Study on the destructive effect to inherent quality of *Fritillaria thunbergii* Miq. (Zhebeimu) by sulfur-fumigated process using chromatographic fingerprinting analysis

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- Ultra-performance liquid chromatography coupled with evaporative light scattering detection
- Sulfur-fumigation process
- Chromatographic fingerprinting

**Abstract**

The after-harvesting sun-dried processing of *Fritillariae thunbergii* bulb (Zhebeimu) was the traditional treatment for commodity. Over recent decades the natural drying process for bulbus of *Fritillariae* has been replaced by sulfur-fumigation for reducing the drying duration and pest control. We used ultra-performance liquid chromatography coupled with evaporative light scattering detection (UPLC–ELSD) fingerprinting analysis and major alkaloids determination to investigate the potential damaging effect of the sulfur-fumigating process. The experimental conditions were as follows: Chromatography was proceeded on Waters Acquity UPLC BEH C\textsubscript{18} column; the linear gradient elution was conducted with mobile phase prepared from acetonitrile–0.02% triethylamine; the drift tube temperature was set at 40 °C with a nitrogen flow-rate of 30 psi, and the spray parameter was set 40%. All calibration curves showed good linear regression (R\textsuperscript{2} > 0.9991) within the tested range. The method was validated for precision, accuracy, limit of detection and quantification. The study also has shown that sulfur-fumigated samples had significant loss of the main active compounds and a more destructive fingerprint profile when compared to the sun-dried samples.

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**Introduction**

*Bulbus Fritillariae* (BF), Beimu in Chinese, derived from the bulbs of several *Fritillaria* species (family Liliaceae), has been used as an antitussive and expectorant herb in traditional Chinese medicine (TCM) for more than 2000 years. In the 2010 edition of Chinese Pharmacopoeia, a total of five groups of Beimu (including eleven *Fritillaria* species), namely Zhe-Beimu (*F. thunbergii* Miq.), Chuan-Beimu (*F. cirrhosa* D. Don, *F. unibracteata* Hsiao et K.C. Hsia, *F. przewalskii* Maxim. ex Batal., *F. delavayi* Franch., *F. taipaiensis* P.Y. Li and *F. unibracteata* Hsiao et K.C. Hsia var. *wabuensis* (S.Y. Tang et S.C. Yue) Z.D. Liu, S. Wang et S.C. Chen), Ping-Beimu (*F. usuriensis* Maxim.), Hubei-Beimu (*F. hupehensis* Hsiao et K.C. Hsia) and Yi-Beimu (*F. walujewii* Regel and *F. pallidiflora* Schrenk), were officially recorded (Chinese Pharmacopoeia Commission 2010). Besides, the bulbs of more than 30 other species were utilized locally as substitutes for BF in some areas of China (Xiao et al. 2007). *Fritillaria thunbergii* Miq. (*F. thunbergii*, family Liliaceae), whose underground bulbs are officially listed in the Chinese Pharmacopoeia as “Zhebeimu” (Chinese Pharmacopoeia Commission 2010), has been used in oriental preparations for a long time. In traditional medicine, *F. thunbergii* is frequently used for the treatment of coughs and asthma. The major active components of the herb are steroidal alkaloids, including peimine, peimine and peiminine (Wagner et al. 2011; Qian and Xu 1985). The after-harvesting sun-dried process of *Fritillariae* thunbergii bulb (Zhebeimu) was traditional treatment for commodity. Over recent decades the natural drying process for bulbus of *Fritillariae* has been replaced by sulfur-fumigation for shortening the drying duration, controlling pests and maintaining a better appearance. However, the sulfur dioxide generated during sulfur-fumigation period would inevitably react with ingredients in the herbal drugs in addition to the drying, insecticide and whitening effects. To date, no related investigations have described the effect of sulfur-fumigation processing on this herb in detail. In the past several years, chromatographic fingerprints have been established using HPLC, HPTLC, GC and CE, and they have been recognized as rapid, reliable methods for the identification and qualification of herbal medicines. (Bauer et al. 1988; Wagner and Bladt 2001;...
Experimental

Chemicals, reagents and materials

The standard compound peimidine was isolated in our laboratory, as published earlier (Cao et al. 2009). The purity (> 98%) was confirmed by chromatographic (HPLC) methods. Hupehenine, imperaline, peimine and peimidine (purity > 98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The chemical structures of these alkaloids are shown in Fig. 1. HPLC grade methanol and acetonitrile were purchased from Fisher (Fisher Scientific, Fairlawn, New Jersey). Triethylamine was obtained from Sigma–Aldrich. Deionized water was prepared using a Purelab Plus UV System (ELGA, UK). Other chemicals were of reagent grade. Samples of F. thunbergii were collected from genuine producing area in Zhejiang province, China. All samples were identified by Prof. Chen Shilin, and the voucher specimens were deposited in the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.

