

EURL-MP-method_002 (version 1)**Determination of pyrrolizidine alkaloids in plant-based food and feed materials, including (herbal) teas, herbal food supplements, fodder and feedstuffs by LC-MS/MS****Analyte group:****Analyte(s):****Plant toxins**

Atropine (At)
Echimidine (Em) and Echimidine-N-oxide (Em-ox)
Echinatine (En) and Echinatine-N-oxide (En-ox)
Erucifoline (Er) and Erucifoline-N-oxide (Er-ox);
Europine (Eu) and Europine-N-oxide (Eu-ox)
Heliosupine (Hs) and Heliosupine-N-oxide (Hs-ox)
Heliotrine (Ht) and Heliotrine-N-oxide (Ht-ox)
Integerrimine (Ir) and Integerrimine-N-oxide (Ir-ox)
Intermedine (Im) and Intermedine-N-oxide (Im-ox)
Jacobine (Jb) and Jacobine-N-oxide (Jb-ox)
Jacoline (Jl)
Jaconine (Jn)
Lasiocarpine (Lc) and Lasiocarpine-N-oxide (Lc-ox)
Lycopsamine (Ly) and Lycopsamine-N-oxide (Ly-ox)
Monocrotaline (Mc) and Monocrotaline-N-oxide (Mc-ox)
Retrorsine (Rt) and Retrorsine-N-oxide (Rt-ox)
Scopolamine (Sc)
Senecionine (Sn) and Senecionine-N-oxide (Sn-ox)
Seneciphylline (Sp) and Seneciphylline-N-oxide (Sp-ox)
Senecivernine (Sv) and Senecivernine-N-oxide (Sv-ox)
Senkirkine (Sk)
Trichodesmine (Td)

Commodity group:

Plant-based food and feed materials, including (herbal) teas, herbal food supplements, fodder and feedstuffs

Commodities validated:

Black tea, peppermint tea, mixed herbal tea, valerian herbal supplement, alfalfa, hay, sunflower expeller, bovine compound feed

Technique:

Liquid chromatography / tandem mass spectrometry (LC-MS/MS)

Modifications compared to previous version:

Not applicable.

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Notices:

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1. Introduction

Pyrrrolizidine alkaloids (PAs) are toxic secondary metabolites found in various weeds, most notably in plants of the family of Asteraceae (genera *Senecio* and *Eupatorium*), the family of Boraginaceae (including the genus *Heliotropium*) and the family of Fabaceae (genus *Crotalaria*). Approximately 600 different PAs have been described in the literature. PAs can exist in two forms: a tertiary amine (free base) form and in a N-oxide form. The PA composition of plants is quite variable, depending on the plant species, chemotype, stage of growth and environmental conditions. PA-containing plants can be present as contaminants in all types of plant-based food and feed materials, including (herbal) teas, herbal food supplements, fodder and feedstuffs.

The tropane alkaloids (TAs) atropine and scopolamine are toxic secondary metabolites found mostly in weeds of the plant family of Solanaceae (genera *Datura* and *Atropa*). They can be present as contaminants in the same types of plant-based food and feed materials as the PAs.

Based on available occurrence data, EFSA has identified a total of 17 PAs as most relevant for monitoring in food in the EU: echimidine, echimidine-N-oxide, intermedine, intermedine-N-oxide, lasiocarpine, lasiocarpine-N-oxide, lycopsamine, lycopsamine-N-oxide, retrorsine, retrorsine-N-oxide, senecionine, senecionine-N-oxide, seneciphylline, seneciphylline-N-oxide, senecivernine, senecivernine-N-oxide, and senkirkine [1].

The European Commission is considering legislation on the presence of PAs in various food products with priority on (herbal) teas and herbal supplements. The EC has identified 21 PAs as relevant for food: the above mentioned 17 PAs and in addition also europine, europine-N-oxide, heliotrine and heliotrine-N-oxide.

For feed commodities no priority list of PAs has yet been identified. Based on in-house occurrence data, and literature data, the following PAs may be of importance: The 21 PAs listed above and in addition: echinatine, echinatine-N-oxide, erucifoline, erucifoline-N-oxide, heliosupine, heliosupine-N-oxide, integerrimine, integerrimine-N-oxide, jacobine, jacobine-N-oxide, jacoline, jacoline, monocrotaline, monocrotaline-N-oxide and trichodesmine.

