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Full Length Research Paper

Chemical fingerprint technique and its application in the classification and quality assessment of the *Gastrodia* tuber

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The *Gastrodia* tuber and its active component, gastrodin, have many pharmacological effects. In this study, optimized high performance liquid chromatography (HPLC) parameters were employed to determine the chemical fingerprints and gastrodin content of nine *Gastrodia* tuber populations. Based on the degree of similarity of the chemical fingerprints, the nine *Gastrodia* tuber populations were grouped into one of the three different classes. Class I *Gastrodia* tubers had the highest content of gastrodin and were thus, regarded as possessing the highest quality. Of the class I *Gastrodia* tuber samples, those from Yichang, Hubei and Shimen, Hunan, were identified as the "best." Close relationships were detected among the chemical fingerprints, gastrodin content and place of origin of the *Gastrodia* tubers. Hence, these findings may be applied in assessing the quality of *Gastrodia* tubers and in identifying and segregating poor quality *Gastrodia* populations from those of good quality.

Key words: Chemical fingerprint, chromatographic classification, *Gastrodia* tuber, gastrodin content, HPLC, quality assessment.

INTRODUCTION

Following its widespread application in the quality control assessment of crude drugs and foods, chemical fingerprinting has recently gained increased attention (Chou et al., 2010; Zhou et al., 2008; Zheng et al., 2009; Locatelli et al., 2009). The major feature of chemical fingerprint technique is its capacity to detect the most chemical components of medicinal plants, determine the content of the all components in each sample according to each peak area of chemical fingerprint, identify the same kind of medicine from different collections and classify them based on similarity degree of chemical fingerprint. Owing to its precision, high performance liquid chromatography (HPLC) has become a popular method used in chemical fingerprinting (Gu et al., 2004; Alaerts et al., 2007; Kong et al., 2009). The application of HPLC fingerprinting in establishing quality control standards for traditional Chinese medicines and crude drugs has been approved by the State Food and Drug Administration (SFDA) of the People's Republic of China (2007).

Several important traditional Chinese medicines have been made from the tuber of *Gastrodia*, a member of the Orchidaceae family. The pharmacological characteristics of the *Gastrodia* tuber and its main active component, gastrodin, include sedation and hypnosis, anticonvulsant properties, intelligence enhancement, brain nourishment, antidementia effects, antioxidant properties, senility postponement, immunological defense enhancement and enhancement of accommodation of the heart and blood vessels (Kim et al., 2001).

However, *Gastrodia* tubers are becoming increasingly difficult to find in the wild. As a consequence, attempts have been made to cultivate *Gastrodia*. In addition, the scarcity and potential pharmacological importance of

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Gastrodia have unfortunately led to the development of imitations and fake products and the replacement of *Gastrodia* tubers gathered in the wild with those that were cultivated. Previously, the quality and authenticity of *Gastrodia* tubers (as well as other herbal medicines) were determined with the naked eye, based on prior experience. Because no scientific techniques for identifying herbal medicines existed, *Gastrodia* tuber populations of poor quality were occasionally identified as being of higher quality. Thus, to establish a precise method for identifying the quality and authenticity of *Gastrodia* tubers, Wang et al. (2007) used an HPLC method to carry out chromatographic fingerprinting analyses of *Gastrodia* tubers. However, the purpose of employing the HPLC method in those studies was not to classify or identify medicinal plant populations or to explore relationships among chemical fingerprints, active component content and place of origin of *Gastrodia* tubers.

Several methods may be applied in the classification of medicinal plants. However, the advantage of classifying medicinal plants based on their chemical fingerprints lies not only in ascertaining the genetic nature of a particular medicinal plant variety (especially reflecting its level of control or regulation of gene expression), but also in directly observing the quantitative character of phenotypes. Thus, the information gained from chemical fingerprinting has both academic and practical significance.

In this study, we determined the most suitable mobile phases and wavelengths of light through comparison and selection. We also used optimized parameters to establish the chemical fingerprints and gastrodin content of nine kinds of *Gastrodia* tuber samples that was gathered from different regions of China. The nine *Gastrodia* tuber populations were grouped into one of three different classes based on the measure of similarity of their chemical fingerprints. Our analysis of the relationships among the chemical fingerprints, active component content and place of origin revealed which populations of *Gastrodia* tubers are of high quality and which regions of China are the most suitable for growing *Gastrodia* tubers.

MATERIALS AND METHODS

Instrumentation and reagents

The Agilent 1100 series HPLC system was from Waldbronn, Germany. The type of column used was the eclipse, XD13-C_8 column (4.6 × 150 mm, 5 µm). The ultrasonic processor (JY92-2D) was from Zhejiang, China. A standard *Gastrodia* tuber (No. 120944-200905) and a gastrodin reference substance (No. 110807-200805, purity >99%) were purchased from China's National Institute for the Control of Pharmaceutical and Biological Products.

Sample preparation

Gastrodia tuber samples were gathered from nine different locations in China: Shimen, Hunan; Yichang, Hubei; Dabieshan, Anhui; Dafang, Guizhou; Chayu, Xizang; Yiliang, Yunnan; Lueyang, Shanxi; Liangshan, Sichuan; Changbaishan, Jilin. For each type of *Gastrodia* tuber under investigation in this study, 12 samples were collected. The samples were identified by specialists from Changde Dehai Medicine Material Development and were stored at -70°C. After the fresh *Gastrodia* tuber samples were steamed and dried, they were ground into powder, passed through an 80-mesh sifter, and then dried in a dry box at 80°C for 1 h. The gastrodin reference substance (5 mg), which was dried at 80°C for 1 h, was accurately weighed with an electronic balance, placed into volumetric flasks (10 ml), diluted with the mobile phase to scale, shaken, passed through a filtration membrane (0.45 µm) and then stored in a refrigerator for later detection.

Extraction of the chemical components of the *Gastrodia* tuber

Aliquots (1 g) of *Gastrodia* tuber powder were accurately measured, placed in an extractor and mixed with 70% ethanol solution. The extractor was placed in a hot water bath at 97°C for heat treatment for 20 min. The samples were then disposed with ultrasonic handling for 25 min (ultrasonic handling for 9 s; intermission for 2 s; power 250 W). Water that was lost was complemented using the same solvent after ultrasonic handling. This process (as previously described) was repeated once again. The processed mixture was passed through filter paper and the filtrate was amassed. The dried extract obtained by evaporation was dissolved using the mobile phase. The solution was passed through a membrane filter (0.45 µm) and then stored in a refrigerator for later detection.

Selection of mobile phases and detection conditions

Two portions (1 g each) of powder were precisely taken from a standard *Gastrodia* tuber and their chemical components were extracted with the aforementioned method. Chemical fingerprints of the *Gastrodia* tuber samples were determined by conducting HPLC (column temperature, 25°C; sample size, 20 µl; flow rate, 1 ml/min), eluting the extract with a 10% acetonitrile solution and a gradient elution program of acetonitrile solution (water elution for 0 to 1.5 min, 5% acetonitrile solution elution for 1.6 to 3.5 min, 10% acetonitrile solution elution for 3.6 to 8 min), monitoring the effluent with light at a wavelength of 221 nm and comparing the effects of the mobile phases.

