Bundesinstitut für Risikobewertung

Determination of pyrrolizidine alkaloids (PA) in plant material by SPE-LC-MS/MS

Method Protocol

BfR-PA-Tea-2.0/2014

CONTENT

1 Scope

Pyrrolizidine alkaloids (PA) are secondary plant metabolites with carcinogenic and genotoxic properties. Currently, more than 600 PA are known. They occur in plants of families of Boraginaceae, Asteraceae and Fabaceae. The worldwide spread of these plants may lead to a contamination of herbal foodstuff, herbal medicines and animal feed (EFSA Panel on Contaminants in the Food Chain (CONTAM) 2011).

This method describes the determination of the following PA in plant material: echimidine (Em), echimidine-N-oxide (EmN), erucifoline (Er), erucifoline-N-oxide (ErN), europine (Eu), europine-N-oxide (EuN), heliotrine (Hn), heliotrine-N-oxide (HnN), intermedine (Im), intermedine-N-oxide (ImN), jacobine (Jb), jacobine-N-oxide (JbN), lasiocarpine (Lc), lasiocarpine-N-oxide (LcN), lycopsamine (La), lycopsamine-N-oxide (LaN), monocrotaline (Mc), monocrotaline-N-oxide (McN), retrorsine (Re), retrorsine-N-oxide (ReN), senecionine (Sc), senecionine-N-oxide (ScN), seneciphylline (Sp), seneciphylline-N-oxide (SpN), senecivernine (Sv), senecivernine-N-oxide (SvN), senkirkine (Sk), trichodesmine (Td).

The limits of determination and quantification for toxins determined during in-house validation are listed in the Annex 8.1.

2 Principle

A test portion of plant material is sonicated twofold in aqueous sulphuric acid solution for PA extraction. After centrifugation an aliquot of the supernatant is purified by solid-phase extraction (SPE) using reversed phase C18 material. PAs are released from the cartridge using methanol. Subsequently, the eluate is evaporated to dryness and reconstituted in methanol/water (initial HPLC conditions).

For chromatographic separation, an RP-HPLC column is used with a binary gradient. Analytes are detected by triple stage quadrupole mass spectrometry. Quantification of pyrrolizidine alkaloids is accomplished by means of matrix matched calibration.

3 Reagents

3.1 General

Please note: Since the use of this method involves reagents harmful to health, appropriate precautionary and protective measures such as avoiding skin contact and using an extractor hood must be taken.
If not specified elsewise, reagents of analytical grade and solvents suitable for HPLC-MS/MS must be used. Water must be distilled in glass vessels or demineralised before use, or must be of equivalent purity.

3.2 Chemicals

3.2.1 echimidine (Em)
3.2.2 echimidine-N-oxide (EmN)
3.2.3 erucifoline (Er)
3.2.4 erucifoline-N-oxide (ErN)
3.2.5 europine (Eu)
3.2.6 europine-N-oxide (EuN)
3.2.7 heliotrine (Hn)
3.2.8 heliotrine-N-oxide (HnN)
3.2.9 intermedine (Im)
3.2.10 intermedine-N-oxide (ImN)
3.2.11 jacobine (Jb)
3.2.12 jacobine-N-oxide (JbN)
3.2.13 lasiocarpine (Lc)
3.2.14 lasiocarpine-N-oxide (LcN)
3.2.15 lycopsamine (La)
3.2.16 lycopsamine-N-oxide (LaN)
3.2.17 monocrotaline (Mc)
3.2.18 monocrotaline-N-oxide (McN)
3.2.19 retrorsine (Re)
3.2.20 retrorsine-N-oxide (ReN)
3.2.21 senecionine (Sc)
3.2.22 senecionine-N-oxide (ScN)
3.2.23 seneciphylline (Sp)
3.2.24 seneciphylline-N-oxide (SpN)
3.2.25 senecivernine (Sv)
3.2.26 senecivernine-N-oxide (SvN)
3.2.27 senkirkine (Sk)
3.2.28 trichodesmine (Td)
3.2.29 formic acid 98 – 100%, e.g. Sigma-Aldrich
3.2.30 methanol (MeOH) in LC-MS quality, e.g. Merck LiChrosolv®
3.2.31 sulphuric acid 98%, e.g. Merck

3.2.32 ammonia 32%, e.g. Merck

3.2.33 ammonium formate in LC-MS quality, e.g. Fluka

3.2.34 acetonitrile, e.g. Merck LiChrosolv®

3.3 Solutions

3.3.1 Extraction solution

2.665 mL of sulphuric acid (H_2SO_4) (3.2.31) are filled up to 1000 mL with water. The final concentration is 0.05 M.

