EURL-MP-method_003 (version 1)
Determination of ergot alkaloids in cereal-based food and feed by LC-MS/MS

Analyte group:	**Mycotoxins**
Analyte(s):	Ergocornine Ergocorninine Ergocristine Ergocristinine α-Ergocryptine β-Ergocryptine α-Ergocryptinine β-Ergocryptinine Ergometrine Ergometrinine Ergosine Ergosinine Ergotamine Ergotaminine
Commodity group:	Cereal-based food and feed materials
Commodities validated:	Food (bread, cereal-based foods for infants and young children, cookies, muesli) and feed (compound feeds, unprocessed cereals)
Technique:	Liquid chromatography / tandem mass spectrometry (LC-MS/MS)

Modifications compared to previous version:
Not applicable

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1. Introduction

Ergot alkaloids (EAs) are produced by fungi of the *Claviceps* genus, most notably by *C. purpurea*, which is a parasite in the seed heads of living plants at the time of flowering. Fungal infections are most commonly found in rye, triticale, wheat, barley, oat and millet. The fungus replaces the developing grain or seed with a characteristic dark coloured crescent shaped alkaloid-containing wintering body, known as ergot or sclerotium. The total ergot alkaloid content of sclerotia may vary considerably, as well as the pattern of alkaloids produced and that are determined by the individual fungal strain in a geographical region and the host plant [1,2]. Sclerotia are harvested together with the cereals or grass and can thus lead to contamination of cereal-based food and feed products with ergots alkaloids. However, ergot alkaloids are also detected in cereals, not only in the sclerotia. Ergotism remains an important veterinary problem, particularly in cattle, horses, sheep, pigs and chicken.

Ergot alkaloids can be sub-classified in two major types: ergopeptines and simple lysergic acid derivatives [1]. There are over 40 ergot alkaloids known, the most important ones are the 8(R)-ergopeptines ergocornine, ergocristine, ergocryptine (which occurs as a mixture of α- and β-isomers), ergosine and ergotamine and the 8(R)-lysergic acid derivative ergometrine. The corresponding 8(S)-epimers are also considered relevant because they can epimerise to the 8(R) analogues under various conditions.

Ergot alkaloids are not regulated in the European Union. Currently, the European Commission is considering legislation on the presence of EAs in various cereal-based food products with priority on barley, wheat, spelt, oats and rye milling products and on processed cereal-based food for infants and young children.

2. Scope

This document describes the confirmation and - by means of standard addition to the sample - the quantification of the ergot alkaloids: i) ergopeptine alkaloids – ergocornine, ergocorninine, ergocristine, ergocristinine, α-ergocryptine, α-ergocryptinine, β-ergocryptine, β-ergocryptinine, ergosine, ergosinine, ergotamine, ergotaminine, and ii) lysergic acid derivatives – ergometrine (synonym: ergonovine), ergometrinine (synonym: ergonovinine) in cereal-based food (cereal products, cookies, muesli, bread) and cereal-based feed ((mixed) grains, compound feeds) using LC-MS/MS. The reporting limit for all individual ergot alkaloids is 1 µg/kg in cereal-based food and 5 µg/kg in cereal-based feed. The method has been successfully in-house validated for the above mentioned ergot alkaloids at 0 to 50 µg/kg in cereal based-food and at 0 to 500 µg/kg in cereal-based feed.

3. Principle

The EAs are extracted by mixing 4 gram 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.

Ergometrine, ergometrinine, ergotamine and ergotaminine are listed as category I drug precursors and for these compounds a special procedure for purchasing, storage and management needs to be followed (Regulation (EC) No 273/2004) [3].

β-Ergocryptine and β-ergocryptinine are currently not available from commercial providers as analytical standards of sufficient purity and quality. In this document α-ergocryptine is used for the determination of β-ergocryptine and α-ergocryptinine is used for the determination of the sum of α- and β-ergocryptinine.

4.1 Ergocornine

4.2 Ergocorninine

4.3 Ergocristine

4.4 Ergocristinine

4.5 α-Ergocryptine

4.6 α-Ergocryptinine

4.7 Ergometrine (maleate)

4.8 Ergometrinine

4.9 Ergosine

4.10 Ergosinine

4.11 Ergotamine

4.12 Ergotaminine

4.13 Methanol, LC-MS grade

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

4.15 Formic acid, 99-100%

4.16 Ammonium carbonate, p.a. quality

4.17 Ammonia, 25%

4.18 Acetonitrile, LC-MS grade

4.19 Extraction solvent: 0.4% formic acid in methanol/water (60:40) (v/v)
Mix 600 ml methanol (4.13), 400 ml water (4.14) and 4 ml formic acid (4.15) in a bottle of 1000 ml. This solution is stored at room temperature and can be used for 3 months.

4.20 Mobile phase A: 10 mM ammonium carbonate in water, pH 9.0
Dissolve 0.96 g ammonium carbonate (4.16) in 1000 ml water (4.14). Check the pH with a pH meter and adjust, when necessary, the pH to 9.0±0.1 by adding formic acid or 25% ammonia (4.17). This solution is stored at room temperature and can be used for 1 month.

