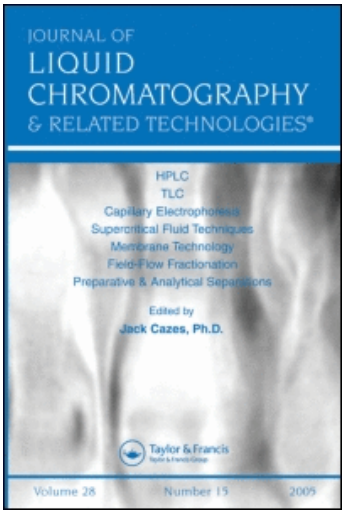


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Zhi-guo Ma[a]; Zhong-lin Yang[a]; Ping Li[a]; Cheng-hua Li[a]
[a] Key Laboratory of Modern Chinese Medicines, Department of Traditional Chinese Medicine, China Pharmaceutical University, Nanjing, P. R. China

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# Simultaneous Determination of Eight Phenylethanoid Glycosides in Different Species of the Genus *Cistanche* by High Performance Liquid Chromatography

**Zhi-guo Ma, Zhong-lin Yang, Ping Li, and Cheng-hua Li**

Key Laboratory of Modern Chinese Medicines, Department of Traditional Chinese Medicine, China Pharmaceutical University, Nanjing, P. R. China



**Abstract:** Although Traditional Chinese Medicine has been used for several thousands of years in clinical practice, the efficacy, quality, and safety of many medical herbs remain uncertain. There is an increasing need for improving analytical methods that can be used for identification of active components and quality control of traditional Chinese medicine. In the present study, a high performance liquid chromatographic (HPLC) method was developed to simultaneously determine eight phenylethanoid glycosides in different species of the genus *Cistanche*, namely acteoside, 2′-acetylacteoside, cistanoside A, cistanoside C, cistanoside F, echinacoside, isoacteoside, and tubuloside A. The HPLC assay was performed on an Akasil $C_{18}$ column with a gradient elution of methanol and 0.1% aqueous methanoic acid for 70 min. The detection wavelength was 330 nm. All of the compounds showed good linearity ($R^2 \geq 0.9991$). The method was reproducible with intra- and inter-day variations of less than 5%. The recovery of the assay was in the range of 94.43–105.44%. The method was successfully applied to the quantification of all the eight constituents in the test samples. The results indicate that the developed assay can be considered as a suitable method for the quality control of the genus *Cistanche*.

**Keywords:** *Cistanche*, Determination, Phenylethanoid glycosides, RP-HPLC

Correspondence: Zhong-lin Yang, China Pharmaceutical University G-03, 1 Shennong Road, Nanjing 210038, P. R. China. E-mail: mzg79@hotmail.com

## INTRODUCTION

The use of traditional medicine in China has a history of several thousands of years and various preventive and therapeutic effects have been shown with various preparations of Traditional Chinese Medicine (TCM). Recently, modernization of TCM has been suggested, focusing on the evidence based on therapeutic and preventive efficacy, better understanding of mechanisms of action, confirmation of active components, and improved quality control of TCM products. However, there are limited analytical methods available for carrying out in depth investigations. Our laboratory has a long standing interest in developing a novel analytical method for TCM analysis and quality control.

*Cistanche* (Orobanchaceae), named 'Roucongrong' in Chinese, is a well known traditional Chinese medicine.[1] It is obtained from various species of the genus *Cistanche*, mainly rhizomes of *Cistanche deserticola* Ma and *Cistanche tubulosa* (Schrenk) Wight, which are two of the most commonly prescribed herbs in traditional Chinese medicine.[2] The stem of the herbaceous *Cistanche* is used to make a medicinal preparation to produce sedation, pain relief, and immunostimulation.[3–5] Previous pharmacological studies have shown that ethanol extracts of *C. deserticola* have anti-aging effects, enhance immune system function, and exert sedative activity.[6,7] Aqueous extracts increase DNA synthesis and SOD activity, and eliminate free radicals.[8] The *n*-butanolic fraction of *C. deserticola* is superior to the aqueous fraction in its anti-nociceptive and anti-inflammatory effects.