3.3.2 Aqueous ammoniacal solution for neutralisation

To prepare the ammonical solution for neutralisation of sample extracts before SPE, 5 mL of ammonia (3.2.32) are filled up to 25 mL with water.

3.3.3 2.5 % ammonia in methanol for SPE elution (SPE elution for black tea and green tea)

To prepare the 2.5 % ammonia solution in methanol 7.8 mL of ammonia (3.2.32) are filled up to 100 mL with methanol (3.2.30). The solution has to be freshly prepared per working day.

3.3.4 HPLC mobile phase

Eluent A:
315 mg ammonium formate (3.2.33) are dissolved in 5 mL of water, 1 mL of formic acid (3.2.29) is added and filled up to 1000 mL with water.

Eluent B:
315 mg ammonium formate (3.2.33) are dissolved in 5 mL of water, 1 mL of formic acid (3.2.29) is added and filled up to 1000 mL with methanol (3.2.30).

3.3.5 Standard solution for calibration

Stock solution (0.1 mg/mL):
To create a stock solution, 1 mg of a pyrrolizidine alkaloid standards are weighed using analytical balance (4.4) and filled up with acetonitrile (3.2.34) in a volumetric flask to make 10 mL. The concentration of the stock solution is 0.1 mg/mL.

Standard working solution (PA mixture, 1 µg/mL)
For preparation of the standard working solution, respective volumes of each PA stock solution (0.1 mg/mL) are pipetted into a volumetric flask and is filled up with acetonitrile (3.2.34), to obtain a concentration of 1 µg/mL.

Preparation of matrix matched standards (MMS)
For a correction of matrix effects a matrix matched calibration is used. In order to obtain the same matrix effect for MMS and for the samples the blank plant material has to be processed as described in section 5. Afterwards, MMS levels have to be prepared according to table 1.

Please note: In order to prepare of a sufficient amount of blank-extract, two times 10 mL of neutralised blank-extract (5.2) from one blank sample can be purified using two SPE-cartridges. Thereby, 2 mL of reconstituted blank-extract for the MMS preparation can be received without extraction of an additional blank sample.

table 1: Matrix matched standards

	Final PA mass concentration in calibration solution ng/mL	**Final PA mass concentration** µg/kg	**Aliquot taken from**	**Aliquot Volume** µL	**Aliquot taken from blank plant material extract** µL
MMS_1	**5,0**	**10,0**	MMS_5	20	280
MMS_2	**10,0**	**20,0**	MMS_8	20	280
MMS_3	**25,0**	**50,0**	MMS_8	20	100
MMS_4	**50,0**	**100,0**	PA-Mix	10	100
MMS_5	**75,0**	**150,0**	PA-Mix	15	185
MMS_6	**100.0**	**200,0**	PA-Mix	20	180
MMS_7	**125,0**	**250,0**	PA-Mix	25	175
MMS_8	**150,0**	**300,0**	PA-Mix	60	340

4 Apparatus

4.1 General

Usual laboratory glassware and equipment should be used and, in particular, the following:

4.2 **centrifugal mill** with 0.5 mm sieve, e.g. Retsch

4.3 **various piston pipettes and multiple dispensers,** e.g. Brand

4.4 **analytical balance,** capable of weighing to 0,0001 g

4.5 **centrifuge** for 50 mL centrifuge tubes, capable of at least 5 000 x g

4.6 **ultrasonic bath**

4.7 **overhead shaker,** e.g. Heidolph

4.8 **laboratory shaker,** e.g. Vortex

4.9 **evaporation station**, e.g. TurboVap

4.10 **centrifuge tube** 50 mL

4.11 **test tubes** 15 mL

4.12 **volumetric flasks,** 10 and 20 mL

4.13 **folded filters,** e.g. Munktell

4.14 **SPE cartridges**: DSC-C18 SPE (Supelco), 500 mg sorbent material, 6mL

4.15 **SPE vacuum chamber**

4.16 **Membrane filter** 0.2 µm, e.g. VWR 0,5 mL centrifugal filters, modified nylon membrane

