“Q-markers targeted screening” strategy for comprehensive qualitative and quantitative analysis in fingerprints of *Angelica dahurica* with chemometric methods

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Chemical compounds:

- prangenin hydrate (PubChem CID: 129710912)
- xanthonoxol (PubChem CID: 65900)
- hydrate oxypeucedanin (PubChem CID: 17536)
- xanthotoxin (PubChem CID: 4114)
- bergapten (PubChem CID: 2355)
- oxypeucedanin (PubChem CID: 160544)
- imperatorin (PubChem CID: 10212)
- phellopterin (PubChem CID: 98608)
- isoimperatorin (PubChem CID: 68081)
- estazolam (PubChem CID: 3261)

**ABSTRACT**

*Angelica dahurica* is a famous functional food and herb. To guarantee quality of *A. dahurica*, a strategy “Q-markers targeted screening” was successfully developed by sufficient extraction of compounds and the targeted screening of qualitative and quantitative markers calculated through chemometric methods based fingerprints. Accelerated solvent extraction was selected due to its prominent advantages exhibiting the maximum extraction yields and varieties of compounds and especially excellent reproducibility (RSD < 1). After extraction, the fingerprints of *A. dahurica* samples were established. For the preliminary herb authenticity, the targeted screening of 23 quantitative markers were performed by similarity analysis and hierarchical cluster analysis based on the fingerprints, which were identified by liquid chromatography tandem mass spectrometry (LC-MS). Subsequently, for further quality control, the targeted screening of nine quantitative markers were done by similarity analysis & linear discriminant analysis, which were determined by LC. Lastly, the strategy was successfully applied to quality assessment of *A. dahurica* samples.

**Abbreviations:** ANOVA, analysis of variance; ASE, accelerated solvent extraction; BBD, Box-Bohnken Design; CID, collision-induced-dissociation; HCA, hierarchical cluster analysis; HRE, heated reflux extraction; IS, internal standard; HPLC-PDA-ESI-ITMS, high performance liquid chromatography-photo diode array-electrospray ionization ion trap mass spectrometry; UV, ultra violet; TOF, time of flight; LDA, linear discriminant analysis; LOD, limits of detection; LOQ, limits of quantification; MAE, microwave-assisted extraction; RSD, relative standard deviation; RSM, response surface methodology; SA, similarity analysis; S/N, signal-to-noise ratio; UAME, ultrasonic-assisted microwave extraction; UE, ultrasonic extraction.

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1. Introduction

Radix Angelicae Dahuricae, the dried root of Angelicae dahurica (Fisch. ex Hoffm) Benth. et Hook. f., is a famous functional food, cosmetic product ingredient and herbal medicines in China, Korea, Japan and Russia due to its antioxidative and skin-whitening functions (Nam, Kim, Sim, & Chang, 2003; Zheng et al., 2010). As a medicine food homology, A. dahurica is also used as an antipyretic and analgesic to treat colds, headaches, toothaches, corzyra, acne (Lee et al., 2011; Li, Dai, Zhang, & Xie, 1991; Pharmacopoeia of the People’s Republic of China, 2020) and alleviate swell and pain from sores and wounds (Lee et al., 2017; Zhang et al., 2017). These functions of the A. dahuricae were guaranteed by their material base. Modern phytochemistry and pharmacological studies reported that the major active constituents in A. dahurica are coumarins, mainly imperatorin, phellopterin and isoimperatorin, which exert various pharmacological activities such as antioxidative, antiinflammatory, antimicrobial, anticancer, antiparkinsonian effects etc. (Bae et al., 2011; Cho et al., 2006; Kang et al., 2019; Yang et al., 2015). In addition, there are many other trace compounds in A. dahuricae, such as oxypeucedanin, bergapten, xanthotoxol, byakangelicin, etc, which also have very extensive pharmacological activities (Chen et al., 2019). To assure the preventable and therapeutic effects of A. dahuricae, quality control in the form of the determination of contents of the multiple chemical ingredients was essential (Gao et al., 2016). The previous study about the quality control of A. dahuricae was focused on the quantification of three main coumarins, namely imperatorin, phellopterin, and oxypeucedanin (Yang, Li, Feng, & Qiu, 2020), or the quantitative analysis of therapeutically important coumarins which can be separated (Chen, et al., 2006), or just chromatographic fingerprint analysis and characterization of furocoumarins in the roots of A. dahurica (Kang, Zhou, Sun, Han, & Guo, 2008). The quantitative compounds were selected only due to their high contents or available reference standards, not due to their great impact on the quality control. Since the selected compounds may not always provide characteristic and representative information, it hardly reflects the quality of A. dahurica. Based on this, we first proposed a new strategy “Q-markers targeted screening”, which integrated and improved our previous scattered and fragmentary research base. The Q-markers, namely qualitative and quantitative markers, were screened according to the influence on quality of A. dahurica which were calculated with the help of chemometric methods. The new strategy provide not only the data about the extraction, separation, quality and quantity of A. dahurica, but also references for the quality control of other food.

