Straightforward Determination of Pyrrolizidine Alkaloids in Honey through Simplified Methanol Extraction (QuPPE) and LC-MS/MS Modes

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ABSTRACT: Contamination of honey with toxic pyrrolizidine alkaloids (PAs) and their N-oxides (PANOs) was assessed by dilution with acidic methanol and analysis through liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) in three different modes. The hybrid linear trap/triple quadrupole (LC-QTRAP) instrument was used in precursor ion scan (PIS), enhanced product ion scan (EPI), and multiple reaction monitoring (MRM) mode. The method was validated: recoveries 86−111%, relative standard deviation (RSD) <20%, at 20 and 40 μg/kg, except retrorsine, which showed a RSD of 30% at 20 μg/kg. Honey samples were analyzed and five of them showed levels of 40 μg/kg for the sum (PAs + PANOs). This approach allows the simultaneous determination of PAs and PANOs in honey, even if their chemical standards are not available.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are a large group of secondary metabolites occurring in Asteraceae, Fabaceae, and Boraginaceae species. Their basic cores are the necines platynecine, heliotridine, retronecine, and otonecine. They are esters that occur in nature in two forms: the tertiary form and its corresponding N-oxides (PANOs). PAs bearing a 1,2 unsaturation in the necine are hepatotoxic, carcinogenic, genotoxic, teratogenic, and sometimes pneumotoxic. The toxicity of unsaturated necines follows the following sequence: macrocyclic, diesters, and monoesters.

The increasing reports on PA contamination of foods such as grain, milk, meat, eggs, or honey, stress the importance of performing a risk assessment on these alkaloids. The main drawback to gather enough data for this study is the lack of appropriate analytical methods to determine them in the different food matrices. This issue has been acknowledged by the Joint FAO/WHO for food standards program of the Codex Alimentarius Commission. Simultaneously, the Panel on Contaminants in the Food Chain (CONTAM Panel) from the European Food Safety Authority (EFSA) stressed the need to generate knowledge about exposure to PAs, in particular due to honey consumption. Honey has shown to be a considerable source of PA exposure. The contamination may be due to nectar and plant pollen rich in PAs collected by bees and transferred to honey.

There is no legal requirement of maximum levels for these natural toxins occurring in honey, bee pollen, and other foodstuffs. Nevertheless, some European countries have introduced acceptable tolerance levels for PAs in herbal preparations and extracts, and these have been applied to the commerce of honey. Germany has introduced a limit of 1 μg of PAs per day for phytopharmaceuticals when consumed during less than 6 weeks and 0.1 μg of PAs per day if the 6-week period of application is exceeded.

In 2017, the Federal Institute of Risk Assessment (Bundesinstitut für Risikobewertung, BfR, Germany) and the UK Committee on Toxicity (COT) has recommended an exposure limit from different foods of 0.007 μg of PAs/kg body weight per day, which corresponds to 0.49 μg per day of PAs for an average 70 kg person. Then, the CONTAM Panel established a revised value of 237 μg/kg body weight per day that has been adopted by the BfR.
Several methods have been developed for the determination of PAs in honey. In 2008, Kempf et al developed a method by gas chromatography-mass spectrometry (GC-MS) that quantifies PAs as a sum parameter after reducing the esters to retronecine. This method was applied to the determination of PAs in honeys used for medicinal purposes, in 2018, Kowalczyk et al employed the same strategy and a clean up by solid phase extraction (SPE) where different sorbents and eluents were tested, achieving a very low limit of quantification.

