SLOVENSKI STANDARD
SIST EN 14123:2008

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Nadomešča:
SIST EN 14123:2003

Živila - Določevanje aflatoksina B1 in vsote aflatoksinov B1, B2, G1 in G2 v arašidih, pistacijah, figah in papriki v prahu - Tekočinska kromatografija visoke ločljivosti s postkolonsko derivatizacijo in imunoafinitetnim kolonskim čiščenjem

Foodstuffs - Determination of aflatoxin B1 and the sum of aflatoxin B1, B2, G1 and G2 in hazelnuts, peanuts, pistachios, figs, and paprika powder - High performance liquid chromatographic method with post-column derivatisation and immunoaffinity column cleanup

Lebensmittel - Bestimmung von Aflatoxin B1 und der Summe von Aflatoxin B1, B2, G1 und G2 in Haselnüssen, Erdnüssen, Pistazien, Feigen und Paprikapulver - Hochleistungsflüssigchromatographisches Verfahren mit Immunaffinitätssäulen-Reinigung und Nachsäulenderivatisierung

Produits alimentaires - Dosage de l'aflatoxine B1 et de la somme des aflatoxines B1, B2, G1 et G2 dans les noisettes, les cacahuètes, les pistaches, les figues et le paprika en poudre - Méthode par purification sur colonne d'immuno-affinité suivie d'une chromatographie liquide à haute performance avec dérivation post-colonne

Ta slovenski standard je istoveten z: **EN 14123:2007**

ICS:

67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products
67.080.01	Sadje, zelenjava in njuni proizvodi na splošno	Fruits, vegetables and derived products in general

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EUROPEAN STANDARD

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English Version

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: rue de Stassart, 36 B-1050 Brussels

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Foreword

This document (EN 14123:2007) 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of an identical text or by endorsement, at the latest by June 2008, and conflicting national standards shall be withdrawn at the latest by June 2008.

This document supersedes EN 14123:2003 with the following changes:

a) Validation data on hazelnut are included.

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.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Standard is applicable to the determination of aflatoxins B_1, B_2, G_1 and G_2 in hazelnuts, figs, pistachios, peanuts and paprika powder. The limit of quantification of the method is 0,8 ng/g for each aflatoxin or better (value derived from in-house and collaborative study), depending on the equipment used.

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

3 Principle

A test portion is either extracted with a solvent solution (methanol/water) or the solvent solution plus hexane (or cyclohexane). The sample extract is filtered, diluted with phosphate buffered saline (PBS) and applied to an immunoaffinity column (IAC) containing antibodies specific to aflatoxins B_1, B_2, G_1 and G_2. The aflatoxins are eluted from the immunoaffinity column with methanol. Aflatoxins are quantified by reverse-phase high performance liquid chromatography (RP-HPLC) with post-column derivatization (PCD) involving bromination followed by fluorescence detection. The PCD is achieved with either electrochemically generated bromine or with pyridinium hydrobromide perbromide (PBPB).

4 Reagents

4.1 General

Use only reagents of recognized analytical grade and water complying with grade 3 of EN ISO 3696, unless otherwise specified.

4.2 Water, complying with grade 1 of EN ISO 3696

4.3 Phosphate buffered saline (PBS), pH = 7,4

Dissolve 0,20 g of potassium chloride, 0,20 g of potassium dihydrogen phosphate, 1,16 g of disodium hydrogen orthophosphate (or 2,92 g of hydrogenphosphate·12 H_20) and 8,00 g of sodium chloride in 0,9 l of water. After dissolution, adjust the pH to 7,4 with HCl (0,1 mol/l) or NaOH (0,1 mol/l) as appropriate. Dilute to 1 l with water.

Commercially available phosphate buffered saline tablets with equivalent properties may be used.

4.4 Sodium chloride (NaCl)

4.5 Pyridinium hydrobromide perbromide (PBPB), [CAS: 39416-48-3]

4.6 Potassium bromide (KBr)

4.7 Acetonitrile, HPLC grade

4.8 Methanol, HPLC grade

4.9 Methanol, p.a. grade

4.10 Toluene

4.11 Extraction solvent mixture of methanol and water

Mix 8 parts per volume of methanol (4.9) with 2 parts per volume of water.

