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SLOVENSKI STANDARD
SIST EN 12955:2000

01-julij-2000

Živila - Določevanje aflatoksina B1 in vsote aflatoksinov B1, B2, G1 in G2 v žitih in lupinastem sadju ter proizvodih iz njih - Metoda tekočinske kromatografije visoke ločljivosti s postkolonsko derivatizacijo in čiščenjem z imunoafinitetno kolono

Foodstuffs - Determination of aflatoxin B1, and the sum of aflatoxins B1, B2, G1 and G2 in cereals, shell-fruits and derived products - High performance liquid chromatographic method with post column derivatization and immunoaffinity column clean up

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Lebensmittel - Bestimmung von Aflatoxin B1 und der Summe von Aflatoxin B1, B2, G1 und G2 in Getreiden, Schalenfrüchten

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Produits alimentaires - Dosage de l'aflatoxine B1 et de la somme des aflatoxines B1, B2, G1 et G2 dans les céréales, les fruits a coque et les produits dérivés - Méthode de chromatographie en phase liquide haute performance avec dérivation post-colonne et purification en colonne d'immuno-affinité

Ta slovenski standard je istoveten z: **EN 12955:1999**

ICS:

67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products
67.060	Žita, stročnice in proizvodi iz njih	Cereals, pulses and derived products
67.080.10	Sadje in sadni proizvodi	Fruits and derived products

SIST EN 12955:2000 **en**

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 12955

July 1999

ICS 67.060; 67.080.10; 67.200.10

English version

Foodstuffs - Determination of aflatoxin B_1, and the sum of aflatoxins B_1, B_2, G_1 and G_2 in cereals, shell-fruits and derived products - High performance liquid chromatographic method with post column derivatization and immunoaffinity column clean up

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Lebensmittel - Bestimmung von Aflatoxin B_1 und der Summe von Aflatoxin B_1, B_2, G_1 und G_2 in Getreiden, Schalenfrüchten und verwandten Produkten - Hochleistungs-flüssigchromatographisches Verfahren mit Nachsäulenderivatisierung und Immunoaffinitätssäulen-Reinigung

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This European Standard was approved by CEN on 7 June 1999.

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This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Central Secretariat: rue de Stassart, 36 B-1050 Brussels

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Foreword

This European Standard has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

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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 January 2000, and conflicting national standards shall be withdrawn at the latest by January 2000.

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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.

NOTE Existing and developing legislation (national or EU) in this area will require a method with lower levels of detection. Such a method is currently being developed as an EU SMT project.

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1 Scope

This European Standard specifies a method for the determination of aflatoxin contents of greater than 8 µg/kg.

The method has been successfully validated in an interlaboratory study according to ISO 5725:1986 on maize containing 24,5 µg/kg, peanut butter containing 8,4 µg/kg and raw peanuts containing 16 µg/kg of total aflatoxins.

Some laboratory experiences have shown that this method can be used to several types of cereals, oilseed products, shell-fruits, dried fruits and derived products, after in-house validation.

2 Normative reference(s)

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 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

The test sample is extracted with a mixture of methanol and water. The sample extract is filtered, diluted with water, and applied to an affinity column containing antibodies specific for aflatoxins B_1, B_2, G_1 and G_2. The aflatoxins are isolated, purified and concentrated on the column then removed from the antibodies with methanol. The aflatoxins are quantified by reverse-phase high performance liquid chromatography (HPLC) with fluorescence detection and postcolumn iodine derivatization.

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WARNING - The method described requires the use of solutions of aflatoxins. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO) [1], [2].

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4 Reagents

4.1 General

During the analysis, unless other wise stated, us only reagents of recognized analytical grade and only water according to grade 1 of EN ISO 3696.

4.2 Sodium chloride

4.3 Iodine, crystalline

4.4 Aflatoxin, in crystal form or as a film in ampoules.

WARNING: Protect the laboratory, where the analyses are done, adequately from daylight. This can be achieved effectively by using Ultraviolet (UV) absorbing foil on the windows in combination with subdued light (no direct sunlight) or curtains or blinds in combination with artificial light (fluorescent tubes are acceptable).

