SLOVENSKI STANDARD
SIST EN 16877:2017

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Krma - Metode vzorčenja in analize - Določevanje toksinov T-2 in HT-2, deoksinivalenola in zearalenona v sestavinah krme in krmni mešanici z LC-MS

Animal feeding stuffs: Methods of sampling and analysis - Determination of T-2 and HT-2 toxins, Deoxynivalenol and Zearalenone, in feed materials and compound feed by LC-MS

Futtermittel - Probenahme- und Untersuchungsverfahren - Bestimmung von T-2- und HT-2-Toxinen, Deoxynivalenol und Zearalenon in Einzelfuttermitteln und Mischfuttermitteln mittels LC-MS

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Dosage par CL-SM des toxines T-2 et HT-2, du déoxynivalénol et de la zéaralénone dans les matières premières pour aliments et les aliments composés

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Animal feeding stuffs: Methods of sampling and analysis - Determination of T-2 and HT-2 toxins, Deoxynivalenol and Zearalenone, in feed materials and compound feed by LC-MS

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Contents

Page

European foreword

This document (EN 16877:2016) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs - Methods of sampling and analysis”, 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of an identical text or by endorsement, at the latest by May 2017, and conflicting national standards shall be withdrawn at the latest by May 2017.

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Introduction

WARNING — The method described in this standard implies the use of reagents that pose a hazard to health. The standard does not claim to address all associated safety problems. It is the responsibility of the user of this standard to take appropriate measures for the health and safety protection of the personnel prior to use of the standard and to ensure that regulatory and legal requirements are complied with.

1 Scope

This method of analysis is applicable to the determination of HT-2 toxin (HT2) in the tested range of 22 µg/kg to 178 µg/kg, T-2 toxin (T2) in the tested range of 7 µg/kg to 50 µg/kg, Deoxynivalenol (DON) in the tested range of 88 µg/kg to 559 µg/kg, and Zearalenone (ZON) in the tested range of 14 µg/kg to 430 µg/kg in cereals and cereal-based compound animal feed. The actual working ranges may extend beyond the tested ranges. It is the responsibility of the laboratory to prove that the limit of quantitation (LOQ) for HT-2 and T-2 toxin is ≤ 10 µg/kg, for DON ≤ 100 µg/kg, and for ZON ≤ 20µg/kg.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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

Finely ground and homogeneous test material is suspended in water. After addition of ethyl acetate the sample is agitated. Then sodium sulphate is added to facilitate phase separation and after a delay the sample is centrifuged to pellet particulate matter at the bottom of the extraction tube. The organic phase is transferred to a clean vial for possible storage. An aliquote of the organic phase is mixed with stable-isotope labelled analogues of the analytes and evaporated to dryness in deactivated glass vials. After reconstitution of the dry extract with organic mobile phase modifier and water, and thorough mixing, the analytes are quantified with a Liquid Chromatography-Mass Spectrometry (LC-MS) system.

4 Reagents

WARNING The method described in this standard implies the use of reagents that pose a hazard to health. The standard does not claim to address all associated safety problems. It is the responsibility of the user of this standard to take appropriate measures for the health and safety protection of the personnel prior to use of the standard and to ensure that regulatory and legal requirements are complied with.

4.1 Water (deionized).

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

4.3 Methanol (LC-MS grade).

4.4 Methanol (p.a.).

4.5 Ethyl acetate (p.a.).

4.6 Formic acid (98-100 %, LC-MS grade).

4.7 Acetonitrile (LC-MS grade).

4.8 Sodium sulfate, anhydrous, granulated.

4.9 Deoxynivalenol (DON).

4.10 HT-2 toxin (HT2).

4.11 T-2 toxin (T2).

4.12 Zearalenone (ZON).

4.13 $^{13}C_{15}$-Deoxynivalenol ($^{13}C_{15}$-DON).

4.14 $^{13}C_{22}$-HT-2 toxin ($^{13}C_{22}$-HT2).

4.15 $^{13}C_{24}$-T-2 toxin ($^{13}C_{24}$-T2).

4.16 $^{13}C_{18}$-Zearalenone ($^{13}C_{18}$-ZON).

4.17 Multitoxin stock solution:

A mixture containing Deoxynivalenol (4.9), HT-2 toxin (4.10), T-2 toxin (4.11), and Zearalenone (4.12) in neat acetonitrile (4.7) at relevant concentrations.

