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SLOVENSKI STANDARD
SIST EN 17194:2020

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Krma: metode vzorčenja in analize - Določevanje deoksinivalenola, aflatoksina B1, fumonizina B1 in B2, toksinov T-2 in HT-2, zearalenona in ohratoksina A v sestavinah krme in krmni mešanici z LC-MS/MS

Animal feeding stuffs: Methods of sampling and analysis - Determination of Deoxynivalenol, Aflatoxin B1, Fumonisin B1 & B2, T-2 & HT-2 toxins, Zearalenone and Ochratoxin A in feed materials and compound feed by LC-MS/MS

Futtermittel: Probenahme- und Untersuchungsverfahren - Bestimmung von Deoxynivalenol, Aflatoxin B1, Fumonisin B1 und B2, T-2- und HT-2-Toxine, Zearalenon und Ochratoxin A in Einzelfuttermitteln und Mischfuttermitteln mittels LC-MS/MS

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Aliments des animaux : Méthodes d'échantillonnage et d'analyse — Détermination du déoxynivalénol, de l'aflatoxine B1, de la fumonisine B1 et B2, des toxines T-2 et HT-2, de la zéaralénone et de l'ochratoxine A dans les matières premières pour aliments et les aliments composés pour animaux par CL-SM/SM

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ICS:

65.120	Krmila	Animal feeding stuffs

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

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EUROPEAN STANDARD

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Animal feeding stuffs: Methods of sampling and analysis - Determination of Deoxynivalenol, Aflatoxin B1, Fumonisin B1 & B2, T-2 & HT-2 toxins, Zearalenone and Ochratoxin A in feed materials and compound feed by LC-MS/MS

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This European Standard was approved by CEN on 9 September 2019.

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European foreword

This document (EN 17194:2019) 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of an identical text or by endorsement, at the latest by May 2020, and conflicting national standards shall be withdrawn at the latest by May 2020.

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

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1 Scope

This document's method of analysis is applicable for the determination of:

— deoxynivalenol (DON) in the tested range of 100 µg/kg to 3 300 µg/kg,

— aflatoxin B1 (AfB1) in the tested range of 2,5 µg/kg to 440 µg/kg,

— fumonisin B1 (FB1) in the tested range of 690 µg/kg to 7 500 µg/kg,

— fumonisin B2 (FB2) in the tested range of 200 µg/kg to 2 500 µg/kg,

— T-2 toxin in the tested range of 7,5 µg/kg to 360 µg/kg,

— HT-2 toxin in the tested range of 14 µg/kg to 1 800 µg/kg,

— zearalenone (ZEN) in the tested range of 30 µg/kg to 600 µg/kg, and

— ochratoxin A (OTA) in the tested range of 10 µg/kg to 230 µg/kg

in cereals and cereal-based compound feed by liquid-chromatography tandem mass spectrometry (LC-MS/MS). The actual working ranges could extend beyond the tested ranges.

2 Normative references

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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.

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

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EN ISO 6498, *Animal feeding stuffs — Guidelines for sample preparation (ISO 6498)*

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 https://www.iso.org/obp.

4 Principle

A test portion of finely ground and homogeneous material is extracted by shaking with a mixture of acetonitrile and aqueous formic acid solution. The extract is centrifuged and an aliquot of the supernatant extract is transferred to a deactivated glass vial, mixed with an appropriate amount of stable-isotope labelled analogues and evaporated to dryness. The reconstituted sample is filtered and quantified with a Liquid Chromatography - Mass Spectrometry (LC-MS) system. Laboratories using this method shall demonstrate the following limits of quantitation (LOQs) in order to be able to apply this method over the whole validation range: for DON ≤ 100 µg/kg, for AfB1 ≤ 2 µg/kg, for FB1 and FB2 ≤ 500 µg/kg (FB1 ≤ 375 µg/kg and FB2 ≤ 125 µg/kg), for T-2 and HT-2 toxin ≤ 10 µg/kg, for ZEN ≤ 20 µg/kg and for OTA ≤ 10 µg/kg.