Protect Aflatoxin containing solutions from light as much as possible (keep in the dark, use aluminium foil or amber-coloured glassware).

4.5 Acetonitrile, for HPLC

4.6 Methanol, for analysis

4.7 Methanol, for HPLC

4.8 Toluene

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4.9 Extraction solvent

Mix 7 parts per volume of methanol (4.6) with 3 parts per volume of water.

4.10 Immunoaffinity column

The immuno affinity (IA) column contains antibodies raised against aflatoxin B_1, B_2, G_1 and G_2. The column shall have a minimim binding capacity of not less than 100 ng of aflatoxin B_1 and shall give a recovery of not less than 80 % for aflatoxin B_1, B_2, G_1 and not less than 60 % for aflatoxin G_2 when a standard solution in 15 ml of a methanol/water-mixture (1 part per volume of methanol and 3,4 parts per volume of water) containing 5 ng of each toxin is applied on the IA column. The IA column should provide an appropriate solvent reservoir, e.g. a syringe with adapter.

4.11 Mobile phase

Mix 3 parts per volume of water with 1 part per volume of acetonitrile (4.5) and 1 part per volume of methanol (4.7). Degas the solution before use.

4.12 Postcolumn derivatization reagent

Dissolve 100 mg of iodine (4.3) in 2 ml of methanol (4.6). Add 200 ml of water, stir for 1 h, and filter through a 0,45 µm filter (5.8). Prepare the solution on the week of use and store the solution in the dark or in a bottle of brown glass. Before use stir the solution for 10 min.

4.13 Toluene/acetonitrile mixture

Mix 98 parts per volume of toluene (4.8) with 2 parts per volume of acetonitrile (4.5).

4.14 Aflatoxin B_1, B_2, G_1 and G_2 stock solutions

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Dissolve aflatoxin B_1, B_2, G_1 and G_2 seperately in the toluene/acetonitrile mixture (4.13) to give seperate solutions containing 10 µg/ml.

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To determine the exact concentration of aflatoxin in each stock solution, record the absorption curve between a wavelength of 330 nm and 370 nm in 1 cm quartz glass cells (5.7) in a spectrometer with toluene/acetonitrile mixture (4.13) in the reference path. Calculate the aflatoxin mass concentration of each aflatoxin, ρ_i, in micrograms per millilitre, using equation (1):

$$\rho_i = \frac{A_{max} \ x \ M_i \ x \ 100}{\epsilon_i \ x \ d} \quad (1)$$

where:

A_{max} is the absorbance determined at the maximum of the absorption curve;
M_i is the relative molecular mass of each aflatoxin, in grams per mol;
ε_i is the molar absorptivity of each aflatoxin in toluene/acetonitrile (4.13), in metres squared per mol;
d is the optical path length of the cell, in centimetres.

M_i and ε_i are given in table 1.

Table 1 — Relative molecular mass and molar absorptivity of aflatoxins B_1, B_2, G_1 and G_2

(Mixture of toluene and acetonitrile 98 + 2)

Aflatoxin	M_i g/mol	ε_i m^2/mol
B_1	312	1930
B_2	314	2040
G_1	328	1660
G_2	330	1790

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4.15 Mixed aflatoxins stock solution

Prepare a stock solution containing 500 ng/ml of aflatoxin B_1, 125 ng/ml of aflatoxin B_2, 250 ng/ml of aflatoxin G_1, and 125 ng/l aflatoxin G_2 in toluene/acetonitrile (4.13). When the solution has to be stored, weigh the flask and record any change in weight when the solution is to be used. Wrap the flask tightly in aluminium foil and store it at approximately 4 ⁻C.

NOTE Normal exposure to UV light during absorbance measurement results in no observable conversion to photoproducts.

4.16 Mixed aflatoxins standard solutions

Transfer each quantity as specified in table 2, of mixed aflatoxins stock solution (4.15) into a series of four 2-ml volumetric flasks (5.5). Evaporate solutions just to dryness under a stream of nitrogen at room temperature. To each flask, add 1 ml of methanol, mix, dilute to the mark with water, and mix. Prepare the solution fresh on the day of use.