4.17 **HPLC vials** 2 mL

4.18 **Glass inserts,** 250 µL conic for HPLC vials

4.19 **chromatographic column,** e.g. Thermo, Hypersil Gold®; 150 x 2.1 mm; 1,9 µm

4.20 **LC-MS/MS system**

5 Procedure

5.1 Sample preparation (grinding of plant material)

To determine the PA-content which is representative for the entire sample, the plant material should meet the following characteristics: uniform particle size and a homogenous distribution of PA or PA-containing material, respectively. Therefore, the entire sample material is mixed with dry ice (ratio 2:1), ground to a particle size of 0.5 mm (4.2) and homogenized for example by shaking over head (4.7). As an alternative to grinding with dry ice (excellent grinding results due to shear forces and porosity of the frozen sample material), the sample material may also be ground to a particle size of 0.25 mm if there is no considerably generation of heat.

If the test material can neither be ground with dry ice nor ground to a particle size of 0.25 mm, it is also possible to increase the weighed sample amount in contrast to 5.2 to at least 10 g (particle size 0.5 mm). In order to keep a constant ratio of sample amount to extraction volume, the used volume of extraction solution (3.3.1) needs to be increased by the same factor as the weighed sample amount.

5.2 Extraction

For the extraction of PA 2.0 g ± 0.1 g of plant are weighed into a centrifuge tube (4.10).

Extraction step 1	For the first extraction step 20 mL of the extraction solution (3.3.1) are added to the sample. The sample material has to be wetted completely before extraction in an ultrasonic bath (4.6) for 15 min at ambient temperature.
Centrifugation	The sample is centrifuged for 10 min ± 2 min at 3800 x g (4.5). The supernatant (extract 1) is transferred into a clean test tube. The sediment is used for the second extraction step.
Extraction step 2	Next, 20 mL of extraction solution (3.3.1) are added to the already extracted sample. The centrifuge tube is shaken vigorously to distribute the sample (the sample can also be stirred if necessary). The sample is again extracted in the ultrasonic bath for 15 min at ambient temperature.
Centrifugation	The sample is centrifuged applying the above mentioned conditions. The supernatant is added to the first extract.
Neutralisation	The combined extracts are set to pH 7 using the neutralisation solution (3.3.2). Control of the pH value is accomplished using indicator strips. Usually, about 500 µL to 1000 µL of the solution are needed.

The complete neutralised extract is passed through a folded filter (4.13). An aliquot of the filtrate is used for SPE. The filtration step prior to SPE can be repeated in case of larger quantities of remaining particles in the solution. Thereby, blockage of SPE cartridges can be avoided.

5.3 SPE-procedure

The Solid Phase Extraction (4.14) is carried out using a vacuum chamber (4.15).

Conditioning step 1	5 mL of methanol (3.2.30)
Conditioning step 2	5 mL of water
Sample load	10 mL sample (filtrate of neutralised extract)
Washing step	2 x 5 mL of water
Drying of cartridges	5 - 10 min (use the vacuum chamber (4.15))
Elution of PA	2 x 5 mL methanol (3.2.30) or in case of green and black tea 2 x 5 mL 2.5 % ammonia in methanol (3.3.3)

The eluate is dried under a nitrogen stream at 50 °C ± 5 °C.

5.4 Reconstitution of the sample

The residue is dissolved in 1 mL of methanol/water (5/95, v/v) by shaking (4.8).

The reconstituted sample extracts are filtered through 0.2 µm membrane filters (4.16). When using centrifugal filters, 500 µL of the sample are centrifuged at 20 000 x g for 10 min ± 3 min. 200 µL of the filtrate are transferred into an HPLC vial (4.17) with a glass insert (4.18).

6 HPLC-MS/MS analysis

6.1 Liquid chromatographic separation

The measurements can be carried out with different high-performance liquid chromatographs (HPLC) and separation columns. The chromatographic conditions can be chosen freely. The acceptable minimum retention time is twice the retention time for the dead volume of the column. Analytes which cannot be distinguished by means of mass spectrometry must be separated chromatographically. The conditions listed in the annex 8.1 using a C18-column (4.19) and the mobile phase described in 3.3.4 have shown to be suitable in pre-trials. However, they are to be seen as examples only.

