



Determination and Chemical Profiling of Toxic Pyrrolizidine Alkaloids in Botanical Samples with UPLC–Q-TOFMS

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Abstract

Pyrrolizidine alkaloids (PAs) and their *N*-oxides (PANOs) can cause liver toxicity and genotoxicity in humans and animals, necessitating the development of screening tools for these alkaloids. Ultra-performance liquid chromatography–electrospray ionization–quadrupole–time-of-flight mass spectrometry (UPLC–ESI–Q-TOFMS) can provide accurate precursor and fragment ion mass information (MS^E mode) as well as simultaneous quantification. A method for rapidly and accurately determining nine PAs with UPLC–ESI–Q-TOF MS was developed and validated herein. Analytes were separated using a C18 column with water containing 0.1% formic acid and acetonitrile, showing high-resolution peaks within 11 min. The validation results showed excellent linearity ($R^2 > 0.9951$), limits of detection (0.4–2.0 ng mL⁻¹), and limits of quantification (0.6–6.0 ng mL⁻¹). Moreover, 70 PAs, their *N*-oxides precursors, and characteristic fragment ions that were generated according to their chemical structure were characterized. A method for the chemical profiling of alkaloids was also proposed using the mass information obtained from the chromatograms of the tested sample. Lycopsamine, senecionine, senkirkine, and echimidine were identified in four potentially PA-containing plants and quantified by matching with authentic standards. Eight PAs and PANOs were also tentatively identified using the mass data from the previously listed alkaloids. This approach will provide a database that can be used to instantly identify alkaloids in botanical samples.

Keywords Pyrrolizidine alkaloids · UPLC–Q-TOF/MS · Chemical profiling · Structural identification

Introduction

Alkaloids are secondary metabolites containing nitrogen, which are present in a variety of organisms including bacteria, fungi, plants, and animals [1], and exhibit physiological activities and toxicity when ingested by humans and animals [1]. Pyrrolizidine alkaloids (PAs) and their *N*-oxides (PANOs) are mainly found in *Asteraceae*, *Fabaceae*, and *Boraginaceae* species and more than 600 PAs have been identified to date [2]. These compounds exhibit toxicity when the 1, 2 carbon atoms are double bonded and esterified with a carboxylic acid on at least one side of the structural

chain, with the toxicity increasing with an increasing number of esters (Fig. 1 [3, 4]) [3, 4]. PAs are naturally toxic substances capable of causing liver damage, mutation, and carcinogenesis [2]. Notably, because PAs are found in a variety of plants, humans can be exposed to toxicity by ingestion of herbal teas, medicinal plants, and dietary supplements [5, 6]. Recently, due to the presence of PAs in honey, pollen, eggs, and milk, the dangers of secondary exposure through PA accumulation are emerging [7]. Therefore, a method for quick, easy, and accurate detection of PAs in containable sources must be developed.

Chromatographic technologies such as HPLC coupled with DAD, ELSD, and MSMS have been used to determine the PA content in various samples [8–10]. In particular, LC–MSMS is preferred over other methods because of its excellent sensitivity, precision, and quantitative properties. However, LC–MSMS analysis requires corresponding reference materials and results in an increased number of false positives [11]. Alternatively, Q-TOF/MS combined with UPLC is an optimal tool for investigating the complex components of plants [12, 13]. UPLC is an upgraded

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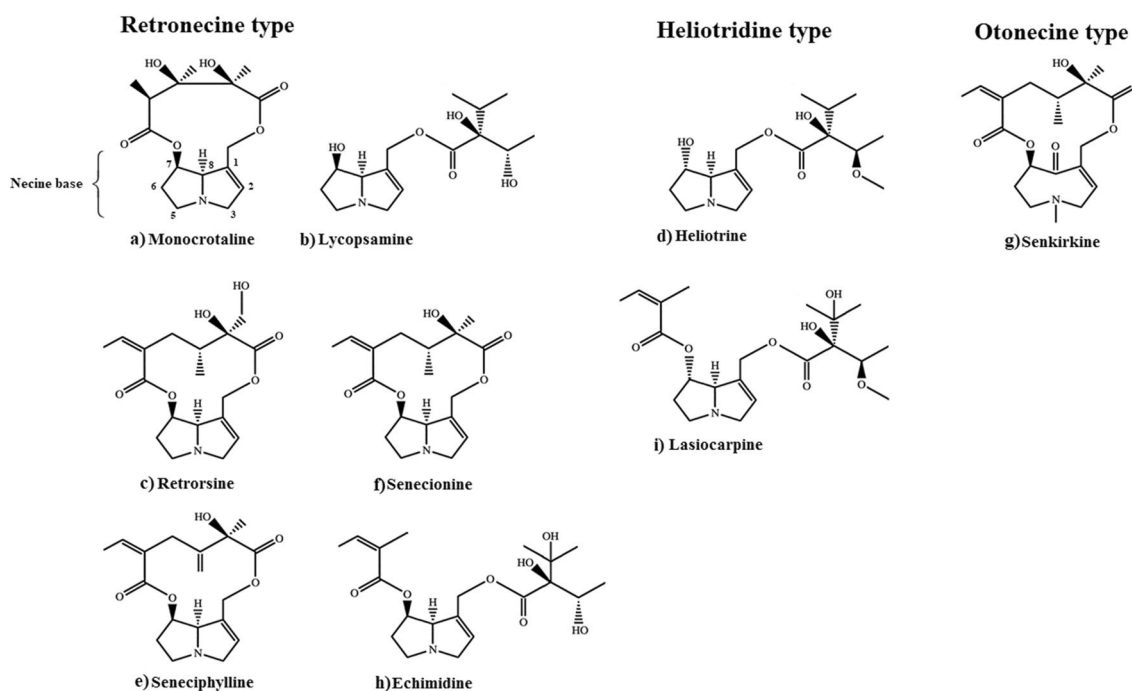


Fig. 1 Chemical structure of pyrrolizidine alkaloids [3, 4]

instrument compared to HPLC that provides rapid analysis, high resolution, and enhanced sensitivity using mobile phase elution with high pressure and narrow inner diameter columns ($\leq 2 \mu\text{m}$) [14]. Q-TOF measures mass data with high accuracy and precision, which can be used to predict molecular formulae with low parts-per-million errors [15]. The unknown components can be predicted tentatively without standard materials from the precursor and fragment ion patterns. This performance can be achieved with a single injection, shortening the analysis time. In addition, when a standard substance can be obtained, the fragment ion pattern measured via CID can be utilized as a part of a library capable of identifying unique components. UPLC-Q-TOF is able to qualify key components by profiling the major components of a sample, such as medicinal plants and decoctions [13, 16].

