

English Version

Foodstuffs - Determination of aflatoxin B<sub>1</sub> and the sum of aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> in hazelnuts, peanuts, pistachios, figs, and paprika powder - High performance liquid chromatographic method with post-column derivatisation and immunoaffinity column cleanup

Produits alimentaires - Dosage de l'aflatoxine B<sub>1</sub> et de la somme des aflatoxines B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> et G<sub>2</sub> dans les noisettes, les cacahuètes, les pistaches, les figues et le paprika en poudre - Méthode par purification sur colonne d'immunoaffinité suivie d'une chromatographie liquide à haute performance avec dérivation post-colonne

Lebensmittel - Bestimmung von Aflatoxin B<sub>1</sub> und der Summe von Aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> und G<sub>2</sub> in Haselnüssen, Erdnüssen, Pistazien, Feigen und Paprikapulver - Hochleistungsflüssigchromatographisches Verfahren mit Immunoaffinitätsäulen-Reinigung und Nachsäulenderivatisierung

This European Standard was approved by CEN on 12 November 2007.

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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of an identical text or by endorsement, at the latest by June 2008, and conflicting national standards shall be withdrawn at the latest by June 2008.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.**

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## 1 Scope

This European Standard is applicable to the determination of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> 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<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. 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<sub>2</sub>O) 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**,  $c(\text{HNO}_3) = 4 \text{ mol/l}$

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<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. The column shall have a maximum capacity of not less than 100 ng of aflatoxin B<sub>1</sub> and shall give a recovery of not less than 80 % for aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and not less than 60 % for aflatoxin G<sub>2</sub> 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<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> 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,  $\rho_i$ , in micrograms per millilitre, using Equation (1):

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

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;

$\varepsilon_i$  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  $\varepsilon_i$  of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> are given in Table 1.

**Table 1 — Molar mass and molar absorption coefficient of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>**  
(In mixture of toluene and acetonitrile (4.18))

Aflatoxin	$M_i$ g/mol	$\varepsilon_i$ m <sup>2</sup> /mol
B <sub>1</sub>	312	1930
B <sub>2</sub>	314	2040
G <sub>1</sub>	328	1660
G <sub>2</sub>	330	1790

#### 4.21 Mixed aflatoxins stock solution

Prepare a mixed aflatoxins stock solution containing 1000 ng/ml of aflatoxin B<sub>1</sub> and G<sub>1</sub>, 200 ng/ml of aflatoxin B<sub>2</sub> and G<sub>2</sub> in the toluene and acetonitrile mixture (4.18) by appropriate dilution of aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) 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<sub>1</sub> and G<sub>1</sub>, 20 ng/ml of aflatoxin B<sub>2</sub> and G<sub>2</sub> 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<sub>1</sub> and G<sub>1</sub>, 20 ng/ml of aflatoxin B<sub>2</sub> and G<sub>2</sub> (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 <sub>1</sub>	B <sub>2</sub>	G <sub>1</sub>	G <sub>2</sub>
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<sub>1</sub> and G<sub>1</sub>, 200 ng/ml of aflatoxin B<sub>2</sub> and G<sub>2</sub>, 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<sub>1</sub> and G<sub>1</sub>, and 40 ng/ml of aflatoxin B<sub>2</sub> and G<sub>2</sub>.

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<sup>1)</sup>, 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  $\leq 0,2$  ng/g (signal-to-noise-ratio = 3) and the limit of quantification (LOQ) is  $\leq 0,5$  ng/g (signal-to-noise-ratio = 6) for each aflatoxin (using the standard solutions).

**5.11.3 RP-HPLC column**, e.g. C<sub>18</sub> 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<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> 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<sup>® 2)</sup> (only to be used with mobile phase B (4.16)).

**5.11.6 Fluorescence detector**, with a wavelength of  $\lambda = 360$  nm excitation filter and a wavelength of  $\lambda = 420$  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<sup>®</sup> 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.

## 6.4 Immunoaffinity clean-up

Pass the filtrate through the column at a flow rate of approximately 3 ml/min (approximately one drop per second) or by gravity. Do not exceed a flow rate of 5 ml/min. Wash the column with approximately 20 ml of water (4.2) applied in little portions of approximately 10 ml at a flow rate of maximum 5 ml/min and dry by applying little vacuum for 5 s to 10 s or passing air through the immunoaffinity column by means of a syringe for 10 s.

Elute the aflatoxins in a two step procedure.

For hazelnut paste apply 0,50 ml of methanol (4.8) on the column and let it pass through by gravity. Collect the eluate in a calibrated volumetric flask of 3 ml (5.10). Wait for 1 min and apply a second portion of 1,0 ml of methanol. Use a syringe to pass air through the column to collect the last few drops. Pass 1,5 ml of water (4.2) through the column and collect this in a calibrated volumetric flask of 3 ml. Fill the flask to the mark with water and shake well and adjust the volume again to the given volume.

For other matrices apply 0,50 ml of methanol (4.8) on the column and let it pass through by gravity. Collect the eluate in a calibrated volumetric flask of 5 ml (5.10). Wait for 1 min and apply a second portion of 0,75 ml of methanol (4.8). Collect most of the applied elution solvent by pressing air through. Fill the flask to the mark with water and shake well and adjust the volume again to the given volume.

If the solution is clear it can be used directly for HPLC analysis. If the solution is not clear, filter through a disposable filter unit (5.12) prior to HPLC injection.

**NOTE** Methods for loading onto immunoaffinity columns, washing the column and elution can vary slightly between column manufacturers. Specific instructions supplied with the columns should be followed.

## 6.5 High performance liquid chromatography (HPLC)

The injection by total loop mode guarantees maximum accuracy. It is recommended (depending on the injection system, i.e. manual injection) to take a sample volume of 3 times the injection loop size and to inject at least 2/3 of this volume into the valve, to ensure that the middle fraction remains in the injection loop. Thus, the loop is rinsed with the injection solvent while enough solvent remains in the valve.

The aflatoxins are separated by isocratic reverse-phase HPLC (RP-HPLC) at room temperature with a reverse-phase column (5.11.3) and an appropriate mobile phase (4.15) or (4.16). The recommended flow rate is 1 ml/min for a column with an inner diameter of 4,6 mm. Thus the flow rate may be adjusted according to the column dimension. The aflatoxins elute in the order G<sub>2</sub>, G<sub>1</sub>, B<sub>2</sub> and B<sub>1</sub> within 16 min and should be baseline resolved. The mobile phase may be adjusted by addition of water, methanol or acetonitrile for maximum peak resolution and chromatographic performance (typical chromatograms are enclosed in Annex A).

