EURL-MP-method_002 (version 2)
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:	**Plant toxins**
Analyte(s):	Atropine (At) Echimidine (Em) and Echimidine-N-oxide (EmNO) Echinatine (En) and Echinatine-N-oxide (EnNO) Erucifoline (Er) and Erucifoline-N-oxide (ErNO) Europine (Eu) and Europine-N-oxide (EuNO) Heliosupine (Hs) and Heliosupine-N-oxide (HsNO) Heliotrine (Ht) and Heliotrine-N-oxide (HtNO) Indicine (Id) and Indicine-N-oxide (IdNO) Integerrimine (Ir) and Integerrimine-N-oxide (IrNO) Intermedine (Im) and Intermedine-N-oxide (ImNO) Jacobine (Jb) and Jacobine-N-oxide (JbNO) Jacoline (Jl) Jaconine (Jn) Lasiocarpine (Lc) and Lasiocarpine-N-oxide (LcNO) Lycopsamine (Ly) and Lycopsamine-N-oxide (LyNO) Monocrotaline (Mc) and Monocrotaline-N-oxide (McNO) Retrorsine (Rt) and Retrorsine-N-oxide (RtNO) Rinderine (Rn) and Rinderine-N-oxide (RnNO) Scopolamine (Sc) Senecionine (Sn) and Senecionine-N-oxide (SnNO) Seneciphylline (Sp) and Seneciphylline-N-oxide (SpNO) Senecivernine (Sv) and Senecivernine-N-oxide (SvNO) Senkirkine (Sk) Spartioidine (St) and Spartioidine-N-oxide (StNO) Trichodesmine (Td) Usaramine (Us) and Usaramine-N-oxide (UsNO)
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:
8 Substances have been added to the scope of the method. The method has been revised to accommodate the analysis of PA isomers. A procedure to quantify PAs in samples present at elevated concentrations has been added.

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Notices:
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1. Introduction

Pyrrolizidine 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 (At) and scopolamine (Sc) 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.

The European Commission is considering legislation on the presence of PAs in various food products with priority on (herbal) teas and herbal supplements, based on a risk assessment conducted by EFSA [1]. The EC has identified 21 PAs as relevant for food: echimidine (Em), echimidine-N-oxide (EmNO), europine (Eu), europine-N-oxide (EuNO), heliotrine (Ht), heliotrine-N-oxide (HtNO), intermedine (Im), intermedine-N-oxide (ImNO), lasiocarpine (Lc), lasiocarpine-N-oxide (LcNO), lycopsamine (Ly), lycopsamine-N-oxide (LyNO), retrorsine (Rt), retrorsine-N-oxide (RtNO), senecionine (Sn), senecionine-N-oxide (SnNO), seneciphylline (Sp), seneciphylline-N-oxide (SpNO), senecivernine (Sv), senecivernine-N-oxide (SvNO), and senkirkine (Sk).
For several of the PAs that have been identified by the EC as relevant for food, also isomeric analogues are known to occur naturally. From a regulatory standpoint it is relevant that in the analytical method these isomeric PA analogues can be separated from the PAs included in legislation. The 14 relevant isomeric PAs are: echinatine (En), echinatine-N-oxide (EnNO), heliosupine (Hs), heliosupine-N-oxide (HsNO), indicine (Id), indicine-N-oxide (IdNO), integerrimine (Ir), integerrimine-N-oxide (IrNO), rinderine (Rn), rinderine-N-oxide (RnNO), spartioidine (St), spartioidine-N-oxide (StNO), usaramine (Us) and usaramine-N-oxide (UsNO).
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 35 PAs listed above and in addition: erucifoline (Er), erucifoline-N-oxide (ErNO), jacobine (Jb), jacobine-N-oxide (JbNO), jacoline (Jl), jaconine (Jn), monocrotaline (Mc), monocrotaline-N-oxide (McNO) and trichodesmine (Td).

2. Scope

This document describes describes the confirmation and -by means of standard addition to the sample- the quantification of the following PAs: echimidine, echimidine-N-oxide, echinatine, echinatine-N-oxide, erucifoline, erucifoline-N-oxide, europine, europine-N-oxide, heliosupine, heliosupine-N-oxide, heliotrine, heliotrine-N-oxide, indicine, indicine-N-oxide, integerrimine, integerrimine-N-oxide, intermedine, intermedine-N-oxide, jacobine, jacobine-N-oxide, jacoline, jaconine, lasiocarpine, lasiocarpine-N-oxide, lycopsamine, lycopsamine-N-oxide, monocrotaline, monocrotaline-N-oxide, retrorsine, retrorsine-N-oxide, rinderine, rinderine-N-oxide, senecionine, senecionine-N-oxide, seneciphylline, seneciphylline-N-oxide, senecivernine, senecivernine-N-oxide, senkirkine, spartioidine, spartioidine-N-oxide, trichodesmine, usaramine and usaramine-N-oxide. The method is also suited for the following tropane alkaloids (TAs): atropine and scopolamine. 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, using, optionally, alkaline or acidic chromatography. PAs are quantified by means of standard addition to the sample. PAs present in a concentration exceeding 200 µg/kg are quantified by an additional spiking produce to the sample extract.