2. Scope

This document describes describes the confirmation and -by means of standard addition to the sample- the quantification of the following PAs: echimidine (Em), echimidine-N-oxide (Em-ox), echinatine (En), echinatine-N-oxide (En-ox), erucifoline (Er), erucifoline-N-oxide (Er-ox), europine (Eu), europine-N-oxide (Eu-ox), heliosupine (Hs), heliosupine-N-oxide (Hs-ox), heliotrine (Ht), heliotrine-N-oxide (Ht-ox), integerrimine (Ir), integerrimine-N-oxide (Ir-ox), intermedine (Im), intermedine-N-oxide (Im-ox), jacobine (Jb), jacobine-N-oxide (Jb-ox), jacoline (Jl), jacoline (Jn), lasiocarpine (Lc), lasiocarpine-N-oxide (Lc-ox), lycopsamine (Ly), lycopsamine-N-oxide (Ly-ox), monocrotaline (Mc), monocrotaline-N-oxide (Mc-ox), retrorsine (Rt), retrorsine-N-oxide (Rt-ox), senecionine (Sn), senecionine-N-oxide (Sn-ox), seneciphylline (Sp), seneciphylline-N-oxide (Sp-ox), senecivernine (Sv), senecivernine-N-oxide (Sv-ox), senkirkine (Sk) and trichodesmine (Td). The method is also suited for the following tropane alkaloids (TAs): atropine (At) and scopolamine (Sc). The method is applicable for plant-based materials in the concentration range of 0 to 1000 µg/kg. PA concentrations are reported from 5 µg/kg.

3. Principle

The method is suited for the confirmation and quantification of PAs in products of plant material. A sample is weighted in duplicate. One of the samples is spiked at 250 µg/kg using PA mixed standard solutions. PAs are extracted with water containing 0.2% formic acid. After centrifuging, an aliquot is further purified using SPE. The SPE eluate is evaporated, re-dissolved in methanol/water 1/9 (v/v) and analysed by LC-MS/MS. PAs are quantified by means of standard addition to the sample.

4. Reagents

Use only reagents of recognized analytical grade. Solvents shall be of quality for LC analysis, unless otherwise specified.

- 4.1 **Water**, deionised MilliQ and with a minimal resistance of 18.2 MΩ/cm.
- 4.2 **Acetonitrile**, LC-MS grade
- 4.3 **Methanol**, LC-MS grade
- 4.4 **Formic acid**, 99-100%
- 4.5 **Ammonia**, 25%
- 4.6 **Ammonium carbonate**, p.a. quality
- 4.7 **Echimidine (Em)**
- 4.8 **Echimidine-N-oxide (Em-ox)**
- 4.9 **Echinatine (En)**
- 4.10 **Echinatine-N-oxide (En-ox)**
- 4.11 **Erucifoline (Er)**
- 4.12 **Erucifoline-N-oxide (Er-ox)**
- 4.13 **Europine (Eu)**
- 4.14 **Europine-N-oxide (Eu-ox)**
- 4.15 **Heliosupine (Hs)**
- 4.16 **Heliosupine-N-oxide (Hs-ox)**
- 4.17 **Heliotrine (Ht)**
- 4.18 **Heliotrine-N-oxide (Ht-ox)**

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- 4.19 Integerrimine (Ir)
 - 4.20 Integerrimine-N-oxide (Ir-ox)
 - 4.21 Intermedine (Im)
 - 4.22 Intermedine-N-oxide (Im-ox)
 - 4.23 Jacobine (Jb)
 - 4.24 Jacobine-N-oxide (Jb-ox)
 - 4.25 Jacoline (Jl)
 - 4.26 Jaconine (Jn)
 - 4.27 Lasiocarpine (Lc)
 - 4.28 Lasiocarpine-N-oxide (Lc-ox)
 - 4.29 Lycopsamine (Ly)
 - 4.30 Lycopsamine-N-oxide (Ly-ox)
 - 4.31 Monocrotaline (Mc)
 - 4.32 Monocrotaline-N-oxide (Mc-ox)
 - 4.33 Retrorsine (Rt)
 - 4.34 Retrorsine-N-oxide (Rt-ox)
 - 4.35 Senecionine (Sn)
 - 4.36 Senecionine-N-oxide (Sn-ox)
 - 4.37 Seneciphylline (Sp)
 - 4.38 Seneciphylline-N-oxide (Sp-ox)
 - 4.39 Senecivernine (Sv)
 - 4.40 Senecivernine-N-oxide (Sv-ox)
 - 4.41 Senkirkine (Sk)
 - 4.42 Trichodesmine (Td)

4.43 Atropine (At)

4.44 Scopolamine (Sc)

4.45 Extraction solvent

Mix 2 ml formic acid with 1000 ml water. This solution is stored at room temperature and can be used for 1 month.

4.46 Neutralisation solution: 1M ammonium carbonate in water

Dissolve 9.6 g ammonium carbonate (4.6) in 100 ml water. This solution is stored at room temperature and can be used for 1 month.

4.47 Formic acid solution (1%)

Mix 1 ml formic acid (4.4) with 100 ml water. This solution is stored at room temperature and can be used for 1 month.

