BRITISH STANDARD

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Foodstuffs — Determination of Deoxynivalenol in animal feed — HPLC method with immunoaffinity column clean-up

ICS 65.120

BSI British Standards

National foreword

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Foreword

This document (EN 15791:2009) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, 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of an identical text or by endorsement, at the latest by March 2010, and conflicting national standards shall be withdrawn at the latest by March 2010.

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1 Scope

This Standard is applicable to the determination of deoxynivalenol (DON) in animal compound feed at concentrations of 150 µg/kg up to at least 4 000 µg/kg.

2 Normative references

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

3 Principle

Deoxynivalenol (DON) is extracted from the commodity using water. The aqueous extract is then cleaned up with an immunoaffinity column to remove impurities from the sample. Subsequently DON is quantitatively determined by HPLC with UV detection.

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and only double-distilled water or water of grade 1 as defined in EN ISO 3696. Solvents shall be of quality for HPLC analysis.

4.1 Acetonitrile

WARNING — Acetonitrile is hazardous and handling shall be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) shall be worn.

4.2 Deoxynivalenol (DON), with a minimum purity of 97 %

WARNING — Deoxynivalenol is highly toxic. Gloves and safety glasses shall be worn at all times and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.3 Methanol

WARNING — Methanol is hazardous and handling shall be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) shall be worn.

4.4 Glacial acetic acid

WARNING — Glacial acetic acid is hazardous and handling shall be carried out inside a fume cupboard. Appropriate safety equipment (lab coat, goggles, gloves) shall be worn.

4.5 Mobile Phase

Mix 15 parts per volume of methanol (4.3) with 84,9 parts per volume of water and 0,1 parts of glacial acetic acid (4.4). The exact amount of methanol used and whether acetic acid will have to be used depends on the HPLC column chosen for analysis and must be adjusted if necessary. Degas this solution before use.

4.6 Wash Solvent

Mix 50 parts per volume of methanol (4.3) with 50 parts per volume of water.

4.7 DON stock solution

250 µg Deoxynivalenol per ml of Acetonitrile.

May be prepared by the following: Add 4,0 ml of acetonitrile (4.1) to 5 mg of DON (4.2) for a solution of 1,25 mg/ml. Dilute 1 000 µl of the 1,25 mg/ml solution to 5,0 ml with acetonitrile for the stock solution of 250 µg/ml. Dilute 200 µl of the 250 µg/ml stock solution in a 2,0 ml volumetric flask (5.11) with acetonitrile to create a diluted stock solution of 25 µg/ml.

To determine the exact concentration record the absorption curve of this 25 µg/ml diluted stock solution with the spectrophotometer (5.15) in the range of 200 nm to 270 nm in a 1 cm quartz cell with acetonitrile (4.1) as reference. Determine the absorption at 220 nm. Calculate the mass concentration of deoxynivalenol, ρ_{Don}, in micrograms per millilitre using equation 1:

$$\rho_{DON}(\sim 25\mu g/ml) = \frac{A_{\max} \times M \times 100}{\kappa \times d} \quad (1)$$

where:

A_{max} is the absorption determined at the maximum of the absorption curve (here: at 220 nm);

M is the molar mass of deoxynivalenol (M = 296,3 g/mol);

κ is the molar absorption coefficient of deoxynivalenol in acetonitrile (4.1), (here: 681 m^2/mol ± 12,6 m^2/mol [1]);

d is the optical path length of the quartz cell in centimetres (here: 1 cm).

Calculate the exact concentration of the 250 µg/ml stock solution by the following equation:

$$\rho_{DON}(\sim 250\mu g/ml) = \rho_{DON}(\sim 25\mu g/ml) \times 10 \quad (2)$$

Stock solution may be stored in the dark for up to 3 months at 4°C to 8°C or at least 6 months at below -18 ºC.

NOTE Stock solution preparation can be carried out gravimetrically by accurately weighing the DON standard material and the solvent used to dissolve it.

4.8 DON spiking solution

Pipette an aliquot of the calibrated DON stock solution (4.7), equivalent to 500 µg DON, into a 5 ml volumetric flask (5.11). Make up to the mark with acetonitrile (4.1). This will result in the spiking solution of 100 µg/ml.