Chemical fingerprints of the standard *Gastrodia* tuber were determined by conducting HPLC (as previously described), eluting the extract with a gradient elution program of acetonitrile solution (as previously described), monitoring the effluent at five different wavelengths (221, 219, 217, 215 and 213 nm) of light and comparing the effects at these five different wavelengths.

Determination of the chemical fingerprints of the *Gastrodia* tuber

The previously discussed methods and conditions and the optimized parameters (the gradient elution program of acetonitrile solution and a wavelength of 219 nm) were applied to determine the chemical fingerprints of nine kinds of *Gastrodia* tubers.

Statistical analysis

The gastrodin content of each sample was calculated using the following equation:

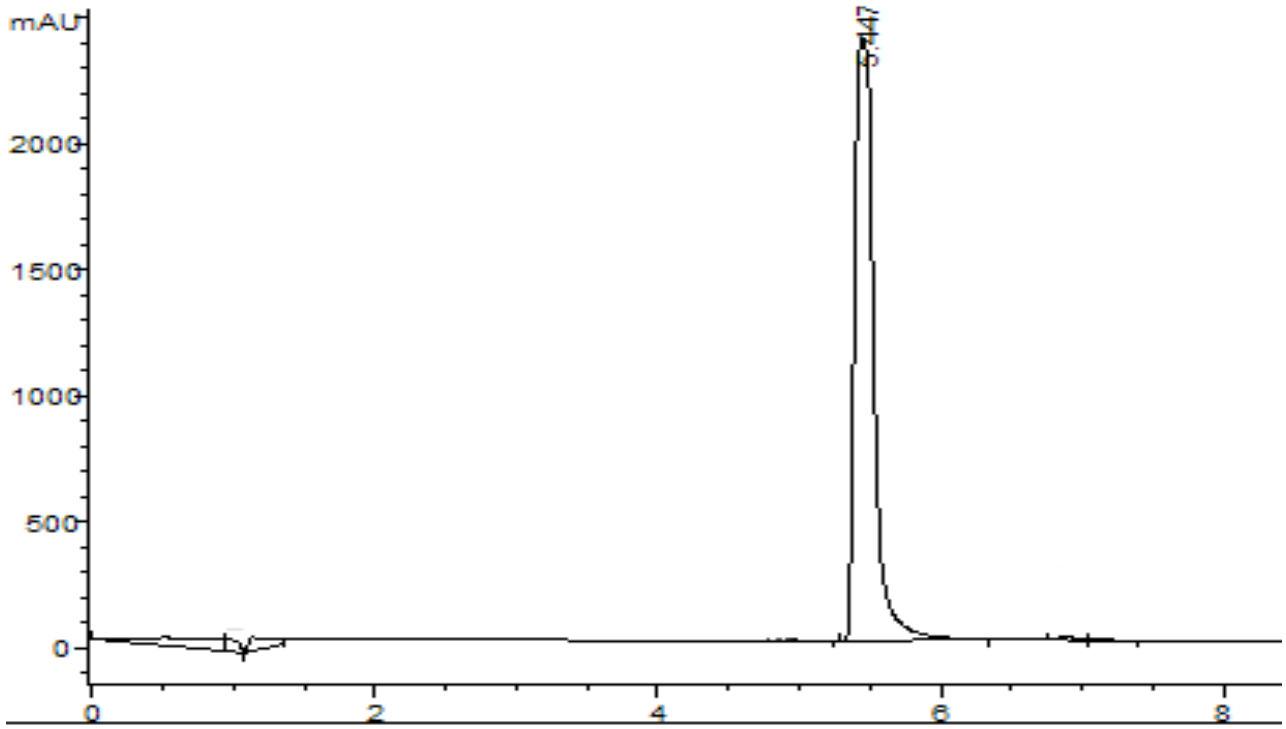


Figure 1. Chromatograms of the gastrodin reference substance obtained by using the gradient eluting program of acetonitrile solution and monitoring the effluent at a wavelength of light of 221 nm. A retention time of gastrodin peak was 5.4 min.

$$CCS = \frac{PACS \times DRSC}{PARSC \times DS}$$

Where, CCS indicates component content of sample; PACS is the peak area of component of sample; DRSC is the dosage of reference substance of component; PARSC is the peak area of reference substance of component; and DS is the dosage of sample.

Linear relationships between sample amount and gastrodin content were analyzed using the sample amount as the abscissa and the gastrodin content as the ordinate (Sun and Xu, 2003). The linear regression equation was subsequently established.

The degree of similarity of the chromatogram fingerprints was determined with software (A evaluation system for the degree of similarity of the chromatogram fingerprints of Chinese traditional medicine 2004A) designed by central south university, according to recommendation of Chinese Pharmacopoeia Commission. Populations of *Gastrodia* tubers were classified using SPSS11.5 software on the basis of the degree of similarity of the fingerprints.

Relationships among chemical fingerprints, active component content and place of origin were analyzed by comparing the chemical fingerprints, gastrodin content and total area of all peak fractions of the *Gastrodia* tubers gathered from different provinces. The significance of the differences in gastrodin content among the groups of *Gastrodia* tubers was established by comparison with the two-group *t* test (Sun and Xu, 2003).

RESULTS AND DISCUSSION

The chromatograms of the gastrodin reference substance were obtained by using a gradient elution program of acetonitrile solution and light at a wavelength of 221 nm (Figure 1). Chemical components from a standard sample of *Gastrodia* tuber were extracted with 70% ethanol. When the extracts were eluted with 10% acetonitrile solution and the effluent was monitored with light at a wavelength of 221 nm, we detected 9 components (Figure 2). However, when the extracts were eluted with a gradient elution program of acetonitrile solution, we detected 18 components (Figure 3).

When the *Gastrodia* tuber extracts were eluted with a gradient elution program of acetonitrile solution and the effluents were monitored at 219, 217, 215, 213 and 221 nm, peak areas for gastrodin were 11 192 mAU*s (an average of three repetitions), 10 966 mAU*s (a 2% reduction), 10 648 mAU*s (4.9%), 10 334 mAU*s (7.7%) and 11 148 mAU*s (0.4%), respectively; the total peak areas calculated at 7.5 min for the extracted components were 67 173, 70 368 (a 4.8% increase), 74 053 (10.2%), 77 718 (15.7%) and 62 921 mAU*s (a 6.3% reduction), respectively. The results showed that when the effluent was monitored at 219 nm, peak areas for gastrodin were the largest. However, the total peak areas were inversely proportional to the wavelength of light.

