## ORIGINAL ARTICLE

# Limitation standard of toxic aconitines in *Aconitum* proprietary Chinese medicines using on-line extraction electrospray ionization mass spectrometry

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**Abstract** Development of rapid analytical methods and establishment of toxic component limitation standards are of great importance in quality control of traditional Chinese medicine. Herein, an on-line extraction electrospray ionization mass spectrometry (oEESI-MS) coupled with a novel whole process integral quantification strategy was developed and applied to direct determination of nine key aconitine-type alkaloids in 20 *Aconitum* proprietary Chinese medicines (APCMs). Multi-type dosage forms (*e.g.*, tablets, capsules, pills, granules, and liquid preparation) of APCM could be determined directly with excellent versatility. The strategy has the characteristics of high throughput, good tolerance of matrix interference, small amount of sample (∼0.5 mg) and reagent (∼240 μL) consumption, and short analysis time for single sample (<15 min). The results were proved to be credible by high performance liquid chromatography−mass spectrometry (LC−MS) and electrospray ionization mass spectrometry, respectively. Moreover, the limitation standard for the toxic aconitines in 20 APCMs was established based on the holistic weight toxicity (HWT) evaluation and the *Chinese Pharmacopoeia* severally, and

| Holistic weight toxicity | turned out that HWT-based toxicity evaluation results were closer to the real clinical applications. Hence, a more accurate and reliable APCM toxicity limitation was established and expected to play an important guiding role in clinics. The current study extended the power of ambient MS as a method for the direct quantification of molecules in complex samples, which is commonly required in pharmaceutical analysis, food safety control, public security, and many other disciplines. |
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## 1. Introduction

Establishment of toxic compound limitation standard is attached great significance in the safe and rational application of toxic traditional Chinese medicines (TCM)[1], which always contains compounds possessing both good activity and toxicity. In *Chinese Pharmacopoeia* (2015 edition), 61 kinds of *Aconitum* proprietary Chinese medicines (APCMs) in multi-type dosage forms, such as pills, tablets, capsules, granules, and ointment, were listed with the properties of good anti-rheumatic and cardiotonic effects[2]. However, the highly toxic aconitines in APCM may produce severe cardiotoxicity, neurotoxicity, and even death[3]. The main active and toxic ingredients in APCM are three types of alkaloids, including the diester-, monoester-, and nonester-aconitines[4,5]. In particular, the toxicity of diester alkaloids is 200−500 times of monoester alkaloids, and 2000−4000 times of non-ester alkaloids[6]. Therefore, it is necessary to carry out a reliable limitation standard of highly toxic diester alkaloids in APCM to ensure medication safety[2]. Unfortunately, up to now, no uniform limitation standard for toxic components in multi-type dosage forms of APCM has been proposed[2].

Accurate qualitative and quantitative analysis of target components in TCM is the prerequisites for quality control[7]. Currently, the liquid chromatography−mass spectrometry (LC−MS)[8−10] and gas chromatography−mass spectrometry (GC−MS)[11] are the most popular platforms in pharmaceutical analysis due to best specificity and sensitivity[12]. However, the multiple-step sample pretreatments such as soak, extraction, centrifugation, and dilution, along with numerous energy and reagent consumption are common obstacles[13]. Furthermore, key chemical information change and loss during the complex procedure always limits its detection throughput[14−16]. On account of solution for defects resulted from complex pretreatment[17], the on-line extraction coupled with LC−MS analysis is currently the mainstream device, which still needs the long separation time[18]. Owing to the fast response sensitivity and minimal sample pretreatment, the ambient ionization techniques increase the simplicity and the throughput of MS analysis. However, the existing ambient MS quantitation strategy has obvious limitations, such as only surface quantification[19], poor versatility[20], or low resistance to matrix interference[21], thus the on-line component enrichment or impurities removal before MS analysis[21] is still indispensable. Hence, for various complicated samples in real-world, the universal, accurate, and sensitive ambient MS method should be developed.

Herein, an on-line extraction electrospray ionization mass spectrometry (oEESI-MS) was developed to realize on-line extraction, real-time determination and accurate quantitative analysis of complicated samples. Internal standard (IS) calibration was performed to significantly improve the precision of the analysis. A novel whole process integral quantification strategy was proposed to accurately quantify the total amount in bulk samples. Based on this strategy, nine key alkaloids in a total of 20 APCMs of different dosage forms were quantified with a wide linear range, low limit of detection, and high precision and accuracy. The quantitative results were proven to be accurate and reliable. The method has the advantages of high throughput, short time (<15 min), accurate quantification, convenient operation, less sample (∼0.5 mg) and solvent consumption (<240 μL). Moreover, the limitation standard for the toxic aconitines in 20 APCMs was established based on the holistic weight toxicity (HWT)[22] analysis and the setting in *Chinese Pharmacopoeia*, and proved that HWT-based toxicity evaluation results were closer to the data from clinical results, which is expected to promote the reasonable and safe application of APCM in clinical practice.

## 2. Materials and methods

### 2.1. Samples, chemicals and reagents

A total of 20 APCMs, Dahuoluo Pill (DHP), Fengshigutong Capsule (FSC), Fufangxiaohuoluo Pill (FXP), Fuguigutong Capsule (FGC), Fuzilizhong Pill (FLP), Goupi Plaster (GPP), Guifulizhong Pill (GLP), Guci Pill (GCP), Qiliqiangxin Capsule (QQC), Sanwujiao Pill (SJP), Shenguilizhong Pill (SLP), Tongrendahuoluo Pill (TDP), Wangbi Granule (WBG), Wangbi Tablet (WBT), Xiaojin capsule (XJC), Xinbao pill (XBP), Yaoxitong Capsule (YXC), Yishenling Granule (YSG), Yunnanhongyao Capsule (YHC), and Shengfuzi Liquor (SFL), were purchased from local pharmacies (Nanchang, China), the manufactures and batch numbers were detailly listed in Supporting Information Table S1. Nine authentic alkaloids including aconitine (AT), mesaconitine (MAT), hypaconitine (HAT), benzoylaconine (BAC), benzoylmesaconine (BMA), benzoylhypaconine (BHA), aconine (AC), mesaconine (MA), and hypaconine (HA) were supplied by Beijing Rongcheng Xinde Technology Development Co., Ltd. (HPLC purity >98%, Beijing, China). Berberine (BB) was supplied by ANPEL Laboratory Technologies (Shanghai) Inc. (HPLC purity >98%, Shanghai, China). De-ionized water (18.2 MΩ/cm) was obtained by Mill-Q water purification system (Billerica, MA, USA). Methanol and dichloromethane (HPLC grade) were purchased from ROE Scientific Inc. (Newark, DE, USA) and were used without further purification.

