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ARTICLES

Determination of Four Lignanoids in Roots, Stems and Leaves of *Zanthoxylum armatum* DC by HPLC-DAD with HPLC-ESI–QTOF-MS Confirmation[1]

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Abstract—A rapid, effective method through an orthogonal design was developed, and four lignanoids were determined by HPLC and confirmed by HPLC coupled with electrospray ionization and quadrupole time-of-flight mass spectrometry (**HPLC-ESI–QTOF-MS**). The roots, stems and leaves of *Zanthoxylum armatum* DC were extracted by methanol for 15 min under reflux. Separation was performed using an UPLC system to quantify four bioactive compounds, namely fargesin, asarinin, planispine A and planispine B, in 12 batches of samples of different origin from China. Furthermore, the samples were analyzed using HPLC-ESI–QTOF-MS to confirm the results. The calibration curves of all four analytes showed good linearity ($R >$ 0.998). Accuracy, precision and repeatability were all within required limits. The mean recoveries measured at the three concentrations were higher than 99% with RSDs lower than 4.1%. The established HPLC-DAD method could serve as a rapid and effective method for quality evaluation of *Zanthoxylum armatum* DC.

Keywords: HPLC-DAD, HPLC-ESI–QTOF-MS, bioactive components, *Z. armatum*

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The genus *Zanthoxylum*, family *Rutaceae*, comprises of 250 species distributed in the tropical and subtropical zones of Asia, Africa, America and Oceania. There are 39 species and 14 varieties in China, occurring nearly everywhere in the country [1]. *Zanthoxylum armatum* DC, a common wild species in the genus, is found in India, Nepal, Malaysia, Pakistan and Japan at altitudes of 1300–1500 m. In China, it is distributed mainly in southeast and southwest and cultivated in some areas. *Z. armatum* is widely used as a Chinese folk medicine for the prevention of stomach ache, toothache, treating cold in the chest and abdomen, preventing snake bites and expelling roundworms [1]. Modern pharmacological studies confirmed that *Zanthoxylum armatum* DC has high biological activity, such as strong analgesic and anti-inflammatory [2, 3], hepatoprotective [4], antioxidant [5, 6], antidiabetic [7], antimicrobial [7], strong sedative-hypnotic [8], anxiolytic [8], and anti-acetylcholine esterase activities [9]. Several types of secondary metabolites including lignans [10, 11], alkaloids [11], amides [12], terpenoids [13], coumarins [13], flavonoids [14], etc. have been reported from various parts of this plant.

The pharmacological activities of *Z. armatum* are mainly attributed to the presence of furofuran lignans, alkaloids and amides. Our previous studies found the ethyl acetate fraction of ethanol extract has significant analgesic and anti-inflammatory activity. We identified eight furofuran lignans as the major constituents of that fraction [2, 15].

Furofuran lignans are one of the largest groups of lignans that are of special interest owing to their powerful antitumor, anti-inflammatory, antioxidant and insecticidal properties, along with phosphodiesterase inhibition and hypercholesterolemia activities in humans [16, 17].

