Arabian Journal of Chemistry (2022) **15**, 104118

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Authentication of herbal medicines from multiple botanical origins with cross-validation mebabolomics, absolute quantification and support vector machine model, a case study of Rhizoma Alismatis

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Received 28 February 2022; accepted 7 July 2022
Available online 13 July 2022

KEYWORDS

Rhizoma Alismatis;
Alisma plantago-aquatica;
Alisma orientale;
Botanical origins;
UHPLC/Orbitrap-MS

Abstract Accurate identification of the botanical origins of Rhizoma Alismatis (RA) is pivotal to its precise clinical usage. We herein present a strategy, by integrating untargeted metabolomics, data cross validation, absolute quantification, and vector machine model, for species discrimination and source recognition of Rhizoma Alismatis for the first time. An ultra-high-performance liquid chromatography/LTQ-Orbitrap mass spectrometry with precursor ions list-including data-dependent acquisition approach was developed for metabolite profiling. Holistic, continuous, and pattern recognition chemometrics could make it feasible to unveil forty-one markers, with "multi-duplicated and traceability samples comparison" cross validation to narrow down to twelve robust markers, some of which were further validated by absolute quantification. A support vector machine model was eventually developed to distinguish these two species and predict origins of commercially available varieties. This was the first report on systematic comparison and discrimi-

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Peer review under responsibility of King Saud University.

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https://doi.org/10.1016/j.arabjc.2022.104118

nation of two original species of RA. This integral strategy, in contrast to conventional approaches, renders more convincing data supporting for the discovery of multi-source chemical makers of traditional Chinese medicines (TCM).

1. Introduction

Alisma plantago-aquatica (AP) or *Alisma orientale* (AO) is a perennial herb and widely distributed in marsh regions of southern China. Their dried rhizomes are important sources of Rhizoma Alismatis (RA, Chinese name Zexie), a well-known diuretic herbal medicine, and hailed as "the first choice of diuresis" attributing to the therapeutic benefits for excreting dampness, eliminating edema and for the therapy of diarrhea. *A. orientale* is officially adopted by Chinese Pharmacopoeia (2015 edition) as the official species of RA (Feng et al., 2021). However, *A. plantago-aquatica*, surprisingly occupies fairly big share of sales, reaching to 70 % in China, owning to wider cultivating areas, lower sale price (54 *vs* 64 yuan) and similarities of names and morphologies to *A. orientale* (Liu et al., 2020a). This confusion phenomenon will lead to an inconvenience in clinical practice or manufacturing of traditional Chinese medicines (TCM) products, which puts *Alisma* species accurate identification to be of great importance. Botanical origins of RA have always been ambiguous, owing to the analogous characteristics both in macroscopic and microscopic aspects, as well as their chemical composition. However, there was few reports to rapidly differentiate these two species and discern the origins of commercial RA samples.

Currently, metabolomics serves as a potent vehicle in the authentication of herbal medicines derived from different congeneric species, collection regions, processing technologies, even from different ages and different grades, and has harvested remarkable progress due to the ongoing development of chromatography, spectroscopy and mass spectrometry, etc. (Hu et al., 2019; Zhao et al., 2020; Meenu and Xu, 2019; Fang et al., 2020). Chromatographic fingerprint is usually used for the authentication that is acquired by thin-layer chromatography (TLC) (Tian et al., 2009; Xu et al., 2021), gas chromatography (GC) (Duan et al., 2016), especially by liquid chromatography (LC) (Liu et al., 2020b; Zhang et al., 2021b). With the popularization of sub-2 μm particles packed columns, more chromatographic methods sprung up, such as ultra-performance liquid chromatography (reversed-phase chromatography) (Tang et al., 2016; Tam Pham et al., 2021), hydrophilic interaction chromatography (HILIC) (Zhang et al., 2021c), supercritical fluid chromatography (SFC) (Tang et al., 2021), and ion exchange chromatography (IEC) (Guyon et al., 2013), and so on. On the other hand, spectroscopic technology (e.g. near infrared spectroscopy, NIR; nuclear magnetic resonance; Raman spectroscopy), by means of direct profiling, makes rapid identification possible (Huck, 2014). However, these approaches face such critical obstacles as ambiguous results and unclear of the so-called diagnostic components, even worse, it is almost impossible to draw a conclusion on various operational instruments and conditions (Wolfender et al., 2015). High resolution MS (e.g. quadrupole/time-of-flight mass spectrometry (QTOF-MS), coupled to the online chromatographic separation, have been a workhorse in source identification, comprehensive profiling and characterization of the multi-components from diverse natural products (Gao et al., 2012; Li et al., 2017; Theodoridis et al., 2012). Studies on the RA's metabolome, geographical origins (Wu et al., 2017), chemical variations (Zhang et al., 2021a) and *in vivo*-metabolites by UPLC-MS (Yan et al., 2021) have been widely reported, however, no satisfactory results have been acquired in geographical origins traceability due to the unambiguity of botanical origin. One deficiency of the UPLC-MS technology is that expensive instrumentation is required, which could be, fortunately, compensated by transferring onto easily accessible instruments, for instance, HPLC, TLC or coupling with low-resolution MS (e.g. QDa) with the aid of diagnostic markers.

