Original article

Predicting the grades of Astragali radix using mass spectrometry-based metabolomics and machine learning

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Abstract

Astragalus radix (AR, the dried root of Astragalus) is a popular herbal remedy in both China and the United States. The commercially available AR is commonly classified into premium graded (PG) and ungraded (UG) ones only according to the appearance. To uncover novel sensitive and specific markers for AR grading, we took the integrated mass spectrometry-based untargeted and targeted metabolomics approaches to characterize chemical features of PG and UG samples in a discovery set (n=16 batches). A series of five differential compounds were screened out by univariate statistical analysis, including arginine, calycosin, ononin, formononetin, and astragaloside IV, most of which were observed to be accumulated in PG samples except for astragaloside IV. Then, we performed machine learning on the quantification data of five compounds and constructed a logistic regression prediction model. Finally, the external validation in an independent validation set of AR (n=20 batches) verified that the five compounds, as well as the model, had strong capability to distinguish the two grades of AR, with the prediction accuracy > 90%. Our findings present a panel of meaningful candidate markers that would significantly catalyze the innovation in AR grading.

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1. Introduction

Astragalus radix (AR, the dried root of Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao or Astragalus membranaceus (Fisch.) Bge.) is a widely used traditional Chinese medicine (TCM) in the treatment of many conditions, such as cardiovascular diseases, stroke, and diabetes [2–4]. In 2018, the National Health Commission of China officially announced that AR could be used as not only medicine but also food in a range of usages and dosages. Meanwhile, a variety of AR preparations are also commercially available in the United States as a general tonic or dietary supplement, to strengthen the immune system, fight viruses and bacteria, and reduce the side effects associated with cancer treatment [5]. Among all the AR products, the most commonly used is the crude dried root which has two grades [6]. The price of premium graded (PG) AR is about double that of ungraded (UG) one. In contrast to the significant difference in price, the classification of AR is still mainly based on the sensory evaluation of TCM workers; the longer and thicker root is usually graded as of better quality. Although assessing the AR quality by appearance is readily available, it is also easily influenced by personal feelings because the specific and quantifiable markers are lacking.

Novel approaches, such as metabolomics that sheds light on the relationship between small molecule compounds and biological system phenotypes [7,8], can serve as powerful tools for screening new potential markers for the evaluation of AR grade. Metabolomics can be divided into untargeted and targeted approaches, which represent the breadth-first and depth-first screening strategies, respectively [9–11]. Untargeted metabolomics provides semi-quantitative assessments of all the detectable compounds including chemical unknowns in a sample, while targeted metabolomics focuses on the accurate measurement of limited groups of compounds.
compounds. Currently, both approaches have been applied for the quality control of AR and related preparations, such as decoction, extraction, and injection [12–15]. However, previous studies have mainly focused on the identification and detection of a defined set of bioactive compounds including saponins and flavonoids, and have not uncovered or verified the compounds indicative of the AR grade [16–18]. For instance, an nuclear magnetic resonance spectroscopy (NMR) based untargeted metabolomics study identified 38 primary metabolites that have high concentrations in AR water extracts, but failed to capture the information of secondary metabolites with low levels [13]. Some liquid chromatography-mass spectrometry (LC-MS) based targeted metabolomics studies compared the differences of chemical compounds of AR from different geographical areas with a list containing no more than 26 compounds [15,19]. It is, therefore, possible that new grading markers cannot be detected with the current methods.

The primary goal of this study was to elucidate the global chemical features of two grades of AR and screen out novel quality markers by using the integrated untargeted and targeted metabolomics approaches with machine learning. Briefly, a discovery set consisting of 9 batches of PG and 7 batches of UG samples was analyzed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) to screen out the differential compounds associated with AR grades. These potential markers were confirmed by a follow-up targeted metabolomics analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and their prediction ability was further validated in an independent validation set of 20 batches of AR samples (11 PG and 9 UG) by building a logistic regression model.

2. Experimental

2.1. Chemicals and reagents

Standards consisting of five compounds and two internal standards were obtained commercially from National Institute for Food and Drug Control (Beijing, China), Chengdu Esite Biotechnology Co., Ltd. (Chengdu, China), Chengdu Mansite Biotechnology Co., Ltd. (Chengdu, China), and Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Analytical grade formic acid was purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Distilled water was purified by Milli-Q system (Millipore, Milford, MA, USA).

