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Fingerprint analysis of *Radix Aconiti* using ultra-performance liquid chromatography–electrospray ionization/ tandem mass spectrometry (UPLC–ESI/MS^n) combined with stoichiometry

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ABSTRACT

A fingerprinting approach was developed by means of UPLC–ESI/MS^n (ultra-performance liquid chromatography–electrospray ionization/mass spectrometry) for the quality control of processed *Radix Aconiti*, a widely used toxic traditional herbal medicine. The present fingerprinting approach was based on the two processing methods recorded in Chinese Pharmacopoeia for the purpose of reducing the toxicity and ensuring the clinical therapeutic efficacy. Similarity evaluation, hierarchical cluster analysis and principal component analysis were performed to evaluate the similarity and variation of the samples. The results showed that the well processed, unqualified processed and the raw *Radix Aconiti* could be clustered reasonably corresponding to the contents of their constituents. The loading plot shows that the main chemical markers having the most influence on the discrimination amongst the qualified and unqualified samples were mainly some monoester diterpenoid aconitines and diester diterpenoid aconitines. Finally, the UPLC–UV and UPLC–ESI/MS^n characteristic fingerprints were established according to the well processed and purchased qualified samples. At the same time, a complementary quantification method of six Aconitine-type alkaloids was developed using UPLC–UV and UPLC–ESI/MS. The average recovery of the monoester diterpenoid aconitines was 95.4–99.1% and the average recovery of the diester diterpenoid aconitines was 103–112%. The proposed combined quantification method by UPLC–UV and UPLC–ESI/MS allows the samples analyzed in a wide concentration range. Therefore, the established fingerprinting approach in combination with chemometric analysis provides a flexible and reliable method for quality assessment of toxic herbal medicine.

1. Introduction

Quality assurance practice is now regarded as a core requirement for international trade, especially for food and pharmaceuticals. Generally, quality assurance practice is applied to processing and products, which are well documented and characterized [1–3]. Important illustrations of such products are the many herbal medicines, which are in common worldwide use [4,5]. A single herb may contain hundreds of components spanning a concentration range of several orders of magnitude. Due to the complexity of herbal medicines, the knowledge about composition of most herbal medicines is still incomplete. This has been reflected in methods for authentication and quality control of herbal medicine [6]. *Radix Aconiti* (monkshood root, *Aconite* Root) is the main roots of *Aconitum carmichaeli Debx.*, which is a genus of flowering plants belonging to the buttercup family and distributed extensively worldwide chief natives of the mountainous parts of the northern hemisphere. In China, the area of producing *Radix Aconiti* is relatively localized, mainly in Sichuan Province. The *Radix Aconite* has long been used as traditional herbal medicine in Asian countries and some Western countries. It is used as an important medicine to treat rheumatic pain, paralysis and skin abscess caused by Staphylococcus aureus bacterial infection in China [7,8].

Aconitine-type alkaloids are the most abundant effective components in *Radix Aconiti* and these alkaloids have anti-inflammatory, diuretics, analgetic and cardiotonic actions. The alkaloids in the *Radix Aconiti* are composed of monoester-diterpenoid aconitines (MDAs), diesterditerpenoid aconitines (DDAs) and Aconitum lipoalkaloids (LDAs) [9]. As a toxic herb, the toxicity of *Radix Aconiti* mainly derives from DDAs that mainly including aconitine (AC), mesaconitine (MA) and hypaconitine (HA). They can be changed into less-toxic MDAs or LDAs through Chinese traditional processing methods, which play an essential role in the reducing the toxicity of *Radix Aconiti* [10]. The improper usage of *Radix Aconiti*

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would present highly toxicological risk and could cause fatal cardiac poisoning. Even now the acute poisoning accidents caused by *Radix Aconiti* in some countries often occurred, especially in some Asian countries. These accidents could be avoided by effective processing procedures of *Radix Aconiti* for ensuring its safe use. But several publications point out that a certain amount of DDAs contributes to the desired clinical effects and that they should not be entirely diminished from the drug [11,12]. Like all kinds of medicines, herbal drugs and their products made thereof have to prove their quality, efficacy and safety prior to gaining access to the market [13]. Therefore, the quality control of *Radix Aconiti* with toxicity is very important. Both reducing the toxicity and ensuring the clinical therapeutic efficacy should be assured through the processing of *Radix Aconiti*. This means that the processing degree of *Radix Aconiti* should be well controlled.

LC–MS is a useful method in the identification of herbal samples, which is widely accepted and is attracting increasing attention [14]. The UPLC (ultra-performance liquid chromatography) technique combined with DAD (diode array detector) and MS takes advantages of chromatography as a separation method and both DAD and MS as an identification method. DAD and MS can provide on-line UV and MS information for each individual peak in a chromatogram. With the help of UV and MS^n information for each individual peak in a chromatogram, in most cases, it could identify the chromatographic peaks by comparison with literature data or with standard compounds, which made the UPLC–DAD and UPLC–ESI/MS^n becomes a powerful approach for the rapid identification of phytochemical constituents in herbal extracts [15–19]. Methods developed for the analysis of Aconitum alkaloids include HPLC, HPLC–ESI/MS^n [9], UPLC–ESI/MS [20], UPLC–Q–TOF–MS [21,22], ESI [23] and Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI MS) [24]. Cui et al. [20] reported a successful UPLC–MS method for the quality control of the *Xiaohuoluo pill* by quantitative and qualitative analysis of Aconitine-type alkaloids. Zhou et al. [21] and Wang et al. [22] reported the profiling approaches that were successfully applied to evaluate chemical constitution between co-decoction and mixed decoction of *Radix aconiti* and *Pinellia praeparata* as well as *Radix aconiti* and *Bulbus fritillariae cirrhosae* using ultra performance liquid chromatography coupled with time-of-fight mass spectrometry (UPLC/Q-TOFMS).

To our knowledge, there are more than 70 techniques for the processing of Aconiti Radix [19]. So many processing methods make the quality control of *Aconiti Radix* more challenging. However, only two processing techniques were recorded in the Chinese Pharmacopoeia 2010 (ChP 2010). A criterion of processing degree was proposed in ChP. Therefore, in this study, in view of the existing problems of the quality control of *Aconiti Radix* a novel fingerprint method was proposed for the quality control of *Aconiti Radix* from its famous-region. The two processing methods of *Aconiti Radix* in ChP were investigated and the UPLC–UV and UPLC–ESI/MS^n fingerprint of *Aconiti Radix* was established through the chemical and chemometric analysis of *Aconiti Radix* in different processing degrees. At the same time, a complementary quantification method of six Aconitine-type alkaloids using UPLC–UV and UPLC–ESI/MS was developed and the combined quantification method by UPLC–UV and UPLC–ESI/MS allows samples in a wide concentration range.

