



# Determination of Pyrrolizidine Alkaloids in Honey with Sensitive Gas Chromatography-Mass Spectrometry Method

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## Abstract

Pyrrolizidine alkaloids (PAs) are secondary metabolites produced by plants as a chemical defense against herbivores. Plants containing PAs are widely distributed in almost all geographical regions posing a risk of honey contamination. To provide safety of honey and decrease the potential risk for the consumers, a sensitive method based on gas chromatography-mass spectrometry enabling determination of a content of 1,2-unsaturated PAs in honey was developed. Honey samples were purified on MCX cartridges, and PAs were eluted with a solvent mixture consisting of ethyl acetate, methanol, ammonia, and triethylamine. Subsequently, 1,2-unsaturated alkaloids were reduced to their common backbone structures and derivatized with heptafluorobutyric anhydride. The method was validated according to SANTE 2015. All received parameters are in consistency with the document requirements as recovery ranged from 73.1 to 93.6%. The repeatability and reproducibility were calculated as relative standard deviation and ranged from 3.9 to 8.6% and from 10.6 to 17.8%, respectively. The limit of quantification was determined as  $1 \mu\text{g kg}^{-1}$ . Good linearity of the method was obtained with the coefficient of determination  $R^2 > 0.99$ . The method was applied to 40 Polish and 14 Asian honey sample analyses.

**Keywords** Pyrrolizidine alkaloids · Honey · Food · GC-MS

## Introduction

Honey is used and consumed worldwide for its sweet taste, nutritive, and pro-health properties. Honey, however, is produced in a natural environment, which is why it can be contaminated with many natural substances (Dubreil-Chéneau et al. 2013). Certain types of flower nectar and pollen have been reported to result in honey that is psychoactive or that can lead to toxicity (Islam et al. 2014). The natural toxic substances that can be transferred to honey are, among others, pyrrolizidine alkaloids (PAs).

PAs are considered to be the most widely spread toxins of natural origin. They are produced by many plants, estimated as 3% of all flowering plants; however, the main sources of PAs are the families *Boraginaceae*, *Asteraceae*, *Fabaceae*, and *Apocynaceae* (Kempf et al. 2008). Toxicity of the compounds

involves unsaturation of the 1,2 position and esterification of at least one of the hydroxyl groups with an acid and increases with the degree of branching and formation of long cyclic diesters (Mudge et al. 2015). However, PAs are pro-toxins as they require metabolic activation to exert toxic effects. Bioactivation takes place in the liver and is induced by the cytochrome P450 (CYP) monooxygenases. Formed pyrroles can rapidly bind to nucleophilic centers in DNA, proteins, and amino acids (Prakash et al. 1999; Fu et al. 2004; Merz and Schrenk 2016).

PAs can cause acute and fatal intoxications in humans and animals. However, the chronic, insidious poisoning caused by intermittent, low-level intake of PAs is more difficult to associate with adverse health effects, especially when they become apparent years after consumption (Boppré 2011). The chronic disease that can be initiated by the low-level dietary exposure to 1,2-unsaturated PAs includes a wide range of cancers, progressive liver disorders leading to cirrhosis, congenital anomalies, and pulmonary arterial hypertension (Edgar et al. 2015).

Despite the recognized toxic effect of PAs and reported cases of human and animal poisoning, no maximum residue limits in food and feed have been established (Valese et al. 2016). Some European countries such as Germany, Austria,

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and the Netherlands have introduced acceptable tolerance levels for herbal preparations and extracts. In Germany, the limit of PA intake has been established as 1  $\mu\text{g}$  per day if consumed for up to 6 weeks, but in the case of longer consumption, the limit has been reduced to 0.1  $\mu\text{g}$  per day (Dübecke et al. 2011).

The Federal Institute of Risk Assessment (Bundesinstitut fuer Riskobewertung, BfR, Germany) and UK Committee on Toxicity have concluded that the exposure to PAs from food should be as low as possible and have recommended an exposure limit from various foods to 0.007  $\mu\text{g}$  of PAs  $\text{kg}^{-1}$  of body weight (b.w.) per day (Oplawska et al. 2014; Merz and Schrenk 2016).

Honey has been assessed as one of the sources of PAs which in combination with other, even more important sources, such as (herbal) teas and plant food supplements for which risk assessment has been recently conducted (Chen et al. 2017), can have an essential contribution of PAs into human diet. Even though, no limits of concentration of PAs in honey have been defined (Bodi et al. 2014). However, some authors have suggested that all honeys need to be assessed for their content of PAs to minimize the dietary exposure of consumers (Edgar et al. 2002; Dübecke et al. 2011; Orantes-Bermejo et al. 2013). In recent years, a growing food safety concern regarding contaminants of honey can be noted, with a special focus on pyrrolizidine alkaloids. Many papers revealing the presence of pyrrolizidine alkaloids, sometimes in very high concentrations, have been published. The content of PAs is strongly related to the genera of plants from which the honey has been produced as well as the country of honey production.

Europe is the second largest global producer of honey. However, it is not self-sufficient and is dependent on the importation of honey from other countries. Around 40% of Europe's consumption needs are met through honey imports (CBI 2017). As it was revealed, the honey of Central and South America or Australia can contain high rates of PA positive samples with high individual PA levels (EFSA 2011; Dübecke et al. 2011; Griffin et al. 2015a; Valesse et al. 2016).

Only two methods based on gas chromatography-mass spectrometry (GC-MS) for the determination of PAs in honey have been described in the literature (Kempf et al. 2008; Cramer and Beuerle 2012). The approach used in GC-MS analysis involves the reduction of 1,2-unsaturated PAs to their respective necine base backbone structures: retronecine and heliotridine. The reduced forms retain the feature of PA toxicity which is 1,2-double bond. This approach can be perceived as very beneficial as far as the safety of honeys is concerned as it does not require any advance information on expected PAs and does not involve the necessity of having analytical standards of all particular alkaloids, because it is more of a non-target screening method (EFSA 2011) that provides the information on the content of almost all 1,2-unsaturated PAs present in the sample.

To ensure appropriate level of consumers' protection, highly sensitive analytical methods have to be developed. For this reason, the aim of the study was to develop a sensitive method based on gas chromatography-mass spectrometry that could be used in a routine laboratory practice.

