



Screening of Alkaloids in *Sophora Flavescens* with Liquid Chromatography Tandem Data-Dependent Acquisition Mass Spectrometry

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Authors' contributions

This work was carried out in collaboration between both authors Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2023/v35i317465

Open Peer Review History:

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/109548>

Original Research Article

Received: 19/09/2023

Accepted: 23/11/2023

Published: 27/11/2023

ABSTRACT

A novel and simple hyphenated method was applied for the screening of alkaloids in *S. flavescens*. The approach combined easy sample extraction with an ultra-high-performance liquid chromatography (UPLC) and a high-resolution mass spectrometry (HRMS) in the data-dependent acquisition (DDA) method. With the optimized chromatographic program, ten alkaloids were screened. Among them, matrine, oxymatrine, oxysophoridine, sophocarpine, and oxysophocarpine were identified and quantified. The other five alkaloids were identified with accurate m/z value in MS¹ and product ions in MS². The quantification method was validated with specificity, linearity, recovery, and precision. The LOD and LOQ were determined. In twelve batches of collected *S. flavescens* roots, the identified ten alkaloids were all observed. The oxymatrine was the most

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abundant component, and sophocarpine was the least abundant in these samples. This comprehensive method is a powerful tool for the biochemical and clinical research of *S. flavescens*. It can also be adopted in resource utilization and related medicine quality control.

Keywords: Alkaloids; *Sophora flavescens*; data-dependent acquisition; identification; quantification.

1. INTRODUCTION

The root of *Sophora flavescens* (*S. flavescens*, also known as Kushen) possesses insecticidal [1] and anti-inflammatory activities [2]. Besides, compound Kushen injection (CKI) was reported to have anti-cancer activities [3–5]. The ingredients of *S. flavescens* include alkaloids, flavonoids, triterpenoids, and lignans [6,7]. Although the mechanism of activities was not clarified, it is believed that alkaloids and flavonoids are the bioactive component [8]. The typical alkaloids are the quinolizidine alkaloids such as matrine, sophocarpine, oxymatrine, sophoridine, sophorine, and oxysophocarpine. They are subdivided into matrine and aloperine type [9,10]. Beyond the compound medicine, individual alkaloids were studied as one-component drugs in clinical practice [11]. For example, matrine was reported to inhibit of matrix metalloproteinase-9 (MMP-9) in hepatocellular carcinoma cells [12]. Whereas, the clinical relevance has yet to be determined. For example, although the mechanisms of CKI for lung cancer have been studied by network pharmacology [13], the molecular mechanisms need investigation [14].

Both for pharmacology studies and metabolite profiles, molecular component identification is always the interesting. However, the components of *S. flavescens* are complicated. The conventional screening protocol employs tedious column isolation and thin layer chromatography (TLC) [15]. Even without consideration of the low identical efficiency, the reagent consumption and time cost are significant. The high-performance liquid chromatography (HPLC) and related tandem techniques were used as improved approaches [16,17]. Among all kinds of hyphenated techniques [18,19], HPLC coupled with mass spectrometry (LC-MS) provides a great improvement in specificity, sensitivity, and quantification [20]. Since the mass spectrometry offers a unique mass-selective capability, baseline separation is not strictly required in most cases [21]. However, the composition of *S. flavescens* is complex [22]. Without proper

pretreatment, the LC-MS is insufficient for profiling *S. flavescens* due to co-eluting, isomers, and matrix effects [23]. Even when one compound is separated, it cannot be identified only by its first stage mass spectrum (MS^1). A second stage mass spectrometry (MS^2 or MS/MS) is always necessary for characterization [20]. The precursor ion for the MS^2 experiment is picked up based on the MS^1 results. Therefore, a large amount of work is needed to carry out for the screening of *S. flavescens*.

Data dependent acquisition (DDA) mode, also known as information dependent acquisition (IDA), is a mode of data collection in tandem mass spectrometry [24]. In DDA mode, the MS^1 and MS^2 scans were achieved simultaneously within one chromatographic procedure. DDA mode is widely used in targeted proteomics studies [25,26]. Sun and coworkers integrated DDA and the data-independent acquisition (DDIA) method to screen hundreds of chemicals in a single ultra-HPLC run coupled with high-resolution mass spectrometry [27].