2. Materials and methods

2.1. Materials

Raw *Radix Aconiti* was purchased from Jiangyou County, Sichuan Province, which is the main productive area of *Radix Aconiti* in China. The raw and the purchased processed *Radix Aconiti* (P1) were purchased from Sichuan Jiangyou Zhongba Monkshood Technology co., LTD. The purchased processed *Radix Aconiti* (P2) was purchased from Jilin Large Pharmacy and P3 was purchased from Changchun TongRenTang Chinese Medicine Store. All these herbal samples were authenticated by Professor Shumin Wang (Changchun University of Traditional Chinese Medicine, China). Aconitine (AC), hypaconitine (HA) and mesaconitine (MA) were purchased from the Chinese Authenticating Institute of Material and Biological Products (Beijing, China). Benzoylmesaconine, benzoylhypacoitine and benzoylaconine were purchased from LanYuan Biological technology Co., LTD (Shanghai, China). Reserpine as the internal standard was purchased from Sigma (USA). Ammonium bicarbonate was purchased from Sigma (USA). Methanol, acetonitrile and acetic acid of HPLC grade were obtained from Fisher Scientific (Loughborough, UK). Diethyl ether was of analytical grade from Beijing Shiji (Beijing, China). Deionized water was prepared using the MilliQ plus (Milford, MA, USA) water purification system.

2.2. Sample preparation

2.2.1. Processing procedures

Radix Aconiti was processed according to ChP 2010: At first, let *Radix Aconiti* be bathed in water until its inside was wet thoroughly. Secondly, the water was exchanged with fresh water and then the *Radix Aconiti* was boiled for one to six hours or steamed for one to eight hours. The samples boiled for four, five and six hours and those steamed for six, seven and eight hours were the qualified *Radix Aconiti*. The other *Radix Aconiti* samples were unqualified ones. Thirdly, the *Radix Aconiti* after boiled or steamed was sliced and dried in 50 °C. At last, the processed *Radix Aconiti* were crushed into powder by a plant pulverizer and screened by a 0.45 mm sieve.

2.2.2. Extraction procedures

The powder of the sample (1.0 g) was accurately weighed and placed in a sealed vessel by adding 0.5 mL of 10% ammonia aqueous solution, then 50 mL of diethyl ether was added and the sealed vessel was extracted by ultrasonic wave for half an hour at room temperature (25 °C). The diethyl ether phase was transferred to a new tube and combined from 3 consecutive extractions. All the diethyl ether parts were evaporated together at 50 °C. All the extracts were stored at −80 °C before use.

The extracts of samples were dissolved in 5 mL methanol–water (1:1, v/v) solvent before analysis, and 0.02 mg/mL reserpine as the internal standard. All final solutions were filtered through a 0.22 μm filter membrane. Finally, 5 μL sample was injected into the UPLC–DAD and UPLC–ESI/MS^n system.

2.3. UPLC–DAD and UPLC–ESI/MS^n analysis

UPLC–UV and UPLC–ESI/MS^n analysis was performed on an Accela UPLC system with an Accela 1250 Pump, an Accela Autosampler, an Accela Diode Array Detector and a LTQ ion trap mass spectrometer (Finnigan, USA) equipped with an electrospray source. A Waters ACQUITY UPLC BEH C18 Column (1.7 μm, 2.1 × 50 mm) was used. The column temperature was maintained at 30 °C. Elution was performed at a flow rate of 0.3 mL min^{-1} by using the mobile phases of water (containing 5 mM ammonium bicarbonate and adjusted to pH 10.5 with ammonia) (A) and methanol (B). Gradient elution profiles were 0–5 min from 65% A (35% B) to 57% A (43% B), 5–10 min fom 57% A (43% B) to 55% A (45% B), 10–30 min from 55% A (45% B) to 40% A (60% B), 30–55 min from 40% A (60% B) to 10% A (90% B), and 55–75 min

from 10% A (90% B) to 5% A (95% B). The DAD detection was performed in the range of 200–600 nm at 1 nm/step. Based on the maximum absorption of the aconitine-type alkaloids in the UV spectra of the three-dimensional chromatograms obtained by DAD detection, the UV detection wavelength was set at 235 nm, where all the aconitine-type alkaloids could be detected and had adequate absorption. The ESI source of the mass spectrometer was connected to the UPLC system via a capillary to the UV cell outlet. Spray voltage was at 4.5 kV in the positive ion mode. Sheath gas flow rate was at 40.5 L h^{-1}, aux gas flow rate was at 90 L h^{-1}, and the capillary temperature was maintained at 250 °C. The scan range was m/z 300–1000 Da. Collision energies for the MS^2 analyses ranged from 25 eV to 40 eV, depending on the mass of the precursor ion. The collision gas used for the MS^n was He.

2.4. Preparation of standard solutions

Stock solutions of Aconitine-type alkaloids, including 3 MDAs (Benzoylmesaconine, benzoylhypacoitine and benzoylaconine) and 3 DDAs (Aconitine, hypaconitine and mesaconitine) were prepared in dichloromethane and were kept in −80 °C. All the solvents used for the dissolution and dilution of the sample and standards in this study were the internal standard solution (0.02 mg mL^{-1} reserpine in 1:1 methanol–water). 500 μl of each stock solution for three standards were mixed and evaporated by N_2 and the mixtures were dissolved in 1 mL of the internal standard solution, a series of solutions with different concentrations (0.001–500 μg mL^{-1}) were prepared by diluting this mixed standard solution with the internal standard solution as the solvent. 5 μL of each sample solution was injected into the UPLC–DAD–MS^n system. The UPLC–UV (235 nm) calibration curve and UPLC–ESI/MS^n calibration curve were constructed using the concentration of standard solution as abscissa and the specific value *As/Ais* (area of sample/ area of internal standard) as *y*-coordinate.

2.5. Chemometrics analysis

2.5.1. Similarity evaluation

The most commonly used standard for evaluation of similarity of the fingerprint, the correlation coefficient, r_{cor}, and the congruence coefficient, r_{con}, were adopted to assess the consistency, and they are formulated respectively as follows [25,26]:

$$r_{cor} = \frac{\sum_{i=1}^{num}(x_i-\overline{x})(y_i-\overline{y})}{\sqrt{\left(\sum_{i=1}^{num}(x_i-\overline{x})^2\right)\left(\sum_{i=1}^{num}(y_i-\overline{y})^2\right)}}$$

$$r_{con} = \frac{\sum_{i=1}^{num}x_i y_i}{\sqrt{\left(\sum_{i=1}^{num}(x_i)^2\right)\left(\sum_{i=1}^{num}(y_i)^2\right)}}$$

$$\overline{x} = \left(\sum_{i=1}^{num}x_i/n\right) \quad ,\overline{y} = \left(\sum_{i=1}^{num}y_i/n\right)$$

Where x_i, y_i were the *i*th element in two different fingerprints, say *x* and *y*, and *num* was the number of the elements in the fingerprints. $\overline{x}$ and $\overline{y}$were the mean values of the *n* elements in fingerprints *x* and *y*, respectively.