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 (EmNO)**

4.9 **Echinatine (En)**

4.10 **Echinatine-N-oxide (EnNO)**

4.11 **Erucifoline (Er)**

4.12 **Erucifoline-N-oxide (ErNO)**

4.13 **Europine (Eu)**

4.14 **Europine-N-oxide (EuNO)**

4.15 **Heliosupine (Hs)**

4.16 **Heliosupine-N-oxide (HsNO)**

4.17 **Heliotrine (Ht)**

4.18 Heliotrine-N-oxide (HtNO)

4.19 Indicine (Id)

4.20 Indicine-N-oxide (IdNO)

4.21 Integerrimine (Ir)

4.22 Integerrimine-N-oxide (IrNO)

4.23 Intermedine (Im)

4.24 Intermedine-N-oxide (ImNO)

4.25 Jacobine (Jb)

4.26 Jacobine-N-oxide (JbNO)

4.27 Jacoline (Jl)

4.28 Jaconine (Jn)

4.29 Lasiocarpine (Lc)

4.30 Lasiocarpine-N-oxide (LcNO)

4.31 Lycopsamine (Ly)

4.32 Lycopsamine-N-oxide (LyNO)

4.33 Monocrotaline (Mc)

4.34 Monocrotaline-N-oxide (McNO)

4.35 Retrorsine (Rt)

4.36 Retrorsine-N-oxide (RtNO)

4.37 Rinderine (Rn)

4.38 Rinderine-N-oxide (RnNO)

4.39 Senecionine (Sn)

4.40 Senecionine-N-oxide (SnNO)

4.41 Seneciphylline (Sp)

4.42 Seneciphylline-N-oxide (SpNO)

4.43 Senecivernine (Sv)

4.44 Senecivernine-N-oxide (SvNO)

4.45 Senkirkine (Sk)

4.46 Spartioidine (St)

4.47 Spartioidine-N-oxide (StNO)

4.48 Trichodesmine (Td)

4.49 Usaramine (Us)

4.50 Usaramine-N-oxide (UsNO)

4.51 Atropine (At)

4.52 Scopolamine (Sc)

4.53 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.

4.54 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.

4.55 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.

4.56 Mobile phase A for alkaline chromatography: 10 mM ammonium carbonate in water, pH 9
Mix 10 ml neutralisation solution (4.54) 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.

4.57 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.

4.58 Strong wash solvent for autosampler
Mix 900 ml methanol (4.3) and 100 ml water.

4.59 Weak wash solvent for autosampler
Mix 100 ml methanol (4.3) with 900 ml water.

4.60 Stock solutions (200 mg/l)
Accurately weight between 3 and 5 mg ± 0.02 mg of standards 4.7 to 4.52. 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 (200 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.

4.61 Mixed standard solution 21 PAs (5 mg/l)
Pipette 500 µl of the stock solutions of echimidine, echimidine-N-oxide, europine, europine-N-oxide, heliotrine, heliotrine-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, 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.

4.62 Mixed standard solution 14 PAs isomers (5 mg/l)
Pipette 500 µl of the stock solutions of echinatine, echinatine-N-oxide, heliosupine, heliosupine-N-oxide, integerrimine, integerrimine-N-oxide, indicine, indicine-N-oxide, rinderine, rinderine-N-oxide, spartioidine, spartioidine-N-oxide, usaramine, usaramine-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.

4.63 Mixed standard solution 9 PAs + 2 TAs (5 mg/l)
Pipette 500 µl of the stock solutions of erucifoline, erucifoline-N-oxide, jacobine, jacobine-N-oxide, jacoline, jaconine, monocrotaline, monocrotaline-N-oxide, 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.

4.64 Mixed standard solution 21 PAs (500 µg/l)
Pipette 1000 µl of the mixed standard solution 21 PAs (5 mg/l) (4.61) 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.

4.65 Mixed standard solution 14 PAs isomers (500 µg/l)
Pipette 1000 µl of the mixed standard solution 14 PAs isomers (5 mg/l) (4.62) 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.

4.66 Mixed standard solution 9 PAs + 2 TAs (500 µg/l)
Pipette 1000 µl of the mixed standard solution 9 PAs + 2 TAs (5 mg/l) (4.63) 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.

4.67 Mixed standard solution 44 PAs + 2 TAs (500 µg/l)
Pipette 1000 µl of the mixed standard solution 21 PAs (5 mg/l) (4.61), 1000 µl of the mixed standard solution 14 PAs isomers (5 mg/l) (4.62) and 1000 µl of the mixed standard solution 9 PAs + 2 TAs (5 mg/l) (4.63) 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.

4.68 Working standard solution 21 PAs (10 µg/l)
Pipette 200 µl of the mixed standard solution 21 PAs (500 µg/l) (4.64) in a volumetric flask of 10 ml. Make up to the mark with 10% methanol. This solution is stored at -20°C and can be used for 12 months.

4.69 Working standard solution 14 PAs isomers (10 µg/l)
Pipette 200 µl of the mixed standard solution 14 PAs isomers (500 µg/l) (4.65) in a volumetric flask of 10 ml. Make up to the mark with 10% methanol (4.59). This solution is stored at -20°C and can be used for 12 months.

4.70 Working standard solution 9 PAs + 2 TAs (10 µg/l)
Pipette 200 µl of the mixed standard solution 9 PAs + 2 TAs (500 µg/l) (4.66) in a volumetric flask of 10 ml. Make up to the mark with 10% methanol (4.59). This solution is stored at -20°C and can be used for 12 months.

4.71 Working standard solution 44 PAs + 2 TAs (10 µg/l)
Pipette 200 µl of the mixed standard solution 44 PAs + 2 TAs (500 µg/l) (4.67) in a volumetric flask of 10 ml. Make up to the mark with 10% methanol (4.59). 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 **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**, 50 ml with screw cap

5.8 **Polypropylene tubes**, 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**, polymeric reversed phase sorbent, e.g. Strata-X 200 mg/6 ml or Oasis HLB 150 mg/6 cc

5.14 **Evaporator** with nitrogen flow, suitable for 12 ml tubes

5.15 **Filtervial** (polytetrafluoroethylene (PTFE), 0,45 µm), with press-on cap, e.g. Mini-UniPrep, Whatman, or equivalent.

5.16 **Compressor** for 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 pH higher than 7 (4.56). 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 g.

6.4 Extraction, clean-up and preparation of test solutions

Depending on the groups of PAs to be analysed (21 PAs; 14 PAs isomers; 9 PAs + 2 TAs; or combinations there off) one or more matrix matched calibration curves as well as quality control samples need to be prepared (for each set or combination a separate MMS and QC should be prepared).

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, for each matrix matched standard curve (21 PAs; 14 PAs isomers; 9 PAs + 2 TAs; or combinations there off), 2 individual test portions of 2.00 ± 0.05 g of the blank plant material in PP tubes of 50 ml (5.7). Extract the samples according to 6.4.5. Combine the supernatants of the 2 samples after centrifugation (5.6). Take, for the preparation of each matrix matched standard curve, 8 aliquots of 5 ml of the blank combined extract and purify the extracts according to 6.4.6. Spike the final extracts according to Table 1.

