Detection wavelength was set at 264 nm. All the experiments were run at room temperature (23 ± 1 °C). The separation voltage was −25 kV.

Background solution (BGS) was composed of 100 mM phosphoric acid, 15 mM SDS and 10% (v/v) THF, with pH 1.82. The solution was filtered through a 0.45 μm membrane (Millipore, Bedford, MA, USA) before use.

2.4. Stock solution of herbal extract

A 0.2 g sample of Coptidis Rhizoma was pulverized and then ultrasonically extracted with 50% ethanol for 20 min. This was followed by centrifugation at 1500 rpm for 10 min. The extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter paper to a 25 ml volumetric flask and diluted to the volume with 50% ethanol. A 0.1 ml of the solution was vacuum dried and 8.5 ml of 50 mM phosphoric acid was added to dissolve the residue. This was stocked for further use.

2.5. Working solution for manipulation of sweeping and separation

Half milliliter of the above stock solution of herbal extract was taken, and then 4.5 ml of 50 mM phosphoric acid was added. This solution was used as the working solution for manipulation of sweeping and separation.

2.6. Standard preparation

Stock solutions of 1 mg/ml (1000 ppm) for berberine, coptisine and palmatine were prepared with de-ionized water. These solutions were diluted to 100 ppm with water. A mixture of the solutions was vacuum dried and then diluted to a concentration of 1 ppm with 50 mM phosphoric acid. The solution was further diluted for use in validation.

2.7. Sample solutions for assay

The above stock solution of herbal extract was used for the assay. For the determination of berberine, 43.8 μl of the above solution was mixed with 20 μl of the internal standard ethidium bromide of concentration 40 μg/ml (diluted from the aqueous stock solution of 1000 μg/ml with 50 mM phosphoric acid) in a 10 ml volumetric flask. Phosphoric acid solution of 50 mM was added to complete the volume. For the determination of coptisine and palmitine, 102.3 μl of the solution was mixed with 20 μl of the internal standard ethidium bromide of concentration 40 μg/ml and worked as in the determination of berberine. The solutions were passed through 0.45 μm membrane filters before injection.

3. Results and discussion

Fig. 1 depicts the sweeping process of positively charged analytes (a) in the presence of very low (nearly zero) electroosmotic flow. In the starting situation (Fig. 1a), sample solution (S) having the same conductivity as the background solution (BGS) is injected. The a_c and a_a are the positively-charged analyte molecules found near the interface between S and BGS zones at the cathodic and anodic ends, respectively. The mc_c and mc_a are the micelles (which bear negative charges) at the cathodic and anodic ends, correspondingly.

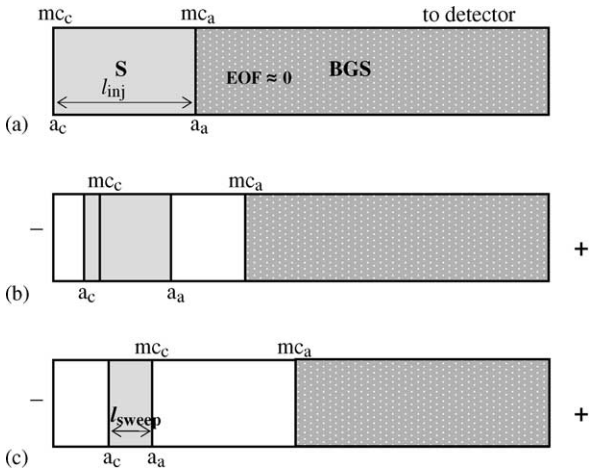

Fig. 1. Evolution of micelles and positively charged analyte molecules during sweeping in the presence of very low electroosmotic flow. (a) Starting situation, injection of S prepared in a matrix having a conductivity similar to that of the BGS; (b) application of voltage at negative polarity, micelles emanating from the cathodic side sweeping analyte molecules; (c) the injected analyte zone is assumed completely swept.

