Metabolomics analysis and rapid identification of changes in chemical ingredients in crude and processed Astragali Radix by UPLC-QTOF-MS combined with novel informatics UNIFI platform

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Available online 20 Sep., 2018

[ABSTRACT] Astragali Radix, the root of Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao or Astragalus membranaceus (Fisch.) Bge., is widely used as a tonic decoction pieces in the clinic of traditional Chinese medicine (TCM). Astragali Radix has various processed products with varying pharmacological actions. There is no modern scientific evidence to explain the differences in pharmacological activities and related mechanisms. In the present study, we explore the changes in chemical components in Astragali Radix after processing, by ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) combined with novel informatics UNIFI platform and multivariate statistical analysis. Our results showed that the crude and various processed products could be clearly separated in PCA scores plot and 15 significant markers could be used to distinguish crude and various processed products by OPLS-DA in UNIFI platform. In conclusion, the present study provided a basis of chemical components for revealing connotation of different processing techniques on Astragali Radix.

[KEY WORDS] Astragali Radix; Processing; UPLC-QTOF-MS; UNIFI; Multivariate statistical analysis; Metabolomics

[CLC Number] R917
[Document code] A

Introduction

Astragali Radix, the root of Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao or Astragalus membranaceus (Fisch.) Bge., commonly referred to as Huang Qi (HQ), is widely used in the clinic of traditional Chinese medicine (TCM). HQ is used for tonifying qi, upraising yang, securing the exterior, stopping sweating, promoting urination, alleviating edema, engendering fluids, nourishing blood, moving stagnation, relieving impediment, expelling toxin and pus, promoting wound healing and promoting tissue regeneration, in accordance with TCM theory [1]. In TCM, processing is a necessary and significant part in preparation of decoction pieces before clinical use. There is a prominent purpose for processing technology, which is to increase efficacy and reduce toxicity. The initial description of processed HQ can be traced back to the Synopsis of Formulas of the Golden Chamber, written by Zhang Zhong-Jing. In this classic, HQ was recorded to deflash rhizome [2]. From Han and Tang to Ming and Qing dynasties in China, there were sixteen processed HQ products and methods documented, including cleansing, cutting, frying, steaming, stir-frying without adjuvant materials, roasting, stir-frying with rice, repeated processing, and processing with rice-wine, vinegar, salt-water, ginger juice, honey, and human milk [3]. Recent studies have shown that the research in processed HQ is mainly focused on the technology of cutting and processing with honey [4-6]. In the present study, we investigated the changes in chemical components of seven kinds of processed HQ with commonly used ancient and modern preparation methods, using metabolomics method, combining UPLC-QTOF-MS analysis with novel informatics UNIFI platform. For data processing, multivariate statistical analysis was employed, consisting of principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA).

The experimentally processed HQ products tested in the
present study included those processed with rice-wine (RHQ), salt-water (SHQ), honey (HHQ), human milk (HMHQ), nine kind of auxiliary materials (NHQ, processed with 9 separate materials), simple parching (SPHQ), and crude HQ. According to TCM theory, RHQ and SHQ contribute to the effective component act on the upper and lower, HHQ and NHQ enhance tonifying qi, and HMHQ enriches the blood.

With the advantage of high selectivity, high sensitivity and high throughput, UPLC-QTOF-MS is mainly applied to elucidate the inherent components in medicinal materials, and to conduct metabolic and pharmacokinetic studies of herbal medicines, in addition to explaining the processing mechanism and providing the scientific basis for processing of TCM, [7-14]. Meanwhile, MS² data acquisition can make a higher degree of mass precision. The UNIFI software possesses a scientific TCM library, following the latest edition of Chinese Pharmacopoeia, which lists all the herbs, including compound name (both in Mandarin and English), chemical structure, molecular formula, average molecular mass and mono-isotopic molecular mass of each compound as well as the plant origins. Additionally, the elemental composition and mass fragment could be speculated according to the acquired mass information in the lack of reference substances [15]. In the present study, we compared the differences between crude and multiple processed HQ products by UPLC-QTOF-MS combined with novel informatics UNIFI platform. It was hoped that the results from this study would help evaluate the effects of the different processing technologies and methods on HQ products and other TCM in general.

