SLOVENSKI STANDARD

SIST EN 16006:2011

01-oktober-2011

Krma - Določanje vsote fumonizinov B1 in B2 v krmni mešanici z imunoafinitetnim čiščenjem in RP-HPLC s fluorescentno detekcijo po pred- ali pokolonski derivatizaciji

Animal feeding stuffs - Determination of the Sum of Fumonisin B1 & B2 in compound animal feed with immunoaffinity clean-up and RP-HPLC with fluorescence detection after pre- or post-column derivatisation

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Futtermittel - Bestimmung der Summe der Fumonisine B1 und B2 in Mischfutter mit Reinigung an einer Immunoaffinitätssäule und RP-HPLC-Verfahren mit Fluoreszenzdetektion nach Vor- oder Nachsäulenderivatisierung

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Aliments pour animaux - Dosage de la somme des fumonisines B1 et B2 dans les aliments pour animaux avec purification par immuno-affinité et RP-HPLC avec détection par fluorescence après dérivation pré- ou post-colonne

Ta slovenski standard je istoveten z: **EN 16006:2011**

ICS:

65.120	Krmila	Animal feeding stuffs

SIST EN 16006:2011 **en,fr,de**

2003-01.Slovenski inštitut za standardizacijo. Razmnoževanje celote ali delov tega standarda ni dovoljeno.

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EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 16006

August 2011

ICS 65.120

English Version

Animal feeding stuffs - Determination of the Sum of Fumonisin B1 & B2 in compound animal feed with immunoaffinity clean-up and RP-HPLC with fluorescence detection after pre- or post-column derivatisation

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This European Standard was approved by CEN on 25 June 2011.

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This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

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

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Foreword

This document (EN 16006:2011) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

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 February 2012, and conflicting national standards shall be withdrawn at the latest by February 2012.

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

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

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

WARNING — Fumonisins are toxic. Gloves and safety glasses should be worn at all times and all standard and sample preparation stages should be carried out in a fume cupboard.

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1 Scope

This European Standard is applicable to the determination of Fumonisin B_1 & B_2 (FB_1 & FB_2) in compound animal feed at levels starting from 3 mg/kg up to 16 mg/kg.

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 835, *Laboratory glassware — Graduated pipettes (ISO 835:2007)*

EN ISO 1042, *Laboratory glassware — One-mark volumetric flasks (ISO 1042:1998)*

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

3 Principle

FB_1 and FB_2 are extracted from the test material with a solution of 50% methanol in phosphate-buffered saline (PBS). The extract is then diluted with PBS and cleaned up using immunoaffinity columns (IAC). FB_1 and FB_2 are eluted from the IAC using methanol and then water. After volume adjustment, the eluate is directly injected into the HPLC and FB_1 and FB_2 are detected by their fluorescence after either pre- or post column derivatisation.

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4 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade. Solvents shall be of HPLC or better quality and only double-distilled water or water of at least grade 2 as defined in EN ISO 3696 shall be used.

4.1 Double distilled or deionised water (EN ISO 3696)

4.2 Methanol, CH_3OH

WARNING — Methanol is hazardous and handling should be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) should be worn.

4.3 Acetonitrile, CH_3CN

WARNING — Acetonitrile is hazardous and handling should be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) should be worn.

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4.4 Potassium chloride, KCl

4.5 Sodium chloride, NaCl

4.6 Disodium hydrogenphosphate dodecahydrate, Na_2HPO_4*12 H_2O

4.7 Disodium tetraborate decahydrate, $Na_2B_4O_7$ *10H_2O

4.8 Sodium carbonate, Na_2CO_3.

4.9 Boric acid, H_3BO_3

4.10 Potassium sulphate, K_2SO_4

4.11 NAC, N-Acetyl-L-Cystein, $C_5H_9NO_3S$

4.12 OPA, o- Phthalaldehyde, $C_6H_4(CHO)_2$

4.13 BME, β-Mercaptoethanol, $HOCH_2CH_2SH$

4.14 Formic Acid, HCO_2H, 98%-100%

WARNING — Concentrated Formic acid is hazardous and handling should be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) should be worn.

