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Short communication

Construction of an optimized method for quality evaluation and species discrimination of Coptidis Rhizoma by ion-pair high performance liquid chromatography combined with response surface methodology

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ABSTRACT

Coptidis Rhizoma (CR), the dried rhizome of three perennial *Coptis* specices, was widely used as a famous herbal medicine in China. Although the quantification of main alkaloids in CR has been extensively conducted, the existing analytical methods suffer from some flaws that restrict the general applicability in the routine quality assessment. In this work, we constructed an optimized method for quality evaluation and species discrimination of CR by ion-pairing high performance liquid chromatography (IP-HPLC) combined with response surface methodology (RSM). By employing Box-Behnken designs (BBD), 30 sets of experimental runs were performed to build the response surface models, and Derringer's desirability was used to optimize the IP-HPLC separation conditions by simultaneously taking resolutions between two pairs of hardly – separated alkaloids and the retention time of the last eluted analyte as optimization criteria. Meanwhile, a single standard to determine multi-components (SSDMC) method based on the optimized IP-HPLC was set up and fully validated, to simultaneously determine six alkaloids including jatrorrhizine (JAT), columbamine (COL), epiberberine (EPI), coptisine (COP), palmatine (PAL) and berberine (BER), using BER as internal standard. Finally, the quantitative data from 33 batches of CR samples were comparatively analyzed, and the ratios of JAT/COL and EPI/JAT were discovered for species classification. Collectively, the established IP-HPLC method can be adopted for comprehensive quality evaluation and species discrimination of CR due to its general applicability.

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1. Introduction

Coptidis Rhizoma (CR), a commonly used traditional Chinese medicine (TCM) known as 'Huang Lian', derives from the dried rhizomes of three perennial *Coptis* specices (Fam. Ranunculaceae), *i.e. Coptis chinensis* Franch., *C. deltoidea* C.Y. Cheng et Hsiao and *C. teeta* Wall., and the commercial decoction pieces are respectively called as 'Wei Lian', 'Ya Lian', and 'Yun Lian'. In TCM clinics, this medicinal herb has been used as an important remedy for the treatment of syndromes incurred by damp-heat, fire and poisoning [1]. At present, it has been manifested that CR can be used to treat infectious diarrhea, gastroenteritis, fever and heat stroke, hypertension and cardiovascular diseases [2]. Phytochemical studies have revealed that protoberberine alkaloids are the main pharmacologically active substances, among which berberine (BER) (Fig. S1) is one of the most representative structures in CR, owing to its relatively high abundance and diverse pharmacological properties, including anti-bacterial, anti-inflammatory, hypolipidemic and anti-diabetic activities [3]. Apart from BER, some other protoberberine alkaloids such as jatrorrhizine (JAT), columbamine (COL), epiberberine (EPI), coptisine (COP), palmatine (PAL) (Fig. S1) are also found to be responsible for the curative efficacies of CR.

The quantification of protoberberine alkaloids in CR has been extensively conducted and many analytical approaches including high-performance liquid chromatography (HPLC) [4–6], ultra-performance liquid chromatography (UPLC) [7], high-performance liquid chromatography-mass spectrometry (HPLC–MS) [8], microemulsion liquid chromatography (MELC) [9], capillary elec-

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trophoresis (CE) [10] and proton nuclear magnetic resonance spectroscopy (^{1}H NMR) [11] have been developed. In comparison with UPLC, HPLC–MS, MELC, CE and NMR, conventional HPLC method has been recognized as the most versatile technique in the routine quality assessment because of general applicability. Even so, the separation of *Coptis* quaternary alkaloids on the silica based stationary phases seems to be a difficult task due to their high hydrophilicity and strong alkalinity ($9 < pKa < 12$), since the ion-exchange interactions between basic analytes and residual surface silanols can give rise to peak tailing. For this reason, a mobile phase at high pH value ($\geq$12) is frequently employed to suppress the ionization of alkaloids [4–6]. However, the strongly basic eluent is definitely detrimental to the stationary phases (resulting in degradation of silica), and consequently cause inferior column-to-column reproducibility of analysis.

