Objective: There are different geographic origins of Aconiti Kusnezoffii Radixs (AKRs) sold in the market with different quality. This study aims to establish a rapid analysis method to distinguish the different geographic origins of AKRs and to realize the rapid evaluation of their quality. Methods: An ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry (UPLC-Q-TOF MS) method was utilized to acquire the constituents' information of AKRs from different geographic origins. MS² data and Progenesis QI software were employed to identify the chemical constituents. Principal component analysis (PCA) was applied to comparing MS data to find the chemical markers of AKRs from different geographic origins. Results: Twenty-three components were detected and 17 out of them were identified, including diester-diterpenoid alkaloids, monoester-diterpenoid alkaloids, and amine-diterpenoid alkaloids. Three pairs of isomers were detected and two of them were distinguished by the retention time of standard samples. Thirteen chemical markers were screened out through PCA and orthogonal partial least square discriminant analysis. Through detecting Napelline or isomer of Napelline (m/z 360.2530) and Aconifine (m/z 662.3170), AKRs from inner Mongolia autonomous could be screened. According to the existence of benzoylaconine (m/z 604.3108) and Indaconitine (m/z 630.3159), it could be confirmed that the AKRs are from Xinjiang Uygur autonomous. AKRs that cannot detect compounds above-mentioned could be from Liaoning or Shanxi Province. Conclusions: The chemical profile could be used not only to distinguish the AKRs from different geographic origins but also to identify the true and false of AKRs. This study lays a foundation for the study of efficacy and toxic of AKRs.

Keywords: Aconiti Kusnezoffii Radix, chemical markers, rapid differentiation, ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry

INTRODUCTION

Aconiti Kusnezoffii Radix (AKR) is the dry root of Aconitum Kusnezoffii Reichb., which has been used as traditional Chinese medicine (TCM) for >2000 years. AKRs are mainly produced in north and northeast China. AKRs were first recorded in ShenNong’s herbal classic, which has analgesic and anti-inflammatory activity and are widely used in rheumatoid arthritis and arthralgia. [1,2] Although AKRs have good clinical efficacy, it is well known for its strong toxicity. [3-5] In China, there are many geographic origins and varieties of aconitum plants. Because of AKRs wide application, some other aconitum plants are confused with AKRs and used in disease treatment. It is difficult to distinguish the decoction pieces.
of different geographic origins according to the appearance, smell, and other external characteristics. However, misuse of these drugs may lead to a decrease in efficacy and unexpected toxicity. Therefore, it is very important to establish a rapid and effective method to identify the AKRs from different areas.

In recent years, the identification of AKRs has developed rapidly, but there are still some defects. The traditional identification of crude drug properties and microscopic identification of medicinal materials were used to identify the medicinal plant. This kind of method always needs professional botany knowledge and has a certain degree of subjectivity, which is not suitable for establishing identification standards. Moreover, infrared spectrophotometry (IR) and X-ray diffraction are not suitable for the identification of TCM because of their high requirements for the purity of samples. The impurities in TCM are easy to affect the experimental results. Nowadays, the identification methods of AKRs mainly focus on its symbolic substances, such as diester-diterpenoid alkaloids (DDAs), monoester-diterpenoid alkaloids (MDAs), and amine-diterpenoid alkaloids (ADAs), which are the main components with both efficacy and toxicity. High-performance liquid chromatography (HPLC) is used to separate and identify chemical components of TCM. It is a common way to establish the HPLC fingerprint of AKRs and quantify the two kinds of alkaloids. HPLC-MS has been widely used in the quality control (QC) of drugs and food safety, especially in the research of TCM in recent years. HPLC-MS can rapidly and simultaneously analyze the complex system of TCM, and identify the chemical components of TCM quickly and accurately by comparing the information of fragment ions scanned with the TCM reference materials or relative literature. As an unsupervised statistical method, principal component analysis (PCA) can cluster unknown samples systematically, while orthogonal partial least square discriminant analysis (OPLS-DA) can quickly screen out distinguishable markers. Therefore, HPLC-MS coupled with PCA, becomes an effective method to analyze and distinguish the components of TCM. For instance, Liu et al. used this method to quickly identify natural Calculus bovis, cultured C. bovis and artificial C. bovis, and screen the different substances that can be used as differentiation. Miao et al. analyzed Fuhi (an Aconitum plant) from a different place of origins and established a fast and effective method of differentiation.

