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Animal feeding stuffs — Determination of zearalenone by immunoaffinity column chromatography and high performance liquid chromatography

Aliments des animaux — Dosage de la zéaralénone par chromatographie à colonne à immunoaffinité et par chromatographie liquide haute performance

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 17372 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

Animal feeding stuffs — Determination of zearalenone by immunoaffinity column chromatography and high performance liquid chromatography

1 Scope

This International Standard is applicable to the analysis of zearalenone in animal feed and feed ingredients, including barley, corn, oats, rye, wheat, soybean meal, canola (rapeseed) meal, corn gluten, dried distillers' grains, lentils, and sugar beet pulp. The limit of quantification is 0,05 mg/kg (50 µg/kg). A lower limit of quantification may be achievable subject to appropriate validation being conducted by the user laboratory.

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.

ISO 565, *Test sieves — Metal wire cloth, perforated metal plate and electroformed sheet — Nominal sizes of openings*

ISO 648, *Laboratory glassware — Single volume pipettes*

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 4788, *Laboratory glassware — Graduated measuring cylinders*

ISO 6498, *Animal feeding stuffs — Preparation of test samples*

3 Principle

Samples are extracted with diluted acetonitrile and clarified by filtration. Then an aliquot of the filtrate is diluted with water or phosphate-buffered saline (PBS) and purified using immunoaffinity column (IAC) chromatography. The purified extracts are analysed by reverse-phase high performance liquid chromatography (HPLC) with fluorescence detection. Suspect positive samples can be confirmed by wavelength ratioing, by using normal phase HPLC analysis, or by using diode-array detection.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

WARNING — Handle all solvents and solutions under a fume hood. Wear safety glasses, protective clothing, and avoid skin contact.

4.1 Water, complying with ISO 3696, grade 1.

4.2 Acetonitrile (CH_3CN), HPLC grade.

4.3 Methanol (CH_3OH), HPLC grade.

4.4 Sodium chloride (NaCl), of purity not less than 99 % by mass.

4.5 Extraction solvent, volume fraction, $\phi(CH_3CN) = 90$ %.

Mix 900 ml of acetonitrile (4.2) with 100 ml water (4.1). Mix well.

4.6 Dilute acetonitrile, volume fraction, $\phi(CH_3CN) = 50$ %.

Combine 1 volume of acetonitrile (4.2) with 1 volume of water (4.1). Mix well. This solution is used for the autosampler syringe, if applicable.

4.7 Dilute methanol, volume fraction, $\phi(CH_3OH) = 30$ %.

Combine 75 ml methanol (4.3) with 175 ml water (4.1). Mix well.

4.8 Disodium hydrogenphosphate (Na_2HPO_4), purity not less than 99 % mass fraction.

4.9 Potassium dihydrogenphosphate (KH_2PO_4), purity not less than 99 % mass fraction.

4.10 Potassium chloride (KCl), purity not less than 99 % mass fraction.

4.11 Sodium hydroxide (NaOH), purity not less than 99 % mass fraction.

4.12 Phosphate-buffered saline (PBS).

Dissolve 8 g sodium chloride (4.4), 1,16 g disodium hydrogenphosphate (4.8), 0,2 g potassium dihydrogenphosphate (4.9), and 0,2 g potassium chloride (4.10) in 1 l water (4.1). Adjust pH to 7,4 with sodium hydroxide solution (4.13). Alternatively, prepared concentrated PBS can be purchased, then diluted for use.

4.13 Sodium hydroxide solution, $c(NaOH) = 0{,}2$ mol/l.

Dissolve 8 g sodium hydroxide (4.11) in 1 l water (4.1).

4.14 HPLC mobile phase.

Add 460 ml acetonitrile (4.2) to 1 l reagent flask, add 460 ml water (4.1) and 80 ml methanol (4.3). Mix well and filter through a filter with a pore size of 0,45 µm (5.14).

4.15 Zearalenone stock standard solution, $\rho(C_{18}H_{22}O_5) \approx 50$ µg/ml.

WARNING — Zearalenone is an oestrogen. Handle with due regard to its biological activity.

