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An analytical platform for the screening and identification of pyrrolizidine alkaloids in food matrices with high risk of contamination

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# Food Chemistry

## An analytical platform for the screening and identification of pyrrolizidine alkaloids in food matrices with high risk of contamination --Manuscript Draft--

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<b>Abstract:</b>	An analytical platform for the detection of pyrrolizidine alkaloids (PAs) in honey, pollen, teas, herbal infusions, and dietary supplements is proposed; it includes a wide-scope suspect screening method, based on a diagnostic product ion filtering strategy for the characterization of PAs, and a target screening and identification method for the high-throughput detection of 118 PAs of a high-resolution mass spectral library. Salting-out assisted liquid-liquid extraction of aqueous extracts combined to ultra-high performance liquid chromatography-high-resolution tandem mass spectrometry was employed. The limit of identification (0.6-30 µg kg <sup>-1</sup> ) of 28 standards were fit-for-purpose in PA-monitoring applications, with a false negative rate < 1.3% at 4 µg L <sup>-1</sup> . The wide-scope suspect screening method allowed the tentative identification of 88 compounds. The screening of 282 commercial samples revealed a broad contamination of the studied matrices, demonstrating the effectiveness of the platform in detecting and identifying both target and untarget PAs.
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1 **An analytical platform for the screening and identification of pyrrolizidine alkaloids in food**  
2 **matrices with high risk of contamination**

3

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18 **Abstract**

19 An analytical platform for the detection of pyrrolizidine alkaloids (PAs) in honey, pollen, teas, herbal  
20 infusions, and dietary supplements is proposed; it includes a wide-scope suspect screening method,  
21 based on a diagnostic product ion filtering strategy for the characterization of PAs, and a target  
22 screening and identification method for the high-throughput detection of 118 PAs of a high-resolution  
23 mass spectral library. Salting-out assisted liquid-liquid extraction of aqueous extracts combined to  
24 ultra-high performance liquid chromatography–high-resolution tandem mass spectrometry was  
25 employed. The limit of identification (0.6-30  $\mu\text{g kg}^{-1}$ ) of 28 standards were fit-for-purpose in PA-  
26 monitoring applications, with a false negative rate  $< 1.3\%$  at  $4 \mu\text{g L}^{-1}$ . The wide-scope suspect  
27 screening method allowed the tentative identification of 88 compounds. The screening of 282  
28 commercial samples revealed a broad contamination of the studied matrices, demonstrating the  
29 effectiveness of the platform in detecting and identifying both target and untarget PAs.

30

31 **Keywords:** Bee products; Dietary supplements; Herbal products; Spectral library; Suspect screening;  
32 Target screening

## 33 **1. Introduction**

34 Pyrrolizidine alkaloids (PAs) are natural toxins produced by different plants (*Boraginaceae*,  
35 *Asteraceae* and *Fabaceae* families) as defense against insects and herbivores. The structures of PAs  
36 consist of the 1-hydroxymethyl pyrrolizidine core (necine base) esterified with one or two aliphatic  
37 acids (necic acids). PAs occur in plants as tertiary amines or N-oxide derivatives (PANOs) (EFSA,  
38 2011; Moreira, Pereira, Valentão, & Andrade, 2018; Schramm, Köhler, & Rozhon, 2019).

39 Because of their wide distribution in plants and their high incidence of contamination in foods and  
40 herbal products, PAs are considered some of the most dangerous classes of phytotoxins in causing  
41 liver damage (Schrenk et al., 2020). In fact, PAs exhibit carcinogenic and genotoxic activities under  
42 chronic exposure and hepatotoxic activity as result of acute toxicity. In particular, PAs/PANOs  
43 containing a 1,2-unsaturated necine base are considered of higher toxicity due to their metabolic  
44 activation into dehydro-pyrrolizidine esters, which can readily react with proteins and form DNA  
45 adducts (Dusemund et al., 2018; EFSA, 2011; Schrenk et al., 2020).

46 Beside the intake of PAs through herbal products containing PA-producing plants (Steinhoff, 2019),  
47 in the last decades, numerous scientific reports have revealed a worrying contamination of PAs from  
48 food products of plant origin (such as beehive and herbal products), mainly due to their accidental  
49 contamination with PA-producing plants during harvest. The high number of PA-producing plants,  
50 their global occurrence and wide distribution as weeds in the agricultural areas (*Crotalaria spp.*,  
51 *Echium spp.*, *Heliotropium spp.*, *Myosotis spp.*, and *Senecio spp.*) make the contamination of PAs a  
52 relevant issue for the food safety and quality of herbal products (EFSA, 2016; Schrenk et al., 2020;  
53 Steinhoff, 2019). In particular, the chronic toxicity due to long-term consumption of PA-contaminated  
54 food or herbal medicines is a current topic for the human health.

55 The European Food Safety Authority (EFSA), in its recent risk assessments, recognized PAs as  
56 undesirable substances in food and concluded that there is a possible human health concern related to  
57 chronic cumulative exposure to contaminated food products, such as teas, herbal infusions, beehive  
58 products and dietary supplements (EFSA, 2011; EFSA 2016; EFSA, 2017). In addition, the European

59 Commission has recently established maximum levels (MLs) for the sum of 21 PAs and 14 of their  
60 co-eluting isomers in certain food products, which are teas, herbal infusions, dried herbs, pollen,  
61 dietary supplements containing herbal ingredients and pollen (European Commission, 2020). To  
62 reduce the chronic exposure to PAs, both the implementation of measures to mitigate their  
63 contamination and the development of new sensitive analytical procedures to evaluate their dietary  
64 exposure and collect occurrence data are important aspects to consider (EFSA, 2016; EFSA 2017).  
65 Currently available methods for the sensitive determination of PAs in various matrices are based on  
66 solid phase extraction (SPE) followed by liquid chromatography coupled to tandem mass  
67 spectrometry (LC-MS/MS) with unit resolution spectrometric analyzers (triple quadrupole and ion  
68 trap). Selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM), is a well-  
69 established MS/MS acquisition mode for the targeted analysis of PAs, due to its high selectivity,  
70 sensitivity, and robustness (Casado, Morante-Zarcelero, & Sierra, 2022; Ma et al., 2018; Mulder et al.,  
71 2018; Picron, Herman, Van Hoeck, & Goscinny, 2018). Although it ensures excellent analytical  
72 performance, which easily meet the quality criteria required in food safety control, this approach  
73 presents limitations on the number of compounds to be analyzed in one run, requires the availability  
74 of reference standards, and it does not provide suitable MS/MS spectra for the screening and structural  
75 elucidation of unknown or suspected compounds (Hird, Lau, Schuhmacher, & Krska, 2014; Righetti,  
76 Paglia, Galaverna, & Dall'Asta, 2016).

77 In recent years, high resolution mass spectrometry (HRMS) has been increasingly used as  
78 complementary method for the analysis of trace-level contaminants in food matrices since it allows  
79 the simultaneous screening of target, suspect, and untarget compounds. Moreover, the acquisition of  
80 accurate MS and MS/MS spectra (resolution < 5 ppm) offers the possibility to detect a theoretically  
81 unlimited number of molecules without the need of a compound-specific tune, carry out the  
82 retrospective data analysis, and perform structural characterization of unknown or suspected  
83 compounds (Hird et al., 2014; Menger, Gago-Ferrero, Wiberg, & Ahrens, 2020; Rajska,  
84 Petromelidou, Díaz-Galiano, Ferrer, & Fernández-Alba, 2021; Righetti et al., 2016).

85 The analysis of PAs is a challenging task as the high variety of both necine bases and necic acids  
86 results in a huge number of different structures and numerous stereoisomers; to date, well over 600  
87 PAs are known (Moreira et al., 2018; Schramm et al., 2019). The regulated list of PAs and PANOs  
88 to monitor is limited and the development of advanced analytical approaches to detect further PAs,  
89 which can potentially contaminate plant-based matrices, is of huge importance to better understand  
90 the presence of these contaminants in food matrices and guarantee their safety (Casado et al., 2022).  
91 This study aims to develop an analytical platform for the rapid and automated screening and  
92 identification of a high number of PAs and PANOs at trace levels in various food matrices to broaden  
93 the knowledge about the distribution of these contaminants in foods. To achieve this goal, an  
94 analytical procedure combining the salting-out assisted liquid-liquid extraction (SALLE) of aqueous  
95 extracts with ultra-high performance liquid chromatography coupled with high resolution tandem  
96 mass spectrometry (UHPLC-HRMS/MS) was established. A systematic workflow, based on a  
97 database (778 molecules) and a diagnostic product ion filtering strategy, was designed to first  
98 characterize PAs and PANOs from PA-producing plants and then create an in-house HRMS/MS  
99 spectral library. Furthermore, two software-assisted processing methods were implemented to  
100 automate and facilitate the detection and characterization of PAs and PANOs (wide-scope suspect  
101 screening method) and perform the rapid and reliable screening of 118 target PAs and PANOs in  
102 commercial samples (high-throughput target screening and identification method). The proposed  
103 platform was validated for six food matrices according to the European guidelines for qualitative  
104 screening methods (Magnusson & Örnemark, 2014; Pihlstrom et al., 2018). Finally, 282 commercial  
105 samples were screened to test the applicability of the screening and identification method and  
106 investigate the contamination profile of the food matrices of interest: honey, pollen, black and green  
107 teas, herbal infusions, and dietary supplements.

108 To the best of our knowledge, this is the first study which proposes an HRMS-based approach for the  
109 target screening analysis of a high number of PAs and PANOs and offers the possibility to detect and  
110 identify new targets of such a vast class of natural toxins.

111 **2. Material and methods**

112 *2.1. Chemicals and standards*

113 Analytical grade acetonitrile (MeCN), methanol (MeOH), magnesium sulfate heptahydrate  
114 ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), sodium sulfate ( $\text{Na}_2\text{SO}_4$ ), sodium hydroxide (NaOH), sulfuric acid ( $\text{H}_2\text{SO}_4$ ) and MS  
115 grade formic acid (HCOOH) were purchased from Merck Chemicals (Milan, Italy). MS grade MeCN  
116 and water ( $\text{H}_2\text{O}$ ) were provided by Romil (Cambridge, UK). Ultrapure water (18 M $\Omega$ ) was prepared  
117 using a Milli-Q purification system (Millipore, Bedford, USA).

118 Reference standards (n = 30) (85-98 % HPLC grade) of echimidine, echimidine N-oxide, erucifoline,  
119 erucifoline N-oxide, europine, europine N-oxide, heliotrine, heliotrine N-oxide, indicine, indicine N-  
120 oxide, intermedine, intermedine N-oxide, jacobine, jacobine N-oxide, lasiocarpine, lasiocarpine N-  
121 oxide, lycopsamine, lycopsamine N-oxide, monocrotaline, monocrotaline N-oxide, retrorsine,  
122 retrorsine N-oxide, senecionine, senecionine N-oxide, seneciophylline, seneciophylline N-oxide,  
123 senkirkine, senecivernine, senecivernine N-oxide, and trichodesmine were provided by Merck  
124 Chemicals (Milan, Italy). Standard stock solutions were prepared for each analyte (1 mg mL<sup>-1</sup>) in  
125 MeOH and stored at -20 °C. Diluted solutions and standard mixtures were prepared in H<sub>2</sub>O/MeOH  
126 7:3 v/v.

127

128 *2.2. PA-producing plants and samples*

129 Ten PAs-producing plants, four of which belonging to the *Asteraceae* family (*Eupatorium*  
130 *cannabinum*, *Petasites hybridus*, *Senecio vulgaris*, *Tussilago farfara*) and the other six to the  
131 *Boraginaceae* family (*Anchusa officinalis*; *Borago officinalis*, *Echium italicum*, *Heliotropium*  
132 *europaeum*, *Lithospermum officinale*, *Symphytum officinale*) were provided by Giardino della  
133 Minerva (Orto botanico della Scuola Medica Salernitana, Salerno, Italy).

134 A total number of 282 commercial samples were analyzed. Honey (n = 72) and pollen (n = 6) samples  
135 from different botanical and geographical origins were obtained from Italian supermarkets, online  
136 shops, and local beekeepers. Herbal infusions (n = 101, including 21 labelled as dietary supplements),

137 black teas (n = 31), green teas (n = 20), and plant-based dietary supplements (n = 44 in solid form  
138 and n = 8 as syrups) were purchased from herbalist's and chemist's shops. Honey samples were stored  
139 at 4 °C until the analysis. Regarding herbal infusions, teas, and solid forms of plant-based dietary  
140 supplements, 50 % of units of each package were combined and milled to form a representative  
141 aggregate sample. Each aggregate sample was appropriately coded and kept in plastic containers at  
142 room temperature and protected from light until the analysis.

