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BSI Standards Publication

Animal feeding stuffs — Determination of Ochratoxin A in animal feed by immunoaffinity column clean-up and High Performance Liquid Chromatography with fluorescence detection

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National foreword

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

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Animal feeding stuffs - Determination of Ochratoxin A in animal feed by immunoaffinity column clean-up and High Performance Liquid Chromatography with fluorescence detection

Aliments pour animaux - Dosage de l'ochratoxine A dans les aliments pour animaux par purification sur colonne d'immuno-affinité et chromatographie liquide à haute performance avec détection par fluorescence

Futtermittel - Bestimmung von Ochratoxin A in Tierfutter durch Reinigung an einer Immunoaffinitätssäule und Hochleistungs-Flüssig-Chromatographie mit Fluoreszenzdetektion

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Foreword

This document (EN 16007:2011) 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of an identical text or by endorsement, at the latest by February 2012, and conflicting national standards shall be withdrawn at the latest by February 2012.

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1 Scope

This European Standard specifies a method for the determination of Ochratoxin A (OTA) in cereal based animal feed using immunoaffinity for clean-up followed by liquid-chromatography with fluorescence detection.

NOTE The validated mass fraction range was 39 µg/kg to 338 µg/kg OTA.

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 1042, *Laboratory glassware — One-mark volumetric flasks (ISO 1042:1998)*

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

3 Principle

OTA is extracted from the test material with a mixture of methanol – 3% aqueous sodium bicarbonate solution. The extract is filtered, diluted with PBS and purified using immunoaffinity columns (IAC). The purified OTA is eluted from the IAC using first methanol and then water, brought to a defined volume with water and quantified by HPLC with fluorescence detection.

4 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade. Solvents shall be of HPLC or better quality.

4.1 Methanol, CH_3OH, technical grade.

4.2 Methanol, CH_3OH, HPLC grade.

4.3 Water, water (EN ISO 3696 grade 1 (HPLC grade)) and water (EN ISO 3696 grade 3), or equivalent.

4.4 Potassium chloride, KCl.

4.5 Sodium chloride, NaCl.

4.6 Disodium hydrogenphosphate dodecahydrate, Na_2HPO_4*12 H_2O .

4.7 Acetonitrile, CH_3CN, HPLC grade.

4.8 Glacial acetic acid, CH_3COOH, 96% minimum.

4.9 Solution of acetonitrile/ glacial acetic acid, acetonitrile (4.7) and glacial acetic acid (4.8) in proportion of 99/1 (v/v).

4.10 Toluene, $C_6H_5CH_3$, analytical grade.

4.11 Toluene/Acetic acid solution, Toluene (4.10) / Glacial acetic acid (4.8) in proportion of 99/1 (v/v).

4.12 Sodium hydrogen carbonate, $NaHCO_3$, minimum 99% purity.

4.13 PBS concentrate, Phosphate buffered saline concentrate.

Dissolve the following in 1 800 ml of water (EN ISO 3696 grade 1):

— 4 g KCl (4.4);

— 160 g NaCl (4.5);

— 72 g Na_2HPO_4*12 H_2O (4.6).

4.14 PBS Ready to use.

Dilute 100 ml of PBS concentrate (4.13) to 1 000 ml with water (EN ISO 3696 grade 1). Adjust to pH 7,4 with 1 mol/l HCl and make up to 2 000 ml with water (EN ISO 3696 grade 1),

or

PBS tablets, Phosphate buffered saline tablets,

One tablet dissolved in 200 ml of water (EN ISO 3696 grade 1) yields 0,01 mol/l phosphate buffer, 0,002 7 mol/l potassium chloride and 0,137 mol/l sodium chloride, pH 7,4, at 25°C (e.g. Sigma P4417).

4.15 3% aqueous sodium hydrogen carbonate solution.

Add 30 g of sodium hydrogen carbonate (4.12) to 1 000 ml of water (EN ISO 3696 grade 3).

4.16 Extraction solvent, methanol / 3% aqueous sodium hydrogen carbonate solution in proportion of 50/50 (v/v).

Add 500 ml of methanol (4.1) to 500 ml of 3% aqueous sodium hydrogen carbonate solution (4.15). Mix well.

