SLOVENSKI STANDARD
oSIST prEN 17279:2018

01-oktober-2018

Živila - Večelementna metoda za pregled ohratoksina A, aflatoksina B1, deoksinivalenola, zearaleona ter fumonizinov B1 in B2 v živilih, razen v hrani za dojenčke in majhne otroke, s HPLC-MS/MS

Foodstuffs - Multimethod for the screening of ochratoxin A, aflatoxin B1, deoxynivalenol, zearalenone and fumonisin B1 and B2 in foodstuffs, excluding foods for infants and young children, by HPLC-MS/MS

Lebensmittel - Multiverfahren mit LC-MS/MS zum Screening auf Ochratoxin A, Aflatoxin B1, Deoxynivalenol, Zearalenon, T2-HT-2-Toxin und Fumonisin B1 und B2 in Lebensmitteln außer Lebensmittel für Säuglinge und Kleinkinder

Produits alimentaires - Multiméthode de dépistage de l'ochratoxine A, de l'aflatoxine B1, du déoxynivalénol, de la zéaralénone et de la fumonisine B1 et B2 dans les produits alimentaires, à l'exception des aliments pour nourrissons et jeunes enfants, par CLHP-SM/SM

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

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European foreword

This document (prEN 17279:2018) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This document is currently submitted to the CEN Enquiry.

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

Introduction

Mycotoxins are fungal metabolites that may occur in various foodstuffs. Cereals and cereal products, peanuts, dried fruits and relevant derived products are most likely to be affected by the mycotoxins covered by this document (aflatoxin B1, deoxynivalenol, fumonisin B1 and B2, ochratoxin A, HT-2 and T-2 toxins, and zearalenone).

WARNING 1 — Suitable precaution and protection measures need to be taken when carrying out working steps with harmful chemicals. The hazardous substances ordinance (EU) 1907/2006 [2], should be taken into account as well as appropriate National statements.

WARNING 2 — The use of this document can involve hazardous materials, operations and equipment. This document does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this document to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This document describes a screening method for the determination of aflatoxin B1, deoxynivalenol, fumonisin B1 and B2, ochratoxin A, HT-2 and T-2 toxins, and zearalenone in foodstuffs by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS).

The aim of the screening method is to test compliance of foodstuff with regulatory limits or to determine whether a certain pre-defined level (the screening target concentration, STC) is exceeded or not. The result of the screening is either "negative" or "suspect". "Negative" (screen negative) means that the targeted mycotoxins are not detected or potentially present but below the STC. "Suspect" (screen positive) means that the established cut-off level is exceeded and the sample can contain one or more mycotoxins at a level higher than the STC.

For full identification and accurate quantification a second confirmatory quantitative analysis method is required which is outside the scope of this document.

The method is suitable for various types of foodstuff and has been validated for representative matrices from four commodity groups:

— high starch and/or protein content and low water and fat content: wheat, cereal mixture, wheat flour and cornflakes;

— high oil content: peanuts;

— high sugar low water content: figs;

— high water content: grape juice.

During validation, cut-off levels were established for the following screening target concentrations:

— aflatoxin B1: 2 µg/kg to 5 µg/kg;

— deoxynivalenol: 250 µg/kg to 865 µg/kg;

— fumonisin B1: 200 µg/kg to 790 µg/kg;

— fumonisin B2: 110 µg/kg to 230 µg/kg;

— ochratoxin A: 4 µg/kg to 9 µg/kg;

— T-2 toxin: 25 µg/kg;

— HT-2 toxin: 25 µg/kg to 50 µg/kg;

— zearalenone: 30 µg/kg to 100 µg/kg.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at http://www.electropedia.org/
- ISO Online browsing platform: available at http://www.iso.org/obp

4 Principle

The mycotoxins are extracted from the homogenized sample material, after addition of water, by shaking with acidified acetonitrile. After a salt-induced phase partitioning and centrifugation, the acetonitrile extract is diluted with water, optionally filtered, and analysed by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). The relative response of each mycotoxin to its isotopic label, added to the final extract at the screening target concentration (STC), is tested against an established cut-off level.

5 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 LC analysis, unless otherwise specified.

5.1 Water, deionised.

5.2 Water, LC-MS grade, double distilled or water of grade 1 as defined in EN ISO 3696:1995.

5.3 Acetonitrile, p.a.

5.4 Acetic acid, purity greater than 98 % (w/w).

5.5 Magnesium sulfate ($MgSO_4$) - anhydrous, p.a.

5.6 Aflatoxin B1 (AB1) e.g. crystalline, as a film or as certified standard solution.

5.7 Deoxynivalenol (DON) e.g. crystalline, as a film or as certified standard solution.

5.8 Fumonisin B1 (FB1) e.g. crystalline, as a film or as certified standard solution.

5.9 Fumonisin B2 (FB2) e.g. crystalline, as a film or as certified standard solution.

5.10 HT-2 toxin (HT2) e.g. crystalline, as a film or as certified standard solution.

5.11 Ochratoxin A (OTA) e.g. crystalline, as a film or as certified standard solution.

5.12 T-2 toxin (T2) e.g. crystalline, as a film or as certified standard solution.

5.13 Zearalenone (ZEN) e.g. crystalline, as a film or as certified standard solution.

5.14 $^{13}C_{17}$ Aflatoxin B1 (U-AB1) e.g. solution ρ = 0,5 mg/l, in acetonitrile.

