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Review Article

Use, history, and liquid chromatography/mass spectrometry chemical analysis of Aconitum

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ABSTRACT

Aconitum and its products have been used in Asia for centuries to treat various ailments, including arthritis, gout, cancer, and inflammation. In general, their preparations and dispensing have been restricted to qualified folk medicine healers due to their low safety index and reported toxicity. In the past few decades, official guidelines have been introduced in Asian pharmacopeias to control Aconitum herbal products. However, these guidelines were based on primitive analytical techniques for the determination of the whole Aconitum alkaloids and were unable to distinguish between toxic and nontoxic components. Recent advances in analytical techniques, especially high performance liquid chromatography (HPLC) and electrophoresis coupled with highly sensitive detectors, allowed rapid and accurate determination of Aconitum secondary metabolites. Reports focusing on liquid chromatography/mass spectrometry analysis of Aconitum and its herbal products are discussed in the current review. This review can be used by the health

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liquid chromatography/mass spectrometry

regulatory authorities for updating pharmacopeial guidelines of *Aconitum* and its herbal products.

1.　Introduction

Aconitum (or monkshood) is a herb native to China and certain parts of Europe [1,2]. It has been used for centuries to treat pain due to arthritis, gout, cancer, inflammation, migraine, and sciatica. Despite such important therapeutic activities, it is highly toxic and ingestion of the raw material can lead to arrhythmia, heart failure, and even death. Even topical applications can be dangerous because its toxic alkaloids can be absorbed through the skin [3]. Toxicity of aconite is related to the C19-norditerpenoid ester alkaloids such as aconitine (AC), deoxyaconitine (DA), mesaconitine (MA), hypaconitine (HA), and yunaconitine (YA) [4]. These alkaloids significantly activate sodium channels and cause widespread membrane excitation in cardiac, neural, and muscular tissues [5–8]. Additionally, muscarinic activation may cause hypotension and bradyarrhythmia. Symptoms of *Aconitum* sp. poisoning include numbness followed by paralysis of the upper and lower extremities. Patients with aconite poisoning, due to the consumption of herbal broth containing a large amount of Fuzi, usually suffer from cardiovascular symptoms including chest pain, palpitation, bradycardia, sinus tachycardia, ventricular ectopics, ventricular tachycardia, and ventricular fibrillation [9,10]. The main causes of death from aconite include ventricular arrhythmias and asystole [11]. There is no specific antidote for aconite poising but only supportive treatment to restore normal heart functions.

The supportive treatment of aconite poisoning includes the use of amiodarone and flecainide as antiarrhythmic agents. Intragastric lavage or oral administration of charcoal can decrease alkaloid absorption. A fatal dose can be as little as 5 mL of aconite tincture, 2 mg of pure aconite, or 1 g of the root [12,13]. For decreasing the toxicity, aconite must be processed prior to use. To date, more than 70 traditional and modern methods have been applied to decrease the toxicity of aconite roots [13,14]. According to the Chinese Pharmacopeia, only two assays are accepted for the quantitative analysis of alkaloids in *Aconitum* sp. Based on these two assays, the maximum allowance of alkaloid content, calculated taking AC into consideration, is 0.15% and 0.20%, respectively. Any herbal product with a lower concentration of the toxic alkaloids can be used in China, but such regulation is not acceptable elsewhere. Microscopic examination of the processed aconite roots (P-ARs) reveals the presence of gelatinized starch masses, which do not appear in the unprocessed aconite root (unP-AR) samples [15].

The plant is rich in diterpene and norditerpene alkaloids as the major active constituents. These compounds exhibit interesting activity toward voltage-gated Na channel either as agonists (e.g. AC and MA) or as antagonists (e.g., lappaconitine and 6-benzoylheteratisine) [16–18]. Activities toward certain neuronal receptors were also noted such as norditerpene alkaloids (e.g., methyllycaconitine). These alkaloids exhibited selective antagonistic activity on the neuronal nicotinic acetylcholine receptor

in a nanomolar range of concentration, rendering it as a potential drug lead in Alzheimer diseases [19–21].

2.　Historical glimpse

The name *Aconitum* comes from the Greek word akonitos, meaning "without struggle" or "without dust," or from the Greek city Acona, where a naturalist in the 3rd century once identified the plant [22]. Other historical sources suggest that the name came from the hill of Aconitus. It is a hill in Greek mythology where Hercules fought with Cerberus, the three-headed dog that guards the entrance to Hades. Saliva from this creature dripped onto the plant, rendering them extremely poisonous. It is also claimed that the cup-shaped flower made the poisonous cup that Medea prepared for Theseus. The plant played an important role in Roman history, as it is assumed that Nero ascended to the throne after poisoning Claudius by tickling his throat with a feather dipped in monkshood. The emperor Trajan (98–117 AD) banned growing of this plant in all Roman domestic gardens [23]. One of the most remarkable pieces describing the role played by this plant in ancient Roman society was described by the writer Ovid. He referred to aconite as the "step-mother's poison." In his work Metamorphoses, he described a certain period of Roman history by the following lines:

> "*Guest was not safe from host, nor father-in-law from son-in-law; even among brothers it was rare to find affection. The husband longed for the death of his wife, she of her husband; and murderous step-mothers brewed deadly poisons, and sons inquired into their fathers' years before the time.*"

Shakespeare highlighted the potency of this herb in his novel Romeo and Juliet, in which he stated that Romeo committed suicide using this poison [22]. In addition, in Macbeth, the witches' brew calling for "tooth of wolf" refers to monkshood. Certain species are also known as wolfsbane because arrows dipped in the poison kill wolves. Until 1930, it was used in the USA and Canada as a painkiller, diuretic, and diaphoretic. It was used externally in the form of ointments to treat rheumatism, neuralgia, and lumbago and as a tincture to lower pulse rate, relieve fever, and treat cardiac failure. Reported cases of toxicity led to the ban of its use in conventional medication.

3.　Liquid chromatography/mass spectrometry techniques for analyzing *Aconitum*-containing samples

Modern analytical techniques have been employed in the last 2 decades to evaluate herbal medicines sold in Asian markets

aiming to improve their safety and reduce adulteration [24—26]. *Aconitum* and its products are strictly monitored due to their extreme toxic components, especially diester-diterpenoid alkaloids (DDAs—AC, MA, and HA). Initially, alkaloidal titration methods were used to monitor these alkaloids. However, compared with the high-performance liquid chromatography (HPLC) method, alkaloidal titration method estimates not only toxic DDAs, but also monoester-diterpenoid alkaloids (MDAs), unesterified alkaloids, and lipo-alkaloids (LPAs) [27], which are less toxic than DDAs and may have potential effect, especially for inflammatory disorders such as arthritis [28]. Therefore, HPLC became the popular method to detect DDAs and MDAs [MDAs such as benzoylaconine (BAC), benzoylmesaconine (BMA), and benzoylhypaconine (BHA)] in recent days.

The degradation of DDAs into MDAs were noted in methanol and ethanol [29,30], especially for AC. Thus, using ethanol or methanol as the extraction solvent or mobile phase in HPLC may lead to inaccurate results. Hydrolysis of DDAs into MDAs is observed especially with a long decoction time (> 120 minutes) [28]. This degradation phenomenon was detected upon using water as the decoction solvent, as well as cow urine and cow milk (Tables 1 and 2) [31]. Furthermore, the stability of DDAs was found to be highly pH dependent and they were stable only in the range of 2.0—7.0. The relative concentrations of DDAs, especially AC and MA, decreases significantly at pH >10 [29]. The evidence showed that storing DDAs in an ambient temperature or at 4°C may cause degradation, and samples are suggested to be kept at −20°C to decrease the degradation rate [32].

To reduce decomposition of DDAs, chloroform and dichloromethane were found to be the most appropriate extraction solvents [29,33]. Although 1% hydrochloride as the extraction solvent could improve the extraction yield, degradation of DDAs occurred [34]. To shorten the extraction time, an ultrasonic bath [35] or a microwave-assisted extraction procedure [36] was applied. The choice of the mobile phase plays an important role in the resolution of alkaloidal peaks on HPLC. Acetonitrile—aqueous 0.1% formic acid [37], acetonitrile—acetic acid solution [38], or acetonitrile—ammonium bicarbonate buffer at pH 10 ± 0.5 [39,40] resulted in excellent peak resolution in several studies.

Recent studies introduced new protocols, which did not require sample preparation, and the herbal products were analyzed in their crude forms. DART-MS (direct analysis in real time mass spectrometry) plus multivariate data analysis method was utilized as a solvent-free method to analyze samples directly in their native condition, without the need of tedious sample manipulation and preparation [41]. Moreover, minimum amounts of samples were analyzed with high accuracy with the introduction of new methods such as ultra-high pressure liquid chromatography with linear trap quadrupole and Orbitrap mass spectrometry system (UHPLC-LTQ-Orbitrap-MS^n) [42].