4.17 Aqueous solution of glacial acetic acid.

Add 30 ml of glacial acetic acid (4.8) to 870 ml of water (EN ISO 3696 grade 1) and filter.

4.18 HPLC mobile phase, acetonitrile / methanol / aqueous solution of glacial acetic acid in proportion of 35/35/30 (v/v/v).

Mix 1 050 ml of methanol (4.2) with 1 050 ml of acetonitrile (4.77) and with 900 ml of aqueous solution of glacial acetic acid (4.1717). Mix well and degas.

4.19 OTA, ochratoxin A.

OTA in pure form as crystals, dried film, powder or in solution.

4.20 OTA Stock Solution.

Prepare from the OTA (4.19) a solution of 20 µg/ml in toluene/acetic acid solution (4.11).

To determine the exact concentration, record the absorption curve of this solution between a wavelength of 300 nm and 370 nm in a 1 cm quartz cell with toluene/acetic acid solution (4.11) as reference using the UV-

spectrophotometer (5.21). Identify the wavelength for maximum absorption. Calculate the mass concentration of OTA, ρ ota, in micrograms per millilitre using Equation (1):

$$\rho_{\text{ota}} = \frac{A_{\max} \times M \times 100}{\varepsilon \times b} \quad (1)$$

where

$A_{\max}$ is the absorption determined at the maximum of the absorption curve (here: at 333 nm);

M is the molar mass, in grams per mol, of OTA (M = 403,8 g/mol);

ε is the molar absorption coefficient, in square metres per mol, of OTA in toluene/acetic acid solution (4.11), (here: 544 m^2/mol);

b is the optical path length, in centimetres, of the quartz cell.

4.21 Nitrogen.

4.22 OTA diluted stock solution for calibration.

Prepare 10 ml of OTA diluted stock solution for calibration by diluting 100 times the OTA stock standard solution (4.20) with mobile phase (4.18). For this pipette 100 µl of the OTA stock standard solution into a volumetric cylinder, evaporate the solution under a slight stream of nitrogen (4.21) at 40 ºC and take up with mobile phase (4.18).

NOTE To ease complete dissolution of the OTA in mobile phase, first fill the volumetric cylinder to approximately 1/3, let the OTA dissolve, and then fill up to the mark and shake.

4.23 Calibration solutions.

From the OTA diluted stock solution for calibration (4.22) prepare six levels of calibration solutions by adding the volumes of diluted stock solution listed below to a volumetric flask of the indicated volume and make up to the mark with mobile phase (4.18):

Table 1 — recommended calibration solutions (4.23) for the determination of OTA

Calibrant	OTA diluted stock solution for calibration (4.22) (µl)	Volumetric flask (5.5) (ml)	Concentration OTA (ng/ml)
1	50	10,0	1
2	250	10,0	5
3	500	10,0	10
4	750	10,0	15
5	1 000	10,0	20
6	1 250	10,0	25

4.24 IAC with antibodies specific to OTA.

The IAC contains antibodies raised against OTA. The column shall have a total capacity of not less than 100 ng of OTA. OTA recovery shall not be less than 85 % when 5 ng OTA is applied in 50 ml of a mixture of 4 parts per volume of extraction solvent (4.16) and 96 parts per volume of PBS Ready to use (4.14).

4.25 Spiking solution.

OTA in a solution of acetonitrile/ glacial acetic acid in proportion of 99/1 (v/v). The OTA concentration of the spiking solution will depend on the spiking level required. The spiking volume should be approximately 500 µl. The solution can be made from the OTA Stock Solution (4.20) in a similar manner as the OTA diluted stock solution for calibration (4.22).

