

**Foodstuffs —
Determination of
ochratoxin A in cereal
based foods for infants
and young children
— HPLC method
with immunoaffinity
column cleanup and
fluorescence detection**

ICS 67.050; 67.230

National foreword

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A list of organizations represented on this committee can be obtained on request to its secretary.

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ICS 67.050; 67.230

English Version

Foodstuffs - Determination of ochratoxin A in cereal based foods
for infants and young children - HPLC method with
immunoaffinity column cleanup and fluorescence detection

Produits alimentaires - Dosage de l'ochratoxine A dans les
aliments à base de céréales pour nourrissons et jeunes
enfants - Méthode CLHP avec purification sur colonne
d'immuno-affinité et détection par fluorescence

Lebensmittel - Bestimmung von Ochratoxin A in Säuglings-
und Kleinkindernahrung auf Getreidebasis - HPLC-
Verfahren mit Reinigung an einer Immunoaffinitätssäule
und Fluoreszenzdetektion

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Foreword

This document (EN 15835:2010) has been prepared by Technical Committee CEN/TC 275 "Food analysis — Horizontal methods", the secretariat of which is held by DIN.

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of an identical text or by endorsement, at the latest by August 2010, and conflicting national standards shall be withdrawn at the latest by August 2010.

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1 Scope

This European Standard specifies a method for the determination of ochratoxin A in cereal based foods for infants and young children by high performance liquid chromatography (HPLC) with immunoaffinity column cleanup and fluorescence detection. This method has been validated in an interlaboratory study via the analysis of both naturally contaminated and spiked samples ranging from 0,050 µg/kg to 0,217 µg/kg. For further information on the validation see Clause 8 and Annex B. Additional studies have shown that this method is applicable to cereal based baby foods containing 8 different types of cereals, honey and cocoa, at levels up to 3,540 µg/kg, see Annex C and [6].

WARNING — The use of this standard can involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

2 Normative references

The following referenced documents are indispensable for the application 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.

EN ISO 3696:1995, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987)*

3 Principle

A test portion is extracted with tert-butyl methyl ether after addition of 0,5 mol/l phosphoric acid / 2 mol/l sodium chloride solution. The extract is evaporated and redissolved in methanol and phosphate buffered saline (PBS) solution. After removal of lipophilic compounds with hexane, the extract is applied to an immunoaffinity column containing antibodies specific to ochratoxin A. The toxin is eluted from the column with methanol. Ochratoxin A is determined by HPLC with enhanced fluorescence detection involving post column reaction with ammonia.

NOTE Some investigations indicate that HPLC can be also performed without the use of ammonia although this results in at least a two-fold decrease of the response for ochratoxin A. In this case, the fluorescence detection conditions need to be changed (excitation wavelength = 333 nm, emission wavelength = 460 nm).

4 Reagents

4.1 General

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696:1995, unless otherwise specified. Solvents shall be of quality for HPLC analysis. Commercially available solutions with equivalent properties to those listed may be used.

WARNING — Dispose of waste solvents according to applicable environmental rules and regulations. Decontamination procedures for laboratory wastes have been reported by the International Agency for Research on Cancer (IARC), see [4].

4.2 Helium purified compressed gas

4.3 Nitrogen

4.4 Disodium hydrogen phosphate, Na_2HPO_4 anhydrous or $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$

4.5 Potassium chloride

4.6 Potassium dihydrogen phosphate

4.7 Sodium chloride

4.8 Sodium hydroxide

4.9 Ammonium hydroxide solution, the mass fraction $w(\text{NH}_4\text{OH}) = 25 \%$ in water (post column reagent)

Degas the solution with a degasser (5.21.7).

4.10 Hydrochloric acid solution, $w(\text{HCl})$ is 37 % (acidimetric)

4.11 Phosphoric acid solution, $w(\text{H}_3\text{PO}_4) = 85 \%$

4.12 Hydrochloric acid solution, $c(\text{HCl}) = 0,1 \text{ mol/l}$

Dilute 8,28 ml of hydrochloric acid solution (4.10) to 1 l of water.

4.13 Sodium hydroxide solution, $c(\text{NaOH}) = 0,1 \text{ mol/l}$

Dissolve 4 g of sodium hydroxide (4.8) in 1 l of water.

4.14 Phosphate buffered saline (PBS) solution, $c(\text{NaCl}) = 120 \text{ mmol/l}$, $c(\text{KCl}) = 2,7 \text{ mmol/l}$,
 $c(\text{phosphate buffer}) = 10 \text{ mmol/l}$, $\text{pH} = 7,4$

Dissolve 8,0 g of sodium chloride (4.7), 1,2 g of anhydrous disodium hydrogen phosphate or 2,9 g of $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ (4.4), 0,2 g of potassium dihydrogen phosphate (4.6) and 0,2 g of potassium chloride (4.5) in 900 ml of water.

