SIST

SLOVENSKI STANDARD
SIST-TS CEN/TS 16187:2011

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Živila - Določevanje fumonizina B1 in fumonizina B2 v predelanih hrani na osnovi koruze za dojenčke in majhne otroke - Metoda HPLC s čiščenjem z imunoafinitetno kolono in fluorescenčno detekcijo po predkolonski derivatizaciji

Foodstuffs - Determination of fumonisin B1 and fumonisin B2 in processed maize containing foods for infants and young children - HPLC method with immunoaffinity column cleanup and fluorescence detection after precolumn derivatization

Lebensmittel - Bestimmung von Fumonisin B1 und Fumonisin B2 in Säuglings- und Kleinkindernahrung auf Maisbasis - HPLC-Verfahren mit Reinigung an einer Immunoaffinitätssäule und Fluoreszenzdetektion nach Vorsäulenderivatisierung

Produits alimentaires - Fumonisine B1 et B2 dans les aliments à base de maïs pour bébés et jeunes enfants

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67.060	Žita, stročnice in proizvodi iz njih	Cereals, pulses and derived products
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English Version

Foodstuffs - Determination of fumonisin B1 and fumonisin B2 in processed maize containing foods for infants and young children - HPLC method with immunoaffinity column cleanup and fluorescence detection after precolumn derivatization

Denrées alimentaires - Dosage de la fumonisine B1 et de la fumonisine B2 dans les aliments pour nourrissons et jeunes enfants contenant du maïs transformé - Méthode par CLHP avec purification sur colonne d'immunoaffinité et détection de fluorescence après dérivation précolonne

Lebensmittel - Bestimmung von Fumonisin B1 und Fumonisin B2 in Säuglings- und Kleinkindernahrung auf Maisbasis - HPLC-Verfahren mit Reinigung an einer Immunoaffinitätssäule und Fluoreszenzdetektion nach Vorsäulenderivatisierung

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Foreword

This document (CEN/TS 16187:2011) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

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Annexes A, B and C are informative.

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1 Scope

This Technical Specification specifies a method for the determination of fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) in processed maize-containing foods for infants and young children by high performance liquid chromatography (HPLC) with immunoaffinity cleanup and fluorescence detection (FLD). This method has been validated in an interlaboratory study via the analysis of both naturally contaminated and spiked samples ranging from 112 µg/kg to 458 µg/kg for FB_1+FB_2, 89 µg/kg to 384 µg/kg for FB_1 and 22 µg/kg to 74 µg/kg for FB_2.

For further information on the validation see Clause 8 and Annex B.

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 3696:1995, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987)*

3 Principle

Fumonisins are extracted from the sample with a mixture of citrate-phosphate buffer with methanol and acetonitrile. The filtered extract is diluted with water and applied to an immunoaffinity column containing antibodies specific to fumonisins. Fumonisins are eluted from the column with methanol and water and quantified by HPLC/FLD with pre-column derivatization with *o*-phthaldialdehyde (OPA) reagent.

4 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696:1995, unless otherwise specified. Solvents shall be of quality for HPLC analysis, unless otherwise specified. Commercially available solutions with equivalent properties to those listed may be used.

WARNING — Dispose of waste solvents according to applicable environmental rules and regulations. Decontamination procedures for laboratory wastes have been reported by the International Agency for Research on Cancer (IARC), see [3].

4.1 Acetonitrile.

WARNING — Acetonitrile is hazardous and samples shall be blended using an explosion proof blender which is housed within a fume cupboard. After blending, samples shall be filtered inside a fume cupboard.

4.2 Methanol.

4.3 O-phthaldialdehyde (OPA).

4.4 Citric acid solution, substance concentration $c(C_6H_8O_7 \cdot H_20) = 0{,}1$ mol/l.

Dissolve 21,0 g of $C_6H_8O_7 \cdot H_20$ in water and dilute to 1 l.

4.5 Disodium hydrogen phosphate solution, $c(Na_2HPO_4) = 0{,}2$ mol/l.

Dissolve 28,4 g of Na_2HPO_4 in water and dilute to 1 l.

4.6 2-mercaptoethanol.

4.7 Citrate-phosphate buffer solution.

Mix one part per volume of citric acid solution (4.4) with one part per volume of disodium hydrogen phosphate solution (4.5).