5 Apparatus

Usual laboratory equipment and, in particular, the following:

5.1 Common laboratory glassware, such as graduated cylinders, beakers, volumetric pipettes

5.2 Analytical balance, capable of weighing to 0,1 mg

5.3 Horizontal or vertical shaker

5.4 Automated SPE Vacuum System

5.5 Volumetric flasks (class A, EN ISO 1042), 5 ml, 10 ml, 100 ml

5.6 Filter paper pre-folded, 30 µm particle retention

5.7 Screw-cap flasks, 250 ml and 500 ml

5.8 Glass funnels, 9 cm ID

5.9 Reservoirs, polypropylene, suitable for attachment to top of IAC, 50 ml to 75 ml size

5.10 Plastic syringes, 5 ml

5.11 Calibrated displacement micropipette, 100 µl, 500 µl and 1 000 µl (variable volume)

5.12 Solvent vacuum filtration system, suitable for 47 mm filter

5.13 Glass microfibre filter, 1,6 µm particle retention

5.14 Vortex mixer

5.15 HPLC syringe filter cartridges, Nylon with 0,45 µm pore size

5.16 Ultrasonic bath

5.17 Amber glass vials, ca 2 ml capacity and crimp caps or equivalent

5.18 HPLC system

The HPLC system comprises the following:

— pump, pulse free, flow capacity 0,5 ml/min to 1,5 ml/min;

— injector system, manual or autosampler, with loop suitable for 100 µl to 300 µl injections;

— fluorescence detector, suitable for measurements with excitation wavelengths 333 nm and emission at 467 nm;

— integrator, or PC workstation.

5.19 Analytical reversed phase HPLC column, C18 RP-column suitable to allow a sufficient separation of OTA from other interfering components; Fully End Capped with column dimensions preferably 250 mm X 4,6 mm ID stationary phase with particle size 5 µm.

5.20 Pre-column (guard-column),, with preferably the same stationary phase material as the analytical column and internal diameter of 4,0 mm, stationary phase with particle size 5 µm.

5.21 UV-Spectrophotometer.

6 Sample preparation

6.1 Extraction of OTA

- Weigh 25,0 g, to the nearest 0,1 g, of the test sample into a large enough container with lid, e.g. 500 ml screw-cap flask (5.67),

NOTE 1 The materials for the interlaboratory study [1] were milled to a particle size of ca. 0,5 mm.

- add 200,0 ml of extraction solvent (4.16), cap, and shake vigorously by hand for a few seconds; so that the material disperses evenly (check visually),
- put on a shaker (5.3) for 40 min. Choose a speed such that the material is mixed well without collecting in the top of the flask. A possible alternative is to ultrasonificate for 15 minutes; in this case shortly shake the extract by hand for a few seconds,
- allow the extracted sample to settle after extraction,
- filter the extract through folded filter paper (5.6) and collect the filtered extract in a screw cap flask of 250 ml (5.7), and
- transfer approx. 10 ml of the filtered extract into the vacuum system and filter through glass microfibre filter (5.13) by applying a slight vacuum (5.12). Discard this volume and filter again the remaining extract in another screw cap flask of 250 ml (5.7) to obtain a clear extract for further analysis. Proceed immediately with the IAC clean-up procedure (6.2).

NOTE 2 Do not apply a strong vacuum in the beginning of the filtration process as this can lead to turbid filtered extract after filtration.

6.2 Clean up and test solution

- Take one IAC (4.24) per extract;
- attach a reservoir (5.9), do not empty storage solution from column;
- transfer 4,00 ml of double filtered extract into a 100 ml volumetric flask (5.5), fill to volume with PBS ready for use (4.14) and shake;
- apply 50,0 ml of the diluted extract to the reservoir on the IAC (see previous point); this is equivalent to 2,00 ml of the double filtered and undiluted extract;
- open the IAC outlet;
- draw extract through the IAC by gravity at a steady flow rate until all extract has passed the immunoaffinity column and the last solvent portion reaches the lower frit of the IAC;
- after the extract has passed completely, wash the IAC with 10 ml of water (EN ISO 3696 grade 1); make sure that the water is pH neutral;
- pass air through the IAC (f.i., using a properly fitted large syringe) in order to expel excess water;
- place a 5 ml volumetric flask (5.5) underneath the IAC and add 0,75 ml of methanol (4.2) to the IAC;
- collect the eluate in the 5 ml volumentric flask;
- after the last drops of methanol have passed through the IAC, allow the methanol to remain on the IAC for approximately 1 min; then add a further 0,75 ml of methanol (4.2) and continue to collect the eluate, followed by 0,50 ml of water (EN ISO 3696 grade 1);
- carefully pass air through the IAC in order to collect most of the applied methanol and water;
- immediately add 1,5 ml of glacial acetic acid solution (4.17) into the volumetric flask; and
- fill the volumetric flask up to the mark with water (EN ISO 3696 grade 1) and shake using a vortex. In case of turbid samples, filter test solutions through a HPLC syringe filter (5.15) with a plastic syringe (5.10). This can be used directly as test solution.