After dissolution, adjust the pH to 7,4 with hydrochloric acid solution (4.12) or sodium hydroxide solution (4.13) as appropriate, then dilute to 1 l with water. Alternatively, a PBS solution with equivalent properties can be prepared from commercially available PBS material.

4.15 Mixture of phosphoric acid solution and sodium chloride solution, $c(\text{H}_3\text{PO}_4) = 0,5 \text{ mol/l}$,
 $c(\text{NaCl}) = 2 \text{ mol/l}$

Dissolve 118 g of sodium chloride (4.7) in approximately 900 ml of water. Add 33 ml of phosphoric acid (4.11) and make up to 1,0 l with water.

4.16 Glacial acetic acid, the mass fraction $\geq 99,7 \%$

4.17 Acetic acid solution, the volume fraction is 9 %

Add 90 ml of glacial acetic acid (4.16) and 910 ml of water.

4.18 Hexane

WARNING — Hexane is highly flammable. Operations involving this solvent shall be performed in a fume cupboard. Serious health problems can be derived from prolonged exposure to this reagent.

4.19 Methanol, gradient grade

4.20 Toluene

4.21 Mixture of methanol and acetic acid solution

Mix 72 parts per volume of methanol (4.19) with 28 parts per volume of acetic acid solution (4.17).

4.22 Tert-butyl methyl ether

WARNING — Tert-butyl methyl ether is hazardous and samples shall be blended using an explosion proof blender which is housed within a fume cupboard. Centrifugation of extracts shall be performed at cool temperature (4 °C to 8 °C).

4.23 Mixture of toluene and glacial acetic acid

Mix 99 parts per volume of toluene (4.20) with one part per volume of glacial acetic acid (4.16).

4.24 HPLC mobile phase A

Acetic acid solution (4.17).

4.25 HPLC mobile phase B

Methanol (4.19).

Degas the mobile phases A and B with for example helium (4.2). Helium can be pumped into the reservoirs of both mobile phases A and B. The pumping rate should be 50 ml/min to 100 ml/min. The use of a degasser is also an acceptable option.

4.26 Immunoaffinity columns

The immunoaffinity column contain antibodies raised against ochratoxin A. The column shall have a capacity of not less than 100 ng of ochratoxin A and shall give a recovery of not less than 85 % when applied as a standard solution of ochratoxin A in a mixture of 15 parts per volume of methanol (4.19) and 85 parts per volume of PBS solution (4.14) containing 3 ng of ochratoxin A.

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

WARNING — Ochratoxin A is a potent nephrotoxin with immunotoxic, teratogenic and potential genotoxic properties. The International Agency for Research on Cancer (IARC) has classified ochratoxin A as a possible human carcinogen (group 2B). Protective clothing, gloves and safety glasses should be worn at all times, and all standard and sample preparation stages should be carried out in a fume cupboard.

4.28 Ochratoxin A stock solution

Prepare a stock solution of ochratoxin A in the mixture of toluene and glacial acetic acid (4.23) with a nominal concentration of 10 µg/ml.

To determine the exact concentration, record the absorption curve between a wavelength of 300 nm and 370 nm in 5 nm steps in 1 cm quartz cells (5.22) in a spectrometer with the solvent mixture (4.23) as reference. Identify the wavelength for maximum absorption and calculate the mass concentration of ochratoxin A, ρ_{ota} , in micrograms per millilitre, using Equation (1):

$$\rho_{ota} = \frac{A_{max} \times M \times 100}{\epsilon \times b} \quad (1)$$

where

- A_{\max} is the absorption determined at the maximum of the absorption curve (here: at 333 nm);
- M is the molar mass, in grams per mole, of ochratoxin A ($M = 403,8$ g/mol);
- ε is the molar absorption coefficient, in square metres per mole, of ochratoxin A in the solvent mixture (4.23), (here: 544 m²/mol);
- b is the path length, in centimetres, of the quartz cell.

Store this solution in a freezer at approximately -18 °C. Allow to reach room temperature before opening. A solution stored in this way is usually stable for 12 months. Confirm the concentration of the solution if it is older than six months.

4.29 Ochratoxin A standard solution

Pipette a volume of ochratoxin A stock solution (4.28) containing exactly 400 ng ochratoxin A into a 10 ml calibrated volumetric flask (5.13) and dilute to 10 ml with the mixture of toluene and glacial acetic acid (4.23) and shake. This gives a standard solution containing 40,0 ng/ml of ochratoxin A.