Aconite is usually combined with other Chinese herbs by practitioners of traditional Chinese medicine (TCM). In order to detect the recovery of toxic alkaloids in these formulas, many liquid chromatography/mass spectrometry (LC/MS)-related techniques were used, such as ultraperformance liquid chromatography—electrospray ionization/mass spectrometry (UPLC—ESI/MS) [43], Ultraperformance liquid chromatography—photodiode array detector (UPLC—PDA) [44], UPLC/MS [45], UPLC—quadrupole time-of-flight mass spectrometry (Q-TOF—MS) [46], ultrafast liquid chromatography--ion trap/time-of-flight mass spectrometry (UFLC/MS-IT-TOF) [47], and UPLC—ESI—Q-TOF—MS [48]. In recent years, various aconite-related medications were introduced into the Chinese market. In order to speed up the quality control process, rapid resolution liquid chromatography coupled with tandem mass spectrometry (RRLC—MS/MS) was applied to analyze the toxic alkaloids in these products such as Shen-Fu formula. This method provides excellent limit of quantification (LOQ) (7—50 pg/mL) as well as limit of detection (LOD) (2.3—17 pg/mL) [49], which are much more sensitive than LC/MS.

There are many *Aconitum* sp. in the world (> 75 species); some of them are used in folk medicine, especially in Asia. The processing methods differ from country to country and from use to use. Determination of fingerprints for raw herbs and medicinal formulas is essential for the establishment of appropriate quality control procedures. Certain studies introduced similarity evaluation, hierarchical cluster analysis, or principal component analysis (PCA) to evaluate the similarity and variation of aconite samples [37,50]. Partial least squares-discriminant analysis (PLS-DA) and orthogonal projection to latent structure analysis were extremely useful in the classification of metabolic phenotypes and in the identification of different metabolites [51].

This review covers recent attempts to establish analytical protocols developed to analyze products containing alkaloids of raw herb or P-ARs using HPLC/MS-related techniques. Significant accomplishments were reported, showing in detail the optimum procedures to evaluate alkaloidal contents of aconite samples. Most of the important findings were reported in the past 15 years starting from the year 2000, and this review summarizes important studies using different HPLC/MS-related laboratory equipment in a chronological order.

Xie et al [39] developed an efficient protocol using HPLC for the separation of six aconite alkaloids, including AC, MA, HA, BAC, BMA, and BHA, in aconite roots and related 12 proprietary Chinese medicines (Tables 1 and 2). They found that ethyl acetate was the optimum solvent for extracting alkaloids from the basified solution. They also evaluated the effect of pH on the separation of alkaloids, and found that all peaks were separated at pH above 9.95 and the optimum pH value for separation was 10.0 ± 0.2. The effect of using different concentrations (5mM, 10mM, or 20mM) of ammonium carbonate, the mobile phase buffer, was studied. It was found that the optimum concentration was 10mM for excellent peak separation and background noise reduction. Alkaloidal peaks were identified by comparing their retention time and UV spectra with the reported data. This method was applied for the identification of 12 aconite-root-containing Chinese medicines, and two P-AR and two unP-AR samples. As expected, P-ARs showed lower levels of the toxic alkaloid AC. However, concentrations of the other toxic alkaloids, HA and MA, were significantly higher than that of AC in the P-AR samples, suggesting that the use of AC concentration as the only marker for toxicity reduction after processing is not enough for general safety guidelines.

Table 1 – Representative sample preparation procedures for aconite roots and their products in recent publications.

Analytes	Samples	Extraction procedures	Analysis	Refs
Monoester-diterpene alkaloids				
BMA	P-AR & its proprietary products containing processed aconite roots	Extract P-AR with 50% EtOH (×3, 60 min each time) via sonication & alkaloids from unP-AR via general method.[a]	HPLC-PDA	[30]
Diester-diterpene alkaloids				
AC, MA, & HA	P-AR & unP-AR	Extract P-AR & unP-AR with 75% EtOH (×3, 30 min each time) via sonication.	HPLC-PDA	[52]
AC, MA, & HA	Aconite roots & decoction pieces containing processed aconite roots	Extract with methanol–water (1:1, v/v) via sonication for 60 min.	HPLC-PDA & HPLC–ESI–MS	[35]
AC, MA, & HA	Aconite roots	Extract using the general extraction procedures.[a]	HPLC-PDA	[40]
AC, MA, & HA	Shen-Fu decoction (Radix ginseng & Fuzi at a ratio of 3:2) containing processed aconite roots	Extract with water under reflux (1:10 v/v, ×3, 60 min each time), & dissolve the corresponding residue in water.	RRLC–ESI–MS/MS	[65]
AC, MA, & HA	Unprocessed aconite roots	Extract P-AR & unP-AR with chloroform (1:10 v/v, for 30 min) via sonication & dissolve the obtained residue in methanol.	UPLC–Q-TOF–MS	[31]
Simultaneous analysis for both monoester & diester diterpene alkaloids				
MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA	P-AR, unP-AR & proprietary Chinese medicines (pills, tablets, & capsules) containing processed aconite roots	Extract by alkaloidal extraction procedures.[a]	HPLC-PDA	[39]
MDAs: BAC, BMA, & BHA	Raw material & P-AR	Extract by alkaloidal extraction procedures.[a] Dissolve the corresponding residue was dissolved in ACN–TEA (75:25, v/v).	HPLC-PDA & HPLC–ESI–MS/MS	[38]
DDAs: AC, MA, & HA				
MDAs: BAC, BMA, & BHA	P-AR & its proprietary products (*Xiaohuoluo* pills)	Add ammonia TS (40% $NH_3 \cdot H_2O$) to each sample & extract with diethyl ether in an ultrasonic bath for 30 min, & dissolve the corresponding residue with methanol. To each sample of pills, add ammonia TS (40% $NH_3 \cdot H_2O$) & extract with isopropanol –ethyl acetate (1:1, v/v) in an ultrasonic bath for 30 min, & dissolve the corresponding residue in acetonitrile.	UPLC–ESI–MS	[37]
MDAs: BAC, BMA, & BHA DDAs: AC, MA, HA, & YA	P-AR, unP-AR, & its patent Chinese medicine products (pills) containing processed aconite roots	Extract with 50% methanol (containing 2.5% formic acid) by microwave for 1 min at a power level of 420 W.	UPLC–ESI–MS/MS	[36]
MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA	Decoction & maceration of Fuzi Xiexin Tang containing P-AR	Extract with water under reflux (1:10 & 1:8 v/v, ×2, for 1 h or 1.5 h) & dissolve the corresponding residue in water. Maceration of FXT: reflux the weighted sample of Radix Aconiti Lateralis Preparata with water (1:10 & 1:8 v/v, ×2, for 1 h or 1.5 h) & combine the corresponding decoction with another prepared maceration of Sanhuang Xiexin Tang to obtain MFXT.	UPLC–ESI–MS	[43]
Simultaneous analysis for the nonester, monoester, & diester diterpene alkaloids				
Nonester type: higenamine MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA	Fuzi decoction (Heishunpian & Baifupian)	Extract with water under reflux (1:10, v/v, ×3, for 60 min each time) & dissolve the corresponding residue in water.	RRLC–ESI–MS/MS	[49]

Nonester type (aminoalcohol-diterpenoid alkaloids): beiwutinine; mesaconine; karakoline; isotalatizidine; aconine; 8-methoxymesaconine; hypaconine; fuziline; 3-deoxyaconine; neoline; 8-methoxyhypaconine; talatizamine; & chasmanine	21 batches of Fuzi from different regions; all the samples were processed from the lateral roots of *A. carmichaelii*	Extract by alkaloidal extraction procedures[a] & solid-phase extraction, & dissolve the corresponding residue in methanol−0.1% formic acid (80:20, v/v).	HPLC−ESI−MS/MS	[66]
Nonester type: songorine; fuziline; neoline; talatisamine; guanfubase H MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA	Shen-Fu injection prepared from red ginseng & processed aconite root	Prepare each sample in acetonitrile−water (1:1, v/v).	UPLC−Q-TOF−MS	[46]

AC = aconitine; BAC = benzoylaconine; BHA = benzoylhypaconine; BMA = benzoylmesaconine; DDA = diester-diterpenoid alkaloid; ESI = electrospray ionization; HA = hypaconitine; HPLC = high-performance liquid chromatography; MA = mesaconitine; MDA = monoester-diterpenoid alkaloid; MFXT = maceration of Fuzi Xiexin Tang; MS = mass spectrometry; MS/MS = tandem mass spectrometry; MSXT = maceration of Fuzi Xiexin Tang; P-AR = processed aconite root; Q-TOF = quadrupole time of flight; RRLC = rapid resolution liquid chromatography; TOF = time of flight; unP-AR = unprocessed aconite root; UPLC = ultra-performance liquid chromatography; YA = yunaconitine; PDA = Photodiode Array Detector; ACN = acetonitrile; TEA = triethylamine; EtOH = ethanol.

[a] Alkaloid extraction procedure: each sample was dissolved/extracted in HCl/ammonia solution by sonication, and further extracted with ethyl acetate/ethyl ether to remove nonalkaloid components. The acidic/basic aqueous solution was basified/acidified with ammonia/HCl solution to adjust the pH value, and extracted with chloroform/ethyl ether to obtain the alkaloid components.

Table 2 — Analytical methods of aconite roots and their proprietary products.

