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Simultaneous determination of pyrrolizidine and tropane alkaloids in honey by liquid chromatography–mass spectrometry

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Abstract

Introduction: Pyrrolizidine alkaloids (PAs) and tropane alkaloids (TAs) are natural contaminants of honey and respectively hepatoxic and neurotoxic compounds. Because honey is a popular constituent of the human diet, it is relevant to warrant the safety of the product. For that reason, a method for simultaneous determination of PAs and TAs in honey based on liquid chromatography–mass spectrometry was developed. **Material and Methods:** The analytical protocol used sulphuric acid extraction and solid-phase extraction purification. The developed procedure was subjected to validation in terms of linearity, selectivity, repeatability, reproducibility, limits of quantification and determination, matrix effect and uncertainty. A total of 29 honey samples were analysed for the determination of PAs and TAs. **Results:** All the evaluated validation parameters fulfilled the requirements of European Commission Decision 2002/657/EC. At least one of the monitored alkaloids was determined in 52% of the samples. Among the most abundant alkaloids were echimidine, intermedine and lycopsamine. The total PA concentrations ranged from 2.2 to 147.0 μg kg^{-1}. Contrastingly, none of the monitored TAs was detected in the analysed samples. An assessment of the dietary exposure to PAs from the consumption of the contaminated honeys showed that three of them would pose a risk to consumers, especially if they were children. **Conclusion:** A sensitive method suitable for simultaneous determination of PAs and TAs in honey was developed and validated. The analysis of 29 honey samples for PAs and TAs revealed that honey destined for retail could pose a risk to consumers.

Keywords: pyrrolizidine alkaloids, tropane alkaloids, honey, liquid chromatography–mass spectrometry (LC-MS), solid-phase extraction.

Introduction

Concerns over natural toxins produced both by plants and fungi have been emphasised in recent years, mostly due to their detrimental effect on food and feed safety. Among plant toxins, different groups of alkaloids have been identified as causes of human and animal intoxication. Alkaloids are natural compounds, produced mainly by plants as their secondary metabolites (22). Toxic effects in general depend on specific dosage, exposure time, and individual characteristics such as sensitivity or site of action. At different times, toxicity effects can be harmful or beneficial depending on the ecological or pharmacological context (26). In the context of food safety, only the harmful effect is considered relevant, and among different groups of alkaloids, pyrrolizidine and tropane compounds have been highlighted as particularly important for their injurious toxicity.

Pyrrolizidine alkaloids (PAs) are some of the most widespread toxins of natural origin. Plants containing them originate mostly from the *Asteraceae*, *Boraginaceae* and *Fabaceae* families and are distributed worldwide, constituting 3% of all flowering plants (19, 27). More than 660 PAs and their *N*-oxides have been identified (6). Structurally, PAs are esters of amino alcohols, consisting of two basic structural elements: a pyrrolizidine-derived moiety necine and various mono- or dicarboxylic acids. The necine base consists of two fused five-membered rings with a nitrogen atom at the bridgehead, which can be saturated or contain a double bond in the 1,2-position (16).

Acute intoxications in humans and animals caused by PAs are highly rare. However, the main health

concern attaches to chronic disease that can be initiated by even low-level dietary exposure to 1,2-unsaturated PAs. The consequences of chronic or intermittent exposure include cancerous diseases, progressive liver disorders leading to cirrhosis, congenital anomalies, and pulmonary arterial hypertension (11).

Plants containing tropane alkaloids (TAs) are found in numerous families such as *Solanaceae* (*Datura*, *Atropa belladonna*), *Erythroxylaceae*, *Convolvulaceae*, *Brassicaceae* and *Euphorbiaceae* (13, 22). Although more than 200 different TAs have been identified in various plants so far, data on their toxicity is limited. Among all identified TAs, the most studied and analysed in different food and feed commodities are (−)-hyoscyamine and (−)-scopolamine. The racemic mixture of (−)-hyoscyamine and (+)-hyoscyamine is called atropine (13, 18). As separation of hyoscyamine enantiomers is very difficult, atropine is often used as a general indicator for both forms (2).

In humans, TAs prevent the interaction of acetylcholine with its receptor, which may affect the heart rate, respiration and functions of the central nervous system. As scopolamine is a competitive antagonist of acetylcholine at both peripheral and central muscarinic receptors, it may impart toxicological effects in humans and animals severe enough in some cases to cause death due to respiratory failure (3).

Pyrrolizidine alkaloid and TA containing plants can be found almost all over the world. Often, they are perceived as invasive and noxious weeds, which can infest cultivated fields, meadows, pastures or open ranges replacing nutritious plants (31). The spread of such weeds from eastern and southern to northern European countries has been observed and is suggested to have occurred as a result of climate change (1). Content of PAs and TAs differs between species, and the TAs and PAs profiles may be different in different parts of the same plant (17, 20). The presence of scopolamine and atropine has also been confirmed in the floral nectar of *Datura* species (5). Pyrrolizidine alkaloids are also present in flower nectar and pollen (14), and thus both types of alkaloids can be transferred into honey through bee foraging and endanger consumers.

