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Pyrrolizidine alkaloids in honey: Quantification with and without standards

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ABSTRACT

Quantification of a large number of compounds in the absence of authentic standards is always a challenge. More than 600 pyrrolizidine alkaloids (PAs) have been found in plants however only limited number of PAs standards are commercially available. As PAs are the most widely distributed natural plant toxins with threat to human health, risk assessment calls for quantitative analytical methods with a wide scope including PAs without available standards. In this study, a method was developed that allows simultaneous quantification of 12 PAs in the honey samples by using HPLC-HRMS with authentic standards. This method was further extended to screen for other potential PAs in the honey using multi-target screening combined with a quantitative prediction model in the cases that authentic PAs were not available. The prediction model was subsequently validated by cross-validation and additional PAs standards which were not included in the model. The maximum concentration prediction error was 50.8%.

KEY WORDS: Pyrrolizidine alkaloids; validation; quantification without standards; screening; honey; HPLC-HRMS

1. INTRODUCTION

Pyrrolizidine alkaloids (PAs) are a group of at least 600 different secondary metabolites that are produced by more than 6000 plant species worldwide. They are suspected to be hepatotoxic and genotoxic carcinogens in humans, since these PAs are activated to electrophilic compounds through the liver's metabolic enzymes (Bolechová, Čáslavský, Pospíchalová, & Kosubová, 2015: Lucchetti et al., 2016). Single or continuous intake of 1, 2-unsaturated PAs can cause hemorrhagic necrosis and hepatic venous occlusion, and also increase the incidence of liver haemangiosarcoma for chronic risk assessment (EFSA, 2017). As approximately 3% of all flowering plants contain PAs compounds (Boppré, 2011), this PAs exposure occurs worldwide, and intoxication by contaminated herbal teas, cereals, herbal medicine, salads, and feed, have been reported (Culvenor, Edgar, Smith, Kumana, & Lin, 1986; Molyneux, Gardner, Colegate, & Edgar, 2011; Tandon, Tandon, & Narndranathan, 1976; Wiedenfeld, 2011). Honey is one of the food products that contain PAs. Bees collect pollen or floral nectar from a wide variety of plants and therefore diverse profiles of PAs are observed in honey (Boppré, Colegate, & Edgar, 2005; Lucchetti et al., 2016). Previous studies have shown a very high frequency of PA positive honey samples, e.g., up to 94.2% samples contain PAs in a study of Spanish raw honey (n = 103) (Martinello, Cristofoli, Gallina, & Mutinelli, 2014; Orantes-Bermejo, Serra Bonvehí, Gómez-Pajuelo, Megías, & Torres, 2013). Hence with regard to the increased awareness of this potential health concern, it is important to clarify the types and concentration of PAs in honey.

Based on the data collected by the European Food Safety Authority (EFSA), a total of 64 PAs were reported in food products (EFSA, 2011), while the lack of certified standards hampers an unequivocal quantification. This is, particularly, a problem in PA analysis, where a complex and variable profile of

PAs can be expected. To date, very limited PA are commercially available and no more than approximately 34 different PAs in one method are reported, while isotopic labeling PAs are still not commercially available (Schenk, Siewert, Toff, 2017). This is unlikely to change in the near future since there is no facile chemical access, *in vitro* biosynthesis and isolation (EFSA, 2011). Therefore measuring the concentrations of a wide variety of PAs within a single experimental detection continues to remain challenging. Many studies about semi-quantification model have been reported, and the ionization efficiency were reported to be correlated to p*K*a, logP, molecular volume, molecular size, surface tension, or the WAPS value as developed by Kruve's group, (Alymatiri, Kouskoura, & Markopoulou, 2015; Hermans, Ongay, Markov, & Bischoff, 2017; Golubović, Birkemeyer, Protić, & Otašević, 2016; Raji, et al., 2010; Oss, Kruve, Herodes, & Leito, 2010; Kruve & Kaupmees, 2017). However, their models either used for specific compounds (such as amino acid, steroids, peptides and sartans) or challenged to calculate the relevant descriptors used in the model. To our knowledge, there are still no quantitative prediction model developed specific for PAs up to now.

Solid-phase extraction (SPE), followed by HPLC-MS analysis is a commonly used sequence to analyze PAs in honey. However, the recovery of PAs from the samples are highly dependent on the types of cartridges, sample loading amount, and elutes used during the extraction procedures (Betteridge, Cao, & Colegate, 2005; Bodi et al., 2014; Chung & Lam, 2018; Colegate, Edgar, Knill, & Lee, 2005). The determination and quantification are generally carried out by a target analysis using GC-MS (with and without derivatization), HPLC-MS or HPLC(GC)-MS/MS (Crews, Berthiller, & Krska, 2010; Lucchetti et al., 2016; Molyneux et al., 2011). Latest LC-MS methods mostly relied on the high selectivity of mass spectrometry operated in the multiple reaction-monitoring (MRM) mode (Chung & Lam, 2018; Kowalczyk & Kwiatek, 2018), where the MRM method can only detect

83 preselected PAs within the correct retention time windows and fragmentation patterns (These, Bodi,
84 Ronczka, Lahrssen-wiederholt, & Preiss-weigert, 2013). Furthermore, the PAs not included in the
85 specific methods, e.g. if authentic standards are not available, will remain undetected and therefore not
86 be quantified.

87 A screening approach (multi-target screening) using high resolution / accurate LC-QTOF-MS with
88 full scan acquisition can achieve both high sensitivity and selectivity. Full-scan MS offer the advantage
89 to determine a virtually unlimited number of analytes simultaneously without preselection of precursor
90 ions. The screening approach also allows a retrospective "targeted" evaluation of data by reconstructing
91 any desired ion chromatogram to explore the data for presence of additional PAs (Heeft, Bolck,
92 Beumer, Nijrolder, Stolker, & Nielen, 2009). These data are however qualitative in character as
93 standards with known concentration is needed to determine the concentration of compounds.

94 The objective of this study is to develop a high-resolution analytical method to detect and estimate
95 the concentrations of a wide range of PAs in honey, even for those without authentic PAs standards.
96 Firstly, we developed a validated method that allows the simultaneous detection and quantification of
97 12 PAs in honey using HPLC-QTOF-MS with pure authentic standards. Secondly, the extracts of
98 different honey samples were analyzed for occurrence of other PAs using a multi-target screening
99 approach. Suspicious signals were verified by recording of product ions using a broadband
100 fragmentation. Consequently, we proposed a semi-quantification strategy for those detected peaks
101 without available standards through a quantitative prediction model (QPM). To evaluate this prediction
102 model, feasibility and reliability have been validated by leave-one-out (LOO) cross validation. In
103 addition, as a test, quantification of four unknown PAs, that were not included in the original dataset,

has also been carried out. Finally, we estimated the contents of PAs in honey samples combining the available reference standards and the QPM.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Milli-Q grade water was used for all analyses (Millipore Corporation, Molsheim, France). Methanol (LC–MS grade), acetonitrile (LC–MS grade), ammonium hydroxide solution (LC–MS grade), and formic acid (LC–MS grade) were purchased from Fluka (Steinheim, Germany). Pyrrolizidine alkaloids (PAs) (7-acetylintermedine, echimidine, heliotrine, heliotrine *N*-oxide, intermedine, intermedine *N*-oxide, lasiocarpine, lasiocarpine *N*-oxide, lycopsamine, lycopsamine *N*-oxide, retrorsine, retrorsine N-oxide, senecionine, senecionine *N*-oxide, seneciphylline, seneciphylline *N*-oxide) were purchased from PhytoLab (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany). Sulfuric acid (analytical grade, 95-97%) was obtained from Merck (Darmstadt, Germany). All other solvents used in this study were of analytical grade.

