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Simultaneous determination of four alkaloids in *Solanum lyratum* Thunb by UPLC-MS/MS method

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Four alkaloids, strychnine, soladulcidine, solamargine and (3-*O*-β-D–glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside-(25ξ)-solanidan-3β,23β-diol) (abbreviation, glycoalkaloid A) were isolated from *Solanum lyratum* Thunb. The structures were elucidated by NMR and measuring physicochemical properties. Then a novel and rapid method using an ultra-performance liquid chromatography coupled with mass spectrometry was developed and validated for the simultaneous determination of these compounds. An acquit UPLC BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm) was used. Acetonitrile and 0.1% formic acid were adopted as mobile phase. Detection was performed on a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer in the positive ion mode using an electrospray source. The multiple reaction monitoring (MRM) mode was used to detect the target compounds. The established method showed a good linearity ($R^2 > 0.999\ 0$) over the investigated concentration ranges, good inter-day and intra-day precisions (less than 2%) and good recoveries (from 99.8% to 100.1%) for all four target compounds. Compared to previous methods employing conventional high performance liquid chromatography (HPLC) separation, the ultra-high-pressure liquid chromatography-tandem mass spectrometry achieved preferable chromatographic parameters and significantly increased sample throughput.

1. Introduction

Solanum lyratum Thunb (Solanaceae) is one of the most valued Traditional Chinese Medicines. It is well known as "Herba *Solani Lyrati*" in mainland of China, which has been used for regulating body immune function and ability (Kim et al. 1999), and has frequently played an important role in clinics for treating cancer for generations (Yang et al. 2010; Teng et al. 2010). As reported previously, the major components in *Solanum lyratum* Thunb were alkaloids and saponins (Han et al. 2006; Kuo et al. 2000) considered as the major effective constituents. We have previously examined the whole plant of *Solanum lyratum* and isolated ten nitrogen-containing compounds (Sun et al. 2011). In the present work, strychnine, soladulcidine, solamargine and (3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyrano syl-(1→4)-β-D-galactopyranoside-(25ξ)-solanidan-3β,23β-diol) (abbreviation, glycoalkaloid A) were quantificated to evaluate the quality of *Solanum lyratum*. Among them, soladulcidine was isolated for the first time from this plant and strychnine was isolated for the first time from Solanaceae (Sun et al. 2011). According to previous studies, strychnine revealed significant inhibitory effects against HepG2 cell (Xu et al. 2006), KB cell lines, HL-60 cell lines and BGC cell lines (Yang et al. 2006). Soladulcidine and its derivatives had the antitumor activities against PC-3 cell line (Zha et al. 2010). Solamargine had shown cytotoxicity in K562 cell proliferation, KB cell proliferation, K562/A02 cell proliferation and KB/VCR cell proliferation (Zhao 2010). Glycoalkaloid A has been reported to show inhibitory activity against JTC-26 cells (Murakami et al. 1985). The quality control methods for soladulcidine and glycoalkaloid A were HPLC-ELSD (Qi et al. 2010; Yang et al. 2010). But the HPLC-ELSD method suffered from long analysis time and low sensitivity. At the same time, it requires large amounts of expensive, toxic and environmentally unfriendly organic solvents. Consequently, an improved method should be developed. With its superior sensitivity and selectivity, high performance liquid chromatography-mass spectrometry (HPLC-MS) has opened up the door to chemical research and quality control of Traditional Chinese Medicine. To shorten the analysis time and enhance sensitivity, an ultra-performance liquid chromatography (UPLC) system with high column effect and peak capacity (Li et al. 2006; Villiers et al. 2006) was adopted in the present study.

The aim of this study was to establish a convenient and effective UPLC-MS/MS method for the quality evaluation of *Solanum lyratum* Thunb. through the quantification of active components. Parameters of both UPLC separation and mass spectrometry were optimized to obtain desirable sensitivity and efficiency. The established method could be applied in the quality control of *Solanum lyratum* Thunb.

2. Investigations, results and discussion

2.1. Optimization of the extraction method

Refluxing and ultrasonic methods were tested to obtain an optimal extraction of the alkaloids from the plant materials. The amount of alkaloids extracted by ultrasonic treatment was a little more than that extracted by refluxing. Therefore, ultrasonic

extraction, which was simple and less time consuming, was selected. Then, different extraction times and extraction solvents were also tested and the extraction efficiency was evaluated. It was found that the target constituents could be efficiently extracted with 90% methanol under ultrasonication for 3 times and 20 min every time.

