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Improved detection of Coptidis alkaloids by field-amplified sample stacking in capillary electrophoresis

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Abstract

To improve the on-line detection of Coptidis alkaloids in capillary electrophoresis the field-amplified sample stacking was studied for them. In this work the peak height enhancements of stacking with hydrodynamic and electrokinetic injections were compared with respect to the conventional sample injection. It was found that the stacking efficiency of electrokinetic injection was more than ten times greater than that of hydrodynamic injection. No peak height enhancement was observed with the pre-injection of a short water plug before sample injection with electrokinetic injection. The concentration limits of detection of berberine, coptisine and palmatine obtained with electrokinetic injection were about 5 ng/ml (ppb), which was approximately 240 times lower than those from conventional sample injections. Baseline separation was also achieved for the main alkaloids. After validation the developed method was applied to determine the quantity of berberine, coptisine and palmatine in a Coptidis Rhizoma sample. The method is simple, rapid and should be able to be used in identification and quantitative evaluation of the crude drugs.

Keywords: Field amplified sample stacking; Capillary zone electrophoresis; Coptidis Rhizoma alkaloids

1. Introduction

Coptidis Rhizoma, the dry rhizome of *Coptis chinensis* Franch (or other plants with the same genus), is one of the most well-known and widely used herbs in traditional Chinese medicine. It is used as a bacteriostatic, antipyretic, and antiphlogistic for the treatment of gastroenteritis, diarrhea, hematemesis, carbuncle, and abscess and as a bitter digestive for the treatment of indigestion [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].

Due to its high resolution, rapid analysis time, and low consumption of sample and reagent, capillary electrophoresis (CE) has gained its status as a powerful tool in natural product analysis. However, because of its small injection volume and short optical path-length the low concentration sensitivity of UV detection was always annoying. The detection limits of some natural products were around ppm (1 ppm = 1 μg/ml) levels, when the samples were treated and injected in conventional ways [3,4]. This concentration sensitivity is in the range of 10^{-5}–10^{-6} M, which is about two-orders higher (worse) than that encountered in high-performance liquid chromatography (HPLC) [5]. To improve the concentration sensitivity of CE in many fields involving trace analysis, the on-line 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. An example of on-line approach is field-amplified sample stacking (FASS), which is connected to the change of electrophoretic velocity due to the electric field across the concentration boundary between the sample zone and the background solution zone. A large number of applications of sample stacking in environmental and biomedical analysis have been reported [6]. In the filed of phytopharmaceutical or natural product analysis, not a few studies concerning

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on-column concentration have also been carried out in recent years [7–13]. Unger and Stöckigt applied CE with field amplified sample injection (FASI) to detect the alkaloids in crude extracts from roots of *Berberis vulgaris* and *Hydrastis Canadensis* [7]. Hu et al. employed FASS to the on-line concentration of the alkaloids from *Sophora flavescens* [8]. With field-enhanced stacking Hu et al. further established an analysis of strychnine and brucine in *Strychnos nux-vomica* [9]. Pospíšilová et al. separated and determined five flavonoids in *Hypericum perforatum* by capillary zone electrophoresis (CZE) with isotachophoretic (ITP) sample pre-treatment using on-line column coupling configuration [10]. Lu et al. developed an analysis of plant hormones in tobacco flowers by micellar electrokinetic chromatography coupled with on-line large volume sample stacking in a reverse electrode polarity-stacking mode [11]. Chen et al. used field-enhanced sample injection with reverse migrating micelles for on-line concentration of the flavonoids in *Epimedium brevicornum* in analysis with micellar electrokinetic chromatography [12]. Sheu et al. separated saikosaponins in crude extract of Bupleuri Radix by the sweeping technique of CE [13].