Materials and Methods

Chemicals, reagents, and materials

Standard substances of astragaloside II, formononetin, calycosin, and calycosin-7-β-D-glucopyranoside were purchased from Must company (Sichuan, China). Medicarpin was bought from Shanghai Yuanye Biotech Co., Ltd. (Shanghai, China). MS-grade acetonitrile and methanol and HPLC-grade formic acid were purchased from Merck KGaA (Darmstadt, Germany). AR-grade ethanol was purchased from Tianjin Ker mol Chemical Reagent Co., Ltd. (Tianjin, China). Ultrapure water was produced by Milli-Q system (18.2 MΩ, Millipore, Billerica, USA). Brand Tower rice wine was purchased from Brand Tower (Billerica, USA). Using an ACQUITY UPLC® BEH C₁₈ column (100 mm × 2.1 mm, 1.7 μm, Waters). The mobile...
phase was consisted of (A) acetonitrile containing 0.1% formic acid and (B) water containing 0.1% formic acid, and the best elution conditions were as follows: 10% to 30% A (0–2 min), 30% to 60% A (2–4 min), 60% to 85% A (4–6 min), 85% to 100% A (6–8 min), (8–10 min) A, and immediately returned to initial mobile phase composition. The flow rate was set at 0.25 mL·min⁻¹. The temperature of column and auto-sampler room were set at 30 and 8 °C, respectively. The injection volume was 2 μL.

Mass spectrometry analysis was performed with a Waters XEVO G2-XS QTOF MS (Waters) with an electrospray ionization (ESI) source in positive ion mode. The desolvation gas (N₂) flow rate was set at 800 L·h⁻¹ with a temperature of 400 °C, the source temperature was set at 100 °C, and the cone gas was set at 40 L·h⁻¹. The capillary and cone voltages were set at 2000 and 20 V, respectively. The ramp collision energy from 20 to 30 V was used. The centroided data of each sample acquired from 50 to 1200 Da, with a scan time of 0.5 s over an analysis time of 10 min. To ensure that the mass accuracy and reproducibility, a LockSpray™ program was used. The [M + H]⁺ ion of leucine enkephalin (200 pg·μL⁻¹ infusion flow rate 10 μL·min⁻¹) at m/z 556.2771 was used as the lock mass in positive ESI mode. The accurate mass and composition for the precursor ions and the fragment ions were calculated using the MassLynx V4.1 software (Waters).

**Statistical analysis**

The peak finding, peak alignment, and peak filtering of ES+ raw data were carried out with the UNIFI software (Waters). The intensity of each detected ion in UPLC-QTOF-MS analysis was normalized with respect to the whole ion count to generate a data matrix that was consisted of the m/z value, retention time, and the normalized peak area. The multivariate data matrix was analyzed by EZinfo 3.0 software (Waters). The entire variables were mean-centered and pareto-scaled prior to PCA and OPLS-DA analysis, in order to identify potential discriminant variables. All the data were plotted as means ± SEM (n = 6).

**Results and Discussion**

**UPLC-QTOF-MS method development**

To develop and optimize the UPLC-QTOF-MS method, we considered the following factors: extractants (methanol, 50% methanol, 75% ethanol, and water), dilution ratio (10 and 100 times), and ion modes (positive and negative). As a result, 75% ethanol, 10-time dilution, and positive ion mode were selected as the optimum conditions, providing abundant chemical compounds, better resolution, and satisfactory peak shapes [17-20] (Fig. 1).

Fig. 1  The BPI mass continuum spectrograms of four kinds of extractants in positive and negative modes. (A) 50% methanol; (B) 75% ethanol; (C) methanol; (D) water; (1) positive; (2) negative

**Samples analysis**

Fig. 2 shows the mass continuum spectrograms of crude sample and five kinds of standard by UPLC-QTOF-MS. The component information was processed with the UNIFI software, and a total of 3409 variables with response value greater than 50 000 counts were exacted and short-listed in csv format file and used in a PCA and OPLS-DA analysis by EZinfo software [21].

