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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