Each alkaloid standard was dissolved in either acetonitrile or acetonitrile/water (50/50 v/v) depending on their solubility to obtain stock solutions at a concentration of 0.5 mg/mL. All the stock solutions were stored at -20 °C. Stock standard mixtures (1 μg/mL) were prepared in acetonitrile/water (50/50 v/v) from the individual stock solutions, and used set up calibration curves.

2.2 Samples

Thirty-two honey samples were included in this study purchased from either local supermarkets or bee keepers, originating from at least 10 different countries. The honey samples were declared to be collected from summer flowers, white clover, wild flowers, creamy flowers, heather flowers, acacia,

fruit trees (apple, plum, rape), and etc. The information of samples: names, geographical and botanical origin, and symbols are listed in **Table** 1.

2.3 Negative control and quality control samples

Honey sample previously analyzed and found not containing PA compounds, was used as blank sample (mountain honey coming from wild flowers of Nicaragua) for method validation. Quality control (QC) samples were made by spiking blank honey samples (25 µL stock standard mixtures to 1.0 g blank honey sample). QC samples were analyzed in each series of samples and were controlled to be in the range of ± 20% of the validation results. If not, the whole set of samples were reanalyzed.

2.4 Solid Phase extraction

The strong cation exchange (SCX) SPE was found to be suitable for plant extracts and for a series of pyrrolizidine alkaloid/N-oxide (PAs/PANOs) representing the major hepatotoxic structural groups (monoesters, diesters and macrocyclic diesters of retronecine, heliotridine and otonecine bases), and also provided efficient capture from honey-based matrices and subsequent release into ammoniated methanol (Betteridge et al., 2005; Colegate et al., 2005). The SPE columns (150 mg/6 mL, Biotage Evolute Express CX) used in our study combines non-polar and SCX functionality for extraction of basic analytes from aqueous samples through a dual, mixed-mode mechanism. Also these specific SPE columns can be used without a preanalysis conditioning and equilibration steps without affecting the recovery significantly.

The extraction of PAs was based on a modified method from German Federal Institute of Risk Assessment (BfR) (BfR, 2013). One gram of honey was weighed into a 10 ml centrifuge tube and dissolved in 3 mL of 0.05 M H_2SO_4 in water at room temperature by shaking for 30 min. The samples were centrifuged at 2000 × g for 10 min at room temperature. The supernatant was transferred to a SPE

147 cartridge by using a vacuum manifold. After loading the sample, the column was washed with 4 mL of
148 0.05 M H_2SO_4 followed by 4 mL of methanol, then the column was eluted with 5 mL of 2.5% (v/v)
149 ammonium hydroxide in methanol at a rate of 0.5-1 mL/min. The eluate was dried under a stream of
150 nitrogen at 45 °C. The residue was dissolved in 0.5 mL acetonitrile/water (5/95, v/v), transferred to an
151 Eppendorf vial, and centrifuged at 10.000 × g for 10 min at room temperature. Then 200 µL of
152 supernatant was transferred into a HPLC-vial with insert.

153 **2.5 HPLC-ESI-QTOF-MS**.

154 Samples were analyzed on an Agilent 1200 HPLC (Agilent Technologies, CA, U.S.) coupled to a
155 Bruker Daltonics micro-TOFq mass spectrometer (Bruker Daltonics, Bremen, Germany). The
156 resolution of the Bruker micro-TOFq is approximately 10000. Samples were injected onto a
157 Nucleoshell C18 reversed-phase column (2.7 µm, 100 × 2 mm, MACHEREY-NAGEL GmbH & Co.
158 KG, Düren, Germany). The mobile phase consisted of water (A) containing 0.1% formic acid and 2.5
159 mM ammonium hydroxide, and acetonitrile (B). The analysis was done using a gradient elution at a
160 flow rate of 0.3 mL/min at 40 °C using the gradient: isocratic at 2% of B for 1.0 min, then increased to
161 10% of B at 4.0 min, to 40% of B at 9 min, and finally to 95% of B at 10.0 min and maintained to 11
162 min, where after the gradient returned to starting conditions 2% of B at 11.2 min followed by 3.8 min
163 equilibration with 2% of B to giving a total runtime of 15 min.

164 An electrospray ion source (ESI) was used in positive ion mode (ESI^+) with the following settings:
165 capillary voltage 4 kV, nebulizer pressure 2 bar, dry gas temperature 180 °C, and dry gas flow rate 10
166 L/min. Spectra were collected from m/z 50 to 800 Da at a rate of 2 Hz. Sodium formate dissolved in
167 50% of 2-propanol was introduced into the ion source between 0.2–0.4 min for internal calibration of
168 the data files. Hexakis (1H, 1H, 2H-perfluoroethoxy) phosphazene was used as the lock mass calibrant.

169 To confirm the presence of pyrrolizidine alkaloids, LC-MS/MS analyses were conducted using
170 broadband collision-induced dissociation (bbCID) with a fragmentation energy of 35 eV and nitrogen
171 as the collision gas.

172 **2.6 Design of validation**

173 Validation parameters such as linearity, specificity, limits of detection (LOD), limit of quantification
174 (LOQ), measurement uncertainty, recovery, and precision were determined for 12 PAs using the
175 established method according to Nordic Committee on Food Analysis (NMKL) procedure No. 4 with
176 minor modification (NMKL, 2009). The spiked samples were prepared according to the procedure
177 described above and analyzed on three different days. All 12 PA compounds were spiked at 4
178 concentration levels: 2 ng/mL, 10 ng/mL, 50 ng/mL, and 150 ng/mL corresponding to 1 μg/kg, 5 μg/kg,
179 25 μg/kg, and 75 μg/kg in the honey samples. At each concentration level at least 10 replicates of the
180 same spiked blank honey samples were analyzed (13 replicates at 10 ng/mL, and 10 replicates for the
181 other 3 levels). Method precision was evaluated by intra- and inter-day analyses. The repeatability
182 (intra-day variability) was tested within one day for each concentration, reproducibility (inter-day
183 variability) was examined on three different days for each concentration (13 replicates for 5 μg/kg, 10
184 replicates for 1 μg/kg, 25 μg/kg and 75 μg/kg).

185 LOD and LOQ were calculated by using the standard deviation of 10 replicates at 2 ng/mL (blank
186 honey spiked before SPE clean-up). LOD = 3 × SD / recovery of each compound, LOQ = 6 × SD /
187 recovery of each compound. Matrix effects (ME) was estimated by adding different levels of standard
188 solution to a sample of the blank honey extracts. Matrix match calibration curves were used to correct
189 for matrix effects (ME), and were made by analysis of each analyte at 7 calibration levels from 2 to 150
190 ng/mL. The standard curves were the peak area versus the concentration of each analyte. ME (%) =

(Slope of matrix match standard curve / Slope of standard curve without honey matrix) × 100%. ME of some other honey samples were also measured at 50 ng/mL spike level to compare the ME difference between different honey samples. The recovery was determined by comparing the analytical results of the extracted PAs from spike honey samples with the results of standards added in the same concentrations to blank matrix (13 replicates for 5 μg/kg, 10 replicates for 1 μg/kg, 25 μg/kg and 75 μg/kg).

2.7 Quantification

All the quantification was performed by integration of peak area of extracted ion chromatograms using exact mass ± 5 mDa. The concentration of PAs in the samples was calculated by QuantAnalysis 2.2 (Bruker Daltonics, Bremen, Germany). All the values of PAs of samples were calculated by using the average value in duplicate samples of the same sequence.

2.8 Software

The Chemdraw Professional 15.0 was used for drawing and displaying chemical structures. R (version 3.3.3), R studio and Origin software were used for all statistical analysis. Jchem version 18.5.0 was used to predict the pK_a, pK_b, logP values, and other properties of molecules.