Before the selection of qualitative and quantitative markers, effective and sufficient extraction of multiple bioactive compounds from matrix is the most foundational step in the process of quality evaluation. Therefore, an advanced extraction method is imperative to fully extract various constituents, guarantee the stability and robustness of extraction. A great deal of research and our previous studies confirmed that accelerated solvent extraction (ASE) is an extraordinary choice for the high automation, low solution and time consumption, and particularly the prominent extraction efficiency in varieties and quantities of compounds, compared to traditional extraction methods (Gao et al., 2013). In addition, the targeted selection of Q-markers, the main influence factors on quality of samples from different resources, is a critical step for subsequent qualitative and quantitative analysis. That needs to be calculated by some reliable chemometric analysis methods based fingerprints (Li, Shen, Yao, & Guo, 2020; Dong et al., 2020). Similarity analysis (SA), hierarchical cluster analysis (HCA) and linear discriminant analysis (LDA) are authoritative chemometric methods with different calculation theories (FDA, 2004; Caoer, Kvalheim, &Ceche, 2018; Zhuo, Xu, Li, Pu, & Ye, 2021). In our previous study, we compared the variation on the classifiers of samples between SA and HCA: SA classified samples through the mean values and HCA classified samples through merging the most similar objects or clusters into a new cluster (Gao et al., 2016). Both chemometric methods have their own advantages and limitations. In this study, the characteristics of SA and HCA were further integrated and utilized. Through the combination of the classification results of the two chemometric methods, some compounds were selected as qualitative markers due to the existence of these compounds has great effect on the samples authenticity. After the preliminary differentiate of the truth and false, the determination of the content of quantitative markers is the final step to comprehensively evaluate the sample quality. The quantitative markers were further chosen according to the common peaks of chromatographic profiles by SA and discriminate peaks according to the group HCA by LDA, which have great contribution to quality. Given the above, the “Q-markers targeted screening” strategy, namely preliminary authentication of herbs based on qualitative markers, subsequent evaluation of herbs quality based on quantitative markers ” provides an innovative perspective for strict and comprehensive quality control of different sources of herbs, and the main characteristics of the new strategy, compared with conventional methods, were listed in supplementary material 3.

2. Materials and methods

2.1. Chemicals and reagents

A total of 14 batches of A. dahuricae from different regions were widely collected throughout China and Dr. Luping Qin (Naval Medical University, Shanghai, China) further confirmed their authenticity. Reference compounds, xanthotoxol, xanthotoxin, bergapten, imperatorin and isoimperatorin were provided by Shanghai yuanye Bio-Technology Co., Ltd. Reference compounds, prangenin hydrate, hydrate oxypeucedanin, oxypeucedanin and phellopterin were isolated and purified by our laboratory from A. dahuricae with their purities over 95% at HPLC level. Internal standard (IS), estazolam was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (Merck company, Darmstadt, Germany) was HPLC grade. Ethanol and formic acid (China Medicine Group Shanghai Chemical Reagent Corporation, Shanghai, China) was analytical grade. Deionized water (18 MΩ, Milli-Q, Millipore, Bedford, MA, USA) was used in the study.

2.2. Extraction and preparation of sample solutions

All of the A. dahuricae were chopped into powdered samples with a particle diameter of less than 0.355 mm. The A. dahuricae sample from Anhui Province was selected for the methods development. A. dahuricae samples was extracted under high pressure using ASE 350 (Dionex Corp., Sunnyvalue, CA). The extraction condition containing temperature, time, static cycles was guided based on the experimental design of single factor experiments and RSM. The residue after extraction was purged with solvents in 60% of the extraction cell volume and then with nitrogen for 60 s. The 66 mL glass vessels were used to collected the extract solution.

The conventional extraction methods, containing heated reflux extraction (HRE), ultrasonic extraction (UE), microwave-assisted extraction (MAE) and ultrasonic-assisted microwave extraction (UAME) were conducted with the ratio of material and solvent fixed at 1:4, and water and 95% ethanol as extraction solvent, respectively. The A. dahuricae samples of 5.0 g adding 20 mL extraction solvent were extracted in a cooled condenser with 2 h at 80 °C for HRE and at an ultrasonic bath with 1 h at room temperature for UE. For MAE and UAME, a simultaneous ultrasonic and microwave extracting apparatus (CW-2000G, Shanghai Xintuo Microwave Instrument Co, Ltd., China) was adopted to extract mixtures with microwave assistance at 80 °C for 20 min and with microwave and ultrasonic assistance at 80 °C for 10 min, respectively. These extraction experiments were performed in triplicate.

Each of the extract was diluted to a final volume of 50 mL after fully filtering and rinsing the residue for three or four times using extractant for quantitative analysis.
2.3. Qualitative and quantitative analysis

The HPLC-UV was applied on a Shimadzu HPLC chromatographic system (Shimadzu Corporation, Kyoto, Japan) for quantitative analysis. For the complete chromatographic separation of compounds from *A. dahuricae* extracts, a YMC-TriaTri C18 column (4.6 mm × 200 mm, 5 µm, YMC, Tokyo, Japan) was employed with the mobile phases composed of 0.1% formic acid (A) and methanol (B) and flow rate at 0.8 mL/min. The gradient elution was as follows: 0–10 min, 15–25% B; 10–12 min, 25–35% B; 12–27 min, 35–40% B; 27–30 min, 40–50% B; 30–40 min, 50–60% B; 40–53 min, 60–70% B; 53–63 min, 70–80% B; 63–65 min, 80–85% B; 65–70 min, 85–90% B; 70–75 min, 90–90% B. The column temperature was set at 25 °C and injection volume was 10 µL. The UV spectra was set at 254 nm as the maximum absorption wavelength of standards and samples. The qualitative analysis was carried on HPLC-PDA-ESI-ITMS® and HPLC-TOF-MS (Supplementary material 1).

