INTERNATIONAL STANDARD

ISO 15141-1

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Foodstuffs — Determination of ochratoxin A in cereals and cereal products —

Part 1:
High performance liquid chromatographic method with silica gel clean up

Produits alimentaires — Dosage de l'ochratoxine A dans les céréales et produits dérivés

Partie 1: Méthode par chromatographie liquide haute performance comprenant une étape d'extraction par chromatographie sur gel de silice

Reference number
ISO 15141-1:1998(E)

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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 15141-1 was prepared by the European Committee for Standardization (CEN) in collaboration with ISO Technical Committee TC 34, *Agricultural food products*, Subcommittee SC 4, *Cereals ans pulses*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Throughout the text of this standard, read "...this European Standard..." to mean "...this International Standard...".

ISO 15141 consists of the following parts, under the general title *Foodstuffs — Determination of ochratoxin A in cereals and cereal products*:

— *Part 1: High performance liquid chromatographic method with silica gel clean up*

— *Part 2: High performance liquid chromatographic method with bicarbonate clean up*

Annexes A and B of this part of ISO 15141 are for information only.

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Foreword

The text of EN ISO 15141-1:1998 has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN, in collaboration with Technical Committee ISO/TC 34 "Agricultural food products".

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by April 1999, and conflicting national standards shall be withdrawn at the latest by April 1999.

This European Standard „Foodstuffs - Determination of ochratoxin A in cereal and cereal products“ consists of two parts:

Part 1: High performance liquid chromatographic method with silica gel clean up

Part 2: High performance liquid chromatographic method with bicarbonate clean up

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Standard specifies a method for the determination of ochratoxin A at levels greater than 0,4 µg/kg.

The method has been successfully validated in 2 interlaboratory studies according to ISO 5725:1996 [1] on wheat whole meal containing 0,4 µg/kg and 1,2 µg/kg of ochratoxin A.

NOTE: Numerous laboratory experiences have shown that this method is also applicable to cereals, dried fruits, oilseeds, pulses, wine, beer, fruit juices and raw coffee, see [2], [3], [4].

2 Normative references

This draft European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this draft European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

EN ISO 3696:1995 Water for analytical laboratory use - Specification and test methods (ISO 3696:1987).

3 Principle

Ochratoxin A (OTA) is extracted with toluene after acidification with hydrochloric acid and after the ionic strength has been increased by adding magnesium chloride. The extract is purified using a mini silica gel column and ochratoxin A is determined by high performance liquid chromatography (HPLC) on a reversed phase column and identified and modified by fluorescence. The result is verified, if required, by derivatization with boron trifluoride in methanolic solution [5], [6].

WARNING: Ochratoxin A causes kidney and liver damage and is a probable carcinogen. Observe appropriate safety precautions [7] 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) [8], [9].

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and only distilled water or water of grade 1 according to EN ISO 3696. Solvent shall be of quality for HPLC analysis.

4.1 Sodium sulfate, anhydrous

4.2 Glacial acetic acid $\varphi(CH_3COOH) \approx 98$ %

4.3 Solution of hydrochloric acid $c(HCl) = 2$ mol/l

4.4 Magnesium chloride solution $c(MgCl_2) = 0,4$ mol/l

4.5 Acetonitrile

4.6 Toluene

4.7 *n*-Hexane

4.8 Dichloromethane

4.9 Acetone

4.10 Methanol

4.11 Solvent mixture I: toluene (4.6) and glacial acetic acid (4.2) 99+1 parts per volume (*V*+*V*)

4.12 Solvent mixture II: acetone (4.9) and toluene (4.6) 5+95 (*V*+*V*)

4.13 Solvent mixture III: toluene (4.6) and glacial acetic acid (4.2) 90+10 (*V*+*V*)

4.14 Mobile phase
Mix 99 volume parts of acetonitrile (4.5) with 99 volume parts of water and 2 volume parts of glacial acetic acid (4.2) and degas this solution before use.

4.15 Boron trifluoride

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

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

4.17 Ochratoxin A, in crystal form or as a film in ampoules

4.18 Ochratoxin A stock solution

Dissolve 1 mg of the ochratoxin A (crystals) (4.17) or the contents of 1 ampoule (if ochratoxin A has been obtained as a film) in solvent mixture I (4.11) 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 (5.5) with solvent mixture I (4.11) 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 equation 1:

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

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 I (here: 544 m^2/mol);

δ is the path length of the cell in centimetres.