In order to improve the chromatographic performance on silica based stationary phases, ion-pair high performance liquid chromatography (IP-HPLC) is increasingly applied to the separation of medicinally important alkaloids. IP-HPLC can effectively eliminate the so-called 'silanol effect' on the silica based stationary phases by addition of ion-pairing reagents (IPR) to the mobile phase (formation of non-charged ion-pairs with basic analytes), thus leading to symmetric peaks and good separation selectivity [12]. Although the practical application of IP-HPLC has been greatly increased during the last decade, the exact retention mechanism remains a matter of considerable debate due to the multiplicity of involved interactions including hydrophobic interactions, electrostatic interactions and dynamic ion-exchange [13]. Consequently, IP-HPLC is practically performed in isocratic elution mode, and the optimization process of chromatographic elution conditions for complex matrices is by no means a trivial task [14].

In fact, an IP-HPLC method is adopted in Chinese Pharmacopeia for the quality control of CR [15], however, the pharmacopeial method suffers from the following flaws: (i) time-consuming chromatographic run (nearly 1 h), (ii) poor peak resolution between JAT and COL (almost co-eluted). Very recently, an attempt to improve the pharmacopeial method in order to quantify six alkaloids was made under the quality by design (QbD) concept [16]. Regrettably, the whole analysis time still exceeded 1 h, which to some extent restricted its applicability. Therefore, the aim of the present study was to further optimize the critical parameters of IP-HPLC method such as the percentage of organic phase, concentrations of IPR and buffer, pH value of the mobile phase, and the response surface methodology (RSM) was employed. Meanwhile, a single standard to determine multi-components (SSDMC) method based on the optimized IP-HPLC was set up and fully validated, to simultaneously determine six protoberberine alkaloids including JAT, COL, EPI, COP, PAL and BER, using BER as internal standard. Finally, the quantitative data from 33 batches of CR samples were comparatively analyzed for species differentiation.

2. Materials and methods

2.1. Materials and reagents

A total of 33 batches of CR samples, originated from *Coptis chinensis* (S01–S13), *C. deltoidea* (S14–S23) or *C. teeta* (S24–S33), were collected or purchased from herbal market in China. All samples were authenticated by Prof. Hui-Jun Li, Department of Pharmacognosy, China Pharmaceutical University. The voucher specimens were deposited in the State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, China.

Analytical grade solvents including sodium heptane sulphonate (SHS), sodium dodecyl sulfate (SDS), hydrochloric acid, phosphoric acid, monobasic potassium phosphate were purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Acetonitrile and methanol, purchased from Tedia Company (Fairfield, OH, USA) and Cinc High Purity Solvents Co., Ltd. (Shanghai, China), were of chromatographic grade. Water was purchased from Wahaha Co., Ltd. (Hangzhou, China). The reference standards of COL, EPI, COP, PAL, BER were supplied by Chengdu Must Biotechnology Co., Ltd. (Chengdu, China), and JAT was from Aladdin-reagent Co., Ltd. (Shanghai, China), their purities were determined to be above 98% by HPLC analysis based on a peak area normalization method.

2.2. Preparation of reference solution and sample solution

The six standards were accurately weighed and individually dissolved with methanol to prepare stock solutions. The concentrations of COL, JAT, EPI, COP, PAL, BER were 24.55, 24.27, 50.49, 97.41, 86.10 and 329.62 μg/mL, respectively. A series of working solutions were prepared by diluting the stock solutions with methanol in order to construct the calibration curves.

All crude CR samples were powdered and sieved through a 60-mesh sieve. Each powdered sample (0.1 g) was extracted with 125 mL of methanol-hydrochloric acid (100:1, *v*/*v*) by ultrasonication at 25 °C for 30 min. Methanol was used to make up a deficiency after the ultrasonic extraction process, and the extraction solution was filtered through a 0.45 μm filter. Finally, an aliquot of 10 μL filtrate was injected into the HPLC system for analysis. All the solutions were stored at 4 °C in a refrigerator before use.

