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Original Article

Chemical profiling of principle active and toxic constituents in herbs containing aristolochic acids

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ABSTRACT

Objective: To clear the amounts of the principal active/toxic components in herbs containing aristolochic acids (HCAAs), which are still used as medicine and/or seasoning in many ethnic minority areas of China.
Methods: In this study, six major active and toxic components in HCAAs were extracted with ultrasonic extraction. With 6-*O*-methyl guanosine as internal standard, the target compounds were analyzed qualitatively and quantitatively by using ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) with multiple reaction monitoring-information dependent acquisition-enhanced production ion scanning mode (MRM-IDA-EPI) combined with dynamic background subtraction (DBS) function.
Results: The method showed good linearity in the linear range of the six analytes. The limit range of detection was from 0.01 ng/mL to 0.27 ng/mL. All of the detection repeatability, extraction repeatability and accuracy of the method were good. After extraction, the samples remained stable at 15 °C within 24 h. Six analytes were all found in samples except aristolactam (AL) in sample 2, and the contents varied greatly. The contents of these compounds decreased in fruits, leaves and stems of *Aristolochia delavayi* successively.
Conclusion: This method has the advantages of less sample dosage, simple operation, short analysis cycle, high sensitivity, specificity and accuracy. It laid a good foundation for guiding the safety of HCAAs, the in-depth study of pharmacological and toxicological effects and the scientific and standardized processing and compatibility of HCAAs.

1. Introduction

Herbs containing aristolochic acids (HCAAs) mainly include *Aristolochia* and *Asarum* of Aristolochiaceae. In China, they are mainly distributed in Yunnan, Guangxi, Sichuan, Guizhou and other ethnic minority areas. The common species are *Aristolochia debilis* Siebold & Zucc, *Aristolochia cinnabaria* C. Y. Cheng & J. L. Wu, *Asarum sieboldii* Miq. and so on. HCAAs have a long medicinal history in China, and most of them have the effects of dispelling wind and dampness, dredging the channels, relieving pain, activating *qi*, promoting blood circulation, clearing heat, promoting urination and relieving cough and asthma (Shao, Zhang, Zheng, Wei, & Li, 2022; Zhang et al., 2019a; Zheng, 2020). It was found that aristolochic acids (AAs), the main active ingredient in HCAAs, had good effects on anti-tumor, anti-bacterial and anti-platelet aggregation. Moreover, AAs could enhance phagocyte activity and treat snakebite (Song, Ren, Yang, Guo, & Du, 2014; Yun, Xu, & Song, 2019; Zhang et al., 2019a).

Studies in the early 1990s found that AAs could lead to strong renal toxicity (But, Bieler, & Cosyns, 2001), including renal interstitial fibrosis and renal failure (Vanherweghem et al., 1993). In the stage of chronic renal failure, some patients were complicated with urinary system tumors mainly located in the pelvis, ureter and bladder (Cosyns, 2003). In addition to urinary tract tumors, anterior gastric tumors were also observed in animals treated with AAs (Liang & Xu, 2005). In 2012, the International Agency for Research on Cancer (IARC) of the World Health Organization listed AAs as a Class I carcinogen, considering that AAs had strong nephrotoxicity, mutagenicity and potential carcinogenicity. Since then, AAs have been more strictly controlled, many countries and regions have taken comprehensive measures to ban HCAAs. *Chi-*

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nese Pharmacopoeia (Chinese Pharmacopoeia Committee, 2015) which was published in 2015 recorded the content of aristolochic acid A (AA-A) as the safety evaluation and quality control guideline of *Aristolochiae Fructus* (Madouling in Chinese), *Aristolochiae Herba* (Tianxianteng in Chinese), and *Asari Radix* et *Rhizoma* (Xixin in Chinese). However, in the 2020 edition (Chinese Pharmacopoeia Committee, 2020), only *Asari Radix* et *rhizoma* was included.

Studies found (Ding et al., 2018) that in addition to AA-A and AA-B, AA-C and AA-D also exist in HCAAs. Ames test results (Cao et al., 2016) showed that AA-B was more mutagenic than AA-A, and AA-D was also a possible genotoxic component in HCAAs. Aristololactam (AL) is not only the active intermediate of AAs to bring genotoxicity *in vivo*, but also has a certain content in HCAAs, which is maybe more toxic than AAs (Michl, Ingrouille, Simmonds, & Heinrich, 2014). Now HCAAs are still widely distributed and applied in ethnic minority areas of China. Mongolians and Tibetans agree that the toxicity of drugs is relative, not absolute. If the doctors diagnose the symptoms correctly and administrate the medicine properly, the effect of the medicine will exactly cope with the situation of the disease, and the medicine will not bring adverse events. In addition, toxicity can be removed or reduced to safeness range by means of processing, compatibility, dosage form, dosage and administration method (Jiang, Yue, & Zhu, 2021; Song, Ren, Yang, Guo, & Du, 2014; Zhang et al., 2019b).

