

Quantitation of Pyrrolizidine Alkaloids in Honey and Herbal Teas by UHPLC/MS/MS

Application Note

Food Testing

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Abstract

This application note describes an easy-to-handle and sensitive analytical method for the quantitation of 28 pyrrolizidine alkaloids (PAs) in honey and herbal tea. The method comprises an acidic extraction and a cleanup by solid phase extraction (SPE) using a strong cation exchange material. The UHPLC separation was performed using an Agilent 1260 Infinity LC coupled to a highly sensitive Agilent 6490 iFunnel triple quadrupole mass spectrometer, operated in positive electrospray ionization with dynamic MRM, acquiring three transitions per compound.

The method was successfully validated for honey and herbal teas. Quantitation was performed based on a matrix-matched calibration to compensate for matrix effects in the electrospray ionization. Limits of quantitation were below the minimum requirements specified by the German Federal Office of Consumer Protection and Food Safety (BVL). Extraction recoveries for most PAs were in the range of 80 to 120 % in the honey samples and between 70 and 85 % in the herbal tea samples. The method has been applied to 24 rooibos tea samples purchased from a local market, and PA concentrations from 143 to 2,300 µg/kg were observed. The senecionine type compounds (Retrorsine, Senecionine, Seneciphylline, and their N-oxides) were the most frequently found PAs in the rooibos teas.



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Introduction

Pyrrrolizidine alkaloids (PAs) are secondary metabolites produced by more than 6,000 plant species from the families *Asteraceae*, *Boraginaceae*, and *Fabaceae*. There are more than 500 different PAs and their N-oxides known. PA-containing plants occur worldwide in almost every biotope, and some seem to be spreading their geographical distribution due to climate change [1]. Food contaminations were reported in 2007 when salad mixes of radicchio, frisee lettuce, and corn salad were accidentally adulterated with common groundsel (*senecio vulgaris*) [2]. Afterwards, it was observed that PAs are transferred into honey containing nectar from composite plants [3,4]. In addition, PA contaminations were found in herbal teas when weeds were inadvertently mixed with crops during harvesting [5]. The German Federal Institute for Risk Assessment (BfR) came to the conclusion that acute and long-term exposure to PAs could result in severe hepatic damage [2].

PAs consist of a 1-hydroxymethyl pyrrolizidine necin base, which is esterified with mono- and dicarboxylic acids. Alkaloids show toxic effects when they contain a double bond in the 1,2-position of the pyrrolizidine ring, which is esterified with at least one branched carboxylic acid [5]. This carboxylic acid can be either at the hydroxymethyl group in the C1 position or at the hydroxyl group in the C7 position (Figure 1).

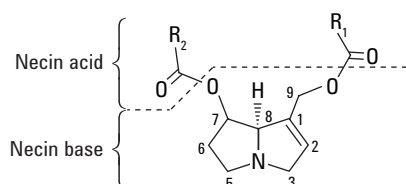


Figure 1. Principal structure of pyrrolizidine alkaloids.

Currently, there are no European Regulations specifying maximum limits for PAs in food and feed. The toxicity assessment done by the European Food Safety Authority (EFSA) is based on the concept of margin of exposure (MOE) [6]. The MOE is the ratio between the dose that causes a small but measurable adverse effect for a given population

and the level of exposure to the substance. MOE values above 10,000 are considered harmless for human health [5]. The BfR has concluded that the total exposure to 1,2-unsaturated pyrrolizidine alkaloids should be as low as reasonably achievable (ALARA principle). A daily intake of 0.007 µg PA per kg body weight should not be exceeded [5].

The determination of individual PAs at low concentrations requires the availability of reliable and accurate analytical methods. LC tandem mass spectrometry has proven to be a powerful tool for the analysis of various residues and contaminants. It has been shown that LC/MS/MS allows the simultaneous determination of multiple PAs in honey and pollen with high sensitivity and selectivity [3]. The accurate quantitation of PAs in complex matrices can be hampered by matrix effects in the electrospray ionization causing signal suppression or enhancement. Differences in the degree of matrix effects cannot only be expected between different commodities such as honey and tea, but also between individual samples of one matrix type.

There are different strategies to compensate for matrix effects such as matrix-matched calibrations, standard addition, the addition of internal standards, or the dilution of the sample. Due to the lack of commercially available isotopically labeled standards for PAs, matrix-matched calibrations are the preferred strategy. However, the ubiquitous occurrence of PAs in some matrices (for example, rooibos tea) and variations in the matrix effects within a commodity are not fully compensated. The dilution of the sample would be attractive, but reduces the sensitivity of the analytical method.

This application note describes a validated analytical method for the quantitation of PAs in honey and herbal teas by UHPLC/MS/MS. Samples are extracted with sulfuric acid followed by a cleanup by solid phase extraction (SPE) using a strong cation exchange material. Finally, samples are measured using UHPLC separation and MS/MS detection applying dynamic MRM. The method was successfully validated for 17 PAs in honey and 28 PAs in herbal teas. In addition, method performance parameters including the determination of linearity, limits of quantitation (LOQs) based on the signal-to-noise ratio (S/N), accuracy, and repeatability have been evaluated.