A number of compounds including phenylethanoid glycosides, iridoids, and lignans have been isolated from the various plants of the *Cistanche* family. Among them, the phenylethanoid glycosides are proven to be responsible for the various biological activities of the herb.[9–11] Some phytochemical studies indicate that phenylethanoid glycosides from *C. deserticola* have antioxidant, hepatoprotective, and neuroprotective activities.[12–14] Acteoside exhibits estrogenic/antiestrotiestrogenic, anti-inflammatory, and antioxidant activities.[15] Echinacoside has anti-inflammatory effects and enhances wound healing.[16] Echinacoside, acteoside, and cistanoside F have vasorelaxant activities.

Hence, quantification of the phenylethanoid glycosides from preparations made of plants in the genus *Cistanche* would be of great significance for the evaluation of the quality of the herb.[17–23] However, previous studies mainly focus on the quantitative determination of single or a few constituents in the genus *Cistanche* by HPLC or HPLC-MS, which may not provide sufficient data for quality control or profiling of this herb. Considering a wide use of this herb, there is a need for a better quality control method. The purpose of this study was to develop a simple and feasible method for the simultaneous quantification of the

eight most important phenylethanoid glycosides isolated from *Cistanche*. The selection of these representative phenylethanoid glycosides in the development of this quality control method was based on their pharmacological activities. Our results not only provide a basis for the quality control of this herbal medicine, but also set an example for the development of modern quality control methods for other traditional Chinese medicines.

## EXPERIMENTAL

### Chemicals and Materials

We used analytical grade methanoic acid (Nanjing Chemical Regent No.1 Factory, Jiangsu, China) and HPLC grade methanol (Hanbang Sci. & Tech. Co., Ltd., Jiangsu, China) for the entire experiment. The deionized water was obtained using a Milli-Q Water purification system (Millifore, MA, USA). All other organic solvents used in this study were of analytical grade and were purchased from Nanjing Chemical Regent Company (Nanjing, China). Herbal samples were collected from the Xingjiang and Neimenggu Provinces of China in 2005. The standards for acteoside and echinacoside were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The standards for 2′-acetylacteoside, cistanoside A, cistanoside C, cistanoside F, isoacteoside, and tubuloside A were isolated previously from 70% ethanol extracts of *C. deserticola* and *C. tubulosa*.[24–27] Their structures were identified by direct comparison of their $^{1}H$ NMR, $^{13}C$ NMR, and MS spectral data with those reported in the literature, and their purities were determined to be not less than 95% by HPLC analysis. Structures of the eight compounds are shown in Figure 1.



### Instrument and Chromatographic Conditions

The analyses were performed using Shimadzu HPLC instrument (Shimadzu Corporation, Kyoto, Japan) consisting of a 20 μL injector loop, a solvent delivery module (LC-20AT) with a double plunger reciprocating pump, and a SPD-20A UV detector. Data was collected and processed using a N2000 chromatography Data System (Zhejiang University Zhida Information and Technologies Corporation, Hangzhou, China). The separation was carried out on an Akasil $C_{18}$ column (150 mm × 4.6 mm, 5 μm). The mobile phase consisted of solvent A (methanol) and solvent B (0.1% aqueous methanoic acid, v/v). Gradient