2.2. Optimization of UPLC conditions

The chromatographic separation was provided by UPLC, which was based on the use of column (ACQUITY UPLCTM BEH-C_{18}, 50 mm × 2.1 mm i.d.) with 1.7 μm particle packing. Considering the best electrospray ionization status and ion suppression which negatively affects several analytical merits, such as detection capability, sensitivity, precision, and accuracy, the ultimate flow rate was optimized at 0.1 mL·min^{-1} in this study. In this work, a series of preliminary experiments was carried on different mobile phases including acetonitrile-water (1), methanol-water (2), methanol-0.1% formic acid (3), acetonitrile-ammonium acetate (4) and acetonitrile-0.1% formic acid (5). The mobile phases 1 and 2 were weak to elute alkaloids. When mobile phase 3 and 4 were used, the best electrospray ionization of sample was not gained and the response of MS was weak. At last, the optimal results were obtained with mobile phase 5, acetonitrile-0.1% formic acid (50:50, *v/v*) by isocratic elution. Results of multiple injections indicated that under such situation nice peak shape and high sensitivity of various alkaloids could be achieved.

2.3. Optimization of MS parameters

All factors related with MS performance including ionization mode, collision energy, gas flow, desolvation temperature and mobile phase additives have been optimized. MS detection of the four standard solutions in positive and negative ionization modes by direct full scan method revealed that the signals obtained from the electrospray ionization source in the positive mode had a better resolution and higher intensity for quantitative measurement. MS parameters, such as capillary voltages (2, 2.5, 3, 3.5, 4.0 kV), cone voltages (25, 35, 40, 50 V), flow rate of desolvation gas (450, 500, 550 and 650 L·h^{-1}), and flow rate of cone gas (30, 40 and 50 L·h^{-1}) were investigated one by one for optimization. The optimal MS parameters were as follows: the desolvation gas and cone gas with a flow rate of 650 L·h^{-1} and 50 L·h^{-1} and optimal MS/MS conditions are shown in Table 1 and Fig. 1. In this study, cross-talk effect produced by different channels in mass system was not observed.

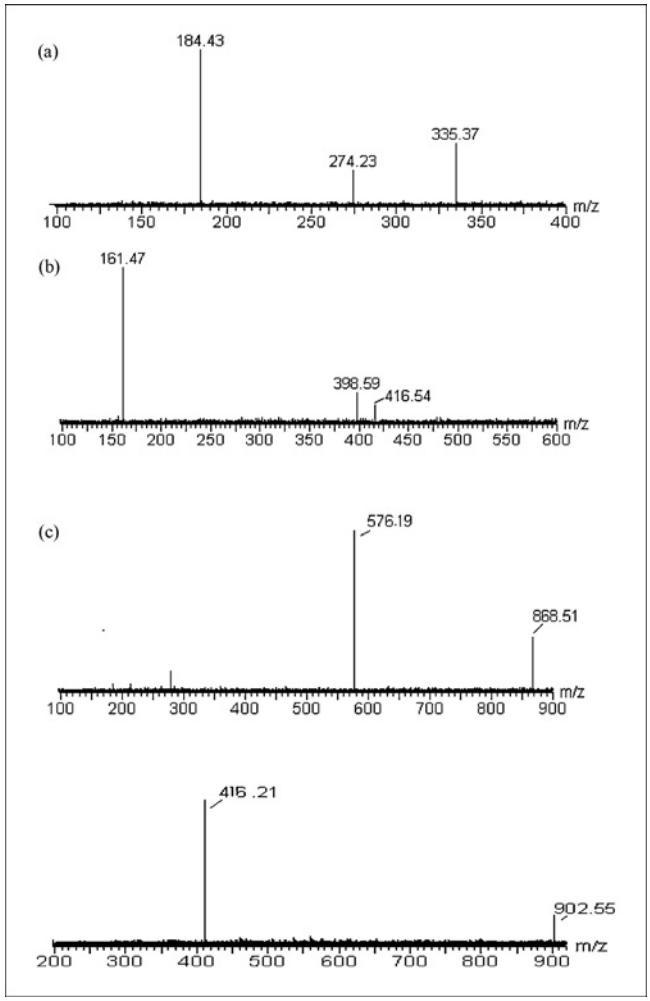

Fig. 1: Full scan MS/MS spectra of strychnine (a), soladulcidine (b), solamargine (c) and glycoalkaloid A (d).

