SLOVENSKI STANDARD
SIST EN 15891:2011

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Živila - Določevanje deoksinvalenola v žitu in žitnih proizvodih in hrani na osnovi žit za dojenčke in majhne otroke - Metoda s HPLC z imunoafinitetnim kolonskim čiščenjem in ultravijolična (UV) detekcija

Foodstuffs - Determination of deoxynivalenol in cereals, cereal products and cereal based foods for infants and young children - HPLC method with immunoaffinity column cleanup and UV detection

Lebensmittel - Bestimmung von Deoxynivalenol in Getreide, Getreideerzeugnissen und Kleinkindernahrung auf Getreidebasis - HPLC-Verfahren mit Reinigung an einer Immunoaffinitätssäule und UV-Detektion

Denrées alimentaires - Dosage du déoxynivalénol dans les céréales, les produits céréaliers, et céréales pour déjeuner en alimentation infantile - Méthode par CLHP avec purification sur colonne d'immunoaffinité et détection UV

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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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EUROPEAN STANDARD

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Foreword

This document (EN 15891:2010) 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of an identical text or by endorsement, at the latest by March 2011, and conflicting national standards shall be withdrawn at the latest by March 2011.

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.

Annexes A and B are informative.

This document has been prepared under a mandate give to CEN by the European Commission and the European Free Trade Association.

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1 Scope

This European Standard specifies a method for the determination of deoxynivalenol (DON) in cereals (grain and flour), cereal based foods and cereal based foods for infants and young children by high performance liquid chromatography (HPLC) with immunoaffinity cleanup and UV detection. This method has been validated in three interlaboratory studies. The first study was for the analysis of samples of wheat, rice flour, oat flour, maize, polenta, and wheat based breakfast cereal ranging from 85,4 µg/kg to 1 768 µg/kg, the second study was for wheat and maize ranging from 165 µg/kg to 4 700 µg/kg and the third study was for cereal based foods for infants and young children ranging from 58 µg/kg to 452 µg/kg.

For further information on the validation, see Clause 9 and Annex B.

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

Deoxynivalenol is extracted from the sample using water. The aqueous extract is cleaned up on an immunoaffinity column to remove impurities from the sample. Deoxynivalenol is then quantitatively determined by HPLC and UV detection.

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 the reagents listed may be used.

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

4.3 Potassium chloride, KCl.

4.4 Potassium dihydrogen phosphate, KH_2PO_4.

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.

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 Polyethylene glycol (PEG), with a molecular mass of approximately 8 000 g/mol.

4.13 Methanol.

4.14 Acetic acid, with a mass fraction of 96 % or glacial acetic acid, with a mass fraction of 100 %.

4.15 Diluent for HPLC analysis.

Mix 9,5 parts per volume of methanol (4.13) with 90,5 parts per volume of water.

4.16 HPLC mobile phase.

Mix 15 parts per volume of methanol (4.13) with 85 parts per volume of water. Add 0,1 parts per volume of acetic acid (4.14).The exact amount of methanol used and the use of acetic acid will depend on the HPLC column chosen for analysis and shall be adjusted if necessary. Degas this solution before use.

4.17 Wash solvent.

Mix 50 parts per volume of methanol (4.13) with 50 parts per volume of water.

4.18 Immunoaffinity (IA) column.

The immunoaffinity column shall contain antibodies raised against DON. The column shall have a capacity of not less than 1 000 ng of DON and shall have a recovery of not less than 80 % when 500 ng of DON are applied in 1 ml to 2 ml of water.

4.19 Deoxynivalenol, purity not less than 97 % mass fraction**.**

WARNING — Deoxynivalenol is highly toxic. 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.

4.20 Deoxynivalenol stock solution 1, mass concentration $\rho \approx 1{,}25$ mg/ml**.**

Add 4,0 ml of acetonitrile (4.11) to approximately 5 mg of deoxynivalenol (4.19) to form a solution with a concentration of approximately 1,25 mg/ml. Alternatively, available commercial solutions with equivalent properties can be used.

Store this solution in a freezer at approximately - 18 °C. A solution stored in this way is stable for 12 months. Confirm the concentration of the solution if it is older than six months.

4.21 Deoxynivalenol stock solution 2, $\rho \approx 250$ µg/ml.

Dilute 800 µl of stock solution 1 (4.20) to 4 ml with acetonitrile (4.11) to form a solution with a concentration of approximately 250 µg/ml.

Store this solution in a freezer at approximately - 18 °C. A solution stored in this way is stable for 12 months. Confirm the concentration of the solution if it is older than six months.