When preparing this solution the certified purities of the mycotoxin reference materials need to be properly accounted for. In any case the purities shall be ≥ 95 %.

NOTE 1 3,2 µg/ml DON, 0,5 µg/ml HT-2 toxin, 0,3 µg/ml T-2 toxin, and 0,3 µg/ml ZON in neat acetonitrile have been used during the collaborative study. This solution is stable for three months in the dark at 2–8 °C.

To compare a new stock solution against an old one add 25 µl of each into separate deactivated vials (5.6) and proceed as described in "Test solution" (6.3).

NOTE 2 If 6.4"Spiking procedure" is executed at least 6 ml of the stock solution are needed.

4.18 Multitoxin working solution:

Dilute Multitoxin stock solution (4.17) with Methanol (4.3) such that the resulting concentration in the working solution is applicable to the calibration range of the different compounds. Only prepare enough volume for one full calibration.

NOTE Adding 188 µl of the Multitoxin stock solution described in 4.17, Note 1 to a 3 ml volumetric flask and making up to the mark with methanol will result in a solution containing 0,2 µg/ml DON, 0,031 µg/ml HT-2 toxin, 0,019 µg/ml T-2 toxin, and 0,019 µg/ml ZON in methanol/acetonitrile (94/6, v/v).

4.19 Multi internal standard (ISTD) stock solution:

A mixture containing $^{13}C_{15}$-DON (4.13), $^{13}C_{22}$-HT-2 toxin (4.14), $^{13}C_{24}$-T-2 toxin (4.15), and $^{13}C_{18}$-ZON (4.16) in neat acetonitrile (4.7) at the same concentrations as the respective native compounds in the Multitoxin stock solution (4.17).

NOTE This solution is stable for three months in the dark at (2–8) °C.

4.20 Calibration:

To six deactivated glass vials (5.6) add different volumes of the Multitoxin working solution (4.18) such that six equidistant calibration levels across the calibration range result. Proceed as described in 6.3, "Test solution".

Table 1 below shows example calibration levels using the solution described in the Note to 4.18 above.

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

Table 1 — Example calibration solutions

Volume of Multitoxin working solution (4.18.) [µl]	Total mass of analyte per vial [ng]			
	DON	**HT-2**	**T-2**	**ZON**
25	5	0,78	0,48	0,48
180	36	5,6	3,4	3,4
335	67	10	6,4	6,4
490	98	15	9,3	9,3
645	129	20	12	12
800	160	25	15	15

4.21 Quality control material:

An appropriate material with natural contamination or fortification of the tested mycotoxins which is sufficiently stable.

5 Apparatus

5.1 Mill:

Single mill or multiple mills capable of comminuting test materials to particle sizes of < 500 µm.

5.2 Mixer capable of sufficiently homogenizing the comminuted test materials.

NOTE A tumble mixer that uses a folding action either through moving paddles or fins, or an end-over-end movement has shown to work well.

5.3 Conical polypropylen screw-cap centrifuge tubes, 50 ml with caps.

5.4 Volumetric flasks: 3, 5, and 10 ml.

5.5 Pipettors: adjustable (10-100) µl and adjustable (100-1 000) µl.

5.6 Deactivated glass vials:

Silanized glass vials, minimum volume 1,5 ml.

5.7 Auto Liquid Sampler (ALS) vials of appropriate size for the Auto Liquid Sampler in use.

5.8 Shaker or Sonicator.

5.9 Evaporator capable of maintaining a stable temperature in the range of 30 - 60 °C with a constant flow of dry nitrogen.

5.10 Centrifuge capable of generating a relative centrifugal force (RCF) of 3 000 g.

5.11 Syringe filter:

Small internal volume, Nylon, Pore size: 0,2 µm Nylon.

5.12 LC-MS:

5.12.1 Solvent delivery system capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

5.12.2 Auto liquid sampler (ALS) capable of injecting an appropriate volume of injection solution with sufficient accuracy, cross-contamination below 0,1 %.

5.12.3 Analytical column capable of separating the four analytes with the following performance:

Peak asymmetry factor at 10 % height: 0,9 < As < 1,4; minimum apparent retention factor for any of the four analytes: k ≥ 2; minimum plate number for any of the four analytes: N ≥ 1200; minimum resolution between two adjacent analyte peaks: Rs ≥ 4.

