Universiteit Leiden
The Netherlands

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Alkaloid profiling of the Chinese herbal medicine Fuzi by combination of matrix-assisted laser desorption ionization mass spectrometry with liquid chromatography–mass spectrometry

Jiangshan Wang[a,b], Rob van der Heijden[b,*], Gerwin Spijksma[b], Theo Reijmers[b], Mei Wang[c], Guowang Xu[a], Thomas Hankemeier[b], Jan van der Greef[b,c]

[a] Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 116023 Dalian, China
[b] Division of Analytical Biosciences, Leiden Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands
[c] TNO, Quality of Life and SU BioMedicine, Utrechtseweg 48, 3700 AJ Zeist, The Netherlands

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ABSTRACT

A matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) method was developed for the high throughput and robust qualitative profiling of alkaloids in Fuzi—the processed lateral roots of the Chinese herbal medicine *Aconitum carmichaeli* Debx (*A. carmichaeli*). After optimization, powdered roots – without any further sample preparation – could be used to screen for the presence of *Aconitum* alkaloids. Furthermore, the semi-quantitative potential of MALDI-MS was confirmed using liquid chromatography–mass spectrometry (LC–MS) as reference. In total over sixty alkaloids were detected by LC–MS and fifteen of them were tentatively identified. Both MALDI-MS and LC–MS analysis revealed significant variation in alkaloid content in different (commercial) samples. LC–MS analysis of three toxic alkaloids in 14 batches of Fuzi resulted in a variation of their concentrations expressed as RSDs of 138%, 99% and 221% for aconitine, hypaconitine and mesaconitine, respectively. The variation in concentrations (expressed as RSD) of about the ninety constituents detected were classified as follows: 13 constituents showed an RSD of 77–100%, 46 with an RSD of 100–150%, 21 with an RSD of 150–200% and 9 constituents with an RSD in concentration of 200–235%. These results demonstrate a strong difference in chemical composition of the various Fuzi and illustrate the necessity of adequate QA/QC procedures for both safety and efficiency of herbal medicine. The described analytical procedures for alkaloid profiling could play a role in these procedures.

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1. Introduction

The root of *Aconitum carmichaeli* Debx (*A. carmichaeli*) is widely used in traditional Chinese medicine (TCM) as an analgesic and cardiotonic herbal medicine. However, it is also a toxic herbal medicine because of the presence of diterpene alkaloids [1–5]. These alkaloids share a common C19 norditerpenoid skeleton and are divided into four types (Table 1), named as non-ester alkaloids (NEAs), monoester-diterpenoid aconitines (MDAs), diester-diterpenoid aconitines (DDAs) and lipoalkaloids [3–6]. The diversity of chemical structures leads to different biological effects. DDAs, mainly, aconitine, hypaconitine and mesaconitine, are well known toxic alkaloids [7].

The adverse reactions caused by TCM may originate from adulteration, misidentification, variability in the amount of active ingredients, and improper processing and preparation [1]. Therefore, the quality control of herbal drugs must at least be performed at two levels. The first level is to minimize adulteration and misidentification. For this, robust methods for identification of the herb, herb preparations and individual constituents are necessary. The second level aims at minimizing the variability of active ingredients to ensure repeatable and reproducible therapeutic effects. At this level quantitative analytical methods are necessary to monitor the variation in herbal medicines during processing and preparation. Processing of TCM herbs comprises the techniques used for the preparation of Chinese raw herbal medicine, including cleaning, drying, steaming and frying, etc., whereas preparation of TCM drugs is referred as making multi-herbal dosage forms including, decoction, tablet, capsula, etc. To guarantee the safety and efficacy of use of *Aconitum* type of herbal medicine, the first prerequisite is to apply comprehensive analytical methods to monitor a large set of active ingredients, i.e. mainly alkaloids.

Fuzi (the processed lateral root of *A. carmichaeli*) needs proper processing in order to reduce the amount of toxic alkaloids. How-

* Corresponding author. Tel.: +31 71 527 4320; fax: +31 71 527 4227.
E-mail address: heijden@lacdr.leidenuniv.nl (R. van der Heijden).

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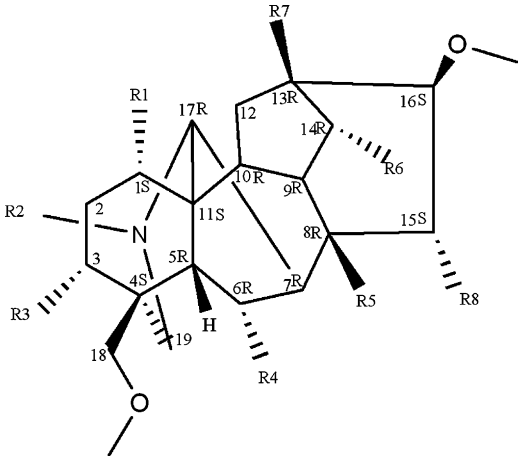
Table 1
Aconitum alkaloids identified by accurate mass and MS/MS fragmentation pattern.



Compound	Formula	Calculated $[M+H]^+$	Observed $[M+H]^+$	R1	R2	R3	R4	R5	R6	R7	R8
Talatizidine (TD)	$C_{23}H_{37}NO_5$	408.2744	408.2741	OH	C_2H_5	H	H	OH	OH	H	H
Talatisamine (TS)	$C_{24}H_{39}NO_5$	422.2901	422.2884	OCH_3	C_2H_5	H	H	OH	OH	H	H
Neoline (NL)	$C_{24}H_{39}NO_6$	438.2850	438.2843	OH	C_2H_5	H	OCH_3	OH	OH	H	H
Fuziline (FL)	$C_{24}H_{39}NO_7$	454.2799	454.2790	OH	C_2H_5	H	OCH_3	OH	OH	H	OH
14-Acetyl-talatisamine (AT)	$C_{26}H_{41}NO_6$	464.3007	464.3005	OCH_3	C_2H_5	H	H	OH	OAc	H	H
Benzoylhypaconine (BH)	$C_{31}H_{43}O_9N$	574.3010	574.2992	OCH_3	CH_3	H	OCH_3	OH	OBz	OH	OH
Benzoyldeoxyaconine (BD)	$C_{32}H_{45}O_9N$	588.3167	588.3144	OCH_3	C_2H_5	H	OCH_3	OH	OBz	OH	OH
Benzoylmesaconine (BM)	$C_{31}H_{43}O_{10}N$	590.2960	590.2913	OCH_3	CH_3	OH	OCH_3	OH	OBz	OH	OH
Benzoylaconitine (BA)	$C_{32}H_{45}O_{10}N$	604.3116	604.3115	OCH_3	C_2H_5	OH	OCH_3	OH	OBz	OH	OH
Hypaconitine (HA)	$C_{33}H_{45}O10N$	616.3116	616.3121	OCH_3	CH_3	H	OCH_3	OAc	OBz	OH	OH
Deoxyaconitine (DA)	$C_{34}H_{47}O_{10}N$	630.3273	630.3258	OCH_3	C_2H_5	H	OCH_3	OAc	OBz	OH	OH
Mesaconitine (MA)	$C_{33}H_{45}O_{11}N$	632.3065	632.3092	OCH_3	CH_3	OH	OCH_3	OAc	OBz	OH	OH
Aconitine (AC)	$C_{34}H_{47}O_{11}N$	646.3222	646.3222	OCH_3	C_2H_5	OH	OCH_3	OAc	OBz	OH	OH
8-Pal-benzoylhypaconine	$C_{47}H_{73}O_{10}N$	812.5307	812.5283	OCH_3	CH_3	H	OCH_3	OPal	OBz	OH	OH
8-Lino-14-benzoylhypaconine	$C_{49}H_{73}O_{10}N$	836.5307	836.5245	OCH_3	CH_3	H	OCH_3	Olino	OBz	OH	OH