5.15 $^{13}C_{15}$ Deoxynivalenol (U-DON) e.g. solution ρ = 25 mg/l, in acetonitrile.

5.16 $^{13}C_{34}$ Fumonisin B1 (U-FB1) e.g. solution ρ = 25 mg/l, in acetonitrile/water.

5.17 $^{13}C_{34}$ Fumonisin B2 (U-FB2) e.g. solution ρ = 25 mg/l, in acetonitrile/water.

5.18 $^{13}C_{22}$ HT-2 toxin (U-HT2) e.g. solution ρ = 25 mg/l, in acetonitrile.

5.19 $^{13}C_{20}$ Ochratoxin A (U-OTA) e.g. solution ρ = 10 mg/l, in acetonitrile.

5.20 $^{13}C_{24}$ T-2 toxin (U-T2) e.g. solution ρ = 25 mg/l, in acetonitrile.

5.21 $^{13}C_{18}$ Zearalenone (U-ZEN) e.g. solution ρ = 25 mg/l, in acetonitrile.

5.22 Extraction solution, acetonitrile containing 1 % of acetic acid.

Add 1 part per volume of acetic acid (5.4) to 99 parts per volume of acetonitrile (5.3) and mix. This solution is stable for six months if stored at room temperature.

5.23 Mixed mycotoxin stock solution.

The individual solutions are either prepared by dissolving neat (solid) standards in an appropriate solvent, or from individual stock solutions purchased as such. The mycotoxins covered in this standard dissolve well in acetonitrile, with the exception of fumonisins for which acetonitrile/water (50+50, v+v) is recommended for preparing individual stock solutions.

Calculate for each of the individual mycotoxins the mass concentration, ρ, in ng/ml according to Formula (1):

$$c = (20/D) \times w_{STC} \quad (1)$$

where

D is the dilution factor of the sample in the final extract (D = 4 by default);

w_{STC} is the screening target concentration (mass fraction) in the sample, in µg/kg.

EXAMPLE For a mycotoxin with an STC of 1 000 µg/kg in the sample, the mass concentration of this mycotoxin in the mixed mycotoxin stock solution is 5 000 ng/ml.

Prepare a mixed mycotoxin stock solution by combining the appropriate volumes of individual mycotoxin solutions, using the appropriate pipets (6.6) and acetonitrile/water (80+20, v+v). This solution is stable for six months if stored at 4 °C.

This mixed mycotoxin stock solution may be used for the preparation of positive control samples (7.3).

5.24 Mixed internal standard (ISTD) intermediate solution (isotopically labelled mycotoxins).

Isotopically labelled mycotoxins are generally available as certified standard solutions. Prepare a mixed internal standard solution in acetonitrile/water (80+20, v+v), containing all isotopically labelled mycotoxins at a mass concentration calculated according to Formula (1).

EXAMPLE For a mycotoxin with an STC of 10 µg/kg in the sample, the mass concentration of the corresponding isotopic label in the internal standard solution is 50 ng/ml.

This solution is stable for six months if stored in the dark at 4 °C.

This solution is used as calibrant and is to be added to each of the sample extracts (7.3).

5.25 Mixed mycotoxin solvent standard solution.

Prepare a mixed mycotoxin solvent standard by combining 1 part per volume of the mixed mycotoxin stock solution (5.23), 1 part per volume of the internal standard solution (5.24), 8 parts per volume of acetonitrile containing 1 % acetic acid (5.22), and 10 parts per volume of water (5.2). A volume of typically 400 µl is freshly prepared for each batch of analysis.

EXAMPLE Preparation in vial:

160 µl acetonitrile containing 1 % acetic acid (5.22);

20 µl mixed mycotoxin stock solution (5.23);

20 µl mixed internal standard intermediate solution (5.24);

200 µl water (5.2).

The mixed mycotoxin solvent standard solution is used to check correct measurement of the mycotoxins and their isotopic labels (7.4.4).

6 Apparatus and equipment

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

6.1 Conical polypropylene screw cap centrifuge tubes, 50 ml with caps.

6.2 Analytical balance, accuracy: 0,01 mg.

6.3 Laboratory balance, accuracy: 0,01 g.

6.4 Adjustable mechanical vertical or **horizontal shaker** or **rotary tumbling machine.**

6.5 Laboratory shaker.

6.6 Pipets, adjustable, e.g. 10 µl to 100 µl and 100 µl to 1000 µl, suited for organic solvents (e.g. positive displacement pipets),, with appropriate tips.