The length of the S zone injected is given as l_{inj}. Upon application of voltage (Fig. 1b), because the analytes are positive charged molecules, the moving directions of analytes and micelles are opposite and the sweeping concentration occur at this time. The migration velocity of a_c follows that in MEKC for charged analytes. However, the migration velocity of a_a is equal to that in CZE because it is not incorporated into any micelle until the time when micelles from the cathodic end reach it. The length of the analyte zone after sweeping is given by l_{sweep}, which is much smaller than l_{inj} (Fig. 1c). The length of l_{sweep} is narrowed down by a factor of $(1 + k)$ of the length of l_{inj}. The greater the k is, the narrower the l_{sweep} will be. In the whole process of sweeping (Fig. 1, a → c), the part of the capillary containing analytes is represented by the gray color, while the part of the capillary containing the BGS at the anodic side is represented by the dotted gray color.

Because berberine, coptisine and palmatine constitute the principal alkaloids of Coptidis Rhizoma, these three compounds were taken as the model compounds to undertake the sweeping process, although there are other minor quaternary alkaloids present in the plant. The structures of these three alkaloids are shown in Fig. 2. Each of them has a positive charge that is ionized independent of the pH of solution.

3.1. Effect of sample matrix on peak height

The sweeping initially proposed by Quirino and Terabe was to be carried out under the condition of relatively constant electric field throughout the capillary [6]. To meet this requirement, the conductivity of the sample solution was adjusted to match that of the BGS. Palmer et al. reported that sample matrix conductivity, two to three times greater than the separation solution conductivity, and devoid of the micelle

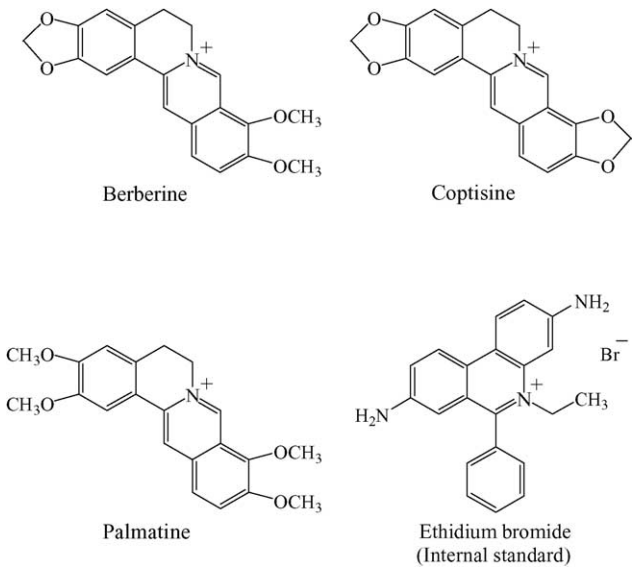

Fig. 2. Structures of the Coptidis alkaloids analyzed and ethidium bromide (internal standard).

improved the focusing effect in MEKC [20]. Quirino et al. later described that the sample matrix could have a lower, similar, or higher conductance than the separation solution [21]. To examine the influence of sample matrix on peak height of the analytes, the sample was dissolved in an aqueous solution containing 50, 100, 150, 200 and 250 mM phosphoric acid, corresponding to about 1 (5.80 mS/cm), 1.5 (9.08 mS/cm), 2 (11.81 mS/cm), 2.5 (14.30 mS/cm) and 3 (16.90 mS/cm) times of conductivities to that of BGS (6.10 mS/cm). The BGS was composed of 100 mM phosphoric acid, 15 mM SDS and 10% (v/v) THF, with pH of the solution being 1.82. Samples were injected at 1000 mbar for 60 s. The electropherograms obtained are shown in Fig. 3. It could be seen that the change of phosphoric acid concentration does not affect the peak height of the analytes. The action of phosphoric acid in

