Pyrrolizidine Alkaloids: Characterization in Botanical and Dietary Supplements using Accurate-Mass Q-TOF LC/MS and All Ions MS/MS

Application Note

Food, Dietary Supplements, Botanicals

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Abstract

Pyrrolizidine alkaloids (PAs) and their *N*-oxides (PANOs) have serious hepatotoxic effects in both humans and animals, necessitating a method capable of detecting, characterizing, and quantifying them using a robust assay. While triple quadrupole LC/MS systems are ideal for targeted detection, targeted analysis requires standards that are not always easily obtained. Only a fraction of known naturally occurring PAs are available as standards. Triple quadrupole analyses are also not useful for screening unknowns. High-resolution mass spectrometers that provide accurate-mass data enable characterization of unknown PAs/PANOs without the need for specific standards.

This application note presents a workflow that uses accurate-mass Q-TOF LC/MS, with All Ions MS/MS and database/mass spectral library searching, for the selective and sensitive characterization, screening, tentative identification, and quantification of PAs and PANOs in botanicals and dietary supplements. Twenty-five PA/PANO standards were characterized using their high-resolution accurate-mass MS/MS spectra. These data were used to create a personal compound database and library (PCDL) that was then used to screen for PAs/PANOs in 44 botanical and dietary supplement samples. Quantitation was performed using Q-TOF LC/MS response. PAs/PANOs not specifically targeted were tentatively identified using characteristic fragment ions and fragmentation patterns. The PAs/PANOs characterized could potentially lead to the discovery of more unique PAs in additional botanical and dietary supplement samples.

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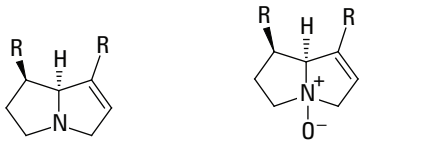
Introduction

Pyrrolizidine alkaloids (PA) are naturally occurring secondary metabolites produced by plants as a defense against insect herbivores. They are found frequently in the genera *Boraginaceae* (forget-me-not and comfrey), *Asteraceae* (sunflower), and *Fabaceae* (pea). PAs and their *N*-oxides (PANOs) are based on pyrrolizidine, a heterocyclic organic compound that forms the central chemical structure (Figure 1).



Pyrrolizidine (PA) Pyrrolizidine *N*-oxide (PANO)

Figure 1. Key skeletal structure of PAs and PANOs.

Detection, characterization, and quantification of PAs/PANOs are of interest because of their hepatotoxic effects in both humans and animals. Over 350 PAs have been identified, and about half of them are toxic [1]. Toxic effects are predominantly due to veno-occlusive disease (VOD), a condition where the small veins of the liver are obstructed. Most of these effects are reversible when the PAs are removed from the diet, but if cirrhosis of liver occurs, it is irreversible. Low-level long-term exposure could be carcinogenic. The German Commission E recommended restricting intake of PAs or their PANOs in herbal medicines to 1.0 µg/day (up to 6 weeks), or 0.1 µg/day (with no time restrictions) [2].

PAs/PANOs are typically concentrated in the seeds and flowering parts of plants [3], and can be transferred to humans in milk, offal, eggs, and meat products. Ragwort is a source of PAs ingested by animals while grazing. Houndstongue is generally not palatable to livestock when grazing; however, when included in pelleted or stored forages, it is eaten [4]. Plant-consuming insects can also accumulate PAs [5], and for this reason honey can contain PAs [6,7]. Other concerns are the health risks associated with the use of medicinal herbs that contain PAs, such as borage leaf, comfrey (used as an herbal tea), thread-leafed groundsel, and coltsfoot. Specific cases have been described [8,9,10].

Given their toxicity and the possibility of exposure, PAs/PANOs pose a significant health risk and present a need for improved testing approaches. Though the structural variety of naturally occurring PAs is a challenge for trace-level analysis and quantification, various analytical methods have been described [11]. Classical methods include colorimetric screening, thin layer chromatography (TLC), nuclear magnetic resonance (NMR), gas chromatography (GC), liquid chromatography (LC), and LC-mass spectrometry (LC/MS). Analysis of PANO's by GC requires derivatization. LC requires fewer sample preparation steps, and enables simultaneous detection of both PAs and PANOs. LC/MS with electrospray ionization (ESI) is the preferred method because of the polarity of PAs and PANOs in particular.