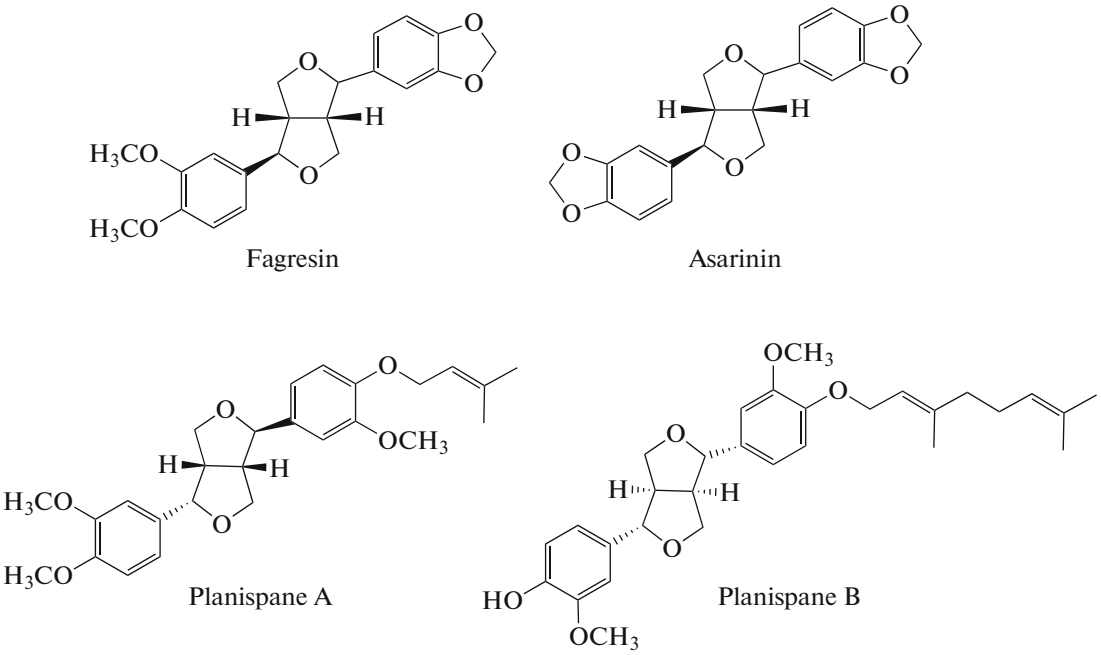
A few analytical methods based on HPLC-DAD have been reported for the determination of isobutylamides and furofuran lignans in *Zanthoxylum* genus [18, 19]. However, no report is available on the quantitative or qualitative analysis of this four furofurans (fargesin, asarinin, planispine A and planispine B) in *Z. armatum*. In this paper, an HPLC-DAD method

[1] The article is published in the original.

has been developed for the determination of four main furofuran lignans (fargesin, asarinin, planispine A and planispine B) in *Z. armatum*. The method was successfully employed to determine the content of these compounds from roots, stems and leaves of *Z. armatum* in three main producing areas of China. Moreover, in order to confirm the identification results, all the samples were also analyzed on HPLC-ESI–QTOF-MS system. This is the first report on the simultaneous qualitative and quantitative analysis of these lignans in *Z. armatum* using HPLC-DAD and HPLC-ESI–QTOF-MS.

EXPERIMENTAL

Materials and reagents. The dried roots, stems and leaves of *Zanthoxylum armatum* DC of different harvest zones were collected from Fuzhou, Fujian province (1), Hunan province (2), Jiangxi province (3) and Tian'e county, Guangxi province (4). The material was identified by vice Prof. Jian-ping Tian of Hainan Medical University. Reference standards of fargesin, asarinin, planispine A and planispine B were isolated and purified by Prof. Guo. On the basis of UV, NMR and MS analysis, the structures of isolated reference standards were confirmed, and their purity determined using HPLC-DAD was over 98.0%. Their structures are presented in Scheme.



Scheme. Chemical structures of the four lignans.

Acetonitrile was HPLC-grade from Merck (Darmstadt, Germany) and deionized water was purified by a Cascada IV super purification system (Pall Corporation, NY, USA). Other reagent solutions were analytical grade (Shanghai Chemical Reagent Company, Shanghai, PR China).

Sample preparation. Twelve samples of *Zanthoxylum armatum* DC were ground into powder with 40 mesh. An aliquot (0.5 g) was weighed precisely and extracted with 10 mL of methanol for 20 min, and finally made to a volume of 10 mL using methanol. Three replicates of the extraction process were carried out on the independent samples. The solution was filtered through 0.22 μm membrane prior to use and a 5 μL aliquot was injected into the HPLC system for analysis.

