SLOVENSKI STANDARD
SIST EN 15835:2010

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Živila - Določevanje ohratoksina A v žitnih kašicah za dojenčke in majhne otroke - Metoda HPLC s čiščenjem z imunoafinitetno kolono in fluorescenčno detekcijo

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

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

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

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ICS:

67.060	Žita, stročnice in proizvodi iz njih	Cereals, pulses and derived products
67.230	Predpakirana in pripravljena hrana	Prepackaged and prepared foods

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of an identical text or by endorsement, at the latest by August 2010, and conflicting national standards shall be withdrawn at the latest by August 2010.

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Annexes A, B and C are informative.

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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 lypophilic 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 $Na_2HPO_4 \cdot 12\ H_2O$

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 $w(NH_4OH)$ = 25 % in water (post column reagent)

Degas the solution with a degasser (5.21.7).

4.10 Hydrochloric acid solution, w(HCl) is 37 % (acidimetric)

4.11 Phosphoric acid solution, $w(H_3PO_4)$ = 85 %

4.12 Hydrochloric acid solution, c(HCl) = 0,1 mol/l

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

4.13 Sodium hydroxide solution, c(NaOH) = 0,1 mol/l

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

4.14 Phosphate buffered saline (PBS) solution, c(NaCl) = 120 mmol/l, c(KCl) = 2,7 mmol/l, c(phosphate buffer) = 10 mmol/l, 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 $Na_2HPO_4 \cdot 12\ H_2O$ (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, $c(H_3PO_4)$ = 0,5 mol/l, c(NaCl) = 2 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 ≥ 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):

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

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 m^2/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 µl, 50 µl, 100 µl, 500 µl and 1 000 µl 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 µl and 1 000 µl 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 µl

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 λ = 390 nm excitation and λ = 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 C_{18}, base deactivated octadecyl silane (ODS), length of 25 cm, inner diameter of 4,7 mm and a particle size of 5 µm, which ensures resolution of ochratoxin A from all other peaks. The maximum overlap of peaks shall be less then 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 × 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;