The gastrodin content of the standard *Gastrodia* tuber samples (4, 8, 12, 16 and 20 µl) was determined with the optimized HPLC parameters. A good linear relationship was established between the gastrodin content and the

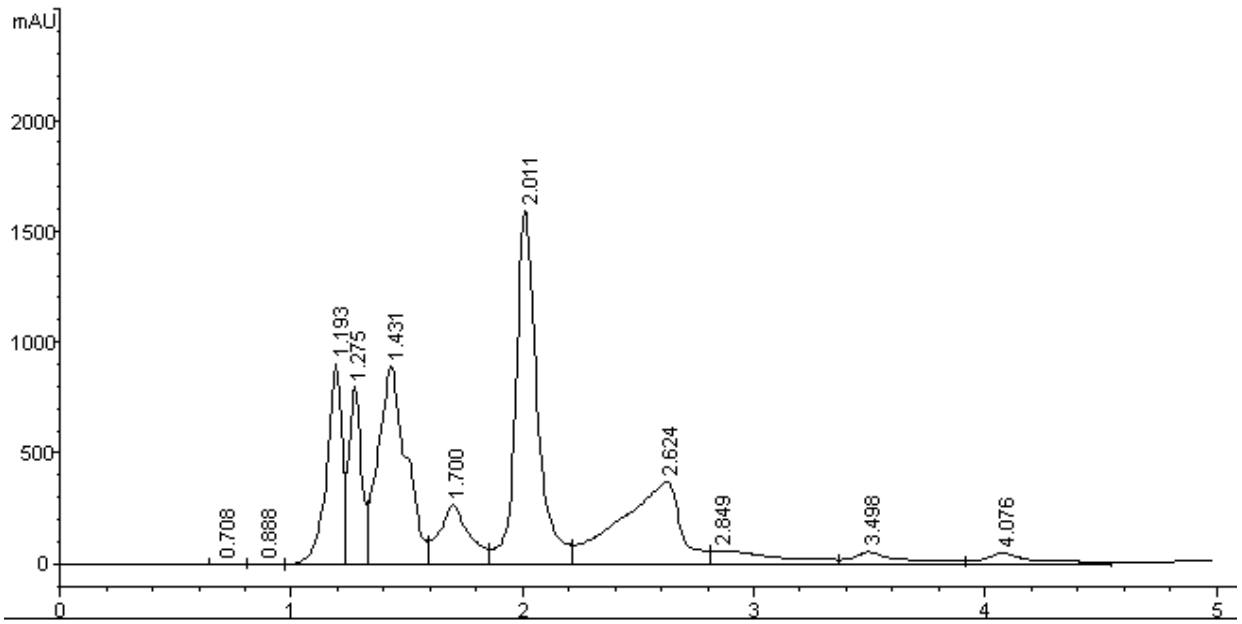


Figure 2. Chromatograms obtained by eluting the extract of standard *Gastrodia* tuber using 10% acetonitrile solution and monitoring the effluent at a wavelength of light of 221 nm. The peak with a retention time of 2.0 min corresponds to gastrodin.

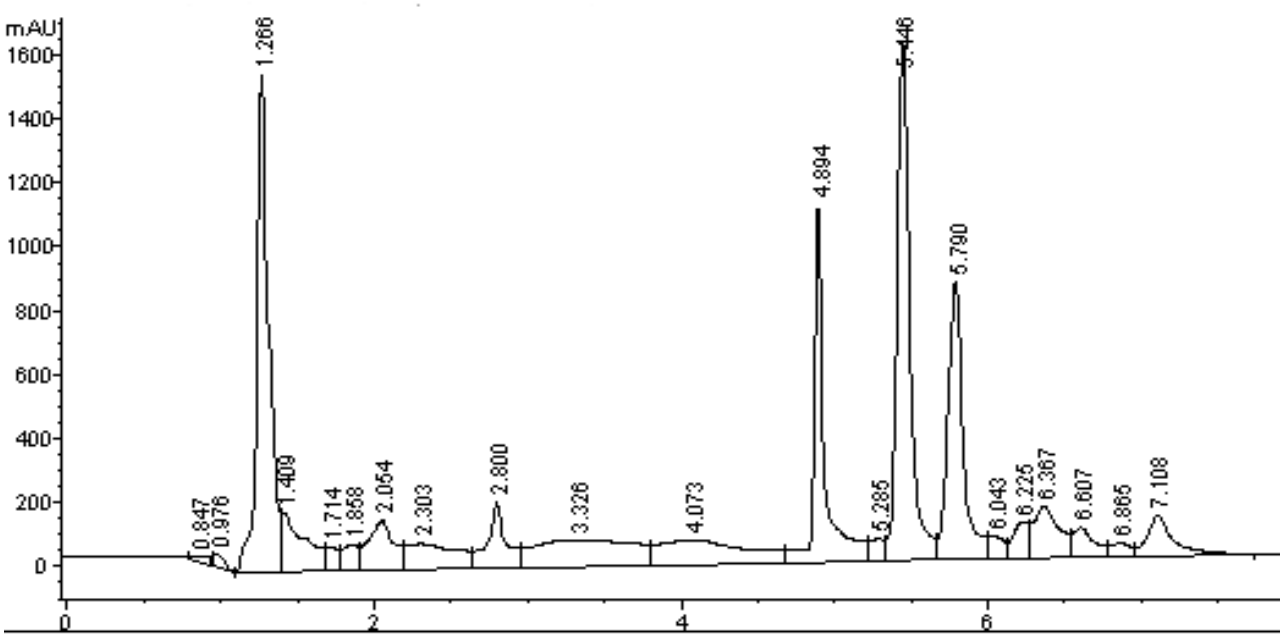


Figure 3. Chromatograms obtained by eluting the extract of standard *Gastrodia* tuber using the gradient eluting program of acetonitrile solution and monitoring the effluent at a wavelength of light of 221 nm. The peak with a retention time of 5.4 min corresponds to gastrodin.

sample amount. The linear regression equation was as follows:

$Y = 0.40795X + 0.3332$, $\gamma = 0.9998$

The gastrodin content of the 20 µl standard *Gastrodia* tuber sample was determined in five consecutive repetitions with the optimized HPLC parameters and the relative standard deviation (RSD) value for the five

repetitions was 1.31%. In addition, the gastrodin content of the 20 µl standard *Gastrodia* tuber sample was determined at 0, 12, 24, 48 and 96 h and the RSD value calculated for the different time points was 1.2%.

When 1 ml of gastrodin reference substance (1 mg/ml) was added to each of the five *Gastrodia* tuber samples of known gastrodin content, the gastrodin recovery rates were measured, yielding an average recovery rate and RSD value of 98.2 and 2.1%, respectively.

In this study, we employed optimized HPLC parameters to determine the chemical fingerprints of *Gastrodia* tuber samples gathered from nine provinces in China (Figure 4). The results showed that the *Gastrodia* tuber samples had different chemical fingerprints and number of peak fractions. However, all the samples had eight common peaks (located at 1.28, 2.70, 4.88, 5.44, 5.83, 6.37, 6.60 and 7.09 min) with similar retention times (average RSD, 1.36%). Gastrodin was particularly stable at the peak time of 5.44 min (RSD, 0.36%). Congeneric *Gastrodia* tuber populations, regardless of whether they were wild or cultivated or whether they were from the same or different provinces, all had very similar fingerprints.