Method	Analytes	Samples	Details	Refs
Analysis methods for monoester diterpene alkaloids				
HPLC-PDA	BMA	P-AR; proprietary products containing processed aconite roots	Alltima RP-C18 (250 × 4.6 mm, 5 μm) was used with the Alltima RP-C18 guard column (7.5 × 4.6 mm) at room temperature. Elution of the alkaloids was carried out using a gradient system of acetonitrile & buffer solution at a flow rate of 1.0 mL/min, detected at 240 nm, validated with linearity, LOD, precision, repeatability, & recovery.	[30]
Analysis methods for diester diterpene alkaloids				
HPLC-PDA	AC, MA, & HA	P-AR & unP-AR	Waters Xterra RP18 column (250 × 4.6 mm, 5 μm) was used with the Xterra RP18 guard column (20 × 3.9 mm) at room temperature. Elution of the alkaloids was carried out using a gradient system of acetonitrile & 10 mM ammonium bicarbonate buffer solution at a flow rate of 1.0 mL/min, detected at 240 nm, validated with linearity, LOD, precision, repeatability, & recovery.	[52]
HPLC-PDA & HPLC–ESI/MS	AC, MA, & HA	Aconite roots & decoction pieces containing processed aconite roots	Waters Xterra RP18 column (250 × 4.6 mm, 5 μm) was used & column temperature maintained at 25 °C. Mobile phase was carried out using a gradient system of 2.5 mM ammonium bicarbonate in water at pH 10 & acetonitrile at a flow rate of 1.0 mL/min, detected at 240 nm & positive ion mode for ESI/MS, validated with linearity, LOD, LOQ, precision, repeatability, & recovery.	[35]
HPLC-PDA	AC, MA, & HA	Aconite roots	Phenomenex Luna C18 column (250 × 4.6 mm, 5 μm) was used & column temperature maintained at room temperature. Mobile phase was carried out using a gradient system of acetonitrile & ammonium bicarbonate buffer solution at a flow rate of 1.0 mL/min, detected at 231 nm, validated with linearity, LOD, LOQ, precision, repeatability, & recovery.	[40]
RRLC–ESI/MS/MS	AC, MA, & HA	Shen-Fu decoction (Radix Ginseng & Fuzi at a ratio of 3:2) containing processed aconite roots	Agilent ZORBAXC18 SB column (100 mm × 2.1 mm, 1.8 μm) was used & column temperature maintained at 40 °C. The gradient mobile phases consisted of water containing 0.05% formic acid & acetonitrile at a flow rate of 0.35 mL/min. The positive ion mode for ESI/MS/MS (totally 10 major components identified, 3 DDAs from Fuzi) was validated & quantitatively analyzed.	[65]
UPLC–Q-TOF–MS	AC, MA, & HA	unP-AR	Separation of components in the samples was performed at 20 °C, using a Waters UPLC C18 analytical column (100 mm × 2.1 mm, 1.7 μm) attached with a C18 precolumn (2.1 mm × 5 mm, 1.7 μm). The gradient mobile phase consisted of a mixture of water & acetonitrile, both containing 0.1% formic acid at a flow rate of 0.4 mL/min. The three analytes of unprocessed aconite roots & the corresponding detoxification process samples were validated & qualified using the UHPLC–Q-TOF–MS in positive ion mode.	[31]
Simultaneous analysis methods for both monoester & diester diterpene alkaloids				
HPLC-PDA	MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA	P-AR, unP-AR, & proprietary Chinese medicines (pills, tablets, & capsules) containing processed aconite roots	Alltima RP-C18 (250 × 4.6 mm, 5 μm) was used as the stationary phase accompanied by Alltima RP-C18 guard column (7.5 × 4.6 mm) at room temperature. Elution of the six alkaloids was carried out using a gradient system of acetonitrile & buffer solution at a flow rate of 1.0 mL/min, detected at 240 nm, validated with linearity, LOD, precision, repeatability, & recovery.	[39]
HPLC-PDA & HPLC–ESI/MS/MS	MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA	Raw material & P-AR	Microsorb C18 column (250 × 4.6 mm, 5 μm) was used with an Econosphere C18 guard column, & column temperature maintained at 45 °C. Mobile phase was carried out using a gradient system of ACN, TEA buffer & THF at a flow rate of 1.0 mL/min, detected at 238 nm, validated with linearity, LOD, LOQ, precision, repeatability, & recovery, & detected & quantified with ESI/MS/MS spectra in positive ion mode.	[38]
UPLC–ESI–MS	MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA	P-AR & its proprietary products (*Xiaohuoluo* pills)	Agilent ZORBAXC18 SB column (100 mm × 2.1 mm, 1.8 μm) was used & column temperature maintained at 35 °C. Mobile phase was carried out using a gradient system of 0.1% aqueous formic acid & acetonitrile (65:35, v/v) at a flow rate of 0.3 mL/min. Quantification analysis was achieved on ESI/MS in positive ion mode & SIR mode, validated, & quantitatively analyzed.	[37]
UPLC–ESI–MS/MS	MDAs: BAC, BMA, & BHA DDAs: AC, MA, HA, & YA	P-AR, unP-AR & its patent Chinese medicine products (pills) containing processed aconite roots	Waters Acquity UPLC BEHC18 column (100 mm × 2.1 mm, 1.7 μm) was used for chromatographic separation & column temperature maintained at 40 °C. The gradient mobile phase consisted of a mixture of 0.1% formic acid aqueous solution & methanol-1,4-dioxane (80:20, containing 0.1% formic acid) at a flow rate of 0.6 mL/min. Quantification analysis was achieved using ESI/MS/MS in positive ion mode & MRM mode, validated, & quantitatively analyzed.	[36]

UPLC—ESI—MS	MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA	Decoction & maceration of Fuzi Xiexin Tang containing processed aconite roots	Waters Acquity UPLC BEHC18 column (50 mm × 2.1 mm, 1.7 μm) was used for the chromatographic separation & column temperature maintained at 45 °C. The gradient mobile phase consisted of 0.1% formic acid aqueous solution & acetonitrile at a flow rate of 0.3 mL/min. Quantification analysis was achieved on ESI/MS in positive ion mode & SIR mode, validated, & quantitatively analyzed.	[43]
Simultaneous analysis for nonester, monoester, & diester diterpene alkaloids				
RRLC—ESI—MS/MS	Nonester type: higenamine MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA e	Fuzi decoction (Heishunpian & Baifupian)	The separation was carried out on an Agilent ZORBAXC18 SB column (100 × 2.1 mm, 1.8 μm) & column temperature maintained at 40 °C. The gradient mobile phase consisted of 0.1% formic acid aqueous solution & acetonitrile at a flow rate of 0.35 mL/min. Quantification analysis was achieved using ESI/MS/MS in positive ion mode & MRM mode, validated, & quantitatively analyzed.	[49]
HPLC—ESI—MS/MS	Nonester type (aminoalcohol-diterpenoid alkaloids): beiwutinine; mesaconine; karakoline; isotalatizidine; aconine; 8-methoxymesaconine; hypaconine; fuziline; 3-deoxyaconine; neoline; 8-methoxyhypaconine; talatizamine; & chasmanine	21 batches of Fuzi from different regions; all the samples were processed from the lateral roots of *A. carmichaelii*	Chromatographic separations the thirteen alkaloids were performed on a Hypersil ODS2 column (4.6 mm × 150 mm, 5 μm), which was conjuncted with a SecurityGuard cartridge (Phenomenex) at ambient temperature. The isocratic mobile phase was composed of methanol-0.1% formic acid (80:20, v/v) at a flow rate of 1.0 mL/min. Quantification analysis was achieved using ESI/MS/MS in positive ion mode & MRM mode, validated & quantitatively analyzed.	[66]
UPLC—Q-TOF—MS & HPLC—ESI/MS	Nonester type: songorine; fuziline; neoline; talatisamine; guanfubase H MDAs: BAC, BMA, & BHA DDAs: AC, MA, & HA e	Shen-Fu injection prepared from red ginseng & P-AR	Chromatographic separation for 11 alkaloids was performed on an Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm) at 30°C. The gradient mobile phase consisted of 10mM ammonium formate—water & 0.1% formic acid acetonitrile at a flow rate of 1.0 mL/min. Quantification analysis was achieved on ESI/MS in positive ion mode & selected ion monitoring mode, validated, & quantitatively analyzed.	[46]

AC = aconitine; BAC = benzoylaconine; BHA = benzoylhypaconine; BMA = benzoylmesaconine; DDA = diester-diterpenoid alkaloid; ESI = electrospray ionization; HA = hypaconitine; HPLC = high-performance liquid chromatography; LOD = limit of detection; LOQ = limit of quantification; MA = mesaconitine; MDA = monoester-diterpenoid alkaloid; MRM = multiple reaction monitoring; MS = mass spectrometry; MS/MS = tandem mass spectrometry; P-AR = processed aconite root; Q-TOF = quadrupole time of flight; RRLC = rapid resolution liquid chromatography; SIR = selected ion recording; TOF = time of flight; UHPLC = ultra-high-performance liquid chromatography; unP-AR = unprocessed aconite root; UPLC = ultra-performance liquid chromatography; YA = yunaconitine.

