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Cereals and cereal products — Determination of ochratoxin A — High performance liquid chromatographic method with immunoaffinity column cleanup and fluorescence detection

Céréales et produits céréaliers — Dosage de l'ochratoxine A — Méthode par chromatographie en phase liquide à haute performance avec purification sur colonne d'immunoaffinité et détection par fluorescence

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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 documents 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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 4, *Cereals and pulses*.

This first edition cancels and replaces ISO 15141-1:1998 and ISO 15141-2:1998, which have been technically revised.

The main change compared to the previous edition is that the principle of the extraction and purification has been changed.

Cereals and cereal products — Determination of ochratoxin A — High performance liquid chromatographic method with immunoaffinity column cleanup and fluorescence detection

1 Scope

This document specifies a high performance liquid chromatographic method with immunoaffinity column cleanup for the determination of ochratoxin A in cereals and cereal products.

The limit of quantification is 0,2 µg/kg. The method detection limit is dependent on the sample matrix as well as on the instrument.

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 terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

4 Principle

Ochratoxin A (OTA) is extracted by acetonitrile-water. The extract is purified using an immunoaffinity column and ochratoxin A is determined by high performance liquid chromatography (HPLC) on a reverse-phase column and fluorescence detection. The result is verified, if required, by derivatization with boron trifluoride in methanolic solution.

WARNING — Ochratoxin A causes kidney and liver damage and is a probable carcinogen. Observe appropriate safety precautions[1] for handling such compounds and in particular 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)[2][3].

5 Reagents

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 HPLC analysis.

5.1 Acetonitrile.

5.2 Methanol.

5.3 Sodium chloride (NaCl).

5.4 Glacial acetic acid, $\varphi(CH_3COOH) \geq 98$ %.

5.5 Tween-20.

5.6 Sodium bicarbonate ($NaHCO_3$).

5.7 Disodium hydrogen phosphate (Na_2HPO_4).

5.8 Potassium dihydrogen phosphate (KH_2PO_4).

5.9 Potassium chloride (KCl).

5.10 Hydrochloric acid, c(HCl) = 12 mol/l.

5.11 Ochratoxin A, in crystal form or as a film in ampoules.

5.12 Extraction solvent, mix 60 volume parts of acetonitrile (5.1) and 40 volume parts of water.

5.13 Phosphate buffered saline (PBS), dissolve 8 g NaCl (5.3), 1,2 g Na_2HPO_4 (5.7), 0,2 g KH_2PO_4 (5.8) and 0,2 g KCl (5.9) in about 990 ml water. Adjust pH to 7 with HCl (5.10) and dilute to 1 l with water.

5.14 Washing solution, dissolve 25 g NaCl (5.3), 5 g $NaHCO_3$ (5.6) and 0,1 ml Tween-20 (5.5) in 1 l water.

5.15 Mobile phase, mix 48 volume parts of acetonitrile (5.1) with 51 volume parts of water and 1 volume parts of glacial acetic acid (5.4) and degas this solution before use.

5.16 Toluene.

5.17 Solvent mixture, mix 99 volume parts of toluene (5.16) with 1 volume parts of glacial acetic acid (5.4).

5.18 Ochratoxin A stock solution.

Dissolve 1 mg of the ochratoxin A (crystals) (5.11) or the contents of 1 ampoule (if ochratoxin A has been obtained as a film) (5.11) in solvent mixture (5.17) to give a solution containing approximately 20 µg/ml to 30 µg/ml of ochratoxin A.

To determine the exact concentration, record the absorption curve between a wavelength of 300 nm and 370 nm in 5 nm steps in a 1 cm quartz cell (6.12) with solvent mixture (5.17) as reference. Identify the wavelength for maximum absorption by recording in 1 nm steps around the maximum as reference.

Calculate the mass concentration of ochratoxin A, ρ_{OTA}, in micrograms per millilitre of solution using Formula (1):

$$\rho_{OTA} = A_{max} \times \frac{M \times 100}{\kappa \times \delta} \quad (1)$$

where

A_{max} is the absorption determined at the maximum of the absorption curve (here: at 333 nm);

M is the relative molecular mass of ochratoxin A (M = 403 g/mol);

κ is the molar absorption coefficient of ochratoxin A, in solvent mixture (here: 544 m^2/mol);

δ is the path length of the cell in centimetres.

Store this solution at approximately –18 °C. A solution stored in this way is usually stable for 12 months. Check the concentration of the solution if it is older than 6 months.

5.19 Ochratoxin A standard solution, ρ_{OTA} = 1 µg/ml.

Evaporate under a nitrogen flow 1 ml of the stock solution (5.18) or the aliquot portion which is equivalent to an absolute amount of 100 µg of ochratoxin A to dryness and dilute to 100 ml with the mobile phase (5.15).