143

### 144 *2.3. Sample preparation*

145 The sample preparation procedure involved the aqueous extraction of PAs and PANOs followed by  
146 Salting-out Assisted Liquid-Liquid Extraction (SALLE). Honey samples were homogenized by  
147 manual stirring (3 min), and a representative aliquot of 25 g was diluted to 100 mL with distilled  
148 water and sonicated for 15 min (Rizzo, Celano, Campone, Rastrelli, & Piccinelli, 2022). Solid  
149 matrices (pollen and solid forms of dietary supplements) were extracted with an acidic water solution,  
150 according to Mulder and co-workers (Mulder et al., 2018). Briefly, 1 g of each sample was extracted  
151 with 20 mL (for pollen) and 10 mL (for dietary supplements) of acidic water (H<sub>2</sub>SO<sub>4</sub>, 0.05 M) by  
152 sonication (15 min) after vortex-mixing (1 min). The supernatant was collected after centrifugation  
153 (5 min at 13,000 rpm) and the solid residue was re-extracted under the same conditions. PA-producing  
154 plants were extracted with the same procedure used for solid matrices (1 g of plant with 20 mL of  
155 acidic water, twice). Herbal infusions, black and green teas were extracted according to the  
156 standardized procedure for teas and infusions (Mulder et al., 2018). In detail, 2 g of each homogenized  
157 sample were brewed with 150 mL of boiling water and left to infuse for 5 min. Then, the solution  
158 was filtered through a fluted filter paper. Syrups were properly diluted with water before being  
159 extracted.

160 Aqueous extracts of each matrix were subjected to the same SALLE procedure, according to our  
161 previous study (Rizzo et al., 2022). Briefly, a 10 mL aliquot of the aqueous solution was brought to  
162 a concentration of 1 M of MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.5 M Na<sub>2</sub>SO<sub>4</sub>, and to a pH value of 9.6; then, it was

163 centrifugated for 5 min (13,000 rpm). Afterwards, 2 mL of the aqueous solution were extracted with  
164 2 mL of MeCN by vortexing the mixture for 1 min. The sample was then centrifugated for 5 min  
165 (13,000 rpm) to achieve the phase separation. The upper organic phase (MeCN) was quantitatively  
166 transferred into a clean tube and left to dry under a gentle nitrogen flow. Afterwards, the dried residue  
167 was redissolved with an appropriate volume of H<sub>2</sub>O/MeOH 7:3 v/v: in 500  $\mu$ L for honey, pollen, and  
168 solid forms of plant-based dietary supplements and 200  $\mu$ L for herbal infusions and teas.

169

#### 170 *2.4 UHPLC-HRMS analysis*

171 The analyses were conducted on an UltiMate 3000 UHPLC system (ThermoFisher Scientific, Milano,  
172 Italy) interfaced via a heated electrospray ionization source (HESI-II) to a Q-Exactive mass  
173 spectrometer (ThermoFisher Scientific, Milano, Italy). The UHPLC system was equipped with a Luna  
174 Omega Polar C18 (2.1  $\times$  100 mm, 1.6  $\mu$ m; Phenomenex, Bologna, Italy) column, operated at 40  $^{\circ}$ C  
175 with a flow rate of 400  $\mu$ L min<sup>-1</sup>. The chromatographic separation was achieved using a binary  
176 gradient of H<sub>2</sub>O (A) and MeCN (B), both containing 0.1 % of formic acid; the elution gradient was  
177 as follows: 0–1 min, 2 % B; 1–5.5 min, 2–8 % B; 5.5–7.5 min, 8 % B; 7.5–9.5 min, 8–12 % B; 9.5–  
178 11 min, 12–18 % B; 11–13 min, 18–20 % B; 13–15 min, 20–40 % B; 15–17 min, 40–60 % B; 17–19  
179 min, 60–80 % B. After each injection, washing (98 % B, 4 min) and re-equilibration of the column (2  
180 % B, 5 min) were performed. The injection volume was set at 5  $\mu$ L.

181 The mass spectrometer operated in positive ionization mode with the following instrument  
182 parameters: spray voltage, 3.5 kV; sheath gas flow rate, 50; auxiliary gas flow rate, 13; capillary and  
183 auxiliary gas heater temperatures, 300  $^{\circ}$ C; S-lens level, 55. Nitrogen was used as collision gas of the  
184 higher-energy collisional dissociation (HCD) cell. Data were acquired in Full MS/dd-MS<sup>2</sup> mode. The  
185 resolution of the Full MS scans (scan range 250–500  $m/z$ ) was set at 70k (FWHM), the Automatic  
186 Gain Control (AGC) target at 3e6, and the maximum IT (Injection time) at 250 ms. Each time the  
187 detector detected a peak corresponding to the accurate mass ( $\pm$  5ppm) of a certain precursor ion of  
188 the inclusion list associated to the method, these ions were isolated in the quadrupole, accumulated

189 in the C-trap, and finally accelerated in the HCD cell to be fragmented. The inclusion list associated  
190 to the acquisition method was filled with 112 masses of precursor ions ( $[M+H]^+$ ) (Table S1). The  
191 fragmentation was performed using the NCE (Normalized Collision Energy) technology, which  
192 applies a stepped collisional energy scheme by combining low, medium, and high collision energies  
193 capable of increasing the diversity of fragment ions generated; a range of collision energies between  
194 40 and 60 was applied in this study. The recording parameters of the dd-MS<sup>2</sup> scans were set as  
195 follows: mass resolution, 17.5 k (FWHM); AGC target, 2e4; maximum IT, 80 ms; isolation window,  
196  $m/z$  1.5; intensity threshold, 1.3e4; and dynamic exclusion: 2.0 s. The TopN parameter, which refers  
197 to the number of ions to be triggered after a Full MS scan, was disabled to prevent precursor ions  
198 other than those contained in the inclusion list from being isolated. Xcalibur software version 4.4  
199 (ThermoFisher Scientific, Milano, Italy) was used for instrument control and data acquisition.

200

## 201 *2.5. Data processing*

202 The data processing was performed using TraceFinder software version 5.1 (ThermoFisher Scientific,  
203 Milano, Italy). In detail, two processing methods were built to automate and facilitate the data  
204 treatment, according to the specific objectives of the study. The first one, named as wide-scope  
205 suspect screening method, was developed to detect and characterize suspect PAs and PANOs from  
206 PAs-producing plants and commercial samples; the second one, named as high-throughput target  
207 screening and identification method, to rapidly perform the screening of a huge number of  
208 commercial samples regarding the presence of 118 target PAs and PANOs of the spectral library.  
209 Both the methods were created using the “Target screening method” workflow of the software.

210

### 211 *2.5.1 Wide-scope suspect screening method*

212 The Compound Database (CD) was built by importing a csv file, containing the list of 112 precursor  
213 ions of the inclusion list, associated with the instrumental acquisition method (Table S1), into the  
214 software. Then, 30 key product ions for the characterization of PAs and PANOs ( $m/z$  120.0808,

215 138.0913, 150.0913, 168.1019, 124.1121, 142.1226, 122.0964, 140.107, 156.1019, 94.0651,  
216 96.0808, 110.0964, 122.0964, 180.1019, 198.1125, 83.0491, 220.1332, 238.1438, 158.1176,  
217 136.0757, 137.0835, 158.1176, 139.0992, 111.0679, 172.0968, 118.0651, 119.0729, 113.0835,  
218 174.1125, 121.0886, 214.1074, 254.1387 (section 3.3.2) were associated with each precursor ion. A  
219 master method was then created with the following processing parameters: a range-integrated  
220 detection type over the entire chromatographic run; a response threshold (peak area) of  $10e5$ ; a mass  
221 tolerance of  $\pm 5$  ppm; and at least three product ions. The suspect compounds were flagged as  
222 “detected” (green flag) when all the criteria were fulfilled. This allowed the method to detect the  
223 presence of PAs/PANOs analogues whenever a peak matched the molecular formula of the relative  
224 precursor ion ( $\pm 5$  ppm) and at least three diagnostic product ions ( $\pm 5$  ppm) over the entire duration  
225 of the chromatographic run.

226

### 227 *2.5.2 High-throughput target screening and identification method*

228 The functioning of the high-throughput screening and identification method was linked to the  
229 construction of an in-house HRMS/MS spectral library. Therefore, the initial step involved the  
230 construction of the library, which was created using mzVault software version 2.3 (ThermoFisher  
231 Scientific, Milano, Italy) by uploading UHPLC-HRMS/MS information of the 118 target PAs and  
232 PANOs (Table 1). Then, the CD was built by importing into the master method a csv containing the  
233 acquired mass spectra information (retention time, molecular formula, precursor ions, five most  
234 abundant product ions and their ratios) of the 118 compounds of the library. The spectral library was  
235 associated to the processing method as additional identification tool. The following identification  
236 criteria were set: a retention time variation of  $\pm 0.2$  min, a response threshold of  $10e4$ , a mass tolerance  
237 of 5 ppm for both precursor and product ions, a minimum of three product ions required for the  
238 identification, and a library match score higher than 70%. The target compounds were flagged as  
239 “identified” (green flag) when all the criteria were fulfilled, “found” when only the precursor ion was

240 encountered at the expected retention time (red flag), and “not found” (yellow flag) when none of the  
241 criteria were met.

242

#### 243 *2.6. Validation of the target screening and identification method*

244 The high-throughput screening and identification method was validated in terms of specificity,  
245 accuracy (expressed as extraction efficiency, EE), limit of identification (LOI), and precision  
246 (expressed as false negative rates), according to the performance criteria of qualitative screening  
247 methods established by the European analytical guidelines (Magnusson & Örnemark, 2014; Pihlstrom  
248 et al., 2018). The validation studies were conducted on 28 out of 30 reference standards (indicine and  
249 indicine N-oxide were excluded for co-elution reasons) in six food matrices: honey, pollen, black and  
250 green teas, herbal infusions, and plant-based dietary supplements. The experiments were performed  
251 on blank samples, previously identified through analysis. A representative sample of herbal infusion  
252 was prepared by mixing the same amount of chamomile, fennel, melissa, mint, and licorice, as these  
253 herbs were the most encountered during the collection of the samples. On the contrary, it was not  
254 possible to select or prepare a representative sample of a plant-based dietary supplement due to the  
255 high variability of their composition. The specificity was evaluated by processing spiked ( $10 \mu\text{g L}^{-1}$ )  
256 and unspiked SALLE extracts of blank samples of each studied matrix. The EEs were determined by  
257 pre- and post-spiking the target analytes at a concentration of  $10 \mu\text{g L}^{-1}$  of the SALLE extract  
258 (corresponding to  $10 \mu\text{g kg}^{-1}$  for honey,  $100 \mu\text{g kg}^{-1}$  for pollen,  $75 \mu\text{g kg}^{-1}$  for teas and herbal  
259 infusions, and  $50 \mu\text{g kg}^{-1}$  for dietary supplements) before and after the sample preparation procedure.  
260 Experiments were conducted in triplicate and EEs were calculated as area ratio of pre- and post-  
261 spiked samples. LOIs of herbal infusions, honey, pollen, black and green teas were evaluated by  
262 fortifying blank samples at eight concentration levels, ranging from  $0.4$  to  $2 \mu\text{g L}^{-1}$  of the SALLE  
263 extracts. LOIs were assigned for each target analyte at the concentration level that met all the  
264 identification criteria of the high-throughput target screening and identification method (section  
265 2.5.2). Regarding plant-based dietary supplements, since it was not possible to find a representative

266 sample, LOIs were estimated as the lowest concentration at which a compound was identified in at  
267 least 95 % of the blank samples. For this purpose, 20 blank samples (10 samples  $\times$  2 replicates) of  
268 different composition were spiked before the extraction at 10 and 20  $\mu\text{g kg}^{-1}$ . The precision of the  
269 method, estimated as false negative rates, was determined by fortifying 72 blank samples (36 samples  
270  $\times$  2 replicates), including honey samples (n = 3), pollen samples (n = 3), herbal infusions (n = 10),  
271 black (n = 5) and green (n = 5) teas, and dietary supplements (n = 10) at a concentration of 2  $\mu\text{g L}^{-1}$   
272 (corresponding to 2  $\mu\text{g kg}^{-1}$  for honey, 20  $\mu\text{g kg}^{-1}$  for pollen, 15  $\mu\text{g kg}^{-1}$  for teas and herbal infusions,  
273 and 10  $\mu\text{g kg}^{-1}$  for dietary supplements) and 4  $\mu\text{g L}^{-1}$  (corresponding to 4  $\mu\text{g kg}^{-1}$  for honey, 40  $\mu\text{g}$   
274  $\text{kg}^{-1}$  for pollen, 30  $\mu\text{g kg}^{-1}$  for teas and herbal infusions, and 20  $\mu\text{g kg}^{-1}$  for dietary supplements) of  
275 the SALLE extracts, which correspond to the tenth and the fifth part of the lower limit of the  
276 Regulation (EU) 2020/2040 (Tea, *Camellia sinensis*, ML of 150  $\mu\text{g kg}^{-1}$ ).