2.5.2. Hierarchical clustering analysis (HCA)

Hierarchical cluster analysis is performed to classify samples based on the similarities of their chemical properties [27]. The results of HCA often give in the way of a tree diagram which is called a dendrogram. All observations constitute the root of the tree and individual observations represent the leaves. Any valid metric may be used as a measure of similarity between pairs of observations. A function of the pairwise distances between observations, which is the linkage criteria, determines the choice of which clusters to merge or split [28,29]. In this part, hierarchical clustering analysis (HCA) of 19 *Radix Aconiti* samples was performed using SPSS statistics software (SPSS for Windows 18.0, SPSS Inc., USA) [30–31]. Hierarchical cluster analysis was based on peak areas of the TIC fingerprints profiles of 19 samples, because the TIC fingerprints can provide more information than the UV chromatogram fingerprints. The complete linkage and squared euclidean distance as pattern similarity measure were selected as measurement for hierarchical cluster analysis. The areas of 48 peaks in the fingerprint data (TIC) of all the 19 *Radix Aconiti* samples consist of a total of 19 × 48 data matrix. Each row (variable) represented a R*adix Aconiti* sample and each column contained the values of 48 characteristic peak areas.

2.5.3. Principal component analysis (PCA)

As an unsupervised method, principal components analysis (PCA) is a method for feature extraction and dimensionality reduction. PCA is the most employed to reduce data dimensionality, and to provide an overview of class separation, clustering and outliers. There is no training set (input data together with the answers) and input data is classified in an "unsupervised" manner [32]. In this part, PCA analysis of the fingerprints data from *Radix Aconiti* samples was performed using SIMCA-P 11.0 software (Umetrics AB, Umea, Sweden). For quality evaluation of *Radix Aconiti*, the areas of 48 peaks in the fingerprint data (TIC) of all the *Radix Aconiti* samples were analyzed by PCA. The PCA analysis was implemented by performing singular value decomposition on the data array of the fingerprints, which consisted of a total of $n \times 48$ data matrix, n was the number of the samples. Each row represented a *Radix Aconiti* sample and each column contained the values of 48 characteristic peak areas. The compounds could be found as the main chemical markers having the most influence on the discrimination amongst different herbal samples on the PCA loading plots.

3. Results and Discussion

3.1. UPLC–ESI/MS^n method for the identification of the alkaloids in the extracts of processed and raw Radix Aconiti

Alkaloids in the extracts of processed and raw *Radix Aconiti* were analyzed by UPLC–ESI/MS^n respectively and the MS^n data was obtained simultaneously. The total ion chromatograms (TIC) of raw *Radix Aconiti* and the processed *Radix Aconiti* were shown in Fig. 1. We found that the number of peaks (compositions) was almost the same between the raw and the processed *Radix Aconiti*. However, the contents of these compositions were changed obviously. 48 characteristic peaks were found in these TICs (shown in Fig. 1) and most of these (35 alkaloids) compounds were identified based on their rule of fragmentation pathway of Aconitum alkaloids according to their MS^2 data and references [9,33–36] (shown in Table 1). As shown from Fig. 1 and Table 1, it is obviously that the chromatogram of *Radix Aconiti* varies a lot after being processed, especially in the region of DDAs (17–44 min).

3.2. Quantitative analysis in raw and processed Radix Aconiti

Because DDAs produced the hydrolyzation, pyrogenation or other chemical changes in the processing of *Radix Aconiti*, the contents of DDAs are usually very low and the contents of MDAs are very high especially the benzoylmesaconine (BMA) in processed *Radix Aconiti*. The quantitative analysis of the three major toxic DDAs by UPLC–UV requires a highly concentrated sample since the limit of quantitation of UPLC–UV is poor for DDAs (> 0.2 μg mL^{-1}). However, a highly concentrated herbal sample extract may cause the chromatographic column and the mass spectrometer overload

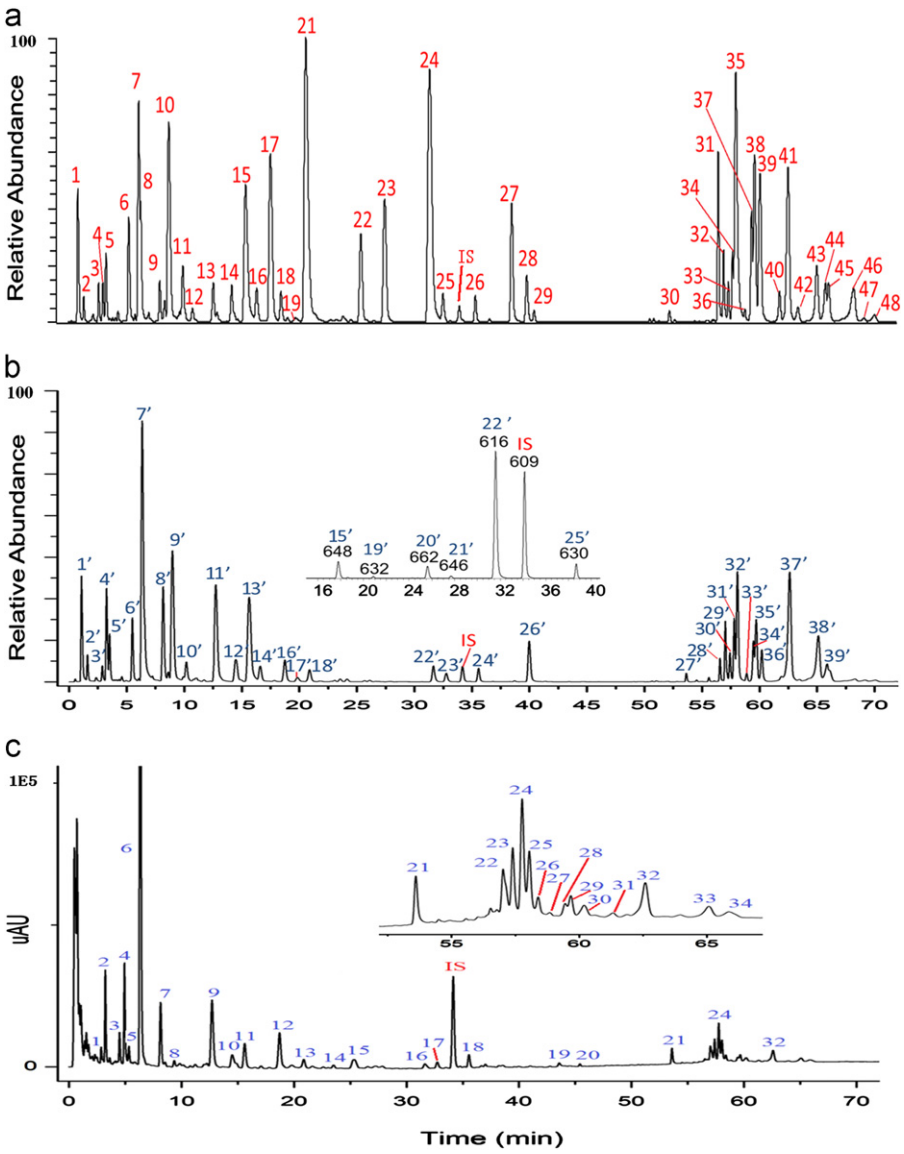


Fig. 1. UPLC–ESI/MS^n (TIC) profiles of *Radix Aconiti*: (a) Raw *Radix Aconiti*. The characteristic fingerprint of the processed *Radix Aconiti*: (b) The UPLC–ESI/MS fingerprint, (c) The UPLC–UV fingerprint.