The purpose of our research is to develop a methodology to authenticate the botanical origin of *A. plantago-aquatica* and *A. orientale* as well as to discover the "Identification Markers" for precise identification by integrating cross-validation metabolomics, absolute quantification and support vector machine (SVM). Firstly, an optimized UHPLC/Orbitrap-MS approach was established for global metabolites profiling of ninety-nine RA samples. Then multivariate statistical analysis was performed using pattern recognition approach to probe the triterpenoid markers diagnostic for differentiating two origins of RA, which was auto-characterized by searching an in-house library by QI software. Secondly, "multi-duplicated samples" and "traceability samples" verification methods were proposed for the first time for cross-validation to obtain the robust biomarkers, as a result, twelve biomarkers for differentiating *A. plantago-aquatica* and *A. orientale* were obtained after removing unstable markers during storage or sample handing, some of which were further validated by absolute quantification. Finally, twelve most important markers were used to establish a SVM model for identifying the remaining 21 commercial samples. The workflow was shown in Fig. 1. This integration strategy, in contrast to traditional approaches, provides more convincing data supporting for the discovery of multi-source chemical makers of TCM.

2. Materials and methods

2.1. 2.1Chemical and reagents

Thirty-five authentic compounds isolated from both *A. plantago-aquatica* and *A. orientale* in our laboratory were implemented here for signal identity confirmation. The chemical structures and detailed information have been offered in Fig. S1. Formic acid and acetonitrile were provided by Merck (Darmstadt, Germany). Other chemicals used in sample pretreatment were analytical grade. Water was produced by Milli-Q water purification system (Millipore, Bedford, MA, USA). Information for the drug materials of two botanical origins of Rhizoma Alismatis has been provided in Table S1-S3, in which 31 batches of *A. orientale* and *A. plantago-aquatica* were identified by morphological identification, TLC and HPLC, and confirmed by DNA barcoding analysis; 47 batches were supplied from local Good Agricultural Practices (GAP) bases, and 21 batches were purchased from the market.

2.2. Sample preparation

The dried RA powers (80 mesh, 300 mg) were extracted with 3 mL methanol and sonicated for 30 min, and centrifuged at 14000 rpm for 10 min. The supernatant was subjected to LC-MS for analysis. QC sample was obtained by pooling equal volume of all solutions. For quantitative assay, the 0.2 g samples of RA were weighed and extracted with 10 mL of

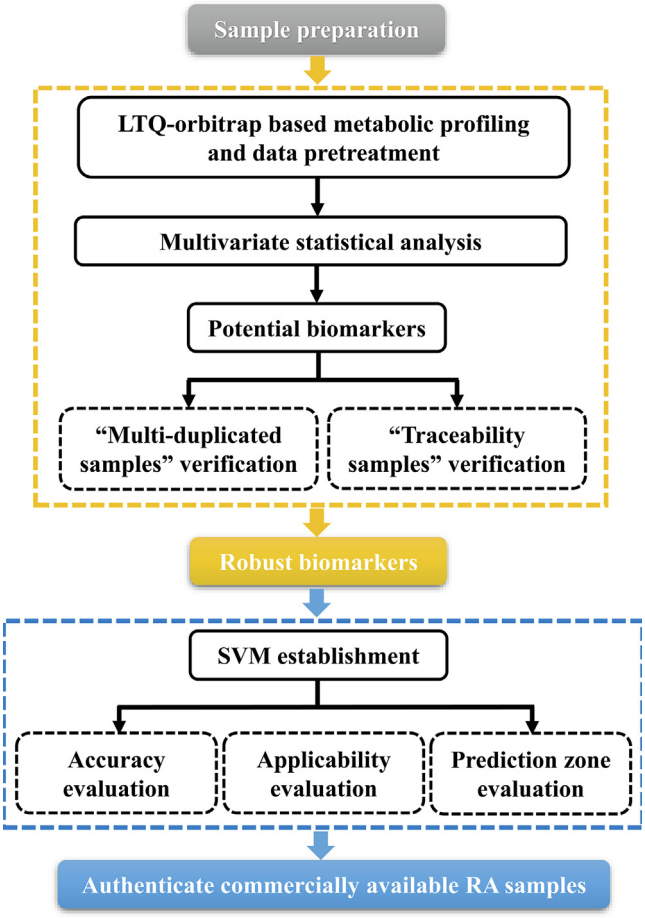

Fig. 1 A general workflow of the strategy for differentiation and recognition of two botanical origins of Rhizoma Alismatis.

solvent, and the test solution was prepared following the same procedure.