277

### 278 3. Results and discussion

#### 279 3.1 UHPLC-HRMS/MS analysis

280 The setting of the UHPLC conditions aimed at solving/minimizing one of the main problems  
281 encountered during the chromatographic analysis of PAs and PANOs, that is the co-elution of  
282 structural isomers impossible to distinguish by their MS/MS spectra. Some examples of these  
283 similarities are offered by the isomeric groups indicine/intermediate/lycopsamine,  
284 echinatine/rinderine, integerrimine/senecionine/senecivernine, echimidine/heliosupine,  
285 seneciophylline/spartioidine, and their N-oxides (Casado et al., 2022; Kaltner, Stiglbauer, Rychlik,  
286 Gareis, & Gottschalk, 2019). Therefore, the chromatographic conditions were carefully optimized on  
287 both the 30 reference standards and the extracts of the 10 PA-producing plants to obtain a better  
288 resolution of the peaks. Thus, it was possible to obtain the separation of both structural isomers of the  
289 reference standards and further isomers. As already reported by Kaltner and co-workers, the best  
290 chromatographic separation conditions of the isomers are obtained using acidified solvents (Kaltner  
291 et al., 2019). The optimized conditions allowed to achieve a good separation for most of the  
292 abovementioned PAs and PANOs isomers, within a run time of 17 min. In addition, many of the  
293 indistinguishable structural isomer pairs, characterized from the extracts of PA-producing plants, (7-  
294 acetylintermediate/7-acetyllycopsamine, amabiline/supinine, asperumine/heliosupine, lasiocarpine/7-  
295 tigloyleuropine, their N-oxides, and neosenkirkine/senkirkine) resulted in well separated peaks.  
296 Indicine/lycopsamine and their N-oxides and integerrimine/senecionine or senecivernine were the  
297 only isomers that couldn't be resolved. Even the isomers putatively identified as echinatine and  
298 rinderine, their 7-acetyl analogues, and their N-oxides were not sufficiently resolved under the  
299 chromatographic conditions used.

300 Different HRMS/MS acquisition modes were considered to evaluate the suitability of the detection  
301 method to the structural characterization and identification of PAs. Eventually, a data-dependent  
302 acquisition mode (Full MS/dd-MS<sup>2</sup>), with an inclusion list of prioritised masses, was selected as it  
303 proved to be efficient in terms of selectivity and ability to detect the target analytes at trace levels and

304 provide high quality HRMS/MS spectra. The quality of the MS/MS spectra is crucial to obtain reliable  
305 identifications of molecules in complex matrices, and the data-dependent acquisition mode provides  
306 MS/MS spectra from specific precursor ions by dismissing the other precursor ions, which reduces  
307 the risk of background noise and signal interferences (Rajski et al., 2021).

308 The Full MS/dd-MS<sup>2</sup> was adapted to the detection and characterization of a wide range of PAs at low  
309 concentration levels (ppb) in complex food matrices. For this purpose, the mass range of the Full MS  
310 scan ( $m/z$  250-550) was defined to cover the entire range of molecules of the internal database (section  
311 3.3.1). The dd-MS<sup>2</sup> scan was triggered on an inclusion list of accurate masses of [M+H]<sup>+</sup> ions  
312 obtained from the internal database. This allowed to fragment the suspected PAs over matrix-  
313 interfering ions, even when they were present as minor compounds. The TopN function, which selects  
314 the most abundant ions of every single Full MS scan, was disabled as it is not suitable for trace  
315 analyses in complex matrices; in fact, in such conditions, the selected ions would correspond to the  
316 matrix interferences. Furthermore, to detect and confirm the target analytes at low contamination  
317 levels, the minimum AGC target of the dd-MS<sup>2</sup> scan was set to a much lower value (10e3) than that  
318 commonly used in Full MS/dd-MS<sup>2</sup> analyses (10e5-10e6). The optimal collision energies were  
319 determined by analyzing the reference standards to obtain fragmentation spectra with significant  
320 product ions. The developed acquisition method allowed to detect (Full MS) and identify (dd-MS<sup>2</sup>)  
321 target PAs and PANOs up to a concentration close to 1  $\mu\text{g L}^{-1}$ , with enough data points across the  
322 chromatographic peaks (Full MS extracted ion chromatogram) and reliable fragmentation profiles.

323

### 324 **3.2 Sample preparation**

325 The development of a sample preparation procedure is challenging for trace-level analysis in complex  
326 matrices. Considering the objectives of the study, a simple, quick, and cheap sample preparation  
327 procedure was carried out. The simultaneous aqueous extraction of PAs and PANOs from the  
328 investigated matrices was performed before subjecting the samples to SALLE, which was used as  
329 clean-up step. All the solid matrices (honey, pollen, and solid forms of plant-based dietary

330 supplements) were extracted with acidified water, an extensively used solvent for the extraction of  
331 these alkaloids from different food matrices due to the ability to provide exhaustive extraction and  
332 cleaner extracts (Casado et al., 2022; Kaltner et al., 2019; Mulder et al., 2018). Teas and herbal  
333 infusions were extracted by infusion with boiling water to simulate the real exposure scenario to these  
334 contaminants (Casado et al., 2022; Mulder et al., 2018; Picron et al., 2018). The SALLE procedure  
335 previously developed for the determination of nine PAs and PANOs in honey and pollen (Rizzo et  
336 al., 2022) was adapted to be applied to further food matrices and a larger pool of analytes (15 PAs  
337 and 13 PANOs). The optimization of the sample preparation procedure was performed by fortifying  
338 the tested matrices at  $10 \mu\text{g L}^{-1}$  of each analyte in SALLE extracts (this level corresponds to  $10 \mu\text{g}$   
339  $\text{kg}^{-1}$  for honey,  $100 \mu\text{g kg}^{-1}$  for pollen,  $75 \mu\text{g L}^{-1}$  for teas and herbal infusions,  $50 \mu\text{g L}^{-1}$  for dietary  
340 supplements). The performances of the procedure were evaluated in terms of extraction efficiency.  
341 Under optimal conditions, the procedure provided exhaustive EEs (69-113 %) (Table 2). The SALLE  
342 procedure was also applied to aqueous extracts of PA-producing plants to evaluate its efficiency in  
343 extracting PAs and PANOs others than the target ones. No differences were observed between the  
344 profiles of the aqueous and SALLE extracts, indicating the efficiency of the procedure in extracting  
345 naturally occurring PAs and PANOs from PA-producing plants.

346

### 347 **3.3 Identification strategy**

348 PAs show a striking variety of chemical structures being the result of the combination of a limited set  
349 of necine bases and many necic acids. The structural diversity of PAs is further amplified by the type  
350 of ester linkage, acetylation, and hydroxylation of necic acids, and the oxidation of the amino group.  
351 Moreover, necine bases can either be fully saturated or 1,2-unsaturated. Based on the necine bases  
352 commonly found in plants, PAs can be classified into six groups: retronecine (R), heliotridine (H),  
353 otonecine (O), supinidine (S), platynecine (P) and trachelanthamidine (T) types (Fig. S1). The first  
354 four types are 1,2-unsaturated PAs, while the types P and T are the corresponding saturated  
355 derivatives of the R/H and S types, respectively. Except for the O type, in which N-oxides cannot be

356 formed, N-oxides of the other types of necine bases naturally occur and often coexist with their PA  
357 form in plant materials. Depending on the linkage between the necine base and the necic acids, PAs  
358 can also be divided into monoesters (m), cyclic diesters (c) and open-chained diesters (d). In PA  
359 diesters, limited to R, H, O, and P types, the esterification occurs at C-7 and C-9 and cyclic diesters  
360 derive from the esterification of the necine base with dicarboxylic necic acids (EFSA, 2011; Moreira  
361 et al., 2018; Schramm et al., 2019).

362

### 363 *3.3.1 Database of PAs and PANOs*

364 A wide database of PAs and PANOs was created from a systematic survey of the literature to support  
365 the identification strategy. The list of known compounds was also implemented with “expected  
366 unknowns”, intended as unreported compounds that can be predicted based on the chemical features  
367 of this class of alkaloids (e. g. N-oxide derivatives). The database (778 molecules) was filled with  
368 structural information (CAS number, elemental composition, molecular weight, accurate mass of  
369 precursor ions), and the groups of PAs with identical molecular formula were further classified into  
370 different subgroups according to the N-oxidation, the necine base and the type of esterification (Table  
371 S2).

372

### 373 *3.3.2 Diagnostic product ions filtering strategy*

374 The structural diversity of PAs appears in the fragmentation patterns that emerge from tandem mass  
375 spectrometry. Depending on the type of necine base, necic acids, esterification type and N-oxidation  
376 of the pyrrolizidine ring, PAs and PANOs show characteristic and predictable product ions with  
377 specific ion ratios. This behavior was used to develop a HRMS/MS approach for their detection and  
378 characterization without the need for reference standards. Thus, a diagnostic product ion filtering  
379 strategy was designed for the characterization of PAs and PANOs through their HRMS/MS spectra.  
380 A systematic flowchart (Fig. 1) was designed to delineate the fragmentation patterns of PAs and  
381 PANOs by studying the HRMS/MS spectra of the reference standards, online spectral libraries, and

382 previous studies (Mädge, Gehling, Schöne, Winterhalter, & These, 2020; Ruan et al., 2012; These,  
383 Bodi, Ronczka, Lahrssen-Wiederholt, & Preiss-Weigert, 2013). Important clarifications regarding the  
384 ion ratios arose during the collection of HRMS/MS spectra of the spectral library. The Fig. S2 and  
385 S3 show the chemical structures, molecular formulas, and exact masses of the key product ions  
386 required for the subdivision of PAs and PANOs into the different groups and subgroups of the Fig.  
387 1. The flowchart was divided in two subsets since the HRMS/MS spectra immediately allowed to  
388 differentiate PAs (Fig. 1A) from PANOs (Fig. 1B).

389 The different necine base types of PAs are easily recognized by the presence of characteristic product  
390 ions:  $m/z$  120.0808 and 138.0910 for both R and H types,  $m/z$  150.0913 and 168.1019 for O type,  $m/z$   
391 122.0964 and 140.1070 for both P and S types, and  $m/z$  124.1121 and 142.1226 for T type (Fig. 1A).  
392 The product ions of higher intensity were placed on the top of each subset by adding in succession  
393 characteristic product ions for each subgroup as far as it was possible. Regarding R and H types, PA  
394 monoesters are easily distinguished from diesters as they show the distinctive product ion at  $m/z$   
395 156.1019. Depending on the base peak, monoesters can be differentiated into R (bp at  $m/z$  94.0654)  
396 and H types (bp at  $m/z$  138.0910). Cyclic and open-chained diesters of R/H type can be differentiated  
397 based on the relative intensity of the product ions at  $m/z$  94.0654, 120.0808, and 138.0910: they show  
398 comparable intensities ( $> 20\%$ ) in cyclic forms, and a base peak at  $m/z$  120.0808 and low intensities  
399 ( $< 10\%$ ) at  $m/z$  94.0651 and 138.0913 in open-chained diester forms. The presence of product ions at  
400  $m/z$  180.1019 and 198.1125 identify an acetyl group at C-7 while product ions at  $m/z$  83.0491,  
401 220.1332, and 238.1438 identify an angeloyl/tigloyl group at the same position. Regarding S types,  
402 the product ions at  $m/z$  94.0654 and 110.0964 are crucial for their identification (Fig. 1A).

403 PANOs show more complex HRMS/MS spectra than PAs and characteristic product ion clusters (Fig.  
404 1B). The cluster 136 to 138 ( $m/z$  136.0757, 137.0835, and 138.0913) identifies R and H PANOs.  
405 Within R and H types, the base peak at  $m/z$  172.0968 and the product ion at  $m/z$  111.0679 identify the  
406 monoester subgroups and allow to distinguish them from the diester subgroups, which show the  
407 cluster 118 to 120 ( $m/z$  118.0651, 119.0729, and 120.0808). Depending on the relative intensities of

408 the cluster 136 to 138, monoesters can be differentiated into R (higher intensities) and H (lower  
409 intensities) types. The same applies to open-chained diesters, albeit in inverted ratios. In both groups,  
410 the product ions at  $m/z$  214.1074 or 254.1387 indicate the presence of an acetyl or angeloyl/tigloyl  
411 group respectively at the C-7 position of the open-chained diesters. S and P types of PANOs are  
412 instead characterized by the cluster 138 to 140 ( $m/z$  138.0913, 139.0992, and 140.1070); the base  
413 peak at  $m/z$  156.1019 and the product ion at  $m/z$  139.0992 allow to differentiate S from P types (Fig.  
414 1B).