In this study, ultra-performance liquid chromatography mass spectrometry (UPLC-MS) combined with PCA is used to identify AKRs from four different geographic origins, using OPLS-DA to screen chemical components in AKRs. Finally, we set up a rapid and effective method to distinguish AKRs.

**Methods**

**Materials**

Raw AKRs were purchased from Zhenyu Chinese Medicine Company and the AKRs were collected from three different Provinces, Liaoning (L), Shanxi (S), and Xinjiang Uygur autonomous (X). AKRs from Inner Mongolia autonomous (N) were collected by Professor Guihua Bao of Inner Mongolia University for Nationalities. All AKRs were identified by Professor Guihua Bao. The batch numbers of these AKRs are listed in Table 1. Analytical grade diethyl ether was purchased from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). Ammonia and ammonium bicarbonate was purchased from Beijing Shiji Company (Beijing, China), which is also an analytical grade. HPLC grade methanol was purchased from Fisher Scientific (Loughborough, UK). The MilliQ plus (Milford, MA, USA) water purification system was used to obtain deionized water. Neoline was obtained from Chengdu Reference Biotechnology Co., Ltd., (Chengdu, China), and 3-deoxyaconitine was purchased from Shanghai Ronghe Pharmaceutical Technology Co., Ltd., (Shanghai, China).

**Sample preparation**

AKRs from different geographic origins were pulverized into powder and passed through a 0.45 nm mesh sieve. 1 g AKR powder was accurately weighed into 50 mL tube and moistened with 0.5 mL of 40% ammonia aqueous solution and then

| Origin of AKRs          | Batch number |
|-------------------------|--------------|
| Liaoning Province       | 181012       |
|                         | 190120       |
|                         | 190712       |
|                         | 190713       |
|                         | 190714       |
| Xinjiang Uygur autonomous| 181012       |
|                         | 181205       |
|                         | 181016       |
|                         | 190305       |
|                         | 190506       |
|                         | 190515       |
|                         | 190610       |
|                         | 190618       |
|                         | 190405       |
|                         | 190406       |
|                         | 190407       |
|                         | 190408       |
|                         | 190409       |
| Shanxi Province         | 190110       |
|                         | 190112       |
|                         | 190120       |
|                         | 190210       |
|                         | 190220       |
|                         | 190610       |
|                         | 190611       |
|                         | 190612       |
|                         | 190613       |
|                         | 190614       |
| Inner Mongolia autonomous| 191011       |
|                         | 191012       |
|                         | 191013       |

**AKRs: Aconiti Kusnezoffii Radixs**
extracted with 50 mL of diethyl ether by ultrasonic extraction for 30 min at 25°C for three times. All extraction solution was evaporated together at 50°C and the residue was dissolved by 5 mL of methanol-water (1:1, v/v). The final solution was filtered with a 0.22 μm filter membrane before UPLC, coupled with time-of-flight MS (UPLC-Q-TOF MS) analysis.

Ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry analysis

Waters acquity UPLC system coupled with a Q-TOF SYNAPT G2 high-definition mass spectrometer (Waters, USA) was used to analyze the samples. Chromatographic separation was archived on an acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 50 mm; Waters) with a column temperature maintained at 35°C and an injection volume of 5 μL. Mobile phases A and B were methanol and deionized water (containing 5 mmol ammonium bicarbonate and adjusted to pH 10.5 with ammonia), respectively. The flow rate was 0.35 mL/min. Gradient elution profiles were 0–5 min from 30%A (70% B) to 35% A (65% B), 5–15 min from 35% A (65% B) to 37% A (63% B), 15–20 min from 37% A (63% B) to 45% A (55% B), 20–32 min from 45% A (55% B) to 53% A (47% B), 32–40 min from 53% A (47% B) to 60% A (40% B), 40–45 min from 60% A (40% B) to 65% A (35% B), 45–50 min from 65% A (35% B) to 79% A (21% B), 50–57 min from 79% A (21% B) to 100% A (0% B), when the system remained 100% A for 5 min, the mobile phase will return to its initial condition for continuous analysis. During the period of analysis, all samples were maintained at 4°C. To avoid the risk of blocking the pipeline caused by ammonium bicarbonate precipitating, deionized water was used to clean the pipeline for 10 min after every five samples. A Q-TOF MS with an ESI source was operated in positive ion mode. MS² data were collected in the full scan mode with a mass range of 200–1000 Da with a scan time of 0.5s. Sodium formate solution was used to set up the mass spectrometer calibration. A lock mass of leucine enkephalin ([M + H]⁺ = 556.2771 in positive ion mode) at a concentration of 2 ng/μL was used with an infusion flow of 5 μL/min through a lock spray interface to ensure accuracy during the MS analysis. Nitrogen was used as a cone, and desolvation gas and the flow rate were set at 50 and 600 L/h, respectively. Argon was used as the collision gas in this study.

Statistical analysis

Progenesis QI software is an effective tool for statistical analysis, and it is widely used in the most study of metabolomics in recent years.²¹,²² Moreover, Progenesis QI can not only screen potential biomarkers in biology study but also play an important role in TCM QC. In this study, it was used to screen the differential compounds of AKRs from different geographic origins. UPLC-Q-TOF MS was used to require MS² data in positive mode, and then recorded high-accuracy MS/MS data of precursor ions. The raw data obtained from the system were imported to Progenesis QI software for automatic data processing. Data processing of Progenesis QI has a series of steps: creation of new experiment, importation of data, alignment peak picking, deconvolution, statistical analysis, and identification.²³ After alignment peak picking, Umetrics EZInfo 2.0 (Waters Corporation, Milford, USA) was used to set up PCA and OPLS-DA model, to identify differences among groups and screen potential chemical markers. In addition, R²Y and Q², these two parameters, can evaluate the quality of the model. VIP values (VIP > 1) and t-test (P < 0.05) is used to screen markers and create tags in Progenesis QI.

Results

Chemical profile analysis of Aconiti Kusnezoffii Radixs from different geographic origins

In this study, the creative mobile phase was used in UPLC-MS analysis. Comparing with common mobile phase, ammonium bicarbonate ammonia buffer solution is more suitable for alkaloids separation, and it has the ability to stabilize the PH value of the mobile phase. In addition, the buffer solution could make chromatogram get better separation, and the shape of peaks could also be better.⁷ The total ion current (TIC) chromatogram of AKRs from four geographic origins is shown in Figure 1. According to retention time (RT) and mass-to-charge ratio (m/z) shown in each peak, we could get the chemical profile of AKRs from different geographic origins. After identification using the reference ofaconitum diterpenoid alkaloids²⁶–²⁸ and an online chemical database (www.chemspider.com), 16 components were identified from 23 detected compounds. All compounds were listed in Table 2. Common Aconitum diterpenoid alkaloids such as MDAs (Compound 5, 6), DDAs (Compound 13, 15, 18, 19, and 22) and ADAs (compound 4, 8, 10, and 12) could be detected in most AKRs, in spite of their abundance has the considerable difference.

Identification of isomers detected in Aconiti Kusnezoffii Radixs

During the data processing and compounds identification, compound 8 and 10, compound 11 and 14, compound 16 and 22, have different RT but have the same mass-to-charge ratio (m/z), whose precursor ions is [M + H]⁺ ion at m/z 438.2846, 360.2530, and 630.3159, respectively. These results inferred that these three pairs of compounds might be isomers. However, isomers cannot be identified only by accurate molecular weight information. To estimate RT of two components, the standard solution containing neoline and 3-deoxyaconitine was prepared at a concentration of 0.02 mg/mL. 5 μL standard solution was injected in UPLC-Q-TOF MS system under the same chromatography condition as AKRs detection. TIC chromatogram containing two standards are shown in Figure 2. According to the above results, Neoline (RT = 14.50 min) was just matched with compound 8, while 3-deoxyaconitine (RT = 45.31 min) was matched with compound 22. According to the accurate molecular weight and peak sequence, compound 10 and 16 were inferred to be Bullatine B and Indaconitine, respectively.
MS/MS data could also confirm the identification, fragment ions of them were consistent with Chemsipder online database and previous reports.\(^\text{[24,25]}\) Compound 11 and 14, whose precursor ions are \([\text{M} + \text{H}]^+\) ion at \(m/z\) 360.2530, could be inferred as Napelline or its isomer according to the reference, a kind of ADAs.\(^\text{[26]}\) However, because of the lack of standard compounds, their structure needs to be further determined.

