



## Study on quality standards for *Chimonanthus nitens*

B. Zhou, S.-Z. Liu, M.-J. Li, Z.-H. Chen, P. Wang and D. Cheng

School of Pharmacy, Jiangxi Science and Technology Normal University,  
Nanchang, China

Corresponding author: B. Zhou  
E-mail: tju\_zhoubin@163.com

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**ABSTRACT.** *Chimonanthus nitens* Oliv. is a commonly used traditional Chinese medicine. Terpenoids, flavonoids, and coumarins are usually considered its main bioactive ingredients. Thus, qualitative and quantitative analyses of these compounds are crucial in quality control studies of *Chimonanthus nitens*. In this study, five compounds were identified by double-development thin layer chromatography (TLC) and the content of four compounds was determined by high performance liquid chromatography; the detection wavelength was set to 344 nm and the column temperature was 40°C. All calibration curves showed good linear regression ( $R^2 > 0.9995$ ). The average recoveries ranged from 97.06 to 104.44%. The RSD was below 4.2%. Four compounds remained stable over 24 h and the relative standard deviation (RSD) of the precision of their measurement was less than 1.5%. The developed method was reproducible, sensitive, and simple, and could be used for quality control of *Chimonanthus nitens*.

**Key words:** *Chimonanthus nitens*; TLC; HPLC; Scopoletin; Scoparone

## INTRODUCTION

*Chimonanthus nitens* (family Calycanthaceae), a specific genus in China, has been used to treat colds and influenza for centuries. It is mainly found in Dexing, Wuyuan of Jiangxi province and Huizhou in the Anhui province. Previous studies have reported that the dry leaf extract shows obvious bioactivities, including cough relief, and anti-inflammatory, anti-pyretic, and anti-bacterial properties (Diao et al., 2002; Li and Shu, 2009). *C. nitens* is rich in volatile oils (Zhan and Xu, 2006; Xu et al., 2006), alkaloids, flavonoids, and coumarins (Xiao et al., 2005; Sun et al., 2009; Shu et al., 2010). Currently, quality control analysis of *C. nitens* is an area of research still undergoing investigation (Li et al., 2010).

Since the polarity of different compounds varies due to the diversity of their molecular structures, simultaneous analysis by one method is difficult. Thin layer chromatography (TLC) is usually used for qualitative analysis and high performance liquid chromatography (HPLC) is used to determine the contents of different components. In order to improve the quality of this kind of herb, double-development high performance thin layer chromatography (HPTLC) was used to identify three terpenoids (cineole,  $\beta$ -caryophyllene, linalool) and two coumarins (scopoletin, scoparone) in this study. HPLC was used to determine the contents of four kinds of compounds; three coumarins (scopoletin, isofraxidin, coparone) and one flavonoid (rutin). The developed method could be applied to quality control of this herb.

## MATERIAL AND METHODS

### Chemicals and materials

Linalool,  $\beta$ -caryophyllene, and cineole were obtained from International Laboratory (Lexington, USA). Scopoletin and isofraxidin were purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China), and scoparone and rutin were isolated and identified in our laboratory. *C. nitens* was bought from Wuyuan (Jiangxi, China). They were identified by associate Professor ke-zong Deng (Jiangxi University of Traditional Chinese Medicine). Double distilled water was prepared using a Millipore Milli Q-Plus system (Millipore, Billerica, MA, USA). Acetonitrile and methanol were bought from Uni-Chem (Belgrade, Serbia and Montenegro).

### Instrumentation

HPLC analysis was carried out on an Agilent HPLC instrument (Agilent Technologies, USA) equipped with a G1312A dual pump, G1332A online degasser, G1316A column temperature controller, G1313A automatic injector, G1315B diode array detector, and Agilent Technologies ChemStation software. The analytical column was an Eliter Hypersil ODS<sub>2</sub> column (250 x 4.6 mm, 5  $\mu$ m) along with an Agilent C<sub>18</sub> guard column (12.5 x 4.6 mm I.D., 5  $\mu$ m).

### Chromatography

The mobile phase consisted of water (A) and acetonitrile (B), and the flow rate was 1 mL/min. The following gradient elution program was adopted: 12% B between 0-26 min, 12 to 13.5% B between 26-28 min, held at 13.5% B from 28-40 min, 13.5 to 12% B during 40-

45 min, and held at 12% B between 45-60 min. The injection volume was 10  $\mu$ L, the column temperature was 40°C, and 344 nm was used as the detection wavelength.

