Analyzing Different Compositions of Polygala from Different Regions Using the X500R QTOF System

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Introduction

Authentic herbs come from specific locations that are traditionally known for these high-quality products. Authentic herbs have become synonymous with traditional Chinese medicine and form a comprehensive material standard for evaluating the quality of Chinese herbal medicines. Authentic herbs thus play a unique and important role in authentication and quality control of Chinese herbal preparations. Authenticity of Chinese medicine has become an important guarantee of high herbal quality.

Polygala is one of the main Chinese herbal medicines, one of 85 traditional Chinese herbal medicine exports, and one of 42 species of level 3 protected wild products in China[1]. The 2010 "Chinese Pharmacopoeia" divides Polygala herbs into those derived from the plant leaves of Polygalaceae and those made from dried Polygala leaves and roots. They have the properties of sedation, promoting heart and kidney circulation, acting as an expectorant, and decreasing swelling. They are used to treat insomnia, excessive dreaming, forgetfulness, and fear caused by poor heart and kidney circulation[2]. The commercial Polygala industry depends on the Polygala supply, which is found in an area bounded by the desert to the south and the Yangtze River to the north. It is grown mainly in Shanxi, Shaanxi, Henan, and Hebei, under the traditional notion of "Shanxi - large quantity, Shaanxi - high quality"[3].

Currently, the identification and analysis of Chinese herbal medicine components is quite challenging. These components underlie the pharmacodynamic efficacy of Chinese medicinal products. Herein lies the key to modernizing Chinese medicine. How to quickly identify the active ingredient and its structure, as well as how to identify the differences between the active ingredients of authentic and inauthentic herbs, are urgent problems that must be solved.

This study used the new SCIEX high resolution X500R QTOF mass spectrometer for data acquisition and used the accompanying MarkerView™ Software to statistically analyze differences between components. This study involved Chinese medicine (e.g., Polygala) that includes components from different regions. This method makes component identification more effective, faster and a better reflection of the integrity and unique nature of the sample tested. In turn, it highlights the differences between Polygala components from different sources and provides a new framework for quality evaluation of Chinese herbal medicines.

The high resolution X500R’s new hardware design, including N-type ion path technology, time of flight tube design, and a stable and durable Turbo V™ ion source, ensures that under routine testing conditions, sample identification is more stable, higher quality, and more reliable for the long term. The X500R’s high-sensitivity, high-resolution analysis and accurate mass-to-charge ratio analysis, combined with the intelligent TOF-MS-IDA-MS/MS acquisition mode, truly achieve the goal of collection of high-quality, accurate primary and secondary mass spectrometry data by single injection, and quickly provide the most accurate qualitative screening results.

SCIEX X500R QTOF mass spectrometry system with ExionLC™ liquid chromatography system and SCIEX OS workstation

Study Design

1. Samples of Polygala herbs from different sources were obtained and assigned to groups, each containing 6 samples.
2. TOF-IDA-MS/MS mode was used for data acquisition; one injection allowed simultaneous collection of various components’ primary ions and secondary daughter ions.
3. MarkerView™ Software was used to analyze differences in components and identify statistically significant differences between groups for use as markers.
4. After entering mass spectrometry data on primary ions and secondary daughter ions into SCIEX OS Software, the components were matched with the SCIEX high resolution MS/MS Chinese medicine database or the ChemSpider online database; differences in components were identified.
Study Design Workflow

1. Acquire high-resolution primary TOF-MS and secondary TOF-MS/MS spectra
2. MarkerView Software, Perform statistical analysis of data, to find variant ions
3. Structural identification software Structural identification and secondary analysis of variant ions

Materials and Methods

This study collected Polygala herbs from 4 regions: Chengcheng, Shaanxi; Shangluo, Shaanxi; Shanxi; and Hebei. After the samples were dried, they were cut into small pieces and dried in the oven at 40 degrees C for 18 h. After removal, they were crushed and filtered through a 20 mesh sieve, placed in the dryer, and then used.

Preprocessing Method

Carefully weigh out about 1.0 g of Polygala powder of consistent weight, add 50 mL of 70% methanol aqueous solution, ultrasonicate 30 min., centrifuge for 10 min at 13000 rpm, and take the supernatant for injection.

Chromatographic Conditions

Chromatographic Column: Phenomenex Kinetex F5, 100*3.0 Mobile phase: A is ultrapure water/B is acetonitrile;

Gradient elution was performed as shown below:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A%</th>
<th>B%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>5.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>15.0</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>20.0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>25.0</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>30.0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>35.0</td>
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<td>35</td>
</tr>
<tr>
<td>40.0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>45.0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>45.1</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td>50.0</td>
<td>90</td>
<td>5</td>
</tr>
</tbody>
</table>

Flow rate: 0.4 mL/min ;
Column temperature: 40°C ;
Amount inserted: 5 μL

Mass Spectrometry Method

Scanning method: TOF-IDA MS/MS qualitative screening;
Ion source: ESI source
Mass spectrum parameters are established in 4 steps:

Figure 1. Typical BPC for four polygala samples from different sources

Chromatographic peak retention reproducibility was very good among the four Polygala samples from different sources. Many baseline analysis separation peaks were obtained on the base
peak chromatogram (BPC), showing good chromatographic separation.