2.4. Method validation

2.4.1. Specificity

The specificity of the method was tested through the analysis of the blank solvent. There was no significant chromatographic interference around the retention times of the analytes (Fig. 2). The retention times of strychnine, soladulcidine, solamargine and glycoalkaloid A were 1.15, 4.23, 1.33 and 1.21 min, respectively.

2.4.2. Linearity range, limits of detection and limits of quantification

The calibration curves were constructed by analyzing at least five different concentrations. All measurements were repeated in triplicate and data were processed by MassLynx software (Version 4.1). The calibration curves were constructed by plotting peak areas versus concentrations. The linearity parameters for the investigated compounds are listed in Table 2. As a result, good linearity ($R^2 > 0.999\ 0$) of the investigated concentration ranges was observed. The LOD and LOQ for each analyte were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. The linearity ranges were adequate for the determinations of four constituents in the samples.

2.4.3. Precision

Intra-day precision was examined with the mixture standard solutions during a single day. The inter-day precision was determined over three consecutive days. The relative standard deviation (RSD %) values varied from 0.21% to 1.5% for intra- and inter-day assays for all the analytes (Table 3).

2.4.4. Repeatability and stability

Injection repeatability was examined through the injection of six samples prepared with the same sample preparation procedure. Stability of the sample solution during 24 h at room temperature was tested. For the repeatability and stability test, the RSD% values varied from 0.86% to 1.9% and 0.93% to 3.3% (Table 3). Results for the stability test indicated that the investigated samples were stable enough for the routine analysis within a day at room temperature.

Table 1: LC-ESI-MS/MS parameters for four alkaloids

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z*)	Collision energy (eV)	Cone Voltage (V)
Strychnine	335.37	184.43	50	55
Soladulcidine	416.54	161.47	35	55
Solamargine	868.51	576.19	50	60
Glycoalkaloid A	902.55	416.21	65	70

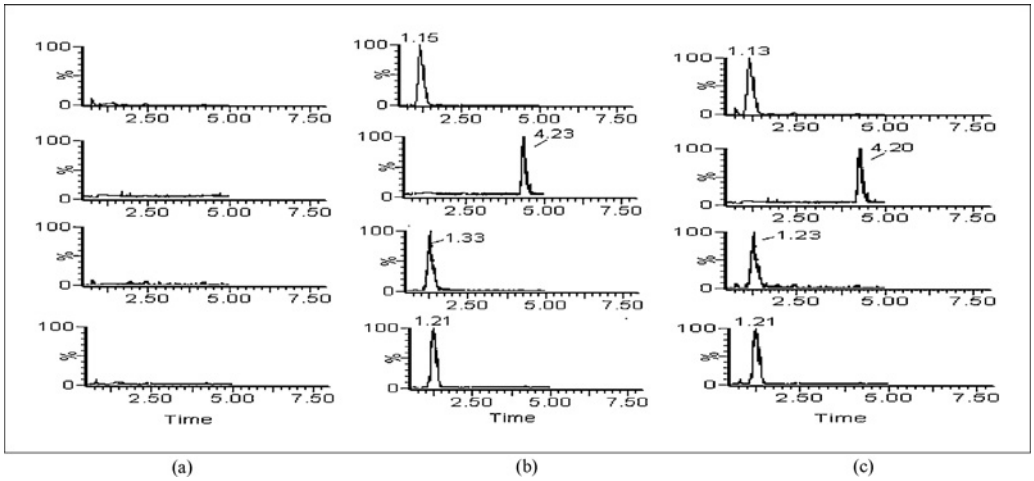

Fig. 2: The MRM chromatogram of four alkaloids in *Solanum lyratum* Thunb a: blank b: standard c: sample. The MRM transitions from top to bottom correspond to strychnine (335.37, 184.43), soladulcidine (416.54, 161.47), solamargine (468.51, 576.19) and glycoalkaloid A (902.55, 416.21)

