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Determination of pyrrolizidine alkaloids in plant material using on-line SPE coupled to UHPLC-MS/MS

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Sven Vedder[1], Anja Grüning[1], Julia Sander[1]
1 Shimadzu Europa, Albert-Hahn-Str. 6-10,
47269 Duisburg, Germany

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Introduction

Pyrrolizidine alkaloids (PAs) are secondary plant metabolites that are supposed to be carcinogenic and genotoxic. They occur mainly in plants of the Boraginaceae, Asteraceae and Fabaceae families. They contain a pyrrolizidine core and make up a large group of heterocyclic alkaloids mainly derived from the 4 Necin bases platynecine, retronecine, heliotridin and ontonecin. PAs are hepatotoxic if they carry a 1,2-double bond as well as an esterified side chain which is a structural prerequisite for their hepatic activation. Plant food and beverage, phytopharmaceuticals or even animal feed can easily be contaminated with PAs and enter the food chain. Currently there are discussions on possible regulatory measures caused by the presence of PAs in honey, tea, herbal infusions and food supplements. Existing methods include laborious sample preparation, e.g. solid-liquid extraction followed by solid phase extraction for clean-up. Here we report an on-line SPE UHPLC-MS/MS method, which overcomes the difficulties of combining low pressure online SPE with high pressure analytical UHPLC.

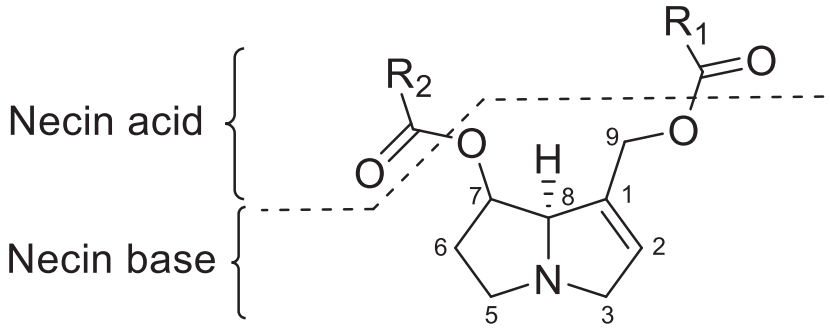

Figure 1 General structure of pyrrolizidine alkaloids

Methods and Materials

Sample Preparation

Tea samples were extracted twice with 0.05M sulfuric acid by sonication. Before centrifugation the pH of the combined extracts was adjusted with ammonium hydroxide.

UHPLC method

Instrument	:	Nexera UHPLC, Shimadzu
Column	:	Shim-pack XR-ODS III, 150 mm x 2.0 mm, 2.2 µm, Shimadzu
Mobile phase A	:	5 mM ammonium formate + 0.1% formic acid
B	:	methanol + 5 mM ammonium formate + 0.1% formic acid
Flow rate	:	0.4 mL/min
Time program	:	B conc. 1% (0-1.6 min) -50% (14.6 min) – 71.5% (18.1 min) – 95% (18.2 min – 20.2 min) -1% (20.3 min – 25 min)
Column temperature	:	30 °C

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Online SPE method

Column	:	EVOLUTE® EXPRESS ABN, 30 x 2.1 mm, Biotage
Mobile phase	:	5 mM ammonium formate + 0.1% formic acid for sample loading methanol / H2O + 5 mM ammonium formate + 0.1% formic acid methanol, isopropanol for washing of SPE column
Flow rate	:	0.2 / 2 mL/min
Injection vol.	:	50 µL
Column temperature	:	RT

MS conditions

Instrument	:	LCMS-8060, Shimadzu
Ionization	:	pos ESI
Nebulizing gas	:	3 L/min
Heating gas	:	15 L/min
Drying gas	:	5 L/min
Interface temperature	:	400 °C
DL temperature	:	300 °C
Heat block temperature	:	400 °C
CID gas	:	270 kPa
Interface voltage	:	1 kV

Result

Method development of the online SPE

The neutralized and centrifuged tea extract samples were put into the autosampler and transferred to the on-line SPE column using an aqueous solution. After washing the sample was eluted with only 10 µL solvent and trapped into a loop. By switching the loop the eluted sample was transferred to the analytical column. A binary gradient separated the PAs for quantification. Due to this hardware set-up UHPLC with high backpressure and on-line SPE which is pressure limited were successfully combined. By careful fine-tuning of the SPE elution and the chromatographic conditions the separation of critical peak pairs could be maintained.