Table 1: Preparation of matrix matched standards (MMS)

	Concentration in blank extract (µg/l)	Concentration in blank matrix (µg/kg)	Mixed standard solution PAs (500 µg/l) (µl) [a]	Mixed standard solution PAs (5 mg/l) (µl) [b]	Methanol (µl)	Water (µl)
MMS 1	0	0	0	0	50	450
MMS 2	5	10	5	0	45	450
MMS 3	12.5	25	12.5	0	37.5	450
MMS 4	25	50	25	0	25	450
MMS 5	50	100	50	0	0	450
MMS 6	125	250	0	12.5	37.5	450
MMS 7	250	500	0	25	25	450
MMS 8	500	1000	0	50	0	450

(a) Use as mixed standard solution: 21 PAs (500 µg/l) (4.64); 14 PAs isomers (500 µg/l) (4.65); 9 PAs + 2 TAs (500 µg/l) (4.66); or combinations there off.

(b) Use as mixed standard solution: 21 PAs (5 mg/l) (4.61); 14 PAs isomers (5 mg/l) (4.62); 9 PAs + 2 TAs (5 mg/l) (4.63); or combinations there off.

6.4.2 Quality control sample limit of quantification (LOQ) (10 µg/kg)

Weigh of the blank plant material used in 6.4.1, depending on the number of PA mixes to be included in the method, 1 to 4 test portions of 2.00 ± 0.05 g in a PP tube of 50 ml (5.7). Add to separate test portions 40 µl of mixed standard solution 21 PAs (500 µg/l) (4.64); 14 PAs isomers (500 µg/l) (4.65); 9 PAs + 2 TAs (500 µg/l) (4.66); or combinations there off. Wait 30 minutes before starting the extraction procedure (6.4.5).

6.4.3 Quality control sample recovery (250 µg/kg)

Weigh of the blank plant material used in 6.4.1, depending on the number of PA mixes to be included in the method, 1 to 4 test portions of 2.00 ± 0.05 g in a PP tube of 50 ml (5.7). Add to separate test portions 100 µl of mixed standard solution of 21 PAs (5 mg/l) (4.61); 14 PAs isomers (5 mg/l) (4.62); 9 PAs + 2 TAs (5 mg/l) (4.63); or combinations there off. Wait 30 minutes before starting the extraction procedure (6.4.5).

6.4.4 Preparation of test samples

Weigh, depending on the number of PA mixes to be included in the method, 2 to 5 test portions of 2.00 ± 0.05 g of the laboratory sample in PP tubes of 50 ml (5.7). One test portion is left unspiked. Add to separate test portions 100 µl of the mixed standard solution of 21 PAs (5 mg/l) (4.61); 14 PAs isomers (5 mg/l) (4.62); 9 PAs + 2 TAs (5 mg/l) (4.63); or combinations there off. The added level is 250 µg/kg. Wait 30 minutes before starting the extraction procedure (6.4.5).

6.4.5 Extraction of samples

Add 40 ml extraction solvent (4.53) 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 (4.54). 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).

6.4.6 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 (6.4.5) onto the cartridge. Wash the cartridge with 6 ml 1% formic acid solution (4.55), 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°C ± 5°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.

7. LC-MS/MS conditions

Choose an analytical column, mobile phase and gradient settings such that the requirement laid out in 5.17 are met. Below examples for the separation of PAs and TAs are given.

LC conditions:

LC system:	Waters Acquity
Column:	Alkaline chromatography: Waters Acquity UPLC BEH C18 1.7 µm 2.1 x 150 mm Acidic chromatography: Waters Acquity UPLC CSH 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) (4.58)
Weak wash:	methanol/water (10/90) (4.59)
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 (4.56) (%)	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 (4.57) (%)	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.

7.1 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 µg/l (4.68, 4.69, 4.70 and/or 4.71). The sensitivity is visually checked for the most critical component in each window.

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

PA	PA group	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	3	290.2	25	93	25	124	20	91	35	7.95	6.95
Im	1	300.2	30	94	35	156	30	138	30	5.60*	4.15
Ly	1	300.2	30	94	35	156	30	138	30	5.65*	4.25*
Id	2	300.2	30	94	35	156	30	138	30	5.65*	4.30*
En	2	300.2	30	138	30	156	30	94	35	6.55	4.40*
Rn	2	300.2	30	138	30	156	30	94	35	6.65	4.40*
Sc	3	304.2	25	138	20	156	25	103	35	8.60	4.80
Ht	1	314.2	30	138	25	156	25	94	35	8.25	6.30
ImNO	1	316.2	30	94	40	172	30	111	40	3.55*	5.00*
LyNO	1	316.2	30	94	40	172	30	111	40	3.60*	5.15
IdNO	2	316.2	30	94	40	172	30	111	40	3.70	5.05*
EnNO	2	316.2	30	111	40	172	30	94	40	3.90	4.85*
RnNO	2	316.2	30	111	40	172	30	94	40	4.00	4.90*
Mc	3	326.2	40	94	35	120	30	121	30	5.75	2.30
Eu	1	330.2	30	94	35	138	30	156	30	6.30	4.40
HtNO	1	330.2	30	111	35	172	25	94	40	5.70	6.90
St	2	334.2	40	120	30	138	30	94	40	9.15	6.35
Sp	1	334.2	40	120	30	138	30	94	40	9.45	6.50
Ir	2	336.2	40	94	40	120	30	138	30	10.15	7.60*
Sn	1	336.2	40	94	40	120	30	138	30	10.40	7.85
Sv	1	336.2	40	94	40	120	30	138	30	10.55	7.65*
McNO	3	342.2	40	120	35	137	30	94	40	2.60	4.25
EuNO	1	346.2	30	172	30	111	40	256	25	3.75	4.85
Er	3	350.2	40	94	40	120	30	138	30	7.40	3.85
StNO	2	350.2	40	94	40	138	30	118	30	5.85	7.15
SpNO	1	350.2	40	94	40	138	30	118	30	5.95	7.30
Jb	3	352.2	40	120	30	155	30	94	40	7.80	4.50
Us	2	352.2	40	94	40	120	30	138	30	8.40	5.90
Rt	1	352.2	40	94	40	120	30	138	30	8.65	6.00
IrNO	2	352.2	40	94	40	120	30	136	30	6.60	8.40
SnNO	1	352.2	40	94	40	120	30	136	30	6.80*	8.60
SvNO	1	352.2	40	94	40	120	30	136	30	6.85*	8.30
Td	3	354.2	40	120	35	222	30	121	30	8.80	5.90
ErNO	3	366.2	40	94	40	118	30	120	30	3.40	4.85
Sk	1	366.2	30	122	30	168	25	150	25	7.20	8.95
JbNO	3	368.2	40	120	30	296	25	119	30	4.50	5.50
UsNO	2	368.2	40	94	40	120	30	119	30	5.35	6.50*
RtNO	1	368.2	40	94	40	120	30	119	30	5.50	6.55*
Jl	3	370.2	40	94	40	138	30	120	30	5.55	2.60
Jn	3	388.2	40	94	40	120	30	138	30	8.95	5.65
Hs	2	398.2	30	120	25	220	20	336	20	10.55	9.00
Em	1	398.2	30	120	25	220	20	83	25	10.70	9.10
Lc	1	412.2	30	120	25	220	20	336	20	11.45	9.65
HsNO	2	414.2	30	94	30	254	30	138	30	7.25	9.90
EmNO	1	414.2	30	254	30	352	25	94	40	7.55	9.20
LcNO	1	428.2	30	138	30	254	25	94	40	8.40	10.65