2.2. Plant materials and sample preparation

A total of 36 batches of AR crude slices, among which 20 were PG and the rest were UG, were produced in Gansu Province, China and purchased from Bozhou Traditional Chinese Medicine Market (Bozhou, China). The length, width and price of each batch of AR samples were recorded, and then the slices were pulverized into homogeneous powders (40 meshes). The microscopic examination of powdered AR samples was observed under the microscope (Olympus CX41, Tokyo, Japan).

The AR powder (10.0 g) was boiled at 100°C two times for 2 h with 100 mL of deionized water. The extracts were filtered, combined and then dried by a freeze-dryer (Labconco, Kansas City, MO, USA) at −83°C. For untargeted metabolomics analysis, a pile of freeze-dried powder containing approximately 100 mg crude slices was mixed with 1,000 μL of methanol/water (85:15, V/V) and filtered through an organic filter for LC-MS/MS analysis.

2.3. LC-HRMS analysis

Untargeted metabolomics analysis was performed on an ultra-fast liquid chromatography ion trap/time-of-flight mass spectrometry (UFLC-IT-TOF/MS; Shimadzu, Tokyo, Japan) system. Samples were separated on a Waters XSelect HSS T3 XP (2.1 mm × 100 mm, 2.5 μm; Agilent, Santa Clara, CA, USA) column at the oven temperature of 40°C. The mobile phase consisted of 0.1% (V/V) formic acid in water (A) and methanol (B) with a flow rate of 0.25 mL/min. A 28-min elution gradient was performed as follows: 0–6 min, 1% B; 6–7 min, 1%–35% B; 7–13 min, 35% B; 13–14 min, 35%–50% B; 14–16 min, 50% B; 16–17 min, 50%–75% B; 17–20 min, 75% B; 20–21 min, 75%–100% B and 21–25 min, 100% B. Finally, the initial conditions were recovered and kept for 3 min.

MS detection was performed by an electrospray ionization (ESI) source operated in both positive and negative modes with the data acquisition range of m/z 100–1000. The chromatographic conditions were as follows: detector voltage 1.8 kV, interface voltage 4.5 kV and 0.1% formic acid in water (B) and 50% formic acid in water (A), 1.8 kV, 4.5 kV, 100°C, and ion accumulation time 20 ms.

2.4. LC-MS/MS analysis

The quantitative analysis of potential markers was achieved using a Shimadzu Nexera UFLC system coupled to an MS-8040 triple quadrupole mass spectrometer system (Tokyo, Japan) equipped with an ESI source. The separation was performed on a Zorbax SB C18 (2.1 mm × 150 mm, 3.5 μm; Agilent, Santa Clara, CA, USA) column with a flow rate of 0.25 mL/min at 40°C. Acetonitrile and 0.1% formic acid in water were used as mobile phase under a gradient program. The MS was operated in positive/negative switching mode with multiple reaction monitoring (MRM). Collision energy and fragment ions were optimized individually for each compound. The method was validated in terms of linearity and range, precision, accuracy, and recovery. The details are shown in Tables S1–S4.

2.5. Data analysis

The raw data obtained from untargeted metabolomics analysis were extracted, preprocessed, statistically analyzed, and identified mainly on the basis of our previous studies [20]. Briefly, data matrix consisting of aligned peaks with m/z, retention time, and intensity were acquired by Profiling Solution software (Ver 1.1, Shimadzu, Tokyo, Japan) and imported to Mathematica (Ver 12.0, Wolfram, Champaign, IL, USA) for further multivariate and univariate statistical analyses. Volcano plot was used to filter important peaks that showed significant fold change (FC > 1.2 or < −1.2) and statistical significance (adjusted P < 0.05) between the PG and UG samples. The free databases, such as TCMSP (http://tcmspw.com) and HMDB (http://www.hmdb.ca), and commercially available standards were used for the identification of compound candidates.

