Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

The Certification of Mass Fractions of Aflatoxin B_1, B_2, G_1 and G_2 in Compound Feedingstuff (low level)

Certified Reference Material ERM®-BE375

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The Certification of Mass Fractions of Aflatoxin B_1, B_2, G_1 and G_2 in Compound Feedingstuff (low level)

Certified Reference Material ERM®-BE375

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this report to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

I. Summary

This report describes the preparation of a compound feeding stuff (ERM-BE375) matrix reference material its characterisation for the aflatoxins B_1, B_2, G_1 and G_2 mass fractions.
The preparation of the material, between bottle homogeneity and stability studies and the characterisation with a discussion of the results is described hereafter. Uncertainties were calculated in compliance with the ISO/IEC Guide 98-3:2008 (GUM) [2] and include contributions from possible hidden between bottle heterogeneity, long-term storage, the characterisation study and the contribution from the common calibrant. The certified values are listed below:

ERM-BE375	Certified value [1)] [µg/kg]	Uncertainty [2)] [µg/kg]	Number of accepted sets of results
B_1	2.6	0.4	7
B_2	0.20	0.04	8
G_1	0.4	0.1	8
G_2	< 0.2 [3)]	n/a [4)]	7

1) These values are the mass fractions based on the unweighted mean of p accepted sets of results.
2) The certified uncertainties are the expanded uncertainties (k = 2) of the values defined in 1).
3) The stated value is based on the limits of quantification of the methods employed, is with a 95 % level of confidence below the stated value.
4) not applicable (see 3)

II. Table of contents

III. Glossary

ACN	Acetonitrile
ANOVA	Analysis of variance
AOTF-NIR	Acousto-optical tuneable filter near-infrared spectrometry
CAS	Chemical Abstracts Service
CRM	Certified reference material
FAO	Food and Agriculture Organization of the United Nations
GUM	Guide to the Expression of Uncertainty in Measurement
HPLC-FLD	High performance liquid chromatography with fluorescence detection
IAC	Immunoaffinity column
IRMM	Institute for Reference Materials and Measurements
IUPAC	International Union for Pure and Applied Chemistry
LOD	Limit of detection
LOQ	Limit of quantification
$MS_{between}$	Mean of squares between groups (ANOVA)
MS_{within}	Mean of squares within groups (ANOVA)
MW	Molecular mass
n	Number of replicates
p	Level of significance
PBPB	pyridinium hydrobromide perbromide
RASFF	Rapid Alert System for Food and Feed of the European Union
RSD	Relative standard deviation
RSD_r	Relative standard deviation calculated from results under repeatability conditions
RSD_{stab}	Relative standard deviation of all results of the stability study
s	Standard deviation
s_{bb}	Between-bottle (in)homogeneity standard deviation
s_{wb}	Within-bottle standard deviation
SI	International Systems of Units
u_{Δ}	Relative, combined uncertainty of certified value and measured value
$u_{\Delta, abs}$	Absolute, combined uncertainty of certified value and measured value
$U_{\Delta, abs}$	Expanded, absolute uncertainty of certified value and measured value
u^{*}_{bb}	Relative standard uncertainty due to the inhomogeneity that can be hidden by the method repeatability
u_{bb}	Relative standard uncertainty due to between-bottle (in)homogeneity
u_{cal}	Relative uncertainty of the mass fraction of the calibrants used
u_{char}	Relative uncertainty of the results of the characterisation exercise
u_{CRM}	Relative, combined uncertainty of certified value
$u_{CRM, abs}$	Absolute, combined uncertainty of certified value
U_{CRM}	Expanded, relative uncertainty of certified value
$U_{CRM, abs}$	Expanded, absolute uncertainty of certified value
u_{lts}	Relative uncertainty of long-term stability
$u_{meas, abs}$	Absolute uncertainty of measurement result
t_{sl}	Pre-defined shelf life
$\bar{x}$	Average of all time points in an isochronous stability study
x_i	Time point i in an isochronous stability study
$\bar{y}$	Average of all results of a homogeneity study