Accurate masses are determined by DI-ESI-MS with internal mass calibration with aconitine (646.3222 $[M+H]^+$) and codeine (300.1594 $[M+H]^+$). OAc, CH_3COO-; OBz, C_6H_5COO; Pal, palmitic acid; Lino, linoleic acids. NL and FL have isomers according to LC–MS analysis.

ever, a large variation in alkaloid content remains, having significant impact on the safety and efficacy of the herbal drug. This is because of the lack of standardized and validated procedures for processing. Until now, the official quality control criteria adopted by the Chinese Pharmacopoeia (2005 edition) are limited with respect to test for aconitine in Fuzi by the use of TLC [8]. Obviously, that is not sufficient to guarantee its quality.

For the purposes of quality control, forensic medicine and therapeutic drug monitoring of the herbal medicines from genus *Aconitum*, many methods have been developed including qualitative and quantitative methods. The advantages of matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and direct-infusion electrospray ionization (DI-ESI-MS) are their high throughput and abilities in identification of components. Therefore, these methods are in principle suitable for the fast profiling of *Aconitum* alkaloids [3–6]. Quantitative methods for *Aconitum* alkaloids focus on the toxic ingredients aconitine, mesaconitine and hypaconitine, in the *A. carmichaeli* roots or preparations. These methods include among others high-performance liquid chromatography (HPLC) [9–11], capillary electrophoresis (CE) [12–14], and gas chromatography–mass spectrometry (GC–MS) [15]. *Aconitum* alkaloids in body fluids and tissues have also been investigated by GC–MS and LC–MS methods [16–21]. In this study, a MALDI-MS based method was developed as a robust and high-throughput technique for the profiling of *Aconitum* alkaloids. Furthermore its quantitative potential was investigated with LC–MS analysis as reference. The high throughput and robust MALDI-MS and LC–MS based profiling method can be used as a complement to other GC, HPLC, and nuclear magnetic resonance (NMR) [22] based quantitative methods for the QA/QC of TCM.

2. Experimental

2.1. Materials: biologicals

In total 14 batches of Fuzi were obtained from different local drug stores in different Chinese provinces (Liaoning, Sichuan, Jiangsu and Hubei). Liuwei Dihuang Pills (preparation contains six herbs, *Rehmanniae*, *Cornus*, *Cortex Moutan*, *Dioscorea opposita* Thunb, *Poria cocos* and *Rhizoma Alismatis*) were purchased form Tai-ji Pharmaceutical Company (Chongqing, China). The preparation used in this study is a Fuzi contained formula Si Ni Tang (homemade). Si Ni Tang contains Fuzi, roots of *Glycyrrhiza uralensis* Fisch. and dry ginger roots and was prepared according to the formula of Chinese Pharmacopoeia [8]. In short, the above mentioned three herbs were weighed (3 g:3 g:2 g, respectively) and boiled twice (the first was for 2 h, the second was for 1.5 h) with 100 mL of water. The decoctions were filtered with absorbent cotton, combined and freeze-dried until dryness.

2.2. Materials: chemicals and reagents

Acetonitrile (ACN), methanol and formic acid (FA) were of Ultra Liquid Chromatography/MS grade (BIOSOLVE, Valkenswaard, The Netherlands). Chloroform was HPLC grade (BIOSOLVE, Valkenswaard, The Netherlands). Trifluoroacetic acid (TFA) was analytical grade (BIOSOLVE, Valkenswaard, The Netherlands). Water was prepared using a Millipore (Billerica, MA, USA) water purification system. Aconitine (the purity claimed by the producer was 95.0% determined by HPLC), atropine sulphate, codeine, scopolamine hydrochloride, reserpiline hydrochloride, sodium chloride,

glucose, starch, methylcellulose, polyethylene glycol 6000 (PEG 6000) were from Sigma (St. Louis, MO, USA). The MALDI matrixes sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Laser Biolab (Sophia-Antipolis Cedex, France). Matrix solutions were DHB, SA or CHCA at 10 mg/mL in acetonitrile/water (1:1, v/v) containing 0.1% (v/v) TFA, or in acetonitrile (direct analysis of powdered samples) containing 0.1% (v/v) TFA.

2.3. LC–MS system

The chromatography was carried out using a Surveyor Plus HPLC System (consisting of vacuum degasser, autosampler and a quaternary pump) (Thermo Fisher Scientific, USA) equipped with a reversed-phase C18 analytical column, 150 mm × 2 mm, 5 μm particle size, ODS-3 (Varian, USA). Column temperature was maintained at 35 °C and the sample tray was kept at 8 °C.

Mobile phases A and B were 0.1% formic acid/acetonitrile (10:1) and acetonitrile, respectively. Gradient elution was as follows: isocratic elution for 1 min at 100% of solvent A, then a linear gradient to 50% solvent B in 10 min, after that, a linear gradient from 50% to 98% B in 2 min and kept constant for 5 min. Next, the solvent was returned to 100% A and the system equilibrated for 3 min. The flow-rate was 0.2 mL/min, and the sample volume injection was 20 μL.

The HPLC system was interfaced to a quadrupole-time-of-flight mass spectrometer (QSTAR Pulsar i, PE/Sciex, Canada) equipped with an electrospray interface operating in the positive ion mode, using the following parameters: capillary voltage, 3500 V; nebulizer pressure, 40 psi; drying gas, 60 psi; gas temperature, 350 °C; FP voltage, 220 V; DP voltage, 35 V; collision gas, N_2. Mass spectra were recorded within the range m/z 100–900. Data acquisition and processing were carried out using Analyst QS 1.1 software (PE/Sciex). The raw data were analyzed using Impress 1.14/Winlin 2.4 (TNO Quality of Life, Zeist) [23,24], and the software allows detection and retention time alignment of the peaks eluting in the chromatograms. The following settings were applied: minimum signal to noise ratio, 2; window (the tolerable retention time shift), 3; minimum peak height, 100.