4.21 Stock solutions (50-100 mg/l)
Accurately weigh into separate amber coloured glass bottles between 2 and 6 mg ± 0.02 mg of the ergot alkaloids (4.1 to 4.12). 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 in such a way that the concentration of the solution is 100 mg/l, except for ergometrine, ergometrinine, ergotamine and ergotaminine, for which the concentration of the solution is 50 mg/l (see Note 1). Take into account the weight, the purity and the appearance form of the standard. The solutions can be used for 12 months when stored in the dark at -18°C.

Note 1: Standards containing a salt may be difficult to dissolve (particularly ergotamine tartrate and ergometrine maleate). In that case the solution can be sonicated for up to 30 min or placed in a water bath of 60°C for up to 30 min.

4.22 Mixed stock solutions of six major EAs and their epimers (25 - 50 mg/l)
The mixed solution of a ergot alkaloid and its epimer is more stable than the separate alkaloid solutions. For this reason the individual stock solutions are mixed in an 1:1 ratio of the epimers in amber coloured glass bottles. This is applied to the stock solutions (100 mg/l) of ergocornine and its epimer ergocorninine, ergocristine and its epimer ergocristinine, α-ergocryptine and its epimer α-ergocryptinine, and ergosine and its epimer ergosinine. This is also applied to the stock solutions (50 mg/l) of ergometrine and its epimer ergometrinine and of ergotamine and its epimer ergotaminine. The solutions can be used for 12 months when stored in the dark at -18°C.

4.23 Mixed standard solution six major EAs and their epimers (5 mg/l)
Pipette 5 ml of the mixed stock solution 50 mg/l (4.22) of ergocornine/ergocorninine, ergocristine/ergocristinine, α-ergocryptine/α-ergocryptinine, ergosine/ergosinine, 10 ml of the mixed stock solution 25 mg/ml (4.22) of ergometrine/ergometrinine and of ergotamine/ergotaminine in a calibrated volumetric flask of 50 ml and make up the volume with acetonitrile and mix. Divide the solution into portions of 10 ml in amber coloured glass bottles. The solution can be used for 12 months when stored in the dark at -18°C.

4.24 Mixed standard solution six major EAs and their epimers (1 mg/l)
Pipette 4 ml of the mixed standard solution 5 mg/l (4.23) in a calibrated volumetric flask of 20 ml and make up the volume with acetonitrile and mix. Transfer the contents to an amber coloured glass bottle of 20 ml. The solution can be used for 3 months when stored in the dark at -18°C (see Note 2).

Note 2: When a new mixed standard solution of 1 mg/l is prepared, new mixed standard solutions of 200, 50 µg/l and 10 µg/l need to prepared as well.

4.25 Mixed standard solution six major EAs and their epimers (200 µg/l)
Pipette 4 ml of the mixed standard solution 1 mg/l (4.24) in a calibrated volumetric flask of 20 ml and make up the volume with acetonitrile and mix. Transfer the contents to an amber coloured glass bottle of 20 ml. The solution can be used for 3 months when stored in the dark at -18°C (see Note 2).

4.26 Mixed standard solution six major EAs and their epimers (50 µg/l)
Pipette 1 ml of the mixed standard solution 1 mg/l (4.24) in a calibrated volumetric flask of 20 ml and make up the volume with acetonitrile and mix. Transfer the contents to an amber coloured glass bottle of 20 ml. The solution can be used for 3 months when stored in the dark at -18°C (see Note 2).

4.27 Working standard solution six major EAs and their epimers (10 µg/l)
Pipette 10 µl of the mixed standard solution 1 mg/l (4.24) in a HPLC vial and add 990 µl extraction solvent (4.19) and mix. Prepare a fresh solution every new day of analysis.

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:

5.1 **Analytical balance**, accuracy: 0.02 mg

5.2 **Balance,** accuracy: 0.02 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 3,500 g. suitable for 12 and 50 ml centrifuge tubes and ultrafilters (5.10)

5.7 **Polypropylene tubes** of 50 ml with screw cap

5.8 **Polypropylene tubes** of 12 ml with screw or plug cap

5.9 **Ultrasonic bath**

5.10 **Ultrafilter** (e.g. Millipore, Amicon Ultra-4 or Amicon Ultra-15, Ultracel 30kD)

5.11 **Amber coloured glass bottle** 20 or 60 ml with screw cap

5.12 **pH meter**

5.13 **Glass HPLC vial**, 2 ml

5.14 Various pipettes. Use positive displacement pipettes for solutions prepared in acetonitrile

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

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

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

5.15.3 Analytical column: capable of retaining the target EAs, preferably capable of baseline separation of EAs 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 (e.g. Waters Acquity BEH C18 1.7 µm, 150*2.1 mm).

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

5.15.5 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 document describes the quantification of EAs in cereal-based food and feed. The steps described in section 6.4 are shown in the format of a checklist in Annex A.

6.2 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 alkaloids 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. Bread samples should be cryogenically milled and stored at -18°C.

6.3 Test portion

The amount of homogenised test sample examined is 4.0 ± 0.1 gram.

6.4 Extraction, clean-up and preparation of test solutions

6.4.1 Calibration standards for cereal-based food

The calibration standards are prepared by addition of standard to the blank sample extract (matrix matched standards (MMS)) according to Table 1. Choose a blank food material, in which according to previous analyses no ergot alkaloids were detected. The blank material should match with most of the samples to be analysed (e.g. bread or cereals).