Weigh 5,0 mg zearalenone to the nearest 0,1 mg. Transfer into a 100 ml one-mark volumetric flask (5.1.2). Dissolve in acetonitrile (4.2) and make up to the mark with the same solvent.

Calibrate the standard solution as follows. Pipette (5.1.3) 4,0 ml of stock standard into a 25 ml one-mark volumetric flask (5.1.2) and make up to the mark with acetonitrile (approximately 8 µg of zearalenone per millilitre).

Measure the ultraviolet (UV) absorbance using a quartz cuvette of pathlength 10 mm.

Determine the concentration, $\rho(C_{18}H_{22}O_5)$, in milligrams per millilitre, by Equation (1):

$$\rho(C_{18}H_{22}O_5) = \frac{M_r \times 1\,000 \times A \times 25}{\varepsilon \times 4} \quad (1)$$

where

M_r is the relative molecular mass, 318,4, of zearalenone;

A is the UV absorbance;

ε is the emissivity, 12 623 $\pm$ 111, at 274 nm.

Record the result to three significant figures.

The stock standard solution is stable for at least 1 year if stored under refrigeration and tightly sealed. Recalibrate whenever fresh diluted standard solutions (4.16 and 4.17) are prepared.

4.16 Spiking standard solution, $\rho(C_{18}H_{22}O_5)$ = 5,0 µg/ml.

Dilute 10,0 ml stock standard solution (4.15) to 100 ml with extraction solvent (4.5). Store in a refrigerator. Prepare fresh every 6 months.

4.17 HPLC standard solutions.

Prepare five standard solutions of zearalenone concentrations shown in Table 1 by diluting the spiking standard solution (4.16) or HPLC standard solution (4.17.1) with HPLC mobile phase (4.14).

Table 1 — Preparation of HPLC standard solutions

HPLC standard solution	Standard solution to dilute	Volume to dilute ml	Final volume ml	Zearalenone concentration µg/ml
4.17.1	4.16	2,0	50	0,20
4.17.2	4.16	1,5	50	0,15
4.17.3	4.16	1,0	50	0,10
4.17.4	4.16	1,0	100	0,050
4.17.5	4.17.1	5,0	50	0,020

Store all HPLC standard solutions in a refrigerator. Prepare fresh every 6 months.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Common laboratory glassware.

5.1.1 Measuring cylinders, complying with ISO 4788, class A.

5.1.2 One-mark volumetric flasks, complying with ISO 1042, class A.

5.1.3 Pipettes, complying with ISO 648, class A.

5.2 UV spectrophotometer.

5.3 Vacuum manifold, to accommodate IACs.

5.4 Conical flasks, of capacities 125 ml and 500 ml.

5.5 Filter paper, of diameter 185 mm, e.g. Whatman No. 41 [1)].

5.6 Glass tube, 5 ml (13 mm × 100 mm) round bottom, or equivalent.

5.7 Centrifuge tubes, of polypropylene or equivalent, of capacity 50 ml.

5.8 Glass funnels, of maximum internal diameter 60 mm and 90 mm.

5.9 Glass microfibre filter paper, of diameter 125 mm, Whatman 934AH [1)].

5.10 Immunoaffinity columns, loading capacity $\geqslant$ 2 µg zearalenone and recovery $\geqslant$ 85 %, ZearalaTest [1)] [standard or WB (wide bore; preferred, as less prone to blockage)] and EASI-EXTRACT [1)].

5.11 Shaker, orbital or wrist action, or equivalent.

5.12 Plastic syringes, of capacity 5 ml.

5.13 Disposable syringe filters, of polyvinyldifluoride (PVDF), of pore size 0,45 µm and diameter 13 mm.

5.14 Solvent filtration system: all glass filter apparatus suitable for a filter of diameter 47 mm (5.15), and a nylon (polyamide) or PTFE filter of diameter 47 mm and of pore size 0,45 µm.

