SLOVENSKI STANDARD
oSIST prEN 17424:2019

01-oktober-2019

Živila - Določevanje aflatoksina v začimbah, razen v papriki, z IAC-čiščenjem in HPLC-FLD s postkolonsko derivatizacijo

Foodstuffs - Determination of aflatoxins in spices other than paprika by IAC clean-up and HPLC-FLD with post-column derivatization

Lebensmittel - Bestimmung von Aflatoxinen in Gewürzen außer Paprika mit IAC Reinigung und HPLC-FLD mit Nachsäulenderivatisierung

Produits alimentaires - Détermination des aflatoxines dans les épices (pour lesquelles un niveau maximal a été établi par l'UE) autres que le paprika

Ta slovenski standard je istoveten z: **prEN 17424**

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products
67.220.10	Začimbe	Spices and condiments

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (prEN 17424:2019) has been prepared by Technical Committee CEN/TC 275 “Food analysis — Horizontal methods”, the secretariat of which is held by DIN.

The document is currently submitted to the CEN Enquiry.

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Introduction

Aflatoxins consist of a group of approximately twenty related fungal metabolites, although only aflatoxins B_1, B_2, G_1 and G_2 are normally found in foods. Aflatoxins B_2 and G_2 are the dihydro derivatives of the parent compounds. Aflatoxins are produced by at least three species of *Aspergillus*, *A. flavus*, *A. parasiticus* and *A. nominus,* and can occur in a wide range of important raw food commodities, including cereals, nuts, spices, figs and dried fruit.

WARNING 1 — Suitable precaution and protection measures need to be taken when carrying out working steps with harmful chemicals. The latest version of the hazardous substances ordinance, Regulation (EC) No 1907/2006 [3], should be taken into account as well as appropriate national statements e.g. such as in [4].

WARNING 2 — The use of this document can involve hazardous materials, operations and equipment. This document does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this document to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

WARNING 3 — Aflatoxins are known to have carcinogenic effects and to be both acutely and chronically toxic.

1 Scope

This document describes a procedure for the determination of aflatoxins B_1, B_2, G_1 and G_2 and total aflatoxins (sum of B_1, B_2, G_1 and G_2) in spices for which EU maximum levels are established, other than paprika, by high performance liquid chromatography (HPLC) with post-column derivatization (PCD) and fluorescence detection (FLD) after immunoaffinity column clean-up.

The method is applicable to the spices capsicum, pepper, nutmeg, ginger, turmeric and mixtures thereof.

The method has been validated for aflatoxins B_1, B_2, G_1 and G_2 and total aflatoxins in a range of test samples that comprised: ginger, pepper, nutmeg, chilli, turmeric as individual spices and mixed pepper+chilli+nutmeg (90+5+5, m+m+m), mixed spice+ginger (6+4, m+m) mixed spice, mixed turmeric+ginger (2+8, m+m).

The validation was carried out over the following concentration ranges: aflatoxin B_1 = 1 µg/kg to 16 µg/kg and total aflatoxins = 2,46 µg/kg to 36,1 µg/kg.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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)*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

4 Principle

Aflatoxins are extracted from the spices with a mixture of methanol, acetonitrile and water. 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. Aflatoxins are quantified by reversed-phase high performance liquid chromatography (HPLC) with PCD followed by FLD.

5 Reagents

Use only reagents of recognized analytical grade, p.a. (pro analysi) and water complying with grade 1 of EN ISO 3696, unless otherwise specified. Solvents shall be of quality for HPLC analysis, unless otherwise specified.

WARNING 1 — Decontamination procedures for laboratory wastes of aflatoxins were developed by the International Agency for Research on Cancer (IARC) [5], [6].

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).

5.1 Methanol, (CH_3OH).

5.2 Acetonitrile, (CH_3CN).

5.3 Potassium bromide (KBr).

5.4 Sodium hydroxide (NaOH).

5.5 Sodium hydroxide solution, substance concentration c(NaOH) = 2 mol/l.

Dissolve 8,0 g of sodium hydroxide (5.4) in 100 ml of water.

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

Dissolve 0,4 g of sodium hydroxide (5.4) in a 100 ml of water.

5.7 Hydrochloric acid solution (HCl), volume fraction φ(HCl) = 37 % (acidimetric).

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

Dilute 8,28 ml of hydrochloric acid solution (5.7) to 1 l with water.

5.9 Sulfuric acid solution, for rinsing glassware, $c(H_2SO_4)$ = 2 mol/l.

5.10 Sodium chloride (NaCl).

5.11 Potassium chloride (KCl).

5.12 Potassium dihydrogen phosphate (KH_2PO_4).

5.13 Disodium hydrogen orthophosphate (Na_2HPO_4).

5.14 Phosphate buffered saline (PBS), pH = 7.4.

Weigh 0,20 g of potassium chloride (5.11), 0,20 g of potassium dihydrogen phosphate (5.12), 1,16 g of disodium hydrogen orthophosphate (5.13) and 8,00 g of sodium chloride (5.10) to the nearest 0,01 g and transfer into a 1 l volumetric flask. Dissolve in water and add 900 ml of water.