4.9 DON working solution

Pipette an aliquot of the calibrated diluted DON stock solution (4.7), equivalent to 50 µg DON, into a 5 ml volumetric flask (5.11). Make up to the mark with acetonitrile (4.1). This will result in the DON working solution of 10 µg/ml.

4.10 DON Calibration solutions

Calibration solutions are prepared from the 10 µg/ml DON working solution (4.9). For example, add the volumes of 10 µg/ml DON working solution (4.9) shown in the table below into 10 ml volumetric flasks (5.11). Fill the flasks up to the mark with mobile phase (4.5). Deviations are permissible as long as the lowest level is above the limit of detection, the highest level does not lead to saturation of the detector signal, and there are at least two more levels equidistantly in between.

Table 1 — Preparation of standard solutions

Calibration solution	DON Working solution (4.9) (µl)	DON concentration ng/ml
1	450	450
2	375	375
3	300	300
4	225	225
5	150	150
6	75	75

4.11 DON immunoaffinity clean-up columns

The immunoaffinity (IA) column contains antibodies raised against deoxynivalenol. The column shall have a capacity of not less than 2 500 ng of DON and shall give a recovery of not less than 70% when 25 ng of DON are applied in 1 ml to-2 ml of water (depending on manufacturer's instructions).

5 Apparatus

Usual laboratory equipment and in particular the following:

5.1 Analytical balance, with d=0,001g for sample weighing, with d=0,01mg for gravimetrical preparation of the DON stock solution (4.7)

5.2 Homogeniser/ High Speed Blender

5.3 Laboratory shaker

5.4 Vortex Mixer, or equivalent

5.5 Mill (various screens)

5.6 Tumble mixer

5.7 Screw cap flasks, with volumes of 250 ml and 500 ml

5.8 Funnels, of appropriate size

5.9 Filter, cellulose with ca. 30 µm pore size

5.10 Filter, binder-free glass microfiber with ca. 2 µm pore size

5.11 Volumetric flasks, with volumes of 2 ml, 5 ml, and 10 ml

5.12 Graduated pipettes, with volumes of 1 ml and 5 ml

5.13 Adjustable Pipettors or gas-tight glass syringes, with volumes of 100 µl and 1 000 µl

5.14 HPLC system consisting of:

5.14.1 Pump, capable at least of generating binary gradients, pulsation-free, at flows appropriate for the analytical column

5.14.2 Analytical column

Any column which allows for sufficient separation of deoxynivalenol from other interfering components is suitable. Examples are: Phenomenex ODS3-Prodigy (15 cm x 4,6 mm i.d.), 5 µm particle size, 100 Å pore size, Octadecylsilane (ODS) 250 mm x 4,6 mm I.D., 3 µm particle size, 80 Å pore size, Octadecyl (C18) 250 mm x 4,6 mm I.D., 5 µm particle size, 180 Å pore size

5.14.3 Pre-column (optional), appropriate for the analytical column used

5.14.4 Autosampler, capable of injecting appropriate volumes with sufficient repeatability

5.14.5 UV detector, capable of measuring at 220 nm

5.14.6 Data collection system

5.15 UV spectrophotometer, for checking the concentration of the DON stock solution (4.7)

5.16 Reservoirs, of appropriate size with adaptors to fit the immunoaffinity columns

5.17 Glass vials, of appropriate size for autosampler (5.14.4) but with minimum volume of 2,0 ml

5.18 Syringe filter unit, polyamide (nylon) with 0,45 µm pore size

5.19 Evaporator, capable of maintaining 50ºC with a steady stream of air or nitrogen

6 Procedure

6.1 Sample preparation

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. Samples should be taken and prepared in accordance with European legislation where applicable [2]. Samples should be finely ground and thoroughly mixed using a mill (5.5) and a tumble mixer (5.6) or another process that has been demonstrated to give complete homogenisation before a test portion is removed for analysis.

In all instances if the sample has been frozen allow it to thaw completely before sampling. Mix the sample thoroughly before removing an analytical test portion.

