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Characteristic ion clusters as determinants for the identification of pyrrolizidine alkaloid *N*-oxides in pyrrolizidine alkaloid–containing natural products using HPLC–MS analysis

Jianqing Ruan,[a,b] Na Li,[a,b] Qingsu Xia,[c] Peter P. Fu,[c]* Shuying Peng,[d] Yang Ye[b,d] and Ge Lin[a,b]*

Pyrrolizidine alkaloid (PA)–containing plants are widely distributed in the world. PAs are hepatotoxic, affecting livestock and humans. PA *N*-oxides are often present together with PAs in plants and also exhibit hepatotoxicity but with less potency. HPLC–MS is generally used to analyze PA-containing herbs, although PA references are unavailable in most cases. However, to date, without reference standards, HPLC–MS methodology cannot distinguish PA *N*-oxides from PAs because they both produce the same characteristic ions in mass spectra. In the present study, the mass spectra of 10 PA *N*-oxides and the corresponding PAs were systemically investigated using HPLC–MS to define the characteristic mass fragment ions specific to PAs and PA *N*-oxides. Mass spectra of toxic retronecine-type PA *N*-oxides exhibited two characteristic ion clusters at *m/z* 118–120 and 136–138. These ion clusters were produced by three unique fragmentation pathways of PA *N*-oxides and were not found in their corresponding PAs. Similarly, the nontoxic platynecine-type PA *N*-oxides also fragmented via three similar pathways to form two characteristic ion clusters at *m/z* 120–122 and 138–140. Further application of using these characteristic ion clusters allowed successful and rapid identification of PAs and PA *N*-oxides in two PA-containing herbal plants. Our results demonstrated, for the first time, that these characteristic ion clusters are unique determinants to discriminate PA *N*-oxides from PAs even without the availability of reference samples. Our findings provide a novel and specific method to differentiate PA *N*-oxides from PAs in PA-containing natural products, which is crucial for the assessment of their intoxication. Copyright © 2012 John Wiley & Sons, Ltd.

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Keywords: pyrrolizidine alkaloid *N*-oxides; pyrrolizidine alkaloid; HPLC–MS analysis; characteristic fragment ions; fragmentation pathway

INTRODUCTION

In the recent decades, the use of herbal products has been rapidly growing worldwide, which makes the safety assessment of these products more challenging than ever before.[1,2] As a result, the safety of natural products has drawn global attention. Among the natural toxins, pyrrolizidine alkaloids (PAs) are probably one of the most significant groups. More than 660 PAs have been identified in more than 6000 plants distributed in many geographical regions worldwide, half of which are hepatotoxic and many are tumorigenic in humans and livestock.[3–5] Consequently, PA poisoning cases resulting from intake of PA-containing herbal medicines or foodstuffs accidentally contaminated with PAs frequently occur worldwide.

Generally, on the basis of the necine bases, PAs are classified into three types: the retronecine type (including its 7-stereoisomer, the heliotridine type), the otonecine type, and the platynecine type (Figure 1A).[3,4] The first and second types of PAs possessing a 1,2-double bond in the unsaturated necine base are hepatotoxic and may ultimately lead to cancer in humans, whereas the platynecine-type PAs with a saturated necine base are generally regarded as nontoxic PAs.[3,5] It has been well established that the metabolism of the two toxic types of PAs is catalyzed by cytochrome P450 enzymes in the liver and produces the highly reactive pyrrolic metabolites that can rapidly bind to cellular proteins or DNA in the liver to form pyrrole–protein or pyrrole–DNA adducts leading to hepatotoxicity or tumors, respectively.[3,4,6–10]

* *Correspondence to: Ge Lin, School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China. E-mail: linge@cuhk.edu.hk*

* *Peter Fu, National Center for Toxicological Research, Jefferson, AR 72079, USA. E-mail: peter.fu@fda.hhs.gov*

Jianqing Ruan and Na Li equally contribute the work and share the first authorship.