2.4. MALDI-MS analysis

A MALDI-TOF mass spectrometer (Biflex III, Bruker Daltonics, Germany) equipped with a 50 Hz nitrogen laser (337 nm) was used in this research. Mass spectra were recorded in the positive ion reflectron mode. The accelerating voltage was 19 kV and the laser power was 31%. FlexControl software (Bruker Daltonics) was used to operate the mass spectrometer. Mass spectra were recorded by accumulating 50 shots on 20 different positions on each sample spot. Three replicates of each sample were measured. FlexAnalysis (Bruker Daltonics) was used to process the raw mass spectra in batch mode and to pick all peaks for a m/z 90–1000 range. Peak picking was performed using the "SNAP" peak picking algorithm. The S/N threshold was set at 6 for the SNAP peak picking algorithm.

2.5. Preparation of standard solutions

Aconitine was accurately weighed and dissolved in chloroform to produce a 0.25 mg/mL stock solution and was stored at 4 °C. Before use, the stock solution was diluted with ACN to 10 μg/mL. For the LC–MS method, calibration curves were obtained using a range of 2 ng/mL to 1 μg/mL by further dilution with mobile phase A. For the MALDI-MS method, calibration curves were established in a range of 0.2–2 μg/mL by further dilution with ACN. Atropine sulphate, codeine, scopolamine HCl and reserpiline HCl were used as internal standards for the MALDI-MS method and as test compounds for the validation, and accurately weighed and diluted with a mixture of water and ACN (70:30, v/v) to 1 mg/mL as stock solutions.

2.6. Method validation

The validation of the LC–MS method was carried out using serial diluted sample extracts and the aconitine chemical standards. The original sample extract was a mixture of extracts obtained from three different batches of Fuzi with 50% aqueous ACN as extraction solvent with a high sample mass to solvent volume ratio (1 g to 5 mL) and using ultrasonication. Linearity was evaluated with aconitine solutions (2, 4, 8, 10, 20, 40, 80, 100, 200, 400, 800, 1000 ng/mL) and with sample extracts, which were obtained by diluting the original sample extract with mobile phase A with varying dilution factors from 1:6.25 to 1:625 times (6.25, 12.5, 31.25, 62.5, 156.25, 312.5 and 625 times, respectively). The precision of retention times of aconitine was investigated in the range of 2–1000 ng/mL. The intra-day and inter-day precisions of peak areas of multiple injections of the 100 ng/mL aconitine standard solutions stored at different temperatures (room temperature, 4 °C and −20 °C) were determined during 3 consecutive days. For the investigation of the effects of ion suppression, atropine, scopolamine, codeine and reserpiline were spiked at a constant concentration (0.8, 0.2, 0.6 and 0.4 μg/ml, respectively) to the serially diluted sample extracts and mobile phase A (used as blank).

Sodium chloride, glucose, starch, methylcellulose, PEG 6000 and Liuwei Dihuang Pills were used to evaluate the robustness of the MALDI-MS against the interferences of mimic concomitant excipients and other herbs. For this purpose, 0.1 g of the above mentioned components were mixed with 0.5 mL undiluted sample extracts (acetonitrile as extract solvent) and ultrasonicated for 30 min and then centrifuged. The supernatants were used for MALDI-MS analysis and each sample was analyzed 3 times. Linearity was evaluated with serial diluted standard aconitine solutions in acetonitrile (0.2, 0.4, 0.6, 0.8, 1, 1.5, and 2 μg/mL) and equal volume internal standard solution (1 μg/mL mixture of atropine, codeine, scopolamine and reserpiline in acetonitrile) added to the above mentioned supernatants. An aconitine standard solution of 2 μg/mL in acetonitrile was used to investigate spot to spot repeatability of MALDI-MS.

2.7. Sample extraction optimization

Eight solvent mixtures (nos. 1–8) were tested for extraction: 1, water:ACN = 3:1; 2, water:ACN = 1:1; 3, ACN; 4, 1% TFA in 50% aqueous ACN; 5, methanol:chloroform = 3:1; 6, methanol:chloroform = 1:1; 7, chloroform; 8, 1% TFA in methanol:chloroform = 1:1. Dried processed roots were ground with pestle and mortar to powder, 0.1 g were weighted and extracted by ultrasonication for 1 h with 1.5 mL the above mentioned solvent mixtures. After extraction, the extracts were centrifuged at 9500 × g for 5 min. The supernatants of 1–4 were diluted with mobile phase A prior to injection into the HPLC. The supernatants of 5–8 were dried under a stream of nitrogen gas, and the residues were redissolved in 50% ACN with 1% TFA and then diluted with mobile phase A prior to injection into the HPLC. For MALDI-MS, after extraction, the supernatants were diluted with acetonitrile and internal standards added.

For evaluation of the extraction efficiency, 19 peaks with different retention times in the LC–MS system were selected across the whole chromatogram. These were peaks with m/z and retention times 342.3 (5.9 min), 408.3 (2.8 min), 422.3 (6.2 min), 454.3 (4.2 min), 464.3 (7.2 min), 466.3 (6.6 min), 486.3 (2.4 min), 500.3 (3.1 min), 530.3 (9.3 min), 558.3 (9.1 min), 570.3 (9.9 min), 574.3 (8.8 min), 588.3 (8.2 min), 590.3 (8.6 min), 604.3 (9.8 min) 616.4 (7.9 min), 620.4 (10.7 min), 700.4 (10.0 min) and 836.6 (14.0 min).

2.8. Sample preparation and data correlation

In the final method, 0.1 g of pulverized powder of Fuzi (or preparation) was extracted with 1.5 mL of ACN at room temperature for 60 min using ultrasonication and the extracts were centrifuged at 9500 × *g* for 5 min. The supernatants were diluted 10 times with mobile phase A prior to injection into the LC–MS. For MALDI-MS analysis, 100 μL ACN was added to 100 μL of the supernatant; of that mixture an aliquot of 100 μL was mixed with 100 μL internal standards solution (10 μg/mL). The samples solutions were mixed with an equal volume of matrix solution, and 0.5 μL of that mixture was applied to the sample plate for MALDI-MS analysis. For accurate mass determination and MS/MS, samples were extracted with 50% ACN/water and diluted with mobile phase A for direct infusion at a flow-rate of 10 μL/min. Codeine and aconitine were used as internal standards for mass calibration of the quadrupole-time-of-flight mass spectrometer. The correlation coefficient of data from LC–MS and MALDI-MS was calculated with Eq. (1).

$$\text{Correl}(X, Y) = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}} \quad (1)$$

where *x* and *y* are the dataset from HPLC–MS and MALDI-MS.

3. Results and discussion

3.1. Identification of Aconitum alkaloids by direct infusion ESI-MS/MS

By direct infusion, a number of alkaloids were tentatively identified by determination of their accurate mass and MS/MS fragmentation (Table 1). Using internal calibrants (aconitine and codeine were added to the extract) the mass spectrometer delivered mass accuracies with a relative error of 5 ppm. From these accurate masses the elemental composition of the alkaloids were deduced. In positive ion mode aconitine is observed as a protonated molecular ion ($[M+H]^+$) at *m*/*z* 646.3 [5,6]. Typical MS/MS spectra of a lipoalkaloid (*m*/*z* 836.6) and a DDA (*m*/*z* 646.3) are shown in Fig. 1.