4.48 Mobile phase A for alkaline chromatography: 10 mM ammonium carbonate in water, pH 9

Mix 10 ml neutralisation solution (4.46) with 1 l water. If necessary, add with a positive displacement pipette ammonia 25% (4.5) and adjust the pH to 9.0 ± 0.1 using a pH meter (5.10). This solution is stored at room temperature and can be used for 1 month.

4.49 Mobile phase A for acidic chromatography: 0.1% formic acid in water

Dissolve 1 ml formic acid (4.4) in 1 l water and mix. This solution is stored at room temperature and can be used for 1 month.

4.50 Strong wash solvent for autosampler

Mix 900 ml methanol (4.3) and 100 ml water.

4.51 Weak wash solvent for autosampler

Mix 100 ml methanol (4.3) with 900 ml water.

4.52 Stock solutions (100 mg/l)

Accurately weight between 3 and 5 mg \pm 0.02 mg of standards 4.7 to 4.44. When the standard is available in a quantity of 5 mg or less, preferably the entire content of the container is used. In that case the weight as reported by the supplier is used. Flush the contents of the container three times with methanol to dissolve and collect all material. Prepare stock solutions (100 mg/l) in methanol, taking into account the constitution of the standard material. These stock solutions are stored at -20°C and can be used for 24 months.

4.53 Mixed standard solution PA free bases and TAs (3.33 mg/l)

Pipette 667 μl of the stock solutions of echimidine, echinatine, erucifoline, europine, heliosupine, heliotrine, integerrimine, intermedine, jacobine, jacoline, jaconine, lasiocarpine, lycopsamine, monocrotaline, retrorsine, senecionine, seneciphylline, senecivernine, senkirkine, trichodesmine, atropine and scopolamine in a volumetric flask of 20 ml and make up to the mark with methanol. This solution is stored at -20°C and can be used for 12 months.

4.54 Mixed standard solution PA N-oxides (3.33 mg/l)

Pipette 667 μl of the stock solutions of echimidine-N-oxide, echinatine-N-oxide, erucifoline-N-oxide, europine-N-oxide, heliosupine-N-oxide, heliotrine-N-oxide, integerrimine-N-oxide, intermedine-N-oxide,

jacobine-N-oxide, lasiocarpine-N-oxide, lycopsamine-N-oxide, monocrotaline-N-oxide, retrorsine-N-oxide, senecionine-N-oxide, seneciophylline-N-oxide, senecivernine-N-oxide in a volumetric flask of 20 ml and make up to the mark with methanol. This solution is stored at -20°C and can be used for 12 months.

4.55 Mixed standard solution PAs (500 µg/l)

Pipette 1500 µl of the mixed standard solution free bases (3.33 mg/l) (4.53) and 1500 µl of the mixed standard solution N-oxides (3.33 mg/l) (4.54) in a volumetric flask of 10 ml and make up to the mark with methanol. This solution is stored at -20°C and can be used for 12 months.

4.56 Working standard solution (10 µg/l)

Pipette 200 µl of the mixed standard solution PAs (500 µg/l) (4.55) in a volumetric flask of 10 ml. Make up to the mark with water. This solution is stored at -20°C and can be used for 12 months.

5. Equipment

Usual laboratory glassware and equipment, in particular, the following:

- 5.1 Analytical balance**, accuracy: 0.01 mg.
- 5.2 Balance**, accuracy: 0.01 g.
- 5.3 Pipets, adjustable**, e.g. 10 µl to 100 µl and 100 µl to 1000 µl, suited for organic solvents (e.g. positive displacement pipets), properly calibrated, with appropriate tips.
- 5.4 Laboratory shaker** (vortex).
- 5.5 Adjustable mechanical vertical or horizontal shaker or rotary tumbling machine.**
- 5.6 Centrifuge**, capable of generating a relative centrifugal force of 3,000 g. suitable for 12 and 50 ml centrifuge tubes
- 5.7 Polypropylene tubes** of 50 ml with screw cap
- 5.8 Polypropylene tubes** of 12 ml with screw or plug cap
- 5.9 Dispenser** 5 - 50 ml
- 5.10 pH meter**
- 5.11 SPE vacuum manifold**
- 5.12 Vacuum pump**
- 5.13 SPE cartridge**, e.g. Strata-X Polymeric reversed phase 200 mg/6 ml
- 5.14 Evaporator** with nitrogen flow, suitable for 12 ml tubes
- 5.15 Filtervial** (polytetrafluoroethylene (PTFE) 0,45 µm), with crimp cap, e.g. Mini-UniPrep,

Whatman, or equivalent.

5.16 Compressor filter vials 6 positions

5.17 LC-MS/MS system, with the following components:

5.17.1 LC pump, capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

5.17.2 Injection system, capable of injecting an appropriate volume of injection solution with sufficient accuracy, and cross-contamination below 0.1%.