## Materials and Methods

### Chemicals and Reagents

All reagents and solvents were of analytical or HPLC grade. Hydrochloric acid 36–38% was purchased from Chempur (Piekary Śląskie, Poland); ammonia solution 25%, sodium sulfate, and zinc dust (particles size 45–150  $\mu\text{m}$ ) were from POCH (Gliwice, Poland); dichloromethane picograde was from LGC Standards (Wesel, Germany); ethyl acetate GC, sodium hydroxide, and triethylamine (TEA) were from Merck (Darmstadt, Germany); methanol and acetonitrile were from J.T. Baker (Deventer, the Netherlands). Pure water was from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Lithium aluminum hydride ( $\text{LiAlH}_4$ ) 1 M solution in tetrahydrofuran (THF) was from Acros Organics (NJ, USA). Heptafluorobutyric anhydride (HFBA) and ethyl acetate anhydrous were purchased from Sigma-Aldrich (St. Louis, MO, USA). All analytical standards of pyrrolizidine alkaloids were purchased from PhytoLab (Vestenbergsgreuth, Germany). Mixed-mode polymeric cartridges Oasis MCX (average particles size 57  $\mu\text{m}$ ) were purchased from Waters (Milford, MA, USA); Bond Elut Plexa PCX (average particles size 45.4  $\mu\text{m}$ ) and HF Bond Elut-SCX (average particles size 134  $\mu\text{m}$ ) cartridges were from Agilent (Santa Clara, CA, USA); Strata SCX (average particles size 52  $\mu\text{m}$ ) and Strata XC (average particles size 30  $\mu\text{m}$ ) cartridges were from Phenomenex (Torrance, CA, USA). All cartridges were of the same 500 mg bed weight and 6 ml volume.

### Sample Preparation

Ten grams of honey, in duplicate, was weighed into a 50-ml polypropylene tube. To one of the sample internal standard (IS), heliotrine was added at the concentration corresponding to 25  $\mu\text{g}$   $\text{kg}^{-1}$ . Honey samples were dissolved in 20 ml of 0.15 M hydrochloric acid. About 2 g of zinc dust was added to reduce *N*-oxides, and the samples were slowly shaken for 1.5 h. Subsequently, the solutions were passed through fast filtering cellulose filters and purified with the use of solid phase extraction. MCX cartridges were preconditioned with 9 ml of methanol and equilibrated with 9 ml of 0.15 M HCl acid. After sample application, the cartridges were washed with 12 ml of  $\text{H}_2\text{O}$  and 12 ml MeOH and vacuum dried for 2 min; then, 6 ml of ethyl acetate was applied. The alkaloids were eluted with 12 ml

of solvent mixture consisting of ethyl acetate, methanol, ammonia solution, and triethylamine (8:2:0.1:0.1 v/v). After evaporation at 40 °C in a nitrogen stream, the residues were reconstituted in 3 ml of ethyl acetate with an addition of 100 µl of MeOH and transferred into smaller vials and evaporated again. The reduction of the alkaloids was performed according to Kempf and Cramer (Kempf et al. 2008; Cramer et al. 2013) with some modifications. First, 10 µl of MeOH was added to solubilize the residue and subsequently 500 µl of LiAlH<sub>4</sub> 1 M solution in THF, and the samples were kept in a refrigerator for an hour. To stop the reduction reaction, 1 ml of dichloromethane and 170 µl of 10% NaOH were added. Samples were vortexed and dichloromethane fraction was passed through a column containing sodium sulfate. The extraction with 1 ml of dichloromethane was repeated two more times. The dichloromethane fractions were evaporated, and to the residues, 40 µl of ethyl acetate anhydrous and 50 µl of HFBA were added. The derivatization was carried out at 70 °C for 30 min in a Pierce block. Subsequently, samples were cooled down and evaporated. The residues were dissolved in 50 µl of ethyl acetate and subjected to instrumental analysis.

### Instrumental Parameters

All analyses were performed on a gas chromatograph Agilent 7890 A GC system (Agilent Technologies, USA) combined with mass spectrometry detector 5975 C VL MSD (Agilent Technologies, USA). The injection port was set on 250 °C; interface, source, and quadrupole temperatures were 300, 230, and 150 °C, respectively. The electron impact ionization was at 70 eV. The temperature program was as follows: 100 °C was held for 1 min; the temperature was increased to 160 °C at 7 °C min<sup>-1</sup>; and then, the temperature was increased to 325 °C at 40 °C min<sup>-1</sup> and held for 2 min.

Two microliters of sample was injected with split ratio 10:1; the helium flow was 0.8 ml min<sup>-1</sup>; and DB-5 MS (30 m × 0.25 mm, 0.25 film thicknesses) column was used for the compound separation. For the selected ion monitoring mode, 547, 334, 136, and 93 m/z ions were chosen, and the quantification was performed with 334 m/z ion.

### Identification and Quantification

The retention indices for retronecine and heliotridine derivatives were calculated according to the linear retention index equation. Relative ion intensities of the retronecine and the heliotridine derivatives were determined on the basis of full mass spectra of the analytical standards (Table 1).

Quantification was achieved using a calibration curve prepared by adding retrorsine standard solution of appropriate concentrations corresponding to 0–200 µg kg<sup>-1</sup> and heliotrine as the internal standard (25 µg kg<sup>-1</sup>) into blank honey samples

before the extraction procedure. Calibration curve was constructed by plotting the ratio of the retronecine derivative peak area to the IS (heliotridine derivative) peak area versus the added retrorsine concentrations. The final PA content is presented as a single sum parameter value, corresponding to the total amount of 1,2-unsaturated retronecine-type PAs in µg kg<sup>-1</sup>.

The analysis of the real samples was performed in duplicate as the internal standard heliotrine and structurally related PAs can occur as contaminants in honey. If in the samples retronecine type of alkaloids were detected, the quantification was done using the sample having heliotrine as IS. Additionally, a sample without IS was used as a control for the presence of heliotridine-type pyrrolizidine alkaloids.

### Method Validation

The in-house validation was carried out according to the guidelines provided in SANTE/11945/2015 document. Validation parameters including method linearity, recovery, repeatability, reproducibility, specificity, limit of quantification (LOQ), matrix effect, robustness, and the stability of PAs in extract and after derivatization were evaluated.