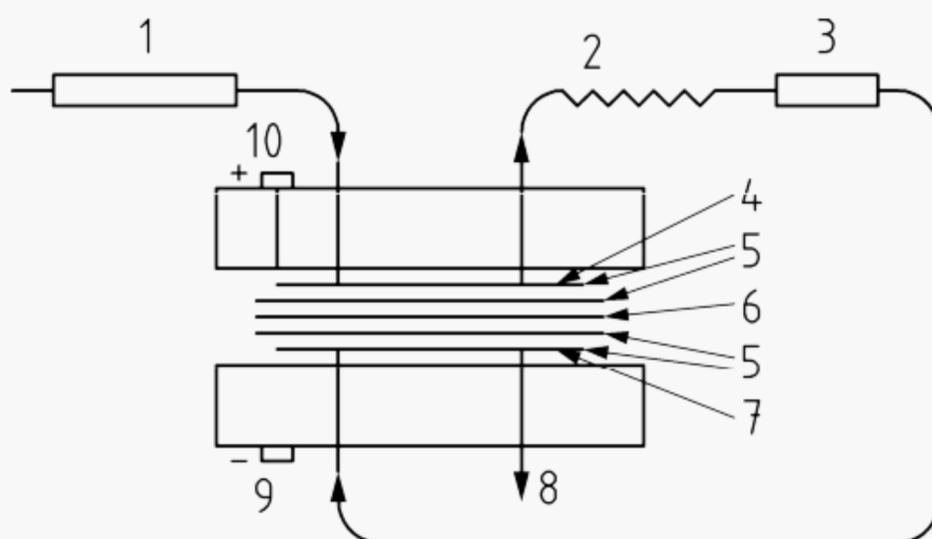
## 6.6 Post-column derivatisation

When using PBPB, mount the mixing T-piece and reaction tubing (see 5.11.4), and then operate using the following parameters:

- flow rate of 1,00 ml/min for the mobile phase (see also 6.5);
- flow rate of 0,30 ml/min for the reagent (flow rate may be adjusted to the flow rate of the mobile phase).

When using electrochemically generated bromine (KOBRA cell<sup>®</sup>) follow the instructions for the installation of the cell as supplied by the manufacturers and operate using the following parameters:

- flow rate of 1,00 ml/min for the mobile phase (see also 6.5). Use polyetheretherketone (PEEK) tubing with a length of 34 cm and inner diameter of 0,5 mm;
- current of 100 µA.



### Key

- 1 — column
- 2 — reaction coil
- 3 — detector
- 4 — working electrode (platinum)
- 5 — spacer
- 6 — membrane
- 7 — counter electrode (stainless steel)
- 8 — waste
- 9 — black
- 10 — red

Figure 1 — KOBRA cell<sup>®</sup> assembly

### 6.7 Calibration curve

Prepare the calibration curves using the mixed aflatoxins calibration solutions described (4.23). These solutions cover the range of 0,5 ng/g to 4,5 ng/g for aflatoxins B<sub>1</sub> and G<sub>1</sub> and the range of 0,1 ng/g to 0,9 ng/g for B<sub>2</sub> and G<sub>2</sub>. Establish the calibration curves prior to analysis according to Table 2 and check the plot for linearity.

If aflatoxin contents in a sample are outside the calibration range, the injection solution for HPLC analysis can be diluted to an aflatoxin content appropriate for the established calibration curve.

### 6.8 Spiking procedures

For the determination of the recovery the spiking procedure has to be carried out using the methanol spiking solution (4.24). The spiking level shall be within the calibration range (preferably mean value). Take care that not more than 2 ml of the spiking solution is added and that the subsequent evaporation takes place in the dark and should last 30 min to 2 h.

### 6.9 Calculation

For those sample preparation procedures that involve the use of *n*-hexane or cyclohexane, the volume of these solvents shall not be taken into account for analysis calculation. The addition of *n*-hexane or cyclohexane to the extraction solvent mixture is necessary only to break possible fat layers (encapsulated aflatoxin). Aflatoxins do not dissolve in *n*-hexane or cyclohexane. Establish a calibration curve using linear

regression and calculate the concentration of aflatoxin, in nanogram per millilitre, in the injection solution for each sample.

Calculate the mass fraction of aflatoxins,  $w$ , in nanogram per gram of the sample using Equation (2):

$$w = \frac{\rho_{\text{smp}} \times V_e \times V_{\text{final}}}{m_s \times V_{\text{iac}}} \quad (2)$$

where:

- $\rho_{\text{smp}}$  is the mass concentration of aflatoxin in the injection solution calculated from linear regression, in nanogram per millilitre;
- $V_e$  is the volume of the extraction solvent, in millilitre ( $V_e = 250$  ml for hazelnut paste,  $V_e = 200$  ml for peanut butter and pistachio paste and  $V_e = 300$  ml for fig paste and paprika powder);
- $V_{\text{final}}$  is the final volume obtained after elution from IAC, in millilitre (3 ml for hazelnut paste and 5 ml for peanut paste, pistachio paste, fig paste and paprika powder);
- $m_s$  is the mass of the sample material taken for analysis, in gram, (here: 50 g). (During the net weigh the water fraction has to be considered which was applied by the sample preparation after 6.1 for the wet homogenization.)
- $V_{\text{iac}}$  is the volume of the sample extract taken for immunoaffinity cleanup, in millilitre (5 ml for hazelnut paste and 10 ml for peanut paste, pistachio paste, fig paste and paprika powder).

## 6.10 Confirmation of aflatoxin B<sub>1</sub> and G<sub>1</sub>

In order to confirm the aflatoxins B<sub>1</sub> and G<sub>1</sub> disconnect the HPLC column from the bromination device and connect it directly to the fluorescence detector. Aflatoxin B<sub>1</sub> and G<sub>1</sub> signals are significantly lower (factor of 10 or more) when the derivatization system is removed. Do not switch off the electrical current with the bromination device still in line due to the possibility of remaining bromine in the cell membrane of the device.

## 7 Precision

### 7.1 General

Details of the interlaboratory test of the precision of the method are summarized in Annex B. The precision data were obtained with two independent interlaboratory studies performed in 2004 for hazelnuts and in 1998 for peanuts, pistachios, figs and paprika powder. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and matrices other than given in Annex B.