6.2 Mass spectrometric operation conditions

The measurements can be carried out with MS/MS devices of different manufacturers. In the annex 8.1, the device-specific settings of one measuring system are given as an example. These conditions have shown to be suitable in pre-trials.

Please note: For the qualitative detection and for quantification, it is necessary to detect and report two substance-specific transitions per analyte.

6.3 Measurement

For a quantitative analysis, the following criteria are defined.

Injection:
Samples and standards are injected in duplicate in order to assess repeatability of MS detection and to check for possible response drift during the sequence.

Sequence
To determine pyrrolizidine alkaloids, the following array of analysis is defined in a sequence.

1. Matrix matched standards (5 – 150 ng/mL)
2. Solvent blank
3. Samples (first injection)
4. Solvent blank
5. Matrix matched standards (5 – 150 ng/mL)
6. Solvent blank
7. Samples (second injection)

7 Calculation

The quantitative determination is performed according to the method of the matrix matched standard by integration of the peak areas in relation to the calibration line.

7.1 Calibration function

Equation1: Calibration function

$$f_{(x)} = y = ax + b$$

where

y	is the peak area of the target analyte
a	is the slope of the calibration function
x	is the concentration of the target analyte [ng/mL] in the MMS
b	is the intercept of the calibration function

7.2 Quantification

Equation2: Calculation of the PA content (analysis equation)

$$PA\ concentration\ = \beta\ x\ DF\ = \left[(y-b)\ x\ \frac{1}{a} \right] x\ \frac{V_{Extract}}{m_{weight}}\ x \frac{1}{V_{Application}}\ x\ V_{sample}$$

where

β	is the analyte concentration [ng/mL] in the sample extract
DF	is the conversion factor from ng/mL to µg/kg
y	is the peak area of the target analyte
a	is the axis intercept from the matrix calibration
a	is the increase from the matrix calibration
V_{Extract}	is the volume of extraction agent [mL]
$m_{\text{Weight-in quantity}}$	is the sample weight in [g]
$V_{\text{Application}}$	is the volume of the extract applied for SPE [mL]
V_{Sample}	is the final sample volume [mL]

7.3 Reporting of results

The results are reported in µg/kg with two significant decimals. To convert the concentration from ng/mL injected solution to µg/kg plant material a factor of 2 is used according to the sample preparation procedure described in chapter 5.

Reference list

DIN ISO 32645. (1994) Chemical Analsysis; Decision limit, Detection limit and determination limit, Estimation in case of repeatability, terms, methods, evaluation. Deutsches Institut für Normung DIN.

EFSA Panel on Contaminants in the Food Chain (CONTAM). (2011) Scientific Opinion on Pyrrolizidine alkaloids in food and feed. The EFSA Journal 9, 1-135

8 Annex

8.1 LC-MS/MS measurement

LC-MS/MS system consisting of

Triple quadrupole mass spectrometer (Thermo TSQ Vantage)

HPLC system — HPLC pump (e. g. Thermo Accela 1250),
Degasser
Autosampler (e. g. CTC Analytics PAL ATS MYX)
Column oven (e. g. MayLab MistraSwitch)

HPLC settings

Eluent A	refer to (3.3.4)
Eluent B	refer to (3.3.4)
Column temperature	40 °C
Flow rate	300 µL/min
Injection volume	10 µL
Column	e. g. Thermo Hypersil Gold; 150 x 2.1 mm, 1.9 µm
Total runtime	15 minutes

Gradient

Time (min)	% A	% B
0.0	95	5
0.5	95	5
7.0	50	50
7.5	20	80
7.6	0	100
9.0	0	100
9.1	95	5
15.0	95	5

MS settings

Ionisation	Electrospray positive (ESI +)
Ion spray voltage [V]	3500 (positive polarity)
Capillary temperature [°C]	270
Vaporiser temperature [°C]	300
Sheath gas pressure [psi]	45.0
Ion sweep gas pressure [psi]	2.0
Aux gas pressure [psi]	10

Substance-specific parameters

The analytes are detected by Selected Reaction Monitoring (SRM). For analyte identification, two PA specific transitions to two product ions are chosen. The relevant transitions and the collision energy (CE) can be found in .
table **2**. The table also lists the retention time per analyte which apply for the HPLC settings described above.