This solution can be stored in a refrigerator at 4 °C. Stability shall be checked.

5.20 Ochratoxin A calibration solutions.

Pipette suitable volumes of ochratoxin A standard solution (5.19), e.g. 0,05 ml, 0,1 ml, 0,25 ml, 0,5 ml and 1 ml into, for example, a 100 ml volumetric flask (6.15) and dilute to the mark with the mobile phase (5.15). The amount of ochratoxin A in the calibration solutions should cover the range of 0,05 ng to 1,0 ng per 100 µl injection volume. The calibration solutions should be freshly prepared from ochratoxin A standard solution (5.19) before each HPLC analysis.

5.21 Sodium hypochlorite solution, ρ(NaOCl) = 4 g/100 ml.

5.22 Boron trifluoride.

5.23 Boron trifluoride in methanol solution, $\rho(BF_3)$ = 14 g/100 ml.

5.24 Dichloromethane.

5.25 Sodium sulfate, anhydrous.

5.26 Elution solvent, mix 98 volume parts of methanol (5.2) and 2 volume parts of glacial acetic acid (5.4).

WARNING — Use a well maintained fume hood. Avoid contact with skin, eyes, and respiratory tract.

6 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

6.1 Analytical balance, accurate to 10 mg.

6.2 Blender, 1 l jar and cover, explosion-proof.

6.3 Filter paper,

a) folded filter paper, or

b) glass microfibre filter.

6.4 Centrifuge tube, 50 ml.

6.5 Membrane filter for aqueous solutions, made of polytetrafluoroethylene (PTFE), with a diameter of 25 mm and a pore size of 0,2 µm.

6.6 Immunoaffinity column, which shall contain antibodies raised against ochratoxin A, ToxinFast® Ochratoxin A Immunoaffinity Column (Huaan Magnech)[1)] or equivalent.

6.7 Glass syringe, 10 ml.

6.8 Vacuum pump.

6.9 Rotary evaporator, with a water bath capable of being controlled between 20 °C and 50 °C.

6.10 Laboratory mill, suitable to grind to 1 mm.

6.11 UV-Spectrometer, suitable for measurement at wavelengths of 300 nm up to 370 nm, having a spectral band width of not more than ± 2 nm.

6.12 Quartz cells, with 1 cm optical path length and no significant absorption between wavelengths of 300 nm and 370 nm.

6.13 Conical flask, 150 ml.

6.14 Sieve, with an aperture size of not more than 1 mm.

6.15 Volumetric flask, 100 ml.

6.16 Microsyringe, of capacity 500 µl.

6.17 HPLC apparatus, comprising

a) **high performance liquid chromatograph**, eluent reservoir, a pump, an injection system, a fluorescence detector with variable wavelength setting and a data processing, e.g. an integrator with plotter, and

b) **analytical reverse-phase HPLC separating column**, C_{18}, which ensures a baseline resolved resolution of the ochratoxin A peak from all other peaks.

1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

length:	150 mm
internal diameter:	4,6 mm
spherical particles of size:	5 µm

NOTE Other length columns that have been found to be suitable can also be used.

6.18 Centrifuge, capable of a centrifugal force of 8 000*g*.

7 Procedure

7.1 General

The whole analytical procedure should be performed in one working day. If several samples are processed at the same time all samples should be analysed during the following night using an automatic sample injector.

7.2 Sampling

Sampling is not part of the method specified in this document. Recommended sampling methods are given in ISO 24333[4].

7.3 Preparation of the test samples

Grind the laboratory sample using a laboratory mill (6.10) until it passes through the sieve (6.14) and mix it thoroughly.

7.4 Extraction of ochratoxin A from the sample

7.4.1 Extraction

Place 25 g (m), weighed to the nearest 0,1 g, of the sample into a conical flask or a blender (6.2), add 100 ml extraction solvent (5.12)(V_1). Cover and shake for 30 min or blend for 3 min. The extract is centrifuged at 8 000*g* for 5 min or filtered through folded filter paper [6.3 a)].

NOTE For light sample (e.g. wheat bran), the recommended weight is 12,5 g and the extraction solvent is 100 ml.

7.4.2 Dilution

Pipet 4,0 ml (V_2) filtered extract into 50 ml centrifuge tube (6.4), and dilute with 26,0 ml (V_3) PBS solution (5.13). The diluted extract is centrifuged at 8000 *g* for 5min, and then collected as extract A.