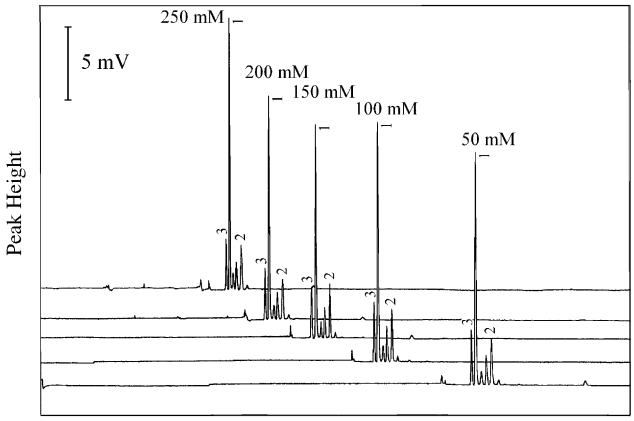

Fig. 3. Effect of sample matrix concentration of phosphoric acid on peak height of coptis alkaloids. Conditions: buffer, 100 mM H_3PO_4/15 mM SDS/10% THF (v/v), pH 1.82; sample matrix, 50–250 mM H_3PO_4; −25 kV; 25 °C; 264 nm; injection, 1000 mbar, 60 s. Peak 1: berberine, peak 2: coptisine, peak 3: palmatine.

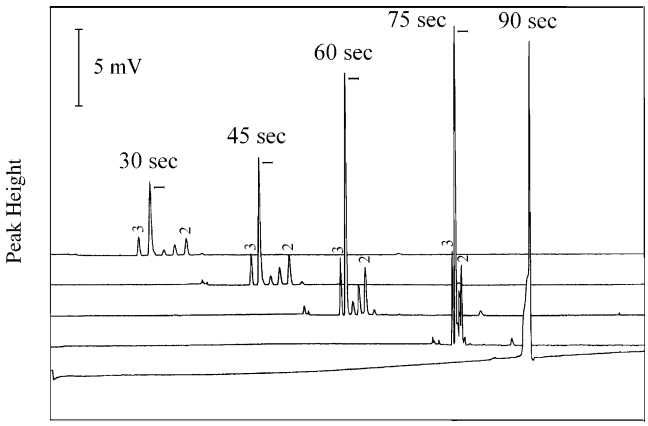

Fig. 4. Effect of sample injection time on peak height and resolution of coptis alkaloids. Conditions: buffer, 100 mM H_3PO_4/15 mM SDS/10% THF (v/v), pH 1.82; sample matrix, 50 mM H_3PO_4; −25 kV; 25 °C; 264 nm; injection, 1000 mbar, 30–90 s. Peak 1: berberine, peak 2: coptisine, peak 3: palmatine.

sweeping is supposed to just provide an acidic environment of low pH so that the EOF could be suppressed. Because the higher phosphoric acid concentration would cause the sharp rise of electric current in the column, the phosphoric acid of 50 mM concentration, which had the same conductivity as the BGS, was chosen in this study to make up the sample matrix.

3.2. Effect of sample injection length on peak height

For sweeping technique, the injected length of an analyte zone is theoretically narrowed by a factor equal to $1/(1+k)$ where k is the retention factor. When k goes to infinity, only the column length restricts injection lengths. In other words, high-k analytes could be concentrated without bounds and separation efficiencies preserved [6]. If the k-value of the analyte is not sufficiently high, it is proposed that the injection sample plug length must be optimized [21]. In this study, under the fixed BGS composition (100 mM H_3PO_4, 15 mM SDS and 10% (v/v) THF, pH 1.82) and sample matrix concentration (50 mM H_3PO_4), the sample solution was injected at 1000 mbar for 30, 45, 60, 75 and 90 s into the column, corresponding to the injected length of 17.5, 26.2, 35.0, 43.7 and 52.4 cm, respectively. The result is shown in Fig. 4. It could be seen that the peak heights increase with the increase of the injected plug length. However, resolutions between the peaks gradually deteriorate following the increase of the injected plug length. At the injection time of 90 s, almost all the peaks are coalesced and no resolution occurs. As a compromise between separation and peak height, the injection time of 60 s was chosen as the optimized condition.