Triple quadrupole LC/MS instruments are used for targeted detection. This is a rigorous approach to identifying and quantifying compounds. However, targeted analysis requires standards not always easily obtained. Only a fraction of the known naturally occurring PAs are commercially available as standards. So, while triple-quadrupole-based analyses are ideal for targeted quantitation, they are not used for screening unknowns. High-resolution mass spectrometers that provide accurate-mass data are a solution to the detection and characterization of unknown PAs and their derivatives when specific standards are not available. The accurate mass of precursor and fragments provides identification capability (but not confirmation), and when accurate-mass spectra are searched using spectral library databases, large numbers of compounds can be screened rapidly. Full-scan mass spectra allow identification of compounds not in databases as well as retrospective analysis of data.

This application note presents a workflow that uses accurate-mass Q-TOF LC/MS, with All Ions MS/MS data acquisition and processing, and spectral library database searching for selective and sensitive characterization, screening, tentative identification, and quantification of PAs and PANOs in botanicals and dietary supplements. Twenty-five PA/PANO standards were characterized using high-resolution accurate-mass MS/MS spectra. These data were used to create a personal compound database and library (PCDL) that was used to screen PAs/PANOs in 44 actual botanical and dietary supplement samples. Quantitation was performed using Q-TOF LC/MS response. PAs/PANOs not specifically targeted were tentatively identified using characteristic fragment ions and fragmentation patterns. The PAs/PANOs characterized could lead to the discovery of more unique PAs/PANOs in other botanical and dietary supplement samples. The complementary research study, "Characterization and screening of pyrrolizidine alkaloids and *N*-oxides from botanicals and dietary supplements using UHPLC-high resolution mass spectrometry", provides a detailed description of the method and results [12].

Experimental

A detailed description of the sources of chemicals, solvents, and experimental procedures can be found in the complementary research published in Food Chemistry [12].

Standards, samples, and sample preparation

The 25 reference standards listed in Table 4 were obtained from Cerilliant Corporation (Round rock, Texas, USA) and prepared at a concentration of 1.0 mg/mL in methanol. Their chemical structures are presented in the complementary research [12]. Noted with asterisks in Table 1, riddelline and retrorsine are among the most toxic PAs at 10 times lower doses.

Table 1. PA/PANO Reference Standards

Monocrotaline	Retrorsine *N*-oxide
Intermedine	Heliotrine
Monocrotaline *N*-oxide	Seneciphylline
Indicine	Heliotrine *N*-oxide
Lycopsamine	Seneciphylline *N*-oxide
Europine	Integerrimine
Europine *N*-oxide	Senecionine
Indicine *N*-oxide	Senecionine *N*-oxide
Riddelline*	Senkirkine
Junceine	Schimidine
Riddelline *N*-oxide	Lasiocarpine
Trichodesmine	Lasiocarpine *N*-oxide
Retrorsine*	

The 44 botanical and dietary supplements samples tested and their sources are listed in the complementary research [12]. The botanicals included: 14 samples of *Senecio* species, five samples of *Eupatorium* species, one sample of *Ageratum conyzoides* (L.) L., one sample of *T. farfara* L., two samples of *Petasites* species, one sample of *H. indicum* L., 11 samples of *Symphytum* species, one sample of *Borago officinalis*, and one sample of *Crotalaria juncea* L. Seven dietary supplements labeled to contain *P. hybridus* were also analyzed.

Because sample extraction procedure can affect PA stability, the ratio of PANOs to PAs, and yield, five sample preparations were examined:

1. Sonication with methanol
2. Sonication and reconstitution with methanol
3. Sonication with MeOH:H_2O and acid
4. Alkaloidal extraction
5. Microwave extraction

Sonication with methanol, where 50 mg of dry plant sample were sonicated in 2.5 mL of methanol for 30 minutes followed by centrifugation for 15 minutes at 959 g, was determined to be the best method. Clear supernatant was collected, the extraction procedure was repeated four more times, and the supernatants were combined into a 10-mL volumetric flask. Prior to injection into the Q-TOF LC/MS system, about 2 mL of extract was passed through a 0.45-µm PTFE membrane filter. Extracts with high levels were diluted 10 times.