4.8 Extraction solvent.

Mix two parts per volume of citrate buffer solution (4.7) with one part per volume of methanol (4.2) and one part per volume of acetonitrile (4.1).

4.9 Glacial acetic acid.

4.10 Phosphate buffered saline (PBS) solution, *c*(NaCl) = 137 mmol/l, *c*(KCl) = 2,7 mmol/l, *c*(phosphate buffer) = 10 mmol/l, pH = 7,4 at T = 25 °C.

Dissolve one tablet of commercially available PBS material in 200 ml of water.

4.11 Mixture of acetonitrile and water A.

Mix one part per volume of acetonitrile (4.1) with one part per volume of water. Use this solvent to prepare spiking solutions.

4.12 Mixture of acetonitrile and water B.

Mix three parts per volume of acetonitrile (4.1) with seven parts per volume of water. Use this solvent to prepare calibration solutions and to redissolve dried extracts from immunoaffinity cleanup.

4.13 Sodium tetraborate solution, $c(Na_2B_4O_7{\cdot}10H_2O)$ = 0,1 mol/l.

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

4.14 OPA reagent solution.

Dissolve 40 mg of OPA (4.3) in 1 ml of methanol (4.2) and dilute with 5 ml of sodium tetraborate solution (4.13). Add 50 µl of 2-mercaptoethanol (4.6) and mix for 1 min. This reagent solution is stable for up to one week at room temperature in the dark in a capped amber vial.

4.15 HPLC mobile phase.

4.15.1 HPLC mobile phase A.

Mix 30 parts per volume of acetonitrile (4.1) with 69 parts per volume of water and one part per volume of glacial acetic acid (4.9).

4.15.2 HPLC mobile phase B.

Mix 60 parts per volume of acetonitrile (4.1) with 39 parts per volume of water and one part per volume of glacial acetic acid (4.9).

4.15.3 Linear gradient settings.

Table 1 — Gradient conditions

Time min	Flow rate ml/min	Mobile phase A %	Mobile phase B %
0,00	1,00	60	40
5,00	1,00	60	40
26,00	1,00	12	88
29,00	1,00	12	88
29,10	1,00	-	100
30,00	1,00	-	100
30,10	1,00	60	40
38,00	1,00	60	40

The gradient programme above has proven to give acceptable resolution for FB_1 and FB_2 by using a Waters C_{18} SymmetryShield™ [1)] column. The use of a different column may necessitate adjustment of these conditions until acceptable resolution is achieved.

4.16 Immunoaffinity column.

The immunoaffinity column shall contain antibodies raised against FB_1 and FB_2. The column shall have a capacity of not less than 5 µg of fumonisins and shall give a recovery of not less than 80 % for the sum of FB_1 and FB_2 when applied as a standard solution in PBS containing 5 µg of fumonisins. The columns shall be warmed up to room temperature before use.

4.17 Certified standard solution of fumonisin B_1 (FB_1), mass concentration $\rho(FB_1)$ = 50 µg/ml in a mixture of one part per volume of acetonitrile and one part per volume of water (e.g. Biopure RK 002003 [1)] or equivalent).

4.18 Certified standard solution of fumonisin B_2 (FB_2), $\rho(FB_2)$ = 50 µg/ml in a mixture of one part per volume of acetonitrile and one part per volume of water (e.g. Biopure RK 002004 [1)] or equivalent).

WARNING — Fumonisins are nephrotoxic, hepatotoxic and carcinogenic to rats and mice and classified as possible human carcinogen by IARC. These compounds should be treated with extreme caution. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.19 Mixed FB_1 and FB_2 stock solution.

Prepare a mixed FB_1 and FB_2 stock solution by pipetting 2 000 µl of the FB_1 certified standard solution (4.17) and 500 µl of the FB_2 certified standard solution (4.18) into a vial. Cap the vial and shake well to obtain a stock solution containing 40,0 µg/ml of FB_1 and 10,0 µg/ml of FB_2.