Quantitation of AC, MA, and HA in different P-AR and unP-AR samples was achieved using LC (Tables 1 and 2) [52]. The effect of the extraction solvent was studied through the use of different concentrations of ethanol (50%, 75%, or 90%). Based on the results, 75% ethanol showed the highest level of HA recovery. In addition, the effects of extraction duration (30 minutes or 60 minutes) and extraction cycles (3 times or 5 times) were studied, which showed no advantages on the alkaloid recovery yields with longer extraction time or more extraction cycles. In the optimization steps of the HPLC protocol, the effect of pH was evaluated, showing that all peaks were efficiently separated at pH above 9.5. The LOD for all alkaloids was 15 ng. Marked differences in alkaloidal contents of the P-AR samples showed lower levels of the three alkaloids compared to those of the unP-AR samples. However, a variation among the P-AR samples was also observed, which might be attributed to the uses of different aconite species, geographical sources, or processing methods. It is noteworthy to mention that despite the fact that AC concentration was lower than the limit established by the Chinese pharmacopeia, concentrations of other alkaloids were higher than that of AC in some samples, suggesting that the detection of multiple alkaloidal markers is needed to clarify the toxicity level of aconite preparations.

The variation in alkaloidal contents among four different species of Aconitum (*Aconitum carmichaelii*, *Aconitum pendulum*, *Aconitum hemsleyanum*, and *Aconitum transsectum*) was studied using HPLC [53]. Different solvents were tested as the mobile phase to separate BMA, MA, AC, HA, and DA from the samples of the four *Aconitum* sp. Methanol—water—chloroform—triethylamine/0.1% trifluoroacetic acid—tetrahydrofuran and methanol—water—acetonitrile/acetonitrile and ammonium hydrogen carbonate buffer were evaluated, and the use of the latter solvent system resulted in the best separation. Separation was optimized at 35°C using a gradient elution. The LOD for the developed method was ≥ 30 ng/mL, with a recovery percentage of > 94.65% for all five alkaloids. The results revealed a significant variation in the alkaloidal contents of the four species, highlighting the importance of using the correct species with known alkaloidal contents in the herbal preparations intended for consumer use.

Tang et al [54] developed a protocol for the determination of AC, HA, and MA in a variety of matrixes, including raw materials, single-ingredient powder extracts, multi-ingredient powder extracts, pills, and capsules, using HPLC coupled with UV detection, and the results were confirmed using tandem mass spectrometry. The authors used liquid-–liquid extraction followed by solid-phase extraction to remove interferences prior to LC separation. The extraction solvent had a significant effect on the alkaloidal yields, with diethyl ether solubilizing more neutral alkaloids compared to the dichloromethane. The use of a mixture of diethyl ether and dichloromethane resulted in a cleaner background but a lower yield. It was also found that the longer the extraction time under alkaline conditions, the more the alkaloids become susceptible to hydrolysis. The effect of the mobile phase additives on the efficiency of separation of alkaloids was studied, which showed that the optimum separation was achieved using 20mM triethylamine (TEA) and 5%

methanol. The results of LC—UV and LC/MS/MS were in close agreement.

The stability of DDAs was studied using HPLC/ESI/MS^n [29]. The information on stability is crucial for the estimation of the herbal products' biological activity as well as their toxicity. The stability of DDAs in different solvents was investigated, the results showing that these alkaloids are stable in dichloromethane but not in methanol. It was found that when ether was used as the extracting solvent, AC concentration declined to 51.8%, but concentrations of the other alkaloids MA and HA did not change more than 10%. This finding suggested that the extraction process should be carried out as soon as possible, to avoid decomposition of AC. Different substituents at the nitrogen atom of DDAs led to different rates of decomposition. Moreover, the effect of pH was evaluated, which showed that the three alkaloids AC, MA, and HA were stable in the pH range of 2.0–7.0. If the pH values of the buffer solutions were in the range of 7–10, the relative concentrations of AC and MA were significantly decreased. If the pH was above 10, the three alkaloids decomposed. The effect of storage on AC concentration was studied, which showed that storing aconite at pH 8 and 25°C for 6 months resulted in 50% reduction of AC concentration.

An HPLC method was developed for estimating the quantity of BMA as the main constituent of aconite alkaloids in Radix Aconiti Lateralis Preparata (Fuzi, aconite roots; Tables 1 and 2) [30]. BMA was reported to possess potent pharmacological activities, such as analgesic and anti-inflammatory activities. Several extracting solvents were evaluated for their efficiency in extracting BMA, revealing that the optimum solvent was 50% ethanol. In the optimization process for the development of the HPLC analytic methods, it was found that the use of acetonitrile and phosphoric acid (0.1%) with triethylamine as the mobile phase improved peak symmetry. Lowering the pH below 2.6 or raising the pH above 4.9 resulted in a longer elution time, so the elution was carried out at pH 3.0. The LOD for BMA was found to be 8 ng with an injection volume of 20 μL. The study also concluded that significant variations in BMA concentrations were observed among different batches of the P-ARs and among different proprietary products, indicating the importance of strict quality control for any herbal product containing aconite.

The use of oxidative-damaged endothelial (ECV304) cells along with LC—MS was applied for the detection of bioactive alkaloids of *Aconitum szechenyianum* [55]. The developed system depends on the interaction of the alkaloidal extract with endothelial cells. The extract was subjected to oxidative stress using H_2O_2, followed by the aggregation of cell membrane proteins through changing the pH to 4.0, to release the special binding components from cell receptors. Separation and analysis of the alkaloidal content were achieved using HPLC, and characterization of these components was performed by LC—MS. In the obtained fingerprint of *A. szechenyianum*, five peaks were detected, and by studying the fragmentation pattern of these compounds, two compounds were identified—MA and AC.

A matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) method was developed for the qualitative profiling of the P-ARs of the Chinese herbal medicine *A.*

carmichaelii, Fuzi [32]. The use of the developed protocol eliminated the need for sample preparation, and the method was applied directly to powdered roots. Results of the MALDI-MS experiments were compared with those of LC—MS, and both results were in agreement with each other. The authors used LC—MS for characterization of Fuzi components resulting in the detection of 60 peaks, which were divided into three groups. One group with retention times below 8 minutes and molecular weight (MW) of 400–500, the second group with retention times between 8 minutes and 12 minutes and MW of 500–800, and the third group with retention times of more than 12 minutes and MW above 800 were detected. The effect of storing the samples at different temperatures was evaluated by measuring the degradation rate in a standard solution of aconite upon storing at $-20°C$, $4°C$, and room temperature. At $-20°C$, the standard solution showed the lowest degree of degradation, suggesting the importance of storing the aconite samples at a low temperature. This study concluded that the tested batches of Fuzi showed significant variation in the concentrations of DDAs, as demonstrated by MALDI-MS and LC—MS.

An HPLC coupled with a diode array detector (HPLC-DAD protocol was developed for the identification and quantification of the three major aconite alkaloids, including AC, MA, and HA, in the roots of *A. carmichaelii* [56]. Contents of LPAs in the roots of *A. carmichaelii* were evaluated using liquid chromatography atmospheric-pressure chemical ionization mass spectrometry (LC-APCI-MS^n), indicating the presence of 26 LPAs. These compounds are interesting from different perspectives, because they possess several pharmacological activities and many AC-type alkaloids are converted to LPAs in the intestine. The authors showed that LPAs can be detected in the P-AR and unP-AR samples. By contrast, the three major AC-type alkaloids, AC, MA, and HA, could not be detected in the P-AR samples. The major alkaloid in the unP-AR sample was MA. In this study, the anti-inflammatory activity of the P-AR and unP-AR samples was evaluated using a COX-inhibitory assay. Both samples showed moderate COX-2 inhibitory activity, with the P-AR samples showing a slightly more potent effect.

A detailed study on the fragmentation pattern of aconite alkaloids was conducted by Yue et al [57]. They identified 111 compounds out of 117 from *A. carmichaelii* using HPLC/ESI—MS/MS^n and Fourier transform ion cyclotron resonance/ESI—MS in the positive ion mode. Among the identified alkaloids, 11 MDAs, 10 DDAs, and 81 LPAs, as well as novel alkaloids, including one MDA, two DDAs, and 48 LPAs, were detected in *A. carmichaelii*. Moreover, one DDA, seven LPAs, and two alkaloids with small MWs that possess C19-norditerpenoid skeleton were reported in *A. carmichaelii* for the first time.

HPLC was also applied for the analysis of an Ayurvedic herbal product, Mahamrutyunjaya Rasa, which is composed of *Aconitum ferox*, *Solanum indicum*, *Piper nigrum*, and *Piper longum* in a ratio of 1:1:1:1 [33]. The marker compounds for these components are AC, solanine, and piperine. The effect of the extracting solvent on alkaloidal recovery was studied through comparing the alkaloidal yields with the use of chloroform, ethyl acetate, or diethyl ether. The optimum solvent for extraction was chloroform. In the optimization process for developing the HPLC method, pH showed a significant effect on the separation of the three marker compounds as well as their separation from other interfering chemicals. The optimum separation was achieved at pH 7.5–8.0. The composition of the mobile phase was studied by evaluating the separation of marker compounds using acetonitrile, KH_2PO_4 buffer, and methanol at different concentrations, including 65:15:15, 60:25:15, and 55:35:15 (v/v), showing the best separation at 60:25:15. The LOD and LOQ for AC were 0.210 μg/mL and 0.693 μg/mL, respectively. The results indicated that the concentration of AC varied across the tested samples and the Relative Standard Deviation (%RSD) values were > 10%. These findings highlighted the importance of strict regulation during the preparation of Ayurvedic herbal products containing aconite.