A number of analytical methods have been used to measure PAs and PANOs in various samples, and in particular, Avula et al. and Picron et al. investigated various foods by simultaneous analysis of 25–30 PAs and PANOs [17, 18]. However, from a literature review, we found that there are 73 toxic PAs and PANOs compounds for which reference materials are not readily available for accurate detection and quantification. Therefore, it is necessary to develop novel methods for screening more PAs and PANOs compounds. Fortunately, it was confirmed via extensive studies that common fragment ions are generated by CID depending on the structure of the PAs and PANOs when using an LC-based high-resolution mass analyzer and triple quadrupole

instruments. Herein, we propose a method that can quickly and easily identify PAs and PANOs peaks obtained via UPLC-O-TOF. PAs and PANOs compounds were grouped according to their chemical structure, and the mass and fragment ions of the expected ionized parent molecules were listed based on the related literature and the nine PAs fragment ions in our library. Furthermore, we established and validated a method for rapid separation and quantification of nine PAs using Q-TOF and applied the above method to botanical samples.

Experimental

Chemicals and Reagents

Analytical reference standards (purity $\geq 95\%$) of monocrotaline, lycopsamine, retrorsine, heliotridine, seneciphylline, senecionine, senkirkine, echimidine, and lasiocarpine were purchased from PhytoLab (Bavaria, Germany). HPLC grade methanol, ethanol, acetonitrile, water, dichloromethane, and ethyl acetate solvents were purchased from Merck (Darmstadt, Germany). Formic acid and ammonium formate (analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Stock solutions of the above listed reference standards were prepared separately in methanol to a final concentration of 1.0 mg mL^{-1} . A mixed standard solution containing

10 $\mu\text{g mL}^{-1}$ was subsequently prepared in methanol and stored at 4 °C.

Chromatographic Separation

The liquid chromatographic system used herein was composed of an Acquity UPLC[®] system (Waters Corp., USA) coupled with a binary solvent delivery pump, auto-sampler, and photodiode array (PDA). UNIFI (ver. 1.8) software (Waters Corp., USA) was used to acquire and process the obtained data. The chromatographic separation of alkaloids was performed on an ACQUITY UPLC BEH C18 column (2.1 \times 50 mm, 1.7 μm ; Waters Corp., USA). The mobile phase solvents were (A) water containing 0.1% formic acid and (B) acetonitrile. The sample parameters of the column were: 40 °C temperature, 0.4 mL min^{-1} flow rate, and 1 μL injection. The gradient elution was performed as follows: 3–14% B from 0 to 8.5 min; 14 to 20% B from 8.5 to 15 min; 20 to 100% B from 15 to 20 min, 100 to 14% B over 4 min.

Q-TOF Analyzer Condition

The mass spectrometer used in the testing was a Xevo G2 Q-TOF/MS (Waters Corp., USA) equipped with an ESI source. The analytes were detected in positive ion mode and the capillary and cone voltages were set to 3.0 kV and 30 V, respectively. The cone and desolvation gas flow rates were set to 60 and 800 L h^{-1} , respectively, and the source temperature was 120 °C. The scan time was set to 0.25 s for obtaining data in the m/z range of 50–1200. To obtain the mass spectrum (MS) and tandem mass spectrum (MS/MS), also known as MS^e data, the low and high collision energies were set as 6 and 35–43 eV, respectively. Leucine enkephalin (m/z 556.2771 in positive ion mode) was used as a reference mass. Sodium formate was used to calibrate the mass spectrometer in positive ion mode.

Sample and Preparation

PA-containing plants *Borago officinalis* (root and arial parts), *Tussilago farfara* (whole), *Lithospermum purpurocaeruleum* (root), and *Symphytum officinale* L. (root and arial parts) were selected for the experiment and analyzed after the samples were sectioned. *B. officinalis* and *T. farfara* were sourced from herb farms in Yeosu-si and Icheon-si (Korea) in March 2018. *L. purpurocaeruleum* was collected from a herbal medicine market in Cheongju-si (Korea) in March 2018. *S. officinale* was provided by the Jeollabuk-do Agricultural Research and Extension Service (Korea) in March 2018. All samples were powder after lyophilization, and then dried plant was grounded to powder by a pulverizer.

Two gram of powdered botanical material was ultrasonically extracted with 20 mL of 70% methanol in an ultra-sonic

water bath for 1 h. The mixtures were subsequently centrifuged at 3000 \times g for 20 min and the supernatant was filtered through a 0.45 μm membrane filter prior to analysis.

Method Validation

The proposed PA analytical methods were validated for linearity, limit of detection (LOD), the limit of quantitation (LOQ), inter- and intra-day precisions, and matrix effects. The nine-compound mixed analytical standard solutions were prepared at six different concentrations (ranging from 10 to 2000 ng mL^{-1}). The calibration curve for each analyte was established by plotting the peak area corresponding to the analyte concentration using least-squares linear regression. The LOD and LOQ values were determined for the analytes under the chromatographic conditions at signal to noise (S/N) ratios of 3 and 10, respectively.