Standard stock solutions were prepared by dissolving appropriate amounts of each compound in water to obtain final concentrations of 4 μg/mL for all the nine target analytes, respectively. Standard solutions with a series concentrations were prepared by diluting the standard stock solution 5, 25, 125, 625,

3,125, 15,625 and 78,125 times respectively. Stock solution of IS was prepared by dissolving BB in the extract solution.

## 2.2. An oEESI-MS device for quantification of multi-type APCM

The oEESI-MS experiments were carried out using a homemade disposable oEESI source installed on a linear trap quadruple (LTQ) mass spectrometer (Thermo Scientific, San Jose, CA, USA). The device for sample extraction and ionization procedure were schematically illustrated in Fig. 1. Charged (+5.5 kV) extraction solvent in syringe (500 μL) was pumped by an injection pump at the flow rate of 16 μL/min. Acetic acid (1%) was added in extraction solvent to enhance ionization while inhibiting the degradation of the diester alkaloids in samples. IS (BB) was added in the extraction solvent (100 ng/mL) to calibrate the target alkaloids signals real-timely. Sample camber was constructed of silicone tube (i.d. 2.0 mm) with a piece of microporous membrane (0.22 μm) at the end and connected with a homemade sample nebulizer using a metal tube (i.d. 0.1 mm). For different types of APCM samples, the detailed operations were developed. The solid preparations, such as granules and capsules, were directly weighed and loaded into the sample camber for on-line extraction and quantification. The bulk and hard solid preparations, including pills and tablets (*e.g.*, SJP), were ground into powder to facilitate extraction effectively. The viscous solid preparations (*e.g.*, GPP) were directly weighed in a small amount (∼0.5 mg) and kneaded into a flat shape for better determination. Only 5 μL of liquid sample (*e.g.*, SFL) was needed and absorbed into cotton fibers for rapid analysis using the same device. The ionizing gas pressure of nitrogen gas and the charged voltage were manually optimized based on the signal intensity. The MS tune method was automatically optimized at positive ion scan mode. After optimization, the parameters were finally set as follow: capillary temperature was 180 °C, the capillary voltage was 38 V and the tube lens was 65 V. Selective reaction monitoring (SRM) mode was selected for automatic scanning and Collision-induced dissociation (CID) collision energy was 15%–30% (Supporting Information Table S2). The MS signal was collected and recorded in real time. The CID fragment ions were used for qualitative analysis and the characteristic fragment ion was selected for quantitative analysis respectively.

## 2.3. Quantitative ability evaluation for oEESI-MS

The series of gradient mixed solution was used for establishment of the standard working curve. Nine target aconitine-type alkaloids and IS were determined simultaneously and sensitively for accurately qualitative and quantitative analysis. The signal
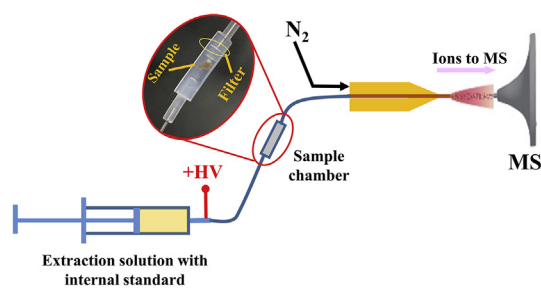
intensity of all the nine alkaloids were first calibrated with IS before quantification to eliminate possible errors and interference. The LOD (limit of detection) and LOQ (limit of quantitation) values were obtained when the signal to noise ratio reached 3 and 10 respectively. The mixed standard solution and the actual sample SJP were separately and repeatedly injected for 6 times for evaluation of precision and accuracy. Each characteristic CID fragment signal of alalytes should be calibrated by IS and then substituted into the calibration equations to calculate the instant concentration. In addition, SJP was used as a model to evaluate the matrix interference and MS saturation effect of oEESI-MS. Since no other pretreatment process, the matrix effect evaluation method also indicated the recovery. A mixed standards solution with IS in it was used as the extractant to extract the model SJP sample and the standard addition method was used for calculating the interference and recovery. The saturation effect of ion source was also determined. The SJP was directly ultrasonically extracted and serially diluted for 5 times, and then the linear relationship between signal intensity and concentration was observed. The final concentration of IS for all analyses was constant at 100 ng/mL.