2.3. UHPLC-Orbitrap conditions for metabolic analysis

Ultimate 3000 UHPLC system coupled with LTQ-Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for sample separation and data collection. Kinetex XB C18 column (Phenomenex, 100 × 2.1 mm, 1.7 μm) was used in this study. The gradient program of mobile phase A (0.1 % formic acid in water) and B (acetonitrile) was as follow: 0–5 min: 35 %B; 5–30 min: 35–78 %B; 30–35 min: 78–95 % B; 35–39 min: 95 %(B). Other conditions: flow rate, 0.3 mL/min; injection volume, 2 μL; column temperature, 30℃; autosampler temperature, 10 °C. MS was carried out in the positive-ion ESI-MS mode with the mass range *m/z* 50–1200. The other operating parameters were optimized as follows: spray voltage, 3.8 kV; capillary temperature, 350℃; source heater temperature, 300℃; sheath gas (N_2), 40 arbitrary units; auxiliary gas (N_2), 10 arbitrary units; and sweep gas (N_2), 2 arbitrary units. As for the targeted MS/MS spectra of possible metabolites, a combined scan method, namely CID/MS^2 (35 %) and HCD/MS^2 (45 %) with dynamic exclusion enabled was used. Dynamic exclusion parameters were set as follow: repeat count, 2; repeat duration, 10 s; exclusion list size, 50; and exclusion duration, 10 s; exclusion mass width low and high, 1.5 Da.

2.4. Multivariate statistical analysis and discriminant analysis

Raw data filtering, peak detection, peak alignment were conducted by using Progenesis QI 2.1 software (Waters, Milford, USA). Next, the processed data that consists of t_R, *m/z* value, and the normalized intensity was subjected to SIMCAP software (version 14.0, Umetrics, Umea, Sweden) for principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Pareto scaling (Par) was performed. The potential makers for the discrimination between AP and AO were filtered out when VIP > 4.0. An intelligent discriminant model, SVM, was established based on twelve most important chemical markers (including alisol B 23 acetate, alisol C 23 acetate, 16-oxoalisol A, alisol A, alisol B, alisol A 24 acetate isomer, alisol C, alisol P isomer, 11-deoxyalisol C 23 acetate, $C_{34}H_{43}O_8$(579.2938), $C_{25}H_{33}O_7N_2$ (579.2938), $C_{30}H_{48}O_8$(535.3279) by using Chempattern Ultimate 1.0 software (Chemmind Technologies, Beijing), to authenticate the remaining unknown RA samples.

2.5. UHPLC-Q-trap MS based quantitative assay of eight abundant compounds

Agilent 1290 Infinity UHPLC system was connected to the 4000QTrap mass spectrometer via an ESI interface. Mobile phase A (waters containing 0.1 % formic acid) and B (acetonitrile) were used as elution solvent at a flow rate of 0.4 mL/min, with the program of 0–5 min:35 %B; 5–10 min: 35–45 %B; 10–25 min: 45 %B; 25–30 min: 45 %-78 % B; 30–34 min: 78 %-95 %B; 34–39 min: 95 %B. Other chromatographic conditions were the same as those used in 2.3. The quantitative method was validated in our previous report (Wu et al., 2017), and quantitative product ion and collision energy of each analyte were provided in Table S4.

3. Results and discussion

3.1. Metabolic analysis and data pretreatment

A total of ninety-nine batches of samples were analyzed in the analytical sequence. Stability was evaluated by analyzing QC samples in every-six injections to diminish the analytical variation resulting from system instability. Before the established untargeted method was used to carry out a metabolomics study, the intraday and interday repeatability were evaluated by testing QC samples in the same day for six times and three consecutive days. The results showed that, in the intra/inter-day precision validation, the RSD values of eight representative compounds varied from 0.01 % to 0.02 % and 1.90 % to 6.42 %, suggesting a good precision of this method (See Table S5-S6). Initial pre-processing of raw full MS data was carried out utilizing Progenesis QI 2.1 software. Multiple adducts ions, including $[M + H]^+$, $[M + Na]^+$, $[M + CH_3CN]^+$, $[M + H\text{-}H_2O]^+$, $[2\ M + Na]^+$, $[2\ M + H]^+$, $[M + K]^+$, $[M + NH_4]^+$ were selected or self-edited to achieve the fusion of precursor ions. $[M + H\text{-}2H_2O]^+$, $[M + H\text{-}3H_2O]^+$, $[M + H\text{-}HAC]^+$, $[M + H\text{-}HAC\text{-}H_2O]^+$,

$[M + H\text{-}HAC\text{-}2H_2O]^+$, $[M + H\text{-}C_4H_{10}O_2]^+$, $[M + H\text{-}C_4H_{10}O_2\text{-}H_2O]^+$, $[M + H\text{-}C_6H_{10}O_2]^+$, $[M + H\text{-}C_6H_{10}O_2\text{-}H_2O]^+$ ions were also added considering the severe in-source decay. Finally, a matrix involving t_R, m/z, and normalized peak area were obtained after centroid peak detection, peak alignment and normalization.