NOTE An alternative to a manual procedure the immunoaffinity is a clean-up and elution. This can be performed with an automatic sample preparation unit, provided that volumes and aliquots remain unchanged.

6.3 Spiking procedure

To determine recovery spike an OTA free representative compound animal feed material with OTA stock solution (4.20) or a dilution thereof. The spiking level should be appropriate and the concentration of the solution used such that approximately 500 µl is added. Leave the spiked sample to stand for a period of 30 min to ensure evaporation of the solvent.

7 Measurements

7.1 HPLC operating conditions

Using the equipment outlined in 5.18, the following conditions have proven to produce satisfying results:

— mobile phase: as in 4.18;

— flow rate: 0,7 ml/min to 1,5 ml/ min;

— injection vol.: 100 µl

— RP-HPLC column, (5.19);

— Pre-colum (guard column): C18(5.20);

— fluorescence detector: Excitation λ: 333 nm (5.18);

— emission λ: 467 nm;

— no temperature control.

7.2 Determination of OTA in test solutions

Inject aliquots of the calibration solutions (4.23) and of the test solutions (6.2) into the chromatograph using the same operating conditions.

7.3 Calibration

Plot the signals (peak area or height) of all the measured calibration solutions against the corresponding concentrations for OTA. Do not use means of the multiple injections. With linear regression estimate slope and intercept of each of the calibration function. Check for significance of the intercept and for linearity (use e.g. a residuals vs. fitted-values plot).

7.4 Peak identification

Identify the OTA peak in the test solution by comparing the retention time with those of the calibration solutions. The signal (peak area or height) of OTA in the test solution must fall within the calibration range. If the OTA signal in the test solution exceeds the signals of the highest calibration solution the test solution shall be diluted to bring it within calibration range, and be reanalysed twice (double injection). The dilution factor must be incorporated into all subsequent calculations.

8 Determination of concentrations

Using the estimated slopes and intercepts from linear regression (7.3) calculate the concentrations of OTA ($c_{T(OTA)}$) in the test solutions (6.2) as follows:

$$c_{T(OTA)} = \frac{\text{signal}_{OTA} - \text{intercept}_{OTA}}{\text{slope}_{OTA}} \text{ (ng/ml)} \quad (2)$$

If the test solution was diluted because of a signal above the calibration range (7.4) multiply the calculated concentrations of OTA ($c_{T(OTA)}$) with the dilution factor.

To calculate the mass fractions (c_{SMP}) of the analyte in the original materials use the following equation:

$$c_{SMP} = \frac{c_T \times Solvent \times Elution}{W \times Aliquot} \text{ (ng/g or µg/kg)} \quad (3)$$

with

c_T = calculated concentration of OTA (2), corrected for dilution if needed (ng/ml);

W = weight of the test material used for extraction (25,0 g);

Solvent = total volume of the extraction solvent (200,0 ml);

Aliquot = extract aliquot used for IAC clean-up (2,0 ml),

(4 ml / 100 ml X 50 ml = 2 ml);

Elution = final volume achieved after elution from IAC (5 ml).

If the weight of the test material and the volumes described herein before are kept the above Equation (3) can be simplified to:

$$c_{SMP} = c_T \text{ x } 20 \text{ (µg/kg)} \quad (4)$$

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.

Table 2 — Precision data repeatability limit

$\overline{x}$ = 39 µg/kg	*r* = 4,54 µg/kg
$\overline{x}$ = 62 µg/kg	*r* = 5,8 µg/kg
$\overline{x}$ = 150 µg/kg	*r* = 18,5 µg/kg
$\overline{x}$ = 240 µg/kg	*r* = 27,7 µg/kg
$\overline{x}$ = 338 µg/kg	*r* = 44,8 µg/kg

The relationship between r and $\overline{x}$ can be sufficiently approximated by the following function:

$r = 2.8 \times 0.045 \times \overline{x}$

meaning that the relative repeatability standard deviation is constant over the working range.