Liu and Sheu developed a separation of eight quaternary alkaloids in Coptidis Rhizoma by capillary electrophoresis [14]. However, the limits of detection of the individual alkaloids had not been described. In this work the application of FASS techniques to the detection of the alkaloids of Coptidis Rhizoma is presented. We compared the two sample-injection modes, the hydrodynamic injection (i.e., pressure injection) and electro-injection, for their magnitude of peak height enhancement factors. The electro-injection that was found to give higher value of enhancement factor was taken to proceed with the determination of the main alkaloids in a Coptidis Rhizoma sample. During the stacking process no less attention was paid to the separation of the individual alkaloids.

2. Experimental

2.1. Chemicals and material

Berberine and tris(hydroxymethyl)aminomethane were purchased from Sigma (St. Louis, MO, USA). Coptisine and palmatine were purchased from Wako (Osaka, Japan). *N*-Benzylcinchonidinium chloride was purchased from Fluka (Buchs, Switzerland). Hydrochloric acid was purchased from Merck (Darmstadt, Germany). Methanol of chromatographic grade was purchased from J.T. Baker (Phillisburg, NJ, 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.

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 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.

A Tris buffer of 400 mM with pH 8.6 (by titrating with 1N HCl) was prepared. Methanol was added to the buffer in 15% (v/v). The resulting solution was filtered through a 0.45 μm membrane before use.

2.4. Standard preparation

Stock solutions of 1 mg/ml (1000 ppm) for berberine, coptisine and palmatine were prepared with deionized water. These solutions were diluted to 100 ppm with water. A mixture of the solutions was then diluted to a concentration of 1 ppm for injections.

2.5. Preparation of herbal extract and assay solution

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 × *g* for 10 min. The extraction was repeated three times. The extracts were combined and filtered through 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 deionized water was added to dissolve the residue. For the determination of berberine, 60 μl of the above solution was mixed with 0.3 ml of a 10 μg/ml internal standard, *N*-benzylcinchonidinium chloride (diluted from the aqueous stock solution of 1000 μg/ml with water) in a 10 ml volumetric flask. Water was added to complete the volume. For the determination of coptisine and palmitine, 200 μl of the solution was mixed with 0.3 ml of the 10 μg/ml *N*-benzylcinchonidinium chloride and worked as in the determination of berberine. This latter solution was also used as the sample solution for the detection of the alkaloids in Coptidis Rhizoma (see Fig. 5). The solutions were passed through a 0.45 μm

membrane filter (Millipore, Bedford, MA, USA) before injection.

3. Results and discussion

Because berberine, coptisine and palmatine constitute the principal alkaloids of Coptidis Rhizoma, these three compounds were taken as the model compounds to undertake the stacking process, although there are other minor quaternary alkaloids present in the plant. The structures of these three alkaloids are shown in Fig. 1. Each of them has a positive charge that is ionized independent of the pH of solution. This feature enabled them to be analyzed with the CZE mode of CE.

In FASS, the sample solution is of lower conductivity than the running buffer. Theoretically, the amount of stacking is proportional to the conductivity difference between the running buffer and the sample solution. This difference is caused by the concentration drop between the two solutions; the larger the drop in concentrations, the narrower the peak and the greater amount of stacking. We chose Tris as the running buffer because it has lower mobility than phosphate and borate buffers thus it generates much smaller current when used at large concentrations. A Tris buffer having 400 mM concentration at pH 8.6 was found to give maximum peak height enhancement for the three analytes (current of 47 μA). To improve resolutions between the three alkaloids as well as between the other protoberberine constituents, 15% methanol (v/v) was added to the Tris buffer.

Two ways of sample injection used in FASS, either hydrodynamically or electrokinetically, were tested in this work to compare which would give higher peak height enhancement.