Their chemical fingerprints were analyzed with the software. Based on the results of this analysis, the nine kinds of *Gastrodia* tubers were grouped into one of three different classes (Figures 5 to 7). Class I *Gastrodia* tubers, characterized by a higher gastrodin peak fraction, consisted of the standard *Gastrodia* tubers and the samples from Hunan (Figure 5), Anhui, Xizang, Guizhou, Shanxi, Hubei and Jilin. Class II included the *Gastrodia* tuber samples from Yunnan (Figure 6); the peak fractions of its fingerprints, as detected at 2.4 min (up to a 38.1% total area) and 7.0 min (up to a 25.2% total area), were very high and large, but the peak fraction for gastrodin was low and small (within only a 4.9% total area). Class III comprised a *Gastrodia* tuber sample from Sichuan (Figure 7); its peak fraction was as large as that of the sample from Yunnan at 2.4 min but lower than that at 7.0 min; however, its gastrodin peak fraction was of medium size.

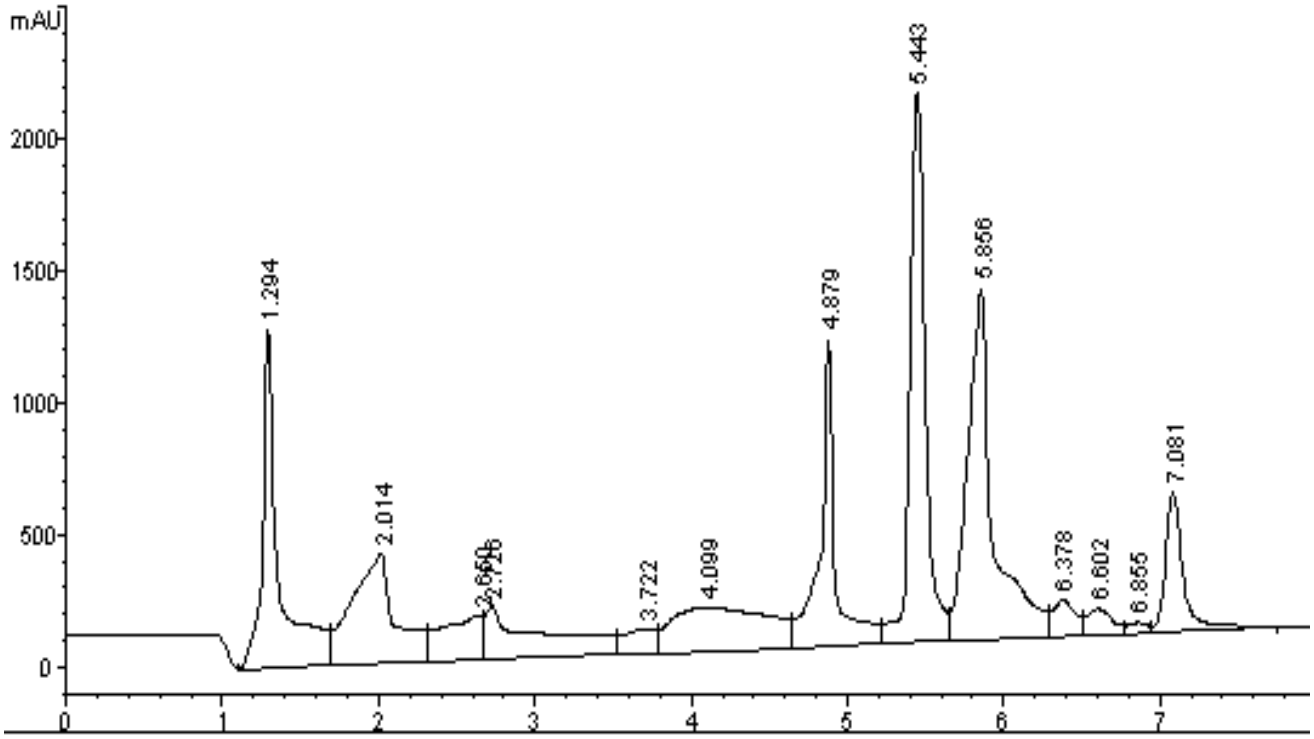
Gastrodia tuber contains the following chemical components: gastrodin, gastroclin aglucon, vanillin, vanillic alcohol, sesquiterpene and purine; gastrodin is the main active component of *Gastrodia* tubers; its content level was one of the most important factors in the quality standardization of the *Gastrodia* tuber samples. The average gastrodin content for the nine kinds of *Gastrodia* tuber samples was calculated using the previously described equation, with the peak area of gastrodin and the average total peak areas of all their extracted components being included (Table 1). The gastrodin content of the *Gastrodia* tuber sample from Hubei was the highest (7.519 mg/g), whereas that of the sample from Yunnan was the lowest (1.703 mg/g). However, the total peak area for the *Gastrodia* tuber sample from Yunnan was very large (67 199 mAU*s), whereas the total peak area for the sample from Changbaishan in Jilin province was very small (34 437 mAU*s).

To obtain the average gastrodin content for each class, the gastrodin content values for the different samples in each class were added and then divided by the number of samples (Table 2). Class I had the highest gastrodin content (5.595 mg/g ± 1.504). Although, class II had the lowest gastrodin content (1.703 mg/g ± 0.357), its total peak area was the largest (up to 67 199 mAU*s ± 7 051). The gastrodin content and total peak area values for class III were in-between those for classes I and II, as were their chemical fingerprints.

The significance of the differences in gastrodin content among the three classes was established with a two-group *t* test. The results showed that the difference in gastrodin content between classes I and II (*t* value = 3.641, *df* = 94), as well as between II and III (*t* value = 2.634, *df* = 22), was statistically significant (*t* value > $t_{0.05}$, $p < 0.05$), but was not between classes I and III (*t* value = 1.179, *df* = 94; *t* value < $t_{0.05}$, $p > 0.05$).

In comparison to the optimized parameter established by Zhang et al. (2007), those established in this study enabled the identification of a greater number of active components in the *Gastrodia* tuber samples and the detection of higher contents of gastrodin and other components (almost twice as much as previously reported) in a shorter period of time (1/6 time). Hence, the optimized parameters established in this study can be applied in the quality assessment of *Gastrodia* tubers and in the identification of higher quality *Gastrodia* tuber populations.