2.3. Apparatus and chromatographic conditions

The HPLC analysis was performed on a Shimadzu LC-20AT HPLC System (Shimadzu Corp., Kyoto, Japan) equipped with a quaternary system, a degasser, an auto-sampler, a quaternary pump, a thermostatically controlled column compartment and a SPD-20A UV detector coupled with an analytical workstation (Labsolution for HPLC). Chromatographic separation was achieved at 25 °C on an Agilent SB-C18 column (250 × 4.6 mm, 5 μm) (Agilent Technologies Inc., Santa Clara, CA, USA). The flow rate was kept at 0.5 mL/min and the detection wavelength was set at 345 nm.

2.4. Experimental design to optimize the IP-HPLC separation conditions

The RSM was developed to describe the relationships between the variables and the response as well as to acquire the optimal conditions for separation. Table S1 summarizes the four independent variables, including (i) X1, the ratio of acetonitrile in the mobile phase; (ii) X2, the concentration of SHS; (iii) X3, the concentration of monobasic potassium phosphate; and (iv) X4, pH of the mobile phase, at three levels in this study. A matrix generated by Box-Behnken Design (BBD) is listed in Table S2, leading to 30 sets of experimental runs. The complete design consisted of 24 combinations and six replicates at central point. All analyses were performed using the CR sample S01. The regression analysis, statistical significance and response surfaces were analyzed using Design-Expert 8.0.6 software (Trial Version, State-Ease Inc., Minneapolis, MN, USA). The *p* values less than 0.05 were considered to be statistically significant.

2.5. Establishment of SSDMC method for simultaneous determination of six alkaloids

In order to increase the applicability of simultaneous determination of six alkaloids for routine analysis, a SSDMC method adopted from our previous study [17] was established. The determination of conversion factor (CF) and relative retention time (RRT) is of the

most importance for the SSDMC method. In this study, BER was selected as internal reference since it was commercially available, cheap and relatively abundant in CR samples.

3. Results and discussion

3.1. Optimization of IP-HPLC condition by RSM

The type of IPR makes great effect on the retention behavior of analyte. SDS and SHS have been widely used as additive in separation of alkaloids. Seen from Fig. S2, it was found that the use of SHS largely shortened the analysis time from 25 to 15 min compared with SDS, even though the resolutions between COL and JAT, JAT and EPI remained undesirable. Similarly, the other crucial factors such as percentage of organic solvent in the mobile phase, ionic strength (concentration of buffer) and pH value were selected and their varying ranges were preliminarily set for further optimization.

There are three critical optimization criteria for the performance in the chromatographic separation of six alkaloids in CR, *viz*. the resolution between COL and JAT (R1), the resolution between JAT and EPI (R2), and the overall analysis time. The resolutions R1, R2 and RT of the last eluted peak (BER) obtained from all the 30 experiments are shown in Table S2.

The experimental data from BBD were analyzed using response surface regression to fit the following quadratic polynomial model:

$$Y = \gamma_0 + \sum_{i=1}^{4} a_i X_i + \sum_{i=1}^{4} a_{ii} X_i^2 + \sum_{i \neq j=1}^{4} a_{ij} X_i X_j \quad (1)$$

In this equation, Y is the predicted response, γ_0 is a constant and a_i, a_{ii} and a_{ij} are the linear, quadratic and interactive coefficients of the model, respectively. Accordingly, X_i and X_j represent the levels of the independent variables, respectively.

