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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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Original

An analytical platform for the screening and identification of pyrrolizidine alkaloids in food matrices with high risk of contamination / Rizzo, Serena; Celano, Rita; Piccinelli, Anna Lisa; Serio, Simona; Russo, Mariateresa; Rastrelli, Luca. - In: FOOD CHEMISTRY. - ISSN 0308-8146. - 406:(2023), p. 135058. [10.1016/j.foodchem.2022.135058]

Availability: This version is available at: https://hdl.handle.net/20.500.12318/140586 since: 2023-11-03T10:41:19Z

Published DOI: http://doi.org/10.1016/j.foodchem.2022.135058 The final published version is available online at:https://www.sciencedirect.

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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--

Manuscript Number:	FOODCHEM-D-22-06697R1
Article Type:	Research Article (max 7,500 words)
Keywords:	Bee products; Dietary supplements; Herbal products; Spectral library; Suspect screening; Target screening
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Abstract:	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 μ g kg–1) of 28 standards were fit-for-purpose in PA-monitoring applications, with a false negative rate < 1.3% at 4 μ g L–1. 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

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18 Abstract

19 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, 20 based on a diagnostic product ion filtering strategy for the characterization of PAs, and a target 21 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 employed. The limit of identification (0.6-30 μ g kg⁻¹) of 28 standards were fit-for-purpose in PA-25 monitoring applications, with a false negative rate < 1.3% at 4 µg L⁻¹. The wide-scope suspect 26 screening method allowed the tentative identification of 88 compounds. The screening of 282 27 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**

Pyrrolizidine alkaloids (PAs) are natural toxins produced by different plants (*Boraginaceae*, *Asteraceae* and *Fabaceae* families) as defense against insects and herbivores. The structures of PAs
consist of the 1-hydroxymethyl pyrrolizidine core (necine base) esterified with one or two aliphatic
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).

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

Beside the intake of PAs through herbal products containing PA-producing plants (Steinhoff, 2019), 46 in the last decades, numerous scientific reports have revealed a worrying contamination of PAs from 47 food products of plant origin (such as beehive and herbal products), mainly due to their accidental 48 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., *Echium spp.*, *Heliotropium spp.*, *Myosotis spp.*, and *Senecio spp.*) make the contamination of PAs a 51 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.

The European Food Safety Authority (EFSA), in its recent risk assessments, recognized PAs as undesirable substances in food and concluded that there is a possible human health concern related to chronic cumulative exposure to contaminated food products, such as teas, herbal infusions, beehive 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 spectrometry (LC-MS/MS) with unit resolution spectrometric analyzers (triple quadrupole and ion 67 68 trap). Selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM), is a wellestablished MS/MS acquisition mode for the targeted analysis of PAs, due to its high selectivity, 69 sensitivity, and robustness (Casado, Morante-Zarcero, & Sierra, 2022; Ma et al., 2018; Mulder et al., 70 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 presents limitations on the number of compounds to be analyzed in one run, requires the availability 73 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).

In recent years, high resolution mass spectrometry (HRMS) has been increasingly used as 77 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 unlimited number of molecules without the need of a compound-specific tune, carry out the 81 82 retrospective data analysis, and perform structural characterization of unknown or suspected compounds (Hird et al., 2014; Menger, Gago-Ferrero, Wiberg, & Ahrens, 2020; Rajski, 83 Petromelidou, Díaz-Galiano, Ferrer, & Fernández-Alba, 2021; Righetti et al., 2016). 84

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 PAs are known (Moreira et al., 2018; Schramm et al., 2019). The regulated list of PAs and PANOs 87 88 to monitor is limited and the development of advanced analytical approaches to detect further PAs, which can potentially contaminate plant-based matrices, is of huge importance to better understand 89 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 the knowledge about the distribution of these contaminants in foods. To achieve this goal, an 93 94 analytical procedure combining the salting-out assisted liquid-liquid extraction (SALLE) of aqueous extracts with ultra-high performance liquid chromatography coupled with high resolution tandem 95 mass spectrometry (UHPLC-HRMS/MS) was established. A systematic workflow, based on a 96 97 database (778 molecules) and a diagnostic product ion filtering strategy, was designed to first characterize PAs and PANOs from PA-producing plants and then create an in-house HRMS/MS 98 spectral library. Furthermore, two software-assisted processing methods were implemented to 99 100 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 commercial samples (high-throughput target screening and identification method). The proposed 102 103 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 teas, herbal infusions, and dietary supplements. 107

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 (MgSO₄·7H₂O), sodium sulfate (Na₂SO₄), sodium hydroxide (NaOH), sulfuric acid (H₂SO₄) and MS 115 grade formic acid (HCOOH) were purchased from Merck Chemicals (Milan, Italy). MS grade MeCN 116 and water (H₂O) were provided by Romil (Cambridge, UK). Ultrapure water (18 M Ω) was prepared 117 using a Milli-Q purification system (Millipore, Bedford, USA).

Reference standards (n = 30) (85-98 % HPLC grade) of echimidine, echimidine N-oxide, erucifoline, 118 erucifoline N-oxide, europine, europine N-oxide, heliotrine, heliotrine N-oxide, indicine, indicine N-119 120 oxide, intermedine, intermedine N-oxide, jacobine, jacobine N-oxide, lasiocarpine, lasiocarpine Noxide, lycopsamine, lycopsamine N-oxide, monocrotaline, monocrotaline N-oxide, retrorsine, 121 retrorsine N-oxide, senecionine, senecionine N-oxide, seneciphylline, seneciphylline N-oxide, 122 123 senkirkine, senecivernine, senecivernine N-oxide, and trichodesmine were provided by Merck Chemicals (Milan, Italy). Standard stock solutions were prepared for each analyte (1 mg mL^{-1}) in 124 MeOH and stored at -20 °C. Diluted solutions and standard mixtures were prepared in H₂O/MeOH 125 126 7:3 v/v.