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NOTE The performance data collected in this study came from laboratories that verified to be able to achieve the above mentioned LOQs. Applicants that could not verify this, were excluded in order to ensure that validation data could be obtained over the targeted concentration range.

5 Reagents

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

WARNING — Environmental regulations and rules apply when disposing of waste solvents. Decontamination procedures for laboratory wastes have been reported by the International Agency for Research on Cancer (IARC), see [1, 2].

5.1 Water (deionized).

5.2 Water, LC-MS grade or of comparable purity (e.g. resistance of 18,2 MΩcm or respectively conductivity of 55 nS/cm at 20 °C).

5.3 Methanol, LC-MS grade.

5.4 Formic acid (FA), 98-100 %.

5.5 Acetonitrile (ACN), LC grade.

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5.6 Extraction solvent composed of 20 parts water (5.1), 79 parts acetonitrile (5.5) and 1 part formic acid (5.4) (v/v/v).

5.7 20 % acetic acid solution, for washing glassware.

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5.8 Aflatoxin B1 (AfB1), purity ≥ 95 %.

5.9 Deoxynivalenol (DON), purity ≥ 95 %.

5.10 Fumonisin B1 (FB1), purity ≥ 95 %.

5.11 Fumonisin B2 (FB2), purity ≥ 95 %.

5.12 HT-2 toxin (HT-2), purity ≥ 95 %.

5.13 T-2 toxin (T-2), purity ≥ 95 %.

5.14 Zearalenone (ZEN), purity ≥ 95 %.

5.15 Ochratoxin A (OTA), purity ≥ 95 %.

5.16 $^{13}C_{17}$-Aflatoxin B1 ($^{13}C_{17}$-AfB1).

5.17 $^{13}C_{15}$-Deoxynivalenol ($^{13}C_{15}$-DON).

5.18 $^{13}C_{34}$-Fumonisin B1 ($^{13}C_{34}$-FB1).

5.19 $^{13}C_{34}$-Fumonisin B2 ($^{13}C_{34}$-FB2).

5.20 $^{13}C_{22}$-HT-2 toxin ($^{13}C_{22}$-HT2).

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5.21 $^{13}C_{24}$**-T-2 toxin** ($^{13}C_{24}$-T2).

5.22 $^{13}C_{18}$**-Zearalenone** ($^{13}C_{18}$-ZEN).

5.23 $^{13}C_{20}$**-Ochratoxin A** ($^{13}C_{20}$-OTA).

5.24 Stock standard solution

A mixture containing Deoxynivalenol (5.9), Aflatoxin B1 (5.8), Fumonisin B1 (5.10) and Fumonisin B2 (5.11), T-2 toxin (5.13) and HT-2 toxin (5.12), Zearalenone (5.14) and Ochratoxin A (5.15) in acetonitrile/water (80/20) with 0,1 % FA at relevant concentrations. When preparing this solution the certified purities of the mycotoxin reference materials need to be properly accounted for.

NOTE 1 6,0 µg/ml DON, 0,040 µg/ml AfB1, 13,5 µg/ml FB1, 4,5 µg/ml FB2, 0,48 µg/ml T-2, 0,6 µg/ml HT-2, 0,9 µg/ml ZEN and 0,45 µg/ml OTA in ACN/H_2O/FA (80/20/0,1, v/v) has shown to work well. This solution is stable up to half a year in the dark at −20 °C or at least three months in the dark at 2 °C to 8 °C.

Compare a new stock solution against the old one by adding 25 µl of each into separate deactivated vials (6.9) and proceeding as described in 7.3.

NOTE 2 If 7.4 is executed at least 4 ml of the stock solution is required.