Weigh a test portion of 4 g in a tube of 50 ml (5.7), add 40 ml extraction solvent (4.19) to the tube and shake vigorously. Place the tubes during 30 minutes in a rotary tumbling machine (5.5). Centrifuge the tubes during 15 minutes at 3500 g (5.6). Transfer 15 ml of the supernatant to an ultrafilter tube with a capacity of 15 ml (5.10) and centrifuge the tubes during 15 - 60 minutes at 3500 g at room temperature (see Note 3). Aliquots of the ultrafiltrate are used to prepare the calibration standards. Transfer an aliquot of 950 µl of the filtrate to a HPLC vial (5.13). Pipette to the vial the amounts of standard solutions and extraction solvent according to Table 1. Mix the contents of the vial well and close the vial.

Table 1: Preparation of calibration standards (MMS) for cereal-based foods

Code	Conc. in extract (µg/l)	Conc. in sample (µg/kg)	Sample extract (6.4.1) (µl)	Mixed standard solution 10 ng/ml (4.27) (µl)	Mixed standard solution 50 ng/ml (4.26) (µl)	Mixed standard solution 200 ng/ml (4.25) (µl)	Extraction solvent (4.19) (µl)
Cal. Std. 1	0.0	0	950	0	0	0	50
Cal. Std. 2	0.05	0.5	950	5	0	0	45
Cal. Std. 3	0.1	1	950	10	0	0	40
Cal. Std. 4	0.25	2.5	950	25	0	0	25
Cal. Std. 5	0.5	5	950	0	10	0	40
Cal. Std. 6	1.0	10	950	0	20	0	30
Cal. Std. 7	2.5	25	950	0	50	0	0
Cal. Std. 8	5	50	950	0	0	25	25

Note 3: The required time will depend on the matrix type to get the desired volume of ultrafiltrate.

6.4.2 Quality control sample limit of quantification (LOQ) (1 µg/kg) for cereal-based food

Weigh 4 g of the blank food material in a tube of 50 ml (5.7). Add 20 µl of mixed standard solution 200 µg/l (4.25) to the sample corresponding to an added level of 1 µg/kg. Wait 30 minutes before starting the extraction procedure (6.4.9).

6.4.3 Quality control sample recovery (25 µg/kg) for cereal-based food

Weigh 4 g of the blank food material in a tube of 50 ml (5.7). Add 100 µl of mixed standard solution 1 mg/l (4.24). Wait 30 minutes before starting the extraction procedure (6.4.9).

6.4.4 Sample preparation for cereal-based food

Weigh two test portions of 4 g of the sample in tubes of 50 ml (5.7). Add 100 µl of mixed standard solution 1 mg/l (4.24), corresponding to an added level of 25 µg/kg. Wait 30 minutes before starting the extraction procedure (6.4.9).

6.4.5 Calibration standards for cereal-based feed

The calibration standards are prepared by addition of standard to the blank sample extract (matrix matched standards (MMS)) according to Table 2. Choose a blank feed material, in which according to previous analyses no ergot alkaloids were detected. The blank material should match with most of the samples to be analysed (e.g. compound feed or cereals).

Weigh a test portion of 4 g in a tube of 50 ml (5.7), add 40 ml extraction solvent (4.19) to the tube and shake vigorously. Place the tubes during 30 minutes in a rotary tumbling machine (5.5). Centrifuge the tubes during 15 minutes at 3500 g (5.6). Transfer 15 ml of the supernatant to an ultrafilter tube with a capacity of 15 ml (5.10) and centrifuge the tubes during 15 - 60 minutes at 3500 g at room temperature (see Note 3). Aliquots of the ultrafiltrate are used to prepare the calibration standards. Transfer an aliquot

of 950 µl of the filtrate to a HPLC vial (5.13). Pipette to the vial the amounts of standard solutions and extraction solvent according to Table 2. Mix the contents of the vial well and close the vial.

Table 2: Preparation of calibration standards (MMS) for cereal-based feeds

Code	Conc. in extract (µg/l)	Conc. in sample (µg/kg)	Sample extract (6.4.2) (µl)	Mixed standard solution 50 ng/ml (4.26) (µl)	Mixed standard solution 200 ng/ml (4.25) (µl)	Mixed standard solution 1000 ng/ml (4.24) (µl)	Extraction solvent (4.19) (µl)
Cal. Std. 1	0	0	950	0	0	0	50
Cal. Std. 2	0.5	5	950	10	0	0	40
Cal. Std. 3	1	10	950	20	0	0	30
Cal. Std. 4	2.5	25	950	50	0	0	0
Cal. Std. 5	5	50	950	0	25	0	25
Cal. Std. 6	10	100	950	0	50	0	0
Cal. Std. 7	25	250	950	0	0	25	25
Cal. Std. 8	50	500	950	0	0	50	0

6.4.6 Quality control sample limit of quantification (LOQ) (10 µg/kg) for cereal-based feed

Weigh 4 g of the blank feed material in a tube of 50 ml (5.7). Add 40 µl of mixed standard solution 1 mg/l (4.24) to the sample corresponding to an added level of 10 µg/kg. Wait 30 minutes before starting the extraction procedure (6.4.9).

6.4.7 Quality control sample recovery (250 µg/kg) for cereal-based feed

Weigh 4 g of the blank feed material in a tube of 50 ml (5.7). Add 200 µl of mixed standard solution 5 mg/l (4.23) to the sample corresponding to an added level of 250 µg/kg. Wait 30 minutes before starting the extraction procedure (6.4.9).

