# Fingerprint Analysis of *Codonopsis Radix* by HPLC Coupled with Chemometrics Analysis

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**Abstract:** **Objective** To establish a validated high performance liquid chromatography (HPLC) for the fingerprint analysis of *Codonopsis Radix* and for the determination of lobetyolin. **Methods** HPLC coupled with diode array detection method was employed to establish the fingerprint profile and quantitative determination of lobetyolin in *Codonopsis Radix*. Principal component analysis method was employed to analyze the 52 *Codonopsis Radix* samples. **Results** The reference chromatogram was generated with 25 common peaks showing good separation from adjacent peaks. **Conclusion** Statistical analysis of the obtained data demonstrates that the developed HPLC fingerprint combined with chemometric is a reliable method for the similar evaluation and quality assessment of *Codonopsis Radix*, and other traditional Chinese herbs.

**Keywords**: chemometrics; *Codonopsis Radix*; fingerprinting analysis; HPLC; quality control
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## Introduction

*Codonopsis Radix* (*Dangshen* in Chinese), the roots of *Codonopsis pilosula* (Franch.) Nannf. or *C. pilosula* var. *modesta* Nannf., is a kind of famous Chinese materia medica (CMM), which has been used as one of the herbal ingredients in prescriptions for replenishing energy deficiency, strengthening immune system, lowering blood pressure, and improving appetite for thousands of years (Qiao *et al*, 2007), and it is often regarded as a tonic equivalent to ginseng. The literatures had demonstrated that the extracts of *Codonopsis Radix* had the beneficial effects to relieve gastrointestinal motility disorders (Wang *et al*, 1997), enhance the function of learning and memory (Singh *et al*, 2004) and neuron regeneration (Chen *et al*, 2010). Traditionally, only a single component or a limited number of pharmacologically active components are selected to assess the authenticity and quality of the herbs (Singh *et al*, 2004). Unlike synthetic drugs, it is well known that the medicinal herbs and their preparations generally exert their therapeutic effects through the synergic effects of the multiple active ingredients and multi-targets (Dong *et al*, 2011; Gomes *et al*, 2010; Liang *et al*, 2010; Lv *et al*, 2012). So it is important to find a more accurate and efficient method for the quality control of herbal medicines.

Chemical fingerprint analysis had been introduced and adopted by World Health Organization (Munich, 1991), State Food and Drug Administration of China (Beijing, 2000), and other authorities as a strategy for the quality assessment of herbal medicines (Duan *et al*, 2012; Lu *et al*, 2009; Tang *et al*, 2010; Wang *et al*, 2007; Yang *et al*, 2007). Using this method, a particular herbal preparation with complex components could be identified and distinguished from other closely related species (Fu *et al*, 2009). Recently, HPLC fingerprint has been widely accepted owing to both the high separation efficiency and high detection sensitivity of the technique (Li *et al*, 2010; Zou *et al*, 2006). Chromatographic fingerprint could give an overall view

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of all components in CMM and is useful in defining the identity and the quality of a given species.

According to *Pharmacopoeia of People's Republic of China 2010*, lobetyolin was used as the only marker for TLC identification of *Codonopsis Radix*, which has been reported to have the protective properties against the injuries of the gastric mucous membrane induced by ethanol (Zheng *et al*, 1998). Some studies on the determination of the active components and finger-printing analysis for *Codonopsis Radix* using HPLC have been published (Feng *et al*, 2005; Qiao *et al*, 2007; Tang *et al*, 2009), few of them involved in the fingerprint analysis coupled with statistical analysis of *Codonopsis Radix* (Rong *et al*, 2011). So it is urgent to set up a reliable and accurate methodology based on the samples collected from Gansu, China to evaluate the quality of *Codonopsis Radix* effectively.

In the present study, we have strategically established an HPLC fingerprinting profile and simultaneous determination of lobetyolin for the assessment of the quality of *Codonopsis Radix* by HPLC coupled with diode array detection (DAD). Combining with principal component analysis (PCA) and discriminant analysis (DA), we efficiently differentiated and evaluated the quality of *Codonopsis Radix*.