Buckwheat honey was used as the blank matrix as different analyzed batches were always negative for PA presence. Linden honey was also tested. However, in some cases, it gave positive results with the content of PAs at the level of 1–2 µg kg<sup>-1</sup>.

### Linearity

For determination of the linearity of the method, blank honey samples were spiked with retrorsine at appropriate concentrations corresponding to 0, 4, 10, 50, 100, and 200 µg kg<sup>-1</sup>. Heliotrine was used as an internal standard at the concentration of 25 µg kg<sup>-1</sup>, and the analysis was performed in triplicate.

### Recovery and Precision

To evaluate the recovery and precision, blank honey samples were spiked with retrorsine *N*-oxide standard solution at three concentration levels corresponding to 4, 50, and 200 µg kg<sup>-1</sup> with six replicates for each level. The samples were analyzed with the same instrument and the same operator. The intra-day precision was calculated as relative standard deviation of the results (%). For the determination of reproducibility also expressed as relative standard deviation (%), another two sets of samples were spiked at the same concentrations as for repeatability and analyzed on different days with the same instrument.

**Table 1** Calculated retention indices, monitored m/z ions, and their relative intensities

Compound	Retention index	m/z ions monitored	Relative intensity (%)
Retronecine derivative	1421	547, 334, 136, 93	20, 100, 18, 59
Heliotridine derivative	1455	547, 334, 136, 93	18, 100, 20, 63

in italics - quantification m/z ion

## Selectivity and Limit of Quantification

Selectivity of the method was determined by the analysis of a set of blank honey samples in order to check the possible presence of interfering peaks at the retention times of retronecine and heliotridine derivatives.

To determine the LOQ, two sets of blank honey samples were spiked at the level of  $1 \mu\text{g kg}^{-1}$ , analyzed on different days, and checked for the quantification with acceptable trueness and precision.

## Matrix Effect and Robustness

The matrix effect was calculated by comparison of the slopes of the calibration curve prepared by spiking blank honey samples and calibration curve in solvent. The robustness of the method was determined on the basis of the Youden procedure. Blank samples fortified at the concentration of  $20 \mu\text{g kg}^{-1}$  were analyzed in order to evaluate the influence of the seven selected variables which were slightly altered. The effect of zinc reduction time (1.5 h, 1 h), SPE cartridge type (MCX, Strata XC), volume of elution mixture (12 ml, 10 ml), volume of  $\text{LiAlH}_4$  (500  $\mu\text{l}$ , 400  $\mu\text{l}$ ), time of refrigeration (1 h, 45 min), dichloromethane volume (3 ml, 2.5 ml), and temperature of derivatization (70 °C, 65 °C) were evaluated.

## Stability Test

To assess the stability of PAs in matrix extract after SPE clean-up but before reduction with  $\text{LiAlH}_4$  and as derivatives for period of 1, 2, 3, 8, 15, and 32 days, blank samples were spiked at the level of  $20 \mu\text{g kg}^{-1}$ . Extracts were stored in glass-closed containers at 4, 20, and  $-18$  °C in the dark. The stability of the derivatives was checked using the first run extracts. The derivatives were also stored at 4, 20, and  $-18$  °C in closed chromatographic vials with internal glass inserts.

## Results and Discussion

### Method Development and Optimization

Honey is one of the most complex naturally produced carbohydrates mixture. It can contain many compounds such as sugars, lactones, nitrogenous compounds, organic and

phenolic acids, flavonoids, proteins enzymes, and other phytochemicals (Islam et al. 2014).

Due to complexity of the matrix and low levels of PAs present, many of the procedures developed for the determination of pyrrolizidine alkaloids in honey have been based on LC-MS/MS methodology (Table 2). Gas chromatographic methods have been popular for analysis of PAs in plants, in which alkaloid concentrations are much higher (Stelljes et al. 1991; Witte et al. 1993). However, GC-MS can also be a useful technique for an analysis of honey samples for the presence of 1,2-unsaturated PAs, especially when the sum parameter approach is used (Kempf et al. 2008, Cramer and Beuerle 2012). The advantage of this approach is that the content of the present 1,2-unsaturated PAs is converted into the common backbone structures (Fig. 1) and expressed as single sum parameter value. The advantages and disadvantages of the sum parameter method have been already described in details (Kempf et al. 2008; Cramer et al. 2013; Kowalczyk and Kwiatek 2017).

As pyrrolizidine alkaloids are polar compounds for dilution of honey samples, 0.05 M sulfuric acid has been mostly used (Kempf et al. 2008; Cramer and Beuerle 2012; Griffin et al. 2013); only in one study 0.5 M sulfuric acid was applied (Orantes-Bermejo et al. 2013). However, in a previously conducted study, 1 M hydrochloric acid was chosen as the most effective extraction solvent of PAs from feed materials (Kowalczyk and Kwiatek 2017); for this reason, HCl solutions were also tested for honey matrix. Orantes-Bermejo et al. (2013) observed that more concentrated acid decreases the viscosity of honey solution and makes the SPE purification less problematic as clogging can be avoided. However, the use of 1 M HCl as the extraction solvent resulted in a very violent reaction when zinc dust was added. For these reasons, 0.15 M HCl was chosen for further study even though 0.05 M  $\text{H}_2\text{SO}_4$  gave comparable recovery results.

Cation exchange cartridges have been the choice of most of the authors presenting results of PA determination in honey (Table 2) and other matrices (Gottschalk et al. 2015). However, it is necessary to test different cartridges as diverse problems can occur during the analysis, e.g., clogging, very slow elution, or different recovery rates (EFSA 2011). That is why SCX, Strata XC, MCX, PCX, and HF Bond cartridges were tested during method development.