5.15 HPLC system consisting of:

5.15.1 Pump, pulse free, of output capacity 0,5 ml/min to 1,5 ml/min.

5.15.2 Injector system, manual or autosampler, with loop suitable for 100 µl injections.

5.15.3 Analytical column, 4 µm or 5 µm C_{18}, 150 mm × 4 mm, e.g. Waters Nova-Pak C_{18} [1)], Inertsil ODS-3 [1)], Lichrospher 100-RP-18 [1)], ACE 3 C_{18} [1)], Waters Symmetry Shield RP18 [1)], and Hypersil ODS/BDS [1)].

5.15.4 Fluorescence detector, suitable for measurements with excitation wavelengths 236 nm and 274 nm, and emission at 440 nm (variable wavelength detector) or 418 nm (detector with emission filter).

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1) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

5.15.5 Integrator or **PC workstation**.

5.15.6 Diode-array detector, optional.

5.16 Glass microfibre filters, of diameter 21 mm, e.g. Whatman GF/D [1)].

5.17 Reservoirs, polypropylene, suitable for attachment to the top of the IAC, of capacity 20 ml and internal diameter 20 mm. An adapter may be required.

5.18 Frits, for reservoirs (5.17), of diameter 20 mm and of pore size 20 µm.

5.19 Sieves, complying with the requirements of ISO 565.

5.20 Nitrogen evaporator, with a bath capable of being maintained at 50 °C ± 5 °C.

5.21 Vortex mixer.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport and storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 [1].

Samples should be stored frozen to prevent changes in mycotoxin levels due to growth of the causitive moulds.

7 Preparation of test sample

Prepare the test sample in accordance with ISO 6498.

Grind the entire laboratory sample so it passes completely through a sieve of nominal opening size 1 mm (5.19). Mix thoroughly.

Studies with other mycotoxins have shown that grinding samples through a 0,5 mm ring sieve vs. a 1,0 mm sieve reduced the variability of replicate analyses and increased the extraction efficiency. Grinding equipment producing a particle size less than 1 mm is recommended; however, care is required to avoid overheating the sample due to the openings of the ring sieve being too small. A 0,75 mm ring sieve is suggested; most of the ground material will pass through a sieve of nominal opening size 0,5 mm.

8 Procedure

8.1 Preparation of quality control sample

The use of a quality control sample is recommended; analysis results should meet the recovery specification, i.e. ⩾ 85 %. Analyse a spiked control sample (0,10 mg/kg) with each sample set. Prepare by pipetting 1,0 ml spiking standard (4.16) into 50 g test portion of blank wheat or corn, and mix. Analyse the blank sample also.

8.2 Extraction

Process each test portion as follows.

Weigh, to the nearest 0,01 g, 50,00 g into a 500 ml conical flask (5.4).

Weigh and add 5 g of sodium chloride (4.4).

Add 150 ml extraction solvent (4.5). Shake for 1 h. Proceed to the IAC cleanup procedure (8.3).

Matrices such as dried silage can absorb most of the 150 ml extraction solvent. In that case, increase the volume of extracting solvent to 200 ml or 250 ml.

If desired, the shaking step can be started at the end of the day using a shaker (5.11) with a timer, shake briefly the next morning, then proceed to step 8.3.

8.3 Immunoaffinity column cleanup

Follow the instructions provided by the manufacturer with IACs, but use a reservoir (5.17) equipped with a frit (5.18) and GF/D filter(s) (5.16) for the ZearalaTest [1)] (8.3.1) and for the EASI-EXTRACT [1)] (8.3.2), columns, and elute with 2,0 ml eluent. For other brands, follow either 8.3.1 or 8.3.2, whichever is more appropriate.

An option is provided for analysing highly pigmented matrices and matrices that present interferences in the chromatogram. Methanol, volume fraction 30 % (4.7), is used to elute interferences without causing antibody denaturation; do not exceed a methanol volume fraction of 35 %. For all types of IAC, to achieve the limit of quantification of 0,05 mg/kg, to reduce variability and to enhance HPLC chromatography, column eluates are evaporated and then dissolved in a minimum volume (2,0 ml) of mobile phase (4.14).

NOTE 1 More than 10 ml of diluted filtrate can be added to the columns in steps 8.3.1.4 and 8.3.2.5 as long as the loading capacity is not exceeded. Blockage of the frit and column may occur with larger volumes; barley is known to be a problem, although the use of two filters on the frit helps to prevent frit blockage. Wide bore columns are less susceptible to blockage.