Samples and standards were spotted on silica gel plates. The width of the spots was 7 mm and the space between two spots was 8 mm. The migration distance was 50 mm with chloroform and methanol (9:1, v/v). After development, the plate was dried by a blower and migrated to 100 mm with a mixture of petroleum ether and ethyl acetate (10:1). The plate was viewed under 365 nm light, then colorized with 1% vanillin-H<sub>2</sub>SO<sub>4</sub> solution and warmed at 110°C.

### Standard preparation

For HPLC, stock solutions of scopoletin (1.133 mg/mL), isofraxidin (1.013 mg/mL), rutin (1.502 mg/mL), and scoparone (1.222 mg/mL) were prepared by dissolving definite amounts of reference compounds in methanol. Blended reference solutions were prepared by mixing the stock solutions with methanol; the concentrations of the four components were 56.65, 24.312, 90.12, and 24.44  $\mu$ g/mL, respectively. Working solutions (1, 2, 4, 6, and 8 mL) were diluted to 10 mL with methanol to obtain calibration standards. The standard solutions were filtered through a 0.45  $\mu$ m membrane filter and then injected into the HPLC system.

For HPTLC, stock solutions of cineole,  $\beta$ -caryophyllene, linalool, scopoletin, and scoparone were obtained by adding 2 mL methanol to accurately weighed amounts of standard compounds.

### Sample preparation

For HPLC, samples (40 meshes, 1.0 g) were accurately weighed into a 100 mL triangular flask and ultrasonically extracted once (1 h) with 25 mL methanol. The extracts were filtered through a 0.45  $\mu$ m membrane, and then 10  $\mu$ L was injected into the instrument.

For HPTLC, the sample (40 mesh, 0.5 g) was accurately weighed into a test tube and 5 mL methanol was added. After the sample had been mixed for approximately 30 s, it was ultrasonically extracted (881 W, 43 kHz) for 60 min at room temperature (25°  $\pm$  2°C). The extracts of samples were centrifuged at 3500 rpm for 10 min and the supernatant was then placed into a tube for analysis.

### Method validation

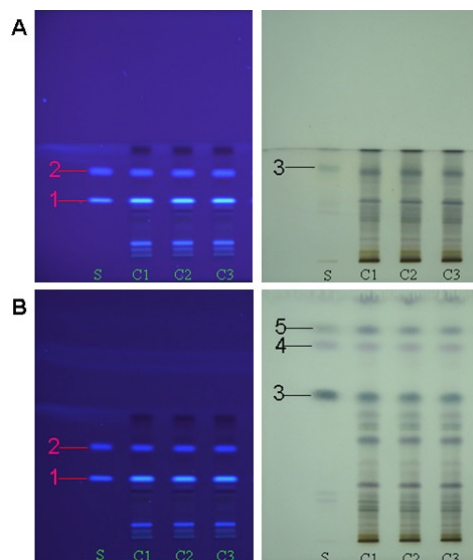
The proposed method for HPLC analysis was validated according to linearity, precision, stability, repeatability, and recovery. The precision was evaluated by determining the concentration of reference solutions six times within one day. Repeatability was measured by determined the contents of analytes in six individual samples solutions. To evaluate stability, the peak areas of reference compounds were measured every 2 h during one day. The precision, repeatability, and stability are expressed by the relative standard deviation (RSD). The accuracy of the method was assessed by determining sample recovery.

## RESULTS

### Chromatography

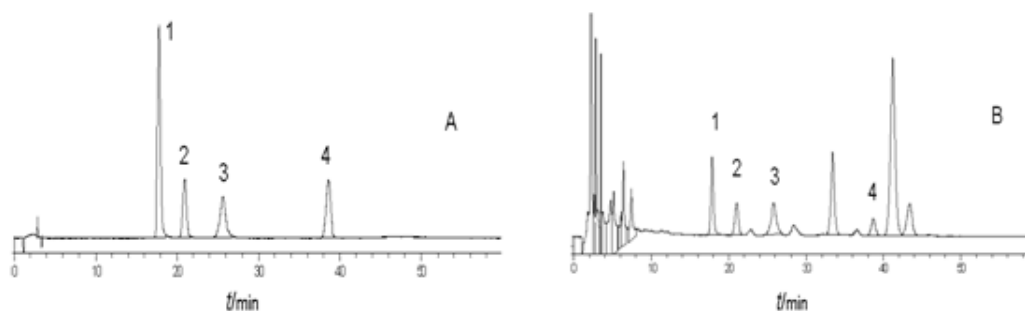
The optimized developing systems were as follows: chloroform and methanol (9:1)

were used for the first development to separate high-polarity compounds scopoletin and scoparone, and a mixture of ethyl acetate and petroleum ether (1:10) was used for the second run to separate linalool,  $\beta$ -caryophyllene, and cineole. TLC identification was highly specific and the spots were clearly separated. Chromatograph charts shown in Figure 1.