Authors used different concentrations of ammonia in methanol for the elution of PAs from cation exchange cartridges (Table 2). However, except for PAs, many additional



**Table 2** Summary of previously published methods which involved cation exchange cartridges, ammonia solution in methanol elution, and LOQ value or range of LOQ values reported for individual alkaloids, with a number of monitored PAs

SPE cartridge	Elution mixture	LOQ	Technique	Reference
Strata SCX	Methanol saturated with ammonia gas	Not reported (15 PAs)	LC-IT-MS	Betteridge et al. 2005
	Methanol saturated with ammonia gas	Not reported (10 PAs)	LC-IT-MS	Boppré et al. 2005
	Methanol saturated with ammonia gas	LOD 50 $\mu\text{g kg}^{-1}$	LC-IT-MS	Cao et al. 2013
	Ammoniated methanol (c.n.m.)	1.21–1.79 $\text{ng/ml}$ (5 PAs)	LC-MS/MS	Mudge et al. 2015
Bond Elut SCX	30 ml ammonia (c.n.m.) in 500 ml methanol	10 $\mu\text{g kg}^{-1}$ , (sum parameter method)	GC-MS	Kempf et al. 2008
	30 ml ammonia (c.n.m.) in 500 ml methanol	1–3 $\mu\text{g kg}^{-1}$ (18 PAs)	LC-MS/MS	Dübecke et al. 2011
	6 ml ammonia (c.n.m.) in 100 ml methanol	6 $\mu\text{g}$ retronecine equivalent $\text{kg}^{-1}$ , (sum parameter method)	GC-MS	Cramer and Beuerle 2012
	2.5% ammonia solution in methanol	0.18–0.62 $\mu\text{g kg}^{-1}$ (17 PAs)	LC-MS/MS	Bodi et al. 2014
	30 ml ammonia (c.n.m.) in 500 ml methanol	1 $\mu\text{g kg}^{-1}$ (5 PAs)	LC-MS/MS	Lucchetti et al. 2016
Discovery DCX-SCX	30 ml ammonia (c.n.m.) in 300 ml methanol	1–3 $\mu\text{g kg}^{-1}$ (16 PAs)	LC-MS/MS	Orantes-Bermejo et al. 2013
Strata XC	0.1% ammonia solution in methanol	30–68 $\mu\text{g kg}^{-1}$ (11 PAs)	LC-IT-MS	Griffin et al. 2013
	3% ammonia solution in methanol	1.4–10.9 $\mu\text{g kg}^{-1}$ (14 PAs)	LC-MS/MS	Griffin et al. 2015a
	3% ammonia solution in methanol	1.8–12.9 $\mu\text{g kg}^{-1}$ (14 PAs)	LC-MS/MS	Griffin et al. 2015b
	5% ammonia solution in methanol	0.25 $\mu\text{g kg}^{-1}$ (5 PAs)	LC-MS/MS	Lucatello et al. 2016

LC-MS/MS, liquid chromatography tandem mass spectrometry; LC-IT-MS, liquid chromatography ion trap mass spectrometry; LOD, limit of detection; c.n.m., concentration not mentioned

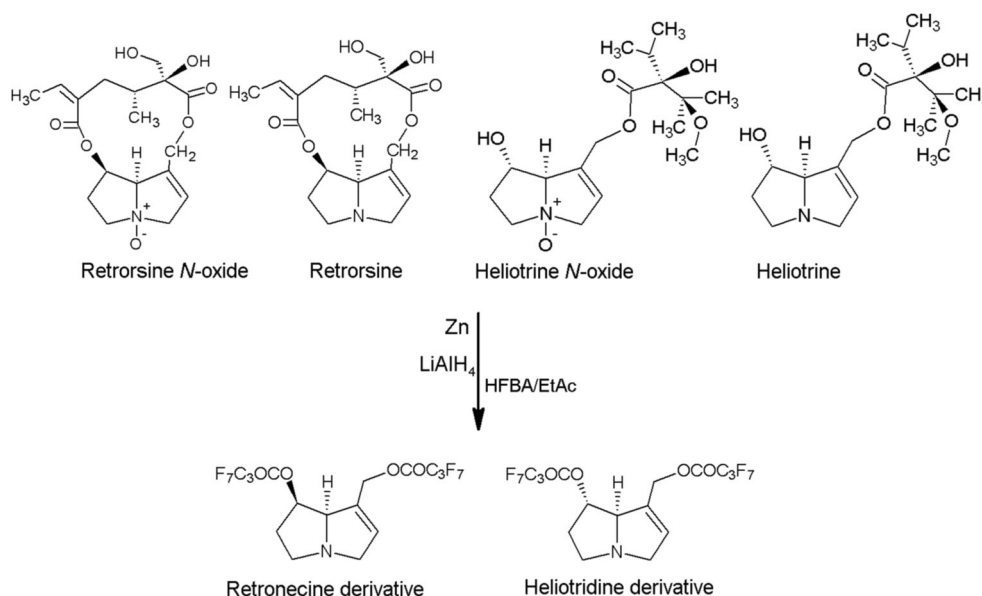
compounds are also eluted, causing some difficulties during instrumental analysis as they can interfere with PAs, especially when PAs are present at very low concentrations (Fig. 2f).