PAs determination evolved from GC-MS to LC-MS methods. This happened because GC methods are applicable only to free bases given that the N-oxides are not stable at the temperatures required for volatilization. On the other hand, LC methods have the advantage that both, bases and PANOs, can be analyzed simultaneously. Liquid chromatography-mass spectrometry (LC-MS) prevents the thermal decomposition of N-oxides and offers important structural information, as PAs are more toxic than PANOs. Most of the LC methods developed for PA determination in honey rely on SPE employing a strong cation exchange resin as sample treatment. The recoveries of PAs from samples are highly dependent on the types of cartridges, sample loading amount, and eluents used during the extraction procedures. Other methods for the determination of trace contaminants have been assayed such as Quick Easy Cheap Effective Rugged Safe (QuEChERS) template. However, a robust method for the simultaneous determination of PAs and PANOs in honey is lacking. QuEChERS is a method originally developed for the multiresidue analysis of pesticides in a wide range of matrices. When it was applied for PA determination, the N-oxides had to be reduced with zinc dust and allowed to react for 1 h and 30 min.

The variant of QuEChERS, Quick Polar Pesticides (QuPPe) is a method developed to determine highly polar pesticides that are not amenable QuEChERS. Up to now, it had not been assayed for the simultaneous determination of PAs and PANOs. The procedure simply involves addition of acidified methanol–water mixture and isotopically labeled standards. The compounds are extracted from the test portion via shaking. After centrifugation, an aliquot of the raw extract could be further cleaned up or analyzed as such by LC-MS/MS.

Tandem hybrid mass spectrometers like Q-traps can work in several operating modes where each of them gives specific structural information, including precursor ion scan (PIS), enhanced product ion (EPI), and multireaction monitoring (MRM) mode. During PIS, all the ions that give rise to a daughter ion of a specific m/z are selected and registered. When using EPI, the ions of specific m/z are selected, fragmented, and driven to the detector focused at the desired mass range, giving the MS^2 spectra. The MRM approach allows monitoring several transitions of parent ions to daughter ions; this is the golden standard for trace quantification purposes (Table 1).

The present work introduces a protocol for the determination of PAs and PANOs in honey. For most of these compounds, standards are not commercially available yet, so the goal of this protocol is to identify and quantify the most toxic PAs, even when analytical standards are not available.

### RESULTS AND DISCUSSION

#### Selection of Sample Treatment
Sample treatment is generally a laborious and time-consuming step that contributes mostly to analytical errors. To simplify PA analysis in honey, two strategies that have proven to be efficient for trace analysis of organic contaminants were considered, QuEChERS and QuPPe.

The recoveries obtained through QuEChERS procedure with a spiked honey were around 100% for the bases but 50% for echimidine N-oxide. In an attempt to improve the N-oxide recovery, 2 g of NaCl was used to increase the salting out effect, but recoveries were not affected. Hence, to recover N-oxides a reduction step is needed, which is troublesome, and therefore the QuEChERS approach was discarded.

QuPPe was tested because it was designed for the determination of polar pesticides residues. Sample dilution with MeOH in acidic media succeeded in recovering both free bases and N-oxides with high percentages and low dispersion. MeOH precipitates the sugars but keeps the target compounds in solution. In its protocol, QuPPe stresses the relevance of using isotopically labeled standards for quantification. In this case, as labeled standards were not available, the conventional approach in the trace analysis of matrix-matched calibration was employed to identify and determine the studied compounds during method evaluation. The uncertainty added by the impossibility of use was evaluated as tolerable for this particular case. Due to the precision and accuracy that QuPPe showed in the trial assays, it was selected as the sample treatment technique. In a single step, it allows the total analysis of the molecular species of PAs present in the sample in trace amounts. The method’s performance characteristics are shown below.

#### LC-MS/MS Workflow
Precursor ion scan (PIS) and enhanced product ion scan modes were executed to identify the compounds. Scheduled multiple reaction monitoring (MRM) mode was applied to quantify them.

Precursor ion scan mode can be used to detect all ions that give rise to a daughter ion of a specific m/z. Toxic and consequently relevant for health, alkaloids contain a 1,2 unsaturated pyrrolizidine nucleus, which yields a characteristic ion at m/z 120 in the case of open-chain mono- and diesters, cyclic diesters, open-chain diester, and cyclic diester N-oxides, and a characteristic ion at m/z 138 in the case of open-chain monoester N-oxides. Monitoring all the parent ions that have a daughter ion at m/z 120 or 138 allows detecting all PAs contained in a sample whether their standards are available or not.