An HPLC-DAD method assisted by similarity hierarchical clustering analysis was applied for the identification of four species of *Aconitum* roots [58]. The root fingerprints were established and compared. The method was validated, showing its potential in differentiating the roots of *Aconitum kusnezoffii* (AKR) from other species. The effect of the extraction methods on the yields of MA, AC, and HA was evaluated. The samples were extracted using an ultrasonic bath with different volumes of diethyl ether at different extraction times. It was found that the optimum results were obtained using 10 mL of ether for 30 minutes. These results were compared with the results of the normal extraction procedures using a percolator. Soaking the roots for 24 hours yielded results similar to the ultrasonic extraction for 30 minutes, and thus the ultrasonic extraction was selected as the optimum extraction method. An isocratic mobile phase composed of acetonitrile—0.25% glacial acetic acid (60:40, v/v), with pH = 10.5 adjusted using ammonia, was selected for elution. Ten samples of AKR grown at different cultivation or wildness regions, in various cultivating environments, or in different harvesting years were analyzed and their fingerprints were compared. Ten peaks were selected as common peaks in all samples. Careful analysis of these peaks revealed that the relative peak areas varied dramatically, but the relative retention times were consistent for all the 10 samples. When the chromatographic profile of *Aconitum karacolicum* was compared with the AKR profile, five common peaks were detected. When the chromatographic profiles of morphologically similar species, *Aconitum austroyunnanense* and *Aconitum contortum*, were compared with the chromatographic profile of AKR, significant differences were observed, as illustrated by the absence of certain characteristic peaks in the profile of *A. austroyunnanense* and *A. contortum*. These findings were confirmed using hierarchical clustering analysis, which showed that the samples could be divided into three clusters. One cluster included all AKR samples, another cluster contained all *A. karacolicum* samples, and the last cluster contained *A. austroyunnanense* and *A. contortum* samples, suggesting the reliability of this method in differentiating AKR samples from other closely related aconite species.

Lu et al [35] investigated the effect of different processing methods on the content of toxic alkaloids in *A. carmichaelii* (Fuzi; Tables 1 and 2). They processed the samples according to the methods reported by the Chinese herbal medicine practitioners. The content of AC, MA, and HA in the tested

samples (84 samples in total) was evaluated by HPLC–DAD and LC–MS. The efficiency of different solvents [methanol, methanol–water (1:1, v/v), and water] in extracting the target alkaloids was evaluated, and methanol–water (1:1, v/v) was found to be the optimum solvent. Effects of the extraction time, volume, and repetitions of the alkaloidal yields were evaluated. To extract 0.5 g of the powder, ultrasonication for 60 minutes using 5 mL of methanol–water (1:1, v/v) was required to achieve 98% recovery of the three alkaloids. The optimum mobile phase was composed of acetonitrile and ammonium bicarbonate–ammonium hydroxide, with pH adjusted at 10. Different wavelengths, 220 nm, 232 nm, and 240 nm, were evaluated for the detection of the three peaks, and 240 nm was found to be the optimum wavelength. The detection limits for AC, MA, and HA were found to be 0.9 mg/kg, 0.6 mg/kg, and 1.3 mg/kg, respectively. The quantitation limit was estimated to be 3.5 mg/kg for AC, 2.2 mg/kg for MA, and 4.8 mg/kg for HA. The results were in agreement with previous reports, suggesting that processing of aconite roots significantly reduces their toxicity as the sum of the toxic alkaloids was 3.91–34.80% of the original value in raw Fuzi.

An HPLC/ESI–MS^n method was developed for the quantification of LPAs in Radix Aconiti, Radix Aconiti Kusnezoffii, and Radix Aconiti Lateralis Preparata [59]. Different solvents were evaluated, and methanol was found to be the optimum mobile phase for the highest resolution of the LPA peaks. The developed method was applied for the analysis of the three herbs, resulting in the identification of 32 alkaloids based on their fragmentation pathway. The average recovery percentages of the alkaloids were 91.1–105.9%.

The concentrations of AC, MA, and HA in A. carmichaelii, A. pendulum, AKR, Aconitum taipeicum, and A. szechenyianum were determined using an efficient HPLC method (Tables 1 and 2) [40]. The extraction process was optimized by L16 (45) orthogonal test and univariant methods. These methods indicated that the optimum extraction procedures of the alkaloids can be achieved by refluxing the sample three times in six volumes of acidic alcoholic solution for 1 hour. The average recovery rates for MA, AC, and HA ranged from 99.49% to 101.9%, from 101.2% to 103.1%, and from 96.62% to 98.43%, respectively. The mobile phase was optimized after testing the effect of using acetonitrile–0.2% acetic acid (adjusted to pH 6.25 with triethylamine), acetonitrile–phosphate buffer (pH = 8.67), acetonitrile–ammonium bicarbonate buffer (pH = 8.50–9.00), or methanol–ammonium bicarbonate buffer (pH = 8.00–10.00) on peak resolution. The best separation was obtained using acetonitrile–ammonium bicarbonate buffer at pH 9.5.

Aconitum-type alkaloids were analyzed in one famous Chinese herbal formula, Yin Chen Si Ni Tang, which is used for the treatment of liver disorders and jaundice [48]. This formula contains Artemisiae scopariae (Yinchenhao), Radix Aconiti Lateralis Preparata (prepared Fuzi), Rhizoma Zingiberis (Ganjiang), and Radix et Rhizoma Glycyrrhizae Preparata Cum Melle (prepared Gancao). Several components in this formula, including flavonoids and coumarins, hindered the analysis of Aconitum-type alkaloids. The developed UPLC–ESI–Q-TOF–MS method along with the postacquisition data processing software, Metabolynx XS, succeeded in the identification of Aconitum-type alkaloids in Yin Chen Si Ni Tang.

Using the developed method, 62 ions were assigned to Aconitum-type alkaloids and identified tentatively by comparing the information on their accurate mass and fragments with that of the authentic standards or by MS analysis and retrieving the reference literatures.

Functionalized analysis was applied for the detection of Aconitum-type alkaloids from A. carmichaelii using vascular endothelial growth factor receptor cell membrane chromatography with LC–MS [60]. This method depends on the detection of the inhibitory effect of the target compounds on vascular endothelial growth factor receptor and thus predicting their potential as future cytotoxic compounds. Using this protocol, factions separated by the vascular endothelial growth factor receptor cell membrane chromatography column (the 1st dimension) were transferred and adsorbed on an enrichment column, which were sent to the LC–MS system (the 2nd dimension) for separation and preliminary identification. The results indicated that the active compounds of A. carmichaelii were MA, AC, and HA.

The efficiency of alkaloidal titration, the method utilized for the quality control of herbal products containing aconite recommended by the Chinese Pharmacopeia, was compared to an HPLC method developed for the identification of MA, AC, and HA in commercial samples of P-ARs [27]. The results showed that no toxic alkaloids were detected in any of the commercial samples, indicating that the processing method was efficient in removing the toxic alkaloids from the samples. The validity of the method was demonstrated through subjecting the samples to in vivo tests showing no signs of toxicity. When the results of the HPLC method were compared to the alkaloidal titration method, a significant discrepancy in the results was observed. The alkaloidal titration method indicated that the samples still contained 0.2% alkaloids. The main drawback of the alkaloidal titration method is the lack of specificity to the toxic alkaloids, because it estimates not only the toxic DDAs, but also the MDAs, unesterified, and LPAs. These results suggested the importance of using different methodologies for estimating the toxicity of herbal products containing Aconitum-type alkaloids.

An HPLC method was developed for the analysis of AC, MA, and HA in the Chinese herbs Caowu (CW) and Chuanwu (CHW) (Tables 1 and 2) [38]. Separation of these alkaloids was highly affected by the concentration of triethylamine phosphate in buffer solution, and the best separation was achieved using 25mM triethylamine phosphate. The average recovery rates of AC, MA, and HA were found to be 91%, 89%, and 87%, respectively. Concentrations of the three alkaloids were less in the processed CHW compared to those in other processed samples. The effects of boiling raw herbs in water for different periods of times were also studied, which showed that AC and MA disappeared after boiling in water for 150 minutes. However, HA was found to survive the heating process, suggesting its importance as a marker for herbal products containing aconite. The validity of the HPLC method was confirmed by comparing its results with the results of an automated analytical system (HPLC) and ESI/MS/MS. The results were comparable, suggesting the future potential application of the developed method in investigating the quality of herbal products containing aconite.