Δ_m.....Difference between measured and certified value
$v_{MSwithin}$.....Degrees of freedom of MS_{within}
v + v .....volume part added to volume part
V-KFT.....Volumetric Karl Fischer titration

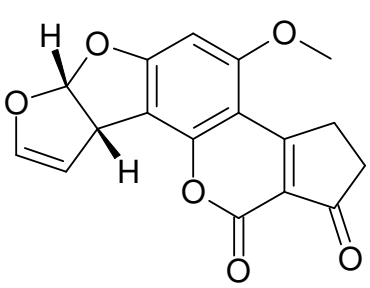
1 Introduction

Mycotoxins are secondary metabolites of moulds. These toxic metabolites can occur in a wide range of food and animal feed from plant origin and are therefore a potential risk to human and animal health. Contamination of food and feed can appear at two stages: during crop cultivation and/or during storage. Moulds infecting food on the field produce different mycotoxins compared to those moulds infecting food during storage [1]. The impact of mycotoxins on agricultural production is massive. The Food and Agriculture Organization of the United Nations (FAO) estimates that 25 % of the world-wide production is affected. The 2008 annual report of the Rapid Alert System for Food and Feed of the European Union (RASFF) [3] also shows that ~ 30 % of all notifications were due to mycotoxin contaminations.

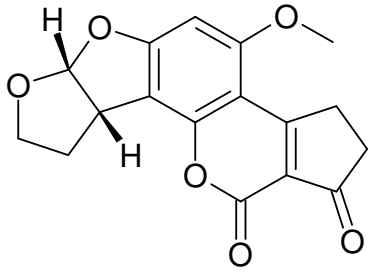
Maximum levels for certain mycotoxins have been introduced in the European Union since 1998 [4]. The maximum level for aflatoxin B_1 in animal feed ranges from 5.0 µg/kg to 20 µg/kg, dependent on the species it is intended for [5]. Aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 (Table 1 and Figure 1) are potent liver carcinogens. The aflatoxins are classified as group 1 carcinogens [6]. Therefore, the Institute for Reference Materials and Measurements (IRMM) has developed, in cooperation with a number of expert laboratories in Europe, a wider set of CRMs with certified values for aflatoxins. IRMM has now produced a new animal feed reference material, based on cereals and copra, certified for the mass fraction of aflatoxins B_1, B_2, G_1 and G_2. IRMM was providing a similar material, produced under the old BCR scheme until recently. The previous material BCR-375 was based on a commercial feed mixed with cattle nut, manioc and citrus pulp.

Table 1. Particulars on aflatoxins

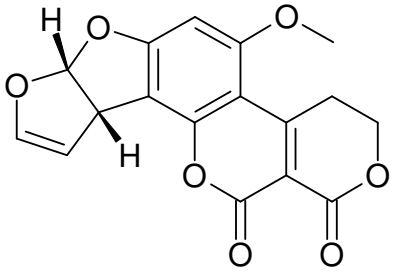
Trivial name	IUPAC name	CAS number	Chemical formula	Molecular mass [g/mol]
Aflatoxin B_1	2, 3, 6aα, 9aα-Tetrahydro-4-methoxycyclopenta [*c*] furo [3´, 2´:4, 5] furo [2, 3-*h*] [*l*] benzopyran-1, 11-dione	1162-65-8	$C_{17}H_{12}O_6$	312.3
Aflatoxin B_2	2, 3, 6aα, 8, 9, 9aα-Hexahydro-4-methoxycyclopenta [*c*] furo [3´, 2´:4, 5] furo [2, 3-*h*] [*l*] benzopyran-1, 11-dione	7220-81-7	$C_{17}H_{14}O_6$	314.3
Aflatoxin G_1	3, 4, 7aα, 10aα-Tetrahydro-5-methoxy-1*H*, 12*H* furo [3´, 2´:4, 5] furo [2, 3-*h*] pyrano [3, 4-*c*] [*l*]-benzopyran-1, 12-dione	1165-39-5	$C_{17}H_{12}O_7$	328.3
Aflatoxin G_2	3, 4, 7aα, 9, 10, 10aα-Hexahydro-5-methoxy-1*H*, 12*H* furo [3´, 2´:4,5] furo [2, 3-*h*] pyrano [3, 4-*c*] [*l*]-benzopyran-1, 12-dione	7241-98-7	$C_{17}H_{14}O_7$	330.3