## Materials and methods

### Chemicals

Lobetyolin was provided by National Institute for Food and Drug Control (Beijing, China). Methanol and acetonitrile were of chromatographic grade and purchased from Merck Co. (Germany). Deionized water was prepared with a water purification system (Shanghai, China). Acetic acid, *n*-butanol, and the other chemicals were of analytical grade and supplied from Tianjin Chemical Reagent Co. (China).

### Plant material and sample preparation

Fifty batches of *Codonopsis Radix* collected from Gansu, Shanxi, and Yunnan provinces in China were investigated, and two batches were purchased from herb markets in Gansu province. These herbal samples were authenticated by Prof. QI Huan-yang. Voucher specimens (DS01—DS52) were deposited in Key Laboratory of Chemistry of Northwestern Plant Resources.

The *Codonopsis Radix* samples were dried, finely powdered, sifted through a 250 μm sieve, and extracted by refluxing for chemical analysis. The dried powder (2 g) was extracted with 40 mL of 90% methanol twice, for 30 min each time. The sample was adjusted to the original mass with 90% methanol. The supernatants were filtered and evaporated to dryness, and then dissolved in 5 mL of methanol. The resulted solutions were filtered through a 0.45 μm filter before use. A 10 L aliquot was injected for HPLC analysis. The lobetyolin standard solution (0.5 mg/mL) was prepared in methanol.

### Chromatographic system

Liquid chromatographic system was consisted of Agilent 1200 HPLC System and DAD System. The analytical $C_{18}$ column was 250 mm × 4.6 mm, 5 μm (Agilent, USA). A binary gradient elution system composed of 0.3% solution of acetic acid in water as solvent A and 0.3% solution of acetic acid in acetonitrile as solvent B were applied for the fingerprint analysis with the linear gradient as follows: 0—5 min, 5%—7% B; 5—18 min, 7% B; 18—19 min, 7%—10% B; 19—31 min, 10%—12% B; 31—33 min, 12%—15% B; 33—40 min, 15% B; 40—54 min, 15%—19% B; 54—70 min, 19%—22% B; 70—80 min, 22%—35% B; 80—90 min, 35%—65% B; 90—93 min, 65% B; 93—97 min, 65%—75% B; 97—105 min, 75% B. The separations were performed with a flow rate of 1.0 mL/min. DAD monitoring was at 268 nm.

### Data analysis

HPLC-DAD data from 30 batches of *Codonopsis Radix* samples were exported from ChemStation software in AIA format and imported to the *Similarity Evaluation System for Chromatographic Fingerprints of TCM* (Version 2004). This system could reflect the similarity of the distribution ratio of the chemical composition accurately, which was recommended by SFDA.

The PCA and orthogonal signal correction partial least squares discriminate analysis (OSC-PLS-DA) were done by SIMCA-P 12.0 software (Umetrics AB, Sweden). The peak areas of 25 common peaks analyzed from 52 *Codonopsis Radix* samples were assigned as the variables and transformed mathematically into 25 uncorrelated principal components, which were used for PCA and OSC-PLS-DA methods.

The DA was performed using SPSS 17 software (USA).

## Results and discussion

### Optimization of chromatographic conditions

In order to achieve good separation as many peaks as possible within a short analysis time for the fingerprint of *Codonopsis Radix*, the mobile phase compositions and gradient elution procedure were optimized. We tried different eluent. The methanol-water, acetonitrile-water, acetonitrile-0.02% and 0.1% phosphoric acid, acetonitrile-0.1%, 0.3%, 0.4%, 0.5%, and 1.0% acetic acid, and acetonitrile-0.3% acetic acid were selected as the most appropriate eluent and a sufficiently large number of peaks on the chromatogram were achieved within 105 min under the optimized linear gradient mode. In the present study, results indicated that the UV maximal absorbances of the main peaks were not at the same wavelength. In order to detect more common peaks, the most appropriate wavelength was chosen as 268 nm.

### Standardization of chromatographic fingerprint

The HPLC fingerprints of 30 samples of *Codonopsis Radix* from the same origin were selected and matched by the *Similarity Evaluation System for Chromatographic Fingerprints of TCM* (Version 2004). As shown in Fig. 1, a good separation and reproducible chromatogram were achieved and 25 peaks were marked as the common peaks in the chromatograms of the 30 raw herbs. Peak 14 was identified as lobetyolin by comparing the UV spectra and retention time with the reference compound.