2.4. Method validation

For method validation, the mixed standard stock solution containing prangenin hydrate, xanthotoxol, hydrate oxypeucedanin, bergapten, oxypeucedanin, imperatorin, phellopterin and iso-imperatorin was prepared and diluted, and 100 µg/mL estazolam (IS) was added as IS. The peak area ratio of each compound with six gradient concentrations versus IS against the concentration were calculated for linear regression, in triplicate. The signal-to-noise ratios (S/N) of compounds at about 3 and 10 were set as the limits of detection (LODs) and quantification (LOQs), respectively. The RSDs (relative standard deviation) of peak area from six replicates of the same sample within one day and twice a day for three consecutive days was analyzed to evaluate the intra-day and inter-day precisions, respectively. The RSDs of peak area from six samples of the same batches after extraction were calculated for the assessment of repeatability. The RSDs of peak area by re-analyzing one prepared sample within 12 h was measured for the sample stability. The standard addition method was adopted for the accuracy of method. Accurate amounts of standard solutions were added to 1.0 g *A. dahuricae*, and then extracted and analysed by ASE and HPLC methods, in six replicates. The recovery (%) = (content detected-originial content) / content added × 100%.

2.5. Statistical analysis

RSM was adopted to optimize the best extraction parameters for ASE using the software of Design Expert Version 8.0.6 (Stat-Ease, Inc., Minneapolis, USA). The process was accomplished step by step, containing the experiment design, model establishment, evaluation of the effects of factors and the determination of the optimum conditions of factors for desirable responses. A Box-Behnken Design (BBD) was constructed consisting of three-variable, three-level, which designed 17 experiments with five center points. The three variables were set as X1 for extraction temperature, X2 for extraction time and X3 for static cycle, each of which was coded –1 for the low level, 0 for the middle level and +1 for the high level. The experiments were randomly arranged to avoid systematic errors. The total yields of imperatorin, phellopterin and iso-imperatorin was averaged as the responses (Y). The second order polynomial model was explained by quadratic equation (1) as flows:

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_i^2 X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \]  

(1)

where Y is the predicted response; \( \beta_0 \) is a constant; \( \beta_i \) and \( \beta_{ij} \) are the regression coefficients of linear, quadratic and interaction, respectively. There were three parameters, namely F-value (the Fisher test value), \( R^2 \) (the coefficients of determination) and the lack of fit calculated by the analysis of variance (ANOVA) to evaluate the adequacy of the model. A factor was considered to be significant with its coefficients of p value lower than 0.05. The combinations of three factors yielding the highest response was selected as the optimum conditions which was experimented to verify the model prediction outcome.

SA was conducted with “Similarity Evaluation System for Chromatographic Fingerprint of TCM” software (China Committee of Pharmacopeia, 2004A version). Thirteen chromatograms from different batches of *A. dahuricae* samples in the form of AIA (*cd*) was introduced into the software and then a “reference chromatogram” a chromatographic pattern of all kind of components in all samples, was established as simulated mean chromatogram. The similarity values were calculated by the chromatograms of each sample relative to the mean chromatogram.

The area of each peak was adopted in hierarchical cluster analysis (HCA) combined with lineal discriminant analysis (LDA) for the species differentiation of the samples. SPSS 10.0 software package (Chicago, IL, USA) was adopt for the data processing.

3. Results and discussion

3.1. Optimization of chromatographic conditions

HPLC parameters were investigated and optimized for a satisfactory separation and the most useful chemical information in the fingerprint chromatograms of *A. dahuricae* in a short time. Through the comparation of different columns, the YMC-TriaTri C18 column could separate the most structural analogues in shorter analysis time. Methanol with hyperviscosity was chosen as organic phase for more separation efficiency. 0.1% formic acid compatible for MS could form molecularly with hydroxyl group of coumarin in *A. dahuricae* to improve peakshape of compounds. In addition, due to the similar polarity and complexity of analytes from *A. dahuricae*, it was highly needed a gradient elution mode for the complete baseline separate and symmetrical peak shape of structural analogous in *A. dahuricae*.

3.2. Comparison of five extraction methods

Effective extraction method is foundational, because the sufficient extraction of bioactive compounds in quality and quantity from matrix was necessary for the comprehensive quality control of food. The comparation of HRE, UE, MAE, UAME and ASE, were performed for their extraction efficiency through varieties of components and the total yields of major compounds, namely imperialorin, phellopterin and iso-imperatorin from *A. dahuricae*. The extraction solvents, namely water and ethanol, were investigated for the extraction effects using the five extraction protocols, respectively. Other extraction conditions were adopted according to the previous literature about sample extraction (Gao et al., 2013). In the case of ASE, the common used extraction conditions were adopted, with the extraction temperature at 125 °C, the extraction time for 5 min and two static cycles.