The above results suggest that many low content active/toxic components in HCAAs may be more toxic, so it is not enough only to detect and control the content of AA-A. HCAAs have wide application and exact curative effect, and various methods have been adopted to reduce and control the toxicity of HCAAs clinically. What's more, the safe evaluation method should be developed. Therefore, the highly sensitive simultaneous analysis method of various main active and toxic components in HCAAs is the basis of the toxicological research of HCAAs. Meanwhile, the method can also provide suggestions and scientific guidance for the processing and compatibility of traditional Chinese medicines to reduce toxicity and increase efficacy (Jiang, Yue, & Zhu, 2021; Song, Ren, Yang, Guo, & Du, 2014; Zhang et al., 2019b).

Currently, high performance liquid chromatography coupled with ultraviolet detector (HPLC-UVD), photodiode array detector (PAD), or fluorescence detector (FLD) (Chan, Lee, Liu, & Cai, 2007; Yuan et al., 2007a; Zhang et al., 2006), and liquid chromatography coupled with mass spectrometry working in selected ion monitoring (SIM) or multiple reaction monitoring (MRM) mode (Chan, Pan, & Chan, 2020; Ding et al., 2018; Guo et al., 2021; Mi et al., 2021; Wei et al., 2005; Yuan et al., 2007b; Zhang et al., 2019b) were the most commonly techniques used in AAs analogues quantification. However, there are problems such as low sensitivity, poor specificity, long analysis cycle and complicated pre-treatment steps (Zhang et al., 2021). In this study, ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) technology was used to establish a highly sensitive and highly specific internal standard quantitative method for six main active/toxic components in HCAAs under multiple reaction monitoring-information dependent acquisition-enhanced product ion (MRM-IDA-EPI) mode. This method was applied to detect the contents of AAs and AL (Chemical structures were shown in Fig. 1) in various HCAAs using 6-*O*-methyl guanosine as internal standard (IS).

2. Materials and methods

2.1. Caution

Aristolochic acids have very definite nephrotoxicity and genotoxicity, so extra care should be taken when crushing plants, handling powders and standard compounds.

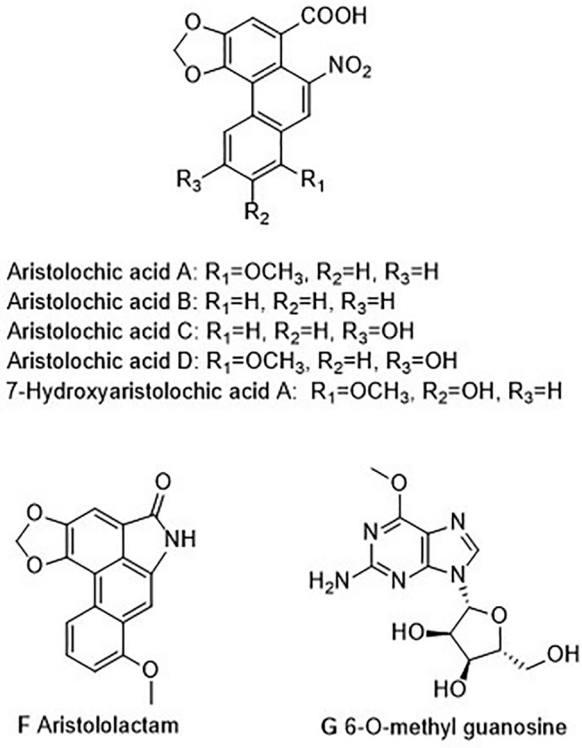

Fig. 1. Chemical structures of five AAs (A–E), AL (F) and IS (G).

2.2. Plant materials

The details about the HCAAs materials used in this research were shown in Table 1 and Fig. 2. Associate professor Xinxin Zhu, who is working in College of Life Sciences, Xinyang Normal University, identified all plant materials used in this research. His research focused on the taxonomy and systematic research of *Aristolochia* genus, and conducted the general survey of plant sources in Dabie Mountain.

2.3. Chemicals and standards

HPLC grade methanol ($\geq$ 99.8%) was purchased from J. T. Baker Chemical Company (New Jersey, USA). Wahaha purified water was produced by Zhejiang Wahaha Drinking Water Co., Ltd. (Hangzhou, China). Glacial acetic acid and other analytical grade reagents used in this research were obtained from Beijing Chemical Works And Sinopharm Chemical Reagents Co., Ltd. (Beijing, China).

Five AAs and aristololactam standards, aristolochic acid A (AA-A, $\geq$ 99%), aristolochic acid B (AA-B, $\geq$ 98%), aristolochic acid C (AA-C, $\geq$ 98%), aristolochic acid D (AA-D, $\geq$ 98%), 7-hydroxyaristolochic acid A (7-OH AA-A) and aristololactam (AL) were HPLC grade and bought from Chengdu Desite Biotechnology Co., Ltd. (Chengdu, China). 6-*O*-Methyl guanosine (IS, > 95%) was provided by Pharmacodia Co., Ltd. (Beijing, China).