**MarkerView™ Data Processing**

The MarkerView™ Software was used for preliminary data extraction of chromatographic peaks. Identification and integration were performed on chromatographic peaks with a retention time of 0 - 50 min; the three-dimensional data was transformed into a two-dimensional data matrix, including variables (m/z RT), number observed (24 samples), and the integral area. This study found 994 variables (m/z RT).

**Principal Component Analysis (PCA)**

All samples underwent supervised PCA analysis, and their Score and Loading chart is as shown in Fig.2:

![Figure 2](image)

**Figure 2** A) Polygala samples from different sources, PCA Score Plot; B) Polygala samples from different sources, PCA Loading Plot;

Fig. 2 Score Plot shows Polygala samples from the 4 different areas are well separated, meaning that there are large differences between groups.

Using Polygala products sourced from different areas, take m/z 667.2 (RT=16.9 min) as an example. For m/z 667.2 in the figure below, showing content differences in samples from different areas, the line plot shows that Chengluo, Shaanxi Polygala has a Tenuifoliside B2 content that is approximately 5 times that of the 3 other areas, as in Fig. 3.

![Figure 3](image)

**Figure 3** Polygala Marker: m/z (667.2), (RT=16.9 min)

Polygala characteristic marker m/z 667.2, retention time 16.9 min, SCIEX OS identification of the marker is: Tenuifoliside B2, C30H36O17, m/z (MS)= 667.1875, m/z (MS/MS) = 461.1288, 367.1035, 239.0557, 205.0498, 190.0265. Using Library search, identification results in

Fig. 4-1 shows that secondary fragment matching is good, with the main fragment structural analysis shown in Fig. 4-2:

![Figure 4-1](image)

**Figure 4-1.** Polygala Marker m/z 667.2 via SCIEX OS structural attribution results

![Figure 4-2](image)

**Figure 4-2.** Polygala Marker m/z 667.2 secondary fragment attribution and main fragment structural analysis

All samples underwent T-test data processing; results are in Fig. 5. Fig. A is the volcano plot, expressed as log fold change vs. p-
value; as the X axis is approached, more ions are located at both ends of the X axis, indicating a greater difference between them. Fig. B is a line plot, and Fig. C is a box plot, showing the content relationships between the samples.

**Figure 5** Log (Fold Change) versus p-values data processing
T-experimentally (p < 0.005) differentiated ion scans appear in line plot B and box plot C. Compound m/z 567.1 (RT 14.8 min) is significantly different in the Shangluo, Shaanxi Polygala, so it is used as a marker. Its structure is identified with SCIEX OS software’s ChemSpider online structural identification for markers. Results are in Fig. 6:

**Figure 6-1.** Polygala Marker: m/z (567.1), (RT 14.8 min)
Marker identified as: Polygalaxanthone III, C_{25}H_{28}O_{15}. m/z (MS)= 567.1359. m/z (MS/MS) = 345.0608, 315.0510, 399.0724, 271.0247; its online secondary fragment matching is good.

**Figure 6-2.** Polygala Marker m/z 567.1 secondary fragment attribution and main fragment structure analysis

**SCIEX OS Compound Structural Identification Process**

Using the SCIEX OS Formula Finder function, based on this ion’s primary mass spectrum exact mass and isotope ratio, the likely molecular formula was identified. At the same time, mass spectrometry fragmentation patterns and the ion’s secondary mass spectrum mass verified the molecular formula.

Using Polygala products sourced from different areas, take m/z 1379.4083 (RT 30.22 min) as an example. With the Formula Finder function, based on an exact mass and isotope distribution, the molecular formula was determined to be C_{62}H_{76}O_{35}. Its TOF MS mass deviation was -0.8 ppm, and 17 TOF MS/MS fragments’ mean mass deviation was 0.9 ppm. Results are shown in Fig. 7:

**Figure 7.** Polygala Marker m/z (1379.4083) molecular formula calculated with Formula Finder
The MS/MS fragmentation molecular formula is shown with green dots; see Fig. 8 for the fragmentation molecular formula, which is consistent with the mass spectrum fragmentation pattern.
This study identified 11 signature markers in Polygala from different areas; of these, No. 1-2 are sucrose esters, No. 3-8 are oligosaccharides, No. 9-10 are triterpenoid saponins, No. 11 is xanthone. A summary is shown in Table 1-1. Table 1-2 shows the content differences in the 11 signature markers.