The extracts obtained were diluted with the same pattern and analyzed by HPLC-UV. The response for the optimization procedure was the total peak area of three major compounds, namely imperatorin, phellopterin and iso-imperatorin in *A. dahuricae*. Fig. 1 showed the significant differences in the extraction efficiencies of the five techniques. The results showed that when water was used as extraction solvents, hydrophilic compounds were the major ingredients, and conversely hydrophobic compounds with pharmaceutical activities were few in each resulting extract obtained by HRE, UE, MAE, UAME (Fig. 1B). While dozens times higher extraction yields of hydrophilic and especially hydrophobic compounds were obtained by ASE with water than by the other four extraction techniques (Fig. 1A and 1B). Moreover, abundant coumarins were extracted by the five extraction methods with ethanol as extraction solvents, and meanwhile ASE provided the most extraction varieties of components and an approximately five fold increase for the extraction yields of the major compounds, namely imperatorin.
phellopterin and isoimperatorin from *A. dahuricae* than the others (Fig. 1IIand III). Importantly, compared to HRE, UE, MAE, UAME, the best reproducibility was observed by adopting ASE as extraction method (Fig. 1IV). The results demonstrated that automatic extraction process of ASE reduced accidental errors and the extracts from *A. dahuricae* were completely entered into glass vessels by high pressure, fresh extraction solvent rinsing and nitrogen purging. Consequently, ASE was selected as the extraction method to extract bioactive compounds of *A. dahuricae* owing to its short time-consumption, high efficiency, good reproducibility and high level automation.

### 3.3. Optimization of ASE

#### 3.3.1. Effect of extraction solvents

To extract the most abundant of constituents from the sample matrix, extraction solvent is the import factor due to the various analytes in physicochemical properties. Water and ethanol were the usually used extractant and thus the effect of the concentration of ethanol (0%, 25%, 50%, 75% and 100%) was investigated on the extraction efficiencies of ASE from *A. dahuricae*. The sample quantity was maintained at 5 g in 10 mL ASE extraction cell and the *A. dahuricae* samples were extracted at 125 °C for 5 min with two static cycles. From Fig. 2IA, it was found that increasing the concentration of ethanol resulted in an improvement of extraction yields for the imperatorin, phellopterin and isoimperatorin in *A. dahuricae* and the most efficient ethanol was 100% ethanol to extract the three main coumarins with weakly polar corresponding to the “like dissolves like” principle. Consequently, ethanol was selected as extraction solvent for the subsequent experiments.

#### 3.3.2. Effect of three extraction factors

The three extraction factors, containing extraction temperature, extraction time and static cycle were the main influence on extraction yields. Initially, different temperatures (50, 75, 100, 125, 150, 175 °C) were explored with ethanol as extraction solvent, 5 min for each cycle and two static cycles. As shown in Fig. 2IB, no significant increase was found in the extraction yields of imperatorin, phellopterin and isoimperatorin when the temperature was below 100 °C. From 100 to 175 °C, temperature had a positive impact on the extraction yields of imperatorin, and meanwhile the extraction yields of phellopterin and isoimperatorin decreased with the increase of temperature before higher than 150 °C. Considering the total yields of the three main coumarins, the temperature range from 125 to 175 °C was adopt for the follow-up optimized experiments. Subsequently, extraction time (3, 6, 9, 12 and 15 min) were investigated with ethanol as extraction solvent, temperature at 150 °C and doubled static cycles. Fig. 2IC showed that the extraction yields of imperatorin, phellopterin and isoimperatorin enhanced when the extraction time increased. Considering that longer extraction time may promote degradation of compounds in *A. dahuricae*, the extraction time within 9–15 min was confined in the next experiments. Finally, the numbers of cycles were explored through the complete extraction of components, which determine the number penetrating matrices through introducing extraction solvent during the extraction process. The experiments were performed through consecutive extractions on the same sample for three times with 150 °C and 15 min each cycle. The total peak area of imperatorin, phellopterin and isoimperatorin were 12,867,522 for the first static cycle, 663,197 for the second, 0 for the third, respectively. Therefore, the numbers of cycles were ranged from one to three due to the no longer increased extraction yields after two cycles of extraction on the same matrix.

#### 3.3.3. Optimisation of extraction conditions of ASE by RSM

According to the single factor experiments, the variable ranges of extraction conditions were set from 125 to 175 °C for extraction temperature, from 9 to 15 min for extraction time, from one to three times for static cycle, which were coded to lie at ±1 for the factorial points, 0 for the center point, respectively. A total of seventeen experiments...
with actual values of the factors were designed by BBD and the consequent responses for the yields of main coumarins were obtained by a HPLC-PDA method (Fig. 2 IIA). Experimental results were analyzed by ANOVA for the validity of the model (Fig. 2 IIB and Supplementary material 2). With the best fitted respond values, the second order polynomial model was used in the subsequent experiments.

Subsequently, the Student’s $F$-test and $p$-value was calculated for the significance of each coefficient. The corresponding coefficient was more significant with the larger $F$-test and the smaller $p$-value. Based on these, extraction temperature ($X_1$), extraction time ($X_2$) and static cycle ($X_3$) were significant influence factors to the total yields of the three main coumarins (Fig. 2 IIB). And the positive and pivotal linear effect of temperature and cycle represented that the extraction yield was promoted with the increase of temperature and cycles.

