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European Union Reference Laboratory

EURL-MP-method_004 (version 1)
Determination of tropane alkaloids in processed cereal-based foods for infants and young children by LC-MS/MS

Analyte group:	**Plant toxins** - tropane alkaloids
Analyte(s):	Atropine Scopolamine
Commodity group:	Processed cereal-based foods for infants and young children
Commodities validated:	Breakfast cereals, biscuits, muesli
Technique:	Liquid chromatography / tandem mass spectrometry (LC-MS/MS)

Modifications compared to previous version:
Not applicable

Method drafted by:
EU Reference Laboratory for mycotoxins & plant toxins in food and feed (EURL-MP)
Wageningen food Safety research (WFSR), part of Wageningen University & Research
Akkermaalsbos 2, 6708 WB, Wageningen, the Netherlands
eurl.mycotoxins-planttoxins@wur.nl

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1 Introduction

Tropane alkaloids (TAs) are secondary metabolites produced by a wide variety of plants from the families of Brassicaceae, Convolvulaceae, Moraceae and Solanaceae. Most important weed species in this respect are *Datura stramonium* (thorn apple) and *Atropa belladonna* (deadly nightshade). TAs are regarded as undesirable substances in food and feed and for that reason have been the subject of an EFSA opinion, published in 2013, in which an ARfD of 0.016 µg kg^{-1} b.w. was derived [1]. More than 200 different TA have been identified in the various plant species. However, sufficient data on toxicity and occurrence in food are available only for the TAs atropine and scopolamine (Figure 1), which are regarded the most important representatives of this class of metabolites. Atropine is the racemic mixture of (-)-hyoscyamine and (+)-hyoscyamine (synonyms D- and L- hyoscyamine) [1]. Atropine and scopolamine are strong antimuscarinic agents.

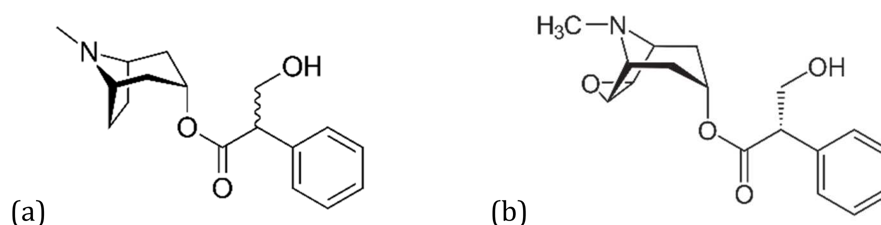

Figure 1 Chemical structures of atropine (a) and scopolamine (b)

Food crops, such as cereals, can be contaminated when TA containing weeds are co-harvested. Common practices for cleaning cereals are not always sufficient to remove the weed plant parts and seeds. Legal limits for TAs in foods were issued in 2016 with Regulation (EU) 2016/239 on maximum levels of TAs in certain cereal-based foods for infants and young children, amending Regulation (EC) No 1881/2006 [2,3]. The maximum limit for these products is 1 µg/kg for atropine and 1 µg/kg for scopolamine.

2 Scope

This document describes the confirmation and – by means of deuterated internal standards added to the sample – the quantification of the TAs atropine and scopolamine in processed cereal-based foods for infants and young children, in the range of 0.5 to 10 µg/kg by liquid chromatography tandem mass spectrometry (LC-MS/MS). The enantiomers of atropine cannot be separated with the method described in this SOP.

3 Principle

The alkaloids are extracted from the sample by mixing 4 g of homogenised sample with 40 ml of methanol/water (60/40, v/v) containing 0.4% formic acid. The mixture is shaken for 30 min on a rotary tumbler. After centrifugation, a portion of the supernatant is further purified by passing it through a 30 kD ultrafilter. The filtrate is transferred to a vial and analysed by injecting 2-5 µl on a reverse-phase column to separate the analytes, followed by MS/MS detection.