The fragmentation pathway of *Aconitum* alkaloids studied by multistage ion trap MS was reported [6]. For low-energy CID, the first step is the elimination of the C8-substituent acetic acid or fatty acid. Then successive losses of CH_3OH, H_2O, CO, benzoic acid and a CH_3 or C_2H_5 (N-substituents) radical can be observed. The CID energy of our mass spectrometer was higher and activated many fragmentation pathways simultaneously; losses of CH_3OH, H_2O, CO, benzoic acid and their combinations were observed. In addition, the ion with *m*/*z* 105 represents the benzoyl cation $[C_7H_5O]^+$, which indicated the presence of a benzoyl group and can be regarded as a diagnostic ion of lipoalkaloids, MDAs and DDAs.

3.2. Profiling of Aconitum constituents by LC–MS and MALDI-MS

The further characterization of the constituents of Fuzi was carried out using LC–MS. Typical chromatograms of a Fuzi extract and the Fuzi containing formula—Si Ni Tang are shown in Fig. 2A and B. For *Aconitum* alkaloids, the increase in molecular weight is generally accompanied with an increased hydrophobicity. Accordingly, the retention time and the molecular mass seem to be correlated. Over sixty peaks could be detected in a single run, of which the majority is considered (based on the nitrogen rule) as alkaloidal. The chromatographic peaks could roughly be divided into three clusters, the retention time below 8 min with M_r 400–500, between 8 and 12 min with M_r 500–800 and above 12 min with M_r above 800. The results also showed that there are several isobaric compounds separated chromatographically; for example at *m*/*z* 454.2 and 422.2 (Fig. 2A).

In a MALDI-MS spectrum of a Fuzi extract using DHB as matrix, most mass peaks above *m*/*z* 400 can be assigned to alkaloids; at *m*/*z* > 800 the lipoalkaloids are showing up, at *m*/*z* 500–800 the DDAs and MDAs are detected, and at *m*/*z* 400–500 various alkaloids are found such as tatatisamine (*m*/*z* 422.3) and neoline (*m*/*z* 438.3, Fig. 3). Aconitine was clearly detected at *m*/*z* 646.3 ($[M+H]^+$). Mass peaks originating from the matrix are found below *m*/*z* 400. Peak assignment based on *m*/*z* of $[M+H]^+$ has been reported previously both with MALDI-MS and ESI-MS for *Aconitum* alkaloids [3–6]. Based on external calibration a mass accuracy of 60 ppm was achieved.

3.3. Validation of LC–MS assay

Target analysis of some *Aconitum* alkaloids from *A. carmichaeli* and biofluids by reversed-phase chromatography using isocratic and gradient elution has been reported [9–11,16,18–21]. The pH of the mobile phase varied from acidic to above 9. Considering potential column degradation at high pH and the detection sensitivity by mass spectrometry, a gradient using ACN and 0.1% formic acid was chosen for our method. After gradient optimization, the total analysis time was 20 min including column re-equilibration. This method was further characterized.

Degradation was observed for aconitine standard solutions stored at room temperature and 4 °C with a 10% and 5% decrease in signals compared with that stored at −20 °C, respectively. Inevitably, the degradation has impact on the inter-day precisions of the peak areas for consecutive 3 days, which are 7.5% (sample stored at room temperature), 5.5% (4 °C) and 4.3% (−20 °C), respectively. The intra-day precision was 2.4% (day 1). The results showed that samples should be stored at −20 °C to obtain good stability and inter-day precision.

The linear regression coefficient (R^2) of a twelve-point calibration curve for a aconitine standard solution over the concentration range of 2–1000 ng/mL was satisfactory, i.e. $R^2 = 0.9965$. For 16 arbitrarily selected endogenous *Aconitum* alkaloids calibration curves were obtained by diluting sample extracts from a dilution factor of 6.25–625. The R^2 for all alkaloids for that range were better than 0.99 for 7 selected *Aconitum* alkaloids. From a dilution factor of 12.5–625, 6 *Aconitum* alkaloids can have a R^2 better than 0.99 and from a dilution factor from 25 to 625, 3 *Aconitum* alkaloids can have a R^2 better than 0.99. If no ion suppression occurs, the response (intensity) of the four test compounds (atropine, scopolamine, codeine and reserpiline, eluting at 5.92, 4.13, 2.88 and 8.09 min, respectively) is expected to be constant and independent of the dilution factor. However, an increase in the response with increase in dilution factor was observed (Fig. 4). Even so, different responses were found for the test compounds. In the most concentrated sample, the responses of atropine, reserpiline, codeine and scopolamine decreased to 67.6%, 77.8%, 28.9% and 34.3% of the pure academic solution, i.e. without extract. When the sample was diluted 62.5 times, ion suppression was less; for reserpiline and scopolamine, the relative peak areas changed to 95.6% and 97.4%, for atropine and codeine, the relative peak areas changed to 92.1% and 91.6%, respectively.

Ion suppression can be a serious problem in certain LC/MS applications. One approach to overcome this challenge is by complete chromatographic separation prior to mass spectrometric analysis. Although chromatographic resolution can be improved by using for example particles of smaller dimensions, longer columns, etc., complete separation of complex biological mixtures is not feasible. As in profiling analysis often concentrations are compared in a relative manner and if suitable dilution (or preconcentration) of samples is applied, ion suppression is less critical if the composi-