### 7.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit  $r$  in not more than 5 % of the cases.

**The values for hazelnut paste are:**

Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,4$ ng/g	$r = 0,11$ ng/g	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 4,2$ ng/g	$r = 0,31$ ng/g	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 2,3$ ng/g	$r = 0,21$ ng/g	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 7,1$ ng/g	$r = 0,69$ ng/g	(naturally contaminated)

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Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,8 \text{ ng/g}$	$r = 0,24 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 12,1 \text{ ng/g}$	$r = 0,80 \text{ ng/g}$	(naturally contaminated)

### The values for peanut butter are:

Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,9 \text{ ng/g}$	$r = 0,25 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 1,9 \text{ ng/g}$	$r = 0,73 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,6 \text{ ng/g}$	$r = 0,31 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 7,9 \text{ ng/g}$	$r = 1,88 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,8 \text{ ng/g}$	$r = 0,14 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 1,3 \text{ ng/g}$	$r = 0,22 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,5 \text{ ng/g}$	$r = 0,28 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 2,2 \text{ ng/g}$	$r = 0,45 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,4 \text{ ng/g}$	$r = 0,36 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 5,0 \text{ ng/g}$	$r = 0,64 \text{ ng/g}$	(naturally contaminated)

### The values for pistachio paste are:

Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,9 \text{ ng/g}$	$r = 0,36 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 2,0 \text{ ng/g}$	$r = 0,67 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,3 \text{ ng/g}$	$r = 0,36 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 7,8 \text{ ng/g}$	$r = 5,10 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,7 \text{ ng/g}$	$r = 0,22 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 0,8 \text{ ng/g}$	$r = 0,28 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,5 \text{ ng/g}$	$r = 0,76 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 1,7 \text{ ng/g}$	$r = 0,87 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 2,9 \text{ ng/g}$	$r = 1,65 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 3,3 \text{ ng/g}$	$r = 1,85 \text{ ng/g}$	(naturally contaminated)

### The values for fig paste are:

Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,1 \text{ ng/g}$	$r = 0,50 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 2,2 \text{ ng/g}$	$r = 1,12 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,6 \text{ ng/g}$	$r = 1,09 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 7,8 \text{ ng/g}$	$r = 2,83 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,3 \text{ ng/g}$	$r = 0,34 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 2,8 \text{ ng/g}$	$r = 0,70 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 2,1 \text{ ng/g}$	$r = 0,34 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 3,8 \text{ ng/g}$	$r = 1,23 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 2,6 \text{ ng/g}$	$r = 1,15 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 5,2 \text{ ng/g}$	$r = 2,52 \text{ ng/g}$	(naturally contaminated)

**The values for paprika powder are:**

Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,9 \text{ ng/g}$	$r = 0,14 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 1,7 \text{ ng/g}$	$r = 0,31 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,4 \text{ ng/g}$	$r = 0,50 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 7,1 \text{ ng/g}$	$r = 2,02 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,8 \text{ ng/g}$	$r = 0,34 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 0,9 \text{ ng/g}$	$r = 0,45 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,4 \text{ ng/g}$	$r = 0,39 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 2,0 \text{ ng/g}$	$r = 0,64 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,0 \text{ ng/g}$	$r = 0,36 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 4,5 \text{ ng/g}$	$r = 0,62 \text{ ng/g}$	(naturally contaminated)

**7.3 Reproducibility**

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit  $R$  in not more than 5 % of the cases.

**The values for hazelnut paste are:**

Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,4 \text{ ng/g}$	$R = 0,28 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 4,2 \text{ ng/g}$	$R = 0,81 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 2,3 \text{ ng/g}$	$R = 0,51 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 7,1 \text{ ng/g}$	$R = 1,35 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,8 \text{ ng/g}$	$R = 0,78 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 12,1 \text{ ng/g}$	$R = 2,08 \text{ ng/g}$	(naturally contaminated)

**The values for peanut butter are:**

Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,9 \text{ ng/g}$	$R = 0,45 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 1,9 \text{ ng/g}$	$R = 0,98 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,6 \text{ ng/g}$	$R = 1,85 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 7,9 \text{ ng/g}$	$R = 4,93 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,8 \text{ ng/g}$	$R = 0,73 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 1,3 \text{ ng/g}$	$R = 1,29 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,5 \text{ ng/g}$	$R = 0,62 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 2,2 \text{ ng/g}$	$R = 0,90 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,4 \text{ ng/g}$	$R = 1,82 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 5,0 \text{ ng/g}$	$R = 2,69 \text{ ng/g}$	(naturally contaminated)

**The values for pistachio paste are:**

Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,9 \text{ ng/g}$	$R = 0,42 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 2,0 \text{ ng/g}$	$R = 1,01 \text{ ng/g}$	(fortified)

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Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,3 \text{ ng/g}$	$R = 2,86 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 7,8 \text{ ng/g}$	$R = 5,10 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,7 \text{ ng/g}$	$R = 0,34 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 0,8 \text{ ng/g}$	$R = 0,48 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,5 \text{ ng/g}$	$R = 1,01 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 1,7 \text{ ng/g}$	$R = 1,18 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 2,9 \text{ ng/g}$	$R = 1,71 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 3,3 \text{ ng/g}$	$R = 2,02 \text{ ng/g}$	(naturally contaminated)

### The values for fig paste are:

Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,1 \text{ ng/g}$	$R = 0,59 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 2,2 \text{ ng/g}$	$R = 2,04 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,6 \text{ ng/g}$	$R = 1,29 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 7,8 \text{ ng/g}$	$R = 3,58 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,3 \text{ ng/g}$	$R = 0,84 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 2,8 \text{ ng/g}$	$R = 2,24 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 2,1 \text{ ng/g}$	$R = 0,87 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 3,8 \text{ ng/g}$	$R = 2,88 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 2,6 \text{ ng/g}$	$R = 2,04 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 5,2 \text{ ng/g}$	$R = 4,37 \text{ ng/g}$	(naturally contaminated)

### The values for paprika powder are:

Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,9 \text{ ng/g}$	$R = 0,25 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 1,7 \text{ ng/g}$	$R = 0,95 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,4 \text{ ng/g}$	$R = 0,98 \text{ ng/g}$	(fortified)
Total aflatoxins:	$\bar{x} = 7,1 \text{ ng/g}$	$R = 2,83 \text{ ng/g}$	(fortified)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 0,8 \text{ ng/g}$	$R = 0,45 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 0,9 \text{ ng/g}$	$R = 0,87 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 1,4 \text{ ng/g}$	$R = 0,67 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 2,0 \text{ ng/g}$	$R = 1,54 \text{ ng/g}$	(naturally contaminated)
Aflatoxin B <sub>1</sub> :	$\bar{x} = 3,0 \text{ ng/g}$	$R = 0,78 \text{ ng/g}$	(naturally contaminated)
Total aflatoxins:	$\bar{x} = 4,5 \text{ ng/g}$	$R = 1,85 \text{ ng/g}$	(naturally contaminated)

