SLOVENSKI STANDARD
SIST EN 15850:2010

01-september-2010

Živila - Določevanje zearalenona v hrani za dojenčke in majhne otroke na osnovi koruze, ječmenove moke, koruzne moke, polente, pšenične moke ter v žitnih kašicah - Metoda HPLC z imunoafinitetnim kolonskim čiščenjem in fluorescenčno detekcijo

Foodstuffs - Determination of zearalenone in maize based baby food, barley flour, maize flour, polenta, wheat flour and cereal based foods for infants and young children - HPLC method with immunoaffinity column cleanup and fluorescence detection

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Lebensmittel - Bestimmung von Zearalenon in Säuglingsnahrung auf Maisbasis, Gerstenmehl, Maismehl, Maisgrieß, Weizenmehl und Lebensmittel auf Getreidebasis für Säuglinge und Kleinkinder - HPLC-Verfahren mit Reinigung an einer Immunoaffinitätssäule und Fluoreszenzdetektion

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Produits alimentaires - Dosage de la zéaralénone dans les aliments à base de maïs pour bébés, dans la farine d'orge, dans la farine de maïs, dans la polenta, dans la farine de blé et dans les aliments à base de céréales pour nourrissons et enfants en bas âge - Méthode par chromatographie liquide haute performance avec purification sur colonne d'immuno-affinité et détection par fluorescence

Ta slovenski standard je istoveten z: **EN 15850:2010**

ICS:

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

SIST EN 15850:2010 **en,fr,de**

2003-01.Slovenski inštitut za standardizacijo. Razmnoževanje celote ali delov tega standarda ni dovoljeno.

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EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 15850

April 2010

ICS 67.060

English Version

Foodstuffs - Determination of zearalenone in maize based baby food, barley flour, maize flour, polenta, wheat flour and cereal based foods for infants and young children - HPLC method with immunoaffinity column cleanup and fluorescence detection

Produits alimentaires - Dosage de la zéaralénone dans la farine d'orge, de maïs et de blé, la polenta et les produits pour nourrissons et jeunes enfants à base de céréales - Méthode par chromatographie liquide haute performance avec purification sur colonne d'immunoaffinité et détection par fluorescence

Lebensmittel - Bestimmung von Zearalenon in Säuglingsnahrung auf Maisbasis, Gerstenmehl, Maismehl, Maisgrieß, Weizenmehl und Lebensmittel auf Getreidebasis für Säuglinge und Kleinkinder - HPLC-Verfahren mit Reinigung an einer Immunoaffinitätssäule und Fluoreszenzdetektion

This European Standard was approved by CEN on 27 February 2010.

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Foreword

This document (EN 15850:2010) 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 October 2010, and conflicting national standards shall be withdrawn at the latest by October 2010.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

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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.

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1 Scope

This European Standard specifies a method for the determination of zearalenone in maize based baby food, barley flour, maize flour, polenta, wheat flour and cereal based foods for infants and young children by high performance liquid chromatography (HPLC) with immunoaffinity cleanup and fluorescence detection. This method has been validated in two interlaboratory studies. The first study was for the analysis of samples of maize based baby food, barley flour, maize flour, polenta and wheat flour ranging from 10 µg/kg to 335 µg/kg, and the second study was for samples of cereal based foods for infants and young children ranging from 9 µg/kg to 44 µg/kg.

Further information on validation, see Clause 9 and Annex B.

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 aqueous acetonitrile or methanol according to the products analyzed. The extract is then diluted with phosphate buffered saline (PBS) to give an aqueous extract that is applied to an immunoaffinity column containing antibodies specific for zearalenone. Zearalenone is purified and concentrated on the column and removed from the antibodies using acetonitrile or methanol as eluent. Zearalenone is quantified by reverse-phase high performance liquid chromatography (RP-HPLC) with fluorescence detection.

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4 Reagents

4.1 General

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

4.2 Disodium hydrogen phosphate, Na_2HPO_4 anhydrous or $Na_2HPO_4 \cdot 12\ H_2O$.

4.3 Potassium chloride (KCl).

4.4 Potassium dihydrogen phosphate, KH_2PO4.

4.5 Sodium chloride (NaCl).

4.6 Sodium hydroxide (NaOH).

4.7 Hydrochloric acid solution, mass fraction w(HCl) = 37 % in water.

4.8 Hydrochloric acid solution, substance concentration c(HCl) = 0,1 mol/l.

Dilute 8,28 ml of hydrochloric acid solution (4.7) to 1 l with water.

