Simultaneous determination of four active components in *Alisma orientale* (Sam.) Juz. by HPLC–DAD using a single reference standard

Yao-Wen Zhang\(^a\)
Qing Li\(^b\)
Chun-Xiao Lv\(^a\)
Xiu-Jia Liu\(^a\)
Xiao-Hui Chen\(^a\)
Kai-Shun Bi\(^a\)

\(^a\)School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, China
\(^b\)National and Local United Engineering Laboratory for Key Technology of Chinese Material Medica Quality Control, Shenyang Pharmaceutical University, Shenyang 110016, China
\(^c\)Liaoning Institute for Drug Control, Shenyang 110036, China

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**Abstract** A rapid, simple and practical high-performance liquid chromatography method coupled with diode array detector (HPLC–DAD) was developed to evaluate the quality of *Alisma orientale* (Sam.) Juz. through a simultaneous determination of four major active triterpenes using a single standard to determine the multi-components (SSDMCs). Alisol B 23-acetate was selected as the reference compound for calculating the relative response factors. All calibration curves showed good linearity ($R^2 > 0.9998$) within test ranges. RSDs for intra- and inter-day of four analytes were less than 3.6% and 2.3%; the overall recovery was 92.1–110.2% (SSDMC). The proposed method was successfully applied to quantify the four components in 20 samples from different localities in China. Moreover, significant variations were demonstrated in the content of these compounds. In addition, hierarchical clustering analysis (HCA) and principal components analysis (PCA) were performed to differentiate and classify the samples based on the contents of Alisol C 23-acetate, Alisol A, Alisol A 24-acetate and Alisol B 23-acetate. This simple, rapid, low-cost and reliable HPLC–DAD method using SSDMC is suitable for routine quantitative analysis and quality control of *A. orientale* (Sam.) Juz.

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1. Introduction

*Alisma orientale* (Sam.) Juz. (Alismataceae) is distributed in Japan, Mongolia, Russia and China (Sichuan and Fujian provinces) [1]. The dried rhizome, known as Alismatis Rhizoma, is a famous Traditional Chinese Medicine (TCM) which has been widely used for diuretic, hypolipidemic, anti-inflammatory and anti-diabetic purposes in China for more than a thousand years [2–6]. The pharmacologically active ingredients in Alismatis Rhizoma have been elucidated to be terpenoids. These terpenoids mainly comprise protostane-type triterpenes, guaiane-type sesquiterpenes, and kaurane-type diterpenes [7–10]. In particular, protostane-type triterpenes such as Alisol A, Alisol B, and Alisol C exist only in Alisma plants which are considered chemotaxonomic markers of the genus [11,12]. These triterpenes have specially been the research focus in recent years because of their relatively high levels of Alismatis Rhizoma and various pharmacological activities [13,14]. Therefore, in order to further effectively utilize it and to enhance the quality of herbal medicines, an accurate and reliable method based on the multiple constituents is urgently needed to be developed for quality control of Alismatis Rhizoma. In the pharmaceutical quality control process, since the separation and preparation of chemical reference substances in TCMs are difficult or unstable, the determination of index components is more difficult, which makes the quality inspection and supervision of TCMs still a challenge. To solve this problem, the SSDMC method was established in this study [15–17]. The SSDMC method is an economical and environment friendly method for the simultaneous assay of multi-components with only one reference standard to determine all the analytes in the sample when the method is officially accepted [18]. It is therefore necessary to develop alternative methods that can overcome this setback. Moreover, Alisol C 23-acetate (C), Alisol A (A), Alisol A 24-acetate (24A) and Alisol B 23-acetate (B) have similar chemical structures, which happen to meet the requirements of SSDMC.

Thus, a selective and sensitive HPLC–DAD for the simultaneous determination of C, A, 24 A and B of Alismatis Rhizoma using SSDMC was developed and validated for the first time in the work. Moreover, HCA and PCA were performed to evaluate and classify the 20 batches of *A. orientale* (Sam.) Juz. collected from different locations in China according to the contents of the four markers which were simultaneously determined by the SSDMC method to evaluate the quality in this study. This will be helpful for improving the quality evaluation of Alismatis Rhizoma.