## 2.4. A novel whole process integral quantification strategy

The main difficulties in on-line quantitative analysis of complicated samples were how to completely extract to accurately quantify the target components and how to establish a functional relationship between the real-time signal intensity and the content of each analyte in samples. Facilitated by the powerful qualitative and quantitative abilities of oEESI-MS, the complete extraction process could be truly reflected such as the curve in Fig. 2, of which the abscissa was the extraction time, and the ordinate was the instant concentration of the analytes. The analytes were selectively extracted when the charged extractant flow through the sample and simultaneously ionized for real-time detection by MS. As time passed, the analytes were gradually extracted and the signal intensity naturally decreased until it disappeared. For the first time, a novel whole process integral quantification strategy was proposed for rapid on-line quantitative analysis of target analytes in complex samples. Hence, the accurately whole content of the analytes could be calculated by integration of the whole extraction process using the Eq. (1):

$$M = \frac{\int C(t)\mathrm{d}t \times r}{1000} \tag{1}$$



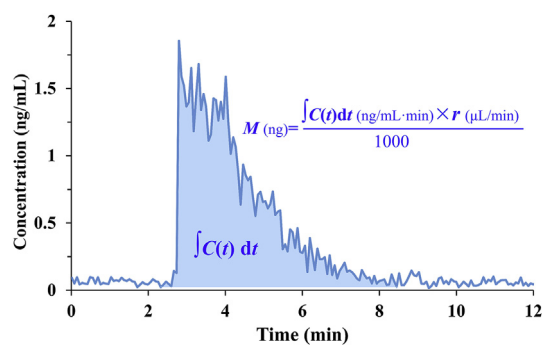**Figure 1** Schematic illustration of oEESI-MS.



**Figure 2** The real-time concentration curves and the whole process integral quantitative schematic diagram.

where $M$ is the content of the analyte (ng), $C$ is the instant concentration (ng/mL), $t$ is the monitoring time (min), $r$ is the flow rate equaled to 16 μL/min.

### 2.5. HWT-based toxic components limitation standard

The HWT was a dimensionless parameter for toxicity evaluation of *Aconitum*-containing system that we proposed in the previous study. According to HWT, the toxicity of the daily medication can be quantified accurately based on the content of HAT, MAT and AT. Hence, the HWT-based toxic components limitation standard (LH) was developed based on the Eq. (2):

$$LH = \frac{1}{HWT} = \frac{1}{(C_H/0.0162 + C_M/0.0035 + C_A/0.0051)/1000/60} \quad (2)$$

where $C_H$ (μg), $C_M$ (μg) and $C_A$ (μg) are the daily oral dose of HAT, MAT and AT; 0.0162, 0.0035 and 0.0051 are the minimum toxic dose (mg/kg) of HAT, MAT and AT, respectively; 60 is the regular adult weight (kg).

In *Chinese Pharmacopoeia*, there are no uniform limitation standards of toxic diester alkaloids for diverse APCM, but for each *Aconitum* herb medicine, the limitation of total diester alkaloids was listed. For different APCM, the *Aconitum* medicines in prescription are different, such as Zhichuanwu, Zhifuzi, etc. Even some drugs contain two or more kinds of *Aconitum* herbs in one prescription. For different *Aconitum* herb medicines, there are also different limitations for total diester alkaloids. Hence, we proposed a new calculation method of APCM limitation standard (LP) based on both limitation of *Aconitum* medicines and the content ratio of different *Aconitum* medicines in each prescription. LP could be calculated by the Eq. (3):

$$LP = \frac{LM \times 10^3}{C_H + C_M + C_A} \quad (3)$$

where LM (mg) is the weighed limitation content of total diester alkaloids for each APCM calculated based on the limitation standard in *Chinese Pharmacopoeia* and the content ratio of different *Aconitum* medicines in each prescription. The maximum clinical dosages of Zhichuanwu, Zhicaowu, and Zhifuzi (Heishunpian) are 3.0, 3.0 and 15.0 g, and the diester alkaloids in them are limited lower than 0.04%, 0.04%, and 0.01%, respectively. Therefore, the LM could be calculated as Eq. (4):

$$LM = (a \times 3 \times 0.04\% + b \times 3 \times 0.04\% + c \times 15 \times 0.01\%) \times 1000 \quad (4)$$

where $a$, $b$, and $c$ are the content ratio of Zhichuanwu, Zhicaowu, and Zhifuzi (Heishunpian) in prescription, respectively. Because there is no dosage limit in the pharmacopoeia of Shengchuanwu, Shengcaowu and Shengfuzi, the APCM containing these medicines, such as SJP, cannot calculate the toxicity limitation according the Eq. (3).

## 3. Results and discussion

### 3.1. Development of the oEESI-MS

First of all, an oEESI-MS device with the characteristics of simple, easy operation, strong versatility and stability was established (Fig. 1). The key parameters, such as extraction solvent, ionization voltage, and spray gas pressure, could be optimized flexibly based on the characteristics of the target analyte for effective extraction, ionization, and determination. The extract solvents optimization result was presented in Fig. 3a−d. Obviously, methanol/methylene chloride system (Fig. 3a) had good extraction efficiency for the target alkaloids (*e.g.*, m/z 604, 590, 574, and 500) and the spectrum was pure with few impurity signals, showing a superiority based on the nice selectivity and few matrix effect. Other solvent systems, such as methanol/dimethyl sulfoxide (Fig. 3b), methanol/water (Fig. 3c), and water/dimethyl sulfoxide (Fig. 3d), either simultaneously extracted a large amount of impurity peaks (*e.g.*, m/z 859, 792, 332) or had low extraction efficiency of the target components. Hence, mixed methanol and methylene chloride (1:1, v/v) was preferred as the extraction solvent.

Inert nitrogen was selected to be the spray gas and the gas pressure was optimized systemically based on the signal intensity (Fig. 3e). Adjusted from 0.2 to 1.0 MPa in sequence, it was obvious that 0.4 MPa could supply the highest signal intensity, indicating the best ionization effect. The ionization voltage was also adjusted sequentially from 3.0 to 6.0 kV for optimization. Interestingly, the signal intensity was positively correlated with voltage from 3.0 to 5.5 kV, but stop continuing to increase from 5.5 to 6.0 kV (Fig. 3f), generating a best choice of the 5.5 kV for further analysis. Finally, facilitated by the abundant adjustable parameters (*e.g.*, ionization voltage, gas pressure, and extraction solvent) and simple structure, the established oEESI-MS device shows obvious advantages of strong versatility, no restriction on sample forms, high extraction efficiency and resistance to matrix interference.

