EURL-MP-method_016 (version 1)
Determination of ochratoxin A in meat and cheese by LC-MS/MS

Analyte group:	**mycotoxins**
Analyte(s):	ochratoxin A
Commodity group:	food
Commodities validated:	cured meat, cured cheese: hard cheese paste, soft cheese, hard cheese rind, edible organ meat (kidney)
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 (EURLMP)
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

Ochratoxin A (OTA) is preliminary produced by the fungal species of *Aspergillus* and *Penicillium*, and may contaminate diverse food and feed materials, like cereals, spices, figs, cocoa, milk or meat products. Moreover, external contamination of stored and/or aging meat and cheese products with OTA producing moulds may result in OTA migration into the depth of the product.
Animals that consume contaminated feed can accumulate OTA in their kidneys, given that the kidneys are the primary target organ. Furthermore, OTA in kidneys can indicate eventual contamination of other edible animal tissues . Among animals, pigs are the most vulnerable animal species for OTA feed to tissue transfer.
To protect human health, European Commission has set legal limits in various plant based food materials [1] and recommended a guidance value in feed [2], to reduce the exposure. So far, there are no harmonized EU OTA limits for animal tissues or animal derived food. Several EU countries screen for OTA in such matrices and have set their national limits, which vary from 1 to 25 µg/kg, depending on the matrix.

2 Scope

This method describes the quantitative determination of ochratoxin A in cured meat and cheese in the range from 0.075 to 11.25 µg/kg and for kidney in the range from 0.05 to 7.5 µg/kg. Limit of quantification is 0.2 µg/kg.

3 Principle

Ochratoxin A is extracted from the sample with methanol, defatted with hexane (cured meat and cheese) and an aliquot of the acetonitrile phase is diluted with buffer. Extracts are cleaned on IAC column dedicated to OTA and analyzed by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). Quantification is carried out by external calibration of standards in solvent after normalization of the response to the isotopically labelled internal standard $^{13}C_{20}$ OTA.

4 Reagents

All reagents must be "pro analysis" quality, or higher quality if stated.

4.1 Analytical standards

4.1.1 Ochratoxin A (OTA), e.g. standard solution in acetonitrile, 10 µg/mL.

4.1.2 $^{13}C_{20}$-OTA, e.g. standard solution in acetonitrile, 10 µg/mL.

4.2 Chemicals

4.2.1 Acetonitrile (ACN), LC-MS grade

4.2.2 Methanol (MeOH), LC-MS grade

4.2.3 Acetic acid (HAc), 99-100%

4.2.4 Water Ultra LCMS, ULC grade

4.2.5 Sodium hydrogen bicarbonate, p.a.

4.2.6 Phosphate Buffered Saline (PBS) pH 7.4; 1 tablet in water to prepare 1 L solution

4.2.7 *N*-Hexane, PEG grade

4.3 Solutions and reagents

4.3.1 Mobile phase A, 0.1% acetic acid in water
Pipette 1.0 mL of acetic acid (4.2.3) in a one-liter flask, fill up to the mark with water (ULC grade, 4.2.4) and mix. The shelf life is one month at room temperature.

4.3.2 Mobile phase B, 0.1% acetic acid in 95% acetonitrile
Pipette 1.0 mL of acetic acid (4.2.3) and 49 mL of water (ULC grade, 4.2.4) in one-liter volumetric flask, fill up to mark with acetonitrile (4.2.1) and mix. The shelf life is three months at room temperature.

4.3.3 Phosphate Buffered Saline (PBS) pH 7.4
Dissolve one tablet of salts for PBS (4.2.6) in 1 L water, store in the refrigerator and in the dark for a maximum of one month.

4.3.4 Reconstitution solvent, 50% methanol/water (v/v) with 0.1% acetic acid
Take 250 mL of water (4.2.4) and add 250 mL of methanol (4.2.2). Add 500 µl glacial acetic acid (4.2.3) and mix. This solution can be used for 3 months stored at room temperature.

4.4 Standard solutions

4.4.1 OTA standard solution 0.1 µg/mL
Add 50 µl of stock standard solution OTA (4.1.1) to 4950 µl reconstitution solvent (4.3.4). Mix well. This solution can be stored in the freezer for a maximum of 12 months.