Q-TOF LC/MS analysis

LC/MS analysis of the standards and sample extracts were performed using an Agilent 1290 Infinity LC system coupled with an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system equipped with an Agilent Jet Stream dual electrospray ionization (ESI) source. The HPLC system included a binary pump, vacuum solvent degasser, autosampler with 108-vial well-plate trays, and thermostatically controlled column compartment. The HPLC and Q-TOF LC/MS parameters are shown in Tables 2 and 3.

Table 2. HPLC Parameters

Instrument	Agilent 1290 Infinity LC system
Column	Agilent Poroshell 120 EC-C18, 2.1 × 150 mm, 2.7 µm (p/n 693775-902)
Mobile phases	A) Water with 0.1% formic acid B) Acetonitrile with 0.1% formic acid
Gradient	0 to 23 minutes, 3 to 4% B; 23 to 45 minutes, 4 to 15% B; 45 to 55 minutes, 15 to 25% B; next 2 minutes to 100% B
Flow rate	0.27 mL/min
Post run column equilibration	5 minutes with 3% B
Column temperature	40 °C
Injection volume	2 µL

Table 3. Q-TOF LC/MS Parameters

Instrument	Agilent 6530 Accurate-Mass Q-TOF LC/MS system
Ionization mode	Positive ion electrospray with Agilent Jet Stream technology
Mass range	50–950 *m/z*
Drying gas	N_2 300 °C at 11 L/min
Sheath gas	325 °C at 10 L/min
Nebulizer gas	35 psi
Fragmentor	125 V
Capillary	3,500 V
Skimmer	65 V
Octopole RF	750 V

To generate data to populate the database and the MS/MS spectral library, the PA/PANO reference standards were analyzed in targeted MS/MS mode using the parameters displayed in Table 4. Multiple collision energies were necessary because of the variety of the fragmentation behavior expected for all 25 compounds.

Table 4. Targeted MS/MS Parameters

Quadrupole isolation width	1.3 amu
Collision energy	10, 20, 30, and 40 eV

Botanical and dietary supplement samples were analyzed in the All Ions MS/MS mode where the quadrupole was set to pass all ions with no isolation. Experiment 1 and experiment 2 were carried out with collision energies of 0 and 30 eV, respectively.

All Ions MS/MS is an acquisition mode and data processing tool for Agilent TOF and Q-TOF LC/MS systems that enables fragmentation without precursor ion isolation. The All Ions MS/MS data acquisition mode alternates between high and low energy scans (Figure 2). All of the ions introduced into the mass spectrometer are first analyzed intact (experiment 1), and then are fragmented (experiment 2) to produce fragment ions that can be subsequently used for compound identification and confirmation.