Experimental

Reagents, honey and tea samples

All reagents and solvents were HPLC or LC/MS grade. Acetonitrile and methanol were purchased from Fisher (Fisher Chemicals, Loughborough, United Kingdom). Ultrapure water was produced using a Barnstead GenPure xCAD system (Thermo Scientific, Braunschweig, Germany). Formic acid was from Fluka (Fluka Sigma-Aldrich, Steinheim, Germany). Sulfuric acid solution (Titrisol 0.05 M) and ammonia (25 %, EMSURE) were obtained from Merck (Merck KGaA, Darmstadt, Germany). Ammonium formate was purchased from Biosolve (ULC/MS, Biosolve BV, Valkenswaard, Netherlands).

The pyrrolizidine alkaloid analytical standards were purchased from Phytolab (Vestenbergsgreuth, Germany), Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (St. Louis, USA), Cfm Oskar Tropitzsch (Markredwitz, Germany), and Latoxan (Valence, France).

For spiking experiments, uncontaminated honey and herbal tea samples were required. For this reason, a rape blossom honey and a spring flower honey were purchased from local German groceries. The herbal tea was a mixture of home-grown herbal teas (chamomile, peppermint, and lemon balm) and a yoghurt mint tea (Kings Crown, Rossmann, Burgwedel, Germany) purchased in a local grocery. The honey samples were just homogenized, and the tea samples were ground with an impact mill (A11 basic analytical mill, IKA, Staufen, Germany) and homogenized. All samples were analyzed for PAs using the same UHPLC/MS/MS method. Background contaminations were below the limits of detection (LODs).

Solutions and standards

A stock solution of 28 PAs was prepared by dissolving 1 mg of the individual PA reference materials in 10 mL acetonitrile, resulting in a concentration of 100 µg/mL. From this stock solution, 100 µL were diluted with acetonitrile to a total volume of 10 mL, resulting in a concentration of 1 µg/mL (working solution 1). Another dilution was prepared with 500 µL of working solution 1 in 10 mL methanol/water (5:95 v/v) (50 ng/mL, working solution 2). The stock solutions for Echimidine, Europine-N-oxide, Lycopsamine-N-oxide, Retrorsine-N-oxide, and Seneciphylline, and working solutions 1 and 2 were stored at 4 °C. The stock solutions for Echimidine-N-oxide, Erucifoline, Erucifoline-N-oxide, Europine, Heliotrine, Heliotrine-N-oxide, Intermedine, Intermedine-N-oxide, Jacobine, Jacobine-N-oxide, Lasiocarpine, Lasiocarpine-N-oxide, Lycopsamine, Monocrotaline, Monocrotaline-N-oxide, Retrorsine, Senecionine, Senecionine-N-oxide, Seneciphylline, Seneciphylline-N-oxide, Senecivernine, Senecivernine-N-oxide, Senkirkin, and Trichodesmine were stored at -20 °C.

A positive control standard with a concentration of 5 ng/mL was prepared by diluting 5 µL of working solution 1 with 995 µL of a mixture of methanol/water (5:95; v/v). For the matrix calibration, working solution 1 was added to the blank material before extraction. Table 1 shows the resulting concentrations and the associated spiking volumes.

Table 1. Concentration of the Spiked Level and Spiking Volume of Working Solution 1

Level	Honey		Herbal tea	
	Concentration (µg/kg)	Spiking volume (µL)	Concentration (µg/kg)	Spiking volume (µL)
0	0	–	0	–
1	1	5	5	10
2	2.5	12.5	10	20
3	5	25	15	30
4	10	50	25	50
5	15	75	50	100
6	20	100	100	200

Sample preparation

Homogenized honey samples ($5 \text{ g} \pm 0.1 \text{ g}$) were weighed in 50-mL centrifuge tubes, and 30 mL of the extraction solvent (sulfuric acid, 0.05 M) was added. Samples were heated to $50 \text{ }^\circ\text{C}$ for 10 minutes in a water bath. Then, the samples were extracted for 30 minutes on an overhead shaker (Heidolph REAX 2, Schwabach, Germany) at room temperature. Next, the samples were centrifuged at 4,000 rpm for 10 minutes (Hettich Centrifuge Rotina 380R, Tuttlingen, Germany). The entire supernatant was then transferred to another 50-mL plastic tube, and heated to $50 \text{ }^\circ\text{C}$ to avoid precipitation of the honey in the subsequent SPE. Agilent Bond Elut SCX cartridges (500 mg, LRC, p/n 14113039) were conditioned with 5 mL of methanol and 5 mL of 0.05 M sulfuric acid. The cartridge was loaded with the entire honey extract, and was washed with 6 mL of water and 6 mL of methanol. After drying the SPE cartridge for 5 to 10 minutes with nitrogen, the PAs were eluted twofold with 5 mL of a mixture of ammonia/methanol (6:94 v/v). The eluate was then evaporated to dryness, and reconstituted in 1 mL of a mixture of methanol/water (5:95 v/v) in a microcentrifuge tube. The extract was centrifuged at 14,000 rpm (Hettich Centrifuge Mikro 120, Tuttlingen, Germany) for 10 minutes. A volume of $50 \text{ }\mu\text{L}$ of the reconstituted honey extract was then transferred into an HPLC vial, and was diluted with $150 \text{ }\mu\text{L}$ methanol/water (5:95 v/v). This procedure resulted in an enrichment factor of 1.25x.