*: isomeric compounds that are not fully chromatographically separated

7.2 Injection sequence

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

- Standard working solution 10 µg/l (4.68, 4.69, 4.70 and/or 4.71)
- Water (4.1)
- MMS in blank plant material (6.4.1)
- Water (4.1)
- Quality control sample LOQ (10 µg/kg) (6.4.2)
- Matrix matched recovery sample (250 µg/kg) (6.4.3)
- Water (4.1)
- Sample extracts (6.4.6)
- Standard working solution 10 µg/l (4.68, 4.69, 4.70 and/or 4.71)

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 and sensitivity of the LC-MS/MS system. For the MMS series, the peak area is plotted as function of the added concentration in the sample extract (µg/l). 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 is equal to or exceeding 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**.

Note: for calculation of the reference ion ratio use only responses with an S/N > 6. For the higher concentrations, exclude peak areas exceeding the linear range from calculation of the reference ion ratio.

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

$$D = \left(\frac{IR_{sample} - IR_{average}}{IR_{average}}\right) \times 100\%$$

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 (%)
IR_{sample} = ion ratio of the analyte in the sample (%) (**Equation III**)
$IR_{average}$ = average ion ratio of the analyte in MMS 3 to MMS 7 (%) (**Equation III**)

Equation III: Ion ratio (IR)

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

Where:
IR = ion ratio (%)
A_{low} = area of the product ion with the lowest intensity (qualifier ion)
A_{high} = area of the product ion with the highest intensity (quantifier ion)

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 10 sample (6.4.2) with **Equation IV**, using the recovery sample QC 250 (6.4.3) as fortified sample. The calculated concentration in the QC 10 sample should be between 70 and 120% of the added level.

Equation IV: Concentration in the sample (C)

$$C = \left(\frac{A_{sample}}{A_{added} - A_{sample}}\right) \times C_{added}$$

Where:
C_{sample} = concentration of the analyte in the sample (µg/kg)
A_{sample} = sum area of the analyte in the sample
A_{added} = sum area of the analyte in the fortified sample

C_{added} = concentration of the analyte added in the fortified sample (µg/kg)

8.3.2 Recovery

Calculate the recovery of the PAs with **Equation V**. The recovery is calculated for information only and is in this method not considered as a critical parameter. The recovery is calculated by comparison of the recovery sample QC 250 (6.4.3) with the MMS 6 (6.4.1).

Equation V: Recovery (R)

$$R = \left(\frac{A_{QC\ 250}}{A_{MMS\ 6}}\right) \times 100\%$$

Where:

R = recovery (%)

A_{QC250} = sum area of the analyte in recovery sample QC 250 (6.4.3), fortified at 250 µg/kg

$A_{MMS\ 6}$ = sum area of the analyte in the MMS 6 sample (6.4.1), spiked at 125 µg/l (corresponding to 250 µg/kg)

8.3.3 Quantification

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

When the calculated concentration of one or more analytes in the sample is higher than 200 µg/kg, it is necessary to reanalyse the sample for these analytes by a spiking procedure to the final extract according to Table 5. Aliquots of 50 (µl) of sample extract are spiked with different concentrations of the analytes, depending on the estimated concentration in the sample extract and methanol and water are added to obtain a dilution factor of 20 calculated from the starting test sample. Dilute MMS extract 1 and 6 (6.4.1) and the recovery sample extract 250 µg/kg (QC 250) (6.4.3) 10 times as well. The MMS 6 and QC 250 samples are used to correct for recovery losses during the sample preparation procedure.

Table 5: Reanalysis of samples containing analytes that exceed 200 µg/kg

	Concentration in sample extract (µg/l)	Concentration in matrix (µg/kg)	Sample extract (6.4.6) (µl)	Mixed standard solution PAs (500 µg/l) (µl) [a]	Mixed standard solution PAs (5 mg/l) (µl) [b]	Methanol (µl)	Water (µl)
S 1	0	0	50	0	0	50	400
S 2	12.5	250	50	12.5	0	37.5	400
S 3	50	1000	50	50	0	0	400
S 4	200	4000	50	0	20	30	400

(a) Use mixed standard solution: 21 PAs (500 µg/l) (4.64); 14 PAs isomers (500 µg/l) (4.65); 9 PAs + 2 TAs (500 µg/l) (4.66); or combinations there off, depending on the PAs that in the sample exceed 200 µg/kg.