3.3. Effect of organic modifiers on separation

Because of the close similarity between the structures of the analytes (see Fig. 2) the BGS without adding of any or-

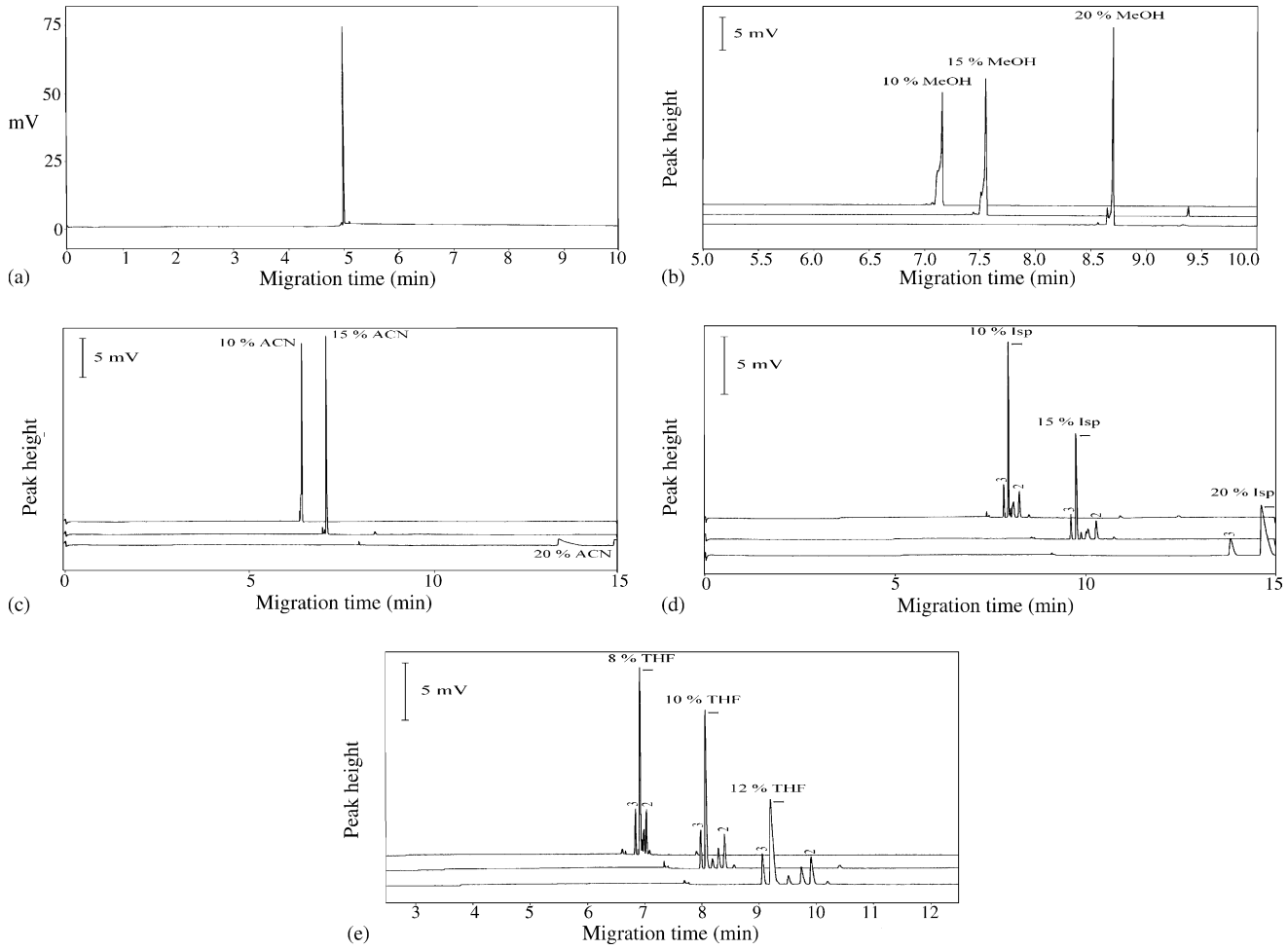

Fig. 5. Effect of organic modifiers on separation of coptis alkaloids: (a) no organic modifier added, (b) methanol added, (c) acetonitrile added, (d) isopropanol added, (e) tetrahydrofuran added. Conditions: buffer, 100 mM H_3PO_4/15 mM SDS/with or without organic modifier, pH 1.82; sample matrix, 50 mM H_3PO_4; −25 kV; 25 °C; 264 nm; injection, 1000 mbar, 60 s. For Fig. 5d and e, peak 1: berberine, peak 2: coptisine, peak 3: palmatine.

ganic solvent (100 mM H_3PO_4, 15 mM SDS, pH 1.82) gave only a single peak (Fig. 5a). Addition of organic solvent could diminish the affinity of the analytes to micellar phase. Various organic solvents with different concentrations were tested for their separation capabilities. Methanol and acetonitrile gave no separation between the analytes at all (Fig. 5b and c). Isopropanol could separate most part of the alkaloids, but some of the peaks were still overlapped or somewhat lowered (Fig. 5d). THF gave the best separations among the tested solvents (Fig. 5e). At 10% (v/v) THF, all the three tested alkaloids were completely separated from the other components in the sample and their peaks still remained sufficiently high.

After testing the various factors which influenced the peak height and separation of the three tested alkaloids, the following conditions had been decided to establish the analytical method: BGS composed of 100 mM phosphoric acid, 15 mM SDS and 10% (v/v) THF, pH 1.82; sample matrix composed of 50 mM phosphoric acid; and sample injection at 1000 mbar for 60 s.