The extraction of the tea samples was slightly different. A $2 \text{ g} \pm 0.1 \text{ g}$ amount of the ground and homogenized samples was weighed in 50-mL centrifuge tubes, and 20 mL of hot sulfuric acid ($50 \text{ }^\circ\text{C}$, 0.05 M) was added. The samples were then extracted for 15 minutes in an ultrasonic bath (Bandelin Sonorex RK 100, Berlin, Germany) at room temperature. Then, they were centrifuged at 4,000 rpm for 10 minutes. The supernatant was filtered over glass wool, and transferred into another 50-mL centrifuge tube. The dry residue was extracted again with 20 mL of hot sulfuric acid, and the two supernatants were combined and centrifuged at 4,000 rpm for 10 minutes. Two milliliters of the tea extract (equivalent to 0.1 g tea) were loaded onto the Bond Elut SCX cartridges (500 mg, LRC, p/n 14113039), which were preconditioned with 5 mL of methanol and 5 mL of 0.05 M sulfuric acid. The cartridge was washed with 6 mL of water and 6 mL of methanol, and dried for 5 to 10 minutes with nitrogen. The PAs were eluted two times with 5 mL of a mixture of ammonia/methanol (6:94, v/v). The extracts were then evaporated to dryness and reconstituted in 1 mL of a mixture of methanol/water (5:95, v/v). The extract was centrifuged and transferred to an HPLC vial without further dilution (enrichment factor 1.0x). Figure 2 shows a schematic overview of the extraction procedure.

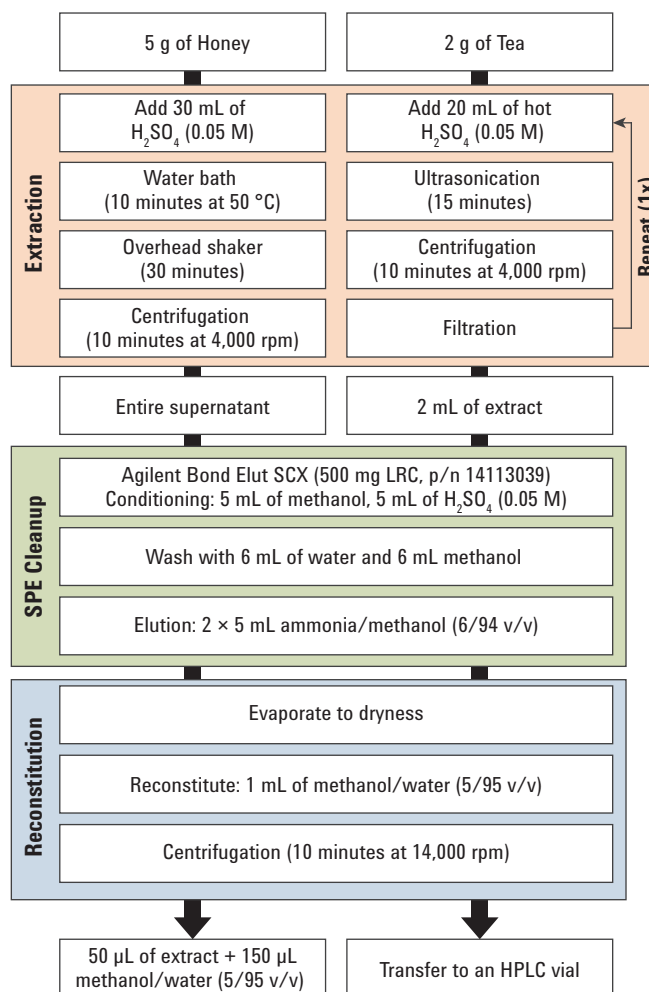


Figure 2. Schematic overview of the sample preparation and extraction procedure for honey and tea samples.

For the evaluation of matrix effects, blank samples were extracted according to the procedure described. The raw extracts were spiked with working solution 2 at six different levels, and were thoroughly mixed. For the evaluation of recoveries, linear working range, LOQs, and repeatability, blank honey and tea samples were spiked before extraction with the appropriate amount of PA working solution 1. Samples were then extracted following the procedure described previously.

Equipment

Separation was carried out using an Agilent 1260 Infinity UHPLC system consisting of an Agilent 1260 Infinity Binary Pump (G1312B), an Agilent 1260 Infinity High Performance Autosampler (G1367E), and an Agilent 1260 Infinity Thermostatted Column Compartment (G1316C). The HPLC system was coupled to an Agilent G6490A Triple Quadrupole mass spectrometer equipped with an Agilent Jet Stream electrospray ionization source. Agilent MassHunter Acquisition software B.07.01 was used for data acquisition, and Agilent MassHunter Workstation software B.07.00 was used for data analysis.

Method

Table 2 summarizes the 1260 Infinity HPLC conditions, and Table 3 shows the G6490A Triple Quadrupole parameters. The analysis was carried out in positive electrospray ionization in dynamic MRM mode using three major transitions per target compound. This was to increase confidence in identification, as in some cases interferences showed up on one or two MRM transitions, especially in the plant material matrix. Negative ionization was evaluated, but was shown to be less sensitive for the PAs. Transitions and conditions for the PAs were optimized using Agilent MassHunter Optimizer software with flow injection using individual solutions for each PA at a concentration of 0.1 ng/mL. Generally, the $[M+H]^+$ species produced the most abundant precursor ion. For many PAs, fragments with a mass of m/z 120 and m/z 138 were selected for the MRM transitions as they were most abundant and indicative for Retronecine-type compounds. Table 4 summarizes all the transitions and conditions.