NOTE 2 The clarity of the filtrate being applied to the IAC affects the column's performance. In addition to causing column blockage, particulates can prevent adsorption of zearalenone on to the antibodies, resulting in variable and low recoveries.

8.3.1 ZearalaTest [1)] immunoaffinity column [standard format and wide bore (WB)]

8.3.1.1 Filter more than 10 ml extract through a fluted filter paper (5.5) into a 125 ml conical flask (5.4).

8.3.1.2 Pipette (5.1.3) 10 ml filtered extract into a 50 ml one-mark volumetric flask (5.1.2) and make up to the mark with water. Mix well. Filter the diluted extract (approximately 25 ml) through glass microfibre filter paper (5.9) into a 50 ml centrifuge tube (5.7).

8.3.1.3 Attach the IAC to the port of the vacuum manifold (5.3). Attach a reservoir (5.17) with frit (5.18) to the top of the column. An adapter is required for WB columns. Insert a glass microfibre filter (5.16).

For standard format columns, two glass microfibre filters are required for some matrices, such as barley. If the filtered solution (8.3.1.2) is clear, no frit or filter is required.

8.3.1.4 Pipette (5.1.3) 10 ml filtrate (8.3.1.2) into the reservoir. Draw extract through the column at a steady flow rate until air comes through the column; the flow rate shall be such that droplets are formed (1 drop per second to 2 drops per second).

8.3.1.5 For pigmented products and samples with interfering peaks, wash column with 15 ml methanol, volume fraction 30 %, (4.7) at a rate of 1 drop per second to 2 drops per second until air comes through the column. For all other types of samples, wash the column with 10 ml water (4.1) at a rate of 1 drop per second to 2 drops per second until air comes through the column.

8.3.1.6 Remove the reservoir, attach a reservoir with no frit (not required for WB columns) and elute zearalenone by passing 2,0 ml methanol (4.3) through the column at a rate of about 1 drop per second, collecting the eluate in a 5 ml tube (5.6).

NOTE The frit can be removed from used reservoirs by use of a wire or narrow rod through the bottom of the reservoir, and the reservoirs can be cleaned and reused.

8.3.1.7 Evaporate to dryness using a nitrogen evaporator with a bath temperature of 50 °C ± 5 °C (5.20). Add 2,0 ml of HPLC mobile phase (4.14). Mix with a vortex mixer (5.21). Continue in accordance with 8.4.

8.3.1.8 Optionally, the test solution may be filtered through a PVDF filter (5.13) using a plastic syringe. The lot of filters shall be checked to verify the absence of interfering peak(s). With the use of frits (5.18) and filters in the reservoirs (5.17), test solutions should be clear and no filtration should be required.

8.3.2 EASI-EXTRACT® immunoaffinity column

8.3.2.1 Filter more than 10 ml extract through a fluted filter paper (5.5) into a 125 ml conical flask (5.4).

8.3.2.2 Pipette (5.1.3) 10 ml filtered extract into a 50 ml one-mark volumetric flask (5.1.2), and make up to the mark with PBS buffer (4.12). Mix well.

8.3.2.3 Filter the diluted extract (approximately 25 ml) through a glass microfibre filter paper (5.9) into a 50 ml centrifuge tube (5.7).

8.3.2.4 Attach the IAC to the port of the vacuum manifold (5.3). Attach a reservoir (5.17) with frit (5.18) to the top of the column using an adapter. Insert a glass microfibre filter (5.16). Wash the column with 10 ml to 20 ml of PBS buffer.

If the filtered solution (8.3.2.3) is clear, no frit or filter is required.

8.3.2.5 Pipette (5.1.3) 10 ml filtrate (8.3.2.3) into the reservoir. Draw the extract through the column at a steady volume flow rate until air comes through the column; the flow rate shall be such that droplets are formed (1 drop per second to 2 drops per second).

8.3.2.6 For pigmented products and samples with interfering peaks, wash column with 15 ml methanol, volume fraction 30 %, (4.7) at a rate of 1 drop per second to 2 drops per second until air comes through the column. For all other samples, wash the column with 20 ml water (4.1) at a rate of 1 drop per second to 2 drops per second until air comes through the column.