The Design-Expert software generated three regression equations which demonstrated the relationship between the three response values and the four statistical parameters as shown in Table 1. In consideration of the adequacy of the assumed model, analysis of variance (ANOVA) was required. A model will be well fitted to the experimental data according to several parameters: (i) a non-significant lack of fit; (ii) the coefficient of R^2; (iii) the coefficient of variation (C.V.) and (iv) the adequate precision values. The three models all present non-significant lack of fit. Of note, to overcome the drawback associated with the use of R^2, the adjusted R^2 is generally used as a replacement. The adjusted R^2 can actually decrease if non-significant terms are added to a model. Therefore, to obtain a simple yet more accurate model, the non-significant terms ($p > 0.05$) were removed from the models through the 'backward elimination' process [18]. In addition, C.V., which is a measure of reproducibility of a model, should generally be less than 10%. The adequate precision values, related to the signal to noise ratios, are found to be more than the ratio limit of 4 [18].

These parameters listed in Table 1 demonstrated that the model in this work was suitable to make precise estimations in the studied experimental area. According to the model, it was concluded that the resolutions R1 and R2 were mainly affected by X2 (the concentration of SHS), while RT of BER was largely determined by X_1 (the ratio of acetonitrile). Additionally, a certain degree of interactions in RT could be observed between X1 and X2, X1 and X3 (the concentration of monobasic potassium phosphate). Notably, the retention behavior of BER seemed not to be significantly affected by pH of the mobile phase. In IP-HPLC, pH of the mobile phase is generally required to lower than two units of the *pKa* value of analyte. In this work, the varying range of pH value (3.5–4.5) is far lower than *pKa* values of six *Coptis* quaternary alkaloids ($9 < pKa < 12$), which allows full ionization of the alkaloids in the mobile phase.

Contour plot images of the models could provide a better understanding of the effects of variables on the separation performance in visual. As can be seen in Table 1, the terms of X1X2, X1X3 are of significant importance in the regression equation of RT. Hence, the contour plots reflecting interactions between X1 and X2 or X3 are deciphered in Fig. 1, where the effects of two factors on the response are shown at one time, while the third factor is kept zero level in all cases.

In more detail, Fig. 1A demonstrates the effects of X1 and X2 on the RT of BER under the concentration of monobasic potassium phosphate and pH of mobile phase keeping constant at their central points of 0.05 mol/L and 4, respectively. As can be seen, the increase of acetonitrile percentage over the studied range results in a pronounced decrease in retention of BER. As respect with the influence of IPR, increase of IPR concentration causes a relatively gradual decrease in retention of BER. Likewise, Fig. 1B represents the effects of X1 and X3 on the RT of BER under the concentration of SHS and pH of mobile phase keeping constant at their central points of 30 g/L and 4, respectively. From the plot, RT of BER is decreased by increasing the acetonitrile percentage, and is decreased as the concentration of monopotassium phosphate increases.

An approach for solving the problem of the optimization of several responses is the use of Derringer's desirability function[19]. The procedure is to construct a function for each individual desirability d_i and then obtain an overall desirability function D that should be maximized for choosing the best conditions of the designed weighted geometric average of the individual desirability (d_i), according to the following equation:

$$D = \left(d_1^{r1} \times d_2^{r2} \ldots\ldots \times d_n^{rn}\right)^{\frac{1}{\sum ri}} = \left(\prod_{i=1}^{n} d_i^{ri}\right)^{\frac{1}{\sum ri}} \quad (2)$$

where r_i is the importance of each variable relative to the others. Therefore, three responses, *i.e.*, R1 (d_1), R2 (d_2) and RT (d_3) were simultaneously optimized by using numerical optimization function of the Design-Expert program. The numerical ranges specified were set to minimize the retention time of BER within 20–60 min, the two resolutions between 1.3 and 1.5. Following the conditions, the optimal calculated parameters were obtained as: X1, 35.82%; X2, 25.24 g/L; X3, 0.05 mol/L, X4, 4.1. The typical IP-HPLC chromatograms of reference solution and sample solutions under the optimized conditions are shown in Fig. 2.