Store this solution in a freezer at approximately -18 °C. Allow to reach room temperature before opening. A solution stored in this way is usually stable for 12 months. Confirm the concentration of the solution if it is older than six months.

4.30 Ochratoxin A spiking solution

Pipette a volume of ochratoxin A stock solution (4.28) containing exactly 2 500 ng ochratoxin A into a 50 ml calibrated volumetric flask (5.13) and dilute to 50 ml with the mixture of toluene and glacial acetic acid (4.23) and shake. This gives a spiking solution containing 50,0 ng/ml of ochratoxin A.

Store this solution in a freezer at approximately -18 °C. Allow to reach room temperature before opening. A solution stored in this way is usually stable for 12 months. Confirm the concentration of the solution if it is older than six months.

5 Apparatus

5.1 General

Usual laboratory glassware and equipment and, in particular, the following:

5.2 High speed blender

5.3 Analytical balance, capable of weighing to 0,000 1 g

5.4 Laboratory balance, capable of weighing to 0,1 g

5.5 Vacuum manifold, to accommodate immunoaffinity columns

5.6 Filter papers, suitable for qualitative analysis

5.7 pH indicator paper, for pH = 0 to pH = 14

5.8 Cooling centrifuge, capable of a centrifugal force of 15 300 g at 4 °C

5.9 Centrifuge bottles, of 250 ml capacity with screw cap, chemically resistant to tert-butyl methyl ether and able to work at 15 300 g without deformation

5.10 Rotary evaporator, with water bath

5.11 Disposable syringe barrels, to be used as reservoirs, of 5 ml, 20 ml and 50 ml capacity, luer locks and attachments to fit to immunoaffinity columns

5.12 Microsyringes, of 25 μ l, 50 μ l, 100 μ l, 500 μ l and 1 000 μ l capacity

5.13 Calibrated volumetric flasks, e.g. of 10 ml, 50 ml and 1 000 ml capacity

5.14 Vacuum system

5.15 Round bottomed flasks, of 100 ml capacity

5.16 Calibrated volumetric pipettes

5.17 Displacement pipettes, of 100 μ l and 1 000 μ l capacity, with appropriate tips

5.18 Glass vials, of GC autosampler type, approximately 1,8 ml capacity and crimp caps

5.19 Separating funnel, of 250 ml capacity

5.20 Vortex mixer

5.21 HPLC apparatus, comprising the following:

5.21.1 Injection system, capable of injecting e.g. 50 μ l

5.21.2 Eluent and post column reagent reservoirs

5.21.3 Mobile phase pump, gradient, capable of maintaining a volume flow rate of 1 ml/min pulse free

5.21.4 Fluorescence detector, able to provide $\lambda = 390$ nm excitation and $\lambda = 440$ nm emission wavelengths

5.21.5 Recorder, integrator or computer based data processing system

5.21.6 Analytical reverse-phase HPLC separating column, for example C₁₈, base deactivated octadecyl silane (ODS), length of 25 cm, inner diameter of 4,7 mm and a particle size of 5 μ m, which ensures resolution of ochratoxin A from all other peaks. The maximum overlap of peaks shall be less than 10 %. A suitable corresponding reverse-phase guard column should be used

5.21.7 Degasser, optional, for degassing HPLC mobile phases (4.24) and (4.25) and the ammonium hydroxide solution (4.9)

5.21.8 Column oven, capable to operate at (50 ± 1) °C

5.21.9 Post-column derivatization system, comprising a second LC pulseless pump, zero dead volume T-piece, reaction stainless steel tubing min. 10 cm \times 0,25 mm internal diameter

5.22 UV spectrometer, with suitable quartz cells

6 Procedure

6.1 Extraction

Stir the sample thoroughly before removing an analytical test portion. Weigh, to the nearest 0,1 g, a 25 g test portion of baby food sample into a centrifuge bottle (5.9). Add 100 ml of the mixture of phosphoric acid solution and sodium chloride solution (4.15). Mix for 1 min on a Vortex mixer (5.20). Add 50 ml of tert-butyl methyl ether (4.22). Blend for 2 min with a high speed blender (5.2). Centrifuge for 10 min at 15 300 g with cooling at approximately 4 °C.

Transfer the upper organic layer to a capped conical flask or measuring cylinder. Re-extract with a second 50 ml portion of tert-butyl methyl ether, blending again for 2 min. After centrifugation under the same conditions, combine the two organic phases.

Pour an aliquot of 75 ml of the combined organic phases into a round bottomed flask (5.15) and evaporate at 35 °C to 40 °C in a rotary evaporator (5.10) until no more solvent distils. Re-dissolve the remaining fatty residue as follows to obtain an extract in a mixture of 15 parts per volume of methanol (4.19) and 85 parts per volume of PBS solution (4.14). Add 3 ml of methanol and thoroughly rinse the walls of the flask. Transfer the methanol extract into a separating funnel (5.19) by using a Pasteur pipette. Repeat this step once again with a new portion of 3 ml of methanol. Combine both methanol extracts in the separating funnel. Dilute by addition of 34 ml of PBS solution (4.14) to the separating funnel and shake vigorously for 1 min.