Instrumentation and chromatographic conditions

UPLC analysis was performed using a Waters Acquity system (Waters, Milford, MA, USA) equipped with a binary solvent delivery pump, an auto sampler and a ELS detector (Waters Acquity model code UPE), connected to a Waters Empower 2 data station. The chromatographic separations were carried out on a Waters Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm, Waters, Milford, MA, USA). The mobile phase consisted of acetonitrile (solvent A) and 0.02% aqueous triethylamine (solvent B) with a linear gradient: 0–3 min (30–60%, A), 3–4 min (60–80%, A), 4–10 min (80–100%, A). The flow rate was 0.25 ml/min, the sample injection volume was 1 µl and the column and sample temperature were 25 °C. The drift tube temperature for the ELSD was set at 40 °C with a nebulizing gas pressure of 30 psi and the spray parameter was set 40% at a gain of 500. Strong needle wash solution (95:5, acetonitrile:water) and weak needle wash solution (10:90, acetonitrile:water) were used.

Preparation of sample solution

All the samples were milled into powder and oven-dried at 30 °C until they reached a constant weight. An accurately weighed sample of 2.0 g powder was refluxed with 60 ml 80% (v/v) chloroform–methanol at a constant temperature of 80 °C for 3 h, then cooled to room temperature. The extract was filtered through glass wool and evaporated, then diluted to volume with methanol in a 2 ml volumetric flask. The sample solution was subsequently filtered through a 0.22 µm membrane and injected into the UPLC system for analysis.

Preparation of standard solution

The standard stock solutions of peimidine (1150 µg/ml), peimine (1095 µg/ml) and peimidine (1030 µg/ml) were prepared in methanol and stored at −4 °C. The calibration curves were prepared at seven different concentration levels. The solutions were brought to room temperature and filtered through a 0.22 µm membrane filter before injection.

Validation of the UPLC method

The newly developed UPLC method was validated in terms of precision, accuracy and linearity according to ICH guidelines (ICH Topic Q2B 1996; Ye et al. 2006; Kong et al. 2009). Assay method precision was carried out by intra- and inter-day tests. The accuracy of the assay method was evaluated in triplicate using three concentration levels designated low, middle and high.

Fig. 1. Structures of five steroidal alkaloids.
Data analysis

Similarity analysis (SA) was performed by the professional software Computer Aided Similarity Evaluation (CASE), which was developed based on chemometrics by the Research Center of Modernization of Traditional Chinese Medicines (Central South University, Changsha, China) and mainly applied in the similarity study of chromatographic and spectral patterns (Gong et al. 2003). All of the fingerprint data were processed on a Pentium IV computer, and the programs for similarity analysis were coded in MATLAB 7.0 (Mathworks). The hierarchical clustering analysis (HCA) and principal components analysis (PCA) were performed on the characteristic chromatographic peaks in the UPLC fingerprints using Unscrambler X 10.0 software from Camo AS (Trondheim, Norway). To establish clusters, Ward’s method was used as the amalgamation rule and the squared Euclidean distance as metric was used as the metric.

Results and discussion
Optimization of chromatographic conditions

The UPLC mobile phase (methanol–water or acetonitrile–water) and the flow rate of the mobile phase (0.1, 0.2 and 0.25 ml/min) were examined for optimization. As a result, acetonitrile–water containing 0.02% triethylamine with a flow rate of 0.25 ml/min was chosen to give the desired separation and offered an acceptable tailing factor. Furthermore, other chromatographic variables were also optimized, including the analytical columns (HSS C18 and BEH C18) and column temperatures (25°C and 30°C). Finally, the optimal separation was achieved on a BEH C18 column (2.1 mm × 100 mm, 1.7 μm) at a column temperature of 25°C.