The ability to identify and quantify samples in the complex mixture makes DDA mode possible to be applied in the screening of *S. flavescens* metabolites. In this study, a novel and simple hyphenated method was applied for the screening of alkaloids in *S. flavescens*. The approach combined an easy sample extraction process with an ultra-high performance liquid chromatography (UPLC) tandem a high-resolution mass spectrometry (HRMS) in DDA method. The screening was achieved by the retention time difference, the high resolution of MS^1 and subsequently the features of MS^2 . Ten alkaloids were identified. Five of them, including matrine [28], oxymatrine [29], sophocarpine, oxysophocarpine, and oxysophoridine [30] (Fig. 1) were quantified with the external standard method simultaneously. The developed method provides a powerful tool for the biochemical and clinical research of *S. flavescens*. It can also be adopted in resource utilization and related medicine quality control.

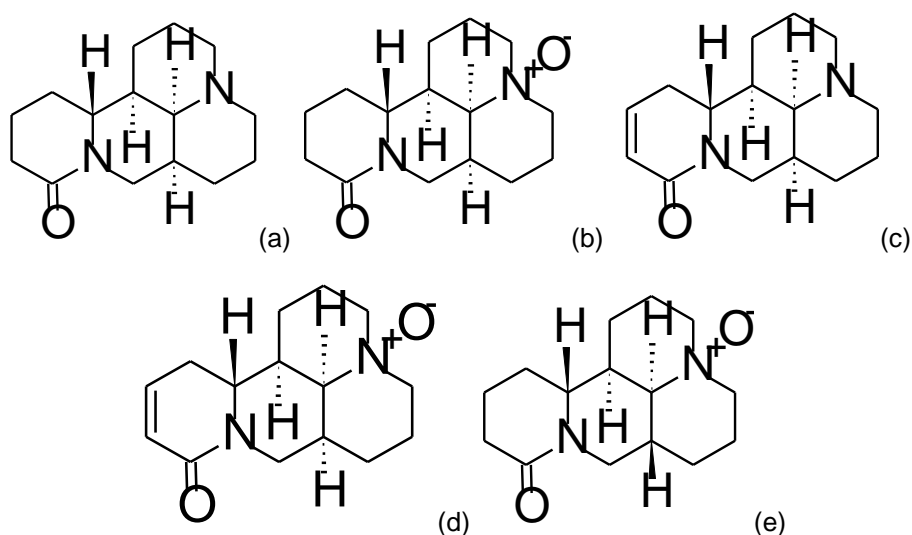


Fig. 1. Chemical structure of (a)matrine, (b)oxymatrine, (c)sophocarpine, (d) oxysophocarpine, and (e) oxysophoridine

2. MATERIALS AND METHODS

2.1 Reagents and Materials

The standard materials (purity >98.0%) of matrine, oxymatrine, oxysophoridine, sophocarpine, and oxysophocarpine were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China).

Acetonitrile and methanol (HPLC grade) were obtained from Tedia Company, Inc. (Fairfield, USA). Formic acid (purity >98.0%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ammonium acetate (purity >98.0%) was bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Water was purified using a Milli-Q system (Molsheim, France).

The *S. flavescens* roots were collected were from Pingshun, Shanxi province, China (*Pingshun* 1, 2, 3), Lichuan, Hubei province, China (*Lichuan* 1, 2, 3), Wenshan, Jiangxi province, China (*Wenshan* 1, 2, 3), and Bozhou, Anhui province, China (*Bozhou* 1, 2, 3). The collected samples were identified as root of *S. flavescens* by Dr. Chengman Bao (School of Marine and Biological Engineering, Yancheng Teachers University).

2.2 Instruments

An UPLC system (Agilent, 1290 Infinity) was employed to carry out the chromatographic separation. A 50mm × 2.1mm, 1.7μm C₁₈ column (Waters, ACQUITY UPLC BEH) was used. A

high-resolution mass spectrometer (AB Sciex, 5600+) was coupled to it.