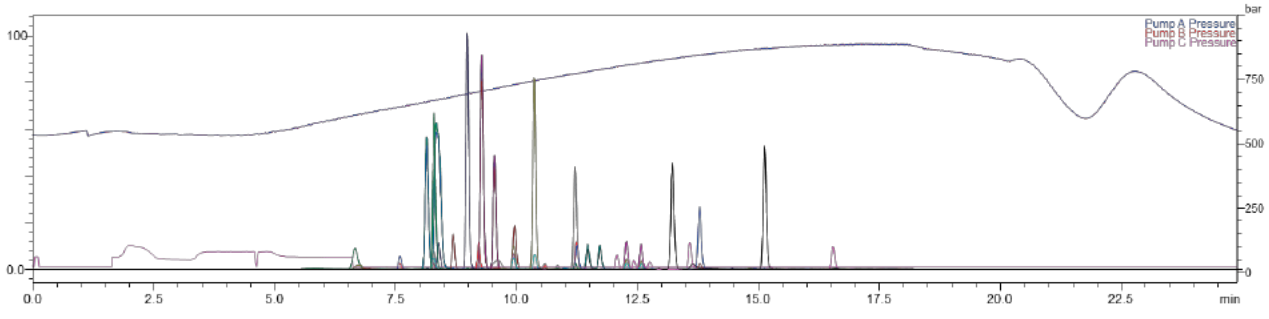

Figure 2 Typical chromatogram of pyrrolizidine alkaloids in tea matrix including the separated pressure curves of the analytical column (Pump A and B pressure) and the online SPE column (Pump C pressure)

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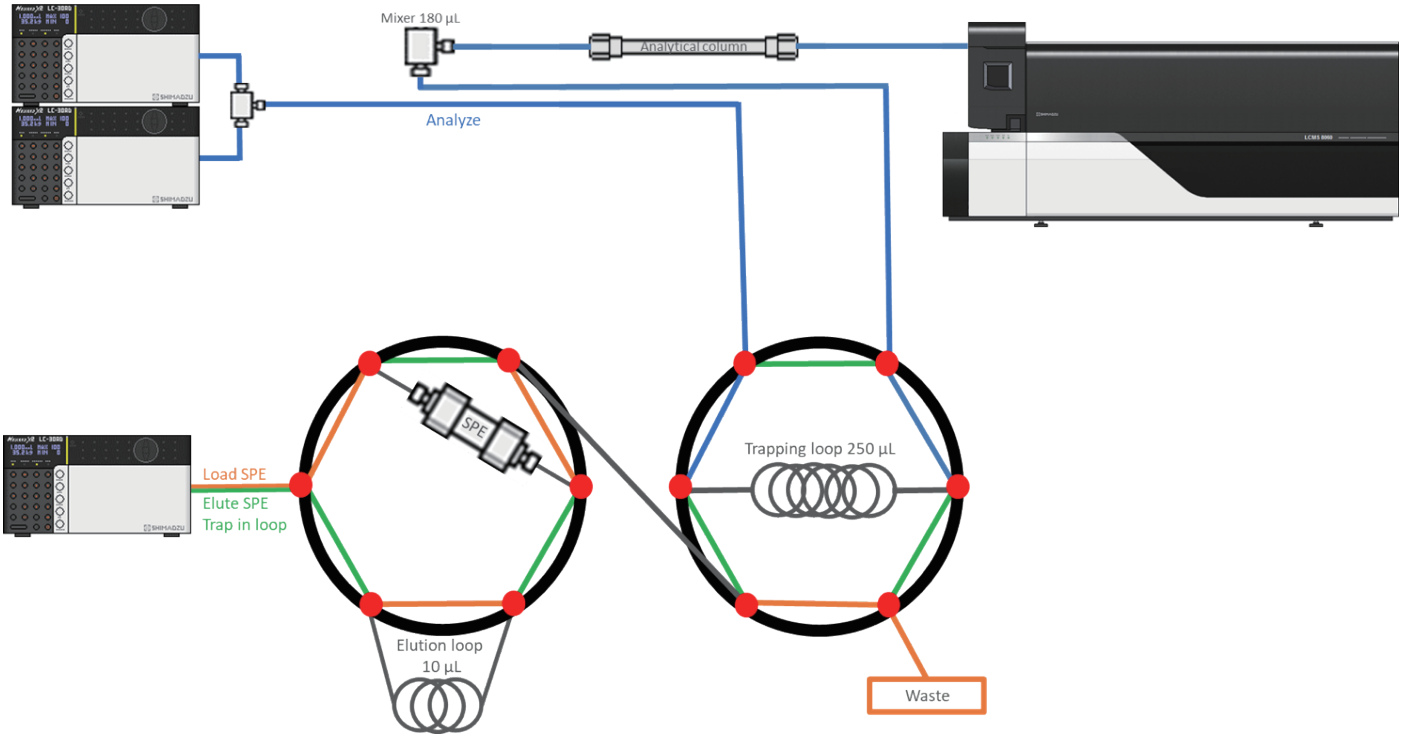