## 8 Test report

The test report shall contain the following data:

- all information necessary for the identification of the sample (kind of sample, origin of sample, designation);
- all information necessary for the identification of the calibrant;
- a reference to this European Standard;

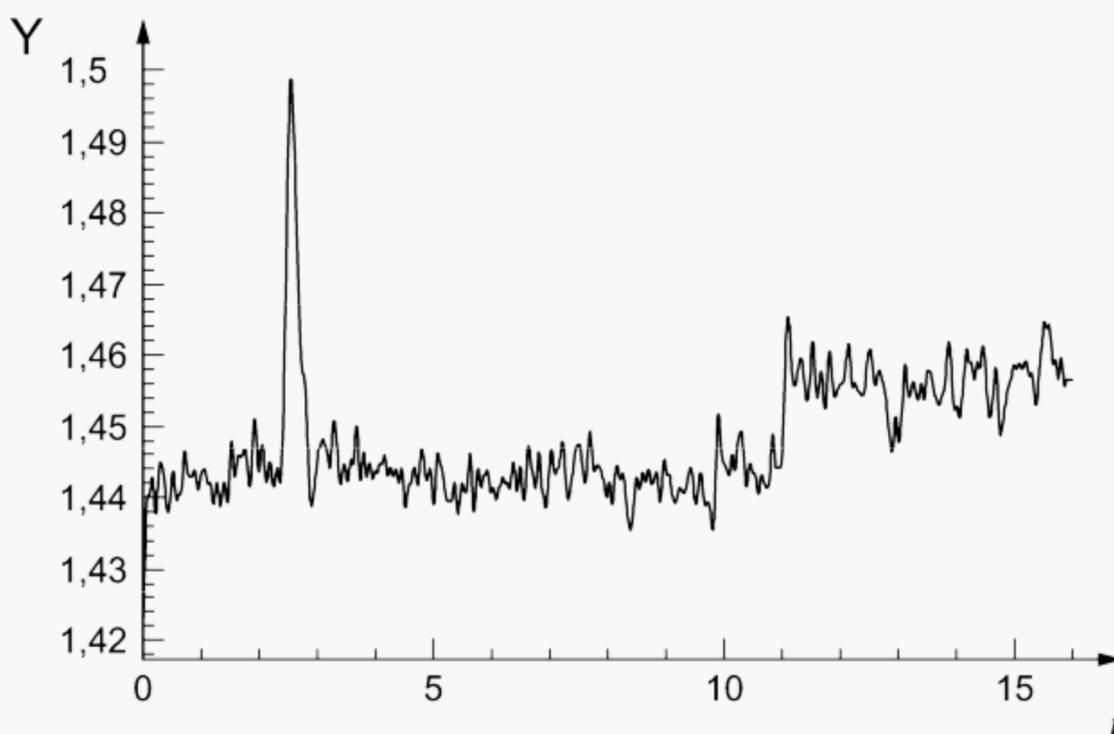
- date and type of sampling procedure (if known);
- the date of receipt;
- the date of test;
- the test results and the units in which they have been expressed;
- any particular points observed in the course of the test;
- any operations not specified in the method or regarded as optional, which might have affected the results.

## Annex A (informative)

### Typical chromatograms

Operating conditions for Figures A.1 and A.2:

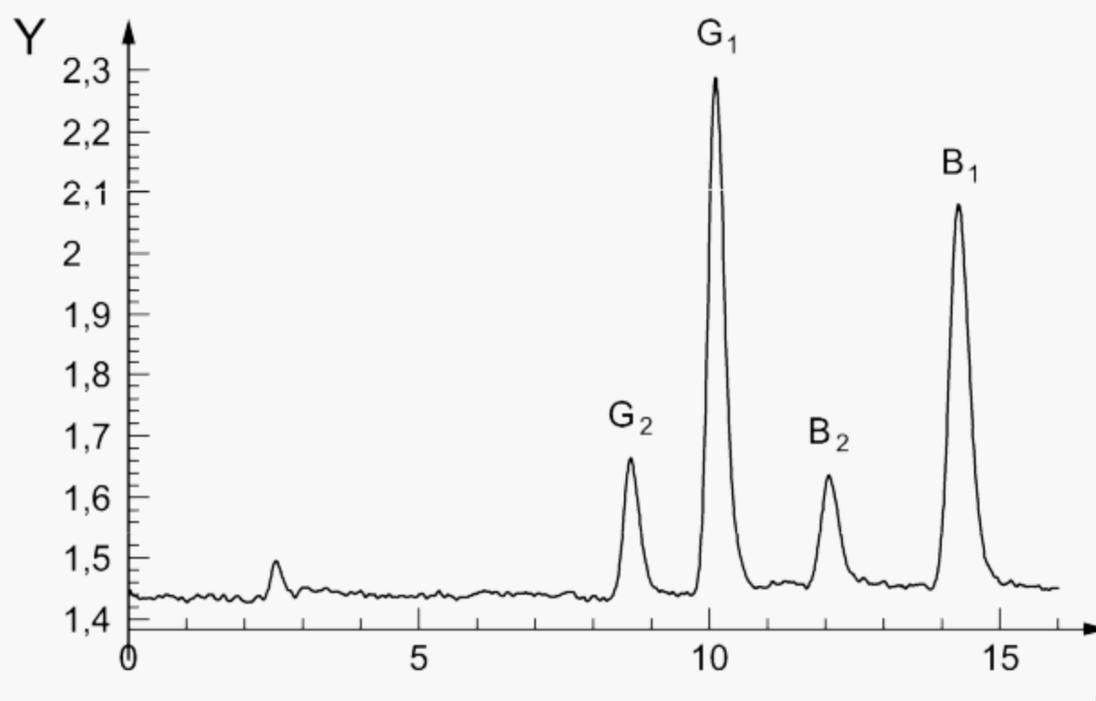
Injection volume:	100 $\mu$ l
Column:	C-18 (length of 25 cm, inner diameter of 4,6 mm and particle size of 5 $\mu$ m)
Flow rate:	1 ml/min
Mobile phase:	water-methanol-acetonitrile (62 + 22 + 16 [V/V/V]) containing 120 mg KBr and 350 $\mu$ l of HNO <sub>3</sub> ( $c(\text{HNO}_3) = 4 \text{ mol/l}$ ) per liter
Derivatization:	electrochemical bromination (KOBRA cell <sup>®</sup> )
Detection:	fluorescence excitation 362 nm, emission. 425 nm for B <sub>1</sub> and B <sub>2</sub> and emission 455 nm for G <sub>1</sub> and G <sub>2</sub>