Add 50 ml of hexane (4.18) to the separating funnel and shake gently for 1 min. Allow the layers to separate, then pour off the lower layer into a second separating funnel and discard the top hexane layer. Repeat this operation with a second portion of 50 ml of hexane. Pour off the lower layer into a centrifuge bottle (5.9) and centrifuge for 10 min at 15 300 g and approximately 4 °C in order to separate any fatty residue. Drain into a measuring cylinder through a funnel and filter paper (5.6).

NOTE Try to avoid the re-mixing of aqueous and organic phases. If draining is difficult, collect and filter at least 35 ml of the aqueous layer by pipetting.

6.2 Immunoaffinity column cleanup

Connect the immunoaffinity column (4.26) to the vacuum manifold (5.5), and attach a reservoir of 50 ml or 20 ml capacity (5.11) to the immunoaffinity column.

Columns should be allowed to reach room temperature prior to conditioning. For conditioning apply 20 ml of PBS solution (4.14) to the top of the column and let it pass at a speed of 2 ml/min to 3 ml/min through the column by gravity. Make sure that a small portion (e.g. 0,5 ml) of the PBS remains on the column until the sample solution is applied.

Transfer 30 ml of the sample extract as obtained in 6.1 to the reservoir and pass through the immunoaffinity column. Do not exceed a flow rate of 3 ml/min. Let it pass by gravity or pushing down slightly with a syringe or applying little vacuum.

CAUTION — If using a vacuum manifold, extra care is necessary to avoid increasing the flow rate through the column to the point where recovery is adversely affected.

6.3 Preparation of the sample test solution

Wash the column with 10 ml of water. Remove the reservoir and eliminate any residual water on the inside of the upper part of the column with a piece of absorbing paper or a cotton stick. Dry the column by applying vacuum for 1 min or blowing air with a syringe.

Attach to the column a reservoir of 5 ml capacity (5.11) and elute the ochratoxin A in a two step procedure:

- Apply 4 ml of methanol on the column and let it pass through by gravity until elution of the first drop. Close the luer lock. Wait for 1 min and slowly elute ochratoxin A from the column into a glass tube;

- collect the elution solvent, push air through to ensure no solvent remains in the immunoaffinity column. Evaporate the eluate to dryness using a gentle stream of nitrogen (for example 0,7 bar to 1,0 bar) in a water bath at approximately 45 °C. Re-dissolve the sample in 300 µl of the mixture of methanol and acetic acid (4.21) and transfer the sample to a glass vial (5.18) or eventually to an autosampler vial in case to proceed to the immediate automatic injection in HPLC.

6.4 Spiking procedure

Weigh, to the nearest 0,1 g, 25 g of blank baby food sample into a centrifuge bottle (5.9). Pipette 40 µl of ochratoxin A spiking solution (4.30) onto the blank baby food. After addition of the spike solution, let the solvent evaporate in a fume cupboard for at least 2 h prior to extraction. Proceed as in 6.1.

7 HPLC analysis

7.1 HPLC operating conditions

When the column (5.21.6) and the mobile phases A (4.24) and B (4.25) were used the following setting were found to be appropriate:

Table 1 — Gradient conditions

Time min	Flow rate ml/min	Mobile phase A %	Mobile phase B %
0	1	40	60
10	1	0	100
11	1	40	60
30	1	40	60

- Column oven temperature (including the guard column) is (50 ± 1) °C;
- injection volume is 50 µl;
- autosampler (optional) temperature is 15 °C to 20 °C;
- flow rate of the ammonium hydroxide solution (4.9) is 0,2 ml/min.

NOTE 1 HPLC under isocratic conditions using a mobile phase formed by a mixture of four parts per volume of acetic acid solution (4.17) and six parts per volume of methanol (4.19) is an acceptable option. Nevertheless, gradient conditions are preferred because they extend the life of the column.

NOTE 2 The eluent should be alkaline (typically pH \approx 9) after the detector. Check it by using pH indicator paper.

NOTE 3 Spread of ammonia vapours should be minimized for example by putting a saturated citric acid solution into the waste reservoir.

NOTE 4 Using the conditions specified in 7.1, ochratoxin A should be completely separated from a small peak which elutes just before it.