3.4. Sensitivity enhancement of the sweeping method

The limits of detection (LOD) for the tested alkaloids were determined at signal to noise ratio equal to 3 with the diluted standard solutions. It was found to be 2.5 ng/ml (ppb) for all the three analytes. The LODs are 1.2, 1.2 and 1.5 μg/ml (ppm) for berberine, coptisine and palmatine, respectively, in a previous report [19], if the compounds are subjected to conventional injection. With the conventional injection the BGS was prepared with a Tris buffer/methanol mixture and the sample was dissolved in the same solution. The detectabilities were lowered about 500 times when the sweeping method in this work is compared with the conventional injection method. The electropherograms are shown in Fig. 6. In the previous experiment using FASS with electrokinetic injection, the LODs for berberine, coptisine and palmatine were 5, 5 and 7.5 ng/ml, respectively [19]. This indicates the sweeping method is even more sensitive than the stacking method.

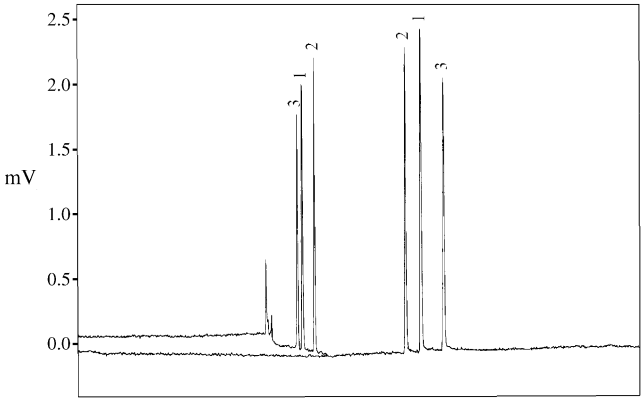

Fig. 6. Comparison of the electropherograms obtained from the sweeping method (left) and from conventional injection (right). The sweeping conditions are as described in Fig. 7.The conditions for conventional injection are as described in the text (see Section 3.4). Concentration of sample injected (standards) for the sweeping conditions: 0.1 μg/ml, concentration of sample injected (standards) for the conventional injection: 50 μg/ml. Peak 1: berberine, peak 2: coptisine, peak 3: palmatine.