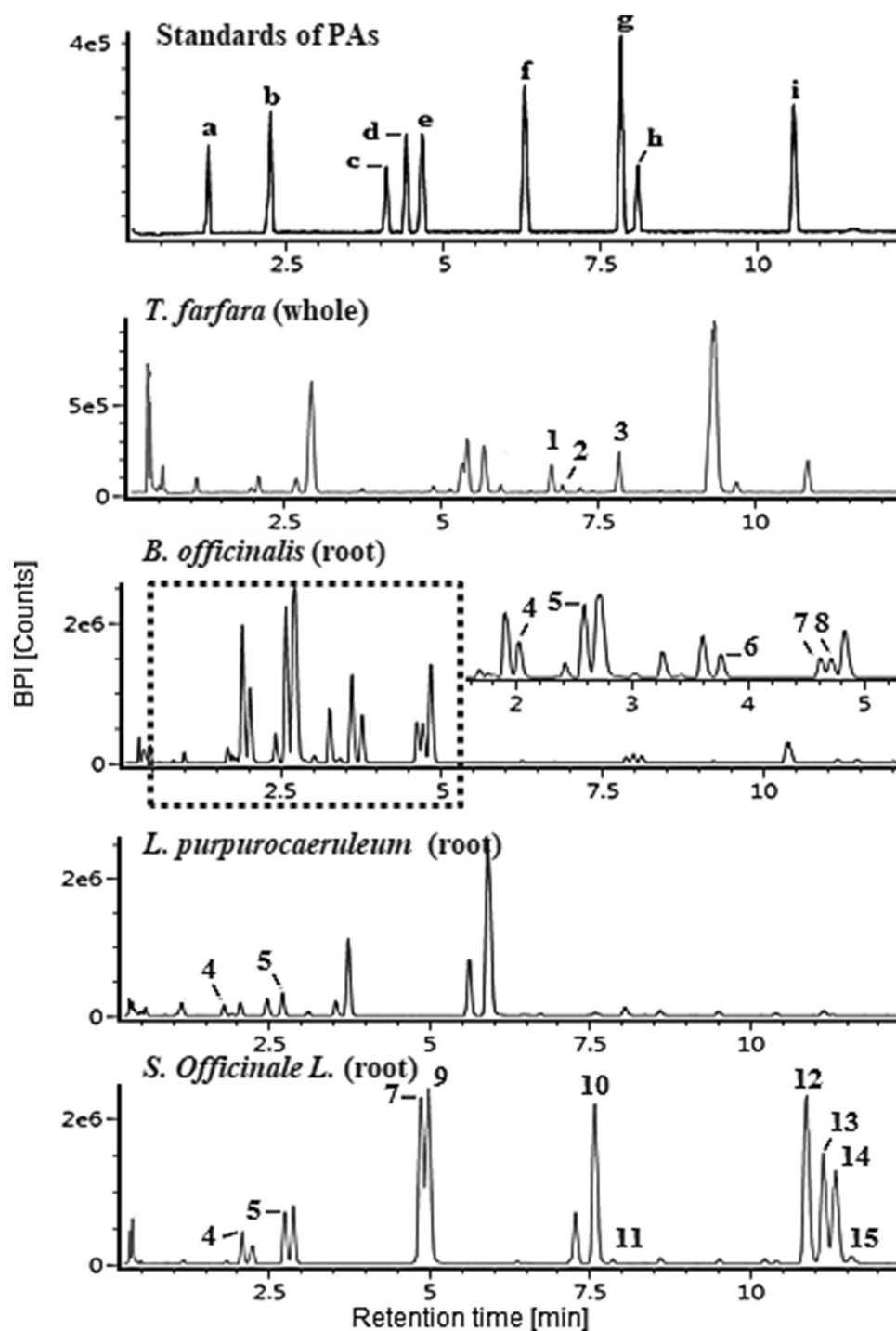
The precisions were determined by analyzing solutions of known concentration for the nine analytical replicates on the same day (intra-day) and three consecutive days (inter-day). The precisions were expressed in terms of relative standard deviation (RSD).

Results and Discussion

Optimization of Chromatographic Separation

The separation of PAs via LCMS was attempted by eluting with acetonitrile or methanol with water at various concentrations using a C18 column [19]. Because alkaloids, including PAs, are basic, their resolution and sensitivity depend on the pH of the aqueous mobile phase. Therefore, their separation time may vary depending on conditions such as column type, slope composition, and flow rate [20]. Several tests using a variety of pH aqueous mobile phases to obtain chromatographic peaks with good resolution within a short time frame instead of relying on results from the literature. The aqueous mobile phase (0.1% formic acid) at pH 2.7 and acetonitrile were eluted on a C18 column to obtain a satisfactory chromatogram, as shown in Fig. 2. Interestingly, the symmetry of the analytical peaks varied depending on the pH of the aqueous portion of mobile phase. The monocrotaline, lycopsamine, and retrorsine peaks were observed to respond to frontal tailing when using an aqueous mobile phase with 0.2% formic acid. Frontal tailing is generated by excess analyte injection or by the acidity (pK_a) of the substance [20, 21]. It is likely that frontal tailing occurred because the basic alkaloid was more dissociated in the lower pH aqueous solution and not due to excess of analyte injection. In contrast, when using an aqueous mobile phase with pH adjusted to 4.7 with 10 mM ammonium formate, frontal

Fig. 2 Chromatograms of standard alkaloids mixture and sample



tailing was improved but the detection sensitivity was reduced. Comprehensively, considering the symmetrical peaks, sensitivity, and analytical times, elution with 0.1% formic acid and acetonitrile was determined to be the ideal separation method.