7.2 Preparation of calibration solutions for HPLC

Prepare five HPLC calibration solutions by distributing with appropriate displacement pipettes (5.17) or microsyringes (5.12) the volumes of the ochratoxin A standard solution (4.29) listed in Table 2 separately into a set of glass vials (5.18). Then evaporate the mixture of toluene and acetic acid solution just to dryness under a stream of nitrogen at room temperature. To each vial, add 1 ml of the mixture of methanol and acetic acid solution (4.21), seal and mix in a Vortex mixer. These solutions cover a range from 0,021 µg/kg to 0,320 µg/kg for ochratoxin A under the conditions of this protocol.

The solutions should be protected from light and can be stored in the freezer at approximately - 18 °C. Check the stability before the next use.

Peak areas corresponding to the same calibration solution injected at regular intervals should be within ± 3 %.

Table 2 — Preparation of HPLC calibration solutions

HPLC calibration solution	Standard solution (4.29) µl	Final ochratoxin A concentration in calibration solution	
		ng/ml	ng/50 µl
1	25	1,00	0,050
2	50	2,00	0,100
3	125	5,00	0,250
4	250	10,00	0,500
5	375	15,00	0,750

NOTE In case that the content of ochratoxin A in the sample is outside of the calibration range, an appropriate calibration curve can be prepared. Alternatively the injection solution for HPLC analysis can be diluted to an ochratoxin A content appropriate for the established calibration curve.

7.3 Calibration curve

Prepare a calibration curve by injecting 50 µl of five different ochratoxin A calibration solutions (7.2) at the beginning of every day of the analysis. Plot the peak area against the mass of injected ochratoxin A and check the curve for linearity.

7.4 Determination of ochratoxin A in test solutions

Inject 50 µl aliquots of the test solutions into the chromatograph using the same conditions used for the preparation of the calibration curve.

7.5 Peak identification

Identify the ochratoxin A peak in the sample test solution by comparing the retention time of the sample with that of the calibration solutions. The concentration of ochratoxin A in the sample test solution shall fall within the calibration range. In the case that the mass concentration of ochratoxin A in the sample test solution is outside the calibration range, an appropriate calibration curve can be prepared. Alternatively the sample test solution can be diluted to a mass concentration of ochratoxin A appropriate for the established calibration curve. The dilution factor shall be incorporated into all subsequent calculations.

8 Calculation

Determine from the calibration curve, the mass in nanograms of the ochratoxin A in the aliquot of sample test solution injected onto the HPLC column. Calculate the mass fraction, w_{ota} , of ochratoxin A in micrograms per kilogram, using Equation (2):

$$w_{ota} = m_a \times \frac{V_1 \times V_3 \times V_5}{V_2 \times V_4 \times V_6 \times m_s} \quad (2)$$

where

m_a is the mass of ochratoxin A, in nanograms, in the aliquot of sample test solution injected and corresponding to the area of the ochratoxin A peak;

- V_1 is the volume, in millilitres, of tert-butyl methyl ether (here: 100 ml);
- V_3 is the volume, in millilitres, of the total extract in the mixture of PBS and methanol (here: 40 ml);
- V_5 is the volume, in microlitres, of the test solution (here: 300 μ l);
- V_2 is the volume, in millilitres, of the tert-butyl methyl ether aliquot taken to dryness before re-dissolution in the mixture of PBS and methanol (here: 75 ml);
- V_4 is the volume, in millilitres, of the extract in the mixture of PBS and methanol passed through the immunaffinity column (here: 30 ml);
- V_6 is the volume, in microlitres, of the test solution injected on the column (here: 50 μ l);
- m_s is the mass, in grams, of sample extracted (here: 25 g).

9 Precision

9.1 General

Details of an interlaboratory test on the precision of the method are given in Table B.1. The values derived from this interlaboratory test may not be applicable to concentration ranges and/or matrices other than those given in Annex B.

9.2 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 cereal based baby food are:

$\bar{x} = 0,023 \mu\text{g/kg}$		(blank)
$\bar{x} = 0,050 \mu\text{g/kg}$	$r = 0,066 \mu\text{g/kg}$	(naturally contaminated)
$\bar{x} = 0,093 \mu\text{g/kg}$	$r = 0,047 \mu\text{g/kg}$	(fortified)
$\bar{x} = 0,096 \mu\text{g/kg}$	$r = 0,122 \mu\text{g/kg}$	(naturally contaminated)
$\bar{x} = 0,217 \mu\text{g/kg}$	$r = 0,218 \mu\text{g/kg}$	(naturally contaminated)

9.3 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.