Table 2: Regression equation, linear range, LOD and LOQ of the method

Constituents	Regression equation	Correlation Coefficient/R^2	Linearity Range/(ng·mL^{-1})	LOD/(ng·mL^{-1})	LOQ/(ng·mL^{-1})
Strychnine	$Y = 1.136 \times 10^3 X + 2.896 \times 10^2$	0.999 4	0.4–10.0	0.13	0.4
Soladulcidine	$Y = 1.158 \times 10^2 X + 49.96$	0.999 7	4.0–100.0	0.68	2.0
Solamargine	$Y = 1.166 \times 10^3 X - 80.08$	0.999 8	0.4–10.0	0.12	0.4
Glycoalkaloid A	$Y = 1.564 \times 10^2 X + 1.344 \times 10^2$	0.999 0	40.0–1000.0	1.4	4.0

2.4.5. Recovery

The recovery test was carried out to evaluate the accuracy of the method. In the recovery test, the proposed method was applied to the samples blended with the mixed standard solution at high, middle and low concentration levels in three replicates. The average recoveries were determined by the following formula: Recovery (%) = (observed amount − original amount)/spiked amount × 100%. The recoveries were between 99.5% and 100.1%. The RSD% values of each concentration level were less than 1.5%.

2.5. Application

The analytical method was utilized for the determination of four alkaloids in *Solanum lyratum* Thunb, and the results are shown in Table 4. The contents of the four compounds varied substantially among the samples. Variation of the amounts in these samples may arise because of different climates and geographical environment. It was believed that the UPLC-MS/MS method would be helpful to improve the quality control of *Solanum lyratum* Thunb.

Table 3: Precision, repeatability and stability

Precision (RSD%)		Repeatability (RSD%)	Stability (RSD%)
Intra-day	Inter-day		
1.0	2.0	1.2	1.7
1.5	1.7	1.6	3.3
0.48	1.4	1.9	0.93
0.21	1.6	0.86	2.1

3. Experimental

3.1. Chemicals and reagents

Strychnine, soladulcidine, solamargine and glycoalkaloid A were isolated from *Solanum lyratum* Thunb in our laboratory (> 98.0% pure). Acetonitrile and formic acid (HPLC grade) were purchased from DIKMA (DIKMA, American). Water used in the experiment was prepared by a Milli-Q water purification system (Millipore, MA, USA). Other reagents of HPLC grade or the highest grade were commercially available. The whole plant of *Solanum lyratum* Thunb were collected from the different regions in China and authenticated by Professor Qi-Shi Sun, College of Chinese Traditional Medicine, Shenyang Pharmaceutical University.

3.2. Extraction and isolation of standards

The dried whole herbs of *Solanum lyratum* Thunb (10 kg) were powered and extracted with 70% EtOH (1.5 hr × 3). The filtrate was concentrated *in vacuou* to provide the ethanolic extract (645.7 g). The aqueous residue was extracted with petroleum ether. The aqueous layer was basified with NH_4OH to pH = 10 to get the alkaloid precipitate (46.5 g). After being filtered and adjusted with 2% HCl at pH 7.0, the supernatant was extracted with $CHCl_3$ and *n*-BuOH to give SL fraction (88.2 g, $CHCl_3$ extract) and SZ fraction (103.6 g, *n*-BuOH extract). The alkaloid precipitate was diluted

Table 4: Quantification of four components in *Solanum lyratum* Thunb (n = 3)

NO.	Strychnine		Soladulcidine		Solamargine		Glycoalkaloid A	
	Content ($\mu g \cdot g^{-1}$)	RSD (%)	Content ($\mu g \cdot g^{-1}$)	RSD (%)	Content ($\mu g \cdot g^{-1}$)	RSD (%)	Content ($\mu g \cdot g^{-1}$)	RSD (%)
1	45.95	1.7	1092.3	0.86	12.13	1.8	11042.2	0.49
2	17.01	0.98	198.0	1.8	63.99	1.2	3491.5	0.75
3	55.49	1.5	401.4	1.1	9.50	1.9	664.3	1.9
4	94.03	1.2	1023.3	1.2	96.13	1.1	2717.5	0.88
5	54.58	1.4	449.0	2.1	65.81	1.4	627.4	1.8
6	94.80	1.8	94.80	1.3	7.10	0.88	1419.2	1.0
7	47.72	0.84	967.9	1.6	32.82	1.4	563.7	1.1
8	63.62	1.6	320.1	1.5	105.3	2.0	551.0	0.88
9	31.38	1.2	191.6	1.3	11.89	1.3	707.9	1.7

with water and partitioned with $CHCl_3$, EtOAc, BuOH successively to provide the fractions: JSL (1.2 g, $CHCl_3$ extract), JSY (1.9 g, EtOAc extract) and JSZ (4.6 g, BuOH extract).