5.25 Working standard solution

Dilute stock standard solution (5.24) in amber glass acid-washed volumetric flask with acetonitrile/water (80/20) with 0,1 % FA such that the resulting concentration in the working solution is applicable to the calibration range of the different compounds. Prepare enough volume for only one full calibration and use freshly prepared. This solution is stable up to one week when stored in dark at −20 °C.

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NOTE Adding 200 µl of the multimycotoxin stock standard solution as given in 5.24, NOTE 1, to a 3 ml volumetric flask and making up to the mark with ACN/H_2O/FA (80/20/0,1, v/v) will result in a solution containing 0,4 µg/ml DON, 0,002 7 µg/ml AfB1, 0,9 µg/ml FB1, 0,3 µg/ml FB2, 0,032 µg/ml T-2, 0,04 µg/ml HT-2, 0,06 µg/ml ZEN and 0,03 µg/ml OTA in ACN/H_2O/FA (80/20/0,1, v/v). Alternatively, 333,3 µl of the multimycotoxin stock standard solution can be diluted in a 5 ml volumetric flask. Pipetting 200 µl of the multimycotoxin stock standard solution and adding 2 800 µl of the above mentioned solvent also gives comparable results.

5.26 Multi Internal standard (IStd) solution, 3,6 µg/ml $^{13}C_{15}$-DON (5.17), 0,02 µg/ml $^{13}C_{17}$-AfB1 (5.16), 3,75 µg/ml $^{13}C_{34}$-FB1 (5.18), 1,25 µg/ml $^{13}C_{34}$-FB2 (5.19), 0,5 µg/ml $^{13}C_{24}$-T-2 toxin (5.21), 0,5 µg/ml $^{13}C_{22}$-HT-2 toxin (5.20), 1,0 µg/ml $^{13}C_{18}$-ZEN (5.22) and 0,4 µg/ml $^{13}C_{20}$-OTA (5.23) in acetonitrile/water (80/20) with 0,1 % FA has shown to work well. This solution is stable up to half a year in the dark at −20 °C or at least three months at 2 °C to 8 °C.

5.27 Calibrations

Add different volumes of the working standard solution (5.25) to five deactivated glass vials (6.9) such that five equidistant calibration levels across the calibration range are obtained. Continue the preparation procedure as described in 7.3.

The solutions should be protected from light and can be stored in the freezer at ca. −20 °C. They are stable up to one week.

NOTE Table 1 below shows example calibration levels using the solutions described in the notes above.

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

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The exact concentrations of the stock standard solution (5.24), the working standard solution (5.25) and the calibration standard solutions shall be derived from the concentration of the reference standard materials (5.8–5.15), taking into account the purity and the uncertainty declared on the certificate, and the volumes used.

Table 1 — Calibration solutions

Calibration sample	Volume of working standard solution [µl]	Total mass of analyte per vial [ng]							
		DON	AfB1	FB1	FB2	T-2	HT-2	ZEN	OTA
Cal 1	20	8,0	0,053	18,0	6,0	0,64	0,8	1,2	0,6
Cal 2	230	92,0	0,613	207,0	69,0	7,36	9,2	13,8	6,9
Cal 3	440	176,0	1,173	396,0	132,0	14,08	17,6	26,4	13,2
Cal 4	650	260,0	1,733	585,0	195,0	20,8	26,0	39,0	19,5
Cal 5	860	344,0	2,293	774,0	258,0	27,52	34,4	51,6	25,8

5.28 Quality control material

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

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6 Apparatus

6.1 Mill, single mill or multiple mills capable of comminuting test materials to particle sizes of < 500 µm.

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6.2 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.

6.3 Conical polypropylene (PP) screw-cap centrifuge tubes 50 ml with caps.

6.4 Balance, with a mass resolution of 0,001 g or better.

6.5 Adjustable vertical or horizontal shaker.

6.6 Centrifuge, capable of generating a relative centrifugal force (rcf) of 2 300 *g*.

6.7 Pipettors, adjustable 10 µl to 100 µl and adjustable 100 µl to 1000 µl, properly calibrated, with appropriate tips.

6.8 Volumetric flasks, amber, deactivated (acid-washed or silanized) glass, or PP, 3 ml (optional), 5 ml and 10 ml.

6.9 Deactivated glass vials (acid-washed or silanized) or PP vials, of appropriate size for the Auto Liquid Sampler (ALS) in use (usually approximately 1,5 ml capacity).

