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Validation of a Method for the Quantitation of Multiple Pyrrolizidine Alkaloids in Herbal Teas and Honey by UHPLC/MS/MS

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Introduction

Pyrrolizidine alkaloids (PAs) are secondary metabolites of plants which have hepatotoxic, cancerogenic and genotoxic effects. PAs are esters of 1-hydroxy-methylpyrrolizidin and aliphatic acids. Currently there are about 800 PAs known which are formed by plants mainly belonging to the genera *Asteraceae*, *Boraginaceae*, and *Fabaceae*. Figure 1 shows the structures of several PAs included in this study.

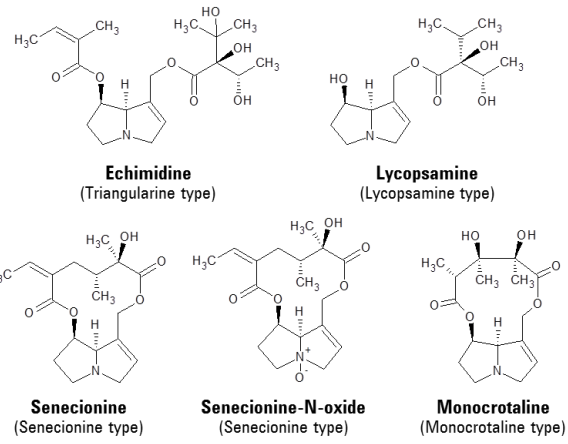


Figure 1. *Chemical structures of some PAs included in this study. The structure of the N-oxides is shown for senecionine as an example.*

In Europe most PA containing plants occur in the Mediterranean but due to climate change a further spread to the north is expected. In Germany there have been cases when PA containing plants have been accidentally mixed up with salads and there have been contaminations with PA containing plants in herbal teas. In July 2013 the Federal Institute for Risk Assessment (BfR) issued a call for action to improve data regarding the occurrence of PAs in herbal teas. PA contaminations have also been reported for honey containing nectar from composite plants.

We show the development and validation of a method for the analysis of the 23 most important commercially available PAs and PA N-oxides in honey and herbal teas based on UHPLC/MS/MS. The concept was to apply the same LC/MS method to both, honey and herbal tea samples, with dedicated extraction and clean up procedures for both sample types. For validation, honey and tea samples were spiked with the PAs before extraction at different concentrations. Due to the lack of isotopically labeled reference compounds, quantitation was based on matrix matched calibrations. Finally the method was applied to honey and tea samples purchased from a local market, and results for the PA contaminations are presented.

Experimental

Sample preparation and cleanup

Honey samples (5 g) were extracted with 0.05 M sulfuric acid for 30 min. Extracts were cleaned up using Bond Elut SCX cartridges (500 mg, LRC, PN 12113013) at an elevated temperature to prevent crystallization of sugar on the cartridge. Cartridges were washed with water and methanol and PAs were eluted using ammoniac methanol. After evaporation, dry residues were reconstituted with methanol/water (5/95, v/v), centrifuged and diluted to a final volume of 200 µL (Enrichment factor: 1.25x).

Herbal teas (2 g) were extracted twice in an ultrasonic bath for 15 min using 0.05 M sulfuric acid. Extracts were combined, filtered and were cleaned up using Bond Elut SCX cartridges (500 mg, LRC, PN 12113013). Cartridges were washed with water and methanol and PAs were eluted using ammoniac methanol. After evaporation, dry residues were reconstituted with methanol/water (5/95, v/v) (Enrichment factor: 1.0x).

Extracts were analyzed using an Agilent 1260 Infinity UHPLC system, coupled to a highly sensitive dual ion funnel triple quadrupole LC/MS system. Further method details are given in table 1.