2. Experimental

2.1. Materials and reagents

Standard: C (MUST-13092811), A (MUST-13052406), 24A (MUST-13040303) and B (MUST-13011608) were all obtained from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). The purity of the standards were all above 98% (Fig. 1).

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fisher Scientific, USA). Phosphoric acid for HPLC was obtained from Kemiou Chemical Reagent Co., Ltd.
Twenty batches of dried rhizomes of *A. orientale* (Sam.) Juz. from different provinces in China were collected and identified by Professor Ying Jia (Shenyang Research Center for Modernization of Traditional Chinese Medicine, Shenyang Pharmaceutical University). Voucher specimens were deposited at Shenyang Pharmaceutical University.

### 2.2. Apparatus

Agilent 1100 HPLC system (Agilent Technologies, USA) consisted of a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and a diode array detector (DAD). System control and data analysis were processed with Agilent ChemStation software. Additionally a different HPLC instrument was used. Agilent 1260 HPLC system (Agilent Technologies, USA) comprised a quaternary solvent delivery system, an on-line degasser, an auto-sampler, and a photodiode array detector coupled with an analytical workstation. A KH5200b sonicated bath (He Chuang, Kun Shan Co., Ltd.) was used for sample preparation.

### 2.3. Preparation of solutions

#### 2.3.1. Preparation of standards solution

Stock solutions of the reference standards (C, A, 24A and B) were prepared by dissolving accurately weighed standards in acetonitrile to yield the concentrations of 0.147, 0.958, 0.752 and 0.711 mg/mL, respectively, and stored in a 10 mL volumetric flask. These solutions were stored at 4 °C before use.

#### 2.3.2. Preparation of sample solutions

Transferred about 1000 mg of Alismatis Rhizoma, finely powdered and accurately weighed to a 100 mL glass-stoppered conical flask and, added accurately 10 mL of 70% acetonitrile–water. Weighed the filled flask with a precision of ±0.01 g and then sonicated for 30 min. After cooling to room temperature, adjusted to the initial weight by adding 70% acetonitrile–water as needed. Passed through a membrane filter having a 0.45-μm porosity; the successive filtrate is used as the sample solution.

### 2.4. Chromatographic conditions

The chromatographic separation was carried out on a YMC-Pack ODS-A (250 mm × 4.0 mm, 5 μm) with a guard column (4.6 mm × 10 mm, 5 μm, Agilent). The mobile phase was a mixture of acetonitrile–methanol (100:7) (A) and 0.05% phosphoric acid in water (B). The eluted gradient is as follows: 58–59% A for 0–10 min, 59–62% A for 10–20 min, 62–72% A for 20–27 min; 72–72% A for 27–48 min, 72–90% A for 48–50 min, 90–90% A for 50–65 min, 90–58% A for 65–65.01 min, 58–58% A for 65.01–75 min. The assay determination runtime was 48 min. The flow rate was set at 0.8 mL/min and the column temperature was at 25 °C with the injection volume of 10 μL. The wavelength program was set at 210 nm and 245 nm with 8 nm bandwidth and no reference wave.

### 2.5. Calculation of relative conversion factor and relative retention time

The relative conversion factor of the reference standard X (*RFX*) was calculated as reported previously based on the linearity data. It was briefly described as the following three equations [19], where *S* is Alisol B 23-acetate and X is other analytes.

$$ Fxi = \frac{Axi}{Cxi}, \quad RFxi = \frac{Fxi}{Fxa} \quad (i = 1 \sim n), \quad RFX = \frac{\sum RFxi}{N} $$

where *Axi* is the peak areas for analytes obtained from the external single standard at the concentration level *i*; *Cxi* is the concentrations of analytes at concentration level *i*; and *N* is the number of linearity data.

The relative retention time of reference standard X (*RRTX*) was calculated as the ratio of retention time of the reference standard X (RTx) to Alisol B 23-acetate (RTs).

### 2.6. Method for HCA of samples

The hierarchical clustering analysis was done by SPSS 19.0 software. Between-group linkage method was applied, and cosin method was selected as a measurement. Dendrogram resulting from the four investigated compounds’ contents was derived from the HPLC profiles of the tested samples.