5.17.3 Analytical column: capable of retaining the target PAs, preferably capable of baseline separation of PAs with identical molecular mass. An analytical column containing high pH-resistant cross-linked C18 reversed phase packing material is required for use with a mobile phase of high pH (4.48). See 7.1 for example conditions.

5.17.4 Column oven, capable of maintaining a constant temperature.

5.17.5 Tandem mass spectrometer (MS/MS), capable of ionisation of the compounds in positive mode, performing Multiple Reaction Monitoring (MRM), and with a sufficiently wide dynamic range and capable of unit mass separation and equipped with a computer based data processing system. Any ionisation source giving sufficient yield may be employed. See 7.2 for example transitions.

6. Procedures

6.1 General

This SOP describes the quantification of PAs in plant materials. The steps described in section 6.4 and 6.5 are shown in the format of a checklist in Annex A.

6.2 Preparation of the test sample

Wet plant samples are dried by freeze-drying. For the preparation of the laboratory sample, the plant sample is finely ground through a sieve of 1 mm. The laboratory sample should be homogenized before it is used to prepare test samples.

6.3 Test portion

The amount of homogenized plant material examined is 2.00 ± 0.05 gram.

6.4 Extraction, clean-up and preparation of test solutions

6.4.1 Matrix matched standards (MMS)

Choose a blank plant material, in which no PAs were detected (<LOD) in previous analyses. The selected blank material should match with (most of) the materials of the samples to be analysed.

Weigh 8 individual test portions of 2.00 ± 0.05 g of the blank plant material in PP tubes of 50 ml (5.7). Add standard solutions according to Table 1. Wait at least 10 minutes before to start the extraction procedure.

Table 1: Preparation of matrix matched standards (MMS)

	Concentration ($\mu\text{g}/\text{kg}$)	Mixed standard solution free bases 3.33 mg/l (4.53) (μl)	Mixed standard solution N-oxides 3.33 mg/l (4.54) (μl)	Mixed standard solution PAs 500 $\mu\text{g}/\text{l}$ (4.55) (μl)
MMS 1	0	0	0	0
MMS 2	10	0	0	40
MMS 3	25	15	15	0
MMS 4	50	30	30	0
MMS 5	100	60	60	0
MMS 6	250	150	150	0
MMS 7	500	300	300	0
MMS 8	1000	600	600	0

6.4.2 Quality control sample limit of quantification (LOQ) (10 $\mu\text{g}/\text{kg}$)

Weigh of the blank plant material used in 6.4.1 a test portion of 2.00 ± 0.05 g in a PP tube of 50 ml (5.7). Add 40 μl of mixed standard solution 500 $\mu\text{g}/\text{l}$ (4.55) to the sample. Wait 30 minutes before starting the extraction procedure (6.4.4).

6.4.3 Preparation of test samples

Weigh two test portions of 2.00 ± 0.05 g of the laboratory sample in PP tubes of 50 ml (5.7). Add to one of the tubes 150 μl of mixed standard solution free bases 3.33 mg/l (4.53) and 150 μl mixed standard solution N-oxides 3.33 mg/l (4.54). The added level is 250 $\mu\text{g}/\text{kg}$. Wait 30 minutes before starting the extraction procedure.

6.4.4 Extraction of samples

Add 40 ml extraction solvent (4.45) to the test portion and mix using a vortex mixer (5.4). Place the tubes during 30 minutes in an overhead shaker (5.5). Centrifuge the tubes during 15 minutes at 3,000 g (5.6). Transfer 5 ml of the supernatant to a PP tube of 12 ml (5.8) and add 300 μl neutralisation solvent (4.46). The pH should be between 7 and 8. Check the pH with pH paper and add more neutralisation solvent when necessary. Centrifuge the tubes during 15 minutes at 3,000 g (5.6).

6.4.5 Solid phase extraction

Activate a Strata-X SPE cartridge (5.13) with 6 ml methanol followed by 6 ml water. Apply the extract resulting from 6.4.4 onto the cartridge. Wash the cartridge with 6 ml 1% formic acid solution (4.47), followed by 6 ml water. Dry the cartridge under vacuum. Elute the compounds with 6 ml methanol in a 12 ml tube. Evaporate the eluate at $50^\circ\text{C} \pm 5^\circ\text{C}$ with nitrogen gas until the extract is dry (5.14). Dissolve the residue first in 50 μl methanol and then add 450 μl water and mix using a vortex (5.4). Transfer the solution to a filtervial (5.15) and close it with help of a compressor (5.16). The extracts are stored by -20°C and can be used for 6 months.