and polluted. Therefore, in order to qualify the MDAs and DDAs simultaneously in a suitable sample concentration, a quantitative analysis method was established by UPLC–UV and UPLC–ESI/MS. The UPLC–UV data could be used mainly for MDAs, the UPLC–ESI/MS data could be used mainly for the DDAs in processed samples. The UPLC–UV and UPLC–ESI/MS chromatograms of the six standards are shown in Fig. 2 and the linear equations were shown in Table 2. The limit of detection (LOD) and the limit of quantification (LOQ) were determined at *S*/*N* ratios of about 3 and 10, respectively. The limit of quantitation (LOQ) was determined to be 0.6–1.0 μg mL^{-1} for DDAs by UV detector. Most of the DDAs cannot be determined accurately by UV detector, because the contents of AC and MA in processed *Radix Aconiti* is below 0.1 and 0.5 μg mL^{-1} (as shown in Table 3). Moreover, in this paper, the UPLC–ESI/MS fingerprints were also used for quantitative analysis based on extracted ion chromatogram (EIC) mode, the linear range of three DDAs in this method was 0.01–22.3, 0.02–22.7 and 0.02–13.2 μg mL^{-1} and it was suitable for the quantitative analysis of the DDAs in processed samples. The linear range of MDAs was 0.1–500 and 0.05–500 μg mL^{-1} by UV detector and it was suitable for the quantitative analysis of the MDAs. In conclusion, the combined quantification method by UPLC–UV and UPLC–ESI/MS allows analytes in a wide concentration range. Moreover, the proposed quantitative analysis method by UPLC–UV and UPLC–ESI/MS was suitable for the simultaneous quantitative analysis of the six Aconitine-type alkaloids in the processed and raw *Radix Aconiti* in a wide concentration range.

Apparatus precision was evaluated by the analysis of a solution of the sample (raw *Radix Aconiti*) for ten consecutive times in three days. The RSDs of overall intra and inter-day variations were less than 3.3% for six analytes, which showed that the precision of the apparatus was satisfactory. Recovery test was used to evaluate the accuracy of this method. The recovery test was done by the standard addition method. Six standards (BMA 790.0 μg g^{-1}, BAC 119.0 μg g^{-1}, BHA 508.0 μg g^{-1}, MA 0.7 μg g^{-1}, AC 0.3 μg g^{-1}, HA 4.0 μg g^{-1}) were added to the known sample (Processed *Radix Aconiti*, boiled for 5 h) and then the extraction and analysis were done as described in Section 2.2.2. The average recovery for the MDAs was 95.4–99.1% and the average recovery for the DDAs was103.0 – 112.0%. Therefore, the method was precise, accurate and sensitive enough for simultaneously quantitative analysis of six Aconitine-type alkaloids in *Radix Aconiti*.

Table 1
Alkaloids in *Radix Aconiti* separated and determined by UPLC–ESI/MS^n.

No.	No.[a]	No.[b]	$t_R \pm$ SD (min)	Precursor ion (*m*/*z*)	Collision energy (eV)	Characteristic fragment (*m*/*z*)	Name
1	1	–	1.10 ± 0.03	486	20	468, 454, 436	Mesaconine
2	2	–	1.59 ± 0.02	500	20	482, 468, 450	Aconine
3	3	1	2.90 ± 0.03	576	20	–	Unknown
4	4	2	3.27 ± 0.01	606	20	574, 556, 524	14-benzoyl-10-OH-mesaconine
5	5	3	3.46 ± 0.02	470	20	438, 420	Hypaconine
–	–	4	4.92 ± 0.02	–	–	–	Unknown
6	6	5	5.49 ± 0.02	408	20	390, 358	Excelsine
7	7	6	6.36 ± 0.03	590	20	572, 558, 540	Benzoylmesaconine
8	–	–	6.56 ± 0.01	454	20	436, 404	Fuziline
9	8	7	8.18 ± 0.01	604	20	586, 572, 554	Benzoylaconine
10	9		8.98 ± 0.03	438	20	420, 388, 356	Neoline
11	10	8	9.43 ± 0.02	438	20	420, 406	Unknown
12			10.18 ± 0.02	438	20	420, 406	Unknown
13	11	9	12.69 ± 0.02	574	20	556, 542	Benzoylhypaconine
14	12	10	14.18 ± 0.01	452	20	420, 388	Chasmanine
15	13	11	15.67 ± 0.02	358	35	340	Unknown
16	14	–	16.60 ± 0.02	360	35	342	Unknown
17	15	–	17.77 ± 0.02	648	15	588, 616, 598	10-OH-mesaconitine
18	16	12	18.70 ± 0.03	700	30	640, 578	Unknown
19	17	–	20.01 ± 0.01	572	20	554	Unknown
20	18	13	20.79 ± 0.01	572	20	554, 540, 508	dehydrated 14-benzoylmesaconine
21	19	14	20.82 ± 0.03	632	20	572, 540	Mesaconitine
22	20	15	25.52 ± 0.02	662	20	644, 602, 542	10-OH–aconitine
23	21	–	27.58 ± 0.02	646	20	586, 554	Aconitine
24	22	16	31.47 ± 0.02	616	20	556. 584	Hypaconitine
25	23	17	32.57 ± 0.03	540	20	522, 462	Unknown
26	24	18	35.53 ± 0.02	770	35	710, 648 622	Unknown
27	25	–	38.55 ± 0.02	630	20	598, 570, 538	Deoxyaconitine
28	26	–	40.00 ± 0.04	300	35	282	Unknown
29	–	–	42.59 ± 0.03	614	20	582, 554, 522	3,13-deoxyaconitine
–	–	19	43.56 ± 0.02	–	–	–	Unknown
–	–	20	45.59 ± 0.02	–	–	–	Unknown
30	27	21	53.63 ± 0.05	798	30	766, 556, 542	8-pdc-benzoylhypaconine
31	28	22	56.41 ± 0.02	850	30	818, 572, 558, 540	8-linolen-14-benzoylmesaconine
32	29	23	56.84 ± 0.03	868	30	836, 572, 558, 540	8-ndn-14-benzoylmesaconine
33	30	24	57.28 ± 0.02	854	30	–	Unknown
34	31	25	57.65 ± 0.04	854	30	–	Unknown
35	32	26	57.90 ± 0.05	852	30	820, 802, 572, 558, 540	8-lino-14-benzoylmesaconine
36	33	27	58.88 ± 0.04	870	30	838, 820, 572, 558, 540	8-ole-10-OH-benzoylmesaconine
37	34	28	59.24 ± 0.03	866	30	834, 816, 586, 572, 554	8-lino-14-benzoylaconine
38	35	29	59.48 ± 0.02	828	30	796, 572, 558, 540	8-pal-14-benzoylmesaconine
39	36	30	59.95 ± 0.02	854	30	822, 804, 572, 558, 540	8-ole-14-benzoylmesaconine
40	–	31	61.60 ± 0.05	868	30	836, 818, 586, 572, 554	8-ole-14-benzoylaconine
41	37	32	62.30 ± 0.02	836	30	804, 556, 542	8-lino-14-benzoylhypaconine
42	–	–	63.15 ± 0.04	856	30	824, 572, 558, 540	8-odc-14-benzoylmesaconine
43	38	33	64.72 ± 0.05	812	30	780, 556, 542	8-pal-14-benzoylhyacaonine
44	39	34	65.31 ± 0.02	838	30	806, 788, 556, 542	8-ole-14-benzoylhypaconine
45	–	–	65.68 ± 0.04	850	30	818, 800, 570, 538	8-lino-14-Benzoyldeoxyaconine
46	–	–	68.20 ± 0.03	680	30	-	Unknown
47	–	–	69.0 ± 0.05	826	30	794, 570, 538	8-pal-14-benzoyldeoxyaconine
48	–	–	69.98 ± 0.05	852	30	820, 802, 570, 538	8-ole-14-Benzoyldeoxyaconine