### 3.2. Qualitative and quantitative performance of oEESI-MS

Full scan MS spectrum and MS/MS spectra of the nine targeted alkaloids and internal standard were shown in Fig. 4. The total 10 alkaloids were properly detected (Fig. 4a) with different molecular ion peaks (*e.g.*, m/z 646, 604 and 500 were AT, BAC and AC, respectively). The MS/MS fragments obtained from the tandem MS experiments on authentic compounds (Fig. 4b−j) were used as the standards to identify the analytes in actual samples. To improve the accuracy of quantitative analysis and avoid the influence from isomerides effectively, the characteristic CID fragment ions (*e.g.*, m/z 586, 572 and 556 for AT, MAT, and HAT, respectively) of the nine alkaloids were used for quantification. IS method was performed to calibrate both the working curves and the actual sample data. BB was selected as the internal standard because of the stable structure and similar ionization behavior[22,23]. The precision was obtained by repeating analysis of the sample for 6 times, and the relative standard deviation (RSD) of all the nine compounds were 3.25%−7.56% (Supporting Information Table S3). After quantitative analysis of the series gradient mixed standard solutions, linear regression fitting was performed to obtain the calibration curves and equations (Supporting Information Table S4). Obviously, all the nine calibration curves have a good linear fit ($R^2 > 0.9960$) and a wide linear range (0.051−4000 ng/mL). Extremely low detection sensitivity on these alkaloids was displayed from the LOD (0.001−0.015 ng/mL) and LOQ (0.003−0.049 ng/mL). The absolute value of RSD for accuracy of the nine alkaloids were lower than 7.5% (Table S3), indicating that the method can accurately characterize the true concentration of the actual sample. Matrix interferences were unavoidable in complex sample analysis and an improved standard addition method was used in this study to evaluate matrix interference. The tested
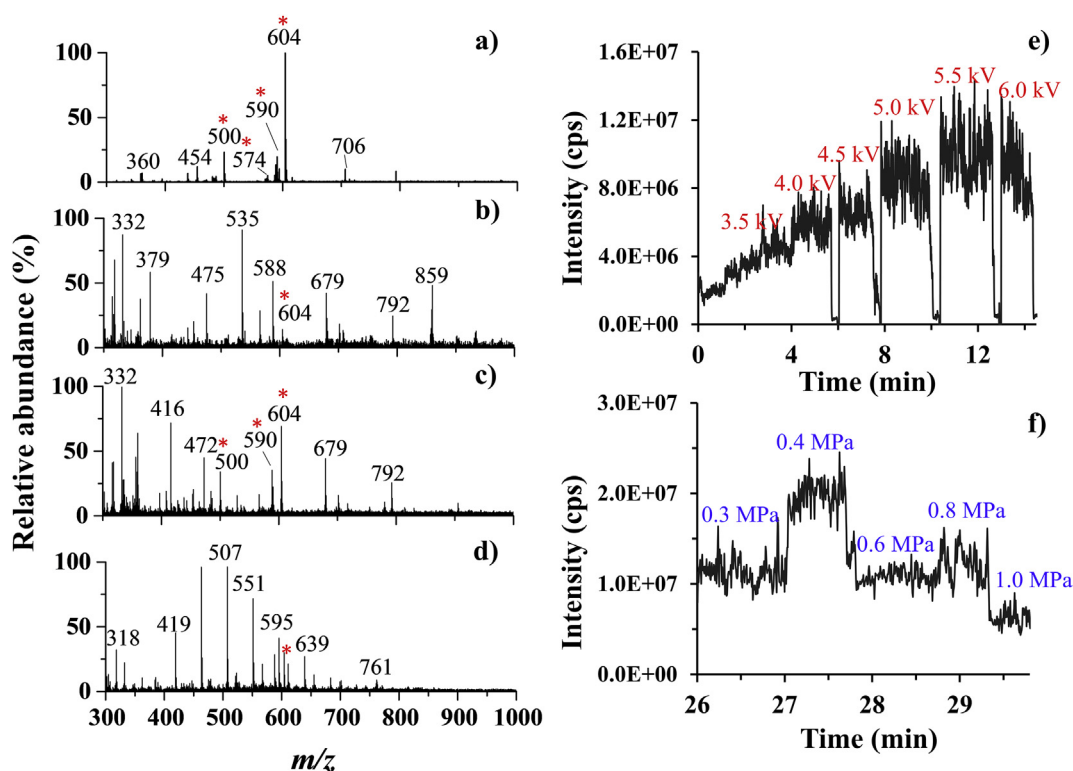
**Figure 3** The MS spetra of oEESI-MS for analytical conditions optimization. The MS spectrum of SJP extracted by dichloromethane and methanol (a), methanol and dimethyl sulfoxide (b), methanol and water (c), water and dimethyl sulfoxide (d). TIC spectrum for optimization of ionization voltage (e) and spray gas pressure (f).

results were all higher than 90% of the true value with RSD<10% (Table S3), revealing that the matrix interference in this method was not significant and the recovery of all the analytes were higher than 90%. Saturation effect of ion source was determined and displayed satisfactory results ($R^2$ = 0.9968, Supporting Information Fig. S1). In summary, the established oEESI-MS approach featured high chemical sensitivity, accuracy, specificity, and matrix tolerance to target analytes in complex samples.

