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## ISO/FDIS 23719

### Cereals and cereal products — Determination of 17 mycotoxins by ultra-high-performance liquid chromatography and tandem mass spectrometry method (UHPLC-MS/ MS)

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## Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 4, *Cereals and pulses*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

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# Cereals and cereal products — Determination of 17 mycotoxins by ultra-high-performance liquid chromatography and tandem mass spectrometry method (UHPLC-MS/MS)

## 1 Scope

This document specifies a method for the quantitative determination of 17 mycotoxins in cereals and cereal products (e.g. wheat, maize, husked rice, rice and their products) using ultra-high-performance liquid chromatography and tandem mass spectrometry method (UHPLC-MS/MS).

The 17 mycotoxins are aflatoxin B<sub>1</sub>, aflatoxin B<sub>2</sub>, aflatoxin G<sub>1</sub>, aflatoxin G<sub>2</sub>, deoxynivalenol, nivalenol, deoxynivalenol-3-Glucoside, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, zearalenone, ochratoxin A, fumonisin B<sub>1</sub>, fumonisin B<sub>2</sub>, fumonisin B<sub>3</sub>, T-2 toxin, HT-2 toxin and sterigmatocystin.

This document does not apply to foods for infants and young children.

This document is applicable to other products (e.g. nuts) provided that the method is validated for each individual case.

The calibration range of the method and the validated range during the interlaboratory study for each mycotoxin are listed in [Table A.1](#).

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

## 3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

## 4 Principle

Mycotoxins are extracted using an acetonitrile-water-acetic acid (70:29:1, v/v/v) solution. After centrifugation, the extract is diluted with water, centrifuged in low temperature and filtered. The final solution is mixed with isotope-labelled internal standard solution and subjected to UHPLC-MS/MS analysis. Quantification is performed by the isotopic dilution approach using <sup>13</sup>C isotopically labelled mycotoxins as internal standards (ISTDs).

**WARNING —** Mycotoxins are generally considered to be carcinogenic, neurotoxic and immunosuppressive. Observe appropriate safety precautions<sup>[1]</sup> for handling such compounds and avoid handling in dry form as the electrostatic nature can result in dispersion and inhalation.

Glassware can be decontaminated with 4 % sodium hypochlorite solution. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO).<sup>[2][3]</sup>

## 5 Reagents

**WARNING — This document requires handling of hazardous substances. Technical, organizational and personal safety measures shall be followed.**

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and only distilled water or water of grade 1 in accordance with ISO 3696. Solvents shall be of quality for LC analysis.

**5.1 Acetonitrile**, LC grade or equivalent.

**5.2 Methanol**, LC grade or equivalent.

**5.3 Ammonium acetate**, American Chemical Society (ACS) grade, > 99 %.

**5.4 Acetic acid**, ACS grade.

**5.5 Formic acid**, ACS grade.

**5.6 Extraction solvent**: Mix 70 volume parts of acetonitrile (5.1) and 29 volume parts of water and 1 volume part of acetic acid (5.4).

**5.7 Solvent of calibration solutions**: Mix 50 volume parts of extraction solvent (5.6) and 50 volume parts of water.

**5.8 Individual stock solutions**, either prepared by dissolving neat (solid) certified standards in an appropriate solvent or from individual stock solutions purchased as such.

The mycotoxins covered in this document dissolve well in acetonitrile, except for fumonisins which are soluble in a mixture of acetonitrile and water (50:50, v/v). The information and concentration of 17 mycotoxins and the isotope-labelled internal standards is shown in [Table B.1](#).

**5.9 Mixed stock solution.**

Prepare a mixed stock solution containing all individual mycotoxins at the concentration given in [Table 1](#), using the appropriate pipettes (6.1) and dilute with deionized water. This solution can be used for six months if stored in the dark at -20 °C.

This mixed stock solution may be used for the preparation of positive control samples (see [7.5](#)).