[a] Common peaks in TIC.
[b] Common peaks in UV chromatograph.

3.3. Fingerprints of Radix Aconiti in different processing times

The extracts of processed *Radix Aconiti* by boiling or steaming in different times were analyzed by UPLC–UV and UPLC–ESI/MS^n, respectively. The fingerprints of their UV chromatograms were shown in S-Fig. 1 and the fingerprints of their total ion chromatograms (TICs) were shown in S-Fig. 2. As shown in S-Fig. 1, S-Fig. 2 and Fig. 1, we could find that the contents of most of the compounds varied a lot during the processing procedure. The identified DDAs have high contents in raw *Radix Aconiti*, such as 10-OH–mesaconitine (No. 17, RT 17.77, *m*/*z* 648), mesaconitine (MA, No. 19, RT 20.82, *m*/*z* 632), 10-OH–aconitine (No. 20, RT 25.52, *m*/*z* 662), aconitine (AC, No. 21, RT 27.58, *m*/*z* 646), hypaconitine (HA, No. 22, RT 31.47, *m*/*z* 616), deoxyaconitine (No. 24, RT 38.55, *m*/*z* 630) and 3,13-deoxyaconitine (No. 25, RT 42.59, *m*/*z* 614). Since DDAs produced the hydrolyzation, pyrogenation or other chemical changes in processing procedure, the contents of the all the DDAs decreased rapidly especially in the first two hours, which means that the toxicity of this herbal medicine drops remarkably when being processed.

The content changes of DDAs and MDAs during processing of *Radix Aconiti* by boiling and steaming method were shown in Fig. 3. The identified MDAs have high contents both in raw and processed *Radix Aconiti* and their contents increased gradually during the processing, such as benzoylmesaconine (BMA, No. 7, RT 6.36, *m*/*z* 590), benzoylaconine (BA, No. 9, RT 8.18, *m*/*z* 604) and benzoylhypaconine (BHA, No. 13, RT 12.69, *m*/*z* 574). This increase is mainly owing to the hydrolysis procedure of DDAs and the hydrolysates are MDAs. The contents of LDAs (No. 26-No. 42) decreased significantly in the first hour of the processing and then remained almost the same during the following processing hours. The level of LDAs decreased probably because the LDAs are hydrolyzed during the processing. There are also the reactions between MDAs or DDAs and the fatty acids, which the

reaction products are LDAs. The formation and decomposition of LDAs must reach equilibrium when the levels of LDAs remained stable.

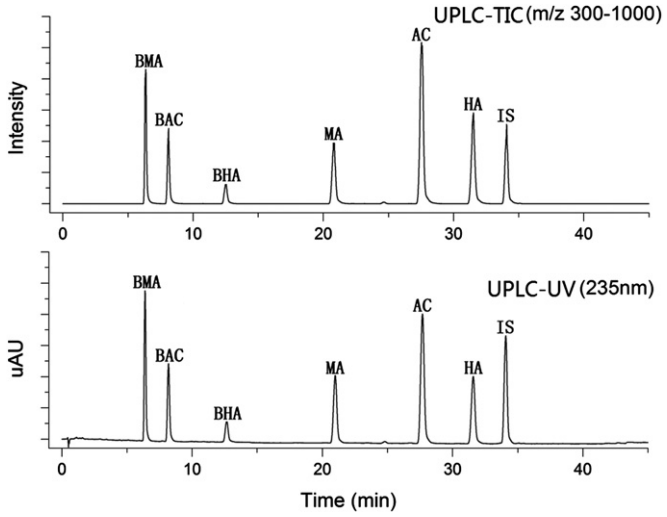


Fig. 2. The UPLC–UV and the TIC chromatograms of the six standards, Benzoylmesaconine (BMA), Benzoylaconine (BA), Benzoylhypaconine (BHA), Mesaconitine (MA), Aconitine (AC), Hypaconitine (HA).

3.4. Quality assessment of Radix Aconiti by hierarchical cluster analysis (HCA)

The dendrogram was presented in Fig. 4 and shows four major clusters, clearly differentiating the *Radix Aconiti* samples through their processing degrees no matter the processing methods (boiling or steaming). The boiling *Radix Aconiti* (4 h, 5 h, 6 h), steaming *Radix Aconiti* (6 h, 7 h, 8 h), and three purchased processed *Radix Aconiti* (P1, P2, P3) were grouped in one cluster "labeled as 'Qualified'" perfectly and these samples were exactly processed. Therefore, the results of the cluster analysis indicated that the quality of processed *Radix Aconiti* by the two processing methods could be controlled by the method of fingerprint analysis and the two processing methods have no obvious difference for the process of *Radix Aconiti*.

3.5. Quality assessment of Radix Aconiti by principal component analysis (PCA)

The PCA results shown in Fig. 5 (a), (c) and (e) were scores plot of different samples. Fig. 5 (b), (d) and (f) were loadings plot of variables corresponding to samples mentioned in Fig. 5 (a), (c) and (e) respectively.

All the *Radix Aconiti* samples, including the samples in different processing times, the purchased samples and the raw samples were investigated by PCA. The scores plot and the loading plot

Table 2
Linear equations of six conitine-type alkaloids by UPLC–UV and UPLC–ESI/MS ($n=3$).