Table 2 — Preparation of standard solutions

Standard solution	Taken from stock solution (µl)	Mass concentration, in ng/ml			
		B_1	B_2	G_1	G_2
1	60	15,0	3,75	7,50	3,75
2	40	10,0	2,50	5,00	2,50
3	20	5,00	1,25	2,50	1,25
4	10	2,50	0,625	1,25	0,625
NOTE The values given are for guidance purpose. The standard range includes the concentrations of the samples.					

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4.17 Sulfuric acid, $c(H_2SO_4) = 2$ mol/l

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5 Apparatus

5.1 Usual laboratory apparatus

Soak laboratory glassware coming into contact with aqueous solutions of aflatoxins in sulfuric acid (4.17) for several hours, then rinse well (e.g. three times) with water to remove all traces of acid. Check the absence of acid with pH paper.

NOTE This treatment is necessary, because the use of non-acid washed glassware may cause losses of aflatoxins. In practice, the treatment is necessary for round bottomed flasks, volumetric flasks, measuring cylinders, vials or tubes used for calibration solutions and final extracts (particularly autosampler vials), and Pasteur pipettes, if these are used to transfer calibration solutions or extracts.

5.2 Blender, with 500 ml blender jar and cover.

5.3 Fluted filter paper, e.g. 24 cm diameter.

5.4 Glass microfibre filter paper, e.g. 11 cm diameter.

5.5 Volumetric flasks, e.g. 2 ml.

5.6 Spectrometer, capable of measurements of wavelengths from 200 nm up to 400 nm.

5.7 Quartz cells, having an optical path length of 1 cm and no significant absorption between wavelengths of 300 nm and 370 nm.

5.8 Membrane filter for aqueous solutions, made of polytetrafluoroethylene (PTFE), with a diameter of 4 mm and a pore size of 0,45 µm

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5.9 HPLC apparatus, comprising the following.

5.9.1 **HPLC pump,** suitable for flow rate at 1 ml/min

5.9.2 **Injection system,** a syringe-loading injection valve with 50 µl loop or equivalent

5.9.3 **Analytical reversed phase seperating column,** e.g. C_{18} which ensures a baseline resolved resolution of the aflatoxin B1, B2, G1 and G2 peaks from all other peaks.

- a length of 250 mm;
- an internal diameter of 4,6 mm;
- spherical particles of size 5 µm

Shorter columns can also be used.

5.9.4 **Postcolumn derivatization system,** consisting of a second pulse free pump and zero-dead volume T piece, with polytetrafluoroethylene (PTFE) or stainless steel tubing with dimensions of length 3000 mm to 5000 mm and internal diameter of 0,5 mm, and heating bath or postcolumn reactor for the iodine reaction.

5.10 **Fluorescence detector** with excitation at a wavelength of 365 nm and emission at wavelength of 435 nm (for filter instruments: emission wavelength > 400 nm). Detection of at least 0,05 ng of aflatoxin B_1 per injection volume (here: 50 µl) shall be possible..

6 Procedure

6.1 Extraction

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Weigh 25 g to the nearest 10 mg of the homogenized test sample into the blender jar, add 5 g of sodium chloride (4.2) and 125 ml of extraction solvent (4.9) and homogenize with a mixer for 2 min at high speed.

NOTE It should be checked that the blending time and speed do not have a negative influence on extraction efficiency.

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Filter through a fluted filter paper (5.3). Pipette 15 ml (V_2) of the filtrate (V_1) into a conical flask of appropriate size with glass stopper. Add 30 ml of water, stopper the flask and mix. Before starting affinity column chromatography, filter the diluted extract through a glass microfibre paper (5.4). The filtrate (V_3) should be clear. If not, refilter. Proceed immediately in accordance with 6.2.

6.2 Clean up

Prepare the IA column and proceed with the clean up procedure in accordance with the manufacturer's instructions. Pipette 15 ml (V_4) of the second filtrate (V_3) into the solvent reservoir of the IA column. Collect the methanol or acetonitrile eluate (depending on the product or manufacturer's instructions) in a 2 ml volumetric flask (5.5) (or another volume as specified by the manufacturer). Dilute to the mark with water (V_5). Mix, and proceed in accordance with 6.3.

The sample solutions and standard solutions for the HPLC determination shall contain the same solvent or solvent mixture.