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4.9 Sodium hydroxide solution, *c*(NaOH) = 0,1 mol/l.

Dissolve 4 g of sodium hydroxide (4.6) in 1 l of water.

4.10 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 (4.5), 1,2 g of anhydrous disodium hydrogen phosphate or 2,9 g of $Na_2HPO_4 \cdot 12\ H_2O$ (4.2), 0,2 g of potassium dihydrogen phosphate (4.4) and 0,2 g of potassium chloride (4.3) in 900 ml of water. After dissolution, adjust the pH to 7,4 with hydrochloric acid solution (4.8) or sodium hydroxide solution (4.9) as appropriate, then dilute to 1 l with water.

Alternatively, a PBS solution with equivalent properties can be prepared from commercially available PBS material.

4.11 Acetonitrile.

WARNING — Acetonitrile is hazardous and samples shall be blended using an explosion proof blender which is housed within a fume cupboard. After blending, samples shall be filtered inside a fume cupboard.

4.12 Methanol, HPLC grade.

4.13 Methanol, technical grade.

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4.14 Extraction solvent A.

Mix 75 parts per volume of acetonitrile (4.11) with 25 parts per volume of water.

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4.15 Injection solvent A for HPLC analysis.

Mix four parts per volume of acetonitrile (4.11) with six parts per volume of water.

4.16 HPLC mobile phase A.

Mix 53 parts per volume of acetonitrile (4.11) with 47 parts per volume of water. Filter and degas the HPLC mobile phase before use.

4.17 Extraction solvent B.

Mix 75 parts per volume of methanol (4.13) with 25 parts per volume of water.

4.18 Washing solvent.

Mix 15 parts per volume of methanol (4.12) with 85 parts per volume of PBS (4.10).

4.19 Injection solvent B for HPLC analysis.

Mix five parts per volume of methanol (4.12) with five parts per volume of water.

4.20 HPLC mobile phase B.

Mix 75 parts per volume of methanol (4.12) with 25 parts per volume of water. Filter and degas the HPLC mobile phase before use.

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4.21 Immunoaffinity column.

The immunoaffinity column shall contain antibodies raised against zearalenone. The column shall have a capacity of not less than 1 500 ng of zearalenone and shall give a recovery of not less than 80 % when 75 ng of zearalenone is applied in 10 ml of a mixture of 15 parts per volume of methanol and 85 parts per volume of PBS.

4.22 Zearalenone, in crystal form or as a film in ampoules, purity not less than 98 % mass fraction or in form of commercially available Zearalenone solution.

WARNING — Zearalenone is an oestrogenic compound and should be treated with extreme caution. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.23 Zearalenone stock solution, $c \approx 200$ µg/ml.

Add 4,0 ml of acetonitrile (4.11) to 5 mg of zearalenone (4.22) to form a solution with a mass concentration of approximately 1,25 mg/ml. Dilute 800 µl of this solution to 5 ml with acetonitrile (4.11) to form a stock solution with a concentration of approximately 200 µg/ml.

Store this solution in a freezer at - 18 °C to - 20 °C. Allow to reach room temperature before opening. A solution stored in this way is usually stable for 12 months. Confirm the mass concentration of the solution if it is older than six months.

4.24 Zearalenone spiking solution, $c \approx 10$ µg/ml.

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Dilute 250 µl of stock solution (4.23) with 4,75 ml of acetonitrile (4.11) to form a solution with a mass concentration of approximately 10 µg/ml.

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To determine the exact concentration, record the absorption curve of this solution between 200 nm to 300 nm in a 1 cm quartz cell in the spectrometer (5.25) with acetonitrile (4.11) as reference. Identify the wavelength for maximum absorption (λ is approximately 274 nm). Calculate the mass concentration of zearalenone, ρ_{zon}, in micrograms per millilitre using Equation (1):

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

where

A_{max} is the absorption determined at the maximum of the absorption curve (274 nm);

M is the molar mass, in grams per mole, of zearalenone (M = 318,4 g/mol);

ε is the molar absorption coefficient, in square metres per mole of zearalenone in acetonitrile (4.11) (1 262 m^2/mol, see [1]);

b is the optical path length, in centimetres, of the quartz cell.