4.4.2 OTA standard solution 0.01 µg/mL
Add 500 µl of OTA solution (4.4.1) to 4500 µl reconstitution solvent (4.3.4). Mix well. This solution can be stored in the freezer for a maximum of 12 months.

4.4.3 Internal standard solution (IS) 0.1 µg/mL
This solution contains the labelled standard used for quantification. This solution is added to the sample before extraction. Add 50 µl of $^{13}C_{20}$-OTA (4.1.2) to 4950 µ of reconstitution solution (4.3.4) and mix well. This solution can be stored in the freezer for a maximum of 12 months.

4.4.4 Calibration solutions in solvent for meat and cheese

Prepare calibration solutions by adding volumes of standard solutions, isotope-labelled standard solution and solvent, as indicated in the table below, to autosampler vials.

Code	Concentrations in meat and cheese (ug/kg)	Concentrations (ng/mL) OTA	OTA 0.1 µg/mL (µl) (4.4.1)	OTA 0.01 µg/mL (µl) (4.4.2)	IS solution 0.1 ug/mL (µl) (4.4.3)	Dilution solution (µl) (4.3.4)
CAL1	0.075	0.15		15	20	965
CAL2	0.1875	0.375	3.75		20	976.2
CAL3	0.375	0.75	7.5		20	972.5
CAL4	0.75	1.5	15		20	965
CAL5	1.5	3.0	30		20	950
CAL6	3	6.0	60		20	920
CAL7	7.5	15.0	150		20	830
CAL8	11.25	22.5	225		20	755

4.4.5 Calibration solutions in solvent for kidney

Prepare calibration solutions by adding volumes of standard solutions, isotope-labelled standard solution and solvent, as indicated in the table below, to autosampler vials.

Code	Concentrations in raw kidney (ug/kg)	Concentrations (ng/ml) OTA	OTA 0.1 µg/mL (µl) (4.4.1)	OTA 0.01 µg/mL (µl) (4.4.2)	IS solution 0.1 ug/mL (µl) (4.4.3)	Dilution solution (µl) (4.3.4)
CAL1	0.05	0.15		15	30	955
CAL2	0.125	0.375	3.75		30	966.2
CAL3	0.25	0.75	7.5		30	962.5
CAL4	0.5	1.5	15		30	955
CAL5	1.0	3.0	30		30	940
CAL6	2	6.0	60		30	910
CAL7	5	15.0	150		30	820
CAL8	7.5	22.5	225		30	745

5 Materials & 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 Materials

5.1.1 Centrifuge tubes, 50 mL, polypropylene, with screw cap

5.1.2 Reaction tubes, 14 mL, polypropylene

5.1.3 Glass or plastic funnel, 7 cm diameter

5.1.4 Total recovery vial or **vial with 300 µl insert and cap**

5.1.5 Filter paper

5.2 Equipment

5.2.1 Balance, accuracy +/- 0.01 g

5.2.2 Analytical balance, accuracy +/- 0.1 mg

5.2.3 Water purification system

5.2.4 Centrifuge, suitable for 50 mL centrifuge tubes

5.2.5 Micropipettes

5.2.6 Vortex mixer

5.2.7 Vacuum manifold

5.2.8 Turbovap

5.2.9 Ultra-Turrax®

5.2.10 Mechanical shaker head-over-head, adjustable

5.2.11 Vacuum pump

5.2.12 Immuno affinity columns

5.2.13 LC-MS/MS system with the following components

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

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

5.2.6.1 **Analytical column**, capable of retaining Ochratoxin A.

5.2.6.1 **Column oven**, capable of maintaining a constant temperature of 40°C.

5.2.6.1 **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 Ochratoxin A in in food of animal origin: cured meat, hard cheese rind, hard cheese paste, soft cheese and pigs kidney by liquid chromatography tandem mass spectrometry (LC-MS/MS). The LOQ for this method is set as 0.2 µg/kg. The steps described in section 6.4 are presented in the format of a checklist in Annex A.1 for cured meat and cheese and as checklist in Annex A.2 for kidney.

6.1 Quality control

Prepare recovery samples as follows:
Use a blank sample and weigh (5.2.1) two portions of meat, kidney or cheese into 50 mL plastic tube (5.1.1).