6.4.6 Matrix matched recovery sample (MMRS 100 $\mu\text{g}/\text{kg}$)

Take 5 ml of blank extract MMS 1 (6.4.1) and adjust the pH with neutralisation solution (4.46) as described under 6.4.4. Purify the extract as described under 6.4.5. Add 50 μl of mixed standard PA solution 500 $\mu\text{g}/\text{l}$ (4.55), mix by vortexing (5.4) and then add 450 μl water and mix using a vortex (5.4). Transfer the solution to a filtervial (5.15) and close it with help of a compressor (5.16). The extract is stored by -20°C and can be used for 6 months.

7. LC-MS/MS conditions

7.1 LC conditions

Choose an analytical column, mobile phase and gradient settings such that the requirements laid out in 5.17.2 are met.

Example LC conditions for separation of the PAs 4.7 until 4.42.

LC system:	Waters Acquity
Column:	Waters Acquity UPLC BEH C18 1.7 µm 2.1 x 150 mm
Column temperature:	50°C
Injection volume:	2(-5) µl
Vial tray temperature:	10°C
Strong wash:	methanol/water (90/10) (4.50)
Weak wash:	methanol/water (10/90) (4.51)
Flow:	0.4 ml/min
Mobile phase:	see Table 2 and Table 3
Gradient:	see Table 2 and Table 3
Run time:	14.2 min
Solvent delay:	0-1.5 and 13.2-14.2 min

Table 2: Gradient for LC-MS/MS analysis with alkaline chromatography:

Time (min)	Mobile phase A (4.48) (%)	Mobile phase B (4.2) (%)	Flow (ml/min)
0.0	100	0	0.4
0.1	95	5	0.4
3.0	90	10	0.4
7.0	76	24	0.4
9.0	70	30	0.4
12.0	30	70	0.4
12.1	100	0	0.4
14.2	100	0	0.4

Table 3: Gradient for LC-MS/MS analysis with acidic chromatography:

Time (min)	Mobile phase A (4.49) (%)	Mobile phase B (4.2) (%)	Flow (ml/min)
0.0	100	0	0.4
0.1	95	5	0.4
6.5	90	10	0.4
10.0	75	25	0.4
12.0	40	60	0.4
12.1	100	0	0.4
14.2	100	0	0.4

See Annex B and C for example chromatograms.

7.2 MS conditions

The conditions given below are guidelines; in practice adjusted settings may be required to obtain an optimal performance of the LC-MS/MS system.

Mass spectrometer:	Waters Xevo TQ-S
Ionization mode:	ESI positive mode
Capillary voltage:	3.0 kV
Source temperature:	150°C
Source offset:	60 V
Desolvation temperature:	600°C
Cone gas flow:	150 L/hr
Desolvation gas flow:	800 L/hr
CID gas:	Argon, $4.3 \cdot 10^{-3}$ mbar (0.17 ml/min)

The precursor ions fragment to structurally related ions. In Table 4 the theoretical monoisotopic masses of the precursor ions and corresponding product ions are shown. Depending on the instrument, a deviation of ± 0.3 D is allowed. The retention times can differ from column to column and depend strongly on the pH of the mobile phase used. The retention times shown in Table 4 are therefore indicative. For individual compounds a third product ion can often be selected for analysis, when one of the product ions mentioned in Table 4 appears to suffer from matrix interferences or when it is less sensitive. The second validation level decides whether it is necessary for a specific compound to select an alternative product ion.

Check the system performance as well as the retention times and time windows of the various PAs. The system should be able to detect the product ion with the lowest intensity with an s/n ratio of at least 50 for the PAs in the working standard solution of 10 $\mu\text{g/l}$ (4.56). The sensitivity is visually checked for the most critical component in each window.

Table 4: MS/MS fragmentation conditions for pyrrolizidine alkaloids.