4 Reagents

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

4.1 Analytical standards

4.1.1 Atropine, e.g. reference solution in acetonitrile or solid substance

4.1.2 Scopolamine (hydrobromide), e.g. reference solution in acetonitrile or solid substance

4.1.3 Atropine-d$_3$, e.g. reference solution in acetonitrile or solid substance

4.1.4 Scopolamine-d$_3$, e.g. reference solution in acetonitrile or solid substance

4.2 Chemicals & solutions

4.2.1 Water, deionised Milli-Q and with a minimal resistance of 18.2 MΩ/cm

4.2.2 Methanol, LC-MS grade

4.2.3 Acetonitrile, LC-MS grade

4.2.4 Formic acid, 99-100%

4.2.5 Ammonia, 25%, p.a. quality

4.2.6 Ammonium carbonate, p.a. quality

4.2.7 Extraction solvent: 0.4% formic acid in methanol/water (60/40, v/v)
Mix 600 ml of methanol (4.2.2) with 400 ml of water (4.2.1) and 4 ml of formic acid (4.2.4).

4.2.8 Mobile phase A, 10 mM ammonium carbonate in water, pH 9.0
Dissolve 0.96 g of ammonium carbonate (4.2.6) in 1000 ml of water (4.2.1). Check pH and adjust, when necessary, to pH 9.0±0.1 by adding formic acid (4.2.4) or 25% ammonia (4.2.5).

4.2.9 Stock solutions 100 mg/l
Accurately weigh into separate amber coloured glass bottles between 2 and 5 mg ± 0.02 mg of the tropane alkaloids and internal standards (4.1.1 to 4.1.4). However, when the standard is only available in a quantity of 3 mg or less, the entire content of the container is used. In that case the weight reported by the supplier is used. Add a volume of acetonitrile (4.2.3) in such a way that the concentration of the solution is 100 mg/l. Take into account the weight, the purity and the appearance form of the standard.

4.2.10 Mixed standard solution 1 mg/l
Pipette 500 µl of the stock solutions 100 mg/l (4.2.9) of atropine and scopolamine in a calibrated volumetric flask of 50 ml, make up the volume with acetonitrile and mix. Transfer the contents to an amber coloured glass bottle of 50 ml. Store the solution in the dark at -18°C

4.2.11 Mixed standard solution 200 µg/l

Pipette 4 ml of the mixed standard solution 1 mg/l (4.2.10) in a calibrated volumetric flask of 20 ml, make up the volume with acetonitrile and mix. Transfer the contents to an amber coloured glass bottle of 20 ml. Store the solution in the dark at -18°C.

4.2.12 Mixed standard solution 10 µg/l

Pipette 1 ml of the mixed standard solution 200 µg/l (4.2.11) in a calibrated volumetric flask of 20 ml, make up the volume with acetonitrile and mix. Transfer the contents to an amber coloured glass bottle of 20 ml. Store the solution in the dark at -18°C.

4.2.13 Mixed internal standard solution 1 mg/l

Pipette 500 µl of the stock solutions 100 mg/l (4.2.9) of atropine-d_3 and scopolamine-d_3 in a calibrated volumetric flask of 50 ml, make up the volume with acetonitrile and mix. Transfer the contents to an amber coloured glass bottle of 50 ml. Store the solution in the dark at -18°C.

4.2.14 Mixed working standard solution 10 µg/l

Pipette 200 µl of the mixed standard solution 1 mg/l (4.2.10) and 200 µl of the mixed internal standard solution 1 mg/l (4.2.13) in a calibrated volumetric flask of 20 ml, make up the volume with extraction solution and mix. Transfer the contents to an amber coloured glass bottle of 20 ml. The solution is prepared fresh for each new series.

5 Equipment

Any reference to type and/or product is only to inform the user and to identify the equipment and does not imply exclusion of similar equipment.

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

5.1 **Analytical balance**, accuracy: 0.01 mg

5.2 **Laboratory balance**, accuracy: 0.01 g

5.3 **Pipets adjustable**, e.g 1 µl to 10 µl, 10 µl to 100 µl, 100 µl to 1000 µl, and 1 ml to 5 ml 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**, adjustable

5.6 **Centrifuge**, capable of generating a relative centrifugal force of 3,500 g. suitable for 12 and 50 ml centrifuge tubes (5.7, 5.8) and ultrafilters (5.9)

5.7 **Polypropylene tubes**, 12 ml with screw cap or plug cap

5.8 **Polypropylene tubes**, 50 ml with screw cap

5.9 **Ultrafilter**, 4 ml (e.g. Millipore, Amicon Ultra-4 Ultracel 30kD)

5.10 **Amber coloured glass bottle**, 20 or 50 ml

5.11 pH meter

5.12 Glass HPLC vial, 2 ml

5.13 Ultrasonic bath

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

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

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

5.14.3 Analytical column, capable of retaining the target TAs and capable of baseline separation of TAs.

5.14.4 Column oven, capable of maintaining a constant temperature of 50°C.

5.14.5 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.

