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Quality evaluation of *Codonopsis Radix* through high performance liquid chromatography fingerprint combined with chemometrics and simultaneous determination of five characteristic ingredients

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ABSTRACT

Codonopsis Radix (CR) is recorded as the roots of *Codonopsis pilosula*, *C. pilosula var. modesta* and *Codonopsis tangshen*. It is difficult to evaluate the quality of CR because of the existence of many original plants. In this paper, a strategy integrating chromatographic analysis and chemometrics for the quality control of CR is proposed. Systematic analysis of the chemical composition of CR was achieved through high performance liquid chromatography (HPLC) fingerprinting. Based on the HPLC fingerprinting data, chemometrics, including unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares-discrimination analysis (OPLS-DA), were applied to classify all CR samples. Components with variable importance in projection values higher than 1 in the OPLS-DA model were selected as potential chemical markers for distinguishing the origins of CR. Finally, an HPLC method was validated for determining the five characteristic ingredients in the CR samples. HPLC characteristic fingerprints showed 17 common peaks for *C. pilosula*, 13 for *C. pilosula var. modesta*, and 9 for *C. tangshen*, and all of them showed good similarity (>0.9). Additionally, there were 9 common peaks for all CR samples with relatively poor similarity, ranging from 0.607 to 0.970. PCA could differentiate CR from the three origins, except for a partial overlap between *C. pilosula* and *C. pilosula var. Modesta*, and the OPLS-DA model achieved excellent classification results. Eight components (peaks 12, 8, lobetyolin, 10, codonopsin I, syringin, 3, and 11) were selected as potential chemical markers. There was a large discrepancy in the contents of the five characteristic ingredients in all samples, with the relative standard deviation ranging from 36.0% (lobetyolin) to 85.9% (atractylenolide III). The average contents of the five characteristic ingredients were similar between *C. pilosula* and *C. pilosula var. modesta* samples and notably higher than those of *C. tangshen* samples. Consequently, a rapid, precise, and feasible strategy was established for the discrimination and quality control of CR with different origins.

KEYWORDS

Codonopsis Radix, HPLC, fingerprints, chemometrics, quality evaluation

INTRODUCTION

Codonopsis Radix (CR), called "dangshen" in Chinese, is a multi-origin traditional Chinese medicine categorized in the family Campanulaceae. In Chinese Pharmacopoeia, it has been recorded to occur in the dried roots of *Codonopsis pilosula* (Franch.) Nannf. (*C. pilosula*), *C. pilosula* Nannf. var. *modesta* (Nannf.) L. T. Shen (*C. pilosula var. modesta*), and *Codonopsis tangshen* Oliv (*C. tangshen*) [1]. It is abundant in Shanxi, Gansu, Sichuan, and Hubei

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provinces. According to the traditional Chinese medicine (TCM) theory, CR can strengthen the spleen, tonify the lung, nourish blood, and engender liquids [2, 3]. Modern research has demonstrated that CR contains various bioactive compounds such as polyacetylenes, phenylpropanoids, flavonoids, lignans, and coumarins [4–8], which have bioactivities that can reinforce immunity and regulate blood pressure [9]. Among them, lobetyolin is an active constituent in CR and has a protective effect on gastric mucosal lesions induced by ethanol, which is consistent with its traditional efficacy of strengthening the spleen and stomach [10–12]. Phenylpropanoid compounds mainly include codonopsin (I–IV) and syringin, which have the bioactive properties of anti-fatigue, anti-cerebral ischemia, and promoting adaptability [13, 14]. Atractylenolide III contains anti-inflammatory bioactivities and is often used as an index component for the quality evaluation of CR [15]. It has been reported that the contents of active components of CR from different origins and production areas vary significantly [16]. *C. pilosula* and *C. pilosula var. modesta* are the main sources of CR used in TCM hospitals and retail medicine stores. *C. pilosula* from the Shanxi province, called "Ludangshen", is considered to be of higher quality based on herbalogical studies, while its market share is lower than that from other production areas owing to low yield [8]. This makes it challenging to guarantee the quality and safety of CR. Thus, to provide scientific evidence for its practice and preference in clinical drugs, the quality evaluation of multi-origin CR is of significant importance.

For the current quality standard of CR in Chinese Pharmacopoeia, similar morphological features of *C. pilosula*, *C. pilosula var. modesta*, and *C. tangshen* make it difficult to authenticate the origins of CR, and no quantitative analysis method for quality control exists. Various detection methods have been developed to monitor the quality of CR. Duan et al. developed a high performance liquid chromatography (HPLC) fingerprint analysis method to evaluate the quality of CR, wherein nine common peaks were detected, and only lobetyolin was identified [17]. Subsequently, to evaluate the quality of CR originating in Shanxi, Guan et al. developed an HPLC characteristic fingerprint of CR, wherein seven common peaks were detected, of which five common peaks were identified [13]. Several researchers have continued CR content determination for one or several components [12, 18]. However, determining one or several marker compounds is challenging for profiling a TCM systematically on the whole. The method of integrating chromatographic techniques and chemometrics is the most extensively applied approach for quality control of TCMs [19–22], and can systematically reflect their quality consistency and stability. Although CR with three origins has been identified to varying degrees, simultaneous systematic evaluation of chemical compositions, origin authentication, and potential marker screening and quantitative analysis for the quality control of CR has not been researched.

The aim of this study was to establish an efficient and reliable approach for the quality evaluation of CR. An HPLC method was developed to identify chemical fingerprints of CR with different origins, obtain relatively comprehensive information regarding its chemical composition, and identify the "sameness" and "differences" among CR with different origins. Chemometrics, including unsupervised and supervised analyses, were performed to accurately discriminate CR with three botanical origins and screen potential markers for origin authentication. An HPLC method was also established for the simultaneous determination of the five characteristic ingredients (Fig. 1) for the quantitative evaluation of CR with different origins.

EXPERIMENTAL

Chemicals, reagents and materials

Standard substances of lobetyolin (purity = 99.21%, Lot number 1506567), atractylenolide III (purity = 98.99%, Lot number 1506568), and syringin (purity = 99.12%, Lot number 1506569) were supplied by Tianjin Nuoer Medical Technology Co., Ltd. (Tianjin, China). Codonopsin I (purity = 98%, Lot number CHB180123), and lobetyolinin (purity = 98%, Lot number CHB200721) were obtained from Chengdu Chroma-Biotechnology Co., Ltd. (Chengdu, China).

HPLC grade acetonitrile, methanol, and phosphoric acid were provided by Tianjin Damao Chemical Reagent Factory (Tianjin, China). Ultrapure water was obtained from Hangzhou Wahaha Group Co., Ltd. (Zhejiang, China).

A total of thirty batches of CR samples including *C. pilosula* (n = 13), *C. pilosula var. modesta* (n = 10), and *C. tangshen* (n = 7) were collected from various provinces in China, and the details were shown in Table 1. All of the samples were authenticated by Professor Cuiling Yang from School of Pharmacy, Shanxi Health Vocational College. Prior to analysis, all the samples were stored in the dry, cool, and sealed conditions.