Table 1. *Major UHPLC and 6490 LC/MS method parameters.*

Column	Agilent Poroshell 120 EC-C18 2.1 x 100 mm, 2.7 µm (p/n 695775-902) with UHPLC Guard (p/n 821725-911) @ 25°C	
Injection volume	10 µL	
Mobile phase	A: 5 mM ammonium formate + 0.025% formic acid B: 5 mM ammonium formate + 0.025% formic acid in methanol	
Flow rate	0.3 mL/min	
Gradient program	Time 0.0 3.0 15.0 18.5 19.0 22.0 22.5 Stop Time Post Time	B% 5.0 5.0 50.0 71.5 95.0 95.0 5.0 23.5 min 5 min
Ion mode	Positive ESI with Agilent Jet Stream	
Number of MRMs	65	
Dwell time	12 ms	
MS1 and MS2 resolution	Unit	

Results and Discussion

Method development

An UHPLC/MS/MS method for the determination of 23 PAs was developed. MRM transitions and conditions were optimized using the MassHunter Optimizer software in FIA mode and a MRM database containing all compounds with at least three transitions has been set up. The most abundant MRM transitions correspond to fragments with a mass of m/z 120 and m/z 138, which are diagnostic for retronecine type compounds.

Figure 2 shows the MRM traces of PAs in a calibration sample. Three MRM transitions have been acquired per compound to improve selectivity as matrix interferences in the plant material showed up on one or even two MRM transitions. The UHPLC separation provides improved chromatographic resolution of isomeric PAs; e.g. it achieves baseline separation of intermedine and lycopsamine as well as their N-oxides.

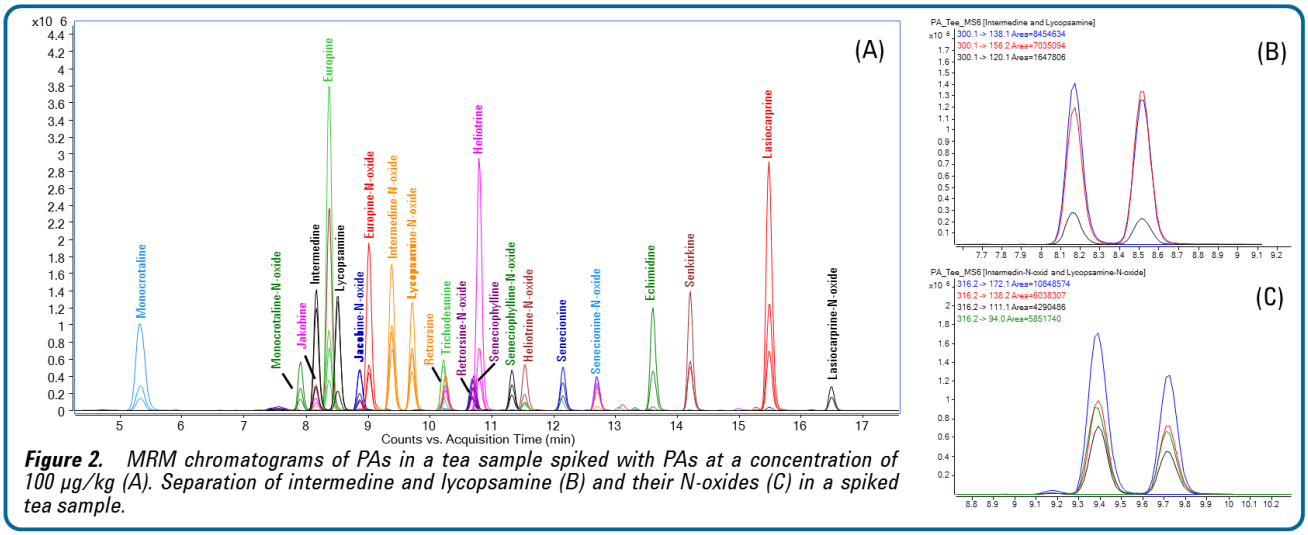


Figure 2. *MRM chromatograms of PAs in a tea sample spiked with PAs at a concentration of 100 µg/kg (A). Separation of intermedine and lycopsamine (B) and their N-oxides (C) in a spiked tea sample.*

Analysis of real world samples

The method was applied to tea and honey samples which were purchased from a local grocery. Figure 3 shows the chromatograms for several PAs in a rooibos tea and a honey sample. In total seven PAs were found in the rooibos and four PAs were found in the honey. Concentrations in these samples ranged between < 5 and 63 µg/kg in the tea and between 2 and 62 µg/kg in the honey. In some samples concentrations of several hundred µg/kg were observed.

