SLOVENSKI STANDARD
SIST EN 14352:2005

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Ģivila - Doloļ evanje fumonizina B1 in B2 v ģivilih na osnovi koruze - Metoda HPLC s ļ iģļ enjem z imunoafinitetno kolono

Foodstuffs - Determination of fumonisin B1 and B2 in maize based foods - HPLC method with immunoaffinity column clean up

Lebensmittel - Bestimmung von Fumonisin B1 und B2 in Maiserzeugnissen - HPLC-Vefahren mit Immunoaffinitätssäulen-Reinigung

Produits alimentaires - Dosage des fumonisines B1 et B2 dans des aliments a base de mais - Méthode CLHP avec purification par colonne d'immunoaffinité

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Foodstuffs - Determination of fumonisin B1 and B2 in maize based foods - HPLC method with immunoaffinity column clean up

Produits alimentaires - Dosage des fumonisines B1 et B2 dans des aliments à base de maïs - Méthode CLHP avec purification par colonne d'immunoaffinité

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

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

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Contents

page

Foreword 3
1 Scope 4
2 Normative references 4
3 Principle 4
4 Reagents 4
5 Apparatus 7
6 Sampling 8
7 Procedure 8
8 Calculation 10
9 Precision 10
10 Test report 12
Annex A (informative) Precision data 14
Annex B (informative) Typical chromatograms 16
Bibliography 18

Foreword

This document (EN 14352:2004) 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of an identical text or by endorsement, at the latest by January 2005, and conflicting national standards shall be withdrawn at the latest by January 2005.

WARNING — Fumonisins are hepatotoxic, nephrotoxic and carcinogenic to rats and mice. Effects on humans are not fully known. Observe appropriate safety precautions for handling fumonisins. Any laboratory spills should be washed with 5 % solution of sodium hypchlorite. Acetonitrile is hazardous and samples shall be shaken using a shaker, which is housed within a fume cupboard.

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 document specifies a method for the detemination of fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) in maize based foods using high performance liquid chromatography (HPLC) and immunoaffinity clean-up, see [1], [2], [3].

The method has been successfully validated in a collaborative study according to AOAC Guidelines for collaborative study procedures [4] to validate characteristics of a method of analysis for the determination of fumonisins in maize flour and corn flakes containing 323 µg/kg to 1414 µg/kg FB_1 and 90 µg/kg to 558 µg/kg FB_2.

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

Fumonisins are extracted from the sample with a mixture of water, methanol and acetonitrile. The filtered extract is purified by immunoaffinity column and fumonisins are eluted with methanol. The extract is evaporated and the residue is redissolved in a mixture of acetonitrile and water and o-phthaldialdehyde-2-mercaptoethanol (OPA-MCE) is added to form fluorescent fumonisin derivatives. The derivatives are analysed by reverse-phase high performance liquid chromatography (RP-HPLC) with fluorescence detection.

4 Reagents

4.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and only distilled water or water of grade 1 according to EN ISO 3696. Solvents shall be of quality for HPLC analysis.

4.2 Methanol

4.3 Acetonitrile

4.4 o-phosphoric acid, volume fraction $\varphi(H_3PO_4)$ = 85 %

4.5 o-phthaldialdehyde (OPA)

4.6 2-mercaptoethanol (MCE)

4.7 Sodium dihydrogen phosphate solution, substance concentration $c(NaH_2PO_4 \cdot 2H_2O)$ = 0,1 mol/l

Dissolve 15,6 g of $NaH_2PO_4 \cdot 2H_2O$ in 1 l of distilled water.

4.8 Disodium tetraborate solution, $c(Na_2B_4O_7 \cdot 10H_2O)$ = 0,1 mol/l

Dissolve 3,8 g $Na_2B_4O_7 \cdot 10H_2O$ in 100 ml of distilled water.

4.9 Sodium chloride (NaCl)

4.10 Disodium hydrogen phosphate (Na_2HPO_4)

4.11 Potassium dihydrogen phosphate (KH_2PO_4)

4.12 Potassium chloride (KCl)

4.13 Hydrochloric acid (HCl), concentrated

4.14 Extraction solvent

Mix 25 volume parts of acetonitrile (4.3) with 25 volume parts of methanol (4.2) and 50 volume parts of water.

4.15 Acetonitrile-water solution, volume fraction $\varphi(CH_3CN)$ = 50 %

Mix 50 volume parts of acetonitrile (4.3) with 50 volume parts of water.

4.16 Phosphate buffered saline (PBS)

Dissolve 8,0 g of sodium chloride (4.9), 1,2 g of disodium hydrogen phosphate (4.10), 0,2 g of potassium dihydrogen phosphate (4.11) and 0,2 g of potassium chloride (4.12) in approximately 990 ml of water. Adjust pH to 7,0 with concentrated hydrochloric acid (4.13) and bring to 1 l with water. Phosphate buffered saline tablet or ready-to-use solutions can also be used.

