Bundesinstitut für Risikobewertung

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

Method Protocol

BfR-PA-Honey-1.0/2013

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 the families of Boraginaceae, Asteraceae and Fabaceae. The pollen of these plants are a potential source for pyrrolizidine alkaloids and may lead to a contamination of honey (EFSA Panel on Contaminants in the Food Chain (CONTAM) 2011).

This method describes the determination of the following PA in honey: senecionine (Sc), senecionine-N-oxide (ScN), seneciphylline (Sp), seneciphylline-N-oxide (SpN), monocrotaline (Mc), monocrotaline-N-oxide (McN), retrorsine (Re), heliotrine (Hn), heliotrine-N-oxide (HnN), trichodesmine (Td), retrorsine-N-oxide (ReN), echimidine (Em), intermedine (Im), lycopsamine (La), senkirkine (Sk), lasiocarpine (Lc) and lasiocarpine-N-oxide (LcN).

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

2 Principles

A test portion is dissolved in aqueous sulphuric acid solution for PA extraction. Dissolved samples are centrifuged to remove particles and afterwards purified by a solid-phase extraction (SPE) using strong cation exchange material. PAs are released from the cartridge using ammoniacal 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 heliotrine (Hn)

3.2.3 heliotrine-N-oxide (HnN)

3.2.4 intermedine (Im)

3.2.5 lasiocarpine (Lc)

3.2.6 lasiocarpine-N-oxide (LcN)

3.2.7 lycopsamine (La)

3.2.8 monocrotaline (Mc)

3.2.9 monocrotaline-N-oxide (McN)

3.2.10 retrorsine (Re)

3.2.11 retrorsine-N-oxide (ReN)

3.2.12 senecionine (Sc)

3.2.13 senecionine-N-oxide (ScN)

3.2.14 seneciphylline (Sp)

3.2.15 seneciphylline-N-oxide (SpN)

3.2.16 senkirkine (Sk)

3.2.17 trichodesmine (Td)

3.2.18 formic acid 98 – 100%, e.g. Sigma-Aldrich

3.2.19 methanol (MeOH) in LC-MS quality, e.g. Merck LiChrosolv®

3.2.20 sulphuric acid 98%, e.g. Merck

3.2.21 ammonia 32%, e.g. Merck

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

3.2.23 acetonitrile, e.g. Merck LiChrosolv®

3.3 Solutions

3.3.1 Extraction solution: 0.05 M sulphuric acid

2.665 mL of sulphuric acid (H_2SO_4) (3.2.20) are filled up to 1 L with water. The final concentration is 0.05 M.

3.3.2 Elution solution for solid phase extraction (SPE)

To prepare a 2.5 % ammoniacal solution for the elution of PA, 7.8 mL ammonia (3.2.21) are filled up to 100 mL with methanol (3.2.19).

Please note: The solution must be prepared every working day.

3.3.3 HPLC mobile phase

Eluent A:
315 mg ammonium formate (3.2.22) are dissolved in 5 mL of water, 1 mL of formic acid (3.2.18) is added and filled up to 1 L with water.

Eluent B
315 mg ammonium formate (3.2.22) are dissolved in 5 mL of water, 1 mL of formic acid (3.2.18) is added and filled up to 1 L with methanol (3.2.19).

3.3.4 Standard solution for calibration

Stock solution (0.1 mg/mL):
To create a stock solution, 1 mg of a pyrrolizidine alkaloid standards are weighed in a volumetric flask using an analytical balance (4.3) and filled up to 10 mL with acetonitrile (3.2.23). 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 filled up with acetonitrile (3.2.23), to achieve 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 strength for MMS as samples the blank honey has to be processed as described in section 5. Afterwards, prepare MMS according to Table 1.