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, procedures involve sample extraction with a mixture of methanol and water, filtration or centrifugation, possible sample dilution with phosphate buffered solution (PBS) or water, loading under pressure onto a possibly pre-washed column, washing of the column with distilled water and elution of aflatoxins with methanol or acetonitrile (depending on the product and manufacturer's instructions).

Take care of not going beyond the maximum capacity of the column.

6.3 HPLC operating conditions

Connect the separation column outlet to one arm of a T piece (5.9.4), using a short piece of tubing with an internal diameter of e.g. 0,25 mm. Connect the outlet of the second pulse free pump which delivers the postcolumn derivation reagent to the second arm of the T piece. Connect one end of a coil of PTFE or stainless steel (5.9.4) to the third arm of the T piece and connect the other end to the detector. Using an oven or water bath, maintain the reaction coil temperature at 70 ⁻C.

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When the column specified in 5.9.3 was used, the following settings were found to be appropriate:

- Flowrate mobile phase (column): 1,0 ml/min;
- Flowrate postcolumn reagent: 0,3 ml/min;
- Volume injected: 50 µl.

Let the entire system run for 10 min to 20 min to stabilize. If an integrator is used, adjust the sensitivity controls of the fluorescence detector or integrator to give a response of signal : noise = 5 : 1 for 0,125 ng of aflatoxin G_2 in 50 µl. If a strip chart recorder is used,adjust the fluorescence detector control to give 30 % to 40 % scale deflection with 0,125 ng of aflatoxin G_2 in 50 µl. Pass the second filtrate (V_3) through the separation column, wash the column as described by the manufacturer's instructions and discard the eluates.

6.4 Identification

Identify each aflatoxin peak in the sample chromatogram by comparing the retention times with those of corresponding reference standards.
Alternatively, the aflatoxins can be identified by simultaneous injection of the sample test solution and standard solutions. Also the disappearance of the aflatoxin B_1 and G_1 peaks if no derivatization reagent is added is helpful for identification.

6.5 Calibration graph

Prepare the calibration graph for each aflatoxin by injecting 50 µl of standard solutions 1, 2, 3 and 4 (see table 2). Check the linearity of the curve [4].

6.6 Determination

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Quantitative determination is performed by the external standard method with integration of the peak area or measurement of the peak height, which is then related to the corresponding value for the standard substance.

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Inject volumes of 50 µl standard mixture into the injection loop following the instructions of the injector manufacturer. Aflatoxins elute in order G_2, G_1, B_2, B_1 with retention times of approximately 6 min, 8 min, 9 min and 11 min, respectively, and should be baseline-resolved. If necessary, adjust the retention times by changing the methanol concentration of the mobile phase solvent (4.11).

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Inject 50 µl (V_6) of purified sample extract (6.2) into the injection loop.

7 Calculation of results

Calculate the mass of the test sample m_t in grams, present in the fraction of the second filtrate taken for the IA column (V_4) using equation (2):

$$m_t = m_o \times \frac{V_2 \times V_4}{V_1 \times V_3} \quad (2)$$

where:

m_0 is the mass of the test portion (6.1), in grams ($m_0 = 25$ g);
V_1 is the total volume of the first filtrate (6.1), in millilitres ($V_1 = 125$ ml);
V_2 is the fraction volume of the first filtrate (6.1), in millilitres ($V_2 = 15$ ml);
V_3 is the total volume of the second filtrate (6.1), in millilitres ($V_3 = 45$ ml);
V_4 is the fraction volume of the second filtrate (6.2), in millitres ($V_4 = 15$ ml).

Calculate the mass fraction of each aflatoxin, w_i, in micrograms per kilogram of the sample using equation (3) (external standard method):

$$w_i = \frac{V_5 \times m_i}{V_6 \times m_t} \quad (3)$$

where:

V_5 is the volume of the eluate (6.2), in microlitres ($V_5 = 2000$ µl);
V_6 is the volume of the eluate injected (6.6), in microlitres ($V_6 = 50$ µl);
m_i is the mass of each aflatoxin i corresponding to the measured peak area or peak height read off the calibration graph, present in the injection volume, in nanogram;