### 3.3. On-line extraction and quantitation of aconitine-type alkaloids in 20 APCMs

Drugs, especially compound preparations containing a variety of materials, are good complicated sample models for determination. Here, 20 APCMs in different preparations such as tablets (*e.g.*, WBT), capsules (*e.g.*, XJC), pills (*e.g.*, DHP), granules (*e.g.*, WBG), liquid preparation (*e.g.*, SFL), etc. were determined. All the preparations were directly loaded into the oEESI-MS device for accurate quantitative analysis without other pretreatment, suggesting the extreme versatility of the approach. An intuitive extraction critical point judgment is attached great importance owing to the accurate quantification resulted from complete extraction. Herein, when the real-time concentration of the analyte was lower than 1% of the highest concentration or LOQ, it could be considered that the absolute extraction has been carried out (*e.g.*, 2.5−8.2 min for AT in Fig. 5a). Obviously, due to the extremely high sensitivity of oEESI-MS (LOQ, 0.003−0.049 ng/mL), only smaller amount of analyte (∼0.5 mg) and extraction

solvent (<240 μL) were needed for rapid extraction and determination. Since liquid−liquid extraction is easier to carry out, solvent was more efficient in extracting liquid samples and could be finished within 2−3 min (Supporting Information Fig. S2). Finally, all the 20 APCMs were online extracted completely and determined within 15 min, respectively. Fig. 5 presented the concentration curves of the nine targeted alkaloids obtained real-timely in XJC. All the analytes except HA in XJC were captured sensitively and BAC (*m/z* 604) presented the most amount with a highest concentration about 10 ng/mL at the beginning. No HA was detected in XJC (lower than 0.006 ng/mL) and other curves were highest at about 2.8 min and complete extracted at about 8 min with the signal returned to the baseline.

The content of the target analytes in the 20 APCMs were shown in Table 1. In almost all the oral medicine, monoester alkaloids (*e.g.*, BAC, BMA, and BHA) presented the most amounts, such as the total proportion was more than 70% in XJC. Diester alkaloids, such as AT, MAT and HAT, generally lower than 10% of the proportion owing to the high toxicity coupled with efficiency[24]. In the external use preparation (*e.g.*, SFL), there were most diester alkaloids contained (>80%), indicating that the diester alkaloids has low toxicity to humans when used externally. Above all, the oEESI-MS coupled with absolute extraction-total integration strategy established in this study overcame the key obstacles in traditional on-line extraction and quantification, featured absolute extraction and accurately quantification of the target analytes, which was expected to be used for absolute rapid quantitative analysis of analytes in various complex samples.
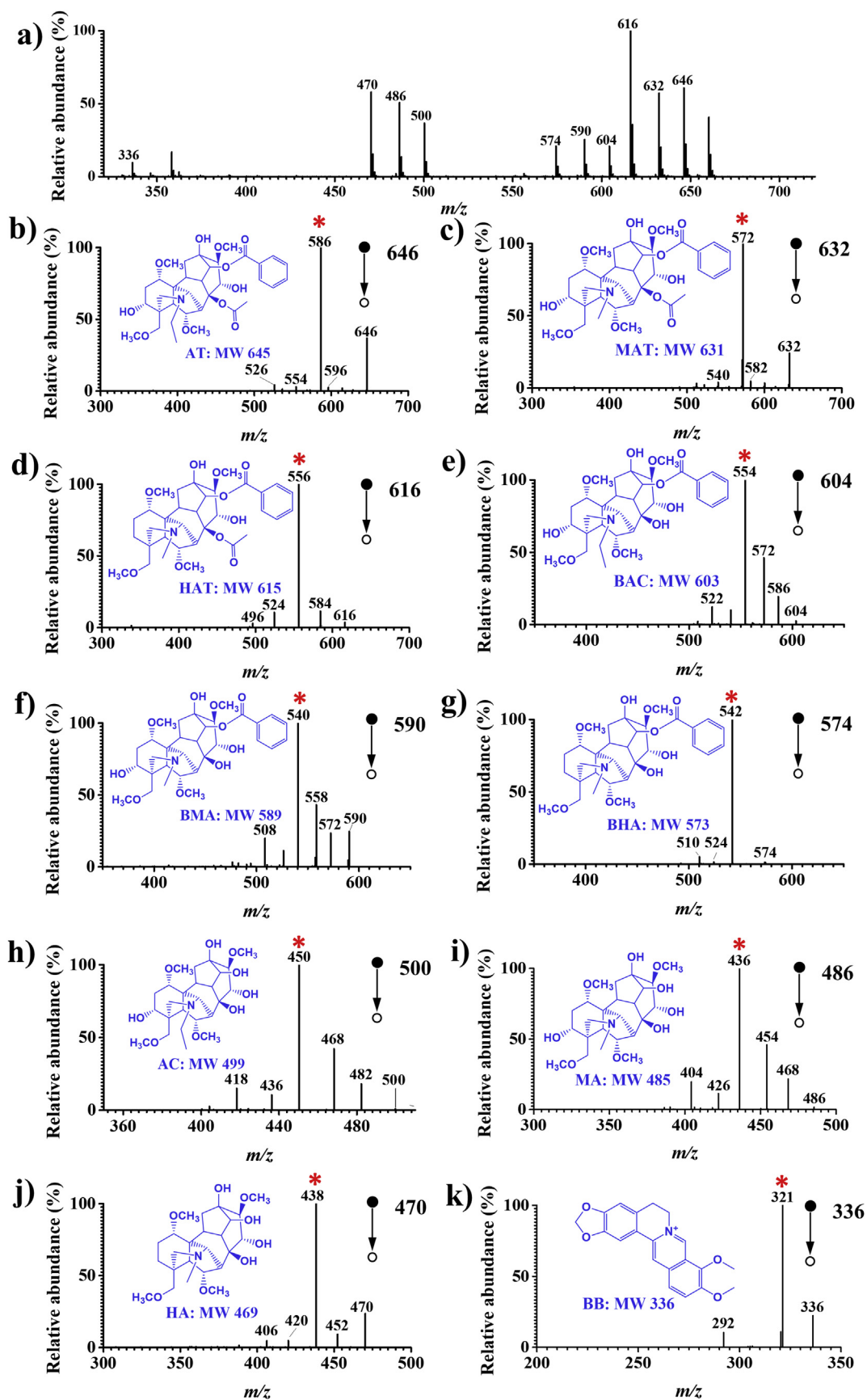
**Figure 4** MS spectra of targeted alkaloids in APCM recorded by oEESI-MS. Full scan of mixed standard solution (a). MS/MS spectrum of AT (b), MAT (c), HAT (d), BAC (e), BMA (f), BHA (g), AC (h), MA (i), HA (j), and BB (k).
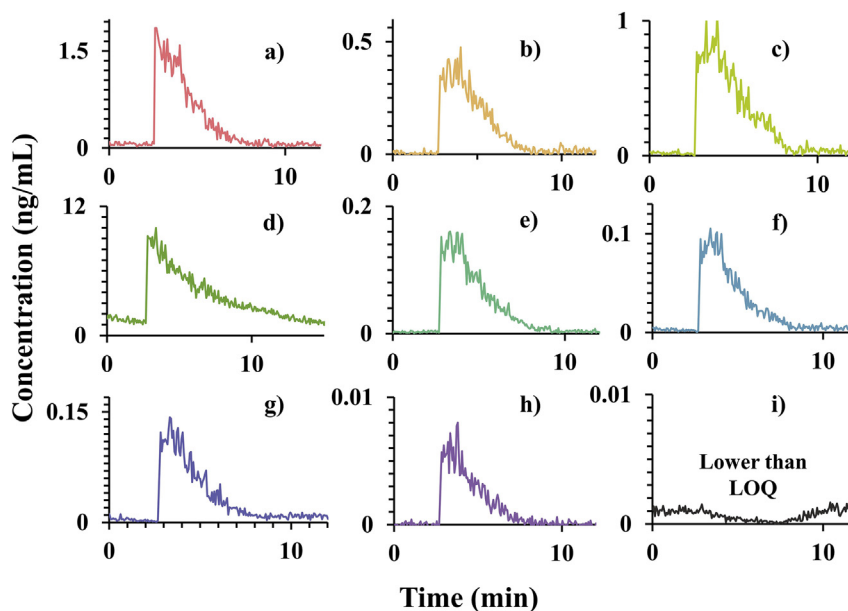
**Figure 5**  Real-time concentration curves of the nine targeted alkaloids in XJC recorded by oEESI-MS. AT (a), MAT (b), HAT (c), BAC (d), BMA (e), BHA (f), AC (g), MA (h), and HA (i).