127

128 *2.2. PA-producing plants and samples*

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

A total number of 282 commercial samples were analyzed. Honey (n = 72) and pollen (n = 6) samples from different botanical and geographical origins were obtained from Italian supermarkets, online

shops, and local beekeepers. Herbal infusions (n = 101, including 21 labelled as dietary supplements),

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

143

144 *2.3. Sample preparation*

The sample preparation procedure involved the aqueous extraction of PAs and PANOs followed by 145 146 Salting-out Assisted Liquid-Liquid Extraction (SALLE). Honey samples were homogenized by manual stirring (3 min), and a representative aliquot of 25 g was diluted to 100 mL with distilled 147 water and sonicated for 15 min (Rizzo, Celano, Campone, Rastrelli, & Piccinelli, 2022). Solid 148 149 matrices (pollen and solid forms of dietary supplements) were extracted with an acidic water solution, according to Mulder and co-workers (Mulder et al., 2018). Briefly, 1 g of each sample was extracted 150 with 20 mL (for pollen) and 10 mL (for dietary supplements) of acidic water (H₂SO₄, 0.05 M) by 151 152 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.

Aqueous extracts of each matrix were subjected to the same SALLE procedure, according to our previous study (Rizzo et al., 2022). Briefly, a 10 mL aliquot of the aqueous solution was brought to a concentration of 1 M of MgSO₄·7H₂O, 1.5 M Na₂SO₄, 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 H₂O/MeOH 7:3 v/v: in 500 μ L for honey, pollen, and 168 solid forms of plant-based dietary supplements and 200 μ L for herbal infusions and teas.

169

170 2.4 UHPLC-HRMS analysis

The analyses were conducted on an UltiMate 3000 UHPLC system (ThermoFisher Scientific, Milano, 171 172 Italy) interfaced via a heated electrospray ionization source (HESI-II) to a Q-Exactive mass spectrometer (ThermoFisher Scientific, Milano, Italy). The UHPLC system was equipped with a Luna 173 174 Omega Polar C18 (2.1 × 100 mm, 1.6 μm; Phenomenex, Bologna, Italy) column, operated at 40 °C with a flow rate of 400 μ L min⁻¹. The chromatographic separation was achieved using a binary 175 176 gradient of H₂O (A) and MeCN (B), both containing 0.1 % of formic acid; the elution gradient was 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; 9.5-177 178 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 % B, 5 min) were performed. The injection volume was set at 5 μ L. 180

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 °C; S-lens level, 55. Nitrogen was used as collision gas of the higher-energy collisional dissociation (HCD) cell. Data were acquired in Full MS/dd-MS² mode. The 184 resolution of the Full MS scans (scan range 250-500 m/z) was set at 70k (FWHM), the Automatic 185 186 Gain Control (AGC) target at 3e6, and the maximum IT (Injection time) at 250 ms. Each time the detector detected a peak corresponding to the accurate mass (± 5ppm) of a certain precursor ion of 187 the inclusion list associated to the method, these ions were isolated in the quadrupole, accumulated 188

in the C-trap, and finally accelerated in the HCD cell to be fragmented. The inclusion list associated 189 190 to the acquisition method was filled with 112 masses of precursor ions $([M+H]^+)$ (Table S1). The fragmentation was performed using the NCE (Normalized Collision Energy) technology, which 191 applies a stepped collisional energy scheme by combining low, medium, and high collision energies 192 capable of increasing the diversity of fragment ions generated; a range of collision energies between 193 40 and 60 was applied in this study. The recording parameters of the dd-MS² scans were set as 194 195 follows: mass resolution, 17.5 k (FWHM); AGC target, 2e4; maximum IT, 80 ms; isolation window, m/z 1.5; intensity threshold, 1.3e4; and dynamic exclusion: 2.0 s. The TopN parameter, which refers 196 to the number of ions to be triggered after a Full MS scan, was disabled to prevent precursor ions 197 198 other than those contained in the inclusion list from being isolated. Xcalibur software version 4.4 (ThermoFisher Scientific, Milano, Italy) was used for instrument control and data acquisition. 199

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

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

138.0913, 150.0913, 168.1019, 124.1121, 142.1226, 122.0964, 140.107, 156.1019, 94.0651, 215 96.0808, 110.0964, 122.0964, 180.1019, 198.1125, 83.0491, 220.1332, 238.1438, 158.1176, 216 136.0757, 137.0835, 158.1176, 139.0992, 111.0679, 172.0968, 118.0651, 119.0729, 113.0835, 217 174.1125, 121.0886, 214.1074, 254.1387 (section 3.3.2) were associated with each precursor ion. A 218 219 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 presence of PAs/PANOs analogues whenever a peak matched the molecular formula of the relative 223 224 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 construction of an in-house HRMS/MS spectral library. Therefore, the initial step involved the 229 230 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 PANOs (Table 1). Then, the CD was built by importing into the master method a csv containing the 232 233 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 ± 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 "identified" (green flag) when all the criteria were fulfilled, "found" when only the precursor ion was 239

encountered at the expected retention time (red flag), and "not found" (yellow flag) when none of thecriteria were met.

242

243 2.6. Validation of the target screening and identification method

The high-throughput screening and identification method was validated in terms of specificity, 244 245 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 et al., 2018). The validation studies were conducted on 28 out of 30 reference standards (indicine and 248 249 indicine N-oxide were excluded for co-elution reasons) in six food matrices: honey, pollen, back 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 possible to select or prepare a representative sample of a plant-based dietary supplement due to the 254 high variability of their composition. The specificity was evaluated by processing spiked (10 μ g L⁻¹) 255 256 and unspiked SALLE extracts of blank samples of each studied matrix. The EEs were determined by pre- and post-spiking the target analytes at a concentration of 10 μ g L⁻¹ of the SALLE extract 257 (corresponding to 10 μ g kg⁻¹ for honey, 100 μ g kg⁻¹ for pollen, 75 μ g kg⁻¹ for teas and herbal 258 infusions, and 50 μ g kg⁻¹ for dietary supplements) before and after the sample preparation procedure. 259 260 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 fortifying blank samples at eight concentration levels, ranging from 0.4 to 2 μ g L⁻¹ of the SALLE 262 263 extracts. LOIs were assigned for each target analyte at the concentration level that met all the identification criteria of the high-throughput target screening and identification method (section 264 2.5.2). Regarding plant-based dietary supplements, since it was not possible to find a representative 265