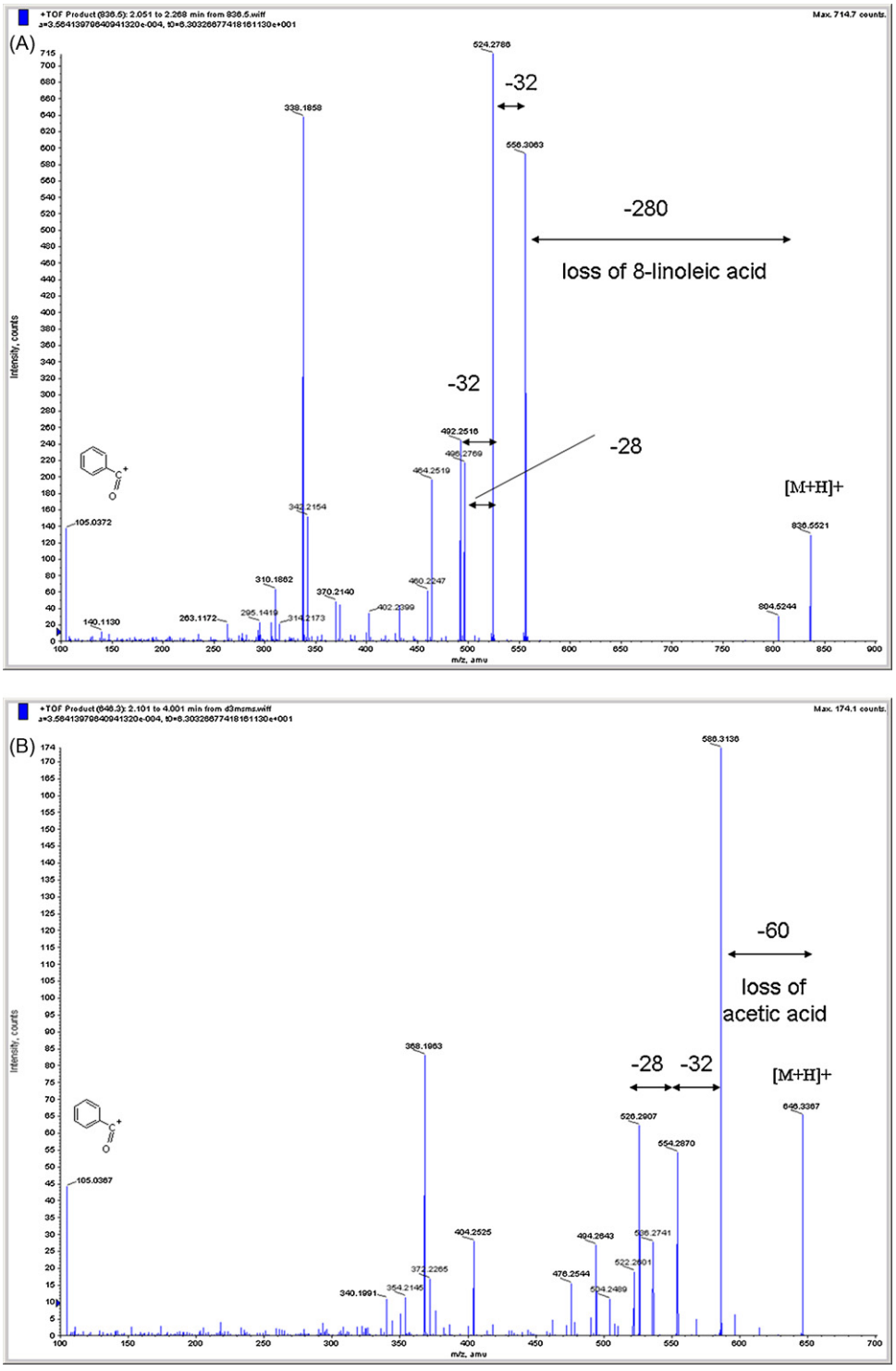


Fig. 1. MS/MS spectra of some selected *Aconitum* alkaloids (and the *m*/*z* of $[M+H]^+$ ion), i.e. (A) 8-Lino-14-benzoylhypaconine (*m*/*z* 836.6), (B) aconitine (*m*/*z* 646.3). Intense fragmentation peaks can be assigned to losses of C19 norditerpenoid skeleton substituents, mainly acetic acid (−60) or fatty acid (−280), CH_3OH (−32), CO (−28), benzoic acid (−122) H_2O and their combination. *m*/*z* 105 indicates the presence of a benzoyl substituent. Sample was introduced by direct infusion.

tion of the sample does not vary too much [25]. Therefore, for the choice of the amount of sample injected, a compromise has to be found between sensitivity (in favour of a large amount of sample aliquot) and reduction of risk of ion suppression (in favour of a small amount of sample aliquot).

The injection repeatability (expressed as RSD, $n = 5$) of the peak areas of standard solutions of 2, 4, 8, and 10 ng/mL aconitine with LC–MS were 12%, 13%, 7% and 9%, respectively. For aconitine solutions with concentration higher than 10 ng/mL, the RSD was better than 4%.

The variation of the retention time of aconitine for the LC–MS analysis of standard solutions at a concentration from 2 to 1000 ng/mL (5 injections per concentration) was maximally 0.16 min and the RSD was 0.45% ($n = 60$).

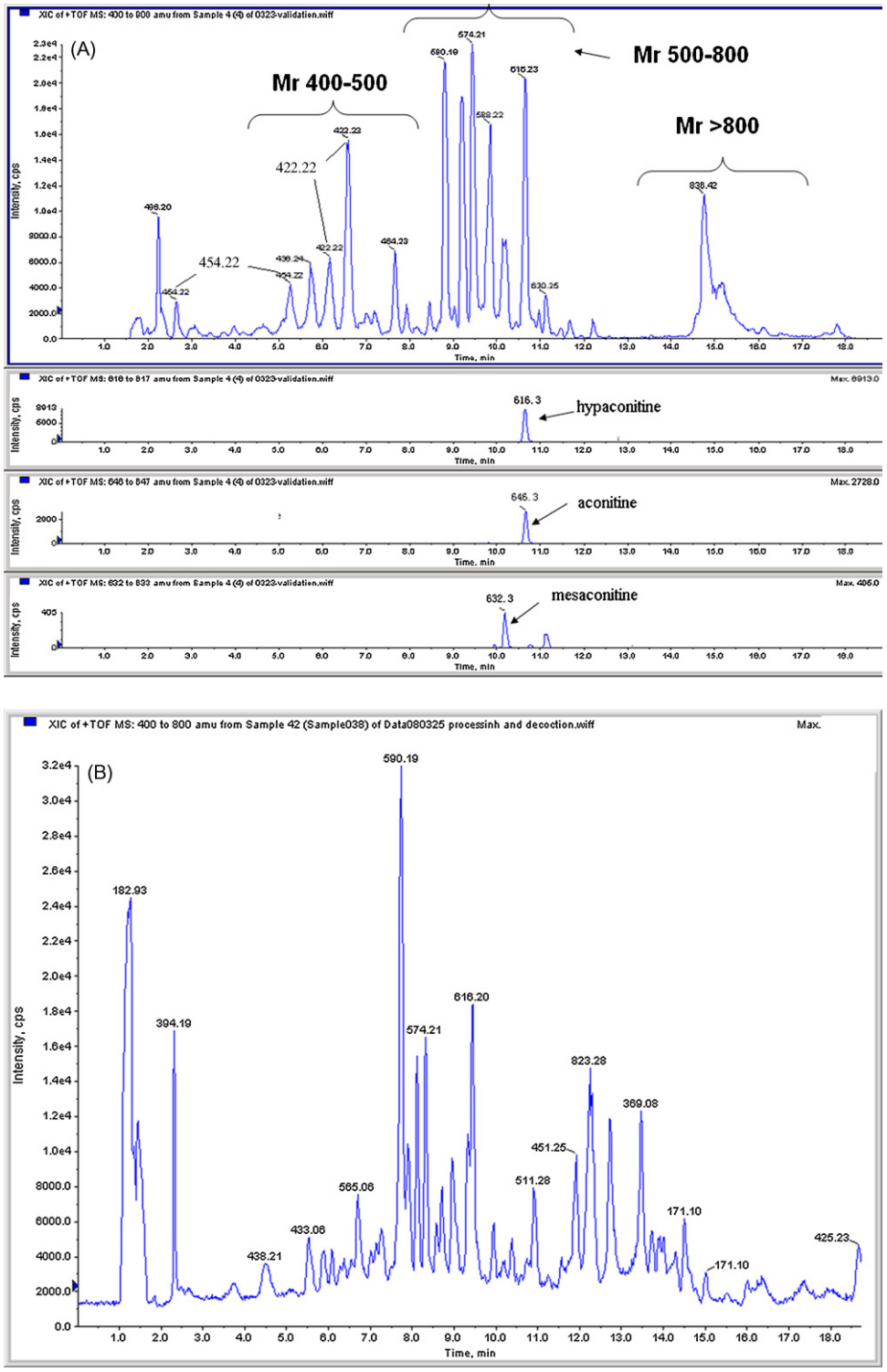


Fig. 2. (A) Typical LC–MS chromatogram of a Fuzi extract. The chromatographic peaks could roughly be divided into three clusters, i.e. retention time below 8 min with M_r of 400–500, retention time from 8 to 12 min with M_r 500–800 and retention time above 12 min with M_r above 800. (B) Typical LC–MS chromatogram of a Si Ni Tang extract containing Fuzi.