3.2. SSDMC method development and validation

The SSDMC method was fully validated for specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision (*i.e.*, repeatability, intra-day and inter-day variability), accuracy, stability and ruggedness. As shown in Tables S3-S5, the developed method was found to be precise, sensitive, accurate and robust, and could be applied to the routine quantitative analysis of CR.

The accurate determination of CFs is a prerequisite for SSDMC method. In Chinese Pharmacopeia [15], the CF values of EPI, COP, PAL and BER were taken for granted as 1.00, however, the real CF values of COL, JAT, EPI, COP, PAL and BER were measured in our study as 1.02, 0.99, 1.10, 1.12, 1.02 and 1.00, respectively (Table S3).

3.3. Quantification and classification of CR species

By using the developed SSDMC method, 33 batches of CR samples were analyzed quantitatively. Seen from Table S6, it was found that the distribution of these six alkaloids highly varied from species to species: for the species of *Coptis chinensis*, BER was the most abundance of alkaloid (5.58–9.61%), followed by COP

Table 1
Refined regression equations of coded factors and statistical parameters for studied responses from the RSM.

Response	Regression equation[a]	C.V. (%)[b]	Adjusted R^2	Predicted R^2	Adequate precision
R1	$1.28 - 0.23X1 - 0.26X2 - 0.005X4^{c} - 0.16X1^2 - 0.074X4^2$	7.36	0.89	0.83	21.95
R2	$1.46 + 0.041X1 + 0.21X2 + 0.001X3^{c} + 0.055X4 - 0.13X1X2 - 0.11X1^2 - 0.055X3^2$	5.65	0.86	0.73	15.23
RT	$39.02 - 25.97X1 - 2.66X2 - 1.46X3 + 2.82X1X2 + 1.52X1X3 + 10.23X1^2 - 0.97X2^2$	2.63	0.99	0.99	68.20

[a] Obtained by applying backward elimination tool to remove non-significant ($p > 0.05$) terms from the full models.
[b] Coefficient of variation.
[c] The non-significant term was included in the equation to maintain model hierarchy.

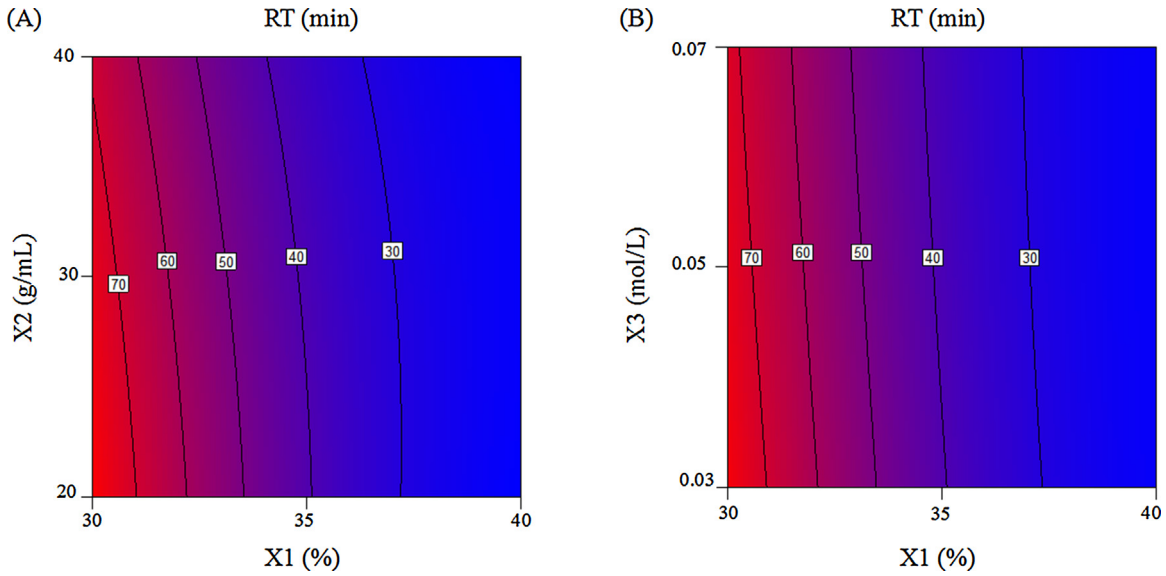

Fig. 1. RSM contour plots representing the retention time of BER as a function of X1(acetonitrile content), X2(concentration of SHS) (A), and of X1, X3(concentration of monobasic potassium phosphate) (B).