2.4. Sample preparation

All naturally dried plant samples were crushed with FW177 Chinese herbal medicine pulverizer (Tianjin Tester Instrument Co., Ltd., Tianjin, China), and the sample powder was accurately weighed using XP205 precision analysis electronic balance (Mettler Toledo, Switzerland), and then ultrasonic extraction was carried out by X6 type 1200 W ultrasonic generator (frequency 28 kHz) (Shenzhen Keli Ultrasonic Cleaning Equipment Co., Ltd., Shenzhen, China). After optimizing by response surface methodol-

Table 1
Details of herb materials used in this research.

Material No.	Chinese names	Botanical names	Sources	Detection sites	Use or efficacy
A	Changye Madouling	*A. championii* Merr. et W. Y. Chun	Bought from Malipo morning market, Wenshan, Yunnan on 28, April 2019 by Dan Zhu and Feng Jiang	Root tuber and fibrous root	Treat stomachache
B	Guangxi Madouling	*A. kwangsiensis* Chun et F. C. How		Root tuber	Treat stomachache
C	Beishesheng	*A. tuberosa* C. F. Liang et S. M. Hwang	Gathered from local courtyard, Malipo, Webshan, Yunnan on 28, April 2019 by Dan Zhu and Feng Jiang	Root tuber	Treat stomachache and gastric ulcer
D	Qiyang Xixin	*Asarum Magnificum* Tsiang ex C. Y. Cheng et C. S. Yang	Gathered from Yingshan, Huanggang, Hubei on 20 April 2019 by Xinxin Zhu	Rhizome	Analgesic and tranquilizing
E	Huluye Madouling	*A. cucurbitoides* C. F. Liang	Gathered from Shanghai Chenshan Botanical Garden in 2021 by Xinxin Zhu	Rhizome	Pain relieving
F	Jinjiang Madouling	*A. jinjiangensis* H. Zhang et C. K. Hsieh		Rhizome	Not clear
G	Guanhua Madouling	*A. tubiflora* Dunn		Rhizome	Pain relieving and detoxification
H	Dabieshan Madouling	*A.dabieshanensis* C. Y. Cheng & W. Yu		Rhizome	Ornamental and medicinal, but the detailed medicinal function has not reported
I	Guanye Madouling	*A. delavayi* Franch.	Bought online in September 2021 by Feng Jiang, produced by Shengshida (Yulong) Biology Science and Technology Co., Ltd.	Fruit	No reports
				Leaf	Seasoning spice, Aromatic stomach tonic, stimulate appetite and treat common cold and malaria
				Stem	No reports