2.9 Statistical model evaluation and validation

Several studies have investigated the relationship between signal response in electrospray ionization-mass spectrometry (ESI-MS) and molecular descriptors (molecular volume, logP, pK_a, polarity) of analytes to estimate analyte concentration (Golubović et al., 2016; Kruve & Kaupmees, 2017; Oss et al., 2010). Seneciphylline was randomly chosen as the reference standard in the method to correct the daily variation. Multi-linear regression (MLR) analysis was carried out to obtain the QPM describing

212 the relationship between relative calibration curves' slope (RS) corresponding to seneciphylline in
213 honey matrix and molecular properties in the following formula:

214 $$\ln RS = \ln\left(\frac{Slope\ of analytes}{Slope\ of\ Seneciphylline}\right) = \sum_{i=1}^{n} \boldsymbol{a_i X_i}$$

215 where $\boldsymbol{X_i}$ is the physicochemical parameters or their combinations, and $\boldsymbol{a_i}$ is the coefficients of these
216 parameters. Experimental values of several parameters such as logP and pK_a are not available for all
217 compounds, therefore these molecular descriptors including Molecular volume, logP, pK_a (strongest
218 acidic), pK_b (strongest basic), and polarizability were computed by using Physico-chemical property
219 predictors plugin of JChem in excel (Szisz, 2017). All the chemical structures of PAs were transferred
220 as the SMILES (Simplified molecular input line entry specification) obtained from PubChem. The
221 molecular descriptors with correlation above 0.9 were removed to avoid the co-linearity in the model.
222 $\boldsymbol{X_i}$ and $\boldsymbol{a_i}$ were obtained by MLR which was calculated in R. Twelve PAs compounds were used to
223 develop the MLR model (exact mass is in the range from 299 to 427 Da, pK_a is in the range from 10.54
224 to 12.37, pK_b is in the range from 2.82 to 8.13, logP is in the range from -1.42 to 1.64). In the gradient
225 elution, all the PAs were eluted from 10% to 37% organic phase, which is considered as a narrow
226 range, thus the corrections with retention parameters or solvent change was ignored in this part.

227 Cross-validation method 'leave-one-out' (LOO) approach was used to validate the obtained results.
228 LOO means that each compound was left out from the model fitting process once; thereafter, the model
229 was used to predict the RS value of the compound not involved in the model development. LOO is
230 equivalent to k-fold cross-validation where k = n (the number of observations in the data set), and LOO
231 for linear regression is exactly equivalent to the PRESS (Predicted Residual Sum of Squares) method
232 based on the leave-one-out or Jackknife technique, detail explanation can be found in Tarpey's study

233 (Tarpey, 2000). Therefore in this study, PRESS and root mean square error of prediction (RMSEP)
234 were used to describe the differences between predicted RS values and measured values.

235 Using the fact of the predicted value for y_i when the i^{th} compound is deleted from the MLR model
236 is equal to

$$y_i - \hat{y}_{(i)} = \frac{\hat{e}_i}{(1 - h_i)} = \hat{e}_{(i)}$$

237 Here $\hat{e}_i = usual\ i^{th}\ residual$ and $h_i =$ potential or leverage value for the ithcompound,
238 y_i and $\hat{y}_{(i)}$ are predicted and measured lnRS value in this study, respectively.

$$PRESS = \sum_{i=1}^{n} \left(y_i - \hat{y}_{(i)}\right)^2$$

239 $$RMSEP = \sqrt{\frac{PRESS}{n}}$$

240 The resulting model was also used to predict the slope of the calibration curves for 4 additional PAs
241 which were not included in the original dataset but within the model coverage to verify the prediction
242 quality. The predicted slopes were used to calculate the concentrations of the other PAs compounds
243 detected in the collected samples.

244 $$C_{\text{predicted}} = \frac{\text{Response}_{from\ sample}}{\text{Slope}_{predicted}}$$

245 $C_{\text{predicted}}$ is the predicted PA concentration, $\text{Response}_{from\ sample}$ is the peak area of the tentatively
246 identified PAs in honey samples, $\text{Slope}_{predicted}$ is the slope of predicted calibration curve of the
247 identified PAs, and it was calculated from the RS value corresponding to the slope value of reference
248 standard (seneciphylline) in honey matrix, assuming that the standard curve pass through zero.

All statistical tests were performed at 95% significant level. The prediction error for additional 4 PAs out of the model was calculated with the following formula:

$$\text{Prediction error}=\left(\frac{\text{RS}_{predicted}-\text{RS}_{measured}}{\text{RS}_{measured}}\right)\times 100\%$$

3 RESULTS AND DISCUSSION

3.1 PART 1. Validation

3.1.1. Specificity

Blank honey samples and blank honey spiked with 5 ng/ml mix standard solution (corresponding to 2.5 μg/kg) were analysed to examine if other compounds in the samples were interfering with these analytes. The HPLC shows good separation of all 12 PA compounds, and there are no apparent interferences observed from the matrix evaluated from the extracted ion chromatograms, thus confirming the specificity. The structures and chromatogram of 12 PAs are shown in in **Figure** 1.

3.1.2. Linearity

A good linear relationship was observed based on correlation coefficients higher than 0.99 (from 2 ng/mL to 150 ng/mL). The average relative residual is less than 25% at all levels, and the intercepts obtained are close to the theoretical value zero, demonstrating good consistency. Information about residues plot can be found in in Supporting Information **Figure** S1.

265 *3.1.3. Precision*

266 The standard deviations of all the PAs at different spiked levels were calculated. Results are shown
267 in **Table** 2. Coefficient of Variation (CV%) for the reproducibility is in the range from 6.3% to 21.6%,
268 and CV% for the repeatability is in the range from 2.6% to 18.9%, which is considered acceptable.

269 *3.1.4. Recovery*

270 The average recovery is 89% for all the 12 PA compounds, ranging from 79.2% to 104.4% with a
271 standard deviation lower than 7.6% (**Table** 2), thus confirming a good reproducibility of the method
272 and sufficient for reliable analysis.

273 *3.1.5 Limit of detection and limit of quantification*

274 Results are shown in **Table** 2. LOD values for the 12 PA compounds are in the range from 0.2 µg/kg
275 to 0.6 µg/kg, LOQ values of the 12 PA compounds are in the range from 0.5 µg/kg to 1.3 µg/kg, which
276 are comparable to those reported in literatures (Bolechová et al., 2015; EFSA, 2012). Although there
277 are studies which present methods with lower LOQs (BfR, 2013; Kowalczyk & Kwiatek, 2018;
278 Lucatello, Merlanti, Rossi, Montesissa, Capolongo, 2016), they all rely on MRM mode rather than full
279 scan detection. These MRM method are very specific however MRM are blind to other PAs that may
280 occur in samples. **Figure** S2 shows samples spiked with 1 ng/mL (corresponding to ~0.5 µg/kg in
281 honey) with peaks still clearly visible for all analytes, indicating that the calculated limits are realistic
282 to detect and quantify for the 12 PA compounds.

283 *3.1.6 Matrix effect*

284 The matrix effect (ME) of spiked blank honey sample with 1 g sample load is in the range from
285 81.0% to 112.3%, indicating that the analytes' signals are only slightly influenced by the honey matrix
286 (**Table** 2). In order to compare the difference of ME between different honey matrix, additional
287 experiments were done to compare the ME. Examining these results showed a RSD% in the range from
288 4 % to 12% from ME of 8 honey samples, which indicates that it is feasible to use one blank matrix to
289 quantify all the other honey samples. Full data are shown in Supporting Information **Table** S1.