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1) Waters C_{18} SymmetryShield™, Biopure RK 002003 and RK 002004 are examples of suitable products available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

4.20 Diluted mixed FB_1 and FB_2 stock solution.

Pipette 500 µl of the mixed stock solution (4.19) into a 10 ml calibrated volumetric flask. Fill up to the mark with the mixture of acetonitrile water B (4.12) and shake well to obtain a diluted mixed stock solution containing 2,0 µg/ml of FB_1 and 0,5 µg/ml of FB_2.

4.21 Mixed FBs calibration solutions for HPLC.

Prepare five HPLC mixed calibration solutions in 5 ml calibrated volumetric flasks by further diluting the diluted mixed FBs stock solution (4.20) according to Table 2. Make up each calibration solution to volume (5 ml) with the mixture of acetonitrile and water B (4.12) and mix well.

Table 2 — Preparation of mixed FBs calibration solutions for HPLC

HPLC calibration solution	Diluted mixed FBs stock solution (4.20)	Final concentration of mixed FBs calibration solutions (4.21)		Sample equivalent levels of FB_1 and FB_2[a]	
	µl	FB_1 µg/ml	FB_2 µg/ml	FB_1 µg/kg	FB_2 µg/kg
1	100	0,04	0,01	20,0	5,0
2	200	0,08	0,02	40,0	10,0
3	400	0,16	0,04	80,0	20,0
4	1 000	0,40	0,10	200,0	50,0
5	2 400	0,96	0,24	480,0	120,0

[a] For a sample extract reconstituted in 500 µl of the mixture of acetonitrile and water B (4.12).

5 Apparatus

Usual laboratory glassware and equipment and, in particular, the following:

5.1 Analytical balance, capable of weighing to 0,000 1 g.

5.2 Laboratory balance, capable of weighing to 0,1 g.

5.3 Thermostated water bath.

5.4 Conical flasks, of 250 ml capacity with screw caps.

5.5 Orbital shaker.

5.6 Centrifuge, capable of a centrifugal force up to 3 000 *g*.

5.7 Centrifuge bottles, of 250 ml capacity with screw caps.

5.8 Calibrated microlitre pipettes or microlitre syringes, of 100 µl, 200 µl or 1 000 µl capacity.

5.9 Displacement pipettes, of 5 ml, 10 ml or 25 ml capacity.

5.10 Vacuum manifold, to accommodate immunoaffinity columns (4.16).

5.11 Reservoirs (of 25 ml capacity) and **attachments** to fit to columns.

5.12 Vacuum pump.

5.13 Filter paper, e.g. qualitative, strong, fast flow, 24 cm diameter, 30 µm pore size, prefolded or equivalent.

5.14 Glass microfibre filter paper, e.g. 1,6 µm pore size or equivalent.

5.15 Heating block with nitrogen or air gas supply.

5.16 Vials, of 4 ml to 12 ml capacity with screw caps.

5.17 HPLC autosampler vials, of 1,8 ml capacity with caps.

5.18 Glass flat bottom vial insert, of 250 µl volume capacity.

5.19 Vortex mixer, or equivalent.

5.20 HPLC apparatus, comprising the following:

5.20.1 Injection system.

5.20.2 Mobile phase pump (binary, ternary or quaternary pump), capable of generating a binary gradient at 1 ml/min.

5.20.3 Autosampler, capable of performing automated pre-column derivatization with OPA reagent according to 6.4.1.

5.20.4 Analytical reverse-phase HPLC separating column, e.g. C_{18} reverse-phase column, 150 mm × 4,6 mm, 5 µm preceded by a suitable pre-column or guard filter, which provides acceptable retention and resolution for FB_1 and FB_2.

Waters C_{18} SymmetryShield™[2], Agilent Zorbax SB-C_{18}[2] or similar have been found to be suitable.

5.20.5 Column oven, capable to operate at 20 °C.

NOTE C_{18} reverse-phase column can also operate at ambient temperature.

5.20.6 Fluorescence detector, fitted with a flow cell and suitable for measurements with excitation wavelength of 335 nm and emission wavelength of 440 nm.

5.20.7 Recorder, integrator or computer based data processing system.

6 Procedure

6.1 Extraction

The sample should be finely ground to pass through 0,5 mm sieve and thoroughly mixed to ensure complete homogenization.