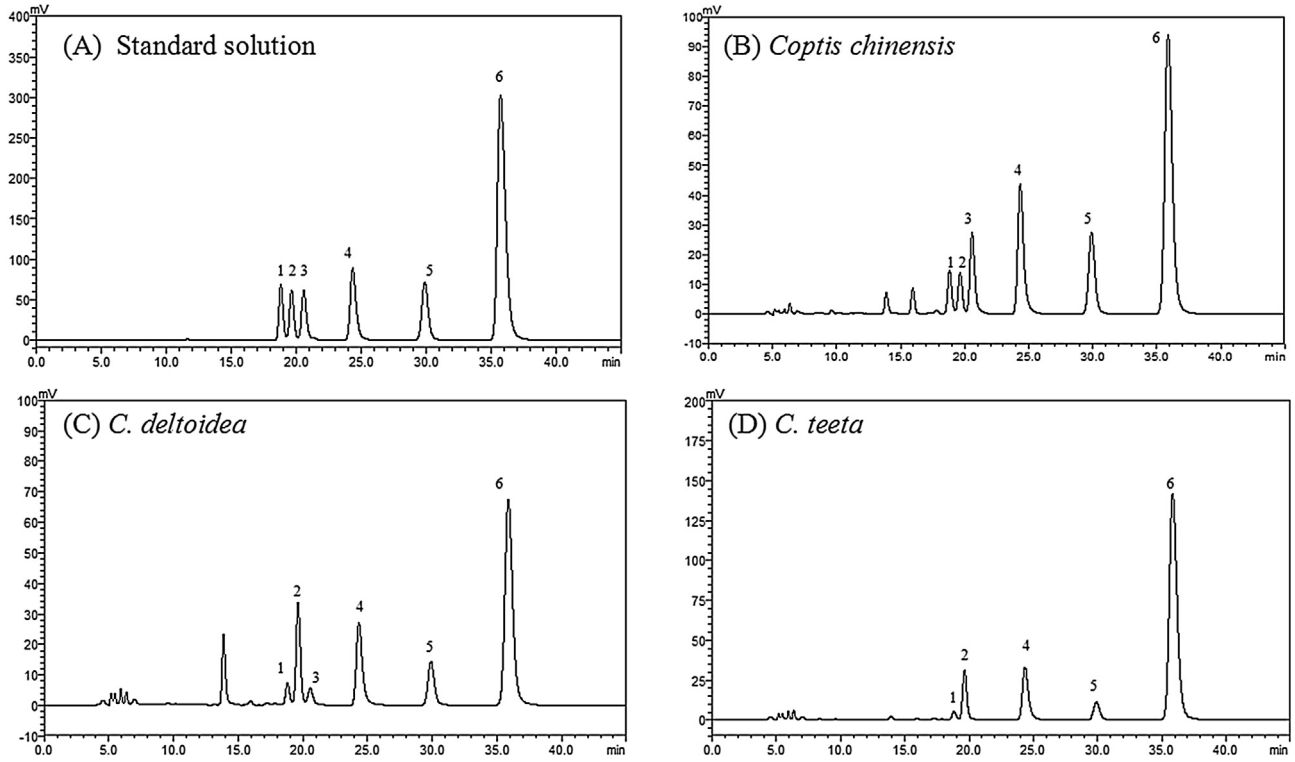

Fig. 2. Typical IP-HPLC chromatograms of mixed standard solution (A), sample solutions of *Coptis chinensis* (B), *C. deltoidea* (C) and *C. teeta* (D). Peak assignments: 1. COL; 2. JAT; 3. EPI; 4. COP; 5. PAL; 6. BER.