- QC_{bl}: use the first portion as blank
- QC_{rec}: add to the second portion an amount of 6 µl of 0.1 µg/mL standard solution (4.4.1) to obtain a spike level of 0.3 µg/kg for meat and cheese and at 0.2 µg/kg in raw kidney

Process the samples according to 6.4.

6.2 Pre-treatment

Samples shall be cryogenically milled or slurried with water (4.2.4) 1:1 ratio before the extraction by using the turrax (5.2.9). Samples will be kept in the freezer and thawed on the day of extraction.

6.3 Trial unit

The test portion to be taken is 2.0 grams, in case of a slurry the amount of a sample will be 4.0 grams.

6.4 Extraction, clean-up and preparation of test solutions

6.4.1 Extraction of ochratoxin A from meat and cheese samples

- Weigh in the sample (6.3) into a 50 mL plastic tube (5.1.1);
- Add 20 µl of internal standard $^{13}C_{20}$-OTA (4.4.3) to each individual sample;
- Add 0.5 mL of water (4.2.4) to a slurried sample (6.3) and 3 mL water (4.2.4) to a not slurried sample (6.3), wait 15 minutes before proceeding;
- Add 7 mL of methanol (4.2.2) and 30 mg of sodium bicarbonate (4.2.5).
- Turrax (5.2.9) course grinded meat and cheese samples for 30 seconds;
- Add 3 mL n-hexane (4.2.7) to the sample and shake on mechanical shaker head-over-head (5.2.10) for 30 min;
- Centrifuge at 3600 rpm for 10 min (5.2.4).
- Discard the upper hexane layer and filter the remaining extract over the fluted filter (5.1.5).
- Transfer 2 mL of the extract to a 50 mL PP centrifuge tube (5.1.1) and dilute to a total of 25 mL with PBS buffer (4.3.3);
- Mix 30 sec on a vortex mixer (5.2.6);

6.4.2 Extraction of ochratoxin A from raw pig kidney

- Weigh in the sample (6.3) into a 50 mL plastic tube (5.1.1);
- Add 20 µl of internal standard $^{13}C_{20}$-OTA (4.4.3) to each individual sample;
- Add 3 mL of water (4.2.4) to a sample (6.3) and wait 15 minutes before proceeding;

- Add 7 mL of methanol (4.2.2) and 30 mg of sodium bicarbonate (4.2.5).
- Turrax (5.2.9) course grinded meat and cheese samples for 30 seconds;
- Mix 30 sec on a vortex mixer (5.2.6);
- Shake on mechanical shaker head-over-head (5.2.10) for 30 min;
- Centrifuge at 3600 rpm for 10 min (5.2.4).
- Transfer 3 mL of the extract to a 50 mL PP centrifuge tube (5.1.1) and dilute to a total of 25 mL with PBS buffer (4.3.3);
- Mix 30 sec on a vortex mixer (5.2.6);

6.4.3 Extraction/clean-up of test solutions by applying immunoaffinity column (IAC)

- Precondition the IAC column (5.2.12) by draining the liquid from the column using the vacuum manifold (5.2.7);
- Load total sample onto the column and let pass by gravity;
- Rinse using 3 mL of water (4.2.4) and remove access water by passing air through the column using vacuum (5.2.7);
- Place plastic tubes (5.1.2) under the columns and apply 3 mL methanol (4.2.2);
- Slowly pass a few drops through the column (let 5 to 6 drops pass through and close the valve), if necessary apply a little overpressure (5.2.7) and wait for 15 minutes for the methanol (4.2.2) to react;
- Slowly (1 drop per 3 seconds) elute the toxins from the column;
- Evaporate to dryness under a stream of nitrogen in a TurboVap (5.2.8) at 55 °C;
- Reconstitute the dry residue in 200 µL of the reconstitution solvent (4.3.4) and vortex for 1 minute (5.2.6);
- Transfer the final extract to a total recovery vial (5.1.4). Store in the freezer until analysis.