4.12 n-Hexane, cyclohexane, p.a. grade

4.13 Nitric acid, $c(HNO_3)$ = 4 mol/l

Dilute 28 ml of nitric acid (volume fraction is 65 %), or 26 ml of nitric acid (volume fraction is 70 %) with water to a final volume of 100 ml.

4.14 Immunoaffinity column

The affinity column contains antibodies raised against aflatoxins B_1, B_2, G_1 and G_2. The column shall have a maximum capacity of not less than 100 ng of aflatoxin B_1 and shall give a recovery of not less than 80 % for aflatoxins B_1, B_2, G_1 and not less than 60 % for aflatoxin G_2 when applied as an aqueous standard solution (10 % of methanol) containing 5 ng of each toxin. The maximum solvent concentration of solutions that can be applied on the column shall not exceed 12 % of methanol.

4.15 HPLC mobile phase solvent (A), for use with PBPB

Mix 6 parts per volume of water (4.2) with 2 parts per volume of acetonitrile (4.7) and 3 parts per volume of methanol (4.8). Degas the solution before use. The mobile phase shall be free of particles and should be filtered prior use.

4.16 HPLC mobile phase solvent (B), for use with electrochemically generated bromine

Mix 6 parts per volume of water (4.2) with 2 parts per volume of acetonitrile (4.7) and 3 parts per volume of methanol (4.8). Add 120 mg of potassium bromide (4.6) and 350 µl of nitric acid (4.13) per litre of mobile phase. Degas the solution before use.

4.17 Post-column reagent

Dissolve 50 mg of PBPB (4.5) in 1 l of water. The solution may be used up to four days if stored in a dark place at room temperature.

4.18 Mixture of toluene and acetonitrile

Mix 98 parts per volume of toluene (4.10) with 2 parts per volume of acetonitrile (4.7).

4.19 Aflatoxins, either in form of crystals or film in ampoules or in form of commercially available aflatoxin solutions

WARNING 1 — Decontamination procedures for laboratory wastes of aflatoxins were developed by the International Agency for Research on Cancer (IARC) [1], [2].

WARNING 2 — Aflatoxins are subject to light degradation. 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) and store at the temperature recommended by the manufacturer (e.g. -18 °C).

4.20 Aflatoxins stock solution

Dissolve aflatoxin B_1, B_2, G_1 and G_2 separately in the mixture of toluene and acetonitrile (4.18) to give separate solutions with a concentration of 10 µg/ml for each aflatoxin. Wrap the flasks tightly in aluminium foil and store them at less than 4 °C.

To determine the exact concentration of aflatoxins in each stock solution, record the absorption curve between a wavelength of 330 nm and 370 nm in 1 cm quartz glass cells in a spectrometer with the mixture of toluene and acetonitrile (4.18) in the reference cell. Calculate the mass concentration of each aflatoxin, ρ_i, in micrograms per millilitre, using Equation (1):

$$\rho_i = \frac{A_{\max} \times M_i \times 100}{\varepsilon_i \times b} \qquad (1)$$

where:

$A_{\max}$ is the absorbance determined at the maximum of the absorption curve;

M_i is the molar mass of each aflatoxin, in grams per mol;

ε_i is the molar absorption coefficient of each aflatoxin in toluene and acetonitrile (4.18), in square metres per mol;

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

M_i and ε_i of aflatoxins B_1, B_2, G_1 and G_2 are given in Table 1.

Table 1 — Molar mass and molar absorption coefficient of aflatoxins B_1, B_2, G_1 and G_2

(In mixture of toluene and acetonitrile (4.18))

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

4.21 Mixed aflatoxins stock solution

Prepare a mixed aflatoxins stock solution containing 1000 ng/ml of aflatoxin B_1 and G_1, 200 ng/ml of aflatoxin B_2 and G_2 in the toluene and acetonitrile mixture (4.18) by appropriate dilution of aflatoxins (B_1, B_2, G_1 and G_2) stock solutions (4.20).

NOTE A commercial total aflatoxins standard solution which is ready to use in a vial containing 1000 ng/ml of total aflatoxin may be used as an alternative.