### 2.7. Method for PCA of samples

The principal components analysis was done by SPSS 19.0 software. In this study, the contents of the four markers analyzed from the 20 samples composed a data matrix with 20 rows and 4 columns, which was used for the PCA analysis after normalization. The first two principal components were extracted, and the scatter plot was obtained by plotting the scores of PC 1 versus PC 2.

## 3. Results and discussion

### 3.1. Optimization of extraction procedure

Several extraction methods, solvents and times were investigated to obtain the best extraction efficiency. The results revealed that sonication was more effective than refluxing for the four analytes, so the further experiments were carried out with sonication. Various solvents including methanol, ethanol and acetonitrile were screened, and acetonitrile was found to be the best solvent, which enabled fewer interfering peaks and provided the highest responses of the four markers. Also 70% acetonitrile was chosen as the highest recovery was provided in the subsequent experiment. The volume of extraction solvent, ratio of acetonitrile and time of sonication were also investigated to optimize the extraction procedure. The results demonstrated that the established extraction method (each sample was extracted by sonication with 10 mL 70% acetonitrile for 30 min) was adequate and appropriate for the analysis.

### 3.2. Optimization of the chromatographic conditions

To obtain chromatograms with better resolution of adjacent peaks within shorter time, the chromatographic conditions were optimized. The resolutions of these compounds were tested and compared with
different reversed phase conditions using a variety of analytical columns such as YMC-Pack ODS-A (250 mm × 4.0 mm, 5 μm), Thermo BDS HYPERGEL C18 (250 mm × 4.0 mm, 5 μm), Waters Spherosorb C18 (250 mm × 4.0 mm, 5 μm) and Inertsil C18 (250 mm × 4.0 mm, 5 μm). Then YMC-Pack ODS-A was selected for the following experiments since results showed that only this column could supply appropriate resolution and response. Considering most of the compounds under investigation have maximum wavelength of absorption compared with methanol. And in this study, acetonitrile–methanol (100:7) was chosen as organic solvent because of its low wavelength threshold of absorption compared with methanol. And in this study, acetonitrile–methanol (100:7) was chosen as organic solvent because this combination gave the best resolution for the analytes. Moreover, 0.02%, 0.05% and 0.1% phosphoric acid were investigated in the experiment. And 0.05% gave the best resolution and eliminated the tailing of the target peak. As a result, a YMC C18 (250 mm × 4.0 mm, 5 μm) and Thermo BDS HYPERSIL C18 (250 mm × 4.0 mm, 5 μm) gave the best resolution and eliminated the tailing of the target peak. As a result, a YMC C18 (250 mm × 4.0 mm, 5 μm) and Thermo BDS HYPERSIL C18 (250 mm × 4.0 mm, 5 μm) was selected as the mobile phase was chosen as the preferred chromatographic conditions, and gradient elution was applied. It also suggested that separation was better when the column temperature was kept at 25°C rather than 30°C, and the runtime of 25°C was shorter time than 20°C. According to the absorption maxima of four reference compounds on the UV spectra with three-dimensional chromatograms of HPLC–DAD detection, the wavelength was set at 210 nm for A, 24A and B and 245 nm for C.

3.3. Selection of reference analyte

In order to generate the relative conversion factor for each analyte to the reference analyte, it is essential to choose a suitable reference analyte. The selected reference analyte should be an index composition which has a clear pharmacological effect or is related to clinical efficacy. The reference substance should be cheap, easily accessible, efficient, and stable in its chemical property. Meanwhile, a good separation under the chromatographic conditions should be achieved. Therefore, Alisol B 23-acetate was selected as the reference analyte because of its low cost, high stability and high content in Alismatis Rhizoma. On the other hand, it is one of the effective components of Alismatis Rhizoma.

3.4. Conversion factors and relative retention time

Using Alisol B 23-acetate as the reference analyte, the conversion factors and relative retention time are listed in Table 1.

The values of the RSDs of RRT (<0.4%) suggested that the RRT obtained on the same instrument was highly repeatable.