The root of Tian'e sample was used to optimize the ultrasound assisted extraction condition of four compounds. The ultrasonic extraction time (*A*), solvent-to-sample ratio (*B*) and solvent (*C*) were optimized. The powder of 0.5 g of the fruit was extracted with three solvents : methanol, 70% ethanol (v/v) and 95% ethanol (v/v). Three solvent-to-sample ratios (10 : 1, 20 : 1, 40 : 1, v/w) and three extraction time (10, 20, and 30 min) were tested. All the factors were investigated using an orthogonal (L933) experimental design, and each extraction was tested in triplicate.

Using the selected optimal extraction conditions, the different harvest time fruits of 0.5 g were accurately weighed and extracted with 10 mL methanol for 20 min. The extraction of each sample was performed in triplicate. The solution was filtered through a 0.22 μm filter before HPLC analysis. The experimental results are listed in Table 1.

The HPLC chromatography conditions. Chromatographic analysis was performed on a Shimadzu LC-

Table 1. Experimental results of the orthogonal test

A (time, min)	*B* (solvent-to-sample ratio)	*C* (solvent)	Yield*, mg/g			
			fargesin	asarinin	planispine A	planispine B
10	10 : 1	Methanol	0.934	0.598	0.651	0.511
10	20 : 1	70% ethanol	0.978	0.600	0.715	0.510
10	40 : 1	95% ethanol	0.867	0.548	0.642	0.478
20	10 : 1	70% ethanol	0.968	0.584	0.929	0.503
20	20 : 1	95% ethanol	0.979	0.598	0.852	0.533
20	40 : 1	Methanol	1.101	0.668	0.819	0.559
20	10 : 1	95% ethanol	0.842	0.534	0.799	0.452
30	20 : 1	Methanol	1.187	0.738	0.924	0.582
30	40 : 1	70% ethanol	0.884	0.567	0.816	0.430

* Yield values are averages of three determinations.

20A HPLC system (Shimadzu Corp., Tokyo, Japan), consisting of a quaternary pump solvent management system, an online degasser, an autosampler and a photo-diode array detector. A Diamonsil C18 column (250 × 4.6 mm, 5 μm) was employed, and the column temperature was maintained at 35°C. The mobile phase was composed of water (A) and methanol (B) using a gradient elution of 50% B at 0 min to 90% B at 40 min with a flow rate set at 1.0 mL/min. The autosampler was conditioned at 4°C and the injection volume was 5 μL.

Mass spectrometry conditions. MS/MS was performed on a Waters Xevo G2 Q-TOF mass spectrometer equipped with a Trizaic nanoTile™ ionization source operating in positive ion mode (Waters Corp.). The parameters of the mass spectrometer under the ESI mode were as follows: capillary voltage 3.0 kV, cone voltage 40 V, source block temperature 120°C, cone gas 50 L/h, desolvation temperature 400°C, desolvation gas 650 L/h. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; leucine-enkephalin was used as the lock mass (m/z = 556.2771) at a concentration of 50 fmol/mL and flow rate 30 mL/min. Data were acquired in centroid mode from 50 to 1000 m/z in MS scanning. All instrument and data acquisition parameters were controlled by MassLynx™ (version 4.1, Waters Corp.) and the data generated were processed using a beta test version of UNIFI™ software (version 1.6, Waters Corp.).

Calibration curves, limits of detection (LOD) and quantification (LOQ). A mixed standard stock solution containing argesin (*1*), asarinin (*2*), planispines A (*3*) and B (*4*) was prepared in acetonitrile. The working standard solutions were prepared by diluting the mixed standard solution with acetonitrile to a series of proper concentrations within the ranges, mg/mL: *1*—0.091–1.365; *2*—0.081–1.215; *3*—0.039–0.581; *4*—0.0204–0.306. The standard stock and working solutions were all stored at 4°C until use and filtered through a 0.22 μm membrane prior to injection.

All series of concentrations of standard solution were prepared for the establishment of calibration curves. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves. LODs and LOQs were determined using diluted standard solution when the signal-to-noise ratios (S/N) of analytes were about 3 and 10, respectively. The S/N was calculated as the peak height divided by the background noise value.