4.22 Deoxynivalenol standard solution A.

Dilute 200 µl of stock solution 2 (4.21) to 2,0 ml with acetonitrile (4.11) to form a solution with a concentration of approximately 25 µg/ml.

To determine the exact mass concentration, record the absorption curve between a wavelength of 200 nm to 270 nm, e.g. in 5 nm steps; in the spectrometer (5.16) against acetonitrile as reference. Identify the wavelength for maximum absorption and calculate the mass concentration of deoxynivalenol, ρ_{DON}, in micrograms per millilitre using Equation (1):

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

where

A_{max} is the absorption determined at the maximum of the absorption curve (here: at 220 nm);

M is the molar mass, in grams per mole, of deoxynivalenol (M = 296,3 g/mol);

ε is the molar absorption coefficient, in square metres per mole, of deoxynivalenol in acetonitrile (4.11) (here: 681 m^2/mol, see [1]);

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

Calculate the mass concentration of the stock solution 2 (4.21), ρ_{DON2}, in micrograms per millilitre using Equation (2):

$$\rho_{DON2} = \rho_{DON} \times 10 \quad (2)$$

Store this solution in a freezer at approximately - 18 °C. A solution stored in this way is stable for 12 months. Confirm the concentration of the solution if it is older than six months.

NOTE Preparation of standard solutions can be carried out gravimetrically by accurately weighing the deoxynivalenol standard material and the solvent used to dissolve it.

4.23 Deoxynivalenol spiking solution, ρ = 100 µg/ml.

Pipette an aliquot of the stock solution 2 (4.21) equivalent to 500 µg of deoxynivalenol in a 5 ml volumetric flask. Dilute to the mark with acetonitrile (4.11).

Store this solution in a freezer at approximately - 18 °C. A solution stored in this way is stable for 12 months. Confirm the concentration of the solution if it is older than six months.

4.24 Deoxynivalenol standard solution B, ρ = 10 µg/ml.

Pipette 500 µl of the spiking solution (4.23) in a 5 ml volumetric flask. Dilute to the mark with acetonitrile (4.11).

Store this solution in a freezer at approximately - 18 °C. A solution stored in this way is 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 Analytical balance, capable of weighing to 0,000 1 g.

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

5.4 High speed blender or homogenizer.

5.5 Laboratory shaker or magnetic stirrer, speed adjustable to approximately 500 min^{-1}.

5.6 Vortex mixer, or equivalent.

5.7 Centrifuge, capable of a centrifugal force of 2 500 *g*.

5.8 Centrifuge tube, of 250 ml capacity.

5.9 Filter paper, qualitative, strong, fast flow, pre-folded and with a diameter of 18,5 cm**.**

5.10 Glass fibre filter, fast flow, fine porosity, retention size 1,6 µm or smaller.

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

5.12 Reservoirs for immunoaffinity columns, of for example 20 ml capacity, with appropriate adaptors.

5.13 Glass vials or assay tubes, of various size.

5.14 Heating block or thermostatic waterbath, capable of maintaining approximately 50 °C.

5.15 HPLC apparatus, comprising the following:

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

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

5.15.3 Analytical reverse-phase HPLC separating column, for example C18 octadecylsilane (ODS), length of 15 cm to 25 cm, inner diameter of 4,6 mm and a particle size of 5 µm, which ensures resolution of deoxynivalenol from all other peaks.

The maximum overlapping of peaks shall be less than 10 %. It can be necessary to adjust the mobile phase for a sufficient baseline resolution. A suitable corresponding reverse-phase guard column should be used.

5.15.4 UV detector, set at 220 nm.

5.15.5 Recorder, integrator or computer based data processing system.

5.15.6 Mobile phase switching unit, or second HPLC pump, if necessary.

5.16 UV spectrometer.

6 Procedure

6.1 General

This method has been validated in three interlaboratory studies. These studies were performed by different laboratories at different times. This is the reason that slightly different procedures were used for extraction and immunoaffinity column cleanup for wheat, rice flour, oat flour, maize, polenta and wheat based breakfast cereal (described in 6.2 and 6.4) and for cereal based food for infants and young children (described in 6.3 and 6.5). The procedures described here are similar to the ones described in the original interlaboratory studies.