Figure 3 Setup of the on-line SPE analytical system

Quantitative Analysis of tea samples

By using the reported instrument set-up, analysis and thus the quantification of 16 PAs and 14 of their related N-Oxides could be performed. Calibration curves in different tea matrices (black tea, green tea and herbal tea) determined in duplicate showed good precision and accuracy and even in a complex matrix like tea we were able to easily quantify the PAs in at least the range of 10 to 400 µg/kg. This is comparable to the established methods using manual sample preparation. For all analytes, weighted regression resulting in r^2 0.99 could be achieved, with S/N >10 for LLOQ levels.

Exemplary calibration curves obtained for the 30 compounds are shown in Figure 4, Chromatograms of exemplary LLOQs are shown in Figure 5, the LLOQs which could be achieved in the different tea matrices are shown in Table 1.

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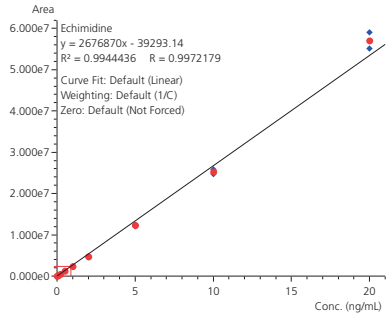
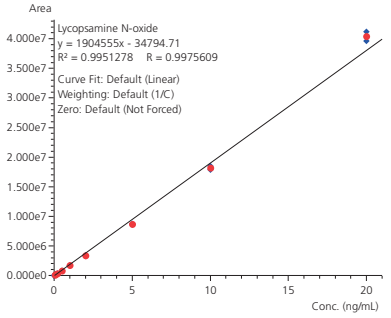
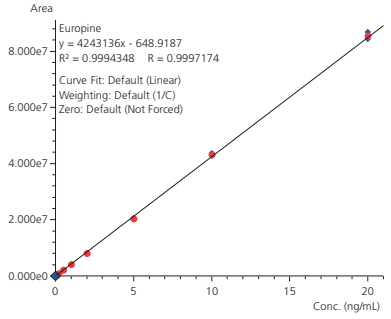


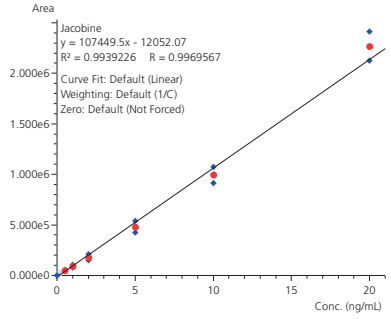
Calibration Curve Echimidine in herbal tea
0.05 - 20 ng/mL (1 – 400 µg/kg)