4.17 Immunoaffinity column (IAC)

The column shall contain antibodies raised against FB_1 and fumonisin FB_2. The column shall have a total capacity of not less than 5 µg of fumonisins and shall give a recovery of not less than 90 % for FB_1 and FB_2 when applied as a standard solution in a mixture of methanol and PBS containing 5 µg of fumonisins. Up to 10 volume parts of methanol or acetonitrile may be used in the mixture with PBS. The columns shall be warmed up to room temperature before use.

4.18 HPLC mobile phase

Mix 77 volume parts of methanol (4.2) with 23 volume parts of sodium dihydrogen phosphate solution (4.7). Adjust to pH 3,35 with o-phosphoric acid (4.4). Filter the solution through a membrane filter (5.14).

The mobile phase composition may have to be adjusted to conform to individual HPLC column characteristics.

4.19 Derivatization reagent

Dissolve 40 mg of OPA (4.5) in 1 ml of methanol (4.2) and dilute with 5 ml of disodium tetraborate solution (4.8). Add 50 µl of MCE (4.6) and mix. The solution is stable for up to one week at room temperature in the dark in a capped amber vial.

4.20 Stock solutions of FB_1 and FB_2

Prepare a stock solution of FB_1 and a stock solution of FB_2 in acetonitrile-water (4.15) at a mass concentration of 50 µg/ml for each standard substance. Store the solutions at approximately 4 °C.

Fumonisin stock solutions are stable for at least 6 months when stored at approximately 4 °C.

4.21 Mixed fumonisins stock solution

Prepare a mixed stock solution by pipetting 1000 µl of the FB_1 stock solution and 500 µl of the FB_2 stock solution into a 5 ml volumetric flask. Dilute to the mark with the acetonitrile-water solution (4.15) and shake well to obtain a mixed stock solution containing 10 ng FB_1/µl and 5 ng FB_2/µl.

This solution is stable at + 4 °C for at least 6 months. Smaller volumes may be used to prepare the mixed fumonisins stock solution.

4.22 Mixed fumonisins standard solutions for HPLC

Prepare four HPLC mixed standard solutions in 5 ml volumetric flasks according to Table 1. Dilute each standard solution to volume (5 ml) with acetonitrile-water solution (4.15) and mix well.

This solution is stable at + 4 °C for at least 6 months. Smaller volumes may be used to prepare the mixed fumonisins standard solution.

Table 1 — Preparation of mixed standard solutions for HPLC

Mixed standard solution	Volume taken from mixed stock solution (µl)	Addition of acetonitrile-water solution (µl)	Final fumonisin concentration of mixed standard solution, ng/µl	
			FB_1	FB_2
1	50	4 950	0,10	0,050
2	125	4 875	0,25	0,125
3	500	4 500	1,00	0,500
4	1 000	4 000	2,00	1,000

5 Apparatus

5.1 Usual laboratory equipment

and, in particular, the following:

5.2 Orbital shaker

5.3 Centrifuge bottle of 250 ml capacity with screw cap

5.4 Centrifuge capable of a centrifugal force up to 2 500 *g*

5.5 Filter papers, with a pore size of 20 µm to 25 µm

5.6 Glass microfiber filter papers, with a pore size of 11 µm

5.7 Reservoir, 25 ml with luer tip connector for immunoaffinity column (IAC)

5.8 Microlitre syringe(s) or calibrated micropipette(s), 25 µl to 1 000 µl

5.9 Laboratory balance, capable of weighing to the nearest 0,01 g

5.10 Analytical balance capable of weighing to the nearest 0,1 mg

5.11 Vacuum manifold to accommodate immunoaffinity columns

5.12 Vortex mixer

5.13 Solvent evaporator, with heating module, or similar.

5.14 Membrane filter for aqueous solutions, with a pore size of 0,45 µm.

5.15 HPLC apparatus, comprising the following

5.15.1 HPLC pump, isocratic, suitable for e.g. 1 ml/min constant flow rate

5.15.2 Injection system capable to deliver e.g. 20 µl

5.15.3 Analytical reverse-phase separating column, for example octyldecylsilane (ODS), which ensures a baseline resolution of the fumonisin peaks from all other peaks, with the following characteristics:

— stainless steel;

— a length of 150 mm;

— an inner diameter of 4,6 mm;

— a stationary phase with particle size of 5 µm;

— a suitable corresponding reverse-phase guard column.

Columns of other dimensions may also be used.

5.15.4 Fluorescence detector, fitted with a flow cell and set at 335 nm (excitation) and 440 nm (emission). Detection of at least 0,5 ng of FB_1 and FB_2 should be possible (signal to noise = 3).