The size of the total peak area of all the extracted components reflects their respective content level, whereby the larger the peak area, the higher the content level. Because gastrodin is the main active component of *Gastrodia* tubers, its content level was one of the most important factors in the quality standardization of the *Gastrodia* tuber samples. Based on the content of gastrodin and total peak area of all the components in each of the *Gastrodia* tuber samples (Kong et al., 2009), we surmise the following: (1) because class I *Gastrodia* tuber samples in this study had the highest content of gastrodin and higher contents of other components, *Gastrodia* tubers with fingerprints categorized as class I were regarded as possessing the highest quality, with *Gastrodia* tubers from Yichang (located in Hubei province) and Shimen (located in Hunan province) identified as being the “best.” This finding suggest that the climate of this mountainous region, which stretches between Hunan in the northwest and Hubei in the southwest, is the most suitable for the growth of *Gastrodia* tubers. Of the *Gastrodia* tuber samples grouped in class I, those from Changbaishan (located in Jilin province) had the lowest content of gastrodin and the lowest total peak area (the height of their highest peak did not exceed 1100 mAU*s); thus, they were considered of the poorest quality. These findings could be related to the fact that the climate of Changbaishan (located in


mAU
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1500
1000
500
0
1.294
2.014
3.722
4.099
4.879
5.443
5.856
6.378
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6.855
7.081
0
1
2
3
4
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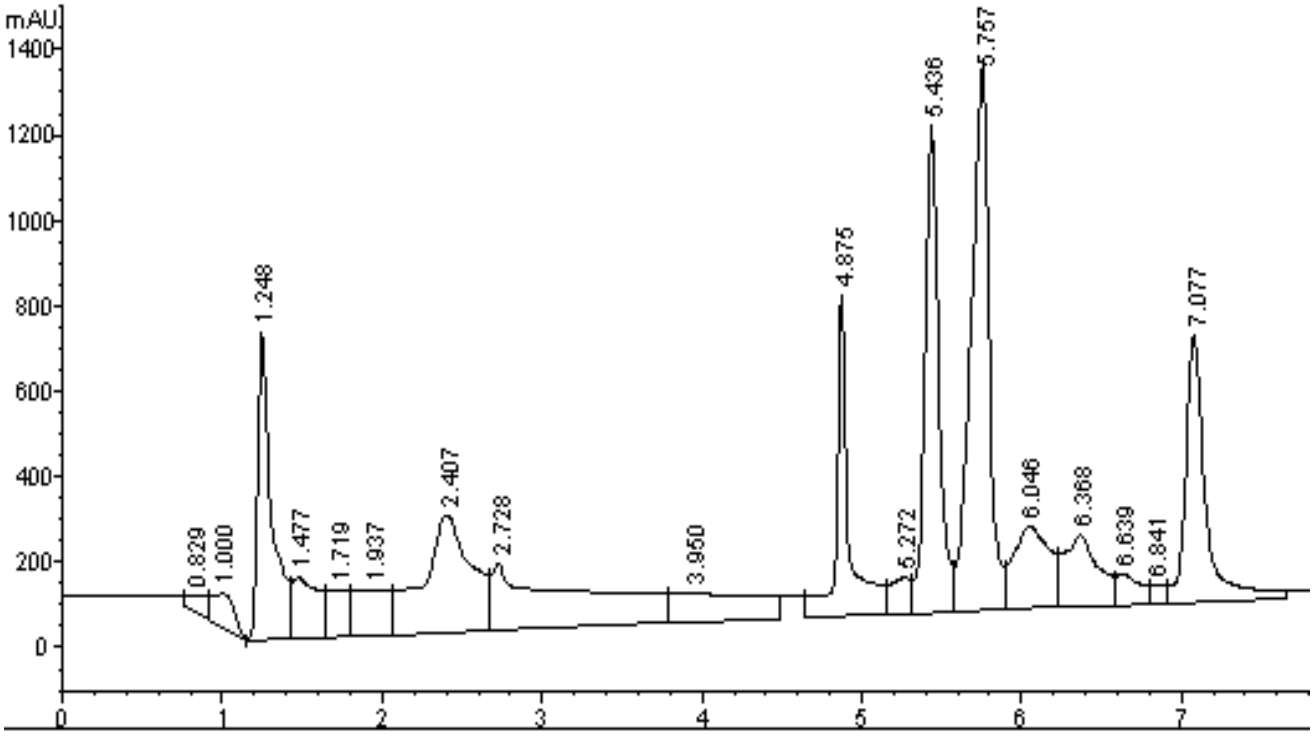
7