sample, LOIs were estimated as the lowest concentration at which a compound was identified in at 266 least 95 % of the blank samples. For this purpose, 20 blank samples (10 samples × 2 replicates) of 267 different composition were spiked before the extraction at 10 and 20 μ g kg⁻¹. The precision of the 268 method, estimated as false negative rates, was determined by fortifying 72 blank samples (36 samples 269 \times 2 replicates), including honey samples (n = 3), pollen samples (n = 3), herbal infusions (n = 10), 270 black (n = 5) and green (n = 5) teas, and dietary supplements (n = 10) at a concentration of 2 μ g L⁻¹ 271 (corresponding to $2 \mu g kg^{-1}$ for honey, $20 \mu g kg^{-1}$ for pollen, $15 \mu g kg^{-1}$ for teas and herbal infusions, 272 and 10 μ g kg⁻¹ for dietary supplements) and 4 μ g L⁻¹ (corresponding to 4 μ g kg⁻¹ for honey, 40 μ g 273 kg⁻¹ for pollen, 30 μ g kg⁻¹ for teas and herbal infusions, and 20 μ g kg⁻¹ for dietary supplements) of 274 275 the SALLE extracts, which correspond to the tenth and the fifth part of the lower limit of the Regulation (EU) 2020/2040 (Tea, *Camellia sinensis*, ML of 150 µg kg⁻¹). 276 277

278 **3. Results and discussion**

279 3.1 UHPLC-HRMS/MS analysis

The setting of the UHPLC conditions aimed at solving/minimizing one of the main problems 280 encountered during the chromatographic analysis of PAs and PANOs, that is the co-elution of 281 282 structural isomers impossible to distinguish by their MS/MS spectra. Some examples of these 283 similarities are offered by the isomeric groups indicine/intermedine/lycopsamine, 284 echinatine/rinderine, integerrimine/senecionine/senecivernine, echimidine/heliosupine, seneciphylline/spartioidine, and their N-oxides (Casado et al., 2022; Kaltner, Stiglbauer, Rychlik, 285 Gareis, & Gottschalk, 2019). Therefore, the chromatographic conditions were carefully optimized on 286 287 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 reference standards and further isomers. As already reported by Kaltner and co-workers, the best 289 290 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 acetylintermedine/7-acetyllycopsamine, amabiline/supinine, asperumine/heliosupine, lasiocarpine/7tigloyleuropine, their N-oxides, and neosenkirkine/senkirkine) resulted in well separated peaks. 295 296 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.

Different HRMS/MS acquisition modes were considered to evaluate the suitability of the detection method to the structural characterization and identification of PAs. Eventually, a data-dependent acquisition mode (Full MS/dd-MS²), with an inclusion list of prioritised masses, was selected as it proved to be efficient in terms of selectivity and ability to detect the target analytes at trace levels and provide high quality HRMS/MS spectra. The quality of the MS/MS spectra is crucial to obtain reliable
identifications of molecules in complex matrices, and the data-dependent acquisition mode provides
MS/MS spectra from specific precursor ions by dismissing the other precursor ions, which reduces
the risk of background noise and signal interferences (Rajski et al., 2021).

The Full MS/dd-MS² was adapted to the detection and characterization of a wide range of PAs at low 308 309 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 3.3.1). The dd-MS² scan was triggered on an inclusion list of accurate masses of [M+H]⁺ ions 311 312 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 analyses in complex matrices; in fact, in such conditions, the selected ions would correspond to the 315 316 matrix interferences. Furthermore, to detect and confirm the target analytes at low contamination levels, the minimum AGC target of the dd-MS² scan was set to a much lower value (10e3) than that 317 commonly used in Full MS/dd-MS² analyses (10e5-10e6). The optimal collision energies were 318 determined by analyzing the reference standards to obtain fragmentation spectra with significant 319 320 product ions. The developed acquisition method allowed to detect (Full MS) and identify (dd-MS²) target PAs and PANOs up to a concentration close to 1 μ g L⁻¹, with enough data points across the 321 322 chromatographic peaks (Full MS extracted ion chromatogram) and reliable fragmentation profiles.

323

324 **3.2 Sample preparation**

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

supplements) were extracted with acidified water, an extensively used solvent for the extraction of 330 331 these alkaloids from different food matrices due to the ability to provide exhaustive extraction and cleaner extracts (Casado et al., 2022; Kaltner et al., 2019; Mulder et al., 2018). Teas and herbal 332 333 infusions were extracted by infusion with boiling water to simulate the real exposure scenario to these contaminants (Casado et al., 2022; Mulder et al., 2018; Picron et al., 2018). The SALLE procedure 334 335 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 the tested matrices at 10 μ g L⁻¹ of each analyte in SALLE extracts (this level corresponds to 10 μ g 338 kg⁻¹ for honey, 100 μ g kg⁻¹ for pollen, 75 μ g L⁻¹ for teas and herbal infusions, 50 μ g L⁻¹ for dietary 339 340 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 profiles of the aqueous and SALLE extracts, indicating the efficiency of the procedure in extracting 344 345 naturally occurring PAs and PANOs from PA-producing plants.