The quantitation of the PAs was based on matrix calibrations. Blank samples of honey and herbal tea were spiked at five different concentrations before the extraction (see Table 1). The extraction recoveries were calculated by comparing the concentrations of blank honey and tea samples spiked before extraction with the matrix-matched calibration standards. LOQs were calculated based on a S/N of 10 for the quantifier trace in the extract of the spiked sample and an S/N of at least 3 for the qualifier trace. The LODs were calculated in a similar way, but using a S/N of 3 for both the quantifier and qualifier transition.

Table 2. HPLC Parameters

Parameter	Value
HPLC column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 μm (p/n 695775-902) with HPLC Guard (p/n 821725-911) at 25 °C
Mobile phase	A) 0.025 % formic acid, 5 mM ammonium formate in water B) 0.025 % formic acid, 5 mM ammonium formate in methanol
Gradient program	Min % B 0 5 3 5 15 50 18.5 71.5 19 95 22 95 22.5 5 Stop time: 23.5 minutes Post time: 5 minutes
Flow rate	0.30 mL/min
Injection volume	10 μL
Needle wash	5 seconds with methanol/water (50/50 v/v)

Table 3. Agilent G6490A Triple Quadrupole Parameters

Parameter	Value
Ionization mode	Positive ESI with Agilent Jet Stream
Scan type	Dynamic MRM
Gas temperature	220 °C
Gas Flow	14 L/min
Nebulizer pressure	30 psi
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Capillary voltage	2500 V (pos)
Nozzle voltage	0 V
High-Pressure RF	150 V
Low-Pressure RF	60 V
Fragmentor	380 V
Cell acceleration	4 V
Cycle time	500 ms
Total number of MRMs	72
Maximum number of concurrent MRMs	24
Minimum dwell time	19.8 ms
Maximum dwell time	165.8 ms
Resolution	Unit (MS1), Unit (MS2)

Table 4. MRM Transitions and Conditions Used for the Analysis of Pyrrolizidine Alkaloids

Compound	RT	Precursor ion	Product ion	Collision energy	Compound	RT	Precursor ion	Product ion	Collision energy
Echimidine	13.2	398.2	120.2	40	Lycopsamine	8.2	300.1	138.2	20
			220.2	16				156.3	32
			118.1	64				120.1	36
Echimidine-N-oxide	13.5	414.2	254.2	36	Lycopsamine N-oxide	9.5	316.2	172.1	28
			352.1	28				94.0	52
			137.1	40				111.1	44
Erucifoline	7.2	350.2	120.1	28	Monocrotaline	4.8	326.2	121.2	32
			94.1	40				237.3	24
			138.2	36				138.2	40
Erucifoline-N-oxide	8.3	366.2	94.1	54	Monocrotaline-N-oxide	7.6	342.1	137.3	32
			119.2	32				119.1	40
			136.1	36				118.1	68
Europine	8.0	330.2	138.1	24	Retrorsine	9.9	352.2	120.0	32
			156.1	32				138.2	32
			94.1	60				324.2	32
Europine-N-oxide	8.7	346.2	172.1	32	Retrorsine N-oxide	10.4	368.1	118.3	44
			94.1	56				136.1	44
			111.2	48				119.3	40
Heliotrine	10.5	314.2	138.2	20	Senecionine	11.0	336.2	120.2	28
			156.2	32				138.2	32
			120.1	40				308.2	28
Heliotrine N-oxide	11.2	330.2	172.1	28	Senecionine N-oxide	11.8	352.2	120.2	44
			138.1	32				136.2	40
			111.1	48				94.0	50
Intermedine	7.8	300.1	138.2	20	Seneciophylline	10.4	334.2	120.3	24
			156.3	32				138.2	32
			120.1	36				306.2	28
Intermedine N-oxide	9.2	316.2	172.1	28	Seneciophylline N-oxide	11.0	350.2	120.1	36
			94.0	52				136.1	40
			111.1	44				118.1	44
Jacobine	7.9	352.2	154.9	32	Senecivernine	11.6	336.2	120.2	28
			120.0	40				138.2	32
			94.4	32				308.2	28
Jacobine N-oxide	8.7	368.2	120.1	40	Senecivernine N-oxide	12.1	352.2	94.0	50
			296.1	24				120.2	44
			94.0	60				136.2	40
Lasiocarpine	15.1	412.2	120.1	32	Senkirkine	13.8	366.2	168.2	32
			220.1	20				150.1	32
			336.1	16				122.1	32
Lasiocarpine N-oxide	16.1	428.2	120.1	40	Trichodesmine	9.3	354.2	222.2	28
			254.2	32				122.2	44
			94.0	56				120.2	44

Results and Discussion

Development of the UHPLC/MS/MS method

Transitions and conditions of all 28 targeted PAs were identified and optimized using MassHunter Optimizer software. Due to the structure of the PAs, which have a 1-hydroxymethylpyrrolizidine moiety as dominant structure element, all compounds formed abundant $[M+H]^+$ ions. The addition of formic acid to the mobile phase improved the ionization efficiency in the electrospray ionization, and improved the chromatographic peak shape.