A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2.728
3.950
4.875
5.272
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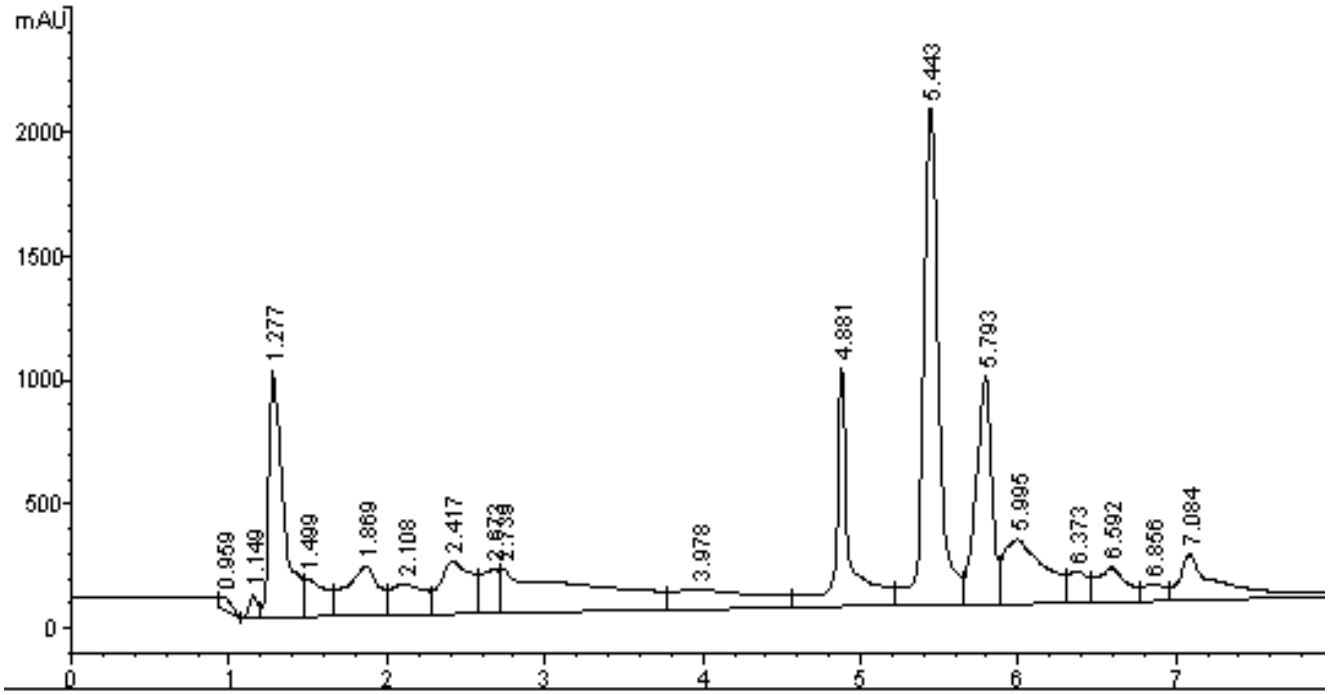
B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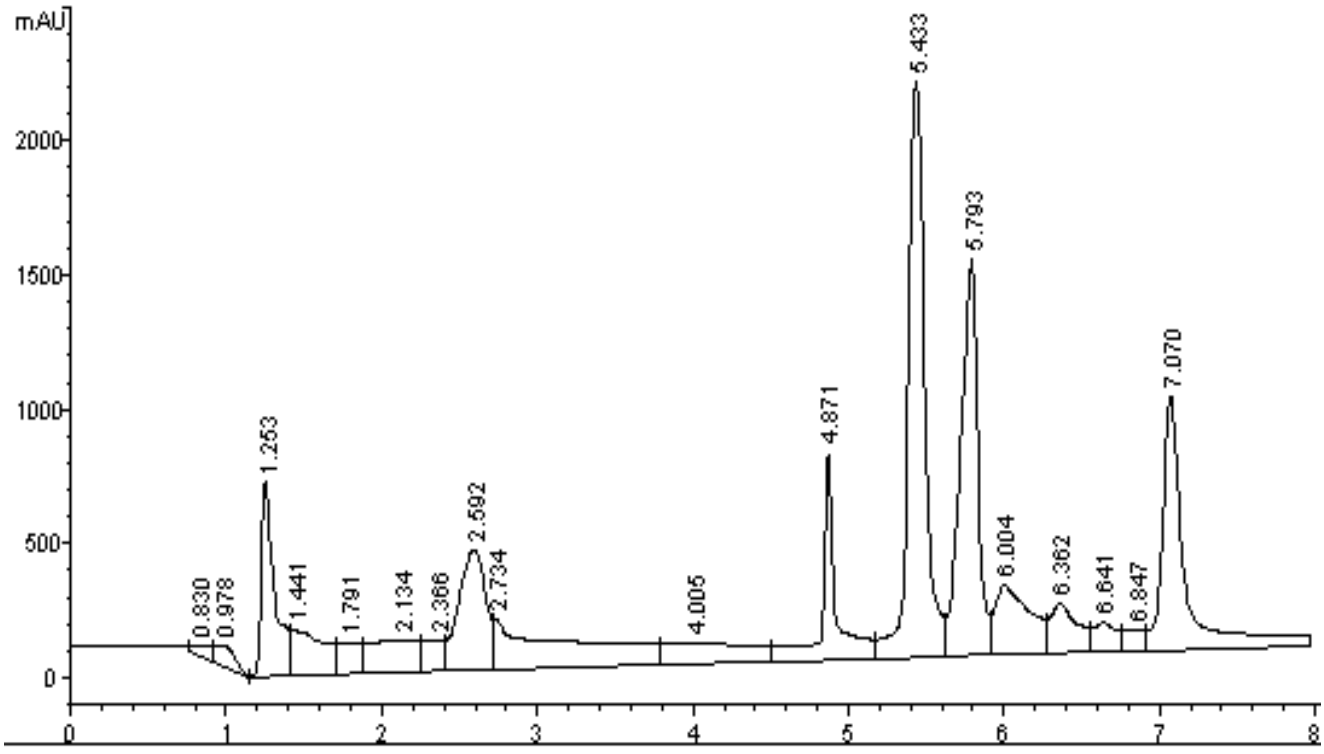
C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5.433
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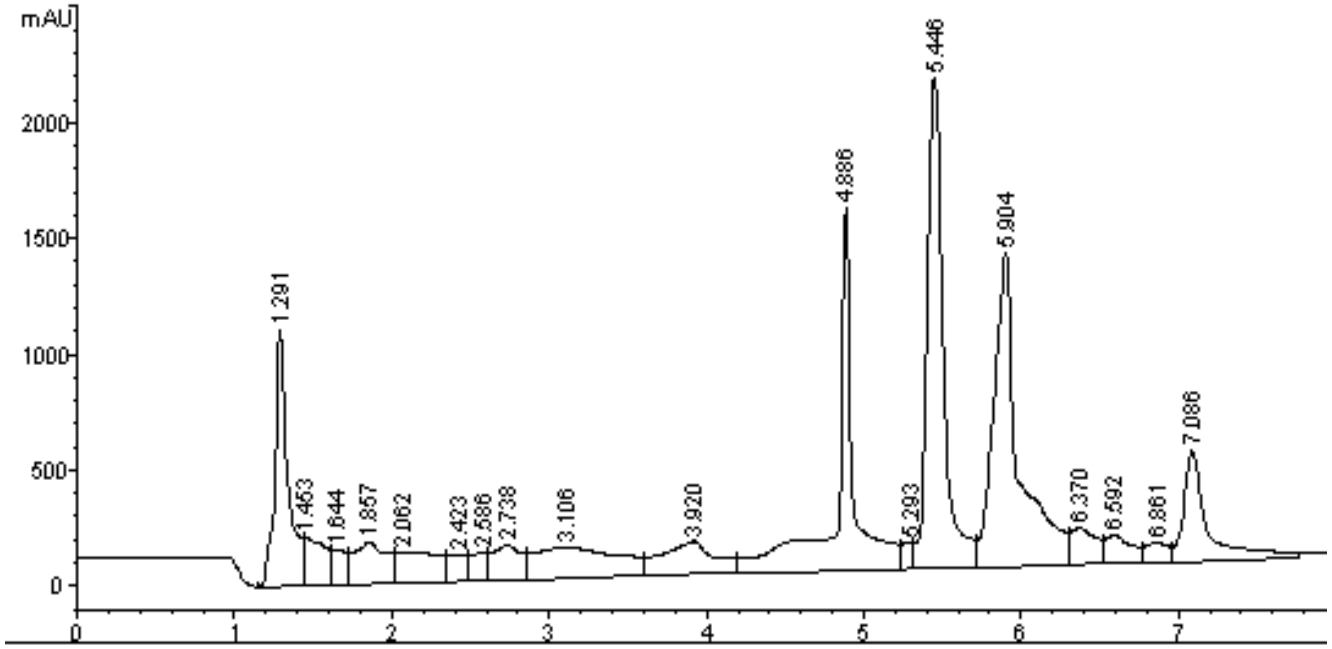
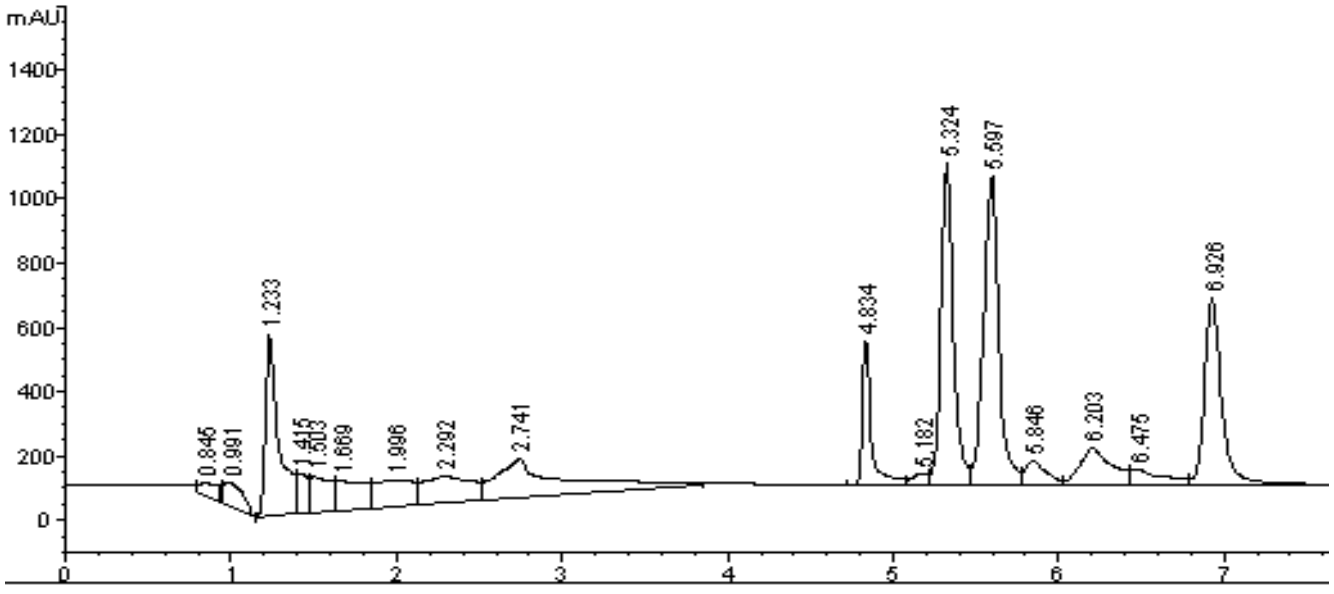
D