Instrumentation and chromatographic conditions

The HPLC analysis was performed on an Agilent 1260 HPLC system (Agilent Technologies, USA) equipped with a diode array detector (DAD). The optimized conditions were as follows: the chromatographic separation was carried out on a Waters XSelect® HSS T3 column (4.6 × 250 mm, 5 μm) maintained at 35°C. The mobile phase was composed of acetonitrile (A) and water with 0.5% phosphoric acid (B) at the following gradient: 0–20 min, 5–10% A; 20–70 min, 10–30% A; 70–100 min, 30–60% A; 100–115 min, 60–85% A and 115–120 min, 85–95% A. The detection was performed at a wavelength of 220 nm under a flow rate of 1.0 mL min^{-1}. An aliquot of 10 μL solution was injected for acquiring chromatograms.

Preparation of standard solution

The stock solutions of syringin, codonopsin I, lobetyolinin, lobetyolin and atractylenoide III were prepared by methanol with the concentrations of 10.0, 2.00, 2.00, 5.00, and 10.0 mg mL^{-1}, respectively. The mixed working solution

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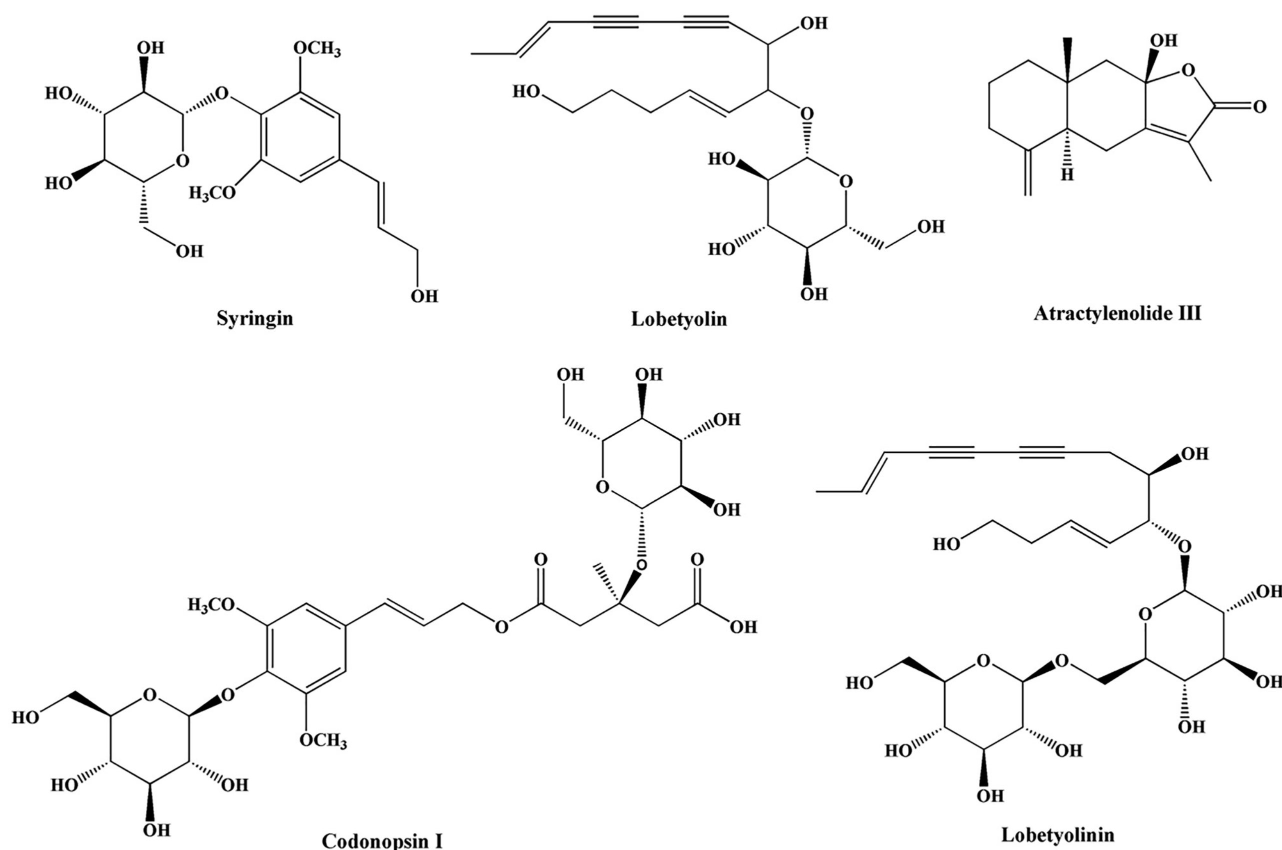

Fig. 1. Chemical structures of five characteristic ingredients in CR samples

Table 1. Sample information of CR samples from different origins

No.	Origin	Production place	No.	Origin	Production place
D1	*C. pilosula*	Shanxi	C3	*C. tangshen*	Chongqing
D2	*C. pilosula*	Shanxi	C4	*C. tangshen*	Chongqing
D3	*C. pilosula*	Shanxi	C5	*C. tangshen*	Chongqing
D4	*C. pilosula*	Shanxi	C6	*C. tangshen*	Hubei
D5	*C. pilosula*	Shanxi	C7	*C. tangshen*	Hubei
D6	*C. pilosula*	Shanxi	S1	*C. pilosula var. modesta*	Gansu
D7	*C. pilosula*	Shanxi	S2	*C. pilosula var. modesta*	Gansu
D8	*C. pilosula*	Shanxi	S3	*C. pilosula var. modesta*	Gansu
D9	*C. pilosula*	Shanxi	S4	*C. pilosula var. modesta*	Gansu
D10	*C. pilosula*	Shanxi	S5	*C. pilosula var. modesta*	Gansu
D11	*C. pilosula*	Shanxi	S6	*C. pilosula var. modesta*	Gansu
D12	*C. pilosula*	Gansu	S7	*C. pilosula var. modesta*	Gansu
D13	*C. pilosula*	Gansu	S8	*C. pilosula var. modesta*	Gansu
C1	*C. tangshen*	Chongqing	S9	*C. pilosula var. modesta*	Gansu
C2	*C. tangshen*	Chongqing	S10	*C. pilosula var. modesta*	Gansu

was prepared by properly diluting the stock solutions with methanol. The serial mixed standard solutions were prepared by diluting the stock solutions with the methanol, including 0.125, 0.250, 0.377, 0.500, 0.741, 1.00, and 2.00 μg mL^{-1} of syringin; 6.25, 12.5, 18.9, 25.0, 37.0, 50.0, and 100 μg mL^{-1} of codonopsin I; 1.25, 2.50, 3.77, 5.00, 7.41, 10.0, and 20.0 μg mL^{-1} of lobetyolinin; 25.0, 50.0, 75.5, 100, 148, 200, and 400 μg mL^{-1} of lobetyolin and 0.500, 0.755, 1.00, 1.48, 2.00, and 4.00 μg mL^{-1} of atractylenoide III, respectively. All the solutions were stored at 4 °C.

Preparation of sample solution

The CR samples were crushed by the pulverizer and passed through a No. 3 sieve (355 μm). Then about 2.0 g of each powdered sample was suspended in 30 mL 90% MeOH in a 150 mL capped conical flask, weighed accurately and sonicated at 20 ± 5 °C for 30 min. After cooling, extraction solution was added to the original weight. All solutions were filtered through a 0.45 μm filter membrane prior to HPLC analysis.