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:

- Retention times should be stable. Inject the standard solution CAL1 at least five times or until stable RTs (less than 0.05 min difference) and sensitivity (less than 10% difference) are obtained;
- The injection volume should be optimized for the column dimension and the sensitivity of the mass spectrometric system. The use of large volume injections may result in distorted peak shape;
- Sensitivity should be sufficient and fit-for-purpose. 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;
- Carry-over: The presence of ochratoxin A in the solvent injection is assessed. If ochratoxin A is present in the solvent injection, it might lead to a false-positive result, the system should be cleaned before starting the analysis series.

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

7.1 Injection sequence

Analyse the sample extracts in the order as given below.

- Standard solution CAL1 at the level of 0.15 ng/mL in solvent (4.4.4./4.4.5), at least 2 times

- Reconstitution solvent (4.3.4)
- Calibration standards (4.4.4./4.4.5, CAL1-8)
- Reconstitution solvent (4.3.4)
- Blank chemicals
- Quality control blank sample (QC_{bl} 6.1)
- Quality control recovery sample (QC_{rec} 6.1)
- Reconstitution solvent (4.3.4)
- Sample extracts (6.4)
- Reconstitution solvent (4.3.4)
- Calibration standards (4.4.4./4.4.5, CAL1-8)
- Reconstitution solvent (4.3.4)

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 calibration solutions CAL1 to 8 (4.4.4 or 4.4.5) are used to determine the linearity of the LC-MS/MS system. Plot the response of the quantifier of all individual calibration solutions (4.4.4 or 4.4.5, calibration solutions 1 to 8) against the corresponding concentrations of OTA in ng/mL. Construct a calibration curve using (weighted) least-square regression with all individual data points obtained. Linearity has been demonstrated and the calibration curve is fit-for-purpose when the deviation of the back-calculated concentrations of the calibration standards from the true concentrations, using the calibration equation, do not exceed 20%.

8.2 Identification of ochratoxin A in the samples

Identify ochratoxin A in the samples by comparing retention time and ion ratio with that of the calibration solutions (4.4.4 or 4.4.5) according to SANTE/11312/2021 [3].

Ochratoxin A is considered present and identified when:

a) in the blank sample (QC_{bl}) (6.1), the peak for the quantifier ion at the retention time of ochratoxin A is below 30% of the limit of quantification;
b) the retention time of the peak observed in the individual calibration standards (4.4.4 or 4.4.5), QC samples (6.1) and the sample extracts (6.4) differs not more than 0.1 min from the average retention time as calculated (**Equation I**) from the calibration standards (4.4.4 or 4.4.5);
c) the ion ratio (IR) in individual calibration standards (4.4.4 or 4.4.5), QC samples (6.1) and sample extracts (6.4) differs not more than 30% from the average ion ratio of the matrix matched calibration standards CAL1-8 (4.4.4 or 4.4.5) (=reference ion ratio) using **Equation II** and **Equation III**.

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.

Equation I: Deviation of the retention time (ΔRT)

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

where:

ΔRT	is the deviation of the retention time (min) of the analyte in the sample extract, compared to the average retention time (min) in the calibration standards CAL1-8 (4.4.4 or 4.4.5)
RT_{sample}	is the retention time of the analyte in the sample extract (min)
RT_{avg}	is the average retention time (min) of the analyte in the calibration standards CAL1-8 (4.4.4 or 4.4.5)

Equation II: Calculation of the ion ratio (IR)

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

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)

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

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 CAL1-8 (4.4.4 or 4.4.5) (%)
IR_{sample}	is the ion ratio of the analyte in the sample (%) (**Equation II**)
IR_{avg}	is the average ion ratio of the analyte in the calibration standards CAL1-8 (4.4.4 or 4.4.5) (%) (**Equation II**)

8.3 Quantification of ochratoxin A in the samples

8.3.1 Recovery

When the response is within the linear range, determine the recovery by calculating the concentration of ochratoxin A in the QC_{rec} sample (6.1) using **Equation IV**.

Equation IV: Calculation of the recovery (Rec)

$$\text{Recovery (Rec)} = \left(\frac{C}{C_{Rec}}\right) \text{x } 100$$

where:

Rec	is the recovery (%)
C	is the concentration of ochratoxin A in the recovery sample (6.1) (µg/L)
A_{QCrec}	is the theoretical concentration of ochratoxin A in the recovery sample (6.1) (µg/L)

The result should be between 70% and 120% of the amount added to the sample.