8.3.2.7 Remove the reservoir and elute zearalenone by passing 2,0 ml acetonitrile (4.2) through the column at a rate of about 1 drop per second, collecting the eluate in a 5 ml tube (5.6).

8.3.2.8 Evaporate to dryness using a nitrogen evaporator with a bath temperature of 50 °C ± 5 °C (5.20). Add 2,0 ml of HPLC mobile phase (4.14). Mix with a vortex mixer (5.21).

8.3.2.9 Optionally, the test solution may be filtered through a PVDF filter (5.13) using a plastic syringe. The lot of filters shall be checked to verify the absence of interfering peak(s). With the use of frits and filters in the reservoirs, test solutions should be clear and no filtration should be required.

8.4 HPLC analysis

8.4.1 HPLC conditions

Mobile phase	see 4.14	
Flow rate	1,0 ml/min	
Injection volume	100 µl	
Column	see 5.15.3, with C_{18} guard column	
Detector wavelength	excitation	274 nm
	emission	440 nm (for a variable wavelength detector)
		418 nm (for a detector with emission filter)

8.4.2 System suitability

8.4.2.1 Resolution

Inject a volume of 0,050 µg/ml HPLC standard solution (4.17.4). A single peak should be observed; however, if an adjacent peak is present, the zearalenone peak shall be baseline resolved.

8.4.2.2 Tailing factor

The United States Pharmacopeia tailing factor, numerically equal to the European Pharmacopoeia symmetry factor, F, shall be less than 1,6.

8.4.3 Determination

8.4.3.1 Inject 100 µl (full loop) of 0,020 µg/ml HPLC standard solution (4.17.5). Make two or more injections of standard solutions to ensure repeatability of peak area is $\sim$ 2 %. Determine the linearity by injecting 100 µl (full loop) of each HPLC standard solution (4.17). Plot peak area vs. zearalenone concentration, in micrograms per millilitre. The correlation coefficient shall be $\geqslant$ 0,999 and the 95 % confidence interval of the y intercept shall include zero (by analysis of residuals).

8.4.3.2 Inject 100 µl test solutions. Inject the five HPLC standard solutions (4.17) at the end of the test solution set, and average the peak area response with those from 8.4.3.1 to prepare a calibration graph. Bracket each group of five to eight test solution injections with 100 µl of a standard solution injection, to check for drift. Dilute the test solution if the peak area is beyond the range of the calibration graph.

8.4.3.3 If the mass concentration of zearalenone in the test sample exceeds 3 mg/kg, to ensure that the IAC is not overloaded, dilute the extract (8.2) 10 times with extraction solvent (4.5), then repeat the procedures specified in 8.3.

8.4.3.4 Suspect positive samples can be confirmed using an excitation wavelength of 236 nm (increased sensitivity will be observed), or by using a diode array detector. Use any of the techniques specified in 8.4.3.4.1, 8.4.3.4.2 or 8.4.3.4.3.

8.4.3.4.1 If using a fluorescence detector with a deuterium lamp, use test solution of 8.3.1.7 or 8.3.2.8. Adjust the detector range or integrator attenuation to obtain similar peak response as in 8.4.3.1. The identity is confirmed if the ratio of the peak areas at 236 nm and 274 nm is consistent for standard and test solutions. Peak area ratios of test sample solutions should be within 5 % of those for the corresponding standard solutions.

8.4.3.4.2 If using a fluorescence detector with a xenon flash lamp, determining the peak ratio using reverse-phase chromatography may not be possible, since the lamp intensity is too low at 236 nm. Verify if 8.4.3.4.1 is applicable for this detector; if not, confirm suspected positive samples using normal phase conditions (see Annex A). Redo 8.3 for the sample(s) and control sample but at the evaporation stages (8.3.1.7 or 8.3.2.8), add 2,0 ml of a mixture of chloroform, hexane, and propan-2-ol, volume ratio 50+50+3, respectively (A.1.2). Proceed according to Clause A.3, using the silica LC column (Clause A.2).