The generated data matrix containing 20,194 metabolite features was further processed by following steps:1) Features with odd nominal mass of m/z were initially ruled out, by the reason that no nitrogen atom has been reported in common chemical components of RA. 2) Filter the ions without neutral loss due to the high frequency of in-source decay in triterpenoids. 3) "80 % rules", by removing features that not existed in 80 % samples, and "30 % rules", detecting unstable ions with RSDs > 30 % in all QC samples, were utilized to filter the variables appeared by accident or unstable response. The finally obtained 7602 variables were imported into the SIMCA-P 14.1 software for pattern recognition chemometrics.

3.2. Chemical marker discovery for differentiating two botanical origins

The MS^E data of samples confirmed by DNA barcoding analysis (involving 24 batches of AP and 7 batches of AO) were statistically analyzed by PCA and OPLS-DA. For PCA, QC samples were gathered in one area which indicated the instrument to be in a good condition (Fig. 2A). To explore potential markers that were largely devoted to the difference in the authentication of AO and AP, OPLS-DA and VIP plots were used. As seen in Fig. 3A, the samples of RA identified by DNA barcode were well gathered and segregated into two different groups scattering in different quadrants. The values of R^2 (0.951) and Q^2 (0.821) demonstrated acceptable fitness and predictability. A chance permutation test suggested the model was not over-fitting (Fig.S2). The variables showing VIP > 4 were considered as potential biomarkers, and resultantly, 41