8.3.2 Quantification

Peak areas normalized to that of the internal standard are used for quantification. Concentrations of the analytes in meat and cheese or kidney samples (6.3-4) are determined by multi-level calibration using the solvent standards (4.4.4 or 4.4.5, respectively, CAL1-8) (matrix-effects are compensated by normalization to the isotope labels) using **Equation V**. The results are expressed in µg/kg.

Equation V: Calculation of the concentration in the sample (C_{sample})

$$C_{sample} = \left(\frac{\frac{R-b}{a} \times V_e}{V_a} \right) \times \frac{V_{extr}}{V_U}$$

where:

C_{sample}	is the concentration of ochratoxin A in the sample (µg/kg)
R	is the relative response of analyte in sample (area analyte/area isotope label*)
a	is the slope of the calibration curve (4.4.4 or 4.4.5, CAL1-8)
b	is the intercept of the calibration curve (4.4.4 or 4.4.5, CAL1-8)
V_a	is the volume of aliquot taken to IAC (2 mL for cheese and meat and 3 mL for raw kidney)
V_{extr}	is the volume of total extract (10 mL)
V_u	is the weight of test portion (2.0 grams)
V_e	is the volume of final extract (0.2 mL)

** concentration of label in standards and final extracts is the same*

8.4 Final result

The concentration of ochratoxin A in the sample is expressed in µg/kg.

9 References

[1] Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006

[2] EU (2006) Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding

[3] DG_SANTE, Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed SANTE/11312/2021. https://ec.europa.eu/food/system/files/2022-02/pesticides_mrl_guidelines_wrkdoc_2021-11312.pdf

Annex A.1 Checklist for extraction of ochratoxin A from cured meat and cheese samples

Technician:	
Date:	
Lab. journal / page:	

A.1.1 Sample preparation and extraction, cured meat and cheese samples (6.1, 6.4.1)

QC Blank (6.1)	QC Rec (6.1)	Samples (6.4.1)	
☐	☐	☐	Weigh in appropriate amount of sample (6.3) into a 50 mL plastic tube (5.1.1)
	☐		Add 6 µl of 0.1 µg/mL standard solution (4.4.1) and wait 30 min
☐	☐	☐	Add 20 µl of internal standard 13C20-OTA (4.4.3)
☐	☐	☐	Add 0.5 mL of water (4.2.4) to a slurried sample (6.3) and 3 mL water (4.2.4) to a not slurried sample (6.3) and wait 15 minutes
☐	☐	☐	Add 7 mL of methanol (4.2.2) and 30 mg of sodium bicarbonate (4.2.5)
☐	☐	☐	Turrax (5.2.9) course grinded meat and cheese samples for 30 seconds
☐	☐	☐	Add 3 mL n-hexane (4.2.7)
☐	☐	☐	Shake on mechanical shaker head-over-head (5.2.10) for 30 min
☐	☐	☐	Centrifuge at 3600 rpm for 10 min (5.2.4)
☐	☐	☐	Discard the upper hexane layer and filter the remaining extract over the fluted filter (5.1.5)
☐	☐	☐	Transfer 2 mL of the extract to a 50 mL PP centrifuge tube (5.1.1) and dilute to a total of 25 mL with PBS buffer (4.3.3)
☐	☐	☐	Mix 30 sec on a vortex mixer (5.2.6)

A.1.2 Extraction/clean-up of test solutions by applying immunoaffinity column (IAC)

QC Blank (6.1)	QC Rec (6.1)	Samples (6.4.1)	
☐	☐	☐	Precondition the IAC column (5.2.12) by draining the liquid from the column
☐	☐	☐	Load total diluted extract onto the column and let pass by gravity
☐	☐	☐	Rinse with 3 mL of water (4.2.4) and remove access water by passing air through the column using vacuum (5.2.7)
☐	☐	☐	Place plastic tubes (5.1.2) under the columns and apply 3 mL methanol (4.2.2)
☐	☐	☐	Let 5 to 6 drops pass through the column and close the valve, wait 15 min
☐	☐	☐	Slowly (1 drop per 3 sec) elute the toxins from the column

