SLOVENSKI STANDARD
oSIST prEN 17280:2018

01-oktober-2018

Živila - Določevanje zearalenona in trihotecena, vključno z deoksinivalenolom (DON) in njegovimi acetiliranimi derivati (3-acetil- DON in 15-acetil-DON), nivalenolom (NIV) in toksinoma T-2 in HT-2, v žitu in žitnih proizvodih z LC-MS/MS

Foodstuffs - Determination of zearalenone and trichothecenes including deoxynivalenol (DON) and its acetylated derivatives (3-acetyl- DON and 15-acetyl-DON), nivalenol (NIV) and T-2 and HT-2 toxin in cereals and cereal products by LC-MS/MS

Lebensmittel - Bestimmung von Zearalenon und Trichothecenen einschließlich Deoxynivalenol (DON) und den acetylierten Derivaten (3-Acetyl-DON und 15-Acetyl-DON), Nivalenol (NIV), T-2- und HT-2-Toxin in Getreide und Getreideerzeugnissen mit LC-MS/MS

Produits alimentaires - Dosage de la zéaralénone et des trichothécènes y compris du déoxynivalénol (DON) et ses dérivés acétylés (3-acétyl-DON et 15-acétyl-DON), du nivalénol (NIV) et des toxines T-2 et HT-2 dans les céréales et les produits céréaliers par CL-SM/SM

Ta slovenski standard je istoveten z: **prEN 17280:2018**

ICS:

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

oSIST prEN 17280:2018 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

DRAFT
prEN 17280

August 2018

ICS 67.060

English Version

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This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 275.

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Recipients of this draft are invited to submit, with their comments, notification of any relevant patent rights of which they are aware and to provide supporting documentation.

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

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Contents

Page

European foreword

This document (prEN 17280:2018) 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 standardization request given to CEN by the European Commission and the European Free Trade Association.

Introduction

The mycotoxins nivalenol, deoxynivalenol, and its acetyl derivatives (3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol), T-2 toxin and its metabolite HT-2 toxin, and zearalenone are produced by various *Fusarium* species. Cereals like maize, wheat, barley, oats, rye and relevant derived products are most likely to be affected.

WARNING 1 — Suitable precaution and protection measures need to be taken when carrying out working steps with harmful chemicals. The hazardous substances ordinance, Regulation (EC) No 1907/2006 [3], 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.

WARNING 3 — *Fusarium* toxins (zearalenone, deoxynivalenol, T-2 and HT-2 toxins) have been implicated as the causative agents in a variety of animal diseases, such as pulmonary oedema, infertility, diarrhoea, vomiting, anorexia, leukopenia, immunosuppression, skin and gastrointestinal irritation, hemorraging, etc., and have been associated to some human diseases. The IARC has defined zearalenone, deoxynivalenol and T-2 as not classifiable as to their carcinogenicity to humans (Group 3) [4].

1 Scope

This document describes a procedure for the determination of nivalenol (NIV), deoxynivalenol (DON) and its acetyl derivatives (3-acetyl-DON and 15-acetyl-DON), HT-2 and T-2 toxins (HT-2, T-2) and zearalenone (ZEA) in cereals and cereal products by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) after cleanup by solid phase extraction (SPE).

The method has been validated with both contaminated and spiked samples of wheat, wheat flour, and wheat crackers.

Validation levels for NIV ranged from 27,7µg/kg to 377,8 µg/kg.

Validation levels for DON ranged from 233,9µg/kg to 2420,0 µg/kg.

Validation levels for 3-acetyl-DON ranged from 18,5µg/kg to 136,5 µg/kg.

Validation levels for 15-acetyl-DON ranged from 11,4µg/kg to 141,8 µg/kg.

Validation levels for HT-2 ranged from 6,6 µg/kg to 133,8 µg/kg.

Validation levels for T-2 ranged from 2,1 µg/kg to 37,6 µg/kg.

Validation levels for ZEA ranged from 31,6µg/kg to 229,7 µg/kg

Laboratory experiences have shown that this method is also applicable to barley and oat flour, and rye based crackers [5], however, this has not been validated in a collaborative study.