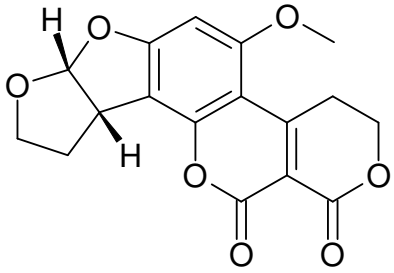
Aflatoxin B_1



Aflatoxin B_2



Aflatoxin G_1



Aflatoxin G_2

Figure 1. Molecular structure of aflatoxins

2 Participants

Project management and evaluation:

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, Geel, BE
(under current scope of ISO Guide 34 and ISO 17025 accreditation; Belac-268-TEST)

Processing:

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, Geel, BE
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Wiertz – Eggert - Joerissen, Hamburg, DE
(accredited to ISO 17025 for measurement of aflatoxins in food and feed; DAP-PL-1453.99)

3 Processing of the compound feedingstuff materials

3.1 ERM-BE375 animal feed (low level)

ERM-BE375 is a compound feeding stuff mixed from copra, wheat, barley, soya, maize and a mineral / vitamin premix (see Table 2 below). The starting materials were obtained from a local feed mill. The copra used was naturally contaminated. The materials were milled individually using an Alpine 160 UPZ impact mill (Alpine, Augsburg, Germany) and sieved through a 500 µm stainless steel sieve (Russel Finex, London, United Kingdom). Two batches of ERM-BE375 have been produced both weighing about 70 kg. The main ingredients of both batches are summarised in Table 2. Each batch has been homogenised for 2 h in a Turbula mixer based on the Paul Schatz principle (WAB, Basel, Switzerland). Around 75.5 g of the powder have been filled in each of about 900 amber glass jars employing an Accurate Feeder (Accurate, White Water, WI, USA). After inserting a polyethene insert the jars were closed manually with plastic screw caps. Prior to labelling a crimp film was placed around the screw cap. Sample numbers were assigned according to the filling sequence and, separated by a hyphen, the batch number (e.g. Sample No. 263-2 for the 263rd filled sample of batch 2). Corresponding sample numbers of batch 1 and 2 were packed together in a plastic bag as a set i.e. that one unit of ERM-BE375 consists of one bottle from batch 1 and one bottle from batch 2.

The material was sterilized by γ-irradiation with 10 to 18 kGy by Isotron (Ede, The Netherlands) to preserve the integrity of the samples. After irradiation the material was stored at -30 °C.

Table 2. Ingredients of ERM-BE375-1 and ERM-BE375-2

Material	BE375-1 [kg]	BE375-2 [kg]
Copra	13.650	13.650
Wheat	20.475	20.475
Barley	10.238	10.238
Soya*	9.897	9.897
Maize	13.650	13.650
Mineral mix*	0.273	0.273

* One third of the Soya was mixed with the mineral mix in a 9:1 premix before adding it to the bulk. The masses mentioned are the final amounts in the prepared bulk material.

3.2 Additional Characterisation measurements

3.2.1 Water content

The water content of five samples from each batch was measured by volumetric Karl Fischer titration (V-KFT) [7]. The results (79 ± 4 g/kg and 73 ± 7 g/kg for batches one and two, respectively) also show no significant difference between the two batches of each material regarding the water content.