3.5. Validation of the analytical method

Run-to-run repeatability ($n = 10$) and day-to-day reproducibility ($n = 3$) of the method in terms of migration times were within 2.21 and 4.18% relative standard deviation (R.S.D.), respectively. Precisions of peak height ratios (with respect to internal standard) were tested at 20 ng/ml. Run-to-run repeatability ($n = 10$) and day-to-day reproducibility ($n = 3$) of peak height ratios were both within 13.6% R.S.D., respectively. Linearity was evaluated by preparing five different concentrations of standard solutions and measuring the relative peak height with respect to internal standard at each concentration level. The regression lines are shown in Table 1. The accuracy of the method was determined by adding a suitable amount of standard to sample solution and expressed as recoveries. The recoveries were 92, 93 and 91% for berberine, coptisine and palmatine, respectively.

3.6. Determination of the alkaloids in the Coptidis Rhizoma sample

The electropherogram obtained with the optimized conditions from the Coptidis Rhizoma sample in the presence of the added internal standard is shown in Fig. 7. In this figure, the three peaks belonging to berberine, coptisine and palmatine are well separated from the other components in the sample. The contents of berberine, coptisine and palmatine in the analyzed sample are 5.77, 1.83 and 1.51%, respectively.

Table 1
Linear relationships between peak-height ratios (y) and concentrations (ng/ml) (x) for the coptis alkaloids

Compound	Linear range (ng/ml)	Intercept	Slope	r^a
Berberine	10–50	3.22×10^{-1}	2.69×10^{-2}	0.9958
Coptisine	10–50	8.86×10^{-2}	2.97×10^{-2}	0.9929
Palmatine	10–50	1.01×10^{-1}	2.54×10^{-2}	0.9967

[a] Correlation coefficient.

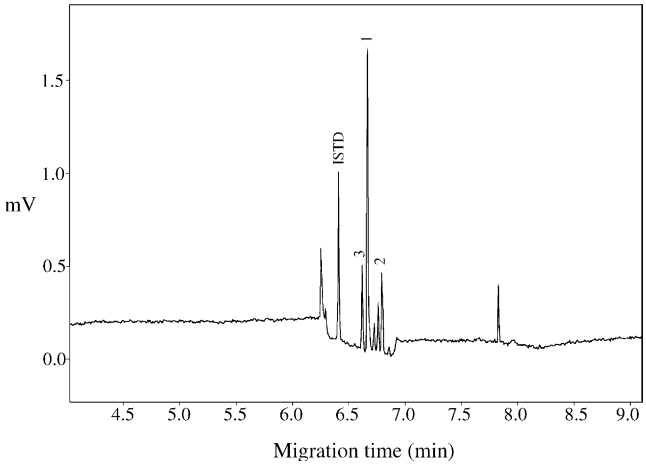

Fig. 7. Electropherogram of the extract of a Coptidis Rhizoma sample. Conditions: buffer, 100 mM H_3PO_4/15 mM SDS/10% THF (v/v), pH 1.82; sample matrix, 50 mM H_3PO_4; −25 kV; 25 °C; 264 nm; injection, 1000 mbar, 60 s. Peak 1: berberine, peak 2: coptisine, peak 3: palmatine, ISTD: internal standard.

4. Conclusions

A sweeping method for the online improvement of detection sensitivity of the main alkaloids of Coptidis Rhizoma has been developed in the work. With the method, the detectabilities (limits of detection) of the alkaloids were lowered to about five hundred times when compared to the conventional sample injection method. For the analysis of herbal drugs or natural products, the methods of high detection sensitivities are required in these days. The developed method is even more sensitive than the method developed previously using the FASS technique. Besides, the method is simple and rapid, which could also be used for the analysis and determination of the main alkaloids of Coptidis herbal drugs.

Acknowledgement

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