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Validation of the qualitative fingerprints in CR samples by HPLC-DAD

HPLC fingerprint analytical method was validated for precision, repeatability and stability. The precision analysis was determined by repeatedly injecting the same sample (D1) solution six times, and it was analyzed at different time periods of 0, 2, 4, 6, 8, 12, and 24 h at 20 ± 5 °C for the sample stability. The repeatability was examined by injecting six independent solutions obtained from the same sample (D1) in parallel. Peaks found in all samples with high response, suitable retention time and good resolution and stability were assigned as references. The ratios of the peak areas and retention times over those of the reference in the same sample solution were calculated as the relative peak area (RPA) and relative retention time (RRT). Precision, repeatability and stability were evaluated by the relative standard deviation (RSD) values of RRT and RPA of common peaks.

HPLC fingerprint establishment and similarity analysis of CR samples

Sample solutions prepared from 30 batches of CR samples were qualitatively analyzed. The HPLC- diode array detection (DAD) chromatograms in the AIA format were imported into the professional software called Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012, National Committee of Pharmacopoeia, China). The HPLC reference fingerprints (R) were obtained through multipoint correction and full spectral peak matching using the median method with a time width of 0.5 min and employing D1 as the reference chromatogram. The similarity results were evaluated through comparison with the reference fingerprint chromatogram.

Chemometrics analysis of CR samples

Based on the HPLC fingerprint data, principal component analysis (PCA) was used as an unsupervised model to visualize the classification trends. It can transform a large number of variables into a few orthogonal synthetic variables [23]. Orthogonal partial least squares-discrimination analysis (OPLS-DA) is a supervised model wherein the users provide the identity of each group of samples to attain maximum variance of the groups in the hyperspace for sample classification [24]. In this study, a 17×30 data matrix was imported into the SIMCA software (Version 14.1, Umetrics, Sweden) for PCA and OPLS-DA analysis. The influence of each chromatographic peak on the discrimination results was determined based on the variable importance in projection (VIP) values. Variables with a VIP score >1 were deemed potential marker compounds for distinguishing between different groups.

Validation of quantitation of five characteristic ingredients in CR samples by HPLC-DAD

To validate the performance of HPLC-DAD for the quantitative determination of five characteristic ingredients (syringin, codonopsin I, lobetyolinin, lobetyolin, and atractylenolide III) in CR samples, their specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, repeatability, stability, and accuracy were measured.

Specificity was confirmed by comparing the chromatograms of the blank sample and CR sample (D1) with those of the reference standard solution.

Linear calibration curves were established and calculated as $Y = aX + b$, where X and Y denote the known concentrations (μg mL^{-1}) and corresponding peak areas of each analyte in the standard solutions, respectively. The LOD and LOQ of each target analyte were obtained by determining the corresponding concentrations based on signal-to-noise ratios (S/N) of 3 and 10, respectively.

Intra- and inter-day precision calculations were performed for one day and three consecutive days, respectively, with six replicate injections of the mixed standard solution (STD 6), and the relative standard deviations (RSDs) of the five components were calculated. Repeatability was determined by parallelly analyzing six independently prepared solutions from the same batch of the CR sample (D1). Stability was evaluated by analyzing the same sample solution stored at 20 ± 5°C for varying timeframes (0, 2, 4, 6, 8, 12, and 24 h). The RSD of the peak area for each compound was calculated to evaluate its precision, repeatability, and stability.

The recovery test was performed on six replicates using the standard addition method. The concentration (approximately equivalent to the concentration of the five ingredients in the matrix) of the mixed standard solution was added to 1 g of D1. Recoveries were then calculated by comparing the determined amount of these standards with the amount originally added.

Sample analysis for the quantitation of five characteristic ingredients in CR samples

Sample solutions of all CR samples parallelly prepared in duplicate were quantitatively analyzed. Then, the contents of five characteristic ingredients in the samples were calculated based on the established calibration curves.

RESULTS AND DISCUSSION

Validation of the qualitative fingerprints in CR samples by HPLC-DAD

To achieve optimal separation, a good peak shape, and appropriate retention time, the chromatographic conditions were investigated. The Agilent ZORBAX Eclipse plus C18 (4.6 × 250 mm, 5 μm), Agilent ZORBAX StableBond C18 (4.6 × 250 mm, 5 μm), and Waters XSelect HSS T3 (4.6 × 250 mm, 5 μm) columns were optimized. The results showed that the HSS T3 column exhibited better retention and separation over a shorter runtime. Good chromatographic behavior was observed when water containing 0.5% phosphoric acid and acetonitrile was selected for the mobile

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phase through a gradient program. Moreover, the results showed that acceptable parameters were obtained when the column temperature was maintained at 35°C, flow rate was set at 1.0 mL min^{-1}, and the injection volume was set to 10 µL. The UV full-scan spectra from 200 to 400 nm of the target compounds were obtained using a DAD detector. The 220 nm wavelength was considered to achieve a higher response and larger peak capacity.

Sample pretreatment conditions were optimized by investigating the effects of extraction solvents, extraction methods, solvent volumes, and extraction time on the extraction efficiencies of the target compounds. In the preliminary studies, different solvent types and proportions of extraction solvents were optimized, and 90% MeOH was discovered to be the most effective solvent for extracting the target compounds. There were no significant differences in the extraction efficiencies of the ultrasonication and heat reflux methods. Ultrasonication was selected for further experimentation because it is more convenient. The appropriate volumes of solvents (20, 30, and 40 mL) and extraction times (15, 30, and 45 min) were also investigated, and the results showed that a solvent volume of 30 mL with an extraction time of 30 min was the appropriate condition for sample preparation.

Lobetyolin was selected as the internal reference peak (S) to calculate the relative retention time (RRT) and relative peak area (RPA). The RSDs of the RPA and RRT were less than 3% for the precision, repeatability, and stability validations (Table 2). The results indicated that the instrument precision was good, the chromatographic conditions were repeatable, and the sample solution was stable in under 24 h.

HPLC fingerprints establishment and similarity analysis of CR samples

The HPLC characteristic fingerprints of CR with different origins were created separately. For HPLC characteristic fingerprint of *C. pilosula*, seventeen common peaks were found (Figs 2A and 3A). There were nine common peaks in *C. tangshen* samples (Figs 2B and 3B). As seen from Figs 2C and 3C, thirteen peaks in common for *C. pilosula var. modesta*. All batches with the same origin showed good similarity (>0.9) (Table 3) compared with the reference fingerprints. The characteristic fingerprints of the same origins were generally consistent and stable, which can be used for the quality control of CR with the same origin. Moreover, the five common peaks of syringin (t_R = 23.417 min), lobetyolinin (t_R = 39.180 min), codonopsin I (t_R = 48.444 min), lobetyolin (t_R = 55.413 min), and atractylenolide III (t_R = 95.414 min) were identified through comparisons with reference substances.

The reference fingerprint and the HPLC fingerprint of 30 samples were also established (Figs 2D and 3D) to evaluate the similarity of CRs from the three origins. The degree of variation among *C. pilosula*, *C. tangshen* and *C. pilosula var. modesta* was larger, with only nine common peaks and similarity values between 0.607 and 0.970 (Table 3). Additionally, the results showed that the RSDs of the RRTs of the 30 batches were very small, whereas the RSDs of the RPAs were extremely large and even reached 165.8%, which indicated that these three origins varied not only in the chemical composition but also in the contents of the chemical components.