415

#### 416 *3.3.2.1 Wide-scope suspect screening method*

417 After delineating the spectral features of PAs and PANOs, A reliable informatic solution was  
418 elaborated to handle with the large amount of HRMS/MS data and to automate and facilitate the  
419 detection and characterization of PAs and PANOs. The wide-scope suspect screening method was  
420 developed by associating each precursor ion of the inclusion list to a set of diagnostic product ions  
421 (section 2.5.1). This allowed the software to process the raw data, flagging as putative PAs/PANOs  
422 the only peaks with a molecular formula corresponding to that of the compounds of the database ( $\pm$   
423 5 ppm) and at least three diagnostic product ions ( $\pm$  5 ppm) (Fig. 2). The product ions ( $m/z$  values and  
424 ion ratios) of suspected peaks were first matched with the information reported in the flowchart (Fig.  
425 1) to establish the group and subgroup of the detected compound, and then the presumed structures  
426 were searched into the database (Table S2) to verify the match with a collected analogue. The  
427 presumed identity of the detected PA/PANO was confirmed by comparison with the reference  
428 standards (MSI, L1 – Metabolomics Standards Initiative, Level 1), or putatively assigned based on  
429 literature studies and online databases (MSI, L2). When no spectrum or literature information was  
430 available, the detected PA/PANO was tentatively assigned to the compound suggested by the  
431 proposed identification strategy, when present (MSI, L3). Fig. 2 shows three examples of application  
432 of the diagnostic product ions filtering strategy during the identification of suspect PAs.

433

### 434 3.4 HRMS/MS spectral library

435 The diagnostic product ions filtering strategy was applied to 10 PAs-producing plants to detect and  
436 identify as much compounds as possible and collect their spectra into an HRMS/MS spectral library.

437 The plant profiles (Table S3) were defined by comparing the information of the diagnostic product  
438 ions filtering strategy with literature information, MS/MS spectra available on online databases and  
439 libraries, and chemotaxonomic data. The latter resulted essential in discriminating structural isomers  
440 with superimposable MS/MS spectra (echiumine in *Echium italicum*, echinatine/rinderine in  
441 *Eupatorium cannabinum*, heliosupine in *Heliotropium europaeum*, and symphytine in *Symphytum*  
442 *officinale*). 84 PAs and PANOs other than the reference standards were detected, including two  
443 “expected unknowns”: canescine/canescenine N-oxide ( $m/z$  416.2275,  $C_{20}H_{34}NO_8^+$ ) and lithosenine  
444 N-oxide ( $m/z$  432.2223,  $C_{20}H_{34}NO_9^+$ ) in *L. officinale*. Their structures, hypothesized on the basis of  
445 the key product ions of R/H open-chained diester type of PANOs (Fig. S4), were further supported  
446 by chemotaxonomic data (El-Shazly & Wink, 2014; Kopp, Abdel-Tawab, & Mizaikoff, 2020).  
447 Moreover, the product ion at  $m/z$  272.1492 ( $C_{13}H_{22}NO_5^+$ ) corresponds to a hydroxyisovaleroyl residue  
448 (typical necic acid of canescine and lithosenine) at the C-7 position. Besides, the clusters at  $m/z$  136  
449 to 138 in the spectra of lithosenine N-oxide (low intensity; < 50%) and canescine N-oxide (high  
450 intensity; > 50%) further supported their assignments as R and H types, respectively (Fig. 1B).

451 A spectral library of 114 total compounds was built (Table 1). Among these, 30 were reference  
452 standards (MSI, L1), 52 were putatively assigned based on their MS similarity with literature  
453 information and online databases (MSI, L2), and 32 were assigned based on the diagnostic product  
454 ions filtering strategy and chemotaxonomic data (MSI, L3). During the analysis of the commercial  
455 samples, four further compounds were characterized, bringing the number of spectra of the library to  
456 118. The library includes all the PAs of the EFSA’s list (28) and the Regulation 2020/2040/EU (21).  
457 103 out of 118 compounds are 1,2 unsaturated PAs, of which the 58 % are R type and the 34 % are  
458 H type.

459

### 460 **3.5 High-throughput target screening and identification method**

461 The in-house spectral library was then associated to the high-throughput target screening and  
462 identification method. The identification criteria were set as follows: the presence of the precursor, a  
463 mass tolerance  $< \pm 5$  ppm, the expected retention time ( $\pm 0.2$  min), at least three product ions ( $\pm 5$   
464 ppm), and a library match score higher than 70%. This post-acquisition data evaluation was combined  
465 with the optimized sample preparation procedure and PA-tailored UHPLC-HRMS/MS method for  
466 the analysis of numerous PAs in food matrices with high risk of contamination. Fig. 3 shows an  
467 example of the method ability to identify europine N-oxide and distinguish it from a close interfering  
468 peak in a dietary supplement sample. As can be seen, europine N-oxide (Fig. 3A) met all the  
469 identification criteria (mass tolerance of the precursor ion, 0.7 ppm; 4 product ions with mass  
470 tolerance  $< 5$  ppm; library match score, 86 %), while the interfering peak (Fig. 3B) only met two of  
471 them (mass tolerance of precursor ion, 0.5 ppm; retention time within the range). These results  
472 highlight that the most stringent identification criteria were those related to the HRMS/MS data and  
473 demonstrate the efficacy of the identification method in detecting the target PAs and PANOs of the  
474 library with high reliability.

475

### 476 **3.6 Qualitative analytical performance**

477 A qualitative validation was performed since the aim of the proposed study was to develop an  
478 analytical platform for the detection and identification of PAs in complex matrices at relevant  
479 contamination levels. The method specificity, LOIs, and precision (false negative rate) were evaluated  
480 on 28 reference standards for all the investigated matrices, according to the performance criteria of  
481 screening methods (Magnusson & Örnemark, 2014; Pihlstrom et al., 2018). Regarding the remaining  
482 PAs and PANOs, for which no reference standards were available, the detection and identification  
483 can be achieved although it is not possible to specify qualitative performance parameters (Pihlstrom  
484 et al., 2018).

485 The method specificity, defined as the ability of the method to distinguish the analyte from any other  
486 matrix interferences, was evaluated by comparison between different blank and spiked samples of the  
487 studied matrices. No interfering peaks were observed at the expected retention time for all the 28  
488 reference standards in honey and pollen samples. On the contrary, some plant-based samples showed  
489 the presence of interfering peaks close to the some of the target analytes. The proposed method  
490 provided satisfactory specificity and the matrix interferents were either chromatographically or  
491 spectrally discriminated; moreover, the number of false positives dropped to zero when all the  
492 identification criteria were considered.

493 To achieve an accurate identification of the target analytes and minimize the risk of false positives,  
494 diagnostic information, that meets the defined criteria, is required (Lehotay, Sapozhnikova, & Mol,  
495 2015). The LOIs, defined as the lowest concentration that fulfill all the identification criteria of the  
496 method, were established to estimate the threshold concentrations at which the identification become  
497 reliable. LOIs of the 28 target analytes in the six tested matrices ranged from 0.6 to 30  $\mu\text{g kg}^{-1}$  (Table  
498 2). The method was able to detect and identify all the target analytes in the SALLE extract at a  
499 concentration of 2  $\mu\text{g L}^{-1}$ , except for echimidine, echimidine N-oxide, erucifoline, jacobine,  
500 monocrotaline, retrorsine, retrorsine N-oxide, seneciophylline N-oxide, senecivernine, senecivernine  
501 N-oxide, which were detected from 4  $\mu\text{g L}^{-1}$  in dietary supplements. The LOIs demonstrated to be  
502 fit-for-purpose regarding PA-monitoring applications; LOIs were much lower than the MLs (17-119  
503 times in pollen, 21-67 times in herbal infusion, 10-50 times in tea, and 20-40 times in dietary  
504 supplement).

505 The precision of the method was calculated as false negative rate. Considering the calculated LOIs  
506 and the regulatory MLs, two cut-off levels, 2 and 4  $\mu\text{g L}^{-1}$  of the SALLE extract, were defined to  
507 achieve the best suited false negative rate; the guidelines require identification methods to accomplish  
508 a false negative rate  $\leq 5\%$  (Lehotay et al., 2015). The overall false negative rate evaluated on 36 blank  
509 samples spiked at the two abovementioned levels and processed in duplicates, was lower than 5 %  
510 (0-1.3 %) at 4  $\mu\text{g L}^{-1}$  of the SALLE extract for all the 28 analytes. However, the method achieved

511 reliable identification results (< 5 % of false negatives) at the lowest level tested (2 µg L<sup>-1</sup>) as well  
512 for most of the analytes, excluding those with a LOI of 20 µg kg<sup>-1</sup> in dietary supplements (Table 2)  
513 and erucifoline N-oxide, senecionine, seneciphylline and senecivernine in teas and infusions.

514

### 515 **3.7 Analysis of commercial samples**

516 A huge number of commercial samples (n = 282) was screened against the 118 target PAs and PANOs  
517 to demonstrate the applicability of the analytical platform and investigate the profile of different food  
518 matrices. The collected samples represent food matrices susceptible to the contamination of PAs and  
519 relevant to consumer intake; they include honey, pollen, black and green teas, herbal infusions, and  
520 plant-based dietary supplements. Qualitative data only are discussed in this study as further studies  
521 will be necessary to test the suitability of the procedure for the quantitative determination of the  
522 analytes in the studied matrices and validate it accordingly.

523 The wide-scope suspect screening method was applied to the commercial samples to interrogate them  
524 regarding the presence of PAs and PANOs other than those already characterized from PA-producing  
525 plants. This allowed to detect four additional PAs: helioamplexine and two isomers of echimidine in  
526 honey samples, and acetylseneciphylline N-oxide in a dietary supplement, which were added to the  
527 HRMS/MS spectral library and to the high-throughput screening and identification method.

528 The qualitative analysis of the samples revealed the presence of 60 PAs/PANOs in 59 % of the  
529 analyzed samples (Table S4); among these, 21 PAs/PANOs were listed in the 2040/2020/EU  
530 Regulation (echimidine, europine, heliotrine, intermedine, lasiocarpine, lycopsamine, retrorsine,  
531 senecionine, seneciphylline, senecivernine, their N-oxides, and senkirkine), 8 belonged to the list of  
532 14 coeluting isomers to be monitored (echinatine, heliosupine, indicine, integerrimine, rinderine,  
533 spartioidine, usaramine, and their N-oxides) and 28 were PAs included in the HRMS/MS spectral  
534 library but not mentioned in the Regulation or in the EFSA's list of relevant contaminants of plant  
535 matrices. Among the studied matrices, honey was found to be the most contaminated one as 89 % of  
536 the samples tested positive to the presence of PAs. In decreasing order of contamination, follow

537 dietary supplements (58 %), pollen (50 %), herbal infusions (46 %), and teas (39 %). Regarding the  
538 contamination profile, Fig. 4 shows the PAs and PANOs detected in honey, herbal infusions, and  
539 plant-based dietary supplements, which turned out to be the matrices with the widest profiles of  
540 contamination (32, 34 and 49 analytes detected, respectively). In detail, the most frequently detected  
541 and identified compounds (> 20 % of the contaminated samples of each matrix) were echimidine and  
542 its two isomers, echimidine N-oxide, echinatine/rinderine, 5-hydroxyindicine, intermedine,  
543 lycopsamine, and symphytines for honey; europine N-oxide, heliotrine N-oxide, lasiocarpine N-  
544 oxide, and senecionine N-oxide for herbal infusions; and echinatine/rinderine, europine, heliotrine,  
545 heliotrine N-oxide, lasiocarpine, and senecionine for dietary supplements. The qualitative data on the  
546 distribution of PAs indicated that the PAs and PANOs of the Regulation 2040/2020/EU contribute to  
547 almost the total content for herbal infusions (86 %) and dietary supplements (83 %). On the other  
548 hand, 47 % of the PAs detected in honey were not included in the lists of relevant PAs to be monitored;  
549 echimidine isomer 1 (55 %) and 2 (59 %), 5-hydroxyindicine (41 %) and the sum of symphytines 1  
550 and 2 (22 %) were the most prevalent.

551

### 552 **3.8 Conclusions**

553 The present study proposes an analytical platform for the rapid and automated detection of  
554 pyrrolizidine alkaloids in food matrices with high risk of contamination. It consists of an easy and  
555 cheap sample preparation followed by a PA-tailored UHPLC-HRMS/MS analysis, which combined  
556 with the identification strategy and a post-acquisition data evaluation allow to detect, identify, and  
557 characterize a wide range of compounds at the required levels.

558 This analytical platform offers the possibility to interrogate the samples on the presence of the 118  
559 target PAs and PANOs of the target screening method, and to identify additional unreported  
560 analogues. The complementary mode of operation of the wide-scope suspect screening method and  
561 the high-throughput target screening and identification method makes the procedure versatile and

562 state-of-the-art. The HRMS/MS spectral library can be continuously implemented with newly  
563 identified compounds according to the proposed strategy.

564 Furthermore, the possibility of adding further molecular masses to the inclusion list of the Full  
565 MS/dd-MS<sup>2</sup> acquisition method, each time a PA is identified, allows to considerably broaden the  
566 identification range since each molecular mass can identify multiple structural isomers. Finally, the  
567 non-dependence of the platform on the purchase of reference standards not only lowers the cost of  
568 the procedure but also solves the problem of the lack of reference standards of these toxins.

569 Further studies are underway to evaluate the suitability of the proposed analytical procedure for the  
570 determination of the PA levels in high-risk food matrices and validate it accordingly.