Optimization of ELSD parameters

The quantitative evaluation of the investigated analytes was achieved by using a waters ELS detector (Waters Acquity, model code UPE). The sensitivity of this method is affected by three instrumental parameters: drift tube temperature, nebulizing gas pressure and photomultiplier gain (Cardenas et al. 1999), each of which plays a prominent role in analyte response. The effect of temperature was evaluated by injection of a standard at different detector temperatures (40°C, 50°C and 60°C). Fig. 2 presents the peak area variation with drift tube temperature. The optimum temperature seemed to be 40°C and the spray parameter was 40%. This temperature was enough to allow a complete solvent evaporation and a negligible baseline noise. With respect to the nebulizer gas pressure, in general the highest signal should be obtained at the lowest nebulizing gas pressure, and increasing the gas pressure results in a marked decrease in the signal response, but in the present study the baseline was unstable when gas pressure was decreased to 25 psi. Therefore, the optimal gas pressure was set at 30 psi because of the effect of the nebulizing gas pressure on S/N of the analytes.

Optimization of extraction conditions

In this study, ultrasonic and reflux extraction methods were investigated in order to obtain the best extraction efficiency. The results suggested that reflux extraction was better than ultrasonic extraction, and was therefore selected for use in further experiments. Various types of extraction solvents, including methanol, ethanol, chloroform and a serial concentration of methanol–chloroform, were evaluated for their extraction efficiency in the reflux method. The results showed that the highest extraction yield was obtained by using 80% (v/v) chloroform–methanol. Other experimental factors, including extraction time (30, 60, 120 and 180 min), temperature (60, 70, 80 and 90°C) and extraction solvent volumes (20, 30, 40 or 60 ml), were also tested and evaluated. All the results suggested that reflux with 80% (v/v) chloroform–methanol (60 ml) at 80°C for 180 min was effective and simple for alkaloid extraction.

UPLC method validation

Calibration curves

A stock solution was diluted with methanol to appropriate concentrations for establishing the calibration curves. Seven concentrations of three analytes solutions were injected in triplicate, and the calibration curves were then plotted on a logarithm using peak area versus concentration for each analyte. Linear regression analysis for each of the three compounds was performed by the external standard method. The range and correlation coefficients are presented in Table 1. All calibration curves showed good linear regression (R > 0.999). The limits of detection (LOD) and quantification (LOQ) were determined by injecting a series of reference solutions until the signal-to-noise (S/N) ratio for each compound was three for the LOD and ten for the LOQ.

Precision

The precision of the method was evaluated by assaying freshly prepared solutions in triplicate. The intra-day and inter-day precision were determined by analyzing the same mixed standard

### Table 1
Linear regression and precision data of the three analytes.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Linear regression</th>
<th>Linear range (μg/ml)</th>
<th>LOQ (μg/ml)</th>
<th>LOD (μg/ml)</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calibration curves</td>
<td></td>
<td></td>
<td></td>
<td>Intra-day RSD (%)</td>
</tr>
<tr>
<td>Peimisine</td>
<td>y = 1.6297x + 1.6800</td>
<td>0.9992</td>
<td>28.75–115.00</td>
<td>11.50</td>
<td>4.60</td>
</tr>
<tr>
<td>Peimine</td>
<td>y = 1.7509x + 1.3807</td>
<td>0.9991</td>
<td>27.375–1095.00</td>
<td>10.95</td>
<td>4.38</td>
</tr>
<tr>
<td>Peimimine</td>
<td>y = 1.8x + 1.2429</td>
<td>0.9994</td>
<td>25.75–1030.00</td>
<td>10.30</td>
<td>4.12</td>
</tr>
</tbody>
</table>

In the calibration curves, y = lg A, x = lg C (A: peak area, C: μg/ml; lg: logarithm).
solution in triplicate within one day and on three consecutive days, respectively. These results are summarized in Table 1.

Accuracy

A recovery test was used to evaluate the accuracy of the method. The standards of three alkaloids were spiked at known amounts into mixed standards of known *F. thunbergii* samples at three different concentration levels (high, medium and low) with three parallels at each level. Nine samples from the same place were extracted and analyzed with the proposed method. The results showed that the recoveries of the three alkaloids ranged from 94.6% to 105.0% with RSD less than 2.4%, which was satisfactory, as shown in Table 2.