6.4.8 Sample preparation for cereal-based feed

Weigh two test portions of 4 g of the sample in tubes of 50 ml (5.7). Add to one of the tubes 200 µl of mixed standard solution 5 mg/l (4.23), corresponding to an added level of 250 µg/kg. Wait 30 minutes before starting the extraction procedure (6.4.9).

6.4.9 Sample extraction

Add 40 ml extraction solvent (4.19) to the tube and shake vigorously. 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 (5.10) and centrifuge the tube during 15 minutes at 3500 g at room temperature. Transfer the filtrate to an HPLC vial (5.13).
In case of QC samples (6.4.2, 6.4.3, 6.4.6, 6.4.7), transfer 950 µl of the filtrate to the HPLC vial, add 50 µl extraction solvent (4.19) and mix the contents of the vial.

6.4.10 Quantification of cereal-based food samples with multi-level standard addition (MLSA)

A sample which contains one or more ergot alkaloids at a concentration higher than 15 µg/kg is reanalysed with standard addition at a higher concentration, e.g. 100 µg/kg.

The sample can also by reanalysed by multi-level standard addition (MLSA). Weigh, depending on the determined indicative levels, 5 to 7 test portions of the investigated sample into separate tubes. Add standard solutions according to Table 3. Wait 30 minutes before starting the extraction procedure (6.4.9).

Table 3: Cereal-based food: Preparation of samples for quantification by multi-level standard addition (MLSA)

Code	Content in sample (µg/kg)	Mixed standard solution 1000 ng/ml (µl) (4.24)
MLSA 1	0	0
MLSA 2	0	0
MLSA 3	5	20
MLSA 4	10	40
MLSA 5	25	100
MLSA 6	50	200
MLSA 7	100	400

6.4.11 Quantification of cereal-based feed samples with multi-level standard addition (MLSA)

A sample which contains one or more ergot alkaloids at a concentration higher than 150 µg/kg is reanalysed with standard addition at a higher concentration, e.g. 1000 µg/kg.

The sample can also be reanalysed by multi-level standard addition (MLSA). Weigh, depending on the determined indicative levels, 5 to 7 test portions of the investigated sample into separate tubes (see Note 4). Add standard solutions according to Table 4. Wait 30 minutes before starting the extraction procedure (6.4.9).

Table 4: Cereal-based feed: Preparation of samples for quantification by multi-level standard addition (MLSA)

Code	Content in sample (µg/kg)	Mixed standard solution 5 mg/l (µl) (4.23)
MLSA 1	0	0
MLSA 2	0	0
MLSA 3	50	40
MLSA 4	100	80
MLSA 5	250	200
MLSA 6	500	400
MLSA 7	1000	800

Note 4: It is recommended, when the first analysis indicates a high concentration of one or more of the analytes, to use a larger sample to extraction solvent ratio, to reduce the risk of saturation of the LC-MS/MS detector. This can be achieved by using a smaller sample size (2.0 ± 0.1 gram). Extract the sample with 40 ml extraction solvent (4.19). The required amounts of mixed standard solution in that case are half the amounts indicated in Table 4.

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 5.15 are met. Below example LC conditions for separation of the EAs are given.

The chosen column dimensions and chromatographic conditions should be appropriate to obtain base line separation of epimers or isomers with the same molecular mass-to-charge ratio. It should be noted that ergocryptine and ergocryptinine may occur in two isomeric forms, α and β. In the LC-MS/MS chromatogram α- and β-ergocryptine are readily separated, while α- and β-ergocryptinine co-elute under the chromatographic conditions used (see Annex C1 and C2).

Satisfactory separation of ergot alkaloid epimers is only achieved using a mobile phase A with a pH > 7. As pH moderator, besides ammonium carbonate buffer pH 9, ammonium carbonate buffer pH 10 and 6 mM ammonia have been shown to work well. The use of a mobile phase system with pH > 7, requires an analytical column that contains a stationary phase that is resistant to high pH values.

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)
Weak wash:	methanol/water (10/90)
Flow:	0.4 ml/min
Mobile phase:	A: 10 mM ammonium carbonate in water pH 9.0; B: Acetonitrile
Gradient:	Table 5
Run time:	15 min
Solvent discard:	0-2 and 13-15 min

Table 5: Gradient for LC-MS/MS analysis of EAs

Time (min)	Mobile phase A (4.22) (%)	Mobile phase B (4.18) (%)
0.0	90	10
11.0	30	70
11.2	30	70
11.3	90	10
15.0	90	10

See Annex C1 and C2 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.

Example MS conditions:

Mass spectrometer:	Waters Xevo TQ-S
Ionization 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 ·10^{-3} mbar (0.17 ml/min)

The precursor ions fragment to structurally related ions. In Table 6 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 systems. The retention times shown in Table 6 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.

Check the system performance as well as the retention times and time windows of the various EAs. The system should be able to detect the product ion with the lowest intensity with an s/n ratio of at least 25 for the EAs in the working standard solution of 10 µg/l (4.27). The sensitivity is visually checked for the most critical component in each window.