#### Key

Y fluorescence in mV

**Figure A.1 — Typical chromatogram of aflatoxins. Blank extract of hazelnut paste after immunoaffinity cleanup (<0,1 ng/g total aflatoxins)**



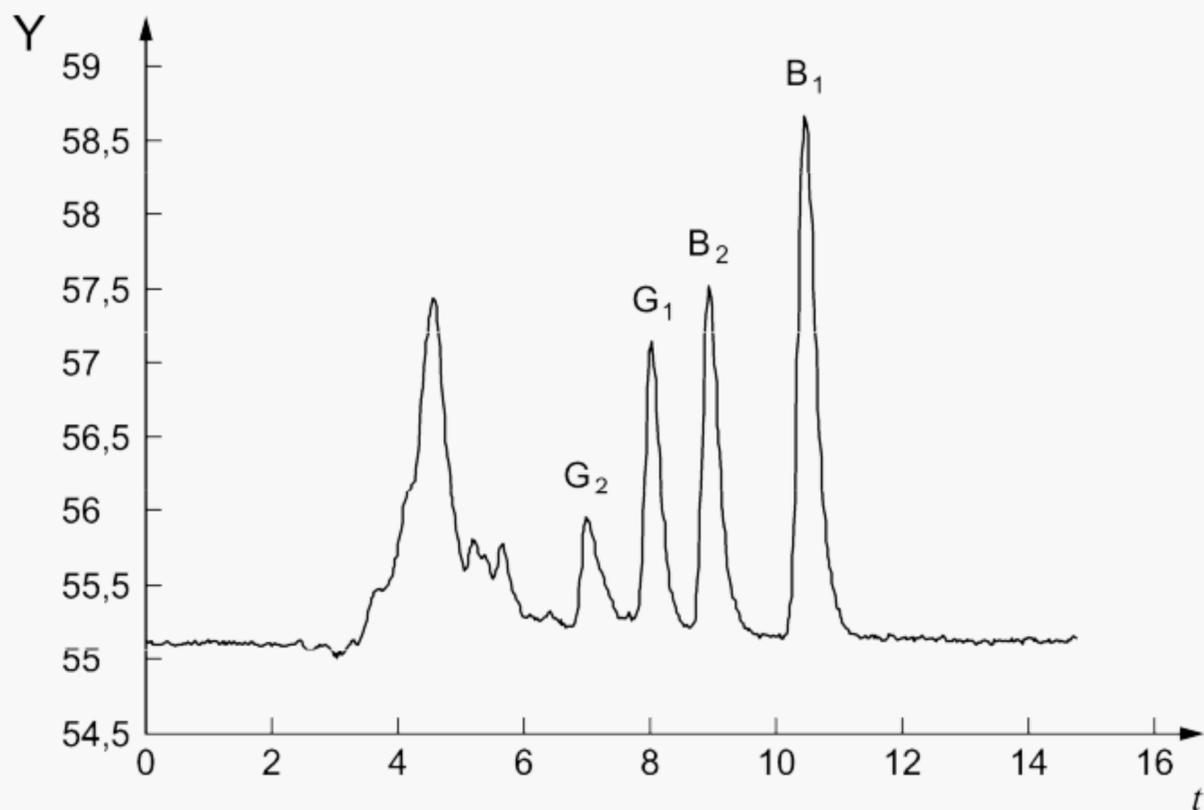
### Key

Y fluorescence in mV

**Figure A.2 — Typical chromatogram of aflatoxins in naturally contaminated hazelnut paste after immunoaffinity cleanup (contamination level 12 ng/g total aflatoxins, 3,8 ng/g aflatoxin B<sub>1</sub>)**

Operating conditions for Figures A.3 to A.6:

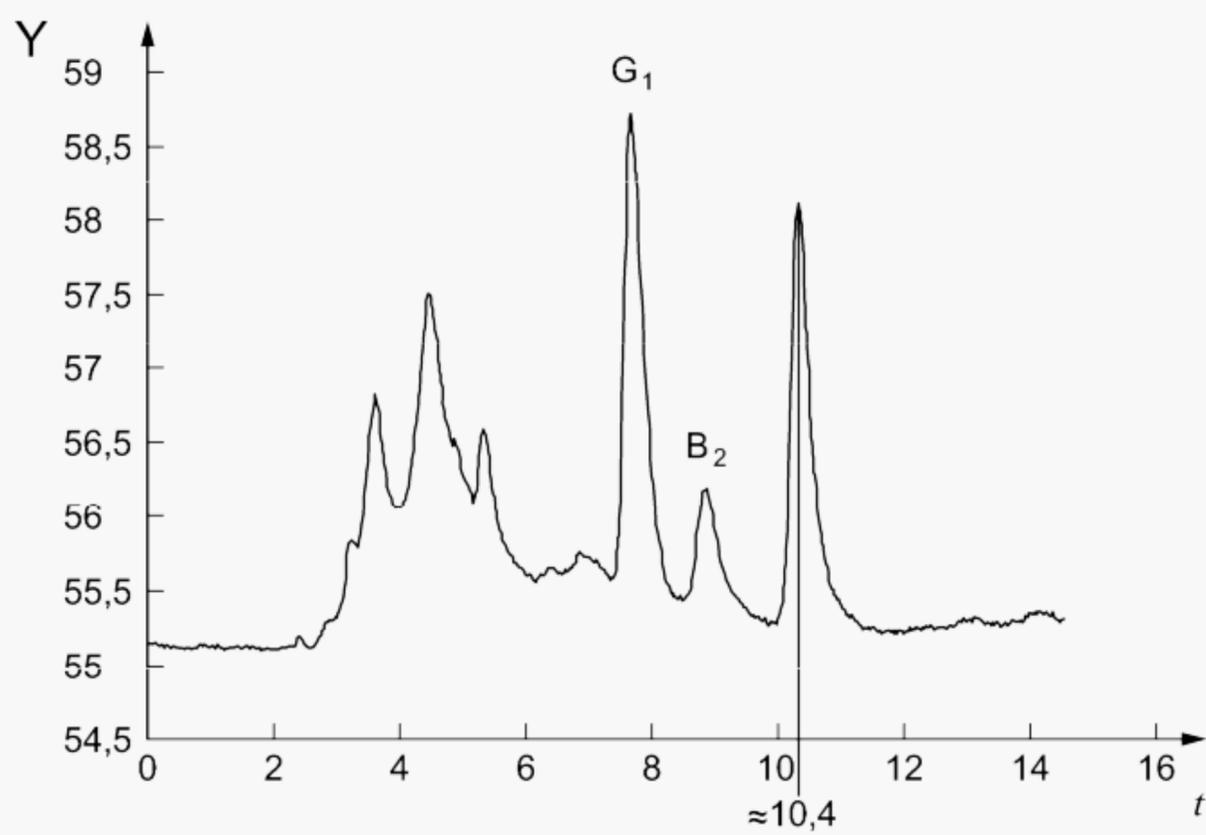
Injection volume: 200  $\mu$ l  
 Column: C-18 (length of 25 cm, inner diameter of 4,6 mm and particle size of 5  $\mu$ m)  
 Flow rate: 1 ml/min  
 Mobile phase: water-methanol-acetonitrile (6 + 3 + 3 [V/V/V]) containing 120 mg KBr and 350  $\mu$ l of HNO<sub>3</sub> ( $c(\text{HNO}_3) = 4 \text{ mol/l}$ ) per liter  
 Derivatization: electrochemical bromination (KOBRA cell<sup>®</sup>)  
 Detection: fluorescence excitation 365 nm, emission 435 nm