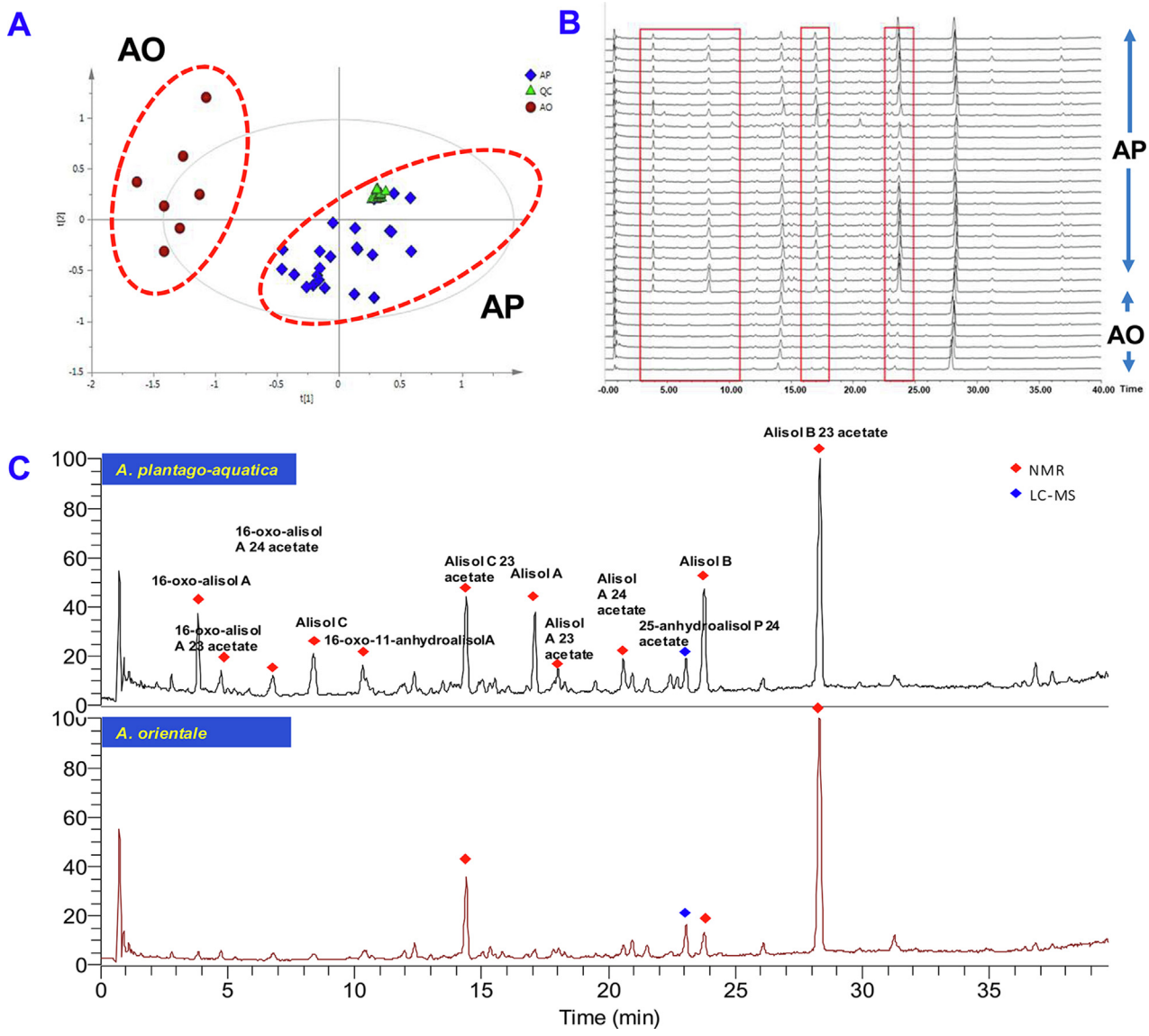

Fig. 2 PCA score plot (A), base peak ion chromatograms (B) and main chemical components (C) of *A.plantago-aquatica* and *A.orientale* identified by DNA barcoding analysis.

potential biomarkers were discovered and identified (Table S7). The main metabolites confirmed by reference compounds (NMR) or LC-MS in two botanical origins were traced back to the fingerprint spectra (Fig. 2C), and significant difference was observed at 3–10 min, 16–18 min and 23–25 min (Fig. 2B).

3.3. "Multi-duplicated and traceability samples comparison" cross validation

Robust markers are needed, especially for those samples that are detected in different instrument conditions or larger sample application, to distinguish different species in production and sales and to differentiate the authentic samples from the adulterant ones. Due to the instable signals of chemical constituents in different runs, it was virtually impossible to acquire the data again to MS spectrum signal of the crude extract. "Multi-duplicated samples", by repeatedly acquiring MS data in a long interval of time (4 months), can screen out the difference biomarkers with better durability and avoid interference caused by the instrument. On the other hand, "traceability samples" directly collected from the origin base, some of which were not verified by DNA barcode due to the impossibility to extract desired DNA fragments from those RA products, were used for further verification. In total, three groups of samples, "DNA barcode samples" (31batches), "multi-duplicated samples" (31batches) and "traceability samples" (46 batches) were utilized to unravel the robust chemical markers to distinguish *A. plantago-aquatica* and *A. orientale*, and the results were shown in Fig. 3.

Despite the similar PCA and OPLS-DA results obtained from "multi-duplicated samples", chemical markers were different. Five differential ions were screened when the VIP cutoff was set at 10.0 (Table S8), which was consistent with section 3.2 except alisol B and 15,16-dihydroalisol B 23 acetate with VIP > 8 (Fig. 3B). This, along with the situations previously discussed, led to a conclusion that alisol B 23 acetate, alisol C 23 acetate, 16-oxoalisol A, alisol A, 25-anhydroalisol P 24 acetate, alisol B and 15,16-dihydroalisol B 23 acetate were the seven most important chemical markers. Twenty-four additional ions were screened when the VIP cutoff was set at 4 to 10, less than the those from "DNA barcode samples", removing 15 compounds with poor durability ultimately.

For "traceability samples" evaluation, although *A. plantago-aquatica* and *A. orientale* were clearly clustered, the PCA and OPLS-DA showed differences owning to the large distinction of samples (Fig. 3C). Based on VIP plot, we were able to identify five potential markers by setting VIP cutoff at 10.0 (Table S9), amongst these differential ions, only four ions (alisol B 23 acetate, alisol A, alisol B, 16-oxoalisol A) were observed in "multi-duplicated samples" evaluation. Thus five markers were confirmed to be the most important ions by cross validation, taking alisol C 23 acetate (VIP > 8) into account. Yet it was worth noting that the markers with 10 > VIP > 4 were extremely different from those obtained from "multi-duplicated samples", with 7 out of 27 were the same (Table S8), indicating that the sample has a greater impact on marker screening than the instrument status.

While considering "DNA barcode samples" (31batches), "multi-duplicated samples" (31batches) and "traceability samples" (46 batches), we finally concluded that, twelve major