290 *3.1.7 Measurement uncertainty*

291 The measurement uncertainty (MU) consists of intra-laboratory reproducibility ("CVR"), bias
292 ("bias") calculated as 100% recovery, standard deviation on bias ("CVRbias/sqrt(n)") calculated as
293 intra-laboratory standard deviation divided by square root of number of results, and standard deviation
294 on matrix effect ("CVmatrix effect") listed in **Table** S1 in the Supporting Information. The method
295 uncertainty was calculated as:

296 $$MU=\sqrt{CVR^2 + bias^2 + \frac{CVRbias^2}{n} + CVRmatrixeffect^2}$$

297 Seneciphylline-N-oxide, with the highest bias (100 – 82.4% = 17.6%) had a MU of 22%

298 $(\sqrt{11.9^2 + 17.6^2 + \frac{11.9^2}{43} + 7.1^2})$.

299 **3.2 PART 2. PA detection and contents in honey samples**

300 3.2.1 PA Quantification and identification

The 12 PAs included as standards in this study were selected based on the ones reported most often and in highest concentrations in honey (EFSA, 2011), and they belong to 3 major PAs chemical groups found in honey:

- **Senecionine-type** PAs (Retrorsine, retrorsine *N*-oxide, senecionine, senecionine *N*-oxide, seneciphylline, seneciphylline *N*-oxide), which occur primarily in the *Senecioneae* spp. (*Asteraceae* family), but are also found in *Crotalaria* spp. (*Fabaceae* family);
- **Lycopsamine-type** PAs (Intermedine, intermedine *N*-oxide, lycopsamine, echimidine), which occur in the *Boraginaceae* family and in the *Eupatorieae spp.* (*Asteraceae* family);
- **Heliotrine-type** PAs (lasiocarpine, lasiocarpine *N*-oxide), which occur in *Heliotropium* spp. (*Boraginaceae* family) (Mulder, López, These, & Preiss-weigert, 2015).

The LC-MS/MS chromatograms of 12 validated PAs in honey samples were characterized by comparing the fragments pattern with authentic standards. The fragmentation pattern of selected PAs, C-1 (monoester or open-chained diester), and C-2 (*N*-oxide open-chained diester) are shown in **Figure** S3 (Avula et al., 2015; These et al., 2013). A compilation of chemical formulas, accurate mass, fragment ions, retention time, and mass error of HRMS detection for 12 reference PAs are shown in **Table** 3. The MS^2 broadband fragmentation spectrums show fragment ions at m/z 172, 156, 138, 136, 120, 118, and 94 which are the most common and characteristic fragments from these PAs. These fragment ions are also well known in unsaturated necine ester type PAs and are similar to fragments reported in the literature (Avula et al., 2015; Beales et al., 2004; Schulz et al., 2015; These et al., 2013). Detailed information about fragment patterns of PAs found in this study are shown in supplemental **Figure** S4.

The analyses showed that one or more of the 12 PA compounds were found in 60% of these samples. The combined results are shown in **Table** 1. The concentration of each PA compound in the positive samples is in the range between 1.4 and 11.1 µg/kg honey and the total PAs contents are in the range of 1.4 to 14.2 µg/kg (the sum of the 12 PAs). The highest total PA content is found in honey H27.

The most frequently found PA is echimidine. Seven of the tested samples are positive with concentrations ranging from 1.4 to 3.4 µg/kg, followed by intermedine and senecionine. These findings are in accordance with the results reported by Lucatello (et al, 2016), most likely because plants from the genus *Echium*, namely *Echium italicum* L., *Echium vulgare* L., and *Echium plantagineum* L. are widely distributed in the European region (Lucatello, Merlanti, Rossi, Montesissa, 2016). For echimidine in H14, H25 intermedine in H3, H23, retrosine *N*-oxide in H12, H23, lycopsamine in H11, H16, H25, H29, and senecionine in H4 and H9, they are below the LOQ value but are still detected in LC-MS. Information about extracted ion chromatograms of these detected PAs are shown in **Figure** S5.

Although PAs are detected in almost half of the samples studied, they are not found in as high concentration as in other studies (Avula et al., 2015; Beales et al., 2004). The levels of the 12 validated and quantified PAs are so low that the honey can be accepted according to both $BMDL_{10}$ of EFSA to assess the carcinogenic risks of PAs (237 µg/kg bw per day for chronic toxicity) and also BFR recommendation (maximal intake of 0.007 µg/kg bw per day) if consumers only consume a limited quantity of honey (see **Table** 1) (Dusemund et al, 2011; EFSA, 2017; BfR, 2013). However, food safety issues frequently originate from compounds that are not included or quantified by the analytical method. Therefore, a multi-target screening method including more PAs compounds is particularly important to predict potential risk from PAs in honey.

3.2.2 PAs screening

A screening method monitoring 85 different PAs compounds based on elemental composition from the literature was used to detect if other PAs could be found in the honey samples. The PAs in the screening list were compiled from literatures and only 1, 2-un-saturated compounds were taken into consideration. Then the suspicious signals with same molecule weight were further analysed by LC-MS/MS. Tentative identification of the PAs was done by comparing the fragments with literatures information (EFSA, 2011). Elemental composition and fragments ion of these tentative identified PAs from the screening method are shown in **Table** 3.

According to the screening results, several other PAs were found in the honey apart from the 12 validated PAs already quantified. Some of these PAs occur in relative high concentration. For example, a chromatographic peak just next to lycopsamine with the same protonated molecular ions (m/z 300.1805 $[M+H]^+$), same fragments pattern, and similar retention time (only 0.1 min difference) is found in 12 out of 32 honey samples. In order to clarify whether this compound is lycopsamine or not, we spiked one of these honey samples with this unknown peak. We proved that this large peak is not lycopsamine, but its peak area is much larger than that of lycopsamine. According to the previous reports and fragments information we obtained (m/z 94.0655, 138.0932. 156.1019), this unknown compound is most likely rinderine (other possibilities are echinatine or indicine), and all of them are belong to lycopsamine-type PA stereoisomers, which include indicine, intermedine, lycopsamine, rinderine and echinatine (Beales et al., 2004; Kempf et al., 2011). All these five PAs are structurally closely related, thus a similar retention behaviour and mass spectrometric response can be assumed to be the same. There are also other large peaks appearing next to senecionine and echimidine again (0.1

364 min retention time difference) with the same molecule weight and fragment pattern, but not the same as
365 verified by standard addition.

366 Several PAs have the similar molecular structures, therefore we will expect similar properties and
367 fragments pattern in LC-MS/MS. For these types of compounds, it is difficult to predict the correct
368 structures without further identification or authentic standard. Therefore, the data can only be seen as
369 indicative. A list of considered PA compounds included in the screening, and their accurate mass data,
370 are shown in Supporting Information **Table** S2.

371 To evaluate the concentrations of these other PAs, which were not included in the validation method,
372 a semi-quantification approach was used to estimate their concentration as discussed in the following
373 section.

374 **3.3 PART 3. QPM and estimation of concentration of other PAs in honey samples**

375 Estimation of the concentration for PAs where standards are unavailable are based on all the
376 information we can obtain from direct measurement of available standards and honey samples, also
377 model calculation. In the initial model, all the previously mentioned molecular properties (molecule
378 volume, p$K_{a,}$ p$K_{b,}$ logP, relative slope, exact mass, and retention time of 12 PAs used in the model and
379 tentative identified PAs in samples are shown in **Table** S3a) were included. While by backward MLR
380 modelling, the best fit for data was achieved with a model containing p$K_{a,}$ p$K_{b,}$ logP, and exact mass.
381 Several authors have found that hydrophobicity (logP, usually expressed as octanol/water partition
382 coefficient) are important characteristics correlated to ESI ionization efficiency, and increasing the
383 extent of nonpolar character in a compound leads to enhanced affinity for the surface phase and higher
384 ESI response (Cech & Enke, 2000; Leito et al., 2008). p*K*a can be expressed as the ability to be

protonated and become a cation, and it has been proved that p*K*a is highly correlated with ionization efficiency in ESI (Oss et al., 2010). Exact mass was characterized by the molecular size or molecular volume, which also has been reported as a parameter that can influence ionization efficiency (Hermans, Ongay, Markov, & Bischoff, 2017; Oss, Kruve, Herodes, & Leito, 2010).