Additionally, the water content of the produced units was measured using a Luminar 4030 instrument (Applitek, Nazareth, Belgium) which was based on acousto-optical tuneable filter near-infrared spectrometry (AOTF-NIR) [8]. The AOTF-NIR was calibrated employing pork meat in amber glass vials. Therefore measurements do not resemble the true water content but are biased due to the material and vials employed for the calibration. The values obtained were used to evaluate any trend of the water content regarding the filling sequence

of the material. No trends in water content regarding the filling sequence could be observed. The values found, 103 ± 6 g/kg and 103 ± 5 g/kg for batches one and two, respectively, also show no significant difference in water content between the two batches.

3.2.2 Particle size measurements

Particle size analyses have been carried out on 10 randomly selected units. The measurements were carried out employing a Sympatec Helos (Sympatec, Clausthal-Zellerfeld, DE) laser diffraction instrument. The material was dispersed in 2-propanol and the particle size distribution recorded over a range of 0.5 µm to 1750 µm. During the 10 s of recording time the sample was stirred with a magnetic stirring bar at 1200 rpm. The particle size distribution of the two batches is shown in Figure 2 and shows that no particles larger then 700 µm are present. The x50 is not below 200 µm. Although a 500 µm sieve was used it is perfectly logical that long fibrous particles may slip through the sieve.

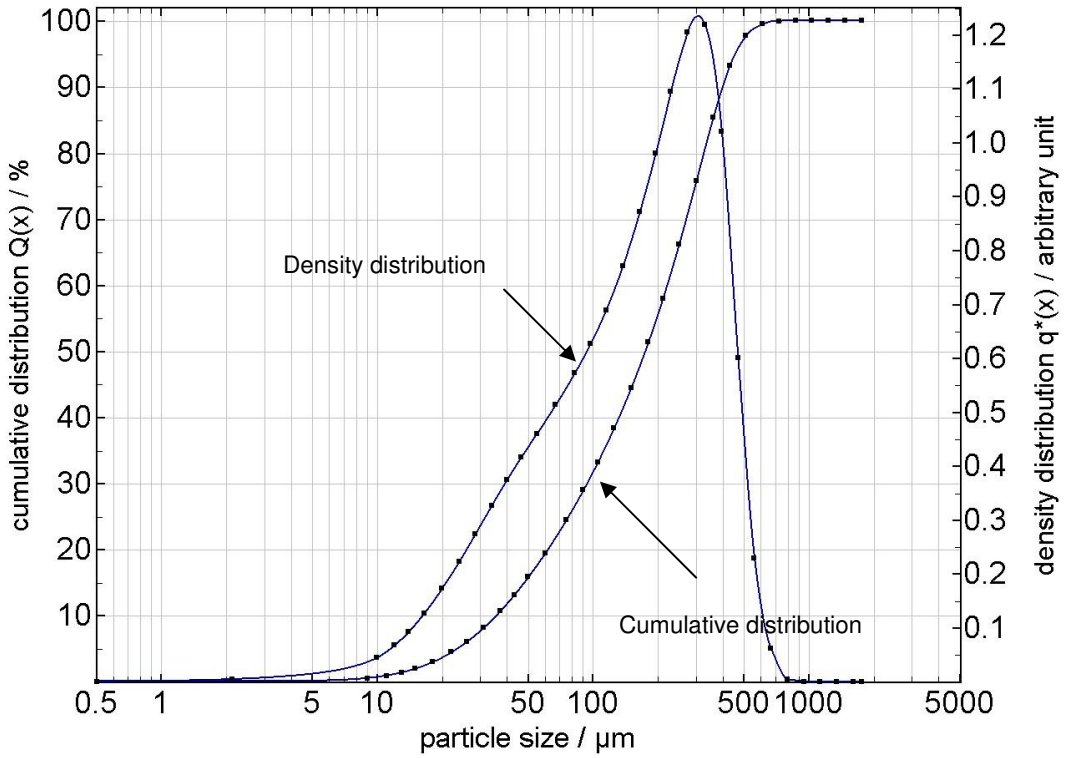

Figure 2. Particle size distribution of ERM-BE375 (average of batch 1 and 2)

4 Homogeneity studies

For the homogeneity study, 10 units of ERM-BE375 were chosen using a random stratified sample picking scheme, and for each unit both bottles were analysed for their aflatoxin content in triplicate. This resulted in 60 analyses as each unit consists of two bottles. Measurements were split over three days. On each day one replicate of each bottle was measured, resulting in 20 measurements per day.