**Key**

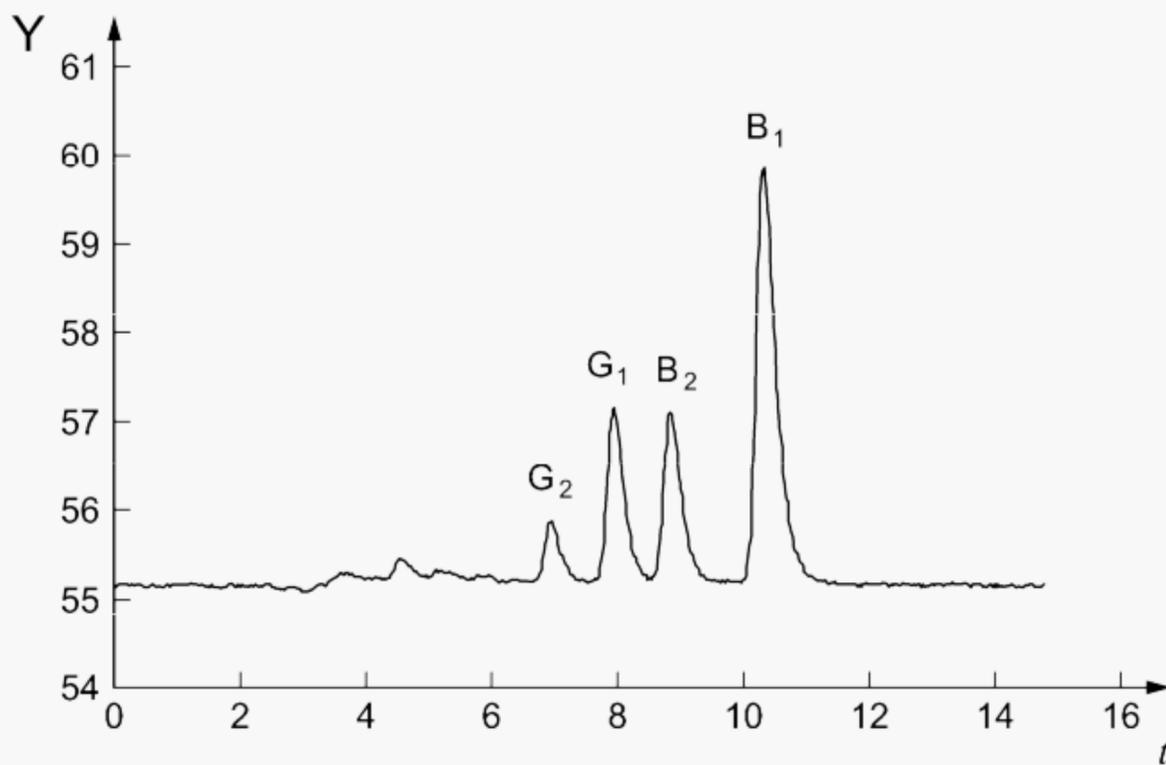
Y fluorescence in mV

**Figure A.3 — Typical chromatogram of aflatoxins in naturally contaminated fig paste after immunoaffinity cleanup (contamination level 1 ng/g aflatoxin B<sub>1</sub>)**