(b) Use mixed standard solution: 21 PAs (5 mg/l) (4.61); 14 PAs isomers (5 mg/l) (4.62); 9 PAs + 2 TAs (5 mg/l) (4.63); or combinations there off, depending on the PAs that in the sample exceed 800 µg/kg.

Calculate the concentration in the diluted extract with **Equation VI**. For concentrations in the range 200 to 800 µg/kg, use S 3 and for concentrations above 800 µg/kg use S 4.

Equation VI: Concentration in the sample (C) using diluted extracts

$$C = \left(\frac{A_{sample}}{A_{added} - A_{sample}}\right) \times S_{added} \times DF \times \frac{A_{MMS\ 6}}{A_{QC\ 250}}$$

Where:

C_{sample} = concentration of the analyte in the sample (µg/kg)
A_{sample} = sum area of the analyte in the diluted sample extract
A_{added} = sum area of the analyte in the spiked sample extract
S_{added} = concentration of the analyte added in the spiked sample extract (µg/l)
DF = dilution factor (ratio between test sample amount and final extract volume)
$A_{MMS\ 6}$ = sum area of the analyte in the diluted MMS 6 sample extract
$A_{QC\ 250}$ = sum area of the analyte in the diluted QC 250 recovery sample extract

8.4 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

- ☐ Weigh for each MMS series 2 portions of 2.00 ± 0.05 g blank plant material in 50 ml polypropylene tubes
- ☐ Extract the samples according to 6.4.5
- ☐ Combine the blank extracts
- ☐ Take 8 aliquots of 5 ml in 12 ml tubes
- ☐ Purify extracts according to 6.4.6
- ☐ Spike final extracts according to Table 1

Table 1: Preparation of MMS extracts

	Concentration in blank extract (µg/l)	Concentration in blank matrix (µg/kg)	Mixed standard solution PAs (500 µg/l) (µl) [a]	Mixed standard solution PAs (5 mg/l) (µl) [b]	Methanol (µl)	Water (µl)
MMS 1	0	0	0	0	50	450
MMS 2	5	10	5	0	45	450
MMS 3	12.5	25	12.5	0	37.5	450
MMS 4	25	50	25	0	25	450
MMS 5	50	100	50	0	0	450
MMS 6	125	250	0	12.5	37.5	450
MMS 7	250	500	0	25	25	450
MMS 8	500	1000	0	50	0	450

(a) The mixed standard solution can be: 21 PAs (500 µg/l) (4.64); 14 PAs isomers (500 µg/l) (4.65); 9 PAs + 2 TAs (500 µg/l) (4.66); or combinations there off.

(b) The mixed standard solution can be: 21 PAs (5 mg/l) (4.61); 14 PAs isomers (5 mg/l) (4.62); 9 PAs + 2 TAs (5 mg/l) (4.63); or combinations there off.

QC sample LOQ (10 µg/kg)

- ☐ Weigh 1 to 4 portions of 2.00 ± 0.05 g blank plant material in 50 ml polypropylene tubes
- ☐ Add to separate test portions 40 µl mix 21 PAs (500 µg/l) (4.64); 14 PAs isomers (500 µg/l) (4.65); 9 PAs + 2 TAs (500 µg/l) (4.66); or combinations there off

Recovery sample (250 µg/kg)

- ☐ Weigh 1 to 4 portions of 2.00 ± 0.05 g blank plant material in 50 ml polypropylene tubes

- ☐ Add to separate test portions 100 µl mix 21 PAs (5 mg/l) (4.61); 14 PAs isomers (5 mg/l) (4.62); 9 PAs + 2 TAs (5 mg/l) (4.63); or combinations there off.

Sample preparation

- ☐ Weigh 2 to 5 portions of 2.00 ± 0.05 g sample in 50 ml polypropylene tubes
- ☐ One test portion is left unspiked. Add to separate test portions 100 µl mix 21 PAs (5 mg/l) (4.61); 14 PAs isomers (5 mg/l) (4.62); 9 PAs + 2 TAs (5 mg/l) (4.63); or combinations there off.