PA	Precursor ion (m/z)	Cone voltage (V)	Product ion 1 (m/z)	Col. energy 1 (eV)	Product ion 2 (m/z)	Col. energy 2 (eV)	Product ion 3 (m/z)	Col. energy 3 (eV)	Alkaline indicative RT (min)	Acidic indicative RT (min)
At	290.2	25	93	25	124	20	91	35	8.15	8.70
Im	300.2	30	94	35	156	30	138	30	5.75*	4.25*
Ly	300.2	30	94	35	156	30	138	30	5.80*	4.35*
En	300.2	30	138	30	156	30	94	35	6.70	4.45*
Sc	304.2	25	138	20	156	25	103	35	8.85	5.80
Ht	314.2	30	138	25	156	25	94	35	8.35	7.60
Im-ox	316.2	30	94	40	172	30	111	40	3.75*	5.15
Ly-ox	316.2	30	94	40	172	30	111	40	3.80*	5.35
En-ox	316.2	30	111	40	172	30	94	40	4.20	4.90
Mc	326.2	40	94	35	120	30	121	30	5.95	3.10
Eu	330.2	30	94	35	138	30	156	30	6.45	4.30
Ht-ox	330.2	30	111	35	172	25	94	40	5.90	8.20
Sp	334.2	40	120	30	138	30	94	40	9.60	8.05
Ir	336.2	40	94	40	120	30	138	30	10.30	9.20
Sn	336.2	40	94	40	120	30	138	30	10.55	9.35*
Sv	336.2	40	94	40	120	30	138	30	10.70	9.30*
Mc-ox	342.2	40	120	35	137	30	94	40	2.85	4.00
Eu-ox	346.2	30	172	30	111	40	256	25	4.00	4.65
Er	350.2	40	94	40	120	30	138	30	7.60	3.80
Sp-ox	350.2	40	94	40	138	30	118	30	6.15	8.55
Jb	352.2	40	120	30	155	30	94	40	8.00	5.15
Rt	352.2	40	94	40	120	30	138	30	8.85	7.05
Ir-ox	352.2	40	94	40	120	30	136	30	6.85	9.45*
Sn-ox	352.2	40	94	40	120	30	136	30	7.05*	9.60
Sv-ox	352.2	40	94	40	120	30	136	30	7.10*	9.45*
Td	354.2	40	120	35	222	30	121	30	8.95	7.15
Jn	388.2	40	94	40	120	30	138	30	9.10	6.05
Er-ox	366.2	40	94	40	118	30	120	30	3.70	4.60
Sk	366.2	30	122	30	168	25	150	25	7.35	9.95
Jb-ox	368.2	40	120	30	296	25	119	30	4.80	5.80
Rt-ox	368.2	40	94	40	120	30	119	30	5.75	7.35
Jl	370.2	40	94	40	138	30	120	30	5.75	3.10
Hs	398.2	30	120	25	220	20	336	20	10.70	9.95*
Em	398.2	30	120	25	220	20	83	25	10.80	10.05*
Lc	412.2	30	120	25	220	20	336	20	11.50	11.00
Hs-ox	414.2	30	94	30	254	30	138	30	7.45	10.45
Em-ox	414.2	30	254	30	352	25	94	40	7.75	9.95
Lc-ox	428.2	30	138	30	254	25	94	40	8.55	11.35

*: isomeric compounds that are not fully chromatographically separated

7.3 Injection sequence

Analyse the MMS and the sample extracts in the order as given below.

- Standard working solution 10 µg/l (4.56)
- Water (4.1)
- MMS in blank plant material (6.4.1)
- Water (4.1)
- Quality control sample LOQ (6.4.2)
- Matrix matched recovery sample (6.4.6)
- Water (4.1)
- Sample extracts (6.4.5)
- Standard working solution 10 µg/l (4.56)

8. Evaluation and calculations

Peak areas are used for all subsequent calculations. For each injection, check peak assignment and integration for all measured transitions and adjust if needed.

8.1 Verification of the linearity of LC-MS/MS measurement

The calibration samples (MMS) are used to determine the linearity of the LC-MS/MS system and to determine if the sample pre-treatment is done correctly. For the MMS series, the peak area is plotted as function of the added concentration in the sample (µg/kg). Apply linear regression using the least squares method. The correlation coefficient of the line should be ≥ 0.990 . The deviation of the back calculated concentrations of the calibration standards from the true concentrations, using the calibration equation, should not exceed 20%.

8.2 Identification of PA in the samples

Identify PAs in the samples by comparing retention time and ion ratio with that of the calibration samples (MMS) according to SANTE/11813/2017 [2].

Calculate for each analyte the deviation of the retention time, and the deviation of the ion ratio. When for an analyte the deviation of the retention time does not exceed 0.1 min, the deviation of the ion ratio does not exceed 30% and the concentration exceeds the LOQ, the identity of the analyte in the sample is confirmed.

- a) Determine the average retention time of the analyte in the calibration samples (MMS) analysed before the sample extracts. The deviation in the individual retention times may not differ more than 0.1 min compared to the average retention time of the analyte in the MMS as stated in SANTE/11813/2017 [2].
- b) The retention time of the analyte observed for the sample extract differs less than 0.1 min from the average retention time as calculated from the MMS, calculated using **Equation I**.

Equation I: Deviation of the retention time (ΔRT)

$$\Delta RT = RT_{sample} - RT_{avg}$$

Where:

- ΔRT = Deviation of the retention time of the analyte in the sample extract, compared to the calibration samples (MMS) (min)
- RT_{sample} = retention time of the analyte in the sample extract (min)
- RT_{avg} = average retention time of the analyte present in the MMS 2 to MMS 8 (min)

- c) The ratio of the area of the quantifier and qualifier transition (lowest area/highest area) for the analyte in the sample extracts deviates less than 30% (relative) from the average ion ratio of the calibration standards (MMS) as stated in SANTE/11813/2017 [2], calculated using **Equation II** and **III**.