Precision. The intra-day and inter-day variations chosen to determine the precision of the developed method were investigated by determining the 9 analytes in six replicates during a single day and by duplicating the experiments on three consecutive days. Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (**RSD**).

Accuracy. A recovery test was used to evaluate the accuracy of this method. The test was performed by adding known amounts of the 9 standards at low (80% of the known amounts), medium (the same as the known amounts) and high (120% of the known amounts) levels. The spiked samples were then extracted, processed, and quantified in accordance with the aforementioned methods. The average recovery percentage was calculated by the formula:

$$\text{Recovery, \%} = (\text{observed amount} - \text{original amount})/\text{spiked amount} \times 100\%.$$

Stability and reproducibility. Reproducibility was confirmed with six independent analytical sample solutions prepared from the same batch of sample (the roots collected from Tian'e county) and variations were expressed by RSD. One of the sample solutions mentioned above was stored at 25°C and injected into the apparatus at 0, 2, 4, 8, 12, and 24 h, to evaluate the stability of solution.

Table 2. Analysis of L_9 $(3)^4$ test results

Parameter*	Yield of fargesin			Yield of asarinin			Yield of planispine A			Yield of planispine B		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
k_1	0.927	0.916	**1.075**	0.582	0.573	**0.668**	0.670	0.794	0.798	0.500	0.489	**0.551**
k_2	**1.017**	**1.049**	0.944	**0.617**	**0.646**	0.584	**0.867**	**0.831**	**0.821**	**0.532**	**0.542**	0.481
k_3	0.972	0.951	0.897	0.614	0.595	0.560	0.847	0.759	0.765	0.488	0.490	0.488
R**	0.090	0.133	0.178	0.035	0.073	0.108	0.197	0.072	0.055	0.032	0.054	0.070
Optimal order	A_2	B_2	C_1	A_2	B_2	C_1	A_2	B_2	C_2	A_2	B_2	C_1

* k_1, k_2, k_3 – the mean values of yield for the factors at levels 1, 2 and 3, respectively. ** $R = k_{max} - k_{min}$.

Table 3. Regression data and LOQs of the analytes

Compound	Range, μg/mL	Linear regression equation	Correlation coefficient	LOD, ng/mL	LOQ, μg/mL
Fargesin	45–910	$y = 14379x + 5049.8$	1.0000	0.6	2.0
Asarinin	40–810	$y = 15692x + 48493$	0.9999	0.6	2.0
Planispine A	20–388	$y = 13987x - 91366$	0.9978	0.3	1.0
Planispine B	10–204	$y = 21439x + 17770$	0.9994	0.3	1.0

RESULTS AND DISCUSSION

Optimization of HPLC conditions. First, the Diamonsil C18 column (250 × 4.6 mm, 5 μm) and Waters Symmetry C18-column (150 × 4.6 mm, 5 μm) were compared using the same mobile phase programme. It was found that planispine B could not be separated completely on the Waters Symmetry C18-column. In addition, the Diamonsil C18 column displayed a better steady baseline under the situation of gradient elution. To achieve good sensitivity and accuracy for quantification, the UV spectra of four analytes were considered carefully. Thus, the DAD detection wavelengths were set at 233 nm according to the maximum absorption wavelength of each compound.

Optimization of the extraction conditions. The parameters obtained from the orthogonal ($L_9 3^3$) test of four compounds extraction were weighted and quantitatively analyzed using evaluation indices *k* (Table 2). The results show that the *R* value of factor *B* was the highest for the fargesin and less significant for the three other components. This indicated the solvent-to-sample ratio was the most important factor among the four parameters. Extraction time had only significant effect on the planispine A. Solvent has importance for planispine B. Based on the *R* values, the factors can be ranked by importance for the overall three types of ingredients as follows: solvent-to-sample ratio > solvent > extraction time.