The formula calculated in R is shown in the following formula (Procedures of calculation is shown in Supporting Information).

$\ln(RS_{predicted})$ = (27.0965 ± 7.6792) + (1.0240 ± 0.3192) × logP - (1.4075 ± 0.4115) × pK_a - (0.3048±0.1010) × pK_b - (0.0285 ± 0.0073) × exact mass

Regarding the calculation, eg., rinderine, $\ln(RS_{predicted})$ of rinderine = 27.0965+1.0240 × (-0.2900) - 1.4075 × 11.3400 – 0.3048 × 7.8200 – 0.0285 × 299.1733 = -0.07149, therefore predicted relative slope of rinderine corresponding to seneciphylline is 0.9310.

The correlation between predicted RS and measured RS is shown in **Figure** 2-a. The R^2 for this model was 0.75 and the residual standard error was 0.21 logarithmic units. By LOO validation, the accuracy of the models can also be described with RMSEP, RMSEP = 0.28 lnRS units. This value shows that on average the mismatch between the predicted and measured RS from LOO is lower than 1.3 times. The prediction errors are in the range from -0.3% to 27.1% for the 12 PA compounds used to develop the model (**Table** S3-b in Supporting Information). To validate the method, the model was also used to quantify 4 additional PAs compounds available as pure standards but not included in the model above in a spiking experiment. The concentrations of these 4 authentic standard compounds were estimated using the model developed by the 12 PAs giving a prediction error in the range between 10.2% and 50.8%. **Figure** 2-b shows the difference in standard curve slope using the predicted concentration and

406 measured concentration of the PAs not included in the model (from 5 to 100 ng/mL). Therefore, a
407 predicting model based on a 12 PAs calibration sets provides acceptable quantification results for a risk
408 assessment. Despite some PAs being of different chemical structures, they share quite similar
409 physicochemical properties, hence this will not have large effect on the predicted concentration. We
410 also calculated the model based on the data we obtained from two different days; and the coefficients in
411 the formula are quite similar as shown in **Table** S3-b. The difference between the two formulas
412 obtained from two different experimental days was compared; the variation for all compounds is within
413 a range of -24.5% to 10.9%. This indicates the feasibility for the future study, to use only one model
414 and one reference PA standard (e.g., Seneciphylline in this study) to predict the concentrations of the
415 other PAs within maximum 50.8% prediction error; as long as we have the structures information of
416 these PAs.

417 In many cases, studies about PAs estimation may choose calibration alkaloids of similar structure
418 using different calibration alkaloids (often an isomer) (Hoogenboom et al., 2011; These et al., 2013). Or
419 just simply reported all alkaloids as equivalent of one alkaloid such as echimidine in Beales' study
420 (Beales et al., 2004). In the approach of PAs estimation with different calibration alkaloids, serveral
421 PAs standards are still necessary. They cover various PAs types, while in the approach of PAs
422 estimation, with only one alkaloid, it is more unpersuasive due to the variation of PAs response which
423 can be nearly 3 times based on our results. Hereby, the difference between the approach of PAs
424 estimation, with only one alkaloid and our prediction model, were compared. It is shown in **Table** S4 in
425 Supporting Information, 3 concentrations (5 ng/ml, 10 ng/ml, 25 ng/ml) were performed, and Max25%
426 (the sum of 25% biggest absolute prediction error, e.g., absolute prediction error of 4 PAs were chosen
427 among 16 PAs in our case) was used to estimate the performance of different approaches. In the

approach simply reported every alkaloid as equivalent of one alkaloid, the Max25% values are in the range of 232.2% ~ 603.1%, 202.5% ~ 502.2%, 170.3% ~ 621.2% in the concentration of 5, 10, 25 ng/ml, respectively. However, when we use the prediction model, the Max25% values are 196.50%, 142.16%, 162.26% separately, which is more accurate and controllable. It has been reported by the EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) that they identify mainly 4 types of PAs of particular importance for food and feed (Senecionine-types, Lycopsamine-type, Heliotrine-type, Monocrotaline-type) (EFSA, 2011). Although there were only a limited number of PAs in our dataset when we developed the QPM, it covers 3 types of most common PAs which exist in honey. Therefore, this model can make relative accurate estimation of PAs covered by these 3 types, at least (only monocrotaline-type PAs was excluded). To make this model more generic, further study can be carried out to include more PAs, such as monocrotaline-type PAs in the model development.

The QPM was used for the estimation of other PAs based on the tentative identification, and prediction error of RS between measured and predicted value are shown in supplemental **Table** S3-b. The results from semi-quantification of these PAs are shown in **Table** 1. Riderine (or Echinatine /Indicine), which elutes just next to lycopsamine and shows the highest level among all the quantified PAs reaching an estimated concentration of about 56 µg/kg in H15. Crotananine shows the concentration of about 51 µg/kg in H8, followed by acetyl-lycopsamine and 3'-acetylindicine, with a concentration of 47 µg/kg and 45 µg/kg both detected in H9.

Although according to assessment of chronic risks referring to a $BMDL_{10}$ of 237 µg/kg bw per day established by EFSA (EFSA, 2017) or acute toxicity data reported by Dusemund et al., (2011), the detected PAs levels are acceptable. However, following the recommendations from the BFR as

449 mentioned before, the oral intake of PAs should not exceed a daily dose of 0.007 µg/kg b.w (BfR,
450 2011). Assuming that a 60 kg body weight and a daily consumption of honey of 20 g, a honey sample
451 should not contain more than 21 µg/kg of PAs. Our estimated concentration of PAs, including all PAs
452 even those where standards are not available, show the total PAs content in honey samples may be as
453 high as: H6 (ca. 26 µg/kg), H7 (ca. 36 µg/kg), H5 (ca. 36 µg/kg), H11 (ca. 36 µg/kg), H18 (ca. 41
454 µg/kg), H8 (ca. 51 µg/kg), H15 (ca. 67 µg/kg) and H9 (ca.117 µg/kg)all exceeding 21 µg/kg. Even if
455 we consider a quantification error of 51% the levels will still be of concern.

456 In conclusion, we proposed a method that can be extended to a general strategy to achieve estimation
457 of quantitative data where authentic standards are not available. By using a prediction model based on
458 physio-chemical parameters, analysis of similar standards, and literature relevant quantitative data for
459 risk assessment can be estimated using HPLC-HR-MS/MS techniques. This approach was
460 demonstrated by the simultaneous quantification of 12 PAs and also the further analysis of honey
461 containing other high content PAs without standards. Future applications of this method could be the
462 quantification or semi-quantification of PAs in other honey samples, and also other PA containing
463 products, such as herbal tea, cereal, feed, and milk.

464 ABBREVIATIONS USED

465 PAs: Pyrrolizidine alkaloids; GC-MS: Gas chromatography–mass spectrometry; LC-MS: Liquid
466 chromatography–mass spectrometry; MRM: multiple reaction-monitoring; LC-QTOF: liquid
467 chromatography-quadrupole-time-of-flight-mass spectrometry; QC: Quality control; LOD: limits of
468 detection; LOQ: limit of quantification; pK_a: pK_a value of strongest acidic; pK_b: pK_a value of strongest
469 basic; SMILE: simplified molecular-input line-entry system; CV%: Coefficient of Variation; ME:

matrix effect; ESI: electrospray ion source; $BMDL_{10}$: the lower confidence limit on the benchmark dose associated with a 10% response

SUPPORTING INFORMATION DESCRIPTION

Supplementary data related to this article can be found in the Supporting Information file.

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TABLES

Table 1. Information of all the tested honey samples: places of production, origins and symbols used in the text.

