INTERNATIONAL STANDARD

ISO 15141-2

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

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

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

Partie 2: Méthode par chromatographie liquide haute performance comprenant une étape d'extraction par une solution de bicarbonate

Reference number
ISO 15141-2:1998(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 15141-2 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 15141-2/1998 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 cereals 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 (OTA) at levels greater than 3 µg/kg.

The method has been successfully validated in interlaboratory studies according to ISO 5725:1986 [1] on whole barley containing 2,9 µg/kg, 3,0 µg/kg, 7,4 µg/kg and 14,4 µg/kg of ochratoxin A, on whole maize containing 8,2 µg/kg and 16,3 µg/kg of ochratoxin A as well as on wheat bran containing 3,8 µg/kg and 4,5 µg/kg of ochratoxin A.

NOTE: Numerous laboratory experiences have shown that this method is also applicable to wheat flour.

2 Normative references

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

3 Principle

Ochratoxin A is extracted from grains with chloroform-aqueous phosphoric acid and isolated by liquid-liquid partitioning into aqueous bicarbonate solution. The solution is applied to a C_{18} cartridge, and ochratoxin A is eluted with ethyl acetate-methanol-acetic acid. Ochratoxin A is separated by reversed phase HPLC and identified and quantified by fluorescence. Chromatography of ochratoxin A methyl ester derivative confirms the identification [2] to [5].

WARNING: Ochratoxin A causes kidney and liver damage and is a probable carcinogen. Observe appropriate safety precautions [6] 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) [7], [8].

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 Chloroform, stabilized with for example 2-methyl-2-butene or ethanol

4.2 Phosphoric acid, $c(H_3PO_4) \approx 0{,}1$ mol/l

4.3 Diatomaceous earth

Soak about 900 g of acid-washed diatomaceous earth e.g. Celite ® 545[1]) overnight in methanol (4.7). Filter through a double layer of paper in a Buchner funnel (5.6), wash with 8 l of water and dry for 12 h at 150 °C.

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[1]) Celite® 545 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.

4.4 Sodium bicarbonate solution, $\rho(NaHCO_3)$ = 30 g/l

4.5 Ethyl acetate

4.6 Toluene

4.7 Methanol

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

4.9 Acetonitrile

4.10 Dichloromethane

4.11 Elution solution: ethyl acetate (4.5), methanol (4.7) and glacial acetic acid (4.8) 95+5+0,5 (*V*+*V*+*V*) .

4.12 Mobile phase: mix acetonitrile (4.9), water and glacial acetic acid (4.8) 99+99+2 (*V*+*V*+*V*) and degas.

4.13 Solvent mixture: toluene (4.6) and glacial acetic acid (4.8) 99+1 (*V*+*V*).

4.14 Boron trifluoride

4.15 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.

4.16 Ochratoxin A, in crystal form or as film in ampoules.

4.17 Ochratoxin A stock solution

Dissolve 1 mg of the ochratoxin A (crystals) (4.16) or the contents of 1 ampoule (if ochratoxin A has been obtained as a film) in solvent mixture (4.13) to give a solution containing approximately 20 µg/ml to 30µg/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 (5.4) with solvent mixture 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 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 relative molar absorption coefficient of ochratoxin A in solvent mixture, (here: 544 m^2/mol);

δ is the path length of the quartz cell in centimetres;

4.18 Ochratoxin A standard solution

Dilute the stock solution (4.17) with solvent mixture (4.13) to obtain a standard solution with a mass concentration of ochratoxin A of 4 µg/ml.

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

4.19 Ochratoxin A calibration solutions

Dispense 5 µl, 10 µl, 25 µl, 50 µl and 100 µl aliquot portions of standard solution (4.18) into separate 4 ml to 5 ml vials (5.15) using fixed-volume syringes (5.16). Evaporate just to dryness under nitrogen. Add 1,0 ml of the mobile phase (4.12) to each vial for final ochratoxin A mass concentrations of 0,5 ng/25 µl, 1 ng/25 µl, 2,5 ng/25 µl, 5 ng/25 µl and 10 ng/25 µl.