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, *Water for analytical laboratory use - Specification and test methods (ISO 3696)*

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

Trichothecenes and zearalenone are extracted from the commodities with a mixture of acetonitrile-water. The extract is filtered and evaporated to dryness. The residue is dissolved with a mixture of methanol and water and applied to a polymeric solid phase extraction column. The mycotoxins are purified and concentrated on the column then released using methanol as eluent. Isotopically labelled mycotoxins are added to the column eluate before evaporating it to dryness. After reconstitution of the dry extract with the injection solvent, the mycotoxins are quantified by reversed phase high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS).

5 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696, unless otherwise specified.

5.1 Nitrogen or **oil-free compressed air.**

5.2 Water, deionized.

5.3 Water, HPLC quality.

5.4 Acetonitrile, HPLC quality.

5.5 Methanol, HPLC quality.

5.6 Ammonium acetate, for mass spectrometry, $c(CH_3COONH_4) \geq 99{,}0$ %.

5.7 Extraction mixture.

Mix 84 parts per volume of acetonitrile (5.4) and 16 parts per volume of water (5.2).

5.8 Solid phase extraction (SPE) columns, containing 60 mg of a balanced hydrophilic/lipophilic polymer able to retain both polar and non-polar compounds (Waters Oasis® HLB is suitable) [2].

5.9 Nivalenol (NIV) e.g. crystalline, as a film or as certified standard solution.

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

5.11 3-Acetyl-DON (3-AcDON) e.g. crystalline, as a film or as certified standard solution.

5.12 15-Acetyl-DON (15-AcDON) e.g. crystalline, as a film or as certified standard solution.

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

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

5.15 Zearalenone (ZEA) e.g. crystalline, as a film or as certified standard solution.

5.16 Nivalenol isotopically labelled internal standard ($^{13}C_{15}$NIV) e.g. nivalenol^{13}C-labelled (fully) ρ = 25 µg/ml, in acetonitrile.

5.17 Deoxynivalenol isotopically labelled internal standard ($^{13}C_{15}$DON) e.g. deoxynivalenol^{13}C-labelled (fully) ρ = 25 µg/ml, in acetonitrile.

5.18 3-Acetyl-DON isotopically labelled internal standard ($^{13}C_{17}$3-AcDON) e.g. 3-acetyl-DON ^{13}C-labelled (fully) ρ = 25 µg/ml, in acetonitrile.

5.19 HT-2 toxin isotopically labelled internal standard ($^{13}C_{22}$HT-2) e.g. HT-2 toxin ^{13}C-labelled (fully) ρ = 25 µg/ml, in acetonitrile.

5.20 T-2 toxin isotopically labelled internal standard ($^{13}C_{24}$T-2) e.g. T-2 toxin ^{13}C-labelled (fully) ρ = 25 µg/ml, in acetonitrile.

5.21 ZEA toxin isotopically labelled internal standard ($^{13}C_{18}$ZEA) e.g. zearalenone ^{13}C-labelled (fully) ρ = 25 µg/ml, in acetonitrile.

5.22 Mixed mycotoxin stock solutions.

Prepare a mixed mycotoxin stock solution in acetonitrile (5.4), containing: NIV (5.9), DON (5.10), 3-AcDON(5.11), 15-AcDON (5.12), HT-2 (5.13), T-2 (5.14),and ZEA (5.15) to be used for spiking purposes (7.5).

5.23 Mixed mycotoxin working solution.

Prepare a mixed mycotoxin working solution in acetonitrile (5.4) containing: NIV (5.9), 1,25 µg/ml; DON (5.10), 6,25 µg/ml; 3-AcDON (5.11), 0,75 µg/ml; 15-AcDON (5.12), 0,75 µg/ml; HT-2 (5.13), 0,25 µg/ml; T-2 (5.14), 0,25, µg/ml; ZEA (5.15), 0,5 µg/ml. This solution is used for calibration purposes (5.25).