In order to acid-wash glassware fill it with 20 % acetic acid (5.7) and leave overnight under a fume hood (ca. 16 h to 24 h). Remove acid and rinse glassware with tap water, then deionized water and finally with

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ultrapure water to reach a pH of 5–7. Usually performing each rinse step three times enough. Dry in an oven at 60 °C to 70 °C.

6.10 Sample concentrator, capable of maintaining a stable temperature in the range of 30 to 60 °C with a constant flow of dry nitrogen.

6.11 Syringe filter, small internal volume, Nylon, pore size: 0,2 µm.

6.12 1 ml syringe with needle.

6.13 Vortex mixer, optional.

6.14 LC-MS/MS:

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

6.14.2 Degasser, optional, for degassing LC mobile phases.

6.14.3 Auto Liquid Sampler, capable of injecting an appropriate volume of injection solution with sufficient accuracy, cross-contamination below 0,1 %.

6.14.4 Column oven, optional, capable to operate in temperatures at least up to 40 °C ± 1 °C.

6.14.5 Analytical column, capable of separating analytes with the following performance:

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— Peak asymmetry factor at 10 % height: $0,9 < A_s < 1,4$;

— Minimum retention factor for the first eluting analyte: $k' \geq 1$;

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— Minimum plate number for any of the eight analytes: $N \geq 1\,200$, where

$$N = 5{,}54\left(\frac{t_R}{w_{1/2}}\right)^2;$$

— Minimum resolution between two adjacent peaks: $R_s \geq 1{,}7$.

6.14.6 Pre-column, optional, with the same stationary phase material as the analytical column, and corresponding dimensions.

6.14.7 Mass spectrometer, capable of performing selected reaction monitoring with a sufficiently wide dynamic range. Any ionization source giving sufficient yield may be employed.

7 Procedure

7.1 Sample preparation

It is important that the laboratory receives a laboratory sample which is truly representative and has not been damaged or altered during transport or storage. Laboratory samples shall be taken and prepared in accordance with EN ISO 6498 unless European legislation [3] applies. The laboratory sample shall be finely ground and thoroughly mixed using a mill (6.1) and a mixer (6.2) or another process for which adequate homogenization has been demonstrated before a test portion is removed for analysis.

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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 shall be taken not to overheat the sample during this process.

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

7.2 Extraction

7.2.1 Introduction

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, acetonitrile and formic acid need to be increased at the same rate, e.g. scale-up by a factor of 2: 10 g test portion, 50 ml extraction solvent (5.6). Regardless of the sample size the sample preparation shall meet the requirements in EN ISO 6498.

7.2.2 Dry samples

Weigh 4,8 g to 5,2 g of the homogenous test portion into a polypropylene screw-cap tube (6.3), round and record the weight to the second decimal. The accuracy of this weight is critical for the accuracy of the final result.

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Add 25,0 ml of acetonitrile/water/formic acid solution (79/20/1, v/v) (5.6), the accuracy of this volume is critical for the accuracy of the final result. Vortex or mix by hand thoroughly until the test portion is completely suspended and shake for 30 min in a shaker (6.5), that ensures thorough mixing of the sample.

Centrifuge sample at 3 000 g for at least 3 min (or 5 min at 2 300 g) to aid settlement of particulate matter.

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If wanted for possible repeats: Transfer the extract into a clean polypropylene vial for storage of up to 7 days at 2 °C to 8 °C in the dark.

Take 500 µl aliquot of crude extract, transfer it to a deactivated glass vial (6.9) for further processing. The accuracy of this volume is critical for the accuracy of the final result.