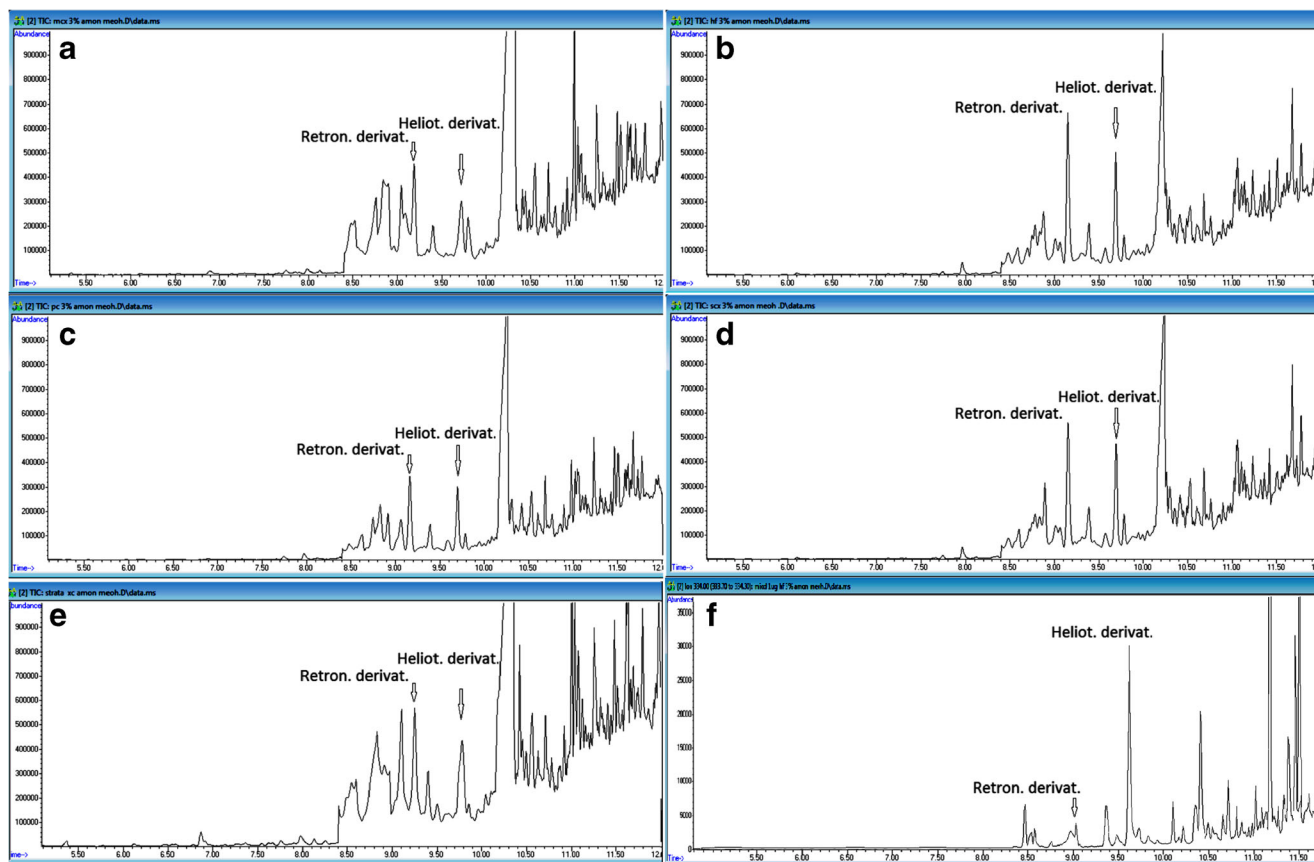
To obtain a cleaner extract, we tried to find a new elution mixture that will provide good recovery of the alkaloids but most of all, a reduction of coeluting compounds. For this reason, different organic solvents and their combinations were tested, e.g., ethyl acetate with acetonitrile and ammonia; ethyl

acetate with methanol and ammonia; ethyl acetate, methanol, and triethylamine; and a mixture of ethyl acetate, methanol, ammonia, and triethylamine.

It was observed that the addition of triethylamine significantly decreases the rate of coeluted impurities. When a combination of ethyl acetate, methanol, and triethylamine (8:2:0.2 v/v) was used, a very clean chromatogram was

**Fig. 1** General scheme of retronecine and heliotridine PA-type conversion leading to formation of the derivatives





**Fig. 2** Comparison of total ion chromatogram (TIC) signals obtained when 3% ammonia in methanol mixture was used for PA ( $25 \mu\text{g kg}^{-1}$ ) elution from tested SPE cartridges. **a** MCX, **b** HF, **c** PCX, **d** SCX, **e** Strata XC, and

**f** extracted ion chromatograms ( $334 \text{ m/z}$ ) obtained for HF cartridge with 3% ammoniated methanol elution (PA concentration  $1 \mu\text{g kg}^{-1}$ ). *Retron. derivat.*, retronecine derivative; *Heliot. derivat.*, heliotridine derivative

obtained. However, recovery rates were low and even lower at higher concentration range.

The best results in terms of recovery and clean chromatograms were obtained with a mixture consisting of ethyl acetate, methanol, ammonia, and triethylamine (8:2:0.1:0.1 *v/v*) in combination with MCX cartridges. In Fig. 3, example chromatograms are presented for each cartridge type with the new elution mixture. Obtained results were also compared with results when 3% ammonia in methanol was used for PA elution (Fig. 2). MCX cartridge was chosen as it provided better recovery rates of the alkaloids compared with SCX or HF cartridges; however, SCX and HF cartridges provided slightly cleaner chromatograms. The advantage of MCX cartridges was also the fact that honey extracts could be passed through the cartridge without the need to keep the extracts at  $40^\circ\text{C}$  to avoid clogging problems. The elution was relatively fast and possible to carry out without the use of back pressure.