There are more than 500 PAs known, covering a wide range of physicochemical properties, among which are many isomeric compounds. The chromatography of this method was optimized to provide an improved resolution of isomeric PAs, and achieved a baseline separation for Intermedine, Lycopsamine, and their N-oxides. The method also achieves baseline separation for Senecionine, Senecivernine, and their N-oxides. Using an Agilent InfinityLab Poroshell 120 EC-C18 UHPLC column improved the chromatographic resolution compared to a fully porous stationary phase, and helped reduce matrix effects. Figure 3 shows the chromatogram of a calibration sample including all 28 PAs. Figure 4 illustrates the separation of the isomeric compounds Intermedine and Lycopsamine and their corresponding N-oxides.

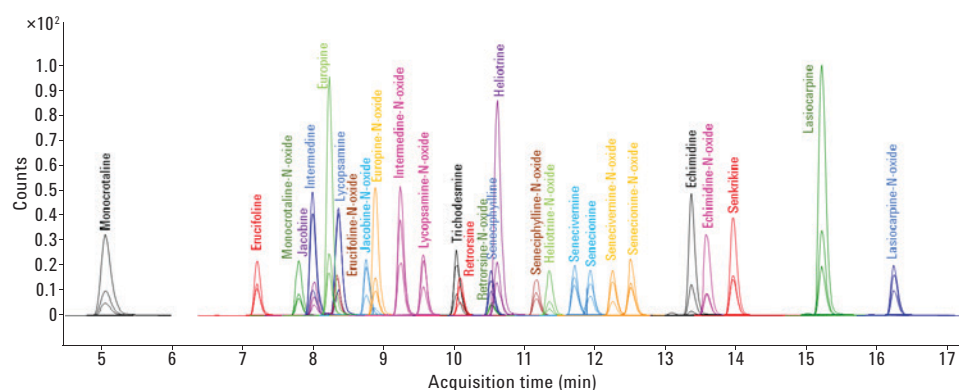


Figure 3. Extracted ion chromatograms for 28 PAs in a calibration standard, illustrating the separation efficiency of the UHPLC method.

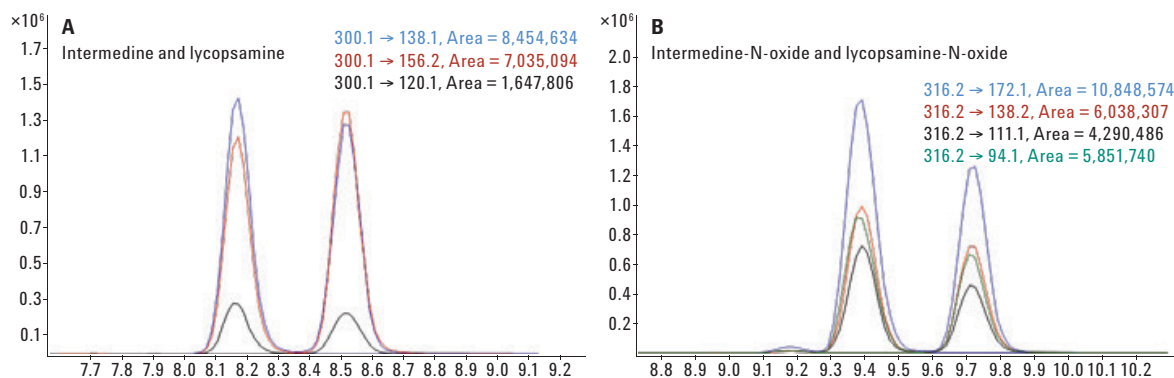


Figure 4. Chromatographic separation of the isomeric compounds intermedine and lycopsamine (A) and their corresponding N-oxides (B) spiked into an herbal tea sample.

Development of the sample preparation methods

The sample preparation method for honey is based on Bettridge *et al.* [4], and the method description provided by the BfR [7]. The extraction of the PAs from honey can be accomplished using acidified water or acidified methanol, and includes a subsequent solid phase extraction with a strong cation exchange phase. Under these conditions, high extraction recoveries are achieved for most of the targeted compounds. Due to improved recoveries and the cleanliness of the extracts, we selected acidified water for the extraction, and Bond Elut SCX cartridges for the SPE cleanup. Because of the high sensitivity of the 6490A Triple Quadrupole LC/MS system, and the improved chromatographic separation of the PAs on the InfinityLab Poroshell 120 column, the amount of sample could be reduced from 10 to 5 g. It was possible to further dilute the final extract for reduced matrix effects.

The method proposed by the BfR for the extraction of herbal teas [8] was optimized. Experiments showed that the extraction efficiency can be improved when hot sulfuric acid is used for extraction. Cleaner extracts were obtained when using the Bond Elut SCX cartridges instead of the proposed polymerically bonded C18 silica sorbent. Finally, the conditions of the SPE cleanup for the herbal tea samples were similar to the cleanup procedure for the honey samples.

Method performance characterization

The validation of the method was done for honey and herbal teas, as PA contaminations are most likely in these matrices. In addition, the BfR have initiated monitoring programs for these two matrices. The method performance was characterized by the linear working range, LODs, and LOQs. Figure 5 shows examples of the calibration curves for the matrix calibration of Intermedine in honey (A), and Senecionine in herbal tea (B).