An analytical balance (Sartorius BSA224S, China) and an ultrasonic cleaner (KQ100DE, China) were used for sample preparation.

2.3 LC-MS Condition

Mobile phase A was an ammonium acetate/water solution (8.0 mmol/L). The mobile phase B was acetonitrile. The total flow rate was 0.25 mL/min. The column temperature was 40 °C. The sample volume introduced for each run was 2.0 μL except with otherwise stated.

The mass spectrometer worked in positive mode. The ionization voltage was set at +5.5 kV with 55 psi for nebulizing gas, 55 psi for auxiliary gas, and 35 psi for curtain gas. The source temperature was set at 550 °C. For MS¹, the TOF scan range covered m/z 100–2000. For the MS² (MS/MS) analysis, the DDA method was adopted. For DDA acquisition, the declustering potential was set at 80 V, the collision energy at 55 eV, the collision energy spread at 10 eV, the ion release delay at 67 ms, and the ion release width at 25 ms.

2.4 Sample Preparation

Stock solutions of each analyte were prepared with acetonitrile at a concentration of 1000 μg/mL. Working standard solutions were diluted from the stock solution with a mobile phase solution (95% A:5% B). Mixture working solutions were also prepared from stock solutions by calculated dilution. The stock solutions were kept

in refrigerator at -20 °C for no more than 30 days. The working solution were prepared freshly every day. .

The collected *Sophora flavescens* roots were dried and ground to pass through a 0.1 mm sieve. The dried powder was stored to avoid light. The obtained powder was then extracted with methanol. 50 mg of powder were carefully weighed and transferred to a 50 mL centrifuge tube. 10 mL of extractant was added. The extractant was a mixture of methanol, water, and formic acid (49:49:2; v/v/v). The sample was sonicated for 60 minutes. After centrifugation, the supernatant was isolated. The extraction was repeated two more times. The supernatants of three extractions were combined, which were then dried by nitrogen blowing. The dried extract was dissolved in a mobile phase solution (95% A:5% B). The reconstituted sample solution is at a concentration of 10 mg/mL of *S. flavescens* powder. The sample solution was filtered through a 0.22 µm polytetrafluoroethylene membrane filter before testing.

3. RESULTS AND DISCUSSION

3.1 Optimization of Chromatography

With the empirical LC method, a pre-experiment was performed. The empirical LC method employed formic acid/water solution (0.2%, v/v) as mobile phase A and methanol as mobile phase B. The flow rate was 0.20 mL/min. The LC program started with 6% B and then increased to 22% within 9 minutes. With this empirical method, most alkaloids were eluted within 3 minutes. In the total ion chromatogram (TIC) of the standards mixture, only four congestive peaks were observed. The tailing of chromatographic peaks was also prominent.

Based on the results of the empirical method, an optimized LC program eventually evolved. The optimized method employed ammonium acetate/water solution (8 mmol/L) as elution phase A and acetonitrile as phase B. The optimized program was listed in Table 1.

With the optimized LC program, the TIC of the standards mixture sample was obtained as shown in Fig. 2. It could be found that the chromatographic peak did not trail manifestly. It was attributed to ammonium acetate, the mobile phase modifier. The ammonium ions compete with the basic group of alkaloidal analytes. As a result, the tailing of the peak diminished.

In Fig. 2, peaks were assigned to compounds with the corresponding retention times of their individual standard samples. The peaks at 1.80, 2.67, 2.96, 3.45, and 4.23 minutes were, respectively, oxysophoridine, matrine, sophocarpine, oxymatrine, and oxysophocarpine. Although the peaks did not achieve baseline separation, identification was easily achieved via accurate first stage mass spectra (MS¹) and corresponding second stage mass spectra (MS²). The MS¹ and MS² spectra were obtained simultaneously in DDA mode.

3.2 Identification

The identification of individual alkaloid in the collected sample depended on the accurate m/z value (MS¹) and product ions (MS²). Since the standards are available, the retention times of matrine, oxymatrine, oxysophoridine, sophocarpine, and oxysophocarpine were also strictly compared. Other compounds were also checked with the reported literature.

Take the sample of *Bozhou 1* as an example, its TIC was shown in Fig. 3.