Fragmentation Behavior of Reference Components

The mass spectral characteristics of the alkaloids separated via UPLC, such as the quasi molecular ions (MS1 spectra) and fragment ions (MS2 spectra) were obtained using an

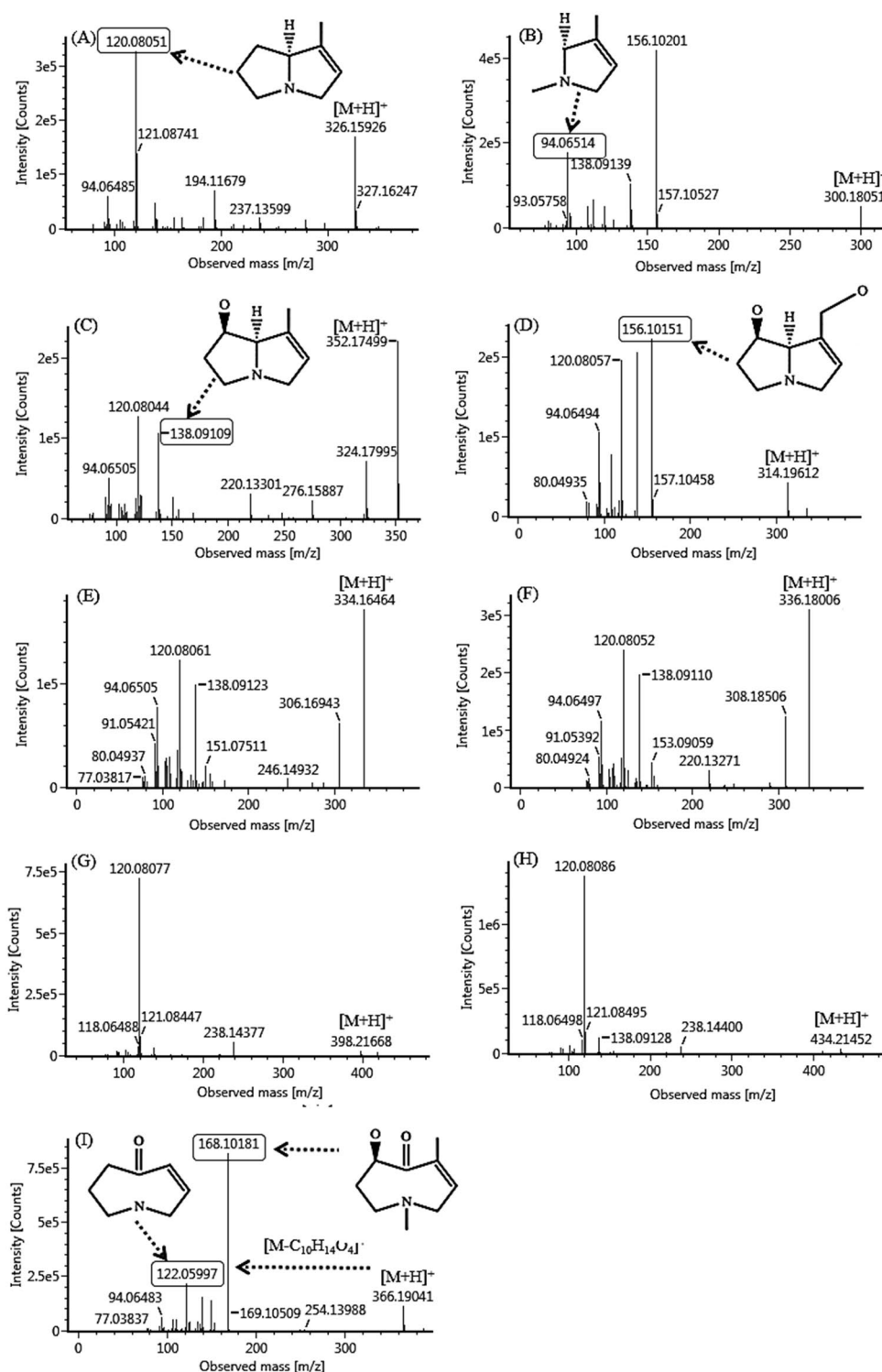
Table 1 Retention time, formula, natural mass, observed molecular ion and fragment ions of nine PAs acquired by UPLC–ESI–Q–TOF

Compound name	Retention time	Formula	Neural mass (Da)	Observed ion (<i>m/z</i>)	Mass error (ppm)	Adduct	Fragment ions <i>m/z</i>
Monocrotaline	1.37	C ₁₆ H ₂₃ NO ₆	325.1525	326.1597	−0.3	[M+H] ⁺	280.1541 (8.2), 237.1346 (11.0), 194.1169 (26.8), 184.0963 (11.0), 156.1016 (7.1), 138.0911 (17.1), 121.0874 (55.1), 120.0805 (100.0), 94.0649 (15.9)
Lycopsamine	2.47	C ₁₅ H ₂₅ NO ₅	299.1733	300.1807	0.5	[M+H] ⁺	156.1020 (100.0), 139.0982 (11.8), 138.0914 (23.7), 120.0807 (15.7), 112.0753 (12.3), 94.0651 (47.3)
Retrorsine	4.29	C ₁₈ H ₂₅ NO ₆	351.1682	352.1756	0.5	[M+H] ⁺	324.1803 (35.7), 276.1589 (10.9), 220.13282 (9.6), 169.0856 (3.7), 151.0750 (10.9), 138.0911 (48.6), 120.0804 (54.6), 94.0651 (18.8)
Heliotrine	4.58	C ₁₆ H ₂₇ NO ₅	313.1889	314.196	−0.5	[M+H] ⁺	156.1015 (96.5), 138.0912 (100), 120.0806 (68.2), 108.0805 (25.1), 94.0649 (33.9)
Seneciphylline	4.8	C ₁₈ H ₂₃ NO ₅	333.1576	334.1646	−0.8	[M+H] ⁺	306.1694 (42.1), 246.1493 (3.5), 151.0751 (13.4), 138.0912 (59.3), 120.0806 (70.2), 108.0806 (13.8), 94.0651 (35.8)
Senecionine	6.32	C ₁₈ H ₂₅ NO ₅	335.1733	336.1806	0.1	[M+H] ⁺	308.1851 (39.6), 220.1327 (7.1), 153.0906 (13.8), 138.0911 (55.4), 120.0805 (61.2), 94.0650 (27.6)
Senkirkine	7.78	C ₁₉ H ₂₇ NO ₆	365.1838	366.191	−0.3	[M+H] ⁺	168.1018 (100), 150.0914 (20.6), 140.0705 (13.9), 122.0600 (8.0), 107.0491 (6.6)
Echimidine	8.02	C ₂₀ H ₃₁ NO ₇	397.2101	398.217	−0.7	[M+H] ⁺	238.1430 (7.1), 138.0913 (3.9), 121.0850 (10.6), 120.0811 (100)
Lasiocarpine	10.26	C ₂₁ H ₃₃ NO ₇	411.2257	412.2326	−1.0	[M+H] ⁺	238.1438 (5.4), 138.0913 (7.9), 121.0845 (12.0), 120.0811 (100)

