EURL-MP-method_008 (version 1)
Determination of citrinin in red yeast rice food supplements by LC-MS/MS

Analyte group:	**mycotoxins**
Analyte(s):	citrinin
Commodity group:	food supplements
Commodities validated:	red yeast rice food supplements
Technique:	Liquid chromatography / tandem mass spectrometry (LC-MS/MS)

Modifications compared to previous version:
Not applicable

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1 Introduction

Red yeast rice food supplements are prepared by fermenting rice with fungus *Monascus purpureus*. If conditions are not monitored carefully, this fungus also produces citrinin during the fermentation process. Therefore, red yeast rice food supplements (RYR) may be contaminated with citrinin. RYR is available to the consumers as a powder packed in a cellulose capsule, dissolved in oil and packed in a gel capsule or pressed into a tablet.

Underlying legislation is Regulation (EC) No 1881/2006 amended by Regulation (EU) 2019/1901: '2.8.1 Food supplements based on rice fermented with red yeast *Monascus purpureus* – limit of 100 µg/kg' (until November 2019 the legal limit was 2000 µg /kg) [1].

2 Scope

This method describes the quantitative determination of citrinin, in red yeast rice food supplements packed as capsules (cellulose or gel) or as tablet. The method was developed and validated for citrinin in the range from 20 to 100 µg/kg. Limit of quantification is 20 µg/kg.

3 Principle

This method starts with determining the weight of the number of whole capsules or tablets intake and subsequently adding the correct volume of dispersion solution and extraction fluid. The samples (whole capsules or tablets) are first dissolved using the correct volume of an acidified aqueous solution and then directly extracted with acidified acetonitrile. After centrifugation, a salt-induced phase partitioning step is performed followed by centrifugation. An aliquot of the acetonitrile phase is diluted and analysed by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). For quantification isotope labelled standards or single-level standard addition, added before phase partitioning, are used.

4 Reagents

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

4.1 Analytical standards

4.1.1 Citrinin, e.g. standard solution in acetonitrile, 100 µg/mL.

4.1.2 13C13-Citrinin, e.g. standard solution in acetonitril, 10 µg/mL.

4.2 Chemicals

4.2.1 Acetic acid (HAc), 99-100%

4.2.2 Acetonitrile (ACN), LC-MS grade

4.2.3 Ammonium acetate (NH4Ac)

4.2.4 Hydrochloric acid (HCl), 37%

4.2.5 Magnesium sulphate (MgSO4)

4.2.6 Methanol (MeOH), LC-MS grade

4.2.7 Sodium chloride (NaCl)

4.2.8 Water Ultra LCMS, ULC grade

4.3 Solutions and reagents

4.3.1 Ammonium acetate solution, 1.0 M
Dissolve 7.7 grams of ammonium acetate (4.2.3) in LC-MS grade water (4.2.8) and make up to 100 ml. The shelf life is six months at room temperature.

4.3.2 Dispersion solution, 10% NaCl in water containing 1% HCl + 1% HAc.
Weight 100 g NaCl (4.2.7) in 1 L-volumetric flask and add 10 mL of HAc (4.2.1) and 10 mL of HCl (4.2.4). Fill with water (4.2.8) up to the mark. The shelf life is one month at room temperature.

4.3.3 Extraction solution, acetonitrile containing 1% HCl + 1% HAc.
In 1L-volumentric flask, pipette 10 mL of HAc (4.2.1) and 10 mL of HCl (4.2.4). Fill up to the mark with acetonitrile (4.2.2). The shelf life is one month at room temperature.

4.3.4 Dilution solution, MeOH/water/HAc (80/18/2 V/V/V)
Mix 80 mL MeOH (4.2.6) with 18 mL of water (4.2.8) and 2 mL of HAc (4.2.1). The shelf life is six months at room temperature.

4.3.5 Mobile phase A, 5 mM NH4Ac / 0.05% HAc in water
Mix 5 mL of ammonium acetate solution 1.0 M (4.3.1) and 0.5 mL acetic acid (4.2.1) with 900 mL LC-MS grade water (4.2.8). Adjust the volume with LCMS grade water (4.2.8) to 1 L and mix well. The shelf life is one month at room temperature.