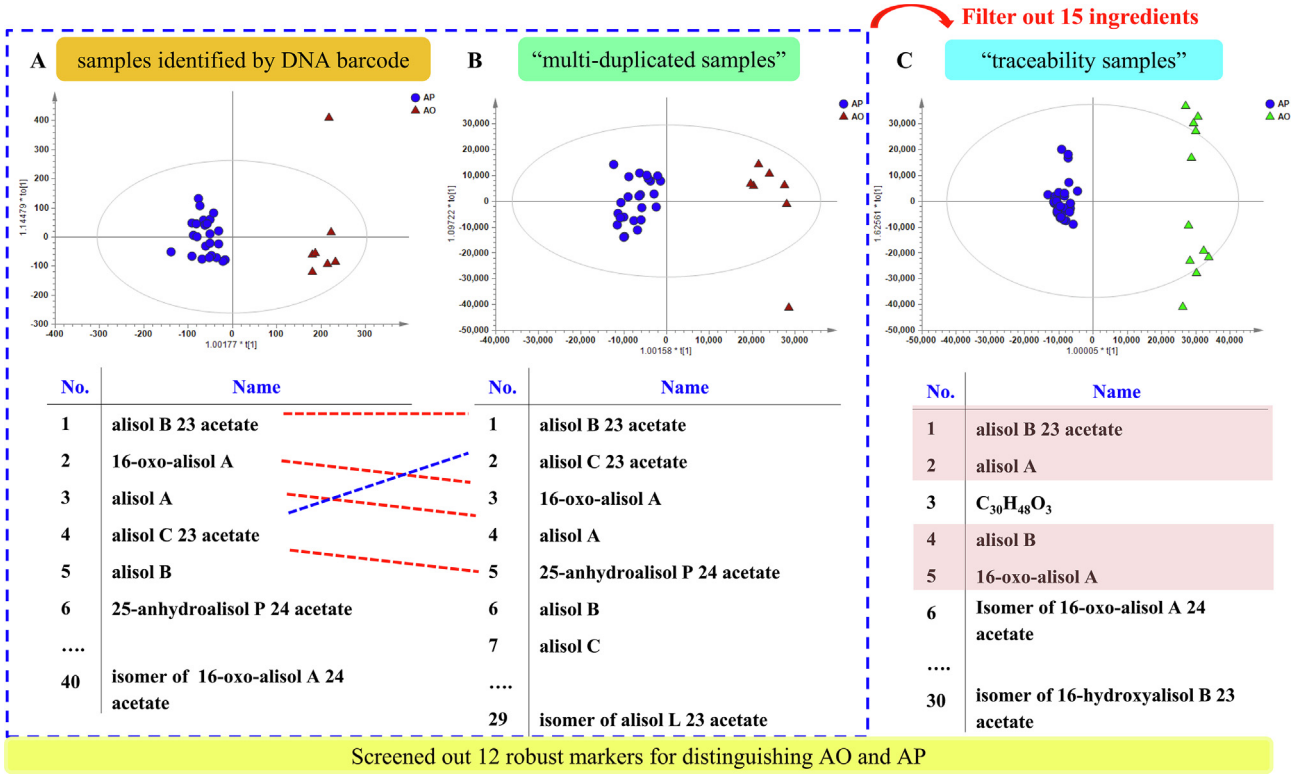

Fig. 3 OPLS-DA score plot and screened markers of *A. plantago-aquatica* and *A. orientale* in samples identified by DNA barcode (A), "multi-duplicated samples" (B) and "traceability samples" (C).

markers (including alisol B 23 acetate, alisol C 23 acetate, alisol B, 16-oxoalisol A, alisol A, alisol A 24 acetate isomer, alisol C, alisol P isomer, 11-deoxyalisol C 23 acetate, $C_{34}H_{43}O_8$(579.2938), $C_{25}H_{33}O_7N_2$(579.2938), $C_{30}H_{48}O_8$ (535.3279), could be selected as the associated markers applicable to differentiate between *A. plantago-aquatica* and *A. orientale* (Fig. 3).

3.4. Verification of chemical markers by targeted LC-MS/MS analysis

Among the twelve robust markers screened above, six markers were identified with the reference substances. The reliability of the twelve markers were further verified by two-way hierarchical clustering analysis (HCA). As shown in Fig. 4A, the samples were directly segregated in two clusters based on remarkable differences of twelve markers. To further verify the credibility of the markers, absolute quantification by LC-MS/MS was adopted. MRM experiments of eight compounds were performed by Q-trap mass spectrometry under the described condition in our previous investigation (Wu et al., 2017). MRM precursor and product ions, and EP, DP, CE, CXP mass spectrometry parameters of eight reference compounds were shown in Table S4. MRM chromatograms were displayed in Fig. 4B, and assay results were in Fig. 4C. The results showed that alisol B 23 acetate was the most abundant compound, followed by alisol B, alisol C 23 acetate, alisol A, alisol A 23 acetate, alisol G and 16-oxo-11-anhydroalisol A and alisol F 24 acetate. The two chemical components with the highest content in AO and AP were alisol B 23 acetate and alisol C 23 acetate, however, no significant difference was observed, speculating that the content of alisol B 23 acetate and alisol C 23 acetate themselves could not be directly used as distinguishing markers. Alisol A 24 acetate and alisol F 23 acetate also showed no difference between *A. plantago-aquatica* and *A. orientale*, which was constant with screening results. In addition, it was found that alisol A and alisol B, alisol G and 16-oxo-11-anhydroalisol A were significantly different among different botanical origins, and the latter two were not screened out in above metabonomics mainly because of their lower contents. The sum of eight compounds in AP was significantly higher than that in AO.

