BS EN 17280:2019

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

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National foreword

This British Standard is the UK implementation of EN 17280:2019.

The UK participation in its preparation was entrusted to Technical Committee AW/275, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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

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

This European Standard was approved by CEN on 5 August 2019.

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European foreword

This document (EN 17280:2019) has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

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of an identical text or by endorsement, at the latest by April 2020, and conflicting national standards shall be withdrawn at the latest by April 2020.

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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 wheat, maize, 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].

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1 Scope

This document specifies 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 and T-2) and zearalenone (ZEN) in cereals and cereal products by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) after clean-up by solid phase extraction (SPE).

The method has been validated with samples of wheat, wheat flour, and wheat crackers. The wheat and the wheat flour were prepared from a mixture of wheat and fungi infected wheat kernels. The wheat crackers were baked from wheat flour and water spiked with the target mycotoxins.

Validation levels for NIV ranged from 27,7 µg/kg to 378 µg/kg.

Validation levels for DON ranged from 234 µg/kg to 2420 µg/kg.

Validation levels for 3-acetyl-DON ranged from 18,5 µg/kg to 137 µg/kg.

Validation levels for 15-acetyl-DON ranged from 11,4 µg/kg to 142 µg/kg.

Validation levels for HT-2 ranged from 6,6 µg/kg to 134 µg/kg.

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

Validation levels for ZEN ranged from 31,6 µg/kg to 230 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 https://www.iso.org/obp

4 Principle

Trichothecenes and zearalenone are extracted from the homogenized sample material with a mixture of acetonitrile and 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 detected by reversed phase HPLC-MS/MS.

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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[1] is suitable).

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

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

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

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

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

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[1] Oasis HLB column is an example available commercially from Waters. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the products named. Equivalent products may be used if they can be shown to lead to the same results.

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

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

5.22 Mixed stock solution.

Prepare a mixed mycotoxin stock solution in acetonitrile (5.4), containing e.g.: NIV (5.9) 12,5 µg/ml; DON (5.10) 62,5 µg/ml; 3-AcDON (5.11) 7,5 µg/ml; 15-AcDON (5.12) 7,5 µg/ml; HT-2 (5.13) 2,5 µg/ml; T-2 (5.14) 2,5 µg/ml; ZEN (5.15) 5,0 µg/ml. This solution can be used for spiking purposes (7.5).

5.23 Mixed standard solution.

Prepare a mixed standard solution in acetonitrile (5.4) containing e.g.: 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; ZEN (5.15), 0,5 µg/ml. This solution is used for calibration purposes (5.25).

5.24 Mixed internal standard (ISTD) solution.

Isotopically labelled mycotoxins are generally available as certified standard solutions in acetonitrile. Prepare a mixed ISTD solution by mixing the commercial individual ISTD solutions to obtain a mixture containing e.g. ^{13}C-NIV (5.16), 1,25 µg/ml; ^{13}C-DON (5.17), 6,25 µg/ml; ^{13}C-3-AcDON (5.18), 0,75 µg/ml; ^{13}C-HT-2 (5.19), 0,25 µg/ml; ^{13}C-T-2 (5.20), 0,25 µg/ml; ^{13}C-ZEN (5.21), 0,5 µg/ml in acetonitrile (5.4).

5.25 Calibration solutions.

Add different volumes of the mixed standard solution (5.23) and the mixed ISTD solution (5.24) to six autosampler vials (6.11) e.g. as listed in Table 1 to obtain six calibration levels across the calibration range. Evaporate to dryness 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 (V_1) of HPLC injection solvent (5.26) and mix thoroughly for at least 10 s.

Table 1 — Example of suitable calibration solutions

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

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5.26 HPLC injection solvent.

The composition of HPLC injection solvent depends on the applied LC conditions. 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, 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 Microlitre syringe(s) or microlitre pipette(s), 10 µl to 1000 µl.

6.9 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.