☐	☐	☐	Evaporate to dryness under a stream of nitrogen (5.2.8) at 55 °C
☐	☐	☐	Reconstitute dry residue in 200 µL of solvent (4.3.4) and vortex for 1 min (5.2.6)
☐	☐	☐	Transfer the final extract to a filter vial (5.1.4)

A.1.3 Preparation of calibration solutions in solvent (4.4.4) applying internal standard

Mix the following solutions:

Name	Conc. in meat and cheese (ug/kg)	Conc.(ng/mL) OTA	OTA 0.1 µg/mL (µl) (4.4.1)	OTA 0.01 µg/mL (µl) (4.4.2)	IS solution 0.1 ug/mL (µl) (4.4.3)	Dilution solution (µl) (4.3.4)
☐ CAL1	0.075	0.15		15	20	965
☐ CAL2	0.1875	0.375	3.75		20	976.2
☐ CAL3	0.375	0.75	7.5		20	972.5
☐ CAL4	0.75	1.5	15		20	965
☐ CAL5	1.5	3.0	30		20	950
☐ CAL6	3	6.0	60		20	920
☐ CAL7	7.5	15.0	150		20	830
☐ CAL8	11.25	22.5	225		20	755

* Conc. = concentration

Annex A.2 Checklist for extraction of ochratoxin A from raw pig kidney

Technician:	
Date:	
Lab. journal / page:	

A.2.1 Sample preparation and extraction, raw pig kidney (6.1, 6.4.2)

QC Blank (6.1)	QC Rec (6.1)	Samples (6.4.2)	
☐	☐	☐	Weigh in appropriate amount of sample (6.3) into a 50 mL plastic tube (5.1.1)
	☐		Add 6 µl of 0.1 µg/mL standard solution (4.4.1) and wait 30 min
☐	☐	☐	Add 20 µl of internal standard 13C20-OTA (4.4.3)
☐	☐	☐	Add 3 mL water (4.2.4) to sample (6.3) and wait 15 minutes
☐	☐	☐	Add 7 mL of methanol (4.2.2) and 30 mg of sodium bicarbonate (4.2.5)
☐	☐	☐	Vortex mix for 30 sec
☐	☐	☐	Add 3 mL n-hexane (4.2.7)
☐	☐	☐	Shake on mechanical shaker head-over-head (5.2.10) for 30 min
☐	☐	☐	Centrifuge at 3600 rpm for 10 min (5.2.4)
☐	☐	☐	Transfer 3 mL of the extract to a 50 mL PP centrifuge tube (5.1.1) and dilute to a total of 25 mL with PBS buffer (4.3.3)
☐	☐	☐	Mix 30 sec on a vortex mixer (5.2.6)

A.2.2 Extraction/clean-up of test solutions by applying immunoaffinity column (IAC)

QC Blank (6.1)	QC Rec (6.1)	Samples (6.4.2)	
☐	☐	☐	Precondition the IAC column (5.2.12) by draining the liquid from the column
☐	☐	☐	Load total diluted extract onto the column and let pass by gravity
☐	☐	☐	Rinse with 3 mL of water (4.2.4) and remove access water by passing air through the column using vacuum (5.2.7)
☐	☐	☐	Place plastic tubes (5.1.2) under the columns and apply 3 mL methanol (4.2.2)
☐	☐	☐	Let 5 to 6 drops pass through the column and close the valve, wait 15 min
☐	☐	☐	Slowly (1 drop per 3 sec) elute the toxins from the column
☐	☐	☐	Evaporate to dryness under a stream of nitrogen (5.2.8) at 55 °C
☐	☐	☐	Reconstitute dry residue in 200 µL of solvent (4.3.4) and vortex for 1 min (5.2.6)
☐	☐	☐	Transfer the final extract to a filter vial (5.1.4)

A.2.3 Preparation of calibration solutions in solvent (4.4.5) applying internal standard

Mix the following solutions:

Name	Conc.* in raw kidney (ug/kg)	Conc. (ng/mL) OTA	OTA 0.1 µg/mL (µl) (4.4.1)	OTA 0.01 µg/mL (µl) (4.4.2)	IS solution 0.1 ug/mL (µl) (4.4.3)	Dilution solution (µl) (4.3.4)
□ CAL1	0.05	0.15		15	30	955
□ CAL2	0.125	0.375	3.75		30	966.2
□ CAL3	0.25	0.75	7.5		30	962.5
□ CAL4	0.5	1.5	15		30	955
□ CAL5	1.0	3.0	30		30	940
□ CAL6	2	6.0	60		30	910
□ CAL7	5	15.0	150		30	820
□ CAL8	7.5	22.5	225		30	745