### Table 1

| Analytes | Regression equation | $R^2$ | Linearity range (μg/mL) | LOQ (μg/mL) | Conversion factors | RRT |
|----------|---------------------|-------|-------------------------|-------------|--------------------|-----|
| C        | $Y=19.75X+0.0011$   | 1.0000 | 1.470–29.40             | 1.24        | 0.49               | 0.28 |
| A        | $Y=10.40X+11.02$    | 0.9999 | 9.58–191.6              | 2.84        | 0.91               | 0.41 |
| 24A      | $Y=9.32X+8.95$      | 0.9998 | 7.520–150.4             | 3.54        | 1.02               | 0.58 |
| B        | $Y=9.68X+1.592$     | 0.9999 | 7.110–142.2             | 4.74        | —                  | —    |

3.5. Validation of the SSDMC method

The SSDMC method adopted to determine C, A, 24A and B in Alismatis Rhizoma was validated based on the following parameters: specificity, linearity, limits of quantification and detection, precision (intra-day and inter-day variability), accuracy, stability, and robustness as guided in USP.

The results obtained by the SSDMC method were compared with the results obtained by the traditional external standard method (ESM).

3.5.1. Specificity

The specificity was assessed by comparing the chromatograms obtained from the analysis of a blank sample, standard solution and sample solution. The integration peak in the chromatogram of the sample solution corresponded in time to the peak in the chromatogram of the standard solution. No peak of that retention time appeared in the chromatogram of the solvent (Fig. 2). The results indicated that the method was specific.

3.5.2. Linearity, limits of quantification and detection

Series of standard solutions of four compounds were freshly prepared in acetonitrile and were used to determine the linear range of the analytes. The results of the standard calibration curves of regression equation and linearity are summarized in Table 1. Good linear relationships and correlation coefficients ($R^2 >0.9998$) were achieved. The limit of detection (LOD) and limit of quantification (LOQ) values of individual compounds (Table 1) clearly indicated that the analytical method was acceptable with excellent sensitivity.

3.5.3. Precision and accuracy

The intra-day variability of precision was analyzed by six replicates at three different concentrations (low, medium, high). The inter-day variability of precision was analyzed by three replicates each day on three consecutive days. The RSDs of intra-day variability were in the range of 1.8–2.3% (SSDMC) and 1.4–2.4% (ESM), while those of inter-day variability were in the range of 1.8–2.3% (SSDMC) and 1.6–2.9% (ESM). The accuracy was evaluated with a recovery test performed by adding all of the reference standards into a sample (0.50 g) of Alismatis Rhizoma powder, followed by extraction and analysis as described in Section 2.3.2. Three concentration levels, covering the ranges of 50–150% of a known amount of analytes in the sample, were established in order to conduct the recovery test. The results of the accuracy tests calculated by the SSDMC method are displayed in Table 2. At the same time, the validation results calculated using the external standard method are also listed in Table 2.
The results indicated that the method was accurate and sensitive.

3.5.4. Stability

The stability test of the analytes was conducted by repeated injection into the instruments at room temperature (25 °C) for different times (0, 2, 4, 12, 24, 48 h). The relative standard deviation (RSD) value of the peak areas of each compound was calculated. The RSDs were less than 1.4%, indicating that the analytes were relatively stable in the mobile phase at room temperature for 48 h.

| Analytes | Original (μg) | Spiked (μg) | ESM | SSDMC |
|----------|---------------|-------------|-----|-------|
|          | Found (μg)    | Recovery (%)| Found (μg) | Recovery (%) | RSD (%) |
| C        | 28.70         | 11.17       | 39.61 | 97.3   | 39.56 | 96.9 | 5.7 |
|          | 29.53         | 22.34       | 50.67 | 92.4   | 50.61 | 92.1 |
|          | 29.86         | 44.69       | 76.31 | 103.4  | 76.22 | 103.2 |
| A        | 685.8         | 366.0       | 1051  | 100.4  | 1044  | 98.5 | 2.0 |
|          | 705.7         | 731.9       | 1485  | 105.5  | 1475  | 104.1 |
|          | 713.7         | 1464        | 2211  | 102.1  | 2196  | 101.1 |
| 24A      | 320.9         | 149.7       | 482.9 | 108.1  | 484.8 | 109.4 | 0.6 |
|          | 330.3         | 299.3       | 657.8 | 109.4  | 660.4 | 110.2 |
|          | 334.1         | 598.6       | 976.3 | 106.9  | 980.2 | 107.6 |
| B        | 306.4         | 147.9       | 457.6 | 103.5  |        |      |
|          | 315.3         | 295.8       | 632.1 | 107.8  |        |      |
|          | 318.9         | 591.6       | 960.1 | 108.4  |        |      |