6 Procedures

This document describes the quantification of the TAs atropine and scopolamine in processed cereal-based foods for infants and young children. Quantification can be performed by matrix matched calibration combined with internal standard correction The steps described in section 6.3 are presented in the format of a checklist in Annex A.

Note: optionally the quantification can be performed by single level standard addition to the sample, e.g. in case internal standards of atropine and scopolamine are not available. *See section 6.3.4.*

6.1 Preparation of the test sample

For screening purposes, the sample needs to be ground through a sieve of 1 mm or smaller. For the confirmation of TAs in the sample, the sample needs to be ground through a sieve of 0.5 mm or smaller. Dry samples are stored at room temperature.

6.2 Test portion

For dry milled samples, the amount of homogenised test sample examined is 4.0 ± 0.1 gram. If desirable, higher amounts can be used, provided that the solutions/reagents mentioned in 6.3 are adjusted proportionally.

6.3 Extraction, clean-up and preparation of test solutions

6.3.1 Preparation of matrix matched calibration standards

The calibration standards are prepared by addition of standards to blank samples before extraction. Choose a blank processed food material, in which according to previous analyses no tropane alkaloids

were detected. The blank material should match with most of the samples to be analysed (e.g. breakfast cereals, rusks or cookies).

Weigh 6 test portions of 4.0 ± 0.1 g in tubes of 50 ml (5.8). Add mixed standard solution and mixed IS solution according to Table 1. Wait 30 minutes before starting the extraction procedure (6.3.4).

Table 1 Preparation of matrix-matched calibration standards (MMC)

Code	Conc. in sample (µg/kg)	Mixed standard solution 200 µg/l (4.2.11) (µl)	Mixed IS solution 1 mg/l (4.2.13) (µl)
MMC 1	0	0	20
MMC 2	0.5	10	20
MMC 3	1	20	20
MMC 4	2.5	50	20
MMC 5	5	100	20
MMC 6	10	200	20

6.3.2 Quality control sample accuracy (1 µg/kg)

Weigh 4.0 ± 0.1 g of the blank food material in a tube of 50 ml (5.8). Add 20 µl of mixed standard solution 200 µg/l (4.2.11) to the sample corresponding to an added level of 1 µg/kg. Add 20 µl of mixed IS solution 1 mg/l (4.2.13). Wait 30 minutes before starting the extraction procedure (6.3.4).

6.3.3 Quality control sample recovery (MMRS) (1 µg/kg)

Weigh 4.0 ± 0.1 g of the blank food material in a tube of 50 ml (5.8). Add 20 µl of mixed IS solution 1 mg/l (4.2.13). Wait 30 minutes before starting the extraction procedure (6.3.4). Transfer an aliquot of 950 µl of the ultrafiltrate to a HPLC vial (5.12). Add 10 µl of mixed standard solution 10 µg/l (4.2.12) and 40 µl extraction solvent (4.2.7) to the vial. Close the vial and mix the contents (5.4).

6.3.4 Sample preparation

Weigh a test portion of 4 g of the sample in a tube of 50 ml (5.8). Add 20 µl of mixed IS solution 1 mg/l (4.2.13). Let the sample equilibrate for 30 min before starting the extraction procedure. Add 40 ml extraction solvent (4.2.7) to the test portion and mix using a vortex mixer (5.4). Place the tubes during 30 minutes in a rotary tumbling machine (5.5). Centrifuge the tube during 15 minutes at 3500 g (5.6). Transfer 2 ml of the supernatant to an ultrafilter tube (5.9) and centrifuge the tube during 15 minutes at 3500 g at room temperature. Transfer 950 µl of the ultrafiltrate to an HPLC vial (5.12). Add 50 µl extraction solvent (4.2.7) to the vial. Close the vial and mix the contents (5.4).