346

347 **3.3 Identification strategy**

PAs show a striking variety of chemical structures being the result of the combination of a limited set 348 349 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 four types are 1,2-unsaturated PAs, while the types P and T are the corresponding saturated 354 derivatives of the R/H and S types, respectively. Except for the O type, in which N-oxides cannot be 355

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

362

363 *3.3.1 Database of PAs and PANOs*

A wide database of PAs and PANOs was created from a systematic survey of the literature to support 364 365 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 of this class of alkaloids (e. g. N-oxide derivatives). The database (778 molecules) was filled with 367 368 structural information (CAS number, elemental composition, molecular weight, accurate mass of precursor ions), and the groups of PAs with identical molecular formula were further classified into 369 370 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. A systematic flowchart (Fig. 1) was designed to delineate the fragmentation patterns of PAs and 380 PANOs by studying the HRMS/MS spectra of the reference standards, online spectral libraries, and 381

previous studies (Mädge, Gehling, Schöne, Winterhalter, & These, 2020; Ruan et al., 2012; These,
Bodi, Ronczka, Lahrssen-Wiederholt, & Preiss-Weigert, 2013). Important clarifications regarding the
ion ratios arose during the collection of HRMS/MS spectra of the spectral library. The Fig. S2 and
S3 show the chemical structures, molecular formulas, and exact masses of the key product ions
required for the subdivision of PAs and PANOs into the different groups and subgroups of the Fig.
1. The flowchart was divided in two subsets since the HRMS/MS spectra immediately allowed to
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 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 390 391 122.0964 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/z395 156.1019. Depending on the base peak, monoesters can be differentiated into R (bp at m/z 94.0654) and H types (bp at m/z 138.0910). Cyclic and open-chained diesters of R/H type can be differentiated 396 397 based on the relative intensity of the product ions at m/2 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 Whitin R and H types, the base peak at m/z 172.0968 and the product ion at m/z 111.0679 identify the

winting and it types, the base peak at *m*/2 172.0908 and the product for at *m*/2 111.0079 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

the cluster 136 *to* 138, monoesters can be differentiated into R (higher intensities) and H (lower intensities) types. The same applies to open-chained diesters, albeit in inverted ratios. In both groups, the product ions at m/z 214.1074 or 254.1387 indicate the presence of an acetyl or angeloyl/tigloyl group respectively at the C-7 position of the open-chained diesters. S and P types of PANOs are instead characterized by the cluster 138 *to* 140 (m/z 138.0913, 139.0992, and 140.1070); the base peak at m/z 156.1019 and the product ion at m/z 139.0992 allow to differentiate S from P types (Fig. 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 detection and characterization of PAs and PANOs. The wide-scope suspect screening method was 419 420 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 of the diagnostic product ions filtering strategy during the identification of suspect PAs. 432

434 **3.4 HRMS/MS spectral library**

435 The diagnostic product ions filtering strategy was applied to 10 PAs-producing plants to detect and identify as much compounds as possible and collect their spectra into an HRMS/MS spectral library. 436 437 The plant profiles (Table S3) were defined by comparing the information of the diagnostic product ions filtering strategy with literature information, MS/MS spectra available on online databases and 438 439 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 officinale). 84 PAs and PANOs other than the reference standards were detected, including two 442 443 "expected unknowns": canescine/canescenine N-oxide (m/z 416.2275, $C_{20}H_{34}NO_8^+$) and lithosenine N-oxide $(m/z 432.2223, C_{20}H_{34}NO_9^+)$ in L. officinale. Their structures, hypothesized on the basis of 444 the key product ions of R/H open-chained diester type of PANOs (Fig. S4), were further supported 445 446 by chemotaxonomic data (El-Shazly & Wink, 2014; Kopp, Abdel-Tawab, & Mizaikoff, 2020). 447 Moreover, the product ion at m/z 272.1492 (C₁₃H₂₂NO₅⁺) corresponds to a hydroxy isovaleroyl residue (typical necic acid of canescine and lithosenine) at the C-7 position. Besides, the clusters at m/z 136 448 449 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

455 samples, four further compounds were characterized, bringing the number of spectra of the library to

ions filtering strategy and chemotaxonomic data (MSI, L3). During the analysis of the commercial

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 identification method. The identification criteria were set as follows: the presence of the precursor, a 462 mass tolerance $\leq \pm 5$ ppm, the expected retention time (± 0.2 min), at least three product ions (± 5 463 ppm), and a library match score higher than 70%. This post-acquisition data evaluation was combined 464 465 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 peak in a dietary supplement sample. As can be seen, europine N-oxide (Fig. 3A) met all the 468 469 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 them (mass tolerance of precursor ion, 0.5 ppm; retention time within the range). These results 471 472 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 screening methods (Magnusson & Örnemark, 2014; Pihlstrom et al., 2018). Regarding the remaining 481 PAs and PANOs, for which no reference standards were available, the detection and identification 482 483 can be achieved although it is not possible to specify qualitative performance parameters (Pihlstrom et al., 2018). 484

The method specificity, defined as the ability of the method to distinguish the analyte from any other 485 486 matrix interferences, was evaluated by comparison between different blank and spiked samples of the studied matrices. No interfering peaks were observed at the expected retention time for all the 28 487 488 reference standards in honey and pollen samples. On the contrary, some plant-based samples showed the presence of interfering peaks close to the some of the target analytes. The proposed method 489 490 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.

To achieve an accurate identification of the target analytes and minimize the risk of false positives, 493 diagnostic information, that meets the defined criteria, is required (Lehotay, Sapozhnikova, & Mol, 494 495 2015). The LOIs, defined as the lowest concentration that fulfill all the identification criteria of the method, were established to estimate the threshold concentrations at which the identification become 496 reliable. LOIs of the 28 target analytes in the six tested matrices ranged from 0.6 to 30 μ g kg⁻¹ (Table 497 498 2). The method was able to detect and identify all the target analytes in the SALLE extract at a concentration of 2 µg L⁻¹, except for echimidine, echimidine N-oxide, erucifoline, jacobine, 499 monocrotaline, retrorsine, retrorsine N-oxide, seneciphylline N-oxide, senecivernine, senecivernine 500 N-oxide, which were detected from 4 μ g L⁻¹ in dietary supplements. The LOIs demonstrated to be 501 502 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).

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

for most of the analytes, excluding those with a LOI of 20 μ g kg ⁻¹ in dietary supplements (and erucifoline N-oxide, senecionine, seneciphylline and senecivernine in teas and infusions	$^{-1}$) as well
and erucifoline N-oxide, senecionine, seneciphylline and senecivernine in teas and infusions	s (Table 2)
	ns.