Alternatively pipet 6,0 ml (V_2) filtered extract into 50 ml centrifuge tube (6.4), and dilute with 39,0 ml (V_3) PBS solution (5.13). The diluted extract is filtered through a glass microfibre filter [6.3 b)], and then collected as extract B.

7.5 Immunoaffnity column cleanup

Pass all extract A or 30,0 ml extract B (V_4) through the OTA immunoaffinity column at a flow-rate of about 1 to 2 drops per second, followed by 10 ml washing solution (5.14) and 10 ml distilled water at 2 drops per second. Elute OTA with 1,5 ml elution solvent (5.26). Evaporate eluate to dryness over steam bath under N_2 cautiously without exceeding 40 °C. Redissolve in 0,5 ml (V_5) mobile phase (5.15). Transfer to liquid chromatography (LC) vial. If necessary the sample can be filtered through

PTFE membrane (6.5) before analysis by LC. Elution of ochratoxin A and the subsequent steps in the procedure described in this clause can depend on the type of immunoaffinity columns used. The elution volume, for example, should be checked to ensure it is appropriate for the type of column used.

Be careful not to overload the immunoaffinity column.

7.6 HPLC operating conditions

When the column [6.17 b)] and the mobile phase (5.15) are used the following settings have been found to be appropriate.

Flow rate:	1 ml/min	
Fluorescence detection:	Excitation wavelength:	333 nm
Emission wavelength:	460 nm	
Injection volume:	50 µl	
Column temperature:	35 °C	

7.7 Calibration graph

Prepare a calibration graph at the beginning of the analysis and whenever the chromatographic conditions are changed.

Inject at least four calibration solutions of different suitable concentrations (see 5.20). Plot the fluorescence values (peak height or peak area) of the ochratoxin A calibration solutions (5.20) against the ochratoxin A mass concentrations in nanograms. Ensure that the linearity check is carried out[5].

7.8 Identification

Identify ochratoxin A by comparing the retention time of the sample with that of the standard substance. Sometimes it can be necessary to identify the ochratoxin A peak by simultaneous injection of sample test solution and standard solution.

7.9 Determination

Chromatograph the sample. To carry out the determination by the external standard method, integrate the peak area or determine the peak height, and compare the results with the corresponding values for the standard substance with the nearest peak area/height, or use a calibration graph. In the case of a calibration graph, additional solutions with concentrations within the linear range may be prepared for the calibration graph.

Inject equal volumes of sample test solution and standard solution used for the calibration graph.

Read off the mass of ochratoxin A, in nanograms, corresponding to the fluorescence of the sample test solution from the calibration graph.

If the ochratoxin A response of the sample is outside the calibration graph, adjust the amount of sample injected by concentrating or diluting the sample test solution.

7.10 Confirmation

If necessary, confirm the identity by disappearance of the peak at the retention time for ochratoxin A and appearance of a new peak at the same retention time as that of standard methyl ester of ochratoxin A.

Take 200 µl of the sample test solution prepared as in 7.5, transfer into a pear-shaped flask and evaporate to dryness in a rotary evaporator (6.9). Take up the residue in 0,5 ml of dichloromethane (5.24), and add 1 ml of boron trifluoride methanol solution (5.23).

Stopper the flask tightly and heat it in a water bath at 50 °C to 60 °C for 15 min. After cooling, transfer the solution into a 50 ml separating funnel containing 15 ml of water, shake three times with 5 ml of dichloromethane each time for 30 s. Combine the organic phases in a second 50 ml separating funnel, add 10 ml of water for washing and shake for 30 s.

Subsequently filter the dichloromethane phase through sodium sulfate (5.25) into a pear-shaped flask, evaporate to dryness, take up in 500 µl of mobile phase (5.15) and subject this solution to chromatographic separation under the conditions as described in 7.6. The completeness of derivatization can be checked from the chromatograms. It is possible with this procedure to verify mass fractions of ochratoxin A of not less than 0,4 µg/kg.

An adequate standard solution (5.20) should be treated separately to check the retention times of the ochratoxin A methyl ester and the completeness of the derivatization.

8 Calculation

Calculate the mass fraction X_1 of ochratoxin A in micrograms per kilogram using Formula (2) (external standard method):

$$X_1 = \frac{c \times V_1 \times (V_2 + V_3) \times V_5}{m \times V_2 \times V_4} \quad (2)$$

where

V_1 is the volume of the solvent used for extraction, in millilitres (V_1 = 100 ml);

V_2 is the volume of the extract used for dilution, in millilitres (extract A V_2 = 4 ml, or extract B V_2 = 6 ml);

V_3 is the volume of the PBS solution used for dilution, in millilitres (extract A V_3 = 26 ml, or extract B V_3 = 39 ml);

V_4 is the volume of the diluted extract used for cleanup, in millilitres (V_4 = 30 ml);

V_5 is the volume of the diluted extract used for cleanup, in millilitres (V_4 = 30 ml);

c is the concentration of OTA in final solution used for LC determination, in µg/l;

m is the mass of the test portion, in grams.