Table 2. Method validation for the qualitative fingerprints of CR by HPLC-DAD

Peak No.	t_R[1] (min)	Precision (n = 6)				Repeatability (n = 6)				Stability (7 time points in 24 h)			
		Mean RRT	RSD (%)	Mean RPA	RSD (%)	Mean RRT	RSD (%)	Mean RPA	RSD (%)	Mean RRT	RSD (%)	Mean RPA	RSD (%)
1	5.39	0.10	0.1	0.63	1.5	0.10	0.1	0.63	0.4	0.10	0.1	0.63	1.7
2	6.78	0.12	0.1	0.08	1.7	0.12	0.1	0.09	1.3	0.12	0.1	0.08	2.4
3	7.12	0.13	0.1	0.18	2.0	0.13	0.1	0.17	0.7	0.13	0.1	0.18	1.9
4	10.22	0.18	0.1	0.15	2.3	0.18	0.1	0.15	0.4	0.18	0.1	0.15	2.1
5	11.09	0.20	0.0	0.41	2.0	0.20	0.1	0.40	2.9	0.20	0.0	0.41	1.9
6	21.34	0.38	0.0	1.48	1.6	0.38	0.1	1.46	0.4	0.38	0.1	1.48	1.5
7 (syringin)	23.51	0.42	0.0	0.06	2.7	0.42	0.1	0.06	1.0	0.42	0.0	0.06	2.9
8	31.64	0.57	0.0	0.06	2.8	0.57	0.0	0.06	0.7	0.57	0.0	0.06	2.6
9 (lobetyolinin)	39.22	0.71	0.0	1.80	1.9	0.71	0.0	1.78	0.5	0.71	0.0	1.79	1.8
10	40.73	0.73	0.0	0.12	1.8	0.73	0.0	0.12	1.4	0.73	0.0	0.12	2.2
11	44.04	0.79	0.0	0.22	1.7	0.79	0.0	0.21	0.8	0.79	0.0	0.22	2.3
12	44.86	0.81	0.0	0.14	1.6	0.81	0.0	0.15	1.4	0.81	0.0	0.15	1.9
13 (codonopsin I)	48.48	0.87	0.0	0.30	1.3	0.87	0.0	0.30	0.6	0.87	0.0	0.30	1.2
14 (lobetyolin)*	55.43	1	0	1	0	1	0	1	0	1	0	1	0
15 (atractylenolide III)	95.41	1.72	0.0	0.06	2.4	1.72	0.0	0.07	0.7	1.72	0.0	0.07	2.9
16	106.19	1.92	0.0	0.06	2.6	1.92	0.0	0.06	1.7	1.92	0.0	0.06	2.5
17	110.49	1.99	0.0	0.24	1.6	1.99	0.0	0.24	1.2	1.99	0.0	0.24	1.5

[1]Retention time.
*Reference peak.

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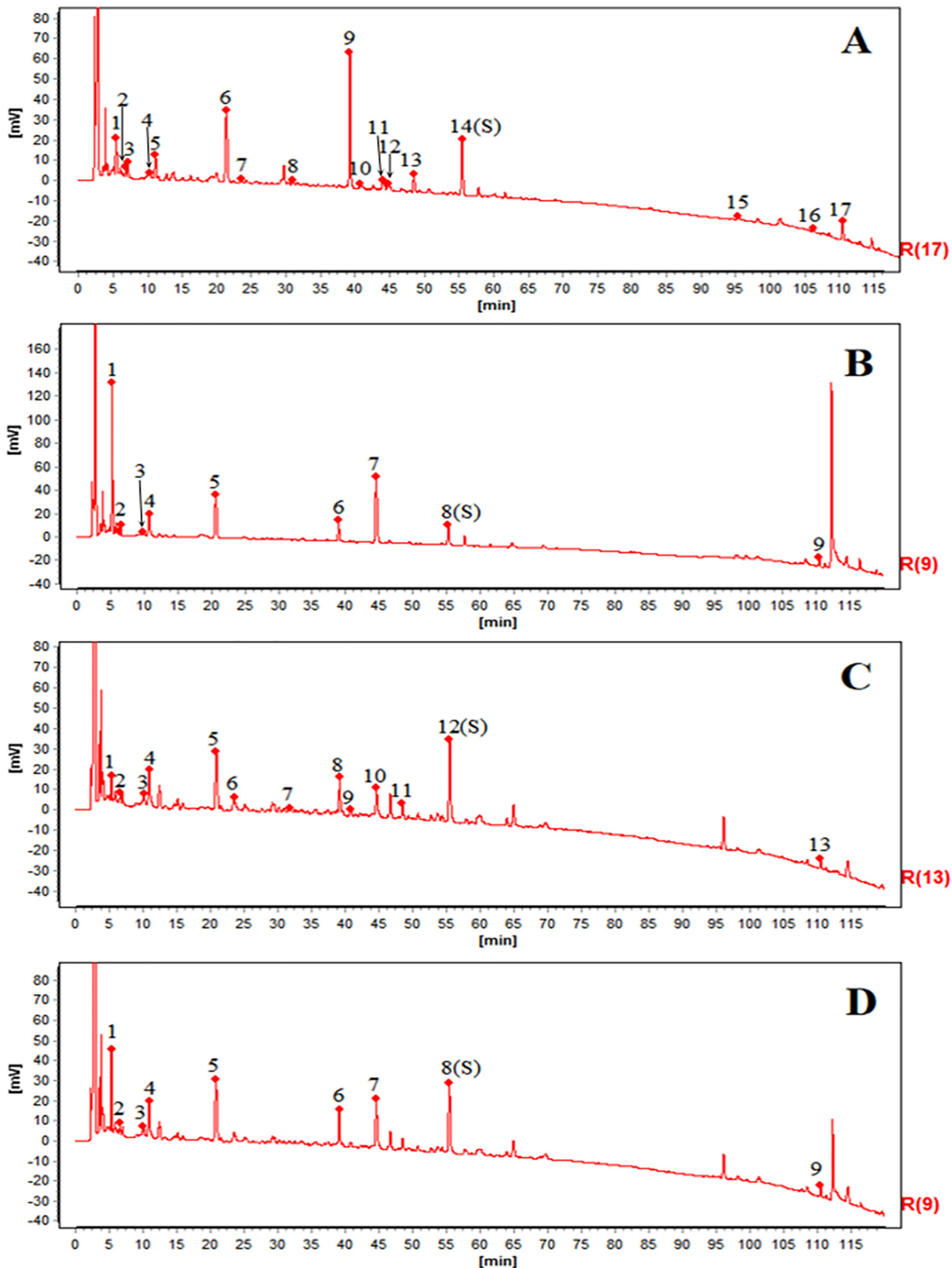

Fig. 2. The reference fingerprints of 13 batches of *C. pilosula* (A); 7 batches of *C. tangshen* (B); 10 batches of *C. pilosul a var. modesta* (C); 30 batches of CR samples with three origins (D)

Chemometrics analysis of CR samples

The HPLC fingerprint results could not efficiently discriminate the CRs from the three origins. Accordingly, chemometrics, including unsupervised (PCA) and supervised (OPLS-DA) models, were used for extracting useful information to achieve good discrimination between samples. The PCA score plot, shown in Fig. 4A, indicates that the *C. tangshen* samples can be clearly separated from those from the other two origins.