The values for cereal based baby food are:

$\bar{x} = 0,023 \mu\text{g/kg}$		(blank)
$\bar{x} = 0,050 \mu\text{g/kg}$	$R = 0,089 \mu\text{g/kg}$	(naturally contaminated)
$\bar{x} = 0,093 \mu\text{g/kg}$	$R = 0,076 \mu\text{g/kg}$	(fortified)
$\bar{x} = 0,096 \mu\text{g/kg}$	$R = 0,122 \mu\text{g/kg}$	(naturally contaminated)
$\bar{x} = 0,217 \mu\text{g/kg}$	$R = 0,273 \mu\text{g/kg}$	(naturally contaminated)

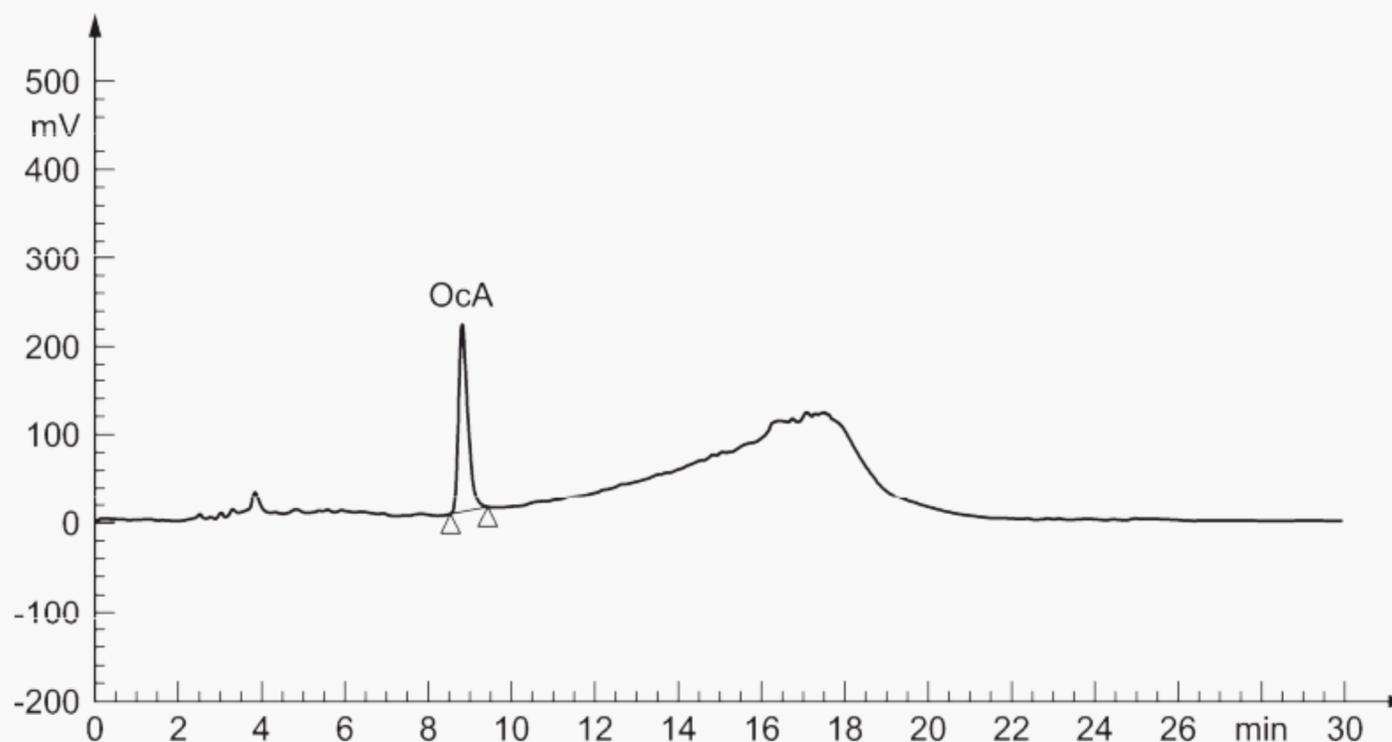
10 Test report

The test report shall contain at least the following data:

- a) all information necessary for the identification of the sample (kind of sample, origin of sample, designation);
- b) reference to this European Standard;
- c) date and type of sampling procedure (if known);
- d) date of receipt;
- e) date of test;
- f) test results and the units in which they have been expressed;
- g) any particular points observed in the course of the test;
- h) any operations not specified in the method or regarded as optional, which might have effected the results.