The SL fraction was subjected to a silica gel column eluted with $CHCl_3$-MeOH (100:0–0:100) to afford 10 fractions (SL-1∼SL-10). SL-2 was chromatographed on a silica gel column to get 5 fractions (SL-2-1∼SL-2-5) using CH_2Cl_2-C_2H_5OH (100:0–0:100). Fraction SL-2-3 afford compound 1 (10.4 mg) using preparing thin layer chromatography (PTLC). The SZ fraction was dissolved in water and passed through macroporous resin D101, and eluted with water, 10%, 30%, 50%, 70% and 95% (*v/v*) methanol successively. The various elutes were evaporated to dryness under vacuum to give 8 fractions (SZ-1∼SZ-8). Fraction SZ-6 was loaded on Sephadex LH-20 column and compound 2 (11.2 mg) was gained after recrystallization. Fraction JSZ was separated on a silica gel column with $CHCl_3$-MeOH (100:0–0:100) and on an ODS column with H_2O-MeOH (5:1–0:100) to yield compound 3 (8.7 mg) and 4 (230.4 mg).

Compound 1: ^{1}H NMR (300 MHz, MeOD) δ: 8.11 (1H, d, J = 8.1 Hz, H-4), 7.29 (1H, d, J = 7.3 Hz, H-1), 7.19 (1H, t, J = 7.7 Hz, H-3), 7.12 (1H, t, J = 7.5 Hz, H-2), 5.93 (1H, brs, H-22), 3.12 (2H, d, J = 14.1 Hz, H-23), 1.50 (1H, d, J = 15.4 Hz, H-8). ^{13}C-NMR (75 MHz, MeOD) δ: 124.3 (C-1), 122.3 (C-2), 135.8 (C-3), 139.9 (C-4), 127.9 (C-5), 132.4 (C-6), 52.6 (C-7), 60.2 (C-8), 169.2 (C-10), 42.7 (C-11), 77.1 (C-12), 48.1 (C-13), 29.7 (C-14), 42.5 (C-15), 60.0 (C-16), 26.7 (C-17), 51.9 (C-18), 31.5 (C-19), 50.3 (C-20), 142.2 (C-21), 128.7 (C-22), 64.5 (C-23). The structure was determined by MS, ^{1}H- and ^{13}C-NMR comparing to the data in literature (Liu et al. 1998). It was strychnine.

Compound 2: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.65 (1H, m, H-9), 0.78 (3H, s, H-18), 0.85 (3H, s, H-19), 0.87 (3H, d, J = 7.4 Hz, H-27), 1.06 (3H, d, J = 6.7 Hz, H-21), 2.22 (1H, m, H-20), 3.54 (1H, m, H-3), 4.22 (1H, m, H-16). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 36.9 (C-1), 31.5 (C-2), 71.2 (C-3), 38.2 (C-4), 44.8 (C-5), 28.6 (C-6), 32.2 (C-7), 35.1 (C-8), 54.5 (C-9), 35.6 (C-10), 21.1 (C-11), 40.2 (C-12), 40.5 (C-13), 56.3 (C-14), 32.1 (C-15), 78.8 (C-16), 62.9 (C-17), 16.6 (C-18), 12.3 (C-19), 41.2 (C-20), 15.2 (C-21), 98.2 (C-22), 34.0 (C-23), 30.2 (C-24), 31.3 (C-25), 47.6 (C-26), 19.3 (C-27). The structure was determined by MS, ^{1}H- and ^{13}C-NMR comparing to the data in literature (Ripperger 1995). It was soladulcidine.