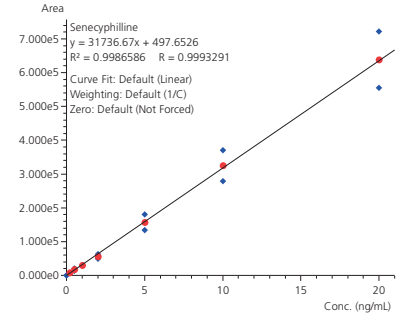
Calibration Curve Lycopsamine–N-oxide in green tea
0.05 - 20 ng/mL (1 – 400 µg/kg)



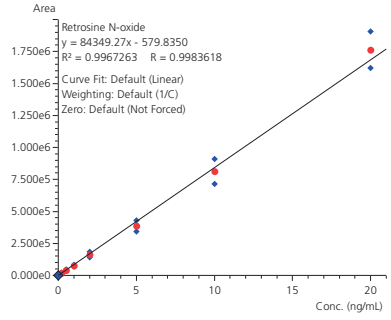
Calibration Curve Europine in black tea
0.05 - 20 ng/mL (1 – 400 µg/kg)



Calibration Curve Jacobine in herbal tea
0.5 - 20 ng/mL (10 – 400 µg/kg)

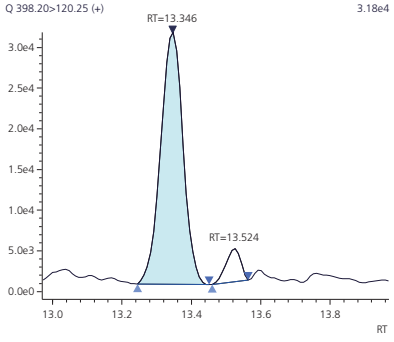


Calibration Curve Senecyphilline in green tea
0.2 - 20 ng/mL (4 – 400 µg/kg)

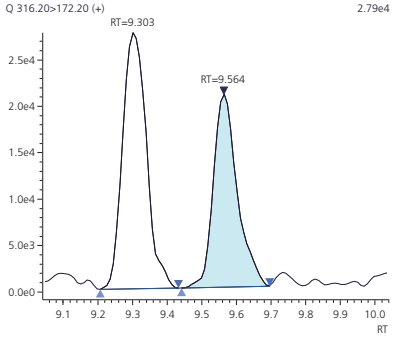


Calibration Curve Retrosine-N-oxide in black tea
0.2 - 20 ng/mL (4 – 400 µg/kg)

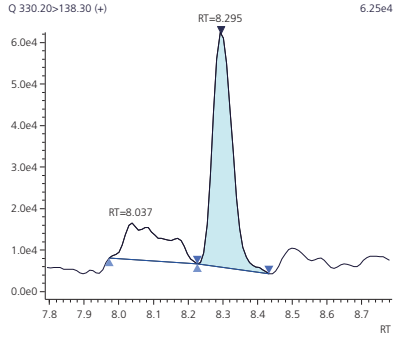
Figure 4 Exemplary calibration curves in different tea matrices



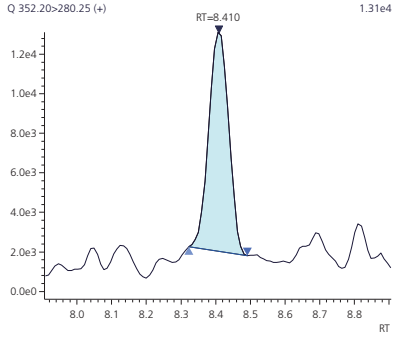
LLOQ of Echimidine in herbal tea / 1.0 µg/kg



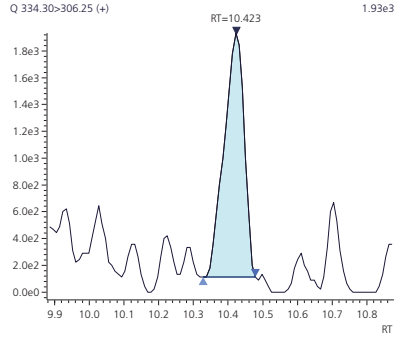
LLOQ of Lycopsamine-N-oxide in green tea / 1.0 µg/kg