An HPLC–Q-TOF–MS method was developed for the analysis of alkaloids in the unprocessed Radix Aconiti and Radix Aconiti Preparata [61]. The effects of the extraction method (soaking or ultrasonic bath), extracting solvent (50% ethyl acetate–isopropanol, 80% ethyl acetate–isopropanol, ethanol, methanol and acetone), solvent volume (50, 100 and 200 mL), extraction time (30, 60 or 120 min) and extraction times (once, twice or three times) were evaluated. The optimum result was obtained using the following conditions: 5.0 g of the ground powder was soaked for 10 minutes in ammonia water (5.0 mL) at pH 10, and then the mixture was extracted with a 100 mL mixture of ethyl acetate–isopropanol (1:1) by ultrasonication (30 minutes). The optimal mobile phase was acetonitrile and 0.1% v/v glacial acetic acid. The developed method was applied for the analysis of the P-AR and unP-AR samples, and the obtained peaks were identified as AC alkaloids. The detected peaks were divided into three groups: (1) alkaloids with MW 400–500, which were named as nonester alkaloids; (2) alkaloids with MW 500–800, which were assigned as DDAs and MDAs; and (3) alkaloids with MW > 800, which were identified as LPAs.

A rapid method was developed for the analysis of YA in the P-AR and unP-AR samples as well as in TCM preparations containing aconite herbs (Tables 1 and 2) [36]. YA is often ignored in the quality control measures of herbal preparations containing aconite despite its reported toxicity. To detect this alkaloid, a UHPLC–MS/MS method was developed, which was able to detect YA as well as AC, MA, HA, BAC, BMA, and BHA. Microwave-assisted extraction was utilized to extract the target alkaloids. The solvent for microwave-assisted extraction was 50% methanol (containing 2.5% formic acid) and the irradiation power was 420 W for 1 minute. Thirty-one samples were analyzed, and the contents of the seven alkaloids were determined. Alarmingly, the content of YA varied significantly in some of the evaluated samples, from 0.015 mg/g to 10.41 mg/g. A concentration of 10.41 mg/g is toxic and should be controlled, suggesting the importance of detecting the concentration of YA in aconite-containing herbal products.

A UHPLC-LTQ-Orbitrap-MS^n method was developed for the analysis of DDAs in *A. carmichaelii* [42]. Using the developed method, the authors were able to detect or characterize 42 DDAs. The fragmentation patterns of the major diagnostic alkaloids, including AC, MA, and HA, were investigated. Using the developed method, 23 new compounds were suggested, including 16 esterified DDAs with short fatty acid esters along with four N-dealkyl-type DDAs. The authors showed the advantages of using UHPLC with its small-particle-size stationary phase (1.7 mm) in comparison with the conventional HPLC (5.0 mm), resulting in improved resolution and shorter analysis time.

The safety of Xiaohuoluo pill, a TCM, which is used in mainland China to treat wind cold damp impediment, limb pains, and numbness, has been the subject of recent investigations (Tables 1 and 2) [37]. This TCM is composed of Radix Aconiti Preparata and Radix Aconiti Kusnezoffii Preparata as the main herbs, accounting for 42% of the entire prescription. It is sold in herbal drug markets and produced by several suppliers without extensive quality control measures. An efficient UPLC–ESI–MS method was developed for the rapid analysis of the Xiaohuoluo pill, and the results of

analyzing different samples were evaluated using chemometric analysis of PCA and orthogonal projection to latent structural discriminant analysis. In the process of developing the analytical methods, it was found that the positive ion mode response was much higher than the response in the negative ion mode for MA, AC, HA, BMA, BHA, and BAC, which might be attributed to the ionization of the nitrogen atom in the alkaloids. The optimum mobile phase was found to be acetonitrile–water containing 0.1% formic acid (35:65, v/v) for the best resolution and peak shapes. Using the developed method, the lower LOQs for MA, AC, HA, BMA, BAC, and BHA were found to be 1.41 ng/mL, 1.20 ng/mL, 1.92 ng/mL, 4.28 ng/mL, 1.99 ng/mL, and 2.02 ng/mL, respectively. Recovery percentages of these alkaloids ranged from 99.7% to 101.7%. The developed method was applied for the analysis of different samples. The results indicated that in the Xiaohuoluo pill, concentrations of the DDAs (MA, AC, and HA) were obviously less than those of the MDAs (BMA, BAC, and BHA), indicating the potent effect of herbal processing in changing the alkaloidal concentrations. The quantitative determination of alkaloids in the Xiaohuoluo pill indicated that MA, AC, and HA concentrations were below the required level, as suggested by the Chinese Pharmacopeia. These results suggested that the studied Xiaohuoluo pill is safe for use, if the indicated dosage and regimen are followed.

A ultra-performance liquid chromatography coupled with quadrupole time-of-flight high-definition mass spectrometry (UPLC–Q-TOF-HDMS^n) method was developed for the analysis of the crude lateral roots of *A. carmichaelii* and three P-AR products, Yanfuzi, Heishunpian (HSP), and Baifupian (BFP), which are used by TCM practitioners [62]. The method utilized PCA for establishing the differences between the metabolic profiles of P-AR and unP-AR samples. The authors were able to select 19 metabolites as biomarkers, and they detected the changes in their concentrations as a result of processing. The results indicated that processing was effective in decreasing the concentrations of DDAs. Concentrations of AC, MA, HA, DA, 10-OH-mesaconitine, in Heishunpian and BFP, were significantly decreased, while those of these alkaloids increased in Yanfuzi. This finding suggested that despite the widely accepted assumption that processing is highly effective in decreasing the toxicity of herbal products containing aconite, P-AR products should be carefully analyzed.

An HPLC–ESI–MS^n method was developed for the identification of alkaloids in the crude and processed *A. carmichaelii* [34]. The alkaloids were extracted from the herb using 1% (v/v) hydrochloric acid, which extracted most of the alkaloids. It was found that the addition of ammonia to the mobile phase depressed tailing of the peaks. BAC and BMA could not be separated if the ammonia concentration was less than 1%. Application of the developed method to the analysis of *A. carmichaelii* led to the identification of 48 AC-type alkaloids by studying their MS^n spectral data. The crude and processed chromatograms were compared, which indicated that the contents of MDAs increased after processing, while the concentrations of DDAs decreased.

An LC–MS method was developed for the analysis of alkaloids in the processed Fuzi decoctions, Baifupian and Heishunpian (Tables 1 and 2) [49]. During the process of method development, the effects of the mobile phase on the

separation of the marker alkaloids were investigated, which showed that the best peak shape and resolution can be achieved using a mixture of acetonitrile and an aqueous 0.1% formic acid solution. Seven alkaloids were detected, including higenamine, BHA, BMA, BAC, AC, HA, and MA. The LOQs were 7.80 pg/mL for higenamine, 25.00 pg/mL for BHA and BAC, 10.00 pg/mL for AC and MA, and 50.00 pg/mL for BMA and HA. The LODs ranged from 2.30 pg/mL to 17.00 pg/mL for the targeting alkaloids. Application of the method for differentiating between Baifupian and Heishunpian decoctions revealed significant variation in the alkaloidal contents among these decoctions. As demonstrated in many other studies, concentrations of certain toxic DDAs decreased significantly with processing; however, this trend was not universal for all DDAs. Contents of AC and MA were much lower than those of BAC and BMA in Heishunpian and Baifupian decoctions. By contrast, the concentration of HA in Heishunpian and Baifupian decoctions was higher than that in BHA. The analysis also indicated that the concentrations of BHA, HA, and BAC in Heishunpian decoction were higher than those in Baifupian decoction. However, contents of MA and BMA in Heishunpian decoction were lower compared with those of Baifupian decoction. These findings supported the notion that the variation in the processing protocols can lead to a significant variation in alkaloidal concentrations.

A detailed investigation on the effect of different processing techniques on the alkaloidal content of Radix Aconiti was achieved using UPLC–ESI/MS[n] [50]. After establishing the fingerprints of the P-AR and unP-AR samples, similarity evaluation, hierarchical cluster analysis, and PCA were performed to evaluate the similarity and variation of the samples. The authors processed the samples according to the Chinese Pharmacopeia, and they labeled the P-AR samples as the qualified Radix Aconiti and the unP-AR samples as the unqualified Radix Aconiti. The total ion chromatograms of the P-AR and unP-AR samples showed significant variations, especially in the region of the DDAs. It was revealed that the content of MDAs was higher in the P-AR samples than that in the unP-AR samples. The results also indicated that BMA was the abundant compound in the P-AR samples. Due to the concentration variations between DDAs and MDAs along with sensitivity limitation of the spectroscopic techniques, the authors suggested that quantitative determination of MDAs can be achieved using UPLC–UV, and DDAs can be detected using UPLC–ESI/MS. Contents of LPAs decreased significantly after the first hours of processing and then remained constant. The decline in the concentration was attributed to the hydrolysis of LPAs under the processing conditions and the afterward stability to the reaction of MDAs, DDAs, and fatty acids forming LPAs. PCA was used for the analysis of the main alkaloidal markers affecting the quality of samples. Fingerprints of the nine analyzed samples were obtained and the characteristic peaks, 39 common peaks from total ion chromatograms of UPLC–ESI/MS[n] and 34 common peaks from UPLC–UV chromatograms, were identified.