Ten alkaloids were identified directly with the developed UPLC-DDA-MS method. No extra sample pretreatment or characterization method was employed. The characterized compounds are listed in Table 2.

3.3 Linear Range

A series of standard solutions was prepared. The concentrations of each analyte were 5, 10, 20, 50, 100, 200, and 300 ng/mL. All samples were analyzed in triplicate. The calibration curve was constructed with the area of the target analyte in its monitored ion chromatogram (MIC) versus the prepared concentration. It could be found that in the range of 20-300 ng/mL, the linear correlation coefficients (R²) for all compounds exceeded 99.73%. The regression equations and corresponding R² are listed in Table 3.

3.4 Limit of Detection and Quantification

The limit of detection (LOD) was calculated based on the signal to noise ratio (s/n) above 3. The LOD (s/n>3) for five compounds were all below 10 ng/mL. Hence, the LOD was set at 10 ng/mL. The limit of quantification (LOQ) of the method was set at 20 ng/mL (s/n > 10). LOQ was selected from the linear range. The lowest concentration level in the linear range was the choice.

Table 1. The optimized elution time program with a flow rate of 0.25 mL/min

Time (min)	%A	%B
0	95	5
15	95	5
25	85	15
30	0	100

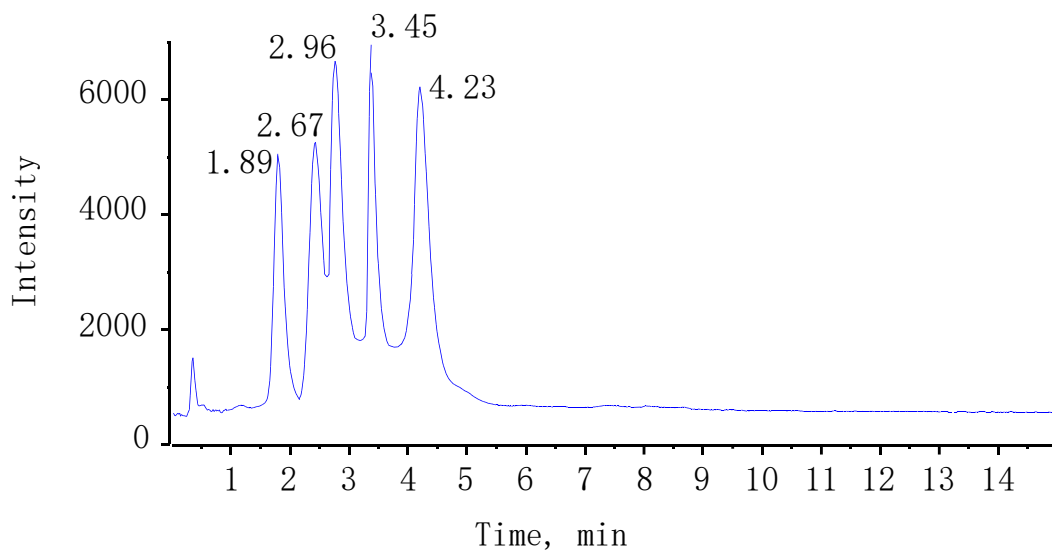


Fig. 2. The TIC of standards mixture with optimized elution program. (oxysophoridine@1.89 min, matrine@2.67 min, sophocarpine@2.96 min, oxymatrine@3.45 min, oxysophocarpine@4.23 min)