Note: optionally the quantification can be performed by single level standard addition to the sample, e.g. in case internal standards of atropine and scopolamine are not available. To this end, weigh two test portions of 4 g of the sample in tubes of 50 ml (5.8). Add to one of the tubes 100 µl of mixed standard solution 200 µg/l (4.2.11) corresponding to an added level of 5 µg/kg. Perform the sample preparation as described above.

7 LC-MS/MS analysis

Chromatographic and mass spectrometric conditions may be chosen freely. The optimal measurement conditions strongly depend on the instrumentation used. However, important criteria and parameters with respect to the chromatographic separation and detection of the analytes are:

The chosen column dimensions and chromatographic conditions should be appropriate to obtain base line separation of atropine and scopolamine from compounds with the same mass-to-charge ratio. For chromatographic separation mobile phases may be used over the range pH 2 to 12. It should be noted that 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.

The injection volume should be optimised for the column dimension and the sensitivity of the mass spectrometric system. The use of large volume injections may result in distorted peak shapes.

The chosen mass spectrometric conditions should be appropriate to measure the analytes with sufficient sensitivity and specificity. Preferably, the protonated molecular parent ion should be selected as precursor ion and the product ions should be specific for the compound. Preferably, product ions that are formed by the loss of water from the protonated molecular parent ion should not be selected. Select at least two precursor-to-product ion combinations to be included in the multiple reaction monitoring (MRM) method. Each chromatographic peak should be composed of at least 10 data points.

The analytical series should not be started before it is verified, by injecting the working standard (4.2.14) at least three times, that the system produces stable analyte retention times and that the sensitivity of the detector is sufficient and stable. The system should be able to detect the product ion with the lowest intensity with an S/N ratio of at least 500 in the working standard solution of 10 µg/l (4.2.14).

Example LC-MS/MS conditions and an example LC-MS/MS chromatogram are given in Annex B.

7.1 Injection sequence

Analyse the sample extracts in the order as given below.

- Standard working solution 10 µg/l (4.2.14)
- Extraction solvent (4.2.7) or mobile phase A (4.2.8)
- Calibration standards (6.3.1)
- Extraction solvent (4.2.7) or mobile phase A (4.2.8)
- Quality control sample accuracy (6.3.2)
- Quality control sample recovery (6.3.3)
- Extraction solvent (4.2.7) or mobile phase A (4.2.8)
- Sample extracts (6.3.4)
- Mixed standard solution 10 µg/l (4.2.14)

Optionally: inject extraction solvent (4.2.7) between the different samples or every 10-20 samples.

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 linearity of LC-MS/MS measurement

The matrix-matched calibration standards (MMC, Table 1) 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 calibration series, the response factor derived from the peak areas of the non-labelled analyte and the isotopically labelled analogue (Equation 4) 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 tropane alkaloids in the samples

Identify atropine and scopolamine in the samples by comparing retention time and ion ratio with that of the calibration standards (MMC) according to SANTE/12682/2019 [4].
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 of 0.5 µg/kg, the identity of the analyte in the sample is confirmed. The blank sample (MMC1) should not show a peak at the retention time of the analyte that exceeds 20% of the reporting limit. The identity of the analyte in MMC2 (0.5 µg/kg) should be confirmed as a verification of the LOQ.

Use the following equations:

Equation I Deviation of the retention time (ΔRT)

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

where:

ΔRT	is the deviation of the retention time of the analyte in the sample extract, compared to the average retention time in the calibration standards (min)
RT_{sample}	is the retention time of the analyte in the sample extract (min)
RT_{avg}	is the average retention time of the analyte in the MMC (min)

Equation II Ion ratio (IR)

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

where:

IR	is the ion ratio (%)
A_{low}	is the area of the product ion with the lowest intensity
A_{high}	is the area of the product ion with the highest intensity

Equation III Relative deviation of the ion ratio (D)

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

where:

D	is the relative deviation of the ion ratio of the analyte in the sample, compared to the average ion ratio of the analyte in the calibration standards (%)
IR_{sample}	is the ion ratio of the analyte in the sample (%) (Equation II)
$IR_{average}$	is the average ion ratio of the analyte in the calibration standards (%) (Equation II)

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 of the mass detector from calculation of the reference ion ratio.

8.3 Quantification of tropane alkaloids in the samples

8.3.1 Quantification by means of isotope labelled internal standard correction

Quantification of atropine and scopolamine is based on isotope labelled internal standard correction. Calculate the response factor (RF) of the analyte in the sample and in the calibration standards according to Equation IV. Calculate the concentration (C) of the analyte in the sample according to Equation V.