4.3.6 Mobile phase B, 5 mM NH4Ac / 0.05% HAc in methanol
Mix 5 mL of ammonium acetate solution 1.0 M (4.3.1) and 0.5 ml acetic acid (4.2.1) with 900 ml methanol (4.2.6). Adjust the volume with methanol (4.3.6) to 1 L and mix well. The shelf life is one month at room temperature.

4.4 Standard solutions

4.4.1 Citrinin standard solution 1.0 µg/mL
Mix 100 µl of citrinin standard solution 100 µg/ml (4.1.1) with 9.9 mL of dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4).

4.4.2 Citrinin standard solution 100 ng/mL
Mix 100 µl of citrinin reference standard solution 1.0 µg/mL (4.4.1) with 0.9 ml of dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4).

4.4.3 Citrinin standard solution 10 ng/mL

Mix 100 µL of citrinin standard solution 1.0 µg/mL (4.4.1) with 9.9 mL of dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4).

4.4.4 13C13-Citrinin standard solution 1.0 µg/mL

Mix 100 µL of 13C13-Citrinin 10 µg/mL (4.1.2) with 0.9 mL of dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4).

4.4.5 13C13-Citrinin standard solution 100 ng/mL

Mix 100 µL of 13C13-Citrinin 10 µg/mL (4.1.2) with 9.9 mL of dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4).

4.4.6 Calibration solutions in solvent applying internal standard

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	***Concen-trations (ng/mL)***		**CIT 10 ng/mL (µl) (4.4.3)**	**CIT 100 ng/mL (µl) (4.4.2)**	**CIT 1000 ng/mL (µl) (4.4.1)**	**13C13-CIT 100 ng/mL (µl) (4.4.5)**	**Dilution solution (µl) (4.3.4)**
	CIT	***13C13 -CIT***					
CAL1	-	-	-		-	10	990
CAL2	*0.20*	*1*	20		-	10	970
CAL3	*0.5*	*1*	50			10	940
CAL4	*1.0*	*1*	-	10	-	10	980
CAL5	*2.5*	*1*	-	25	-	10	965
CAL6	*5.0*	*1*	-	50	-	10	940
CAL7	*10*	*1*	-	-	10	10	980
CAL8	*25*	*1*	-	-	25	10	965
CAL9	*50*	*1*	-	-	50	10	940

4.4.7 Calibration solutions in solvent applying single point standard addition

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	***Concen-trations (ng/mL) CIT***	**CIT 10 ng/mL (µl) (4.4.3)**	**CIT 100 ng/mL (µl) (4.4.2)**	**CIT 1000 ng/mL (µl) (4.4.1)**	**Dilution solution (µl) (4.3.4)**
CAL1	-	-		-	1000
CAL2	*0.20*	20		-	980
CAL3	*0.5*	50			950
CAL4	*1.0*	-	10	-	990
CAL5	*2.5*	-	25	-	975
CAL6	*5.0*	-	50	-	950
CAL7	*10*	-	-	10	990
CAL8	*25*	-	-	25	975
CAL9	*50*	-	-	50	950

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 PP bottle, 500 mL

5.1.3 PTFE Filter, mini-UniPrep, 0.45 µm

5.2 Equipment

5.2.1 Balance, accuracy +/- 0,01 g

5.2.2 Analytical balance, accuracy +/- 0,1 mg

5.2.3 Mechanical shaker head-over-head, adjustable

5.2.4 Vortex mixer

5.2.5 Centrifuge, suitable for 50 mL centrifuge tubes

5.2.6 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 citrinin and capable of baseline separation of citrinin.

5.2.6.1 Column oven, capable of maintaining a constant temperature of 50°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 citrinin in red yeast rice food supplements. The target level is 100 µg/kg. The steps described in section 6.4 are presented in the format of a checklist in Annex A.1 when applying internal standards and Annex A.2 when applying single standard addition.