6.14.3 LC column, capable of retaining the target mycotoxins, preferably with a retention factor of at least two. The two isomers 15-AcDON and 3-AcDON cannot be distinguished individually by specific Multiple Reaction Monitoring (MRM) transitions. Therefore the LC column and gradient shall be able to separate both compounds. Examples of suitable LC columns and gradients are reported in Annex B.

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 analysers or Parallel Reaction Monitoring (PRM) in case of MS/high resolution mass spectrometry (HRMS) analysers.

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Any ionization source providing sufficient yield may be employed.

6.14.6 Data evaluation system.

7 Procedure

7.1 Preparation of the test sample

Finely grind the laboratory sample and homogenize it.

7.2 Extraction

Weigh 10,0 g to the nearest 0,1 g into a 100 ml conical flask (6.5). Add 50 ml (V_3) 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 (V_4) of the filtered extract into a 10 ml tube (6.9) and evaporate to dryness under a stream of air or nitrogen (5.1) at approximately 40 °C.

Re-dissolve the residue by adding first 100 µl of methanol (5.5) and shake for approximately 1 min. Then add 900 µl 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 by applying a gentle air flow.

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 solution (5.24) to the SPE eluate.

Evaporate the SPE eluate to dryness 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 (V_1) of HPLC injection solvent (5.26) and mix thoroughly for at least 10 s.

Filter the re-dissolved residue through a syringe filter or centrifuge filter (6.12).

Transfer the sample test solution into autosampler vials (6.11).

7.5 Spiking procedure

For the determination of the recovery, carry out a spiking procedure using the mixed stock solution (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 solution is added, and distribute the solution evenly over the sample materials. Evaporate the spiked solution at room temperature.

Alternatively, a certified reference material can be applied.

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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.

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

Chromatographic separation of 3-AcDON and 15-AcDON is necessary to provide individual data on the two isomers. 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 Injection 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 calibration range from the least concentrated to the most concentrated.

Then inject a solvent blank to check for possible carry over.

Subsequently inject the sample test solutions.

At the end of the batch, inject the low-end point, to ensure that the end-of-series sensitivity is the same as at the beginning, and to ensure that there has been no change in retention times.

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 retention time and the ion ratio of the two peaks shall match that of the standard substance. Confirm the identity of the sample peaks using the retention time ± 0,2 min of the mean observed for the calibration standards [6]. Ion ratios, defined as the response of the peak with the lower area/response of the peak with the higher area, shall match (± 30 % of average) the calibration standards from the same sequence [6].

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-3-AcDON.

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

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8 Calculation

Calculate the mass fraction of each mycotoxin, w, in microgram per kilogram, of the sample according to Formula (1):

$$w = \left(\frac{R}{a} - \frac{b}{a}\right) \times \frac{V_1}{V_2} \times \frac{1000}{m_{SPE}} \quad (1)$$

where

R is the peak area ratio of the relevant analyte and the corresponding internal standard in the sample test solution;

a is the slope of the calibration curve from calibration data (7.8), in μg^{-1};

b is the intercept of the calibration curve from calibration data (7.8);

V_1 is the volume of the reconstituted extract after clean-up, here: 0,4 ml;

V_2 is the injection volume of the reconstituted sample extract, in millilitre;

1000 is a conversion factor;

m_{SPE} is the sample equivalent weight purified on SPE column, here: 1 g.

Calculate the sample equivalent weight (m_{SPE}) according to Formula (2):

$$m_{SPE} = \frac{m \times V_4}{V_3} \quad (2)$$

where

m is the mass of the extracted test portion, here: 10 g;

V_3 is the volume of the extraction mixture, here: 50 ml;

V_4 is the volume of filtered extract dried before clean-up, here: 5 ml.

9 Precision

9.1 General

Details of the interlaboratory test of the precision of the method are summarized in Annex D. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and matrices other than given in Annex D.

9.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in Tables 2 to Table 8 in not more than 5 % of the cases.

9.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit R in Tables 2 to Table 8 in not more than 5 % of the cases.