#### Table 1. Optimized Conditions for EPI and MRM Experiments

| Alkaloid                  | Transition | DP (V) | CE (KeV) |
|---------------------------|------------|--------|----------|
| acetyllycopsamine         | 342.3−198.4| 53     | 38       |
|                           | 342.3−138.3| 36     |          |
|                           | 342.3−120.2| 36     |          |
|                           | 342.3−94.2 | 60     |          |
| echimidine                | 398.3−220.3| 75     | 22       |
| echimidine N-oxide        | 414.4−396.4| 80     | 35       |
| echiumine                 | 382.5−220.3| 51     | 25       |
| senecionine               | 336.0−138.2| 60     | 42       |
|                           | 336.0−120.2|        |          |
not. Figure 1 shows the precursor ion spectrum of a honey sample for the daughter ion of m/z 138.

Once this experiment reveals possible parent ions, it is necessary to confirm if they are PAs. At this step, an enhanced product ion (EPI) experiment is performed. The ions of specific m/z are selected at Q1 and collected in the collision cell where they are fragmented and driven to the detector, giving a MS\(^2\) spectrum that provides the structural information needed to unequivocally identify the compound (Figure 2).

The presence of the ions of m/z 120, 138, 156, 172, and 220 reveals the presence of lycopsamine.

Afterward, the compounds are quantified by multireaction monitoring protocol using two ions, a qualifier that corresponds to the daughter ion giving the less intense signal and the most intense one, the quantifier (Figure 3).\(^{25}\)

The results are expressed as acetyllycopside for the open esters, retrorsine for the cyclic esters, and echimidine N-oxide for the N-oxides, using a five-point matrix-matched calibration curve of acetyllycopside, retrorsine, and echimidine N-oxide, respectively.

Since there are more than 300 PAs reported and only a few standards are commercially available, this approach is of utmost relevance.

Method’s Performance Characteristics. The following performance characteristics were evaluated:

Linearity was evaluated through a matrix-matched five-point calibration curve in the range of 3.8–20 \(\mu\)g/L for acetyllycopside, 18–126 \(\mu\)g/L for retrorsine, and 5.6–29.8 \(\mu\)g/L for echimidine N-oxide. A linear calibration function, \(a = 12819995C - 8172, R^2 = 0.993; a = 1260294C - 8580, R^2 = 0.9949; \) and \(a = 7898688C - 24203, R^2 = 0.9934, \) where “a” is area and “C” the concentration in \(\mu\)g/L was found to fit the experimental data for acetyllycopside, retrorsine, and echimidine N-oxide, respectively.

Precision (repeatability) was evaluated at two levels by repeating five times the entire procedure in a honey sample.
with no detectable amounts of PAs, so it was spiked at the 20 and 40 μg/kg of honey level. The relative standard deviations are shown in Table 2. A standardized alkaloid extract of *Echium plantagineum* was employed to spike the sample to evaluate the repeatability for echiumine and echimidine. The values of $s_r$ (%) were 5.8 and 6.1, respectively (the amount of echiumine and echimidine added was 21.8 and 21.4 μg/kg of honey, respectively).

Quantiﬁcation limits were assessed according the Eurachem criteria employing the standard deviation of the results obtained for each compound ($n = 5$) at the low concentration level. The LOQs were 8.6, 13.0, and 18.4 μg/kg in honey for acetylicopsamine, echimidine N-oxide, and retrorsine, respectively.

**Evaluation of Matrix Effect.** Matrix effects for the specific standards used for quantitation were evaluated comparing the response from standards in MeOH with that from the standards in the extract. An open-chain diester (acetylicopsamine) a macrocyclic diester (retrorsine) and an N-oxide (echimidine N-oxide) were employed. The matrix effects calculated as the relative difference in percentage between the slopes of the curves obtained in the extract and in MeOH were 213, 438, and 108%; hence, matrix-matched calibrations were performed.