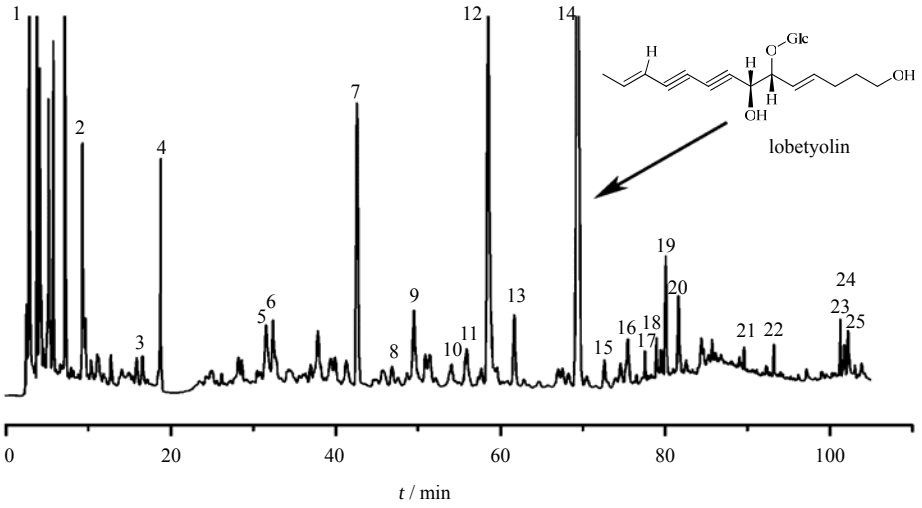


**Fig. 1 Chromatogram of *Codonopsis Radix***

### Method validation

Lobetyolin was used as a chemical marker for TLC identification of *Codonopsis Radix* in *Pharmacopoeia of People's Republic of China 2010*. In this study, the HPLC-DAD fingerprint method was explored for the authentication of *Codonopsis Radix* and also for the content of lobetyolin in 52 *Codonopsis Radix* samples. The standard curve was obtained using the linear regression method and the peak areas were plotted versus concentration. The regression equation was $y$ = 9315.2 $x$ − 228.93 ($r$ = 0.9996) in the concentration range of 0.045 — 0.900 mg/mL for the reference lobetyolin. Good linearity with a high correlation coefficient in the given calibration range was observed for lobetyolin. The limit of detection (LOD) and the limit of quantification (LOQ) were 2.2 and 7.6 μg/mL, respectively, which indicated that the analytical method was acceptable with excellent sensitivity. Variable amounts of lobeytolin ranging from 0.147 to 1.604 mg/g were determined in the 52 *Codonopsis Radix* samples. Standard addition method was used to evaluate the recovery rate of extraction protocol for lobetyolin. The sample of *Codonopsis Radix* (No. DS16) was accurately spiked with known amount of lobetyolin (1.3 mg/g), and then extracted and processed according to the procedures above. Good recovery from the analysis was obtained as (98.7 ± 2.8)% ($n$ = 3).

Intraday precision, repeatability, and stability of

the method used were determined and expressed by the RSD value of the average relative retention times (RRT) and relative peak areas (RPA) of the 25 common characteristic peaks with the respect to the reference peak (peak 14) at retention time ($t_R$) of 69.825 min. Using the optimized conditions described above, the method repeatability was obtained by assaying six replicates of identical sample. The variation of the RRT and RPA of the characteristic peaks did not exceed 1% and 5%, respectively. The intraday precision variation of the RRT and RPA of the characteristic peaks was not more than 1% and 5%, respectively, by analyzing the six injections on the same day. The stability test was performed by analyzing the sample solution on the same day at different time intervals (0, 2, 4, 6, 8, and 24 h) and the RSD values of the $t_R$ and peak areas of the characteristic peaks were both less than 5%. The similarity of these results indicated that the sample remained stable during this period.

**Similarity analysis**

Correlation coefficients of 30 samples of *Codonopsis Radix* were calculated by the *Similarity Evaluation System for Chromatographic Fingerprints of TCM* (Version 2004) (Fig. 2). The correlation coefficients of the samples were all higher than 0.82. This indicated that the chemical constituents of the 30 batches *Codonopsis Radix* samples were similar and the common pattern of the 30 test samples could be applied as a reference HPLC fingerprint.