571

572 **CRedit author statement**

573 **Serena Rizzo:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology;  
574 Software; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Rita**  
575 **Celano:** Conceptualization; Investigation; Methodology; Supervision; Validation; Roles/Writing -  
576 original draft; Writing - review & editing. **Simona Serio:** Formal analysis; Software. **Anna Lisa**  
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579 editing. **Mariateresa Russo:** Conceptualization; Funding acquisition; Writing - review & editing.  
580 **Luca Rastrelli:** Conceptualization; Funding acquisition; Project administration; Resources; Writing  
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675

676 **Figures captions.**

677 **Fig. 1.** Flowchart of key product ions for the identification of pyrrolizidine alkaloids (A) and their N-  
678 oxides (B).

679

680 **Fig. 2.** Mode of operation of the diagnostic product ions filtering strategy for the identification of (A)  
681 senkirkine (MSI, L1), (B) heliosupine N-oxide (MSI, L2), and (C) thesinine (MSI, L3).

682

683 **Fig. 3.** Specificity of the high-throughput target screening and identification method in (A) identifying  
684 europine N-oxide and (B) distinguishing it from a close interfering peak in a dietary supplement.

685

686 **Fig. 4.** Contamination profiles of (A) honey, (B) herbal infusions, and (C) dietary supplements. The  
687 percentage on each bar represents the prevalence of each PA/PANO in positive samples. Only PAs  
688 present in more than 5 % of the positive samples are shown.

**Table 1.** HRMS/MS spectral library of the 118 target PAs and PANOs.

Name <sup>a</sup>	Necine base <sup>b</sup>	Necic acid <sup>c</sup>	Molecular formula	[M+H] <sup>+</sup> (m/z) <sup>d</sup>	TR (min)	Diagnostic product ions, m/z (relative abundance)	IL <sup>e</sup>
7-Tigloyl/retronecine NO	R	m	C <sub>13</sub> H <sub>19</sub> NO <sub>4</sub>	254.1387	7.2	106.0653 (100); 83.0496 (34); 111.0679 (28); 136.0757 (21); 94.0654 (18)	2
7-Angeloyl/retronecine NO	R	m	C <sub>13</sub> H <sub>19</sub> NO <sub>4</sub>	254.1387	7.4	106.0654 (100); 83.0496 (46); 136.0758 (32); 94.0655 (31); 111.0680 (30)	2
9-Tigloyl/retronecine NO	R	m	C <sub>13</sub> H <sub>19</sub> NO <sub>4</sub>	254.1387	10.4	93.0577 (88); 136.0757 (77); 138.0913 (35); 137.0833 (30); 94.0654 (30); 108.0809 (20)	2
9-Angeloyl/retronecine NO	R	m	C <sub>13</sub> H <sub>19</sub> NO <sub>4</sub>	254.1387	10.7	93.0577 (93); 136.0757 (66); 154.0861 (56); 83.0496 (46); 94.0654 (36); 137.0834 (34); 138.0913 (33)	2
Supinine	S	-	C <sub>13</sub> H <sub>23</sub> NO <sub>4</sub>	284.1856	8.1	122.0965 (100); 140.1069 (90); 70.0657 (28); 110.0967 (12); 94.0655 (11)	2
Amabiline	S	-	C <sub>13</sub> H <sub>23</sub> NO <sub>4</sub>	284.1856	8.4	122.0964 (100); 140.1069 (90); 70.0657 (28); 110.0967 (12); 94.0655 (13)	2
Spilanthine	T	-	C <sub>13</sub> H <sub>23</sub> NO <sub>4</sub>	284.1856	9.2	142.1227 (100); 124.1122 (56); 96.0811 (8); 70.0657 (6)	2
Viridiflorine	T	-	C <sub>13</sub> H <sub>27</sub> NO <sub>4</sub>	286.2013	7.9	142.1227 (100); 125.1198 (7); 70.0657 (6); 124.1121 (5); 96.0814 (1)	2
Cynaustaline	T	-	C <sub>13</sub> H <sub>27</sub> NO <sub>4</sub>	286.2013	8.2	142.1225 (100); 124.1120 (52); 70.0657 (12); 86.0968 (9); 96.0810 (5)	2
Thesinine	T	-	C <sub>17</sub> H <sub>31</sub> NO <sub>3</sub>	288.1594	13.7	147.0441 (100); 142.1227 (40); 124.1122 (39); 119.0493 (18); 96.0812 (5)	3
Heleurine	S	-	C <sub>16</sub> H <sub>27</sub> NO <sub>4</sub>	298.2013	12.0	122.0965 (100); 140.1069 (57); 94.0655 (13); 110.0966 (12); 70.0657 (11)	2
Intermedine	R	m	C <sub>13</sub> H <sub>23</sub> NO <sub>5</sub>	300.1805	5.3	94.0654 (100); 156.1018 (47); 138.0912 (39); 120.0807 (16); 82.0656 (7)	1
Indicine	R	m	C <sub>13</sub> H <sub>23</sub> NO <sub>5</sub>	300.1805	5.5	94.0654 (100); 156.1016 (46); 138.0912 (35); 120.0808 (18); 82.0655 (7)	1
Lycoposamine	R	m	C <sub>13</sub> H <sub>23</sub> NO <sub>5</sub>	300.1805	5.6	94.0654 (100); 156.1018 (55); 138.0912 (35); 120.0807 (18); 82.0656 (7)	1
Rinderine	H	m	C <sub>13</sub> H <sub>23</sub> NO <sub>5</sub>	300.1805	5.9	138.0913 (100); 156.1019 (50); 120.0808 (31); 94.0655 (24); 82.0656 (12)	2
Echinatine	H	m	C <sub>13</sub> H <sub>23</sub> NO <sub>5</sub>	300.1805	6.0	138.0912 (100); 156.1018 (55); 120.0808 (33); 94.0654 (26); 82.0656 (13)	2
Supinine NO	S	-	C <sub>13</sub> H <sub>23</sub> NO <sub>5</sub>	300.1805	8.7	156.1020 (100); 139.0992 (34); 120.0809 (24); 122.0965 (22); 121.0887 (9); 138.0913 (7); 96.0812 (7)	2
Amabiline NO	S	-	C <sub>13</sub> H <sub>23</sub> NO <sub>5</sub>	300.1805	9.1	156.1019 (100); 139.0991 (32); 120.0808 (26); 122.0965 (26); 121.0887 (10); 138.0913 (8); 96.0812 (7)	3
Curassavine	T	-	C <sub>16</sub> H <sub>29</sub> NO <sub>4</sub>	300.2169	11.9	142.1227 (100); 124.1122 (75); 156.1022 (12); 70.0658 (12); 96.0812 (10)	2
Dihydroechinatine (rinderine)	P	m	C <sub>13</sub> H <sub>27</sub> NO <sub>5</sub>	302.1962	6.5	158.1176 (100); 140.1069 (56); 122.0963 (41); 96.0812 (18)	3
Dihydrointermedine	P	m	C <sub>13</sub> H <sub>27</sub> NO <sub>5</sub>	302.1962	6.6	158.1175 (100); 140.1068 (55); 122.0964 (36); 96.0811 (14)	3
Dihydrolycoposamine	P	m	C <sub>13</sub> H <sub>27</sub> NO <sub>5</sub>	302.1962	6.7	158.1175 (100); 140.1069 (55); 122.0965 (34); 96.0810 (12)	3
Viridiflorine NO	T	-	C <sub>13</sub> H <sub>27</sub> NO <sub>5</sub>	302.1962	8.5	158.1176 (100); 124.1121 (16); 141.1148 (9); 140.1071 (7); 122.0966 (2)	3
Helioamplexine	R	m	C <sub>16</sub> H <sub>27</sub> NO <sub>5</sub>	314.1962	7.4	94.0655 (100); 156.1019 (36); 138.0914 (30); 120.0809 (17); 82.0657 (9)	2
Heliotrine	H	m	C <sub>16</sub> H <sub>27</sub> NO <sub>5</sub>	314.1962	9.2	138.0912 (100); 156.1017 (43); 94.0654 (29); 120.0808 (27); 82.0656 (14); 108.0809 (9)	1
Heleurine NO	S	-	C <sub>16</sub> H <sub>27</sub> NO <sub>5</sub>	314.1962	12.4	156.1019 (100); 120.0808 (20); 139.0991 (18); 122.0965 (16); 138.0914 (8); 121.0889 (5); 96.0811 (3)	2
5'-Hydroxyindicine	R	m	C <sub>13</sub> H <sub>23</sub> NO <sub>6</sub>	316.1755	1.9	94.0654 (100); 138.0912 (43); 156.1017 (25); 120.0808 (18); 82.0656 (5); 108.0809 (2)	2
5'-Hydroxyintermedine (lycoposamine)	R	m	C <sub>13</sub> H <sub>23</sub> NO <sub>6</sub>	316.1755	3.0	94.0654 (100); 138.0912 (40); 156.1018 (26); 120.0808 (16); 82.0655 (5); 108.0809 (2)	3
5'-Hydroxyechinatine (rinderine)	H	m	C <sub>13</sub> H <sub>23</sub> NO <sub>6</sub>	316.1755	3.5	138.0913 (100); 94.0653 (28); 72.0813 (23); 156.1017 (21); 120.0809 (8)	2
Rinderine NO	H	m	C <sub>13</sub> H <sub>23</sub> NO <sub>6</sub>	316.1755	6.4	172.0965 (100); 138.0912 (19); 111.0680 (18); 94.0654 (18); 136.0757 (7); 137.0835 (3); 155.0939 (18)	2
Echinatine NO	H	m	C <sub>13</sub> H <sub>23</sub> NO <sub>6</sub>	316.1755	6.6	172.0965 (100); 138.0912 (20); 111.0680 (20); 94.0654 (19); 136.0757 (7); 137.0833 (3); 155.0939 (16)	2
Intermedine NO	R	m	C <sub>13</sub> H <sub>23</sub> NO <sub>6</sub>	316.1755	6.9	172.0965 (100); 138.0912 (53); 94.0654 (37); 111.068 (25); 155.0938 (18); 136.0756 (15); 137.0836 (6)	1
Indicine NO	R	m	C <sub>13</sub> H <sub>23</sub> NO <sub>6</sub>	316.1755	7.2	172.0964 (100); 138.0912 (53); 94.0654 (37); 111.0679 (23); 136.0754 (18); 137.0833 (7); 155.0938 (1)	1
Lycoposamine NO	R	m	C <sub>13</sub> H <sub>23</sub> NO <sub>6</sub>	316.1755	7.2	172.0966 (100); 138.0912 (64); 94.0654 (41); 111.0681 (24); 136.0758 (19); 155.0939 (18); 137.0834 (6)	1
Dihydrointermedine NO	P	m	C <sub>13</sub> H <sub>27</sub> NO <sub>6</sub>	318.1911	7.6	174.1122 (100); 113.0837 (20); 96.0810 (3); 140.1069 (2); 138.0913 (1); 139.0990 (1)	3
Dihydrolycoposamine NO	P	m	C <sub>13</sub> H <sub>27</sub> NO <sub>6</sub>	318.1911	7.7	174.1122 (100); 113.0837 (20); 96.0811 (3); 140.1070 (2); 138.0912 (1); 139.0990 (1)	3