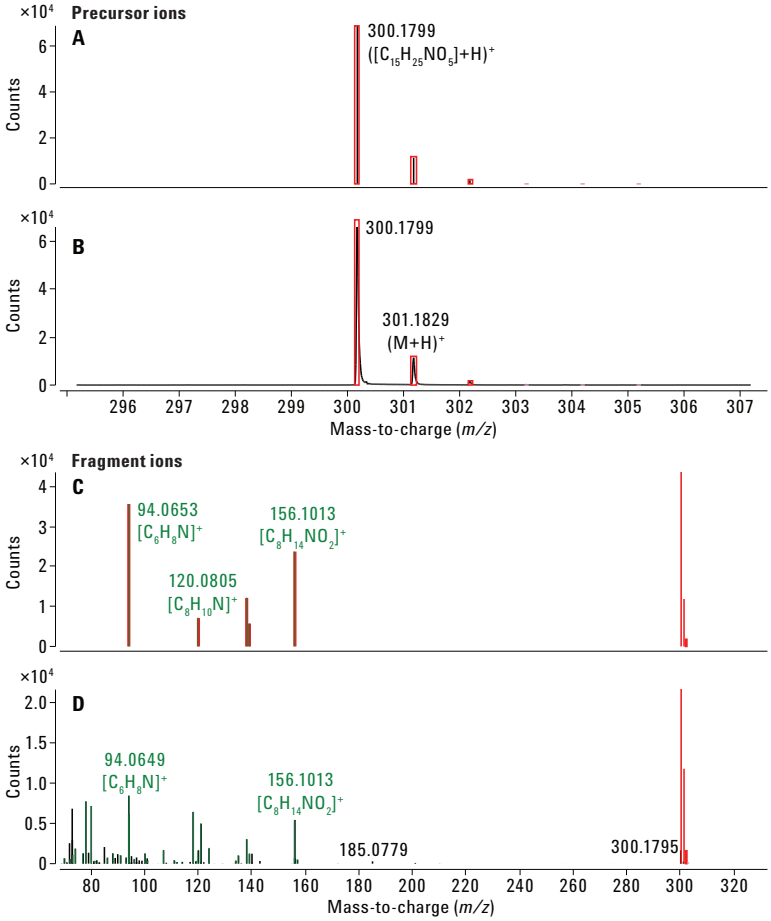


Figure 2. A and B show the $M+H^+$ *ions obtained at 0 eV collision energy, and identified by the Agilent MassHunter Qualitative Analysis software Find by Formula algorithm. A is the background subtracted centroid mass spectrum, and B is the raw profile mass spectrum. The red boxes indicate the theoretical position and abundance of the isotopes. C and D show the spectra obtained at 30 eV collision energy. C is the centroid mass spectrum of the ions matching the MS/MS library spectrum. D is the raw profile spectrum and contains ions that may be from other precursors.*

Accurate-mass measurements were obtained with reference ion correction using reference masses at *m/z* 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine, or HP-921] in positive ESI mode. The reference solution was delivered to the ESI source through a T-junction using an Agilent Series 1200 isocratic pump with 100:1 split at a flow rate of 20 µL/min.

Data analysis

Agilent MassHunter Workstation software was used to acquire and process data (Acquisition version A.05.01, Qualitative Data Analysis version B.06.00).

The MassHunter PCDL manager (Version B.04.00) was used to build the PA/PANO database and library. After the chromatographic peaks were found, spectra were generated by averaging the peak, then importing them into the MassHunter PCDL Manager.

MassHunter Qualitative Analysis software uses its Find by Formula algorithm to extract precursor ions from the All Ions MS/MS data using the accurate-mass database within the Agilent PCDL. Figure 3 illustrates a coelution plot of indicine. The All Ions MS/MS software automatically correlates the extracted chromatographic peaks with fragment ions stored in the accurate-mass MS/MS library. The precursor and corresponding fragment ion peaks are plotted to evaluate their coelution, and are assigned a coelution score. The coelution score and coelution plot indicate the quality of correlation between precursor and fragment ions for each compound.