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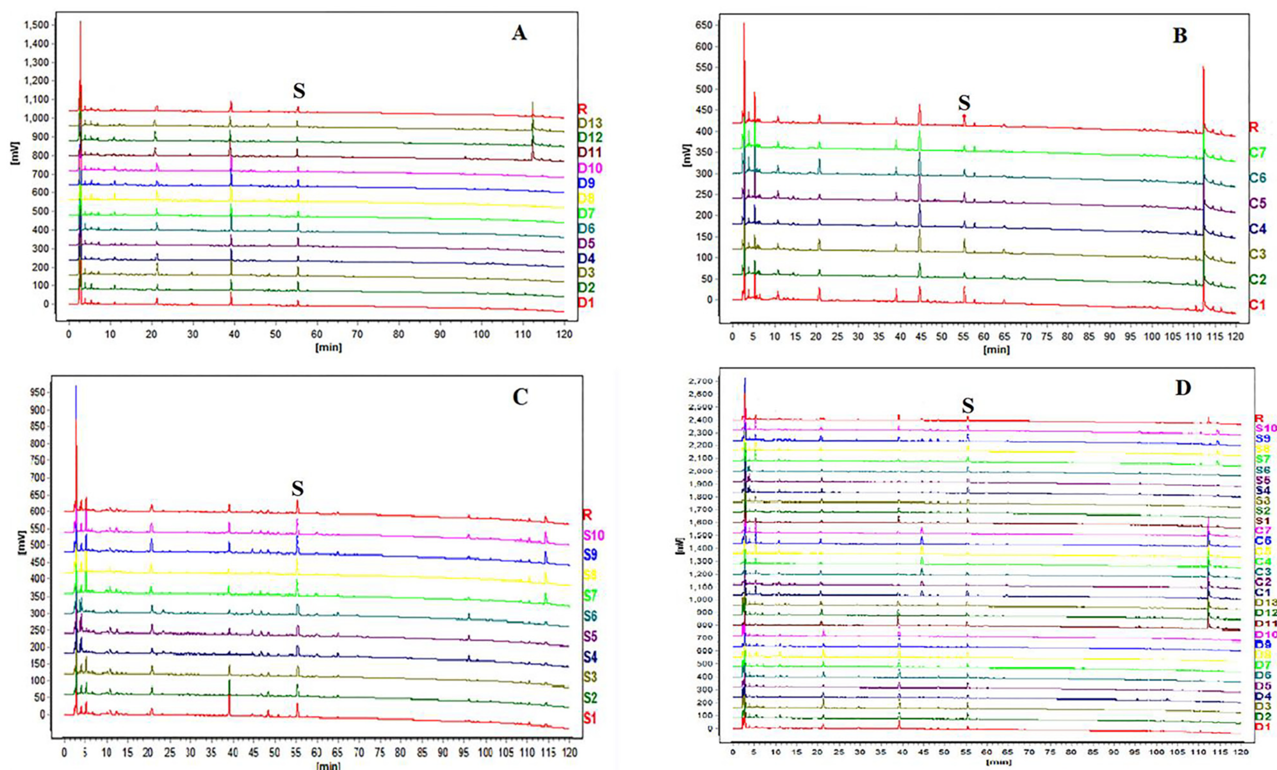


Fig. 3. The HPLC fingerprints of 13 batches of *C. pilosula* (A); 7 batches of *C. tangshen* (B); 10 batches of *C. pilosula var. modesta* (C); 30 batches of CR samples with three origins (D)

Table 3. Similarity analysis results for CR samples

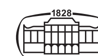
No.	Similarity[1]	Similarity[2]	No.	Similarity[1]	Similarity[2]
D1	0.989	0.939	C3	0.954	0.767
D2	0.935	0.970	C4	0.963	0.607
D3	0.975	0.921	C5	0.979	0.667
D4	0.986	0.923	C6	0.966	0.735
D5	0.931	0.907	C7	0.969	0.699
D6	0.985	0.906	S1	0.903	0.928
D7	0.989	0.926	S2	0.966	0.947
D8	0.973	0.939	S3	0.976	0.946
D9	0.955	0.901	S4	0.911	0.765
D10	0.971	0.866	S5	0.943	0.849
D11	0.971	0.877	S6	0.904	0.780
D12	0.962	0.926	S7	0.948	0.855
D13	0.975	0.939	S8	0.965	0.873
C1	0.937	0.918	S9	0.986	0.929
C2	0.971	0.698	S10	0.974	0.902

[1]The similarities were calculated by comparing the fingerprints of CR sample from the same origin to the their corresponding characteristic fingerprints, respectively

[2]The similarities were calculated by comparing the fingerprints of 30 batches of CR to the reference fingerprints obtained from the three origins, respectively

However, an overlap can be observed between the *C. pilosula* and *C. pilosula var. modesta* samples. The results indicated that PCA could not provide accurate and reliable origin clustering based on the common peaks of the HPLC-DAD chromatogram.

For further discrimination analysis, OPLS-DA was employed. In the OPLS-DA model, three parameters, R^2X, R^2Y, and Q^2, are typically used to assess the performance of the model. R^2X and R^2Y represent the explanatory capability of variables in the X and Y matrices, respectively, and Q^2



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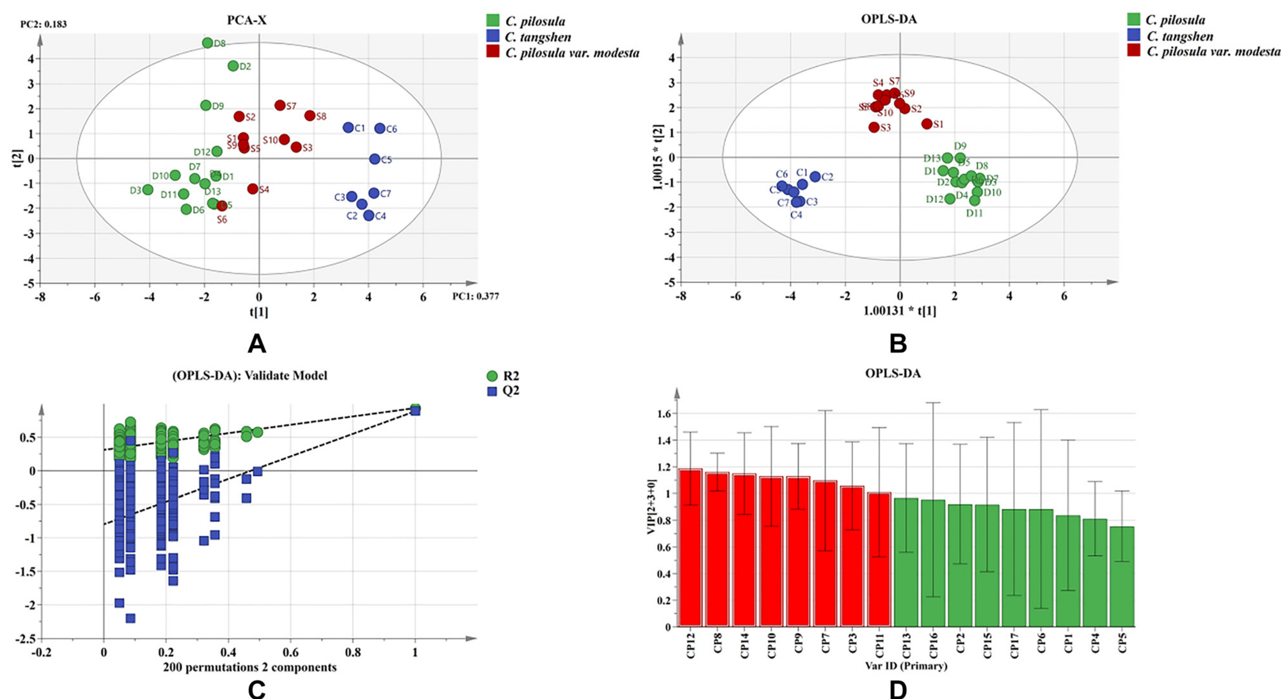