6.2 Extraction

Weigh a 25,0 g test portion into a 250 ml or 500 ml screw cap flask (5.7). Add 200 ml of deionised water, cap and shake for 1 hour with a shaker (5.3).

Prepare a funnel (5.8) with filter paper (5.9). Filtrate the extracted sample into a clean 250 ml or 500 ml screw cap flask (5.7).

6.3 Immunoaffinity Column Clean-up

Attach a reservoir (5.16) to an immunoaffinity column. Add 8 ml of deionised water. Then transfer 2,0 ml of the filtered extract (see above; 0,5 ml in case of analytical results above 4 000 µg/kg; see section 8) into the reservoir (5.16). Allow this solution to pass slowly through the column by gravity at a rate of 1 drop/s to 2 drops/s. When the extract has passed completely through the immunoaffinity column, pass 5 ml of deionised water through the column. Remove residual liquid by passing nitrogen or air through the column for about 5 seconds. Discard all the eluates from this stage of the clean-up procedure.

Finally, place a HPLC autosampler vial (5.17) under the column and pass 0,5 ml of methanol (4.3) through the column by gravity collecting the eluate. After the last drops of methanol have passed through the column allow the methanol to remain on the column for approximately 1 minute. Then add another 1,0 ml of methanol (4.3) and continue to collect the eluate. Carefully pass nitrogen or air through the column in order to collect any residual eluate.

NOTE Alternatively to the manual procedure (6.4) the immunoaffinity clean-up and elution may be performed with an automatic sample preparation unit, provided that volumes and flow rates remain unchanged.

6.4 Preparation of the test solution for HPLC analysis

Place the vial with the eluate in the evaporator (5.19) and carefully evaporate to dryness under nitrogen or air at ca. 50ºC. Immediately afterwards cool the HPLC vial to ambient temperature and reconstitute the residue with 0,50 ml of HPLC mobile phase (4.5). Mix well with vortex mixer (5.4) for at least 30 seconds to ensure the residue is completely re-dissolved. In case of turbidity filtrate the test solution through a syringe filter unit (5.18).

6.5 Spiking procedure

To determine the recovery spike a deoxynivalenol-free material with the spiking solutions (4.8). The spiking level should be within the calibration range (preferably mid-range). Leave the spiked sample to stand for a minimum of 30 minutes to ensure evaporation of the solvent.

7 HPLC determination

7.1 Calibration graph

Prepare a calibration graph at the beginning of every day of analyses by injecting calibration solutions (4.10) at different suitable concentrations into the chromatograph. Establish the calibration curve prior to analysis of test samples by plotting the concentration of DON [ng/ml] (x-axis) against the peak signal as area or height (y-axis), determine slope and possible intercept with linear regression, and check the plot for using appropriate diagnostics.

7.2 Determination of deoxynivalenol in the test solution

Inject aliquots of the test solutions into the chromatograph using the same conditions used for the preparation of the calibration graph.

7.3 Peak identification

Identify the deoxynivalenol peak in the test solution by comparing the retention time with that of the nearest HPLC calibration solution (4.10) injected in the HPLC analyses batch. The concentration of deoxynivalenol in the test solution must fall within the calibration range. If the deoxynivalenol level in the test solution exceeds the concentration of the highest calibration solution the test solution shall be diluted with HPLC mobile phase to bring it within calibration range and be reanalysed. The dilution factor must be incorporated into all subsequent calculations.

7.4 HPLC operating conditions

Using the equipment outlined in 5.14 the following conditions have shown to provide adequate separation:

Injection volume:	100 µl - 300 µl
UV detection wavelength:	220 nm
Flow rate mobile phase (column):	1,0 ml/min

If the HPLC pump delivered mobile phase (4.5) through channel A and wash solvent (4.6) through channel B the gradient profile would look as follows:

Time [min]	Channel A [%]	Channel B [%}
0 to 15	100	0
15 to 25	0	100
25 to 35	100	0

NOTE Mobile phases prepared with acetonitrile and water have also been shown to be suitable alternatives. Such mobile phases may be used provided sufficient separation is achieved.

8 Calculation

Determine from the calibration graph the mass concentration in ng/ml of the deoxynivalenol in the test solution injected onto the HPLC column.