4.22 Diluted mixed aflatoxins stock solution

Prepare a diluted mixed aflatoxins stock solution containing 100 ng/ml of aflatoxin B_1 and G_1, 20 ng/ml of aflatoxin B_2 and G_2 in the toluene and acetonitrile mixture (4.18) by pipetting exactly 1,0 ml of the mixed aflatoxins stock solution (4.21) into a 10 ml calibrated volumetric flask (5.10), fill to the mark with the toluene and acetonitrile mixture (4.18) and mix well.

Wrap the flask tightly in aluminium foil and store it at less than 4 °C or in a freezer. Before use, do not open the flask until the contents have reached room temperature to avoid incorporation of water by condensation.

4.23 Mixed aflatoxins calibration solutions

Use the diluted mixed aflatoxins stock solution containing 100 ng/ml of aflatoxin B_1 and G_1, 20 ng/ml of aflatoxin B_2 and G_2 (see 4.22) for pipetting the volumes as given in Table 2 into a set of 10 ml volumetric flasks (5.10). Evaporate the toluene/acetonitrile solution just to dryness under a stream of nitrogen at room temperature. To each flask, add 4 ml of methanol, let aflatoxins dissolve, dilute to 10 ml with water, and shake well. Methanol and water are subject to volume contraction when mixed, so adjust the volume again to the given volume.

Table 2 — Preparation of mixed aflatoxins calibration solutions

Calibration solution	Taken from diluted stock solution (4.22) µl	Mass concentration of calibration solution ng/ml			
		B_1	B_2	G_1	G_2
1	40	0,400	0,080	0,400	0,080
2	120	1,200	0,240	1,200	0,240
3	200	2,000	0,400	2,000	0,400
4	280	2,800	0,560	2,800	0,560
5	360	3,600	0,720	3,600	0,720

4.24 Spiking solution

Prepare a spiking solution by pipetting 2 ml of the mixed aflatoxins stock solution (containing 1000 ng/ml of aflatoxin B_1 and G_1, 200 ng/ml of aflatoxin B_2 and G_2, see 4.21) into a 10 ml calibrated volumetric flask. Evaporate the toluene/acetonitrile solution just to dryness under a stream of nitrogen at room temperature. Dilute to the mark with methanol and shake well. The concentration of this spiking solution is 200 ng/ml of aflatoxin B_1 and G_1, and 40 ng/ml of aflatoxin B_2 and G_2.

Wrap the flask tightly in aluminium foil and store it at less than 4 °C. Before use, do not open the flask until the contents have reached room temperature to avoid incorporation of water by condensation.

5 Apparatus

5.1 General

All glassware coming into contact with aqueous solutions of aflatoxins shall be washed with acid solution before use. Many laboratory washing machines do this as part of the washing program. Otherwise soak such laboratory glassware in sulfuric acid (2 mol/l) for several hours (e.g. 15 h overnight), then rinse well (e.g. three times) with water to remove all traces of acid. Check the absence of acid with pH paper.

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 Usual laboratory apparatus and, in particular, the following

5.3 Laboratory mill, or explosion proof high speed blender[1)] , necessary for the production and extraction of pastes from hazelnuts, peanuts, pistachios and figs, with suitable blender jar

5.4 Adjustable vertical or horizontal shaker, needed for the analysis of paprika powder

5.5 Paper filter, e.g. 24 cm diameter, prefolded

5.6 Conical flask, with screw top or glass stopper

1) Contact your National Standardization Institute for appropriate high speed blenders.

5.7 Glass microfiber filter paper, retention size 1,6 µm or smaller

5.8 Reservoir, 75 ml with luer tip connector for immunoaffinity column (IAC)

5.9 Hand pump, 20 ml syringe with luer lock or rubber stopper for IAC

5.10 Volumetric glassware, flasks of e.g. 3 ml, 5 ml, 10 ml and 20 ml, with an accuracy of at least 0,5 %

5.11 HPLC system, consisting of

5.11.1 HPLC pump, suitable for flow rate at 1,0 ml/min

5.11.2 Injection system, capable for total loop injection. A 100 µl loop is recommended.

In the case that a different loop size than recommended is used it shall be guaranteed that the limit of detection (LOD) for the system is ≤ 0,2 ng/g (signal-to-noise-ratio = 3) and the limit of quantification (LOQ) is ≤ 0,5 ng/g (signal-to-noise-ratio = 6) for each aflatoxin (using the standard solutions).