5.24 Mixed internal standard (ISTD) working solution.

Isotopically labelled mycotoxins are generally available as certified standard solutions in acetonitrile. Prepare mixed ISTD working solution by mixing the commercial individual ISTD solutions to obtain a mixture containing $^{13}C_{15}$NIV (5.16), 1,25 µg/ml, $^{13}C_{15}$DON (5.17), 6,25 µg/ml, $^{13}C_{17}$3-AcDON (5.18), 0,75 µg/ml, $^{13}C_{22}$HT-2 (5.19), 0,25, µg/ml,$^{13}C_{24}$T-2 (5.20), 0,25, µg/ml, and $^{13}C_{18}$ZEA (5.21), 0,5 µg/ml in acetonitrile (5.4).

5.25 Calibration solutions.

Add different volumes of the mixed mycotoxin working solution (5.23) to six autosampler vials (6.11) as listed in Table 1 to obtain six calibration levels across the calibration range. Proceed as described in 7.4.

Table 1 — Example of suitable calibration solutions

		Mass concentration of calibration solutions (µg/ml)						
Calibration solution	**Mixed mycotoxin working solution**	**NIV**	**DON**	**3-AcDON**	**15-AcDON**	**HT-2**	**T-2**	**ZEA**
	µl	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
1	25	0,078	0,391	0,047	0,047	0,016	0,016	0,031
2	50	0,156	0,781	0,094	0,094	0,031	0,031	0,063
3	100	0,313	1,563	0,188	0,188	0,063	0,063	0,125
4	200	0,625	3,125	0,375	0,375	0,125	0,125	0,250
5	400	1,250	6,250	0,750	0,750	0,250	0,250	0,500
6	600	1,875	9,375	1,125	1,125	0,375	0,375	0,750
		Mass concentration of isotopically labelled analyte (µg/ml) in all calibration solutions						
		0,316	1,563	0,188	0,188	0,063	0,063	0,125

Once it has been shown that there is linearity, the number of levels may be adjusted accordingly to local needs and requirements.

5.26 HPLC injection solvent.

The composition of HPLC injection solvent depends on the applied LC conditions (see 7.1). Examples of eluents suitable for LC-MS/MS systems are given in Annex B.

6 Apparatus and equipment

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

6.1 Analytical balance, accuracy of 0,01 mg.

6.2 Laboratory balance, accuracy of 0,01 g.

6.3 Adjustable mechanical vertical or horizontal shaker,for solvent extraction with suitable 100 ml flasks.

6.4 Paper filter, pore size 20 µm to 25 µm.

6.5 Conical flasks, with screw top or glass stopper.

6.6 Vacuum manifold to accommodate solid phase extraction columns.

6.7 Single marked pipettes, 5 ml capacity.

6.8 Calibrated microlitre syringe(s) or microlitre pipette(s), 10 µl to 1000 µl.

6.9 Glass tubes of 10 ml with caps.

6.10 4 ml vials with caps.

6.11 Autosampler vials, with caps.

6.12 Disposable filter unit, with pore size of 0,2 µm, regenerated cellulose.

6.13 Concentration evaporator workstation.

6.14 LC-MS/MS system with the following components:

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

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

6.14.3 LC column, capable of separating the seven analytes and ensuring a baseline resolution of mycotoxins monitored in positive from those monitored in negative ion mode. The minimum acceptable retention time for the analytes under examination shall be at least twice the retention time corresponding to the void volume of the column.

6.14.4 Column oven, capable of maintaining a constant temperature.

6.14.5 Tandem mass spectrometer (MS/MS), capable of ionization of the mycotoxins (either resulting in positive or negative ions), performing Selected Reaction Monitoring (SRM) in case of MS/MS analyzers or Parallel Reaction Monitoring (PRM) in case of MS/high resolution mass spectrometry (HRMS) analyzers.

Any ionization source providing sufficient yield may be employed.

7 Procedure

7.1 Preparation of the test sample

Finely grind the laboratory sample and homogenize it.

7.2 Extraction of mycotoxin from the sample

Weigh 10,0 g, to the nearest 0,1 g (6.2), of the test sample into a 100 ml conical flask (6.5). Add 50 ml of extraction mixture (5.7) and shake vigorously for 60 min with a shaker (6.3).