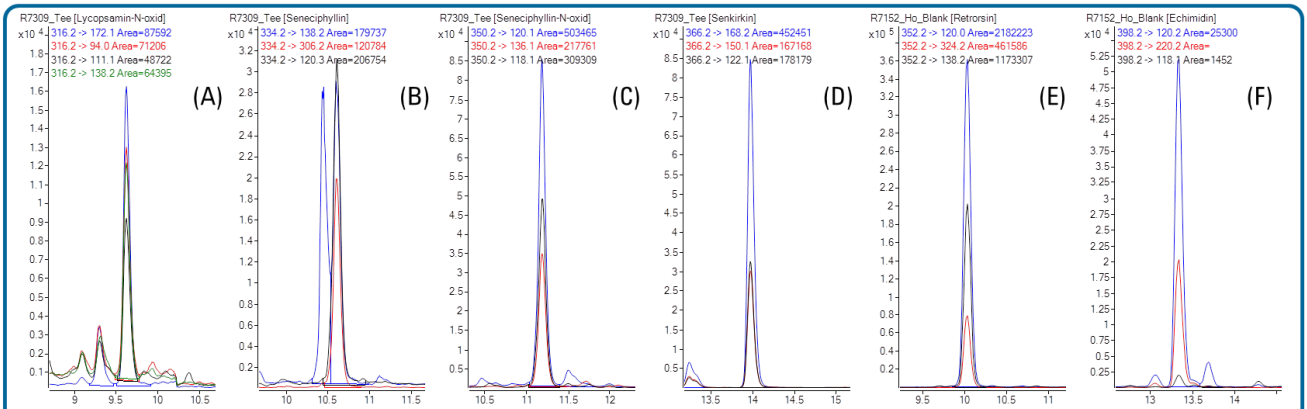
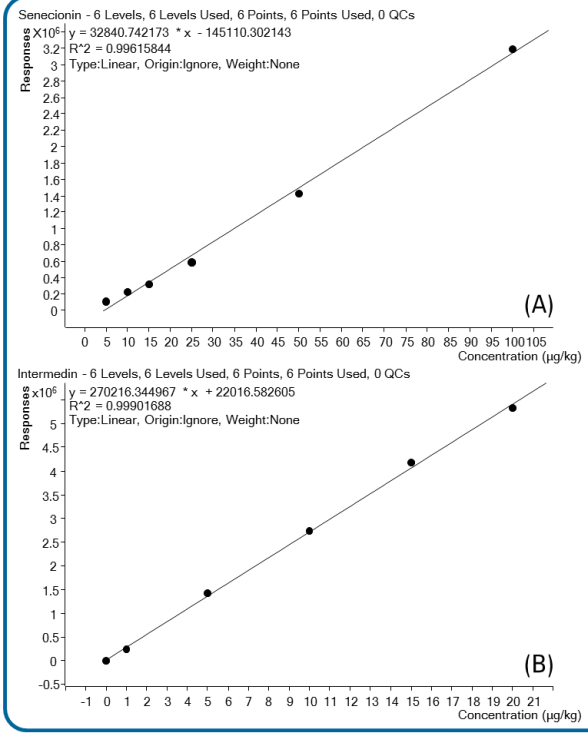