7.2.3 Slurry

Adjust the amount of slurry to 5 g of feed sample, e.g. for 1:1 slurry weigh in 10 g, for 1:2 slurry weigh in 15 g (equivalent to 5 g feed sample) of homogenized test portion into a polypropylene screw-cap tube (6.3) or another appropriate container. The mass shall be recorded to 2 decimal places. If more than 5 ml of water is in the slurry portion, remember to scale-up the amount of solvents to reach the final ratio of extraction solvent composition acetonitrile/water/formic acid of 79/20/1 (v/v).

Add to the slurry an appropriate amount of formic acid (5.4), vortex or mix by hand for 5 s and then add appropriate amount of acetonitrile (5.5), vortex or mix by hand thoroughly for 10 s and then shake for 30 min in a shaker (6.5).

From this point follow the procedure as for dry samples.

7.3 Test solution

Add 25 µl of labelled IStd solution (5.26) to the aliquot of extract and/or the calibration solutions and evaporate to dryness at 50 °C under a gentle stream of nitrogen.

NOTE 1 To reduce the possibility of cross-contamination, labelled IStd solution can be added to the vial before the aliquot of extract/calibration solution is added.

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NOTE 2 Leaving the solutions under a gentle stream of nitrogen for up to 20 min longer than just necessary for complete evaporation did not show to have any negative effect on the final results.

Allow the vial to cool down to room temperature and add 250 µl of LC mobile phase B (e.g. see Annex B) to the dry residue for reconstitution.

Cover the vial (with a cap) and vortex thoroughly for at least 15 s, making sure that also the upper part of the vial is thoroughly rinsed by the solvent.

Add 250 µl of LC mobile phase A (e.g. see Annex B) to the reconstituted extract.

Vortex thoroughly for at least 5 s.

Transfer the test solution into an ALS vial (6.9) for analysis; if the solution is turbid it shall be filtered through a syringe filter (6.11).

7.4 Spiking procedure

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

Only for recovery determination, the sample size is reduced to 2 g in order to reduce the amount of standard solution used.

To three 2 g portions of a material ideally free of all analytes (in case no blank sample can be identified, use a sample with as little contamination as possible) add three different volumes of the Multi-mycotoxin stock standard solution (5.24), such that three contamination levels across the calibration range are obtained. Distribute the solutions evenly over the materials, mix to further distribute the spike and leave containers open in a dark fume hood overnight (ca. 17 h to 24 h) with air flow on at room temperature to allow for solvent evaporation. In case of centrifuge tubes, these can be placed either vertically (e.g. in a rack) or on a clean surface in horizontal position. Both have been tested for sufficient evaporation of the spiking solution solvent. Proceed to 7.2, but remember to use 10 ml of extraction solvent (5.6) to keep the sample-to-solvent ratio constant. Calculate the recovered concentrations from the formula of calibration (1).

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NOTE Addition of 180 µl, 590 µl and 1000 µl of the Multitoxin stock standard solution (5.24) with the concentrations described in 5.24, Note 1 gives satisfactory results. Volumes of 250 µl and 1000 µl were used in method validation study and have shown to give satisfactory results.

8 Measurements

8.1 General

The LC-MS system shall meet the requirements laid out in 6.14 and subclauses.

8.2 LC conditions

A combination of analytical column, mobile phase, gradient settings and injection volume should be such that it allows obtaining acceptable separation, as mentioned in 6.14.5 and reliable results at the required levels (for examples see Annex B).

8.3 MS conditions

Choose an ion source with sufficient ionization yield for the eight analytes and ion source settings such that a stable spray is achieved.

Choose for each analyte an appropriate precursor ion (adducts of the molecule with a proton, sodium, ammonia, etc. in positive mode, or deprotonation, etc. in negative mode). If more than one precursor ion per analyte is detectable then choosing the strongest is a good starting point. Be aware that the choice of precursor ion will affect the repeatability and, by that, Limits of Detection and Quantification (LOD and LOQ).