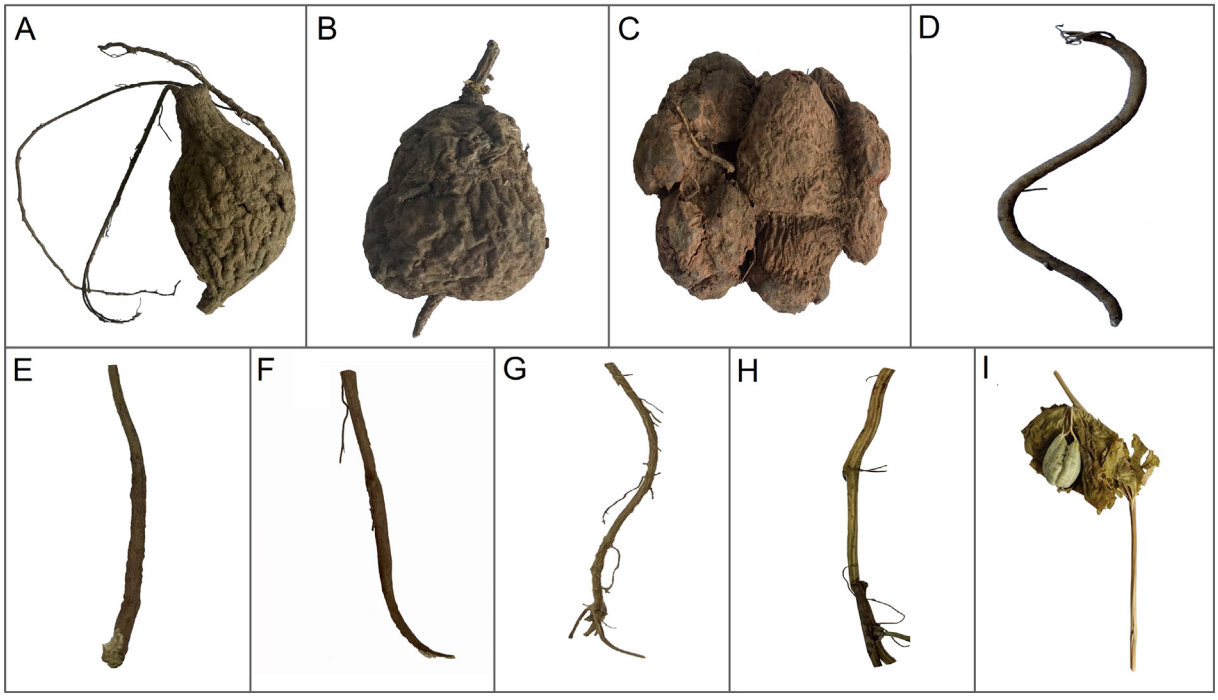

Fig. 2. Plant materials used in this study (A, *A. championii*; B, *A. kwangsiensis*; C, *A. tuberosa*; D, *Asarum Magnificum*; E, *A. cucurbitoides*; F, New species, *A. jinjiangensis*; G, *A. tubiflora*; H, *A. dabieshanensis*; I, *A. delavayi*).

ogy, the ultrasonic extraction conditions were determined as follows (Yue, Yang, Jiang, Yang, & Zhu, 2021): The ratio of liquid to material was 20 mL/g, the extraction temperature was 30 °C, the extraction solution was 53% methanol aqueous, the extraction time was 59 min, and the ultrasonic power was 85%. The extraction was diluted to the concentration of linear range and filtered by 0.22 μm microporous membrane prior to the instrumental analysis.

2.5. Instrumentation and analytical conditions

An AB SCIEX Exion AD UPLC system (Milford, USA) consisting a temperature controllable autosampler, thermostatted column compartment and binary pump and equipped with a Waters ACQUITY UPLC®BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm, Milford,USA) was applied for the separation of analytes. The mobile phases consisted of 0.1% acetic acid aqueous solution (A) and HPLC-grade methanol (B), and the isocratic elution procedure A: B = 1:1 was performed during 4 min analysis period. The flow rate was 0.3 mL/min, and the injection volume was 2 μL.

MS detection was executed in the positive ESI mode on an AB SCIEX QTrap 5500 mass spectrometer (AB SCIEX, Foster, USA) equipped with Turbo V™ ion source. The curtain gas was 241.32 kPa, the collision gas was Medium, both of the spray gas (ion source gas 1) and auxiliary heating gas (ion source gas 2) were 344.74 kPa, ionspray voltage was 5 500 V and ion source temper-

ature was 500 °C, respectively. Quantification was performed with MRM-IDA-EPI mode combined with dynamic background subtraction (DBS) function. Entrance potential was 12 V and collision cell exit potential was 17 V for all analysts. Other MS parameters were listed in Table 2.

2.6. Method validation

The peak area ratio of the analyte and the internal standard as the ordinate, and the concentration ratio of the analyte and the internal standard as the abscissa, the linearity was evaluated by $1/x$ weighted linear least squares regression analysis of calibration curves. The sensitivity was established by determining the limit of detection (LOD, S/N = 3) and the lower limit of quantification (LLOQ, S/N = 10). The precision was determined by the intra-day and inter-day variations of three concentrations quality control (QC) samples for seven times in 1 d and on five consecutive days. The accuracy was expressed as the mean ± standard deviation (SD) of percentage ratios, and the calculation formula was (found concentration − concentration in sample) × 100% / the standard solution concentrations spiked in HCAAs sample matrix. The stability at 15 °C in 24 h was determined as the relative standard deviation (RSD) of determined value. The extraction repeatability was determined by five parallel samples of the same plant material powder and extract under the same conditions.

3. Results and discussion

3.1. Method development

3.1.1. MS conditions

At the beginning, the MS parameters of seven compounds (five AAs, AL and IS) were optimized for the highest sensitivity by flow injection, and the standard solution was injected continuously by the needle pump. Then, the first order mass spectrometry (Q1 Scan) and the production scan were performed successively. The mass spectrometry responses of the positive and negative ion modes were compared, and it was found that the seven compounds could obtain high sensitivity and stable precursor and production pairs in the positive ion mode. Among them, the $[M + NH_4]^+$ of five AAs, $[M + H]^+$ of AL and IS were stable and had high response. The base peak of mass spectrum corresponding m/z was selected as the quantitative ion and the strongest and subintense fragment ions as the qualitative ion. Therefore, the quantitative precursor/product ion pairs of m/z 359.4 → m/z 298.1, m/z 329.0 → m/z 268.0, m/z 345.2 → m/z 282.0, m/z 375.0 → m/z 312.0, m/z 375.3 → m/z 314.0, m/z 294.3 → m/z 279.0, and m/z 298.4 → m/z 166.3 were used as multiple reaction monitoring (MRM) transitions of compounds A to E (Fig. 1), respectively. Then, the collision energy (CE) and declustering potential (DP) of each compound were optimized using compound optimization function by connecting two-way instead of chromatography column, and the results were shown in Table 2. The mass spectra of five AAs, AL and IS were shown in Fig. 3.