ESI–Q–TOF MS instrument (Table 1). All analytes were ionized as protonated [M+H]⁺ ions in positive ion mode, and their mass error range was −1.0 to 0.5 ppm, similar to their actual mass values. Furthermore, as compound identification is more accurately confirmed as more MS2 spectra are generated, we selected a collision energy of 35–43 eV, which allowed us to confirm various fragmentation patterns via preliminary experimentation. The obtained MS2 spectra are shown in Fig. 3. First, the characteristic monocrotaline fragment ion, a macrocyclic diester PAs, was observed at *m/z* 280, where CHO₂ was lost at the ester bond at the C9 position and the ion fragment broke from the molecular ion. Next, fragment ions at *m/z* 237, 194, and 138 were formed by the losses of −C₂H₃O, −C₆H₇O₂, and −C₇H₁₀O₃ from the *m/z* 280 ion, respectively. The loss of H₂O and −C₂H₂ from the *m/z* 138 fragment ion resulted in the *m/z* 120 and 93 ions, respectively, where the *m/z* 120 ion showed the highest peak intensity. The monoester PAs, lycopsamine and heliotrine,

exhibited different spectral peak intensities, but commonly exhibited principle fragment ions at *m/z* 156, 138, 120, and 94. The spectral peak at ion *m/z* 156 was produced from the molecular ions of lycopsamine and heliotridine via losses of −C₇H₁₁O₃ and −C₈H₁₃O₃, respectively. Furthermore, the fragmentation pathway of the other fragment ions was similar to that of monocrotaline. Retrorsine, seneciphylline, and senecionine, and the macrocyclic diester PAs produced product ions at *m/z* 324, 334, and 336, respectively, with losses of −CO at the C1 carbon position of the molecular structure. Characteristic fragment ions at *m/z* 138, 120, and 94 were observed to be similar to those of the above listed alkaloids. The characteristic fragment ions of the diester PAs, echimidine and lasiocarpine, were observed at *m/z* 238, 138, and 120. In echimidine and lasiocarpine, ions at *m/z* 238 were formed via losses of −C₇H₁₁O₄ and −C₈H₁₁O₄, respectively, and the *m/z* 238 ion subsequently produced ions at *m/z* 138 and 120 with losses of −C₅H₈O₂ and H₂O, respectively.

Fig. 3 Fragmented structure and MS2 spectra and of nine PAs as determined by UPLC–ESI–QTOF in positive mode. **a** Monocrotaline, **b** lycopsamine, **c** retrorsine, **d** heliotridine, **e** seneciophylline, **f** senecionine, **g** echimidine, **h** lasiocarpine and **i** senkirkine



Notably, the spectral peak at m/z 120 exhibited the highest intensity, and this ion was found to be easily fragmented by low-collision energies [22]. Finally, senkirkine, a macrocyclic ester otonecine-type PAs, formed principle fragment ions at m/z 168, 150, 140, and 122. The spectral peak at m/z 168, which was the most intense peak in the spectrum, was

produced via $-C_3$ loss from the parent molecule, and fragment ions at m/z 150 and 140 were generated from by the further loss of $-H_2O$ and methyl group, respectively. Furthermore, the spectral peak at m/z 122 was produced by the loss of $-H_2O$ from the m/z 150 ion.

Method Validation

The results of the method validation via the analysis of linearity, LOD, LOQ, and precision are listed in Table 2. The calibration curves of all compounds showed excellent linearity with R values of >0.9951 and the LOD and LOQ values ranging from 0.2 to 2.0 ng mL⁻¹ and 0.6–6.0 ng mL⁻¹, respectively. The intra- and inter-day precisions of the analytes were $<4.58\%$ RSD.

Application to Botanical Samples

As previously mentioned, the PAs produced common fragment ions by CID according to their chemical structure. Zhu et al. proposed a method for quantification without reference materials using the m/z 136 and 120 ions, which are commonly produced by retronecine-type PAs and PANOs [5]. However, otonecine-type and mono- and di-ester PAs and PANOs were not amenable to this method. Although it is difficult to prepare and quantify a large number of PA standards, we assumed that the alkaloids could be rapidly screened using the characteristic fragment ions produced. Based on a literature review [17, 23–28] and our database, structural classifications, predicted molecular ion adducted with hydrogen, and fragmental patterns were obtained for 73 PAs and PANOs, and we listed and drawn them (Refer to Online Resource 1 and 2). We subsequently investigated whether the chemical profile of the alkaloids could be measured in botanical samples. The mass information of the peaks identified tentatively is shown in Table 3 and the spectra and fragmentation pathways observed at the peak of the material are shown in Fig. 4.