6.1 Quality control

Prepare recovery samples as follows:
Use a blank sample and weigh 2 portions of tablets or capsules into a PP flask of 500 mL (5.1.2)

- QC_{bl}: use the first portion as blank
- QC_{rec}: add to the second portion an amount of citrinin standard solution 100 µg/mL (4.4.1) to obtain a spike level of 100 µg/kg

Process the samples according to 6.4.

6.2 Pre-treatment

No pre-treatment of the samples is required.

6.3 Trial unit

The number of capsules for analysis is based on Regulation (EC) No 401/2006, amended with Regulation (EU) No 519/2014 [2].

6.4 Extraction, clean-up and preparation of test solutions

6.4.1 Extraction, clean-up and preparation of test solutions applying internal standard

- Weigh in the number of tablets or capsules needed into a PP bottle of 500 mL (5.1.2);
- Register the total weight of the tablets or capsules used;
- Add per 1 g of sample 2.5 mL of dispersion solution, 10% NaCl in water containing 1% HCl + 1% HAc (4.3.2);
- Shake on a mechanical shaker head-over-head (5.2.3) for 15 min;
- Add per 1 g of sample 5.0 mL extraction solution, acetonitrile containing 1% HCl + 1% HAc (4.3.3);
- Shake on mechanical shaker head-over-head (5.2.3) for 30 min;
- Transfer 15 mL of the extraction mixture to a 50 mL PP centrifuge tube (5.1.1) (equivalent to 2 g sample);
- Add 100 µl of 13C13-citrinin standard solution 1.0 µg/mL (4.4.4);
- Add 4 g magnesium sulphate (4.2.5) and 1 g sodium chloride (4.2.7);
- Mix 30 sec on a vortex mixer (5.2.4);
- Centrifuge the tubes at 3500 rpm for 10 minutes (5.2.5);
- Dilute the extract (ACN phase) 10x with dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4);
- Transfer the final extract to a filter vial (5.1.3).

6.4.2 Extraction, clean-up and preparation of test solutions applying single level standard addition at e.g. 150 µg/kg

In this option matrix effects are compensated for on an individual sample basis. The final extract is split in two aliquots. One aliquot is analysed as such. To the other aliquot, a small

volume of solvent standard is added before phase separation. The concentration in the sample extract is calculated from the 1-point standard addition. A disadvantage of this option as described here is that each sample requires typically 2 injections.

Note: a single-level standard addition has been shown to give comparable results as multi-level standard addition when: a) the response obtained after standard addition is at least two times the response of the peak in the extract without addition, b) the response of extracts with and without addition are within the linear range of the detection. The latter can be verified through a calibration curve in solvent.

- Weigh in the number of tablets or capsules needed into a PP bottle of 500 mL (5.1.2);
- Register the total weight of the tablets or capsules used;
- Add per 1 g of sample 2.5 mL of dispersion solution, 10% NaCl in water containing 1% HCl + 1% HAc (4.3.2);
- Shake on a mechanical shaker head-over-head (5.2.3) for 15 min;
- Add per 1 g of sample 5.0 mL extraction solution, acetonitrile containing 1% HCl + 1% HAc (4.3.3);
- Shake on mechanical shaker head-over-head (5.2.3) for 30 min;
- Transfer two aliquots of 15 mL of the extraction mixture (ACN phase) to a 50 mL PP centrifuge tube (5.1.1) each;
- Spike one of the two aliquots with 300 µl of citrinin standard solution 1.0 µg/mL (4.4.1)
- Add 4 g magnesium sulphate (4.2.5) and 1 g sodium chloride (4.2.7);
- Mix 30 sec on a vortex mixer (5.2.4);
- Centrifuge the tubes at 3500 rpm for 10 minutes (5.2.5);
- Dilute the extract (ACN phase) 10x with dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4);
- Transfer the final extract to a filter vial (5.1.3).

7 LC-MS/MS analysis

Chromatographic and mass spectrometric conditions may be chosen freely. The optimal measurement conditions strongly depend on the instrumentation used.
The LC-MS system is conditioned and the CAL2 is injected twice, followed by the injection of solvent. These injections should meet the following criteria:

- Retention times should be stable.
- Sensitivity should be sufficient and fit-for-purpose.: Sensitivity is sufficient if citrinin can be measured at the reporting limit level (0.36 ng/mL).
- Carry-over: The presence of the target compounds in the solvent injection is assessed. If the target compounds are present in the solvent injection, which might lead to a false-positive result, the specialist should be informed 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.