No	Geographical origin	Botanical origin	PAs (µg/kg)
H1	Spain	Mandarin honey	-
H2	Romania	Creamy flowers honey	Echimidine (1.4)
H3	Mixture from outside the EU	Not clear	Intermedine (<LOQ), Rinderine//Echinatine/Indicine (ca. 6.1)
H4	Argentina and Ukraine	Not clear	Echimidine (3.4), Senecionine (<LOQ)
H5	China	Mountain honey (many wild flowers)	Integerrimine/ Senecivernine (ca.1.6), Trichodesmine (ca. 33.9)
H6	Mexico	Not clear	Intermedine (3.5), Lycopsamine (1.7), Retrorsine (4.3), Rinderine//Echinatine/Indicine (ca.16.4)
H7	Brazil	Not clear	Echimidine (1.6), Intermedine (2.3), Lycopsamine (2.2), Retrorsine (1.6), Senecionine (2.4), Rinderine//Echinatine/Indicine (ca.16.5), Integerrimine/ Senecivernine (ca.9.5)
H8	Denmark	Clover and rape	Crotananine (~51.0)
H9	Denmark	Heather flowers honey	Senecionine (<LOQ), Crotananine (ca.24.7), 3'-acetylindicine (ca.45.4), Acetyl-lycopsamine (ca.46.8)
H10	Denmark	Fruit trees (apple, plum, rape)	Crotananine (~14.2)
H11	Denmark	Summer flowers and white clover honey	Lycopsamine (<LOQ), Myoscorpine *N*-oxide/Echiumine *N*-oxide/ Echiupinine-*N*-oxide (ca. 2.7-3.2), Rinderine//Echinatine/ Indicine (ca.10.3), Crotananine (ca.7.8), 3'-acetylindicine (ca.8.8),Acetyl-lycopsamine (ca.6.7)
H12	Brazil	Not clear	Retrorsine-*N*-oxide (<LOQ)
H13	Nicaragua	Mountain honey (many	Crotananine (ca.5.2)

		wild flowers)	
H14	Ukraine	Fruit trees	Echimidine (<LOQ)
H15	Mixture from countries inside and outside the EU	Not clear	Intermedine (8.4), Lycopsamine (2.0), Rinderine//Echinatine/Indicine (ca.56.4),
H16	Ukraine	Not clear	Echimidine (2.2)
H17	Mixture from outside the EU	Not clear	Crotananine (ca.3.4)
H18	South America	Not clear	Intermedine (8.0), Lycopsamine (0.8), Rinderine//Echinatine/Indicine (ca.31.7)
H19	Denmark	Fruit trees	Crotananine (ca.7.7), Rinderine//Echinatine/Indicine (ca.1.1)
H20	Ukraine	Not clear	Echimidine (2.0), Crotananine (~2.0)
H21	Mixture from countries inside and outside the EU	Acacia	Riddelline/Jacozine (ca.1.5-4.9)
H22	Mixture from outside the EU	Not clear	Retrorsine (8.2), Crotananine (ca. 2.6), Rinderine//Echinatine/Indicine (ca. 2.5)
H23	Mixture from outside the EU	Not clear	Intermedine (<LOQ), Retrorsine (8.2), Retrorsine-*N*-oxide (<LOQ), Crotananine (ca. 4.4), Rinderine//Echinatine/Indicine (ca. 3.6)
H24	Mixture from outside the EU	Acacia honey with hazelnuts	Crotananine (ca. 1.8)
H25	Argentina and Ukraine	Not clear	Echimidine (<LOQ), Lycopsamine (<LOQ),
H26	China	Acacia	-
H27	Mixture from outside the EU	Not clear	Intermedine (11.1), Lycopsamine (3.1)
H28	Denmark	Not clear	Crotananine (ca. 5.3), Riddelline/ Jacozine (ca. 2.3-7.5), Rinderine//Echinatine/Indicine (ca. 1.5)

H29	Mixture from countries inside and outside the EU	Wild flower	Echimidine (2.7), Lycopsamine (<LOQ), Crotananine (ca. 2.1)
H30	Mixture from countries inside and outside the EU	Acacia	-
H31	Mixture from outside the EU	Not clear	Echimidine (1.6), Intermedine (1.4), Lycopsamine (0.7), Crotananine (ca. 12.8), Rinderine//Echinatine/Indicine (ca. 3.3)
H32	Mixture from outside the EU	Not clear	-

Table 2. Validation results.

Compounds	Conc		Inter-day reproducibility			Intra-day Repeatability			Recovery	LOD	LOQ	ME
			mean	std	CV %	mean	std	CV %	%	µg/kg		
Echimidine	1.00	µg/kg	0.89	0.19	21.6	0.89	0.17	18.8	93.5	0.54	1.07	101.92
	5.00	µg/kg	5.22	0.64	12.3	5.22	0.52	10.0	104.4			
	25.00	µg/kg	24.20	2.88	11.9	24.20	2.08	8.6	96.8			
	75.00	µg/kg	67.96	5.80	8.5	67.96	1.80	2.6	90.6			
Intermedine	1.00	µg/kg	0.81	0.08	10.3	0.81	0.07	8.0	89.4	0.41	0.81	90.50
	5.00	µg/kg	4.06	0.69	17.0	4.06	0.46	11.3	81.2			
	25.00	µg/kg	24.21	2.12	8.8	24.21	0.81	3.4	96.8			
	75.00	µg/kg	72.45	7.74	10.7	72.45	3.38	4.7	96.6			
Intermedine-*N*-oxide	1.00	µg/kg	0.96	0.16	16.9	0.96	0.16	16.9	95.6	0.52	1.04	94.05
	5.00	µg/kg	4.01	0.76	18.9	4.01	0.76	18.9	80.2			
	25.00	µg/kg	22.77	2.53	11.1	22.77	1.48	6.5	91.1			
	75.00	µg/kg	72.71	7.53	10.4	72.71	4.54	6.2	96.9			
Lasiocarpine	1.00	µg/kg	0.81	0.11	13.4	0.81	0.07	8.6	80.5	0.22	0.45	112.28
	5.00	µg/kg	4.19	0.59	14.1	4.19	0.24	5.8	83.8			
	25.00	µg/kg	23.57	3.20	13.6	23.57	1.30	5.5	94.3			
	75.00	µg/kg	68.47	8.95	13.1	68.47	3.65	5.3	91.3			
Lasiocarpine-*N*-oxide	1.00	µg/kg	0.81	0.15	18.6	0.81	0.15	18.6	81.4	0.60	1.20	97.10
	5.00	µg/kg	4.23	0.57	13.5	4.23	0.41	9.6	84.7			
	25.00	µg/kg	22.42	2.14	9.5	22.42	1.16	5.2	89.7			
	75.00	µg/kg	68.65	4.58	6.7	68.65	2.91	4.2	91.5			
Lycopsamine	1.00	µg/kg	0.83	0.10	12.3	0.83	0.08	9.4	82.7	0.28	0.56	90.68
	5.00	µg/kg	4.65	0.61	13.1	4.65	0.54	11.5	93.0			
	25.00	µg/kg	22.68	3.86	17.0	22.68	0.81	3.6	90.7			
	75.00	µg/kg	71.06	8.94	12.6	71.06	1.87	2.6	94.7			
Retrorsine	1.00	µg/kg	0.96	0.14	15.1	0.96	0.14	15.1	95.5	0.34	0.67	91.55
	5.00	µg/kg	4.66	0.93	20.0	4.66	0.62	13.3	93.1			
	25.00	µg/kg	22.94	3.05	13.3	22.94	2.59	11.3	91.8			