The fitted polynomial model was demonstrated as three-dimensional response surface curves to visualize the relationship between responses and the experimental levels of each variables and meanwhile the optimum extraction conditions and maximum efficiency were deduced. The curves were established against each two of independent variables while the remaining one was fixed at its middle level (Fig. 2 III). As depicted in Fig. 2 III A and Fig. 2 III B, a higher extraction yield was obtained with the extraction temperature increasing to the middle level. At higher temperature, a negative interaction effect of the temperature and cycle were observed through the specific curvature of the surfaces, which demonstrated that the increasing extraction temperature not always favored the response. Extraction time had little influence on the response and the trend of curvature of the response surfaces was similar to extraction temperature (Fig. 2 III A and Fig. 2 III C). Fig. 2 III B and Fig. 2 III C reflected the negative interaction influence of static cycles and the other two factors. Nevertheless, there was an inconspicuous influence on response when the cycles exceeded two, which agreed with the single-factor experiments. Consequently, a stationary point at the quadratic experimental model was confirmed as the predictive yield with the maximal response. The optimum extraction condition of *A. dahuricae* were recommended as follows: 174 ºC for extraction temperature, 13 min for extraction time and two cycles for static cycle. The RSM is more reliable, visual and comprehensive than single-factor experiments to synthetically reflect the potential interactions among extraction parameters and globally optimize the variable factors for the maximal response.

![Fig. 2. Optimization of ASE extraction method. I: Extraction efficiencies of imperatorin, phellopterin and isoimperatorin in *A. dahuricae* affected by extraction solvents (A), extraction temperatures (B), extraction time (C) using ASE. II: Response surface design and corresponding response values (A) and analysis of variance for the second order response surface model (B) by response surface methodology. III: Response surface plots representing interaction of two variables and their response to the total yields of imperatorin, phellopterin and isoimperatorin.](image-url)
3.3.4. Validation of the optimal condition of ASE

Subsequently, an experimental rechecking of the deduced optimal condition was tested for the comparison between the predicted values and practical ones. The result showed that there was a 0.055% deviation of the total yield of the three main coumarins while no significant difference exited within 95% confidence internal \((p = 0.851,\) calculated by Bonferroni test) between measured value of \(1.48181E + 7\) \((n = 3)\) and the predictive value of \(1.48099E + 7\). The good correlation meant that the response model was adequate to reflect the expected optimization.

3.4. Selection of qualitative markers of A. dahuricae samples based on SA and HCA with fingerprints

3.4.1. Establishment of HPLC fingerprint of A. dahuricae

Besides a high-efficiency extraction method, a comprehensive statistical analysis is highly necessary for the strict quality control of A. dahuricae. The fingerprint analysis technique has been used as an effective and powerful tool and has been internationally acknowledged for the quality control of TCM (FDA, 2004). Based on this, the HPLC fingerprints of thirteen batches of A. dahuricae samples from different sources of China were obtained under the optimal HPLC conditions, which were then standardized by the Similarity software for further analysis. According to the pharmacological experiment for analgesic effect we have conducted, the hydrophilic compounds corresponding to the peaks within 30 min in the chromatogram were inactive. To remove the interference, the 13 chromatograms were analyzed by the software without the peaks within 30 min. There were 34 peaks in chromatographic profiles (Fig. 3).

3.4.2. SA and HCA

To distinguish the truth and false of A. dahuricae, the qualitative markers were selected according to the combination of the samples classification results of the two chemometric methods. SA was widely used to assess the similarity (represented by the correlation coefficients) between two chromatograms in fingerprint analysis. Fig. 3 showed the correlation coefficients of 13 A. dahuricae samples compared to the

![Fig. 3. Statistical analysis based fingerprint of 13 A. dahuricae samples. I: HPLC fingerprints of 13 A. dahuricae samples extracted by ASE. II: The similarities (correlation coefficients) of 13 A. dahuricae samples from different sources. III: The hierarchical cluster dendrogram of 13 A. dahuricae samples obtained by HCA.](image)
reference chromatogram, respectively. Generally, the two chromatograms were more similar with the correlation coefficients closer to 1.0. HCA, another chemometric method with a different idea from SA, was also adopted to distinguish the samples into different clusters. HCA computation was used to calculate hierarchical and squared Euclidean distance in the cluster dendrogram. The shorter distance representing the degree of association among samples shows the higher degree of relationship. The hierarchical and squared Euclidean distance were obtained convert the entire chromatograms of 13 A. dahuricae samples (in row) containing 34 peaks (in column) into a total of 13 × 34 data matrix and then singular value decomposition was performed on the statistical structures (Fig. 3 III). Due to the fact that not all 34 peaks have great effect on the samples quality, which were characteristics peaks from reference chromatogram, which was established as simulated mean chromatogram by “similarity Evaluation System for Chromatographic Fingerprint of TCM” software, the qualitative markers should be selected based on the analysis of fingerprints with the different similarities by SA and the fingerprints in different clusters by HCA from the 34 characteristics peaks.