Store this solution in a freezer at - 18 °C to - 20 °C. Allow to reach room temperature before opening. A solution stored in this way is usually stable for 12 months. Confirm the mass concentration of the solution if it is older than six months.

4.25 Zearalenone standard solution A, ρ = 2 µg/ml, for maize based baby food, barley flour, maize flour, polenta and wheat flour.

Transfer an aliquot of the spiking solution (4.24) equivalent to 10 µg of zearalenone into either a vial (5.9) or a calibrated volumetric flask (5.10). Add acetonitrile (4.11) to make the total volume up to 5 ml.

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Store this solution in a freezer at - 18 °C to - 20 °C. Allow to reach room temperature before opening. A solution stored in this way is usually stable for 12 months. Confirm the mass concentration of the solution if it is older than six months.

4.26 Zearalenone standard solution B, ρ = 0,4 µg/ml, for cereal based foods for infants and young children.

Transfer an aliquot of the spiking solution (4.24) equivalent to 2 µg of zearalenone into either a vial (5.9) or a calibrated volumetric flask (5.10). Add acetonitrile (4.11) to make the total volume up to 5 ml.

Store this solution in a freezer at - 18 °C to - 20 °C. Allow to reach room temperature before opening. A solution stored in this way is usually stable for 12 months. Confirm the mass 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 or homogenizer.

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

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

5.5 Adjustable vertical or horizontal shaker.

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5.6 Vortex mixer, or equivalent.

5.7 Mills, various screens.

5.8 Tumble mixer.

5.9 Glass vials, of various sizes

5.10 Volumetric flasks, of 3 ml or 5 ml and 10 ml capacity.

5.11 Beaker, of 250 ml capacity.

5.12 Conical flask, with screw cap or glass stopper of 100 ml, 250 ml and 500 ml capacity.

5.13 Filter paper, e.g., qualitative, strong, fast flow, 24 cm diameter, 30 µm pore size, prefolded or equivalent.

5.14 Glass microfibre filter, e.g. 1,6 µm retention size or equivalent.

5.15 Pipettes, of e.g. 25 µl to 250 µl, 1 ml, 5 ml and 10 ml capacity.

5.16 Displacement micropipettes or syringes, gas tight of e.g. 100 µl, 500 µl, 1 000 µl capacity.

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5.17 Vacuum manifold or automated system, to accommodate immunoaffinity columns.

5.18 Reservoirs, of 50 ml to 75 ml capacity, and attachments for immunoaffinity columns.

5.19 Plastic syringes, 5 ml.

5.20 Vacuum pump, capable of for example pulling a vacuum of 1 kPa and or pumping 18 l/min.

5.21 Solvent vacuum filtration system, fitted with 47 mm glass microfibre filter.

5.22 Disposable syringe filter unit, nylon with a pore size of 0,45 µm.

Prior to usage, verify that no zearalenone losses occur during filtration (recovery testing).

NOTE There is a possibility that various filter materials retain zearalenone.

5.23 Ultrasonic bath.

5.24 HPLC apparatus, comprising the following:

5.24.1 Injection system, capable of injecting e.g. 100 µl to 300 µl.

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5.24.2 Mobile phase pump, pulse free, capable of maintaining a volume flow rate of 0,5 ml/min to 1,5 ml/min.

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5.24.3 Analytical reverse-phase HPLC separating column, that allows a sufficient baseline separation of zearalenone from other interfering components. The maximum overlap shall be less than 10 % peak height.

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Phenomenex Prodigy® ODS 3 [1] (150 mm × 4,6 mm internal diameter, 5 µm particle size, 25 nm pore size) or Spherisorb® [1] ODS-2 Excel (250 mm × 4,6 mm internal diameter, 5 µm particle size, 25 nm pore size) have been found to be suitable when used with mobile phase A (4.16).

Supelcosil® [1] (C_{18}), fully end capped (250 mm × 4,6 mm internal diameter, 5 µm particle size, 18 nm pore size), carbon loading of 12 %, or similar, has been found to be suitable when used with mobile phase B (4.20).