Consumption of honey is constantly increasing worldwide, and in the case of Poland, it has doubled within the last 15 years (35). Even though the number of bee colonies and the production of Polish honey have been growing, the demand cannot be fully covered by domestic production. Like many other countries, Poland imports honey from all over the world, and in 2018 the amount of imported honey was estimated at 25,712 tonnes (28). It was found that honey originating from Central and South America or Australia can contain elevated rates of PAs. Testing of samples from these regions revealed some to be contaminated with high individual PA levels (9, 16, 34). Because a considerable part of the honey available on the market is imported and some foreign honey has been demonstrated to have alkaloid impurities as noted, there is a need to test the product for the presence of different contaminants including PAs and TAs. For that reason, suitable analytical methods are needed.

Numerous methods for the determination of only PAs in honey can be found in the literature (4, 15, 21, 24, 25), and several have also been developed for the simultaneous determination of PAs and TAs in plant based foods (10, 30, 33). However, according to the authors' knowledge, a very limited number of protocols have been designed for PAs and TAs analysis in honey (25). Procedures for the determination of single TAs group in honey could also only be found in inconsiderable number (29, 32). Most of the developed methods were based on high-resolution mass spectrometry (HRMS) combined with liquid chromatography, which enables high selectivity and specificity; however, the drawback of the technique is still its high price. The technique is not always affordable for official laboratories focused on routine analysis.

The study aimed to develop and validate an alternative method based on liquid chromatography–mass spectrometry suitable for the determination of PAs and TAs alkaloids in honey. The selection of the compounds to determine sought to comprehend representatives of particular types of PAs, including jacobine, erucifoline, retrorsine, senecionine, senecivernine and seneciphylline as senecionine types; lycopsamine, intermedine, and echimidine as lycopsamine types; europine, heliotrine and lasiocarpine as heliotrine types; and monocrotaline and trichodesmine as monocrotaline types. Senkirkine was also included. Scopolamine and atropine were selected as representatives of the TAs.

Material and Methods

Chemicals and reagents. Water was purified with the Milli-Q water purification system (MilliporeSigma, Burlington, MA, USA). Ammonia in 25% solution was purchased from POCH (now Avantor Performance Materials, Gliwice, Poland). Ethyl acetate of gas chromatography grade was obtained from Merck (Darmstadt, Germany), and sulphuric acid (95%) from Chempur (Piekary Śląskie, Poland). Formic acid, zinc dust and the TA standards of scopolamine and atropine were ordered from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were received from J.T. Baker (now Avantor Performance Materials, Deventer, the Netherlands). Mixed mode Oasis MCX cation exchange cartridges for solid-phase extraction (SPE) of 500 mg bed weight and 6 mL volume were supplied by Waters (Milford, MA, USA). PAs standards used in the study, which were monocrotaline, erucifoline, europine, intermedine, lycopsamine, jacobine, heliotrine, retrorsine, trichodesmine, seneciphylline, senecivernine, senecionine, echimidine,

lasiocarpine and senkirkine, were procured from PhytoLab (Vestenbergsgreuth, Germany).

Standard solutions. Standard stock solutions of each compound were prepared in methanol at a concentration of 1,000 µg/mL. All solutions were stored at −20°C. Mixed working standard solutions were prepared by adding an appropriate amount of each single stock solution in order to reach a concentration of 10 µg/mL, and the mixed solution was further diluted with methanol to obtain a lower concentration of 1 µg/mL.

Honey samples. Honey samples were collected as regular veterinary inspection procedure. The analysed honeys were of Polish and foreign origin, including Cuban, Ukrainian and Vietnamese. The samples represented various types of honey: monofloral including buckwheat, rape, and acacia; multifloral and honeydew.

Sample preparation. Honey samples were heated in a water bath at 40–45°C and then homogenised. A 10 g mass was weighted into 50 mL polypropylene tubes and 20 mL of 0.05 M sulphuric acid was added. The samples were shaken on a horizontal shaker until complete dissolution, and 1 g of zinc dust was added to each. Samples were mixed by hand and left for 24 h at room temperature. Subsequently, samples were additionally shaken on a horizontal shaker for 0.5 h and centrifuged for 10 min (4,000 x *g*). The supernatants were filtered through cellulose filters and the total volume of extracts was subjected to SPE. Mixed-mode cation exchange cartridges were conditioned with 12 mL of MeOH and 12 mL of 0.05 M H_2SO_4. Subsequently honey extracts were introduced. The SPE cartridges were washed with 10 mL of purified water and 10 mL of methanol, and then dried under vacuum for 2 min, after which 6 mL of ethyl acetate was added to the cartridges. The alkaloids were eluted with 10 mL of solvent mixture containing ethyl acetate, methanol, acetonitrile, ammonia solution and triethylamine (8:1:1:0.3:0.1, *v/v*). After evaporation in a nitrogen stream, the residues were reconstituted in 0.2 mL of purified water and 0.2 mL of methanol, passed through a PVDF filter and subjected to the instrumental analysis.