E



F

Figure 4. Fingerprints obtained by eluting the extract of different *Gastrodia* tuber samples using gradient concentration acetonitrile solution as the mobile phase and monitoring the effluent at 219 nm wavelengths of light. The peak with retention times 5.4 was gastrodin. A, Fingerprint of planted *Gastrodia* tuber from Anhui; B, fingerprint of wild *Gastrodia* tuber from Xizang; C, fingerprint of planted *Gastrodia* tuber from Guizhou; D, fingerprint of planted *Gastrodia* tuber from Shanxi; E, fingerprint of planted *Gastrodia* tuber from Hubei; F, fingerprint of planted *Gastrodia* tuber from Jilin.

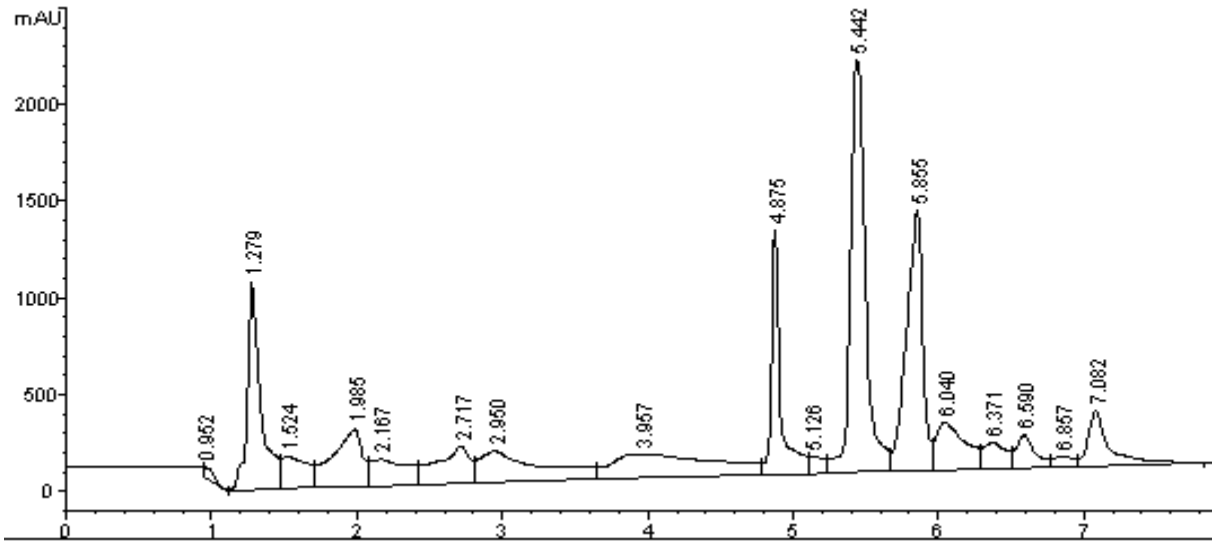


Figure 5. Chemical fingerprints obtained by eluting the extract of *gastrodia* tuber samples from Hunan (class I) using the gradient eluting program of acetonitrile solution and monitoring the effluent at a wavelength of 219 nm. The peak with a retention time of 5.4 min corresponds to gastrodin.

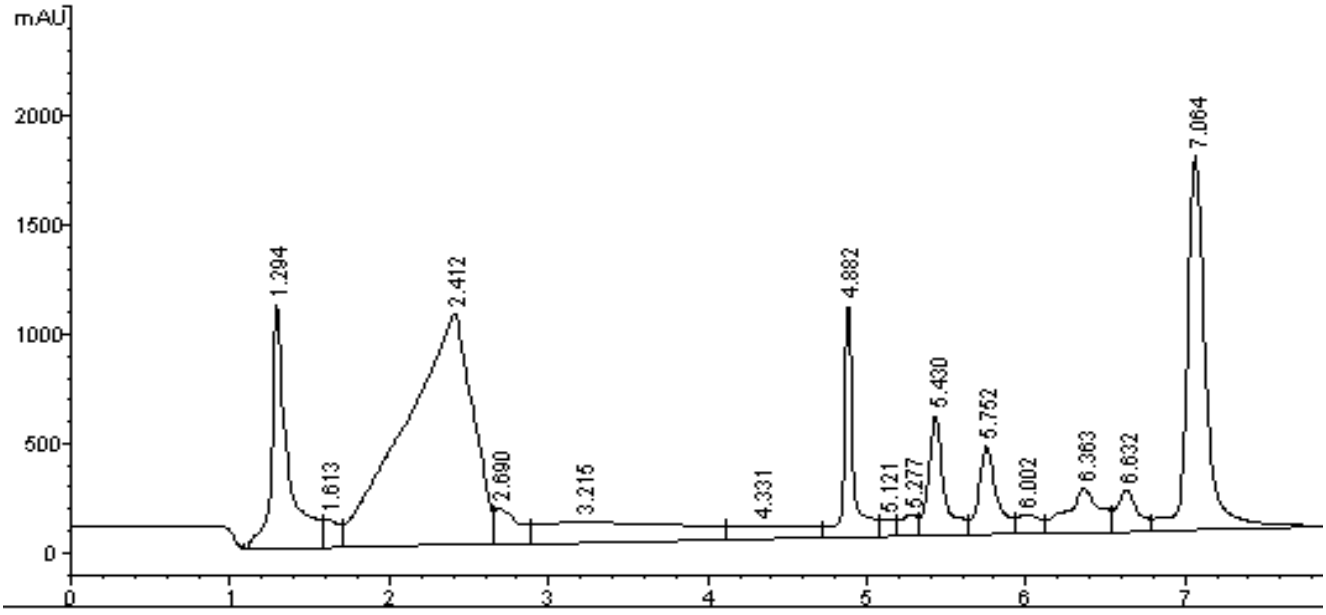


Figure 6. Chemical fingerprints obtained by eluting the extract of *Gastrodia* tuber samples from Yunnan (class II) using the gradient eluting program of acetonitrile solution and monitoring the effluent at a wavelength of 219 nm.