**Trueness.** Trueness was assessed by spike recoveries ($n = 5$) at 20 and 40 μg/kg of honey levels.

The average recovery percentage for each compound is presented in Table 3. The recoveries obtained for echimidine and echiumine were 86.3 and 92.5%, respectively.

![Figure 3. MRM transitions employed for lycopsamine N-oxide quantification m/z 138 qualifier (red) and m/z 120 quantifier (blue).](#)

| Table 2. Precision (repeatability) Estimated as $s_r$ (%) |
|----------------------------------------------------------|
| **compound** | **$s_r$ (%)** | **μg/kg of added to honey** |
|----------|----------------|--------------------------|
| **Low Level** | | |
| acetylicopsamine | 5.4 | 16.5 |
| echimidine N-oxide | 6.3 | 23.0 |
| retrorsine | 31 | 21.9 |
| senecionine | 11 | 20.0 |
| seneciphylline | 3.3 | 16.5 |
| **Mid-Level** | | |
| acetylicopsamine | 20 | 33.0 |
| echimidine N oxide | 15 | 46.0 |
| retrorsine | 18 | 43.8 |
| senecionine | 6.7 | 40.0 |
| seneciphylline | 7.8 | 33.0 |

![Table 2. Precision (repeatability) Estimated as $s_r$ (%)](#)

| Table 3. Trueness Evaluated as Recovery Percentage |
|------------------------------------------------|
| **compound** | **average recovery (%)** |
|----------|--------------------------|
| **Low Level** | | |
| acetylicopsamine | 95.8 |
| echimidine N-oxide | 90.7 |
| retrorsine | 86.4 |
| senecionine | 94.5 |
| seneciphylline | 98.3 |
| **Mid-Level** | | |
| acetylicopsamine | 96.4 |
| echimidine N-oxide | 111 |
| retrorsine | 103 |
| senecionine | 88.7 |
| seneciphylline | 89.9 |
PA Determination in Commercial Honey Samples.

Five honey samples were analyzed in duplicates, and the results are presented in Table 4.

| Sample  | PA       | µg/kg | PA       | µg/kg | PA       | µg/kg |
|---------|----------|-------|----------|-------|----------|-------|
| 1       | echiu.*  | 14    | Lyc.*    | 10    | Lyc N-ox*| 19    |
| 2       | echim.*  | 15    | Lyc.     | 10    | unknown  | 34    |
| 3       | echim.*  | 44    | Lyc.     | 9     | AcLyc    | 22    |
| 4       |         | 22    | Lyc.     | 25    | Lyc N-ox| 19    |
| 5       | AcLyc*   | 22    | Lyc.     | 19    | Lyc N-ox| 10    |

*Echiu: echiumine, Echim: echimidine, Lyc: lycopsamine, Lyc N-ox, lycopsamine N-oxide, and AcLyc: acetyllycopsamine.

Senecionine was detected in samples 1 and 5, but it was below its limit of quantification.

The combination of different strategies and operational modes of LC-MS/MS allowed the determination of several PAs and PANOs in honey without employing individual standards. An “in house” standard can be built up with this protocol, which is useful for further analysis and method development.

The LC-MS/MS proposed protocol is generally applicable for the characterization of families of compounds in extracts that bear common structural features such as pyrrolizidine alkaloids. The PIS mode available on hybrid linear trap/triple quadrupole (QTRAP) LC-MS/MS configurations allowed detecting every compound that has an m/z 120 or 138 daughter ion. Then, the presence of PAs was confirmed through their MS² spectrum obtained by enhanced product ion scan using the linear trap. Finally, compound quantitation was performed with high sensitivity and selectivity through target analysis acquired in the MRM mode.