Table 6: Example MS/MS fragmentation conditions for ergot alkaloids (in order of elution)

Component	Indicative RT (min)	Cone Voltage (V)	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)	Product ion 4 (m/z)	Col. energy 4 (eV)
Ergometrine	4.83	30	326.2	223.1	25	208.1	30	180.1	30		
Ergometrinine	6.13	30	326.2	208.1	30	223.1	20	180.1	30		
Ergosine	8.17	30	548.4	223.1	30	208.1	40	268.1	25	277.1	25
Ergotamine	8.43	30	582.4	223.1	35	208.1	40	268.1	25	277.1	25
Ergocornine	9.04	30	562.4	268.1	25	223.1	35	268.1	25	277.1	25
α-Ergocryptine	9.47	30	576.4	223.1	35	268.1	25	268.1	25	305.2	30
β-Ergocryptine	9.58	30	576.4	223.1	35	268.1	25	268.1	25	305.2	30
Ergocristine	9.58	30	610.4	223.1	35	268.1	25	208.1	40	305.2	30
Ergosinine	9.83	30	548.4	223.1	30	208.1	40	268.1	25	277.1	25
Ergotaminine	10.14	30	582.4	223.1	25	277.1	35	268.1	25	277.1	25
Ergocorninine	10.55	30	562.4	223.1	35	305.2	25	268.1	25	277.1	25
α-Ergocryptinine	11.01	30	576.4	223.1	35	305.2	30	268.1	25	305.2	30
β-Ergocryptinine	11.01	30	576.4	223.1	35	305.2	30	268.1	25	305.2	30
Ergocristinine	11.17	30	610.4	223.1	35	305.2	30	268.1	25	305.2	30

7.3 Injection sequence

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

- Standard working solution 10 µg/l (4.27)
- Extraction solvent (4.19) or mobile phase A (4.20)
- Calibration standards (6.4.1 or 6.4.5)
- Extraction solvent (4.19) or mobile phase A (4.20)
- Quality control sample limit of quantification (6.4.2 or 6.4.6)
- Quality control sample recovery (6.4.3 or 6.4.7)
- Extraction solvent (4.19) or mobile phase A (4.20)
- Sample extracts (6.4.9, 6.4.10 or 6.4.11)
- Standard working solution 10 µg/l (4.27)

Inject extraction solvent between the various samples when confirmation by MLSA is performed.

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 linearity of LC-MS/MS measurement

The calibration standards (MMS; see Tables 1 and 2) 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 sum of the peak areas 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 EAs in the samples

Identify EAs in the samples by comparing retention time and ion ratio with that of the calibration standards (MMS) according to SANTE/11813/2017 [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, the identity of the analyte in the sample is confirmed.

a) Determine the average retention time of the analyte and its deviation in the calibration standards 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 or MLSA as stated in SANTE/11813/2017 [4].

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 in the calibration standards or MLSA series (min)
RT_{sample} = retention time of the analyte in the sample extract (min)
RT_{avg} = average retention time of the analyte in the calibration standards or MLSA series (min)

b) Calculate an average ion ratio and deviation of ion ratio of the analyte, using **Equations II** and **III**.

Equation II: Ion ratio (IR)

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

where:
IR = ion ratio (%)
A_{low} = area of the product ion with the lowest intensity
A_{high} = 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 = 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 (MMS) or MLSA series (%)
IR_{sample} = ion ratio of the analyte in the sample (%) (**Equation II**)
$IR_{average}$ = average ion ratio of the analyte in the calibration standards (MMS) or MLSA series (%) (**Equation II**)

c) For confirmation of the identity on the basis of the ion ratio, EU criteria are applied as described in SANTE/11813/2017[4]. Calculate the average ion ratio of the analyte in the calibration standards (MMS) (1 – 50 µg/kg cereal-based food; 5 – 500 µg/kg cereal-based feed). The deviation in the ion ratio of the individual calibration standards compared to the average should not exceed 30%.

d) For confirmation of analytes by MLSA, calculate the average ion ratio of the fortified samples (concentrations 5 – 100 µg/kg cereal-based food; 50 – 1000 µg/kg cereal-based feed). The deviation in the ion ratio of each fortified sample compared to the average should not exceed 30%.

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 EAs in the samples

8.3.1 Quantification based on single level standard addition

The concentration of an EA in the sample is calculated based on single level standard addition, according to **Equation IV**. Note that for cereal-based food products the concentration added to the sample is 25 µg/kg. For cereal-based feed products the concentration added is 250 µg/kg.

Equation IV: Calculation of the analyte concentration in the sample with single standard addition

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

where:

C_{sample} = concentration of the analyte (µg/kg)
A_{added} = sum of the area of the product ions of the analyte in the fortified sample
A_{sample} = sum of the area of the product ions of the analyte in sample without added standard addition
C_{added} = concentration of the analyte added to the sample (µg/kg)

*Note: For quantification of β-ergocryptine, α-ergocryptine is used. Calculate the concentration of β-ergocryptine according to **Equation V**.*

Equation V: Calculation of the concentration of β-ergocryptine in the sample

$$C_{\beta-ergocryptine} = \left(\frac{A_{\beta-ergocryptine}}{A_{\alpha-ergocryptine}} \right) x\ C_{\alpha-ergocryptine}$$

where:

$C_{\beta\text{-}ergocryptine}$ = Concentration of β-ergocryptine in the sample (µg/kg)
$A_{\beta\text{-}ergocryptine}$ = sum of the area of the product ions of β-ergocryptine in the sample without standard addition
$A_{\alpha\text{-}ergocryptine}$ = sum of the area of the product ions of α-ergocryptine in the sample without standard addition
$C_{\alpha\text{-}ergocryptine}$ = Concentration of α-ergocryptine in the sample (µg/kg)

Note: α-Ergocryptinine is used for quantification of the sum of α-ergocryptinine and β-ergocryptinine.