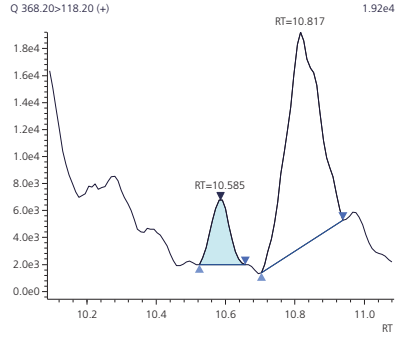
LLOQ of Europine in black tea / 1.0 µg/kg



LLOQ of Jacobine in herbal tea / 10 µg/kg



LLOQ of Senecyphilline in green tea / 4.0 µg/kg



LLOQ of Retrosine-N-oxide in black tea / 10µg/kg

Figure 5 Exemplary chromatograms of LLOQs different tea matrices

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Table 1 LLOQs of the pyrrolizidine alkaloids in different tea matices

	Black tea LLOQ		Green tea LLOQ		Herbal tea LLOQ	
	ng/mL	µg/kg	ng/mL	µg/kg	ng/mL	µg/kg
Echimidine	0.05	1.0	0.05	1.0	0.05	1.0
Echimidine-N-oxide	0.05	1.0	0.05	1.0	0.05	1.0
Erucifoline	0.20	4.0	0.20	4.0	0.50	10.0
Erucifoline-N-Oxide	0.10	2.0	0.20	4.0	0.10	2.0
Europine	0.05	1.0	0.05	1.0	0.05	1.0
Europine -N-Oxide	0.05	1.0	0.05	1.0	0.05	1.0
Heliotrine	0.05	1.0	0.05	1.0	0.05	1.0
Heliotrine N-oxide	0.05	1.0	0.05	1.0	0.05	1.0
Intermedine	0.05	1.0	0.05	1.0	0.05	1.0
Intermedine N-oxide / Indicine N-oxide	N.A	N.A	0.05	1.0	0.05	1.0
Jacobine	0.20	4.0	0.50	10.0	0.50	10.0
Jacobine N-oxide	0.05	1.0	0.10	2.0	0.10	2.0
Lasiocarpine	0.05	1.0	0.10	2.0	0.05	1.0
Lasiocarpine N-oxide	0.10	2.0	0.10	2.0	0.05	1.0
Lycopsamine / Indicine	N.A	N.A	0.05	1.0	0.05	1.0
Lycopsamine N-oxid	0.05	1.0	0.05	1.0	0.05	1.0
Monocrotaline	0.50	10.0	0.20	4.0	0.50	10.0
Monocrotaline-N-oxide	0.10	2.0	0.10	2.0	0.10	2.0
Retrosine	0.10	2.0	0.05	1.0	0.10	2.0
Retrosine N-oxide	0.20	4.0	0.50	10.0	0.50	10.0
Senecionine	0.05	1.0	0.50	10.0	0.50	10.0
Senecionine N-oxide	0.05	1.0	0.20	4.0	0.50	10.0
Senecyphilline	0.10	2.0	0.20	4.0	0.50	10.0
Senecyphilline N-oxide	0.05	1.0	0.20	4.0	0.10	2.0
Senecivernine	0.20	4.0	0.50	10.0	0.20	4.0
Senecivernine N-oxide	0.05	1.0	0.20	4.0	0.10	2.0
Senkirkine	0.05	1.0	0.05	1.0	0.05	1.0
Trichodesmine	0.05	1.0	0.10	2.0	0.20	4.0

A total of 29 commercially available tea samples were analyzed. Among these samples there were 6 samples of green tea, 10 samples of black tea and 13 samples of herbal tea. In 59% of all analyzed tea samples one or more of the pyrrolizidine alkaloids could be detected above their LLOQ. 3 out of 6 green tea samples, 5 out of 10 black tea samples and 9 out of 13 herbal tea samples where contaminated with pyrollizidine alkaloids.

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Conclusions

An on-line SPE method for high-sensitivity analysis was successfully developed for PA analysis in plant material. The manual sample preparation could be reduced to a minimum as the set up of on-line SPE followed by UHPLC-MS/MS saves additional clean-up steps without compromising the performance of the assay.

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