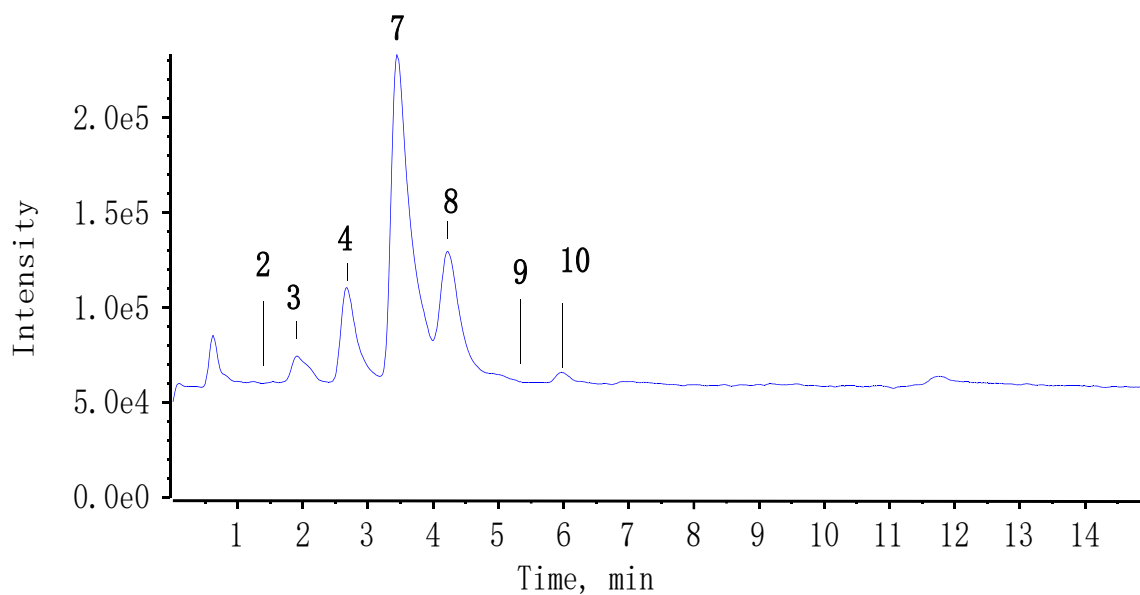


Fig. 3. The TIC of the sample *Bozhou 1*

Table 2. Characterization of the alkaloids in collected sample by UPLC-DDA-MS

No.	Compound	R. Time (min)	molecular formula	Theoretical m/z	detected m/z	Error (ppm)	MS ² ions
1	5 α ,9 α -dihydroxy matrine [31]	1.28	C ₁₅ H ₂₄ N ₂ O ₃	281.1860	281.1869	3.2	281.18, 263.17, 148.11
2	Oxysophoranol [17]	1.52	C ₁₅ H ₂₄ N ₂ O ₃	281.1860	281.1863	1.1	281.18, 263.17, 136.11
3	Oxysophoridine	1.89	C ₁₅ H ₂₄ N ₂ O ₂	265.1905	265.1913	3.0	247.22, 205.16, 150.15
4	Matrine	2.67	C ₁₅ H ₂₄ N ₂ O	249.1955	249.1951	-1.6	249.19, 150.15, 120.10
5	Sophoramine [32]	2.81	C ₁₅ H ₂₀ N ₂ O	245.1648	245.1642	-2.4	245.18, 189.13, 148.11
6	Sophocarpine	2.96	C ₁₅ H ₂₂ N ₂ O	247.1799	247.1804	2.0	245.20, 150.15, 136.13
7	Oxymatrine	3.45	C ₁₅ H ₂₄ N ₂ O ₂	265.1905	265.1915	3.8	265.23, 247.22, 205.16
8	Oxysophocarpine	4.23	C ₁₅ H ₂₂ N ₂ O ₂	263.1748	263.1739	-3.4	245.20, 203.15, 136.13
9	5 α ,9 α -dihydroxy isomatrine [33]	5.57	C ₁₅ H ₂₄ N ₂ O ₃	281.1860	281.1855	-1.8	281.19, 263.18, 162.11
10	N-methylcytisine [33]	5.98	C ₁₂ H ₁₆ N ₂ O	205.1335	205.1329	-2.9	205.13, 175.08, 108.08

Table 3. Linear range (20-300 ng/mL)

	Equation	R ²
Oxysophoridine	$y = 348.42x + 3230.2$	0.9974
Matrine	$y = 211.92x + 2528.9$	0.9999
Sophocarpine	$y = 278.69x - 2105$	0.9973
Oxymatrine	$y = 440.03x + 18444$	0.9992
Oxysophocarpine	$y = 236.76x + 4140.2$	0.9987

Table 4. Mean recoveries of matrine, oxymatrine, sophocarpine, oxysophocarpine, and oxysophoridine