**Figure 1.** TLC results of reference substances and samples for (A) first and (B) second runs on plates observed at  $\lambda = 365$  nm (left) and colored with 1% vanillin- $H_2SO_4$  solution (right). S is the mixed reference substance and C1-C3 are three batches of samples. 1. Scopoletin. 2. Scoparone. 3. Linalool. 4.  $\beta$ -caryophyllene. 5. Cineole.

The developed HPLC method was set up and validated. It could determine simultaneously the content of four bioactive components in *Chimonanthus nitens*. Different proportions of methanol, acetonitrile, and water were tested and comparison showed that all of the analytes were separated with good resolution using the acetonitrile-water system and a wavelength of 344 nm. Typical HPLC chromatograms of standards and samples of *Chimonanthus nitens* are shown in Figure 2. They show that the peaks of different components in *Chimonanthus nitens* did not interfere with each other.



**Figure 2.** HPLC chromatograms of standards (A) and samples of *Chimonanthus nitens* (B). 1. Scopoletin. 2. Isofraxidin. 3. Rutin. 4. Scoparone.

## Method validation

The calibration curves had good linearity for each analyte ( $R > 0.9992$ ). Precision, stability, and repeatability were measured for these compounds (Table 1). The RSD of both precision and repeatability for the four compounds was below 2.50%. The sample solutions were stable over 24 h. All recoveries for the four compounds were between 97.66-104.44% (Table 1).

**Table 1.** Results for the four compounds tested in *Chimonanthus nitens*.

Analyte	Regression			Precision (RSD, %)	Stability RSD (%)	Repeatability RSD (%)	Detection recovery (RSD%, N = 6)
	Linear regression equation ( $y = ax + b$ )	R	Linear range ( $\mu\text{g/mL}$ )				
Scopoletin	$y = 21.111x - 5.20$	0.9999	5.665 - 56.65	0.26%	2.60%	0.86%	97.06 (1.62)
Isofraxidin	$y = 16.375x - 0.4160$	0.9999	2.4312 - 24.312	0.64%	1.88%	0.88%	102.86 (0.93)
Rutin	$y = 3.2261x - 14.052$	0.9992	2.444 - 24.44	1.48%	2.66%	1.53%	104.44(4.2)
Scoparone	$y = 20.218x - 0.3280$	0.9999	9.012 - 90.12	0.33%	1.65%	2.21%	102.18(1.81)

## Application

*Chimonanthus nitens* is a classic herb that is well used in China. In the study, we established a fast and accurate method that can simultaneously analyze the content of four major active components in *Chimonanthus nitens*. The results are shown in Table 2.

**Table 2.** Contents of four components in samples of *Chimonanthus nitens*.

Analyte	Contents of Analyte ( $\mu\text{g/g}$ )					Average Content	RSD (%)
	1	2	3	4	5		
Scopoletin	264.83	263.79	267.12	262.42	261.68	263.97	0.81
Isofraxidin	162.95	161.42	162.97	163.06	160.23	162.13	0.78
Rutin	849.88	855.8	864.13	837.23	838.85	849.18	1.34
Scoparone	88.49	87.29	87.45	91.35	88.75	88.67	1.84

## DISCUSSION

For optimization, different proportions of ethyl acetate, formic acid, and water (8:1:1, 7:1.5:1.5), ethyl acetate and methanol (7:3, 8:2), and petroleum ether and ethyl acetate (9:1, 10:1, 15:1) were studied. Due to the differences in polarity, it is difficult to separate the components in one development. Thus, double-development was adopted. Since the polarity of different compounds vary, it is very difficult to separate them well using isocratic elution. Experimental conditions such as the mobile phase and wavelength were optimized to obtain good separation.

Terpenoids are the main effective components of volatile oil, and flavonoids and coumarins have definite pharmacological effects in the natural plant (Yi, 2012; Kong et al., 2012). Thus, qualitative and quantitative analyses of these compounds in traditional Chinese medicine are of great importance. This developed method has good reproducibility, is sensitive and simple, and could be used for quality control of *C. nitens*. In the future, further development of a TLC method for qualitative analysis of flavonoids and a HPLC method for quantitative analysis of terpenoids will be required.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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