514

515 **3.7 Analysis of commercial samples**

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

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

The qualitative analysis of the samples revealed the presence of 60 PAs/PANOs in 59 % of the 528 529 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, spartioidine, usaramine, and their N-oxides) and 28 were PAs included in the HRMS/MS spectral 533 534 library but not mentioned in the Regulation or in the EFSA's list of relevant contaminants of plant matrices. Among the studied matrices, honey was found to be the most contaminated one as 89 % of 535 the samples tested positive to the presence of PAs. In decreasing order of contamination, follow 536

dietary supplements (58 %), pollen (50 %), herbal infusions (46 %), and teas (39 %). Regarding the 537 538 contamination profile, Fig. 4 shows the PAs and PANOs detected in honey, herbal infusions, and plant-based dietary supplements, which turned out to be the matrices with the widest profiles of 539 contamination (32, 34 and 49 analytes detected, respectively). In detail, the most frequently detected 540 541 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, heliotrine N-oxide, lasiocarpine, and senecionine for dietary supplements. The qualitative data on the 545 546 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 hand, 47 % of the PAs detected in honey were not included in the lists of relevant PAs to be monitored; 548 echimidine isomer 1 (55 %) and 2 (59 %), 5-hydroxyindicine (41 %) and the sum of symphytines 1 549 and 2 (22 %) were the most prevalent. 550

551

552 **3.8 Conclusions**

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

This analytical platform offers the possibility to interrogate the samples on the presence of the 118 target PAs and PANOs of the target screening method, and to identify additional unreported analogues. The complementary mode of operation of the wide-scope suspect screening method and the high-throughput target screening and identification method makes the procedure versatile and state-of-the-art. The HRMS/MS spectral library can be continuously implemented with newlyidentified compounds according to the proposed strategy.

Furthermore, the possibility of adding further molecular masses to the inclusion list of the Full MS/dd-MS² acquisition method, each time a PA is identified, allows to considerably broaden the identification range since each molecular mass can identify multiple structural isomers. Finally, the non-dependence of the platform on the purchase of reference standards not only lowers the cost of the procedure but also solves the problem of the lack of reference standards of these toxins. 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.

572 **CRediT author statement**

Serena Rizzo: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; 573 Software; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. Rita 574 575 Celano: Conceptualization; Investigation; Methodology; Supervision; Validation; Roles/Writing -576 original draft; Writing - review & editing. Simona Serio: Formal analysis; Software. Anna Lisa 577 Piccinelli: Conceptualization; Data curation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & 578 editing. Mariateresa Russo: Conceptualization; Funding acquisition; Writing - review & editing. 579 580 Luca Rastrelli: Conceptualization; Funding acquisition; Project administration; Resources; Writing - review & editing. 581

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676	Figures	cantions.
070	riguics	captions.

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

679

680	Fig. 2. Mode of o	peration of the diagnostic	product ions filtering strategy	for the	identification of ((A)
	2		0 01			. /

senkirkine (MSI, L1), (B) heliosupine N-oxide (MSI, L2), and (C) thesinine (MSI, L3).

682

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.