Instrumental parameters. HP 1200 series separation modules from Agilent Technologies (Santa Clara, CA, USA) were used for the analysis. These modules consisted of a degasser system, binary pump, automatic injector and column thermostat. The compounds were detected with a 6140 mass spectrometer (Agilent Technologies). The separation of the alkaloids was carried out on a Kinetex C18, 2.6 µm, 100 mm × 4.6 mm column coupled with a C18 guard column (both from Phenomenex, Torrance, CA, USA). The column was thermostatted at 30°C. The mobile phase containing 0.3% formic acid in water (A) and a mixture of methanol and acetonitrile (2:1, *v/v*) (B) was used in the following gradient mode: 0 min, 5.5% B; 0–2 min 6% B; 2–6 min, 15% B; 6–8 min, 18% B; 8–11.5 min, 20% B; 11.5–13 min, 30% B; 13–15.5 min, 40% B; 15.5–16.5 min, 45% B; 16.5–17 min, 45% B; 17–18 min, 85% B; 18–19 min, 90% B; and 19–22 min, 5.5% B up to 26 min. The flow rate was 0.6 mL min^{-1} and the injection volume was 2 µL. Electrospray ionisation (ESI) was set in a positive mode, the capillary voltage was set at 2,000 V and the nebulizer pressure was 35 psi. The drying gas flow and temperature were 11.0 L/min and 300°C, respectively. The fragmentor voltage was set at 100 V for all monitored alkaloids. Selected ion monitoring was used for the detection, and the protonated molecular (M + H)+ ions (m/z) monitored are listed in Table 1.

Identification and quantification. Identification was made by comparison with the relevant reference standard by the retention time and the protonated molecular ion (M + H)+. Quantification was based on the calibration curves prepared by spiking blank honey samples with standard solution before the extraction procedure to obtain concentrations in the range 5–200 µg/kg for each compound. Blank honey samples were samples in which none of the monitored alkaloids was detected in the previous analysis. Calibration curves were constructed by plotting the peak area versus the alkaloid concentrations. Fifteen PAs were quantitatively determined directly, and fourteen corresponding *N*-oxides were quantified in an indirect way after their reduction to the basic form.

Evaluation of the *N*-oxides reduction rate. To evaluate the rate of *N*-oxides' reduction to their corresponding basic forms, blank honey samples were spiked with the *N*-oxide standards. After zinc addition, samples were left for 24 h at room temperature. Subsequently, samples were subjected to the sample preparation procedure. The obtained results were compared with the results of this reduction in blank honey samples spiked at the same concentration level with the standards of the free base forms of the PAs. In studies previously carried out by the authors (23), the best conversion results were obtained after 24 h, and therefore in this study only this period was evaluated. The evaluation included *N*-oxides of monocrotaline, erucifoline, europine, intermedine, lycopsamine, jacobine, heliotrine, retrorsine, seneciphylline, senecivernine, senecionine, echimidine and lasiocarpine. The conversion of tropane alkaloids and trichodesmine *N*-oxides to their basic forms was not investigated in this study.

Method validation. The presented method was validated in-house according to the requirements of European Commission Decision 2002/657/EC. Recovery, repeatability, reproducibility, linearity, selectivity, matrix effect, limit of detection (LOD) and limit of quantification (LOQ) and uncertainty were determined during the validation process. Honey samples in which none of the monitored PAs and TAs were detected were used as the blank samples for the preparation of fortified samples used for validation.

Linearity, selectivity, matrix effect, LOD and LOQ. Evaluation of the method's linearity was based on the analysis of the matrix calibration curves and the

assignment of an R^2 determination coefficient. Blank honey samples were fortified to concentrations of 5, 10, 20, 50, 100 and 200 μg/kg before the extraction procedure. The linearity was proved if the value of R^2 was greater than 0.98. In order to determine the selectivity of the method, a set of honey samples was analysed to check the possible presence of interferences resulting from the endogenous matrix composition in the retention times of the monitored alkaloids. For the matrix effect assessment, blank honey samples were spiked with the standard solutions after the sample SPE purification procedure, and standard solutions of the alkaloids at the same concentration in solvents were prepared. The matrix effect (%) was calculated as the ratio of the analyte peak area in the extract of the blank sample spiked with the standard solution to the analyte peak area of the standard solution in solvents, multiplied by 100. The limit of detection and limit of quantification were evaluated based on the signal-to-noise ratio (3 for LOD and 10 for LOQ).