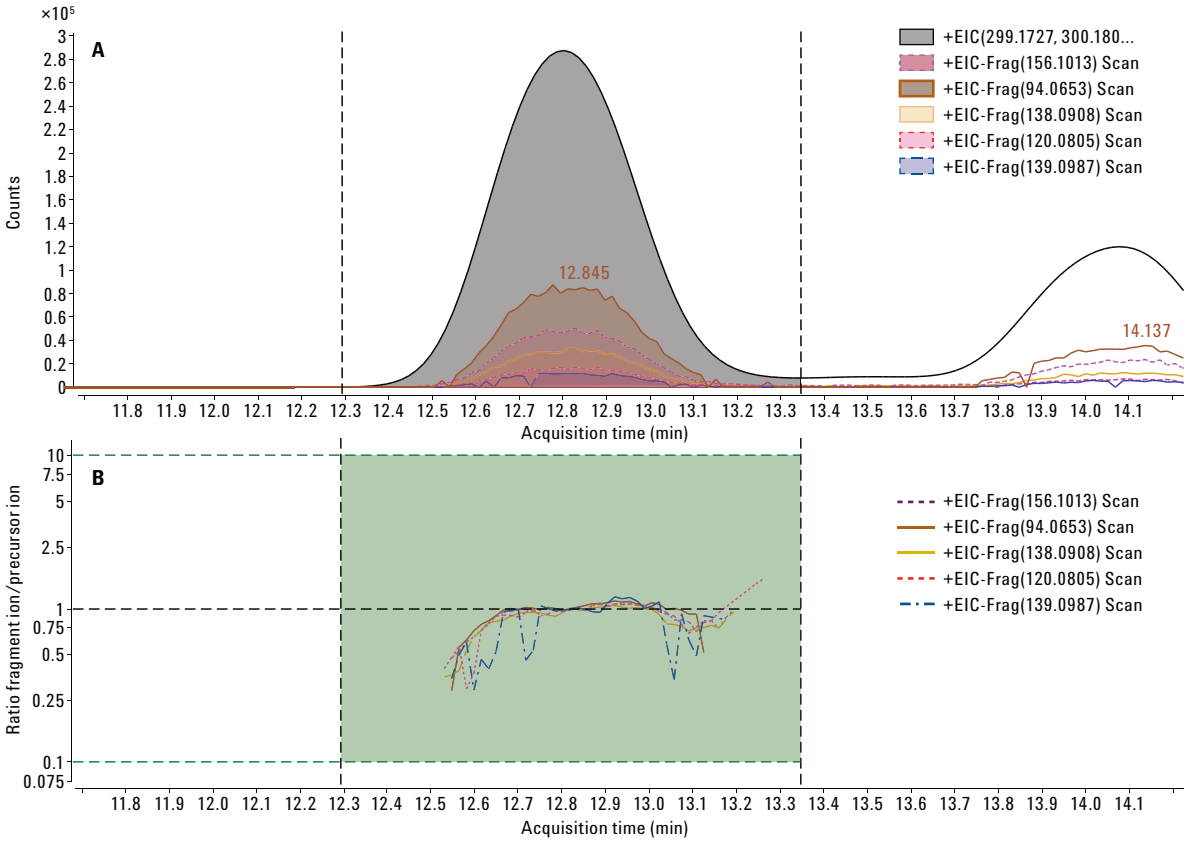


Figure 3. Coelution plot of indicine from S. officinalis. A) Precursors and product ions are extracted and overlaid in ion chromatograms. B) The calculated coelution scores are plotted.

Results and Discussion

Chromatographic results

As presented in the complementary research, the MS1-level base peak chromatogram of the standard mix and a botanical from each genus analyzed showed separation of all the standards except for indicine at *m/z* 299.2 and europine at *m/z* 329.2 [12]. These compounds were easily distinguished using accurate-mass MS and MS/MS data. Mass accuracy better than 2 ppm was achieved.

Fragmentation of standards

The MS/MS fragmentation experiments performed on the reference standards provided characteristic CID fragment ions to aid in the identification of specific PAs/PANOs in the sample extracts. The most abundant fragment ions were observed at *m/z* 94, 120, 138, 168, 172, and 254. For example, ions at *m/z* 94.06, 120.08, 138.09, and 254.14 were observed in the spectra shown in Figures 4A and 4B. Many of the characteristic fragment ions observed in the PAs were also observed in their respective *N*-oxide spectra. The fragmentation pathways of riddelline and riddelline *N*-oxide are presented in the complementary research [12].

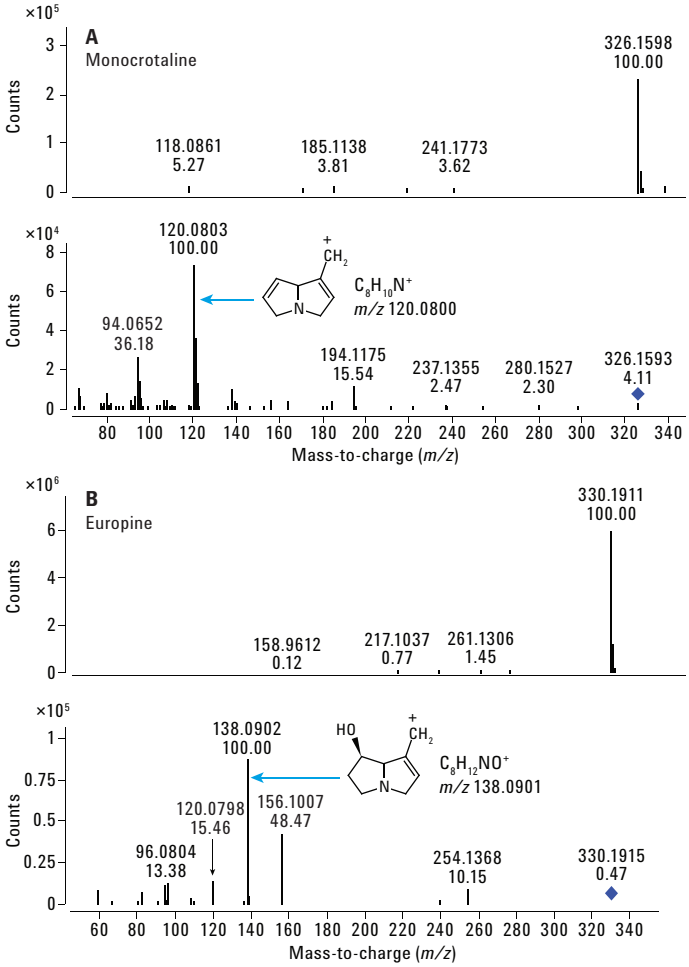


Figure 4. MS (no isolation or collision energy) and MS/MS library spectra collected at 40 eV collision energy of (A) monocrotaline and (B) europine showed characteristic ions at m/z 96.08, 120.08, 138.09 and 254.14.