8.3.2 Quantification based on multi-level standard addition

When multi-level standard addition (MLSA) is used, the sum of the peak areas is plotted as function of the added concentration to the sample (µg/kg). Apply linear regression using the least squares method. Use three consecutive standard addition levels to calculate the concentration in the sample, preferably the middle addition level being close to the level present in the sample. The correlation coefficient of the line should be ≥ 0.990. Calculate the concentration in the sample according to **Equation VI**.

Equation VI: Calculation of the analyte concentration in the sample with multi-level standard addition

$$C_{sample} = b/a$$

where:

C_{sample} = concentration of the analyte (µg/kg)
b = intercept of the MLSA calibration curve (from the linear regression curve*)
a = slope of the MLSA calibration curve (from the linear regression curve*)

* Plot the sum of the area of the product ions as function of the added levels. Calculate the linear regression using the least squares method.

8.4 Verification of Limit of Quantification

8.4.1 LOQ in cereal-based food samples

In the blank sample material (Cal Std 1) (6.4.1) no analytes should be detected (<LOD). Calculate the concentration of the analytes in the LOQ QC1 sample (6.4.2) with **Equation IV**, using the recovery sample QC 25 (6.4.3) as fortified sample. Note that the concentration added to the sample is 24 µg/kg. The calculated content should be between 70 and 120% of the added content.

8.4.2 LOQ in cereal-based feed samples

In the blank sample material (Cal Std 1) (6.4.5) no analytes should be detected (<LOD). Calculate the concentration of the analytes in the LOQ QC1 sample (6.4.6) with **Equation IV**, using the recovery sample QC 25 (6.4.7) as fortified sample. Note that the concentration added to the sample is 240 µg/kg. The calculated content should be between 70 and 120% of the added content.

8.5 Verification of Limit of Quantification

8.5.1 Recovery in cereal-based food samples

Calculate the recovery of the analytes with **Equation VII**. The recovery is calculated by comparison of the recovery sample QC 25 (6.4.3) with the Cal Std 25 µg/kg (6.4.1). 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%.

Equation VII: Calculation of the recovery (extraction efficiency) QC 25 µg/kg for cereal-based food

$$R_{QC25} = \left(\frac{A_{QC25}}{A_{CS25}}\right) x\ 100\%$$

where:

R_{QC25} = recovery in sample QC 25 µg/kg
A_{QC25} = sum of the area of the product ions from the analyte in sample QC 25
A_{CS25} = sum of the area of the product ions from the analyte in Cal. Std. 25 µg/kg (MMS level 7)

8.5.2 Recovery in cereal-based feed samples

Calculate the recovery of the EAS with **Equation VIII**. The recovery is calculated by comparison of the recovery sample QC 250 (6.4.7) with the Cal Std 250 µg/kg (6.4.5). 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%.

Equation VIII: Calculation of the recovery (extraction efficiency) QC 250 µg/kg for cereal-based feed

$$R_{QC250} = \left(\frac{A_{QC250}}{A_{CS250}}\right) x\ 100\%$$

where:

R_{QC250} = recovery in sample QC 250 µg/kg
A_{QC250} = sum of the area of the product ions from the analyte in sample QC 250
A_{CS250} = sum of the area of the product ions from the analyte in Cal. Std 250 µg/kg (MMS level 7)

8.6 Final result

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

9. References

[1] EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific Opinion on Ergot alkaloids in food and feed. EFSA Journal 2012;10(7):2798 [158 pp.].

[2] Mulder, P.P.J.; Raamsdonk, L.W.D. van; Voogt, H.J.; Brakel, M.W. van; Horst, G.M. van der; Jong, J. de. Dutch survey ergot alkaloids and sclerotia in animal feeds. RIKILT Report 2012.005 [45 pp.].

[3] Regulation (EC) No 273/2004 of the European Parliament and of the Council of 11 February 2004 on drug precursors. Official Journal of the European Union L 47/1-10.

[4] 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 for cereal-based food samples

Analyst:

Date:

Lab. journal / page:

Preparation of calibration standards for food samples

- ☐ Weigh 1 portions of 4.0 ± 0.1 g of blank food material in a 50 ml polypropylene tube
- ☐ Add 40 ml of extraction solvent (0.4% formic acid in methanol/water (60:40) (v/v)) to the test portion and shake vigorously;
- ☐ Place the tube in a rotary tumbling machine and extract for 30 minutes;
- ☐ Centrifuge the tube for 15 minutes at 3500 g at room temperature;
- ☐ Transfer 15 ml of the supernatant to an ultrafilter tube with a capacity of 15 ml;
- ☐ Centrifuge the tube during 15 - 60 minutes at 3500 g at room temperature;
- ☐ Transfer 8 aliquots of 950 µl of the filtrate to HPLC vials;
- ☐ Spike the aliquots according to Table 1;
- ☐ Mix the contents of the vial well and close the vial.