4.15 PBS concentrate, Phosphate buffered saline concentrate

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Dissolve the following in 1 800 ml of water (4.1):

- 4 g KCl (4.4);
- 160 g NaCl (4.5);
- 72 g Na_2HPO_4*12 H_2O (4.6).

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Adjust to pH 7,4 with 10 mol/l HCl and make up to 2 000 ml.

4.16 PBS Ready to use

Dilute 100 ml of PBS concentrate (4.15) to 1 000 ml with water (4.1),

or

PBS tablets, Phosphate buffered saline tablets

one tablet dissolved in 200 ml of water (4.1) yields 0,01 mol/l phosphate buffer, 0,002 7 mol/l potassium chloride and 0,137 mol/l sodium chloride, pH 7,4, at 25°C (e.g. Sigma P4417).

4.17 Diluent

Mix 50 parts per volume methanol (4.2) with 50 parts per volume water (4.1).

4.18 Extraction solvent

Mix 50 parts per volume methanol (4.2) with 50 parts per volume of PBS (4.16).

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4.19 Reaction buffer

4.19.1 Post-column derivatisation (0,006 mol/l OPA, 0,006 mol/l NAC, 0,384 mol/l sodium carbonate, 0,216 mol/l boric acid and 0,108 mol/l potassium sulphate):

- Dissolve 40,7 g sodium carbonate (4.8), 13,4 g boric acid (4.9) and 18,8 g potassium sulphate (4.10) per 1,0 l of water (4.1);
- stir for 10 min;
- add 800 mg of OPA (4.12) per 1,0 l of the above solution;
- add 1 g of NAC (4.11) per 1,0 l of the above solution;
- stir for 10 min;
- sonicate for 15 min;
- stir for 10 min;
- sonicate again for 15 min and
- filter the solution through a 0,45 µm nylon filter (5.17).

Proper dissolution of the OPA is very important!

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The reaction buffer should not be changed within a sequence of HPLC runs.

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Prepare fresh for every sequence of HPLC runs.

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4.19.2 Pre-column derivatisation (0,05 mol/l OPA, 0,12 mol/l BME, 0,08 mol/L disodium tetraborate, 16,7% methanol):

- Dissolve 40 mg OPA (4.12) in 1,0 ml methanol (4.2);
- mix until completely dissolved;
- add 5,0 ml of a 0,1 mol/l solution of disodium tetraborate decahydrate (3,8 g / 100 ml; 4.7);
- mix thoroughly;
- add 50 µl of BME (4.13), and
- mix thoroughly.

Alternatively:

- Phthaldialdehyde Reagent.

4.20 FB_1 & FB_2 stock solution:

- A certified solution of Fumonisin FB_1 and FB_2 of ca. 50 µg/ml each in an appropriate solvent. Take exact concentration from certificate;

or

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- Separate certified solutions of Fumonisin FB_1 and FB_2 in appropriate solvents that will be mixed such that a stock solution containing ca. 50 µg/ml of each is obtained. Calculate exact concentrations from certificates and dilutions.

NOTE 1 The above solutions (4.20) may also be prepared gravimetrically by accurately weighing the dry substance and the solvent used to dissolve it. Accurately measuring the volume of the solvent is also allowed.

NOTE 2 The above solutions may be stored for up to six months at below -18°C in the dark.

4.21 FB_1 & FB_2 diluted stock solution for calibration

- Add 160 µl of the FB_1 & FB_2 stock solution (4.20) to a 2 ml volumetric flask (5.13), and
- Make up to mark (2,0 ml) with diluent (50% methanol, 4.17).

This will result in 2,0 ml of a solution containing ca. 4 µg/ml FB1 & FB2 each in mostly methanol /water (50/50, v/v).

4.22 Calibration solutions

From the diluted stock solution for calibration (4.21) prepare five levels of calibration solutions by adding the volumes of diluted stock solution listed in the following table to a volumetric flask (5.13) of the indicated volume and make up to the mark with diluent (4.17).