Tussilago farfara

Tussilago farfara is a medicinal plant belonging to Asteraceae used for bronchial therapy in Asian folk therapy. However, it is a well-known PA-containing plant [29]. When the extracts of the root and upper part of this plant were

analyzed, both senkirkine and senecionine were detected by matching the retention time, parent molecular ion, and fragment ion with standard material. In addition, as a result of matching with the PA information in the database, the parent molecular ion m/z 352.1752 estimated as jacobine, madurensine, retrorsine, usaramine, and senecionine-*N*-oxide was observed in the chromatogram of the root extract, with major fragment ions of m/z 136.0750, 120.0802, 118.0549, and 94.0647 (Table 3). The four matched PAs except senecionine-*N*-oxide are macrocyclic diester retronecine-type PAs similar to retrorsine. Their fragment ions were similar to retrorsine, but those at m/z 136 and 118 were not observed. The m/z 136 and 118 fragment ions were presumed to be senecionine-*N*-oxide because they were observed in the macrocyclic diester PANOs [17, 23, 24]. In addition, senecionine-*N*-oxide exhibited a longer retention time than senecionine under the same conditions eluting with acetonitrile and water added formic acid on C18 column [17, 23]. This was considered to be an accurate identification because these compounds are often found in *Tussilago farfara*.

Borago officinalis

Lycopsamine was detected in the root extract of the *Borago officinalis* by matching with the corresponding reference material, and additional peaks 5, 6, 7 and 8, which were likely alkaloids, were observed. The protonated molecular ion $[M+H]^+$ of peak 5 was m/z 316.1754, which was tentatively identified as an *N*-oxide of lycopsamine, indicine, intermedia, and echinatine. In particular, the m/z 172, 155 and 111 fragment ions were formed through fragmentation pathways of monoester PAs, such as lycopsamine and heliotrine, but they contained oxygen atoms bonded to the nitrogen. Peak 6 (RT = 3.76 min, m/z 300.1803) was presumed to correspond to the monoesterified PAs such as heliotrine and lycopsamine, indicine, echinatine, intermedine, and renderine, as they are isomers. However, since indicine and intermedine elute earlier than lycopsamine in the pH 2–3 mobile phase, they are unlikely to be the corresponding alkaloids

Table 2 Result of method validation for nine pyrrolizidine alkaloids

Compound name	Regression equation	R^2	LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹)	Precision (RSD, %)	
					Inter-day	Intra-day
Monocrotaline	$Y = 1.07817e5 \times X + 434$	0.9998	0.4	1.2	1.97	3.99
Lycosamine	$Y = 1.55941e5 \times X + 664$	0.9998	0.4	1.2	4.20	2.58
Retrorsine	$Y = 1.02622e5 \times X + 596$	0.9998	0.7	2.1	4.58	1.75
Heliotrine	$Y = 9.93138e4 \times X + 899$	0.9997	0.7	2.1	4.36	0.89
Seneciophylline	$Y = 1.54612e5 \times X + 1491$	0.9996	0.4	1.2	1.54	0.90
Senecionine	$Y = 1.73587e5 \times X + 5301$	0.9951	0.2	0.6	3.45	1.86
Senkirkine	$Y = 2.36870e5 \times X + 3314$	0.9993	0.2	0.6	3.46	3.82
Echimidine	$Y = 9.10475e4 \times X + 718$	0.9999	2.0	6.0	3.43	0.95
Lasiocarpine	$Y = 1.66907e5 \times X + 320$	0.9994	0.4	1.2	4.57	0.40

Table 3 Compounds identified tentatively and by matching with reference compounds from medicinal plant by UPLC-ESI-Q-TOF/MS

Peak no.	Compound name	RT	Formula	Neural mass (Da)	Observed m/z	Mass error (ppm)	Adduct	Fragment ions m/z (relative intensity, %) [17, 22–25]	Plant part	Amount ($\mu\text{g g}^{-1}$)
<i>T. farfara</i> (whole)										
1	Senecionine	5.96	$\text{C}_{19}\text{H}_{27}\text{NO}_6$	335.1733	336.1800	-1.8	[M+H] ⁺	352.1757 (72.3), 324.1796 (2.9), 220.1326 (8.1), 138.0905 (21.4), 136.0751 (79.7), 120.0802 (100), 118.0649 (89.4), 94.0647 (66.9)	Whole	0.58±0.01 _b
2	Senecioine N-oxide	6.42	$\text{C}_{18}\text{H}_{25}\text{NO}_6$	351.1682	352.1751	-1.0	[M+H] ⁺			
3	Senkirkine	7.49	$\text{C}_{19}\text{H}_{27}\text{NO}_6$	365.1838	366.1909	-0.5	[M+H] ⁺			7.99±0.31
<i>B. officinalis</i> (root, aerial part and flower)										
4	Lycopsamine	2.01	$\text{C}_{15}\text{H}_{25}\text{NO}_5$	299.1733	300.1803	-0.8	[M+H] ⁺		Root	6.32 ±0.78
5	Lycopsamine N-oxide	2.58	$\text{C}_{15}\text{H}_{25}\text{NO}_6$	315.1682	316.1754	-0.1	[M+H] ⁺	316.1753 (92.2), 172.0965 (100), 155.0935 (23.1) 138.0907 (40.1) 136.075 (27.4) 111.0676 (52.5) 94.0648 (59.9)	Root	_b
6	Indicine	3.76	$\text{C}_{15}\text{H}_{25}\text{NO}_5$	299.1733	300.1802	-1.1	[M+H] ⁺	300.1802 (18.3) 156.1013 (100) 139.0983 (55.7) 138.0907 (26.6) 122.0959 (77.5) 120.0803 (62.5) 108.0802 (16.8) 94.0646 (5.7)	Root	_b
7	7-Acetylycopsamine	4.62	$\text{C}_{17}\text{H}_{27}\text{NO}_6$	341.1838	342.1913	0.5	[M+H] ⁺	300.1806 (11.67), 198.1120 (9.5), 156.1015 (10.58), 120.0806 (100), 118.0646 (10.8), 94.0649 (6.6), 120.0806 (100) 94.0650 (11.7)	Root	_b
8	Uplandicine	4.7	$\text{C}_{17}\text{H}_{27}\text{NO}_7$	357.1788	358.1863	0.7	[M+H] ⁺	358.1862 (22.7) 137.0830 (23.5) 120.0806 (100) 94.0650 (11.7)	Root	_b
<i>L. purpurocaeeruleum</i> (root)										
4	Lycopsamine	2.05	$\text{C}_{15}\text{H}_{25}\text{NO}_5$	299.1733	300.1805	-0.2	[M+H] ⁺		Root	2.09 ±0.15
5	Lycopsamine N-oxide	2.75	$\text{C}_{15}\text{H}_{25}\text{NO}_6$	315.1682	316.1758	1.1	[M+H] ⁺	316.1756 (35.86), 172.0965 (100), 155.0930 (17.93) 138.0908 (12.1) 136.0748 (7.38) 111.0676 (52.5) 94.0648 (59.9)	Root	_b
<i>S. officinale</i> (root, stem and leaves)										
4	Lycopsamine	2.03	$\text{C}_{15}\text{H}_{25}\text{NO}_5$	299.1733	300.1805	-0.1	[M+H] ⁺			0.27±0.01
5	Lycopsamine N-oxide or indicine-N-Oxide	2.70	$\text{C}_{15}\text{H}_{25}\text{NO}_6$	315.1682	316.1757	0.9	[M+H] ⁺	316.1755 (87.3), 172.0963 (100), 155.0933 (21.9) 138.0908 (40.6) 136.0750 (22.0) 111.0674 (52.9) 94.0646 (53.9)	All part	_b
7	7-Acetylycopsamine	4.74	$\text{C}_{17}\text{H}_{27}\text{NO}_6$	341.1838	342.1915	1.1	[M+H] ⁺	198.1112 (6.9), 120.0803 (100), 118.0865 (1.8)		_b