3.5. Authentication of different species of RA by SVM

To accomplish a convenient and reliable identification of two different parts of RA, SVM was used to build discriminant model using twelve robust biomarkers as selected above (Fig. 5A). Normalized ion intensity of robust biomarkers was used as the variables for data modeling as it could improve the prediction confidence. All the ninety-nine samples were split at random into two groups: two-thirds of the samples (36 AP and 11AO) were selected as training set, whereas the remaining 1/3 as the test. 7-fold cross-validation was used in model. The pattern recognition results were shown in Fig. 5B and Table S10. In the classification results of test set, all selected samples (31 batches) representing the corresponding species were successfully divided into their own groups which was consistent with the real situation. To validate the suitability of SVM model, 50 %, 100 %, and 150 %

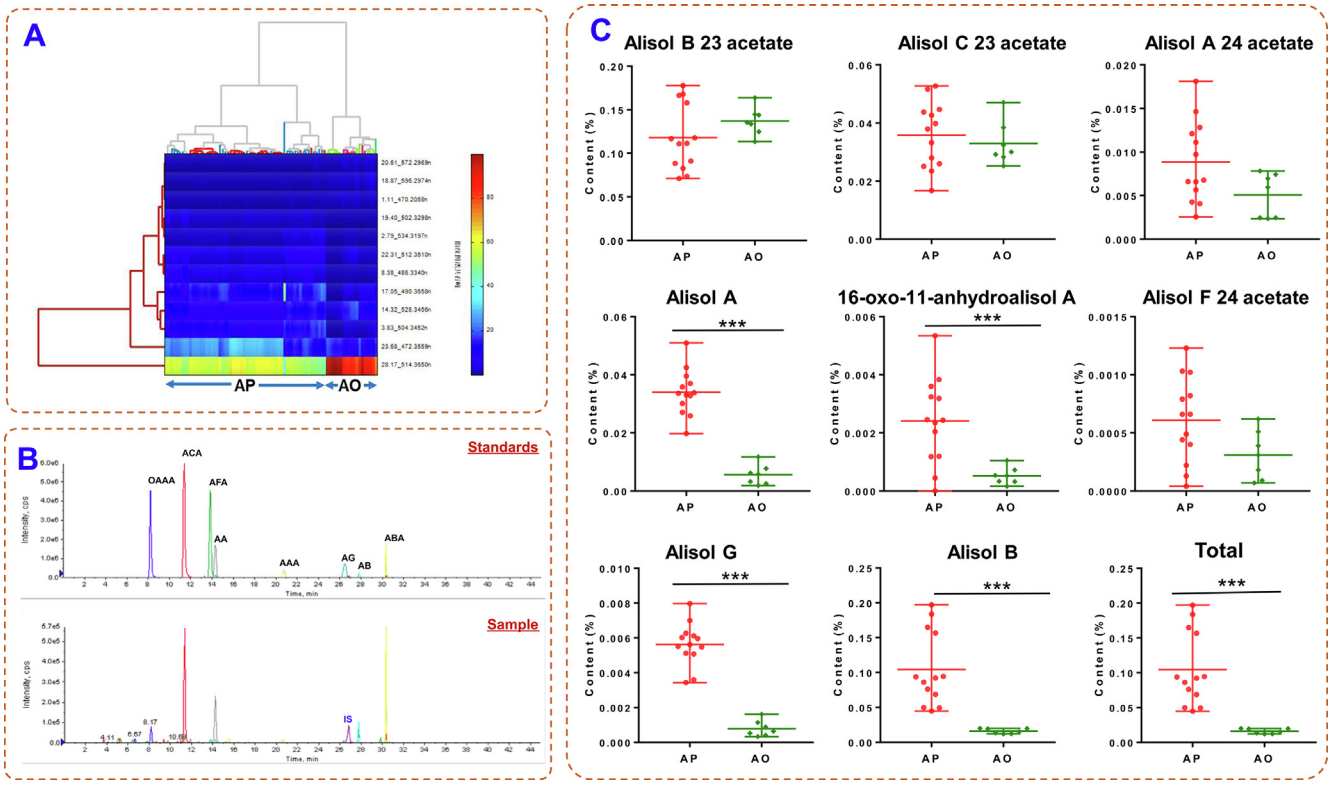

Fig. 4 The heatmap of two-way hierarchical clustering analysis based on the abundances of twelve potential discriminating markers in AP and AO samples (A), MRM chromatograms (B), and assay results (C) of eight main compounds by UHPLC-QTrap-MS in AP and AO.