**Table 1 — Concentration of stock solution for 17 mixed mycotoxins**

Compounds	Concentration µg/ml	Compounds	Concentration µg/ml
NIV	20	FB <sub>1</sub>	12,5
DON	15	FB <sub>2</sub>	7,5
DON-3G	2,5	FB <sub>3</sub>	9,0
3-AcDON	4	T-2	0,2
15-AcDON	2	HT-2	1
AFB <sub>1</sub>	0,1	ZEN	2

**Table 1** (continued)

Compounds	Concentration µg/ml	Compounds	Concentration µg/ml
AFB <sub>2</sub>	0,1	OTA	0,2
AFG <sub>1</sub>	0,1	ST	0,1
AFG <sub>2</sub>	0,1	-	-

### 5.10 Mixed isotope-labelled internal standard (ISTD) solution.

Isotopically labelled mycotoxins are generally available as certified standard solutions. Prepare a mixed ISTD solution in a mixture of acetonitrile and water (30:70, v/v), containing all isotopically labelled mycotoxins at the concentration given in [Table 2](#). This solution can be used for six months if stored in the dark at –20 °C.

**Table 2 — Concentration of isotope-labelled internal standard stock solution for 17 mixed mycotoxins**

Compounds	Concentration µg/ml	Compounds	Concentration µg/ml
[ <sup>13</sup> C <sub>15</sub> ]-NIV	2,5	[ <sup>13</sup> C <sub>34</sub> ]-FB <sub>1</sub>	0,5
[ <sup>13</sup> C <sub>15</sub> ]-DON	2	[ <sup>13</sup> C <sub>34</sub> ]-FB <sub>2</sub>	0,3
[ <sup>13</sup> C <sub>21</sub> ]-DON-3G	1	[ <sup>13</sup> C <sub>34</sub> ]-FB <sub>3</sub>	0,3
[ <sup>13</sup> C <sub>17</sub> ]-3-AcDON	1	[ <sup>13</sup> C <sub>24</sub> ]-T-2	0,05
[ <sup>13</sup> C <sub>17</sub> ]-15-AcDON	1	[ <sup>13</sup> C <sub>22</sub> ]-HT-2	0,125
[ <sup>13</sup> C <sub>17</sub> ]-AFB <sub>1</sub>	0,01	[ <sup>13</sup> C <sub>18</sub> ]-ZEN	0,2
[ <sup>13</sup> C <sub>17</sub> ]-AFB <sub>2</sub>	0,01	[ <sup>13</sup> C <sub>20</sub> ]-OTA	0,04
[ <sup>13</sup> C <sub>17</sub> ]-AFG <sub>1</sub>	0,01	[ <sup>13</sup> C <sub>18</sub> ]-ST	0,025
[ <sup>13</sup> C <sub>17</sub> ]-AFG <sub>2</sub>	0,01	-	-

### 5.11 Calibration solutions.

Transfer appropriate volumes of the mixed stock solutions ([5.9](#)) into six separate 1,5 ml injection vials as described in [Table 3](#). The concentrations for the calibration solutions are shown in [Table 4](#). Store them at –20 °C for no longer than two weeks before use.

Transfer a 180 µl aliquot of the calibration solutions into an injection vial ([6.3](#)), individually. Add 20 µl mixed ISTD solution ([5.10](#)) into all the vials. The same ISTD solution as used in sample preparation shall be used. Cap and mix.