Calculate the mass fraction of deoxynivalenol, w_{DON}, in nanograms per gram or micrograms per kilogram to one decimal place using the equation below:

$$w_{DON} = c_{DON} \times \frac{V_3}{V_2} \times \frac{V_1}{m_s} \qquad (3)$$

where

c_{DON} is the mass concentration of deoxynivalenol as determined through calibration (7.1);

V_3 is the total volume of the test solution (0,5 ml; 6.4);

V_2 is the volume of the aliquot of extract used for clean-up (2,0 ml or 0,5 ml; 6.3);

V_1 is the total volume of the extraction solvent (200 ml; 6.2);

m_s is the mass of the extracted test portion (m_s = 25,0 g; 6.2);

The above equation can be simplified if the described masses and volumes have been used:

$$w_{DON} = c_{DON} \times 2 \qquad \text{(2 ml of extract were cleaned-up)} \qquad (4)$$

Should the above calculation render a value above 500 then a new clean-up with 0,5 ml of the sample extract should be prepared (see section 6.3). The simplified equation is then as follows:

$$w_{DON} = c_{DON} \times 8 \qquad \text{(0,5 ml of extract were cleaned-up)} \qquad (5)$$

9 Precision

9.1 Interlaboratory study

Details of an interlaboratory study on the precision of the method are shown in [1]. The values derived from this interlaboratory study may not be applicable to concentration ranges and/or matrices other than those given.

9.2 Repeatability

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

$\bar{x}$ = 229 µg/kg	*r* = 87,5 µg/kg
$\bar{x}$ = 401 µg/kg	*r* = 97,2 µg/kg
$\bar{x}$ = 188 µg/kg	*r* = 31,9 µg/kg
$\bar{x}$ = 505 µg/kg	*r* = 233,2 µg/kg
$\bar{x}$ = 1 013 µg/kg	*r* = 232,9 µg/kg

9.3 Reproducibility

The absolute difference between two single test results on identical test materials reported by two laboratories will exceed the reproducibility limit *R* in not more than 5% of the cases.

$\bar{x}$ = 229 µg/kg	*R* = 100,6 µg/kg
$\bar{x}$ = 401 µg/kg	*R* = 184,7 µg/kg
$\bar{x}$ = 188 µg/kg	*R* = 132,8 µg/kg
$\bar{x}$ = 505 µg/kg	*R* = 329,2 µg/kg
$\bar{x}$ = 1 013 µg/kg	*R* = 299,0 µg/kg

10 Test report

The test report shall contain the following data:

a) information necessary for the identification of the sample (kind of sample, origin of sample, designation);

b) a reference to this European Standard;

c) the date and type of sampling procedure (if known);

d) the date of receipt;

e) the date of test;

f) the test results and the units in which they have been expressed;

g) whether the repeatability has been verified;

h) particular points observed in the course of the test;

i) operations not specified in the method or regarded as optional, which might have affected the results.

Annex A
(informative)

Precision data

The following data were obtained in an interlaboratory study [3] according to AOAC Guidelines for collaborative study procedures to validate characteristics of a method of analysis [4].

Table A.1 — Precision data

Sample	**Animal feed**	**Animal feed**	**Animal feed**	**Animal feed**	**Animal feed**	**Animal feed**
Year of inter-laboratory study	2005	2005	2005	2005	2005	2005
Number of laboratories	11	11	13	13	13	12
Number of laboratories retained after eliminating outliers	10	10	12	11	13	12
Number of outliers (laboratories)	1	1	1	2	0	0
Number of accepted results	10	10	12	11	13	12
Mean value $\bar{x}$, µg/kg	229	401	<30	188	505	1 013
Repeatability standard deviation s_r, µg/kg	31,2	34,7	n.a. [c]	11,4	83,3	83,2
Coefficient of variation of repeatability, CV(r), %	13,7	8,7	n.a. [c]	6,1	16,5	8,2
Repeatability limit r [a], µg/l	87,5	97,2	n.a. [c]	31,9	233,2	232,9
Reproducibility standard deviation s_R, µg/kg	35,9	66,0	n.a. [c]	47,4	117,6	106,8
Coefficient of variation of reproducibility, CV(R), %	15,7	16,5	n.a. [c]	25,2	23,3	10,5
HorRat value	0,8	0,9	n.a. [c]	1,2	1,3	0,7
Reproducibility limit R [b], µg/l	100,6	184,7	n.a. [c]	132,8	329,2	299,0
Recovery, %	100	93	n.a. [c]	n.a. [c]	n.a. [c]	n.a. [c]