Fig. 4. Chemometrics analysis of 30 batches of CR samples from three origins. PCA score plot (A), OPLS-DA score plot (B), R2 and Q2 intercept values from 200 permutations (C), Variable importance in projection (VIP) plot (D)

indicates the predictive ability of the model. Their obtained values were close to 1.0, indicating an excellent fitness for the method. Generally, a Q^2 value greater than 0.5 is acceptable, and the difference between R^2 and Q^2 values should be less than 0.3 [25]. As shown in Fig. 4B, all samples are within a confidence interval of 95% (within the oval circle) and can be clearly separated into three groups based on their origins. The OPLS-DA model with R^2X value of 0.806, R^2Y of 0.936, and Q^2 of 0.833 was established, which indicated that the model fit the data well and had good prediction accuracy. Even after 200 permutations, the R^2Y and Q^2Y values of the original OPLS-DA model were still significantly higher than those of the permuted models. The results revealed that this OPLS-DA model was not overfitted because the intercept value was negative (Fig. 4C). Therefore, the OPLS-DA technique was more effective than the PCA approach in providing an accurate classification of 30 batches of samples based on the characteristic component information, which can also be applied to evaluate the quality of CR samples from different origins.

All the characteristic peaks were ranked on the loading diagram according to their contribution values in the OPLS-DA model. As illustrated in Fig. 4D, eight characteristic compounds with peaks 12, 8, 14 (lobetyolin), 10, 9 (codonopsin I), 7 (syringin), 3, and 11 showed considerable contribution to the classification of origins with VIP >1. Therefore, they were selected as potential chemical markers for the origin authentication of CR. Moreover, it has been proven that the activities of lobetyolin, codonopsin I, and syringin are closely related to the traditional efficacy of CR [10–14]. Hence, these components were selected as potential marker compounds to control the quality of CR samples of different origins. Our team is currently identifying unknown potential markers through ultra-high liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS), which will be a good supplement in the systematic quality control of CR.

Method validation for the quantitation of five characteristic ingredients of CR samples by HPLC-DAD

Specificity. As displayed in Fig. 5, good separation of the five characteristic ingredients in the complex sample was obtained under this chromatographic condition, The retention time of five ingredients in the chromatogram of the sample solution was consistent with that in the chromatogram of the standard solution, and there was no interference in the blank sample at the corresponding retention time.

Linearity, LOD and LOQ. The results are summarized in Table 4. Each ingredient had a wide linear range and the correlation coefficients were not less than 0.9990, which indicated the calibration curves were considered effective for quantitative analysis within the test concentration ranges. The LOD and LOQ suggested that the method was sufficiently sensitive for the quantitation analysis.

Precision, repeatability and stability. The results are listed in Table 5. The RSDs of intra- and inter-day precision ranged from 0.4 to 2.9% and from 0.9 to 2.3%, indicating that the instrument had a good performance in terms of consecutive analysis. For the repeatability test, the RSDs ranged from 0.3 to 2.6%, showing that the chromatographic condition was repeatable. The results showed that the RSDs

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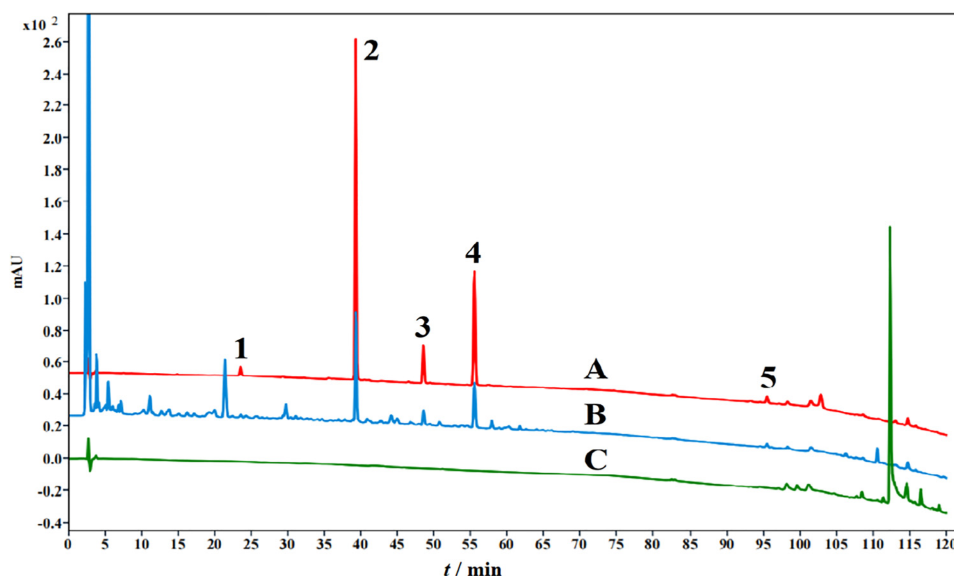

Fig. 5. HPLC-DAD chromatographic specificity of the quantitative method for CR. Mixed standard solution (A), Sample solution (B), Blank sample (C). Syringin (1), Codonopsin I (2), Lobetyolinin (3), Lobetyolin (4), Atractylenoide III (5)

Table 4. The results of linearity, LOD and LOQ of five characteristic ingredients in CR samples

Analytes	Linearity equation	Range (μg mL^{-1})	r	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Syringin	$y = 38.84x - 0.9870$	0.125–2.00	0.9998	0.0144	0.0432
Codonopsin I	$y = 23.68x + 0.1249$	6.25–100	1.0000	0.0182	0.0546
Lobetyolinin	$y = 17.48x - 0.9384$	1.25–20.0	1.0000	0.0592	0.178
Lobetyolin	$y = 2.852x + 2.093$	25.0–400	1.0000	0.113	0.339
Atractylenoide III	$y = 17.61x - 1.942$	0.500–4.00	0.9990	0.0852	0.256

Table 5. The precision, repeatability and stability of the quantitative method for CR samples

Marker compounds	Precision (RSD, %)		Repeatability (RSD, %, $n = 6$)	Stability (RSD, %, 7 time points)
	Intra-day ($n = 6$ per day)	Inter-day ($n = 18$ in 3 d)		
Syringin	1.2	1.4	0.7	1.1
Codonopsin I	2.9	2.3	1.0	3.0
Lobetyolinin	0.4	0.9	2.6	1.7
Lobetyolin	1.1	1.0	0.8	1.0
Atractylenoide III	1.4	1.0	0.3	1.6

of stability varied from 1.0 to 3.0%, indicating that the sample solutions were stable within 24 h.

Recovery. The recovery results are shown in Table 6. The average recoveries of five ingredients were between 90.4 and 108.8%, and the RSDs ranged from 2.2 to 3.1%.

Sample analysis for the quantitation of five characteristic ingredients in CR samples

Codonopsin I, syringin, and lobetyolin were considered as potential markers, while codonopsin I and lobetyolin were also the index components of high content in CR samples. Although atractylenolide III and lobetyolinin were not identified as distinguishing markers for origin authentication, they were abundant in most CR samples. Atractylenolide III can be used for the quality evaluation of CR from different origins and is an effective anti-inflammatory agent, as reported in the literature [15]. Lobetyolinin is also a characteristic component of CR samples and shows anti-arrhythmic activity [26]. Thus, these five components were considered to be the characteristic ingredients in the CR samples.