* Conc. = concentration

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 conditions for the LC system

LC system:	Waters Acquity Ultra LCMS (ULC grade)
Analytical column:	Acquity BEH C18, 1.8 µm, 2.1 x 100 mm
Column temperature:	40 °C
Mobile phase solvent A:	0.1% acetic acid in water (4.3.1)
Mobile phase solvent B:	0.1% acetic acid in 95% acetonitrile (4.3.2)
Flow rate:	0.4 mL/min
Injection volume:	10 µL
Injection temperature	10 °C
Gradient program:	Table B.1

Table B.1 Gradient for the LC system

Time (min)	Mobile phase A (4.3.1) %	Mobile phase B (4.3.2) %
0.0	90	10
1.0	90	10
6.0	0	100
7.7	0	100
8	90	10
8.5	90	10

See Annex B.3 for an example LC-MS/MS chromatogram.

B.2 MS conditions

The conditions given in Table B.2 are guidelines; in practice adjusted settings may be required to obtain an optimal performance of the LC-MS/MS system.

Table B.2. Example for MS conditions

Parameter	***Qtrap 6500***	***Xevo TQ-S***
Ionisation mode	ESI positive	ESI positive
Scan type	MRM	MRM
capilary voltage	4.5 kV	2.5 kV
Source temperature	Fixed	150 °C
desolvation temp	400 °C	500 °C
desolvation gas	GS1 and GS2: 50	1000 L/hr

The precursor ions fragment to structurally related products ions. In Table B.2.2 the theoretical masses of the precursor ion and corresponding product ions are shown. Depending on the instrument, a deviation of ± 0.3 Da is allowed. All transitions shown in Table B.2.2 are included in the MS method installed on the LC-MS/MS. The retention times can differ from column to column and between LC systems. The retention times shown in Table B.2.2 are therefore indicative.

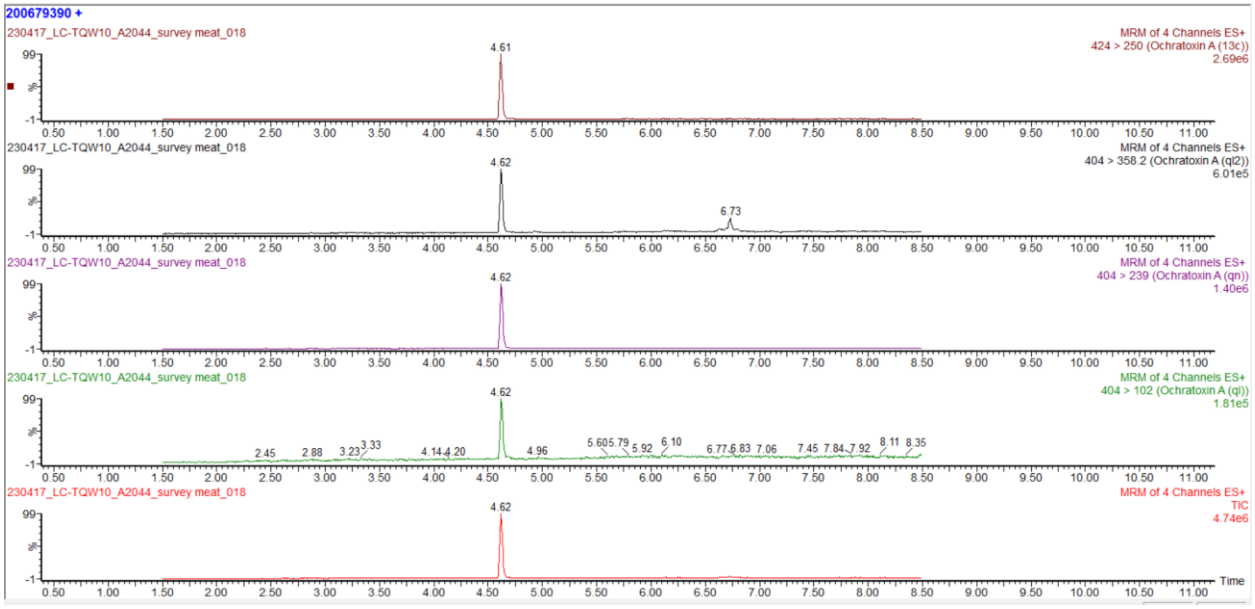
Table B.2.2 MS/MS fragmentation conditions for ochratoxin A on Sciex Qtrap 6500