5.11.3 RP-HPLC column, e.g. C_{18} or ODS-2 (length of 25 cm, inner diameter of 4,6 mm and particle size of 5 µm), which ensures a baseline resolution of the aflatoxin B_1, B_2, G_1 and G_2 peaks from all other peaks. The maximum overlapping of peaks shall be less than 10 %. It could be necessary to adjust the mobile phase for a sufficient baseline resolution. A suitable pre-column should be used.

5.11.4 Post-column derivatisation system, with PBPB (only to be used with mobile phase A (4.15))

Consisting of an HPLC pulseless pump, zero-dead volume T-piece, reaction tubing min. 45 cm x 0,5 mm internal diameter PTFE.

5.11.5 System for derivatisation with electrochemically generated bromine, e.g. KOBRA cell® [2] (only to be used with mobile phase B (4.16).

5.11.6 Fluorescence detector, with a wavelength of λ = 360 nm excitation filter and a wavelength of λ = 420 nm cut-off emission filter, or equivalent (e.g. a detector with an adjustable monochromator).

Recommended settings for adjustable detectors are 365 nm (excitation wavelength), 435 nm (emission wavelength) and a bandwidth of 18 nm.

5.12 Disposable filter unit, of pore size 0,45 µm

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

NOTE There is a possibility that various filter materials can retain aflatoxins.

5.13 Pipettes, 2 ml, 5 ml and 10 ml capacity, with an accuracy of at least 0,5 %

5.14 Analytical balance, capable of weighing to 0,1 mg

5.15 Laboratory balance, capable of weighing to 0,01 g

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2) KOBRA cell® is the trade name of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.16 Calibrated microliter syringe(s) or microliter pipette(s), 10 µl to 1000 µl

5.17 Vacuum manifold, optional

6 Procedures

6.1 Sample preparation

Homogenize a suitable amount (e.g. 10 kg, see European legislation [3]) of pistachios, peanuts, hazelnuts and figs appropriately to give a paste, e.g. using a high speed blender (5.3). Information on sample sizes and sampling is given in [3].

6.2 Conditioning of immunoaffinity columns

Allow the immunoaffinity columns (4.14) to reach room temperature prior to conditioning. Connect the immunoaffinity column to the vacuum manifold (5.17) and attach the reservoir (5.8) to the immunoaffinity column.

For conditioning transfer 10 ml of PBS (4.3) on the top of the column and let it pass at a speed of 2 ml/min to 3 ml/min through the column (e.g. by gravity). Make sure that a small portion (0,5 ml) of the PBS remains on the column until the sample solution is applied.

Different conditioning procedures shall be considered in accordance with the manufacturer's instructions.

6.3 Extraction

6.3.1 General

For the extraction of hazelnut paste, fig paste, peanut butter and pistachio paste a high speed blender shall be used, since the fatty commodities (hazelnut paste, peanut butter and pistachio paste) need to form an emulsion to break the fatty layers and allow a sufficient extraction. In addition, fig paste needs to break down in the solvent, which cannot be guaranteed if a shaker is used, due to its consistency. Paprika powder can be extracted by shaking (provided that the powder is ground sufficiently to a particle size up to 500 µm) to process several samples simultaneously and reduces the risk of cross contamination.

Before weighing the homogenized test portion for extraction, stir the sample container well if it is a paste to overcome segregation of the matrix particles in the container.

6.3.2 Hazelnuts

Weigh, to the nearest 0,1 g, approximately 50 g of the homogenized test portion (6.1) into a blender jar. Add 4 g of sodium chloride (4.4) and 100 ml of water (4.2). Blend for 1 min with a high speed blender (5.3) to produce a slurry. Add 150 ml of methanol (4.8) and blend again for 2 min with the high speed blender.

Filter the extract using a paper filter (5.5) and collect the filtrate in a 100 ml conical flask. Transfer 5 ml of the clear filtrate (equivalent to 1 g of sample) into a glass beaker and add 15 ml of PBS (4.3) solution. Add the diluted sample extract to the reservoir connected to the conditioned immunoaffinity column (4.14) and proceed as described in 6.4.