**Principal component analysis and orthogonal partial least square discriminant analysis about Aconiti Kusnezoffii Radixs from different geographic origins**

PCA score is used to evaluate the degree of difference among these four groups of AKRs. PCA score is shown in Figure 3. QC samples were mixture of all AKRs; each sample was taken 20 \(\mu\)L and mixed together by vortex mixer. QC samples were located in the center of the PCA score shows that the analytical system is reliable. Samples that belong to the same group were gathered together. AKRs from Shanxi and Liaoning can be obviously distinguished from Inner Mongolia autonomous and Xinjiang Uygur autonomous. However, there is no significant difference between Shanxi and Liaoning, which makes their position in the PCA score very close. These conclusions are consistent with the information obtained from TIC chromatograms.

OPLS-DA model was established to find out the representative differential compounds among different groups, which was processed automatically by Progenesis QI. Due to the similarity chemical profiles between AKRs from Liaoning and Shanxi, these two kinds of AKRs were gathered

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**Figure 1:** TIC chromatogram in positive ion mode from four origins. (a) Liaoning, (b) Shanxi, (c) Inner Mongolia autonomous, (d) Xinjiang Uygur autonomous

**Figure 2:** TIC chromatogram of Neoline (\(m/z\) 438.2846) and 3-Deoxyaconitine (\(m/z\) 630.3159)
Table 2: Components that detected in Aconiti Kusnezoffii Radixs from different geographic origins

| NO | Source          | Identification                                  | Formula     | RT/min | [M+H]+ | Error (ppm) | MS/MS (m/z)        |
|----|-----------------|------------------------------------------------|-------------|--------|---------|-------------|-------------------|
| 1  | L and S and N   | Unknown                                        | -           | 0.56   | 342.1700 | -           | 330.7575,314.7858 |
| 2  | L and S and N   | Mesaconine                                     | C$_7$H$_{14}$NO$_6$ | 1.16   | 486.2706 | -           | 468.2226,454.2051,436.2024 |
| 3  | L and S and N   | Aconine                                        | C$_7$H$_{14}$NO$_6$ | 1.67   | 500.2855 | 0.18        | 468.7048,450.2101 |
| 4  | L and S and N   | Fuziline                                       | C$_7$H$_{14}$NO$_6$ | 9.25   | 454.2798 | -0.31       | 436.2389,404.2174 |
| 5  | L and S and N   | Benzoylmesaconine                              | C$_7$H$_{14}$NO$_6$ | 10.07  | 590.2960 | 0.01        | 572.2049,558.1934,540.1843 |
| 6  | L and S and N   | Benzoyleaconine                                | C$_7$H$_{14}$NO$_6$ | 13.67  | 604.3108 | -1.26       | 586.2208,574.2049,554.2059 |
| 7  | L and S         | Unknown                                        | -           | 13.81  | 394.2591 | -           | 376.2162       |
| 8  | L and S and N   | Neoline                                        | C$_7$H$_{14}$NO$_6$ | 14.49  | 438.2846 | 47.39       | 420.2719,388.2147 |
| 9  | X               | Unknown                                        | -           | 16.96  | 586.5326 | -           | 480.2388       |
| 10 | L and S and N   | 10-OH Talatizamine                             | C$_7$H$_{14}$NO$_6$ | 17.86  | 438.2881 | -           | 420.2288,388.2092 |
| 11 | N               | Napelline or Isomer of Napelline               | C$_7$H$_{14}$NO$_6$ | 20.24  | 360.2530 | -0.76       | 342.2095       |
| 12 | L and S and X   | Songorine                                      | C$_7$H$_{14}$NO$_6$ | 23.58  | 358.2381 | 1.26        | 340.2000       |
| 13 | L and S and N   | Beiwutine                                      | C$_7$H$_{14}$NO$_6$ | 27.32  | 648.3019 | 0.72        | 598.1923,588.2026 |
| 14 | L and S and N   | Napelline or Isomer of Napelline               | C$_7$H$_{14}$NO$_6$ | 27.87  | 360.2530 | -0.76       | 342.2095       |
| 15 | L and S and N   | Mesaconitine                                   | C$_7$H$_{14}$NO$_6$ | 30.29  | 632.3066 | 0.08        | 572.2153,540.2945 |
| 16 | X               | Indaconitine                                   | C$_7$H$_{14}$NO$_6$ | 34.01  | 630.3159 | -1.19       | 570.2314,536.2162 |
| 17 | L and S and N   | Aconifine                                      | C$_7$H$_{14}$NO$_6$ | 35.38  | 662.3170 | -0.16       | 602.2062,342.1968 |
| 18 | L and S and N   | Aconifine                                      | C$_7$H$_{14}$NO$_6$ | 36.63  | 646.3217 | -0.88       | 586.2208,554.2059 |
| 19 | L and S and N   | Hypaconitine                                   | C$_7$H$_{14}$NO$_6$ | 39.12  | 616.3118 | 0.37        | 556.2236,584.2127 |
| 20 | L and S         | Unknown                                        | -           | 41.19  | 540.2956 | -           | 522.2209       |
| 21 | L and S         | Unknown                                        | -           | 43.88  | 770.3182 | -           | 648.1894       |
| 22 | L and S and N   | 3-Deoxyaconitine                               | C$_7$H$_{14}$NO$_6$ | 45.31  | 630.3159 | -1.19       | 570.2927,538.2653 |
| 23 | L and S         | Unknown                                        | -           | 48.26  | 300.2320 | -           | -              |