Dihydroindrine NO	P	m	C <sub>13</sub> H <sub>27</sub> NO <sub>6</sub>	318.1911	8.0	174.1123 (100); 96.0811 (26); 113.0837 (23); 140.1068 (22); 138.0913 (10); 139.0994 (2)	3
Dihydroechinatine NO	P	m	C <sub>13</sub> H <sub>27</sub> NO <sub>6</sub>	318.1911	8.1	174.1123 (100); 96.0811 (41); 140.1068 (25); 113.0837 (25); 138.0912 (13); 139.0994 (2)	3
Monocrotaline	R	c	C <sub>16</sub> H <sub>23</sub> NO <sub>6</sub>	326.1598	2.7	120.0808 (82); 121.0885 (80); 94.0654 (31); 237.1358 (21); 194.1175 (20); 280.1540 (16); 138.0911 (15); 298.1642 (6)	1
Europine	H	m	C <sub>16</sub> H <sub>27</sub> NO <sub>6</sub>	330.1911	5.9	138.0913 (100); 156.1019 (41); 94.0655 (22); 120.0809 (18); 254.1383 (11); 82.0657 (11); 108.0810 (5)	1
Helioamplexine NO	R	m	C <sub>16</sub> H <sub>27</sub> NO <sub>6</sub>	330.1911	9.2	172.0966 (100); 138.0913 (83); 94.0654 (65); 111.0681 (32); 155.0939 (29); 136.0757 (24); 137.0837 (8)	3
Heliotrine NO	H	m	C <sub>16</sub> H <sub>27</sub> NO <sub>6</sub>	330.1911	10.2	172.0964 (100); 111.0679 (17); 138.0912 (13); 94.0654 (12); 155.0938 (6); 136.0754 (6); 137.0834 (3);	1
5'-Hydroxyechinatine (rinderme) NO	H	m	C <sub>15</sub> H <sub>23</sub> NO <sub>7</sub>	332.1704	1.9	172.0965 (100); 111.0681 (19); 155.0937 (7); 136.0757 (2); 137.0837 (3); 138.0912 (1); 94.0654 (1)	2
5'-Hydroxyintermedine (lycopsamine) NO	R	m	C <sub>15</sub> H <sub>23</sub> NO <sub>7</sub>	332.1704	3.0	172.0965 (100); 138.0912 (30); 94.0654 (24); 111.0680 (19); 155.0938 (16); 136.0757 (11); 137.0835 (5)	2
Spartioidine	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>5</sub>	334.1649	9.8	120.0807 (67); 94.0654 (41); 138.0912 (35); 306.1696 (21)	2
Seneciphylline	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>5</sub>	334.1649	10.1	120.0807 (58); 94.0654 (50); 138.0912 (38); 306.1697 (22)	1
Senecivermine	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>5</sub>	336.1805	12.1	120.0808 (41); 138.0913 (27); 308.1855 (23); 94.0654 (18)	1
Senecionine	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>5</sub>	336.1805	12.3	120.0808 (51); 94.0654 (45); 138.0912 (33); 308.1851 (19)	1
Monocrotaline NO	R	c	C <sub>16</sub> H <sub>23</sub> NO <sub>7</sub>	342.1547	5.2	137.0833 (76); 119.0729 (39); 120.0808 (33); 136.0755 (24); 118.0651 (23); 236.1278 (19); 94.0654 (19); 296.1491 (8); 138.0913 (7); 314.1587 (4)	1
3'-Acetylintermedine	R	m	C <sub>17</sub> H <sub>27</sub> NO <sub>6</sub>	342.1911	8.8	94.0655 (100); 138.0914 (22); 120.0809 (20); 156.1017 (15); 282.1697 (6)	2
3'-Acetylirinderme	H	m	C <sub>17</sub> H <sub>27</sub> NO <sub>6</sub>	342.1911	8.9	138.0913 (100); 120.0809 (36); 94.0655 (27); 156.1017 (19); 282.1707 (6)	2
7-Acetylirinderme	H	d	C <sub>17</sub> H <sub>27</sub> NO <sub>6</sub>	342.1911	9.6	120.0808 (100); 180.1016 (6); 94.0657 (4); 138.0913 (3);	3
7-Acetylechinatine	H	d	C <sub>17</sub> H <sub>27</sub> NO <sub>6</sub>	342.1911	9.7	120.0808 (100); 138.0913 (6); 198.1123 (5); 94.0653 (3); 282.0629 (2); 180.1021 (2)	3
3'-Acetylycopsamine	R	m	C <sub>17</sub> H <sub>27</sub> NO <sub>6</sub>	342.1911	9.8	94.0656 (100); 138.0913 (27); 120.0810 (20); 156.1020 (14); 282.1695 (4)	2
3'-Acetylechimatine	H	m	C <sub>17</sub> H <sub>27</sub> NO <sub>6</sub>	342.1911	10.2	138.0912 (100); 120.0809 (28); 94.0654 (26); 156.1018 (14); 282.1696 (3)	2
7-Acetylintermedine	R	d	C <sub>17</sub> H <sub>27</sub> NO <sub>6</sub>	342.1911	10.5	120.0807 (100); 198.1122 (6); 94.0654 (5); 180.1016 (5); 138.0912 (3)	2
7-Acetylycopsamine	R	d	C <sub>17</sub> H <sub>27</sub> NO <sub>6</sub>	342.1911	10.7	120.0807 (100); 94.0654 (7); 198.1123 (7); 180.1014 (4); 138.0913 (3)	2
Europine NO	H	m	C <sub>16</sub> H <sub>27</sub> NO <sub>7</sub>	346.1860	6.5	172.0965 (100); 111.0680 (16); 155.0939 (12); 256.1175 (12); 138.0912 (10); 94.0655 (10); 136.0754 (5); 137.0834 (2)	1
Erucifoline	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	350.1598	5.5	120.0808 (67); 138.0911 (39); 94.0653 (33); 322.1643 (5)	1
Riddelline	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	350.1598	7.1	120.0807 (62); 94.0654 (48); 138.0912 (42); 322.1644 (23)	2
Spartioidine NO	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	350.1598	11.0	120.0807 (91); 118.0652 (84); 119.0729 (77); 94.0654 (72); 136.0756 (39); 138.0912 (25); 322.1647 (10); 137.0833 (4)	2
Seneciphylline NO	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	350.1598	11.2	120.0808 (85); 94.0654 (79); 118.0652 (68); 119.0729 (54); 136.0756 (38); 138.0913 (25); 322.1646 (8); 137.0832 (5)	1
Retrorsine	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	352.1755	8.8	120.0808 (51); 94.0654 (36); 138.0912 (34); 324.1798 (18)	1
Jacobine	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	352.1755	10.4	120.0808 (100); 155.1065 (62); 122.0964 (57); 123.1043 (37); 94.0655 (34); 280.1547 (28); 140.1068 (11); 138.0913 (9)	1
Senecivermine NO	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	352.1755	12.6	120.0807 (57); 118.0652 (54); 94.0654 (53); 119.0731 (35); 136.0757 (21); 138.0912 (15); 324.1804 (9); 137.0838 (3)	1
Integerrimine NO	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	352.1755	12.7	118.0652 (68); 120.0807 (56); 94.0654 (52); 119.0729 (50); 136.0756 (41); 138.0912 (16); 324.1799 (8); 137.0833 (4)	2
Senecionine NO	R	c	C <sub>18</sub> H <sub>23</sub> NO <sub>6</sub>	352.1755	12.8	118.0651 (55); 120.0808 (50); 94.0654 (50); 136.0756 (43); 119.0730 (37); 138.0913 (17); 324.1799 (6); 137.0836 (5)	1
Trichodesmine	R	c	C <sub>18</sub> H <sub>27</sub> NO <sub>6</sub>	354.1911	8.1	222.1487 (100); 120.0808 (83); 94.0654 (30); 164.1069 (19); 308.1850 (17); 138.0912 (16)	1
Uplandicine	R	d	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	6.2	120.0808 (100); 94.0655 (6); 180.1018 (4); 198.1127 (3); 138.0915 (2)	2
3'-Acetylirinderme NO	H	m	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	9.9	172.0966 (100); 298.1646 (37); 138.0912 (28); 94.0655 (26); 111.0681 (26); 155.0939 (18); 136.0756 (11); 137.0834 (4)	2

7-Acetylintermediate NO	R	d	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	10.7	214.1070 (100); 137.0834 (50); 180.1016 (43); 136.0756 (24); 120.0808 (22); 119.0731 (19); 118.0651 (14)	2
3'-Acetylintermediate NO	R	m	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	10.8	172.0967 (100); 138.0913 (73); 94.0655 (61); 298.1649 (54); 111.0682 (33); 136.0757 (23); 155.0939 (21); 137.0837 (8)	3
3'-Acetylchimatine NO	H	m	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	10.9	172.0965 (100); 138.0912 (37); 298.1647 (35); 94.0654 (33); 111.0680 (23); 155.0938 (17); 136.0756 (16); 137.0833 (4)	3
7-Acetylycopsamine NO	R	d	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	11.0	214.1071 (100); 180.1015 (53); 137.0835 (51); 136.0757 (25); 120.0807 (23); 119.0732 (16); 118.0652 (14)	2
7-Acetylinderine NO	H	d	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	11.3	214.1070 (100); 137.0835 (81); 119.0730 (75); 120.0807 (62); 136.0756 (28); 118.0650 (25); 180.1019 (10); 298.1652 (5); 138.0911 (5)	3
7-Acetylchimatine NO	H	d	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	11.4	214.1072 (100); 137.0835 (76); 120.0808 (62); 119.0731 (60); 106.0654 (50); 136.0757 (25); 118.0653 (22); 298.1662 (3); 138.0915 (6)	3
3'-Acetylycopsamine NO	R	m	C <sub>17</sub> H <sub>27</sub> NO <sub>7</sub>	358.1860	11.7	172.0968 (100); 138.0914 (87); 94.0656 (77); 298.1648 (53); 111.0682 (33); 136.0758 (29); 155.0939 (17); 137.0837 (9)	2
Erucifoline NO	R	c	C <sub>18</sub> H <sub>25</sub> NO <sub>7</sub>	366.1547	6.2	118.0651 (93); 119.0730 (86); 94.0654 (80); 120.0808 (78); 136.0755 (68); 137.0835 (9); 138.0913 (10)	1
Riddelline NO	R	c	C <sub>18</sub> H <sub>25</sub> NO <sub>7</sub>	366.1547	7.7	120.0808 (100); 94.0654 (99); 118.0652 (78); 119.0730 (70); 136.0757 (51); 138.0913 (26); 338.1598 (7); 137.0832 (6)	2
Neosenkirkine	O	c	C <sub>19</sub> H <sub>27</sub> NO <sub>6</sub>	366.1911	12.9	168.1021 (100); 150.0915 (52); 122.0603 (25)	2
Senkirkine	O	c	C <sub>19</sub> H <sub>27</sub> NO <sub>6</sub>	366.1911	13.3	168.1018 (100); 150.0911 (34); 122.0600 (34); 348.1821 (2)	1
Jacobine NO	R	c	C <sub>18</sub> H <sub>25</sub> NO <sub>7</sub>	368.1704	7.3	120.0808 (100); 296.1488 (62); 94.0654 (28); 118.0651 (25); 119.0729 (22); 139.0992 (10); 138.0914 (8)	1
Retrorsine NO	R	c	C <sub>18</sub> H <sub>25</sub> NO <sub>7</sub>	368.1704	9.4	120.0808 (68); 118.0652 (67); 94.0654 (67); 136.0757 (53); 119.0731 (43); 138.0912 (24); 340.1743 (7); 137.0835 (7)	1
Uplandicine NO	R	d	C <sub>17</sub> H <sub>27</sub> NO <sub>8</sub>	374.1809	6.2	214.1070 (100); 137.0835 (35); 180.1015 (19); 136.0756 (16); 120.0807 (15); 119.0731 (9); 118.0651 (6)	2
Acetylseneciphylline NO	R	c	C <sub>20</sub> H <sub>35</sub> NO <sub>6</sub>	376.1755	16.0	118.0653 (100); 120.0809 (94); 94.0655 (76); 119.0731 (57); 136.0758 (47); 352.1490 (21); 138.0915 (16); 137.0594 (2)	2
Symphytine isomer 1	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>6</sub>	382.2224	15.4	120.0808 (100); 83.0496 (22); 238.1436 (6); 138.0914 (4); 94.0654 (2)	2
Symphytine isomer 2	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>6</sub>	382.2224	15.6	120.0808 (100); 83.0496 (61); 138.0914 (8); 238.1432 (8); 94.0655 (4); 220.1331 (1)	2
Echiumine	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>6</sub>	382.2224	15.7	120.0807 (100); 138.0912 (41); 94.0654 (15); 83.0496 (5); 220.1332 (5); 238.1439 (2)	2
5'-Acetylleuropine NO	H	m	C <sub>18</sub> H <sub>29</sub> NO <sub>8</sub>	388.1966	11.3	172.0965 (100); 137.0834 (58); 328.1749 (38); 111.0679 (11); 138.0911 (11); 136.0753 (5)	2
7-Angeloylhelitroine	H	d	C <sub>21</sub> H <sub>33</sub> NO <sub>6</sub>	396.2381	16.4	120.0809 (100); 138.0913 (6); 94.0654 (3)	3
Asperumine	H	d	C <sub>20</sub> H <sub>31</sub> NO <sub>7</sub>	398.2173	13.1	120.0809 (100); 138.0913 (6); 94.0653 (3); 238.1425 (2); 83.0495 (1)	2
Echimidine isomer 1	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>7</sub>	398.2173	13.2	120.0808 (100); 83.0497 (15); 238.1438 (2); 138.0916 (2); 94.0656 (2); 220.1332 (1)	3
Heliosupine	H	d	C <sub>20</sub> H <sub>31</sub> NO <sub>7</sub>	398.2173	13.3	120.0809 (100); 138.0915 (4); 238.1434 (2); 220.1333 (2); 94.0654 (2); 83.0496 (2)	2
Echimidine isomer 2	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>7</sub>	398.2173	13.4	120.0808 (100); 83.0495 (63); 138.0913 (5); 238.1424 (3); 94.0654 (3);	3
Echimidine	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>7</sub>	398.2173	13.4	120.0808 (100); 83.0496 (20); 238.1431 (2); 138.0913 (2); 94.0656 (2);	1
Symphytine NO	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>7</sub>	398.2173	15.5	254.1383 (100); 83.0496 (86); 137.0834 (53); 220.1330 (49); 120.0807 (39); 136.0758 (36); 119.0729 (27); 118.0652 (23)	2
Echiumine NO	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>7</sub>	398.2173	15.7	83.0496 (100); 254.1385 (75); 137.0834 (40); 220.1329 (38); 136.0757 (37); 120.0809 (29); 119.0730 (17); 118.0651 (15); 138.0913 (6)	2
Canescine (canescenine)	H	d	C <sub>20</sub> H <sub>33</sub> NO <sub>7</sub>	400.2330	12.4	120.0809 (100); 94.0655 (9); 138.0914 (5); 256.1535 (3); 83.0496 (1)	3
7-Tigloyllepupine	H	d	C <sub>21</sub> H <sub>35</sub> NO <sub>7</sub>	412.2330	14.9	120.0809 (100); 138.0915 (4); 94.0655 (3); 238.1434 (2); 220.1334 (1); 83.0495 (1)	2
Lasiocarpine	H	d	C <sub>21</sub> H <sub>35</sub> NO <sub>7</sub>	412.2330	15.1	120.0807 (100); 138.0911 (5); 94.0654 (4); 238.1437 (3); 220.1321 (2); 83.0495 (1)	1
7-Angeloylhelitroine NO	H	d	C <sub>21</sub> H <sub>33</sub> NO <sub>7</sub>	412.2330	16.7	120.0809 (100); 94.0655 (87); 138.0912 (52); 254.138 (51); 119.0731 (48); 136.0757 (47); 137.0836 (30); 118.0653 (25)	2