Alkaloids	Linear equations	Correlation coefficient	LOD(ng mL^{-1})	LOQ(ng mL^{-1})	Linear range(μg mL^{-1})
Benzoylmesaconine	[a]$Y=0.00284+0.00799$ X	0.9998	82.0	329.0	0.1–500
	[b]$Y=-0.00304121+0.342321$ X	0.9960	5.5	12.0	0.02–13.2
Benzoylaconine	[a]$Y=0.02125+0.0256$ X	0.9995	176.0	500.0	0.1–500
	[b]$Y=-0.00360155+0.236332$ X	0.9973	4.5	12.0	0.02–28.3
Benzoylhypaconine	[a]$Y=0.01238+0.01587$ X	0.9998	600.0	1500.0	0.05–500
	[b]$Y=-0.005342+0.133099$ X	0.9970	4.5	12.0	0.06–21.2
Mesaconitine (MA)	[a]$Y=0.00767+0.02581$X	0.9990	200.0	630.0	0.2–500
	[b]$Y=-0.00588546+0.408199$ X	0.9974	4.0	10.0	0.01–22.3
Aconitine (AC)	[a]$Y=0.00664+0.02897$ X	0.9993	280.0	800.0	0.2–500
	[b]$Y=-0.00667755+0.468236$ X	0.9993	6.0	15.0	0.02–22.7
Hypaconitine (HA)	[a]$Y=0.02529+0.02767$ X	0.9996	300.0	1000.0	0.1–500
	[b]$Y=-0.00691247+0.514219$ X	0.9955	3.0	9.0	0.02–13.2

[a] Quantified by UV chromatograph (235 nm).
[b] Quantified by UPLC–ESI/MS (EIC).

Table 3
The contents of the six conitine-type alkaloids in *Radix Aconiti* ($n=3$).

	Processing times (hours)	Benzoylmesaconine (μg g^{-1})	Benzoylaconine (μg g^{-1})	Benzoylhypaconine (μg g^{-1})	Mesaconitine (MA) (μg g^{-1})	Aconitine (AC) (μg g^{-1})	Hypaconitine (HA) (μg g^{-1})
Unprocessed	0	422.8	35.0	77.2	734.0	87.9	514.4
Steeped	0	375.4	36.7	85.8	476.2	84.7	496.4
Boiled	1 h	816.8	77.5	219.7	25.5	4.4	192.5
	2 h	954.1	103.0	291.3	5.0	1.1[a]	51.3
	3 h	1157.2	162.9	403.9	1.7[a]	0.8[a]	22.7
	4 h	885.4	153.1	428.5	0.8[a]	0.3[a]	14.3
	5 h	871.0	128.7	476.5	0.5[a]	0.2[a]	4.8
	6 h	792.1	125.2	474.3	0.4[a]	0.1[a]	4.4
Steamed	1 h	941.1	97.7	173.6	32.4	7.5	118.2
	2 h	982.6	104.9	215.0	8.4	2.6[a]	79.5
	3 h	1032.9	135.8	427.5	4.9	1.7[a]	71.2
	4 h	1206.5	117.5	495.1	3.4[a]	0.6[a]	46.4
	5 h	1093.6	113.1	518.0	2.7[a]	0.6[a]	39.6
	6 h	1050.0	121.4	504.3	0.5[a]	0.2[a]	7.2
	7 h	1039.8	137.4	505.2	0.50[a]	0.2[a]	7.3
	8 h	1027.4	142.2	503.4	0.5[a]	0.2[a]	8.0

[a] Quantified by UPLC–ESI/MS (EIC).

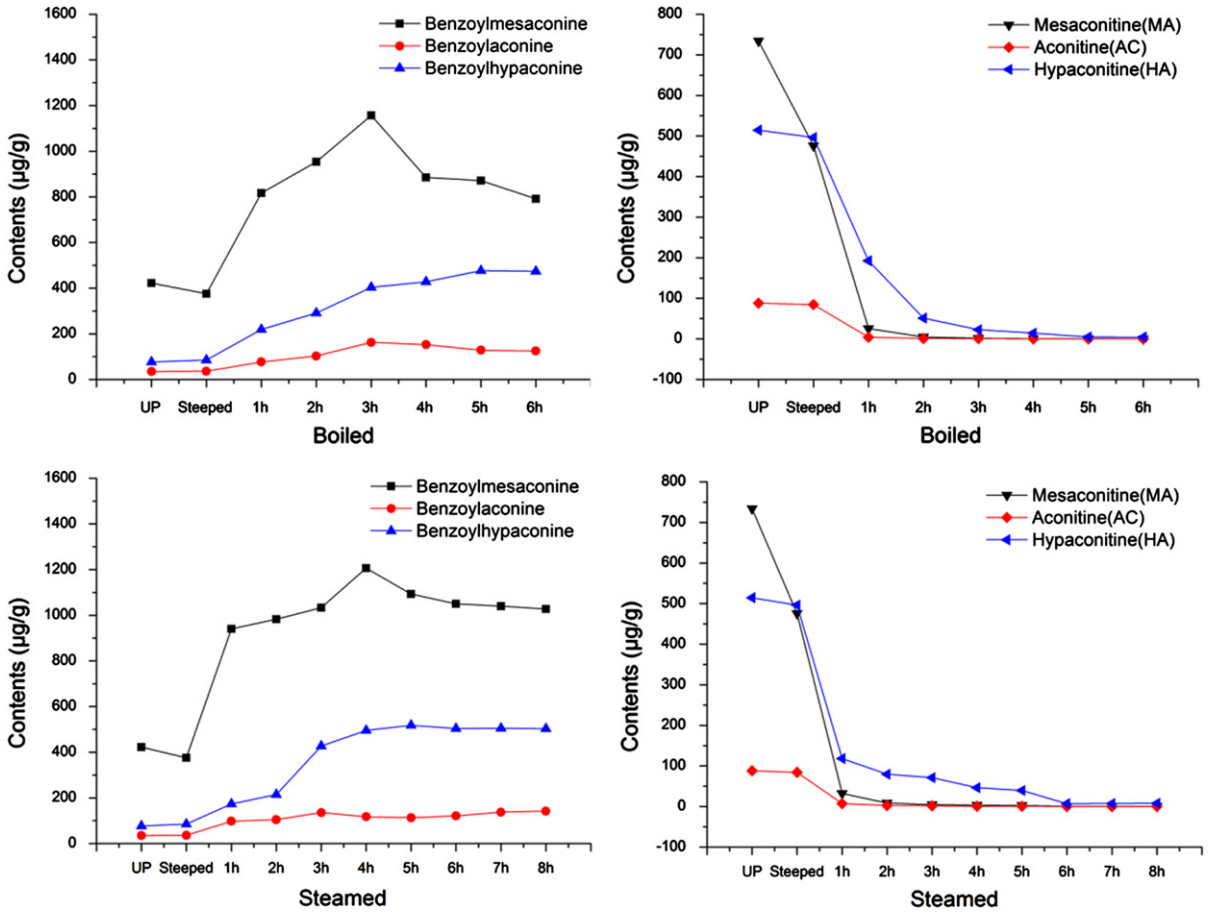


Fig. 3. The contents of DDAs and MDAs in different processing time of *Radix Aconiti* by boiling and steaming method.