4.20 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** and a **sieve** with a 1 mm aperture size

5.2 **High-speed blender**. 1250 ml jar with cover

5.3 **Spectrometer**, for measurements of wavelengths between 300 nm and 370 nm, having a spectral band width of not more than ± 2 nm

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

5.5 **Glass fibre filters**, 0,3 mm thickness, 1,5 µm pore retention, 9,0 cm diameter (or equivalent)

5.6 **Buchner funnels**, of suitable diameters, e. g. of 9 cm and 25 cm

5.7 **Fluted filter paper**

5.8 **Separation funnels**, 25 ml and 100 ml

5.9 **Centrifuge**, with 100 ml tubes or flasks

5.10 **Adsorption cartridge**, disposable 3 ml polypropylene tube containing 500 mg of 40 µm C_{18} silica

5.11 **Vacuum manifold** with stopcocks at each port for holding C_{18} columns. May be replaced by a syringe (5 ml to 10 ml) with a suitable adapter (Luer)

5.12 **Test tubes**, e.g. 10 ml with polytetrafluoroethylene (PTFE)-lined screw cap

5.13 **Membrane filter**, of pore size of approximately 0,45 µm

5.14 **HPLC apparatus** comprising the following

5.14.1 High performance liquid chromatograph, eluent reservoir, pump with adjustable flow from 0,5 ml/min to 5 ml/min, injection valve with e.g. 25 µl loop, fluorescence detector, compatible recorder or integrator.

5.14.2 HPLC reverse phase analytical column, e.g. from Supelco[2]).

- length: 250 mm
- inner diameter: 4,6 mm
- packing: spherical 5 µm C_{18} material or equivalent

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

5.15 Vials, approximately 5 ml, with PTFE-lined screw cap, or appropriate sealable container.

5.16 Fixed-volume syringe

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 sample

Grind the sample and mix it thoroughly until it passes a 1 mm sieve (5.1) using a laboratory mill (5.1) or mixer and mix thoroughly.

6.3 Extraction of ochratoxin A from the sample

Weigh, to the nearest 0,1 g, a 50 g test portion prepared as in 6.2 into a blender jar (5.2) and add first 250 ml of chloroform (4.1) and then 25 ml of phosphoric acid (4.2). Blend for 3 min at medium speed. Near the end of blending add 10 g (45 ml) of diatomaceous earth (4.3). Filter the extract through glass fibre paper (5.5) covered with about 10 g of diatomaceous earth on a 9 cm Buchner funnel (5.6), or through a 32 cm fluted paper (5.7). Collect at least 50 ml of filtrate.

6.4 Partition

Transfer 50 ml of the filtrate to a 100 ml separation funnel (5.8). Add 10 ml of sodium bicarbonate solution (4.4) and shake gently. Allow the phases to separate. If an emulsion forms, centrifuge for 2 min at 2000 min^{-1}. Collect the upper aqueous phase for cartridge extraction.

6.5 Cartridge preparation

Attach the C_{18} cartridges (5.10) to vacuum manifold ports (5.11) with 25 ml conical flasks or beakers inside the manifold for collecting conditioning and washing solvents. Wash each cartridge twice with about 2 ml of methanol (4.7), 2 ml of water, and 2 ml of sodium bicarbonate solution (4.4). **DO NOT**

[2]) Supelco 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

ALLOW THE CARTRIDGE TO RUN DRY. To speed elutions, apply gentle suction. This procedure may also be performed manually by applying pressure with a 5 ml to 10 ml syringe fixed to the top of the cartridge. Leave about 2 mm of solvent on top of the frit.

6.6 Cartridge extraction

Pipette 5 ml of the bicarbonate extract obtained as given in 6.4 to the C_{18} cartridge. **DO NOT ALLOW THE CARTRIDGE TO RUN DRY**. Wash with 2 ml of phosphoric acid solution (4.2) followed by 2 ml of water. Discard the washing liquids.

Elute ochratoxin A with 8 ml of elution solution (4.11). Collect the eluate in a 10 ml test tube (5.12) containing 2 ml of water. Shake or stir the eluate with a glass rod to mix the two phases. Pipette the ochratoxin A extract (upper phase) into another 10 ml test tube with a screw-cap (5.12). Rinse the remaining upper phase from the first tube twice with 1 ml of ethyl acetate (4.5) and add to the ochratoxin A phase in the second tube. Evaporate just to dryness under nitrogen. Immediately dissolve in 500 µl (V_T) of the mobile phase (4.12) and filter through a 0,45 µm microfilter (5.13) into a 5 ml screw-cap vial (= sample test solution).