5.15.5 Data system

6 Sampling

It is important that the laboratory receives a sample, which is truly representative and has not been damaged during transport or storage.

7 Procedure

7.1 Preparation of the test sample

Grind the sample to pass through a 1 mm sieve and homogenize the sample.

7.2 Extraction

Weigh, to the nearest 0,1 g, a 20 g test sample into a 250 ml centrifuge bottle (5.3) and add 50 ml of extraction solvent (4.14). Cover centrifuge bottle and shake for 20 min with orbital shaker (5.2). Centrifuge (5.4) for 10 min at 2 500 g and filter the supernatant through filter paper (5.5) avoiding transferring solid material on the filter. Extract again the remaining solid material by adding 50 ml of extraction solvent to the centrifuge bottle and shake again for 20 min. Centrifuge for 10 min at 2500 *g* and filter the extract through the filter paper.

Mix the two extract filtrates and pipette 10 ml of filtrate into 100 ml flask. Add 40 ml of PBS (4.16) to the 10 ml filtrate and mix well. Filter the diluted extract through microfiber filter (5.6) and collect 10 ml of filtrate (equivalent to 0,4 g test sample) that will be cleaned-up through immunoaffinity column (4.17).

7.3 Immunoaffinity clean-up

Remove top cap from column and connect with reservoir (5.7). Remove end cap from column and attach to vacuum manifold (5.11). Pipet 10 ml aliquot portion of the filtrated sample extract into reservoir. Let filtrate flow through column at approximately 1 to 2 drops per second and discard the eluate. Wash column with 10 ml PBS (4.16) at a rate of 1 to 2 drops per second until air comes through the column. Discard washing and place a 4 ml vial under column. Elute fumonisins with 1,5 ml methanol (4.2), at a flow rate not more than 1 drop per second. Evaporate (5.13) the eluate to dryness under a stream of nitrogen at approximately 60 °C or less. Retain the dried residue at approximately 4 °C until derivatization and HPLC analysis.

7.4 Derivatization

7.4.1 Calibration solutions

Transfer 50 µl of each mixed fumonisin standard solution (4.22) to the base of a small test tube and add 50 µl of the derivatization reagent (4.19) just before analyzing each standard solution. Mix the solutions vigorously for 30 s with a vortex mixer (5.12) and proceed with the HPLC separation (7.5.2) at a reproducible time within 3 min after addition of the derivatization reagent. Alternatively, derivatization could be performed in an automatic system. The final fumonisin concentrations in the calibration solutions are listed in Table 2.

Table 2 — Final concentration of calibration solutions after derivatization

Calibration solution	Final fumonisin concentration in calibration solution (ng/µl)	
	FB_1	FB_2
1	0,050	0,025
2	0,125	0,063
3	0,500	0,250
4	1,000	0,500

7.4.2 Sample test solution

Redissolve the purified sample residue from 7.3 in 200 µl of acetonitrile-water solution (4.15). Transfer 50 µl of extract to the base of a small test tube and add 50 µl of the derivatization reagent (4.19) just before analyzing each extract solution. Mix the solutions vigorously for 30 s with a vortex mixer (5.12) and proceed with the HPLC separation (7.5.3) at a reproducible time within 3 min after addition of the derivatization reagent.

7.5 HPLC

7.5.1 HPLC operating conditions

When a column meeting the specification in 5.15.3, (dimensions 4,6 mm x 150 mm with 5 µm particle size) and the mobile phase specified in 4.18 were used, the following settings were found to be appropriate.

— Flow rate mobile phase (column): 1,0 ml/min;

— Fluorescence detection, emission wavelength: 440 nm;

— excitation wavelength: 335 nm;

— Injection volume: 20 µl.

7.5.2 Calibration graph

Prepare a calibration graph for FB_1 and FB_2 at each day of analysis or whenever the chromatographic conditions have changed by using the fumonisin calibration solutions 1, 2, 3 and 4 according to Table 2.

Inject 20 µl of each derivatized calibration solutions (7.4.1) into the HPLC system at a reproducible time within 3 min after addition of the derivatization reagent (4.19).

The repeatability coefficient of variation of HPLC injections for FB_1 and FB_2 shall be less than 5 % based on 10 replicates injections of the derivatized mixed fumonisins standard solution number 3 (see Table 1).

7.5.3 Sample test solution

Inject for example 20 µl (i.e. 0,02 g of test sample) according to 7.4.2 into the HPLC system at a reproducible time within 3 min after addition of the derivatization reagent (4.19).

7.5.4 Identification

Identify fumonisin B_1 and fumonisin B_2 by comparing the retention times of each sample with the standard solution.