4.19 Ochratoxin A standard solution ρ_{OTA} = 1 µg/ml

Evaporate under a nitrogen flow 1 ml of the stock solution (4.18) 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 (4.14).

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

4.20 Ochratoxin A calibration solutions

Pipette suitable volumes of ochratoxin A standard solution (4.19), e.g. 1 ml, 2,5 ml, 4 ml and 5 ml into e.g. a 100 ml volumetric flask (5.12) and dilute to the mark with the mobile phase (4.14). The amount of ochratoxin A in the calibration solutions should cover the range of 0,2 ng to 1,0 ng per 20-µl-injection volume.

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

5 Apparatus and equipment

Usual laboratory equipment and, in particular, the following:

5.1 Laboratory mill, suitable to grind to 1 mm

5.2 Rotary evaporator, with a water bath capable of being controlled between 20 ºC and 50 ºC

5.3 Mechanical shaker

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

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

5.6 Centrifuge tubes, e.g. of capacity 250 ml, plastic made of high density polyethylene (HDPE), with screw cap

5.7 Cooling centrifuge, preferably a refrigerated centrifuge, capable of producing a gravitational force of at least 3500 *g* at the base of the centrifuge tubes (5.6)

5.8 Solid phase extraction columns, e.g. SEP-PAK®[1]) disposable silica gel

After the pack has been opened, condition at 105 ºC for 2 h and store over activated silica gel with moisture indicator. Before use, wash with 10 ml of toluene (4.6). Check the recovery with each new batch. In the case of use of SEP-PAK columns, the cartridges have the following specification:

- mean mass of the packing material: 690 mg
- pore size: 12,5 nm
- particle size: 55 µm to 105 µm

in a 3 ml polypropylene tube.

5.9 Solvent containers, such as syringes, e.g. of 50 ml capacity with central opening and stop-cock

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[1]) SEP PAK® is an example of a suitable product available commercially. This information is given for the convenience of users of this Standard and does not constitute an endorsement by CEN of these products.

5.10 Pear-shaped flasks, 50 ml, with ground glass joint

5.11 Separating funnel, 50 ml

5.12 Volumetric flask, 100 ml

5.13 Membrane filter for aqueous solutions, made of polytetrafluoroethylene (PTFE), with a diameter of 4 mm and a pore size of 0,45 µm

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

5.15 Vials with crimped caps or screw cap vials

5.16 Microsyringe, of capacity 500 µl

5.17 HPLC apparatus, comprising the following

5.17.1 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.

5.17.2 Analytical reversed phase HPLC separating column, C_{18}, e.g. Lichrospher® 100 RP 18 [2]) which ensures a baseline resolved resolution of the ochratoxin A peak from all other peaks.

- length: 250 mm
- internal diameter: 4 mm
- spherical particles of size: 5 µm

NOTE: Shorter columns can also be used (e.g. a column with a length of 120 mm to 150 mm)

5.17.3 Precolumn, C_{18},

- length: 40 mm
- internal diameter: 4 mm
- spherical particles of size: 5 µm

6 Procedure

6.1 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.

6.2 Preparation of the test samples

Grind the laboratory sample using a laboratory mill (5.1) until it passes through the sieve (5.14) and mix it thoroughly.

NOTE: Grinding is not necessary for wheat flour with a maximum size of 250 µm.

[2]) Lichrospher® 100 RP 18 is an example of a suitable product available commercially. This information is given for the convenience of users of this Standard and does not constitute an endorsement by CEN of these products.

6.3 Extraction of ochratoxin A from the sample

Place 20 g (m_0), weighed to the nearest 0,1 g, of the sample prepared as in 6.2 in a centrifuge tube (5.6). For ochratoxin A contents of more than 5,0 µg/kg repeat the analysis using a test portion of 10 g, otherwise the risk of reduced recovery has to be taken into account. Successively add 30 ml of hydrochloric acid solution (4.3), 50 ml of magnesium chloride solution (4.4), stir with a glass rod, and add 100 ml of toluene (4.6) (V_1).

Shake for 60 min and subsequently centrifuge the suspension. The centrifugation time depends on the efficiency of the centrifuge, while cooling prevents loss of toluene. Remove 50 ml (= toluene aliquot portion V_2) from the upper toluene layer and load it onto the solid phase mini disposable column which has been prepared as in 5.8 and to which the syringe (5.9) is attached as solvent reservoir.