Reserve the remaining sample test solution to identity confirmation by formation of methyl ester (see 6.11).

6.7 HPLC operating conditions

When the column according to 5.14.2 and the mobile phase according to 4.12 were used the following settings were found to be appropriate:

Flow rate:	1 ml/min
Fluorescence detection (with gratings):	Excitation wavelength 333 nm Emission wavelength 460 nm
Fluorescence detection (with filters):	420 nm cut-off filter
Injection volume (V_i):	20 µl to 25 µl and use at least 50 µl to fill the 25 µl loop.

6.8 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 4.19).

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

Ensure that the linearity check is carried out [9].

6.9 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.

6.10 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, 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.

6.11 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 the standard methyl ester of ochratoxin A (about 15 min later).

Quantitatively transfer the remaining sample test solution (see 6.6) to a 25 ml separation funnel (5.8), using 3 times 1 ml of dichloromethane (4.10) to rinse the vial. Shake and allow the layers to separate. Collect the lower layer into a vial (5.15) and evaporate to dryness.

Transfer 100 µl of standard ochratoxin A solution (4.18) to another vial (5.15) and evaporate to dryness.

Add 0,5 ml of boron trifluoride methanol solution (4.15) to each vial, cap and heat for 15 min in a 50 °C to 60 °C water bath. Evaporate to dryness on a steam bath under nitrogen.

If water is present, add 1 ml of acetonitrile (4.9) and continue evaporation to dryness. Cool and dilute with the mobile phase (4.12) to the same volume as used for HPLC analysis of the underived sample test solution (see 6.7.3) and subject this solution to chromatographic separation under the conditions as described in 6.7.

The completeness of derivatization can be checked from the chromatograms.

7 Calculation

Calculate the mass fraction, w_{ota}, of ochratoxin A in micrograms per kilogram using equation 2:

$$w_{OTA} = \frac{R_S \times V_T}{R_A \times V_I \times m} \qquad (2)$$

where:

m is the mass of test sample in the final extract, here: 50 g x 50 ml x 5 ml/250 ml x 10 ml = 5 g;

R_S is the response of test sample solution injected measured as peak area or peak height;

R_A is the calculated mean normalized response (response for 1 ng of ochratoxin A) of the five different calibration solutions (see 4.19);

V_T is the final volume of test sample solution (here: 500 µl);

V_I is the volume of test sample injected (here: 25 µl).

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 inter-laboratory test 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 are:

Barley:	$\bar{x}$ =	7,4 µg/kg	r =	- µg/kg
Barley:	$\bar{x}$ =	14,4 µg/kg	r =	3,1 µg/kg
Maize:	$\bar{x}$ =	8,2 µg/kg	r =	- µg/kg
Maize:	$\bar{x}$ =	16,3 µg/kg	r =	9,2 µg/kg
Barley:	$\bar{x}$ =	3,0 µg/kg	r =	1,28 µg/kg
Barley:	$\bar{x}$ =	2,9 µg/kg	r =	1,37 µg/kg
Wheat bran:	$\bar{x}$ =	4,5 µg/kg	r =	2,16 µg/kg
Wheat bran:	$\bar{x}$ =	3,8 µg/kg	r =	2,23 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 are:

Barley	$\bar{x}$ =	7,4 µg/kg	R =	5,6 µg/kg
Barley:	$\bar{x}$ =	14,4 µg/kg	R =	10,6 µg/kg
Maize:	$\bar{x}$ =	8,2 µg/kg	R =	4,8 µg/kg
Maize:	$\bar{x}$ =	16,3 µg/kg	R =	12,9 µg/kg
Barley:	$\bar{x}$ =	3,0 µg/kg	R =	1,9 µg/kg
Barley:	$\bar{x}$ =	2,9 µg/kg	R =	1,74 µg/kg
wheat bran:	$\bar{x}$ =	4,5 µg/kg	R =	3,35 µg/kg
wheat bran:	$\bar{x}$ =	3,8 µg/kg	R =	2,58 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 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.