a *School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China*

b *Joint Research Laboratory for Promoting Globalization of Traditional Chinese Medicines between Shanghai Institute of Materia Medica, Chinese Academy of Sciences and The Chinese University of Hong Kong*

c *National Center for Toxicological Research, Jefferson, AR 72079, USA*

d *Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China*

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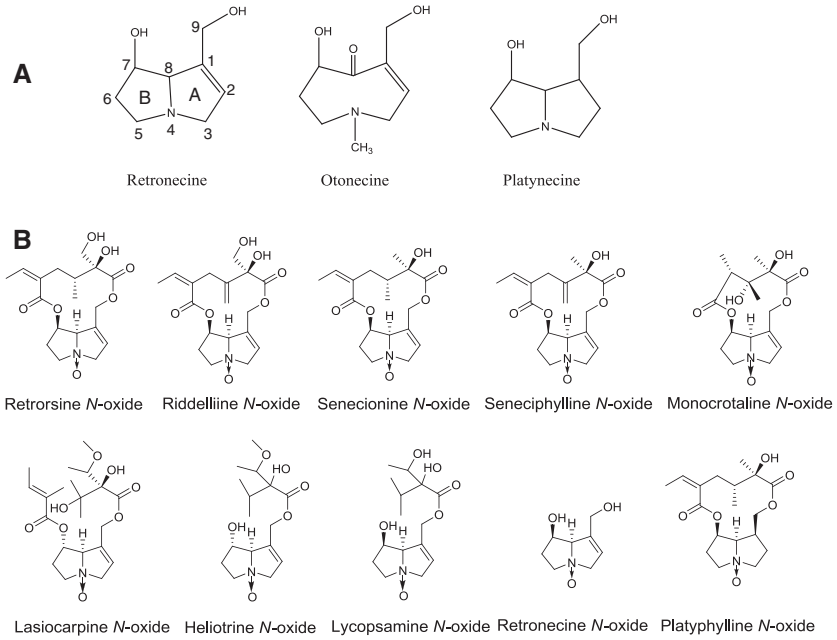

Figure 1. Structures of (A) the common necine bases of PAs and (B) PA *N*-oxides investigated in the present study.

Except for the otonecine type, in which *N*-oxide cannot be formed, *N*-oxides of other two types of PAs also naturally occur and often coexist with PAs in plant materials. The quantities of PA *N*-oxides are found to be equal to, greater, or less than their corresponding PAs.[11] It has been reported that retronecine-type PA *N*-oxides are hepatotoxic, but with less potency than the corresponding PAs because of the necessity for metabolic reduction to the corresponding PAs in gastrointestinal tract and/or in the liver.[4,12] The results of human and rat liver microsomal metabolism of riddelliine *N*-oxide, monocrotaline *N*-oxide, and retrorsine *N*-oxide as well as *in vivo* metabolism of these three PA *N*-oxides in F344 rats showed that pyrrole–DNA adducts were also generated from these PA *N*-oxides but in lesser amounts than those formed from the corresponding PAs, apparently because reduction of PA *N*-oxides to the parent is not the sole metabolism pathway.[13–15] Moreover, an *in vivo* study showed that intraruminal infusion of riddelliine *N*-oxide alone to cattle can induce typical signs of hepatotoxicity similar to that caused by the original toxic plant *Riddell groundsel*.[16] Therefore, in addition to the analysis of PAs, the determination of PA *N*-oxides in natural products is indispensable for the safety assessment of PA-containing products.

HPLC–MS is commonly used for qualitative and quantitative analysis of PAs and PA *N*-oxides in the PA-containing products and biological samples.[17–25] Our previous study[20] demonstrated that in the positive mode, the mass spectra of retronecine-type, otonecine-type, and platynecine-type PAs each showed two characteristic fragmentation ions at *m/z* 120 and 138, 150 and 168, and 122 and 140, respectively. These characteristic fragment ions can be used for the identification of different types of PAs even in the absence of reference standards. It was recently reported that both retronecine-type and platynecine-type PA *N*-oxides produced the same characteristic fragment ions of their corresponding types of PAs.[21,25] Consequently, a convenient mass analytical methodology needs to be developed for distinguishing PA *N*-oxides from the corresponding PAs in PA-containing herbal plants and herbal products. In the present study, we determine that PA *N*-oxides exhibit novel and unique characteristic mass fragment ion clusters that are not formed from the PAs. We also establish that these characteristic ion clusters can be used to distinguish PA *N*-oxides from PAs present in PA-containing plants and to identify different types of PA *N*-oxides.