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.

Table 3 — Precision data reproducibility limit

$\overline{x}$ = 39 µg/kg	*R* = 14,8 µg/kg
$\overline{x}$ = 62 µg/kg	*R* = 24,9 µg/kg
$\overline{x}$ = 150 µg/kg	*R* = 61,2 µg/kg
$\overline{x}$ = 240 µg/kg	*R* = 93,8 µg/kg
$\overline{x}$ = 338 µg/kg	*R* = 144 µg/kg

The relationship between R and $\overline{x}$ can be sufficiently approximated by the following function:

$R = 2.8 \times 0.147 \times \overline{x}$

meaning that the reproducibility standard deviation is constant over the working range.

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)

Example of a chromatogram

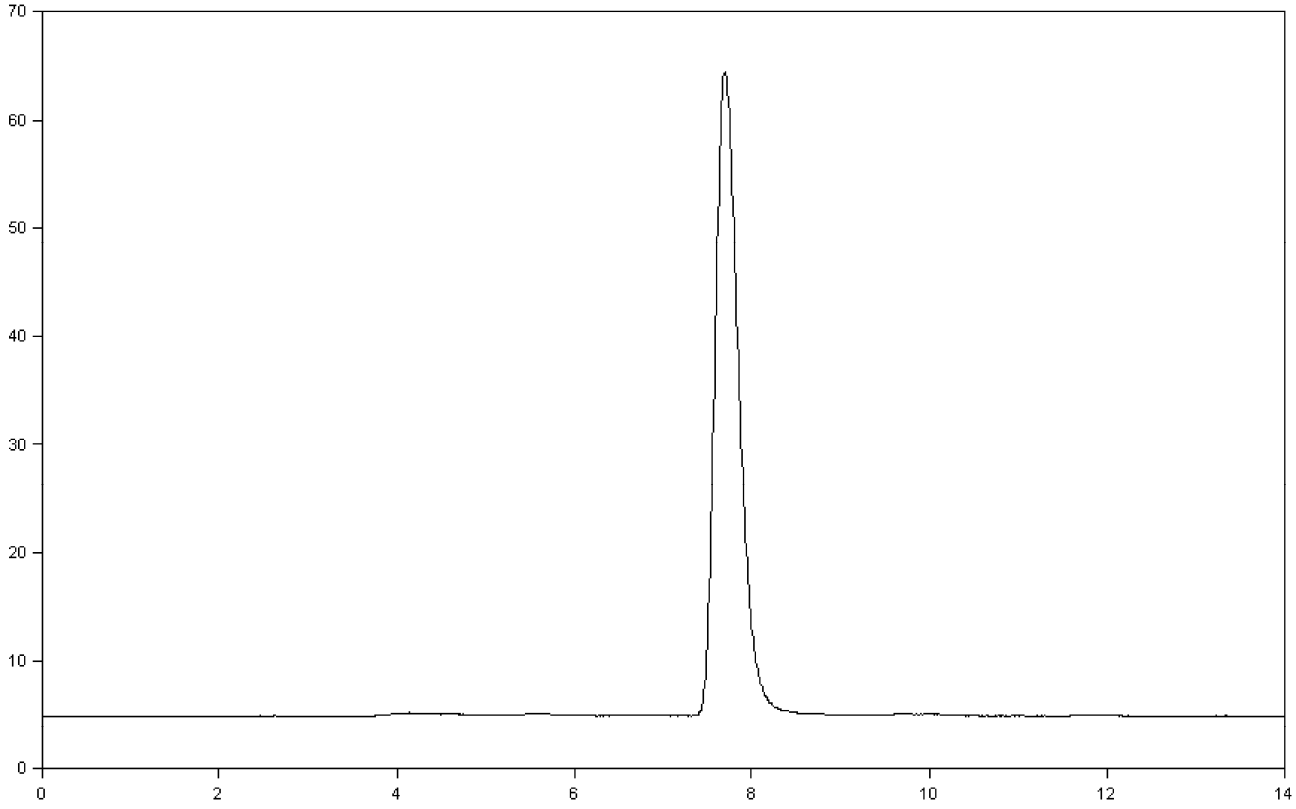

Figure A.1 — Example chromatogram (~200 µg/kg OTA in animal feed)

Annex B
(informative)

Results of the interlaboratory study

The data given in Table B.1 and Table B.2 were obtained in an interlaboratory study [1] according to AOAC Guidelines for collaborative study procedures to validate characteristics of a method of analysis [2], [3]. The test samples are described in Table B.3.