6.3.3 Figs

Weigh, to the nearest 0,1 g, approximately 50 g of the homogenized test portion (6.1) into a 500 ml conical flask (5.6) or blender jar. Add 5 g of sodium chloride (4.4) and 300 ml of extraction solvent mixture (4.11). Blend for 3 min with a high speed blender (5.3).

Filter the extract using a paper filter (5.5). Pipette 10,0 ml of the clear filtrate into a 100 ml glass beaker (or similar) and dilute with 60 ml of PBS (4.3). Add the diluted sample extract to the reservoir connected to the conditioned immunoaffinity column (4.14) and proceed as described in 6.4.

Slurries or larger test portions may be used, provided that ratios (sample-to-extraction solvent as well as the extraction solvent composition for slurries) are maintained.

6.3.4 Peanuts

Weigh, to the nearest 0,1 g, approximately 50 g of the homogenized test portion (6.1) into a 500 ml conical flask (5.6) or blender jar. Add 5 g of sodium chloride (4.4), 200 ml of extraction solvent mixture (4.11) and 100 ml of n-hexane or cyclohexane (4.12). Blend for 3 min with a high speed blender (5.3).

Filter the extract using a paper filter (5.5). In case of a solvent layer separation carry on with the lower phase. Pipette 10,0 ml of the clear filtrate into a 100 ml glass beaker (or similar) and dilute with 60 ml of PBS (4.3). Add the diluted sample extract to the reservoir connected to the conditioned immunoaffinity column (4.14) and proceed as described in 6.4.

Solvent layer separation should not occur if filtration takes place immediately after blending since n-hexane/cyclohexane will be retained in the filter. A filter phase separator may be used if needed.

Larger test portions may be used, provided that the sample-to-extraction solvent ratio is maintained.

6.3.5 Pistachios

Weigh, to the nearest 0,1 g, approximately 50 g of the homogenized test portion (6.1) into a 500 ml conical flask (5.6) or blender jar. Add 5 g of sodium chloride (4.4), 200 ml of extraction solvent mixture (4.11) and 100 ml of n-hexane or cyclohexane (4.12). Blend for 3 min with a high speed blender (5.3).

Filter the extract using a paper filter (5.5). In case of a solvent layer separation carry on with the lower phase. Pipette 10,0 ml of the clear filtrate into a 100 ml glass beaker (or similar) and dilute with 60 ml of PBS (4.3). Add the diluted sample extract to the reservoir connected to the conditioned immunoaffinity column (4.14) and proceed as described in 6.4.

If significant precipitation occurs diluting with PBS, alternatively pipette 20 ml of the sample filtrate into a 250 ml glass beaker (or similar) and dilute with 140 ml of PBS (4.3) and then filter to a filter paper (5.7). In this case add 70 ml of this filtered sample extract to the reservoir connected to the conditioned immunoaffinity column (4.14) and proceed as described in 6.4.

Solvent layer separation should not occur if filtration takes place immediately after blending since n-hexane/cyclohexane will be retained in the filter. A filter phase separator may be used if needed.

Larger test portions may be used, provided that the sample-to-extraction solvent ratio is maintained.

6.3.6 Paprika powder

Weigh, to the nearest 0,1 g, approximately 50 g of the homogenized test portion (6.1) into a 500 ml conical flask (5.6). Add 5 g of sodium chloride (4.4) and 300 ml of extraction solvent mixture (4.11). Shake vigorously by hand for the first 15 s to 30 s and then for 30 min with a shaker (5.4). For various types of shakers (e.g. horizontal platform shaker or vertical wrist shaker) the motion speed shall be adjusted to obtain maximum agitation of the extraction mixture.

Filter the extract using a paper filter (5.5). Pipette 10,0 ml of the clear filtrate into a 100 ml glass beaker (or similar) and dilute with 60 ml of PBS (4.3). Add the diluted sample extract to the reservoir connected to the conditioned immunoaffinity column (4.14) and proceed as described in 6.4.

Larger test portions may be used, provided that the sample-to-extraction solvent ratio is maintained.