Table 1: Matrix matched standards

	Final PA mass concentration in calibration solution	**Final PA mass concentration**	**Aliquot taken from**	**Aliquot Volume**	**Aliquot taken from blank honey extract**
	ng/mL	µg/kg		µL	µL
MMS_1	**1.0**	**0.1**	MMS_3	20	180
MMS_2	**5.0**	**0.5**	MMS_5	20	180
MMS_3	**10.0**	**1.0**	MMS_5	40	160
MMS_4	**25.0**	**2.5**	Standard working solution (1 µg/mL)	5	195
MMS_5	**50.0**	**5.0**	Standard working solution (1 µg/mL)	10	190
MMS_6	**75.0**	**7.5**	Standard working solution (1 µg/mL)	15	185
MMS_7	**100.0**	**10.0**	Standard working solution (1 µg/mL)	20	180

4 Apparatus

4.1 General

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

4.2 various piston pipettes and multiple dispensers, e.g. Brand

4.3 analytical balance, capable of weighing to 0,0001 g

4.4 compartment drier

4.5 centrifuge for 50 mL centrifuge tubes, 5000 x g

4.6 laboratory shaker, e.g. Vortex

4.7 overhead shaker, e.g. Heidolph

4.8 evaporation station, e.g. TurboVap

4.9 centrifuge tube 50 mL

4.10 test tubes 15 mL

4.11 volumetric flasks, 10 and 20 mL

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

4.13 SPE vacuum chamber

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

4.15 HPLC vials 2 mL

4.16 Glass inserts, 250 µL conic for HPLC vials

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

4.18 LC-MS/MS system

5 Procedure

5.1 Extraction (Betteridge et al. 2005; modified)

For the extraction of PA 10.0 g ± 0.1 g of honey are wheighed into a centrifuge tube (4.9).

The sample is dissolved in 30 mL of extraction solution (3.3.1) at room temperature by shaking (4.7) for 30 min. Sample solutions are centrifuged at 3,800 x g for 10 min ± 2 min (4.5). The supernatant is transferred to a clean vessel and afterwards used for SPE.

5.2 SPE procedure

SPE (4.12) is carried out using a vacuum chamber (4.13) which is placed in a compartment drier (4.4) at 40 °C ± 5 °C. By means of the elevated temperature the cristallization of honey at SPE cartridges can be avoided.

Conditioning step 1	5 mL of methanol (3.2.19)
Conditioning step 2	5 mL 0.05 M H_2SO_4 (3.3.1)
Sample load	30 mL sample (supernatant), rinse vessel with 5 mL 0.05 M H_2SO_4 (3.3.1) and add solution to the cartridge
Washing step 1	6 mL of water
Washing step 2	6 mL of methanol (3.2.19)
Drying of cartridges	5 - 10 min (use the vacuum chamber (4.13))
Elution of PA	2 x 5 mL 2.5% ammonia in methanol (3.3.2)

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

5.3 Reconstitution of the sample

The residue is dissolved in 1 mL of methanol/water (5/95, v/v) by shaking (4.6).

The reconstituted sample extracts are filtered through a 0.2 µm membrane filter (4.14). 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 (4.15) with a glass insert (4.16).

6 HPLC-MS/MS

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 C-

18 column (4.17) and the mobile phase described in 3.3.3 have shown to be suitable in pre-trials. However, they are to be seen as examples only.

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

6.2 Mass spectrometric operating 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 measurement system are given as an example. These conditions have shown to be suitable in pre-trials.

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 (1 – 100 ng/mL)
2. Solvent blank
3. Samples (first injection)
4. Solvent blank
5. Matrix matched standards (1 – 100 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 results

The results are reported in µg/kg with two significant decimals. To convert the concentration from ng/mL injected solution to µg/kg honey a factor of 0.1 is used according to the sample preparation procedure described in chapter 5.

Reference list

Betteridge K, Cao Y, Colegate SM (2005) Improved method for extraction and LC-MS analysis of pyrrolizidine alkaloids and their N-oxides in honey: application to Echium vulgare honeys. J Agric Food Chem 53 (6):1894-1902.

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 (Thermo Accela 1250),
Degasser
Autosampler (CTC Analytics PAL ATS MYX)
Column oven (MayLab MistraSwitch)