Name ^a	Necine _{base} ^b	Necic acid ^c	Molecular formula	₄ [H+W]	TR (min)	Diagnostic product ions, m/z (relative abudance)	Ш°
7-Tiglovlretronecine NO	R	a a	C13H10NO4	2.54 1387	7.2	106 0653 (100): 83 0496 (34): 111 0679 (28): 136 0757 (21): 94 0654 (18)	2
	: -		C II NO	F0C1.15C	1		1 c
/-Angeloyiretronecine INO	K	E	C13H19INU4	1001.402	1.4	(nc) noon (11) (11) (ccan 44 (7c) sc/nc1 (0) 44 (nc) + (nc	7
9-Tigloylretronecine NO	R	ш	$C_{13}H_{19}NO_{4}$	254.1387	10.4	93.0577 (88); 136.0757 (77); 138.0913 (35); 137.0833 (30); 94.0654 (30); 108.0809 (20)	2
9-Angeloylretronecine NO	R	ш	$C_{13}H_{19}NO_4$	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	1	$C_{15}H_{25}NO_4$	284.1856	8.1	122.0965 (100); 140.1069 (66); 70.0657 (23); 110.0967 (12); 94.0655 (11)	2
Amabiline	S	ı	$C_{15}H_{25}NO_4$	284.1856	8.4	122.0964 (100); 140.1069 (90); 70.0657 (28); 110.0967 (16); 94.0655 (13)	2
Spilanthine	Ţ		$C_{15}H_{25}NO_4$	284.1856	9.2	142.1227 (100); 124.1122 (56); 96.0811 (8); 70.0657 (6)	2
Viridiflorine	F		$C_{15}H_{27}NO_4$	286.2013	7.9	142.1227 (100); 125.1198 (7); 70.0657 (6); 124.1121 (5); 96.0814 (1)	2
Cynaustraline	F	1	$C_{15}H_{27}NO_4$	286.2013	8.2	142.1225 (100); 124.1120 (52); 70.0657 (12); 86.0968 (9); 96.0810 (5)	2
Thesinine	F	1	$C_{17}H_{21}NO_3$	288.1594	13.7	147.0441 (100); 142.1227 (40); 124.1122 (39); 119.0493 (18); 96.0812 (5)	3
Heleurine	s	1	$C_{16}H_{27}NO_4$	298.2013	12.0	122.0965 (100); 140.1069 (57); 94.0655 (13); 110.0966 (12); 70.0657 (11)	2
Intermedine	R	ш	C ₁₅ H ₂₅ NO ₅	300.1805	5.3	94.0654 (100); 156.1018 (47); 138.0912 (39); 120.0807 (16); 82.0656 (7)	-
Indicine	R	ш	C ₁₅ H ₂₅ NO ₅	300.1805	5.5	94.0654 (100); 156.1016 (46); 138.0912 (35); 120.0808 (18); 82.0655 (7)	-
Lycopsamine	R	ш	C ₁₅ H ₂₅ NO ₅	300.1805	5.6	94.0654 (100); 156.1018 (55); 138.0912 (35); 120.0807 (18); 82.0656 (7)	1
Rinderine	Н	ш	C ₁₅ H ₂₅ NO ₅	300.1805	5.9	138.0913 (100); 156.1019 (50); 120.0808 (31); 94.0655 (24); 82.0656 (12)	2
Echinatine	Н	ш	C ₁₅ H ₂₅ NO ₅	300.1805	6.0	138.0912 (100); 156.1018 (55); 120.0808 (33); 94.0654 (26); 82.0656 (13)	2
Supinine NO	s		C ₁₅ H ₂₅ NO ₅	300.1805	8.7	156.1020 (100); 139.0992 (34); 120.0809 (24); 122.0965 (22); 121.0887 (9); 138.0913 (7); 960812 (7)	2
Amabiline NO	s	I	C ₁₅ H ₂₅ NO ₅	300.1805	9.1	156.1019 (100); 139.0991 (32); 120.0808 (26); 122.0965 (26); 121.0887 (10); 138.0913 (8); 960812 (7)	3
Curassavine	Г	1	$C_{16}H_{29}NO_4$	300.2169	11.9	142.1227 (100); 124.1122 (75); 156.1022 (12); 70.0658 (12); 96.0812 (10)	7
Dihydroechinatine (rinderine)	Р	ш	$C_{15}H_{27}NO_5$	302.1962	6.5	158.1176 (100); 140.1069 (56);122.0963 (41); 96.0812 (18)	3
Dihydrointermedine	Р	ш	$C_{15}H_{27}NO_5$	302.1962	9.9	158.1175 (100); 140.1068 (55); 122.0964 (36); 96.0811 (14)	ю
Dihydrolycopsamine	Р	ш	C ₁₅ H ₂₇ NO ₅	302.1962	6.7	158.1175 (100); 140.1069 (55); 122.0965 (34); 96.0810 (12)	3
Viridiflorine NO	Г	1	$C_{15}H_{27}NO_5$	302.1962	8.5	158.1176 (100); 124.1121 (16); 141.1148 (9), 140.1071 (7); 122.0966 (2)	θ
Helioamplexine	R	ш	$C_{16}H_{27}NO_5$	314.1962	7.4	94.0655 (100); 156.1019 (36); 138.0914 (30); 120.0809 (17); 82.0657 (9)	7
Heliotrine	Н	ш	$C_{16}H_{27}NO_5$	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	1	$C_{16}H_{27}NO_5$	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)	7
5'-Hydroxyindicine	R	ш	$C_{15}H_{25}NO_6$	316.1755	1.9	94.0654 (100); 138.0912 (43); 156.1017 (25); 120.0808 (18); 82.0656 (5); 108.0809 (2)	7
5'-Hydroxyintermedine (lycopsamine)	R	ш	$C_{15}H_{25}NO_6$	316.1755	3.0	94.0654 (100); 138.0912 (40); 156.1018 (26); 120.0808 (16); 82.0655 (5); 108.0809 (2)	Э
5'-Hydroxyechinatine (rinderine)	Η	ш	$C_{15}H_{25}NO_6$	316.1755	3.5	138.0913 (100); 94.0653 (28); 72.0813 (23); 156.1017 (21); 120.0809 (8)	7
Rinderine NO	Н	в	$C_{15}H_{25}NO_6$	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)	7
Echinatine NO	Η	ш	$C_{15}H_{25}NO_6$	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)	7
Intermedine NO	R	ш	$C_{15}H_{25}NO_6$	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	ш	$C_{15}H_{25}NO_6$	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
Lycopsamine NO	R	ш	$C_{15}H_{25}NO_6$	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	Р	ш	$C_{15}H_{27}NO_6$	318.1911	7.6	174.1122 (100); 113.0837 (20); 96.0810 (3); 140.1069 (2); 138.0913 (1); 139.0990 (1)	3
Dihydrolycopsamine NO	Р	ш	$C_{15}H_{27}NO_6$	318.1911	7.7	174.1122 (100); 113.0837 (20); 96.0811 (3); 140.1070 (2); 138.0912 (1); 139.0990 (1)	3