Equation IV Response factor (RF)

$$RF = \frac{A_x}{A_{IS}}$$

where:

RF	is the response factor
A_x	is the sum of the peak areas of the product ions of the analyte in the sample
A_{IS}	is the peak area of the isotope labelled analogue in the sample

Equation V Concentration in the sample (C)

$$C_{sample} = \left(\frac{RF_{sample} - b}{a}\right)$$

where:

C_{sample}	is the concentration of the analyte in the sample (µg/kg)
RF_{sample}	is the RF obtained for the analyte in the sample
b	is the intercept of the matrix matched calibration curve
a	is the slope of the matrix matched calibration curve

Note: optionally, the area of the quantifier ion only may be used in the calculations of analyte concentrations.

8.3.2 Quantification by means of single level standard addition

In case internal standards of atropine and scopolamine are not available the quantification can be performed by single level standard addition to the sample. Use Equation VI to calculate the concentration in the sample:

Equation VI Concentration in the sample (C) by means of single level standard addition to the sample

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

where:

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

Note: optionally, the area of the quantifier ion only may be used in the calculations of analyte concentrations.

8.3.3 Quality control sample accuracy

Calculate the content in the QC sample accuracy (1 µg/kg) (6.3.2) as described in section 8.3.1. The analytical result should be between 80% and 120% of the amount added to the sample.

8.3.4 Recovery (extraction efficiency)

Calculate the recovery (extraction efficiency) of the tropane alkaloids with Equation VII. The recovery is calculated by comparison of the QC recovery sample MMRS 1 (6.3.3) with the MMC 3 (6.3.1).

Equation VII Recovery

$$R = \left(\frac{RF_{MMC\,3}}{RF_{MMRS\,1}}\right) \times 100\%$$

where:

R	is the recovery (%)
$RF_{MMC\,3}$	is the response factor of the analyte in MMC 3 (6.3.1), fortified at 1 µg/kg
$RF_{MMRS\,1}$	is the response factor of the analyte in recovery sample MMRS 1 (6.3.3), spiked at 0.1 µg/l (corresponding to 1 µg/kg)

Note: The recovery is calculated for information only and is in this method not considered as a critical parameter. The recovery should preferably be between 50 and 120%.

8.4 Final result

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

9 References

[1] EFSA, Scientific Opinion on tropane alkaloids in food and feed. EFSA Panel on Contaminants in the Food Chain. EFSA Journal, 2013. 11(10): 3386(10): pp. 113.

[2] European Commission, Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Communities, 2006. L364: p. 5-24.

[3] European Commission, Commission Regulation (EU) 2016/239 amending Regulation (EC) No 1881/2006 as regards maximum levels of tropane alkaloids in certain cereal-based foods for infant and young children. Official Journal of the European Union, 2016. L45: p. 3-5.

[4] DG_SANTE, Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed SANTE/12682/2019. https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2019-12682.pdf

Annex A Checklist for sample preparation

Analyst:

Date:

Lab. journal / page:

A.1 Preparation of calibration standards (MMC) (6.3.1)

- ☐ Weigh 6 portions of 4.0 ± 0.1 g blank processed cereal-based food material in 50 ml polypropylene tubes (5.8);
- ☐ Spike the samples according to Table A.1;
- ☐ Follow the steps described in the sample preparation procedure (6.3.4) (Annex A.4).

Table A.1 Preparation of matrix-matched calibration standards

Code	Concentration in blank matrix (µg/kg)	Mixed standard solution 200 µg/l (4.2.11) (µl)	Mixed IS solution 1 mg/l (4.2.13) (µl)
MMC 1	0	0	20
MMC 2	0.5	10	20
MMC 3	1	20	20
MMC 4	2.5	50	20
MMC 5	5	100	20
MMC 6	10	200	20

A.2 Quality control sample accuracy (1 µg/kg) (6.3.2)

- ☐ Weigh 4.0 ± 0.1 g of the blank food material in a tube of 50 ml;
- ☐ Add 20 µl of mixed standard solution 200 µg/l (4.2.11) to the sample, corresponding to an added level of 1 µg/kg;
- ☐ Add 20 µl of the mixed internal standard solution 1 mg/l (4.2.13);
- ☐ Follow the steps described in the sample preparation procedure (6.3.4) (Annex A.4).