NOTE 1: Care should be taken not to overload the column.

Wash the column twice with 10 ml of n-hexane (4.7) and again, twice with 10 ml of solvent mixture II (4.12). Subsequently wash with 5 ml of toluene. Discard all the washings.

Elute ochratoxin A with two 15 ml portions of solvent mixture III (4.13) into a 50 ml pear-shaped flask (5.10). Evaporate the eluate under reduced pressure to dryness cautiously without exceeding 40 ºC. Take up the residue by pipetting 1 ml (V_3) of the mobile phase (4.14) into the pear-shaped flask and filter through a membrane (5.13) into a vial (5.15) (= sample test solution).

NOTE 2: Elution of ochratoxin A and the subsequent steps in the procedure described in this clause can depend on the type of solid phase extraction columns that is used. The elution volume for example should be checked to be appropriate for the type of column that is used.

NOTE 3: The size and/or shape of the flask can have a negative influence on the recovery.

6.4 HPLC operating conditions

When the column according to 5.17.2 and the mobile phase according to 4.14 were used the following settings were found to be appropriate.

Flow rate:	1 ml/min	
Fluorescence detection:	Excitation wavelength:	330 nm
	Emission wavelength:	460 nm
Injection volume:	20 µl (V_4)	

6.5 Calibration graph

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

Inject at least four calibration solutions of different suitable concentrations (see 4.20).

Plot the fluorescence values of the ochratoxin A calibration solutions (4.20) against the ochratoxin A mass concentrations in nanograms.

Ensure that the linearity check is carried out [10].

6.6 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.

6.7 Determination

Immediately 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, (m_1), 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.

6.8 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 500 µl of the extract prepared as in 6.3, transfer into a pear-shaped flask and evaporate to dryness in a rotary evaporator (5.2). Take up the residue in 1 ml of dichloromethane (4.8), and add 2 ml of boron trifluoride methanol solution (4.16).

Stopper the flask tightly and heat it in a water bath at 50 ºC to 60 ºC for 15 min. After cooling, transfer the solution into a 50 ml separating funnel containing 30 ml of water, shake 3 times with 10 ml of dichloromethane each time for 30 s. Combine the organic phases in a second 50 ml separating funnel, add 20 ml of water for washing and shake for 30 s.

Subsequently filter the dichloromethane phase through sodium sulfate (4.1) into a pear-shaped flask, evaporate to dryness, take up in 500 µl of mobile phase (4.14) and subject this solution to chromatographic separation under the conditions as described in 6.4. 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 (4.19) should be treated separately to check the retention times of the ochratoxin A methyl ester and the completeness of the derivatization.

7 Calculation

Calculate the mass fraction w_{OTA} of ochratoxin A in micrograms per kilogram using equation (2) (external standard method):

$$w_{OTA} = \frac{V_1 \times V_3 \times m_1}{V_2 \times V_4 \times m_0} \qquad (2)$$

where

V_1 is the volume of the solvent used for extraction (6.2), in millilitres, here: 100 ml;

V_2 is the volume of the centrifugate (toluene aliquot portion), in millilitres, here: 50 ml;

V_3 is the total volume of the sample test solution, in millilitres, here: 1 ml;

V_4 is the injection volume, in millilitres;

m_1 is the mass of ochratoxin A corresponding to the measured peak area or peak height read off the calibration graph, in nanograms;

m_0 is the mass of the test portion, in grams.

Report the result according to current legislation and after rounding to two decimal places.

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

8 Precision

8.1 General

Details of the interlaboratory test of the precision of the method according to ISO 5725:1986 [1] 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.

8.2 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.

The values for wheat whole flour are:	$\bar{x}$ = 0,41 µg/kg	r = 0,18 µg/kg
	$\bar{x}$ = 1,23 µg/kg	r = 0,70 µg/kg

8.3 Reproducibility

The absolute difference between two single test results on identical test material reported by two laboratories will exceed the reproducibility limit R in not more than 5 % of the cases.

The values for wheat whole flour are:	$\bar{x}$ = 0,41 µg/kg	R = 0,30 µg/kg
	$\bar{x}$ = 1,23 µg/kg	R = 1,10 µg/kg

9 Test report

The test report shall contain at least the following data:

- all information necessary for the identification of the sample;
- a reference to this draft European Standard or to the method used;
- the results and the units in which the results have been expressed;
- date and type of sampling (if known);
- date of receipt of the laboratory sample;
- date of 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.