Table 1-1: Summary of signature ions of 4 Polygala samples from different sources

<table>
<thead>
<tr>
<th>No.</th>
<th>m/z (MS)</th>
<th>RT (min)</th>
<th>Ion Formula</th>
<th>Tentative identification</th>
<th>Major fragment ion (MS/MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>507.1874</td>
<td>16.9</td>
<td>C36H43O20</td>
<td>Tenuifoliose B2</td>
<td>345.3044</td>
</tr>
<tr>
<td>2</td>
<td>753.3242</td>
<td>17.38</td>
<td>C52H59O29</td>
<td>Tenuifoliose S</td>
<td>223.0602</td>
</tr>
<tr>
<td>3</td>
<td>1203.3621</td>
<td>27.3</td>
<td>C50H65O31</td>
<td>Tenuifoliose F</td>
<td>175.0401</td>
</tr>
<tr>
<td>4</td>
<td>1245.6042</td>
<td>25.76</td>
<td>C52H59O29</td>
<td>Tenuifoliose S</td>
<td>223.0602</td>
</tr>
<tr>
<td>5</td>
<td>1255.6032</td>
<td>25.86</td>
<td>C54H67O32</td>
<td>Tenuifoliose F</td>
<td>223.0602</td>
</tr>
<tr>
<td>6</td>
<td>1289.6035</td>
<td>30.22</td>
<td>C54H67O32</td>
<td>Tenuifoliose F</td>
<td>223.0602</td>
</tr>
<tr>
<td>7</td>
<td>1405.6036</td>
<td>25.76</td>
<td>C52H59O29</td>
<td>Tenuifoliose L</td>
<td>223.0602</td>
</tr>
<tr>
<td>8</td>
<td>1450.6036</td>
<td>25.76</td>
<td>C54H67O32</td>
<td>Tenuifoliose F</td>
<td>223.0602</td>
</tr>
<tr>
<td>9</td>
<td>1450.6036</td>
<td>25.76</td>
<td>C54H67O32</td>
<td>Tenuifoliose F</td>
<td>223.0602</td>
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<tr>
<td>10</td>
<td>1489.6037</td>
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<td>223.0602</td>
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<tr>
<td>11</td>
<td>567.1309</td>
<td>14.85</td>
<td>C36H43O20</td>
<td>Polygalaxanthone III</td>
<td>223.0602</td>
</tr>
</tbody>
</table>

Note: Numbers within the table show only the fold-relationship of the same compound (e.g., Shangluo, Shaanxi Tenuifoliside B2 content is 5 times that of Chengcheng, Shaanxi; Shanxi; and Hebei).

Experimental Conclusion

The X500R high-resolution LC system was used to analyze 24 Polygala samples, and grouped analysis found 11 signature markers responsible for sample variation. These included 2 x sucrose esters, 6 x oligosaccharides, 2 x triterpenoid saponins, and 1 x xanthone.

Results

Loading results and T-experimental results are combined to find the differentiated ions and the exact mass number and retention time of the potential biomarker are shown in Table 1.

Figure 8. Polygala Marker m/z (1379.4083) secondary mass spectrum element component fitting

ChemSpider online structural identification, an additional verification of the structure, identified this marker as: Tenuifoliose A2, C_{62}H_{76}O_{35}, m/z (MS)= 1379.4083, m/z (MS/MS) = 1203.3603,1337.3977, 1161.3507, 1143.3406, 795.2362, 175.0401. Results are shown in Fig. 9.

Figure 9-1. Polygala Marker m/z (1379.4083)

Figure 9-2. Polygala Marker m/z (1379.4083) secondary fragment attribution and main fragment structure analysis
Analysis of the differences among the 11 markers revealed that the content of Shangluo, Shaanxi Polygala is clearly different from that of other locations. Main component analysis software MarkerView™ helped to differentiate between Polygala samples, for example using the 11 signatures that differentiate Shangluo, Shaanxi Polygala from that of the 4 other locations.

Summary

This study showed the value of applying the X500R high resolution LC system to Chinese medicine component analysis. It obtained high resolution spectrometric data (TOF-MS and TOF-MS/MS) and delivered high efficiency, rapid, integrated solutions, giving users stronger data to support their Chinese medicinal component identification. The main technical features of this method are as follows:

5. SCIEX OS is a comprehensive software package for data acquisition and analysis. Its simple data acquisition and processing features avoid the need for tedious switching between multiple software packages.

6. SCIEX OS software brings together simpler analytic methods; following automated calculation of a molecular formula, it can be matched to items in the SCIEX high resolution Chinese medicine MS/MS database, or the structure may be deduced using ChemSpider. Secondary fragment analysis can be performed to dramatically reduce time and effort laboratory personnel must invest to identify compounds.

7. Data entry and PCA analysis in MarkerView™ software is rapid. T-experimental statistical analysis enables users to quickly identify differentiated ions among groups and alleviates the difficulty of analyzing massive data sets. The X500R QTOF is an important tool for origin analysis and group analysis of Chinese medicines.

References