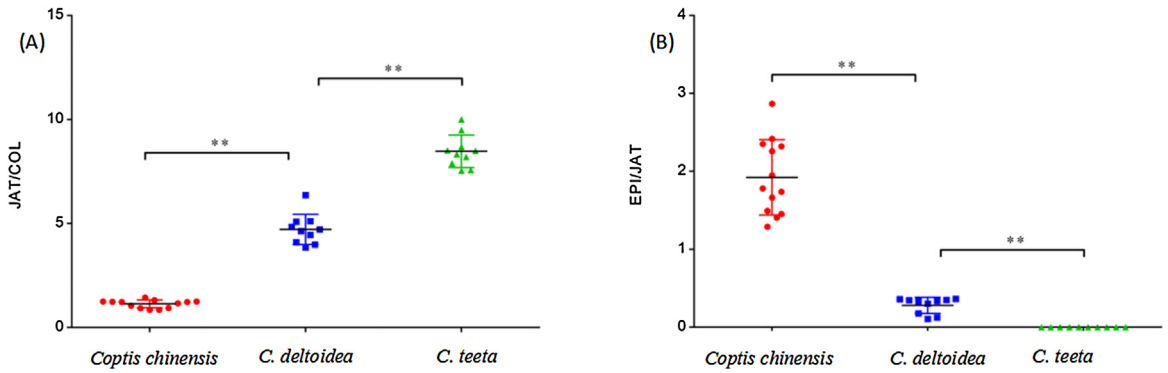

Fig. 3. Species classification of CR samples by the ratios of JAT/COL (A) and EPI/JAT (B). Each point represents an individual sample. ** represents $p < 0.05$, based on *Sidak* – test.

(1.65–2.64%), PAL (1.20–2.28%), EPI (0.95–1.76%), while COL and JAT occurred in relatively lower contents (below 1%); for *C. deltoidea*, the first, second, third and fourth alkaloids with highest concentrations were BER, COP, JAT and PAL, respectively, whereas the levels of COL and EPI were comparatively lower; for *C. teeta*, the most intense constituent was BER, whose amount reached up to 10.84%, and conversely, EPI was absent. Similarly, Fig. S3 visually characterizes the distinct occurrence of six alkaloids among CR species, proving the necessity of comprehensive analysis for precise quality evaluation of multi-origin TCM herbs.

Although several previous reports [5,11] had successfully employed professional chemometrics tools including hierarchical clustering analysis (HCA) and principal component analysis (PCA) to distinguish CR species based on the contents of these six alkaloids, in the present study, two simple but significantly distinctive indicators, namely the ratio of JAT to COL and the ratio of EPI to JAT, were firstly discovered for species classification. In terms of the ratio of JAT/COL (Fig. 3A), *C. teeta* has the highest ratio ranging from 7.54 to 10.01, followed by *C. deltoidea* from 3.99 to 6.37, and *C. chinensis* from 0.86 to 1.32. In terms of the ratio of EPI/JAT (Fig. 3B), *C. chinensis* possesses the highest ratio ranging from 1.29 to 2.86, followed by *C. deltoidea* from 0.17 to 0.37, and *C. teeta* with the value of 0. The comparative results indicated that minor constituents might play a more crucial role in discrimination of closely-related plant species than those major ones.

4. Conclusion

This work established an IP-HPLC method of simultaneously determining six alkaloids in CR by applying the response surface methodology strategy based on the Box-Behnken design. Under the optimized conditions, a SSDMC method was set up fully validated to simultaneously quantify six alkaloids in CR as an economical way. When applied to the analysis of CR samples, the method was found to give satisfactory applicability. Furthermore, the ratios of JAT/COL and EPI/JAT were firstly discovered as distinctive markers for discrimination of the species of CR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2018.02.019.

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