Table 2 The results of recovery tests analyzed by the SSDMC method and the external standard method (ESM) (n=9).
3.5.5. Robustness

The ruggedness test was performed in order to introduce the SSDMC method to different laboratories. The factors studied were adjusted subjectively for one variable at a time. In this study, seven experimental conditions, which were slightly varied, included column temperature, flow rate, acid concentration, ratio of organic phase, time of gradient, wavelength and injection volume. Each experimental condition was selected at three levels. The maximal difference of relative conversion factor to each factor is shown in Fig. 3. The value of each factor was calculated by subtracting the relative conversion factor in level (+$1$) from that in $-1$ level at each factor.

From Fig. 3, it can be observed that (1) the variability of wavelength was more remarkable to the relative conversion factors of $C$ ($RF_C$) than the other two. $C$ has maximum absorption at 245 nm, the change of WL1 does not affect its absorption, meanwhile $FB$ has a great change, so RF$^C$ is very high. It also showed that the factor of wavelength was a prominent factor among the seven factors. (2) In this method, pH also had an enormous impact on relative conversion factors. (3) Influence of mobile phase ratio on the three analytes was similar. (4) The column temperature had little impact on the experiment. (5) Flow rate had a significant impact only on $FC$.

In addition, some factors were often not described in the experimental procedure, such as instruments and columns sources.

![Fig. 3](image)

The max difference of conversion factor obtained by subtracting the conversion factor in +1 level from that in −1 level at each factor. CT: column temperature; FR: flow rate; pH: pH of mobile phase; MP: ratio of components in mobile phase; TP: time programs of mobile phase; WL1: wavelength 1: 210 nm; WL2: wavelength 2: 245 nm; IV: injection volume.

### Table 3

| Samples | Origin          | ESM (mg/g) | SSDMC (mg/g) |
|---------|-----------------|------------|--------------|
|         |                 | C A 24 A B | C A 24 A Total | C A 24 A Total |
| 1       | Sichuan         | 0.054 1.719 | 0.938 0.622 3.333 | 0.055 1.700 0.942 3.319 |
| 2       | Shandong        | 0.132 1.268 | 0.466 1.095 2.961 | 0.134 1.254 0.468 2.951 |
| 3       | Sichuan         | 0.038 1.472 | 0.872 0.496 2.878 | 0.039 1.455 0.876 2.866 |
| 4       | Sichuan         | 0.061 1.582 | 0.934 0.638 3.215 | 0.062 1.564 0.938 3.202 |
| 5       | Dujiangyan, Sichuan | 0.070 1.345 | 0.623 0.571 2.609 | 0.070 1.330 0.626 2.598 |
| 6       | Taihangshan, Henan | 0.137 1.142 | 0.499 1.090 2.868 | 0.139 1.129 0.501 2.859 |
| 7       | Fujian          | 0.041 1.403 | 0.742 0.380 2.566 | 0.041 1.387 0.745 2.554 |
| 8       | Mianyang, Sichuan | 0.053 1.601 | 0.803 0.608 3.066 | 0.053 1.583 0.807 3.052 |
| 9       | Fuzhou, Fujian  | 0.081 1.017 | 0.501 0.503 2.103 | 0.082 1.005 0.504 2.094 |
| 10      | Pengshan, Sichuan | 0.056 1.373 | 0.749 0.472 2.650 | 0.056 1.358 0.752 2.639 |
| 11      | Heilongjiang    | 0.167 1.502 | 0.413 1.196 2.828 | 0.169 1.491 0.415 2.820 |
| 12      | Shicheng, Jiangxi | 0.114 1.378 | 0.727 0.861 3.080 | 0.115 1.363 0.731 3.070 |
| 13      | Nanjing         | 0.151 0.953 | 0.287 0.731 2.121 | 0.153 0.942 0.288 2.114 |
| 14      | Hebei           | 0.111 1.200 | 0.616 0.999 2.926 | 0.112 1.186 0.619 2.916 |
| 15      | Sichuan         | 0.047 1.611 | 0.869 0.558 3.085 | 0.048 1.593 0.873 3.071 |
| 16      | Sichuan         | 0.031 1.163 | 0.603 0.234 2.030 | 0.031 1.150 0.606 2.020 |
| 17      | Hangzhou, Zhejiang | 0.103 1.293 | 0.673 0.726 2.794 | 0.104 1.278 0.676 2.784 |
| 18      | Dujiangyan, Sichuan | 0.017 1.351 | 0.677 0.291 2.335 | 0.017 1.335 0.680 2.323 |
| 19      | Pengshan Sichuan | 0.068 1.186 | 0.599 0.487 2.340 | 0.069 1.173 0.602 2.331 |
| 20      | Fuzhou, Fujian  | 0.058 1.616 | 0.732 0.618 3.024 | 0.058 1.598 0.735 3.010 |
The relative conversion factors might be changed with the above environmental factors when the analysis procedure was used in different laboratories. Two columns were used on the two HPLC instruments separately in this study. The RRTs and RFs from different columns and instruments were almost the same, indicating a good robustness of the RRTs and RFs.