5.24.4 Pre-column, with preferably the same stationary phase material as the analytical column, an internal diameter of 4 mm, 5 µm particle size.

5.24.5 Fluorescence detector, fitted with a flow cell and suitable for measurements with excitation wavelength of 274 nm or 275 nm and emission at 446 nm or 450 nm.

5.24.6 Recorder, integrator or computer based data processing system.

5.24.7 Degasser (optional).

5.25 UV spectrometer, with suitable quartz cells.

1) Phenomenex Prodigy®, Spherisorb®, Supelcosil® are examples of a suitable products available commercially. This information is given for the convenience of the users of this European Standard and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can show to lead to the same results.

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6 Procedure

6.1 Extraction

6.1.1 Extraction for barley flour, maize flour, wheat flour, polenta and maize based baby food

Weigh, to the nearest 0,1 g, a 25 g test portion of the ground sample into a beaker (5.11). Add 100 ml of extraction solvent A (4.14). Homogenize for 3 min with a homogenizer (5.2) set at high speed. Filter the extract through a folded filter paper (5.13). Transfer 88 ml of PBS (4.10) to a conical flask (5.12). Pipette (5.15), add 12 ml of the filtrate and mix well by shaking by hand. If on addition of the filtrate to PBS the sample becomes cloudy filter through a glass microfibre filter paper (5.14) prior to cleanup.

6.1.2 Extraction for cereal based foods for infants and young children

Weigh, to the nearest 0,1 g, a 20 g test portion into a screw capped conical flask (5.12). Add 150 ml of extraction solvent B (4.17). Mix shortly by hand for a few seconds to obtain a homogeneous suspension, then either shake for 1 h in a shaker (5.5) or sonicate for 15 min in an ultrasonic bath (5.23) and shake on a shaker (5.5) for another 15 min.

Filtrate the extract through a folded filter paper (5.13) and collect the extract in a conical flask of 100 ml (5.12). Transfer exactly 30 ml of the filtrate extract into a 150 ml volumetric cylinder with stopper. Dilute the extract in the cylinder with PBS (4.10) to achieve a final volume of 150 ml. Shake and filter approximately 20 ml of this diluted extract through a glass microfibre filter (5.14) into a glass beaker by applying a slight vacuum (5.21). Discard this 20 ml and filter again a further approximately 70 ml for analysis.

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NOTE Do not apply a strong vacuum in the beginning of the filtration process, as this can lead to turbid filtered extracts after filtration.

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Proceed immediately with the immunoaffinity column cleanup procedure (6.2).

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6.2 Immunoaffinity column cleanup

Allow the immunoaffinity column to reach room temperature prior to conditioning. Connect the immunoaffinity column (4.21) to the vacuum manifold (5.17), and attach the reservoir (5.18) on top of the immunoaffinity column. Precondition the immunoaffinity column with 20 ml of PBS (4.10) using a flow rate of 3 ml/min to 5 ml/min. Transfer 50 ml of diluted (and possibly filtered) sample extract (see 6.1.1 or 6.1.2) into the reservoir. Draw the extract through the column by gravity at a steady flow rate until all the extract has passed through the column and the last solvent portion reaches the frit of the column. The flow rate should result in a dropping speed of one drop per second to two drops per second.

After the extract has passed through the column, wash the column with 20 ml of water or for baby food 5 ml of washing solvent (4.18) followed by 15 ml of water at a rate of one drop per second to two drops per second.

Remove residual water from the immunoaffinity column for example by passing 3 ml of air or nitrogen through the column. Discard all the eluent from this stage of the cleanup procedure.

NOTE 1 The conditions above for preconditioning and washing elution can be altered to comply with individual column manufacturer's instructions for use.

NOTE 2 Care should be taken not to exceed the capacity of the immunoaffinity column.

6.3 Preparation of the sample test solution

6.3.1 Preparation for barley flour, maize flour, wheat flour, polenta and maize based baby food

Place a vial (5.9) under each immunoaffinity column. Pipette 1,5 ml of acetonitrile (4.11) to the column reservoir (5.18). Allow the solvent to remain in contact with the immunoaffinity column for 1 min then elute the