northeastern China) is comparatively colder than the climates of the places from which the other class I *Gastrodia* tuber samples were gathered. Our findings are in line with those of several previous studies reporting on the correlation between the content of active components of a crude drug and its habitat (Bai et al., 2008); (2) class II *Gastrodia* tuber samples in this study had the lowest content of gastrodin, but the highest content of other components; (3) the quality of class III *Gastrodia* tuber samples in this study was categorized as in-between that of class I and that of class II.

When the gastrodin content of different sizes of *Gastrodia* tuber samples were compared with each other, no clear connection was established between gastrodin content and size of the *Gastrodia* tuber. However, medium-sized *Gastrodia* tubers in our study generally

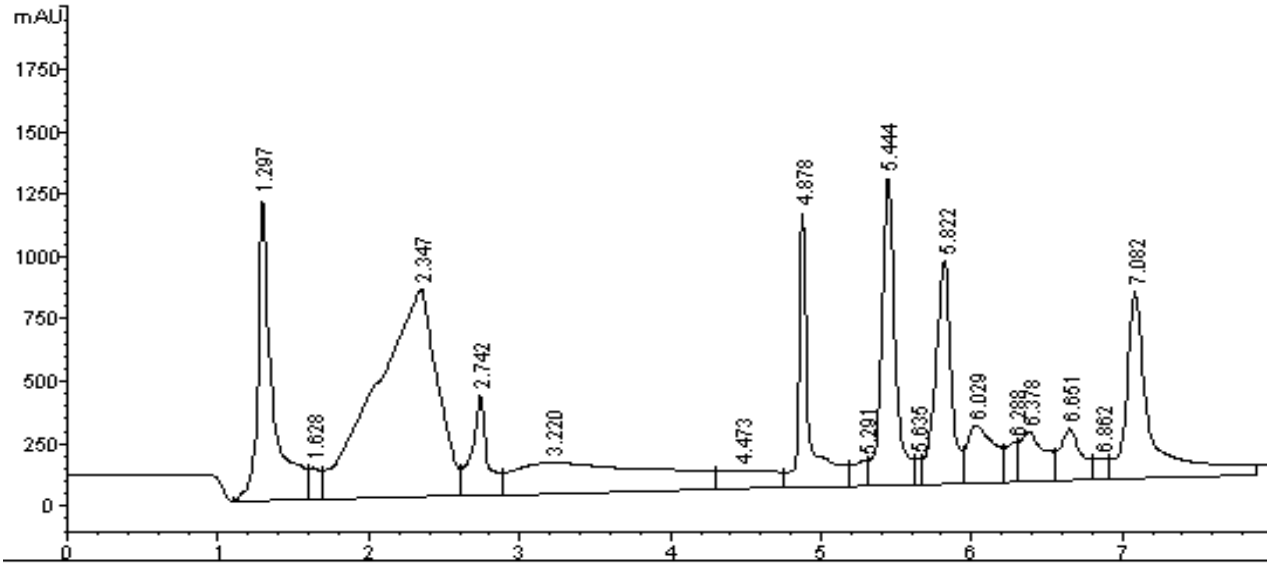


Figure 7. Chemical fingerprints obtained by eluting the extract of different *gastrodia* tuber samples from Sichuan (class III) using the gradient eluting program of acetonitrile solution and monitoring the effluent at a wavelength of 219 nm.

Table 1. Comparison of gastrodin content and total area of all peak fractions of nine kinds of *Gastrodia* tubers.

Code of variety	Average gastrodin content, mg/g	Gastrodin content ranking	Average total peak area, mAU*s	Peak area ranking
YH[a]	7.519 ±2.155	1	79453 ±13417	1
SH	7.255 ±1.235	2	67583 ±9001	2
LSH	6.625 ±1.345	3	55388 ±8678	7
DG	6.092 ±1.206	4	62952 ±8564	6
DA	5.196 ±1.563	5	67025 ±9062	4
CX	4.530 ±1.519	6	54678 ±16005	8
LS	4.078 ±1.659	7	66456 ±15055	5
CJ	2.466 ±0.896	8	34437 ±5269	9
YY	1.703 ±0.357	9	67199 ±7051	3

[a]YH = *Gastrodia* tuber from Yichang in Hubei; SH = *Gastrodia* tuber from Shimen in Hunan; LSH = *Gastrodia* tuber from Lueyang in Shanxi; DG = *Gastrodia* tuber from Dafang in Guizhou; DA = *Gastrodia* tuber from Dabieshan in Anhui; CX = *Gastrodia* tuber from Chayu in Xizang; LS = *Gastrodia* tuber from Liangshan in Sichuan; CJ = *Gastrodia* tuber from Changbaishan in Jilin; YY = *Gastrodia* tuber from Yiliang in Yunnan.

Table 2. Comparison of average gastrodin content and average total peak area of three classes of *Gastrodia* tuber.

Class	Average gastrodin content, mg/g	Gastrodin content ranking	Average peak area, mAU*s	Peak area ranking
I	5.595 ±1.504	1	60987±10788	3
II	1.703 ±0.357	3	67199 ±7051	1
III	4.078 ±1.659	2	66456 ±15055	2

displayed comparatively higher content of gastrodin and of other components (estimated according to their peak areas) and were consequently categorized as being of higher quality.

Conclusions

In this study, we determined the optimal method for separating extracts from the *Gastrodia* tubers to be the

gradient eluting program of acetronitrile solution and the optimal wavelength of light for detecting their chemical components to be 219 nm. We investigated nine different *Gastrodia* tuber populations and grouped them into one of three different classes (I, II, III) based on the degree of similarity of their fingerprints. Our results showed that class I *Gastrodia* tubers had the highest content of gastrodin and comparatively higher contents of other components. Consequently, populations of *Gastrodia* tubers with class I fingerprints were regarded as possessing the highest quality, with samples from Yichang (located in Hubei province) and Shimen (located in Hunan province) identified as the "best." A strong interrelationship was established among chemical fingerprints, content of active components and place of origin of the *Gastrodia* tubers. Hence, the findings of this study can be applied in assessing the quality of *Gastrodia* tubers, in identifying and selecting better *Gastrodia* tuber populations for cultivation and in determining the most appropriate regions for cultivating *Gastrodia* tubers. These results may also be applied when determining the criteria for the quality control of *Gastrodia* tubers and when conducting similar correlative studies on other medicinal and food plants.

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