# HPLC–MS Analysis of Ethanol Extract of *Corydalis yanhusuo* and Simultaneous Determination of Eight Protoberberine Quaternary Alkaloids by HPLC–DAD

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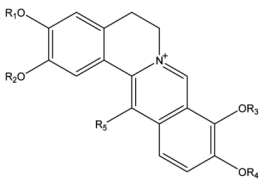
## Abstract

**An accurate and simple analysis method by high-performance liquid chromatography (HPLC)-electrospray tandem mass spectrometry (ESI-MS–MS) for the ethanol extract of *Corydalis yanhusuo* was established in this study. In this method, a $C_{18}$ column was used, and the mobile phase was water (0.2% acetic acid, 0.1% triethylamine, v/v)–acetonitrile (24:76, v/v). Under the optimal analysis conditions established in this study, eight protoberberine quaternary alkaloids in the ethanol extract of *Corydalis yanhusuo* were well separated and identified, respectively. Eight regression equations showed a good linear relationship between the peak area of each marker and concentration ($r$ = 0.9990–0.9995). The various other aspects of analysis (i.e., peak purity, precision, recovery, and repeatability) were also validated. For the eight components, the recoveries were found to be 98.9%, 99.2%, 105.0%, 97.7%, 101.4%, 98.6%, 98.5%, and 98.7%, respectively. The limits of detection were 14.6, 42.5, 22.4, 17.9, 25.4, 10.0, 18.7, 3.9 ng/mL, respectively, and the limits of quantitation were 48.6, 141.7, 74.6, 59.6, 84.8, 33.2, 62.3, and 12.9 ng/mL, respectively. The developed method is useful for the simultaneous determination of these alkaloids in a large number of ethanol extracts of *Corydalis yanhusuo*.**

## Introduction

*Corydalis yanhusuo* W.T. Wang, which is also called Rhizoma corydalis, is one of the important crude drugs in traditional Chinese medicines (TCM) (1) and has been employed as an analgesic agent for treating spastic pain, abdominal pain, menstrual pain, and pain due to injuries. It also has been widely used to promote blood circulation and treat coronary heart diseases (2,3). Alkaloids are acknowledged to be major active components in *Corydalis yanhusuo*. Amongst alkaloids, the main quaternary active principles of *C. yanhusuo* showed stronger activity than tertiary (4,5). The objective of our research was to investigate the kinds and contents of quaternary alkaloids present in *Corydalis yanhusuo*. Previous papers have reported liquid chromatography (LC)–UV or LC–mass spectrometry (MS) methods used for the qualitative determination of alkaloids in *Corydalis yanhusuo* (6,7,8). However, among them, very few quaternary alkaloids have been detected and identified. For the purpose of convenience and accuracy, in this study, a new method by high-performance liquid chromatography (HPLC)–electrospray ionization (ESI)–MS is reported for analysis of the ethanol extract of *Corydalis yanhusuo*, which used the common $C_{18}$ column and water–acetonitrile as the mobile phase. By this method, eight




| | Components | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|---|
| 1 | columbamine | $CH_3$ | H | $CH_3$ | $CH_3$ | H |
| 2 | coptisine | -$CH_2$- | | -$CH_2$- | | H |
| 3 | 13-Methyldehydrocorydalmine | $CH_3$ | $CH_3$ | $CH_3$ | H | $CH_3$ |
| 4 | Dehydrocorybulbine | $CH_3$ | H | $CH_3$ | $CH_3$ | $CH_3$ |
| 5 | 13-Methylpalmatrubine | $CH_3$ | $CH_3$ | H | $CH_3$ | $CH_3$ |
| 6 | palmatine | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | H |
| 7 | berberine | -$CH_2$- | | $CH_3$ | $CH_3$ | H |
| 8 | Dehydrocorydalmine | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ |

**Figure 1.** Chemical structures of the quaternary alkaloids 1–8.

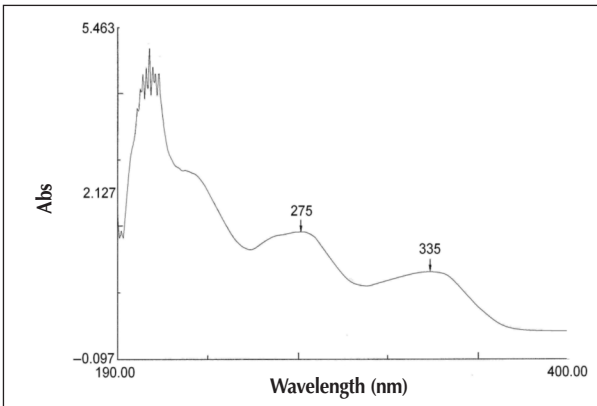



**Figure 2.** The UV-vis spectrum of the sample solution.

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protoberberine quaternary alkaloids in the ethanol extract were successfully separated and identified by ESI–MS data. Considering it was sensitive enough to detect these alkaloids with UV detector, the eight protoberberine quaternary alkaloids in the ethanol extract of *Corydalis yanhusuo* were quantitatively determined by HPLC–diode array detection (DAD), under the optimal chromatographic conditions established in this study.

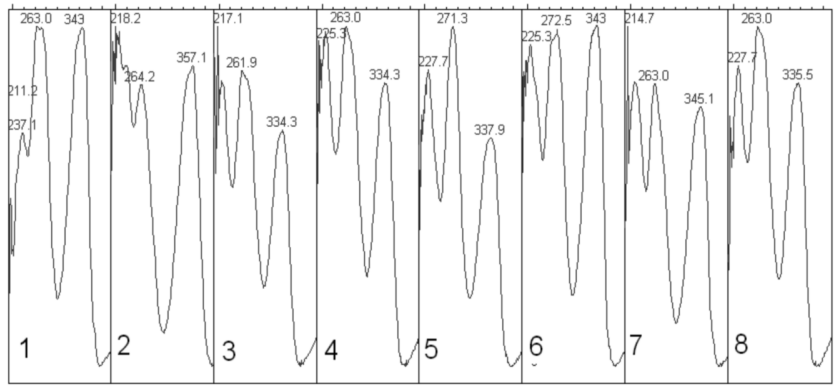


**Figure 3.** The UV-vis spectrum beteen 200 and 400 nm of each analyte. Analyte numbers for the spectrum are: columbamine, 1; coptisine, 2; 13-Methyldehydrocorydalmine, 3; Dehydrocorybulbine, 4; 13-Methylpal-matrubine, 5; palmatine, 6; berberine, 7; Dehydrocorydalmine, 8.