Table 1: Preparation of calibration standards for cereal-based foods

Code	Conc. in extract (µg/l)	Conc. in sample (µg/kg)	Sample extract (6.5.2) (µl)	Mixed std sol. 10 ng/ml (4.27) (µl)	Mixed std sol. 50 ng/ml (4.26) (µl)	Mixed std sol. 200 ng/ml (4.25) (µl)	Extraction solvent (4.19) (µl)
Cal. Std. 1	0.0	0	950	0	0	0	50
Cal. Std. 2	0.05	0.5	950	5	0	0	45
Cal. Std. 3	0.1	1	950	10	0	0	40
Cal. Std. 4	0.25	2.5	950	25	0	0	25
Cal. Std. 5	0.5	5	950	0	10	0	40
Cal. Std. 6	1.0	10	950	0	20	0	30
Cal. Std. 7	2.5	25	950	0	50	0	0
Cal. Std. 8	5	50	950	0	0	25	25

Annex A. Checklist (continued)

Quality control limit of quantification (LOQ) (1 µg/kg) for cereal-based food

- ☐ Weigh 4 g of the blank food material in a tube of 50 ml;
- ☐ Add 20 µl of mixed standard solution 200 µg/l (4.25) to the sample, corresponding to an added level of 1 µg/kg;
- ☐ Follow the steps described in the sample preparation procedure.

Quality control sample recovery (25 µg/kg) for cereal-based food

- ☐ Weigh 4 g of the blank food material in a tube of 50 ml;
- ☐ Add 100 µl of mixed standard solution 1 mg/l (4.24) to the sample, corresponding to an added level of 25 µg/kg;
- ☐ Follow the steps described in the sample preparation procedure.

Sample preparation for analysis of cereal-based food samples

- ☐ Weigh two test portions of 4 g of the blank food material in a tubes of 50 ml;
- ☐ Add 100 µl of mixed standard solution 1 mg/l (4.24) to the sample, corresponding to an added level of 25 µg/kg;
- ☐ Wait 30 minutes before starting the extraction procedure;
- ☐ Add 40 ml of extraction solvent (0.4% formic acid in methanol/water (60:40) (v/v)) to the test portion and shake vigorously;
- ☐ Place the tube in a rotary tumbling machine and extract for 30 minutes;
- ☐ Centrifuge the tube for 15 minutes at 3500 g at room temperature;
- ☐ Transfer 2 ml of the supernatant to an ultrafilter tube with a capacity of 4 ml;
- ☐ Centrifuge the tube during 15 minutes at 3500 g at room temperature;
- ☐ Transfer an aliquots of 950 µl of the filtrate to HPLC vials;
- ☐ Add 50 µl of extraction solvent;
- ☐ Mix the contents of the vial well and close the vial.

Annex B. Checklist for cereal-based feed samples

Analyst:

Date:

Lab. journal / page:

Preparation of calibration standards for feed samples

- ☐ Weigh 1 portion of 4.0 ± 0.1 g of blank feed material in a 50 ml polypropylene tube
- ☐ Add 40 ml of extraction solvent (0.4% formic acid in methanol/water (60:40) (v/v)) to the test portion and shake vigorously;
- ☐ Place the tube in a rotary tumbling machine and extract for 30 minutes;
- ☐ Centrifuge the tube for 15 minutes at 3500 g at room temperature;
- ☐ Transfer 15 ml of the supernatant to an ultrafilter tube with a capacity of 15 ml;
- ☐ Centrifuge the tube during 15 - 60 minutes at 3500 g at room temperature;
- ☐ Transfer 8 aliquots of 950 µl of the filtrate to HPLC vials;
- ☐ Spike the aliquots according to Table 2;
- ☐ Mix the contents of the vial well and close the vial.

Table 2: Preparation of calibration standards for cereal-based feeds

Code	Conc. in extract (µg/l)	Conc. in sample (µg/kg)	Sample extract (µl) (6.5.2)	Mixed std sol. 50 ng/ml (µl) (4.26)	Mixed std sol. 200 ng/ml (µl) (4.25)	Mixed std sol. 1000 ng/ml (µl) (4.24)	Extraction solvent (µl) (4.19)
Cal. Std. 1	0	0	950	0	0	0	50
Cal. Std. 2	0.5	5	950	10	0	0	40
Cal. Std. 3	1	10	950	20	0	0	30
Cal. Std. 4	2.5	25	950	50	0	0	0
Cal. Std. 5	5	50	950	0	25	0	25
Cal. Std. 6	10	100	950	0	50	0	0
Cal. Std. 7	25	250	950	0	0	25	25
Cal. Std. 8	50	500	950	0	0	50	0

Annex B. Checklist (continued)

Quality control limit of quantification (LOQ) (10 µg/kg) for cereal-based feed

- ☐ Weigh 4 g of the blank feed material in a tube of 50 ml;
- ☐ Add 40 µl of mixed standard solution 1 mg/l (4.24) to the sample, corresponding to an added level of 10 µg/kg;
- ☐ Follow the steps described in the sample preparation procedure.