	75.00	µg/kg	69.08	6.10	8.8	69.08	2.35	3.4	92.1			
Retrorsine-*N*-oxide	1.00	µg/kg	0.79	0.10	12.9	0.79	0.10	12.9	79.2			
	5.00	µg/kg	4.31	0.77	17.9	4.31	0.77	17.9	86.2	0.64	1.27	81.01
	25.00	µg/kg	23.69	3.42	14.4	23.69	1.47	6.2	94.8			
	75.00	µg/kg	70.80	9.91	14.0	70.80	3.47	4.9	94.4			
Senecionine	1.00	µg/kg	0.81	0.13	16.5	0.81	0.13	16.5	81.4			
	5.00	µg/kg	4.19	0.83	19.9	4.19	0.76	18.2	83.9	0.45	0.91	82.95
	25.00	µg/kg	22.91	2.92	12.7	22.91	1.03	4.5	91.6			
	75.00	µg/kg	69.81	11.96	17.1	69.81	5.01	7.2	93.1			
Senecionine-*N*-oxide	1.00	µg/kg	0.80	0.14	17.8	0.80	0.14	17.8	79.8			
	5.00	µg/kg	4.18	0.81	19.5	4.18	0.52	12.5	83.5	0.42	0.84	99.93
	25.00	µg/kg	22.58	2.66	11.8	22.58	1.98	8.8	90.3			
	75.00	µg/kg	68.89	6.05	8.8	68.89	3.41	5.0	91.9			
Seneciphylline	1.00	µg/kg	0.79	0.05	6.5	0.79	0.05	6.5	79.2			
	5.00	µg/kg	4.12	0.63	15.2	4.12	0.52	12.7	82.4	0.23	0.45	81.55
	25.00	µg/kg	22.20	2.18	9.8	22.20	1.46	6.6	88.8			
	75.00	µg/kg	67.64	4.50	6.6	67.64	2.57	3.8	90.2			
Seneciphylline-*N*-oxide	1.00	µg/kg	0.84	0.15	17.5	0.84	0.13	15.4	84.4			
	5.00	µg/kg	4.17	0.52	12.5	4.17	0.52	12.5	83.5	0.60	1.19	88.53
	25.00	µg/kg	19.80	2.26	11.4	19.80	1.31	6.6	79.2			
	75.00	µg/kg	61.75	3.90	6.3	61.75	2.70	4.4	82.3			

Table 3. Molecular ions and selected MS/MS fragment ions of major PAs.

RT	PAs (µg/kg)	Formula	$[M+H]^+$ calculated	$[M+H]^+$ experimental	Fragment pattern and abundancy	Error (ppm)
7.6	Echimidine	$C_{20}H_{31}NO_7$	398.2173	398.2170	120.0810 (48), 220.1301 (2)	-0.75
4.3	Intermedine	$C_{15}H_{25}NO_5$	300.1805	300.1800	138.0916 (33), 156.1021(100)	-1.66
5.2	Intermedine *N*-oxide	$C_{15}H_{25}NO_6$	316.1755	316.1751	138.0914(42), 172.0961(100)	-1.27
8.2	Lasiocarpine	$C_{21}H_{33}NO_7$	412.2330	412.2333	120.0815(100), 238.1427(5)	-0.73
8.5	Lasiocarpine *N*-oxide	$C_{21}H_{33}NO_8$	428.2279	428.2262	94.0651(19), 120.0806 (73), 138.0908 (51)	-3.96
4.5	Lycopsamine	$C_{15}H_{25}NO_5$	300.1805	300.1805	138.0917(28), 156.1020(100)	0
6.1	Retrorsine	$C_{18}H_{25}NO_6$	352.1755	352.1744	120.0822 (100), 138.0894 (58)	-3.12
6.3	Retrorsine-*N*-oxide	$C_{18}H_{25}NO_7$	368.1704	368.1707	118.0648(100), 136.0765 (52)	0.81
7.2	Senecionine	$C_{18}H_{25}NO_5$	336.1805	336.1792	120.0810 (98) 138,0906 (33)	-3.87
7.4	Senecionine *N*-oxide	$C_{18}H_{25}NO_6$	352.1755	352.1753	94.0645(26), 118.0649 (94), 136.0741(77)	-0.56
6.5	Seneciphylline	$C_{18}H_{23}NO_5$	334.1649	334.1655	120.1818(100), 138.0911(66)	1.79
6.8	Seneciphylline *N*-oxide	$C_{18}H_{23}NO_6$	350.1598	350.1595	120.0827 (100), 136.0748 (41)	-0.85
			Tentative identified PAs			
7.8	3'-acetylindicine	$C_{17}H_{27}NO_6$	342.1911	342.1910	94.0699 (7), 121.0680	-0.29

					(81), 120.0813 (25)	
8.1	Acetyl-lycopsamine	$C_{17}H_{27}NO_6$	342.1911	342.1911	120.0791 (46), 156.0903 (8)	0
7.1	Crotananine	$C_{17}H_{25}NO_5$	324.1805	324.1793	120.0768(92), 138.0907 (18)	-3.70
4.6	Echinatine /Indicine/Rinderine	$C_{15}H_{25}NO_5$	300.1805	300.1795	138.0912 (100). 156.0986 (28)	-1.67
7.1	Integerrimine/ Senecivernine	$C_{18}H_{25}NO_5$	336.1805	336.1813	120.0796 (100), 138.0904 (54)	2.38
6.1	Jacozine /Riddelline	$C_{18}H_{23}NO_6$	350.1598	350.1605	120.0772 (33.9), 138.0963 (6)	2
7.5	Myoscorpine *N*-oxide/Echiumine *N*-oxide	$C_{20}H_{31}NO_7$	398.2173	398.2184	120.0807 (56), 136.0693(2), 172.1412 (15)	3.26
5.2	Trichodesmine	$C_{18}H_{27}NO_6$	354.1911	354.1911	94.0700(17), 120.0810 (100)	0

Note: the corresponding chromatograms of LC-MS/MS are shown in Figure S1 in supporting information.

FIGURE CAPTIONS

Figure 1. Structures and chromatogram of 12 PAs

(a) Chemical structures of 12 PAs.

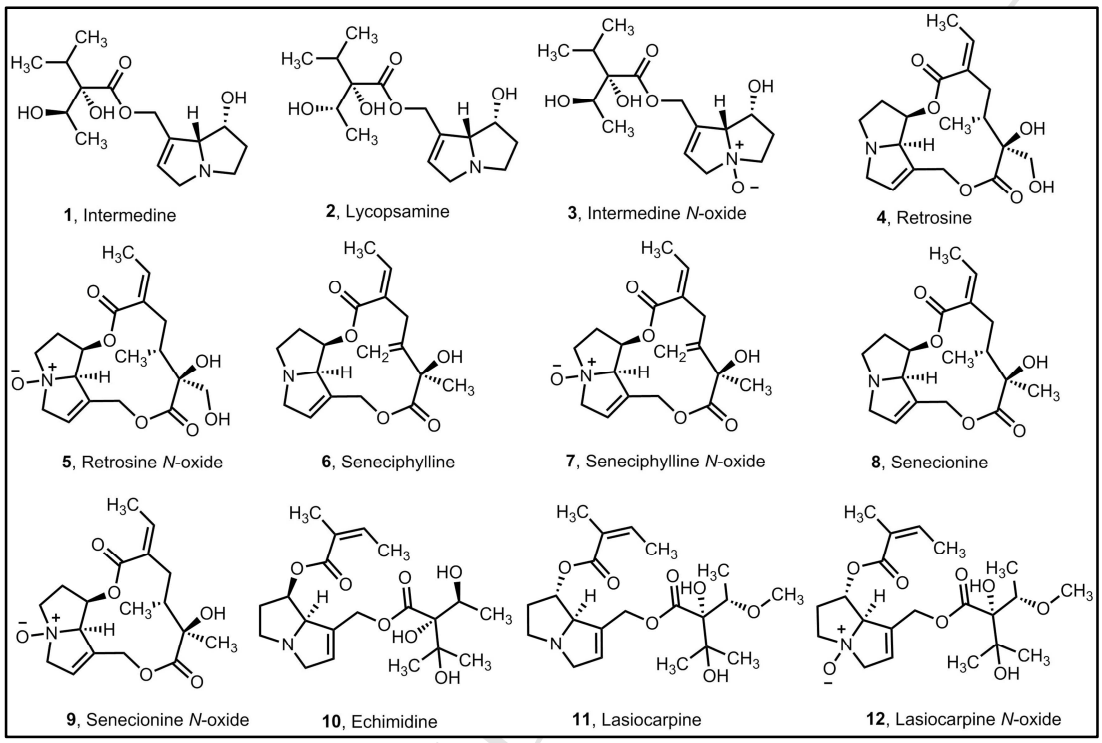
(b) Chromatogram of 12 PAs in 5 ng/ml spiked blank honey sample: 1. Intermedine, 2. Lycopsamine, 3. Intermedine *N*-oxide, 4. Retrosine, 5. Retrosine *N*-oxide, 6. Seneciphylline, 7. Seneciphylline *N*-oxide, 8. Senecionine, 9. Senecionine *N*-oxide, 10. Echimidine, 11. Lasiocarpine, 12. Lasiocarpine *N*-oxide.