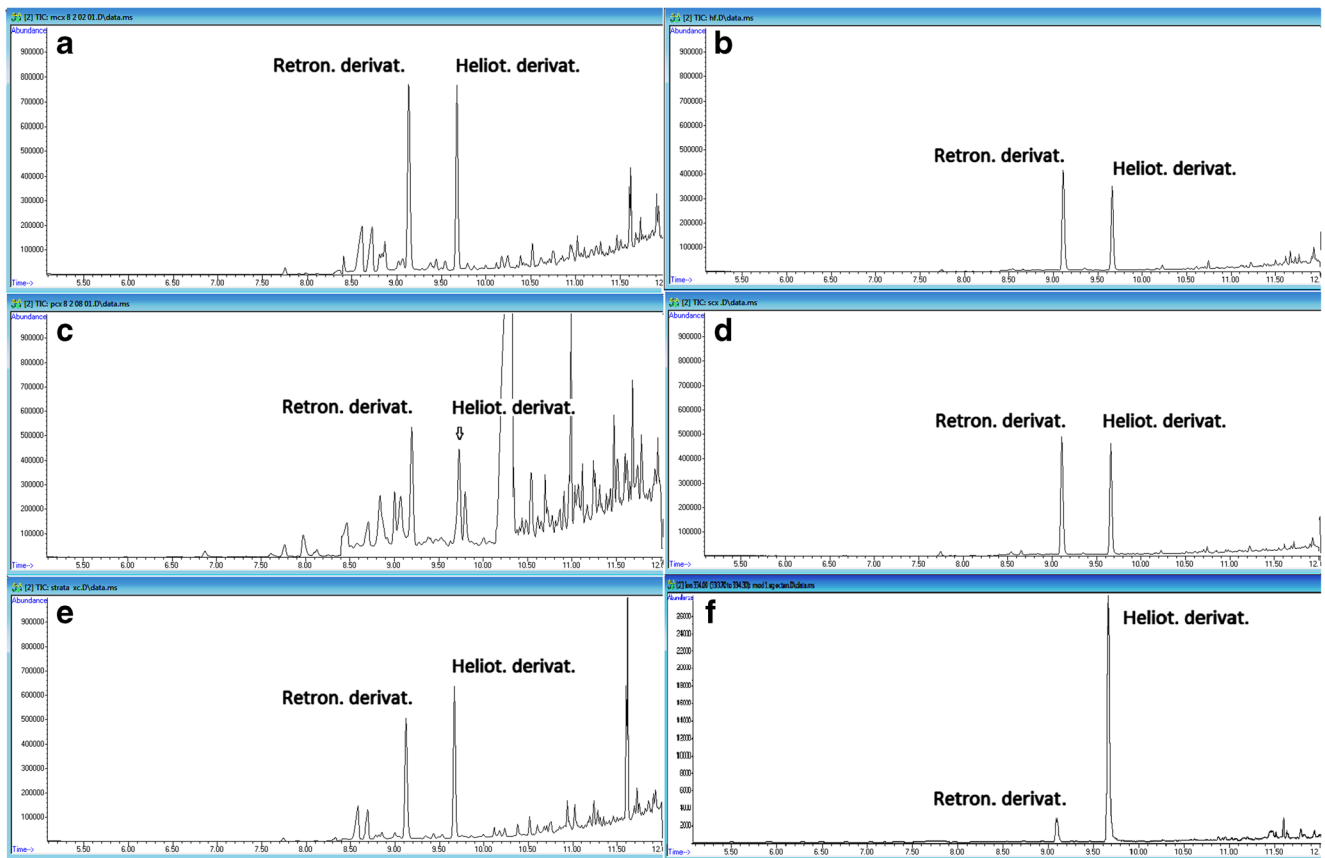
Cramer et al., 2013 eliminated the *N*-oxide zinc reduction step by increasing the amount of  $\text{LiAlH}_4$  and also decreased the time of refrigeration and confirmed the efficiency of reduction of PAs to the backbone structure experimentally. However, it was observed that the zinc reduction step reduces

the viscosity of the honey extract. Moreover, this step also provides cleaner chromatograms compared to chromatograms of samples without the Zn reduction step. For this reasons, the Zn reduction step has been left in sample preparation procedure.

The efficiency of reduction reaction to the common backbone structure was checked for intermedine, lycopsamine, jacobine, retrorsine, seneciphylline, senecionine, echimidine, heliotrine, lasiocarpine, and *N*-oxides of retrorsine, senecionine, and echimidine. All tested alkaloids revealed very comparable conversion results. The retrorsine *N*-oxide was chosen for the validation study as it covers all sample preparation steps.

Gas chromatograph parameters were also optimized. Many parameters are the same as applied in the previous study (Kowalczyk and Kwiatek 2017), such as quadrupole and ion source temperatures; however, a different oven temperature ramp was used which was more suitable for the honey matrix.

The optimization of derivatization step and other GC-MS parameters were described in details elsewhere (Kowalczyk and Kwiatek 2017).



**Fig. 3** Comparison of TIC obtained when elution mixture consisting of ethyl acetate, methanol, ammonia, and triethylamine (8:2:0.1:0.1 v/v) was used for PA ( $25 \mu\text{g kg}^{-1}$ ) elution from tested SPE cartridges. **a** MCX, **b**

**HF**, **c** PCX, **d** SCX, **e** Strata XC, and **f** extracted ion chromatograms (334 m/z) obtained for MCX cartridge (PA concentration  $1 \mu\text{g kg}^{-1}$ )

## Validation Results

Matrix calibration curve was linear in a concentration range corresponding to 0–200  $\mu\text{g kg}^{-1}$ . The linearity was confirmed as the coefficient of determination was higher than 0.99.

Intra- and inter-day analyses of spiked blank honey samples at appropriate concentration levels (4, 50, and 200  $\mu\text{g kg}^{-1}$ ) were used for the determination of validation parameters of the method (Table 3). The recovery ranged from 73.1 to 93.6%. The repeatability was calculated as relative standard deviation and ranged from 3.9 to 8.6%. The reproducibility was in the range from 10.6 to 17.8%. All determined parameters are in agreement with SANTE criterion as recovery should be in the range of 70–120% and relative standard deviation  $\leq 20\%$ .

The method is selective as no interfering peaks were determined in the retention time of the retronecine and heliotridine derivatives.

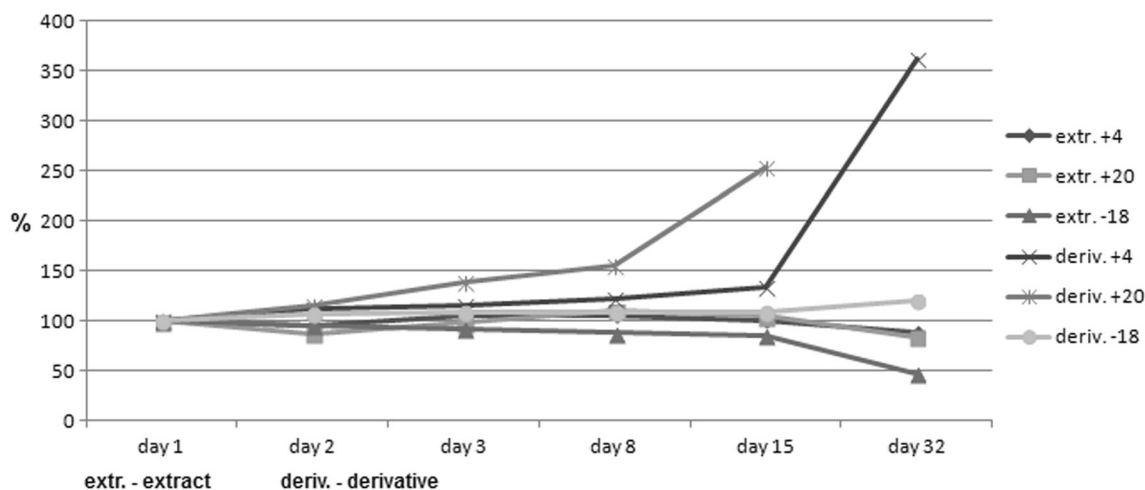
Matrix effect was in the range  $\pm 20\%$  which is in compliance with SANTE 2015 requirements.