3.4. Extraction of Aconitum alkaloids

The ultimate goal of profiling analysis is the unbiased (relative) quantification of every component in a biological system [26] and such profiling can reveal the quality of TCM from a holistic perspective [27]. Different sample preparation methods have been applied for alkaloid extraction from roots of *A. carmichaeli*. Straightforward methods comprise the use of aqueous ethanol [6,7] or the use of alkalized organic solvents as methanol, chloroform and diethyl ether [3,13,14]. More complex methods involve liquid–liquid or solid-phase extraction (SPE) procedures [10,11].

For the extraction of *Aconitum* alkaloids a series of solvent mixtures (Section 2.7) were evaluated. Using MALDI-MS analysis, compared with the other solvents, ACN was the best extraction sol-

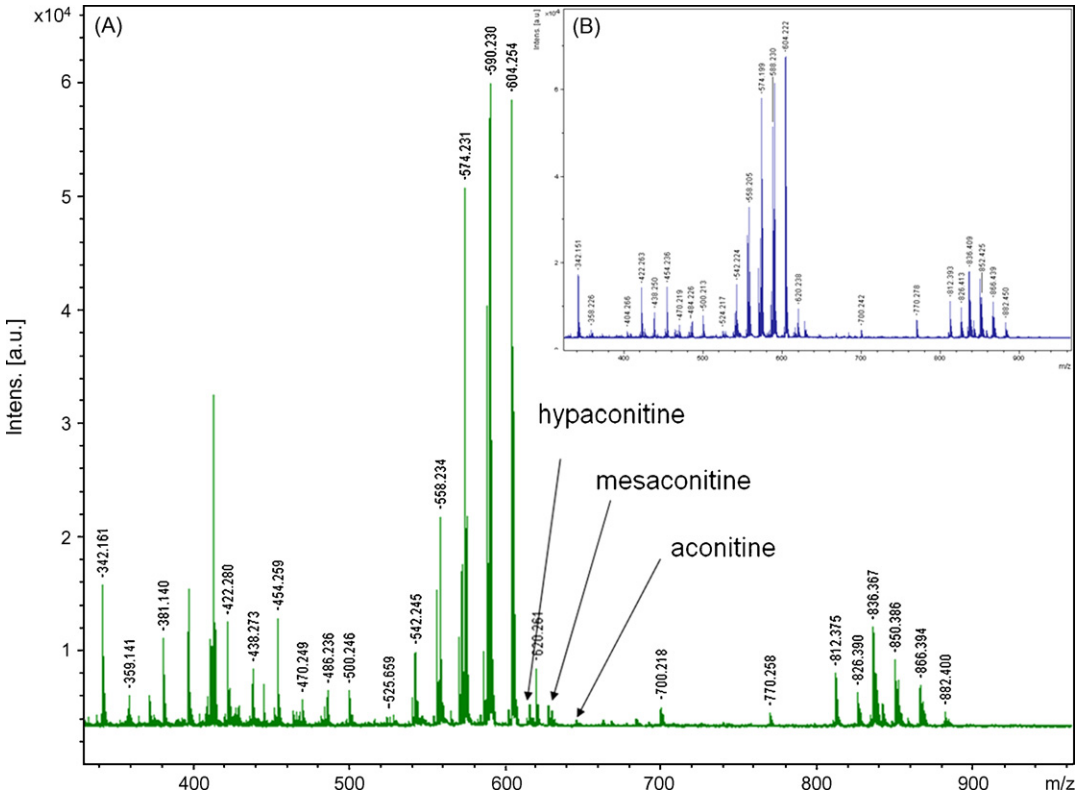


Fig. 3. (A) Typical MALDI-MS spectrum of *Aconitum* alkaloids from *A. carmichaeli* root. (B) Typical MALDI-MS spectrum of *Aconitum* alkaloids by direct analysis.

vent and can provide highest signals for the representative alkaloid with *m*/*z* 574.3 (Fig. 5A) and almost all selected peaks (18 out of 19, Section 2.7). Further, these extracts by different solvent mixtures were subjected to the validated LC–MS analysis (described in Section 3.3), the use of water/ACN mixture (1:1) as extraction solvent provided the most intense signal for *m*/*z* 574.3 (Fig. 5A) and other selected peaks. The reason for this difference could be found in ion suppression effects in MALDI-MS analysis. Due to its polarity, water/ACN (1:1) extracts many polar components such as inorganic salt and sugar, which will interfere with the ionization of the alkaloids resulting in lower signals or even in complete suppression. Actually, the internal standards (atropine, codeine, scopolamine and reserpiline) added to the extract prior to MALDI analysis, were hardly visible for the water/ACN extract. In conclusion, ion suppression is more critical for the MALDI-MS method. In this research, an identical sample extraction method is favoured to compare HPLC–MS with MALDI-MS analysis. Furthermore, alkaloids profiling aims at qualitative analysis or relative quantification and

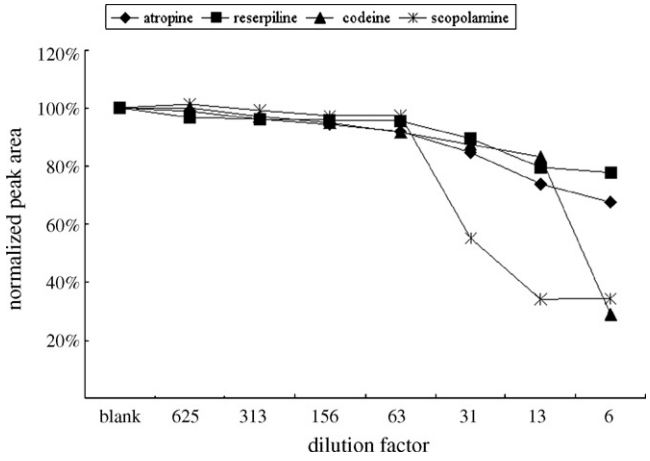


Fig. 4. Evaluation of matrix effects on spiked internal standards atropine, reserpiline, codeine and scopolamine. The normalized peak area of the internal standards as obtained by LC–MS versus the dilution factors of the sample extract is shown. The blank sample did not contain any extract.