The quantitative data collected from targeted LC-MS/MS analysis were also imported to Mathematica for constructing a machine learning-based prediction model with logistic regression algorithms. The machine learning process mainly involved two steps: the data from discovery set (16 batches of AR) were used to train the model and subsequently the data from validation set (20 batches of AR) were employed to evaluate the performance of the model.
model with the area under the receiver operating characteristic curve (AUC-ROC).

3. Results

3.1. Appearances of two grades astragali radix

We compared the morphological and microscopic characteristics of PG and UG samples. As shown in Fig. 1, the length and width of PG crude slices were about twice those of UG samples, while the microscopic characteristics of the two grades powdered samples under normal and polarized light microscopy were almost the same. These results indicated that there were deficiencies in the method of grading AR by appearance, especially for the powder.

3.2. Discovery of differential compounds

Untargeted metabolomic analysis was conducted in the discovery set to uncover differential compounds between PG and UG groups. After screening of the detected peaks using "QC variation < 30%" and "80% rule", a total of 777 peaks were extracted from LC-HRMS data. The unsupervised multivariate principle component analysis (PCA) was subsequently applied to characterize chemical patterns of two grades of AR. As illustrated by the PCA scatter plot (Fig. 2A), PG samples were mostly separated from UG samples with only one overlap. Totally 97 differential peaks were screened out with adjusted \( P < 0.05 \) and \(|\text{FC}| > 1.2 \) (Fig. 2B). Eventually, five differential compounds including arginine, calycosin, ononin, formonononetin, and astragaloside IV were identified and confirmed using commercial standards (Table S5 and Fig. S1). Their detailed information, including \( m/z \), retention time, adjusted \( P \) value, and fold change, is listed in Table S6. In addition, on the basis of semi-quantitative data, it was found that four of the five identified differential compounds were accumulated in PG samples, whereas the level of astragaloside IV decreased (Fig. S2).

3.3. Targeted analysis and prediction model construction

We measured the five differential compounds in all samples of the discovery set by LC-MS/MS. Similar results (Fig. 3A) with the untargeted study were observed, showing a marked increase in the concentration of arginine, calycosin, ononin, and formonononetin in the PG group (\( P = 0.0004, 0.022, 0.016, \) and 0.021, respectively) as well as a significant decrease of astragaloside IV (\( P = 0.019 \)). Therefore, we performed machine learning on the quantitative data, and built a logistic regression model to predict the grade of AR samples. The equation is as follows:

\[
\text{Logit} (P) = -116.7 + 0.0505 \text{arginine} + 0.4323 \text{calycosin} \\
+ 1.2689 \text{ononin} + 0.6649 \text{formononinetin} \\
- 0.4299 \text{astragaloside IV}
\]

where \( \text{Logit} (P) \) is the log-odds of the probability of the event that the AR sample is PG, and the sign of each coefficient indicates the direction of the relationship between a compound and the log-odds.

3.4. Validation of predictive ability

To further validate the capabilities of the five differential compounds in the prediction of the AR grade, we also quantified and compared their levels in an independent set of 20 batches of AR crude slices, including 11 PG and 9 UG samples. We confirmed the findings from the discovery set (Fig. 3B), and more importantly, the classification tests indicated that the five differential compounds showed a good potential to discriminate PG samples from UG samples in both discovery and validation sets \( \text{AUC} = 0.949 \) and \( \text{AUC} = 0.949 \) respectively: Figs. 4A and B). The prediction probability values of them are shown in Figs. 4C and D. At the traditional cut-off value of 0.5, 9 of 9 and 10 of 11, PG samples were correctly classified when respectively compared with UG samples in the discovery and validation sets, giving a composite accuracy of 90% to 100%. These results indicated that the five differential compounds could serve as quality markers for accurate grading of AR crude slices, even if they were powdered.