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

Dihydrorinderine NO	Р	ш	$C_{15}H_{27}NO_6$	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	Ρ	ш	$C_{15}H_{27}NO_6$	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	$C_{16}H_{23}NO_{6}$	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	Н	ш	$C_{16}H_{27}NO_6$	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)	-
Helioamplexine NO	R	ш	$C_{16}H_{27}NO_6$	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	Н	ш	$C_{16}H_{27}NO_6$	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 (rinderine) NO	Η	ш	$C_{15}H_{25}NO_7$	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)	7
5'-Hydroxyintermedine (lycopsamine) NO	R	ш	$C_{15}H_{25}NO_7$	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	$C_{18}H_{23}NO_5$	334.1649	9.8	120.0807 (67); 94.0654 (41); 138.0912 (35); 306.1696 (21)	7
Seneciphylline	R	c	$C_{18}H_{23}NO_5$	334.1649	10.1	120.0807 (58); 94.0654 (50); 138.0912 (38); 306.1697 (22)	1
Senecivernine	R	ပ	$C_{18}H_{25}NO_5$	336.1805	12.1	120.0808 (41); 138.0913 (27); 308.1855 (23); 94.0654 (18)	-
Senecionine	R	ပ	$C_{18}H_{25}NO_5$	336.1805	12.3	120.0808 (51); 94.0654 (45); 138.0912 (33); 308.1851 (19)	
Monocrotaline NO	R	c	$C_{16}H_{23}NO_7$	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)	-
3'-Acetylintermedine	R	ш	$C_{17}H_{27}NO_6$	342.1911	8.8	94.0655 (100); 138.0914 (22); 120.0809 (20); 156.1017 (15); 282.1697 (6)	7
3'-Acetylrinderine	Η	в	$C_{17}H_{27}NO_6$	342.1911	8.9	138.0913 (100); 120.0809 (36); 94.0655 (27); 156.1017 (19); 282.1707 (6)	2
7-Acetylrinderine	Н	p	$C_{17}H_{27}NO_6$	342.1911	9.6	120.0808 (100); 180.1016 (6); 94.0657 (4); 138.0913 (3);	e
7-Acetylechinatine	Н	p	$C_{17}H_{27}NO_6$	342.1911	9.7	120.0808 (100); 138.0913 (6); 198.1123 (5); 94.0653 (3); 282.0629 (2); 180.1021 (2)	ŝ
3'-Acetyllycopsamine	R	н	$C_{17}H_{27}NO_6$	342.1911	9.8	94.0656 (100); 138.0913 (27); 120.0810 (20); 156.1020 (14); 282.1695 (4)	7
3'-Acetylechinatine	Н	ш	$C_{17}H_{27}NO_6$	342.1911	10.2	138.0912 (100); 120.0809 (28); 94.0654 (26); 156.1018 (14); 282.1696 (3)	2
7-Acetylintermedine	R	p	$C_{17}H_{27}NO_6$	342.1911	10.5	120.0807 (100); 198.1122 (6); 94.0654 (5); 180.1016 (5); 138.0912 (3)	7
7-Acetyllycopsamine	R	p	$C_{17}H_{27}NO_6$	342.1911	10.7	120.0807 (100); 94.0654 (7); 198.1123 (7); 180.1014 (4); 138.0913 (3)	7
Europine NO	Н	ш	$C_{16}H_{27}NO_7$	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	ပ	$C_{18}H_{23}NO_6$	350.1598	5.5	120.0808 (67); 138.0911 (39); 94.0653 (33); 322.1643 (5)	-
Riddelliine	R	c	$C_{18}H_{23}NO_6$	350.1598	7.1	120.0807 (62); 94.0654 (48); 138.0912 (42); 322.1644 (23)	7
Spartioidine NO	R	с	$C_{18}H_{23}NO_6$	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)	7
Seneciphylline NO	R	c	$C_{18}H_{23}NO_6$	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)	
Retrorsine	R	ပ	$C_{18}H_{25}NO_6$	352.1755	8.8	120.0808 (51); 94.0654 (36); 138.0912 (34); 324.1798 (18)	-
Jacobine	R	S	$C_{18}H_{25}NO_6$	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
Senecivernine NO	R	с	$C_{18}H_{25}NO_6$	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	ပ	$C_{18}H_{25}NO_6$	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)	7
Senecionine NO	R	ပ	$C_{18}H_{25}NO_6$	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	с	$C_{18}H_{27}NO_6$	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	р	$C_{17}H_{27}NO_7$	358.1860	6.2	120.0808 (100); 94.0655 (6); 180.1018 (4); 198.1127 (3); 138.0915 (2)	5
3-Acetylrinderine NO	Н	E	$C_{17}H_{27}NO_7$	358.1860	6.6	172.0966 (100); 298.1646 (37); 138.0912 (28); 94.0655 (26); 111.0681 (26); 155.0939 (18); 136.0756 (11); 137.0834 (4)	5
						32	

7-Acetvlintermedine NO	R	p	$C_{17}H_{27}NO_7$	358,1860	10.7	214 1070 (100): 137 0834 (50): 180 1016 (43): 136 0756 (24): 120 0808 (22): 119 0731 (19): 118 0651	7
		1				(14)	I
3'-Acetylintermedine NO	R	ш	$C_{17}H_{27}NO_7$	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)	ю
3'-Acetylechinatine NO	Н	E	$C_{17}H_{27}NO_7$	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)	б
7-Acetyllycopsamine NO	К	p	$C_{17}H_{27}NO_7$	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-Acetylrinderine NO	Н	р	$C_{17}H_{27}NO_7$	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);	ŝ
7-Acetylechinatine NO	Н	p	$C_{17}H_{27}NO_7$	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)	ŝ
3'-Acetyllycopsamine NO	R	E	$C_{17}H_{27}NO_7$	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)	7
Erucifoline NO	R	c	$C_{18}H_{23}NO_7$	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)	
Riddelliine NO	R	c	$C_{18}H_{23}NO_7$	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)	7
Neosenkirkine	0	c	$C_{19}H_{27}NO_6$	366.1911	12.9	168.1021 (100); 150.0915 (52); 122.0603 (25)	2
Senkirkine	0	c	$C_{19}H_{27}NO_6$	366.1911	13.3	168.1018 (100); 150.0911 (34); 122.0600 (34); 348.1821 (2)	
Jacobine NO	R	c	$C_{18}H_{25}NO_7$	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)	
Retrorsine NO	Я	ပ	$C_{18}H_{25}NO_7$	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	р	$C_{17}H_{27}NO_8$	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	ပ	$C_{20}H_{25}NO_6$	376.1755	16.0	118.0653 (100); 120.0809 (94); 94.0655 (76); 119.0731 (57); 136.0758 (47); 332.1490 (21); 138.0915 (16); 137.0594 (2)	7
Symphytine isomer 1	R	р	$C_{20}H_{31}NO_6$	382.2224	15.4	120.0808 (100); 83.0496 (22); 238.1436 (6); 138.0914 (4); 94.0654 (2)	2
Symphytine isomer 2	R	p	$C_{20}H_{31}NO_6$	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	p	$C_{20}H_{31}NO_6$	382.2224	15.7	120.0807 (100); 138.0912 (41); 94.0654 (15); 83.0496 (5); 220.1332 (5); 238.1439 (2)	7
5'-Acetyleuropine NO	Н	ш	$C_{18}H_{29}NO_8$	388.1966	11.3	172.0965 (100); 137.0834 (58); 328.1749 (38); 111.0679 (11); 138.0911 (11); 136.0753 (5)	7
7-Angeloylheliotrine	Н	р	$C_{21}H_{33}NO_6$	396.2381	16.4	120.0809 (100); 138.0913 (6); 94.0654 (3)	3
Asperumine	Н	p	$C_{20}H_{31}NO_7$	398.2173	13.1	120.0809 (100); 138.0913 (6); 94.0653 (3); 238.1425 (2); 83.0495 (1)	5
Echimidine isomer 1	R	p	$C_{20}H_{31}NO_7$	398.2173	13.2	120.0808 (100); 83.0497 (15); 238.1438 (2); 138.0916 (2); 94.0656 (2); 220.1332 (1)	3
Heliosupine	Н	р	$C_{20}H_{31}NO_7$	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	р	$C_{20}H_{31}NO_7$	398.2173	13.4	120.0808 (100); 83.0495 (63); 138.0913 (5); 238.1424 (3); 94.0654 (3);	3
Echimidine	R	p	$C_{20}H_{31}NO_7$	398.2173	13.4	120.0808 (100); 83.0496 (20); 238.1431 (2); 138.0913 (2); 94.0656 (2);	-1
Symphytine NO	К	p	$C_{20}H_{31}NO_7$	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)	7
Echiumine NO	R	p	$C_{20}H_{31}NO_7$	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)	0
Canescine (canescenine)	Н	р	$C_{20}H_{33}NO_7$	400.2330	12.4	120.0809 (100); 94.0655 (9); 138.0914 (5); 256.1535 (3); 83.0496 (1)	e
7-Tigloyleuropine	Н	р	$C_{21}H_{33}NO_7$	412.2330	14.9	120.0809 (100); 138.0915 (4); 94.0655 (3); 238.1434 (2); 220.1334 (1); 83.0495 (1)	2
Lasiocarpine	Н	р	$C_{21}H_{33}NO_7$	412.2330	15.1	120.0807 (100); 138.0911 (5); 94.0654 (4); 238.1437 (3); 220.1321 (2); 83.0495 (1)	-
7-Angeloylheliotrine NO	Н	р	$C_{21}H_{33}NO_7$	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