Recovery, repeatability, and within-laboratory reproducibility. The values of recovery and repeatability were estimated by the analysis of blank samples spiked at three concentration levels: 5, 50 and 200 μg/kg (six samples for each level). Recovery was determined according to the following equation: % recovery = 100 × measured content/fortification level. To evaluate the repeatability, the coefficient of variation (CV %) was calculated for each analysed level. Within-laboratory reproducibility was assessed by spiking two other sets of blank honey samples at the same concentrations as for repeatability and analysing them on different days with the same instrument; CV (%) values were calculated.

Uncertainty. The uncertainty of the method was estimated by identification and quantification of the uncertainty components of the whole analytical process according to the guidelines of Eurachem and Co-Operation on International Traceability in Analytical Chemistry (12). The expanded uncertainty was expressed as a percentage value (P = 0.05, k = 2).

Results

Method validation results. Several parameters were evaluated in the validation process. The proposed procedure was linear in the range of 5–200 μg/kg and the determination coefficients of the matrix-spiked curves were above 0.98 (Table 1). The method also proved to be selective, as no interfering peaks were detected in the retention times of the analysed alkaloids.

All matrix effect results are presented in Table 1. For most of the investigated compounds signal enhancement was observed, with the highest for atropine and heliotrine. Slight signal suppression was noticeable for senecionine, jacobine, erucifoline retrorsine, seneciphylline and senecivernine.

The LODs and LOQs ranged from 0.05 to 0.17 μg/kg and from 0.17 to 0.58 μg/kg, respectively. The LODs of scopolamine and atropine were 0.15 and 0.11 μg/kg and the LOQs were 0.49 and 0.36 μg/kg, respectively. Recovery of the analysed compounds varied from 81.2 % to 106.3 % for PAs and from 83.9 % to 102.5 % for TAs.

Adequate repeatability expressed as the coefficient of variation was observed for all validation assays, with CVs between 1.5% and 13.3% for analysed PAs and between 3.5% and 10.4% in the case of TAs, depending on each concentration level investigated. Coefficients of variations for reproducibility were in the range of 3.3% to 17.8% in case of PAs and for TAs ranged from 5.1% to 10.3% (Table 2).

The uncertainty of the method depending on the compound varied from 5.6% to 25.5%. The highest value of 25.5% was adopted as the overall method uncertainty.

As far as the reduction rates of *N*-oxides are concerned, most of the *N*-oxide forms were completely reduced 24 h after treatment with the zinc powder.

Honey sample alkaloid presence results. In 15 out of 29 samples, at least one of the analysed PAs was detected (Table 3). The most abundant alkaloids were echimidine, intermedine, lycopsamine (the lycopsamine type) and senecionine. Echimidine was present in 31% of all analysed honeys, while intermedine, lycopsamine and senecionine contaminated 24% (Fig. 1). Seneciphylline, retrorsine, senecivernine and erucifoline were the other detected alkaloids; however, they had much lower incidence rates as their presence was confirmed only in two samples or single samples.

Echimidine occurred in a concentration ranging from 2.2 to 120.0 μg/kg. Intermedine and lycopsamine content ranged from 2.2 to 23.3 μg/kg and from 2.3 to 22.5 μg/kg, respectively. Senecionine, senecivernine, seneciphylline, retrorsine and erucifoline occurred in relatively low concentrations, mostly of below 5 μg/kg (Fig. 2).

With respect to the total content of PAs, only in two samples were high concentrations of the alkaloids determined, those contents being 147.0 μg/kg and 120 μg/kg. Total PA concentrations in a range of 11.8–16.7 μg/kg were detected in five analysed honeys, and one sample contained 31.6 μg/kg of PAs. In addition, five other samples were contaminated in a range of 5.8–9.2 μg/kg and two samples revealed relatively low contamination of 2.2 μg/kg and 2.5 μg/kg. The average concentration was evaluated as 14.2 μg/kg and the median as 2.2 μg/kg. Scopolamine and atropine were not detected in any of the analysed samples. The average and median content of PAs in Polish honey were 5.3 μg/kg and 0 μg/kg, and the content range was 2.2–31.6 μg/kg. The average and median concentrations of PAs in honeys of foreign origin were 37.6 μg/kg and 9.5 μg/kg, respectively. The total content of the monitored PAs in all foreign honeys was in the range of 5.8–147.0 μg/kg.