	Conc. level(ng/mL)	Mean recovery	RSD (n=6)
Matrine	50	96.7%	0.99%
	150	97.3%	0.63%
	300	98.2%	0.09%
Oxymatrine	50	101.6%	1.28%
	150	100.3%	0.61%
	300	100.2%	0.12%
Sophocarpine	50	95.6%	1.08%
	150	97.0%	0.63%
	300	95.3%	0.08%
Oxysophocarpine	50	98.3%	0.08%
	150	98.1%	0.07%
	300	98.6%	0.05%
Oxysophoridine	50	96.1%	0.17%
	150	97.1%	0.09%
	300	96.8%	0.09%

3.5 Recovery and Precision

The recovery of an analytical method was determined with the standard addition method. At three concentration levels, a 5% standard was added. The difference between the results of spiked sample and before is calculated as a percentage. The results are listed in table 4. The recoveries of this method are between 95.3% and 101.6%. Therefore, the accuracy of this developed method is accepted.

The within-laboratory reproducibility was also conducted at three concentration levels. The precision of six parallel analyses is shown in Table 4 as well. The relative standard deviation (RSD) results of five analytes are all less than 1.28%. The developed method was precise.

3.6 Contents in Sample

With the developed method, 12 batches of collected *S. flavescens* were analyzed. The ten identified alkaloids were all found in each sample. In order to quantify the matrine, oxymatrine, sophocarpine, oxysophocarpine, and oxysophoridine, the samples were diluted along with the assay process. Thereby, the concentration of analytes could fall within the linear range of the method.

Take *Bozhou 1* as an example, it was analyzed with the developed UPLC-DDA-MS method. The monitored ion chromatograms (MIC) of five target alkaloids are shown in Fig. 4. The MS² spectra obtained are shown in Fig. 5

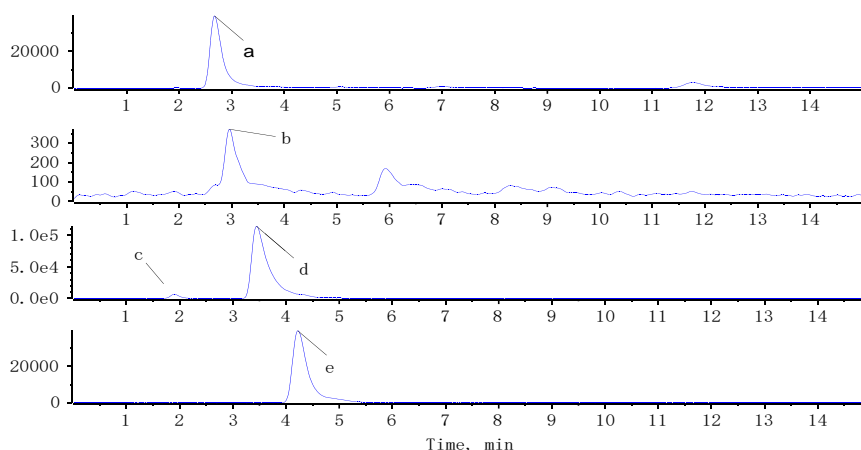


Fig. 4. The monitored ion chromatograms of (a) matrine (m/z 249.19), (b) sophocarpine (m/z 247.18), (c)oxysophoridine (m/z 265.19), (d)oxymatrine (m/z 265.19), and (e) oxysophocarpine (m/z 263.18)