From the robustness test, it could be concluded that there was no critical variable that dominated the outcome of the results, but that all chosen variables contributed, as was evidenced by the use of the Student's *t* test.

Stability tests in extract and as derivative revealed that extracts and derivatives can be stored in a refrigerator and in a freezer for 5 days without significant change in a concentration. On the other hand, storage at room temperature of extracts, particularly the derivatized

**Table 3** Validation results

	Concentration level ( $\mu\text{g kg}^{-1}$ )	Recovery (%)	Repeatability RSD (%)	Reproducibility RSD (%)
LOQ	1	110.6	10.9	15.8
Retrorsine <i>N</i> -oxide	4	93.6	8.6	15.4
	50	73.9	8.5	10.6
	200	73.1	3.9	17.8



**Fig. 4** Stability of the PA extracts and of the derivatized extracts kept at different temperatures for up to 1 month. Results are the mean of three replicates. The area ratio of retronecine derivative to heliotridine

derivative at day 1 is set at 100%. A relative ratio of more or less than 100% indicates a relative instability of the heliotridine derivative and the retronecine derivative, respectively

extracts, resulted in significant changes in the calculated concentrations. This is most likely due to the heliotridine derivative being less stable than the retronecine derivative, resulting in an increase of the apparent retronecine PA concentration (Fig. 4).

A LOQ of  $1 \mu\text{g kg}^{-1}$  retronecine PAs was established based on the acceptable recovery and precision data obtained at this concentration (Table 3, SANTE 2015). This low LOQ could be achieved due to use of the new elution mixture consisting of ethyl acetate, methanol, ammonia, and triethylamine (8:2:0.1:0.1). The obtained LOQ is significantly lower than that reported by Kempf et al. (2008) and Cramer and Beuerle (2012) which were  $10 \mu\text{g kg}^{-1}$  and  $6 \mu\text{g kg}^{-1}$  retronecine equivalent  $\text{kg}^{-1}$ , respectively. The LOQ of  $1 \mu\text{g kg}^{-1}$  is comparable to those reported for individual PAs in LC-MS/MS methods (Table 2). However, our method is in fact more sensitive as the LOQ corresponds to total content of retronecine type of PAs in honey.

## Real Sample Application

Europe is the largest global consumer of honey, being responsible for more than 20% of the total global consumption. Polish honey is becoming more and more popular on the foreign market, and in the years 2008–2014, the export of Polish honey increased by about 230%. At the same time, the import of honey from mostly non-European countries to Poland also increased considerably (Portal spozywczy.pl 2017).

As it has been revealed by other authors, honey from non-European countries can be contaminated with considerably higher concentrations of PAs (Dübecke et al. 2011; Griffin et al. 2015a, b). To provide an appropriate level of consumer protection, before introduction into the market, honey should be checked for the presence of PAs.

The developed method was applied to an analysis of 54 honey samples. Forty samples were from Polish territory and were collected directly from apiarists. The honeys were polyfloral; no other information concerning the samples was available. The remaining 14 honeys were of Asian origin and were available in our laboratory from an earlier study.

The 68% of Polish samples were positive for the presence of PAs. The content of detected alkaloids ranged from  $1.0$  to  $20.2 \mu\text{g kg}^{-1}$ . The mean concentration (excluding negative samples and results below LOQ) and median were  $4.5$  and  $2.7 \mu\text{g kg}^{-1}$ , respectively. When all samples were considered, the mean and the median PA concentration were  $2.9$  and  $1.6 \mu\text{g kg}^{-1}$ , respectively. Only in two samples elevated PA concentrations ( $20.0$  and  $20.2 \mu\text{g kg}^{-1}$ ) were detected (Fig. 5).

Much higher PA concentrations were detected in the honey of Asian origin. Only one out of 14 samples was PA negative. The concentration range of detected alkaloids was from  $4.0$  to  $64.1 \mu\text{g kg}^{-1}$ . The mean and median values for positive samples were  $22.6$  and  $12.0 \mu\text{g kg}^{-1}$ , respectively.

Results obtained for Polish honeys are relatively low and are consistent with results published by others authors, reporting the concentration of PAs in European honeys. Amounts of PAs in honey (six alkaloids monitored) from the Vento Region of Italy ranged from  $0.6$  to  $17.6 \mu\text{g kg}^{-1}$  (Lucatello et al. 2016). Griffin et al. (2015b) reported that five analyzed Irish honeys were negative for the presence of 14 PAs, including *N*-oxides or the concentrations were below the LOD. Bodi et al. (2014) published results of PA concentration in honeys taken from German and Austrian apiarists. The maximum value was  $28.2 \mu\text{g kg}^{-1}$  with mean and median concentration  $6.1$  and  $1.4 \mu\text{g kg}^{-1}$ , respectively (17 PAs and *N*-oxides monitored). Dübecke et al. (2011) observed substantial differences in the amount of PAs found in honeys depending on their country of origin. The mean PA concentration in 381 European honeys was  $17 \mu\text{g kg}^{-1}$ , including negative samples. Honeys