Solvent-to-sample ratio of 20 : 1 gave a higher yield for all the four ingredients than other ratios. The effect of extraction solvent was slightly different for the four components. The methanol solvent gave the highest yields for fargesin, asarinin and planispine B, while a second high yield for planispine A. Extraction time of 20 min provided the highest yield for all the four components. So the 20 : 1 methanol during 20 min was chosen for the extraction of four compounds from *Zanthoxylum armatum* DC. Typical chromatograms are shown in Fig. 1.

Analytical method validation. The proposed HPLC method for quantitative analysis was validated by determining the linearity, LOD, LOQ, intra-day and inter-day precisions, stability, and accuracy. As shown in Table 3, all calibration curves showed good linearity ($r > 0.998$) within the test ranges, and the overall LODs and LOQs were in the range of 0.3–0.6 and 1.0–2.0 μg/mL, respectively. The RSD values of intra- and inter-day variations, repeatability and stability of the nine analytes were all less than 5% (Table 4). The overall recoveries lay between 98.99 and 104.24% with RSD less than 5%. All the results mentioned above indicated that the established method was accurate.

Sample analysis. The proposed HPLC method was applied to determine four chemical markers including fargesin, asarinin, planispine A, planispine B from the 12 samples of *Zanthoxylum armatum* DC. The results are given in Table 5.

HPLC-ESI–QTOF-MS confirmation. The full-scan product ion spectra of the four constituents were shown in Table 4. The precursor–product ion reactions selected were *m/z* 371.1473, 355.1155, 427.2112 and 495.2742 for fargesin, asarinin, planispine A, planispine B, respectively.

All 12 samples were analyzed on an HPLC-ESI–QTOF-MS system to confirm the results. Figure 2 shows the mass spectra for the four constituents in sample Tian'e. The MS spectra show the protonated

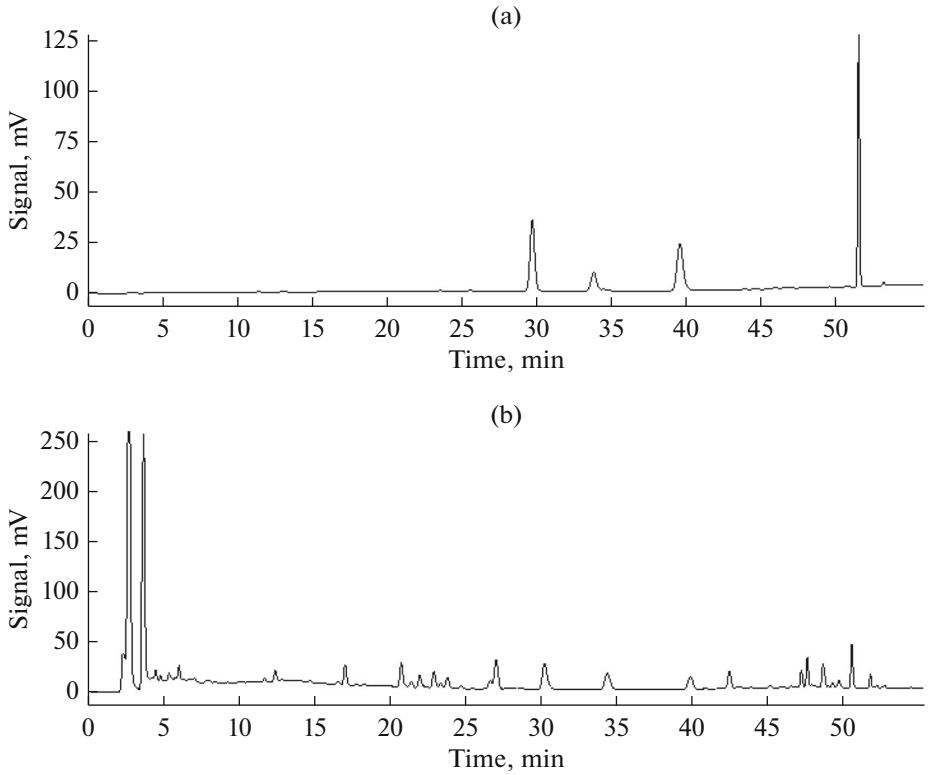


Fig. 1. Typical HPLC-DAD chromatograms of the mixed standards (a) and extract of sample ZA-10 (b).