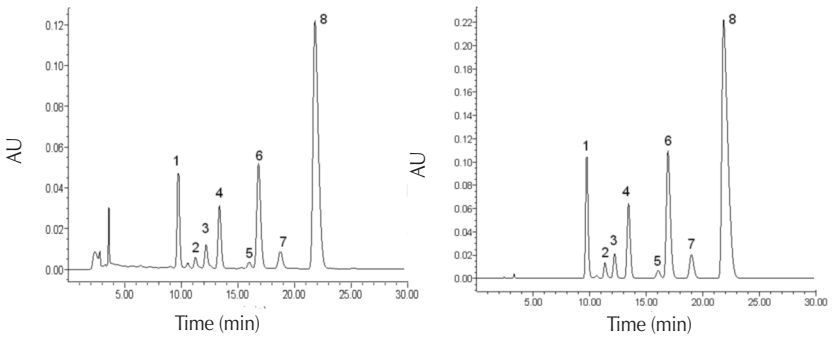


**Figure 4.** The chromatograms at 335 nm of the ethanol extract of *Corydalis yanhusuo* (A) and a standard mixture (B).

## Experimental

### Materials and chemicals

The ethanol extract and its raw materials were provided by a Pharmaceutical Factory (Beijing, china). The reference standards: columbamine, 1; coptisine, 2; 13-methyldehydrocorydalmine, 3; dehydrocorybulbine, 4; 13-methylpalmatrubine, 5; palmatine, 6; berberine, 7; and dehydrocorydalmine, 8 (99.37%, 95.01%, 99.10%, 98.35%, 98.14%, 98.15%, 99.09%, and 99.64%, respectively) were prepared from the ethanol extract of *Corydalis yanhusuo* in our laboratory in a previous study (9,10). Among these alkaloids, compound 3 was a new compound; 5 was a new natural compound (11–14); 4 was not previously found in this species. Their chemical structures (Figure 1) were identified by several spectral analyses such as UV, IR, ESI-MS, HRMS, 1HNMR, $^{13}$CNMR, HMBC, and NOESY. Acetonitrile (HPLC-grade) was purchased from Honeywell Burdick & Jackson Company (Morristonw, NJ). Other reagents were analytical grade and obtained from Beijing Chemical Reagents Company (Beijing, China). The water used was double distilled.

### HPLC-MS instrument and conditions

An Agilent HPLC system (Agilent, Palo Alto, CA) coupled to an API 3000 triple quadrupole LC–MS–MS from MDS Sciex/Applied Biosystems (Foster City, CA) was used. Data acquisition and processing were performed using Analyst software from Applied Biosystems. An ESI interface with positive mode was employed. The ESI conditions were as follows: capillary voltage = 3800 V, nebulizer gas = 8 (arbitrary units), curtain gas = 12 (arbitrary units), collision gas = 6 (arbitrary units), nebulizer current = 3 (arbitrary units), and temperature = 450°C, declustering potential = 20

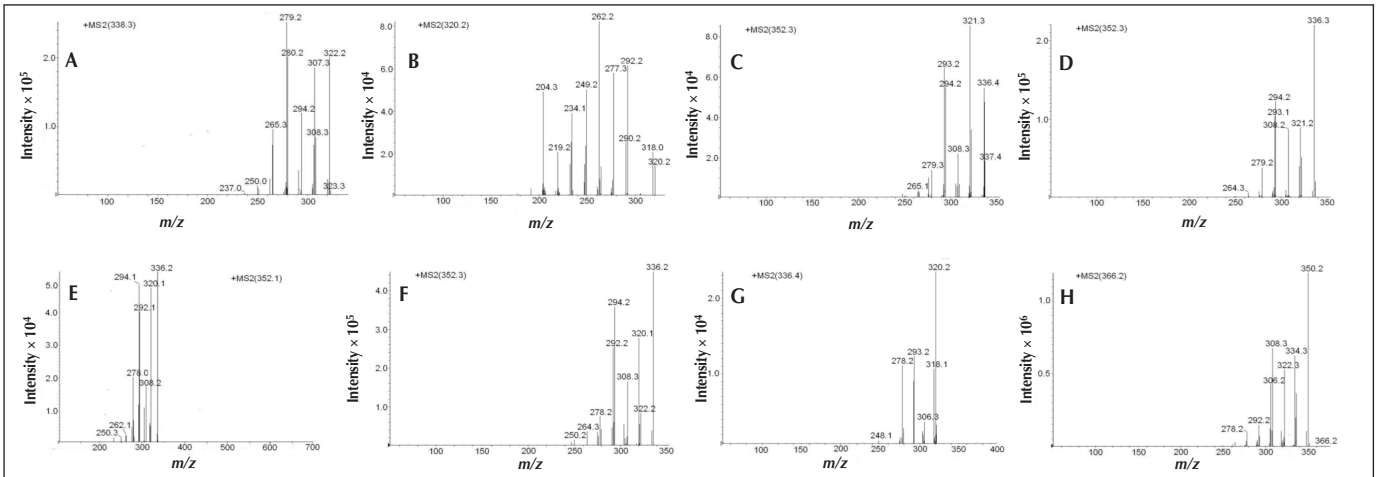


**Figure 5.** Mass spectrum of peaks from Figure 4: 1, A; 2, B; 3, C; 4, D; 5, E; 6, F; 7, G; 8, H.

(arbitrary units), focusing potential = 375 (arbitrary units), entrance potential = 10 (arbitrary units), collision energy = 50 eV. Other chromatographic conditions were: analytical column, Diamonsil $C_{18}$ column (250- × 4.6-mm i.d., 5 µm) (Dikma Technologies); column temperature, 30°C; mobile phase, water (0.2% acetic acid, 0.1% triethylamine, v/v)–acetonitrile (24:76, v/v); flow rate, 1.0 mL/min; λ = 335 nm; sample injected, 10 µL.