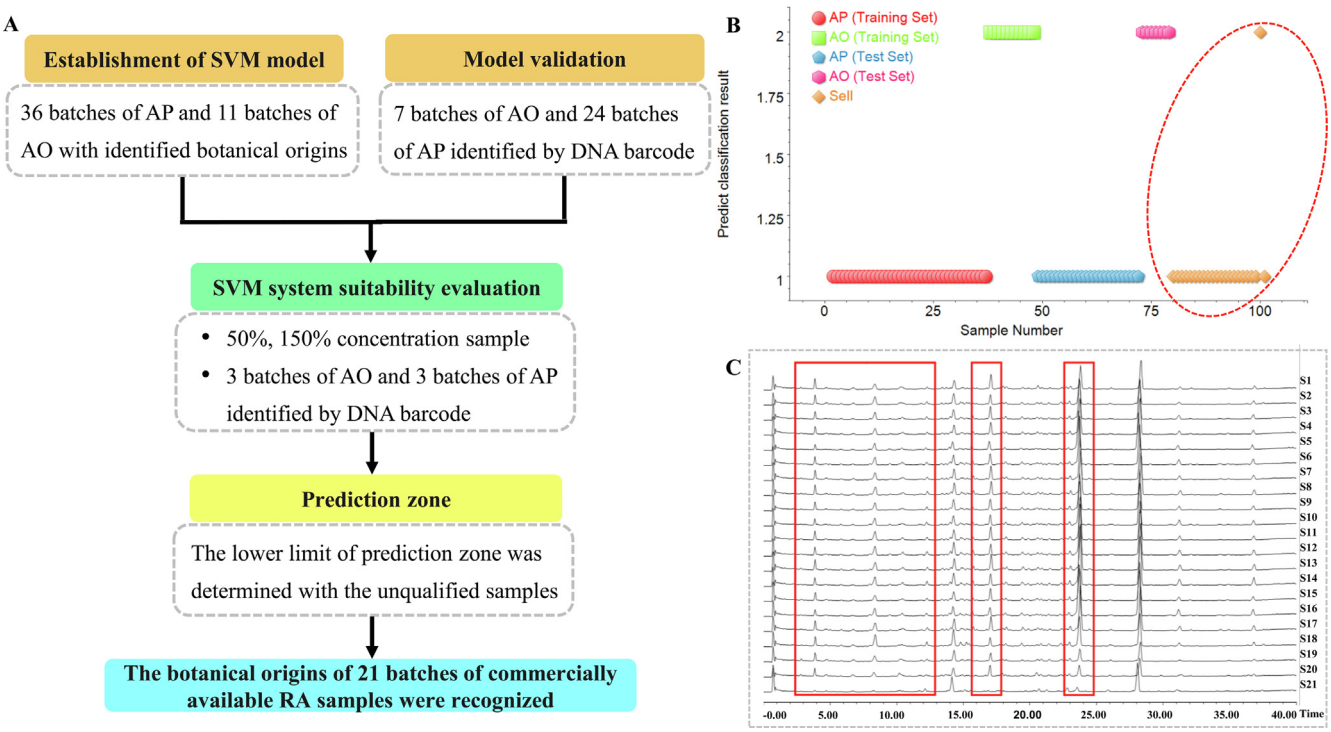

Fig. 5 A workflow of SVM model establishment and verification (A), SVM evaluation results (B) and the base peak ion chromatograms of twenty-one batches commercial samples (C).

concentration of samples (three batches of AP and AO identified by DNA barcoding) were tested, and the results showed that the model have an excellent predication accuracy with prediction value 0.75–0.09 for AP and 0.49–0.78 for AO, respectively (Fig. S3 and Table S11). Moreover, the lower limit of prediction value was defined by seven inferior products in the model, and ultimately these products were all judged as AP with prediction values of 0.14–0.34, suggesting that the model results will not be reliable when the prediction value was below 0.34 (Fig. S4 and Table S12). Finally, we utilized this SVM model to predict 21 batches of commercial unknown samples, as a result, only one were predicted as AO, and the others as AP with the prediction values of 0.47 and 0.92–1.14, respectively (Fig. 5C and Table S13). From the results of 99 batches of RA, it can be concluded that botanical origins of RA cultured in Fujian, called Jian-Zexie, was *A. orientale* which was also formally recognized by Chinese Pharmacopoeia (2015 edition). While the RA samples from Sichuan and Jiangxi Provinces of China, called Chuan-zexie, were *A. plantago-aquatica*, which occupied fairly big share of sales.

4. Conclusion

We integrated untargeted metabolite profiling based on UHPLC-Orbitrap, data cross validation, absolute quantification, and vector machine model, as a strategy for the classification and prediction of "one-to-multiple" herbal medicine, exemplified by RA in the current work. The untargeted metabolite profiling showed remarkable differences of chemical constituents visualized by base peak ion chromatograph and PCA model, and 41 potential markers were identified by pattern recognition OPLS-DA. Particularly, twelve compounds could render robust information used to differentiate *A. orientale* from *A. plantago-aquatica* after "multi-duplicated and traceability samples comparison" cross validation. By using these twelve markers, SVM model could give direct discrimination in the accurate authentication of RA from the market. This was the first report on systematic comparison and discrimination of two original species of RA. It is concluded that the combination of multiple analytical methods offers a practical and potent vehicle favoring the authentication of herbal medicines with multiple botanical origins.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This research was supported by National Natural Science Foundation of China (Grant No. 82003940), and Chief scientist of Qi-Huang Project of National Traditional Chinese Medicine Inheritance and Innovation "One Hundred Million" Talent Project (2020).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2022.104118.

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