To further investigate origin differences, five characteristic components were simultaneously determined in the 30 batches of CR samples. The contents of the five

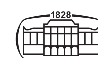

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Table 6. Average recoveries of the quantitative method for CR samples ($n = 6$)

Marker compounds	Original amounts (μg)	Spiked amounts (μg)	Detected amounts (μg)	Recovery (%)	Average recovery (%)	RSD (%)
Syringin	13.0	12.0	23.4	87.0	90.4	2.7
		12.0	23.9	90.7		
		12.0	24.1	92.5		
		12.0	24.1	92.5		
		12.0	24.0	91.9		
		12.0	23.5	87.6		
Codonopsin I	537.1	520.0	1026.2	94.1	96.8	2.6
		520.0	1032.6	95.3		
		520.0	1034.3	95.6		
		520.0	1063.6	101.2		
		520.0	1043.7	97.4		
		520.0	1040.9	96.9		
Lobetyolinin	123.4	110.0	232.4	99.1	100.3	2.2
		110.0	230.4	97.3		
		110.0	232.8	99.4		
		110.0	237.4	103.6		
		110.0	234.8	101.3		
		110.0	234.4	100.9		
Lobetyolin	2468.0	2300.0	4749.8	99.2	101.6	2.3
		2300.0	4765.5	99.9		
		2300.0	4783.5	100.7		
		2300.0	4893.5	105.5		
		2300.0	4836.2	103.0		
		2300.0	4803.4	101.5		
Atractylenoide III	30.8	30.0	63.1	107.7	108.8	3.1
		30.0	65.1	114.3		
		30.0	63.6	109.3		
		30.0	62.8	106.5		
		30.0	63.9	110.3		
		30.0	62.1	104.4		

characteristic components are listed in Table 7, wherein we can observe that there is a large discrepancy in the contents of the five components in all samples. Syringin, lobetyolinin, and atractylenolide III contents ranged from not detectable (ND) to 0.0240 mg g^{-1}, from ND to 0.196 mg g^{-1}, and from ND to 0.0836 mg g^{-1}, respectively, whereas codonopsin I and lobetyolin contents were within a range of 0.0370–0.788 mg g^{-1} and 0.958–4.87 mg g^{-1}, respectively, which were discovered in all samples with higher concentrations. The RSDs of the contents of five characteristic ingredients in the 30 batches ranged from 36.0% (lobetyolin) to 85.9% (atractylenolide III), and varied greatly across different batches. These results imply that it is challenging to control the contents of the five characteristic ingredients in CR samples to within a stable range when using herbs from different origins.

To evaluate the quality of samples from the three origins, the average contents of five characteristic ingredients are depicted in Fig. 6 (using 1/10 and 1/50 of the actual average contents as the y-axis for codonopsin I and lobetyolin owing to their higher contents). Substantial levels of lobetyolin were present in samples of all three origins. The average lobetyolin content was the highest in *C. pilosula var. modesta* samples, followed by *C. pilosula* and *C. tangshen* samples. The average lobetyolinin content was similar between *C. pilosula* and *C. pilosula var. modesta* samples and notably higher than that in *C. tangshen* samples. The average contents of codonopsin I, syringin, and atractylenolide III were the highest in *C. pilosula* samples and the lowest in *C. tangshen* samples, and syringin and atractylenolide III were not detected in some *C. tangshen* and *C. pilosula var. modesta* samples. Monitoring one or two components does not fully reflect the CR quality. Therefore, the quantitative analysis of multiple indicators provides a reliable method for quality control.

CONCLUSION

In this study, the general characteristics of the chemical components in CR were obtained using the HPLC fingerprint chromatographic technique. The internal quality of CR with different origins was different, whereas the samples with the same botanical origin showed good similarity. The characteristic fingerprints can be used to distinguish the different origins of CR through common peaks. The chromatographic data combined with chemometrics tools efficiently distinguished the three origins of the CR samples, and eight components were selected as potential chemical markers for the origin authentication of CR. Finally, the HPLC method

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Table 7. Contents of five characteristic ingredients of 30 batches of CR samples from three origins

Origin	Batch No.	Contents (mg g^{-1})				
		Syringin	Codonopsin I	Lobetyolinin	Lobetyolin	Atractylenoide III
C. pilosula	D1	0.0117	0.492	0.114	2.26	0.0265
	D2	0.00830	0.410	0.110	3.76	0.0648
	D3	0.0119	0.649	0.166	4.08	0.0174
	D4	0.0114	0.448	0.0840	2.24	0.0417
	D5	0.00550	0.421	0.125	3.66	0.0251
	D6	0.0124	0.652	0.146	3.32	0.0310
	D7	0.00640	0.473	0.145	2.40	0.0350
	D8	0.0115	0.596	0.140	2.99	0.0836
	D9	0.00870	0.671	0.0910	2.66	0.0330
	D10	0.00400	0.717	0.0730	1.87	0.0199
	D11	0.00770	0.788	0.0470	3.05	0.0390
	D12	0.00570	0.426	0.102	2.09	0.0376
	D13	0.00130	0.416	0.196	2.53	0.0188
	Mean ± SD	0.00819 ± 0.00350	0.551 ± 0.133	0.118 ± 0.0407	2.84 ± 0.700	0.0364 ± 0.0189
C. tangshen	C1	0.00310	0.223	0.0345	3.41	0.0128
	C2	ND	0.0370	0.0145	0.958	ND
	C3	0.00300	0.105	0.0124	2.40	ND
	C4	ND	0.0736	0.0233	1.05	0.00940
	C5	0.00230	0.128	0.0551	1.60	ND
	C6	0.00250	0.119	ND	1.22	ND
	C7	0.00250	0.155	ND	0.854	ND
	Mean ± SD	0.00268 ± 0.00310	0.120 ± 0.223	0.0200 ± 0.0345	1.64 ± 3.41	0.00317 ± 0.0128
C. pilosula var. modesta	S1	0.0116	0.475	0.195	3.33	0.0384
	S2	0.0240	0.311	0.181	3.32	0.0253
	S3	0.00390	0.214	0.113	2.99	ND
	S4	0.0181	0.0611	0.0851	3.71	0.0170
	S5	0.0145	0.120	0.0979	3.43	0.00970
	S6	0.0223	0.0946	0.0813	3.64	ND
	S7	0.00430	0.163	0.0796	3.74	0.0325
	S8	ND	0.0952	0.102	3.98	0.0387
	S9	0.00430	0.226	0.139	4.87	0.0184
	S10	0.00560	0.197	0.114	3.52	0.0186
	Mean ± SD	0.0121 ± 0.00805	0.196 ± 0.124	0.119 ± 0.0407	3.65 ± 0.508	0.0248 ± 0.0107
30 batches of CR samples	Mean ± SD	0.00762 ± 0.00628	0.332 ± 0.227	0.0955 ± 0.0555	2.83 ± 1.02	0.0231 ± 0.0199
	RSD (%)	82.5	68.3	58.1	36.0	85.9

ND, Not detectable.