6.2 Extraction for wheat, rice flour, oat flour, maize, polenta and wheat based breakfast cereal

Weigh, to the nearest 0,1 g, a 25 g (m_s) test portion and 5 g of PEG (4.12) into a centrifuge tube (5.8). Add 200 ml (V_1) of water (or another volume as specified by the IA column manufacturer) and homogenize at high speed for 3 min using a homogenizer (5.4). Alternative extraction procedures have been shown to give equivalent results. Either shake the sample and the extraction solvent on a wrist action shaker for 2 h or add a magnetic stirrer bar to the flask, cap it and place it on a magnetic stirrer (5.5) to mix at medium-high speed for 30 min. In both cases, shake the sample and reagents together thoroughly by hand to ensure they are well mixed before placing on shaker. Centrifuge the homogenized sample for 15 min at 2 500 *g*. After centrifugation filter the sample with a glass fibre filter (5.10).

NOTE It has been shown during the validation study that for some matrices (maize), samples that have been extracted on a magnetic stirrer do not require centrifugation.

6.3 Extraction for cereal based food for infants and young children

Weigh, to the nearest 0,1 g, a 25 g (m_s) test portion into a 250 ml or 500 ml conical flask. Add 200 ml (V_1) of water, cap and shake for 1 h on a laboratory shaker (5.5). Allow the sample to settle after shaking and transfer 50 ml of supernatant to a centrifuge tube (5.8) and centrifuge for 15 min at 2 500 *g*.

Prepare a funnel and filter paper (5.9). Pour the extracted sample after centrifugation into a 250 ml or 500 ml conical flask through the prepared funnel and filter paper.

6.4 Immunoaffinity column cleanup for wheat, rice flour, oat flour, maize, polenta and wheat based breakfast cereal

Prepare the IA column and proceed with the cleanup procedure in accordance with the manufacturer's instructions. Transfer 2,0 ml (V_3) of the filtered aqueous extract (6.2) onto the immunoaffinity column (4.18). Pass the extract completely through the column at a rate of about one drop per second. Wash the column with 5 ml of water or PBS (4.10). Dry the column by pushing air through it. Using a pipette, transfer 2 ml of acetonitrile (4.11) or methanol (4.13), depending on manufacturer's instructions, to the column reservoir. Allow elution solvent to pass slowly into the column. Stop the flow then wait 1 min before eluting deoxynivalenol from the column at a rate of one drop per second and collect in a 4 ml vial or assay tube (5.13). Carefully pass air through the column in order to collect any final drops.

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

6.5 Immunoaffinity column cleanup for cereal based food for infants and young children

Prepare the IA column and proceed with the cleanup procedure in accordance with the manufacturer's instructions. Transfer 10 ml (V_3) of the aqueous layer (6.3) into the reservoir (5.12) of an immunoaffinity column (4.18). Allow this solution to pass slowly through the column at a rate of approximately one drop per second. When the extract has passed completely through the immunoaffinity column, pass 5 ml of water or PBS (4.10) through the column. Dry the column by passing nitrogen through the column for about 5 s. Then discard all the eluent from this stage of the cleanup procedure. Finally, place a 2,0 ml to 2,5 ml vial (5.13) under the column and pass 0,5 ml of methanol (4.13) through the column, collecting the eluate. Allow the methanol to remain on the column for approximately 1 min before allowing to pass through. Then add a further 1,0 ml of methanol and continue to collect the eluate. Carefully pass air through the column in order to collect any final drops.

NOTE Methods for loading onto IA columns, washing and elution vary slightly between column manufacturers and the specific instructions supplied with the columns should be followed precisely. In general for deoxynivalenol, procedures involve sample extraction with water, centrifugation, filtration, loading under pressure onto a possibly pre-washed column, washing the column with distilled water or (phosphate buffered saline PBS) and elution of deoxynivalenol with methanol or acetonitrile. Take care not to exceed the maximum loading volume or capacity of the column.

6.6 Preparation of sample test solution

Place the vial in a heating block or thermostatic waterbath (5.14) and evaporate under nitrogen at no more than 50 °C. Re-dissolve the residue at ambient temperature in a final volume of 0,5 ml of HPLC diluent (4.15) or mobile phase (4.16). Mix well to ensure the residue is completely re-dissolved by shaking for at least 30 s, for example with a vortex mixer (5.6).

NOTE 1 If necessary the sample can be filtered before analysis by HPLC. A check should be made with a standard solution to assess any loss of deoxynivalenol due to this filtration step.

NOTE 2 After evaporation, the residue can be stored one week at (4 ± 2) °C protected from light.

6.7 Spiking procedure

To determine the recovery carry out the spiking procedure using the spiking solution (4.23). The spiking level should be within the calibration range (preferably mid-range). Add the spiking solution to a test portion of material previously shown not to contain deoxynivalenol and leave to stand for 30 min before adding the extraction solvent.