Sample analysis

The developed UPLC–ELSD method was applied to the simultaneous determination of the three alkaloids in *F. thunbergii* samples processed by different methods and collected from various locations in China. The target compounds were identified by comparing retention times with those of standards. Representative UPLC–ELSD chromatograms of the standard solution and the extracts of these samples are shown in Fig. 3. The mean content of each sample was analyzed in triplicate and calculated by the external standard method, and the results are presented in Table 3. The results in Table 3 showed that the contents of alkaloids of *F. thunbergii* samples were various, with the contents of three most common alkaloids in *F. thunbergii* samples in the order peimine > peiminine > peimisine, except for the sample (S4) collected from Panan, Zhejiang. This discrepancy indicated the differences in *F. thunbergii* quality and bioactivity. Imperialine and hupehenine were not detected in *F. thunbergii*, which was in agreement with the reported literature (Li et al. 2001).

Through comparison with the qualified sample’s content limit of *Fritillariae thunbergii* bulbus stated in the Chinese Pharmacopoeia (2010 edition, 0.08% (w/w) total contents of peimine and peiminine), three unqualified samples (30% of total samples) were found. That is, the total contents (w/w) of peimine and peiminine were 0.17% (S1), 0.12% (S3, S7), 0.10% (S2, S9), 0.14% (S8), 0.13% (S5), 0.02% (S4), 0.01% (S6) and 0.00% (S10). Therefore, S4, S6 and S10 were the samples below the limit for *Fritillariae thunbergii* bulb stated in the Chinese Pharmacopoeia. There were two possible reasons for this. One is that two of the samples (S4, S10) were fumigated by sulfur dioxide, which might have destroyed the chemical constituents. Another reason could be that S6 was stored too long.

**Table 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial amount (µg)</th>
<th>Spiked amount (µg)</th>
<th>Found (µg)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peimisine</td>
<td>124.44</td>
<td>69.00</td>
<td>192.06</td>
<td>98.0</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>124.44</td>
<td>115.00</td>
<td>245.19</td>
<td>101.0</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>124.44</td>
<td>172.50</td>
<td>293.66</td>
<td>99.7</td>
<td>1.96</td>
</tr>
<tr>
<td>Peimine</td>
<td>108.50</td>
<td>30.90</td>
<td>159.75</td>
<td>94.6</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>108.50</td>
<td>72.10</td>
<td>217.12</td>
<td>99.2</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>108.50</td>
<td>103.00</td>
<td>338.23</td>
<td>104.9</td>
<td>0.75</td>
</tr>
<tr>
<td>Peiminine</td>
<td>71.56</td>
<td>54.75</td>
<td>96.77</td>
<td>97.9</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>71.56</td>
<td>109.50</td>
<td>131.20</td>
<td>96.5</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>71.56</td>
<td>164.25</td>
<td>233.79</td>
<td>105.0</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* a Recovery (%) = [(detected amount – original amount)/spiked amount] × 100.

* b RSD (%) = (SD/mean) × 100.

Fig. 3. Typical UPLC–ELSD chromatogram of mixed standards (A) and sample extracts (B). Peak 6: peimine; 7: peiminine; 9: peimisine; 10: imperialine; 11: hupehenine.
Sulfur-fumigated Zhebeimu is resistant against pest, microbial and mold growth, and has a fresh, white color that is extremely appealing. However, sulfur residue remaining in the raw herbs has been shown to be harmful. Additionally, treatment with sulfur caused the chemical constituent to change greatly. These results indicated the instability of the herb quality in relation to the different processing methods, storing time and producing areas.

**UPLC fingerprint of F. thunbergii and similarity analysis (SA)**

To obtain the standard fingerprint, 10 batches of *F. thunbergii* samples were analyzed with the developed UPLC–ELSD analysis procedure. Peaks that existed in all 10 samples with reasonable heights and good resolution were assigned as “characteristic peaks” for the identification of the plant. There were eight characteristic peaks (1–8) within 10 min, shown in Fig. 3(b), and the assignment of three peaks in the profile was confirmed by comparison of the retention times of reference substances. The assignment of these three peaks was: peimine (6), peimisine (7) and peiminine (9). The UPLC profile of sulfur-fumigated Zhebeimu demonstrated all the major alkaloids were significantly decreased, with peak 6 (peimine) reduced by about 80% and peak 7 (peimisine) almost disappearing (Fig. 4).