For examples, in SA, the samples 5, 10, 11 (correlation coefficient < 0.95) were sorted to a group and other samples (correlation coefficient ≥ 0.95) were clustered into another group. In HCA, all A. dahuricae samples were grouped into cluster A containing samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 12 and 13, and cluster B containing samples 10 and 11. The qualitative markers were selected from 34 characteristics peaks from reference chromatogram through the comparison of fingerprints of samples from different groups. From the Fig. 3I, some difference was observed. There were no peaks 1, 2, 3, 4, 7, 8, 11, 12, 17, 24 and 33 in samples 10 and 11. Moreover, the areas of peak 9, 13, 14, 15, 16, 20, 27, 28, 30 and 31 in samples 10 and 11 were lower than those in other samples, but the areas of peak 19, 22 in samples 10 and 11 were higher, compared to other samples. For sample 5 (Fig. 3I), the differences in varieties of compounds were not found. But the areas of some peaks were distinct from others. For examples, the area of peak 22 in sample 5 was higher than that in other samples. By that analogy, a total of 23 peaks were selected as qualitative markers based on the analysis of fingerprints with the different similarities by SA and the fingerprints in different clusters by HCA. The HPLC-PDA-ESI-ITMS5/HPLC-TOF-MS were adopted for the identification of qualitative markers in A.dahuricae extracts and the 23 quantitative markers were tentatively identified (Fig. 4I and Supplementary Material 1).

3.5. Selection of quantitative markers of A. dahuricae samples based on SA and LDA with fingerprints

3.5.1. SA and LDA

It is insufficient to assess quality of samples through difference in qualitative markers, and determination of qualitative markers were overwhelming and unpractical. Based on the fact, the targeted selection of quantitative markers, on the basis of qualitative markers, is central for the quality control of food. The reference chromatogram from SA could not be used to group different samples through the similarities, but still could provide a chromatographic pattern containing common components. In the reference chromatogram of 13 A. dahuricae samples, seven common peaks (Peaks 9, 15, 16, 20, 27, 28 and 31) was discriminated and considered to be a direction to next quantify ingredients.

Furthermore, according to the results of HCA, LDA was adopted to generate two linear discriminant functions representing the two clusters, which was as follows Eqs. (2) and (3):

\[
A = 1.897E^{-3} \text{Peak7} - 2.426E^{-3} \text{Peak9} + 6.306E^{-4} \text{Peak15} - 1.654E^{-3} \text{Peak22} - 9.892E^{-3} \text{Peak25} + 8.556E^{-3} \text{Peak26} - 1.612E^{-4} \text{Peak31} - 5.865E^{-3} \text{Peak34} - 3.692E^{-7}
\]

\[
B = 8.568E^{-3} \text{Peak8} - 1.620E^{-4} \text{Peak10} + 6.327E^{-4} \text{Peak11} - 7.855E^{-3} \text{Peak12} - 9.892E^{-3} \text{Peak14} + 1.612E^{-4} \text{Peak16} - 5.865E^{-3} \text{Peak17} - 3.692E^{-7}
\]

Fig. 4. The qualitative and quantitative analysis of A. dahuricae samples. I: The representative HPLC chromatogram at 254 nm (A) and the TIC chromatogram (B) of A. dahuricae extract. II: HPLC chromatograms of mixed standard (A), A. dahuricae extract (B) and A. dahuricae extract spiked with internal standard (C). III: The contents of nine quantitative markers from 13 A. dahuricae samples (A) and the contents of three main compounds, namely imperatorin, phellodendrin and isoimperatorin from 13 A. dahuricae samples (B).
B = -1.228E-3 Peak7 + 1.664E-3 Peak9 - 4.234E-3 Peak15 +1.119E-3 Peak22 + 6.677E-2 Peak25 - 5.796E-2 Peak26 + 1.082E-3 Peak31 + 3.828E-2 Peak34 – 1.652E4  

(3)

The linear discriminant functions contained eight variables, demonstrating that the eight ones have good discrimination ability to classify different samples and were regarded as discriminate peaks, which were Peaks 7, 9, 15, 22, 25, 26, 31 and 34.

To comprehensively reflect the quality of *A. dahurica*, the quantitative markers were selected among the seven common peaks by SA and eight discriminate peaks by LDA, taking into account the different calculation theories of SA and LDA. Due to the fact that there are no or few compounds 25, 26 and 34 in *A. dahurica* samples, a total of nine quantitative markers were determined to be applied in quality control of the thirteen *A. dahurica* samples, namely prangenin hydrate (peak 7), xanthotoxol (peak 9), and hydrate oxypeucedanin (peak 15), xanthotoxin (peak 16), bergapten (peak 20), oxypeucedanin (peak 22), imperatorin (peak 27), phellopterin (peak 28) and isoimperatorin (peak 31).

3.5.2. Validation of quantitative method

The HPLC-UV chromatograms of mixed standard solution of nine quantitative markers, *A. dahurica* extract and *A. dahurica* extract spiked with IS are shown in Fig. 4 II. A series of tests for the validation of the HPLC method were performed and the results were shown in Table 1A. There was excellent linearity of each compounds with correlation coefficients (R²) higher than 0.9989 in appropriate concentration ranges in accordance with their levels in samples. The results of LOQs and LODs indicated that the HPLC-UV method was sensitive for the quantitative detection. The RSD values of intra-day and inter-day, within 2.58% and 2.88% respectively, showed that the precisions of the nine compounds met the requirement. The reproducibility and stability of the method were acceptable with the RSDs within 2.71 and 2.11, respectively. The average recovery rates were between 96.10% and 104.24% with the RSDs all less than 3.16%, which demonstrated that the method was accurate. The established HPLC-UV method was validated and appropriate for simultaneously quantitative determination of the nine compounds in *A. dahuricae*.