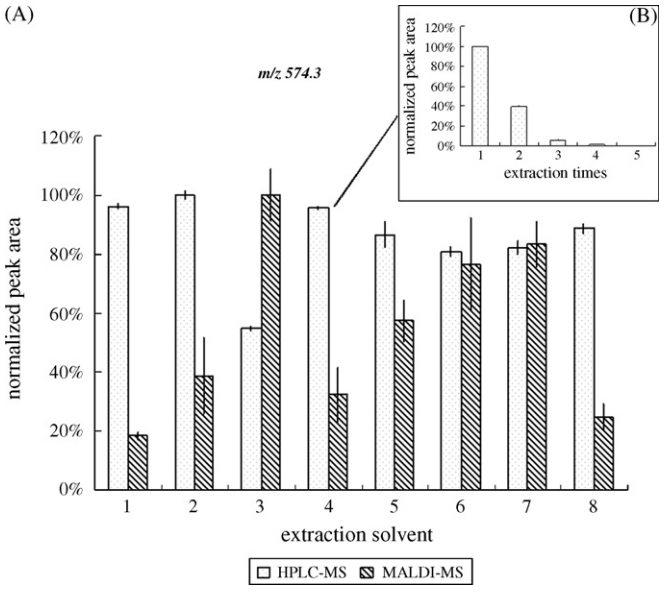


Fig. 5. (A) The influence of extract solvents on the signals of representative *Aconitum* alkaloid with *m*/*z* 574.3 measured by HPLC–MS and MALDI-MS. Peak areas are normalized by the highest peaks of MALDI-MS and HPLC–MS respectively. The numbers 1–8 in *x* axis indicate the extract solvent mixtures listed in Section 2.7. (B) The influence of extraction times on the remains of the *Aconitum* alkaloid with *m*/*z* 574.3 in sample. Peak areas are normalized by the peak of the first extraction. The numbers 1–5 in *x* axis indicate extraction times, i.e. the 1st, 2nd, 3rd extraction.

complete extraction is not critical. Actually, complete extraction only can be achieved by multiple extraction. For the representative alkaloids with *m*/*z* 574.3, after 4 times extractions by 1% TFA in 50% ACN (extract solvent 4), it was undetectable in the extract (inserted Fig. 5B). As the ACN was the best extraction solvent for MALDI MS analysis, and as the repeatability of extraction with ACN for the 19 selected test compounds was good with LC–MS (RSD for 13 peaks below 5% and for 6 peaks 5–10%), ACN was selected as extraction solvent and a sample was extracted once.

3.5. Validation of MALDI-MS assay

The commonly used matrices, DHB, CHCA and SA were evaluated for their applicability in profiling of *Aconitum* alkaloid extracts with MALDI-MS. Using DHB and CHCA good quality spectra were obtained, showing a similar MS profile. SA was less effective as a matrix and required higher laser energy. Compared with CHCA, DHB showed a higher sensitivity, for example, the peak intensity of *m*/*z* 590.3 was 2–4 times higher for the same sample extract. Furthermore DHB was more sensitive in the detection of higher mass range alkaloids (*m*/*z* > 700).

In TCM, Fuzi is always used in a formulation. Therefore, a screening method for *Aconitum* alkaloids has to be able to tolerate the presence of excipients and other herbs. It was found that PEG 6000 severely interferes with the detection of selected *Aconitum* alkaloids (i.e. *m*/*z* 574.3, 590.3 and 604.3). PEG 6000 partly dissolved in the extraction solvent acetonitrile and interfered with the formation of matrix/sample crystals. After decreasing the concentration of PEG from 200 to 10 mg/mL, the selected *Aconitum* alkaloids could be detected. For the other mimic interferences (sodium chloride, glucose, starch, methylcellulose and Liuwei Dihuang Pills) compared with control samples (without interference) the intensity of three selected peaks varied from 56% to 160%, and were considered no interferences.

Solid sampling technique for direct MALDI-MS has been applied in polymer analysis. The polymer powders were suspended with matrix solution, and after mixing, the sample suspension was deposited on a flat stainless steel MALDI plate and allowed to air-dry prior to analysis [28]. Besides the premix method, the most popular method in direct tissue analysis and MALDI imaging techniques is to apply the matrix solution directly onto the surface of the sample [4,29].

In this research, a solid sample analysis method has been investigated by applying the matrix solution onto the powdered samples directly on the MALDI plate. Compared with the above mentioned two solid sampling methods, our method omits the procedure of premixing. Because the sample is a fine powder, it is supposed to have a large surface area for alkaloids extraction.

The ratio of sample mass to DHB matrix volume has a significant impact on the mass spectrum. When the ratio was 10 μg to 0.5 μl, the solid sampling method generated similar mass spectra as the conventional solvent extraction method for the same sample (Fig. 3A and B). For direct analysis of the Si Ni Tang only weak signals were obtained. The matrix solvent, acetonitrile/water/TFA (5:5:0.01) will co-extract other constituents which may lead to ion suppression. Using ACN:TFA (100:0.1) as solvent increased the sensitivity twenty times, and good profiling spectrum could be acquired.

MALDI-MS is in general not regarded as a quantitative method, largely because of the poor shot to shot repeatability. Calibration using internal standards could be an effective way to improve quantification by MALDI-MS [30,31]. For our MALDI-MS method, atropine, scopolamine and codeine were used as internal standards for quantification. To achieve a good shot to shot repeatability, it is essential to select a proper "hotspot" to be shot and only spots generating a significant signal for the internal standards (i.e. with a S/N above 100) were taken into account. In total, mass spectra from 15 spots (50 shots each) were acquired. The RSD of the signal of the aconitine peak (*m*/*z* 646.3) for the 15 spots was relatively high, i.e. 36%, whereas with the calibration of internal standards, the RSD was less than 10%. The linearity of aconitine standard solutions was also significantly improved from R^2 0.92 to 0.94–0.98 after calibrating with internal standards. In conclusion, with spot quality control and internal standards calibration, the MALDI-MS based method can be used as a semi-quantitative method.

3.6. Application of alkaloid profiling

3.6.1. Application of alkaloid profiling to Fuzi by MALDI-MS and validation by LC–MS

The Chinese Pharmacopoeia applies thin layer chromatography to guarantee the limitation of aconitine content [8]. However, the above described LC–MS and MALDI-MS methods reveal the presence of many, so far unidentified alkaloid-type compounds, which are overlooked by target analysis, but have consequences for QC strategies and bioactivity studies.

With the MALDI-MS method described above, significant differences in alkaloids content were found in 14 batches of Fuzi, and in three batches only traces of alkaloids could be detected. For the toxic DDAs, aconitine and mesaconitine could be detected in 8 samples and hypaconitine was found in 10 samples. The most intense peaks that are characteristic and are probably suited for the identification of Fuzi were MDAs (*m*/*z* 590.3, 588.3, 574.3, 604.3), DDAs (*m*/*z* 616.3) and lipoalkaloids (*m*/*z* 812.6, 836.6, 852.6) (for assignment see, Table 1). The characteristic alkaloid of Fuzi, fuziline (*m*/*z* 454.2) was also detected [3].