Calculate the concentrations of FB_1 & FB_2 for the different calibration levels by dividing the certified or calculated concentrations of the stock solution (4.20) by the final dilution stated below. Should you observe saturation of the detector signal at the highest calibration level dilute 250 µl of diluted stock solution into 2,0 ml for a final dilution of 100.

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Table 1 — Recommended calibration solutions (4.22) for the determination of the sum of Fumonisin B1 & B2

Calibrant	Diluted stock solution (4.21) (µl)	Volumetric flask (5.13) (ml)	Final dilution of stock solution (4.20)	Approx. concentration of FB1 & FB2 each (ng/ml)
1	50	20,0	5 000	10
2	125	10,0	1 000	50
3	125	5,0	500	100
4	500	2,0	50	1 000
5	1 000	2,0	25	2 000

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These calibration levels are recommendations and may be adjusted to the individual needs. The exact concentrations of the calibration levels should be calculated based on the final dilution and the exact concentration of the stock solution (4.20).

NOTE The above solutions may be stored for up to 5 days at 6 °C ± 2°C in the dark.

4.23 IAC (Immunoaffinity column)

The immunoaffinity columns must contain a stationary phase with immobilized monoclonal antibodies specific to, at least, Fumonisin B_1 and B_2. To be suitable for this method they must meet the requirements stated below:

An aliquot of more than 5 ml of an extract of a fumonisin-free representative compound animal feed material is spiked with FB_1 & FB_2 in equal parts at either 920 (high) ng/ml or 110 (low) ng/ml for the sum of both. Then dilute 5,0 ml of that spiked extract to a total volume of 50,0 ml (see 6.2).

Following the procedures described in 6.3 and 6.4 this will result in expected concentrations in the injection solutions of either 460 ng/ml or 55 ng/ml for the sum of FB_1 & FB_2.

After measuring (Clause 7) these solutions the observed concentrations of FB_1 & FB_2 can be calculated with Equation (1) and Equation (2) of Clause 8. Dividing the sum of the observed concentrations of FB_1 & FB_2 by the expected concentrations will result in the yield of the immunoaffinity columns.

These yields must be 99 % ± 18 % (U, k=2) at the high level and 118 % ± 18 % (U, k=2) at the low level.

The above column test should be performed for each level on at least three randomly selected columns of every new batch of immunoaffinity columns which will be used. Should the tested batch not meet the above requirements either a new batch which does should be obtained or the conditions described in 6.3 need to be adjusted such that the requirements are met (the user instructions supplied with the columns are a good starting point).

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Any changes to the clean-up procedures will necessitate a revalidation of the clean-up and all subsequent steps (chromatography).

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5 Apparatus

Usual laboratory equipment and, in particular, the following:

5.1 Mill

5.2 Tumble mixer

Creates a folding motion of the material through, for instance, a rotating drum with internal fins and paddels or moving a closed container head-over-heels.

5.3 Vortex mixer

5.4 Laboratory shaker

5.5 250 ml flasks with screw caps

5.6 Graduated cylinders, 5 ml, 50 ml, 1 000 ml and 2 000 ml

5.7 Graduated pipettes (Class A, EN ISO 835) 2 ml, 10 ml and 50 ml

5.8 Analytical balance (d= 0,01g)

5.9 Glass micro fibre filter, binder-free with ca. 2 µm pore size

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5.10 Filter funnel, of appropriate size

5.11 Auto sampler vials, of appropriate size with caps

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5.12 Reservoirs for IACs

Of appropriate size with adapter for connection to top of IACs.

5.13 Volumetric flasks (Class A, EN ISO 1042) 2 ml, 5 ml, 10 ml and 20 ml

5.14 Gastight glass syringes and/or direct displacement pipettors

Capable of precisely dispensing the following volumes: 5 µl, 50 µl, 125 µl, 160 µl, 500 µl, and 1 000 µl.

5.15 Support stand for immunoaffinity columns, of appropriate size

5.16 HPLC instrumentation, comprising the following:

5.16.1 Solvent delivery system

Capable of generating a binary gradient with sufficient precision at the required pressures.

5.16.2 Auto sampler

Capable of injecting sufficient volumes of injection solution with sufficient repeatability and, for pre-column derivatization, capable of mixing reagent and sample solution before injection.