- Dilution solution (4.3.4)
- Calibration solutions CAL (4.4.6 or 4.4.7)
- Dilution solution (4.3.4)
- Blank chemicals
- Quality control blank sample (QC_{bl} 6.1)
- Quality control recovery sample (QC_{rec} 6.1)
- Sample extracts (6.4.1)
- Dilution solution (4.3.4)
- Calibration solutions CAL (4.4.6 or 4.4.7)

Optionally: injected calibration solution CAL4 (2.5 ng/mL) (4.4.6) at least after every 10 sample extracts.

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 CAL 1 to 9 (4.4.6 or 4.4.7) are used to determine the linearity of the LC-MS/MS system. Plot the response of the quantifier of all individual calibration solutions (4..4.6 or 4.4.7, calibration solutions 1 to 9) against the corresponding concentrations 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 citrinin in the samples

Identify citrinin in the samples by comparing retention time and ion ratio with that of the calibration solutions (4.4.6 or 4.4.7) according to SANTE/12682/2019 [3].

Citrinin is considered present and identified when:

a) in the blank sample (QC_{bl}), the peak for the quantifier ion at the retention time of the mycotoxin is below 30% of the limit of quantification;
b) the retention time of the peak observed for the sample extracts differs less than 0.1 min from the average retention time as calculated from the calibration solutions;
c) the ratio of the area of the quantifier and qualifier transition (lowest area/highest area) for the mycotoxin in the sample extracts deviates less than 30% (relative) from the average ion ratio of the calibration standards (=reference ion ratio).

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 citrinin in the samples

8.3.1 Quantification with isotope labelled internal standard

When the response is within the linear range, the concentration of citrinin in the sample is calculated based on 1-point bracketing calibration and the use of an isotope labelled internal standard. Calculate the concentration of citrinin in the sample according to Equation I

Equation I: Calculation of the concentration of citrinin in the sample

$$C_s = \frac{R_s}{R_{av}} \times \frac{V_e}{i} \times D \times C_{std} \times \frac{C_{label\ sample}}{C_{label\ std}}$$

Where:

C_s	=	is the concentration of the analyte in the sample in µg/kg
R_s	=	is the response ratio of the quantifier peak area of citrinin and the quantifier peak area of its isotope labelled internal standard obtained from the sample
R_{av}	=	is the average of the response ratios of the quantifier peak area of citrinin and the quantifier peak area of its isotope labelled internal standard in the calibration standard injected before and after the sample
V_e	=	is extraction volume, here volume extraction solution added (mL)
i	=	is sample intake (g)
D	=	is the dilution factor
C_{std}	=	is the concentration of the citrinin in the calibration solution in ng/mL (4.4.6)
$C_{label\ sample}$	=	is the concentration of isotope-labelled standard in the final sample extract in ng/mL
$C_{label\ std}$	=	is the concentration of isotope-labelled standard in the calibration solution in ng/mL

8.3.2 Quantification by means of single level standard addition

In case an internal standard for citrinin is not available the quantification can be performed by single level standard addition to the sample. Use Equation II to calculate the concentration in the sample:

Equation II 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 area of the quantifier in the sample
A_{added}	is the area of the quantifier in the spiked sample
C_{added}	is the concentration of citrinin in the final extract of the fortified sample in ng/ml. (here: 0.3 ml x 1,0 µg/ml/11 ml* x 1000/10 = 2.7 ng/ml)

* total extraction volume is 11 ml due to increase by the extraction procedure (QuEChERS method)

8.3.3 Recovery

Determine the recovery by calculating the concentration of citrinin in the QC_{rec} sample (6.1), as described in section 8.3.1 or 8.3.2. The result should be between 90% and 110% of the amount added to the sample.

8.4 Final result

The concentration of citrinin in the sample red yeast rice food supplement is expressed as µg/kg.