table 2: Substance-specific parameters of the LC-MS/MS method

analyte	precursor	fragment	CE	S Lens	retention time [min]
Mc	326.2	120.3	35	130	4.25
		237.3	25	130	
McN	342.1	118.3	37	141	4.99
		137.4	29	141	
ErN	366.1	136.1	30	129	5.16
		120.1	33	129	
Jb	352.1	120.1	36	110	5.25
		155.2	29	120	
Eu	330.1	138.1	20	89	5.34
		156.2	28	89	
Im	300.1	138.3	18	112	5.40
		156.3	28	112	
JbN	368.1	120.1	32	110	5.51
		296.1	23	110	
La	300.1	138.3	18	112	5.53
		156.3	28	112	
EuN	346.1	111.2	41	91	5.63
		172.1	31	91	
ImN	316.1	111.2	37	95	5.91
		138.1	26	95	
LaN	316.1	111.2	37	95	6.02
		138.1	26	95	
Td	354.2	120.3	35	137	6.37
		222.3	28	137	
ReN	368.2	136.2	30	145	6.41
		118.2	40	145	
Sp	334.2	120.3	26	138	6.56
		138.4	28	138	
Hn	314.2	138.3	19	119	6.72
		156.3	28	119	
Er	350.2	120.3	32	110	4.87
		138.1	30	110	
SpN	350.2	118.2	36	135	6.79
		136.3	32	135	
HnN	330.2	138.2	22	121	7.03
		172.1	27	121	
Sv	336.2	120.1	27	135	7.26
		138.1	27	135	
Sc	336.2	120.2	27	130	7.33
		138.2	29	130	
SvN	352.1	118.1	30	110	7.42
		120.1	36	110	
Re	352.2	120.3	27	140	7.54
		138.3	29	140	

analyte	precursor	fragment	CE	S Lens	retention time [min]
ScN	352.2	118.1	28	116	7.54
		136.3	27	116	
EmN	414.2	254.1	32	129	8.01
		352.1	27	129	
Em	398.2	120.3	23	139	8.02
		220.3	17	139	
Sk	366.2	150.3	24	132	8.19
		168.2	28	132	
Lc	412.2	120.2	30	139	8.99
		336.3	17	139	
LcN	428.2	136.1	29	135	9.33
		254.1	27	135	

table 3: Limits of detection (LOD) and limits of quantification (LOQ) determined during in-house validation of the described method*

analyte	LOD [µg/kg]	LOQ [µg/kg]
Mc	0,9	2,8
McN	1,7	5,4
ErN	1,2	3,8
Jb	1,3	4,0
Eu	0,7	2,1
Im	1,0	3,1
JbN	1,3	4,2
La	2,0	6,4
EuN	0,7	2,3
ImN	1,2	3,8
LaN	1,5	4,9
Td	1,0	3,1
ReN	1,4	4,6
Sp	1,3	4,0
Hn	0,5	1,7
Er	0,6	1,9
SpN	0,9	2,7
HnN	0,6	2,0
Sv	1,7	5,3
Sc	1,8	5,9
SvN	0,8	2,6
Re	0,8	2,7
ScN	0,9	2,9
EmN	1,9	6,1
Em	0,8	2,6
Sk	0,8	2,4
Lc	0,8	2,4
LcN	0,9	2,8

* LOD and LOQ were determined according to DIN EN ISO 32645 Calibration method (DIN ISO 32645 1994)