EXPERIMENTAL

Chemicals and solvents

The reference compounds monocrotaline, retrorsine, and retrorsine *N*-oxide were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Senecionine and seneciphylline were purchased from Extrasynthesese (Genay, France). Lycopsamine was purchased from PhytoLab (Dutendorfer, Germany). Platyphylline was a gift from Prof. Hai Shen Chen from the Department of Phytochemistry, The Secondary Military Medical University, China. Riddelliine was obtained from Dr Po-Chuen Chan, the National Toxicology Program (NTP). Lasiocarpine is gift from Dr John A. Edgar, CSIRO Livestock Industries, Australia. Heliotrine was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY, USA). All the other PA *N*-oxides used in this study were synthetically prepared by *N*-oxidation of the corresponding PAs with 30% hydrogen peroxide.[14,26] HPLC-grade acetonitrile and methanol (E. Merck, Darmstadt, Germany) were used for HPLC analyses.

HPLC–MS conditions

HPLC–MS analysis was performed on an Agilent 1200 series LC system (Agilent Technologies, Santa Clara, CA, USA), equipped with a binary solvent delivery system, an autosampler, connecting with an API 2000Q-Trap mass spectrometer (AB Sciex, USA). The separation of individual compounds was achieved using Agilent Extend C_{18} column (250 × 4.6 mm, 5 μm) coupled with Extend C_{18} guard column (12.5 × 4.6 mm, 5 μm). The flow rate was set at 0.8 ml/min, and the injection volume was 10 μl. The mobile phases consisted of 0.2% ammonia water (A) and acetonitrile (B) and a gradient elution was adopted as follows: 0–15 min, 10%–20% B; 30–40 min, 35%–35% B; 45–60 min, 35%–90% B. The mass spectrometer was operated in positive ion mode using an electrospray ionization