Echihumilime NO	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>8</sub>	414.2122	13.2	254.1387 (100); 137.0836 (44); 83.0496 (44); 120.0808 (26); 136.0757 (23); 220.1331 (16); 119.0732 (13); 118.0655 (11)	3
Echimidine NO	R	d	C <sub>20</sub> H <sub>31</sub> NO <sub>8</sub>	414.2122	13.4	254.1384 (100); 83.0496 (40); 137.0834 (34); 120.0807 (32); 136.0756 (21); 220.1331 (20); 119.0730 (17); 118.0653 (8); 138.0913 (3)	1
Vulgarine NO	R	m	C <sub>20</sub> H <sub>31</sub> NO <sub>8</sub>	414.2122	13.5	172.0967 (100); 256.1178 (49); 94.0656 (43); 138.0914 (42); 136.0757 (27); 111.0682 (17); 155.0938 (11)	3
Asperumine NO	H	d	C <sub>20</sub> H <sub>31</sub> NO <sub>8</sub>	414.2122	13.7	119.0731 (100); 120.0809 (76); 137.0836 (74); 94.0655 (64); 254.1384 (59); 136.0757 (56); 138.0913 (35); 121.0889 (34); 118.0652 (34)	3
Heliosupine NO	H	d	C <sub>20</sub> H <sub>31</sub> NO <sub>8</sub>	414.2122	14.1	94.0655 (100); 119.0731 (81); 137.0836 (81); 120.0809 (80); 254.1384 (75); 138.0915 (76); 136.0758 (74); 118.0652 (30)	2
Lithosamine	R	d	C <sub>20</sub> H <sub>33</sub> NO <sub>8</sub>	416.2279	8.3	120.0807 (100); 94.0654 (8); 138.0913 (4); 256.1540 (1)	3
Canescine (canescenine) NO	H	d	C <sub>20</sub> H <sub>33</sub> NO <sub>8</sub>	416.2279	12.7	272.1491 (90); 137.0835 (58); 136.0757 (41); 120.0809 (33); 119.0730 (23); 118.0651 (23); 138.0913 (21)	3
7-Tigloy/leuropine NO	H	d	C <sub>21</sub> H <sub>33</sub> NO <sub>8</sub>	428.2279	15.6	119.0730 (100); 120.0808 (95); 254.1381 (83); 137.0834 (81); 136.0756 (65); 118.0651 (32); 138.0913 (33)	2
Lastiocarpine NO	H	d	C <sub>21</sub> H <sub>33</sub> NO <sub>8</sub>	428.2279	15.8	94.0654 (100); 254.1384 (91); 120.0808 (90); 119.0731 (83); 136.0757 (77); 137.0835 (76); 138.0913 (72); 118.0652 (60)	1
Lithosamine NO	R	d	C <sub>20</sub> H <sub>33</sub> NO <sub>9</sub>	432.2228	8.7	272.1490 (100); 137.0835 (46); 120.0809 (26); 136.0757 (25); 119.0731 (18); 138.0916 (14); 118.0652 (11)	3
Thesimine-4'-ramnoside	T	-	C <sub>23</sub> H <sub>31</sub> NO <sub>7</sub>	434.2173	13.6	147.0440 (100); 142.1227 (26); 124.1121 (25); 119.0493 (17); 288.1594 (15)	3
3'-Acetylheliosupine	H	d	C <sub>22</sub> H <sub>33</sub> NO <sub>8</sub>	440.2279	15.0	120.0809 (100); 138.0913 (4); 238.1438 (2); 83.0495 (2); 220.1335 (1)	2
3'-Acetyllechiamine NO	R	d	C <sub>22</sub> H <sub>33</sub> NO <sub>8</sub>	440.2279	16.7	83.0496 (100); 254.1387 (40); 380.2062 (34); 220.1331 (34); 136.0755 (32); 137.0835 (30); 120.0809 (30); 118.0652 (18); 119.0730 (15)	3
Thesimine-4'-glucoside	T	-	C <sub>23</sub> H <sub>31</sub> NO <sub>8</sub>	450.2122	11.1	147.0440 (100); 142.1222 (20); 124.1122 (20); 119.0493 (17); 288.1591 (6)	3
5'-Acetylasiocarpine	H	d	C <sub>23</sub> H <sub>33</sub> NO <sub>8</sub>	454.2435	16.4	120.0808 (100); 138.0912 (7); 238.1439 (6); 94.0654 (3); 220.1333 (2); 83.0495 (2)	2
3'-Acetylheliosupine NO	H	d	C <sub>22</sub> H <sub>33</sub> NO <sub>9</sub>	456.2228	15.6	94.0655 (100); 119.0731 (95); 138.0913 (83); 120.0808 (61); 136.0759 (55); 254.1386 (52); 137.0836 (38); 118.0652 (35); 396.2015 (3)	2
5'-Acetylasiocarpine NO	H	d	C <sub>23</sub> H <sub>33</sub> NO <sub>9</sub>	470.2385	16.7	94.0655 (100); 120.0809 (87); 254.1382 (75); 138.0914 (73); 136.0757 (65); 119.0731 (63); 137.0834 (58); 118.0650 (30); 410.2153 (25)	2

<sup>a</sup> NO, N-oxide; <sup>b</sup> R, retronecine; H, heliotridine; O, otonecine; T, trachelanthamide; P, platynecine; S, supinidine; <sup>c</sup> m, monoester; d, open-chained diester; c, cyclic diester; <sup>d</sup> exact mass; <sup>e</sup> IL, identification level according to Metabolomics Standards Initiative.

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693 **Table 2.** Extraction efficiencies (EEs) and Limits of identification (LOIs) of the 28 target PAs in different food matrices.

Compound	Honey		Pollen		Black tea		Green tea		Herbal infusion		Dietary supplement	
	EE (SD)	LOI ( $\mu\text{g kg}^{-1}$ )	EE (SD)	LOI ( $\mu\text{g kg}^{-1}$ )	EE (SD)	LOI ( $\mu\text{g kg}^{-1}$ )	EE (SD)	LOI ( $\mu\text{g kg}^{-1}$ )	EE (SD)	LOI ( $\mu\text{g kg}^{-1}$ )	EE (SD)	LOI ( $\mu\text{g kg}^{-1}$ )
Echimidine	80.1 (2.8)	0.6	94.8 (0.4)	10.4	89.6 (3.2)	3.0	94.9 (8.9)	3.2	83.5 (8.2)	3.0	95.5 (1.6)	20.0
Echimidine NO	98.7 (2.4)	0.6	100.3 (2.8)	10.4	109.1 (5.0)	3.0	94.0 (7.2)	9.4	106.0 (6.5)	3.0	105.5 (0.6)	20.0
Erucifoline	94.3 (7.8)	0.6	95.6 (8.3)	15.6	88.8 (5.3)	7.5	89.6 (7.6)	11.7	93.5 (6.2)	3.0	101.7 (5.5)	20.0
Erucifoline NO	92.9 (4.4)	0.6	87.2 (7.2)	10.4	89.8 (7.1)	4.5	91.3 (2.4)	9.4	94.3 (5.9)	3.0	85.1 (2.3)	10.0
Europine	96.4 (4.9)	1.3	97.7 (5.7)	15.6	96.5 (5.6)	9.4	92.1 (2.7)	9.4	90.4 (3.2)	9.4	91.1 (4.4)	10.0
Europine NO	71.4 (4.5)	0.6	81.4 (9.1)	15.6	72.6 (5.6)	6.0	75.9 (5.8)	9.4	73.1 (6.9)	7.5	70.8 (3.2)	10.0
Heliotrine	87.1 (6.3)	0.6	98.5 (4.0)	4.2	91.5 (6.3)	3.0	90.9 (5.2)	4.7	96.1 (7.1)	3.0	93.8 (2.6)	10.0
Heliotrine NO	90.9 (3.0)	0.6	84.2 (1.0)	12.5	88.6 (4.9)	7.5	88.0 (6.1)	7.8	87.2 (5.7)	3.0	86.4 (0.6)	10.0
Intermedine	91.2 (1.6)	0.6	90.6 (1.5)	8.3	93.3 (0.9)	4.5	95.4 (3.7)	9.4	88.9 (0.9)	3.0	89.8 (6.9)	10.0
Intermedine NO	75.3 (0.8)	0.6	78.3 (3.7)	8.3	73.1 (3.7)	6.0	80.4 (4.3)	9.4	76.5 (1.4)	3.0	70.8 (2.6)	10.0
Jacobine	91.8 (8.0)	0.8	94.3 (11.1)	15.6	95.2 (3.9)	9.4	85.9 (9.1)	11.7	89.7 (7.1)	3.0	104.5 (6.0)	20.0
Jacobine NO	86.1 (5.6)	0.6	95.4 (6.2)	12.5	87.6 (5.9)	4.5	89.7 (3.9)	7.8	92.8 (2.4)	3.0	88.2 (1.6)	10.0
Lasiocarpine	89.8 (2.7)	0.6	99.2 (5.1)	10.4	100.2 (5.1)	3.0	99.1 (7.1)	4.7	95.7 (5.7)	3.0	96.9 (5.9)	10.0
Lasiocarpine NO	90.5 (0.8)	0.6	89.8 (4.6)	12.5	95.0 (4.3)	4.5	93.8 (2.3)	15.0	92.0 (6.2)	3.0	97.4 (4.0)	10.0
Lycopsamine	91.1 (0.4)	0.6	92 (2.1)	4.2	93.5 (1.6)	3.0	91.6 (2.4)	3.2	87.3 (0.5)	3.0	91.9 (4.0)	10.0
Lycopsamine NO	69.7 (5.7)	0.6	73.6 (0.7)	4.2	68.8 (4.5)	3.0	69.3 (5.8)	3.2	72.4 (5.3)	3.0	70.2 (6.1)	10.0
Monocrotaline	94.2 (4.9)	1.3	89.9 (8.4)	15.6	91.2 (3.0)	6.0	93.3 (6.1)	11.7	90.8 (7.1)	4.5	98.7 (5.5)	20.0
Monocrotaline NO	74.9 (5.7)	1.3	76.7 (0.9)	15.6	75.3 (6.9)	7.5	77.4 (5.3)	9.4	77.0 (5.4)	4.5	79.6 (6.9)	10.0
Retrorsine	100.7 (2.5)	0.6	80.0 (3.3)	15.6	98.8 (3.3)	4.5	104.2 (13.2)	6.2	74.1 (7.9)	4.5	99.2 (1.2)	20.0
Retrorsine NO	83.2 (3.1)	0.6	108.3 (5.2)	30.0	106.2 (3.2)	15.0	90.2 (8.6)	11.7	112.9 (4.0)	9.4	95.9 (1.6)	20.0
Senecionine	77.8 (2.6)	0.6	90.7 (9.3)	15.6	88.8 (3.3)	6.0	96.4 (5.7)	11.7	102.3 (5.4)	3.0	99.5 (2.6)	10.0
Senecionine NO	101.8 (3.4)	0.6	99.5 (6.8)	20.0	101.1 (2.8)	3.0	87.1 (5.8)	11.7	102.4 (4.2)	3.0	93.6 (1.0)	10.0
Seneciphylline	84.9 (3.6)	0.6	89.5 (7.1)	15.6	93.3 (0.8)	3.0	89.1 (4.8)	9.4	88.9 (0.9)	3.0	102.4 (3.9)	10.0
Seneciphylline NO	91.9 (6.4)	0.6	101.4 (4.0)	10.4	88.8 (4.1)	4.5	89.6 (4.6)	9.4	93.5 (6.2)	3.0	91.5 (0.5)	20.0
Senecivermine	81.3 (0.0)	0.6	86.0 (9.1)	20.0	91.6 (5.0)	9.4	88.3 (5.3)	7.8	93.4 (4.3)	3.0	100.9 (6.4)	20.0
Senecivermine NO	94.3 (5.6)	0.6	90.9 (8.4)	4.2	96.9 (3.1)	3.0	96.0 (3.7)	7.8	91.5 (6.4)	3.0	92.8 (0.9)	20.0
Senkirkine	99.2 (8.7)	0.6	102.6 (0.6)	4.2	96.8 (8.7)	3.0	97.2 (7.9)	3.2	94.3 (3.9)	3.0	98.1 (5.2)	10.0
Trichodesmine	95.2 (8.2)	1.3	97.3 (0.2)	15.6	90.5 (5.0)	4.5	92.3 (7.1)	7.8	88.2 (3.2)	3.0	96.2 (3.1)	10.0