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Table 2 — Validation data for nivalenol

Sample	$\bar{x}$ µg/kg	r µg/kg	R µg/kg
wheat	194,1	31,76	91,81
wheat	87,12	81,12	85,02
wheat	186,9	57,12	21,06
wheat	377,8	151,2	268,6
wheat flour	116,0	19,79	59,80
wheat flour	52,89	50,46	49,26
wheat flour	107,7	12,63	28,41
wheat flour	214,8	21,26	58,50
wheat crackers	70,71	16,39	40,33
wheat crackers	27,69	11,62	24,65
wheat crackers	64,87	11,27	53,26
wheat crackers	123,8	20,14	75,89

Table 3 — Validation data for deoxynivalenol

Sample	$\bar{x}$ µg/kg	r µg/kg	R µg/kg
wheat	1212	124,8	322,5
wheat	635,4	53,91	263,0
wheat	1201	355,7	366,4
wheat	2420	146,1	435,3
wheat flour	692,5	97,12	188,3
wheat flour	351,0	65,68	313,9
wheat flour	613,0	64,50	244,7
wheat flour	1234	114,2	354,4
wheat crackers	448,7	34,98	235,4
wheat crackers	233,9	92,80	158,2
wheat crackers	487,6	68,32	204,5
wheat crackers	1004	374,1	389,1

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Table 4 — Validation data for 3-acetyl deoxynivalenol

Sample	$\bar{x}$ µg/kg	r µg/kg	R µg/kg
wheat	136,5	21,74	30,56
wheat	49,10	4,074	37,27
wheat	83,68	21,99	18,40
wheat	67,68	12,14	14,64
wheat flour	67,01	16,07	75,53
wheat flour	30,22	5,800	27,09
wheat flour	58,95	16,57	52,23
wheat flour	96,22	14,32	26,37
wheat crackers	44,96	7,433	38,08
wheat crackers	18,47	8,603	13,24
wheat crackers	26,22	4,924	14,89
wheat crackers	49,96	16,21	28,30

Table 5 — Validation data for 15-acetyl deoxynivalenol

Sample	$\bar{x}$ µg/kg	r µg/kg	R µg/kg
wheat	141,84	33,87	138,28
wheat	35,64	5,788	44,38
wheat	41,48	10,75	28,79
wheat	30,14	6,023	32,26
wheat flour	68,25	11,40	59,06
wheat flour	12,87	3,744	12,22
wheat flour	24,38	9,390	28,63
wheat flour	28,64	7,720	29,14
wheat crackers	46,24	11,14	47,57
wheat crackers	11,41	8,049	14,36
wheat crackers	16,42	4,751	15,53
wheat crackers	32,34	5,374	30,35

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Table 6 — Validation data for HT-2 toxin

Sample	$\bar{x}$ µg/kg	r µg/kg	R µg/kg
wheat	46,40	9,544	42,51
wheat	17,33	3,209	12,53
wheat	36,29	31,25	35,92
wheat	133,8	38,34	100,7
wheat flour	21,60	3,660	5,981
wheat flour	6,63	4,813	7,110
wheat flour	14,18	3,530	11,64
wheat flour	32,97	8,489	24,71
wheat crackers	10,73	1,427	7,924
wheat crackers	7,988	3,133	6,835
wheat crackers	19,59	8,357	11,30
wheat crackers	38,02	11,22	32,62

Table 7 — Validation data for T-2 toxin

Sample	$\bar{x}$ µg/kg	r µg/kg	R µg/kg
wheat	45,89	15,55	35,82
wheat	27,50	8,670	24,92
wheat	47,79	44,93	55,16
wheat	18,01	5,930	17,72
wheat flour	20,85	3,691	20,58
wheat flour	11,53	2,347	10,51
wheat flour	26,85	4,770	6,392
wheat flour	37,57	7,590	38,14
wheat crackers	11,05	2,162	9,664
wheat crackers	4,530	1,544	4,796
wheat crackers	8,091	2,350	8,806
wheat crackers	15,88	2,181	13,43

Documents
Document Sharing Hub
Full document details: BS EN 17280:2019