3.1.2. UPLC conditions

The stationary phase and mobile phase were also optimized for high sensitivity, fast analysis speed and good peak shape. At first, ACQUITY UPLC®BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm, USA) was used for the analysis, and good separation was obtained. However, the long analysis time of 10 min decreased the analysis throughout. Subsequently, ACQUITY UPLC®BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm, USA) was employed for good separation, rapid analysis and high sensitivity.

Various mobile phase compositions were evaluated using HPLC-grade methanol as organic solvents, and purified water or purified water containing formic acid (0.1%), or formic acid (0.1%) and ammonium formate (5 mmol/L), or acetic acid (0.1%). The chromatography behavior of the analyte in the latter two aqueous phases was similar, which could obtain good sensitivity and selectivity. Considering the convenience of operation, 0.1% acetic acid aqueous and HPLC-grade methanol were selected as mobile phases. Furthermore, the influence of different gradients on the analysis was investigated. It was found that under the condition of methanol concentration close to the extraction solution, good separation and peak shape could be obtained in a short analysis time. Thus, the mobile phase was finally determined as the water phase to organic phase 50:50 (volume percent). The addition of acetic acid modifier can not only prevent the formation of anions in analytes, but also effectively inhibit the $[M + Na]^+$ peak, making $[M + NH_4]^+$ become the main molecular ion peak, thus significantly improving the response of mass spectrometry. Chromatograms of reference substances of AAs, AL, and IS in UPLC-MS/MS using MRM mode were shown in Fig. S1.

3.1.3. Determination of internal standard

Few researches used IS in similar analysis methods, for example, acacetin (Ding et al., 2018) and tribenzylamine (Yuan et al., 2007b). In consideration of the accuracy and reliability, internal standard method was applied. Although inferior to the isotope internal standard, compared with the external standard, the conventional internal standard can eliminate the instability of the sample and instrument, reduce the influence of matrix effect, and obtain results that are more accurate. In this study, some available compounds in our laboratory, such as Irbesartan, 7-

Table 2
Mass spectrum parameters.

Analytes	Molecular weight	Precursor ion	Product ion	Declustering Potential (V)	Collision Energy (V)
AA-A	341.3	359.3 $[M + NH_4]^+$	298.0* $[M\text{-}CO_2 + H]^+$ 324.3 $[M\text{-}H_2O + H]^+$	95	15
AA-B	311.2	329.5 $[M + NH_4]^+$	268.4* $[M\text{-}CO_2 + H]^+$ 294.3 $[M\text{-}H_2O + H]^+$	80	15
AA-C	327.2	345.2 $[M + NH_4]^+$	282.3* $[M\text{-}CO\text{-}H_2O + H]^+$ 310.4 $[M\text{-}H_2O + H]^+$	70	15
AA-D	357.0	375.5 $[M + NH_4]^+$	312.2* $[M\text{-}CO\text{-}H_2O + H]^+$ 281.3 $[M\text{-}CH_3O\text{-}NO_2 + H]^+$	65	18
7-OH AA-A	357.3	375.3 $[M + NH_4]^+$	314.0* $[M\text{-}CH_3\text{-}NO_2 + H]^+$ 340.3 $[M\text{-}H_2O + H]^+$	60	15
AL	293.3	294.2 $[M + H]^+$	279.2* $[M\text{-}CH_3 + H]^+$ 251.1 $[M\text{-}CH_3\text{-}CO + H]^+$	160	36
IS	297.3	298.3 $[M + H]^+$	166.2* $[M\text{-}C_5H_8O_4 + H]^+$ 282.4 $[M\text{-}CH_4 + H]^+$	80	25

Note: * Quantification ion.