Table B.1 — Performance Characteristics from the collaborative study OTA in animal feed (neat results)

Parameter	Ochratoxin A					
Sample Set (nc = naturally contaminated)	1	2	3	4(nc)	5(nc)	6(nc)
Year of interlaboratory test	2007					
Number of laboratories	29					
Number of samples (duplicates)	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	29	28	26	28	29	29
Number of outliers	n.a.	1	2	1	0	0
Number of accepted results	29	28	26	28	29	29
Mean value (µg/kg)	n.d	62	240	39	150	338
Repeatability standard deviation s_r (µg/kg)	n.a.	2,07	9,91	1,62	6,60	16,0
Repeatability relative standard deviation RSD_r (%)	n.a.	3,33	4,12	4,15	4,40	4,74
Repeatability limit r (r = 2,8 x s_r) (µg/kg)	n.a.	5,80	27,7	4,54	18,5	44,8
Reproducibility standard deviation s_R (µg/kg)	n.a.	8,90	33,5	5,30	21,9	51,2
Reproducibility relative standard deviation RSD_R (%)	n.a.	14,3	13,9	13,6	14,6	15,2
$HORRAT_R$ (Modified calculation according to Thompson [4])	n.a.	0,6	0,7	0,6	0,7	0,8
Reproducibility limit R (R = 2,8 x s_R) (µg/kg)	n.a.	24,9	93,8	14,8	61,3	143
Recovery (%)	-	82	79	-	-	-

Sample set 1 was blank material, sets 2 and 3 were fortified materials, sets 4, 5 and 6 were naturally contaminated. n.d = not detected. The level reported for N.D. (or „smaller than“ or <LOD) varies from the achieved LOD in each case. n.a. = not applicable.

Table B.2 — Performance Characteristics from the collaborative study OTA in animal feed (corrected for recovery – only for information)

Parameter	Ochratoxin A		
Sample Set (nc = naturally contaminated)	4(nc)	5(nc)	6(nc)
Year of interlaboratory test	2007		
Number of laboratories	29		
Number of samples (duplicates)	1	1	1
Number of laboratories retained after eliminating outliers	26	29	30
Number of outliers	4	1	0
Number of accepted results	26	29	30
Mean value (µg/kg)	48,7	186	420
Repeatability standard deviation s_r (µg/kg)	1,76	8,90	19,1
Repeatability relative standard deviation RSD_r (%)	3,61	4,79	4,55
Repeatability limit r (r = 2,8 x s_r) (µg/kg)	4,93	24,9	53,5
Reproducibility standard deviation s_R (µg/kg)	2,73	10,1	26,8
$HORRAT_R$ (Modified calculation according to Thompson [4])	0.3	0.3	0.4
Reproducibility relative standard deviation RSD_R (%)	5,60	5,46	6,38
Reproducibility limit R (R = 2,8 x s_R), (µg/kg)	7,64	28,3	75,0

NOTE Sample sets 4, 5 and 6 were naturally contaminated. Results shown are those after correction for recovery with the mean recovery value generated from sample sets 2 and 3 (see Table A). This information has been added purely for control purposes. Results must be compared on a recovery corrected basis.

Table B.3 — Collaborative Trial Test Material Composition [1]

Test Material	Ingredients and Amount (%)
1 - 3	Rye 26%, Rice 19%, Linseed 8%, Soy 11%, Maize 25%, Sugar beet pellets 11%
4(nc)	Oat flakes 10 % Rye 1 % Sugar beet pellets 7 % Maize 54 % Linseed 4 % Rice 24 %
5(nc)	Rye 12 % Sugar beet pellets 17 % Maize 23 % Soy 31 % Rice 17 %
6(nc)	Rye 9 % Sugar beet pellets 14 % Maize 40 % Linseed 1 % Rice 14 % Cereal flakes 22 %

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[4] *Thompson, M. (2000), Analyst, 125 p. 385-386*

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