After dissolution adjust the pH to 7,4 with hydrochloric acid solution (5.8) or sodium hydroxide solution (5.6) as appropriate, then fill up to the mark with water.

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

5.15 Polysorbate 20, e.g. Tween® 20[1], lauric acid ≥ 40 %.

5.16 PBS/Polysorbate 20 solution.

Mix Polysorbate 20 (5.15) with PBS (5.14) (1+9, v+v).

[1)] Tween 20 is a trade name of a polysorbate 20-type nonionic surfactant available from various suppliers. This information is given for the convenience of users of this European standard and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.

5.17 Extraction solution.

Mix acetonitrile (5.2), methanol (5.1) and water (40+35+25, v+v+v).

5.18 Nitric acid solution (HNO_3), $c(HNO_3)$ = 4 mol/l.

Slowly add 25,5 ml of concentrated HNO_3 (70 %, mass concentration $\rho(HNO_3)$ = 1,413 g/ml at 25 °C) to 25 ml water, adjust the final volume to 100 ml with water.

5.19 Pyridinium bromide perbromide solution (PBPB), ρ(PBPB) = 50 mg/l.

Weigh 50 mg ± 10 mg of PBPB into a 20 ml glass vial. Dissolve in approximately 20 ml water, use an ultra-sonic bath if required, and transfer quantitatively into a 1 l amber glass bottle. Fill up to 1 l with water. The solution can be stored in a dark place at room temperature for four days.

5.20 Aflatoxin B_1 e.g. crystalline, as a film or as certified standard solution.

5.21 Aflatoxin B_2 e.g. crystalline, as a film or as certified standard solution.

5.22 Aflatoxin G_1 e.g. crystalline, as a film or as certified standard solution.

5.23 Aflatoxin G_2 e.g. crystalline, as a film or as certified standard solution.

5.24 Aflatoxins (B_1, B_2, G_1 and G_2) stock solutions.

Dissolve aflatoxin B_1 (5.20), B_2 (5.21), G_1 (5.22) and G_2 (5.23) separately in acetonitrile (5.2) to give separate solutions with a mass concentration of 10 µg/ml for each aflatoxin. Transfer the stock solutions to amber vials and store them below 4 °C when not in use.

To determine the exact mass 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 using a spectrometer (6.5) with acetonitrile (5.2) as reference. Calculate the mass concentration of each aflatoxin, ρ, in µg/ml, according to Formula (1):

$$\rho = \frac{A_{max} \times M \times 100}{\delta \times \varepsilon} \quad (1)$$

where

A_{max} is the maximum extinction value determined from the absorption curve (here: 350 nm);

M is the molar mass of each aflatoxin, in g/mol;

δ is the path length of the quartz cell, in cm;

ε is the molar absorption coefficient of each aflatoxin in acetonitrile (5.2), in m^2/mol.

M and ε of aflatoxins B_1, B_2, G_1 and G_2 are given in Table 1 [7].

Table 1 — Molar mass and molar absorption coefficient of aflatoxins B_1, B_2, G_1 and G_2 in acetonitrile (5.2)

Aflatoxin	***M*** g/mol	ε m2/mol
B_1	312	2 070
B_2	314	2 250
G_1	328	1 760
G_2	330	1 890

5.25 Mixed stock solution.

Prepare a mixed stock solution containing 1 000 ng/ml of aflatoxin B_1 and G_1, 500 ng/ml of aflatoxin B_2 and G_2 in acetonitrile (5.2) by appropriate dilution of aflatoxins (B_1, B_2, G_1 and G_2) stock solutions (5.24).

A certified mixed aflatoxins stock standard solution which is ready to use may be used as an alternative.

Protect the solution from light and store below 4 °C when not in use. This solution can be stored under these conditions for two months.

5.26 Mixed intermediate solution.

Prepare a mixed intermediate solution by pipetting 2,0 ml of the mixed stock solution (5.25) into a 10 ml volumetric flask. Dilute to the mark with acetonitrile (5.2) and shake well. The concentration of this mixed intermediate solution is 200 ng/ml of aflatoxin B_1 and G_1, and 100 ng/ml of aflatoxin B_2 and G_2.

Protect the solution from light and store below 4 °C when not in use. This solution can be stored under these conditions for two months.

5.27 Mixed standard solution.

Pipette 1,0 ml of the mixed intermediate solution (5.26) into a 10 ml volumetric flask, fill to the mark with the acetonitrile (5.2) and mix well to give a solution containing 20 ng/ml of aflatoxin B_1 and G_1, 10 ng/ml of aflatoxin B_2 and G_2 for preparation of calibration solutions.