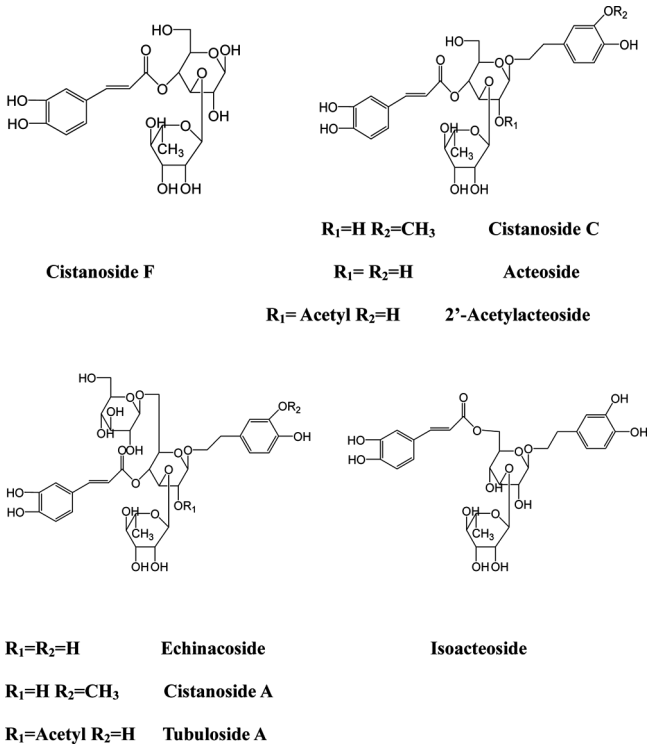


*Figure 1.* Structures of eight phenylethanoid glycosides.



elution was as follows: initial 0–36 min, linear change from A-B (20:80, v/v) to A-B (30:70, v/v); 36–42 min, maintain A-B (30:70, v/v); 42–48 min, linear change to A-B (35:65, v/v); 48–60 min, linear change to A-B (40:60, v/v). The UV absorption was monitored at 330 nm. The column temperature was set at 30°C and the flow rate was 1.0 mL/min.

The stock solution containing all eight reference standards was prepared by dissolving the reference standards in 60% methanol to a final concentration of 232.0 μg/mL for acteoside, 269.6 μg/mL for 2′-acetylacteoside, 56.8 μg/mL for cistanoside A, 61.2 μg/mL for cistanoside C, 48.8 μg/mL for cistanoside F, 882.0 μg/mL for echinacoside, 106.4 μg/mL for isoacteoside, and 160.4 μg/mL for tubuloside A, respectively. Then the stock solution mixture was diluted to appropriate concentrations to establish the calibration curves. Each calibration curve consisted of six different concentrations and was performed in triplicate. All calibration curves were constructed from peak areas of the reference standards versus their concentrations.

### Sample Preparation

The dried powders of samples (0.5 g)[1] were accurately weighed and extracted by ultrasonication with 25 mL 70% aqueous methanol solution for 70 min. Then, the resultant mixture was adjusted to the original weight, and aliquots of the supernatant were filtered through a 0.45 μm membrane before HPLC injection.

## RESULTS AND DISCUSSION

### Extraction Method

In order to obtain satisfactory extraction efficiency, the best extraction solvent, extraction method, and extraction time were investigated. To determine the best extraction solvent, we evaluated 30% methanol, 50% methanol, 70% methanol, and 100% methanol. As shown in Figure 2, 70% methanol was the most suitable extraction solvent. Ultrasonic and reflux extraction were also compared, and the ultrasonic method was found to lead to better extraction. Finally, 0.5 g samples were extracted with 25 mL 70% methanol by ultrasonication for 30, 50, 70, and 90 min, respectively, to determine optimal extraction time. As shown in Figure 3, the compounds were almost completely extracted within 70 min. Hence, 70 min was chosen as optimal extraction time.



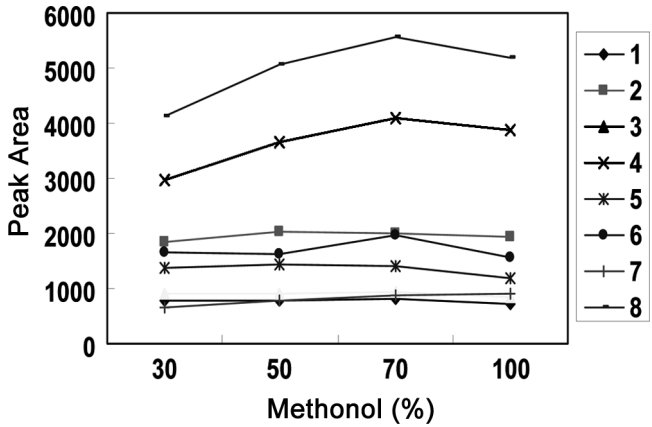


*Figure 2.* Extraction efficiency of different solvents: (1) cistanoside F; (2) echinacoside; (3) cistanoside A; (4) acteoside; (5) tubuloside A; (6) isoacteoside; (7) cistanoside C; (8) 2′-acetylacteoside.