8.4.3.4.3 If using a diode array detector, use the test solution of 8.3.1.7 or 8.3.2.8. Record the spectrum (200 nm to 400 nm) of the peak at the zearalenone retention time and compare to the spectrum of a similar standard solution. The three absorbance maxima (236 nm, 274 nm, and 316 nm) shall be visible, and the relative intensities shall be similar for sample and standard solutions. Sample maxima shall be within $\pm$ 2 nm of the standard maxima. A threshold value of $>$ 950 out of 1 000 for peak spectra similarity also confirms the presence of zearalenone.

NOTE It is possible that diode array detectors are not sensitive enough for levels near the method limit of quantification. Additional run time can be necessary to avoid late eluting peaks, and modification of the mobile phase (4.14) can be required to obtain adequate selectivity due to additional peaks that can occur with UV determination.

9 Calculation of results

Calculate the sample zearalenone mass fraction, w_s, in milligrams per kilogram, using either Equation (1) or Equation (2):

$$w_s = \rho_t \times \frac{150}{m} \times \frac{50}{10} \times \frac{2}{V} \times f_d \quad (1)$$

$$w_s = \frac{A_t}{A_{st}} \times \rho_{st} \times \frac{150}{m} \times \frac{50}{10} \times \frac{2}{V} \times f_d \quad (2)$$

where

ρ_t is the zearalenone concentration, in micrograms per millilitre, in the test solution derived from the calibration graph;

A_t is the peak area of zearalenone in the test solution;

A_{st} is the peak area of zearalenone in the HPLC standard solution (average of bracketing standards);

ρ_{st} is the zearalenone concentration, in micrograms per millilitre, in the HPLC standard solution;

m is the mass, in grams, of the test portion;

V is the volume, in millilitres, of filtrate in 8.3.1.4 or 8.3.2.5;

f_d is the dilution factor (equal to 1, unless the final volume is different from 2 ml).

Report the result to three significant figures.

EXAMPLE

A_t	65 224
A_{st}	72 578
ρ_{st}	0,051 0 µg/ml
m	50,10 g
Final volume	2,0 ml
V (8.3.1.4)	10 ml
f_d	1

$$w_s = \frac{65\,224}{72\,578} \times 0{,}051\,0 \times \frac{150}{50{,}10\ \text{g}} \times \frac{50}{10} \times \frac{2{,}0}{10} = 0{,}137 \quad (3)$$

10 Precision

10.1 Interlaboratory test

Repeatability and reproducibility values were derived from the results of a 2005 interlaboratory test carried out in accordance with References [6] and [7] (see Annex B). The single laboratory validation showed that the method is applicable to all types of feeds and cereal grains, soybean meal, canola (rapeseed) meal, corn gluten, dried distillers' grains, lentils, and sugar beet pulp.

10.2 Repeatability

The absolute difference between two independent single test results, expressed as a percentage of the mean determined value, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the relative repeatability limit, r_{rel}, derived from Equation (4):

$$r_{rel} = 2{,}8 \times CV(r) \quad (4)$$

where CV(r) is the coefficient of variation of repeatability.

10.3 Reproducibility

The absolute difference between two single test results, expressed as a percentage of the mean determined value, obtained using the same method on identical test material different laboratory with different operators using different equipment will in not more than 5 % of cases exceed the relative reproducibility limit, R_{rel}, derived from Equation (5):

$$R_{rel} = 2{,}8 \times CV(R) \quad (5)$$

where CV(R) is the coefficient of variation of reproducibility.

Repeatability and reproducibility criteria derived from Table B.2 are given in Table 2.

Table 2 — Repeatability and reproducibility criteria

Parameter	Value %
CV(r), maximum	12,1
CV(r), arithmetic average [a]	9,25
r_{rel}, maximum	34,0
r_{rel}, average [a]	25,9
CV(R), maximum	19,7
CV(R), arithmetic average [a]	15,4
R_{rel}, maximum	55,3
R_{rel}, average [a]	43,1

[a] Determined by analysis of variance. The study indicates that the analysis variability is independent of the matrix and zearalenone concentration.