Compound database and library

The MS/MS spectral library was built using the data obtained from analysis of the 25 reference standards. Only singly-charged positive pseudo molecular ions ($[M+H]^+$) were selected to produce MS/MS spectra. After the chromatographic peaks were found, spectra were generated by averaging across the peaks, and then imported into the MassHunter PCDL Manager (Figure 5).

The usefulness of the library was tested on the botanical and dietary supplement sample extracts. Spectral matching was performed by searching corresponding peaks in the library and unknown spectra. When all fragments in the library spectra were also present in the sample, within the accurate mass tolerance specified and with perfect chromatographic coelution, a match score of 100 was given.

Often only the precursor and one fragment with the correct retention time are needed to provide a true positive in complex samples. For complex sample analyses, All Ion MS/MS can significantly reduce the number of false negatives obtained in a data-dependent MS/MS experiment caused by a precursor of a PA not being selected. This can occur during a data dependent analysis (Auto MS/MS) when too many compounds coelute.

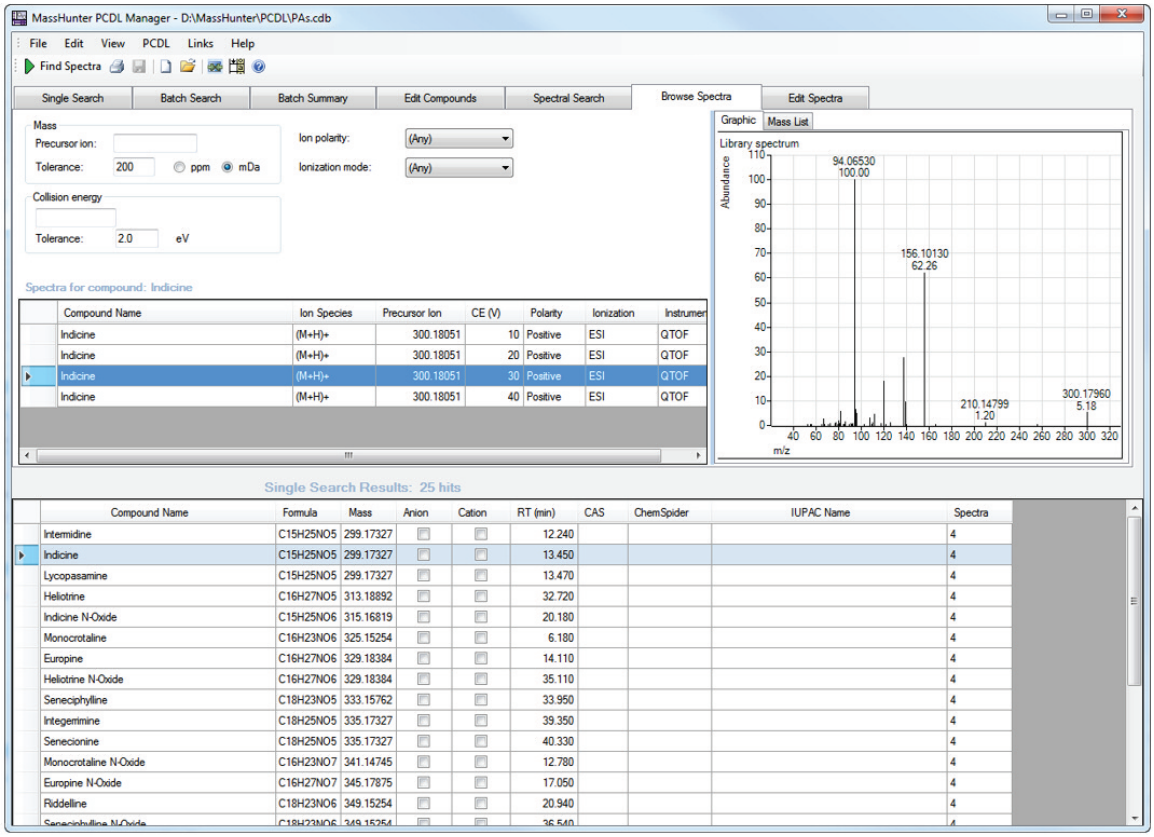


Figure 5. The PA/PANO-specific MS/MS spectral library was built using the data obtained from analysis of the 25 reference standards and the MassHunter PCDL manager.