HPLC settings

Eluent A refer to (3.3.3)
Eluent B refer to (3.3.3)
Column temperature 40 °C
Flow rate 300 µL/min
Injection volume 10 µL
Column 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 Lense	Retention time [min]
Monocrotaline	326.2	120.3 237.3	35 25	130 130	4,41
Monocrotaline-N-oxide	342.1	118.3 137.4	37 29	141 141	5,18
Intermedine	300.1	138.3 156.3	18 28	112 112	5,66
Lycopsamine	300.1	138.3 156.3	18 28	112 112	5,78
Retrorsine	352.2	120.3 138.3	27 29	140 140	6,60
Trichodesmine	354.2	120.3 222.3	35 28	137 137	6,68
Retrorsine-N-oxide	368.2	136.2 118.2	30 40	145 145	6,71
Seneciphylline	334.2	120.3 138.4	26 28	138 138	6,89
Heliotrine	314.2	138.3 156.3	19 28	119 119	7,08
Seneciphylline-N-oxide	350.2	118.2 136.3	36 32	135 135	7,12
Heliotrine-N-oxide	330.2	138.2 172.1	22 27	121 121	7,42
Senecionine	336.2	120.2 138.2	27 29	130 130	7,77
Senecionine-N-oxide	352.2	118.1 136.3	28 27	116 116	7,98
Echimidine	398.2	120.3 220.3	23 17	139 139	8,53
Senkirkine	366.2	150.3 168.2	24 28	132 132	8,71
Lasiocarpine	412.2	120.2 336.3	30 17	139 139	9,50
Lasiocarpine-N-oxide	428.2	136.1 254.1	29 27	135 135	9,75

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]
Monocrotaline	0.22	0.59
Monocrotaline-N-oxide	0.20	0.55
Intermedine	0.13	0.35
Lycopsamine	0.09	0.24
Retrorsine	0.13	0.35
Trichodesmine	0.23	0.62
Retrorsine-N-oxide	0.11	0.29
Seneciphylline	0.10	0.27
Heliotrine	0.13	0.37
Seneciphylline-N-oxide	0.17	0.47
Heliotrine-N-oxide	0.11	0.28
Senecionine	0.15	0.42
Senecionine-N-oxide	0.13	0.35
Echimidine	0.15	0.42
Senkirkine	0.10	0.27
Lasiocarpine	0.07	0.21
Lasiocarpine-N-oxide	0.06	0.18

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

8.2 Typical chromatogram

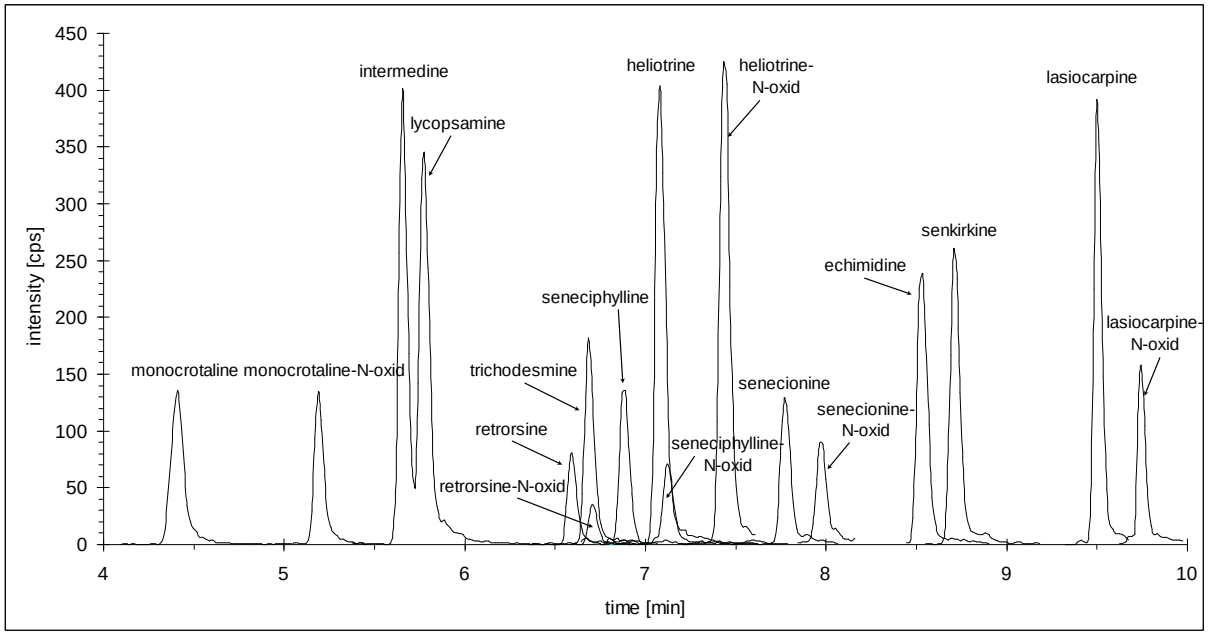


figure 1: Typical chromatogram of a PA standard mixture (10 ng/mL), TIC of SRM-transitions