10.4 Limit of quantification

The limit of quantification is 0,05 mg/kg (50 µg/kg).

11 Test report

The test report shall specify:

a) the information required for the complete identification of the sample;

b) the sampling method used, if known:

c) the test method used, together with reference to this International Standard;

d) any operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s).

e) the test result obtained and, if the repeatability has been checked, the final quoted result(s) obtained.

Annex A
(normative)

Confirmation using normal phase chromatography

A.1 Reagents

A.1.1 HPLC mobile phase

Prepare by adding volumes in the proportions 485+484+50+1 of dichloromethane, hexane, propan-2-ol and acetic acid, respectively, and mixing.

A.1.2 Solvent for HPLC standards

Prepare by adding volumes in the proportions 50+50+3 of chloroform, hexane, and propan-2-ol, respectively, and mixing.

A.1.3 HPLC standard solutions for confirmation

Prepare five standard solutions of zearalenone concentrations shown in Table A.1 by diluting the spiking standard solution (4.16) or HPLC standard solution (A.1.3.1) with solvent (A.1.2).

Table A.1 — Preparation of HPLC standard solutions

HPLC standard	Standard solution to dilute	Volume to dilute	Final volume	Zearalenone concentration
		ml	ml	µg/ml
A.1.3.1	4.16	2,0	50	0,20
A.1.3.2	4.16	1,5	50	0,15
A.1.3.3	4.16	1,0	50	0,10
A.1.3.4	4.16	1,0	100	0,050
A.1.3.5	A.1.3.1	5,0	50	0,020

Store all HPLC standards in a refrigerator. Prepare fresh every 6 months.

A.2 Column

In addition to the HPLC equipment specified in 5.15, a 5 µm Zorbax SIL [2)] column, of length 250 mm and diameter 4,6 mm, or equivalent.

2) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

A.3 HPLC analysis

A.3.1 HPLC conditions

Mobile phase	see A 1.1	
Flow rate	1,0 ml/min	
Injection volume	100 µl	
Guard column	silica	
Detector wavelengths (for dual monochromator detector)	excitation	236 nm
	emission	440 nm

A.3.2 System suitability

Inject 100 µl of 0,050 µg/ml standard solution (A.1.3.4; 100 µl contains 5,0 ng zearalenone). If necessary, modify the mobile phase (by adjusting the propan-2-ol content) to obtain a retention factor, $k \geqslant 2{,}0$.

Make two or more injections of standard solutions to ensure repeatability of peak response is ~2 %.

Determine the linearity by injecting 100 µl of each HPLC standard solution (A.1.3). Plot peak area vs. mass, in nanograms, of zearalenone. The correlation coefficient shall be $\geqslant$ 0,999, and the 95 % confidence interval of the y intercept shall include zero.

A.3.3 Determination

Inject 100 µl test solutions (diluted if necessary, according to concentration determined by reverse phase). Bracket each two test solution injections with 100 µl of the appropriate standard solution.

The identity is confirmed if the ratio of peak responses at 236 nm and 274 nm is consistent for standard and sample solutions. Peak response ratios of samples shall be within 5 % of that for the standard solution.

Annex B
(informative)

Results of an interlaboratory test

B.1 Pocedure and samples

An interlaboratory test was organized and carried out in accordance with References [6] and [7], in which nine materials were selected as test samples. Between 2,5 kg and 5 kg of each material was ground using a Retsch SR3 mill [3)] equipped with a 1,0 mm sieve (60 % to 80 % of the ground material passed a 0,5 mm sieve). The ground material was vector mixed for 2 h, then divided into 50 g to 60 g laboratory samples using a Retsch rotary PTZ divider [3)]. Homogeneity of the laboratory samples was verified by analysing five or more randomly selected laboratory samples. The description and homogeneity test results for each material are shown in Table B.1.