9 References

[1] EU (2006) Regulation (EC) No 1881/2006 amended with Regulation (EU) 2019/1901.
[2] EU (2006) Regulation (EC) No 401/2006 amended with Regulation (EU) No 519/2014.
[3] 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/system/files/2020-01/pesticides_mrl_guidelines_wrkdoc_2019-12682.pdf**

Annex A.1 Checklist for sample preparation red yeast rice food supplements applying internal standard

Technician:	
Date:	
Lab. journal / page:	

Annex A.1 Checklist for sample preparation red yeast rice food supplements applying internal standard

A.1.1 Preparation of calibration solutions in solvent (4.4.6) applying internal standard

Mix the following solutions:

Code	*Concentrations (ng/mL)*		CIT 10 ng/mL (µl) (4.4.3)	CIT 100 ng/mL (µl) (4.4.2)	CIT 1000 ng/mL (µl) (4.4.1)	13C13-CIT 100 ng/mL (µl) (4.4.5)	Dilution solution (µl) (4.3.4)
	CIT	*13C13-CIT*					
CAL1	-	-	-		-	10	990
CAL2	*0.20*	*1*	20		-	10	970
CAL3	*0.5*	*1*	50			10	940
CAL4	*1.0*	*1*	-	10	-	10	980
CAL5	*2.5*	*1*	-	25	-	10	965
CAL6	*5.0*	*1*	-	50	-	10	940
CAL7	*10*	*1*	-	-	10	10	980
CAL8	*25*	*1*	-	-	25	10	965
CAL9	*50*	*1*	-	-	50	10	940

A.1.2 Quality control recovery QC REC sample (100 µg/kg) (6.1)

- ☐ Weigh correct number of blank red yeast rice material in a PP bottle of 500 mL (5.1.2);
- ☐ Register the weight of the tablets or capsules used;
- ☐ Add correct amount of the citrinin standard solution 100 µg/mL (4.1.1) to make a QC_{rec} sample of 100 µg/kg (6.1);
- ☐ Continue with A1.3.

Annex A.1 Checklist for sample preparation red yeast rice food supplements applying internal standard

A.1.3 Sample preparation for analysis (6.4.1)

- ☐ Weigh in the number of tablets or capsules needed into a PP flask of 500 mL (5.1.2);
- ☐ Register the total weight of the tablets or capsules used;
- ☐ Add per 1 g of sample 2.5 mL of dispersion solution, 10% NaCl in water containing 1% HCl + 1% HAc (4.3.2);
- ☐ Shake on a mechanical shaker head-over-head (5.2.3) for 15 min;
- ☐ Add per 1 g of sample 5.0 mL extraction solution, acetonitrile containing 1% HCl + 1% HAc (4.3.3);
- ☐ Shake on mechanical shaker head-over-head (5.2.3) for 30 min;
- ☐ Transfer 15 mL of the extraction mixture to a 50 mL PP centrifuge tube (5.1.1);
- ☐ Add 100 µl of 13C13-Citrinin standard solution 1.0 µg/mL (4.4.4);
- ☐ Add 4 g magnesium sulphate (4.2.5) and 1 g sodium chloride (4.2.7);
- ☐ Mix 30 sec on a vortex mixer (5.2.4);
- ☐ Centrifuge the tubes at 3500 rpm for 10 minutes (5.2.5);
- ☐ Dilute the extract (ACN phase) 10x with dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4);
- ☐ Transfer the final extract to a filter vial (5.1.3).

Annex A.2 Checklist for sample preparation red yeast rice food supplements applying single level standard addition

Technician:	
Date:	
Lab. journal / page:	

Annex A.2 Checklist for sample preparation red yeast rice food supplements applying single-level standard addition

A.2.1 Preparation of calibration solutions in sovent (4.4.7) applying single-level standards addition

Mix the following solutions

Code	*Concentrations (ng/mL) CIT*	CIT 10 ng/mL (µl) (4.4.3)	CIT 100 ng/mL (µl) (4.4.2)	CIT 1000 ng/mL (µl) (4.4.1)	Dilution solution (µl) (4.3.4)
CAL1	-	-		-	1000
CAL2	*0.20*	20		-	980
CAL3	*0.5*	50			950
CAL4	*1.0*	-	10	-	990
CAL5	*2.5*	-	25	-	975
CAL6	*5.0*	-	50	-	950
CAL7	*10*	-	-	10	990
CAL8	*25*	-	-	25	975
CAL9	*50*	-	-	50	950