Table 4. The results of precision and recovery tests for HPLC determination

Compound	Precision (RSD, %)		Repeatability (RSD, %; $n = 6$)	Stability (RSD, %; $n = 6$)	Recovery (%, $n = 3$)	
	intra-day ($n = 6$)	inter-day ($n = 18$)			mean	RSD
Fargesin	3.1	2.2	3.5	3.5	100.12	3.63
Asarinin	3.0	3.1	3.6	4.0	101.15	4.12
Planispine A	2.6	3.0	3.0	4.0	98.99	3.46
Planispine B	3.7	2.7	3.6	3.0	104.24	3.91

Table 5. Four lignans contents (mg/g) in fruits of different harvest time

Sample	Harvest zone	Part of plant	Fargesin	Asarinin	Planispine A	Planispine B
ZA-1	Fuzhou	Root	2.7137	0.3100	0.8798	1.1147
ZA-2		Stem	1.3874	0.0630	1.3375	–
ZA-3		Leaf	0.7616	0.4237	0.6595	0.4126
ZA-4	Huanan	Root	0.6650	–	0.1832	0.1006
ZA-5		Stem	0.5538	–	0.1823	0.2306
ZA-6		Leaf	0.2338	0.1027	0.5121	0.1315
ZA-7	Jiangxi	Root	0.6013	–	0.4636	0.1043
ZA-8		Stem	0.2782	–0.0483	0.4122	–
ZA-9		Leaf	0.2731	0.2276	0.5911	0.1330
ZA-10	Tian'e	Root	1.7891	1.9629	0.5407	–
ZA-11		Stem	0.5307	0.2608	0.2906	0.0024
ZA-12		Leaf	5.9898	0.6683	0.7670	0.0327