8.2 Typical Chromatogram

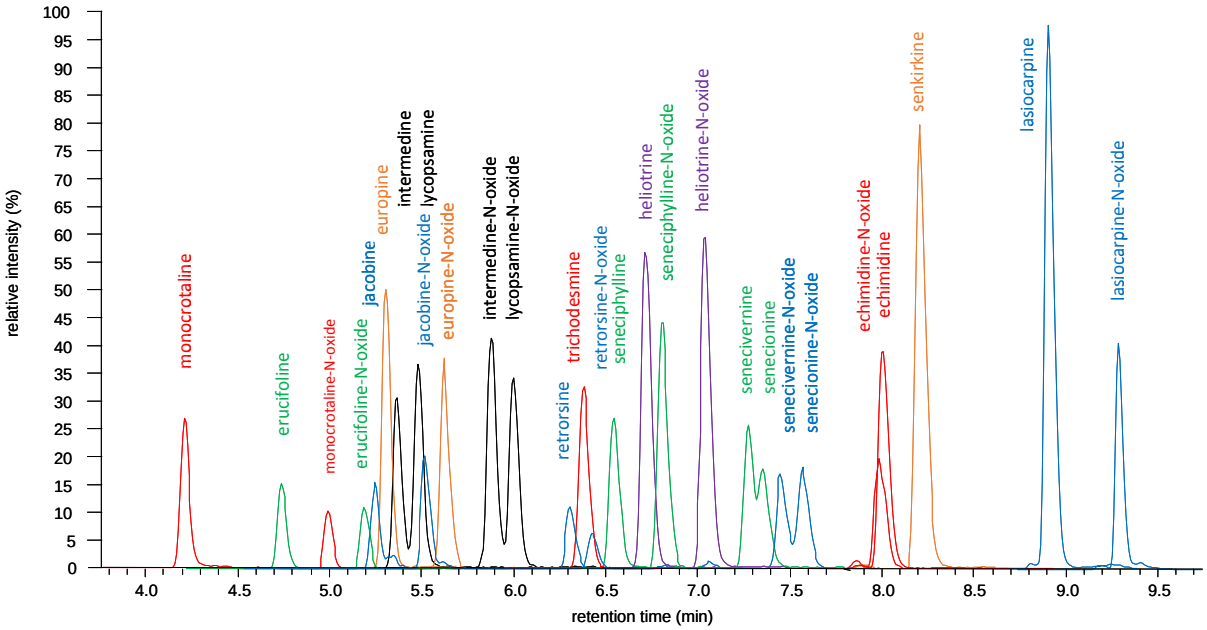

Figure 1: Typical chromatogram of a PA standard mixture (1 ng/mL), TIC of SRM-transitions

8.3 Provider of PA-Standards

pyrrolizidine alkaloid	mass	CAS	provider	order code
echimidine	397,47	520-68-3	Oskar Tropitzsch PhytoLab* PlantaAnalytica	7550006 89553 -
erucifoline	349.38	40158-95-0	Carl Roth Oskar Tropitzsch PhytoLab*	1657.1 7550021 83446
erucifoline-N-oxide	365,37	123864-94-8	Carl Roth PhytoLab*	1664.1 83434
europine-hydrochloride	365,86	570-19-4	Carl Roth PhytoLab*	1676.1 83237
europine-N-oxide	345,39	65582-53-8	AppliChem Carl Roth Oskar Tropitzsch PhytoLab*	A9574,0010 1687.1 7500063 83238
heliotrine	313,40	303-33-3	AppliChem Carl Roth Latoxan* Oskar Tropitzsch PhytoLab	A9583,0020 1929.1 L6007 7550511 80403
heliotrin-N-oxide	329,39	6209-65-0	AppliChem Carl Roth Oskar Tropitzsch* PhytoLab	A9590,0010 1944.1 755054 83236
indicine-hydrochloride	335,83	1195140-94-3	Carl Roth Oskar Tropitzsch PhytoLab	1960.1 7500069 83234
indicine-N-oxide	315,36	41708-76-3	AppliChem Carl Roth Oskar Tropitzsch PhytoLab	A9593,0010 1961.1 7500070 83235
intermedine	299,37	10285-06-0	Carl Roth Oskar Tropitzsch PhytoLab*	1962.1 7501610 82424
intermedine-N-oxide	315,36	95462-14-9	PhytoLab*	83434
lasiocarpine	411,49	303-34-4	AppliChem Carl Roth Oskar Tropitzsch* PhytoLab	A9596,0010 2090.1 7500019 80412
lasiocarpine-N-oxide	457,5	127-30-0	AppliChem Carl Roth Oskar Tropitzsch* PhytoLab	A9600,0010 2202.1 7501284 83220
lycopsamine	299,37	10285-07-1	Carl Roth Oskar Tropitzsch PhytoLab*	2208.1 7501080 89726
lycopsamine-N-oxide	315,36	95462-15-0	Oskar Tropitzsch PhytoLab*	7501358 83447