Compound 3: ^{1}H NMR (300 MHz, pyridine-d_5) δ: 5.747 (1H, s), 5.186 (1H, s), 4.319 (1H, d, J = 7.8 Hz). ^{13}C-NMR (75 MHz, pyridine-d_5) δ: 37.6 (C-1), 30.3 (C-2), 78.1 (C-3), 39.0 (C-4), 148.0 (C-5), 121.9 (C-6), 32.7 (C-7), 31.8 (C-8), 50.4 (C-9), 37.2 (C-10), 21.3 (C-11), 40.2 (C-12), 40.7 (C-13), 56.8 (C-14), 32.5 (C-15), 78.9 (C-16), 63.6 (C-17), 16.7 (C-18), 19.5 (C-19), 41.7 (C-20), 15.8 (C-21), 98.5 (C-22), 34.8 (C-23), 31.2 (C-24), 31.7 (C-25), 48.2 (C-26), 19.9 (C-27), 102.1 (C-1′), 72.6 (C-2′), 72.9 (C-3′), 74.2 (C-4′), 69.6 (C-5′), 18.8 (C-6′), 102.9 (C-1″), 72.6 (C-2″), 72.8 (C-3″), 74.0 (C-4″), 70.5 (C-5″), 18.6 (C-6″), 100.3 (C-1‴), 78.0 (C-2‴), 77.8 (C-3‴), 78.6 (C-4‴), 77.0 (C-5‴), 61.3 (C-6‴). The structure was determined by MS, ^{1}H- and ^{13}C-NMR comparing to the data in literature (Ripperger and Porzel 1993). It was solamargine.

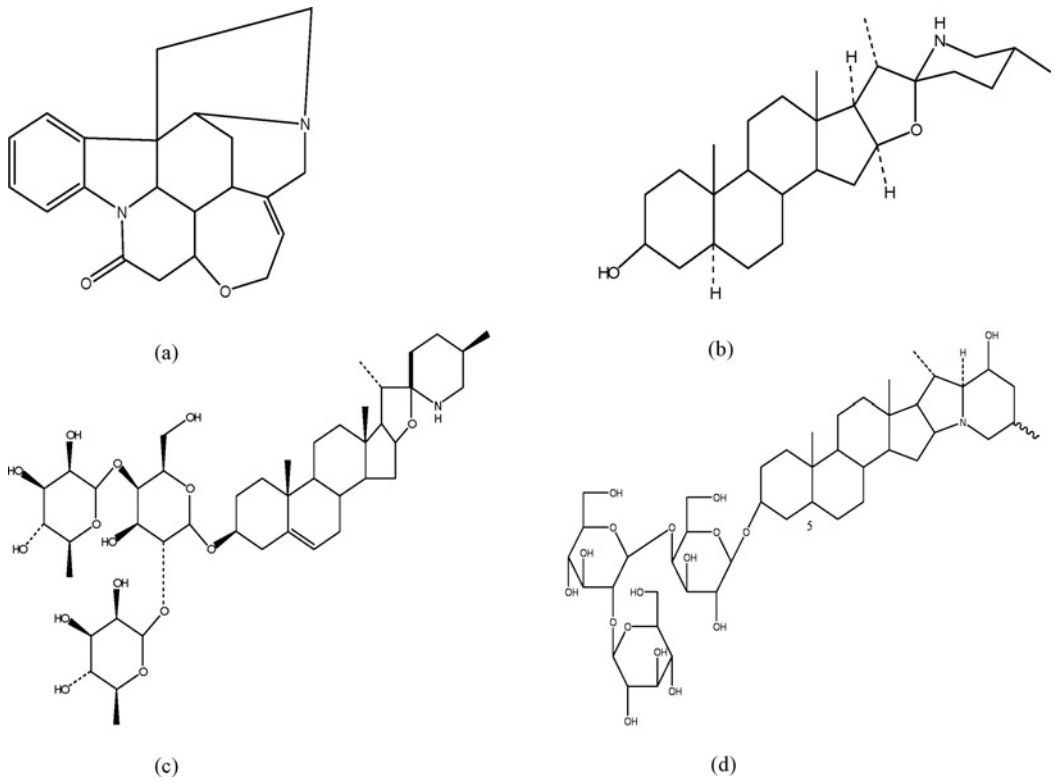

Fig. 3: Chemical structures of strychnine (a), soladucidine (b), solamargine (c) and glycoalkaloid A (d) in *Solanum lyratum* Thunb