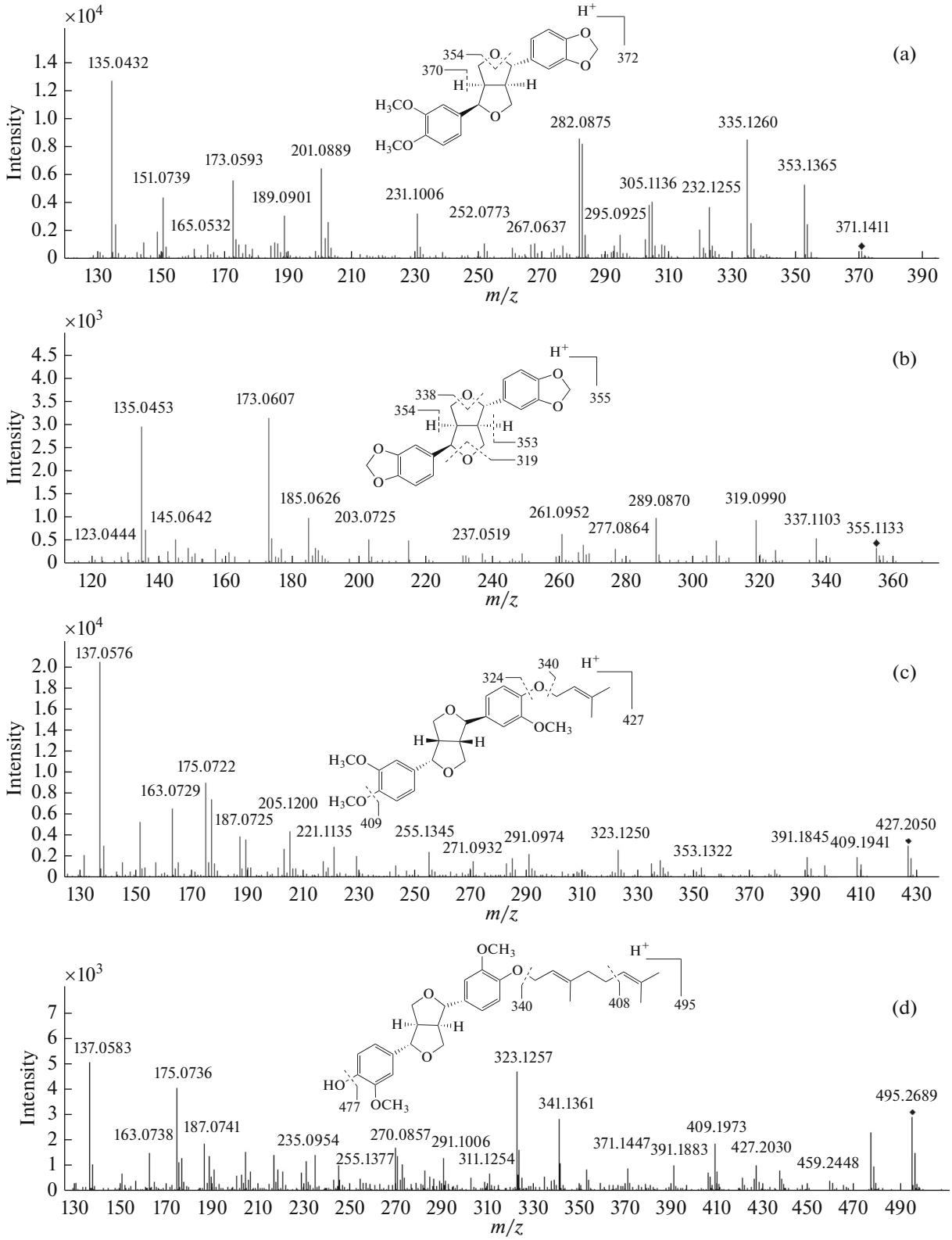


Fig. 2. MS spectra of sample ZA-10: fargesin (a), asarinin (b), planispine A (c), and planispine B (d).

Table 6. The mass data of four lignans acquired by UPLC-ESI–QTOF-MS

t_R, min	Compound	Molecular formula	Theoretical mass of $[M + H]^+$, Da	Measured mass of $[M + H]^+$, Da	Error, ppm	Precursor ion/ production ion
29.8	Fargesin	$C_{21}H_{22}O_6$	371.1495	371.1473	5.93	371.1473/353.1391
33.9	Asarinin	$C_{20}H_{18}O_5$	355.1182	355.1155	7.60	355.1155/337.1077
39.7	Planispine A	$C_{25}H_{30}O_6$	427.2121	427.2112	2.11	427.2112/409.2008
51.6	Planispine B	$C_{30}H_{38}O_6$	495.2747	495.2742	1.01	495.2742/409.2012

molecular $[M + H]^+$ and/or sodium adduct molecule $[M + Na]^+$ of the analytes. The observed accurate mass and isotopic pattern of precursors and their fragment ion are close to the theoretical values, which mean that they can reflect the extract elemental composition (Table 6 and Fig. 2). In addition, retention times, UV spectra of the reference substances and UV data of reported compounds were used as complementary data for the identification. The confirmatory results were sufficient and reliable.

A HPLC-MS/MS and HPLC-DAD method was proposed and validated as a reliable and powerful technique for simultaneous detection and quantification of four compounds in *Zanthoxylum armatum* plants. This method was applied to investigate the contents of four compounds harvested at different zones. The data showed the contents of four chemical markers varied greatly with the harvester zone and the tissue of plants, and this kind of phenomenon is common that the difference of ingredients content is very large in different tissues of plant, and even some components can not be found [20, 21]. This is the case for asarinin and planispine B that could not be detected in some samples (e.g., asarinin in ZA-4, ZA-5 and ZA-7; planispine B in ZA-2, ZA-8 and ZA-10). The results mentioned above showed that our work could offer a general analytical method for the quality control of *Zanthoxylum armatum*.

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