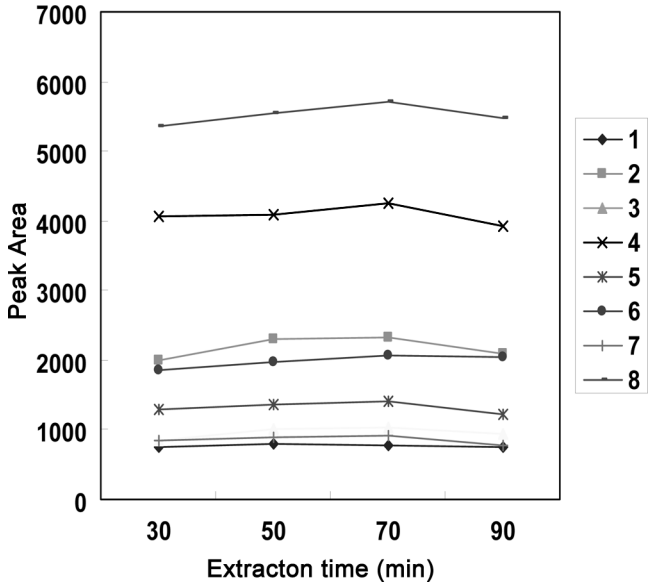


*Figure 3.* Extraction efficiency at different times: (1) cistanoside F; (2) echinacoside; (3) cistanoside A; (4) acteoside; (5) tubuloside A; (6) isoacteoside; (7) cistanoside C; (8) 2′-acetylacteoside.



## Optimization of Chromatographic Conditions

To obtain chromatograms with a good separation and resolution of adjacent peaks within a short analysis time, the mobile phase, column temperature, and flow rate were optimized. The mobile phase was prepared to be acidic in order to suppress the ionization of phenolic hydroxyl groups. This acidification was beneficial, leading to better retention times. Different mobile phase compositions (such as $CH_3$ CN–$H_2O$–HOAc, $CH_3OH$–$H_2O$–HOAc, and $CH_3OH$–$H_2O$–methanoic acid) were evaluated to determine the best mobile phase to separate the investigated components. $CH_3$ OH and water containing 0.1% methanoic acid was chosen as the eluting solvent system, because it was possible to achieve efficient separation of peaks and desired peak shapes using this solvent system. Under isocratic elution modes, the eight compounds could not be separated effectively. The gradient elution mode was, therefore, used and afforded effective separation of the eight compounds. The effect of temperature on the separation was also investigated in the range of 25–40°C; 30°C was found to be optimal. The most suitable flow rate was found to be 1.0 mL/min.