### 3.4.  Method validation by UPLC−MS and ESI-MS

To validate the quantitative accuracy of oEESI-MS for practical analysis, traditional UPLC−MS and ESI-MS determination were performed on the 20 APCMs, respectively. The experimental process and conditions were detailed in Supporting Information, and the determination results for nine alkaloids in 20 APCMs were listed in Supporting Information Tables S5 and S6. To visually compare the accuracy of the data obtained by two methods, linear regression was performed on oEESI-MS and LC−MS, along with oEESI-MS and ESI-MS data, respectively (Fig. 6). In Fig. 6a, all the oEESI-MS data were set as the abscissas ($x$) and the corresponding UPLC−MS data were chose as the ordinate ($y$). Obviously, the smaller the difference in the $x$ and $y$ was, the more consistent the results of the two methods were. Fig. 6b was the linear regression result between oEESI-MS and ESI-MS. In general, the data obtained from oEESI-MS have good consistency with both UPLC−MS data and ESI-MS data, as the slopes were 0.9798 and 0.9662, respectively. This illustrated the accuracy of the whole data higher were than 96%. Only a small number of

**Table 1**  Quantitation results of the 9 aconitine-type alkaloids in 20 APCMs by oEESI-MS (ng/mg).

| APCM | Content (ng/mg) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | HA | MA | AC | BHA | BMA | BAC | HAT | MAT | AT |
| TDP | 0.05 | 0.13 | 0.18 | 0.19 | 0.23 | 0.61 | 0.04 | 0.01 | 14.41 |
| WBT | 0.03 | 0.11 | 0.14 | 0.79 | 1.42 | 0.71 | 0.07 | − | 0.08 |
| FGC | 0.15 | 3.79 | 0.69 | 14.74 | 67.24 | 9.39 | 0.61 | 0.15 | 0.28 |
| SLP | − | 0.07 | 0.04 | 0.68 | 3.37 | 1.26 | 0.60 | 0.87 | 1.09 |
| GLP | 0.02 | 0.10 | 0.06 | 1.00 | 4.98 | 1.86 | 0.89 | 1.29 | 1.62 |
| QQC | − | 0.19 | 0.07 | 1.62 | 5.41 | 1.54 | 0.25 | 0.12 | 0.72 |
| DHP | 0.02 | 0.04 | 0.01 | 0.19 | 0.49 | 0.10 | 0.04 | 0.02 | 0.23 |
| FSC | 0.96 | 3.35 | 0.76 | 34.52 | 52.95 | 27.01 | 3.41 | 1.34 | 1.39 |
| FXP | 0.04 | 0.14 | 0.56 | 2.23 | 6.37 | 41.63 | 0.64 | 0.31 | 2.34 |
| WBG | 0.05 | 0.19 | 0.07 | 1.14 | 2.94 | 0.67 | 0.23 | 0.05 | 0.18 |
| YXC | 0.42 | 1.71 | 0.26 | 2.26 | 6.46 | 0.76 | 1.19 | 0.32 | 3.92 |
| YSG | − | − | − | 0.08 | 0.24 | 0.05 | 0.02 | 0.02 | 0.08 |
| GPP | 0.21 | − | 0.01 | 0.21 | 0.23 | 0.16 | 0.22 | 0.05 | 0.24 |
| FLP | 0.20 | 0.15 | 0.69 | 0.76 | 1.85 | 2.65 | 0.28 | 0.10 | 5.61 |
| SJP | 2.94 | 0.35 | 1.90 | 0.36 | 6.35 | 17.54 | 0.33 | 0.06 | 0.03 |
| GCP | − | 0.09 | 0.18 | 1.38 | 1.79 | 2.37 | 0.29 | 0.08 | 0.46 |
| XJC | − | 0.03 | 0.85 | 0.67 | 1.10 | 112.09 | 0.11 | 0.05 | 0.11 |
| XBP | 0.07 | 0.43 | 0.10 | 2.14 | 11.43 | 3.03 | 0.55 | 0.10 | 0.06 |
| YHC | 0.10 | 0.07 | 0.36 | 0.82 | 1.17 | 25.02 | 0.70 | 0.25 | 3.47 |
| SFL | 0.05 | 0.02 | 0.05 | 0.09 | 0.04 | 0.16 | 0.73 | 0.20 | 0.27 |