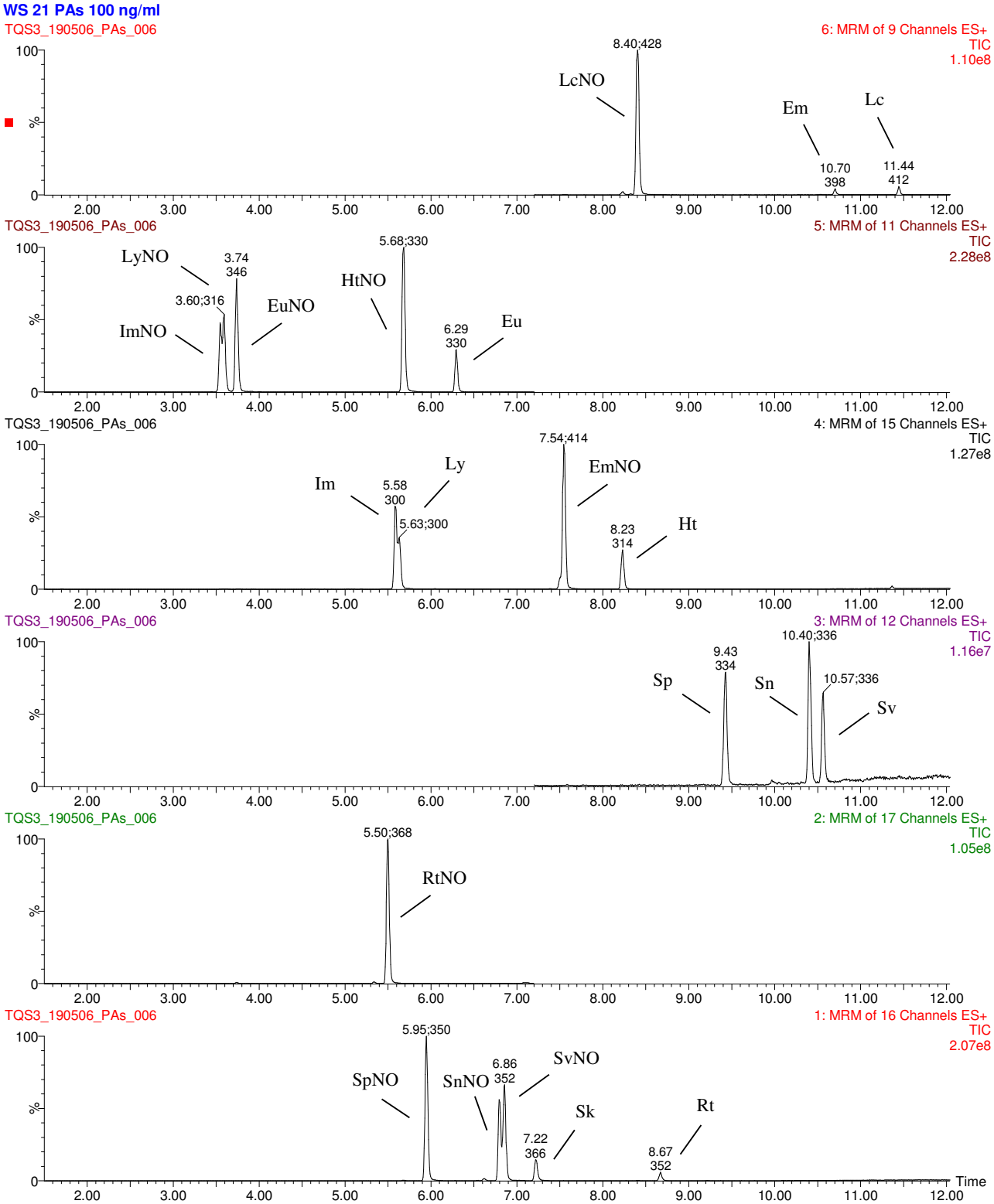
Sample extraction

- ☐ Wait 30 min
- ☐ Add 40 ml of 0.2% formic acid solution (6.55)
- ☐ 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

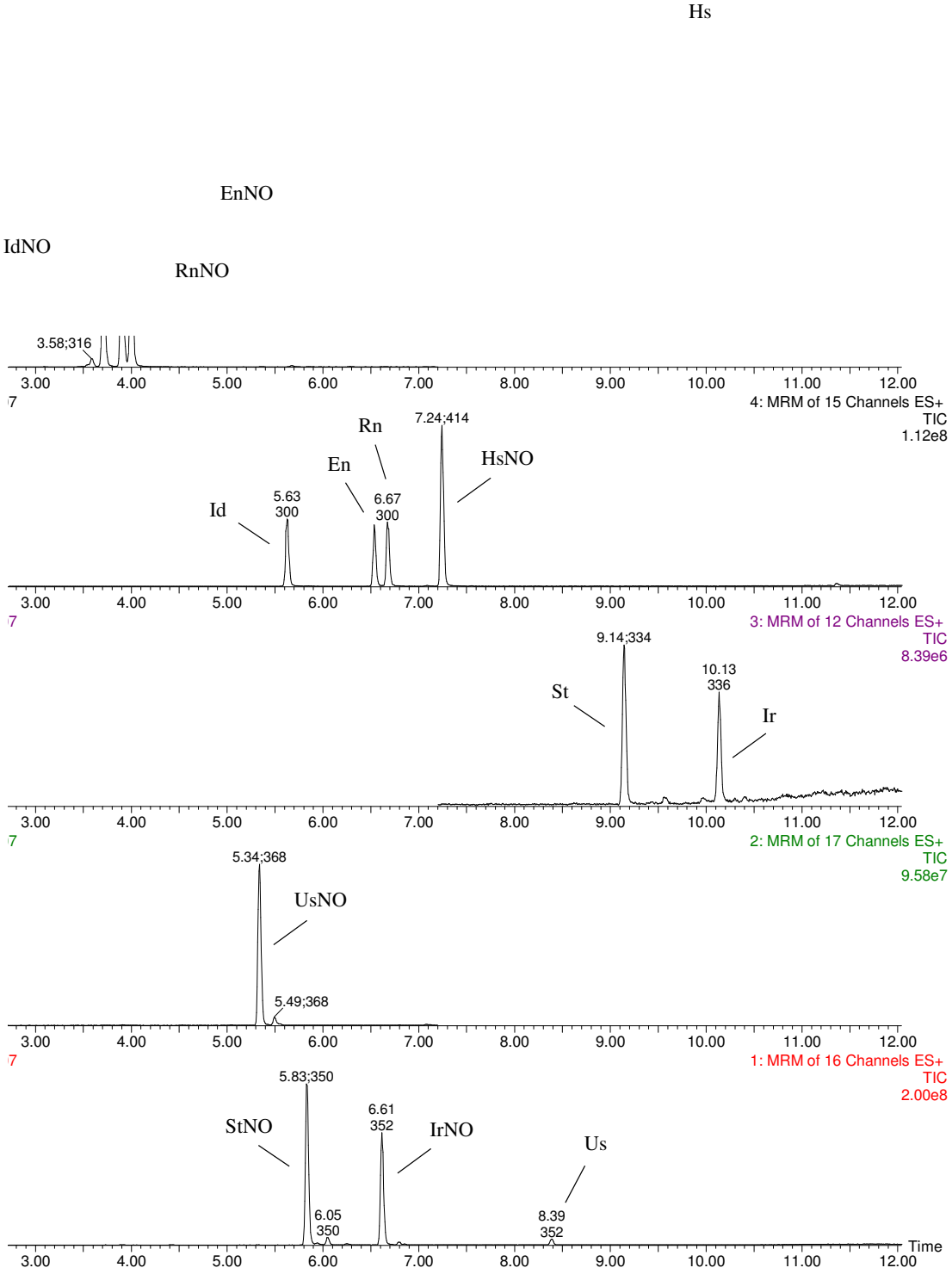
Solid phase purification

- ☐ Activate SPE cartridge 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 N_2 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