Equation II: Relative deviation of the ion ratio (D)

$$D = \left(\frac{R_{\text{sample}} - R_{\text{avg}}}{R_{\text{avg}}} \right) \times 100\%$$

Where:

- D = relative deviation of the ion ratio of the analyte in the sample, compared to the average ion ratio of the analyte in MMS 3 to MMS 7 (%)
- R_{sample} = ion ratio of the analyte in the sample (%) (**Equation III**)
- R_{avg} = average ion ratio of the analyte in MMS 3 to MMS 7 (%) (**Equation III**)

Equation III: Ion ratio (R)

$$R = \left(\frac{A_{\text{low}}}{A_{\text{high}}} \right) \times 100\%$$

Where:

- R = ion ratio (%)
- A_{low} = area of the product ion with the lowest intensity
- A_{high} = area of the product ion with the highest intensity

Note: for calculation of the reference ion ratio use only responses with an $S/N > 10$. For the higher concentrations, exclude peak areas exceeding the linear range from calculation of the reference ion ratio.

8.3 Quantification of PAs in the samples

8.3.1 First line control and LOQ

In the blank sample material (MMS 1) no analytes should be detected (<LOD). Calculate the concentration of the analytes in the LOQ QC sample with **Equation IV**, using MMS 5 (250 $\mu\text{g}/\text{kg}$) as fortified sample. The calculated concentration in the LOQ QC sample should be between 70 and 120% of the added level.

Equation IV: Concentration in the sample (X)

$$X = \left(\frac{RF_{\text{sample}}}{RF_{\text{spiked}} - RF_{\text{sample}}} \right) \times 250$$

Where:

- X = concentration of the analyte in the sample ($\mu\text{g}/\text{kg}$)
- RF_{sample} = sum area of the analyte in the sample
- RF_{spiked} = sum area of the analyte in the fortified sample of 250 $\mu\text{g}/\text{kg}$

8.3.2 Quantification based on one-point standard addition

The concentration of a PA in the sample is calculated based on standard addition to the sample, according to **Equation IV**.

When the concentration of one or more PAs in the sample is higher than the standard addition level, the procedure is repeated by analysis of the sample in duplicate and by using one or more appropriate standard addition levels (e.g. 250, 1000, 2500 µg/kg).

It may be necessary to dilute the final extracts by an appropriate factor to stay within the linear range of the mass detector.

8.4 Recovery

Calculate the recovery of the PAs with **Equation V**. The recovery is calculated for information only and is not considered as a critical parameter.

Equation V: Recovery (Rec)

$$Rec = \left(\frac{RF_{MMS100}}{RF_{MMRS}} \right) \times 100\%$$

Where:

Rec = recovery (%)

RF_{MMS100} = sum area of the analyte in MMS 4, fortified at 100 µg/kg

RF_{MMRS} = sum area of the analyte in the MMRS sample

8.5 Final results

The concentration of the PAs in the sample is expressed as µg/kg.

9. References

1. EFSA, *Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements*. EFSA Journal 2017;15(7):4908, 2017: p. pp. 34.
2. DG_SANTE, *Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed SANTE/11813/2017*. https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2017-11813.pdf, 2017: p. p. 46.

Annex A. Checklist

Analyst:

Date:

Labjournal / page:

MMS-series and QC samples

- Weigh 8 portions of 2.00 ± 0.05 g blank plant material in 50 ml polypropylene tubes
- Spike the tubes according to Table 1

Table 1: Preparation of MMS series and QC sample

	<i>Concentration ($\mu\text{g}/\text{kg}$)</i>	<i>Mix free bases 3.33 mg/l (4.53) (μl)</i>	<i>Mix N-oxides 3.33 mg/l (4.54) (μl)</i>	<i>Mix PAs 500 $\mu\text{g}/\text{l}$ (4.55) (μl)</i>
MMS 1	0	0	0	0
MMS 2	10	0	0	40
MMS 3	25	15	15	0
MMS 4	50	30	30	0
MMS 5	100	60	60	0
MMS 6	250	150	150	0
MMS 7	500	300	300	0
MMS 8*	1000	600	600	0
QC LOQ	10	0	0	40

Sample preparation

- Weigh 2 portions of 2.00 ± 0.05 g sample in 50 ml polypropylene tubes
- Spike one tube with 150 μl of mix PA free bases (4.53) and with 150 μl of mix PA N-oxides (4.54)

Sample extraction

- Wait 30 min
- Add 40 ml of 0.2% formic acid solution
- Rotate samples for 30 min (rotary tumbler)
- Centrifuge 15 min at 3000 g
- Transfer 5 ml supernatant to a 12-ml tube
- Adjust pH to 7-8 with 300 μl 1M ammonium carbonate solution, mix well, check with pH indicator strips
- Centrifuge 15 min at 3000 g