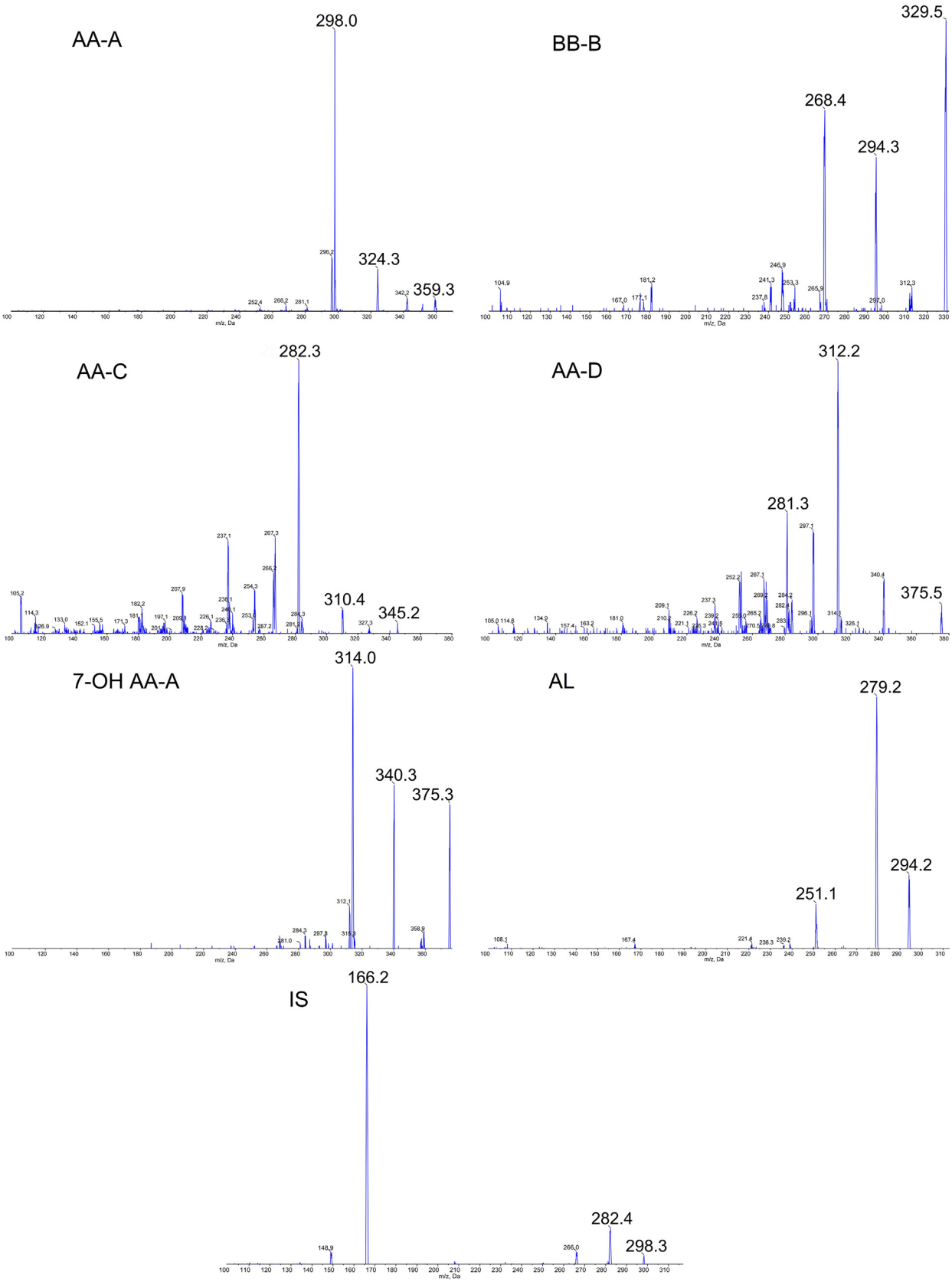

Fig. 3. Mass spectrum of six analytes.

methyl guanosine and 6-*O*-methyl guanosine were investigated as internal standard, and the results showed that the chromatography behavior of Irbesartan was quite different with that of the target compounds, and the purity of 7-methyl guanosine was not high enough. Therefore, 6-*O*-methyl guanosine was selected as the internal standard.

3.1.4. MRM-IDA-EPI mode

In order to qualitative and quantitative the target compounds at the same time and avoid false positives, MRM triggered enhancer ion scanning mode was adopted. As AA-D and 7-OH AA-A are isomers with the same precursor ion *m*/*z* and similar chromatography behavior, both of the retention times are about 1.26 min under the above chromatography conditions, and the two compounds flow out together, so baseline separation cannot be achieved. Fortunately, there are significant differences in their mass spectroscopic fragmentation patterns and characteristic fragment ions. Therefore, it becomes feasible to trigger the strongest and sub-strong parent ions in IDA Criteria level and start dynamic background subtraction in real time.

3.2. Method validation

3.2.1. Linearity and sensitivity

The calibration curves obtained were linear over the concentration ranges with the correlation coefficient *r* over 0.99 (Table 3). The lowest LOD was 0.01 ng/mL for 7-OH AA-A and AL, the highest LOD was 0.27 ng/mL for AA-C (approximately 40–540 fg on-column), and the LLOQ defined as the lowest concentration that could be measured with a precision of less than 15% (RSD). Two LOD were listed in Table 3, the first LOD represented the lowest detection concentration of the sample solution, which can directly reflect the sensitivity of the analytical method. The second LOD reflected that the minimum AAs' amounts can be detected in the medicinal materials, which has reference value for establishing quality standards of herbal medicines.

The detection sensitivity was comparable to that of reference using HPLC-FLD (Chan, Lee, Liu, & Cai, 2007) and higher than that of other methods in references (Chan, Pan, & Chan, 2020; Ding et al., 2018; Guo et al., 2021; Mi et al., 2021; Wei et al., 2005; Yuan et al., 2007a,b; Zhang et al., 2006; Zhang et al., 2019b), but extraction, derivation and solid phase extraction (SPE) purification steps had to be go through, which were tedious and time-consuming, and the derivative products should be evaluated by a specific method.