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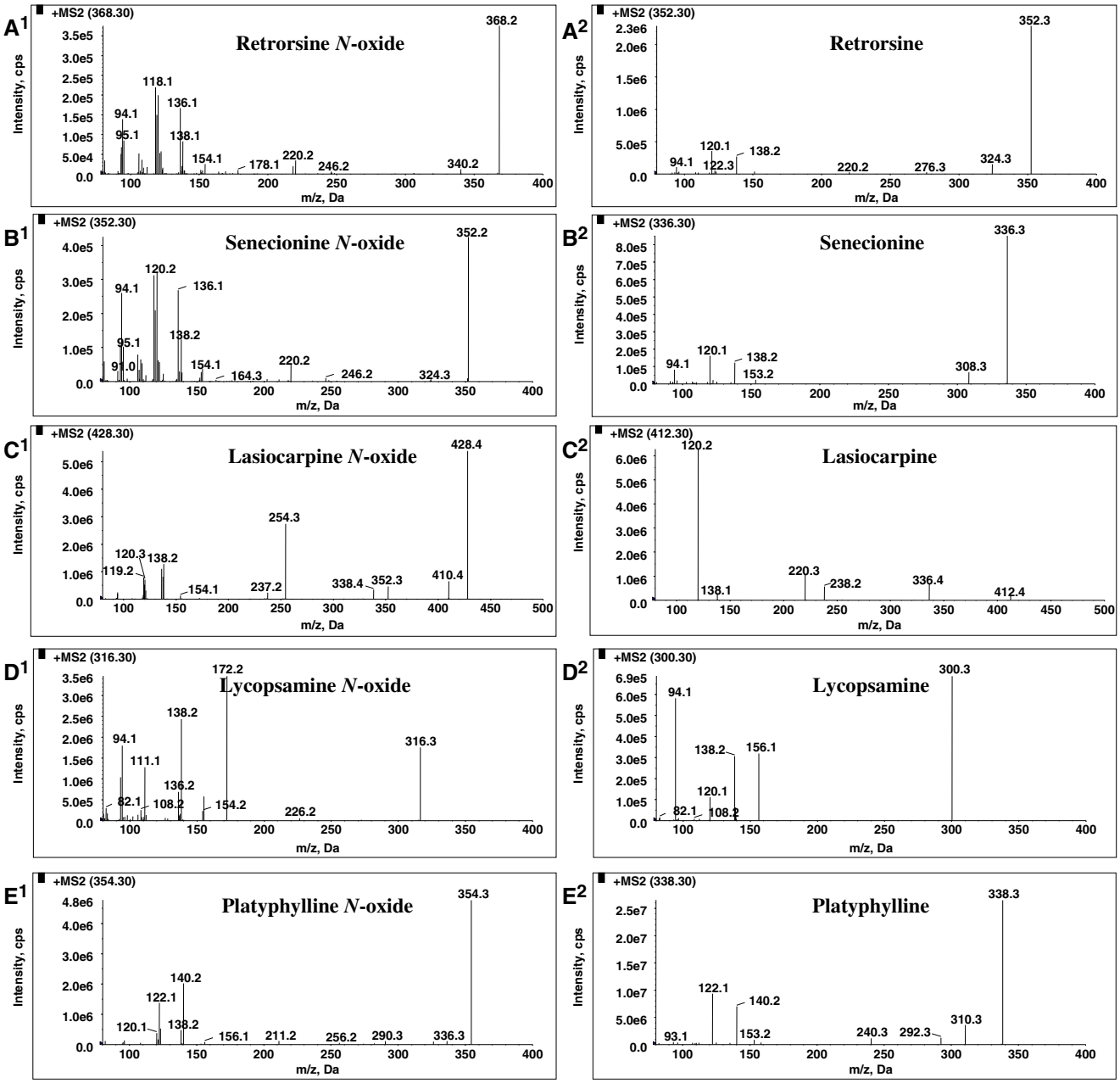

Figure 2. MS2 spectra of different representative PA *N*-oxides and their corresponding PAs.

interface with the following working parameters: ion spray voltage, 5500 V; curtain gas, 20 psi; gas 1, 30 psi; gas 2, 70 psi; source temperature, 400 °C. All gases used were nitrogen. Declustering potential was set at 70 V, and entrance potential was set at 10 V for all modes. Precursor ion scan (PIS) acquisition for detecting characteristic product ions at *m/z* 120 and 138 or 122 and 140 was used to identify peaks corresponding to retronecine-type PAs and their *N*-oxides or platynecine-type PAs and their *N*-oxides and consequently unveil the corresponding parent ions to produce the MS2 (MS/MS) spectra. The collision energy and collision cell exit potential were set at 30 and 2 V, respectively, for MS2 analysis.

Extraction of herbs

Two PA-containing herbal plants, *Gynura pseudochina* and *Gynura japonica*, were purchased at Nujiang in Yunnan Province, China, in August 2010. PAs and PA *N*-oxides present in the plants were extracted according to our previously developed method,[27] with minor modification. Briefly, powdered roots (0.5 g) of individual herbal plants were soaked in a 10-ml capped conical flask with 3 ml of 2.0 % sulfuric acid for 30 min. After soaking, the sample was extracted under supersonic washer (50 Hz) for 1 h, followed by filtration through a 0.22-μm membrane and subject to HPLC/MS analysis.