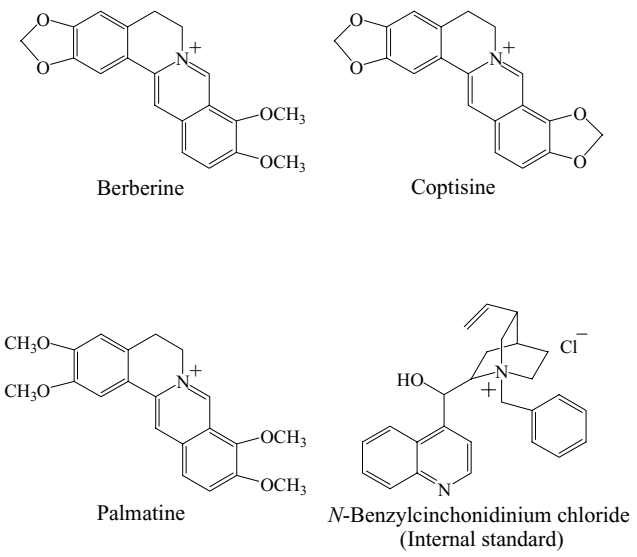

Fig. 1. Structures of the Coptidis alkaloids analyzed and *N*-benzylcinchonidinium chloride (internal standard).

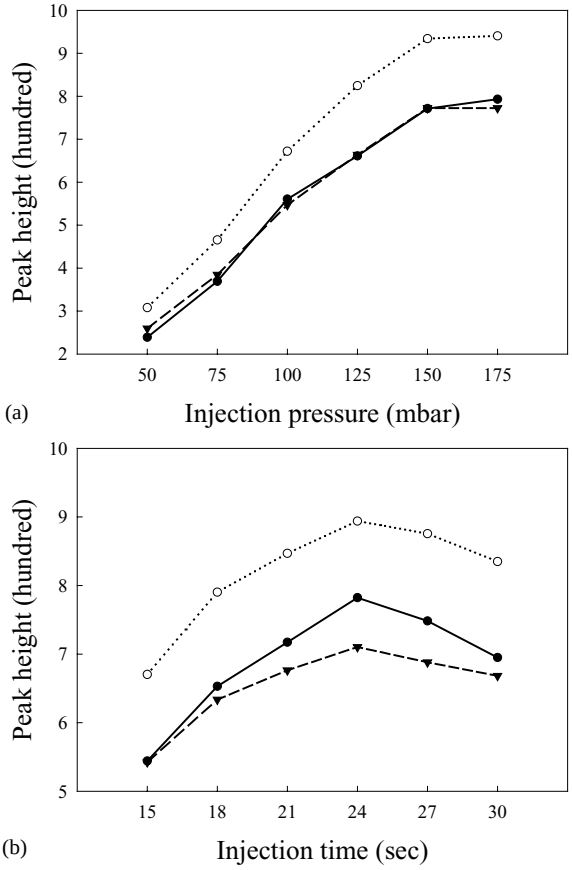

Fig. 2. Influence of injection pressure and time of sample solution on peak heights of the tested alkaloids in FASS hydrodynamic injection, (a) at injection time of 24 s, (b) at injection pressure of 150 mbar. (○) Berberine; (●) coptisine; (▼) palmatine.

3.1. FASS of hydrodynamic injection of samples

In hydrodynamic injections of FASS, a certain volume of sample solution is injected by pressure before the application of high voltages for separation. The larger the volume of sample solution is injected, the greater the stacking amount should be. However, owing to the mismatch of electroosmotic flow between the running buffer and sample solution, the laminar flow generates which broadens the peaks and consequently corrupts the stacking [15,16]. Under such circumstances, there should be a compromise between the volume of sample injected and the peak broadening; in other words, there should exist an optimum for the sample volume injected. Because the injected sample volume is proportional to the injection pressure and injection time, these two parameters were varied to search for their optimum values. It was found that the peak height increased almost linearly from 50 to 150 mbar of injection pressure; however, over 150 mbar, the peak heights did not increase any more (Fig. 2a). The injection time was tested between 15 and 30 s. It was found that the maximum peak heights for the three alkaloids were all located at 24 s (Fig. 2b). These two optimum values (150 mbar and 24 s) were combined together