The similarities in the chromatograms of the 10 samples with the reference fingerprint, which was the median of all chromatograms, are shown in Table 4. The closer the cosine values were to 1, the more similar the two chromatograms were (Wei et al. 2010). The similarity values of all 7 samples were more than 0.80, except S4, S10 and S6. Compared to the sun-dried Zhebeimu, the similarity of the sulfur-fumigated samples was lower than 0.80.

If similarity value over a certain value, 0.8 for example, were regarded as the threshold for qualification, it was easy to identify the qualified samples based on the chromatographic fingerprint. The fingerprint patterns of the samples were different, and by comparing each fingerprint pattern with the mean chromatogram, we can obtain the similarity value of each fingerprint pattern. This value could then help us evaluate the quality of the different samples (Wei et al. 2010). The plot of PCA also revealed that the two types of Zhebeimu were neatly partitioned (Fig. 5). This observation indicated that sulfur-fumigation destroyed the constituents to varying degrees. Fig. 4, for example, clearly shows these differences between UPLC fingerprints for sulfur-fumigation and sun-dried processing methods.

Peak 6 was an important active component of *F. thunbergii*, and was chosen to calculate the relative retention time (RRT) and relative peak area (RPA). The resulting RRT and RPA of the eight characteristic peaks in the 10 samples are shown in Table 5.

**Quality assessment by hierarchical clustering analysis (HCA)**

In order to evaluate the resemblance and differences in these samples, hierarchical cluster analysis (HCA) was performed based on 8 constituent alkaloids in the HPLC profiles. The results of HCA are shown in Fig. 6. It was clear from the data that the samples could be divided into three clusters. Cluster I was formed by the...
TABLE 5
The relative retention time (RRT) and relative peak area (RPA) of characteristic peaks for 10 F. thunbergii samples.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>RRT</th>
<th>RSD (%)</th>
<th>RPA</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09</td>
<td>3.09</td>
<td>0.82</td>
<td>143.28</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.85</td>
<td>4.18</td>
<td>172.55</td>
</tr>
<tr>
<td>3</td>
<td>0.55</td>
<td>1.36</td>
<td>18.25</td>
<td>205.58</td>
</tr>
<tr>
<td>4</td>
<td>0.57</td>
<td>1.42</td>
<td>2.06</td>
<td>182.13</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
<td>1.70</td>
<td>0.82</td>
<td>146.97</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0.00</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>7</td>
<td>1.05</td>
<td>0.16</td>
<td>0.36</td>
<td>42.53</td>
</tr>
<tr>
<td>8</td>
<td>1.19</td>
<td>0.21</td>
<td>0.27</td>
<td>160.42</td>
</tr>
</tbody>
</table>

Moreover, the results from PCA were largely consistent with that of the similarity evaluation, as these samples were determined to be outliers by similarity evaluation in view of their low similarity indexes (below 0.8). Therefore, for the F. thunbergii samples studied in this work, the internal quality of the samples processed by sun-drying was determined to be better than the samples sulfur-fumigated by analyzing the total contents of peimisine, peimine, peimine in these samples using UPLC–ELSD.

Conclusions

This work developed for the first time a UPLC method for the fast, simultaneous determination of peimine, peimine, peimine and the UPLC chemical fingerprint profile in F. thunbergii. Chemometrics methods, such as SA, PCA and HCA, were successfully applied to the data from the UPLC technique to identify the method of processing of F. thunbergii samples. The results of this study concluded that the sulfur-fumigation process brought about significant loss of the main active constituents. Furthermore, the analytical method described here is simple, sensitive and selective with good accuracy and precision. Considerable reductions in analysis time were observed with excellent separation quality compared to traditional HPLC. This UPLC assay provides an important reference, and can be readily utilized as a suitable method for the rapid and accurate quality evaluation for F. thunbergii and its related medicinal materials or preparations.

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