5.12.4 Mass spectrometer:

An instrument capable of performing selected reaction monitoring (SRM) with a sufficiently wide dynamic range. Any ionization source giving sufficient yield may be employed.

5.13 Balance with readability d = 0,001 g or better.

6 Procedures

6.1 Sample preparation

Laboratory samples should be taken and prepared in accordance with European legislation ([1], [2]) where applicable or, in any other case, with EN ISO 6498. The laboratory sample should be finely ground and thoroughly mixed using a mill (5.1) and a mixer (5.2) or another process for which complete homogenization has been demonstrated before a test portion is removed for analysis.

The recommended way is to comminute the laboratory sample in several steps. Beginning with the totality of the laboratory sample each step consists of taking a representative aliquot of the previous step after sufficient homogenization. This aliquot is then comminuted to the next smaller particle size until a subsample of ca. 50 g of the final particle size is obtained. It is of utmost importance that the test portion is taken from a subsample which is sufficiently homogenous with a particle size of ≤ 500 µm. Care should be taken to not overheat the sample during this process.

In all instances everything should be at room temperature before any kind of manipulation takes place.

6.2 Extraction

Some of the steps described below are more critical for the accuracy of the results than others. These steps are marked as such and should be carried out with the necessary attention. A scale-up of the test portion size is deemed to be acceptable if such a need is assumed. In that case the amounts of added water, ethyl acetate, and sodium sulphate need to be increased at the same rate, f.i. scale-up by factor of 2: 4 g test portion, 16 ml water, 32 ml ethyl acetate, 16 g sodium sulphate. In no way shall a scale-up be seen as replacement for proper sample preparation (6.1).

— For the test portion weigh 1,9 to 2,1 g of the homogeneous sample into a conical polypropylene screw-cap tube (5.3), round and record the weight to the second decimal (the accuracy of this weight is critical for the accuracy of the final result!).

— Add 7,2 to 8,8 ml of deionized water (4.1).

— Vortex thoroughly until test portion is completely suspended. Do not let this suspension stand for more than 15 min to prevent effects due to enzymatic activities.

— Add 16,0 ml of ethyl acetate (4.5).

The accuracy of this volume is critical for the accuracy of the final result!

— Extract for 27 to 33 min in a sonicator or by vigorously shaking (5.8).

— Add between 7,2 and 8,8 g of sodium sulphate (4.8).

— Instantly shake hard for 5 s.

— Let stand for 10 to 20 min.

— Centrifuge (5.10) at RCF 3 000 g for at least 1 min to aid settlement of particulate matter and phase separation.

— If wanted for possible repeats: Transfer the extract (organic layer) into clean glass vial for storage of up to 7 d at 2 °C to 10 °C in the dark.

— Transfer 500 µl of the extract (organic layer) into a deactivated glass vial (5.6) for further processing (the accuracy of this volume is critical for the accuracy of the final result!).

6.3 Test solution

— Add 25 µl of the Multi ISTD stock solution (4.19) to the aliquot of the extract and/or the calibration solutions (4.20) (the accuracy of this volume is critical for the accuracy of the final result!).

— Dry down the aliquot of the extract and/or the calibration solutions in an evaporator (5.9) with a gentle stream of dry nitrogen at 60 °C.

— Add 250 µl of the organic mobile phase modifier used for LC-MS to the dry residue for reconstitution.

— Vortex thoroughly for at least 10 s.

— Add 250 µl deionized water (4.1) to the reconstituted extract.

— Vortex thoroughly for at least 5 s.

— Transfer the test solution into an ALS vial (5.7); if solution is turbid it may be filtered through a syringe filter (5.11).

NOTE It has been shown that even very turbid samples can be injected without any negative effects to the life time of column and LC provided that appropriate in-line filters or guard columns are used.

6.4 Spiking procedure

If recovery needs to be determined execute the following in duplicate:

To three times 2 g of a material free of DON, HT2, T2, and ZON add three different volumes of the Multitoxin stock solution (4.17) such that 3 contamination levels across the calibration range result. Distribute the solutions evenly over the materials, mix to further distribute the spike, and leave for a minimum of 5 h to a maximum of 18 h. Proceed to 6.2 “Extraction” second step.

NOTE Addition of 360, 980, and 1 600 µl of the Multitoxin stock solution described in 4.17, Note 1 has been shown to work well.