Solid phase purification

- Activate StrataX cartridge (200 mg, 6 cc) with 6 ml methanol
- Condition cartridge with 6 ml water
- Apply extract to the column
- Wash with 6 ml 1% formic acid
- Wash with 3 ml water
- Dry cartridge 10 min under vacuum
- Elute with 6 ml methanol
- Evaporate under N₂ at 50°C ± 5°C
- Redissolve in 50 µl methanol and 450 µl water and mix
- Filter over 0.45 µm using a 500 µl filtervial
- Press and close vial

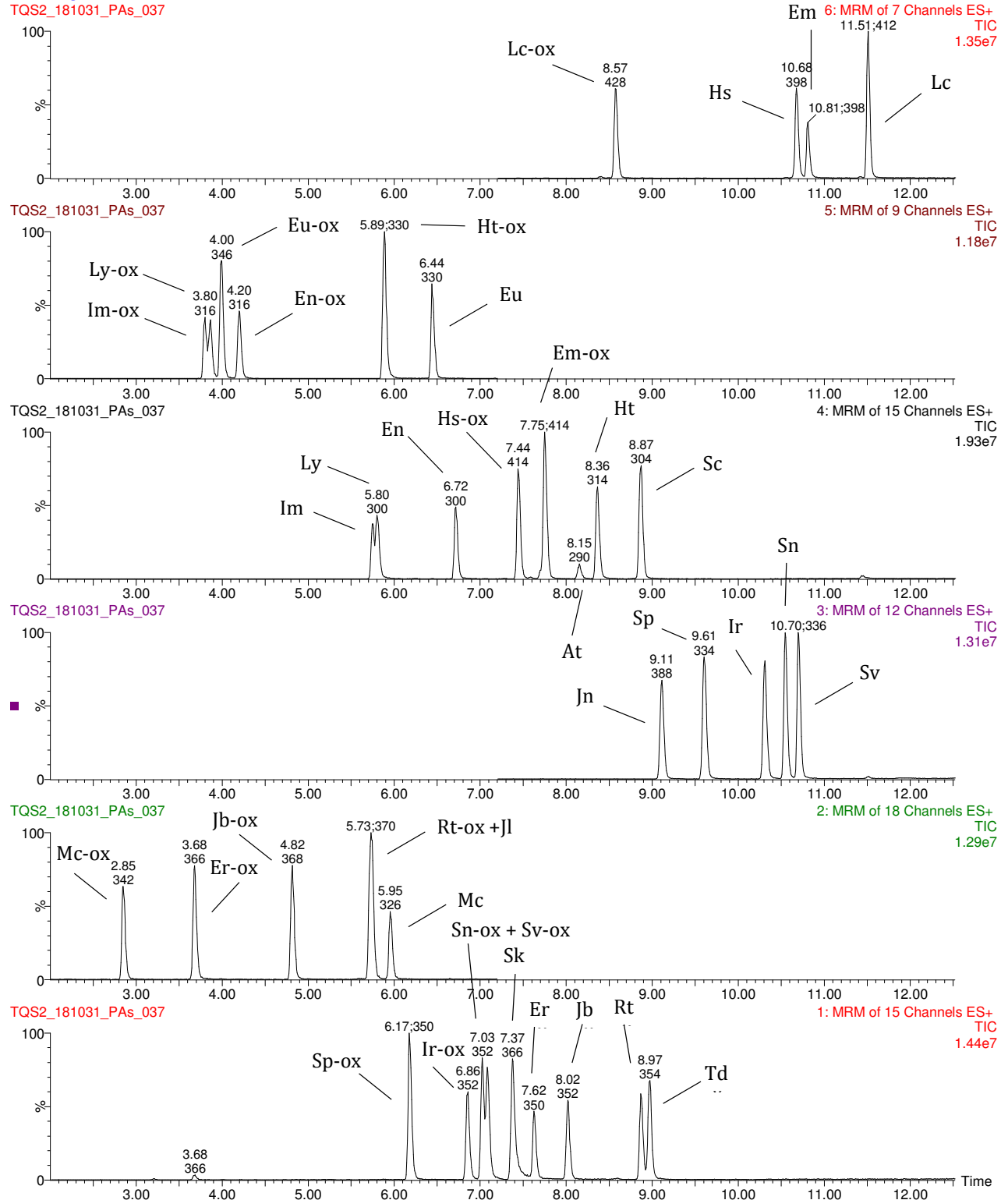
Matrix matched recovery sample

- Process additional portion of 5 ml of blank extract (MMS 1)
- Redissolve in 50 µl PA mix 500 µg/l (4.55) and 450 µl water and mix
- Filter over 0.45 µm using a 500 µl filtervial
- Press and close vial

Annex B. LC-MS/MS chromatogram (alkaline method)

Ws 10 ug/ml

TQS2_181031_PAs_037



Annex C. LC-MS/MS chromatogram (acidic method)

WS 10 ng/ml

TQS2_181101_PAs_003