RESULTS AND DISCUSSION

The mass spectra of retronecine-type PAs and PA *N*-oxides

In the present study, nine retronecine-type PA *N*-oxides (Figure 1B), which include three subtypes, diester, monoester, and nonester PA *N*-oxides, were investigated. For comparison, all the corresponding PAs were also examined in parallel except retronecine. Given the

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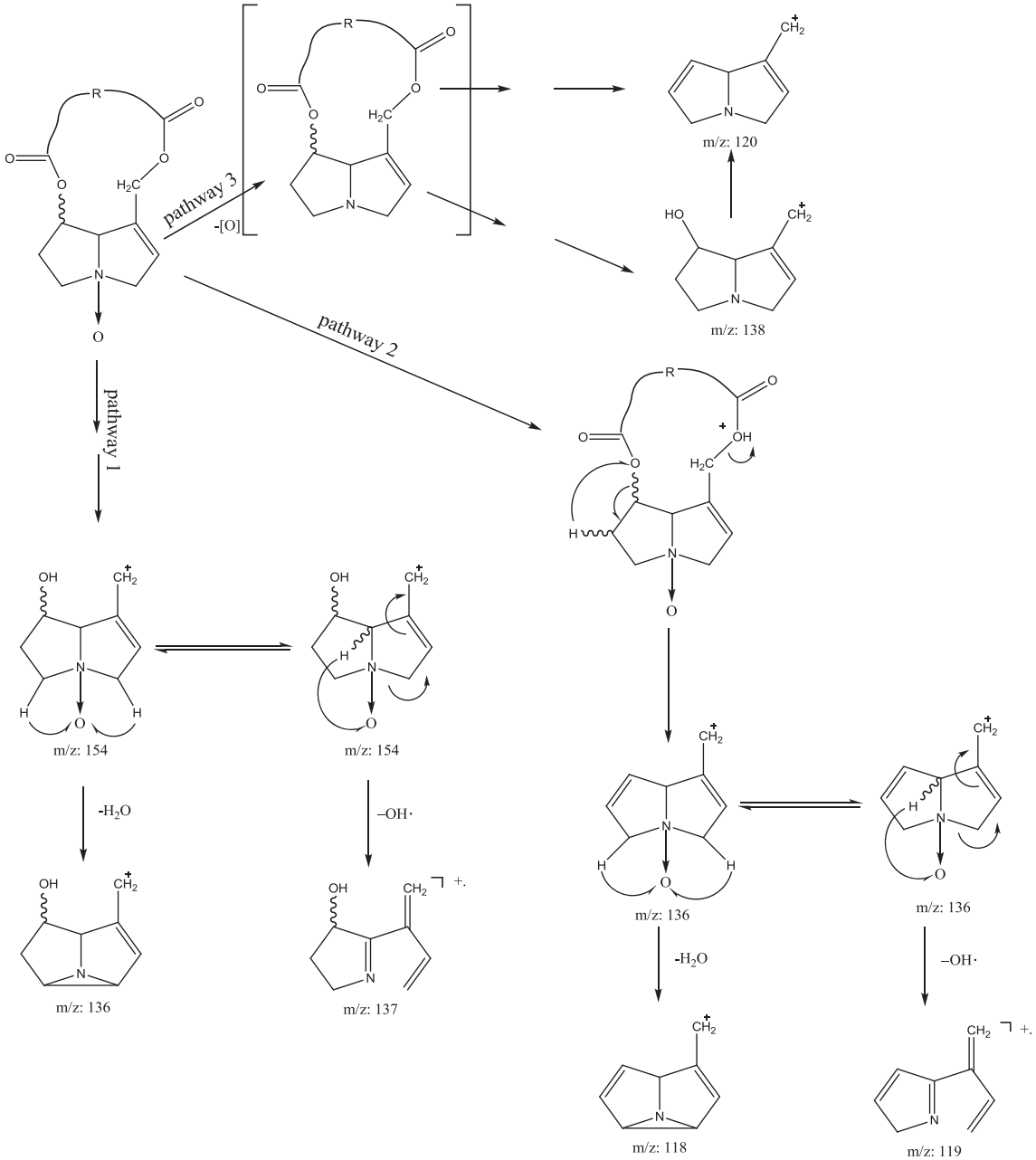