Protect the solution from light and store below 4 °C when not in use. This solution can be stored under these conditions for two months.

5.28 Calibration solutions.

Add different volumes of the mixed standard solution (5.27) to five 10 ml volumetric flasks as listed in Table 2 to obtain five calibration levels across the calibration range. The values in Table 2 were used in the validation study.

Evaporate the acetonitrile just to dryness under a stream of nitrogen at room temperature. To each flask, add 4 ml of methanol (5.1), dissolve the aflatoxins by mixing, then dilute to 10 ml with water, and shake well. Methanol and water are subject to volume contraction when mixed, so adjust the volume with water again to the given volume.

These calibration solutions cover the range from 0,48 µg/kg to 9,6 µg/kg for aflatoxins B_1 and G_1 and the range from 0,24 µg/kg to 4,8 µg/kg for B_2 and G_2.

Table 2 —Preparation of HPLC calibration solutions

Calibration solution	Mixed standard solution (5.27)	Targeted mass concentration per analyte			
		B_1	B_2	G_1	G_2
	µl	ng/ml	ng/ml	ng/ml	ng/ml
1	10	0,02	0,01	0,02	0,01
2	20	0,04	0,02	0,04	0,02
3	50	0,10	0,05	0,10	0,05
4	100	0,20	0,10	0,20	0,10
5	200	0,40	0,20	0,40	0,20

These solutions should be freshly made on each day of analysis.

5.29 HPLC mobile phase solvent A.

Mix water, acetonitrile (5.2) and methanol (5.1) (56+30+14, v+v+v). Degas the solution before use if an online system is not available on the HPLC (6.13).

5.30 HPLC mobile phase solvent B.

Mix water, acetonitrile (5.2) and methanol (5.1) (56+30+14, v+v+v). Add 350 µl of nitric acid solution (5.18) and 119 mg of potassium bromide (5.3) per litre of mobile phase. Degas the solution before use if an online system is not available on the HPLC (6.13).

5.31 Immunoaffinity column (IAC).

The affinity column contains antibodies raised against aflatoxins B_1, B_2, G_1 and G_2. The column shall have a capacity of not less than 100 ng of aflatoxin B_1.

6 Apparatus and equipment

The use of non-acid washed glassware may cause losses of aflatoxins. It is recommended that all glassware coming into contact with aqueous solutions of aflatoxins should 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 (5.9) for several h (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.

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.

Usual laboratory apparatus and, in particular, the following:

6.1 Laboratory balance, accuracy of 0,01 g.

6.2 Analytical balance, accuracy of 0,1 mg.

6.3 Centrifugation bottle, e.g. 250 ml.

6.4 Centrifuge, suitable for relative centrifugal force of 2 000 *g* to 2 500 *g*.

NOTE $g = 9{,}81\ m \cdot s^{-2}$.

6.5 UV-spectrometer with quartz cuvettes.

6.6 Laboratory blender, e.g. Ultra Turrax®[2].

6.7 Laboratory shaker, adjustable.

6.8 pH meter.

6.9 Filter paper, e.g. 190 mm diameter, pre-folded, wet strengthened, nominal particle retention rating of 30 µm.

6.10 Pipettes, 25 µl, 50 µl, 250 µl, 1 ml, 10 ml capacity.

6.11 Syringe, plastic disposable, 5 ml capacity.

6.12 Syringe filter, 0,45 µm polytetrafluoroethylene (PTFE).

6.13 HPLC-FLD system, with the following components:

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

6.13.2 Injection system, capable for total loop injection (a 100 µl loop is recommended).

6.13.3 HPLC column, e.g. C18 or ODS-1 (length of 250 mm, 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. A suitable pre-column shall be used.

6.13.4 Column oven.

6.13.5 Post-column derivatization system, with PBPB (only to be used with mobile phase A (5.29)). Consisting of an HPLC pulseless pump, zero-dead volume T-piece, reaction tubing minimum 34 cm × 0,5 mm internal diameter PTFE.

6.13.6 System for derivatization with electrochemically generated bromine, e.g. KOBRA® CELL[3] (only to be used with mobile phase B (5.30)) and reaction tubing minimum 34 cm × 0,5 mm internal diameter PTFE.

6.13.7 Alternative System for derivatization by photochemical reaction, e.g. Photochemical Reactor for Enhanced Detection (PHRED™[4]), only to be used with mobile phase A (5.29). The photochemical reactor is inserted between the HPLC column and the detector inlet. The knitted reactor consists of 25 m of PTFE, 1/16 inch OD × 0,25 mm ID tubing.

6.13.8 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).

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[2] Ultra Turrax® is a trade name of a product commercially available from various suppliers. 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.

[3] KOBRA® CELL is a trade name of a product commercially available. 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.

[4] PHRED™ is a trade name of a product commercially available. 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.