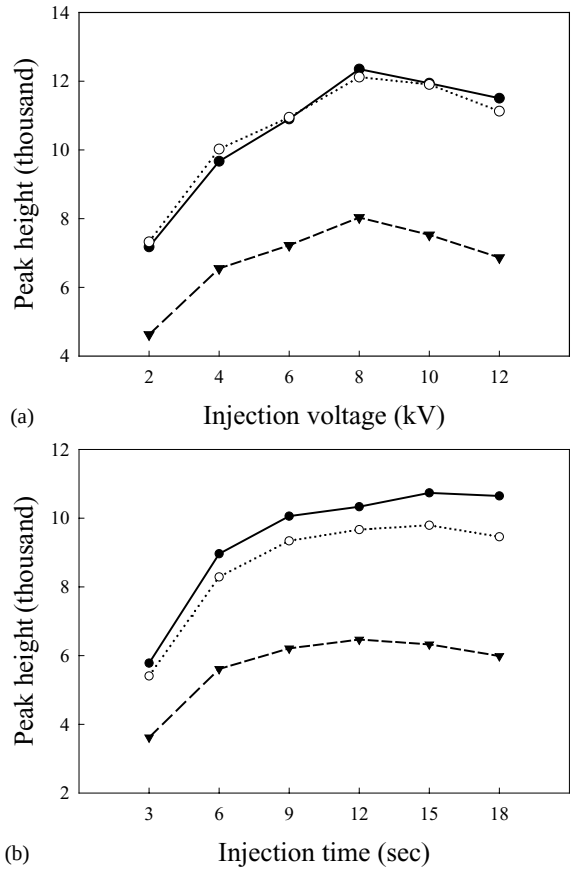

Fig. 3. Influence of injection voltage and time of sample solution on peak heights of the tested alkaloids in FASS electrokinetic injection, (a) at injection time of 12 s, (b) at injection voltage of 8 kV. (○) Berberine; (●) coptisine; (▼) palmatine.

to compose the optimum conditions for the hydrodynamic injections of FASS.

3.2. FASS of electrokinetic injection of samples

For electrokinetic injections (i.e., electro-injections) of FASS, the injection voltage and the injection time are the factors that can be worked to increase the stacking amount [17]. These two factors were tested in this work. The injection voltage was varied from 2 to 12 kV (Fig. 3a) and it was found that at 8 kV the peak height enhancement attained its maximum for all the three tested compounds. The injection time was also tested from 3 to 18 s (Fig. 3b). The peak heights increased from 3 to 12 s and then fell off. Injection voltage 8 kV and injection time 12 s were therefore selected to constitute the optimum conditions for the electro-injections of FASS. Chien and Burgi as well as Zhang and Thorman proposed to inject a short plug of water before sample injection so that during injection the buffer boundary at the end of the column was not disturbed and a proper electric field enhancement at the injection point was ensured from the beginning of injection [18,19]. It was also suggested that an empty region to concentrate sample ions deeper into the column and away from the injection point was rendered by such pre-injection of water plugs [20]. In this experiment, we also injected a short water plug before sample injections; however, no peak height enhancement was observed. Quirino and Terabe had conducted experiments where the direction of the electroosmotic flow (EOF) and electrophoretic mobility of the sample ion were the same (our experiment being similar) and found the presence of a water plug did not improve the peak shape [6]. Their sample was also dissolved in water.