Table 3 (continued)

Peak no.	Compound name	RT	Formula	Neural mass (Da)	Observed m/z	Mass error (ppm)	Adduct	Fragment ions m/z (relative intensity, %) [17, 22–25]	Plant part	Amount ($\mu\text{g g}^{-1}$)
9	7-Acetylgyco-psamine <i>N</i> -oxide	4.84	$\text{C}_{17}\text{H}_{27}\text{NO}_7$	357.1788	358.1862	0.6	$[\text{M}+\text{H}]^+$	214.1074 (48.13), 180.1017 (24.54), 137.0831 (32.84), 136.0754 (19.52), 120.0803 (16.73), 106.0649 (18.77), 101.0595 (18.22), 94.0645 (5.33)	– ^b	– ^b
10	Heliosupine <i>N</i> -oxide	7.28	$\text{C}_{20}\text{H}_{31}\text{NO}_8$	413.2050	414.2122	0.0	$[\text{M}+\text{H}]^+$	254.1390 (100), 220.1331 (17.30), 154.0859 (10.90), 141.0908 (14.65), 137.0831 (23.91), 136.0754 (13.88), 120.0805 (22.29), 106.0648 (9.38)	– ^b	– ^b
11	Echimidine	7.87	$\text{C}_{20}\text{H}_{31}\text{NO}_7$	397.2101	398.2172	–0.3	$[\text{M}+\text{H}]^+$	– ^a	610.28±22.76	– ^b
12	Echiumine <i>N</i> -oxide	10.88						254.1390 (57.78), 220.1331 (24.17), 154.0859 (15.05), 141.0908 (38.43), 137.0831 (37.31), 136.0754 (21.84), 120.0805 (44.39), 106.0648 (18.10)	– ^b	– ^b
13	Symphytine <i>N</i> -oxide						$[\text{M}+\text{H}]^+$		– ^b	– ^b
14	Symviridine	11.23	$\text{C}_{20}\text{H}_{31}\text{NO}_6$	381.2151	382.2225	0.3	$[\text{M}+\text{H}]^+$	238.14311 (6.9), 120.08038 (100)	– ^b	– ^b
15	Symviridine <i>N</i> -oxide	11.31	$\text{C}_{20}\text{H}_{31}\text{NO}_7$					254.1390 (57.78), 220.1331 (24.17), 154.0859 (15.05), 141.0908 (38.43), 137.0831 (37.31), 136.0754 (21.84), 120.0805 (44.39), 106.0648 (18.10)	– ^b	– ^b

^aThe same as the fragment ion of the reference material^bComponents. Therefore, the amount can not be expressed