**Table 3 — Example of calibration solutions**

No.	Calibration standard solution no.	Calibration standard solution	Volume of standard solution µl	Volume of acetonitrile- water-acetic acid (35:64.5:0.5, v/v/v) ( <a href="#">5.7</a> ) µl
Step 1	SS1	mixed stock solution ( <a href="#">5.9</a> )	50	950
Step 2	SS2	mixed stock solution ( <a href="#">5.9</a> )	20	980
Step 3	SS3	SS1	200	800
Step 4	SS4	SS2	200	600
Step 5	SS5	SS3	200	600
Step 6	SS6	SS4	200	600

Table 4 — Concentration of calibration solutions

Dimensions in µg/l

Mycotoxin	SS6	SS5	SS4	SS3	SS2	SS1
NIV	25	50	100	200	400	1 000
DON	18,75	37,5	75	150	300	750
DON-3G	3,125	6,25	12,5	25	50	125
3-ACDON	5	10	20	40	80	200
15-ACDON	2,5	5	10	20	40	100
AFB <sub>1</sub>	0,125	0,25	0,5	1	2	5
AFB <sub>2</sub>	0,125	0,25	0,5	1	2	5
AFG <sub>1</sub>	0,125	0,25	0,5	1	2	5
AFG <sub>2</sub>	0,125	0,25	0,5	1	2	5
FB <sub>1</sub>	15,625	31,25	62,5	125	250	625
FB <sub>2</sub>	9,375	18,75	37,5	75	150	375
FB <sub>3</sub>	11,25	22,5	45	90	225	450
T-2	0,25	0,5	1	2	4	10
HT-2	1,25	2,5	5	10	20	50
ZEN	2,5	5	10	20	40	100
OTA	0,25	0,5	1	2	4	10
ST	0,125	0,25	0,5	1	2	5

## 6 Apparatus and equipment

The usual laboratory equipment and, in particular, the following shall be used.

**6.1 Automatic pipettes**, suitable for handling volumes of 1 µl to 20 µl, 10 µl to 100 µl, 200 µl to 1 000 µl and 1 ml to 10 ml.

Instead of the latter, 10 ml graduated glass pipettes may be used as an alternative.

**6.2 Centrifuge tubes**, 1,5 ml and 10 ml.

**6.3 Injection vials**, 1,5 ml, suitable for an LC autosampler, if necessary, with 400 µl micro-inserts.

**6.4 Vortex mixer**.

**6.5 Orbital shaker**, adjustable mechanical vertical or horizontal shaker, capable to shake at 30 min

**6.6 Polytetrafluoroethylene (PTFE) filters**, syringe filters, 0,2 µm pore size, 13 mm internal diameter.

**6.7 UHPLC-MS/MS system**, with the components given in [6.7.1](#) to [6.7.7](#).

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

**6.7.2 Degasser**, optional, for degassing LC mobile phases.

**6.7.3 Injection system**, capable of injecting an appropriate volume of injection solution with sufficient accuracy.



**6.7.4 LC column**, capable to retain the first eluting analyte at least twice the retention time corresponding to the void volume of the column. Examples of suitable columns and gradients are given in [Annex C](#).

**6.7.5 Column oven**, capable to maintain a constant temperature.

**6.7.6 Tandem mass spectrometer** (e.g. triple quadrupole or quadrupole linear ion trap), equipped with an electrospray ionization (ESI) interface and operated in multiple reaction monitoring (MRM) mode. Any ionization mode (typically negative or positive) giving sufficient yield may be employed.

**6.7.7 Computer-based instrument control and data evaluation system.**

**6.8 Mill equipped with 0,5 mm sieves.**

**6.9 Conical flask**, 250 ml.

**6.10 Blender**, 1 jar and cover, explosion-proof.

**6.11 Ceramic abrasive particles**, oblique elliptical cylinder diamond, 1,5 cm (length) × 0,65 cm (outer diameter).

**6.12 Centrifuges:**

- a) suitable for the centrifuge tubes (10 ml and 50 ml) and capable of achieving at least 3 500*g*;
- b) suitable for the centrifuge tubes (1,5 ml) and capable of achieving at least 7 500*g* at 4 °C.

**6.13 Laboratory balance**, accuracy of 0,01 g.

**6.14 Analytical balance**, accuracy of 0,1 mg.

**6.15 Disposable syringe**, 1,5 ml.

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## 7 Procedure

### 7.1 Sampling and preparation of the laboratory sample

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 24333<sup>[4]</sup>.