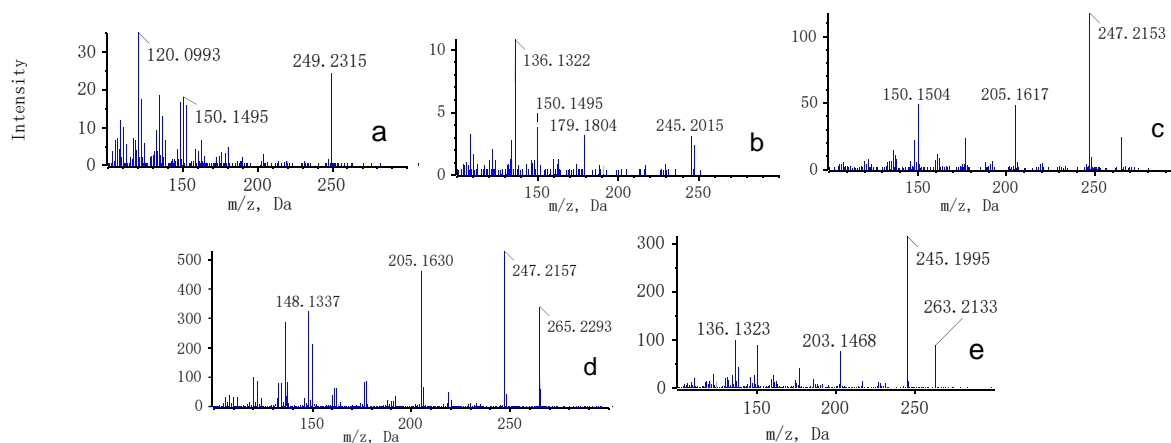


Fig. 5. The MS² spectra of (a) matrine, (b) sophocarpine (c)oxysophoridine, (d)oxymatrine, and (e) oxysophocarpine obtained by DDA method

Table 5. The content of five alkaloids in 12 *S. flavescens* root (mg/g)

Sample no	Oxysophoridine	Matrine	Sophocarpine	Oxymatrine	Oxysophocarpine
<i>Pingshun 1</i>	0.44	3.25	0.03	12.69	5.67
<i>Pingshun 2</i>	0.81	4.83	0.04	12.92	5.78
<i>Pingshun 3</i>	0.878	5.465	0.07	13.03	5.82
<i>Lichuan 1</i>	1.21	5.87	0.03	11.59	6.01
<i>Lichuan 2</i>	1.32	5.56	0.08	11.04	5.36
<i>Lichuan 3</i>	1.29	5.49	0.07	10.28	5.93
<i>Wenshan 1</i>	1.06	6.72	0.11	13.32	4.07
<i>Wenshan 2</i>	0.95	6.05	0.06	13.50	5.81
<i>Wenshan 3</i>	0.89	6.17	0.05	12.78	6.22
<i>Bozhou 1</i>	0.66	5.39	0.07	10.39	4.59
<i>Bozhou 2</i>	0.79	5.31	0.07	10.85	5.31
<i>Bozhou 3</i>	0.92	5.03	0.09	10.80	5.92

The peak area in MIC of each analyte was used to calculate the concentration. The contents of them in the raw roots were calculated according to the sample preparation process. The results are listed in Table 5. The oxymatrine was the most abundant alkaloid in all *S. flavescens*.

Although high-content alkaloids such as oxymatrine, matrine, and oxysophocarpine were detected and quantified by the analysis method developed in this study, their subsequent further studies need to obtain their respective monomer compounds by isolation. The chromatographic method of this study can be used as a reference for the subsequent chromatographic method of their monomer preparation.

4. CONCLUSION

In this study, the developed method for screening alkaloids in *S. flavescens* was validated. The approach combines an easy sample extraction with a comprehensive ultra-high performance liquid chromatography (UPLC) tandem a high-resolution mass spectrometry (HRMS) in the DDA method. After chromatography optimization, the peak tailing was diminished. Ten alkaloids were identified within 20 minutes. Since the standards are available, matrine, oxymatrine, oxysophoridine, sophocarpine, and oxysophocarpine were identified with both retention time and MS spectra. The other five alkaloids were identified with the accurate m/z value in MS¹ and product ions in MS². The quantification of matrine, oxymatrine, oxysophoridine, sophocarpine, and oxysophocarpine was investigated. In the range of 20-300 µg/L, the linear correlation coefficients (R²) for five target compounds exceeded 99.73%. The LOD of the method was set at 10 ng/mL, while the LOQ was 20 ng/mL. Recovery

and precision were accepted. Twelve batches of collected *S. flavescens* roots were analyzed. The ten identified alkaloids were all found in each sample. Oxymatrine was the most abundant component and sophocarpine was the least. The developed method provides a powerful tool for the biochemical and clinical research of *S. flavescens*. It can also be adopted in resource utilization and related medicine quality control.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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