Linear calibration curves were obtained for all targeted PAs over the relevant concentration ranges. These ranges were 1 to 20 µg/kg for honey and 5 to 100 µg/kg for herbal tea. There are different calibration ranges because of the different amounts of PAs expected in the samples. PA contaminations in honey are caused by the transfer of nectar and pollen from PA-containing plants into the honey through bees. Although PAs are presumably protectants against insect herbivores, there is no evidence that honey bees do avoid contact to PA plants. Higher levels of PA contamination can be expected for herbal teas due to the automated harvesting process.

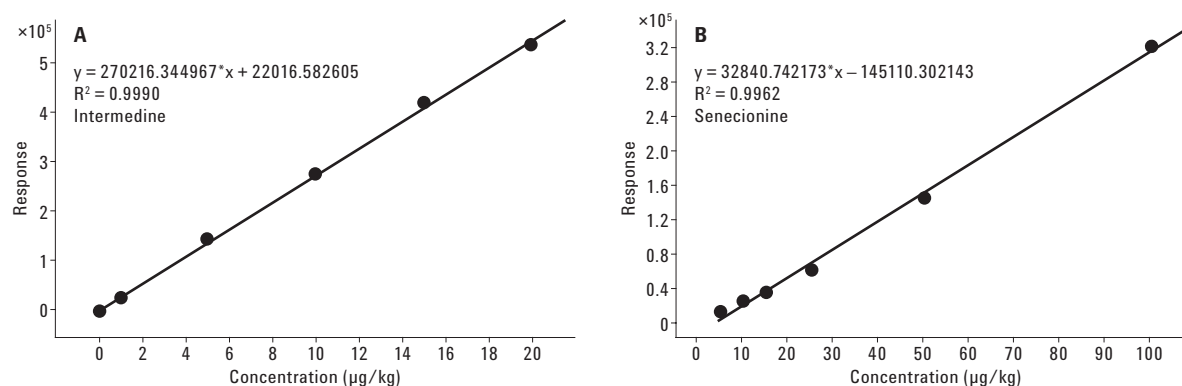


Figure 5. Calibration curves for the matrix calibration of Intermedine in honey (A) and Senecionine in herbal tea (B).

Based on the proposed target value of a daily intake of 0.007 µg PA/kg body weight [2] and typical daily intakes, concentrations in food products should not exceed certain values. Table 5 shows the maximum allowable PA concentrations in honey and tea for different intakes for adults and children.

Table 5. Maximum PA Content in Honey and Tea with Regards to the Target Value of 0.007 µg PA/(kg BW-d)

Daily intake	Adult (body weight 70 kg) (µg/kg)	Child (body weight 14 kg) (µg/kg)
20 g honey	24.5	4.9
0.2 L tea = 1 cup (= 2 g plant material)	245	49
0.5 L tea (= 5 g plant material)	98	20
1 L tea (= 10 g plant material)	49	9.8

Table 6. Method Performance Parameters Observed for Pyrrolizidine Alkaloids in Honey Matrix

Analyte	Linear working range (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	Recovery level 20 µg/kg
Echimidine	1–100	0.5	1	116 ± 9
Heliotrine	1–100	0.5	1	84 ± 10
Heliotrine-N-oxide	1–100	0.5	1	113 ± 23
Intermedine	1–100	0.5	1	95 ± 9
Lasiocarpine	1–100	0.5	1	93 ± 8
Lasiocarpine-N-oxide	1–100	0.5	1	106 ± 17
Lycopsamine	1–100	0.5	1	91 ± 8
Monocrotaline	1–100	0.5	1	93 ± 6
Monocrotaline-N-oxide	1–100	0.5	1	122 ± 25
Retrorsine	1–100	0.5	1	89 ± 9
Retrorsine-N-oxide	1–100	0.5	1	108 ± 39
Senecionine	1–100	0.5	1	81 ± 11
Senecionine-N-oxide	1–100	0.5	1	135 ± 34
Seneciphylline	1–100	0.5	1	82 ± 7
Seneciphylline-N-oxide	1–100	0.5	1	120 ± 30
Trichodesmine	1–100	0.5	1	92 ± 9
Senkirkine	1–100	0.5	1	110 ± 4

Table 6 gives the LOQs and LODs, as well as the linear ranges of the method for honey, and Table 7 gives the LOQs and LODs, as well as the linear ranges of the method for herbal tea. Figure 6 shows the chromatograms of Senecionine and Monocrotaline spiked into blank honey and a blank mixed tea at concentrations corresponding to the lowest calibration levels of the matrix calibrations.

Table 7. Method Performance Parameters Observed for Pyrrolizidine Alkaloids in Herbal Tea Matrix