Table 1. Monitored ions (m/z), retention time, matrix effect, determination coefficient (R^2), limit of detection (LOD) and limit of quantification (LOQ) of analysed pyrrolizidine and tropane alkaloids

	Monitored ion (m/z)	Retention time (min)	Matrix effect (%)	Linearity R^2	LOD µg kg^{-1}	LOQ µg kg^{-1}	Uncertainty (%)
Monocrotaline	326.1	5.26	101	0.9924	0.16	0.52	9.8
Erucifoline	350.1	7.60	95	0.9933	0.10	0.35	12.9
Intermedine	300.1	8.14	115	0.9900	0.09	0.31	19.0
Europine	330.1	8.37	115	0.9929	0.17	0.58	24.3
Lycopsamine	300.1	8.54	116	0.9877	0.09	0.28	22.1
Jacobine	352.1	9.07	94	0.9922	0.13	0.44	17.6
Retrorsine	352.1	11.49	97	0.9904	0.09	0.29	21.4
Trichodesmine	354.1	11.65	101	0.9945	0.13	0.44	13.7
Heliotrine	314.1	12.19	119	0.9952	0.11	0.37	17.7
Seneciphylline	334.1	12.79	96	0.9944	0.10	0.34	13.7
Senecivernine	336.1	15.47	98	0.9940	0.13	0.43	14.2
Senecionine	336.1	15.73	91	0.9901	0.09	0.29	17.1
Echimidine	398.1	17.25	103	0.9941	0.16	0.53	25.5
Senkirkine	366.1	17.44	113	0.9939	0.05	0.17	5.6
Lasiocarpine	412.1	18.89	115	0.9935	0.06	0.21	13.4
Scopolamine	304.1	10.28	105	0.9922	0.15	0.49	15.5
Atropine	290.1	14.36	127	0.9942	0.11	0.36	14.7

Table 2. Validation results for recovery, repeatability and within-laboratory reproducibility

	Recovery (%)			Repeatability CV (%)			Reproducibility CV (%)		
Concentration	5	50	200	5	50	200	5	50	200
Monocrotaline	92.6	96.5	91.2	5.7	3.8	4.6	7.9	8.6	7.6
Erucifoline	90.6	96.9	94.9	5.4	4.1	8.0	8.6	6.2	7.9
Intermedine	100.2	98.2	88.6	5.2	9.6	13.3	7.8	9.8	12.6
Europine	98.6	95.9	81.2	3.9	11.4	12.9	8.5	11.1	17.4
Lycopsamine	97.1	95.6	84.5	4.7	11.6	12.4	9.5	11.8	15.4
Jacobine	94.0	100.6	92.1	3.3	4.5	10.0	8.0	6.7	9.6
Retrorsine	83.9	97.7	92.1	7.2	12.7	11.0	17.8	13.9	12.8
Trichodesmine	94.7	97.2	89.4	9.0	5.0	7.3	11.9	7.5	8.8
Heliotrine	99.9	100.3	92.8	7.4	5.7	9.7	6.7	7.5	9.8
Seneciphylline	90.3	99.4	95.5	7.7	3.5	8.2	9.9	8.0	8.1
Senecivernine	100.0	104.0	96.1	3.5	2.7	8.3	5.7	6.5	8.8
Senecionine	96.2	100.1	90.9	1.7	7.0	8.9	7.8	8.2	11.1
Echimidine	98.9	99.6	89.0	6.5	9.1	13.3	8.3	9.8	16.4
Senkirkine	99.4	104.2	92.9	3.8	1.5	7.6	6.5	3.4	7.6
Lasiocarpine	106.3	103.1	93.0	4.4	4.3	6.8	5.0	4.6	10.6
Scopolamine	83.9	102.5	90.7	8.9	3.5	7.8	9.4	8.1	7.8
Atropine	88.6	102.2	89.0	6.4	3.6	10.4	7.3	5.1	10.3

Table 3. Alkaloid contamination results for the honey samples tested

	Intermedine	Lycopsamine	Senecionine	Senecivernine	Echimidine	Retrorsine	Seneciphylline	Erucifoline	Total
02/p		3.0	1.9		6.9				11.8
03/p	2.2	2.8	2.2		7.3				14.5
06/f	23.3	22.5			101.2				147.0
07/p	2.2	3.2	1.9						7.3
09/f	2.9				10.2				13.1
10/p		3.0	2.1				4.1		9.2
11/f			1.8				4.0		5.8
14/f					120.0				120.0
15/p					5.9				5.9
16/p	9.2	14.1	2.0		6.3				31.6
19/f			2.7	3.0		4.3		4.5	14.5
22/p	7.1	5.3				4.3			16.7
25/p	8.7								8.7
27/p					2.2				2.2
28/p					2.5				2.5

p – honey sample of Polish origin; f – honey sample of foreign origin. Results expressed in µg/kg