A representative sample should be sent to the laboratory. It should not have been damaged or changed during transport and storage. Grind the laboratory sample using a laboratory mill ([6.8](#)) until it passes through the sieve (0,5 mm) and mix it thoroughly.

### 7.2 Test portion weighing

Weigh a test portion of 25,00 g (*m*) of the homogeneous laboratory sample to the nearest 0,01 g into a conical flask ([6.9](#)) or a blender ([6.10](#)).

For samples with good homogeneity (e.g. flour), a minimum test sample amount of 5 g and an extraction solvent volume of 20 ml may be used.

### 7.3 Extraction

Add 100 ml extraction solvent ([5.6](#)) (*V<sub>1</sub>*). Cover and shake for 30 min ([6.5](#)) or blend for 3 min ([6.10](#)). Pipette 5 ml supernatant into a 10 ml centrifuge tube ([6.2](#)) and centrifuge at 3 500*g* for 5 min. Add 0,5 ml supernatant

( $V_2$ ) into a 1,5 ml centrifuge tube (6.2), dilute with 0,5 ml water ( $V_3$ ) and shake on a vortex mixer (6.4) for 1 min. Then centrifuge (7 500g, 4 °C, 10 min, 6.12 b). The supernatant shall be filtrated through a 0,2 µm PTFE filter (6.6).

## 7.4 Preparation of the sample test solution

Transfer a 180 µl aliquot of the filtrate obtained in 7.3 into an injection vial (6.3). Add 20 µl mixed ISTD solution (5.10) into each vial. The same ISTD working solution as used in the preparation of the sample and standard curve shall be used. Cap and mix.

## 7.5 Preparation of control samples

With each sample batch, one reagent blank and one positive control shall be used.

To create a reagent blank, perform extraction (see 7.3) and subsequent steps without adding the sample test portion.

The positive control should be a sample free of the target mycotoxins (non-detectable) which is spiked with 10 µl mixed stock solution (5.9) per 1 g. The spiking concentration level in samples for 17 mixed mycotoxins are shown in Table 5. Alternatively, a reference material known to contain the target mycotoxins may be used.

**Table 5 — Spiking concentration level in samples for 17 mixed mycotoxins**

Compounds	Concentration µg/kg	Compounds	Concentration µg/kg
NIV	200	FB <sub>1</sub>	125
DON	150	FB <sub>2</sub>	75
DON-3G	25	FB <sub>3</sub>	90
3-AcDON	40	T-2	2
15-AcDON	20	HT-2	10
AFB <sub>1</sub>	1	ZEN	20
AFB <sub>2</sub>	1	OTA	2
AFG <sub>1</sub>	1	ST	1
AFG <sub>2</sub>	1	-	-

## 7.6 Analysis

### 7.6.1 Operating conditions

Optimize analytical parameters (selection of the ionization mode, selection of the masses of precursor and product ions, optimization of source parameters and collision energies) by infusion of each individual mycotoxin standard solution. At least two ion transition reactions should be measured for each unlabelled mycotoxin. The ion transition reaction giving the largest signal-to-noise ratio shall be selected as the quantifier one. Choose the MRM transition reaction of the internal standard that corresponds to it, taking into account the degree of isotope labelling.

A combination of analytical column, mobile phase composition, gradient settings and injection volume shall be selected so that it allows for obtaining an acceptable separation and reliable results at the required levels, with sufficient selectivity. Annex C gives some suitable parameters.

### 7.6.2 Injection sequence

An example is as follows:

- Start a batch of measurements by injecting an aliquot of the diluting solution (5.7) to prove non-contamination of the system.

- Then inject the calibration solutions from SS6 to SS1 (5.11) and carefully check that all mycotoxins and their respective ISTDs are visible at the lowest calibration level (SS6).