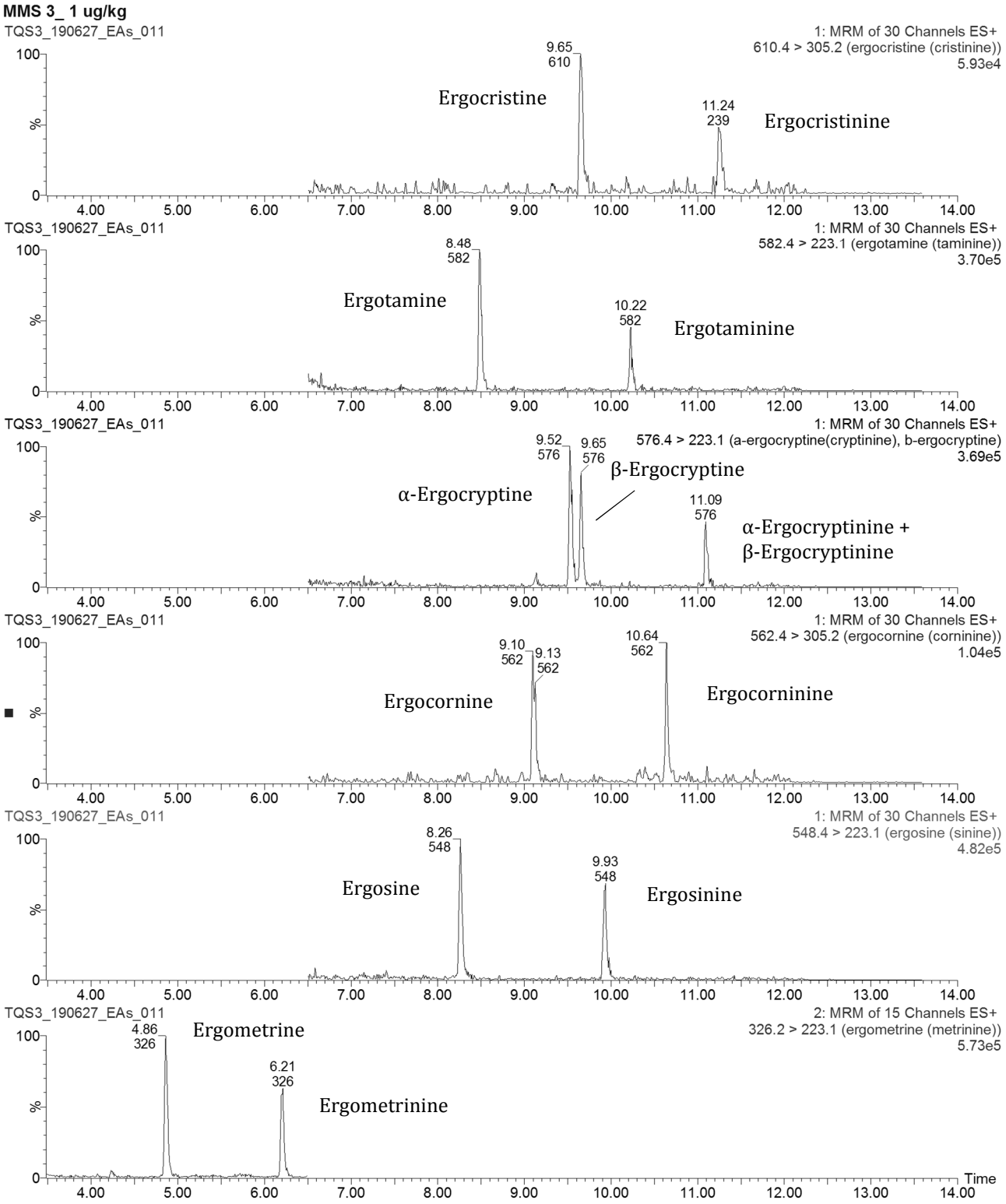
Quality control sample recovery (250 µg/kg) for cereal-based feed

- ☐ Weigh 4 g of the blank feed material in a tube of 50 ml;
- ☐ Add 200 µl of mixed standard solution 5 mg/l (4.23) to the sample, corresponding to an added level of 250 µg/kg;
- ☐ Wait 30 minutes before starting the extraction procedure;
- ☐ Follow the steps described in the sample preparation procedure

Sample preparation for analysis of cereal-based feed samples

- ☐ Weigh two test portions of 4 g of the blank feed material in a tubes of 50 ml;
- ☐ Add to one of the tubes 200 µl of mixed standard solution 5 mg/l (4.23) to the sample, corresponding to an added level of 250 µg/kg;
- ☐ Wait 30 minutes before starting the extraction procedure;
- ☐ Add 40 ml of extraction solvent (0.4% formic acid in methanol/water (60:40) (v/v)) to the test portion and shake vigorously;
- ☐ Place the tube in a rotary tumbling machine and extract for 30 minutes;
- ☐ Centrifuge the tube for 15 minutes at 3500 g at room temperature;
- ☐ Transfer 2 ml of the supernatant to an ultrafilter tube with a capacity of 4 ml;
- ☐ Centrifuge the tube during 15 minutes at 3500 g at room temperature;
- ☐ Transfer an aliquots of 950 µl of the filtrate to HPLC vials;
- ☐ Add 50 µl of extraction solvent;
- ☐ Mix the contents of the vial well and close the vial.

Annex C1. LC-MS/MS chromatogram: Babyfood spiked with 1 µg/kg EAs



Annex C2. LC-MS/MS chromatogram: Wheat spiked with 50 µg/kg EAs