### UV–VIS and HPLC–DAD instrument

Absorption spectra were obtained with a Shimadzu UV-2000 spectrophotometer (Kyoto, Japan). A Waters HPLC system, consisting of a Waters 600 pump, a 2996 diode array detector (DAD), and an LC workstation equipped with Empower software for data collection (Milford, MA) was used for quantitative determination. The UV spectra were recorded from 200 to 400 nm by DAD. Other chromatographic conditions were the same as those of HPLC–MS analysis.

### Preparation of the ethanol extract

The dried tubers of the plant were extracted with 80% ethanol under reflux for 4 h, and the alcohol extract was chromatographed over a macroporous resin column with 0%, 50%, and 95% EtOH in $H_2O$ as eluants. The fraction eluted with 50% EtOH in $H_2O$ was concentrated and dried under reduced pressure to obtain the ethanol extract.

### Preparation of the sample solution

The sample solution was extracted from the ethanol extract with 80% methanol by ultrasonic extraction for 5 min at room temperature. Sample solutions were filtered though a 0.45-µm Millipore filter (Kaide, Tianjin, China) and injected for HPLC analysis.

**Table I. Regression Analysis of Calibration Curves of Eight Alkaloids**

| Alkaloids | $t_R$ (min) | Regression equation | Correlation coefficient (*r*) | Linear range (µg/mL) |
|---|---|---|---|---|
| 1 | 9.62 | $y = 20726x - 21191$ | 0.9995 | 3.96–79.20 |
| 2 | 11.24 | $y = 11721x - 4662.7$ | 0.9993 | 0.99–19.86 |
| 3 | 12.13 | $y = 19555x - 7706.1$ | 0.9992 | 1.00–19.96 |
| 4 | 13.38 | $y = 15552x - 15718$ | 0.9995 | 3.99–79.84 |
| 5 | 16.07 | $y = 15038x - 2833.5$ | 0.9995 | 0.51–10.25 |
| 6 | 16.98 | $y = 31945x - 37681$ | 0.9995 | 0.42–8.32 |
| 7 | 19.04 | $y = 26655x - 8742.2$ | 0.9994 | 0.99–19.90 |
| 8 | 22.15 | $y = 31994x - 102557$ | 0.9995 | 12.69–253.75 |

* *y* = Peak area

**Table II. Recovery and Precision of the Method (*n* = 6)**

| Alkaloids | Recovery(%) | | Precision RSD(%) | |
|---|---|---|---|---|
| | Mean | RSD | Intraday | Interday |
| 1 | 98.9 | 2.0 | 0.9 | 1.3 |
| 2 | 99.2 | 1.7 | 0.6 | 0.9 |
| 3 | 105.0 | 1.8 | 0.9 | 0.9 |
| 4 | 97.7 | 1.2 | 1.1 | 1.3 |
| 5 | 101.4 | 1.9 | 2.2 | 2.4 |
| 6 | 98.6 | 1.1 | 1.0 | 1.2 |
| 7 | 98.5 | 0.8 | 0.7 | 1.0 |
| 8 | 98.7 | 1.4 | 1.0 | 1.7 |

## Result and Discussion

### Selection of the HPLC conditions

*Selection of the detection wavelength*

In this study, the UV detection wavelength was set at 335 nm. The UV spectra of the sample solution (Figure 2) showed strong absorptions at 275 and 335 nm. And the UV spectra of eight compounds, 1–8, in the ethanol extract (Figure 3) showed that there were very strong absorptions at 211.2–227.7, 261.9–272.5, and 334.3–357.1 nm. For the sensitivities and separations of eight alkaloids, the UV detection wavelength was set at 335 nm.