Inject an aliquot of the diluting solution (5.7) to check for possible carryover.

Inject the sample extracts (see 7.4) and regularly inject an aliquot of the diluting solution (5.7) to check for possible carryover.

For each injection sequence, a reagent blank shall be injected (7.5).

For each injection sequence, a quality control sample may be included. A reference material known to contain the targeted mycotoxins may be used. Alternatively, an extract of a sample free of the targeted mycotoxins (e.g. non-detectable levels) spiked with the mycotoxins within the range of the measurements may be used.

End the sequence by re-injecting the calibration solutions from SS6 to SS1 (5.11).

### 7.6.3 Data treatment

Process the data using an appropriate integration software. Peak areas are used for subsequent calculations. Check peak area assignment and integration for the measured transition reactions. Annex D gives some example chromatograms of mycotoxins and ISTDs.

## 8 Identification

Mycotoxins are considered as positively identified in the sample when the following criteria are fulfilled:

- A peak with an S/N (signal/noise) ratio  $\geq 3$  should be observed in at least two selected ion transition reactions.
- The retention time of the mycotoxin should correspond to that of its labelled internal standard within a tolerance of  $\pm 0,1$  min.
- The peak area ratio of the two product ions from sample extracts should be within  $\pm 30$  % (relative) of average of calibration solutions from same sequence.<sup>[5]</sup>

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## 9 Calculations

Construct the calibration curve by plotting the quantifier peak area ratio of each mycotoxin and its ISTD (y-axis) against concentration of each mycotoxin (x-axis) using calibration solutions from SS6 to SS1. To improve the precision on the low concentration, a weighing factor (e.g.  $1/x$  or  $1/x^2$ ) should be used for the calibration curve. Alternatively, the regression curve can be forced to zero. Calculate the slope and the intercept by linear regression. Check that the deviation of the back-calculated concentration of the calibrants standards from the true concentration, using the calibration curve, shall be not more than  $\pm 20$  %. If higher deviations or nonlinearity are observed, identify the cause and, if necessary, re-run the analyses. Calculate the slope ( $S$ ) and the intercept ( $I$ ) of the calibration curve.

Each mass fraction,  $X$ , of the mycotoxins, in micrograms per kilogram, shall be calculated according to Formula (1):

$$X = \frac{(A - I) \times V_1 \times (V_2 + V_3)}{S \times m \times V_2} \quad (1)$$

where

- $X$  is the final concentration of samples, in  $\mu\text{g}/\text{kg}$ ;
- $A$  is the peak area ratio of a given mycotoxin and its IS (quantifier transition reaction);
- $I$  is the intercept of the (weighted) regression line;
- $S$  is the slope of the (weighted) regression line;
- $V_1$  is the volume of the of sample extract, in ml (=100);
- $V_2$  is the volume of the supernatant for dilution, in ml (= 0,5);
- $V_3$  is the volume of the water for dilution, in ml (= 0,5);
- $m$  is the mass of the test portion, in g (=25).

## 10 Precision

### 10.1 General

Details of the interlaboratory test of the precision of the method in accordance with ISO 5725-2:2019<sup>[6]</sup> are summarized in [Annex E](#). The values derived from the interlaboratory tests are not necessarily applicable to analyte concentration ranges and matrices other than those given in [Annex E](#).

### 10.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit  $r$  in not more than 5 % of the cases (see [Tables E.1 to E.17](#)).

### 10.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit  $R$  in not more than 5 % of the cases (see [Tables E.1 to E.17](#)).

## 11 Test report

The test report shall contain at least the following information:

- all information necessary for the identification of the sample;
- a reference to this document, including its year of publication, e.g. ISO 23719;
- the results and the units in which the results have been expressed;
- the date and type of sampling (if known);
- the date of receipt of the laboratory sample;
- the date of the test;
- any points observed during the test;
- any operations not specified in the method or regarded as optional which can have affected the results.