Figure 3. Proposed fragmentation pathways of retronecine-type diester PA *N*-oxides.

mass spectra shown in Figure 2 and Supplementary Figure 1, all PAs showed two characteristic ions at *m/z* 120 and 138, which are in good agreement with our previously established mass spectrometric patterns for retronecine-type of PAs.[20] For the *N*-oxides of diester PAs, including both open chain diester (lasiocarpine *N*-oxide) and macrocycle diester (*N*-oxides of retrorsine, riddelliine, senecionine, seneciphylline and monocrotaline) PA *N*-oxides, their mass spectra all exhibited two additional characteristic ion clusters at *m/z* 118–120 and 136–138 (Figure 2 and Supplementary Figure 1). We propose the mechanism that these characteristic fragment ion clusters are generated through three individual fragmentation pathways (Figure 3). Pathway 1 involves the cleavage of two ester groups at C-7 and C-9 positions to produce a fragmentation ion at *m/z* 154, which is further fragmented to generate the ions at *m/z* 136 and 137 through the loss of H_2O and OH·, respectively. Pathway 2 involves the simultaneous loss of these two ester groups and a molecule of water to generate the ion at *m/z* 136 followed by further loss of a molecule of H_2O or an OH· group, leading to the fragment ions at *m/z* 118 and 119, respectively. Pathway 3 involves the loss of the oxygen atom attached to the nitrogen of the necine base to form the corresponding PA. Although the relatively intensity of the corresponding ion $[M + H\text{-}O]^+$ for most of the PA *N*-oxides tested were very low in the present study, it is a common thermal degradation pathway for *N*-oxides of different types of chemicals under the conditions of mass spectrometric analysis.[28] The subsequent fragmentation is identical to the spectrometric pattern of the retronecine-type PAs and thus form the typical characteristic ions at *m/z* 120 and 138.[20] Consequently, these three pathways yield the two ion

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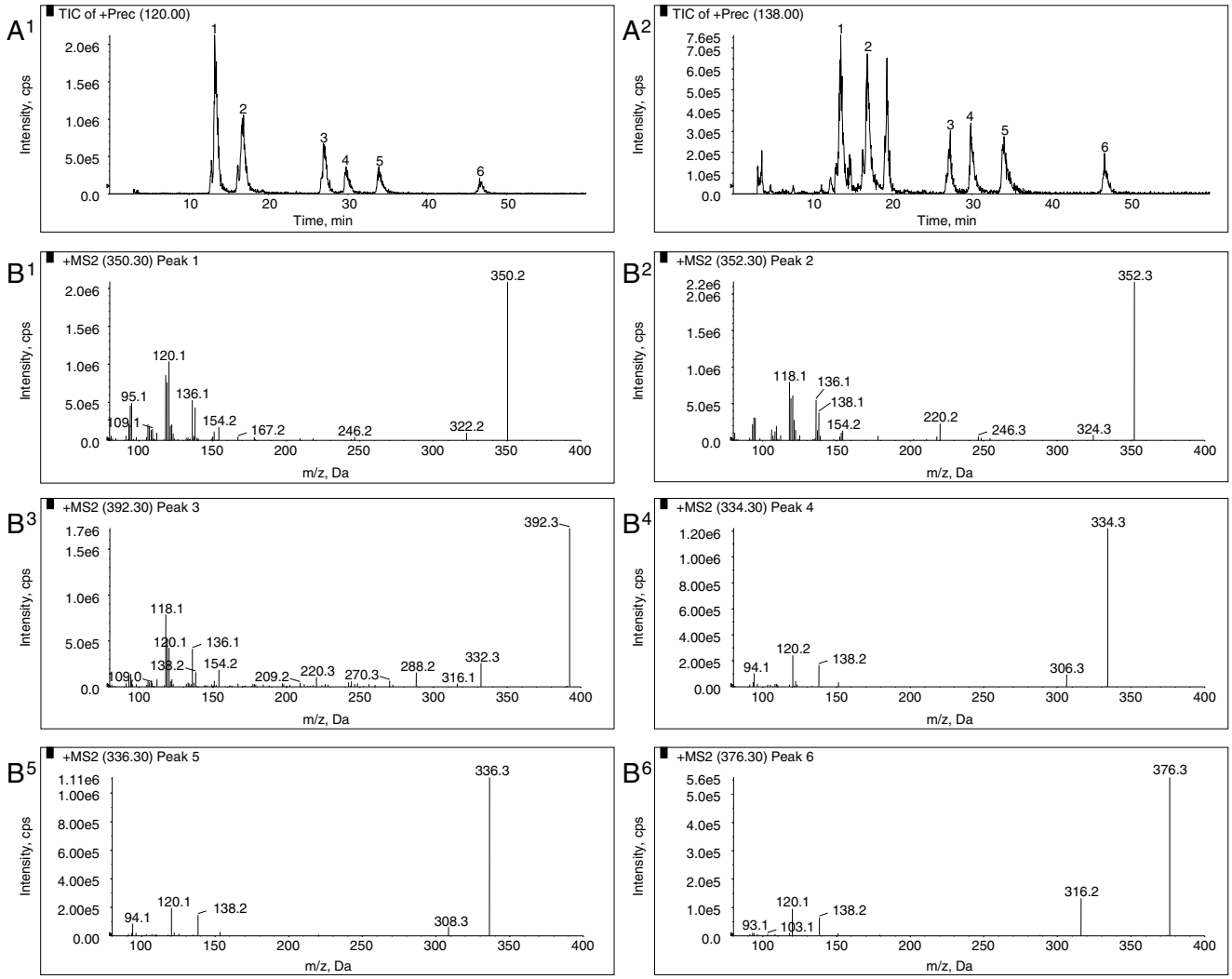