A.3 Quality control sample recovery MMRS (1 µg/kg) (6.3.3)

- ☐ Weigh 4.0 ± 0.1 g of the blank food material in a tube of 50 ml;
- ☐ Add 20 µl of the mixed internal standard solution 1 mg/l (4.2.13);
- ☐ Follow the steps described in the sample preparation procedure (6.3.4) (Annex A.4);
- ☐ Transfer an aliquot of 950 µl of the filtrate to HPLC vials (5.12);
- ☐ Add 10 µl of mixed standard solution 10 µg/l (4.2.12) and 40 µl of extraction solution (4.2.7);
- ☐ Close the vial and mix the contents of the vial (5.4).

Annex A Checklist for sample preparation, continued

A.4 Sample preparation for analysis (6.3.4)

- ☐ Weigh a portion of 4.0 ± 0.1 g sample in a 50 ml polypropylene tube (5.8);
- ☐ Add 20 µl of the mixed internal standard solution 1 mg/l (4.2.13) and let the sample equilibrate for 30 min;
- ☐ Add 40 ml of extraction solution (4.2.7) and mix the contents on a rotary shaker (5.4);
- ☐ Place the tube in a rotary tumbling machine (5.5) and extract for 30 minutes at room temperature;
- ☐ Centrifuge the tube for 15 minutes at 3500 g (5.6) at room temperature;
- ☐ Transfer 2-4 ml of the supernatant to an ultrafilter tube (5.9);
- ☐ Centrifuge the tube during 15 minutes at 3500 g (5.6) at room temperature;
- ☐ Transfer an aliquot of 950 µl of the filtrate to HPLC vials (5.12);
- ☐ Add 50 µl of extraction solution (4.2.7);
- ☐ Close the vial and mix the contents of the vial (5.4).

Note: In case of absence of internal standards, single level standard addition to the sample is used:

- ☐ *Weigh two portions of 4.0 ± 0.1 g sample in 50 ml polypropylene tubes (5.8);*
- ☐ *Add 100 µl of the mixed standard solution 200 µg/l (4.2.11) to one of the tubes, corresponding to 5 µg/kg, and let the sample equilibrate for 30 min;*
- ☐ *Continue with the steps described in 6.3.4 (Annex A.4).*

Annex B Example of LC-MS/MS conditions

B.1 LC conditions

The equipment and measuring conditions shown here are provided as an example. Other analytical equipment, columns, mobile phases and gradient conditions may work equally well.

Example LC conditions

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, v/v)
Weak wash:	methanol/water (10/90, v/v)
Flow:	0.4 ml/min
Mobile phase:	A: 10 mM ammonium carbonate in water pH 9.0 (4.2.8); B: acetonitrile (4.2.3)
Gradient:	Table B.1
Run time:	15 min

Table B.1 Gradient for LC-MS/MS analysis

Time (min)	Mobile phase A (4.2.8) (%)	Mobile phase B (4.2.3) (%)
0.0	90	10
11.0	30	70
11.2	90	10
14.2	90	10

See Annex B.3 for an example chromatogram.

B.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.

Example MS conditions

Mass spectrometer:	Waters Xevo TQ-S
Ionisation mode:	ESI positive mode
Capillary voltage:	3.0 kV
Cone voltage	30 V
Source temperature:	150°C
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 B.2 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 slightly differ from column to column and between LC-MS/MS systems. The retention times shown in Table B.2 are therefore indicative. The fragmentation

behaviour can also differ between instruments. For individual compounds two product ions with sufficient sensitivity and selectivity on the instrument should be chosen for analysis.

Table B.2 MS/MS fragmentation conditions for tropane alkaloids

Component	Indicative RT (min)	Precursor ion (m/z)	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)
Atropine	4.27	290.2	124.0	25	93.0	25	91.0	35
Atropine-d_3	4.18	293.2	127.0	20	93.0	25		
Scopolamine	4.49	304.2	138.0	20	156.0	25	103.0	25
Scopolamine-d_3	4.46	307.2	141.0	20	159.0	25		

B.3 LC-MS/MS example chromatogram of a breakfast cereals sample spiked at 1 µg/kg