Report the result in the relevant format and after rounding to two decimal places.

NOTE Relevant legislation can apply.

Indicate whether or not a correction for recovery has been applied.

9 Precision

9.1 General

Details of the interlaboratory test of the precision of the method in accordance with ISO 5725:1986[6] are summarized in Annex A. The values derived from the interlaboratory tests may not be applicable to analyte concentration ranges and matrices other than given in Annex A.

9.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will not be greater than the repeatability limit, *r*, given in Formula (3) in more than 5 % of cases:

$$r = 2{,}8\, s_r = 2{,}8\, (0{,}044\, 3\, \bar{x} - 0{,}016\, 0) \tag{3}$$

where

s_r is the repeatability standard deviation;

$\bar{x}$ is the mean concentration of OTA (µg/kg).

9.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will not be greater than the reproducibility limit, *R*, given in Formula (4) in more than 5 % of cases:

$$R = 2{,}8\, s_R = 2{,}8\, (0{,}122\, 8\, \bar{x} - 0{,}017\, 8) \tag{4}$$

where

s_R is the reproducibility standard deviation;

$\bar{x}$ is the mean concentration of OTA (µg/kg).

10 Test report

The test report shall contain at least the following data:

— all information necessary for the identification of the sample;

— 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 particular points observed in the course of the test;

— any operations not specified in the method or regarded as optional which might have affected the results.

Annex A
(informative)

Results of interlaboratory tests

A.1 General

The values of the repeatability limit and reproducibility limit for this method have been derived from the results of an international interlaboratory test programme carried out in accordance with ISO 5725:1986[6].

The tests were carried out on 10 samples of cereals and cereal products (naturally and fortified samples). Fourteen laboratories in three countries took part. The test programme was organized by the Academy of State Administration of Grain, China in March 2016.

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1:1994[7], ISO 5725-2:1994[8] and ISO 5725-6:1994[9] to give the precision data shown in Table A.1. Because the heterogeneous distribution of OTA in naturally contaminated samples is an important and inseparable part of the variability of the test results, the relationship between precision values and the mean level (Figure A.1 and Table A.2) is established on the naturally contaminated samples, to show the statistical results of the "real" samples. The fortified samples in the interlaboratory tests were used to evaluate the recoveries and validate the applicability in different matrix.

A.2 Precision data

Table A.1 — Interlaboratory test results for OTA content

Sample	Naturally contaminated wheat			Naturally contaminated maize			Fortified samples			
	High	Low	Blank	High	Low	Blank	Husked rice	Barley	Wheat bran	Wheat flour
Year of interlaboratory test	2016	2016	2016	2016	2016	2016	2016	2016	2016	2016
Number of laboratories	14	14	14	14	14	14	14	14	14	14
Number of samples	3	3	3	3	3	3	3	3	3	3
Number of laboratories retained after eliminating outliers	14	14	14	14	13	14	13	13	12	14
Number of outliers (laboratories)	0	0	0	0	1	0	1	1	2	0
Number of accepted results	42	42	42	42	39	42	39	39	36	42
Mean value, $\bar{x}$ (µg/kg)	6,7	2,8	< 0,2	4,1	2,4	< 0,2	4,2	4,2	4,3	1,7
Repeatability standard deviation, s_r (µg/kg)	0,28	0,10	—	0,17	0,09	—	0,11	0,09	0,09	0,06
Coefficient of variation of repeatability, $C_{V,r}$	4,2 %	3,6 %	—	4,2 %	3,8 %	—	2,6 %	2,1 %	2,1 %	3,6 %
Repeatability limit, r (µg/kg)	0,79	0,28	—	0,49	0,26	—	0,31	0,27	0,25	0,18
Reproducibility standard deviation, s_R (µg/kg)	0,79	0,26	—	0,54	0,29	—	0,33	0,28	0,19	0,19
Coefficient of variation of reproducibility, $C_{V,R}$	11,80 %	9,50 %	—	13,30 %	12,30 %	—	7,90 %	5,60 %	4,40 %	11,30 %
Reproducibility limit, R (µg/kg)	2,24	0,74	—	1,52	0,82	—	0,94	0,79	0,54	0,55
Recovery	—	—	—	—	—	—	91 %	93 %	93 %	92 %
HorRat value[10][11]	0,53	0,43	—	0,60	0,56	—	0,36	0,30	0,20	0,51