RT: Retention time

Figure 3: PCA score of AKRs from different origins. (●) QC samples, (●) L (Liaoning), (♦) N (Inner Mongolia autonomous), (▲) S(Shanxi), (▼) X (Xinjiang Uygur autonomous).

Together to be a new group and were selected as the standard comparison object. OPLS-DA was performed with Inner Mongolia autonomous (N vs. S and L) and Xinjiang Uygur autonomous (X vs. S and L), respectively. The OPLS-DA score is shown in Figure 4a and b.

VIP > 1.0 and the significance level $P < 0.05$ were identified as the potential chemical markers. Finally, 13 compounds were identified through Chemspider online database and references, which are listed in Table 3. Through detecting Napelline or isomer of Napelline (m/z 360.2530) and Aconifine (m/z 662.3170), AKRs from Inner Mongolia autonomous could be screened. According to the existence of Benzoyleaconine (m/z 604.3108) and Indaconitine (m/z 630.3159), it could be confirmed that the AKRs are from Xinjiang Uygur autonomous. Other compounds could be detected in all AKRs, while the contents have a significant difference. Therefore, if four compounds that mentioned above cannot be detected in AKRs, it could be Liaoning or Shanxi Province. These components can be used as representative chemical markers to distinguish the geographic origin of AKRs.

**Discussion**

UPLC-Q-TOF MS were successfully used to distinguish different AKRs samples rapidly, which can bring in better separation and shorter detection time. Three pairs of isomers were identified through fragmentation of characteristic ions and the difference of RT. PCA can completely classify different geographic origins of AKRs, while OPLS-DA can be used to screen the chemical markers with the character of VIP > 1 and $P < 0.05$ and the geographic origin of AKRs can be quickly identified by these chemical markers. The establishment

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The chemical profile of AKRs lay a foundation for the study of its effective and toxic substances. In addition, this rapid distinguishing method can be used to prevent shoddy AKRs misuse or other toxic medicinal plants to fake AKRs, so as to prevent the misuse of poisonous medicinal plants and provide safeguard for the drug safety of AKRs.

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Conflicts of interest

There are no conflicts of interest.

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