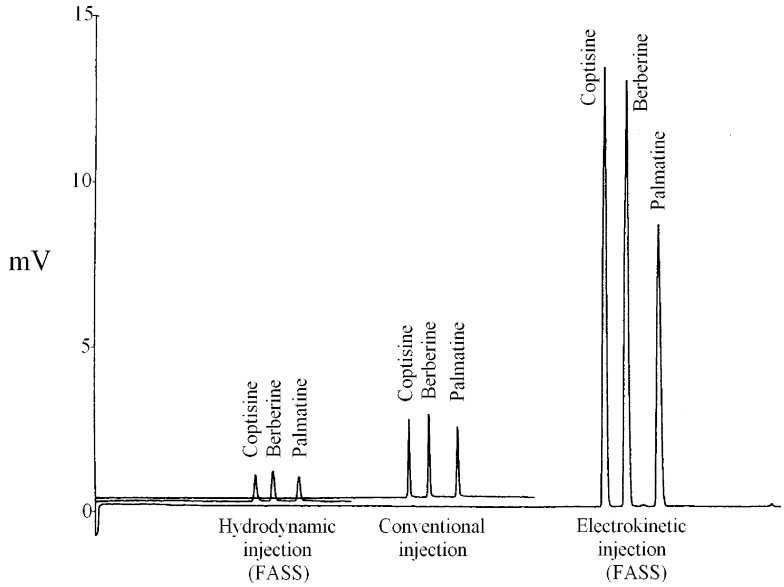

Fig. 4. Comparison of peak heights obtained from FASS/electrokinetic injection, FASS/hydrodynamic injection, and conventional injection. Concentration of each alkaloid in FASS/electrokinetic injection and FASS/hydrodynamic injection is 1 ppm; in conventional injection it is 50 ppm. Conditions see the text.

3.3. Stacking efficiencies of FASS of hydrodynamic and electrokinetic injections

The stacking efficiencies (peak height enhancement factors) of the above pressure- and electro-injections are obtained with respect to the conventional injection of samples. For the conventional injection of samples the samples were dissolved in running buffer and then hydrodynamically injected for 1.2 s (0.02 min). The electropherograms for the three injection modes are shown in Fig. 4. It should be noted that in Fig. 4 the concentrations of the three alkaloids in FASS pressure- and electro-injections are 1 ppm (μg/ml), while in conventional injection the concentrations are 50 ppm. The stacking efficiencies were calculated by multiplying the peak height ratios with the concentration dilution factors. Compared with the conventional injection, the FASS electro-injection gave a stacking efficiency of 262, 243 and 191-fold for coptisine, berberine and palmitine, respectively; the FASS pressure-injection gave a stacking efficiency of 17.9, 16.9 and 16.6-fold for berberine, palmitine and coptisine, respectively. The sensitivity enhancement of the FASS electro-injection is more than 10 times larger than that of the FASS pressure-injection. This is explained by the fact that in pressure-injection (in-column FASS), the volume of the sample solution that can be injected into the capillary limits the injected amount of sample; however, this is not a problem in electro-injection (head-column FASS) since analyte molecules are introduced electrophoretically [15].

Because the FASS of electrokinetic injection provided the greatest sensitivities for the detection of the alkaloids, its conditions were taken consequently as the method for the determination of these alkaloids in the herbal medicine. Before carrying on the quantitative analysis, the method should be going through the validation process.

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

Compound	Linear range (ng/ml)	Intercept	Slope	r^a
Berberine	20–100	−0.1152	0.0362	0.9961
Coptisine	20–100	−0.1576	0.0314	0.9827
Palmatine	20–100	−0.1075	0.0232	0.9963

[a] Correlation coefficient.

3.4. Validation of the method

Run-to-run repeatability ($n = 6$) and day-to-day reproducibility ($n = 3$) of the method in terms of migration times were both within 1.45% relative standard deviation (R.S.D.). Precisions of peak height ratios (with respect to *N*-benzylcinchonidinium chloride used as internal stadard) were tested at the lowest concentration level of each calibration curve (20 ng/ml). Run-to-run repeatability and day-to-day reproducibility of peak height ratios were within 13.95 and 14.86% R.S.D., respectively. These large values are due to the variability of electro-injections. 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 limits of detection (LOD, S/N = 3) of the method are 5, 5 and 7.5 ng/ml (ppb) for berberine, coptisine and palmatine, respectively. The LODs are 1.2, 1.2 and 1.5 μg/ml (ppm) for berberine,