6.7 Centrifuge, capable of generating a relative centrifugal force of 3 000 *g*.

6.8 Vials, 1,5 ml to 2 ml, made of glass vials or polypropylene with screw cap.

6.9 Syringe filter or **centrifuge filter,** 0,20 µm to 0,45 µm, made of nylon or polytetrafluoroethylene (PTFE).

6.10 Auto sampler vials, of appropriate size for the auto sampler in use, e.g. glass with insert vials, filter vials (polytetrafluoroethylene (PTFE) 0,45 µm), with crimp cap or equivalent.

6.11 LC-MS/MS system, with the following components:

6.11.1 LC pump, capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

6.11.2 Injection system, capable of injecting an appropriate volume of injection solution with sufficient accuracy, and cross-contamination below 0,1 %.

6.11.3 LC column, capable of retaining the target mycotoxins, preferably with a retention factor of at least two.

6.11.4 Column oven, capable of maintaining a constant temperature.

6.11.5 Tandem mass spectrometer (MS/MS), capable of ionization of the mycotoxins (either resulting in positive or negative ions), performing Selected Reaction Monitoring (SRM), and with a sufficiently wide dynamic range.

NOTE Instruments capable of alternating measurement of positive and negative ions (pos/neg switching) are beneficial because these can cover all target analytes within one run.

6.11.6 Data evaluation system

7 Procedure

7.1 Preparation of control samples

With each batch of samples, one negative control and one positive control is used.

The negative control is either a sample free of the target mycotoxins (non-detectable or < 10 % of the STC), or, if not available, a reagent blank.

The positive control is a sample free of the target mycotoxins (non-detectable or < 10 % of the STC) which is spiked with the mycotoxins at STC. Alternatively, a reference material known to contain the target mycotoxins at a level close to the STC is used.

For preparation of a positive control sample: spike a sample free of the target mycotoxins by adding 1,0 ml of mixed mycotoxin solution (5.23).

7.2 Extraction of mycotoxin from the samples

Finely grind the laboratory sample and homogenize it.

The amount of homogenized test sample examined is 5 g. For samples homogenized by slurry mixing, the amount of slurry corresponding to 5 g of the original sample is used.

Weigh the test portion indicated in Table 1, to the nearest 0,01 g (6.3), into a 50 ml centrifuge tube (6.1).

Add water (5.1) and acidified acetonitrile (5.22) to the sample as indicated in Table 1. Close the tube, and shake thoroughly by hand. Make sure dry samples are suspended into the liquid.

Table 1 — Composition

Sample	Test portion g	Added water ml	Extraction solvent (5.22)[d] ml	$MgSO_4$ (5.5)[e] g
Sample with moisture content < 15 %[a]	5	10	10	5
Slurried sample 1+1[b]	10	5	10	5
Slurried sample 1+1,5[b]	12,5	2,5	10	5
Slurried sample 1+2[b]	15	0	10	5
Slurried sample 1+3[b]	20	0	10	7,5
Samples with moisture content > 85 %[c]	5	5	10	5

[a] e.g. dry milled cereals.

[b] x + y means: x g sample slurried with y ml of water.

[c] most fresh fruits/vegetables, liquids .

[d] the ratio of water/extraction solvent is 1+1, v+v, except for slurry 1+3 (1+0,67, v+v).

[e] the amount of magnesium sulfate is 0,5 g per ml of total water (from sample + added) in the extraction tube.

Place the tubes in a mechanical shaker (6.4) and shake for 30 min.

Open the tube, add the amount of magnesium sulfate (5.5) indicated in Table 1 to the tube, close the tube, immediately shake for approximately 5 s to avoid formation of lumps of magnesium sulfate. Shake the tube vigorously for approximately 30 s by hand or in a mechanical shaker (6.5).

Centrifuge the tube at approximately 3 000 *g* (6.7) for at least 5 min to aid settlement of particulate matter and phase separation.

NOTE After phase partitioning, the volume of the acetonitrile phase (upper layer) is approximately 10,7 ml and contains approximately 17 % water [5].

7.3 Test solution

Using the appropriate pipets, add10 parts per volume of extract into a vial (6.8 or 6.10), furthermore add 1 part per volume of mixed internal standard solution (5.24) and 9 parts per volume of water (5.1). Mix using a shaker (6.5) for at least 5 s.

NOTE 1 Volumes of 200 µl of extract, 20 µl of internal standard solution (5.24), and 180 µl of water have shown to work well.

The final extract can be turbid. Turbid extracts can be injected without adverse effects on the analysis. In case precipitates occur, these shall be removed by centrifugation or by filtration, either using a filter vial (6.10), centrifuge filter, or a syringe filter (6.9). In the latter case, the total volume of 400 µl indicated above might be too small.

NOTE 2 The final extract composition is acidic acetonitrile/water (approximately 1+1, v+v). A lower content of organic solvent can result in solubility limitations of higher levels of less water soluble mycotoxins such as zearalenone.