The method may seem laborious at first glance, but the possibility of expanding it to new PA findings in honey is one of its major features and advantages. Its target is not a closed list of compounds but an open one. In the absence of a PA standard, the MS² spectrum is the definitive tool to identify the unknown. Nevertheless, careful inspection has to be done to not inform false positives. The very low LOQs reached allow accomplishing the concepts of consumers’ food safety.

MATERIALS AND METHODS

All Chemicals Employed Were High-Performance Liquid Chromatography (HPLC) or P.A. Grade. PA standards (acetyllycopsamine, echimidine N-oxide, senecionine, and senechylidine) were purchased from PhytoLab GMBH (Vestenbergsgreuth, Germany), and retrorsine was purchased from Sigma-Aldrich (Steinheim, Germany). Echiu mine and echimidine were obtained from a characterized (LC−(electrospray ionization (ESI))MS/MS) extract of Echium plantagineum L. prepared as described before.26

Stock solutions of the pure standards of 500–5000 mg/L were prepared in acetonitrile. The solutions were stored at −18 °C, and the working standards were prepared daily.

Samples of raw honey were taken directly from the producer and stored at ambient temperature.

Equipment. A 1200 Infinity (Agilent Technologies, Santa Clara) high-pressure liquid chromatograph fitted with a ZORBAX Eclipse XDB C18 column (150 × 4.6 mm², 5 µm) and coupled to a 4000 QTRAP system (AB Sciex, Ontario, Canada) equipped with an electrospray (ESI) source was employed to perform LC-MS/MS analysis.

Sample Preparation. QuEChERS. Ten milliliters of water and 10 mL of acetonitrile were added to 5 g of sample and shaken by hand. Then, 4 g of MgSO₄ and 1 g of NaCl were added under vigorous shaking (1 min). After centrifugation (5 min, 3500 rpm) and filtration, an aliquot was analyzed by LC-MS/MS.

QuPPe. Five grams of honey was dissolved in 10 mL of water under vigorous manual shaking for 1 min. Ten milliliters of 1% (v/v) formic acid in MeOH was added, and the mixture was shaken vigorously for another min. Following centrifugation (5 min, 3500 rpm), the supernatant was filtered and analyzed by LC-MS/MS.

LC Analysis. The mobile phase was composed of (A) 0.1% formic acid in water and (B) acetonitrile at a flow rate of 0.5 mL/min. The LC conditions were same as those described previously based on the protocol from Avula et al.28 with some modifications. The elution gradient was 0 min, 87% A/13% B isocratic for 1 min, in next 7 min to 50% A/50% B, and in next 7 min to 100% B. Each run was followed by a 3 min equilibration period with 87% A/13% B. The injection volume was 10 µL.

MS/MS. The ESI source was operated in a positive-ion mode with a capillary voltage of 3500 V and air as the nebulizer gas (70 psi), as well as the drying gas (dry air at 350 °C/50 psi). The curtain gas was N₂ (30 psi). The operating conditions were optimized with pure standards and with characterized extract.

PIS was performed employing a declustering potential (DP) ramp from 50 to 120 V and a collision energy (CE) ramp from 13 to 60 KeV.

The optimized conditions for EPI experiment and MRM of the known compounds are shown in Table 1.

For others compounds identified as possible PAs, DP and CE employed for tertiary bases either for EPI experiment or for their quantification by MRM was 60 V and 35 keV, respectively (average of DP and CE of the known compounds). DP employed for N-oxides was 80 V.

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Author Contributions

A.S. (PhD student) contributed in collection of plant sample and identification, chromatographic analysis, analysis of the data, and drafting of the paper. S.N. helped in the LC-MS experimental design and determinations, as well as in the analysis and discussion of the data. H.H. participated in the experiment planning, supervised the laboratory work, and contributed to critical reading and improvement of the manuscript. All the authors have read the final manuscript and approved its submission.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the Agencia Nacional de Investigación e Innovación (ANII) and Programa de Ciencias Básicas (PEDECIBA) for financial support.
REFERENCES

(1) Wiedenfeld, H. Plants containing Pyrrolizidine Alkaloids - Toxicity and Problems. Food Addit Contam Part A. 2011, 28, 282–292.