were shown in Fig. 5a and Fig. 5b. On the scores plot, the blue ones (marked as "2") were the samples which were well processed (qualified), the red ones (marked as "1") were the samples which had not been processed sufficiently and the black ones (marked as "3") were raw samples. The processed and the raw samples were classified into two groups obviously as shown in Fig. 5a. Fig. 5a also shows the variation tendency corresponding to the processing times (explained by both PC_1 and PC_2). From top to bottom in the cluster of the processed samples were the samples arranged by processing times. The samples at the top half part of the cluster were those boiled for 1-3 h and steamed for 1-5 h. Because the contents of the constituents in these samples changed rapidly in these processing times, the spots that represented these samples were relatively scattered (seen in Fig. 3). The samples at the bottom half part of the cluster were those boiled for 4–6 h and steamed for 6–8 h, and the spots represented that these samples were relatively intensive since the fingerprint of these samples were similar and contents of their constituents changed a little in these processing times. Fig. 5b shows the main chemical markers having the most influence on the discrimination of the samples mentioned, there were mainly MDAs and DDAs in the first principal component (PC_1), i.e. benzoylmesaconine (BMA, m/z 590), benzoylaconine (BA, m/z 604), benzoylhypaconine (BHA, m/z 574), mesaconitine (MA, m/z 632), hypaconitine (HA, m/z 616), aconitine (AC, m/z 646) and 10-OH–mesaconitine (m/z 648). BMA (m/z 590), HA (m/z 616) and MA (m/z 632) have the most influence on the discrimination of the *Radix Aconiti* samples in different processing times. Structures of the main chemical markers were

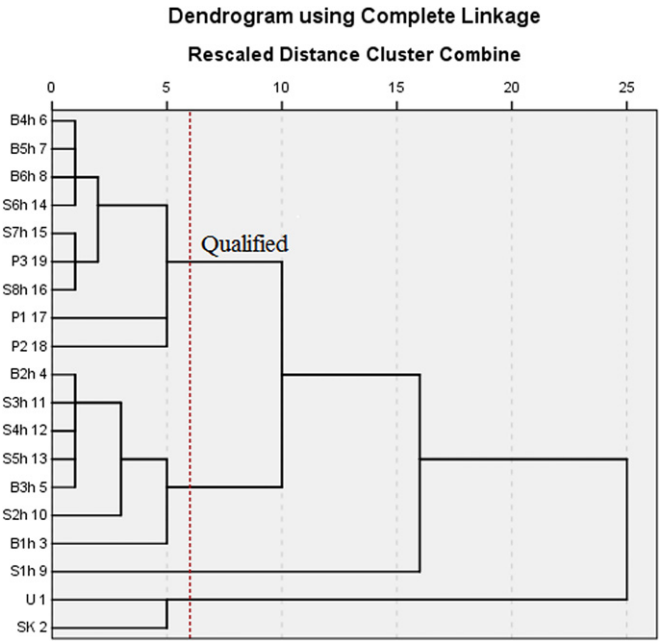


Fig. 4. Cluster dendrogram constructed from the TIC peak area data for 19 *Radix Aconiti* samples. (U) Raw *Radix Aconiti*; (SK) Soaked without boiling or steaming; B1h to B6h stand for "boiling for 1 h" to "boiling for 6 h"; S1h to S8h stand for "steaming for 1 h" to "steaming for 8 h"; P1,P2 and P3 were purchased processed *Radix Aconiti*.

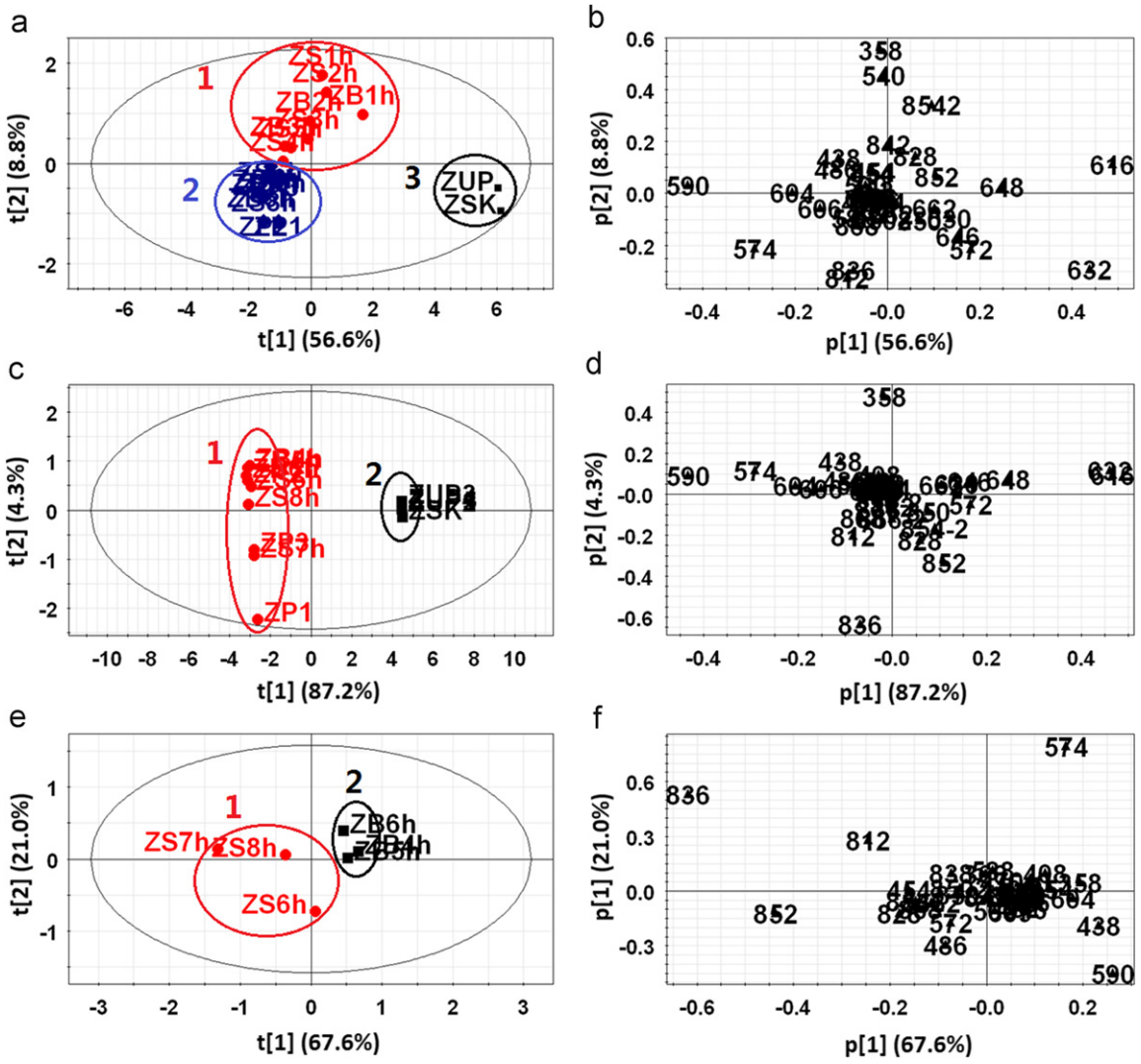
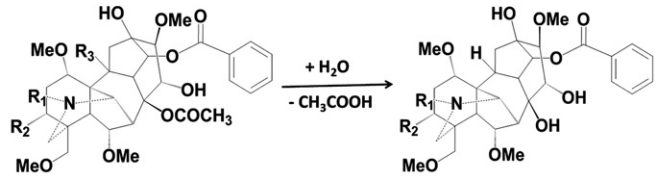