Analyte	Linear working range (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	Recovery level 20 µg/kg
Echimidine	5–500	2.5	5	72 ± 5
Echimidine-N-oxide	10–500	5	10	na
Erucifoline	10–500	5	10	na
Erucifoline-N-oxide	10–500	5	10	na
Europine	5–500	2.5	5	80 ± 7
Europine-N-oxide	5–500	2.5	5	70 ± 33
Heliotrine	5–500	2.5	5	72 ± 10
Heliotrine-N-oxide	10–500	5	10	72 ± 25
Intermedine	5–500	2.5	5	79 ± 6
Intermedine-N-oxide	5–500	2.5	5	70 ± 31
Jacobine	10–500	5	10	81 ± 16
Jacobine-N-oxide	5–500	2.5	5	79 ± 48
Lasiocarpine	5–500	2.5	5	84 ± 14
Lasiocarpine-N-oxide	10–500	2.5	10	83 ± 34
Lycopsamine	10–500	5	10	64 ± 11
Lycopsamine-N-oxide	5–500	2.5	5	79 ± 38
Monocrotaline	5–500	2.5	5	68 ± 9
Monocrotaline-N-oxide	15–500	5	15	74 ± 43
Retrorsine	10–500	5	10	59 ± 7
Retrorsine-N-oxide	10–500	5	10	80 ± 34
Senecionine	15–500	10	15	77 ± 23
Senecionine-N-oxide	5–500	2.5	5	82 ± 34
Seneciphylline	5–500	2.5	5	76 ± 11
Seneciphylline-N-oxide	5–500	2.5	5	85 ± 25
Senecivernine	20–500	10	20	na
Senecivernine-N-oxide	20–500	10	20	na
Trichodesmine	10–500	5	10	60 ± 8
Senkirkine	5–500	2.5	5	72 ± 13

na = not analyzed

The matrix effect in the electrospray ionization needs to be compensated by matrix-matched calibrations or standard addition. Figure 7 shows the differences of the peak areas between the analytes in solvent (methanol/water (5:95 v/v)) and in matrix. In honey, ion suppression of up to 60 % was observed for Seneciophylline N-oxide, while matrix effects in herbal tea were even stronger, and caused up to 90 % suppression for Lasiocarpine. This is the reason for the slightly lower LOQs in the tea matrix compared to honey.

The lowest spiking concentration in honey was 1 µg/kg for all compounds. An S/N above 10 was achieved for all PAs at that level. Therefore, the lower limit of quantitation (LLOQ) was specified as 1 µg/kg in the matrix honey. The LOD was defined as 50 % of the LOQ, although lower levels would be achievable for most of the compounds.

The lowest calibration level in the herbal tea matrix was 5 µg/kg. For half of the evaluated PAs, the S/N at the lowest calibration level was above 10, and for those compounds 5 µg/kg was specified as the method LOQ. For the remaining compounds, the LOQ was set to 10 µg/kg, and only four compounds had an LOQ between 15 and 20 µg/kg. These

are excellent results considering the reduced sample amount and the additional dilution of the final extract. The results are in good agreement with the performance requirements published in the Federal Office of Consumer Protection and Food Safety (BVL) monitoring manual 2015 for the herbal tea matrix. In this manual, maximum LOQs of 15 µg/kg are specified for most of the compounds, and 20 µg/kg for Senecionine and Retrorsine and their N-oxides.

The herbal tea extracts contained a large number of matrix constituents even after SPE cleanup. These constituents also affected the extraction recoveries. When calculating recoveries for the samples spiked before extraction based on a matrix calibration, recoveries between 81 and 135 % were obtained for the honey matrix (20 µg/kg) and between 59 and 85 % for the herbal tea (100 µg/kg). The recoveries for the free bases were generally between 70 and 120 %, whereas the additional cleanup steps negatively affected recovery of the N-oxides.

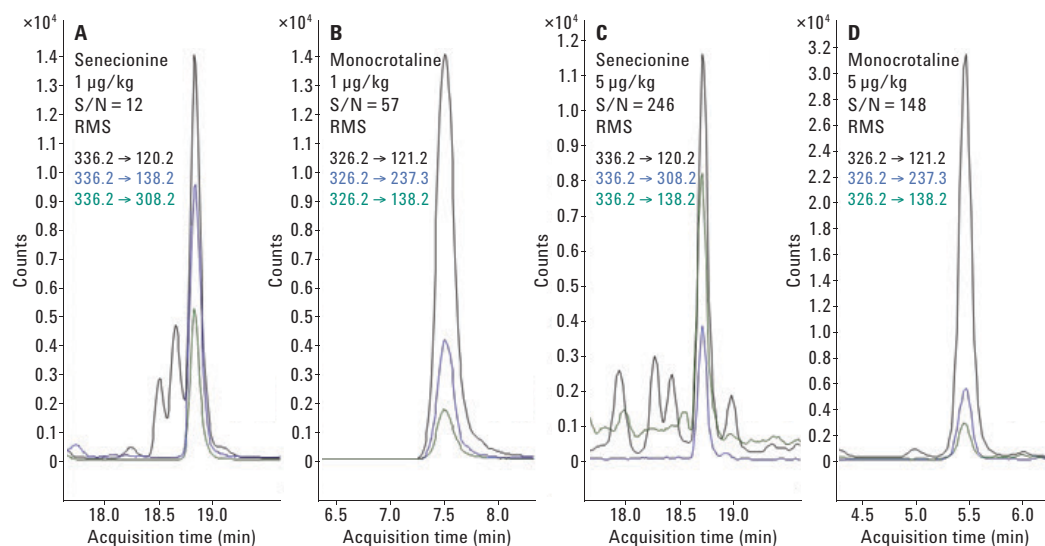


Figure 6. Chromatograms of senecionine (A and C) and monocrotaline (B and D) spiked into blank honey (left) and a blank mixed tea (right) before extraction corresponding to the lowest concentration levels of the matrix calibration.