(2) Centre for Food Safety. Pyrrolizidine Alkaloids in Food - Centre for Food Safety. https://www.cfs.gov.hk/english/programme/programme_rafs/files/Pyrrolizidine_Alkaloids_in_Food_e.pdf (accessed Jun 10, 2017).

(3) Codex Alimentarius Commission. REP11/CFJ01NT FAO/WHO FOOD STANDARDS PROGRAMME... Discussion Paper on Pyrrolizidine Alkaloids. https://www.cfs.gov.hk/english/programme/programme_rafs/files/Pyrrolizidine_Alkaloids_in_Food_e.pdf (accessed Jun 10, 2017).

(4) European Food Safety Authority. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the European Commission related to pyrrolizidine alkaloids as undesirable substances in animal feed. EFSA J. 2007, 5273.

(5) Kempf, M.; Wittig, M.; Schönfeld, K.; Cramer, L.; Schreier, P.; Beuerle, T. Pyrrolizidine alkaloids in food: downstream contamination in the food chain caused by honey and pollen. Food Addit Contam., Part A 2011, 28, 325–331.

(6) Bundesinstitut für Risikobewertung. Pyrrolizidine Alkaloids in Herbal Teas And Teas. BfR Opinion No. 018/2013 of 5 July 2013, http://www.bfr.bund.de/cm/349/pyrrolizidine-alkaloids-in-herbal-teas-and-teas.pdf (accessed Jun 21, 2017).

(7) Bundesinstitut für Risikobewertung. Analytik und Toxizität von Pyrrolizidinalkaloiden sowie eine Einschätzung des gesundheitlichen Risikos durch deren Vorkommen in Honig. http://www.bfr.bund.de/cm/343/analytik-und-toxizitaet-von-pyrrolizidinalkaloiden.pdf (accessed Jul 22, 2019).

(8) Committee on Toxicity of Chemicals in Food, Consumer Products and Environment. COT Statement on Pyrrolizidine Alkaloids in Food. https://www.cfs.gov.hk/english/programme/programme_rafs/files/Pyrrolizidine_Alkaloids_in_Food_e.pdf (accessed Jul 10, 2019).

(9) European Food Safety Authority. Risk for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. EFSA J. 2017, 15, 4908 4932 DOI: 10.2903/j.efsa.2017.4908.

(10) Kempf, M.; Beuerle, T.; Bhringer, M.; Denner, M.; Trotz, D.; von der Ohe, K.; Bhavanam, V. B. R.; Schreier, P. Pyrrolizidine alkaloids in honey: Risk analysis by gas chromatography-mass spectrometry. Mol. Nutr. Food Res. 2008, 52, 1193–1200.

(11) Cramer, L.; Beuerle, T. Detection and Quantification of Pyrrolizidine Alkaloids in Antibacterial Medical Honeyes. Planta Med. 2010, 77, 196–198.

(12) Kowalczyk, E.; Sieradzki, Z.; Kwiatek, K. Determination of Pyrrolizidine Alkaloids in Honey with Sensitive Gas Chromatography-Mass Spectrometry Method. Food Anal. Method 2018, 11, 1345–1355.

(13) Zhu, L.; Wang, Z.; Wong, L.; He, Y.; Zhao, Z.; Ye, Y.; Fu, P. P.; Lin, G. Contamination of hepatotoxic pyrrolizidine alkaloids in retail honey in China. Food Control 2018, 85, 484–494.

(14) Griffin, C.; T.; Danaher, M.; Elliott, C. T.; Kennedy, G.; Furey, A. Detection of pyrrolizidine alkaloids in commercial honey using liquid chromatography–ion trap mass spectrometry. Griffin, C. T. Food Chem. 2013, 136, 1577–1583.