a - $r = 2{,}8 \times s_r$
b – $R = 2{,}8 \times s_R$
c – not applicable

Table A.2 – Material composition

Test Material	Ingredient	Amount (kg)	Composition[1]
Blank	Oats (peeled)	1,5	Oats
	Oats (black)	1,5	Oats
	Soya beans	1,5	Soya
	Rabbit feed	1,5	Mixed cereals, carrots
	Bird feed mix	3	Sunflower seeds, maize, oats, wheat, wild seeds, linseed
Level 1	Compound pig feed	8	Peas, soya, wheat, barley, tapioca, cabbage seeds , animal fat, corn, calcium carbonate
Level 2	Compound horse feed	8	Barley flakes, oat flakes, maize flakes, plant oil, alfalfa, horse-mix pellets
Level 3	Horse muesli	2	Oat, barley flakes, cornflakes, peas, molasses, plant oil
	Rabbit feed	2	Wheat, alfalfa, sunflower seeds, cabbage seeds, straw molasses, barley, roasted soya
	Compound pig feed	4	Peas, soya, wheat, barley, tapioca, cabbage seeds , animal fat, corn, calcium carbonate
	Horse sweets	0,5	Unknown composition with carrots and vitamins A, D3 & E
	Alfalfa	1	Alfalfa
	Wheat	0,7	Wheat
	Soya/corn mix	2	Soya/corn, 50% each

[1] In decreasing amounts.

Annex B
(informative)

Chromatogram

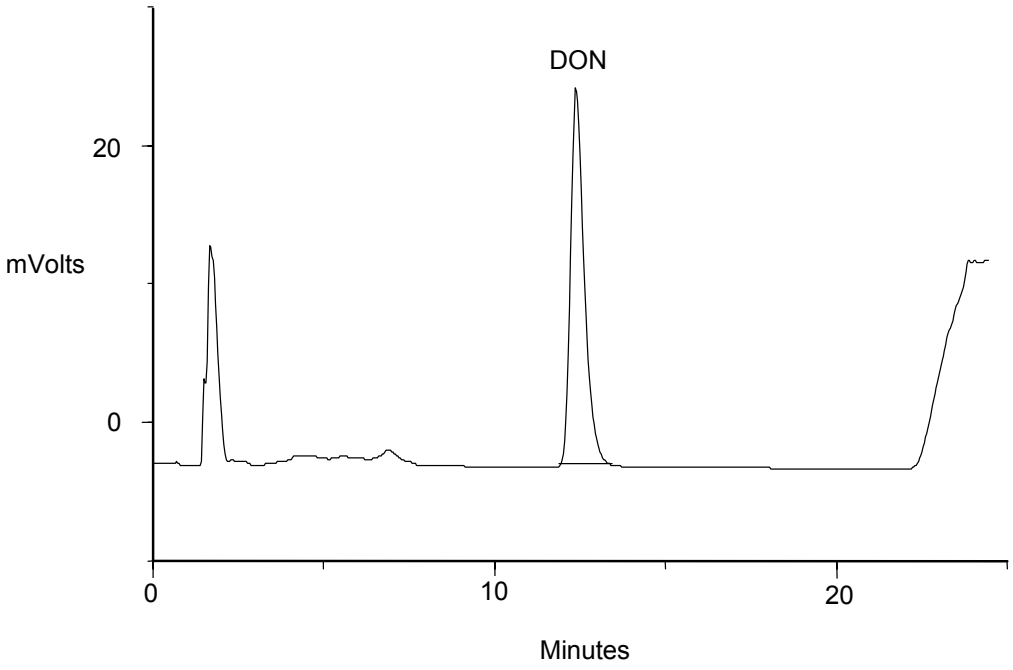

Figure B.1 — Naturally contaminated animal feed sample at approximately 2 mg/kg

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