Analyte	Indicative RT (min)	Precursor ion (m/z)	Product ion (m/z)	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
OTA (qn)	4.6	404.0	239.0	40	10	33	16
OTA (ql)	4.6	404.0	102.0	40	10	91	14
13C20 OTA	4.6	424.0	250.0	40	10	33	16

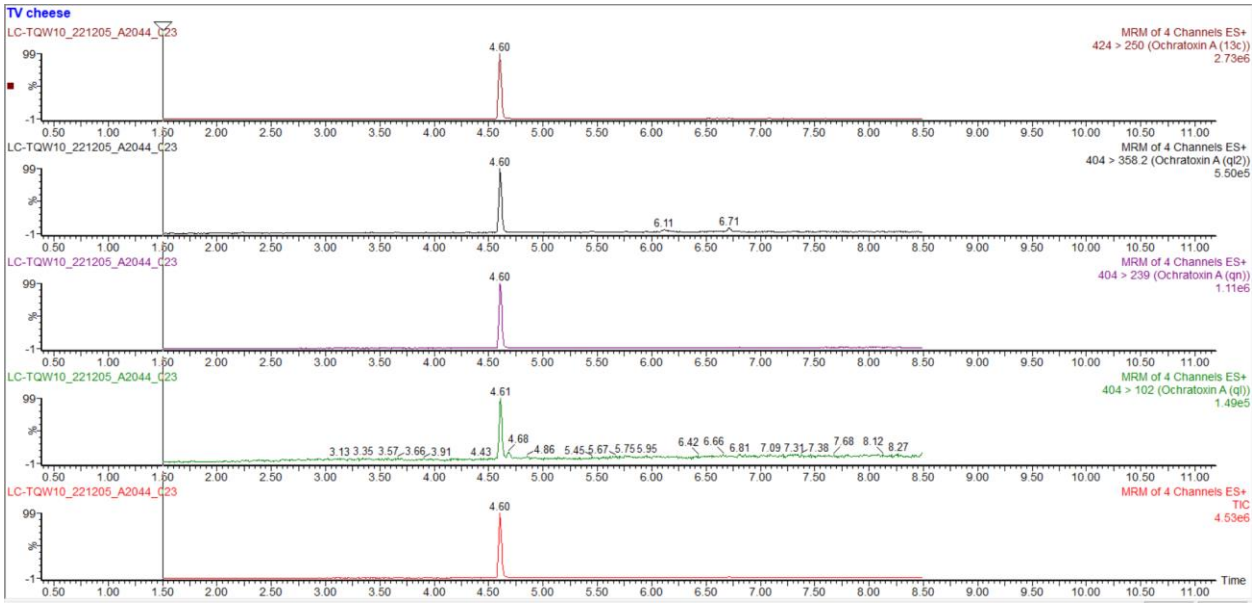
Table B.2.3 MS/MS fragmentation conditions for ochratoxin A on Waters Xevo TQ-S

Analyte	Indicative Rt (min)	Precursor Ion (m/z)	Product ion (m/z)	Dwell (s)	Cone (V)	Collision (eV)
OTA (qn)	4.6	404.0	239.0	0.08	20	25
OTA (ql)	4.6	404.0	102.0	0.08	20	45
OTA (ql2)	4.6	404.0	358.2	0.08	20	16
$^{13}C_{20}$ OTA	4.6	424.0	250.0	0.08	20	25

B.3.1 LC-MS/MS example chromatogram of cured meat fortified at 0.3 µg/kg



B.3.2 LC-MS/MS example chromatogram of cured cheese fortified at 0.3 µg/kg



B.3.3 LC-MS/MS example chromatogram of raw kidney fortified at 0.2 µg/kg