Figure 4. Chromatograms of *Gynura pseudochina* extract analyzed by HPLC–MS using PIS mode, (A^1–A^2) total ion chromatograms at *m/z* 120 and 138, respectively; (B^1–B^6) MS2 spectra of peaks 1–6, respectively.Peak 1, seneciphylline *N*-oxide; peak 2, senecionine *N*-oxide; peak 3, seneciphyllinine *N*-oxide; peak 4, seneciphylline; peak 5, senecionine; and peak 6, seneciphyllinine.

clusters at *m/z* 118–120 (118 and 119 from pathway 2 and 120 from pathway 3) and *m/z* 136–138 (136 and 137 from pathway 1 and 138 from pathway 3).

Furthermore, it is noteworthy that only one ion cluster at *m/z* 136–138 was observed in monoester (heliotrine and lycopsamine *N*-oxide) and nonester (retronecine *N*-oxide) PA *N*-oxide (Figure 2 and Supplementary Figure 1), which further confirms that the ester substitution at C-7 position is essential for the formation of ion cluster at *m/z* 118–120 for the retronecine-type PAs. As a result, the presence or absence of the ion cluster at *m/z* 118–120 can be used as determinants to further differentiate retronecine-type diester PA *N*-oxides from nonester and monoester PA *N*-oxides with the ester substituted at C-9 position.

The mass spectra of platynecine-type PAs and *N*-oxides

Platyphylline was chosen as the representative platynecine-type PA in the present study. Platyphylline with the protonated molecular ion at *m/z* 338 ($[M+H]^+$) exhibited two specific fragment ions at *m/z* 122 and 140 (Figure 2), which have been previously proved to be the characteristic ions for this type PAs.[20] Its *N*-oxide with the protonated molecular ion at *m/z* 356 exhibited not only the same two characteristic ions but also the two additional specific ion clusters at *m/z* 120–122 and 138–140. Similarly to retronecine-type PA *N*-oxides, these two characteristic ion clusters are also expected to be generated from the similar three fragmentation pathways as illustrated in Supplementary Figure 2. Therefore, the fragmentation ions at *m/z* 120 and 121 (pathway 2) and 122 (pathway 3) and 138 and 139 (pathway 1) and 140 (pathway 3) formed two ion clusters at *m/z* 120–122 and 138–140, respectively. These two unique ion clusters all had two mass units greater than that of retronecine-type *N*-oxides and therefore can also be used to differentiate platynecine-type PA *N*-oxides from retronecine-type PA *N*-oxides.