(15) Bodi, D.; Ronczka, S.; Gottschalk, C.; Behr, N.; Skibba, A.; Wagner, M.; Lahrsen-Wiedeholt, M.; Preiss-Weiger, A.; These, A. Determination of pyrrolizidine alkaloids in tea, herbal drugs and honey. Food Addit Contam., Part A 2014, 31, 1886–1895.

(16) Lorena, L.; Roberta, M.; Alessandra, R.; Clara, M.; Francesca, C. Evaluation of Some Pyrrolizidine Alkaloids in Honey Samples from the Veneto Region (Italy) by LC-MS/MS. Food Anal. Methods 2016, 9, 1825–1836.

(17) Lucchetti, M. A.; Glauser, G.; Kilchenmann, V.; Diubecce, A.; Beckh, G.; Praz, C.; Kast, C. Pyrrolizidine alkaloids from Echium vulgare in honey originate primarily from floral nectar. J. Agric. Food Chem. 2016, 64, 5267–5273.

(18) Orantes-Bermejo, F. J.; Serra Bonvehí, J.; Gómez-Pajuelo, A.; Megías, M.; Torres, C. Pyrrolizidine alkaloids: their occurrence in Spanish honey collected from purple viper’s bugloss (Echium spp.). Food Addit Contam., Part A 2013, 30, 1799–1806.

(19) Diubecce, A.; Beckh, G.; Illemann, C. Pyrrolizidine alkaloids in honey and bee pollen. Food Addit Contam., Part A 2011, 28, 348–358.

(20) Wang, T.; Frandsen, H. L.; Roed Christiansson, N.; Rosendal, S. E.; Pederse, M.; Smidsgaard, J. Pyrrolizidine alkaloids in honey: Quantification with and without standards. Food Control 2019, 98, 227–237.

(21) Mudge, E. M.; Jones, A. M. P.; Brown, P. N. Quantification of pyrrolizidine alkaloids in North American plants and honey by LC-MS: single laboratory validation. Food Addit Contam Part A 2015, 32, 2068–2074.

(22) Anastassiades, M.; Lehotay, S. J.; Stainbäher, D.; Schenck, F. J. Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and “Dispersive Solid-Phase Extraction” for the Determination of Pesticide Residues in Produce. J. AOAC Int. 2004, 86, 412–431.

(23) Martimello, M.; Borin, A.; Stella, R.; Bovo, D.; Biancotto, G.; Gallina, A.; Mutinelli, F. Development and validation of a QuECHERS method coupled to liquid chromatography and high resolution mass spectrometry to determine pyrrolizidine and tropane alkaloids in honey. Food Chem. 2017, 234, 295–302.

(24) These, A.; Bodi, D.; Ronczka, S.; Lahrsen-Wiedeholt, M.; Preiss-Weige, A. Structural screening by multiple reaction monitoring as a new approach for tandem mass spectrometry: presented for the determination of pyrrolizidine alkaloids in plants. Anal. Bioanal. Chem. 2013, 405, 9375–9383.

(25) European Commission. Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed. https://ec.europa.eu/food/sites/sites/files/plants/docs/pesticides_mrl_guidelines_wrdoc_2017-11813.pdf (accessed Set 03, 2019).

(26) Sixto, A.; Perez-Parada, A.; Niell, S.; Heinen, H. GC–MS and LC–MS/MS workflows for the identification and quantitation of pyrrolizidine alkaloids in plant extracts, a case study: Echium plantagineum. Rev. Bras. Farmacogn. 2019, 29 (4), 500–503.

(27) Eurachem. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf (accessed Jan 18, 2019).

(28) Avula, B.; Wang, Y.-H.; Wang, M.; Smillie, T. J.; Khan, I. A. Simultaneous determination of sesquiterpenes and pyrrolizidine alkaloids from the rhizomes of Petasites hybrids (L) G.M. et Sch. and dietary supplements using UPLC-UV and HPLC-TOF-MS methods. J. Pharm. Biomed. Anal. 2012, 70, 53–63.