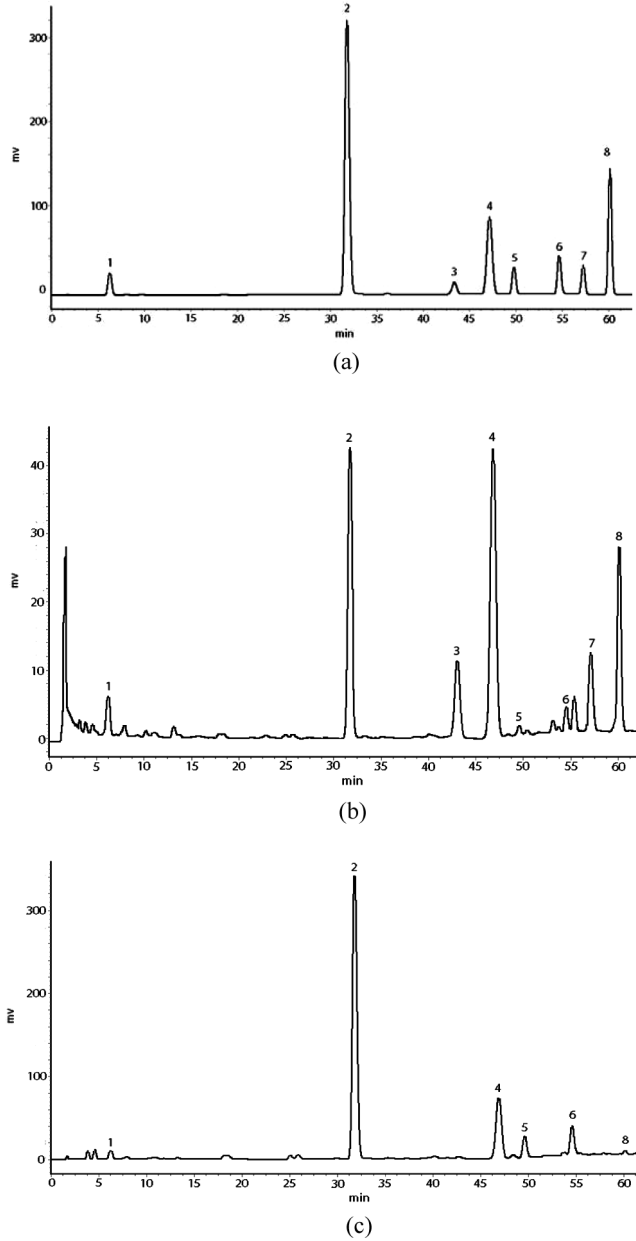


***Figure 4.*** HPLC chromatograms of standard mixture (a) *Cistanche deserticola* (b) *Cistanche tubulosa* (c). (1) cistanoside F; (2) echinacoside; (3) cistanoside A; (4) acteoside; (5) tubuloside A; (6) isoacteoside; (7) cistanoside C; (8) 2′-acetyl-acteoside.

***Table 1.*** Calibration curves of the eight constituents of *Cistanche*

| Compound | Regression equation | $R^2$ | Linear range (μg/mL) | LOD (μg/mL) | LOQ (μg/mL) |
|---|---|---|---|---|---|
| Acteoside | y = 40249x–139523 | 0.9991 | 9.28–232.00 | 0.093 | 0.371 |
| 2′-Acetylacteoside | y = 31910x–23698 | 0.9998 | 10.78–269.60 | 0.054 | 0.194 |
| Cistanoside A | y = 27391x–10813 | 0.9995 | 2.27–56.80 | 0.114 | 0.409 |
| Cistanoside C | y = 37151x–20022 | 0.9995 | 2.45–61.20 | 0.082 | 0.294 |
| Cistanoside F | y = 35440x–11077 | 0.9999 | 1.95–48.80 | 0.098 | 0.390 |
| Echinacoside | y = 29437x–227920 | 0.9991 | 35.28–882.00 | 0.106 | 0.353 |
| Isoacteoside | y = 30092x–48376 | 0.9994 | 4.26–106.40 | 0.106 | 0.383 |
| Tubuloside A | y = 13602x–17413 | 0.9992 | 6.42–160.40 | 0.160 | 0.577 |

y, peak area; x, concentration of compound (μg/ml); limit of detection, S/N = 3; limit of quantification, S/N = 10.



On the basis of the UV spectra of the eight components recorded from 200 nm to 370 nm, 330 nm was selected for monitoring. Chromatograms of standard mixture (a) and samples (b, c) are shown in Figure 4.