**Key**

Y fluorescence in mV

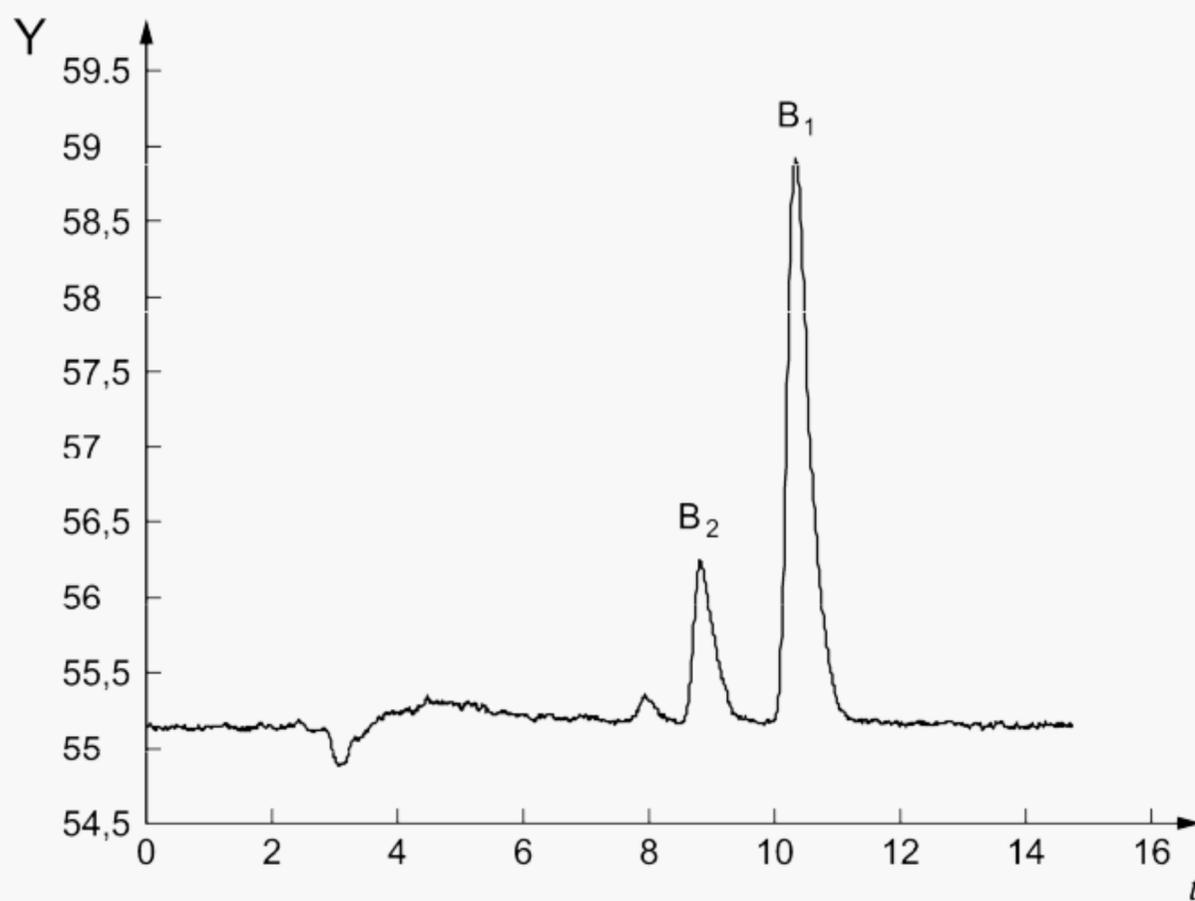
**Figure A.4 — Typical chromatogram of aflatoxins in naturally contaminated paprika powder after immunoaffinity cleanup (contamination level 1 ng/g aflatoxin B<sub>1</sub>)**



**Key**

Y fluorescence in mV

**Figure A.5 — Typical chromatogram of aflatoxins in naturally contaminated peanut butter after immunoaffinity cleanup (contamination level 1 ng/g aflatoxin B<sub>1</sub>)**

**Key**

Y fluorescence in mV

**Figure A.6 — Typical chromatogram of aflatoxins in naturally contaminated pistachio paste after immunoaffinity cleanup (contamination level 1 ng/g aflatoxin B<sub>1</sub>)**

## Annex B (informative)

### Precision data

The data given in Table B.1 were obtained in an interlaboratory study organized by TÜBITAK-Ankara Test and Analysis Laboratory, Turkey. Samples of hazelnut paste both naturally contaminated and spiked with aflatoxins, were included in the study [4].

**Table B.1 — Precision data for hazelnut paste**

Parameter	Aflatoxin B <sub>1</sub>						Total Aflatoxins					
	1 <sup>a</sup>	2 <sup>b</sup>	3 <sup>c</sup>	4 <sup>d</sup>	5 <sup>e</sup>	6 <sup>f</sup>	7 <sup>a</sup>	8 <sup>g</sup>	9 <sup>h</sup>	10 <sup>d</sup>	11 <sup>e</sup>	12 <sup>f</sup>
Sample number	1 <sup>a</sup>	2 <sup>b</sup>	3 <sup>c</sup>	4 <sup>d</sup>	5 <sup>e</sup>	6 <sup>f</sup>	7 <sup>a</sup>	8 <sup>g</sup>	9 <sup>h</sup>	10 <sup>d</sup>	11 <sup>e</sup>	12 <sup>f</sup>
Year of interlaboratory test	2004						2004					
Number of laboratories	14						14					
Number of laboratories retained after eliminating outliers	14	13	13	13	13	13	14	13	13	12	13	12
Number of outliers	0	1	1	1	1	1	0	1	1	2	1	2
Number of accepted results	28	26	26	26	26	26	28	26	26	24	26	24
Mean value $\bar{x}$ , ng/g	0,01	0,89	2,15	1,4	2,3	3,8	0,04	3,6	8,7	4,2	7,1	12,1
Repeatability standard deviation $s_r$ , ng/g	n.a. <sup>i</sup>	-	-	0,040	0,074	0,086	n.a.	-	-	0,112	0,245	0,285
Repeatability relative standard deviation $RSD_r$ , %	n.a.	-	-	2,9	3,2	2,2	n.a.	-	-	2,7	3,4	2,3
Repeatability limit $r$ [ $r = 2,8 \times s_r$ ], ng/g	n.a.	-	-	0,11	0,21	0,24	n.a.	-	-	0,31	0,69	0,80
Reproducibility standard deviation $s_R$ , ng/g	n.a.	-	-	0,10	0,18	0,28	n.a.	-	-	0,29	0,48	0,74
Reproducibility relative standard deviation $RSD_R$ , %	n.a.	-	-	7,4	7,8	7,3	n.a.	-	-	7,0	6,7	6,1
Repeatability limit $R$ [ $R = 2,8 \times s_R$ ], ng/g	n.a.	-	-	0,28	0,51	0,78	n.a.	-	-	0,81	1,35	2,08
Recovery, %	n.a.	89	86	n.a.	n.a.	n.a.	n.a.	89	87	n.a.	n.a.	n.a.
<sup>a</sup> Blank	<sup>d</sup> Naturally contaminated, low level						<sup>g</sup> Spike 1, 4 ng/g					
<sup>b</sup> Spike 1, 1 ng/g	<sup>e</sup> Naturally contaminated, medium level						<sup>h</sup> Spike 2, 10 ng/g					
<sup>c</sup> Spike 2, 2,5 ng/g	<sup>f</sup> Naturally contaminated, high level						<sup>i</sup> Not applicable					

The data given in Table B.2, B.3, B.4 and B.5 were obtained in an interlaboratory study organized by the European Communities, Standards Measurement and Testing Programme in accordance with ISO 5725-2, -4 and -6. Samples of peanut butter, pistachio paste, fig paste and paprika powder, both naturally contaminated and spiked with aflatoxins, were included in the study [5].