Fig. 5. (a), (c) and (e) distribution of samples on scores plot. ZUP1 to ZUP5 stand for Raw *Radix Aconiti*; (ZSK) Soaked without boiling or steaming; ZB1h to ZB6h stand for "boiling for 1 h" to "boiling for 6 h"; ZS1h to ZS8h stand for "steaming for 1 h" to "steaming for 8 h"; ZP1, ZP2 and ZP3 were purchased processed *Radix Aconiti*. (a) The scores plot obtained from PCA of all the samples. (c) The scores plot obtained from PCA of all the qualified samples and the raw samples. (e) The scores plot obtained from PCA of all the qualified processed samples. (b), (d) and (f) are the loadings plot of variables corresponding to samples mentioned in (a), (c) and (e) respectively. The numbers in these figures are related to the mass to charge ratio (m/z) of each common peak.



DDAs

R_1	R_2	R_3	*Aconitum* alkaloids	$[M+H]^+$
CH_3	H	H	Hypaconitine	616
CH_3	OH	H	Mesaconitine	632
C_2H_5	OH	H	Aconitine	646
CH_3	OH	OH	10-OH-mesaconitine	648

MDAs

R_1	R_2	*Aconitum* alkaloids	$[M+H]^+$
CH_3	H	Benzoylhypaconine	574
CH_3	OH	Benzoylmesaconine	590
C_2H_5	OH	Benzoylaconine	604

Fig. 6. Structures of the main chemical markers and the detoxification of diester diterpenoid alkaloids by hydrolysis during the processing.

shown in Fig. 6. The DDAs contents of the raw samples are higher than that of processed ones; it is already known that the detoxification of *Radix Aconiti* is bound to a hydrolysis procedure and the hydrolysates are MDAs (shown in Fig. 6). Therefore, the contents of DDAs and MDAs change a lot during the processing and that may be the reason why DDAs and MDAs have the most influence on the discrimination of the samples in different processing times.

In order to observe the difference between the well processed and the raw samples, fingerprint data was analyzed by PCA, which is from the six well processed samples, three qualified purchased samples and six raw samples. As shown from Fig. 5c, the well processed samples (marked as "1") and the raw samples (marked as "2") were classified into two groups obviously by PC_1. Fig. 5d was the loading plot directly bound up with Fig. 5c. Fig. 5d shows almost the same main chemical markers which have the most influence between the raw and processed samples in different processing times in Fig. 5b. BMA (m/z 590), HA (m/z 616) and MA (m/z 632) were the alkaloids which have the most influence on the discrimination between the qualified processed *Radix Aconiti* and raw *Radix Aconiti*. Furthermore, fingerprint data of the well processed samples by the two processed methods was also analyzed by PCA. As shown in Fig. 5e and Fig. 5f, the two kinds of well processed samples could be differentiated but their difference was tiny which indicated that the processed *Radix Aconiti* by steaming and boiling were similar. Fig. 5f shows that

the main chemical markers which have the most influence on the discrimination of the well processed samples. Those were mainly some LDAs in the first principal component (PC_1), i.e. 8-lino-14-benzoylhypaconine (m/z 836) and 8-lino-14-benzoylmesaconine (m/z 852). As a useful tool for the evaluation of the fingerprints data, PCA gives a simplified representation of the data and is generally useful to the quality assurance of herbal medicine.

3.6. Similarity analysis

The relationship among a set of chromatographic fingerprints could be currently analyzed through comparison in terms of similarity of the objects with a certain reference. Correlation coefficient and congruence coefficient were used as similarity measure in our work to examine the similarity of the samples which were clustered in group "Qualified". The similarity analysis of each chromatogram to their average chromatogram was investigated. It is considered as the best, the better or the worst drug with a corresponding correlation coefficient above 0.9, between 0.8 and 0.9, or below 0.8, respectively. The correlation coefficients and the congruence coefficients of the nine samples were more than 0.96, which indicated that their chromatographic fingerprints of applicable processed *Radix Aconiti* were stable and consistent.

3.7. Characteristic fingerprint of the processed Radix Aconiti

To standardize the fingerprint, the chromatographic data of all the nine qualified samples with high similarity were used. The peaks existing in all these chromatograms were identified as described in 3.2 and assigned as "common peaks". The 39 common peaks form TIC of UPLC–ESI/MS^n and 34 common peaks from UPLC–UV chromatogram, which were well isolated and identified could be found in all these well processed *Radix Aconiti* in their UPLC–UV and UPLC–ESI/MS^n fingerprints.

The characteristic fingerprints were obtained by calculating the average value of total ion chromatograms or UPLC–UV chromatograms of nine batches of qualified samples. The established UPLC–UV and UPLC–ESI/MS characteristic fingerprint of processed *Radix Aconiti* marked with the common peaks was shown in Fig. 1b and Fig. 1c. The using of the characteristic fingerprint provides a new basis of practice for the quality control of processed *Radix Aconiti* by comparing some of the unique characteristic peaks.

4. Conclusions

The quality control of traditional herbal medicines plays an important role in the safety and effectivity of clinical application. The present fingerprinting approach for quality control of processed *Radix Aconite* was established. The UPLC combined UV and ESI/MS^n offers a powerful tool for quantifying and separating the individual compounds and creates characteristic fingerprint profile. In the UPLC–UV and UPLC–ESI/MS^n fingerprint study, 39 characteristic TIC peaks and 34 UV chromatogram peaks in the common pattern were identified to further characterize the chromatographic fingerprint and contribute to the quality control of processed *Radix Aconite*. Chemometrics analysis such as hierarchical clustering analysis, principal component analysis and similarity evaluation was applied to establish and evaluate the fingerprints. All the results indicated that the present fingerprinting approach in combination with chemometrics provide a very flexible and reliable method for quality assessment of toxic herbal medicines, and the present approach is an appropriate means of quality control for processed Aconite Radix.

Because of the high toxicity of *Radix Aconiti* and the difficulties in treating *Radix Aconiti*-poisoning, accurate qualitative and quantitative determination of Aconitine-type alkaloids in Aconite Radix is of great importance. A complementary quantification method of Aconitine-type alkaloids using UPLC–UV and UPLC–ESI/MS^n was developed. As showed in the results of UPLC–UV and UPLC–ESI/MS^n quantification method can be effectively applied to identify and quantify DDAs and MDAs. It is highly recommended that the determination of these Aconitine-type alkaloids is done as a routine measurement. This would provide a safe application to patients in clinics, and would meet the needs as the standards of quality control in good manufacturing practice.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.10.006.

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