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Foodstuffs — Determination of aflatoxin B_1, and the total content of aflatoxins B_1, B_2, G_1 and G_2 in cereals, nuts and derived products — High-performance liquid chromatographic method

Produits alimentaires — Dosage de l'aflatoxine B_1 et détermination de la teneur totale en aflatoxines B_1, B_2, G_1 et G_2 dans les céréales, les fruits à coque et les produits dérivés — Méthode par chromatographie liquide à haute performance

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 16050 was prepared by Technical Committee ISO/TC 34, *Food products*. It is based on EN 12955:1999 elaborated by CEN/TC 275, *Food analysis — Horizontal methods*.

Foodstuffs — Determination of aflatoxin B_1, and the total content of aflatoxins B_1, B_2, G_1 and G_2 in cereals, nuts and derived products — High-performance liquid chromatographic method

WARNING — The use of this standard involves hazardous materials and operations. 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 practice and to determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard specifies a reverse-phase high-performance liquid chromatographic method, with immunoaffinity column clean-up and post-column derivatization, for the determination of aflatoxins in cereals, nuts and derived products. The limit of quantification for aflatoxin B_1, and for the sum of aflatoxins B_1, B_2, G_1 and G_2, is 8 µg/kg.

The method has been validated for maize containing 24,5 µg/kg, for peanut butter containing 8,4 µg/kg, and for raw peanuts containing 16 µg/kg of total aflatoxins. It has also been shown that this method can be used for oilseed products, dried fruits and derived products.

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.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

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 post-column derivatization.

4 Reagents

Use only reagents recognized analytical grade, unless otherwise stated.

4.1 Water, according to grade 1 of ISO 3696:1987.

4.2 Sodium chloride.

4.3 Iodine, crystalline, or as an alternative, **pyridinium hydrobromide perbromide** (PBPB)[1].

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

WARNING — Aflatoxins are carcinogenic to human subjects. Attention is drawn to the statement made by the International Agencies for Research on Cancer (WHO) (see [1], [2]).

Adequately protect from daylight the laboratory where the analyses are carried out. This may 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).

4.5 Acetonitrile, HPCL grade.

4.6 Methanol, analytical grade.

4.7 Methanol, HPLC grade.

4.8 Toluene, analytical grade.

WARNING — Toluene is highly flammable and harmful. Standard preparation involving this solvent shall be performed in a fume cupboard. Operations outside the fume cupboard, such as measurement of standards by UV spectrometry, shall be performed with the standards in closed containers.

4.9 Toluene/acetonitrile mixture

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

4.10 Extraction solvent

Mix 7 parts per volume of methanol (4.6) with 3 parts per volume of water (4.1).

Other extraction solvent mixtures which are compatible with the mobile phase may also be used if proved to be more effective or recommended by the manufacturer of the immunoaffinity (IA) column.

4.11 Mobile phase

Mix 3 parts per volume of water (4.1) 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 Post-column derivatization reagent

Dissolve 100 mg of iodine (4.3) in 2 ml of methanol (4.6). Add 200 ml of water (4.1), stir for 1 h, then filter through a 0,45 µm membrane filter (5.8). Prepare the solution the week of use and store the solution in the dark or in a brown glass bottle. Before use, stir the solution for 10 min.

As an alternative, dissolve 50 mg of PBPB (4.3) in 1 000 ml of water. This solution may be used for up to 4 days if stored in a dark place at room temperature.

4.13 Aflatoxin B_1, B_2, G_1 and G_2 stock solutions

WARNING — Protect solutions containing aflatoxin from light as far as possible (keep in the dark, use aluminium foil or amber-coloured glassware).

1) CAS: 39416-48-3 (CAS = Chemical Abstract Service).

Dissolve aflatoxin B_1, B_2, G_1 and G_2 separately in the toluene/acetonitrile mixture (4.9) to give separate solutions containing 10 µg/ml.

To determine the exact concentration of aflatoxin in each stock solution, record the absorption curve at a wavelength between 330 nm and 370 nm in 1 cm quartz glass cells (5.7) using a spectrometer (5.6) with a toluene/acetonitrile mixture (4.9) as reference. Calculate the aflatoxin concentration of each aflatoxin, ρ_i, in micrograms per millilitre, using Equation (1):

$$\rho_i = \frac{A_{\max} \times M_i \times 1000}{\varepsilon_i \times d} \quad (1)$$

where

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

M_i is the molecular mass of each aflatoxin, in grams;

ε_i is the molar absorption coefficient of each aflatoxin in toluene/acetonitrile;

NOTE This value is determined in a solution that contains c = 1 mol/l of aflatoxin and in a cell with the optical pathlength d = 1 cm. The molar absorption coefficient (ε) is usually given without a unit of measurement, but from the equation $A = \varepsilon \times c \times d$, the following unit can be derived for it: $l \cdot mol^{-1} \cdot cm^{-1}$.

d is the optical pathlength of the cell, in centimetres.