A microcalorimetric assay along with a UPLC method were applied for the analysis of five different species of aconite, including Radix Aconiti, Radix Aconiti Singularis, Radix Aconiti Kusnezoffii, Radix Aconiti Lateralis Preparata, and Radix Aconiti Brachypodi [63]. Using the developed UPLC method, fingerprints of the five *Aconitum* plants were established. Biological effect of the alkaloids in the tested *Aconitum* sp. on *Escherichia coli* metabolism was studied using a microcalorimetric assay. The metabolic process of *E. coli* was studied, and it was found that the process can be divided into different phases: the first exponential phase (A–B), the lag phase (B–C), the second exponential phase (C–D), and the decline phase (D–E). The effect of using different concentrations of *Aconitum* sp. was studied, which showed significant changes in the metabolic curve with various concentrations of plant samples. A comparison of the fingerprints of the aconite samples led to the identification of 15 common peaks. Among these peaks, those corresponding to AC, HA, and MA were identified. The correlation between these peaks and the changes in *E. coli* metabolism was established, which showed that MA and HA had a negative effect on metabolism, while AC was found to promote bacterial metabolism.

A UPLC–Q-TOF-HDMS method was developed for the analysis of alkaloidal contents in the roots of AKR (CW), the mother root of *A. carmichaelii* (CHW), and the daughter or lateral roots of *A. carmichaelii* ["Shengfuzi" in Chinese] [51]. The results indicated that the optimum mobile phase was 0.1% formic acid in water and 0.1% formic acid in acetonitrile. After analyzing the samples using the developed method, certain statistical tools were applied, including PCA, PLS-DA, and orthogonal projection to latent structure analysis. These tools helped in the classification of the metabolic phenotypes and identification of the differentiating metabolites. The PCA results are shown as score plots indicating the scatter of the samples. If the score plots are clustered together, this indicates similar metabolomic compositions, whereas they are considered compositionally different if the score plots are dispersed. PLS-DA predicts a list of metabolites through measuring the distance from different groups. The S-plot is utilized to identify metabolites according to the orders of their contributions to the separation of clustering. Using these statistical tools, 22 metabolites between Shengfuzi and CHW and 13 metabolites between CHW and CW were identified as biomarkers. Interestingly, concentrations of MA and AC were higher in CW. This phenomenon was attributed to the fact that CW is grown in cold weather, which may induce the production of toxic alkaloids. Moreover, it was found that songorine, carmichaeline, and isotalatizidine were absent in CW, despite their presence in Shengfuzi and CHW.

The effect of adding *Cinnamomum cassia* to the alkaloidal content of Sini Tang, which is formed of *Zingiber officinale*, *Glycyrrhiza uralensis*, and *A. carmichaelii*, was studied using HPLC-DAD [64]. Certain complexes were formed, which were analyzed using proton nuclear magnetic resonance (^{1}H-NMR) and UV/Vis spectroscopy. To clearly study the effect of complexation on toxic alkaloids of *A. carmichaelii*, eight batches of the P-AR and one batch of the unP-AR *A. carmichaelii* roots, as well as one batch of *G. uralensis* roots, *Z. officinale* rhizome, and *C. cassia* bark were tested. *A. carmichaelii* roots were processed by repeatedly soaking them in salt water and boiling until the sliced roots turned black before drying in an oven. The processed samples of *A. carmichaelii* were analyzed using HPLC. Based on the obtained chromatograms, AC and MA contents were below the LOD in all batches. Only HA was detected and thus was selected as the marker compound.

Effect of the extracting solvent was studied, which showed that HA could be detected only if MeOH was used as the extracting solvent; however, other organic solvents were unable to extract HA. HPLC analysis of the unprocessed batch of *A. carmichaelii* extracted with MeOH indicated the presence of HA (269.34 + 0.58 μg/g). The use of 1% HCl yielded 251.12 μg/g HA, MeOH:H_2O (1:1) furnished 199.48 μg/g HA, and 71.32 μg/g HA was obtained using H_2O. However, analysis of the prepared *A. carmichaelii* decoction did not show any traces of HA. The effect of combining other herbs with *A. carmichaelii*, such as *G. uralensis*, *Z. officinale*, and *C. cassia*, on HA content was studied. No HA was detected in the presence of *G. uralensis*, but it was detected when *Z. officinale* or *C. cassia* or both were combined with *A. carmichaelii*; HA concentration was below 40% of the original concentration in *A. carmichaelii*. The effect of combining singular components of *G. uralensis*, *Z. officinale*, and *C. cassia* on HA concentration was also studied, which showed that liquiritin and isoliquiritin were able to reduce the concentration of HA. Other components did not affect HA concentration. When isoliquiritin was mixed with *A. carmichaelii*, the shape of the UV spectra changed. Formation of a supramolecular structure was suggested, which was found to possess a defined stoichiometry, binding constant, and molecular structure. Binding constants of HA with liquiritin in different D_2O/MeOD mixtures were determined for the first time using ^{1}H-NMR titration experiment.

A detailed study of the effect of decoction time on reducing AC alkaloids toxicity was conducted using HPLC. *A. carmichaelii* roots were processed according to the Chinese Pharmacopeia [28]. First, the roots were washed with water and soaked in edible mother liquor of mineral salts for several days. Second, the mixture was boiled and rinsed with water. Third, the roots were peeled, sliced, and soaked and rinsed in water. After steaming and drying, P-ARs were obtained and named as processed Fuzi or Baifupian (BFP). The P-ARs were decocted over different time intervals (30 minutes, 60 minutes, or 120 minutes) forming three different decoctions (DBFP-30, DBFP-60, and DBFP-120, respectively). Each decoction was analyzed using HPLC, and the obtained chromatograms were compared to those of the raw root and BFP. The raw root was found to possess the highest concentrations of AC, MA, and HA. In BFP, concentrations of these three alkaloids were lower than those in raw Fuzi but higher than those in DBFP. The results showed a stepwise decrease in the concentration of each alkaloid with increasing decoction time. In DBFP-120, AC was undetectable and the concentrations of the other two alkaloids reached the lowest values. Interestingly, the content was almost similar to the total alkaloidal content in the three prepared decoctions, suggesting that the toxic alkaloids were successfully converted to nontoxic alkaloids. The toxic effect of the prepared decoction was evaluated using male and female Kunming mice. The median lethal dose (LD_{50}), maximal tolerance dose (MTD), minimal lethal dose (MLD), and no-observed-adverse-effect level (NOAEL) were determined for each decoction. The results indicated that with an increase in the decoction time, acute toxicity of the detoxified Fuzi decreased in the following order: DBFP-30 (LD_{50} 145.1 g/kg, MTD 70 g/kg, MLD 100 g/kg, NOAEL 70 g/kg) > DBFP-60 (very large LD_{50}, MTD 160 g/kg, MLD 190 g/kg, NOAEL 100 g/kg) > DBFP-120 (no LD_{50}, unlimited MTD, unlimited MLD,

NOAEL 130 g/kg). Additionally, adjuvant arthritis rats were used to assess the pharmacological effect of detoxified Fuzi roots. Adjuvant arthritis rats are special experimental models that develop rheumatoid arthritis symptoms including anorexia and body weight loss. Restoration of body weight can only be achieved using detoxified Fuzi roots. The results indicated no significant difference in the pharmacological effects of the three different decoctions. Based on these findings, the authors recommended the use of DBFP-120 compared to other aconite forms because it exhibited the same pharmacological effect without any acute toxicity.

An RRLC–MS/MS method was developed for analyzing the components of an ancient TCM, Shen-Fu (Tables 1 and 2) [65]. The herbal formula is composed of Radix ginseng and Fuzi (Radix Aconiti Lateralis Preparata) at a ratio of 3:2, and it is prescribed for the treatment of diseases associated with the signs of Yangqi decline and Yang exhaustion. The effect of the mobile phase on the separation of alkaloids was evaluated, and the results showed that the best peak shape and resolution were obtained using a mixture of acetonitrile and an aqueous 0.05% formic acid solution. The LOD ranged from 0.01 ng/mL to 1.25 ng/mL, and the recovery percentage ranged from 91.13% to 111.97% for all components including AC, MA, and HA. The results indicated that AC was the least abundant component among all the analytes.

A UPLC–ESI/MS method was developed for the identification of the constituents of complex herbal preparations used in TCM including Sanhuang Xiexin Tang (SXT) and Fuzi Xiexin Tang (FXT). SXT is composed of Rhei Radix et Rhizoma (Polygonaceae family, rhizomes of *Rheum officinale*), Scutellariae Radix (Labiatae family, roots of *Scutellaria baicalensis*), and Coptidis Rhizoma (Ranunculaceae family, rhizomes of *Coptis chinensis*) (Tables 1 and 2) [43]. FXT possesses similar composition in addition to Aconiti Lateralis Radix Preparata (Ranunculaceae family, roots of *A. carmichaelii*). SXT and FXT are prepared either through maceration or through decoction. The developed method was applied for the analysis of Aconiti Lateralis Radix Preparata decoction, SXT, FXT, macerated SXT, macerated FXT, SXT decoction, and FXT decoction. The results indicated a significant variation in the compositions of the evaluated samples. Specifically, DDAs (AC, HA, and MA) were not detected in FXT decoction and macerated FXT. However, HA was detected in Aconiti Lateralis Radix Preparata decoction, but without AC and MA. In general, more constituents were found in the decoction products compared to the maceration products, which suggested more potent pharmacological activity of the decoction. The results also revealed possible drug–drug interaction due to the complexity of the herbal preparation and differences in their preparation procedures.