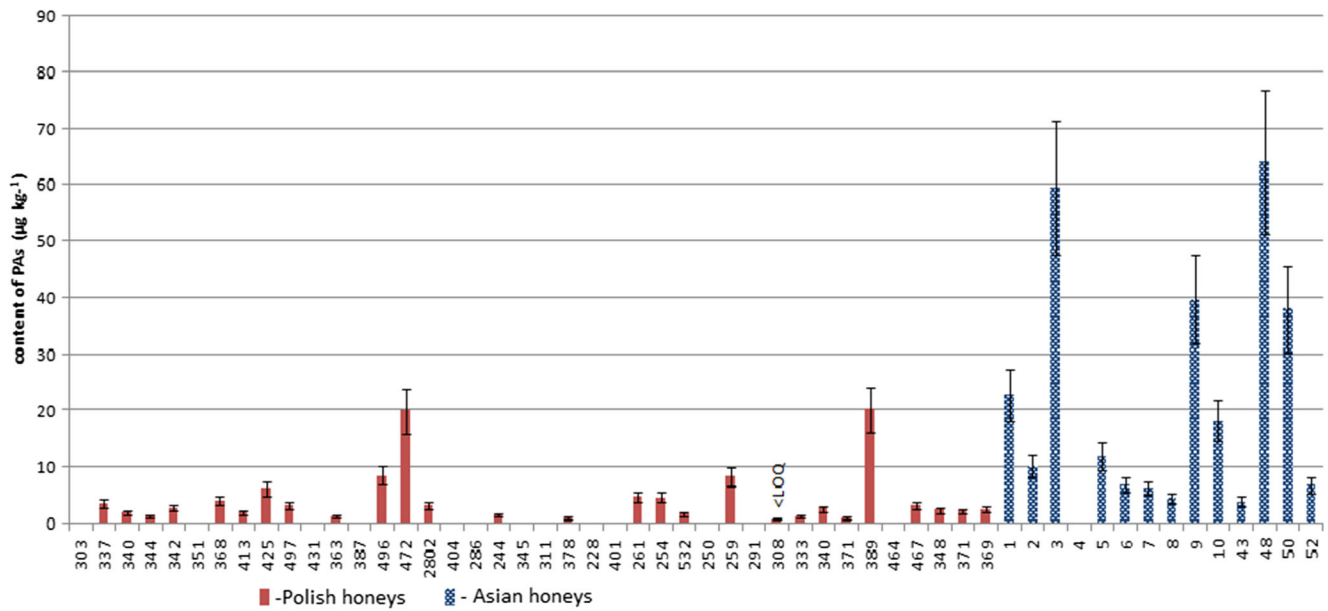


Fig. 5 The content of pyrrolizidine alkaloids in Polish and Asian honey samples

from Germany, Bulgaria, and Romania showed lower levels of PAs (1–43 µg kg<sup>-1</sup>) compared to honeys from Italy and Spain (concentrations up to 225 µg kg<sup>-1</sup>), as honeys from these regions often reveal a high number of *Echium* pollen grains (18 PAs and *N*-oxides monitored) (Dübecke et al. 2011; Kast et al. 2014). Martinello et al. (2014) reported higher concentrations of PAs (nine alkaloids monitored) in honeys that were blends of EU and non-EU for which determined mean concentration was 17.5 µg kg<sup>-1</sup>. The mean content of PAs in EU honeys was 3.1 µg kg<sup>-1</sup>. All reported results, however, could be underestimated due to limited number of monitored PAs.

The estimated average dose that should not cause a health concern of 0.007 µg kg<sup>-1</sup> of b.w. per day for chronic consumption of 1,2-unsaturated PAs, translates for an individual weighting 60–70 kg to 0.42–0.49 µg of PAs per day. Considering the average daily consumption of 20 g of honey, samples with the content up to 21–24.5 µg kg<sup>-1</sup> of PAs seems

to be safe. Taking into consideration the concentration of PAs determined in analyzed samples, all Polish and nine non-European honeys would not exceed the recommended maximum intake limit if consumed in the amount of 20 g per day.

In the EFSA report published in 2016, the consumers of honey were divided into groups based not only on their age but also on the frequency (chronic and acute) and amount of honey consumed (average and high) (EFSA 2016). Considering only the upper values of the range of honey chronic consumption amounts determined in EFSA report and assuming an average adult weight as 60 kg and an average young consumer weight as 15 kg, the concentration of PAs in honey that could be consumed without exceeding recommended maximum daily intake limit of 0.007 µg kg<sup>-1</sup> b.w. per day has been calculated (Table 4). The calculated acceptable concentrations in honey, especially in the case of high consumers, are much lower than estimated above for the average consumption of 20 g. Nevertheless, most Polish honey

Table 4 Consumption of honey (adult and young consumers) according to EFSA (2016) and the calculated maximum acceptable PA concentration in honey

		Consumption	Upper value of honey amount consumption (g kg <sup>-1</sup> b.w. per day)	Maximum PA concentration in honey (µg kg <sup>-1</sup> )
Chronic consumption	Adult population	Average	0.06	116.7
		High	0.32	21.9
	Adult consumers only	Average	0.27	25.9
		High	0.64	10.9
	Young population	Average	0.14	50
		High	0.49	14.3
	Young consumers only	Average	0.98	7.1
		High	1.13	6.2

would be safe to be consumed. In the case of average consumption, all analyzed Polish and most of analyzed Asian honey could be consumed without any potential risk.

In 2017, EFSA published a new report on PAs where new BMDL<sub>10</sub> (benchmark dose) of 237  $\mu\text{g kg}^{-1}$  b.w. per day has been selected (EFSA 2017). Based on this new BMDL<sub>10</sub>, the recommended maximum daily intake limit of 0.007  $\mu\text{g kg}^{-1}$  b.w. per day can be raised to 0.0237  $\mu\text{g kg}^{-1}$  b.w. per day. Consequently, taking an amount of 20 g of honey, this would correlate to a PA concentration of 71.1  $\mu\text{g kg}^{-1}$ . In that case, all analyzed honey could be consumed without any potential risk.

## Conclusions

The increase of honey imports from different parts of the world to Europe may expose consumers to increased levels of pyrrolizidine alkaloids. Special care must be taken to decrease the risk of exposure for consumers and some measures like established limits of PAs content, and the laboratory control of honey introduced into the market should be implemented. To provide appropriate control, sensitive and reliable analytical methods should be developed.

This presented method fulfills the requirements especially in the context of sensitivity. The method was successfully validated according to SANTE 2015 document. It was also successfully applied to the analysis of 54 honey samples; therefore, it can be stated that this developed method can be a useful tool for the monitoring of honeys for the presence of pyrrolizidine alkaloids.

## Compliance with Ethical Standards

**Conflict of Interest** The corresponding author Ewelina Kowalczyk, the second author Zbigniew Sieradzki and the third author Krzysztof Kwiatek declare that they have no conflict of interest.

**Ethical Approval** This article does not contain any studies with humans and animals performed by any of the authors.

**Informed Consent** Not applicable.

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