*Effect of the mobile phase composition*

The mobile phase was water (0.2% acetic acid, 0.1% triethylamine, v/v)–acetonitrile (24:76, v/v). To study the effect of the mixture ratio on the separation, some HPLC conditions were set as follows: column temperature, 30°C; λ = 335 nm; flow rate, 1.0 mL/min. Several solvent systems based on the various mixtures of acetonitrile or methanol with water and ratios were tested in order to achieve optimal separation in a relatively short time. With respect to the separation efficiency and sensitivity, better results were achieved with acetonitrile instead of methanol. However, the mixtures consisting only of water and acetonitrile were not sufficient to separate completely for the components in the ethanol extract, and some peaks overlapped. The use of 0.2% acetic acid and 0.1% triethylamine in the solvent system could reduce the ionization of alkaloids and then give the more symmetrical peak and a better separation (Figure 4). Therefore, the

**Table III. Data of LOD and LOQ**

| Alkaloids | LOD(ng/mL) | LOQ (ng/mL) |
|---|---|---|
| 1 | 14.6 | 48.6 |
| 2 | 42.5 | 141.7 |
| 3 | 22.4 | 74.6 |
| 4 | 17.9 | 59.6 |
| 5 | 25.4 | 84.8 |
| 6 | 10.0 | 33.2 |
| 7 | 18.7 | 62.3 |
| 8 | 3.9 | 12.9 |

**Table IV. Percent Contents of Eight Alkaloids in the Extract of *Corydalis yanhusuo* (%)**

| Alkaloids | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| 1 | 4.47 | 3.59 | 4.12 |
| 2 | 0.82 | 0.81 | 0.86 |
| 3 | 1.57 | 0.95 | 1.50 |
| 4 | 4.37 | 3.57 | 4.04 |
| 5 | 0.75 | 0.10 | 0.55 |
| 6 | 4.38 | 3.84 | 4.08 |
| 7 | 0.82 | 0.72 | 0.78 |
| 8 | 15.07 | 13.08 | 14.00 |

optimum mobile phase was achieved with water (0.2% acetic acid, 0.1% triethylamine, v/v)–acetonitrile (24:76, v/v).

**HPLC–MS qualitative analysis**

In order to identify the structures of main constituents in the ethanol extract of *Corydalis yanhusuo*, the sample was analyzed by an HPLC–MS techniques. ESI in both negative and positive mode were tried. The results showed that ESI in positive mode was sensitive to quaternary alkaloids. All alkaloids marked were well detected and exhibited their molecular ions [M]+ (shown in Figure 5). By careful studying on the molecular ions and fragment ions of these compounds and comparing with the MS data of standards, 8 common peaks (Figure 4) in the extract of *Corydalis yanhusuo* were identified.

**HPLC quantitative analysis**

*Calibration and linearity*

The calibration curves were obtained by plotting the peak area against the concentration of corresponding standards. Equations for a linear least square regression fit of each analyte of interest along with the concentration range and correlation coefficients are provided in Table I.

*Precision*

To assess the precision of the method, the sample solution was injected six times within 24 h and over a 3-day analysis period. The coefficient variations of intra- and inter-day were both less than 3.0% (Table II).

*Recovery*

The recovery of the method was performed by adding a known amount of the standard amounts to the material and then undergoing analysis according to the method. The results of the recoveries of compounds 1–8 ranged from 98% to 105%. The relative standard deviation (RSD) recoveries of the eight alkaloids ranged between 0.8% and 2.0% (Table II).

*Limit of detection and quantitation*

The use of a DAD starting with the maximum noise assisted with the rapid and accurate investigation of the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ values were calculated for compounds 1–8 based on 3 and 10 times of the noise level, respectively. The values of the LOD and LOQ are given in Table III.

*Determination of contents*

The contents of eight alkaloids in the ethanol extract of *Corydalis yanhusuo* were determined in three samples, simultaneously. The percent contents of compounds 1–8 in the ethanol extract of *Corydalis yanhusuo* are reported in Table IV.

## Conclusion

In the reported method, eight protoberberine quaternary alkaloids in the ethanol extract of *Corydalis yanhusuo* can be successfully separated, identified, and quantitated by HPLC–ESI–MS and HPLC–DAD. The method, which provides baseline separation of the test alkaloids, is simple, rapid, and precise. The procedure reported here could be used for the rapid screening of the *Corydalis yanhusuo* plant for genotypic quality assessment, drug analysis, etc.

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