## Linearity and the Limit of Detection

Linear regression analysis for each of the eight compounds was performed by the external standard method. Under the optimized chromatographic conditions, all 8 calibration curves showed good linearity ($R^2 \geq 0.9991$)

***Table 2.*** Precison of the method for quantifying eight constituents of *Cistanche*

| Compound | Concentration (μg/mL) | R.S.D. (%) | |
|---|---|---|---|
| | | Intra-day precision | Inter-day precison |
| Acteoside | 92.80 | 0.79 | 3.28 |
| 2′-Acetylacteoside | 107.84 | 0.43 | 3.52 |
| Cistanoside A | 22.72 | 0.74 | 3.03 |
| Cistanoside C | 24.48 | 0.92 | 4.09 |
| Cistanoside F | 19.52 | 0.18 | 2.43 |
| Echinacoside | 352.80 | 0.08 | 1.23 |
| Isoacteoside | 42.56 | 0.44 | 2.10 |
| Tubuloside A | 64.16 | 0.54 | 4.92 |

R.S.D. (%) = (standard deviation/mean) × 100.

(Table 1). The limit of detection (LOD) and limit of quantification (LOQ) were in the range of 0.054–0.160 and 0.194–0.577 μg/mL, respectively, for the eight compounds.

## Precision and Stability

The intra- and inter-day variations were measured to determine the precision of the method. The intra-day variability was examined for five individual samples on 1 day, and inter-day variability was determined on 3 independent days. The results are presented in Table 2. The relative standard deviations were 0.08–0.92% and 1.23–4.92%, respectively. To examine the stability of the compounds, the same sample solution was analyzed at different times for 24 h when the solution was stored at room temperature. The R.S.D. values of the peak areas were all lower than 1.26%, suggesting that the compounds were sufficiently stable and that they can be stored for 1 day prior to analysis, if necessary.



## Accuracy

To further evaluate the accuracy of the method, a recovery test was performed by spiking known quantities of the mixed standard solution into *C. deserticola* samples of known concentrations. The resultant samples were then extracted and analyzed with the described HPLC method. The accuracy was calculated using the value of the detected samples, versus added concentrations. The recovery for the method was in the range of 94.43–105.44%, with R.S.D. less than 3.06% (Table 3). Considering these results, the method was deemed to be accurate.

***Table 3.*** Analysis of the recovery of compounds

| Compound | Amound added (mg) | Amount detected (mg) | Recovery (%) | R.S.D. (%) |
|---|---|---|---|---|
| Acteoside | 1.114 | 1.052 | 94.43 | 1.77 |
| 2′-Acetylacteoside | 2.696 | 2.630 | 97.55 | 1.82 |
| Cistanoside A | 0.568 | 0.560 | 98.59 | 2.29 |
| Cistanoside C | 0.294 | 0.310 | 105.44 | 2.15 |
| Cistanoside F | 0.234 | 0.232 | 99.14 | 1.45 |
| Echinacoside | 0.882 | 0.905 | 102.61 | 1.12 |
| Isoacteoside | 0.106 | 0.102 | 96.23 | 2.39 |
| Tubuloside A | 0.160 | 0.161 | 100.62 | 3.06 |

n = 5, R.S.D. (%) = (standard deviation/mean) × 100.