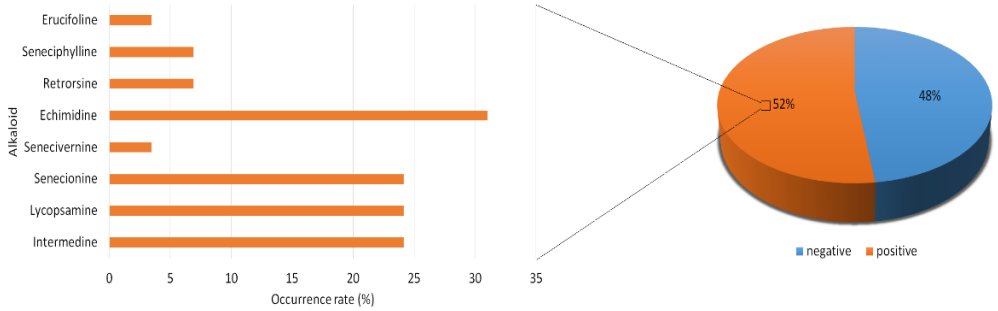

Fig. 1. Results of honey sample alkaloid investigation expressed as percentage of samples contaminated and not contaminated and percentage distribution of determined alkaloids in the contaminated samples

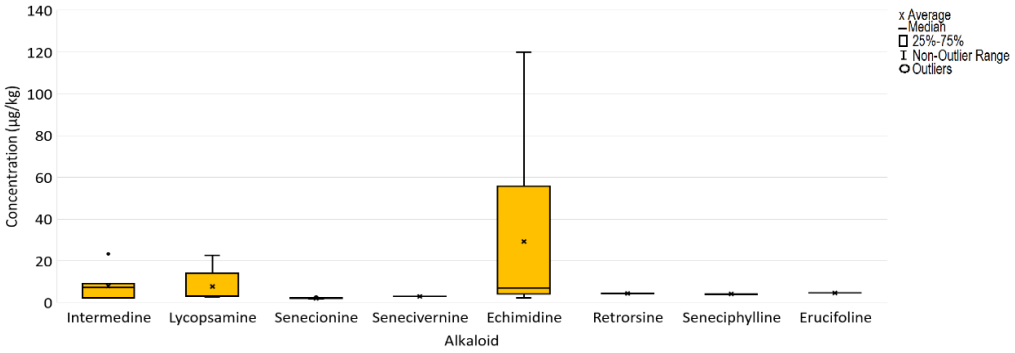

Fig. 2. Boxplots of the concentration of determined alkaloids

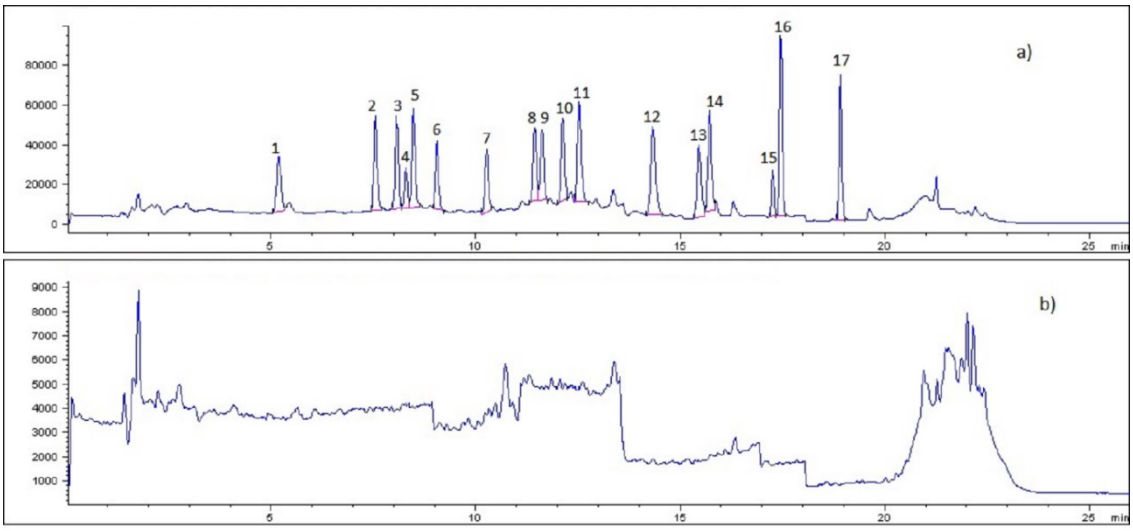

Fig. 3. a) SIM chromatogram obtained for honey matrix spiked at a concentration of 5 µg kg^{-1}. 1 – monocrotaline, 2 – erucifoline, 3 – intermedine, 4 – europine, 5 – lycopsamine, 6 – jacobine, 7 – scopolamine, 8 – retrorsine, 9 – trichodesmine, 10 – heliotrine, 11 – seneciphylline, 12 – atropine, 13 – senecivernine, 14 – senecionine, 15 – echimidine, 16 – senkirkine, 17 – lasiocarpine; b) chromatogram of a blank honey sample

Discussion

A founding premise of this research was that it was considered desirable to achieve chromatographic resolution of all investigated compounds. However, because of the occurrence of some PAs in the form of isomers, their chromatographic separation was expected to pose additional challenges.