M_i and ε_i are given in Table 1.

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

Aflatoxin	M_i	ε_i
B_1	312	19 300
B_2	314	20 400
G_1	328	16 600
G_2	330	17 900

NOTE A mixture of toluene and acetonitrile (98 + 2) is used as solvent.

4.14 Stock solution of mixed aflatoxins

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/ml of aflatoxin G_2 in toluene/acetonitrile (4.9). If the solution has to be stored, weigh the flask before storage. Wrap the flask tightly in aluminium foil and store it at approximately 4 °C. Immediately before use, reweigh the flask and record any change in mass after storage.

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

4.15 Standard solution of mixed aflatoxins

Transfer each quantity, as specified in Table 2, of mixed aflatoxin stock solution (4.14) into a series of four 2 ml volumetric flasks (5.5). Evaporate the solutions just to dryness under a stream of nitrogen at room temperature. To each flask, add 1 ml of methanol (4.6). Dissolve the dry residue in it, dilute the solution to the mark with water (4.1) and mix. Prepare the solution freshly on the day of use.

Table 2 — Preparation of standard solutions

Standard solution	Volume taken from stock solution	Concentration of aflatoxin ng/ml			
	µl	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 only. The standard range includes the concentrations of the samples.					

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

5 Apparatus

Soak laboratory glassware coming into contact with aqueous solutions of aflatoxins in sulfuric acid (4.16) 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 can 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.

Usual laboratory apparatus and, in particular, the following.

5.1 Immunoaffinity (IA) column

The IA column contains antibodies raised against aflatoxin B_1, B_2, G_1 and G_2. The column shall have a minimum binding capacity of not less than 100 ng of aflatoxin B_1. It 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 methanol (4.6) and 3,4 parts water (4.1) (by volume)] containing 5 ng of each toxin is applied to the IA column. The IA column should be equipped with an appropriate solvent reservoir (e.g. a syringe with adapter).

It is advisable to carry out recovery experiments for every matrix that the method is used for.

5.2 Blender, with 500 ml blender jar and cover.

The use of a high-speed blender is recommended.

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

5.4 Glass microfibre filter paper[2)], e.g. 11 cm diameter.

5.5 Volumetric flasks, class A grade, of capacity 2 ml.

5.6 Spectrometer, capable measuring wavelengths between 200 nm and 400 nm.

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2) For example, Whatman 934AH is appropriate for this purpose. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Other products may be used if they can be shown to give comparable results.

5.7 Quartz glass cells, of optical path length 1 cm, and with 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.

5.9 HPLC apparatus, comprising the following.

5.9.1 HPLC pump, capable of producing a 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 reverse-phase separating column, e.g. C_{18}, which ensures a baseline resolved resolution of the aflatoxin B_1, B_2, G_1 and G_2 peaks from all other peaks, with the following characteristics:

— length: 250 mm;

— internal diameter: 4,6 mm;

— spherical particle size: 5 µm.

Shorter columns may be used.

5.9.4 Post-column derivatization system, consisting of a pulse-free pump and very low dead-volume T-piece, with polytetrafluoroethylene (PTFE) or stainless-steel tubing of length 3 000 mm to 5 000 mm and internal diameter of 0,5 mm, and a heating bath or post-column reactor for the iodine reaction.

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

6 Procedure

6.1 General

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

6.2 Extraction

Weigh, to the nearest 0,1 g, 25 g of the homogenized test sample into the blender jar (5.2). Add 5 g of sodium chloride (4.2) and 125 ml of extraction solvent (4.10) and homogenize with a mixer for 2 min at high speed. Check that the blending time and speed do not have a negative influence on the extraction efficiency. Filter the mixture through a fluted filter paper (5.3) (V_1).

Pipette 15 ml (V_2) of the filtrate 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 filter paper (5.4). The filtrate (V_3) should be clear. If not, refilter it. Proceed immediately in accordance with 6.3.

A centrifuge may also be used to obtain a clear solution.