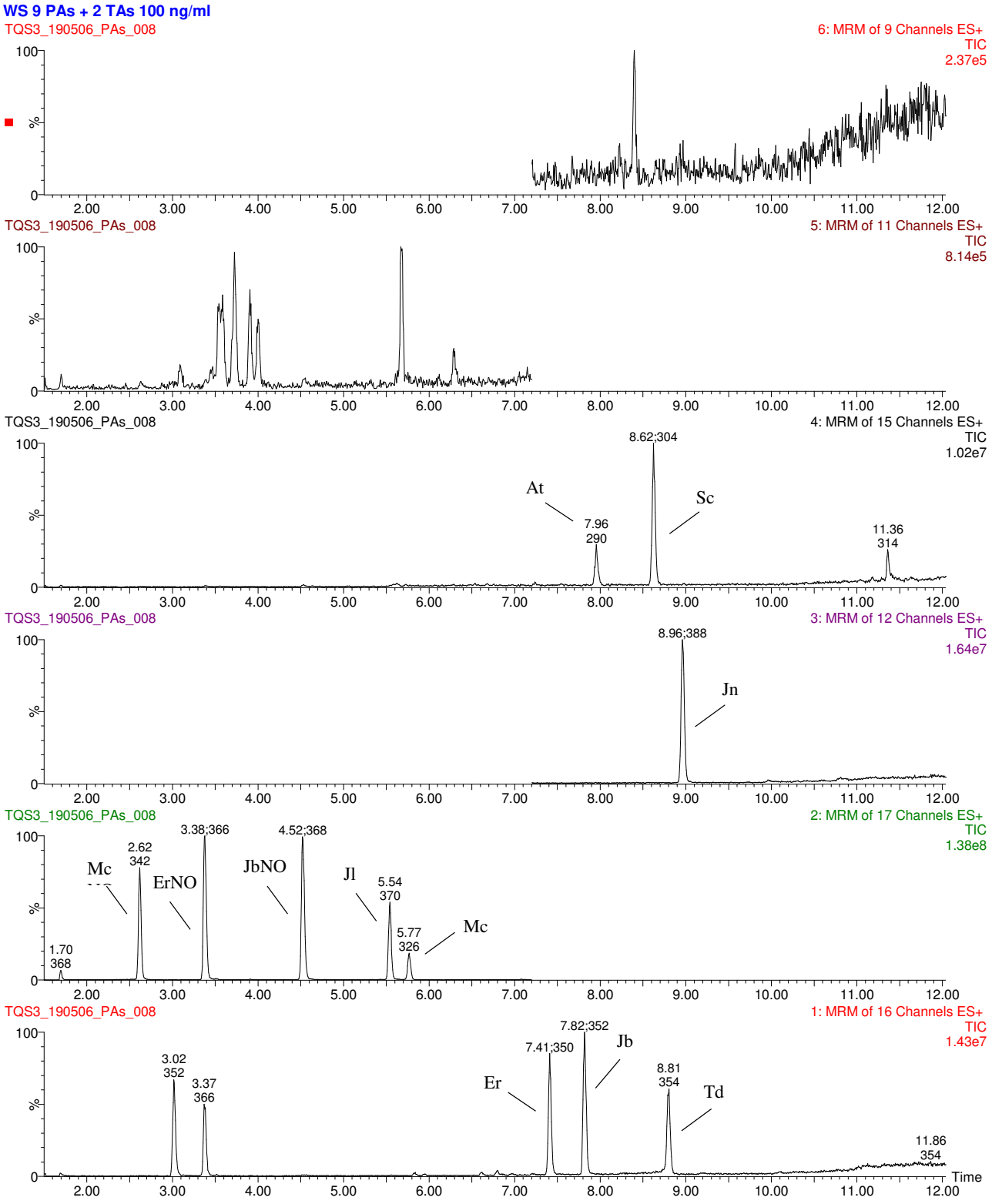
Annex B1. LC-MS/MS chromatogram (alkaline method): mix 21 PAs



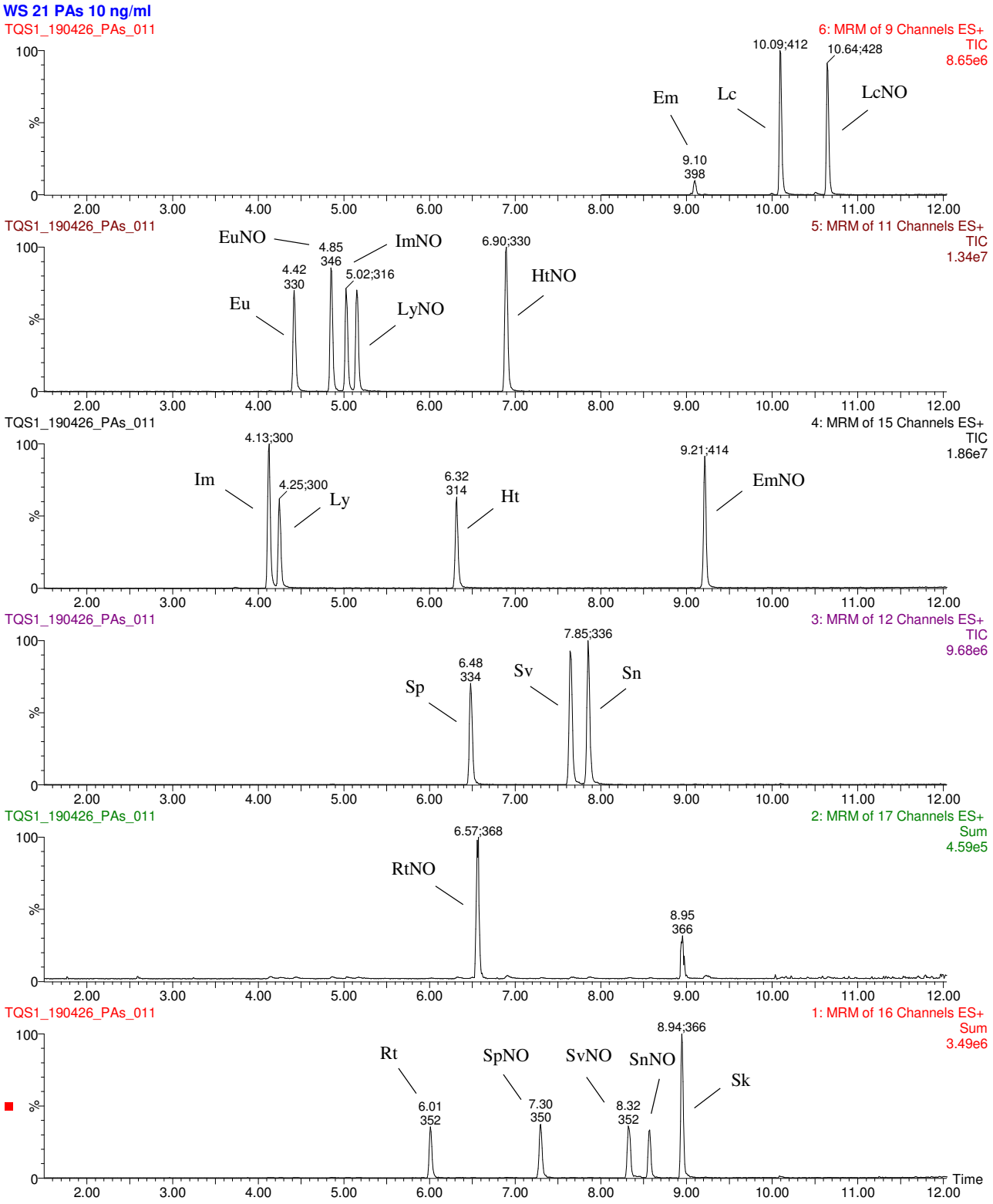
Annex B2. LC-MS/MS chromatogram (alkaline method): mix 14 PAs isomers