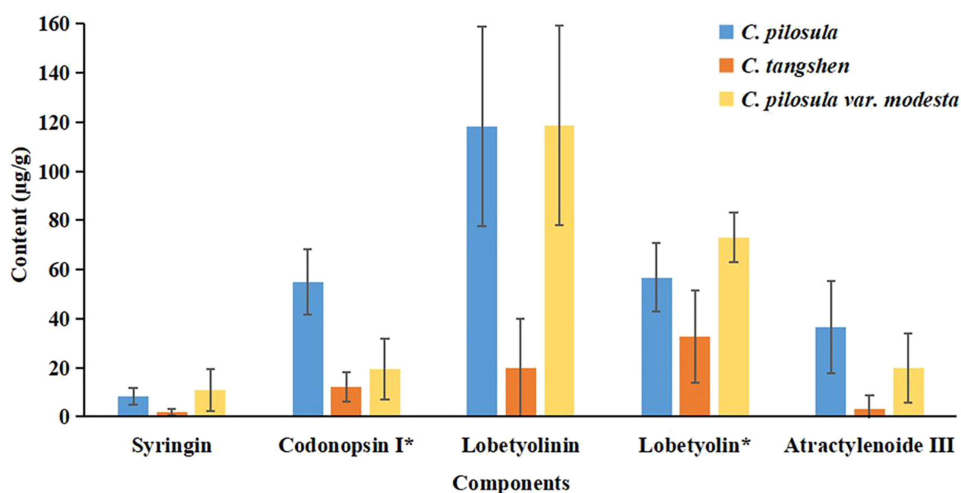

Fig. 6. The average contents of five characteristic ingredients in CR from different origins. (mean ± SD)
*Note: The average contents of codonopsin I and lobetyolin displayed in the figure were 1/10 and 1/50 as the actual average contents, respectively.

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was validated for the simultaneous determination of five characteristic ingredients in 30 batches of CR samples. Thus, the three origins of CR were objectively and clearly distinguished by comparing the contents of the five characteristic ingredients. In summary, the method of combining HPLC fingerprints with chemometrics and multi-component determination for origin authentication and quality evaluation of CR is practical, efficient, and reliable.

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REFERENCES

1. China Pharmacopoeia Committee. Pharmacopoeia of the People's Republic of China (I); China Medical Science Press: Beijing, **2020**; pp. 293–4.
2. Yang, D. D.; Chen, Y.; Guo, F. X.; Huang, B. T.; Okyere, S. A.; Wang, H. *Ind. Crops Prod.* **2019**, *142*, 111844.
3. Gu, C.; Cao, L. Y.; Su, Q.; Guan, L. J.; Yang, J.; Gao, J. P. *J. Chin. Med. Mate.* **2016**, *39*(8), 1716–22.
4. Gao, S. M.; Liu, J. S.; Wang, M.; Cao, T. T.; Qi, Y. D.; Zhang, B. G.; Liu, H. T.; Sun, X. B.; Xiao, P. G. *Chin. Herb. Med.* **2019**, *11*, 160–8.
5. Zhang, X.; Li, J. K.; Zhao, Y. J.; Gao, J. P. *Chin. J. Exp. Tradit. Med. Form.* **2018**, *24*(24), 53–9.
6. Zhang, J.; Xu, X. J.; Xu, W.; Huang, J.; Bai, J. Q.; Zhu, D. Y.; Qiu, X. H. *Chin. J. Exp. Tradit. Med. Form.* **2015**, *21*(9), 59–63.
7. Li, Z. Y.; Wang, A. N.; Liu, X. J.; Xue, S. Y.; Qin, X. M.; Guo, X. Q.; Zhang L. Z. *J. Shanxi Med. Univ.* **2011**, *42*(1), 56–62.
8. Gao, S. M.; Liu, J. S.; Sun, T.; Liu, F. S.; Jing, H.; Qi, Y. D.; Zhang, B. G.; Liu, H. T.; Xiao, P. G. *China J. Chin. Mater. Med.* **2016**, *41*(20), 3753–60.
9. Li, R. Y.; Lai, L. N.; Nie, L.; Yan, X. X. *Chin. J. Exp. Tradit. Med. Form.* **2019**, *25*(11), 133–8.
10. Huang, Y. Y.; Zhang, Y.; Kang, L. P.; Yu, Y.; Guo, L. P. *Chin. Tradit. Herbal Drugs* **2018**, *79*(1), 239–50.
11. Feng, P. P.; Li, Z. X.; Yuan, Z. *J. Shenyang Pharm. Univ.* **2012**, *29*(4), 307–11.
12. Wang, X. X.; Zhuang, P. Y.; Chen, J. M.; Yang, Y. K.; Lin, X. Y.; Zhang, D. Y. *Chin. Tradit. Herbal Drugs* **2017**, *48*(9), 1719–23.
13. Feng, Y. J.; Wang, X. X.; Zhuang, P. Y.; Zhang, D. Y.; Gao, L.; Chen, J. M.; Han, G. *J. Chin. Med. Mate.* **2017**, *42*(1), 135–9.
14. Chen, Q. F.; Deng, X. Y.; Zhu, H. F. *Sci. Technol. Food Ind.* **2016**, *37*(6), 64–7.
15. Gong, D, J.; Chen, P. Y.; Pang, Y. P.; Wang, X. Y. *Linshizhen Med. Mater. Med. Res.* **2015**, *26*(1), 70–2.
16. Yang, J.; Su, Q.; Liu, E. L.; Gao, J. P. *J. Shanxi Med. Univ.* **2010**, *41*(8), 698–702.
17. Duan, Q. M.; Liang, Z. S.; Yang, D. F. *Chin. Tradit. Herbal Drugs* **2012**, *43*(5), 995–9.
18. Wang, R. S.; Liu, Y. Q.; Guo, J. L.; Pang, W. R.; Zhao P. *Chin. J. Pharm. Anal.* **2007**, *27*(8), 1229–31.
19. Du, Y. Y.; Li, Q.; Liu, J. J.; Yin, Y. D.; Bi K. S. *Anal. Methods* **2014**, *6*, 5891.
20. Song, J.; Chen, F.; Liu, J.; Zou, Y.; Luo, Y.; Yi, X.; Meng, J.; Chen, X. *Phcog. Mag.* **2017**, *13*, 180–7.
21. Lei, Y.; Wang, Y.; Sun, Z.; Lin, M. Y.; Cai, X.; Huang, D.; Luo, K.; Tan S.H.; Zhang Y.; Yan J. Y.; Xia, X. H. *J. Sep. Sci.* **2020**, *43*, 1382–92.
22. Lin, L.; Li, H. H.; Xie, H.; Yan, G. J.; Hu, Y. T.; Lu, T. L.; Mao, C. Q. *Chin. Tradit. Herbal Drugs* **2021**, *52*(6), 1751–8.
23. Nurfarhana, H.; Tahir, A.; Norhidayu, M. Z. *J. Food Sci.* **2018**, *83*(12), 2903–11.
24. Ren, M. J.; Hu, Y. F.; Zhu, Y. B.; Qiu, P.; Li, Z.; Li, W. L. *Chin. Tradit. Herbal Drugs* **2022**, *53*(5), 1338–44.
25. Liao, Y. J.; Ao, M. Y.; Peng, Y.; Hu, C. J.; Chen, Z. M.; Xu, R. C. *Chin. Tradit. Herbal Drugs* **2021**, *52*(11), 3224–33.
26. Liu, H.; Chen, X. C.; Zhao, X. P.; Zhao, B. C.; Qian, K.; Shi, Y.; Baruscotti, M.; Wang, Y. *Front. Pharmacol.* **2018**, *9*, 1288. https://doi.org/10.3389/fphar.2018.01288.

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