A UHPLC–Q-TOF/MS method was developed to compare the efficiency of detoxification mechanisms, as described in Ayurveda and TCM, of the roots of *Aconitum heterophyllum*, *A. carmichaelii*, and AKR [31]. In Ayurveda, the detoxification mechanism or Shodhana is accomplished by treating the herbal products with cow urine or cow milk. In TCM, the most general protocol for the detoxification of herbal products containing *Aconitum* sp. is the use of water decoction. The developed method was validated, and the LODs of AC, MA, and HA were found to be 0.383 ng/mL, 0.438 ng/mL, and

0.088 ng/mL, respectively. The LOQs for AC, MA, and HA were 1.15 ng/mL, 1.31 ng/mL, and 0.264 ng/mL, respectively. Samples treated with cow milk, cow urine, or water were analyzed using the developed method. The results demonstrated that the three detoxification mechanisms were effective in reducing the concentrations of DDAs in all samples. However, treating samples with cow urine was found to be less effective than the other two protocols.

An efficient analytical method was developed for the determination of aminoalcohol-diterpenoid alkaloids in the lateral roots of *A. carmichaelii* (Fuzi) using solid-phase extraction followed by chromatography–tandem mass spectrometry [66]. For the solid-phase extraction experiment, a good recovery of aminoalcohol-terpenoid alkaloids was achieved using 8% ammonia in methanol as the elution solvent. The optimum mobile phase was found to be methanol–0.1% formic acid in the ratio of 80:20 (v/v). This mobile phase yielded a good peak shape, acceptable separation, and a small tailing factor. Specificity of the developed protocol was evaluated by analyzing the blank solvent, mixed standard solution, reference solutions of the determined alkaloids, internal standard, and sample solution. No interference was detected among the solvent, alkaloids, or any other tested samples, suggesting the specificity of the method. The developed method was successfully applied for the determination of 13 aminoalcohol-diterpenoid alkaloids in Fuzi samples obtained from different sources and subjected to different processing conditions.

A UFLC/MS-IT-TOF method was developed for the analysis of alkaloidal constituents of Fuzi and Fuzi–Gancaoherb pair (FG), which consists of *A. carmichaelii* (Fuzi) and Roast Radix Glycyrrhizae (*Glycyrrhiza glabra*, Gancao in Chinese) [47]. Diazepam was used as the internal standard in the developed protocol. The optimum mobile phase for the separation of alkaloids was found to be ammonium acetate and acetonitrile. Application of the developed protocol for the analysis of Fuzi and FG resulted in the detection of 60 common peaks in both samples. Among these peaks, those corresponding to 51 alkaloids were identified by accurate mass measurements and fragmentation pathways. A semiquantitative analysis of the samples, which was achieved through comparing the obtained alkaloidal peak areas with the IS peak areas, provided a clearer picture on the differences of alkaloidal contents between FG and Fuzi. It was found that the concentrations of DDAs and aminoalcohol-diterpenoid alkaloids were higher in FG decoction, while the concentrations of MDAs were lower.

A quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF–MS) method was developed for the analysis of a traditional herbal medicine, Wu-tou decoction, which is used to treat rheumatic arthritis [67]. It is composed of Aconiti Radix Cocta, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix, and Glycyrrhiza Radix Preparata, indicating the complexity of structures and difficulties in the determination of mixture components. The optimum mobile phase for the developed method was found to be acetonitrile–water with 0.1% formic acid. Application of the developed method led to the identification of 74 components, including alkaloids, monoterpene glycosides,

triterpene saponins, flavones, and flavone glycosides. The components were confirmed by comparing the obtained chromatogram with the chromatograms of individual herbs and standard available components. Among the detected compounds, 43 alkaloids, including Ephedra alkaloids as well as aconite alkaloids, were detected in the positive ion mode. Fragmentation pattern of the identified alkaloids was studied and compared with that of the previous literature, which aided in their identification. The developed method can be added to the quality control tools that might be used for the analysis of Wu-tou decoction.

A microcalorimetric method along with UPLC was developed for the analysis of Fuzi and three different P-AR products including Yanfuzi, Heishunpian, and Paofupian [68]. A microcalorimetric method can provide valuable information on the growth and metabolic status of cells under the effect of the tested compounds. Under certain growth conditions, cellular heat production and growth progress of cells produce unique power–time curves. From these curves, the effect of certain compounds can be evaluated, suggesting the potential action of these compounds. Using UPLC, the fingerprints of Fuzi and its three P-AR products were developed and compared. The effect of these samples on the metabolism of rat mitochondria was investigated using the developed microcalorimetric method. Finally, the results of the UPLC and calorimetric method were correlated using a canonical correlation analysis model. A study of the metabolism of mitochondrial rats suggested that the metabolic cycle can be divided into four stages: Stage I, Stage II, Stage III, and Stage IV. Certain thermokinetic parameters were also calculated, including k, Q, P_{max}, t_{max}, t_{lag}, and P_{av}. Among these parameters, the most important one was "k", the exponential growth rate of Stage II. It was found that metabolism reached its peak when a sample concentration of 4.0 mg/mL was used, and any increase in the concentration led to a decrease in metabolism. The use of 4.0 mg/mL of the samples did not produce any significant difference in the "k" values among Heisunpian and Paofupianor Yanfuzi, which suggested lower efficiency compared to the unprocessed Fuzi but higher safety. By comparing the chromatograms of the analyzed samples, 26 common peaks were detected in the four samples. The effect of these alkaloids on metabolism could be studied by the use of the canonical correlation analysis model. It was found that benzoylhypaconitine, MA, and HA had a positive effect on the promotion of mitochondrial metabolism. On the other hand, benzoylaconitine had a negative effect on metabolism.

An HPLC–Q-TOF–MS method was developed for the analysis of Shen-Fu injection, which is a widely used Chinese herbal formulation for cardiac diseases (Tables 1 and 2) [46]. It is composed of red ginseng and P-ARs. The developed method was highly sensitive and did not require sample pretreatment. The LOQs ranged from 0.4 ng/mL to 18 ng/mL for DDAs. The method was applied to nine batches of Shen-Fu injection, and it showed high batch-to-batch reproducibility. Application of the developed method led to the identification of 44 compounds and quantification of 24 major alkaloids and ginsenosides.

A functionalized analytical method was developed for the analysis of Fuzilizhong pills. These pills are a modified form of

a famous TCM Lizhong Wan described in Treatise on Febrile Diseases. It consists of *Panax ginseng* (Ren Shen), *A. carmichaelii* Debx. (Fu Zi, Zhi), *G. uralensis*, *Glycyrrhiza inflata* or *G. glabra* (Gan Cao), *Atractylodes macrocephala* (Bai Zhu), and *Z. officinale* (Gan Jiang) [45]. This medication is prescribed for dyspnea and pulmonary edema. For the analysis of Fuzilizhong pills, a UPLC−MS method and a luciferase reporter assay system to simultaneously screen nuclear factor kappa beta (NF-κB) inhibitors and Beta-2 Adrenergic Receptor (β2AR) agonists were applied. Inhibition of NF-κB is related to lower inflammatory reaction. Stimulation of β2AR is related to the activation of adenylyl cyclase, cyclic adenosine monophosphate (cAMP), and protein kinase A, and phosphorylation of numerous effector proteins, which lead to the relaxation of tracheal smooth muscles. The use of 250 μg/mL or 1000 μg/mL of Fuzilizhong pills led to the inhibition of tumor necrosis factor alpha-induced NF-κB production and activation of β2AR signal. The results indicated that MA, flaconitine, BMA, AC, and HA are potent NF-κB inhibitors.

4.   Conclusion

Aconitum and its products have played an important role in human history. It was one of the most feared plants in all ancient civilizations. Despite its extreme toxicity, it was sought for its healing and mysterious power. Indian and Chinese civilizations studied this plant extensively and introduced detailed treatises on how to prepare nontoxic decoctions and extracts from this plant. More than 70 different preparation methods were reported in ancient scriptures, and most of these methods are still used today. The Chinese Pharmacopeia established certain criteria for the total contents of toxic alkaloids, which should be referenced by any producer preparing herbal products containing *Aconitum*. Despite these measurements, a wide variation in concentrations of toxic alkaloids has been recorded in the marketed preparations, exposing the public to unnecessary risks. The recent advances in analytical techniques, especially HLPC, MS, and CE, allowed the accurate determination of each alkaloid in herbal mixtures, thus establishing more precise limits for the toxic alkaloids in aconite preparations intended for human consumption. Theses analytical techniques are available in quality control laboratories and can easily be applied for assessing the quality of aconite products. Based on the summary presented in this review, it is highly recommended to change the original measures set in pharmacopeias that focused only on the determination of AC and replace the tests with advanced analytical methods establishing the accurate concentrations of every aconite alkaloids.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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