694 <sup>a</sup>NO: N-oxide

**Highlights:**

- An analytical platform for the detection of PAs/PANOs in food matrices
- A diagnostic product ions filtering strategy for the characterization of PAs/PANOs
- An internal database of 779 known and expected unknown PAs/PANOs
- A spectral library containing HRMS/MS information of 118 PAs/PANOs
- The platform offers the possibility to detect both target and untarget PAs/PANOs

Fig. 1

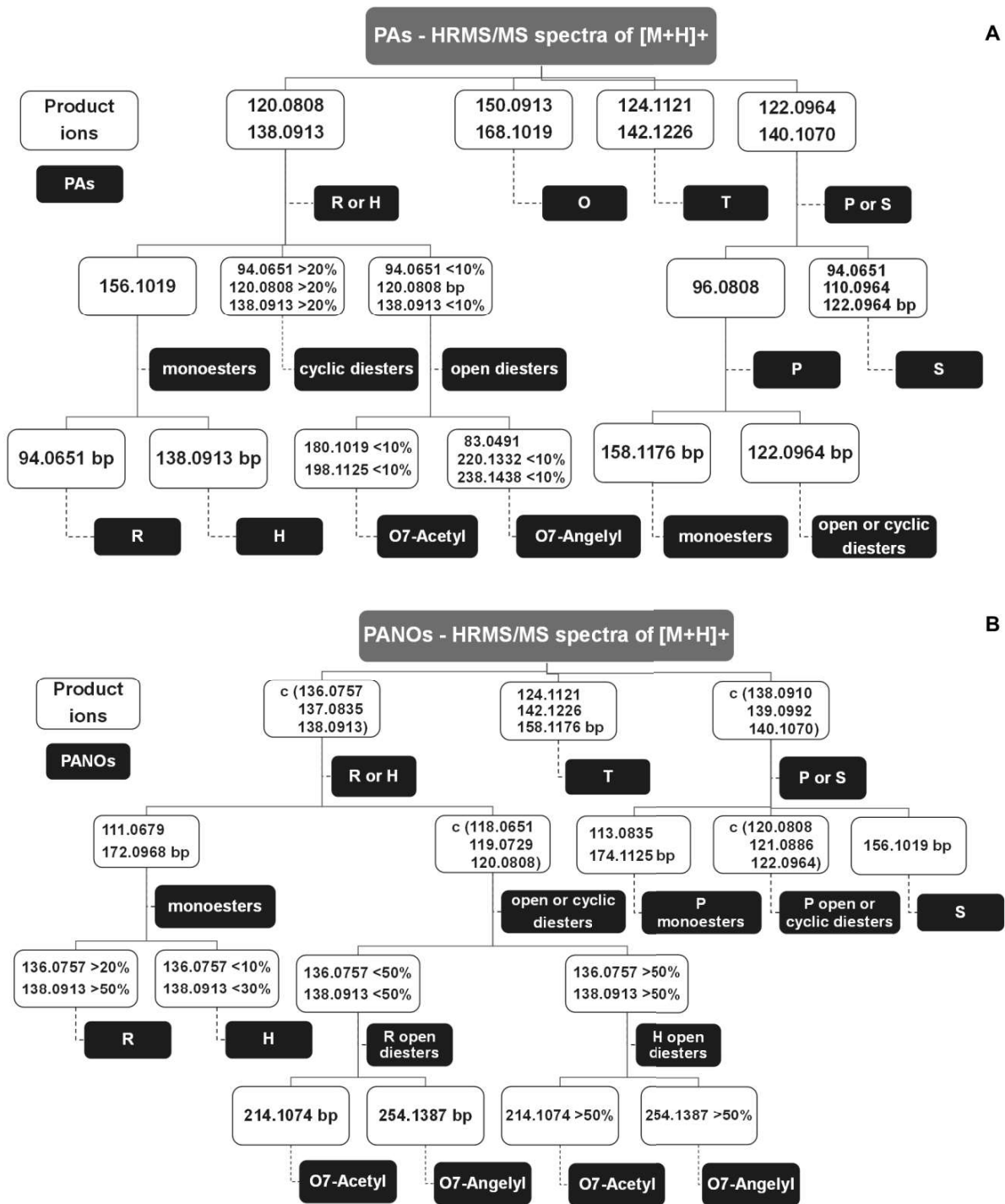
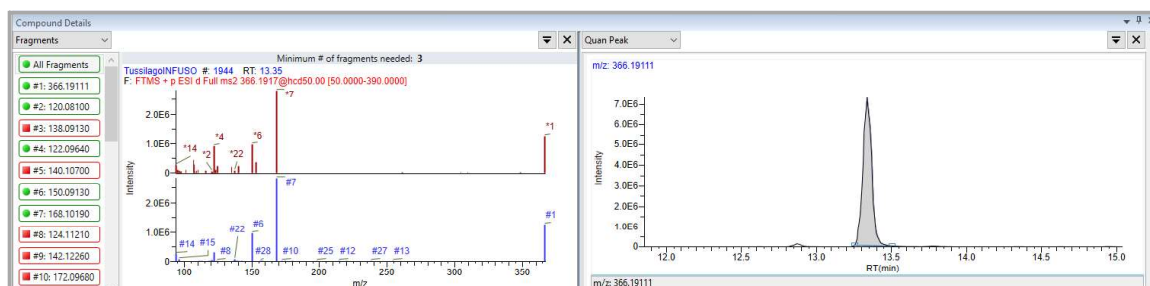


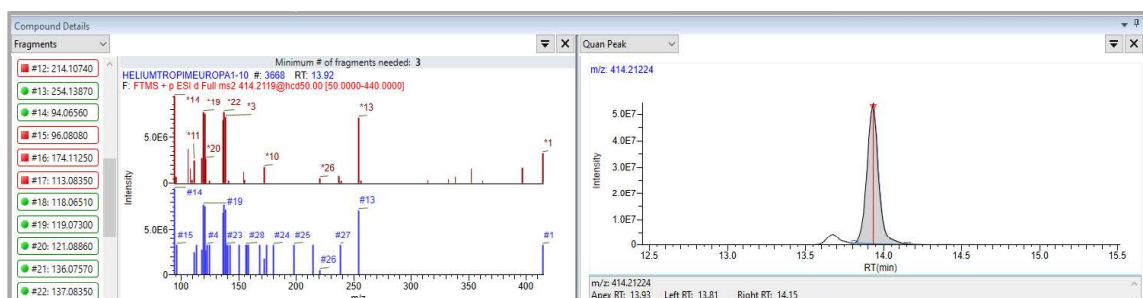
Fig. 2



A

Flowchart: Otonecine type PA

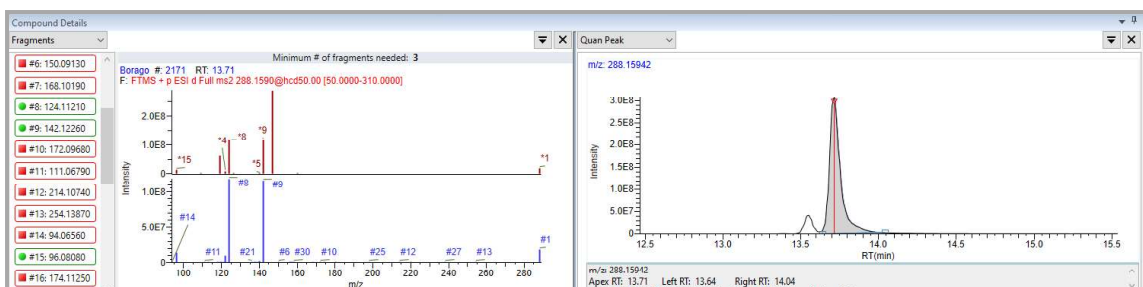
Name	Expected Unknowns	CAS Registry Number	Molecular formula <sup>b</sup>	Molecular Weight	[M+H] <sup>+</sup> (calculated m/z)	Necine base type <sup>c</sup>	Necic acid type	N-oxide	Isomer group	Isomer subgroup
Crotaverrine		60827-69-2	C19H27NO6	365.1833	366.1911	otonecine	cyclic diester		47	A
Emiline		36506-99-7	C19H27NO6	365.1833	366.1911	otonecine	cyclic diester		47	A
Neosenkirkine		57194-70-4	C19H27NO6	365.1833	366.1911	otonecine	cyclic diester		47	A
Senkirkine		2318-18-5	C19H27NO6	365.1833	366.1911	otonecine	cyclic diester		47	A



B

Flowchart: Heliotridine open-chained diester type PANO

Name	Expected Unknowns	CAS Registry Number	Molecular formula <sup>b</sup>	Molecular Weight	[M+H] <sup>+</sup> (calculated m/z)	Necine base type <sup>c</sup>	Necic acid type	N-oxide	Isomer group	Isomer subgroup
2'-epi-Heliosupine N-oxide		2171456-54-3	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	X	81	D
Asperumine N-oxide		54324-54-8	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	X	81	D
Heliosupine N-oxide (Cynoglossopine N-oxide)		31701-88-9	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	X	81	D



C

Flowchart: Trachelanthamide type PA

Name	Expected Unknowns	CAS Registry Number	Molecular formula <sup>b</sup>	Molecular Weight	[M+H] <sup>+</sup> (calculated m/z)	Necine base type <sup>c</sup>	Necic acid type	N-oxide	Isomer group	Isomer subgroup
Theshine		488-02-8	C17H21NO3	287.1516	288.1594	trachelanthamide	-		9	A

Fig. 3.

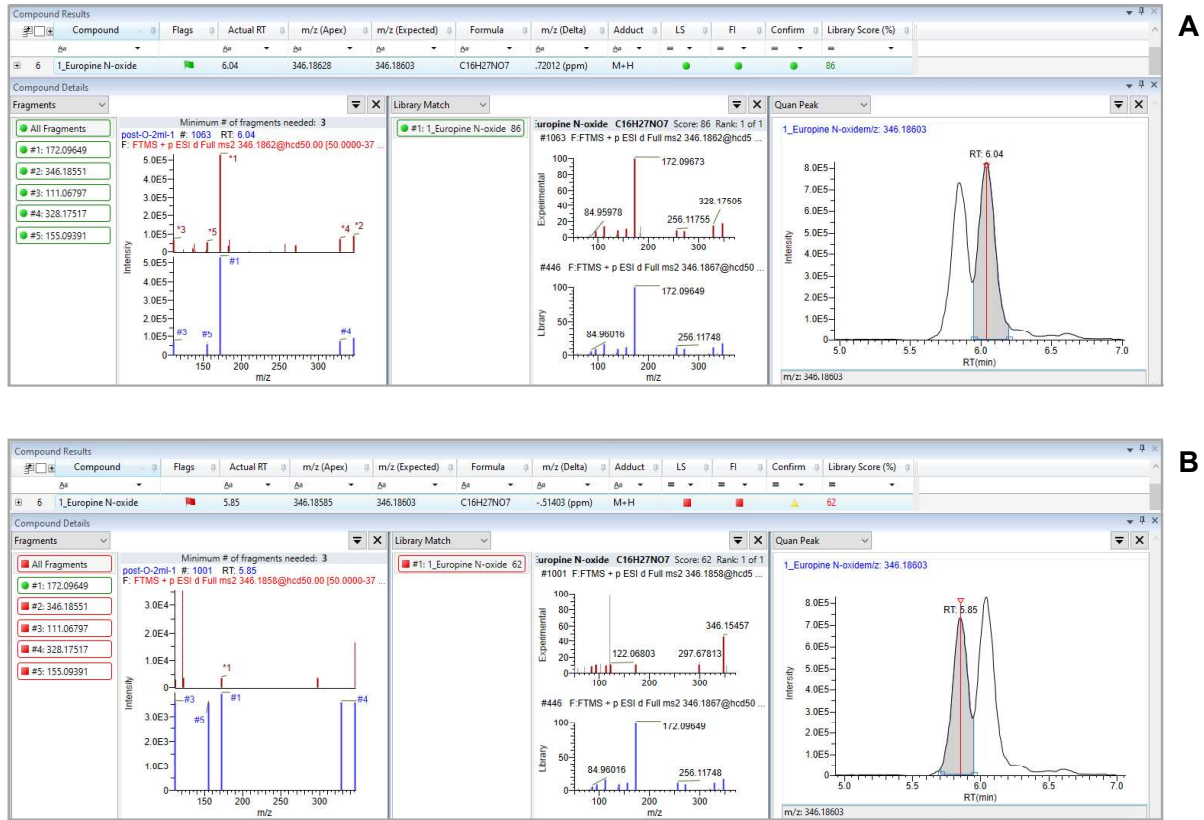


Fig. 4.