3.2.2. Precision, recovery, repeatability and stability

The results of intra-day and inter-day precision, recovery, repeatability and stability were shown in Table 4. The intra-day assay RSD values were less than 15% with the values ranging from 0.96% to 13.23%, and the inter-day assay precision was less than 20% with values ranging from 0.46% to 18.83%.

The good recoveries at three different concentration levels ranged from 80.38% to 111.94%. Although the recovery rate was measured based on the standard spiked in the sample matrix, the results showed that the recovery rate was good and the matrix effect was almost negligible. The reason may be the very high content of the target compound in the sample, which required dilution of 100 times or more to be analyzed, and the diluted sample had almost no matrix effect.

According to the repeatability and stability results, the ultrasonic extraction method had good repeatability. After extraction, the samples could remain stable at 15 °C for 24 h.

To sum up, the UPLC-MS/MS method established in this paper had the advantages of qualitative accuracy, high sensitivity, and strong specificity. The low ionization efficiency and matrix effect were compensated by adding modifiers and internal standard quantification. MRM-IDA-EPI mode was used to achieve simultaneous qualitative and quantitative.

Table 3
Linear regression parameters of calibration curves and method sensitivity.

Compounds	Retention time (min)	Linear equation	*r*	Linear range (ng/mL)	LOD (ng/mL)	LOD (ng/g)
AA-A	2.84	$y = 0.176\,82\,x + 0.003\,53$	0.994 3	0.49–250	0.12	2.4
AA-B	2.18	$y = 0.348\,22\,x + 0.006\,87$	0.993 7	0.44–224	0.11	2.2
AA-C	1.01	$y = 0.171\,65\,x + 0.004\,53$	0.994 7	1.09–280	0.27	5.4
AA-D	1.27	$y = 0.442\,60\,x + 0.001\,99$	0.993 6	0.11–227	0.06	1.2
7-OH AA-A	1.26	$y = 2.087\,23\,x + 0.003\,54$	0.993 3	0.02–200	0.01	0.2
AL	3.50	$y = 1.105\,11\,x + 0.003\,89$	0.998 2	0.02–800	0.01	0.2

Table 4
Results of intra-day, inter-day precision and recovery, repeatability and stability.

Compounds	Precision*			Recovery (*n* = 3)		Repeatability (%) (*n* = 5)	Stability (%)* (*n* = 5)		
	Concentration added (ng/mL)	Intra-day precision (%, *n* = 7)	Inter-day precision (%, *n* = 5)	Concentration added (ng/mL)	Recovery (%) (mean ± SD)		Leaf	Stem	Fruit
AA-A	0.49	10.25	4.60	1.95	107.27 ± 14.26	2.06	8.76	8.94	6.91
	7.81	3.87	8.68	15.63	104.58 ± 2.40				
	250.00	1.68	9.09	62.50	100.33 ± 4.33				
AA-B	0.44	6.41	7.48	1.75	80.38 ± 5.79	1.98	8.74	3.81	4.56
	7.00	2.89	0.46	14.00	80.40 ± 4.10				
	224.00	1.01	0.91	56.00	99.54 ± 1.80				
AA-C	1.09	13.23	5.88	2.19	97.42 ± 6.84	3.99	13.47	3.34	5.50
	8.75	1.73	7.79	17.50	90.51 ± 2.67				
	280.00	0.96	7.49	70.00	99.31 ± 2.10				
AA-D	0.44	12.53	9.86	1.77	110.23 ± 14.49	5.86	14.92	7.03	6.17
	7.09	6.44	8.85	14.18	103.82 ± 5.79				
	226.80	0.96	8.32	56.70	111.94 ± 2.34				
7-OH AA-A	0.39	9.39	4.54	1.56	107.17 ± 2.73	4.64	10.53	8.00	11.03
	6.25	1.97	8.97	12.50	89.81 ± 3.24				
	200.00	1.16	6.52	50.00	98.03 ± 3.44				
AL	0.02	11.98	18.83	1.56	81.57 ± 1.96	3.62	11.91	9.69	4.60
	6.25	2.06	10.25	12.50	91.10 ± 3.00				
	200.00	0.99	8.80	50.00	86.61 ± 1.51				

Note: *Expressed as the relative standard deviation (RSD).

Table 5
Detection results of analysts in HCAAs plant samples (mean ± SD, n = 3).