Annex A (informative)

Typical chromatograms

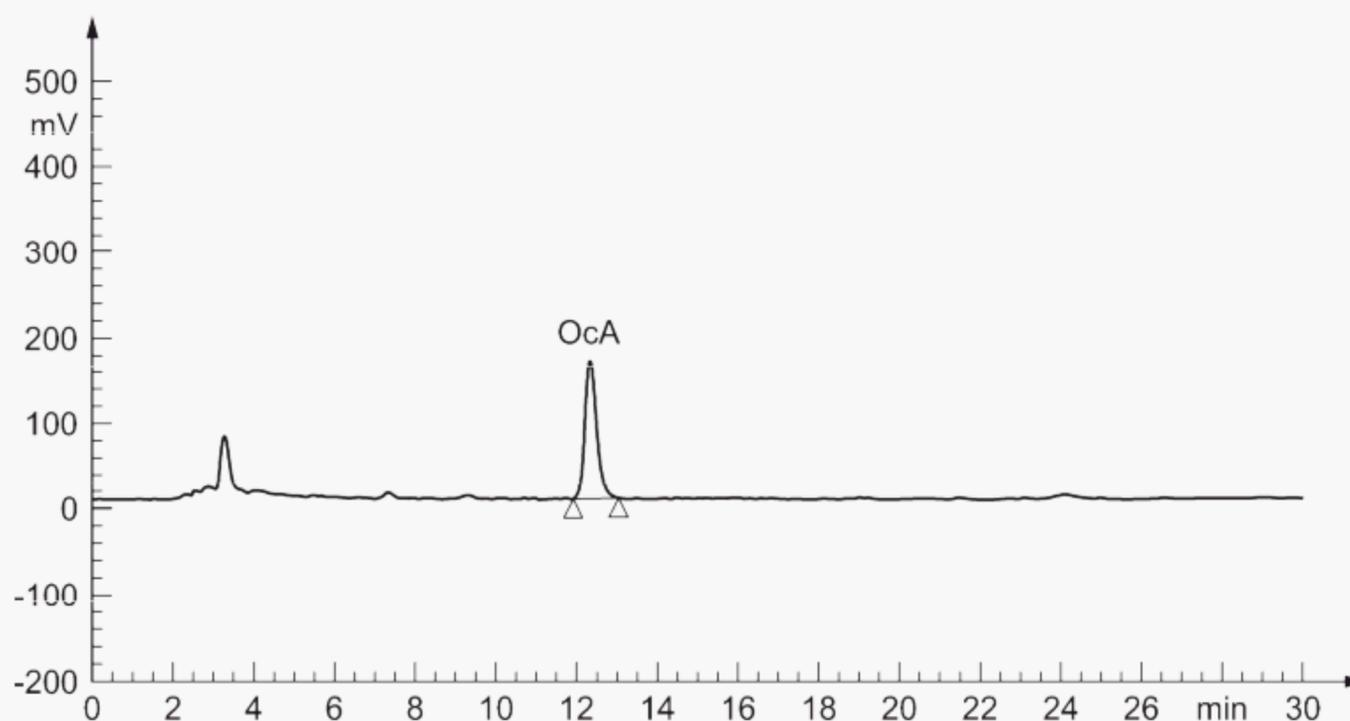


Key

x time, in minutes
y fluorescence

Operating conditions: see 7.1, gradient conditions.

Figure A.1 — Chromatogram from the interlaboratory study of ochratoxin A in naturally contaminated cereal based baby food after immunoaffinity cleanup (contamination level is 0,191 µg/kg)



Key

X time, in minutes
Y fluorescence

Operating conditions: mobile phase (isocratic conditions): methanol - 9 % acetic acid (60 + 40, v/v).

Figure A.2 — Chromatogram from the in-house study of ochratoxin A in naturally contaminated cereal based baby food containing honey, after immunoaffinity cleanup (contamination level is 0,290 µg/kg)

Annex B (informative)

Precision data

The data given in Table B.1 were obtained in an interlaboratory study organized by the European Communities, Standards, Measurement and Testing Programme in accordance with ISO 5725-2, -4 and -6 (see [1], [2] and [3]). Samples of cereal based baby foods, both naturally contaminated and spiked with ochratoxin A, were included in the study [5].

Table B.1 — Precision data interlaboratory study for baby food

Sample	Blank	Low level	Medium level	High level	Blind spike
Year of interlaboratory test	2000	2000	2000	2000	2000
Number of laboratories	12	12	12	12	12
Number of noncompliant results	3	1	0	1	0
Number of outliers (laboratories)	0	0	0	1	2
Number of accepted results	9	11	12	10	10
Mean value, \bar{x} , $\mu\text{g/kg}$	0,023	0,050	0,096	0,217	0,093
Repeatability standard deviation s_r , $\mu\text{g/kg}$	-	0,023	0,044	0,078	0,017
Repeatability relative standard deviation, RSD_r , %	-	47	45	36	18
Repeatability limit r [$r = 2,8 \times s_r$], $\mu\text{g/kg}$	-	0,066	0,122	0,218	0,047
Reproducibility standard deviation s_R , $\mu\text{g/kg}$	-	0,032	0,044	0,097	0,027
Reproducibility relative standard deviation, RSD_R , %	-	63	45	45	29
Reproducibility limit R [$R = 2,8 \times s_R$], $\mu\text{g/kg}$	-	0,089	0,122	0,273	0,076
Spiking level, $\mu\text{g/kg}$	-	-	-	-	0,085
Recovery, %	-	-	-	-	108
HorRat value, according to [7]	-	0,9	0,7	0,8	0,4
HorRat value, according to [8]	-	2,9	2,0	2,0	1,3