The first attempts involved the separation of the compound with a Gemini 3 µm NX-C18, 150 mm × 4.6 mm column, (Phenomenex,) and a mobile phase consisting of 0.2% formic acid in water (A) and a mixture of methanol and acetonitrile (1:1, *v/v)* (B) (23). The separation of scopolamine and atropine did not pose any major difficulties. However, the isomeric tendencies of some compounds resulted in the overlapping of some of them. Intermedine, europine, lycopsamine, trichodesmine and retrorsine in particular were not separated. Co-elution also occurred in the case of senecionine and senecivernine. For this reason, different columns and new compositions of the mobile phase had to be used to achieve the resolution of all targeted alkaloids.

Among the columns evaluated were the same Gemini column; Kinetex C18, 2.6 µm, 100 mm × 4.6 mm and Kinetex C8, 2.6 µm, 100 mm × 4.6 mm. In optimising the mobile phase we evaluated various concentrations of formic acid in water as phase A, with phase B consisting of different volume ratios of

methanol and acetonitrile. A combination of varying proportions of methanol and acetonitrile effectively modified the elution strength of the mobile phase. With the appropriate gradient, elution strength control allowed the separation of all PAs and TAs. Separation was achieved with a mobile phase consisting of 0.3% formic acid in water (A) and a mixture of methanol and acetonitrile (2:1, *v/v*) (B) and a Kinetex C18, 2.6 µm, 100 mm × 4.6 mm column (Fig. 3a).

For the extraction and purification of the extracts, different approaches have been described in the literature. The procedure used by Martinello *et al.* (25) for the extraction of TAs and PAs was based on the quick, easy, cheap, effective, rugged and safe (QuEChERS) approach and purification with primary and secondary amines (PSA). Also, in their method for the determination of TAs in honey, Romera-Torres *et al.* (29) used dispersive solid-phase extraction (d-SPE) for the purification of the extracts with graphitised black carbon. However, as our first experience had shown, the QuEChERS and d-SPE approach was not sufficient for adequate purification of honey extracts in LC-MS analysis of PA content (23). Therefore, we decided to evaluate SPE with an in-house developed elution mixture for the clean-up of the honey extracts to leave them utilisable in both PAs and TAs analysis.

As the first specification, MCX cartridges with an elution mixture consisting of ethyl acetate, methanol, acetonitrile, triethylamine and ammonium (8:1:1:0.1:0.1 *v/v*) were tested for sample clean-up. Even though the clean-up effect was good, the recoveries of europine and erucifoline were unacceptable. To improve the recovery rates of all compounds, especially of europine and erucifoline and the analysed TAs, new compositions of the elution mixture and other cation exchange cartridges were evaluated. More satisfactory recovery was attempted with larger volumes of ammonia, methanol and acetonitrile and alternative cartridges, including MCX, Bond Elut Plexa - PCX and HF Bond Elut (Agilent Technologies), strong cation exchange - SCX and polymeric strong cation - Strata-X-C (Phenomenex).

Among the solvents, only the increase of ammonia volume improved the recovery rates of all the analysed alkaloids. However, it also caused the elution of some other matrix constituents, leading to the deterioration of the chromatogram quality. A satisfactory balance between acceptable recoveries, especially of europine and erucifoline, and the quality of chromatograms was achieved when ammonia was used in a volume of 0.3 mL.

The MCX and PCX columns gave some of the best results for both groups of analysed alkaloids; however, with PCX cartridges the clean-up effect, and therefore the quality of the chromatograms obtained, were worse than those offered by MCX or HF cartridges.

To enhance the sensitivity of the method, a relatively high volume of honey extract was subjected to SPE purification and concentration. For this reason, the clogging problem was considered as another relevant factor affecting the choice of SPE cartridges. Most of the tested SPE columns, including SCX, Strata-X-C, and PCX, became clogged while the honey solution was being passed through them, decreasing the efficiency of the SPE step. Thus, the selection of the most effective cartridge was a compromise between recovery rate, purification effect and avoidance of clogging. Only MCX cartridges provided acceptable recovery rates of all analysed alkaloids, good clean-up effect, and crucially, the capacity to pass extracts without clogging.

Even though the *N*-oxides can be determined in a direct way, we decided to reduce them to their free base forms. The reduction step improved the clean-up effect. Moreover, it reduced the viscosity of the honey extracts, which was especially important in the elimination of the clogging of the SPE cartridges.