Samples	Sample No.	Content of compounds (μg/g)						
		AA-A	AA-B	AA-C	AA-D	7-OH AA-A	AL	Total
Material A	1	991.42 ± 26.89	5.75 ± 0.94	26.95 ± 3.03	6.59 ± 0.93	0.32 ± 0.03	0.38 ± 0.04	1 031.41 ± 23.86
Material B	2	25.81 ± 0.59	3.79 ± 0.13	0.84 ± 0.03	0.65 ± 0.03	0.04 ± 0.00	Not detected	31.13 ± 0.78
Material C	3	5 146.82 ± 28.15	1 921.23 ± 8.58	339.07 ± 3.77	22.74 ± 0.46	6.66 ± 0.16	6.62 ± 0.13	7 443.14 ± 40.06
Material D	4	2.47 ± 0.06	0.40 ± 0.05	0.10 ± 0.03	0.38 ± 0.01	0.02 ± 0.00	12.77 ± 1.02	16.14 ± 1.16
Material E	5	1 056.92 ± 73.34	17.34 ± 0.69	31.05 ± 0.25	16.69 ± 0.31	0.82 ± 0.01	0.62 ± 0.01	1 123.44 ± 73.64
Material F	6	2 194.18 ± 330.80	149.54 ± 6.76	11.67 ± 0.69	9.97 ± 0.30	0.24 ± 0.02	0.05 ± 0.00	2 365.65 ± 325.05
Material G	7	1 515.88 ± 12.39	139.88 ± 1.93	424.12 ± 2.61	89.04 ± 0.96	31.70 ± 0.67	2.28 ± 0.08	2 202.90 ± 30.09
Material H	8	3 937.06 ± 487.05	192.47 ± 16.08	144.31 ± 15.01	162.56 ± 18.45	8.79 ± 0.93	0.31 ± 0.02	4 445.50 ± 550.01
Fruit of Material I	9	1 114.80 ± 26.60	40.79 ± 1.13	47.10 ± 0.90	58.31 ± 2.22	0.27 ± 0.01	0.05 ± 0.00	1 261.31 ± 30.86
Leaf of Material I	10	30.64 ± 0.07	2.03 ± 0.01	3.03 ± 0.10	4.12 ± 0.67	3.11 ± 0.12	3.79 ± 0.06	46.68 ± 0.66
Stem of Material I	11	23.78 ± 1.30	2.48 ± 0.20	7.84 ± 0.34	3.57 ± 0.24	0.24 ± 0.01	0.01 ± 0.00	37.91 ± 2.08

3.3. Method application

In this study, all materials were collected or purchased by team members during the research process. HCAAs are still widely used for medicinal and/or food use in ethnic minority areas of China, which can be easily obtained from vegetable and fruit markets or online. In addition to wild plants, there are also medicinal farmers growing HCAAs and even breeding new varieties.

UPLC-ESI-MS/MS technology was used to conduct qualitative and quantitative analysis of AAs and AL in HCAAs materials in Table 1 and Fig. 2 in MRM-IDA-EPI mode. The results were listed in Table 5, chromatograms of AAs, AL, and IS of samples were shown in Fig. S2. Except that AL was not detected in sample (No. 2), six target compounds were all detected in the other ten samples of eight materials. The AL content in No. 4 sample was 12.77 μg/g, which was the highest among the six compounds. The AA-A content was 2.47 μg/g, which was less than the 0.001% (i.e 10 μg/g) restricted in *Chinese Pharmacopoeia* (Chinese Pharmacopoeia Committee, 2015; Chinese Pharmacopoeia Committee, 2020). This result showed that even if AA-A content met the standard requirements, there was still a great security risk. In other *Aristolochia* materials, the content of AA-A was the highest, but the content difference was large, the lowest was 23.78 μg/g, the highest was 5 146.82 μg/g. Among the root tuber materials, the No. 3 sample with the highest AAs content was compact dark brown colloid interior, while within the No. 1 and No. 2 sample were loose and light yellow fiber with lower AAs contents.

The second highest concentration of the compound varied from material to material. The total content of target compounds in fruit, leaf and stem decreased in sequence. It was worth noting that compared with fruit and stem, the contents of 7-OH AA-A and AL in leaves were the highest, and the contents of AA-B and AA-C were the lowest. *A. delavayi* is abundant in Lijiang of Yunnan Province, China. Naxi and Tibetan people like to add the leaves of *A. delavayi* to their food to improve its flavor, but the higher 7-OH AA-A and AL contents also have potential risks. Surprisingly, there are no reports of nephrotoxicity caused by long-term consumption of leaves of *A. delavayi* in these areas, and the reason is worthy of further study.

4. Conclusion

A highly sensitive and specific UPLC-MS/MS quantification method for mainly active and toxic compounds in HCAAs was established and validated completely, which was in MRM-IDA-EPI analysis mode coupled with dynamic background subtraction function. The discovery and confirmation of aristolochic acids were completed, as well as quantitative and qualitative analysis to ensure the authenticity of the results, reduce false positive and improve the accuracy of the results. This method has shown satisfactory sensitivity, precision, and accuracy, and it can be applied to determine the AAs and AL in HCAAs successfully. The analysis method will provide guidance for the safety of HCAAs medication, and lay a good foundation for further study of pharmacological and toxicological effects of HCAAs and scientific and standardized processing compatibility.

CRediT authorship contribution statement

Lijun Yue: Conceptualization, Methodology, Writing – original draft. **Kaijun Yang:** Investigation, Data curation. **Feng Jiang:** Resources, Writing – review & editing. **Shuai Dong:** Visualization. **Kang Yang:** Investigation. **Dan Zhu:** Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2023.02.008.

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