8.3 Provider of PA standards

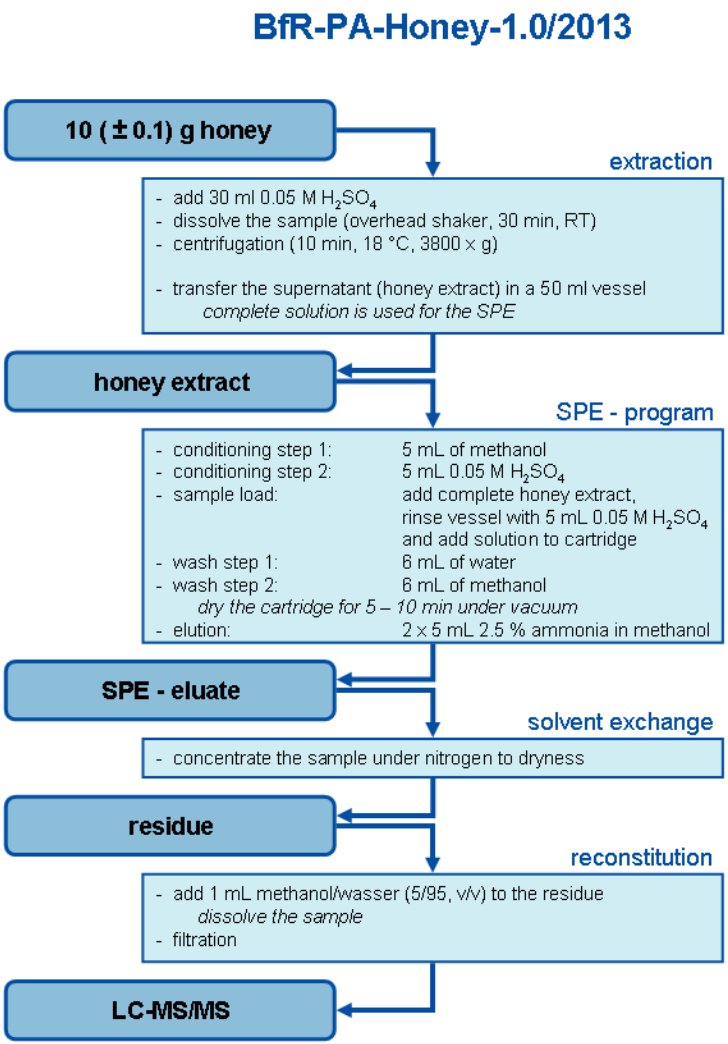
pyrrolizidine alkaloid	mass	CAS	provider	order code
echimidine	397,47	520-68-3	Oskar Tropitzsch PhytoLab* PlantaAnalytica	7550006 89553 -
erucifoline	349.38	40158-95-0	Carl Roth Oskar Tropitzsch PhytoLab*	1657.1 7550021 83446
erucifoline-N-oxide	365,37	123864-94-8	Carl Roth PhytoLab*	1664.1 83434
europine-hydrochloride	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

pyrrolizidine alkaloid	mass	CAS	provider	order code
lycopsamine-N-oxide	315,36	95462-15-0	Oskar Tropitzsch	7501358
			PhytoLab*	83447
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 Biotechnology	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

pyrrolizidine alkaloid	mass	CAS	provider	order code
senecivernine	335.40	72755-25-0	Carl Roth Oskar Tropitzsch PhytoLab*	2209.1 7550066 83436
senecivernine-N-oxide	351,39	101687-28-9	Carl Roth PhytoLab*	2215.1 83437
senkirkine	365,43	2318-18-5	AppliChem Carl Roth Fluka Oskar Tropitzsch PhytoLab*	A6765,0010 4934.1 37032 7500441 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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apparatus
overhead shaker; centrifuge; 50 ml vessel

solvent
0.05 M H_2SO_4 – solution

apparatus
SPE – vacuum-chamber

materials
HF Bond Elute LRC SCX; 500 mg

solvents
methanol; 0.05M H_2SO_4 – solution; water; 2,5 % ammonia in methanol

apparatus
evaporator

materials
HPLC-vials; membrane filter (0.2 µm)

solvent
methanol/wasser; wasser

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