Compound 4: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.75 (3H, s, H-18), 0.84 (3H, s, H-19), 0.88 (3H, d, J = 7.5 Hz, H-27), 1.14 (3H, d, J = 6.9 Hz, H-21), 4.88 (1H, d, J = 7.5 Hz, H-1′), 5.06 (1H, d, J = 7.2 Hz, H-1″), 5.13 (1H, d, J = 7.2 Hz, H-1‴). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 36.7 (C-1, 24), 30.0 (C-2), 71.6 (C-3), 38.3 (C-4), 44.2 (C-5), 28.5 (C-6), 34.2 (C-7), 35.0 (C-8), 54.0 (C-9), 35.4 (C-10), 20.7 (C-11), 39.8 (C-12), 40.9 (C-13), 57.0 (C-14), 31.9 (C-15), 69.1 (C-16), 61.7 (C-17), 16.8 (C-18), 12.2 (C-19), 29.2 (C-20), 18.6 (C-21), 78.3 (C-22), 64.2 (C-23), 27.2 (C-25), 58.1 (C-26), 21.4 (C-27), 103.4 (C-1′), 75.0 (C-2′), 73.6 (C-3′), 79.5 (C-4′), 76.5 (C-5′), 60.2 (C-6′), 104.7 (C-1″), 84.0 (C-2″), 77.2 (C-3″), 73.2 (C-4″), 76.7 (C-5″), 61.9 (C-6″), 101.0 (C-1‴), 75.9 (C-2‴), 76.6 (C-3‴), 73.5 (C-4‴), 76.5 (C-5‴), 61.2 (C-6‴). The structure was determined by MS, ^{1}H- and ^{13}C-NMR comparing to the data in literature (Murakami et al. 1985). It was glycoalkaloid A.

The purities of these chemical standards were evaluated to be more than 98.0% by HPLC-ELSD analysis based on a peak area normalization method. Their structures are showed in Fig. 3.

3.3. Preparation of standard solutions

A stock standard solution containing strychnine (20 ng·mL^{-1}), soladulcidine (200 ng·mL^{-1}), solamargine (20 ng·mL^{-1}) and glycoalkaloid A (2000 ng·mL^{-1}) were prepared in water-acetonitrile (50:50, *v/v*). The stock solution was appropriately diluted to obtain a series of working solutions. The solutions were stored at -4 °C and were brought to room temperature before use.

3.4. Preparation of sample solutions

Dried *Solanum lyratum* Thunb was ground into powder (40 mesh). An aliquot (1.0 g) of the powder was accurately weighed and macerated with 5 mL of ammonium hydroxide for 1 h, then extracted by sonication (20 min × 3) with 20 mL 90% methanol. The extract was filtered and concentrated to dryness *in vacuou* at 70 °C and the residue was dissolved with water-acetonitrile (50:50, *v/v*) in a 25 mL volumetric flask. Then 0.1 mL solution was diluted in 50 mL volumetric flask and filtered through a 0.22 μm membrane filter before introduced to UPLC-MS/MS system.

3.5. Instrumentation and UPLC-MS parameters

The experiments were performed on a Waters Acquity UPLC system, equipped with a quaternary pump system (Milford, MA, USA). Separations were performed on a 2.1 mm × 50 mm column packed with 1.7 μm particles (Acquity UPLC BEH-C_{18} column, Waters) designed to withstand 15000 psi. Optimum separation was achieved with a binary mobile phase at a flow rate of 0.1 mL·min^{-1}. The mobile phase consisted of acetonitrile and 0.1% formic acid water (50:50, *v/v*). The column temperature was maintained at 25 °C. The sample injection volume was 10 μL. Detection was performed on a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer (Waters, Manchester, UK) in the positive ion mode using an electrospray source. The ionization source conditions were as follows: source temperature 110 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation gas and cone gas with a flow rate of 650 L·h^{-1} and 50 L·h^{-1}. Detection was carried out in multiple reaction monitoring (MRM) mode. Other parameters of analytes are shown in Table 1. All data were processed using MassLynx V 4.1 soft-ware with a QuanLynx program (Waters).

3.6. Method validation

The analytical method was validated in terms of specificity, linearity, precision, accuracy, repeatability and stability. The relative standard deviation (RSD %) was taken as an evaluation of precision, repeatability and stability.

3.7. Sample analysis

In this study, the samples were obtained from nine regions (1–9). These nine batches of dried herb materials were collected from Shanghai (1), Zhengzhou (2), Anhui (3), Hunan (4), Henan (5), Fujian (6), Jiangsu (7), Guangzhou (8) and Wuhan (9). Samples were aliquoted and stored in darkness at room temperature before analysis. All samples were prepared in three replicates.

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