Characterization of PAs and PA *N*-oxides in herbal plants

The developed HPLC–MS method using PIS approach was further applied to analyze toxic retronecine-type PAs and their PA *N*-oxides in two PA-containing herbal samples, *G. pseudochina and G. japonica*. The total ion chromatograms of *G. pseudochina* extract at PIS mode is showed in Figure 4A. The six peaks with the retention time at 13.4 min (peak 1), 16.9 min (peak 2), 27.1 min (peak 3), 29.8 min (peak 4), 34.0 min (peak 5), and 46.6 min (peak 6) all generated both characteristic ions at *m/z* 120 and

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138; therefore, they were retronecine-type PAs and/or PA *N*-oxides. Furthermore, the MS2 spectra (Figure 4B) of peaks 1, 2, and 3 all exhibited characteristic ion clusters at *m/z* 118–120 and 136–138, suggesting that they were all retronecine-type diester PA *N*-oxides. The MS2 spectra of peaks 4, 5, and 6 did not only show the typical *N*-oxide ion clusters and thus were retronecine-type PAs. These results further confirmed that the established characteristic ion clusters of PA *N*-oxides can be successfully used to identify rapidly PA *N*-oxides in PA-containing natural products even without requiring the reference standards.

Furthermore, with the availability of reference standards, four ingredients in *G. pseudochina* were unambiguously identified as seneciphylline *N*-oxide (peak 1), senecionine *N*-oxide (peak 2), seneciphylline (peak 4), and senecionine (peak 5). According to their molecular weight and PAs found in *Gynura* genus,[23] peaks 3 and 6 were identified as seneciphyllinine *N*-oxide seneciphyllinine. The structures of seneciphyllinine and its *N*-oxide are shown in Supplementary Figure 3. Similarly, the herbal extract of *G. japonica* was also analyzed by HPLC–MS method, and interestingly the same three PAs and three PA *N*-oxides were identified (data not shown).

Previously, using HPLC–MS analysis, several PA *N*-oxides have been identified by our research group[21] and others,[22,23] and the identification of all these PA *N*-oxides was mainly based on the corresponding protonated molecular ion ($[M+H]^+$) and/or protonated molecular dimer adduct ion ($[2M+H]^+$) and further confirmed with the comparison of the reference standards. With the mass spectra reported in these studies, we have retrospectively scrutinized mass spectra of all PA *N*-oxides identified and definitively identified the characteristic ion clusters at *m/z* 118–120 and 136–138 in the mass spectra of retrorsine *N*-oxide, senecionine *N*-oxide, and seneciphylline *N*-oxide in the study of *Senecionis scandentis*,[21] adonifoline *N*-oxide in the study of *in vitro* rat microsomal metabolism of adonifoline,[22] and seneciphyllinine *N*-oxide in the study of *Gynura segetum*.[23] These findings further support our novel approach to use the two characteristic mass spectrometric characteristic ion clusters at *m/z* 118–120 and 136–138 for the determination of toxic retronecine-type PA *N*-oxides in different samples.

CONCLUSIONS

Our study has, for the first time, found that the characteristic ion clusters were unique determinants to distinguish between PA *N*-oxides and PAs and also identify different subtypes among retronecine-type PA *N*-oxides. Our findings provide a novel and specific analytical method to differentiate PA *N*-oxides from PAs in PA-containing natural products. The discrimination of PA *N*-oxides from PAs will allow predict the risk of PA intoxication and make suitable quality control of PA-containing products and thus reducing the potential high risk of PA poisonings.

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Supporting Information

Supporting information may be found in the online version of this article.

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