3.6. Quality control of different *A. dahuricae* samples

The thirteen batches of the *A. dahurica* samples from different origins were extracted and evaluated using the developed ASE coupled with HPLC-UV method. The contents of nine quantitative markers in 13 sample extracts were determined and summarized in Table 1B and Fig. 4 III. From Fig. 4 III A, the quality of samples could be comprehensively evaluated. In samples 10 and 11, there were differences in quality and quantity, with few prangenin hydrate and xanthotoxol, the higher content of oxypeucedanin and the lower contents of imperatorin, phellopterin and isoimperatorin than other samples. In samples 6 and 12, there are no oxypeucedanin and smaller amounts of the other eight quantitative markers. Based on the analysis, the samples 6, 10, 11 and 12 were of poor quality. Combined with the results of SA and HCA, there were also differences in samples 5. However, the differences were generated just for the higher content of oxypeucedanin in sample 5. Moreover, the quality of the samples 3, 4 and 5 is higher due to the abundant contents of quantitative markers. If the quality control focused on quantitation of major compounds with high content, such as imperatorin, phellopterin and isoimperatorin (in Fig. 4 III B), the poor quality of sample 6, 10 and 12 could be observed. The results could not reflect the poor quality of sample 11 and the differences in varieties of components, which were also inconsistent with the results of chemometric analysis. Chemometric analysis has the ability to screen the low quality samples, but the distinguishability was insufficient. Therefore, the targeted selection of quantitative markers based on the chemometric analysis and the determination of the content of the quantitative markers were more comprehensive, reliable and rigorous to represent sample quality.

Additionally, the fourteenth *A. dahurica* samples (from Sichuan) were introduced and analyzed to validate the robustness of the “Q-markers targeted screening” strategy through preliminary authentication of herbs based on quantitative markers, subsequent evaluation of herbs quality based on quantitative markers. For the preliminary authentication of herbs, the fingerprints of the fourteenth samples were shown in Supplementary Material 4. Among 23 qualitative markers, a total of 21 peaks were observed and there were no peaks 14 and 17. Those means the fourteenth samples was correct *A. dahurica* plats, but maybe not of good quality. For the further evaluation of herbs quality, the contents of nine quantitative markers from the fourteenth samples was shown in Table 1B. The contents of oxypeucedanin (peak 22) was not detected because the area of peak 22 was below LOQ. Furthermore, the contents of prangenin hydrate was the higher than that in sample 10 and 11, and the contents of hydrate oxypeucedanin was only higher than that in samples 11. The total contents of imperatorin, phellopterin and

### Table 1A
Quantitative determination of the *A. dahuricae* samples. A: regression equation, linear range, determination coefficient (R²), limit of quantitation (LOQ), limit of detection (LOD), reproducibility, stability and recovery of nine quantitative markers.

| Peak no. | Compounds           | Regression equation | Linear range (μg/mL) | R²    | LOQ (μg/mL) | LOD (μg/mL) | Intra-day precisions (n = 6) | Inter-day precisions (n = 3) | Reproducibility (n = 6) RSD (%) | Stability (n = 6) RSD (%) | Recovery (n = 3) RSD (%) | Recovery (n = 3) Mean (%) |
|----------|---------------------|---------------------|----------------------|-------|-------------|-------------|-----------------------------|-----------------------------|-------------------------------|--------------------------|--------------------------|---------------------------|
| 7        | prangenin hydrate   | Y = -0.0515X + 0.021 | 1-9                  | 0.9996 | 0.25        | 0.05        | 2.58                        | 2.88                        | 1.99                          | 0.68                     | 96.10                    | 2.39                      |
| 9        | xanthotoxol         | Y = -0.0223X + 0.0308| 10-50                | 0.9995 | 0.25        | 0.05        | 1.75                        | 1.99                        | 2.01                          | 1.20                     | 102.6                    | 1.95                      |
| 15       | hydrate oxypeucedanin xanthotoxin | Y = -0.0260X + 0.0211 | 10-90                | 0.9989 | 0.1         | 0.02        | 2.02                        | 1.89                        | 2.11                          | 2.11                     | 101.9                    | 2.55                      |
| 16       | bergapten           | Y = -0.0252X + 0.0261| 10-30                | 0.9997 | 0.05        | 0.1         | 1.89                        | 2.10                        | 1.95                          | 0.89                     | 98.61                    | 1.89                      |
| 20       | oxypeucedanin       | Y = -0.0192X + 0.0104| 5-25                 | 0.9991 | 0.05        | 0.1         | 2.01                        | 1.00                        | 1.64                          | 0.88                     | 99.81                    | 2.59                      |
| 22       | phellopterin        | Y = -0.0223X + 0.0152| 5-45                 | 0.9998 | 0.1         | 0.02        | 2.55                        | 2.41                        | 2.71                          | 1.61                     | 103.5                    | 3.16                      |
| 27       | imperatorin         | Y = -0.0179X + 0.3978| 100-300              | 0.9996 | 0.5         | 0.1         | 1.52                        | 2.31                        | 0.59                          | 0.85                     | 98.71                    | 2.61                      |
| 28       | isoimperatorin      | Y = -0.0157X + 0.1051| 5-60                 | 0.9997 | 0.25        | 0.05        | 1.04                        | 1.54                        | 1.62                          | 1.11                     | 104.2                    | 1.88                      |
| 31       |                     | Y = -0.019X + 0.0109 | 10-90                | 0.9997 | 0.05        | 0.01        | 0.98                        | 1.21                        | 2.71                          | 1.64                     | 99.04                    | 2.67                      |
isoimperatorin were highest compared with other samples. Combined with the sample 10 and 11 of poor quality, the quality of sample 14 was unsatisfied, despite the three major compounds with high content.