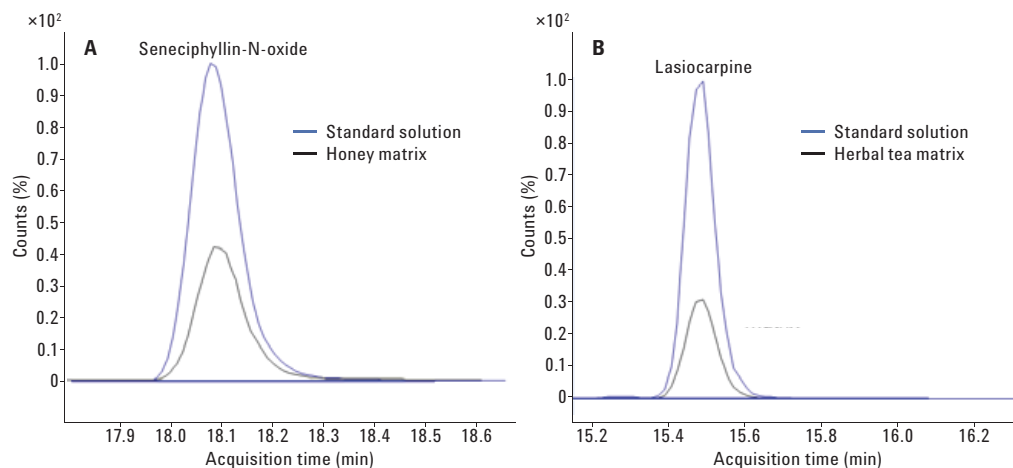


Figure 7. Chromatograms illustrating the matrix suppression for seneciphylline N-oxide spiked in honey at the concentration 5 ng/mL compared to a solvent standard (A). As well as for lasiocarpine spiked in herbal tea at the concentration 20 ng/mL compared to a solvent standard (B).

Conclusions

A fast and simple UHPLC-based multitarget method for the quantitation of pyrrolizidine alkaloids in honey and herbal tea was developed and validated. The method comprises an easy and fast solvent extraction combined with a strong cation exchange SPE cleanup. The combination of an Agilent 1260 Infinity LC and an Agilent InfinityLab Poroshell 120 EC-C18 column provided improved chromatographic resolution and reduced run-time with a reasonable backpressure even when using methanol as the mobile phase.

The method takes full advantage of the increased ionization efficiency of the Agilent Jet Stream ionization source and the innate sensitivity of the Agilent 6490 iFunnel triple quadrupole LC/MS. This is of particular importance in achieving low LOQs for the pyrrolizidine alkaloids.

The method was successfully validated for honey and herbal teas. Quantitation was done based on a matrix-matched calibration to compensate for matrix effects in the electrospray ionization. Herbal tea is known to produce complex extracts, which can cause matrix effects and matrix interferences. For improved confidence in the identification of PAs in the herbal extracts, three MRM transitions per compound were monitored. For confirmatory analysis, quantitation by standard addition is recommended. A method that performs well for herbal tea can most likely be applied to other plant materials.

The method was applied to 24 rooibos tea samples purchased from a local market. PA concentrations from 143 to 2,300 $\mu\text{g}/\text{kg}$ were observed, and PAs belonging to the Senecionine type (Retrorsine, Senecionine, Seneciphylline, and their N-oxides) were most frequently found. A challenge for the routine analysis of PAs is the inhomogeneous distribution of the PAs in the sample, which requires the use of larger sample amounts and special care during sample homogenization.

References

1. M. Boppré. "The ecological context of pyrrolizidine alkaloids in food, feed and forage: an overview" *Food additives & contaminants* **28(3)**, 260–281 (2011).
2. BfR Opinion 028/2007: Salad mix contaminated with groundsel containing pyrrolizidine alkaloids. http://www.bfr.bund.de/cm/349/salad_mix_contaminated_with_groundsel_containing_pyrrolizidine_alkaloids.pdf (accessed 22 February 2016).
3. M. Kempf, *et al.* "Pyrrolizidine alkaloids in honey: comparison of analytical methods" *Food additives & contaminants* **28(3)**, 332–347 (2011).
4. K. Betteridge, Y. Cao, S. M. Colegate. "Improved method for extraction and LC-MS analysis of pyrrolizidine alkaloids and their N-oxides in honey: Application to *Echium vulgare* honeys" *J. Agric. and Food Chem.* **53**, 1894–1902 (2005).
5. BfR Opinion 018/2013: Pyrrolizidine alkaloids in herbal teas and teas. <http://www.bfr.bund.de/cm/349/pyrrolizidine-alkaloids-in-herbal-teas-and-teas.pdf> (accessed 25 July 2016).
6. EFSA Panel on Contaminants in the Food Chain (CONTAM). (2011) "Scientific Opinion on Pyrrolizidine alkaloids in food and feed" *The EFSA Journal* **9(11):2406**, 1-135 (2011).
7. Method protocol BFR-PA-Honey-1.0/2013: Determination of pyrrolizidine alkaloids (PA) in honey by SPE-LC-MS/MS. <http://www.bfr.bund.de/cm/349/determination-of-pyrrolizidine-alkaloids-pa-in-honey.pdf> (accessed 22 February 2016).
8. Method protocol BFR-PA-Tea-2.0/2014: Determination of pyrrolizidine alkaloids (PA) in plant material by SPE-LC-MS/MS. <http://www.bfr.bund.de/cm/349/determination-of-pyrrolizidine-alkaloids-pa-in-plant-material.pdf> (accessed 22 February 2016).

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