SLOVENSKI STANDARD
oSIST prEN 17194:2018

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Krma: metode vzorčenja in analize - Določevanje deoksinivalenola, aflatoksina B1, fumonizina B1&B2, toksinov T-2 & 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 - Multimethode für Mycotoxine in Einzelfuttermitteln und Mischfuttermitteln mittels LC-MS/MS

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

Ta slovenski standard je istoveten z: **prEN 17194**

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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

Aliments des animaux : Méthodes d'échantillonnage et d'analyse - Multiméthode pour la détermination par CL-SM/SM, des mycotoxines présentes dans les matières premières pour aliments et les aliments composés pour animaux

Futtermittel - Probenahme- und Untersuchungsverfahren - Multimethode für Mycotoxine in Einzelfuttermitteln und Mischfuttermitteln mittels LC-MS/MS; Deutsche und Englische Fassung prEN 00327097:2017

This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 327.

If this draft becomes a European Standard, CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

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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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European foreword

This document (prEN 17194:2017) 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 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.

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 European Standard method of analysis is applicable for the determination of Deoxynivalenol (DON) in the tested range of 96,2 µg/kg to 3 269 µg/kg, Aflatoxin B1 (AfB1) in the tested range of 2,62 µg/kg to 444 µg/kg, Fumonisin B1 (FB1) in the tested range of 693 µg/kg to 7 529 µg/kg, Fumonisin B2 (FB2) in the tested range of 203 µg/kg to 2 465 µg/kg, T-2 toxin in the tested range of 7,47 µg/kg to 360 µg/kg and HT-2 toxin in the tested range of 13,9 µg/kg to 1 758 µg/kg, Zearalenone (ZON) in the tested range of 34,3 µg/kg to 593 µg/kg and Ochratoxin A (OTA) in the tested range of 10,8 µg/kg to 228 µg/kg in cereals and cereal-based compound feed by liquid-chromatography tandem mass spectrometry (LC-MS/MS). The actual working ranges may extend beyond the tested ranges.

2 Normative reference

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 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

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 deactivated glass vial, mixed with an appropriate amount of stable-isotope labelled analogues and evaporated to dryness. 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: DON ≤ 100 µg/kg, for AfB1 ≤ 2 µg/kg, for FB1&FB2 ≤ 500 µg/kg (FB1 ≤ 375 µg/kg and FB2 ≤ 125 µg/kg), for T-2 and HT-2 toxin is ≤ 10 µg/kg, for ZON ≤ 20 µg/kg and for OTA ≤ 10 µg/kg,

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 — Dispose of waste solvents according to applicable environmental rules and regulations. Decontamination procedures for laboratory wastes have been reported by the International Agency or 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 55nS/cm at 20°C).

5.3 Methanol, LC-MS grade.

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

5.5 Acetonitrile (ACN), LC grade.

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

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 (ZON), purity ≥ 95 %.

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

5.16 $^{13}C_{17}$-Aflatoxin B1 ($^{13}C_{15}$-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).

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

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

5.23 $^{13}C_{20}$-Ochratoxin A ($^{13}C_{18}$-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 ZON 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 – 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 "Test solution" (7.3).

NOTE 2 If 7.4 "Spiking procedure" is executed at least 4 ml of the stock solution are needed.

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.

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, 0,0027 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 ZON 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 abovementioned 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 $^{13}C_{18}$-ZON (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 – 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.

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 into 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	ZON	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.

6 Apparatus

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

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 10-100 µl and adjustable 100-1000 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 –24 h). Remove acid and rinse glassware with tap water, then deionized water and finally with ultrapure water to reach a pH of 5–7. Usually rinsing 3 × each step is enough. Dry in an oven at 60 °C –70 °C.

6.10 Sample concentrator, capable of maintaining a stable temperature in the range of 30 - 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:

Peak asymmetry factor at 10 % height: 0,9 < As < 1,4; minimum retention factor for the first eluting analyte: k' ≥ 2; 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: Rs ≥ 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 should be taken and prepared in accordance with European legislation where applicable [3, 4], 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 (6.2) or another process for which adequate 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 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

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 factor of 2:

10 g test portion, 50 ml extraction solvent (5.6). In no way shall a scale-up be seen as replacement for proper sample preparation.

Dry samples: Weigh 4,8 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!

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 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.

If wanted for possible repeats: Transfer the extract into 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 deactivated glass vial (6.9) for further processing. The accuracy of this volume is critical for the accuracy of the final result!

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 should 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 ACN/H_2O/FA 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.

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 A) to the dry residue for reconstitution.

Cover vial (with 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 A) to the reconstituted extract.

Vortex thoroughly for at least 5 s.

Transfer the test solution into an ALS vial (6.9) for analysis; if solution is turbid it should 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 standards used.