3.6. Sample analysis

Twenty representative samples of Alismatis Rhizoma from different locations were analyzed using two methods (SSDMC and ESM). The contents of the four investigated compounds were calculated (Table 3). There were no remarkable differences between the contents obtained from the two methods. Also, the results showed that there were remarkable differences among the contents of the four analytes in different Alismatis Rhizoma samples. For example, although Alisol A was the highest content constituent in almost all Alismatis Rhizoma samples, its contents varied from 0.942 to 1.700 mg/g. The same variation could also be found in other constituents. In fact, the differences in the content of the analytes among different Alismatis Rhizoma samples may be attributed to many factors, including genetic variation, plant origin, harvest time, and climate or geography (soil or minerals) [15,20].

3.7. HCA of the samples

In order to evaluate the variation of Alismatis Rhizoma, HCA was performed based on the contents of the four analytes from HPLC profiles. The results (Fig. 4) showed that the 20 tested samples of Alismatis Rhizoma were divided into two main clusters (I and II) according to their contents. The results indicated that the samples collected from similar cultivation regions were classified in one cluster, such as samples 8, 5, 1, 4 and so on (from Sichuan, Fujian provinces of China) were classified in the same cluster, and samples 6, 11 and 2 and so on (from other regions which are non-genuine producing areas) were classified in another cluster, which indicates that the influence of Alismatis Rhizoma cultivation regions on the contents of Alisol C 23-acetate, Alisol A, Alisol A 24-acetate and Alisol B 23-acetate is very obvious.

3.8. PCA of the samples

The determination results of 20 samples were further analyzed and classified by PCA. The two principal components (PC 1 and PC 2) with more than 99.6% of the whole variances were extracted for analysis. PC 1 accounted for 89.7% variances and PC 2 accounted for 9.9%. The scatter plot is shown in Fig. 5, where each sample was represented as a marker. It was noticeable that the samples were clearly clustered into two domains. Samples 2, 6, 11, 12, 13, 14 and 17 were in domain I, and the others were in domain II. Actually, these results were exactly the same as those of the HCA. All the samples in domain I are from non-genuine producing areas; on the contrary, all the samples in domain II are from genuine producing areas.

4. Conclusion

A validated and sensitive HPLC method with high precision, stability, and repeatability was developed for simultaneous quantitation of four analytes in Alismatis Rhizoma, and the method was successfully applied to 20 samples from different locations of China. Four peaks were identified by comparison with standards based on their UV spectra and the relative retention time for each analyte. The results demonstrated that this method was feasible for the comprehensive quality evaluation of Alismatis Rhizoma. Compared with accurate values obtained from ESM, the SSDMC method was proved to be a fast, convenient, and accurate method to determine the contents in Alismatis Rhizoma. The method is ideal for rapid, routine analysis, especially for those laboratories where the standards are not readily available.

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