A total of 20 laboratories from 13 European countries, Canada, the USA, Japan, and Uruguay were invited to participate in the interlaboratory test. The study was done in two phases: a familiarization study involving the analysis and confirmation of a spiked sample and two contaminated samples, and the collaborative study phase involving the analysis of blind duplicate samples. A total of 13 candidate laboratories were selected for the collaborative study on the basis of results from the familiarization study. In all, 20 laboratory samples were provided; the nine samples in Table B.1 as blind duplicates, and a blind blank corn and a blind blank wheat sample. A sample of blank wheat was provided for control sample preparation. All laboratories returned acceptable data within the required timeframe. Two laboratories experienced interfering peaks in the chromatograms, indicating contamination. The source of the interferences could not be identified, but changes to the mobile phase or change of the HPLC column allowed for reporting of results. Consequently some of the data from these laboratories were rejected in the statistical evaluation. For all laboratories, the two blank samples were reported as “zearalenone not detected” or at trace level ($<$ 0,01 mg/kg). Acceptable recoveries (89 % to 116 % mass fraction) were reported by all laboratories for two spiked wheat control samples (average recovery, 102,6 %; s_{rel}, 6,88 %).

Table B.1 — Samples

Sample No. and description	Homogeneity test results			IAC wash solvent
	n	Arithmetic average	s	
		mg/kg	%	
1. Barley	5	0,111	6,72	Water (4.1)
2. Barley	5	0,161	1,58	Water (4.1)
3. Corn	5	0,076 5	6,50	Water (4.1)
4. Corn	6	0,120	6,74	Water (4.1)
5. Corn	5	0,281	3,66	Water (4.1)
6. Dairy feed	5	0,142	5,60	Methanol, volume fraction 30 % (4.7)
7. Dried distillers' grains	5	0,279	2,09	Methanol, volume fraction 30 % (4.7)
8. Swine feed	5	0,115	2,57	Water (4.1)
9. Wheat	5	0,202	5,74	Water (4.1)

3) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

B.2 Statistical analysis of results

The results of the blind duplicate sample analyses were examined for evidence of individual systematic error using Cochran and Grubbs tests progressively, by procedures described in References [6] and [7] using the AOACI Statistical Program 2001 for Blind Replicate Spreadsheets. Calculations for repeatability, r, and reproducibility, R, as defined by the guidelines were carried out after removal of outliers. HorRat values, the ratios of the coefficient of variation of reproducibility to the predicted coefficient of variation of reproducibility, were calculated. HorRat values between 0,5 and 1,5 are the limits for performance acceptability (References [6] and [7]). The calculated method performance data (repeatability, reproducibility and HorRat values) are presented in Table B.2.

Table B.2 — Results of interlaboratory test

Parameter	Sample No.								
	1 Barley	2 Barley	3 Corn	4 Corn	5 Corn	6 Dairy feed	7 Dried distillers' grains (corn)	8 Swine feed	9 Wheat
Number of laboratories retained after elimination of outliers	13	13	13	13	12	13	12	12	13
Target zearalenone mass fraction, µg/kg	111	161	76,5	120	281	142	279	115	202
Mean zearalenone mass fraction, µg/kg	115	166	79,6	122	273	134	250	120	189
Repeatability standard deviation, s_r, µg/kg	14,0	12,1	9,13	10,2	18,2	13,2	14,6	11,9	18,4
Coefficient of variation of repeatability, CV(r), %	12,1	7,28	11,5	8,36	6,67	9,84	5,84	9,92	9,72
Repeatability limit, r, µg/kg	39,1	33,8	25,6	28,5	51,0	37,0	40,9	33,4	51,5
Relative repeatability limit, r_{rel}, of mean determined content, %	34,0	20,4	32,2	23,4	18,7	27,6	16,4	27,8	27,2
Reproducibility standard deviation, s_R, µg/kg	20,4	22,1	11,9	18,4	37,5	22,3	33,4	23,7	23,6
Coefficient of variation of reproducibility, CV(R), %	17,7	13,3	14,9	15,1	13,8	16,6	13,4	19,7	12,5
Reproducibility limit, R, µg/kg	57,1	61,8	33,2	51,6	105	62,4	93,6	66,4	66,1
Relative reproducibility limit of mean determined content, R_{rel}, %	49,7	37,2	41,7	42,3	38,5	46,6	37,4	55,3	35,0
HorRat value	0,80	0,63	0,64	0,69	0,71	0,77	0,68	0,90	0,61

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