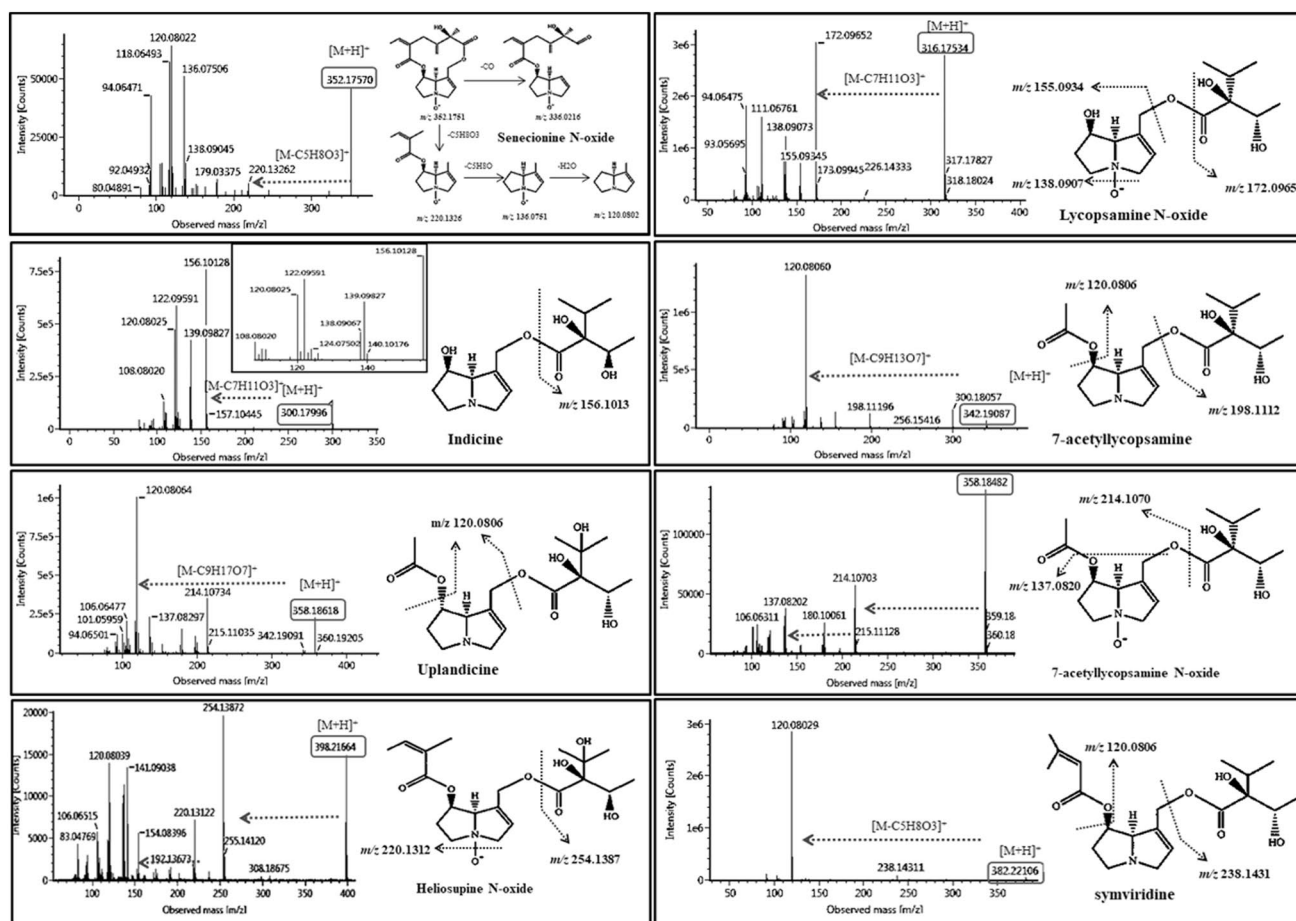


Fig. 4 MSMS spectra acquired from the sample and proposed fragmentation pathway for tentatively identified compounds

[23]. Therefore, although the remaining two compounds are estimated, an additional module is required to completely separate the PA and PAOX isomers. Peaks 7 and 8 were tentatively identified as 7-acetyllycopsamine and uplandicine, respectively. Their protonated molecular ions were accurate to 0.5 and 0.7 ppm, respectively, when compared to the theoretical masses, and m/z 120.0806 ion produced by the diester PAs was observed with high intensity.

Lithospermum erythrorhizon

The roots of *Lithospermum erythrorhizon* are commonly used as antipyretics and antidotes in Oriental medicine. We tentatively identified lycopsamine and lycopsamine-*N*-oxide in this root extract and lycopsamine was quantified by content as $2.09 \mu\text{g g}^{-1}$.

***Symphytum officinale* L.**

Symphytum officinale L. (comfrey) is a well-known medicinal plant, but it has been reported to contain PAs and PANOs

[28, 30]. Lycopsamine and echimidine were detected in the root and arial extracts by matching with the corresponding reference materials and showed more echimidine content in the root. In similarity to *Borago officinalis*, lycopsamine-*N*-oxide (or its isomers) and 7-acetyllycopsamine (or 7-acetylintermediate) were tentatively identified in both extracts. In addition, the new peaks 9, 10, 11, and 12 were observed and assumed to be alkaloids. The relevant mass information is listed in Table 2. The molecular ion m/z 342.19 of peak 9 was consistent with 7-acetyllycopsamine-*N*-oxide or 7-acetylintermediate-*N*-oxide. The MS2 spectra of this peak showed m/z 214.1074, 137.0831, 136.0753, 120.083, 106.0649, 101.0591, and 94.0645 ions, with a particularly high-intensity fragment ion at m/z 214 generated in acetylated PAOX at C-7 [26]. Peak 11 was tentatively identified as symviridine and symphytine, and a m/z 120 fragment ion derived from diester PA was exhibited. Peaks 10 and 12 were tentatively identified as heliosupine-*N*-oxide and symviridine-*N*-oxide, respectively, and their MS2 spectrum intensities differed but showed the same fragmentation pattern. Among the generated fragment ions, the m/z 254 and

220 fragment ions were derived from a diester PAOX, such as echimidine-*N*-oxide, and exhibited high intensities [17]. Heliosupine-*N*-oxide can be mistaken for isomeric echimidine-*N*-oxide, but according to Mudge et al. [27] although heliosupine is an isomer with the same molecular weight as echimidine, heliosupine exhibits low retention times under the conditions used herein. Moreover, because PAOXs have a longer retention time than PAs, the peak is more likely to be heliosupine-*N*-oxide than echimidine-*N*-oxide.

Conclusion

Herein, we developed and validated a method for simultaneous determination of nine toxic PAs in botanical samples using UPLC–ESI–Q–TOF MS, obtaining satisfactory validation results. Although PA and PANO have different molecular weights except for their isomers, they were ionized to $[M+H]^+$ in the positive mode of ESI, and showed a common fragmentation pattern and ions depending on structural characteristics. We listed mass spectral information of 70 alkaloids through a literature review and our in-house database. The proposed method quickly quantified four PAs and tentatively identified eight alkaloids in four PA-containing plants. Moreover, the accurately measured mass values minimized the ambiguity of the spectral interpretation. As there are many isomeric forms of these toxic alkaloids, accurate identification would require additional modules, but the C18 column yields retention times that can be used for isomeric identification in some cases. Our approach was immediately applicable to the data obtained from plant extracts and will be useful for PA and PANO screening of unexamined samples.

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Compliance with Ethical Standards

Conflict of interest The authors declare there are no conflicts of interest regarding the publication of this paper.

Ethical approval This article does not contain any studies with human participants or animals performed by authors. The authors confirm compliance with ethical standards.

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