pyrrolizidine alkaloid	mass	CAS	provider	order code
monocrotaline	325,35	315-22-0	Carl Roth	3418.1
			Fluka	37024
			Sigma	C2401
			Oskar Tropitzsch	7550522
			PhytoLab*	89251
			R&D Chemicals	7351
			Santa Cruz Biotechnology	sc-211921
monocrotaline-N-oxide	341,36	35337-98-5	Carl Roth	2249.1
			Oskar Tropitzsch	7501658
			PhytoLab*	82629
retrorsine	351,40	480-54-6	AppliChem	A4922,0020
			Carl Roth	1213.1
			Fluka	37025
			Oskar Tropitzsch	7550659
			PhytoLab	89775
			Santa Cruz Biotechnolog	sc-215805
			Sigma*	R0382
retrorsine-N-oxide	367,40	15503-86-3	AppliChem	A8668,0010
			Carl Roth	6733.1
			Oskar Tropitzsch	7500347
			PhytoLab*	82630
senecionine	335,40	130-01-8	AppliChem	A2071,0020
			Carl Roth*	2261.1
			Fluka	37031
			Oskar Tropitzsch	7550292
			PhytoLab*	89789
			R&D Chemicals	1828
			Sigma	17806
			Santa Cruz Biotechnology	sc-286770
senecionine-N-oxide	351,40	13268-67-2	AppliChem	A8678,0010
			Carl Roth	6734.1
			Oskar Tropitzsch	7500301
			PhytoLab*	82631
seneciphylline	333,39	480-81-9	AppliChem	A2072,0020
			Carl Roth*	6414.1
			Fluka	37033
			R&D Chemicals	1850
			Santa Cruz Biotechnology Inc.	sc-229697
			ABCR GmbH	AB167974
			PhytoLab*	89275
seneciphylline-N-oxide	349,38	38710-26-8	AppliChem	A8684,0010
			Carl Roth	6735.1
			Oskar Tropitzsch	7500573
			PhytoLab*	82632
senecivernine	335.40	72755-25-0	Carl Roth	2209.1
			Oskar Tropitzsch	7550066
			PhytoLab*	83436

pyrrolizidine alkaloid	mass	CAS	provider	order code
senecivernine-N-oxide	351,39	101687-28-9	Carl Roth	2215.1
			PhytoLab*	83437
senkirkine	365,43	2318-18-5	AppliChem	A6765,0010
			Carl Roth	4934.1
			Fluka	37032
			Oskar Tropitzsch	7500441
			PhytoLab*	89274
trichodesmine	353,41	548-90-3	Latoxan*	L6049

* substances were used for the in-house validation by BfR

8.4 Flow chart of the sample preparation procedure

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2 (± 0.1) g plant material

extraction

- add 20 mL 0.05 M H_2SO_4
- 1st extraction (ultrasonic bath, 15 min, RT)
  → centrifugation (10 min, 3800 x g), supernatant in a 50 mL vessel
- 2nd extraction (ultrasonic bath, 15 min, RT)
  → centrifugation (10 min, 3800 x g)
  combine both supernantants, adjust pH value to 6-7, filtration
  10 ml of the solution is used for the SPE

apparatus
ultrasonic bath; centrifuge; 50 ml vessel, 10ml pipette, filter

solvent
0.05 M H_2SO_4 – solution, NH_3-solution

plant extract for the SPE

SPE - program

- conditioning step 1: 5 mL of methanol
- conditioning step 2: 5 mL of water
- sample load: 10 mL of sample
- wash step: 2 x 5 mL of water

dry the cartridge for 5 – 10 min under vacuum

- elution: 2 x 5 mL of methanol or
  in case of black/green tea: 2 x 5 mL of 2.5% ammonia in methanol

apparatus
SPE – vacuum chamber

materials
DSC-C18 SPE cartridge; 500 mg

solvent
methanol; water; 2.5% ammonia in methanol

SPE - eluate

solvent exchange

- evaporate the sample to dryness (nitrogen stream)

apparatus
evaporator

residue

reconstitution

- add 1 mL methanol/wasser (5/95, v/v) to the residue
  dissolve the sample
- filtration

apparatus
HPLC-vials; membrane filter (0.2 µm)

solvent
methanol/water (5/95, v/v)

LC-MS/MS

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