**PCA method**

It is known that lobetyolin is one of the main constituents in chromatographic cluster of *Codonopsis Radix*. Furthermore, the contents largely differ in batches. However, the usefulness of this difference for identifying *Codonopsis Radix* is limited, since the chemical contents in herbal samples varied greatly and solid the presence of largely unknown peaks in the chromatogram. So it was by no means easy to assign the identity of *Codonopsis Radix* by visual inspection of the chromatograms.

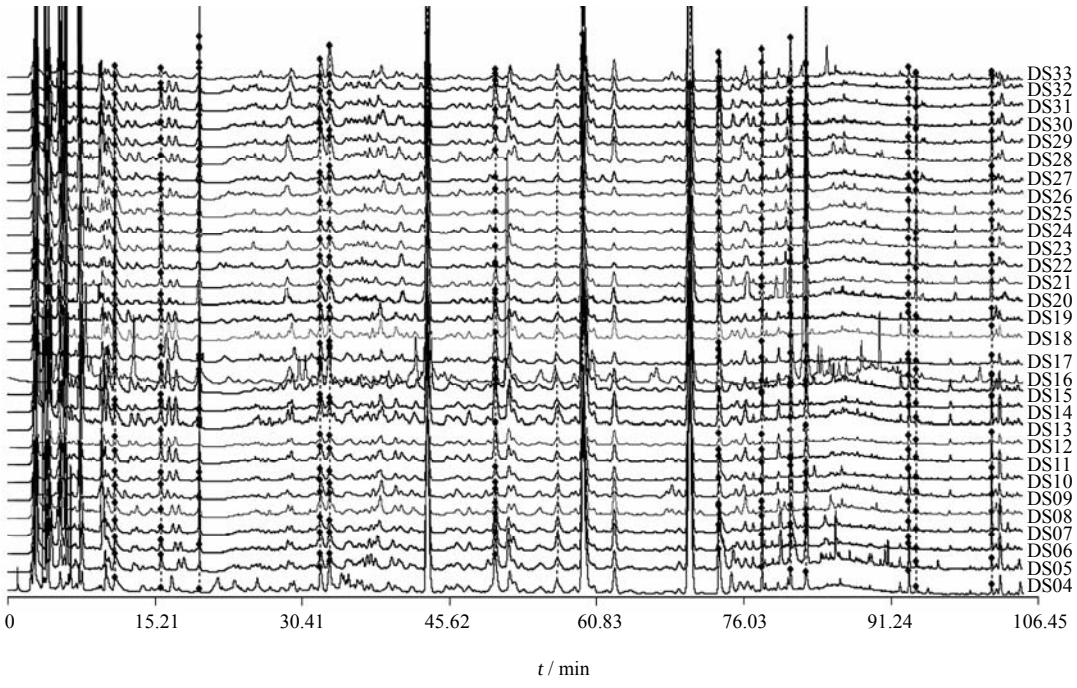


**Fig. 2 Chromatographic fingerprint of *Codonopsis Radix* collected from 30 origins**

To obtain a comprehensive view of the distinction, an unsupervised PCA method was employed to analyze the 52 *Codonopsis Radix* samples. It is shown in Fig. 3A that a trend of separation could be observed from the score plot of PCA model. In order to eliminate the effect of the inter-subject variability among the samples, the technique of OSC-PLS-DA was applied (Liu *et al*, 2011). Fig. 3B showed the score plot of OSC-PLS-DA model. A better classification result was obtained, and good and bad samples were located in different regions. The model parameters were calculated by the first two principal components that could explain 56.4% of the