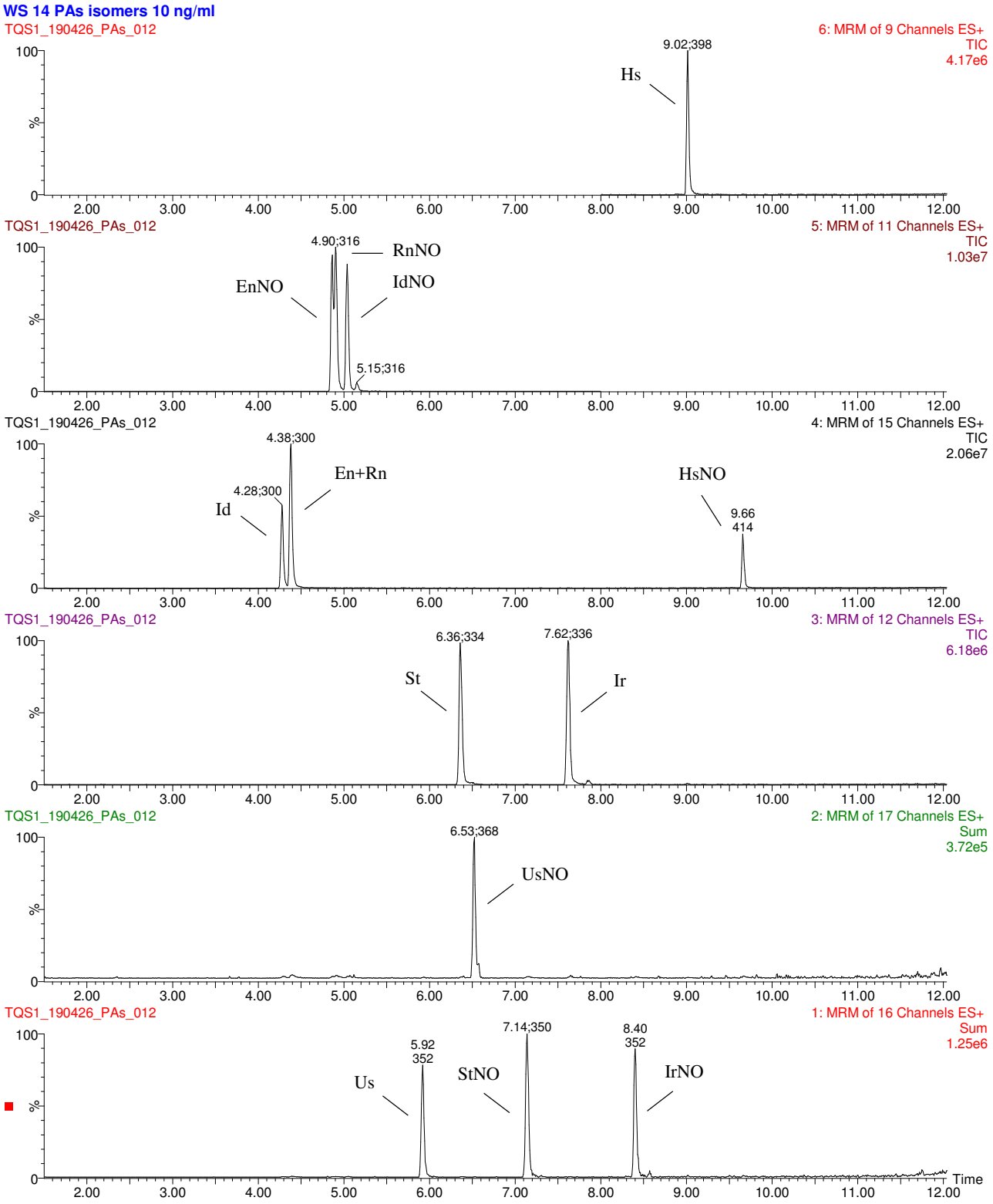
Annex B3. LC-MS/MS chromatogram (alkaline method): mix 9 PAs + 2 TAs



Annex C1. LC-MS/MS chromatogram (acidic method): mix 21 PAs



Annex C2. LC-MS/MS chromatogram (acidic method): mix 14 PAs isomers



Annex C3. LC-MS/MS chromatogram (acidic method): WS 9 PAs + 2 TAs