Detection of PAs in botanical and dietary supplements

By comparing their LC retention times and ESI mass spectra with those of the reference standards captured in the PDCL, numerous PAs/PANOs were detected in the botanical and dietary supplement sample extracts. The complete list of findings, including specific compounds and amount detected, are presented in the complementary research [12]. Identification of PAs/PANOs were based on the exact mass of protonated molecules and characteristic fragment ions. Even without specific reference standards, accurate-mass and characteristic MS/MS fragments indicated that additional PAs/PANOs were present. For this reason, the method could be used to screen for the presence of PA-like target fragments without standards.

Figure 6 shows the All Ions MS/MS extracted ion chromatogram of a dietary supplement sample extract containing Butterbur (genus *Petasites*, commonly known as Sweet Coltsfoot). It shows how nontargeted PAs/PANOs were found using characteristic ions at *m/z* 138, 136, and 120. Figure 7 presents the All Ions MS/MS spectra collected at 0 and 30 eV collision energy for the peak at the retention time of 23 minutes. The psuedomolecular ion was produced when 0 eV was applied, while a rich fragmentation pattern was produced at 30 eV. It is important to note that the fragments shown could be the product of more than one psuedomolecular ion. The results show a PA-indicative fragment is present; determination of the molecular formula with interpretation of other fragments present in peaks that were not in the database could lead to identification of unknown PA/PANO compounds.

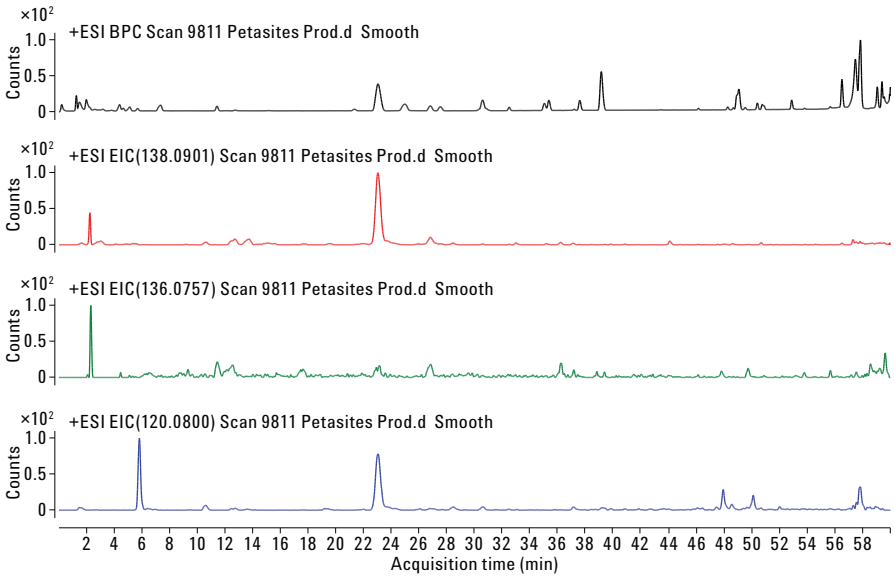


Figure 6. All Ions MS/MS extracted ion chromatogram of a dietary supplement sample extract containing Butterbur (genus Petasites, commonly known as Sweet Coltsfoot.) Nontargeted PAs were found using the characteristic ions at m/z 138, 136, and 120.