Echihumiline NO	R	р	$C_{20}H_{31}NO_8$	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	q	$C_{20}H_{31}NO_8$	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)	-
Vulgarine NO	R	Ε	$C_{20}H_{31}NO_8$	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)	ε
Asperumine NO	Η	q	$C_{20}H_{31}NO_8$	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)	9
Heliosupine NO	Н	q	$C_{20}H_{31}NO_8$	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)	7
Lithosenine	R	p	$C_{20}H_{33}NO_8$	416.2279	8.3	120.0807 (100); 94.0654 (8); 138.0913 (4); 256.1540 (1)	3
Canescine (canescenine) NO	Н	q	$C_{20}H_{33}NO_8$	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)	c,
7-Tigloyleuropine NO	Н	q	$C_{21}H_{33}NO_8$	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)	5
Lasiocarpine NO	Н	q	$C_{21}H_{33}NO_8$	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)	-
Lithosenine NO	R	р	$C_{20}H_{33}NO_9$	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):	ю
Thesinine-4'-ramnoside	Τ	I	$C_{23}H_{31}NO_7$	434.2173	13.6	147.0440 (100); 142.1227 (26); 124.1121 (25); 119.0493 (17); 288.1594 (15)	3
3'-Acetylheliosupine	Η	p	C ₂₂ H ₃₃ NO ₈	440.2279	15.0	120.0809 (100); 138.0913 (4); 238.1438 (2); 83.0495 (2); 220.1335 (1)	2
3-Acetylechiumine NO	R	р	$C_{22}H_{33}NO_8$	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)	ε
Thesinine-4'-glucoside	Τ	I	$C_{23}H_{31}NO_8$	450.2122	11.1	147.0440 (100); 142.1222 (20); 124.1122 (20); 119.0493 (17); 288.1591 (6)	ŝ
5'-Acetyllasiocarpine	Η	p	$C_{23}H_{35}NO_8$	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	Н	р	$C_{22}H_{33}NO_9$	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'-Acetyllasiocarpine NO	Н	р	$C_{23}H_{35}NO_9$	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
^a NO N-ovide ^{, b} R retrone	rine. H	heliotri	dine. O oto	necine. T	trache	anthamidine. D nlatynecine. S suminidine. ^c m monoester. d onen-ch	ined

^a NO, N-oxide; ^v R, retronecine; H, heliotridine; O, otonecine; T, trachelanthamidine; P, platynecine; S, supinidine; ^v m, monoester; d, open-chained diester; c, cyclic diester; ^d exact mass; ^e IL, identification level according to Metabolomics Standards Initiative. 691

Ξ

	Hon	ey	Poll	en	Black	tea	Green	tea	Herbal in	ufusion	Dietary sup	plement
Compound	EE (SD)	LOI	EE (SD)	LOI	EE (SD)	LOI	EE (SD)	LOI	EE (SD)	LOI	EE (SD)	TOI
		$(\mu g \ kg^{-1})$		$(\mu g \ kg^{-1})$		$(\mu g \ kg^{-1})$		$(\mu g kg^{-1})$		$(\mu g kg^{-1})$		$(\mu g k g^{-1})$
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	(6.0) 6.88	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	(0.0)	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
Senecivernine	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
Senecivernine 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
^a 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







Flowchart: Heliotridine open-chained diester type PANO

Name	Expected Unknowns -	CAS Registry Number 👻	Molecular formula ^b	Molecular Weight →	[M+H] ⁺ (calculated m√T	Necine base type ^c	Necic acid type 🚽	N-oxide	lsomer grou[→	lsomer subgroup -
2'-epi-Heliosupine N-oxide		2171456-54-3	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	х	81	D
Asperumine N-oxide		54324-54-8	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	х	81	D
Heliosupine N-oxide (Cynoglossophine N-oxide)		31701-88-9	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	Х	81	D



Fig. 3	5.
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21 PAs of the Reg. 2040/2020/EU 14 co-eluting PAs of the Reg. 2040/2020/EU PAs not included in the Reg. 2040/2020/EU