−Not detected.
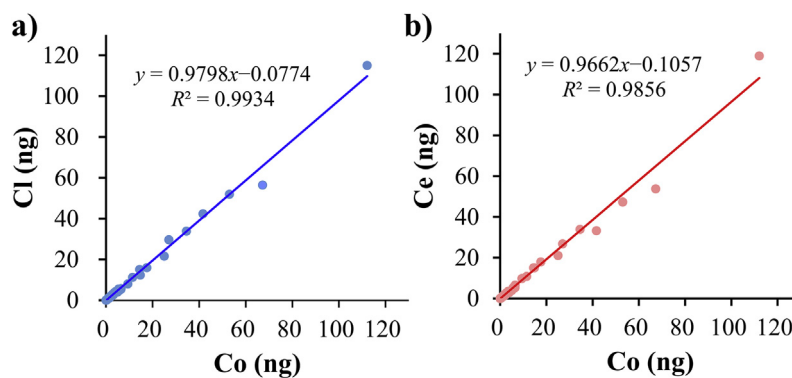
**Figure 6** Linear regression performed on oEESI-MS, UPLC−MS and ESI-MS data. (a) oEESI-MS and UPLC−MS data, (b) oEESI-MS and ESI-MS data. Co was the content determined by oEESI-MS, Cl was the content determined by UPLC−MS, and Ce was the content determined by ESI-MS.

high concentration points deviated from the regression line, such as BAC in XJC (112.09 ng/mg determined by oEESI-MS, and 118.99 ng/mg determined by ESI-MS), but the error was still within the allowable range (<10%). Obviously, UPLC−MS and oEESI-MS have better consistency, which may come from the smaller error compared to the relatively strong ion suppression of direct ESI-MS at high concentrations.

The traditional extraction method requires a large amount of solvent for repeated extraction, which easily leads to the dilution of trace components and lose of chemical information in the sample. In contrast to the traditional deficient extraction method, while oEESI-MS only need a small amount of solvent and analyzed rapidly in real time. In addition, the whole process curve integral quantification was adopted to avoid large system deviation caused by instantaneous fluctuation of MS. Therefore, it can be seen from the table that oEESI-MS had higher sensitivity than ESI-MS and UPLC−MS as more analytes were determined (e.g.,

MAT in TDP, and HA in GLP). Moreover, the sum time of the pretreatment process and detection of ESI-MS and UPLC−MS for a sample was more than 60 and 90 min in this study, which were approximately 4 and 6 times of oEESI-MS (<15 min). Due to repeated extraction, filtration/centrifugation, transfer, etc., the process was extremely complicated and the sample and solvent consumption were much higher.

### 3.5. Toxic aconitines limitation standard of Aconitum drugs based on HWT

Quality control and evaluation of TCM is one of the key obstacles limit the modernization progress[25]. For the Aconitum herb medicines, various toxicity evaluation and quality control strategy have been proposed, such as toxic constituents index (TCI)[26], bioassay-based toxicity evaluation[27], and premature ventricular contractions-based biological assess methods[28]. However, for

**Table 2** Limitation standard and toxic level of the 20 APCMs.

| APCM | HM | SM (g) | DM (g) | LM (mg) | LP | HWT | LH | TL | TR |
|------|-----|--------|--------|---------|------|-------|---------|------|-----|
| TDP | Zhicaowu, Zhifuzi | 4 | 8 | 1.35 | 11.67 | 0.1258 | 7.95 | **** | 2 times, safe[30] |
| WBT | Heishunpian | 2 | 6 | 1.50 | 1666.67 | 0.0007 | 1499.46 | * | Nontoxic[31] |
| FGC | Zhifuzi, Zhichuanwu | 2 | 6 | 1.40 | 224.36 | 0.0045 | 221.54 | ** | 130 times, lethal[32] |
| SLP | Zhifuzi | 12 | 24 | 1.50 | 24.41 | 0.0666 | 15.02 | **** | 11 times, nontoxic[33] |
| GLP | Zhifuzi | 9 | 18 | 1.50 | 21.93 | 0.0741 | 13.49 | **** | — |
| QQC | Heishunpian | 1.2 | 3.6 | 1.50 | 382.26 | 0.0038 | 261.93 | ** | Nontoxic[34] |
| DHP | Zhicaowu | 3.5 | 7 | 1.20 | 591.13 | 0.0021 | 482.61 | ** | 40 times, safe[35] |
| FSC | Zhichuanwu, Zhicaowu | 1.2 | 2.4 | 1.20 | 81.43 | 0.0115 | 86.62 | *** | 60 times, toxic[36] |
| FXP | Zhichuanwu, Zhicaowu | 6 | 12 | 1.20 | 30.40 | 0.0391 | 25.56 | **** | 7.25 times, lethal[37] |
| WBG | Heishunpian | 6 | 18 | 1.50 | 181.16 | 0.0064 | 156.80 | *** | Nontoxic[31] |
| YXC | Zhicaowu | 0.6 | 1.8 | 1.20 | 122.77 | 0.0093 | 107.12 | *** | 16 times, safe[38] |
| YSG | Zhifuzi | 20 | 60 | 1.50 | 208.33 | 0.0075 | 132.54 | *** | — |
| GPP | Caowu, Chuanwu | — | — | — | — | — | — | — | — |
| FLP | Zhifuzi | 9 | 18 | 1.20 | 11.13 | 0.1146 | 8.73 | **** | 25 times, lethal[39] |
| SJP | Caowu, Chuanwu, Fuzi | 5 | 10 | — | — | 0.0088 | 114.15 | *** | — |
| GCP | Zhicaowu, Zhichuanwu | 9 | 18 | 1.20 | 80.32 | 0.0131 | 76.36 | *** | — |
| XJC | Zhicaowu | 3 | 6 | 1.20 | 740.74 | 0.0014 | 703.49 | ** | — |
| XBP | Fuzi | 0.36 | 1.08 | 1.50 | 1956.18 | 0.0004 | 2243.56 | * | — |
| YHC | Zhicaowu | 0.75 | 2.25 | 1.20 | 120.66 | 0.0099 | 100.63 | *** | 48 times, toxic[29] |
| SFL | Fuzi | — | — | — | — | — | — | — | — |