Weigh, to the nearest 0,1 g, a 20 g test portion of the ground sample into a conical flask (5.4). Add 100 ml of extraction solvent (4.8) and close the flask with screw cap. Heat at 55 °C (static condition) in the thermostated

2) Waters C_{18} SymmetryShield™ and Agilent Zorbax SB-C_{18} are examples of suitable products available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

water bath (5.3) for 60 min. Put the flask on the orbital shaker (5.5) and shake for 30 min at room temperature. Transfer the sample into a centrifuge bottle (5.7) and centrifuge (5.6) at 3 000 *g* for 15 min. Filter through a filter paper (5.13) and collect 10 ml filtrate. Dilute the 10 ml filtrate with 40 ml of water and mix. Filter through a microfibre filter (5.14), collect 25 ml filtrate (equivalent to 1 g of test sample) and proceed rapidly (within the next hour) to immunoaffinity cleanup.

6.2 Immunoaffinity column cleanup

Accommodate the immunoaffinity column (4.16) to the vacuum manifold (5.10) and attach the reservoir. Do not empty storage solution from column. Add the 25 ml of the diluted filtered extract to the reservoir. Elute the extract at a flow rate of about one drop per second. Wash the column with 10 ml of PBS solution (4.10) followed by 2 ml of water at a flow rate of about one drop per second. Pass air or nitrogen for 5 s through the column to expel water. Place a vial or tube under the column.

Elute fumonisins with two times 1 ml of methanol (4.2) and 2 ml water in the following way.

Add 1 ml of methanol on the column. Press the liquid through the column with slight over pressure of air (e.g. with Pasteur balloon or syringe) until the first drop comes out. Close the stopcock immediately. Wait for 1 min. Open the stopcock. Allow elution to take place by gravity force, until the liquid reaches the top layer of the column. Close the stopcock immediately and wait for 1 min. Add the second 1 ml of methanol. Open the stopcock and let half of the liquid (approximately 500 µl) flow through the column by gravity force. Close the stopcock and wait 1 min. Open the stopcock and elute the remaining liquid by gravity force. Pass air or nitrogen through the column to remove the last methanol from the column.

Add 2 ml of water and elute the liquid through the column by gravity force. Pass air or nitrogen through the column to remove the last liquid from the column. Evaporate the eluates to dryness under a stream of air or nitrogen at ca 55 °C (5.15). Redissolve the residue in 500 µl of the mixture of acetonitrile and water B (4.12). Cap the vial and shake on a vortex mixer (5.19) for 30 s, making sure the lower part of the vial is thoroughly rinsed by the solvent and store at 4 °C until HPLC analysis.

6.3 Spiking procedure

To determine recovery spike a fumonisin-free representative ground baby food material with a spiking solution prepared by appropriately diluting the mixed FB_1 and FB_2 stock solution (4.19) with the mixture A of acetonitrile and water (4.11). No more than 1 ml of spiking solution should be added to each test portion. The fumonisin mass concentration of the spiking solution should be prepared according to the spiking level chosen. Leave the spiked sample to stand overnight to ensure evaporation of the solvent.

6.4 Derivatization and HPLC determination

6.4.1 Automated pre-column derivatization programme

Transfer 110 µl of extract to an HPLC autosampler vial containing a glass flat bottom vial insert (5.18). Put the vial containing 110 µl of sample extract in the autosampler.

The following conditions have proven to produce satisfying results.

Aspirate an air segment of 5 µl to separate the wash solvent in the buffer tubing from the OPA reagent (4.14). Aspirate 50 µl of OPA reagent to flush the tubing and needle. Unload the syringe to the syringe-waste position. Aspirate 110 µl of OPA reagent and dispense it to the vial insert containing 110 µl of sample extract or calibration solution. Rinse the buffer tubing and needle with the mixture of acetonitrile and water A (4.11). Aspirate 55 µl of derivatized solution and dispense it back into the vial insert; repeat this four times. Rinse the buffer tubing and needle with wash solvent (4.11). Wait for 20 s. Inject 50 µl in full loop mode. Rinse the buffer tubing and needle with wash solvent (4.11) five times. This automated derivatization procedure takes 2,5 min.

NOTE Different pre-column derivatization conditions (volumes, derivatization programme, etc.) could be used according to your autosampler, bearing in mind that the signals (peak area or height) of FB_1 or FB_2 in the sample extract should fall within the calibration range.