4. Conclusion

This research offers a comprehensive, reliable and strict strategy “Q-markers targeted screening” for quality control of A. dahurica, containing high-efficiency ASE extraction method, comprehensive selection and identification of qualitative markers by SA & HCA and HPLC-PDA-ESI-ITMS/TOF-MS, and reliable selection and determination of quantitative markers by SA & LDA and HPLC-UV (Fig. 5). ASE method with optimized conditions extracted the maximum amounts and varieties of components with especially excellent reproducibility from matrix, which could better respond to quality. With optimal extraction, the SA & HCA chemometric analysis based on HPLC fingerprints divided 13 batches of samples into different groups according to the different theories. Due to the fact that compounds varied from groups to groups, 23 qualitative markers with the great impact on the groups were selected for the preliminary differentiate of the truth and false of A. dahurica and then identified by HPLC-PDA-ESI-ITMS/HPLC-TOF-MS methods. The chromatogram and mass rules for the differentiation of furocoumarins isomers were refined. For further quality control, the quantitative markers were subsequently screened according to the influence on quality of A. dahurica with the help of SA & LDA, which were determined in 14 batches of A. dahurica samples. With the new strategy, The quality of different A. dahurica samples was successfully assessed and validated. Compared to the conventional methods that direct selection of quantitative indexes according to the compounds with high contents or available reference standards in herbs, the “Q-markers targeted screening” strategy is innovative because of the targeted screening of qualitative markers with SA&HCA for herbs authentication, and then the targeted screening of quantitative markers with SA&LDA for herbs quality. In all, the study involving extraction, the targeted screening of qualitative and quantitative markers based fingerprints could provide a valuable and potential reference for quality control of other related food and so on.

CRediT authorship contribution statement

Fang-Yuan Gao: Conceptualization, Funding acquisition, Investigation, Validation, Writing – original draft, Writing – review & editing. Hai-Yan Chen: Data curation, Formal analysis, Writing – review & editing. Yu-Sha Luo: Investigation, Methodology, Writing – review & editing. Ji-Kuai Chen: Software. Lang Yan: Supervision. Jiang-Bo Zhu: Funding acquisition. Guo-Rong Fan: Conceptualization, Funding acquisition. Ting-Ting Zhou: Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial

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Table 1B

| Sample no. | prangenin hydrate | xanthotoxol | hydrate oxypeucedanin | xanthotoxin | bergapten | oxypeucedanin | imperatorin | phellopterin | isoimperatorin |
|------------|------------------|-------------|------------------------|-------------|-----------|--------------|-------------|--------------|---------------|
| 1          | 2.928            | 4.103       | 6.870                  | 2.244       | 1.450     | 0.467        | 11.15       | 4.722        | 4.515         |
| 2          | 3.811            | 3.018       | 6.547                  | 2.427       | 1.753     | 0.420        | 17.03       | 5.357        | 6.643         |
| 3          | 8.902            | 7.990       | 13.18                  | 5.374       | 4.081     | 2.090        | 44.19       | 12.31        | 11.98         |
| 4          | 6.391            | 6.037       | 10.79                  | 4.501       | 3.077     | 0.936        | 38.69       | 10.90        | 11.78         |
| 5          | 6.982            | 2.301       | 10.31                  | 3.644       | 2.466     | 3.822        | 24.18       | 7.716        | 8.190         |
| 6          | 2.692            | 2.168       | 3.866                  | 1.204       | 0.961     | ND           | 9.317       | 2.937        | 4.561         |
| 7          | 3.828            | 4.073       | 7.696                  | 2.545       | 1.726     | 0.677        | 13.62       | 4.480        | 6.827         |
| 8          | 3.627            | 4.324       | 7.024                  | 2.532       | 2.032     | 0.387        | 15.24       | 4.684        | 5.233         |
| 9          | 4.043            | 4.796       | 5.910                  | 2.008       | 1.747     | ND           | 10.64       | 3.233        | 6.029         |
| 10         | ND               | ND          | 5.240                  | 2.432       | 1.926     | 12.471       | 7.781       | 2.895        | 2.880         |
| 11         | ND               | ND          | 1.692                  | 1.037       | 1.612     | 9.788        | 15.33       | 3.250        | 4.367         |
| 12         | 2.895            | 2.934       | 5.218                  | 1.526       | 0.995     | ND           | 6.641       | 2.638        | 4.789         |
| 13         | 3.481            | 4.152       | 6.213                  | 2.230       | 1.574     | 0.364        | 13.83       | 3.841        | 4.203         |
| 14         | 0.683            | 6.918       | 2.617                  | 6.171       | 3.344     | ND           | 59.321      | 5.588        | 9.828         |

ND: not detected.

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Fig. 5. The graphical abstract of the paper.
interests or personal relationships that could have appeared to influence
the work reported in this paper.

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Appendix A. Supplementary data

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