Figure 2. (a) Correlation between relative predicted slope and measured slope in all PAs corresponding to Seneciphylline. (b) Correlation between predicted concentration and measured concentration in PAs (from 5 to 100 ng/mL) out of the model. Note: Lycopsamine *N*-oxide, heliotrine, heliotrine *N*-oxide, 7-acetylintermedine are 4 other PAs out of the 12 PAs in the model that used for verification of the prediction model.

FIGURE GRAPHICS

Figure 1.

(a)



(b)

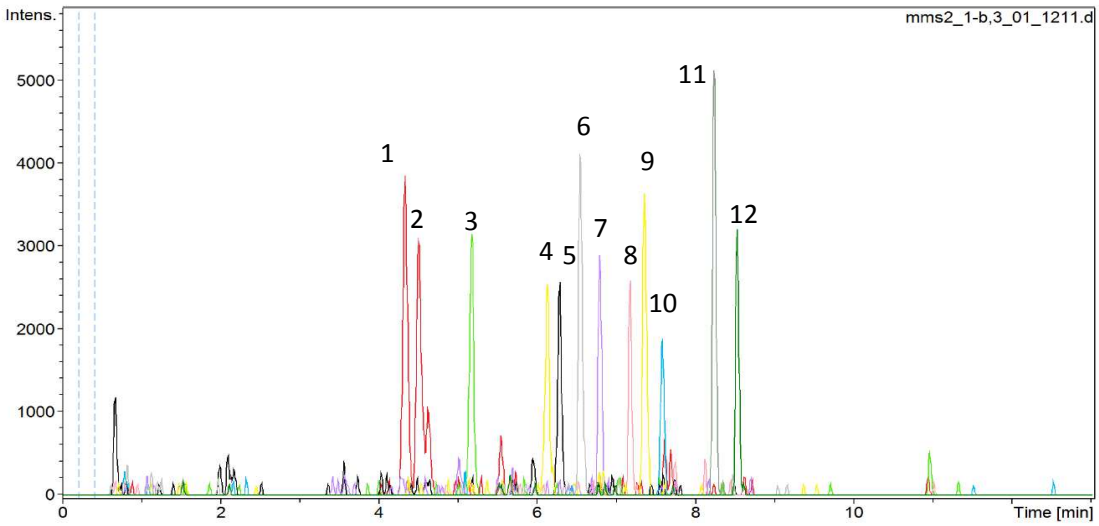
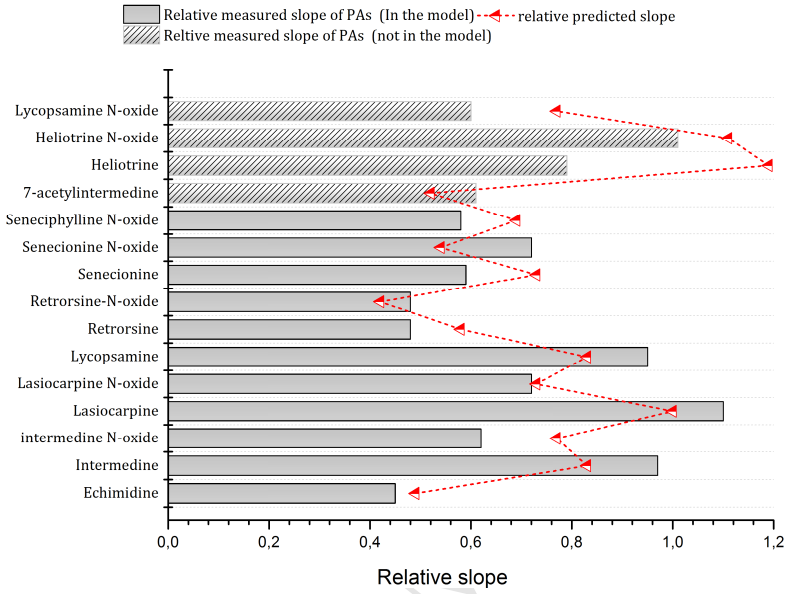
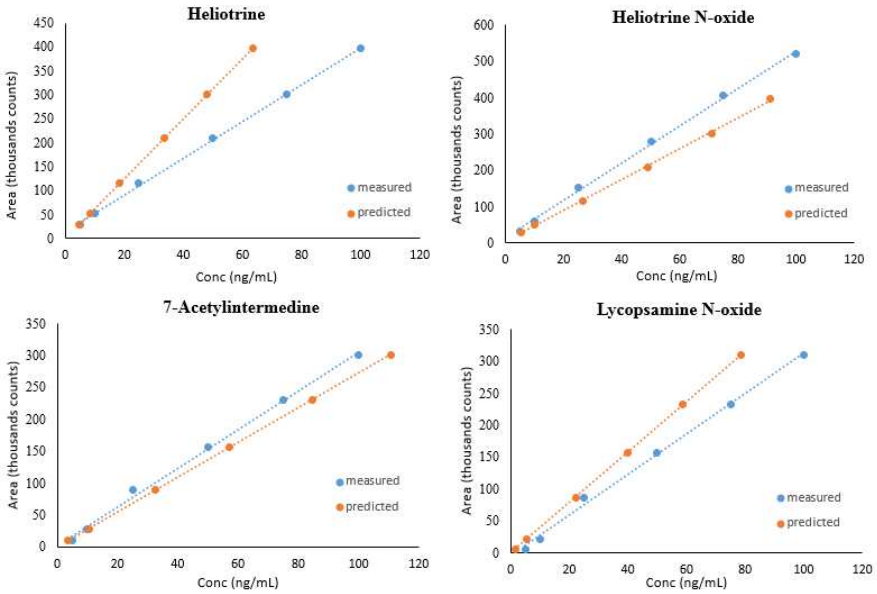


Figure 2.

(a)


Relative measured slope of PAs (In the model)
relative predicted slope
Reltive measured slope of PAs (not in the model)
Lycopsamine N-oxide
Heliotrine N-oxide
Heliotrine
7-acetylintermedine
Seneciphylline N-oxide
Senecionine N-oxide
Senecionine
Retrorsine-N-oxide
Retrorsine
Lycopsamine
Lasiocarpine N-oxide
Lasiocarpine
intermedine N-oxide
Intermedine
Echimidine
0,0
0,2
0,4
0,6
0,8
1,0
1,2
Relative slope

(b)


Heliotrine
Heliotrine N-oxide
7-Acetylintermedine
Lycopsamine N-oxide
Area (thousands counts)
Conc (ng/mL)
measured
predicted

- Semi-quantification of other PAs by quantitative prediction model without standards
- Method validation for the analysis 12 PAs in honey
- Multi-target screening of potential PAs in honey samples

GRAPHIC ABSTRACT