***Table 4.*** Phenylethanoid glycoside contents in the genus *Cistanche*

| | Concentration of Constituent (%)[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample (%) | Cistanoside F | Echinacoside | Cistanoside A | Acteoside | Tubuloside A | Isoacteoside | Cistanoside C | 2′-acetylacteoside | Total |
| *C. deserticola*[b] | 0.046±0.001 | 0.378±0.006 | 0.197±0.005 | 0.433±0.000 | 0.026±0.001 | 0.032±0.001 | 0.083±0.001 | 0.205±0.002 | 1.400±0.008 |
| *C. deserticola*[b] | 0.103±0.002 | 0.349±0.005 | 0.178±0.005 | 0.534±0.008 | 0.060±0.000 | 0.050±0.001 | 0.110±0.002 | 0.838±0.006 | 2.222±0.018 |
| *C. deserticola*[b] | 0.063±0.001 | 0.649±0.011 | 0.246±0.007 | 0.173±0.002 | 0.046±0.001 | 0.045±0.001 | 0.125±0.002 | 0.119±0.001 | 1.466±0.010 |
| *C. deserticola*[b] | 0.018±0.001 | 0.320±0.005 | 0.251±0.007 | 0.037±0.001 | <LOQ | <LOD | 0.043±0.001 | 0.006±0.000 | 0.675±0.009 |
| *C. deserticola*[c] | 0.077±0.002 | 0.168±0.003 | 0.054±0.002 | 0.225±0.003 | 0.051±0.001 | 0.021±0.000 | 0.031±0.001 | 0.478±0.004 | 1.105±0.008 |
| *C. deserticola*[c] | 0.053±0.001 | 0.611±0.015 | 0.102±0.003 | 0.396±0.005 | 0.029±0.001 | 0.019±0.001 | 0.059±0.002 | 0.442±0.005 | 1.711±0.020 |
| *C. tubulosa*[c] | 0.158±0.004 | 5.511±0.104 | <LOD | 0.492±0.005 | 1.111±0.016 | 0.170±0.003 | <LOD | 0.028±0.001 | 7.470±0.082 |
| *C. tubulosa*[c] | 0.070±0.002 | 3.285±0.072 | <LOD | 0.718±0.008 | 0.536±0.008 | 0.332±0.006 | <LOD | 0.029±0.001 | 4.970±0.056 |
| *C. tubulosa*[c] | 0.112±0.002 | 3.833±0.085 | <LOD | 0.561±0.008 | 0.812±0.010 | 0.257±0.004 | <LOD | 0.023±0.001 | 5.598±0.071 |

[a] Data were expressed as mean ± S. D. of three experiments.

[b] Samples collected from Neimenggu province.

[c] Samples collected from Xinjiang province.

### Sample Analyses

The optimized analytical method was then applied to simultaneously determine the eight constituents in six *C. deserticola* and three *C. tubulosa* samples obtained from Neimenggu and Xinjiang provinces. Representative chromatograms are shown in Figure 4. The content of each compound in 9 samples was quantified. There were substantial differences in the amounts of the eight phenylethanoids glycosides in the two species, suggesting that each medicinal plant has its own chemical characteristics. There were 2–11-fold variations in the concentrations of the 8 investigated phenylethanoids glycosides between the two species (the highest; *C. tubulosa*, 4.970–7.470%) and (the lowest; *C. deserticola*, 0.675–2.222%). We observed that echinacoside was the dominant compound in all of the *C. tubulosa* samples. Its content ranged from 3.285 to 5.511%, making up 66.1–73.8% of the total amount of the eight compounds. In all of the *C. deserticola* samples, its content ranged from 0.168 to 0.649%, with echinacoside making up 15.2–47.4% of the total amount of the eight compounds. In all of the *C. deserticola* samples, the amount of Cistanoside A ranged from 0.054–0.251% and Cistanoside C ranged from 0.031–0.125%; while in *C. tubulosa* samples, Cistanoside A and Cistanoside C were not detected. Therefore, it appears that the characteristics of the constituents, especially cistanoside A and cistanoside C can be used as markers to differentiate between extract from *C. deserticola* and *C. tubulosa*.



## CONCLUSIONS

In the present study, we developed and validated a simple, rapid, and accurate HPLC method for the determination of bioactive constituents extracted from plants in the genus *Cistanche*. To our best knowledge, this represents the first report of the simultaneous quantification of these eight major constituents in this herb. The HPLC method was also successfully applied to analyze samples from herbs of different species, revealing differences in the chemical characteristics of two species of the genus *Cistanche* that could be used to differentiate extract from the plants. Based on their chemical characteristics, the identification and quality of the two species can be controlled, helping to ensure the safety and efficacy of medicines made from these plants. The concept frame, principle, and practice employed in this study may have a value in developing analytical methods for other TCMs in the future.

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Manuscript 6325