4. Discussion

Using untargeted and targeted metabolomics, we identified specific chemical compounds that were significantly affected due to the grade of AR. Previous variability studies \([21–23]\) have suggested that high-quality herbs usually contain higher levels of active substances that can trigger beneficial physiological effects or enhance the taste, which is consistent with our observations of flavonoids (calycosin, ononin, and formononetin) in two grades of AR. Studies have shown that these flavonoids have antioxidant, anti-inflammatory, anti-diabetic, anti-cancer, anti-obesity, and...
cardioprotective effects [24,25]. Specifically, our data also demonstrated a reduction in the concentration of astragaloside IV that associates with high-quality AR crude slice (Fig. 4). Astragaloside IV has been reported to have protective effects on the cardiovascular, immune, digestive, and nervous systems [25,26]. It is believed to be one of the bioactive as well as representative compounds in AR, and works as a widely used marker for the quality control of various AR preparations such as crude drugs, injections, and oral solutions [15,27,28]. A recent tissue-specific study has shown that the concentration of saponins decreases from the outer layer toward the center of AR slices, especially the periderm accumulates more than 80% of them [29]. Thus, it is mechanistically feasible that a lower level of astragaloside IV in PG samples compared to UG samples may be associated with the smaller proportion of periderm caused by the relative greater size (Fig. 1).

Another critical feature between the UG and PG samples was the significant difference of arginine level, which was rarely reported as AR quality marker. We not only screened out arginine but also quantified that its concentration in the PG group was 1.51 and 1.27 times higher than that in the UG group in the discovery and validation sets, respectively (Fig. 3). Arginine can act as a vasodilator by releasing nitric oxide for the treatment of cardiovascular conditions.

Fig. 2. Statistical analysis for the data obtained from discovery set. (A) PCA score plot of two grades of AR; (B) volcano plot of all ions. UG: ungraded; PG: premium graded.

Fig. 3. Concentration differences of five potential markers between two grades of AR in the (A) discovery and (B) validation sets. UG: ungraded; PG: premium graded.
and erectile dysfunction, having similar effects as AR to reinforce the vital energy [30]. However, previous studies generally employed the AR-specific compounds including flavonoids and saponins as markers to achieve the high specificity of quality evaluation [12,15]. The only exception is a chemical composition study that determined 17 amino acids in different AR species but failed to use them as a conclusive distinction [31]. Therefore, our subsequent ROC analysis for the first time suggested that arginine could distinguish PG samples from UG samples, with 81.8% sensitivity and 55.6% specificity (Table S7), providing a supplementary role to flavonoids and saponins.

On the basis of our systematic analysis in the discovery set and linking the findings to an independent validation set, we confirmed the classification ability of a panel of five compounds and constructed a logistic regression-based prediction model that provided more than 90% accuracy (Fig. 4). To further explore the effect of each compound in grading AR, we examined the potential importance of them via the logistic regression coefficient and ROC analysis. It was found that ononin had the greatest coefficient as well as the highest AUC among the five compounds (Table S7). Ononin is an isoflavone glycoside found in many herbs and plants such as radix glycyrrhizae [32], and therefore previous studies always measured it together with other isoflavones to characterize the chemical features in different species or parts of AR [15,19]. Our findings suggest that ononin could serve as a high importance marker for the discrimination of two grades of AR, which is worth special attention and further exploration.

Our study has several strengths. We used a state-of-the-art metabolomics methodology that possesses good reproducibility and quantitative capability, and applied the machine learning to construct a prediction model. The five markers we found as well as the model may provide a new possibility not only for the classification of AR crude slices but also for the quality control of astragalus-containing herbal preparations. However, there remain certain limitations. We used the crude slices of authentic AR produced from only one geographical region (Gansu, China) and the sample size was relatively small (10–20 samples per class). Moreover, we screened out a panel of five potential markers without uncovering the underlying biological mechanism. Thus, larger validation experiments and in-depth mechanistic studies may be necessary to confirm our findings and further clarify the association between the five compounds and AR grade.

5. Conclusion

In summary, we demonstrated that the integration of untargeted and targeted metabolomics approaches and machine learning could benefit the discovery of quality markers of AR. Specifically, we found that a panel of five compounds, arginine, calycosin, ononin, formononetin, and astragaloside IV, had a high capability in the discrimination of commercial PG and UG samples, and constructed a logistic regression model on the basis of the quantitative data that provided more than 90% prediction accuracy. From a translational perspective, the five potential markers, as well as the prediction model presented here, could help develop novel, sensitive, and specific tools for AR grading.
Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2020.07.008.

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