Annex C (informative)

Data on the in-house study

The data given in Table C.1 were obtained during the in-house development of the method under the project SMT-CT96-2045, funded by the European Commission, Standards, Measurement and Testing Programme (4th Framework Programme). The data in Table C.2 were obtained in an in-house study organized by the Centro Nacional de Alimentación in Spain. Three kinds of commercial samples of cereal based baby foods containing eight different types of cereals, honey or cocoa, respectively, in their list of ingredients were included in the study. Samples both naturally contaminated and spiked with ochratoxin A, were included in the study [6].

Table C.1 — Limit of quantification (lowest validated level)

Limit of quantification (signal/noise = 6)	7,00 ng/kg
Limit of quantification tested (fortified)	6,98 ng/kg
Level found	8,20 ng/kg
Repeatability limit <i>r</i>	0,30 ng/kg
Recovery	117,5 %

Table C.2 — Precision data in-house study

Sample	Mean value µg/kg	Repeatability µg/kg	Internal reproducibility µg/kg	Recovery %
Values for eight cereal based baby food:				
(blank, naturally contaminated)	0,133	n.a.	n.a.	n.a.
(fortified)	0,541	0,016	n.a.	n.a.
(fortified)	2,620	0,126	n.a.	n.a.
(fortified)	0,556	n.a.	0,025	n.a.
(fortified)	2,520	n.a.	0,194	n.a.
Spiking level 0,5 µg/kg	n.a.	n.a.	n.a.	90,0 %
Spiking level 3,0 µg/kg	n.a.	n.a.	n.a.	81,7 %

Table C.2 (continued)

Sample	Mean value µg/kg	Repeatability µg/kg	Internal reproducibility µg/kg	Recovery %
Values for cereal based baby food with honey:				
(blank, naturally contaminated)	0,382	n.a.	n.a.	n.a.
(fortified)	0,871	0,017	n.a.	n.a.
(fortified)	3,540	0,028	n.a.	n.a.
(fortified)	0,857	n.a.	0,024	n.a.
(fortified)	3,410	n.a.	0,218	n.a.
Spiking level 0,5 µg/kg	n.a.	n.a.	n.a.	100,8 %
Spiking level 3,0 µg/kg	n.a.	n.a.	n.a.	103,8 %
Values for cereal based baby food with cocoa:				
(blank, naturally contaminated)	0,750	n.a.	n.a.	n.a.
(fortified)	1,230	0,046	n.a.	n.a.
(fortified)	3,500	0,098	n.a.	n.a.
(fortified)	1,200	n.a.	0,046	n.a.
(fortified)	3,480	n.a.	0,077	n.a.
Spiking level 0,5 µg/kg	n.a.	n.a.	n.a.	96,3 %
Spiking level 3,0 µg/kg	n.a.	n.a.	n.a.	93,4 %
n.a.: not applicable.				

Bibliography

- [1] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*
- [2] ISO 5725-4:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 4: Basic methods for the determination of the trueness of a standard measurement method*
- [3] ISO 5725-6:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 6: Use in practice of accuracy values*
- [4] Castegnaro M., Barek J., Fremy J.M., Lafontaine M., Sansone E.B. and Telling G.M., Laboratory decontamination and destruction of carcinogens in laboratory wastes: some mycotoxins. IARC Scientific Publication No. 113, International Agency for Research on Cancer, Lyon (France), 1991, p. 63
- [5] Burdaspal P.A., Legarda T.M. and Gilbert J., (2001), Determination of Ochratoxin A in Baby Food by Immunoaffinity Column Clean-up with Liquid Chromatography: Interlaboratory Study. *Journal of AOAC International.*, vol. 84: 1445-1452
- [6] Burdaspal P.A. and Legarda T.M., (2004), Determination of ochratoxin A in processed cereal based baby foods by immunoaffinity column cleanup with liquid chromatography: surveillance data. *Alimentaria*, vol. 350: 11-18
- [7] Horwitz W. and Albert R, (2006), The Horwitz Ratio (HorRat): A useful Index of Method Performance with Respect to Precision, *Journal of AOAC International*, vol. 89, 1095-1109
- [8] Thompson M., (2000), Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing, *Analyst*, vol. 125: 385-386

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