The same samples were also subjected to LC–MS analysis. To reduce possible ion suppression, a lower sample mass to extract solvent ratio (0.1 g to 1.5 mL, rather than 1 g to 5 mL as described in Section 2.6) was used in alkaloids extraction and a further dilution of 1:10 was chosen. Also with LC–MS, significant differences in alkaloid content were found. In the same three batches (as for the MALDI method) only traces of alkaloids were detected. For the other eleven samples, the most intense signals were from monoester-diterpenoid aconitines with *m*/*z* 590.3, 588.3, 574.3 and 604.3. The toxic DDAs, aconitine was detected in 10 samples; hypaconitine in 11 samples and mesaconitine was detected in 9 samples. In summary, these results were rather consistent with the results of MALDI-MS. Using LC–MS, the variation in the content of the various alkaloids in the 14 samples was determined. The significant variation in contents of aconitine, hypaconitine and mesaconitine in roots of *A. carmichaeli* has been reported [10,11,20], in this research the variations (expressed as RSD) of above mentioned three DDAs were 138%, 99% and 221%, respectively. For the 89 compounds (peaks) detected with LC–MS the variation of each peak (expressed as RSD) in the 14 samples was determined; for 13 peaks the variation was 77–100%, for 46 peaks 100–150%, for 21 peaks 150–200% and for 9 peaks 200–235%.

It was investigated to what extent the semi-quantitative data obtained with MALDI-MS using an internal standard were comparable to those found with LC–MS. 24 peaks were detected in 11 samples by both MALDI-MS and LC–MS. Therefore, the correlation of the same peak determined in different samples by LC–MS and MALDI-MS was calculated (see Section 2). It showed that the correlation coefficient was dependent on the intensity obtained for the peak with LC–MS (Fig. 6A). For the highly abundant peaks with *m*/*z* 422.3, 438.3, 574.3, 588.3, 590.3 and 604.3, the correlation coefficients were above 0.9. As an example, the linear correlation plot for the peak with *m*/*z* 574.3 was established between LC–MS and MALDI-MS (Fig. 6B). For the three toxic alkaloids (aconitine, hypaconitine, measaconitine), the correlation coefficients were 0.79, 0.78 and 0.79, respectively. In conclusion, MALDI-MS can be used for

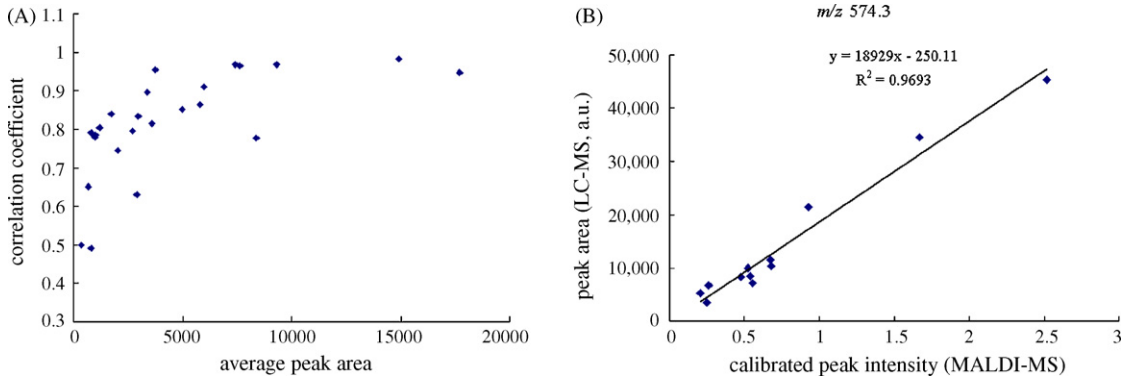


Fig. 6. (A) Pearsons correlation coefficients between the signal of the same alkaloid measured with LC–MS and MALDI-MS in 11 different samples. For 24 peaks the correlation coefficient is given in dependence of its average peak area in 11 samples determined by LC–MS. This plot indicates that correlation between LC–MS and MALDI-MS was better for the higher abundant peaks. (B) As an example the correlation between LC–MS peak area (arbitrary units, a.u.) and MALDI-MS peak intensity calibrated by internal standard (atropine) is for benzoylhypaconine (*m*/*z* 574.3) for 11 herbal samples.

the semi-quantitative profiling of those alkaloids which are clearly showing up in MALDI-MS.

3.6.2. Alkaloid profiling of formulation

The alkaloid contents in plants are influenced by many factors [32]. This variation in content (qualitatively as well quantitatively) is fortified by commonly practiced processing of the herbal medicine. In TCM practice, Fuzi is processed by boiling and always used in combination formulas with several other herbs. This procedure of preparation is necessary for the reduction of the toxicity. The LC–MS method was used to monitor the changes in the alkaloids during the preparation of a Si Ni Tang. In total 57 peaks, as detected in Fuzi, were monitored. In general, the alkaloid content was lower in Si Ni Tang than in Fuzi. Of the 57 peaks, 17 peaks could not be detected in the decoction, the majority of them were DDAs and lipoalkaloids. These results indicated that DDAs and lipoalkaloids were readily hydrolyzed (to probably less toxic alkaloids)[7]. During the processing the content of hypaconitine was reduced 38 times. Contents in a typical MDA (benzoylhypaconitine *m*/*z* 574.3) and NEA (talatisamine *m*/*z* 422.3) were reduced 8.1 and 5.5 times respectively.

In general, the amount of alkaloids were reduced by the processing, for 24 alkaloid peaks the amount of alkaloid was reduced 4–10 times, for 15 alkaloids (mainly MDAs or NEAs) the amount was reduced 10–24 times.

4. Conclusions

The therapeutic application of TCM is mainly based on a combination of herbs, comprising a multitude of constituents. Hence a profiling approach should be ideal for TCM quality control. Fuzi is widely used in TCM formula. In this study, for the purpose of quality control, two analytical methods were developed which could find application in the following areas. The first is in the identification of Fuzi. For this purpose, the MALDI-MS method was developed as a fast method for alkaloid screening and identification. For the main alkaloids present at higher concentrations the MALDI-MS method performed in a semi-quantitative manner. The method is robust; it is tolerant to a number of interferences and it even handles solid samples, which makes it a high-throughput method. The second application is to provide a platform for characterizing the chemical constituents of Fuzi in a (semi-) quantitative way. MALDI-MS and LC–MS based *Aconitum* alkaloid profiling covers a wide range of different types of *Aconitum* alkaloids. Compared with the MALDI-MS method, better quantitative information is obtained by LC–MS. The LC–MS analysis of 14 samples obtained from different local Chinese drug stores showed that the contents of *Aconitum* alkaloids were significantly different. The causes leading to these differences are not clear; insufficient exact information on the history of the samples was not available. However, the developed methods have clearly demonstrated to be able to distinguish the different samples and thus could be used for (alkaloid) profiling purposes. The third area is in the manufacturing to monitor the effects of processing and preparation on the alkaloid content. Eventually this may lead to standardized processing and formulation protocols. In this study, the impact of processing of Fuzi and Fuzi formulations is clearly illustrated. For this, it is highly recommended to implement *Aconitum* alkaloid profiling methods to provide a safe application and good manufacture practices. The methodology described here could be a basis for that.

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