Figure 3. *PAs detected in a commercial tea (A to D) and honey samples (E and F): (A) lycopsamine-N-oxide (< 5 µg/kg), (B) seneciphyllin (11 µg/kg), (C) seneciphyllin-N-oxide (22 µg/kg), (D) senkirkine (9 µg/kg), (E) retrorsine (62 µg/kg), and (F) echimidine (8 µg/kg).*

Results and Discussion

Method performance characterization



Analyte	Tea			Honey		
	LOD (in µg/kg)	LOQ (in µg/kg)	Recovery (in %)	LOD (in µg/kg)	LOQ (in µg/kg)	Recovery (in %)
Echimidin	2.5	5	91 ± 9	0.5	1	123 ± 12
Europin	2.5	5	102 ± 8	0.5	1	n.a.
Europin-N-oxid	2.5	5	62 ± 24	0.5	1	n.a.
Heliotrin	2.5	5	105 ± 11	0.5	1	91 ± 16
Heliotrin-N-oxid	5	10	80 ± 23	0.5	1	75 ± 8
Intermedin	2.5	5	118 ± 8	0.5	1	93 ± 12
Jacobin	5	10	119 ± 9	0.5	1	n.a.
Lasiocarpin	2.5	5	85 ± 20	0.5	1	103 ± 12
Lasiocarpin-N-oxid	2.5	10	63 ± 25	0.5	1	76 ± 10
Monocrotalin	2.5	5	103 ± 11	0.5	1	96 ± 10
Monocrotalin-N-oxid	5	15	81 ± 17	0.5	1	50 ± 18
Retrorsin	5	10	101 ± 5	0.5	1	99 ± 14
Retrorsin-N-oxid	5	10	72 ± 26	0.5	1	41 ± 27
Senecionin	10	15	109 ± 17	0.5	1	111 ± 20
Senecionin-N-oxid	2.5	5	57 ± 26	0.5	1	49 ± 22
Seneciphyllin	2.5	5	105 ± 10	0.5	1	96 ± 15
Seneciphyllin-N-oxid	2.5	5	63 ± 30	0.5	1	34 ± 22
Senkirkin	2.5	5	83 ± 10	0.5	1	112 ± 9
Trichodesmin	5	10	98 ± 5	0.5	1	91 ± 17
Lycopsamin	5	10	101 ± 6	0.5	1	85 ± 12

Figure 4. *Matrix matched calibration for senecionine in herbal tea (A) and for intermedine in honey (B) as well as method performance characteristics for the analysis of PAs in tea and honey samples.*

Quantitation was based on matrix matched calibrations to compensate for matrix effects in the electrospray ionization. Linear calibration curves were observed for all PAs in the relevant concentration range of 5 to 100 µg/kg in tea and 1 to 20 µg/kg in honey. For validation honey and tea samples were spiked with the PAs before extraction at different concentrations and were analyzed with the described method. Calibration curves as well as method performance characteristics are shown in figure 4. Limits of quantification (LOQs) were in the sub or low µg/kg range for the evaluated PAs in the honey and slightly higher in the herbal teas as matrix effects were stronger in the complex plant matrices. Recoveries were generally between 70 and 120% except for the N-oxides, particularly in the honey matrix.

Conclusions

The presented method for the quantification of PAs in herbal teas and honey comprises an extraction with sulfuric acid, a clean-up using Bond Elut SCX and subsequent injection of the final extracts into the UHPLC/MS/MS system. The method benefits from the enhanced chromatographic resolution offered by the Poroshell 120 column and the outstanding sensitivity of the dual ion funnel triple quadrupole LC/MS system.

The method was successfully validated for herbal teas and honey. Extraction recoveries were generally between 70 and 120% except for the N-oxides in the honey samples. Limits of quantitation were in the low µg/kg range for honey and slightly higher for the more complex tea samples. The analysis of tea and honey samples from a local grocery revealed PA contaminations from a few µg/kg up to several hundred µg/kg. Some samples contained several of the targeted PAs simultaneously.