Fig. 3 shows the crude and processed product samples that were distinctly divided into 2 main clusters. The crude HQ on the one side, and the processed products on the other side. Meanwhile, the various processed products also respectively made a division in the PCA scores plot. This phenomenon indicated that crude HQ was processed with auxiliary materials or heating process could bring about significantly quantitative and/or qualitative changes in the chemical
ingredients, which might result in a wide range of pharmacological actions. In the recorded summary on TCM processing methods of all ages often has a description: “The HQ products processed with rice-wine, salt-water, and honey were mainly made for a better use of entering the upper energizer, lower energizer and tonifying qi, respectively.”

The h-o were selected and returned to online Chemspider for inference. However, h-o were just a prediction, but not confirmed, so we marked them as “unknown” for the moment. All the results of OPLS-DA demonstrated significant differences between crude and processed HQ products.

Fig. 5 shows the variable average plot of the markers in the crude and processed HQ products. In the diagrams, some compounds showed quantitative changes after processed with heating and auxiliary materials, which may be the reason for that crude and processed products can be clearly separated in PCA scores plot, such as a-g. We might conclude that the c, d, and e were reduced after processing as the result of heating. Additionally, newly generated compounds appeared in various processed HQ products, which might result in the separation of the processed HQ products in PCA scores plots, such as h-o.

Potential markers

In order to identify the potential discrimination markers between crude and processed products, we performed OPLS-DA analysis and generated the S-plots (Fig. 4). In the S-plots, each point represented an ion pair \((t_R-m/z)\). The points at the two ends of “S” curves were mostly deemed as the conspicuously characteristic markers with a high confidence level in distinguishing markers. Ultimately, the points at the two ends of “S” or the values of variable importance plot (VIP) greater than 1.0 were selected as the markers for discrimination. A total of 15 significant ions were chosen as the markers in distinguishing between crude and varied processed products (Table 1). The components of a-o were identified by UNIFI. Meanwhile, a-g were searched and identified in the internal TCM library under HQ; furthermore, a, b, c, e, and f were further confirmed by corresponding standard substances.
Table 1  Components identified from crude and processed HQ samples