Filter through a paper filter (6.4).

Pipette a 5 ml aliquot (6.7) of the filtered extract into a 10 ml glass tube (6.9) and evaporate to dryness under a stream of air or nitrogen (5.1) at approximately 40 °C.

Redissolve the residue by adding first 100 µl (6.8) of methanol (5.5) and shake for approximately 1 min. Then add 900 µl (6.8) of water (5.3) and shake again for approximately 1 min.

7.3 Solid phase extraction clean up

Connect the SPE column (5.8) to the vacuum manifold (6.6).

Activate and condition the SPE column (5.8) by passing through 2 ml of methanol (5.5), then 2 ml of water (5.3).

Pass the whole volume (1 ml) of reconstituted extract at a flow rate of about one drop per second through the column and discard the eluate. Dry the column.

Wash the column with 1 ml of water (5.3) and discard the eluate. Dry the column.

Elute the mycotoxins with 1 ml of methanol (5.5). Collect the eluate in a 4 ml vial (6.10). Pass air through the column to completely recover the eluate.

7.4 Preparation of the sample test solution

Add 100 µl of the mixed ISTD working solution (5.24) to the SPE eluate and/or the relevant volumes of mixed mycotoxin working solution (5.23) as listed in Table 1.

Evaporate to dryness the SPE eluate and/or the mixed mycotoxin working solution volumes in an evaporator (6.13) under a stream of air or nitrogen (5.1) at approximately 40 °C.

Re-dissolve the dried residue by adding 400 µl of HPLC injection solvent (5.26) and mix thoroughly for at least 10 s.

Filter the solution through a syringe or centrifuge filter (6.12).

Transfer the solution into autosampler vials (6.11).

7.5 Spiking procedure

For the determination of the recovery, carry out a spiking procedure using the mixed mycotoxin stock solutions (5.22). The spiking level shall be within the calibration range and preferably shall correspond to the middle concentration of the calibration curve. Take care that no more than 1 ml of the spiking solvent is added, and distribute the solution evenly over the materials. Evaporate the spiking solvent at room temperature.

7.6 LC-MS/MS analysis

7.6.1 General

Optimize analytical parameters (selection of the ionization mode, selection of the masses of precursor and product ions, optimization of cone voltages and collision energies) by infusion and injection of standard solutions of the analytes.

A combination of analytical column, mobile phase composition, gradient settings and injection volume shall be such that it allows obtaining acceptable separation and reliable results at the required levels, with sufficient selectivity to obtain an acceptably low false suspect rate.

Annex A illustrates some example chromatograms, and Annex B gives some suitable parameters.

Some additional examples of LC columns and relevant operating conditions allowing the separation between 3-AcDON and 15-AcDON are given in Annex C.

7.6.2 Batch composition and analytical sequence

Always start a batch of measurements by injecting a LC injection solvent (5.26) to prove non-contamination of the system. Then inject the highest calibration solution followed by a reagent blank to check for possible carry over.

Subsequently inject the sample test solutions. At the end of the batch, re-inject the calibration series. For larger batches of samples, inject calibration solution number 3 after every approximately 10 samples.

7.7 Identification

Identify each mycotoxin by comparing retention times of calibration solution with that of the sample test solution. Identify the analyte on the basis of at least two mass transitions. The area ratio of the two peaks shall match that of the standard substance.

7.8 Calibration

For each injection calculate the ratio of the peak area of each analyte to the peak area of the respective labelled analogue. These peak area ratios are used in all subsequent calculations.

Divide the peak area of 15-AcDON by the peak area of $^{13}C_{17}$ 3-AcDON.

Prepare a calibration curve for each of the seven analytes (NIV, DON, 3-AcDON, 15-AcDON, HT-2, T-2, and ZEA) by plotting the peak area ratios of each analyte calculated in the calibration solutions against the corresponding amount (µg) of analyte injected on column. Estimate slope and possible intercept of each of the seven calibration curves by using linear regression.