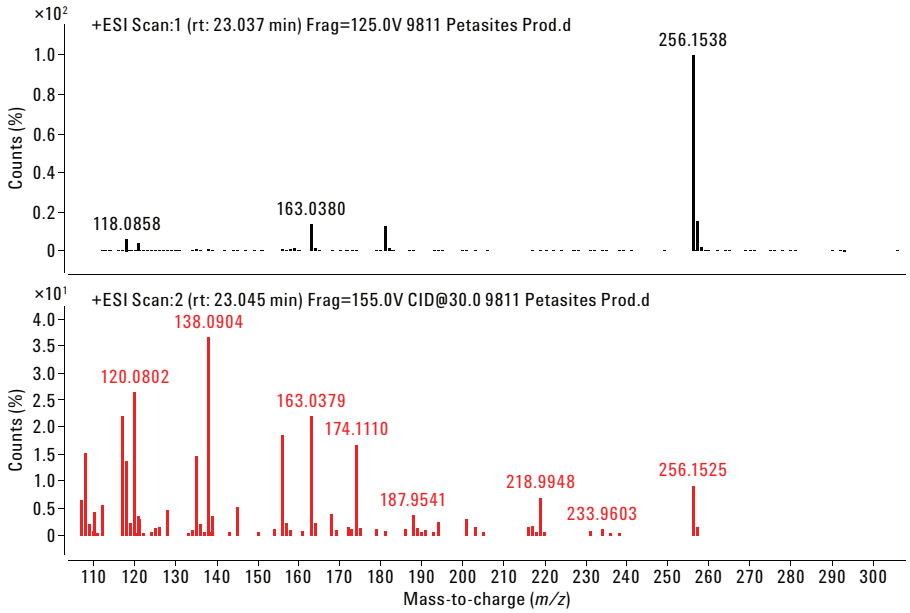


Figure 7. All Ions MS/MS spectra taken at a retention time of 23 minutes from the analysis of the dietary supplement extract shown in Figure 5. When 0 eV collision energy was applied, the psuedomolecular ion at m/z 256.1538 was obtained. At 30 eV collision energy, a rich fragmentation pattern including characteristic PA fragments was observed.

Quantitation of PAs

From analysis of the reference standards, calibration curves were developed for the ranges from 0.5 to 500 ng/mL for low level compounds, and from 500 to 5,000 ng/mL for others with $R^2 > 0.99$, except for riddelline *N*-oxide, trichodesmine, and retrorsine. Here the ranges were from 5 to 2,500 ng/mL, 20 to 2,500 ng/mL, and 1 to 2,500 ng/mL, respectively. The limits of detection (LOD) were between 0.05 and 0.1 ng/mL except for the three compounds listed above; here the LODs were 1, 5, and 0.4 ng/mL, respectively.

Quantification of sample extracts was performed using the Q-TOF LC/MS in single MS mode. For the specific PAs/PANOs, extracted ion chromatograms (EICs) were generated for the pseudo molecular ion ($[M+H]^+$), and the area was determined by integration.

Selectivity was provided by the Q-TOF LC/MS system's accurate-mass and high-resolution capabilities. Quantitative results for selected botanicals and supplements are provided in the complementary research [12]. Once the PAs/PANOs present in a particular botanical are fully characterized, a targeted triple-quadrupole based method could be used for quantification. However, if the identity of the botanical is unknown, Q-TOF LC/MS is the method of choice. Because dietary supplements may contain any number of unintentional contaminants, the Q-TOF LC/MS method would be preferred to reduce false negatives.

Conclusion

Known to have hepatotoxic effects in humans and animals, PAs and PANOs are commonly found in many plants including those of the genera *Boraginaceae*, *Asteraceae*, and *Fabaceae*. Another concern is the health risks associated with the use of PA/PANO-containing herbs and supplements. For this reason, the detection, characterization, and quantification of PAs/PANOs is of great importance.

While triple quadrupole LC/MS systems are ideal for targeted detection, targeted analysis requires standards that are not always easily obtained. In addition, triple quadrupole analyses are not useful for unknown screening. The Agilent 6530 Accurate-Mass Q-TOF LC/MS system with All Ions MS/MS capability provided a solution to screen for the presence of

fragments indicative of PAs/PANOs without need for specific reference standards. Twenty-five PA/PANO reference standards were characterized using their high-resolution accurate-mass MS/MS spectra. These data were used to create a PCDL that enabled the authors to screen for PA/PANO-type compounds in botanical and dietary supplement samples. PAs not specifically targeted were tentatively identified using characteristic fragment ions and fragmentation patterns. The results showed the method is a sensitive, selective, and effective approach to the characterization of known and unknown PAs/PANOs in botanicals and dietary supplements in a single chromatographic run. The PAs characterized could potentially lead to the discovery of more unique PAs in additional samples.

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