<table>
<thead>
<tr>
<th>No.</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; min</th>
<th>Identification</th>
<th>Formula</th>
<th>[M + H]&lt;sup&gt;+&lt;/sup&gt;/[M + Na]&lt;sup&gt;+&lt;/sup&gt; (Calcd.)</th>
<th>Detected in extracts (m/z)</th>
<th>Mass accuracy (ppm)</th>
<th>Existed in samples</th>
<th>Quantitative or qualitative</th>
</tr>
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<tr>
<td>a</td>
<td>2.860</td>
<td>Calycosin-7-O-β-D-glucopyranoside</td>
<td>C&lt;sub&gt;22&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;O&lt;sub&gt;10&lt;/sub&gt;</td>
<td>446.121 30</td>
<td>447.129 56</td>
<td>1.0</td>
<td>Crude vs NHQ and SPHQ (in crude, NHQ and SPHQ)</td>
<td>△</td>
</tr>
<tr>
<td>b</td>
<td>4.054</td>
<td>Calycosin</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
<td>284.068 48</td>
<td>285.076 79</td>
<td>1.7</td>
<td>Crude vs NHQ, RHQ, HHQ, SPHQ, HMHQ and SHQ (in crude, RHQ, HHQ, HMHQ and SHQ)</td>
<td>△</td>
</tr>
<tr>
<td>c</td>
<td>4.862</td>
<td>Formononetin</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
<td>268.073 56</td>
<td>269.081 75</td>
<td>1.3</td>
<td>Crude vs NHQ, RHQ, HHQ, SPHQ, HMHQ, and SHQ (in crude)</td>
<td>△</td>
</tr>
<tr>
<td>d</td>
<td>4.090</td>
<td>(6αR, 11αR)-10-Hydroxy-3, 9-dimethoxy-pterocarpan</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
<td>300.099 78</td>
<td>301.107 90</td>
<td>1.0</td>
<td>Crude vs NHQ, RHQ, HHQ, SPHQ, HMHQ, and SHQ (in crude and HHQ)</td>
<td>△</td>
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<tr>
<td>e</td>
<td>4.675</td>
<td>Astragaloside II</td>
<td>C&lt;sub&gt;43&lt;/sub&gt;H&lt;sub&gt;70&lt;/sub&gt;O&lt;sub&gt;15&lt;/sub&gt;</td>
<td>826.471 48</td>
<td>849.466 50</td>
<td>6.3</td>
<td>Crude vs HHQ (in crude)</td>
<td>△</td>
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<tr>
<td>f</td>
<td>5.004</td>
<td>Medicarpin</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
<td>300.099 78</td>
<td>301.107 90</td>
<td>2.5</td>
<td>Crude vs NHQ, HHQ and SPHQ (in crude, NHQ, HHQ and SPHQ)</td>
<td>△</td>
</tr>
<tr>
<td>g</td>
<td>6.818</td>
<td>Linolenic acid</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>278.224 58</td>
<td>279.232 83</td>
<td>1.5</td>
<td>Crude vs NHQ, RHQ, HHQ, SPHQ, HMHQ and SHQ (in crude, NHQ, RHQ, HHQ, HMHQ and SHQ)</td>
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<td>h</td>
<td>7.943</td>
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<td>—</td>
<td>—</td>
<td>599.503 04</td>
<td>—</td>
<td>Crude vs NHQ and HMHQ (in NHQ and HMHQ)</td>
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<tr>
<td>i</td>
<td>3.542</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>489.140 65</td>
<td>—</td>
<td>Crude vs RHQ (in RHQ)</td>
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<td>j</td>
<td>8.580</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>965.625 19</td>
<td>—</td>
<td>Crude vs RHQ (in RHQ)</td>
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<tr>
<td>k</td>
<td>8.218</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>804.555 57</td>
<td>—</td>
<td>Crude vs HHQ (in HHQ)</td>
<td>△</td>
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<tr>
<td>l</td>
<td>4.259</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>473.145 06</td>
<td>—</td>
<td>Crude vs SPHQ (in SPHQ)</td>
<td>△</td>
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<tr>
<td>m</td>
<td>8.492</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>637.480 53</td>
<td>—</td>
<td>Crude vs SPHQ (in SPHQ)</td>
<td>△</td>
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<tr>
<td>n</td>
<td>8.238</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>758.572 99</td>
<td>—</td>
<td>Crude vs HMHQ (in HMHQ)</td>
<td>△</td>
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<tr>
<td>o</td>
<td>8.547</td>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>786.602 75</td>
<td>—</td>
<td>Crude vs SHQ (in SHQ)</td>
<td>△</td>
</tr>
</tbody>
</table>

▲, represent quantitative changed after processing; ◊, represent qualitative changed after processing
Fig. 4  The S-plots of OPLS-DA between crude and processed HQ samples. (A) Crude and NHQ; (B) Crude and RHQ; (C) Crude and HHQ; (D) Crude and SPHQ; (E) Crude and HMHQ; (F) Crude and SHQ. The first quadrant region represents processed HQ samples, third quadrant region represents crude HQ.

Fig. 5  The variable averages plot of the 15 markers in the crude and processed HQ samples. (A) NHQ and Crude; (B) RHQ and Crude; (C) HHQ and Crude; (D) SPHQ and Crude; (E) HMHQ and Crude; (F) SHQ and Crude. The left group represent processed HQ samples, right group represent crude.
Conclusion

The present study explored the differences in chemical constituents between crude and processed HQ products by UPLC-QTOF-MS and multivariate statistical analysis. The PCA scores plots and OPLS-DA S-plots demonstrated that the HQ processed with heating and auxiliary materials showed the changes in compound compositions, which provided a scientific basis for various pharmacological effects of the HQ products. Further research is needed to determine the relationships between the changes in compounds and pharmacological effects, the transformation mechanisms, and identification of qualitative changes in HQ components after processing. The results from the present study would provide a better reference for a reasonable selection of different HQ products for clinical application in the treatment of different disorders.

References