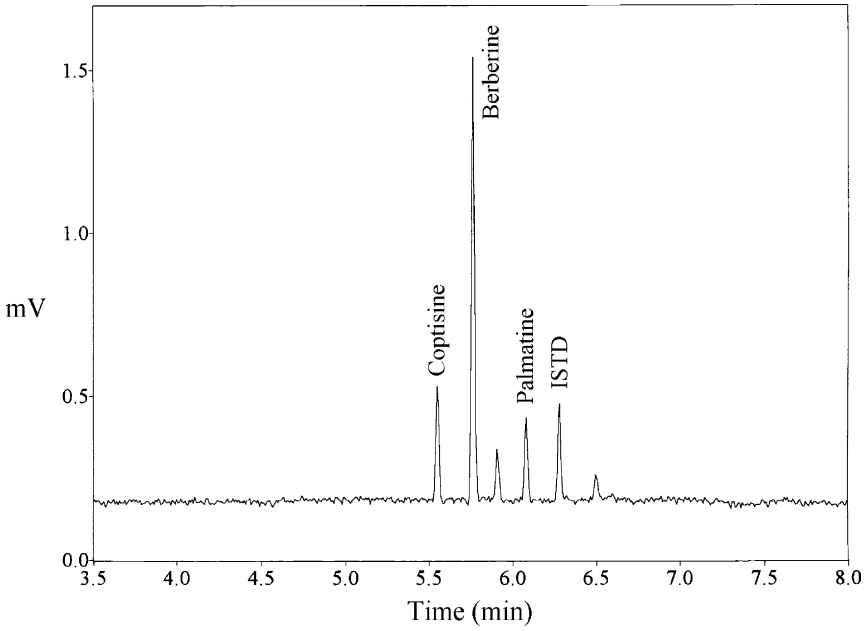

Fig. 5. Electropherogram obtained from the extract of a Coptidis Rhizoma sample, Conditions: fused-silica capillary, 72 cm × 50 μm i.d., 60 cm detection length; 400 mM Tris, pH 8.6, with the addition of 15% (v/v) MeOH to buffer; 25 kV; ambient (ca. 23 °C); 264 nm; injection, 8 kV, 12 s; sample dissolved in water. ISTD: internal standard.

coptisine and palmatine, respectively, if the compounds are subjected to conventional injection. This corresponds to a sensitivity enhancement of more than 200-fold for the developed method with respect to the conventional injection. The accuracy of the method was determined by adding a suitable amount of standard to sample solution and expressed as recoveries. The recoveries were 94, 93 and 89% for berberine, coptisine and palmatine, respectively.

3.5. Determination of the alkaloids in the Coptidis Rhizoma sample

On applying the optimum conditions to the Coptidis Rhizoma sample, the electropherogram obtained is shown in Fig. 5. In this figure, five constituents of the sample were detected; beside the peaks of berberine, coptisine and palmatine, the third and the last migrated-peaks might be due to epiberberine and berberasine, respectively, which are also non-phenolic protoberberines. Because of the injection bias associated with the electrokinetic injection, the phenolic protoberberines, such as columbamine and jatrorrhizine, in Coptidis Rhizoma were not detected. These compounds are all minor constituents as compared with the three tested compounds. The contents of berberine, coptisine and palmatine in the analyzed sample are 6.06, 2.60 and 2.15%, respectively.

4. Conclusions

A head-column FASS for the on-line improvement of detection sensitivity of the main alkaloids of Coptidis Rhizoma has been developed in the work. With the method, 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. For the analysis of herbal drugs, or even natural product researches, the methods of high detection sensitivities are required nowadays. The developed method provides a sensitive detection for the analysis and determination of the main alkaloids of the Coptidis herbal drugs.

Acknowledgements

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