According to European Commission Decision 2002/657/EC (7), the recovery should be in the range of 70% to 110% for spike levels between 1.0 and 10.0 µg/kg, and in the range of 80%–110% for concentrations greater than or equal to 10.0 µg/kg. All the obtained recovery values are in line with these requirements, proving the efficient extraction of the compounds. Also, adequate repeatability and within-laboratory reproducibility (CV %) values below 20% were achieved. The method proved to be selective (Fig. 3b) and linear in the investigated range ($R^2 > 0.98$).

Matrix effects are generally expressed as a suppression or enhancement of the analytical signal due to co-eluting matrix components. Matrix effects have been extensively studied and documented as a source of error in quantitative liquid chromatography–mass spectrometry analysis of food samples (8). For most of the investigated compounds, signal enhancement was observed, as the matrix effect values exceeded 100%. The highest signal enhancement was visible in the cases of atropine and heliotrine; however, slight signal suppression was also observed for some of the compounds.

The obtained LOQ values for PAs and TAs were relatively low, and are comparable to the LOQ of other methods based on HRMS (25) and even lower than the LOQ obtained for tropane alkaloids by Romera-Torres *et al.* (29). All parameter values found during validation were in line with the performance requirements of European Commission Decision 2002/657/EC, proving the utility of the method for the determination of PAs and TAs in honey.

The finding that echimidine, intermedine, and lycopsamine were among the most abundant alkaloids is in concurrence with the results reported by the European Food Safety Authority, Huybrechts *et al.* (15) and Callebaut *et al.* (21) and Martinello *et al.* (25). In all studies, echimidine and lycopsamine were reported as the most abundant PAs.

The PA concentrations determined in Polish honey, which fell in a 2.2–31.6 µg/kg range, are comparable with the results obtained in the authors' previous study on honey from Poland (23). These results are also consistent with the findings of other authors who tested

honey of European origin. Bodi *et al.* (4) studied honey samples from Germany. The average total concentration of the analysed PAs was 6.1 μg/kg in samples from local beekeepers, with a concentration range of 0.4–28.2 μg/kg. Concentrations of PAs in the honey of Italy reported by Lucatello *et al.* (24) were also comparable and ranged from 0.6 to 17.6 μg/kg. The highest concentration values were obtained for honey of origin outside the European Union. Also, the average and median concentrations of PAs in this honey were higher than in Polish honey. Similarly, other authors observed higher contamination of honey originating from non-European countries (9, 34).

However, contrary to the findings of Martinello *et al.* (25), Romera-Torres *et al.* (29) and Thompson *et al.* (32), atropine or scopolamine was found in none of the analysed honeys. Martinello *et al*. (25) reported the presence of atropine in 9 out of 40 analysed samples; however, scopolamine was not observed. Romera-Torres *et al.* (29) confirmed the presence of scopolamine in one honey and determined the level as 27 μg/kg but did not detect atropine in any of the 19 honeys analysed. Thompson *et al.* (32) determined hyoscyamine at the level of 0.012 μg/kg in two honey samples, one of which also contained 0.012 μg/kg of scopolamine.

As all analysed honeys were free of TAs, the safety assessment was focused on PAs only. To assess the safety of analysed honeys, a benchmark dose lower confidence limit for a 10% excess cancer risk ($BMDL_{10}$) of 237 μg/kg b.w. per day was used as a reference point. This was derived from the study of the incidence of liver haemangiosarcoma in female rats exposed to riddelliine implementing margin of exposure (MOE) of 10,000 (14). For the calculation of dietary exposure, an average consumption of 20 g of honey, an average adult weight of 70 kg and an average child weight of 20 kg were adopted. Taking into consideration the $BMDL_{10}$ and MOE, it was assessed that the maximum content of PAs in honey should not exceed 83.0 μg kg^{-1} for adults and 23.7 μg kg^{-1} for children. Based on the determined concentrations of PAs, it can be stated that only two samples would exceed the threshold of 83.0 μg/kg. Three analysed honeys were contaminated above the safe level of 23.7 μg/kg, and could pose a risk to children if consumed in the amount of 20 g per day or more. Most of the analysed honeys contained PA concentrations below 23.7 μg/kg, and therefore ingestion of 20 g should not pose any risk to child or adult consumers.

A sensitive method suitable for simultaneous determination of PAs and TAs in honey was developed and validated. All the assessed parameters met the prerequisites for analytical methods, which proved the utility of the developed protocol. The method was applied in the analysis of 29 honey samples, of which 52% were positive for the presence of at least one of the monitored PAs. Echimidine, lycopsamine, intermedine and senecionine were the most abundant alkaloids; however, scopolamine and atropine were not detected in any of the analysed honey. The risk assessment revealed that the ingestion of three analysed honeys, could pose a potential risk to their consumers, especially children. All three honeys were bound for retail distribution; in light of the demonstrable potential for this product to be injurious to health, it is hoped that the analytical method developed will contribute to food safety surveillance in the honey category.

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