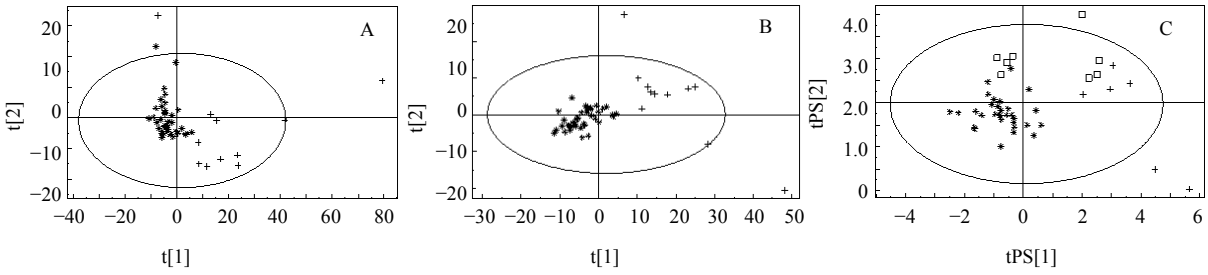


**Fig. 3 PLS-DA score plot of *Codonopsis Radix* (A), score and corresponding loading plots from OSC-PLS-DA models based on *Codonopsis Radix* samples (B), and T predicted scatter plots from *Codonopsis Radix* samples (C)**

Two groups were separated along PC1: Superior (*) *vs* Inferior (+); Superior (*), Inferior (+), and no class (□)

variables. The differences among the two classes were expressed as $R^2Y$ and $Q^2Y$, representing the percentage of the total variances within the data explained by the first two principal components. The cumulative values of $R^2Y$ and $Q^2Y$ were close to 1, indicating an excellent model, and the values of $R^2Y$ and $Q^2Y$ were 0.837 and 0.768, respectively, which are close to 1. Cross validation (seven times) and response permutation test were used to assess the models to avoid over-fitting due to chance correlation. According to Eriksson *et al* (Martinon, 2010), the values of $R^2$-intercept and $Q^2$-intercept should not respectively exceed 0.4 and 0.05, indicating that the model had no over-fitting. The values of $R^2$-intercept and $Q^2$-intercept were 0.198 and –0.441, respectively, indicating that the models were reliable in explaining and predicting the variations in *X* and *Y* matrixes. Subsequently, we evaluated the prediction levels for good and bad quality *Codonopsis Radix* models. Data from four good and four bad samples comprised the prediction set. The predicted result was shown in Fig. 3C in visual form and the correction level was 100%, which indicated that the OSC-PLS-DA model for *Codonopsis Radix* is possible to prevent the misfit samples.

**DA method**

DA method was aimed at classifying and predicting the group *Codonopsis Radix* samples based on observed characteristics of each sample. The construction of DA function was carried out considering the common peaks of each sample. This procedure generated the discriminant function based on the linear combinations of the predictor variables that provided the best discrimination between the two groups. The function was generated from the samples with the known classification; then the function could be applied to new cases with unknown classification for identification.

We collected 37 samples classified into two groups (superior and inferior), and the 25 common peaks established by the fingerprint were selected as the variables of each *Codonopsis Radix* sample to establish the discriminant function. The procedure will generate the discriminant function only by the effective predictor variables. The discriminant function of *Codonopsis Radix* generated from two classifies were as follows:

$$Func = -0.257 + 0.001 \times Var\ 2 - 0.002 \times Var\ 5 + 0.002 \times Var\ 14 + 0.001 \times Var\ 19$$

Where *Func* is the superior *Codonopsis Radix* sample and *Var* is the variable

There are only four variables in the discriminant function to generate the function, and the four variables represent the common peak areas of No. 2, 5, 14, and 19, respectively. Then we collected 15 batches of samples as ungroups for testing the discriminant function. We put the values of the four variables into the function and the sample belongs to the superior group where the calculated value of the function is less than 1. The result of DA method is shown in Table 1.

**Table 1 Results of DA**

| Average linkage | | Predicted group membership | | Total |
|---|---|---|---|---|
| | | Superior | Inferior | |
| Initial Count | Superior | 32 | 0 | 32 |
| | Inferior | 0 | 5 | 5 |
| | Ungrouped cases | 10 | 5 | 15 |
| Percentage of predicted count / % | Superior | 100 | 0 | 100 |
| | Inferior | 0 | 100 | 100 |
| | Ungrouped cases | 66.7 | 33.3 | 100 |

## Conclusion

In the present study, a simple, accurate, and reliable method was developed to evaluate the quality of *Codonopsis Radix* through the established chromatographic fingerprint and the determination of bioactive compound, lobetyolin. The HPLC method showed the good linearity, precision, and reproduciblity, and so was suitable for the quality evaluation of *Codonopsis Radix* samples. The fingerprinting analysis using similarity, PCA, and DA approaches has produced the desirable results with high accuracy.

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