HM is the Aconitum herbs in prescription of each drug. SM and DM are the single and daily oral maximum dose, respectively. LM is the toxic dose of total diester alkaloids based on Chinese Pharmacopoeia. TL is the toxicity level, ****represents high toxicity, ***represents low toxicity, **represents quite little toxicity, and *represents no toxicity. TR is the toxicity reported in literature. −Not applicable.

various APCMs, no uniform and reliable limitation standard of toxic components is proposed before. At present, toxicity was commonly evaluated by the total amount of three main diester alkaloids according to *Chinese Pharmacopoeia*. This strategy neglected the toxicity difference among the three alkaloids[26], which may present potential risks in clinics. Hence, we established a novel HWT-based and *Chinese Pharmacopoeia*-based calculation method to evaluate the toxicity of the 18 oral APCMs, and the limitation standard was proposed accordingly (Table 2).

In general, all drugs were safe at the dosage recommended, but the toxicity was varied. TDP and FLP had strong toxicity, and the poisoning doses were only 7.95 and 8.72 times of the prescribed dose, respectively. WBT and XBP were almost non-toxic, and the toxic doses were more than 1000 and 2000 times of the prescribed dose, respectively. The calculation results of LP and LH were close on the whole, but some still showed a large difference. For example, the toxic dose of YHC is 100.63 times of clinical dose based on LH while 120.66 times based on LP, the difference may come from the high proportion of stronger toxic diester alkaloids, such as AT and MAT. Thus LH based on HWT took the difference of toxicity among multi-component into account, which remarkably increased the accuracy of evaluation on the whole toxicity of APCM. It was reported that 48 times of prescribed dose could cause a toxic reaction[29], which was closer to LH results compared to LP, reveling the higher accuracy of LH for toxicity evaluation. Notably, for row *Aconitum* herb medicines, such as Sheng-chuanwu, Shengcaowu, and Shengfuzi, no clinical dosage limit was recorded in *Chinese Pharmacopoeia*, so when they are included in the APCM prescription (*e.g.*, SJP), the toxicity cannot be calculated based on LP. Hence, LH is more versatile than LP due to the neglect of different herbal materials and only focused on three toxic diester alkaloids.

Accordingly, the toxicity of APCM was divided into four levels. The first level is whose toxic dose within 30 times of prescribed dose (LH < 30), represented by TDP, GLP, and FLP, which possess high toxicity thus extra attention should be paid to the specification in clinical. The second level is whose toxic dose ranges from 30 to 200 times (30 < LH < 200), such as FSC, GCP with a low toxicity. The third level is whose toxic dose ranges from 200 to 1000 times (200 < LH < 1000), which means quite little toxicity. The fourth level is whose toxic dose beyond 1000 times of prescribed dose (LH > 1000), which can be considered as nontoxic like WBT, XBP. Further attention should be paid on that the toxicity of APCM is not limited to aconitines. For example, in YHC, there are other toxic herbal materials in the prescription, such as *Dactylicapnos scandens*, *Rhizoma paridis*, and *Psammosilene tunicoides*, so the reported toxic dose (48 times) was lower than calculated (100.63 times). In summary, novel uniform toxicity limitation standard for APCM was proposed based on HWT and *Chinese Pharmacopoeia*. Combined with the rapid and accurate quantitative strategy of APCM, a safer medication guidance for APCM was proposed, which may contribute to more reasonable and safer use of APCM and the toxicity evaluation of other drugs.

## 4. Conclusions

The distinctive feature of the presented toxicity limitation standard filled in the blank of the toxicity limit of APCM, and provided important basis for the rational clinical use and quality control of APCM. The HWT-based limitation standard could predict the toxicity of drugs more accurately, which was expected to have a wide range of application in other toxic drugs. The developed ambient oEESI-MS strategy allows precise molecular quantification of analytes in complex samples universally. For the first time, an absolute extraction-total integration strategy was used to achieve rapid on-line quantitative analysis of target components in complex solid and liquid samples accurately. Nine aconitone-type alkaloids in 20 APCMs with multi-type preparations such as tablets, capsules, pills, powders, granules, and liquid preparations, were successfully quantitatively determined by oEESI-MS. The linear range of detection was about 0.051−4000 ng/mL ($R^2$ > 0.9960) and limit-of-detection varied from 0.001 to 0.015 ng/mL. No significant matrix interference (>90%) was observed in the oEESI-MS strategy with high precision (RSD<8.0%) and accuracy (RSD<7.5%). The analytical results were validated by ESI-MS and UPLC−MS, showing a higher accuracy and sensitivity of oEESI-MS. The strategy greatly expanded the quantitative analysis capability of ambient MS, which is commonly required in pharmaceutical analysis, food safety control, public security, and many other disciplines.

## Author contributions

Zi-Dong Qiu, Xu-Ya Wei, and Rui-Qi Sun performed the experiment and wrote the manuscript; Jin-Long Chen, Ting Tan, and Guang-Hong Cui analyzed the data; Jia-Quan Xu, Tong Chen, and Juan Guo contributed to writing; Lu-Qi Huang and Chang-Jiang-Sheng Lai designed this work.

## Conflicts of interest

The authors have no conflicts of interest to declare.

## Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2019.12.009.

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