A.2.2 Quality control recovery QC REC sample (100 µg/kg) (6.1)

- ☐ Weigh correct number of blank red yeast rice material in a PP bottle of 500 mL (5.1.2);
- ☐ Register the weight of the tablets or capsules used;
- ☐ Add correct amount of the citrinin standard solution 100 µl/mL (4.1.1) to make a QC_{rec} sample of 100 µg/kg (6.1);
- ☐ Continue with A.2.3.

Annex A.2 Checklist for sample preparation red yeast rice food supplements applying single-level standard addition

A.2.3 Sample preparation for analysis (6.4.2)

- ☐ Weigh in the number of tablets or capsules needed into a PP flask of 500 mL (5.1.2);
- ☐ Register the total weight of the tablets or capsules used;
- ☐ Add per 1 g of sample 2.5 mL of dispersion solution, 10% NaCl in water containing 1% HCl + 1% HAc (4.3.2);
- ☐ Shake on a mechanical shaker head-over-head (5.2.3) for 15 min;
- ☐ Add per 1 g of sample 5.0 mL extraction solution, acetonitrile containing 1% HCl + 1% HAc (4.3.3);
- ☐ Shake on mechanical shaker head-over-head (5.2.3) for 30 min;
- ☐ Transfer two aliquots of 15 mL of the extraction mixture to a 50 mL PP centrifuge tube (5.1.1) each;
- ☐ Spike one of the two aliquots with 300 µl of citrinin standard solution 1.0 µg/mL (4.4.1)
- ☐ Add 4 g magnesium sulphate (4.2.5) and 1 g sodium chloride (4.2.7);
- ☐ Mix 30 sec on a vortex mixer (5.2.4);
- ☐ Centrifuge the tubes at 3500 rpm for 10 minutes (5.2.5);
- ☐ Dilute the extract (ACN phase) 10x with dilution solution MeOH/water/HAc (80/18/2 V/V/V) (4.3.4);
- ☐ Transfer the final extract to a filter vial (5.1.3).

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 HSS T3 1.8 µm 100 x 2.1 mm
Column temperature:	40 °C
Mobile phase solvent A:	5 mM NH4Ac / 0.05% HAc in water (4.3.5)
Mobile phase solvent B:	5 mM NH4Ac / 0.05% HAc in methanol(4.3.6)
Flow rate:	0.4 mL/min
Injection volume:	5 µL
Injection temperature	10 °C
Gradient program:	Table B.1

Table B.1 Gradient for the LC system

Time (min)	Mobile phase A (4.3.5) %	Mobile phase B (4.3.6) %
0	95.0	5.0
0.1	95.0	5.0
4.1	15.0	85.0
4.2	0.0	100
7.0	0.0	100
7.1	95.0	5.0
9.0	95.0	5.0

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.2.1 Example for MS conditions

Parameter	Settings
Capillary voltage	2.80 kV
Cone voltage	20.0 V
Source Offset (V)	30 V
Source temperature	150°C
Desolvation temperature	450°C
Cone gas flow	150 L/hr
Desolvation gas flow	650 L/hr
Collision Gas Flow (mL/Min)	0.18 (mL/min)
Nebuliser Gas Flow (Bar)	7.00 (Bar)

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 citrinin

Analyte	Indicative RT (min)	Precursor ion (m/z)	Cone voltage (V)	Product ion (m/z) (eV)	Collision energy (eV)	Dwell time (s)
Citrinin (qn)		281.2	20	249.2	30	0.080
Citrinin (ql1)		281.2	20	205.2	25	0.080
Citrinin (ql2)		281.2	20	177.1	20	0.080
^{13}C13-Citrinin		294.2	20	262.2	30	0.080

B.3 LC-MS/MS example chromatogram of a red yeast rice food supplement cellulose capsule/gel capsule fortified at 100 µg/kg