Table B.2 — Precision data for peanut butter

Parameter	Aflatoxin B <sub>1</sub>					Total Aflatoxins				
	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Sample number	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Year of interlaboratory test	1998					1998				
Number of laboratories	16					16				
Number of samples (duplicates)	1	1	1	1	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	15	13	15	14	14	15	15	15	13	14
Number of outliers	1	3	1	2	2	1	1	1	3	2
Number of accepted results	15	13	15	14	14	15	15	15	13	14
Mean value $\bar{x}$ , ng/g	0,87	3,65	0,80	1,52	3,40	1,9	7,9	1,3	2,2	5,0
Repeatability standard deviation $s_r$ , ng/g	0,09	0,11	0,05	0,10	0,13	0,26	0,67	0,08	0,16	0,23
Repeatability relative standard deviation $RSD_r$ , %	10	3	6	6	4	13	9	6	7	5
Repeatability limit $r$ [ $r = 2,8 \times s_r$ ], ng/g	0,25	0,31	0,14	0,28	0,36	0,73	1,88	0,22	0,45	0,64
Reproducibility standard deviation $s_R$ , ng/g	0,16	0,66	0,26	0,22	0,65	0,35	1,76	0,46	0,32	0,96
Reproducibility relative standard deviation $RSD_R$ , %	19	18	32	14	19	18	22	34	14	19
Reproducibility limit $R$ [ $R = 2,8 \times s_R$ ], ng/g	0,45	1,85	0,73	0,62	1,82	0,98	4,93	1,29	0,90	2,69
Recovery, %	87	91	-	-	-	81	82	-	-	-
<sup>a</sup> Spiked sample										
<sup>b</sup> Naturally contaminated sample										

Table B.3 — Precision data for pistachio paste

Parameter	Aflatoxin B <sub>1</sub>					Total Aflatoxins				
	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Sample number	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Year of interlaboratory test	1998					1998				
Number of laboratories	16					16				
Number of samples (duplicates)	1	1	1	1	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	15	12	13	15	14	14	14	13	15	14
Number of outliers	1	4	3	1	2	2	2	3	1	2
Number of accepted results	15	12	13	15	14	14	14	13	15	14
Mean value $\bar{x}$ , ng/g	0,94	3,29	0,74	1,54	2,93	2,0	7,8	0,8	1,7	3,3
Repeatability standard deviation $s_r$ , ng/g	0,13	0,13	0,08	0,27	0,59	0,24	1,82	0,10	0,31	0,66
Repeatability relative standard deviation $RSD_r$ , %	14	4	11	18	20	12	23	12	18	20
Repeatability limit $r$ [ $r = 2,8 \times s_r$ ], ng/g	0,36	0,36	0,22	0,76	1,65	0,67	5,10	0,28	0,87	1,85
Reproducibility standard deviation $s_R$ , ng/g	0,15	1,02	0,12	0,36	0,61	0,36	1,82	0,17	0,42	0,72
Reproducibility relative standard deviation $RSD_R$ , %	16	31	17	23	21	18	23	21	24	22
Reproducibility limit $R$ [ $R = 2,8 \times s_R$ ], ng/g	0,42	2,86	0,34	1,01	1,71	1,01	5,1	0,48	1,18	2,02
Recovery, %	94	82	-	-	-	83	81	-	-	-
<sup>a</sup> Spiked sample										
<sup>b</sup> Naturally contaminated sample										

Table B.4 — Precision data for fig paste

Parameter	Aflatoxin B <sub>1</sub>					Total Aflatoxins				
	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Sample number	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Year of interlaboratory test	1998					1998				
Number of laboratories	16					16				
Number of samples (duplicates)	1	1	1	1	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	15	15	16	14	16	15	15	16	16	16
Number of outliers	1	1	0	2	0	1	1	0	0	0
Number of accepted results	15	15	16	14	16	15	15	16	16	16
Mean value $\bar{x}$ , ng/g	1,10	3,60	1,32	2,07	2,55	2,2	7,8	2,8	3,8	5,2
Repeatability standard deviation $s_r$ , ng/g	0,18	0,39	0,12	0,12	0,41	0,40	1,01	0,25	0,44	0,90
Repeatability relative standard deviation $RSD_r$ , %	17	11	10	6	16	18	13	9	12	17
Repeatability limit $r$ [ $r = 2,8 \times s_r$ ], ng/g	0,5	1,09	0,34	0,34	1,15	1,12	2,83	0,7	1,23	2,52
Reproducibility standard deviation $s_R$ , ng/g	0,21	0,46	0,30	0,31	0,73	0,73	1,28	0,80	1,03	1,56
Reproducibility relative standard deviation $RSD_R$ , %	19	13	23	15	29	32	17	28	29	30
Reproducibility limit $R$ [ $R = 2,8 \times s_R$ ], ng/g	0,59	1,29	0,84	0,87	2,04	2,04	3,58	2,24	2,88	4,37
Recovery, %	109	90	-	-	-	92	81	-	-	-
<sup>a</sup> Spiked sample										
<sup>b</sup> Naturally contaminated sample										

Table B.5 — Precision data for paprika powder

Parameter	Aflatoxin B <sub>1</sub>					Total Aflatoxins				
	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>a</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Sample number	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>a</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Year of interlaboratory test	1998					1998				
Number of laboratories	16					16				
Number of samples (duplicates)	1	1	1	1	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	14	15	15	15	14	13	15	16	16	14
Number of outliers	2	1	1	1	2	3	1	0	0	2
Number of accepted results	14	15	15	15	14	13	15	16	16	14
Mean value $\bar{x}$ , ng/g	0,86	3,41	0,84	1,39	3,02	1,7	7,1	0,9	2,0	4,5
Repeatability standard deviation $s_r$ , ng/g	0,05	0,18	0,12	0,14	0,13	0,11	0,72	0,16	0,23	0,22
Repeatability relative standard deviation $RSD_r$ , %	6	5	14	10	4	6	10	17	12	5
Repeatability limit $r$ [ $r = 2,8 \times s_r$ ], ng/g	0,14	0,5	0,34	0,39	0,36	0,31	2,02	0,45	0,64	0,62
Reproducibility standard deviation $s_R$ , ng/g	0,09	0,35	0,16	0,24	0,28	0,34	1,01	0,31	0,55	0,66
Reproducibility relative standard deviation $RSD_R$ , %	10	10	19	17	9	20	14	34	28	15
Reproducibility limit $R$ [ $R = 2,8 \times s_R$ ], ng/g	0,25	0,98	0,45	0,67	0,78	0,95	2,83	0,87	1,54	1,85
Recovery, %	86	85	-	-	-	71	74	-	-	-
<sup>a</sup> Spiked sample										
<sup>b</sup> Naturally contaminated sample										

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