Samples were measured in a random order to allow distinction between an analytical trend and a trend in the filling sequence. A reversed phase high performance liquid chromatography (HPLC) method with fluorescence detection after immunoaffinity clean-up was used for the measurements.

The means of bottle 1 and bottle 2 were tested for significant difference with a t-test on each day. In order to exclude the influence of the day-to-day variance and to estimate the uncertainty contribution from possible heterogeneity the two-way analysis of variance (ANOVA) was applied. Individual results are presented in the Annex A.

Within unit variability (s_{wb}), an estimate for the method repeatability, expressed as a relative standard deviation is given in equation given below:

$$s_{wb} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$

MS_{within}: mean square within a bottle from an ANOVA
$\bar{y}$: average of all results of the homogeneity study

Between-unit variability (s_{bb}) expressed as a relative standard deviation is given by the following equation:

$$s_{bb} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$

$MS_{between}$: mean square among bottles from an ANOVA
n: average number of replicates per bottle

The heterogeneity that can be hidden by method repeatability is defined as follows:

$$u^*_{bb} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{v_{MSwithin}}}$$

$v_{MSwithin}$: degrees of freedom of MS_{within}

The larger value of s_{bb} or u^*_{bb} were used as uncertainty estimation for possible present heterogeneity, u_{bb}. The distribution of sample averages was checked employing normal probability plots for normal distribution and histograms for unimodal distribution. Data were checked for single and double outliers employing the Grubbs' test at a level of confidence of 95 % and 99 %.

Conclusions:

No significant difference between batch 1 and batch 2 was found employing the t-test. Therefore the results for bottle 1 and bottle 2 were combined. No significant trends regarding filling sequence were detected. The individual contributions of heterogeneity to the uncertainty budget are summarized in Table 3.

Table 3. Results of homogeneity study

BE375	s_{wb} [%]	s_{bb} [%]	u^*_{bb} [%]	**u_{bb}[1] [%]**
aflatoxin				
B_1	2.9	0.6	0.8	**0.8**
B_2	9.6	0.8	2.7	**2.7**
G_1	14.7	2.5	4.1	**4.1**

[1] higher value u^*_{bb} or s_{bb} taken as contribution of heterogeneity

The smallest sample intake employed during the characterisation study was 10 g. These results have similar or better repeatability then those results with a higher sample intake. Therefore the minimum sample intake is 10 g.

5 Stability studies

Two stability studies were performed, one 4 weeks isochronous study to evaluate stability of the material during transport and one 18 months isochronous study to evaluate stability during storage.

For the short-term study samples were stored in the dark at 4 °C, 18 °C, 60 °C and for reference at -70 °C. For the 18 months long-term study samples were stored in the dark at -20 °C, -30 °C, and for reference at -70 °C. Two units were stored at each temperature for 0, 1, 2 and 4 weeks for the short-term study and 0, 6, 12 and 18 months for the long-term study. After the indicated periods the samples were transferred to storage at -70 °C until analysis.

At the end of each isochronous sample storage scheme the samples were measured together under repeatability conditions in a random order in duplicate for the short term study and in five replicates for the long term study. The laboratory employed its in-house HPLC-FLD methods based on reversed phase chromatography and post column bromination. The mycotoxins were quantified using an external calibration and the peak area. The results were not corrected for recovery.

Results (Annex B) were tested for significant trends (degradation, fungal enrichment) due to the storage conditions. Therefore the data points were plotted against time and the regression line was calculated.

The uncertainty of related to possible hidden instability u_{lts} of the material upon long-term storage was then calculated for a set shelf life as:

$$u_{lts} = \frac{RSD_{stab}}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot x$$

with RSD_{stab} being the relative standard deviation of all 40 individual results of the relevant long-term stability study, x_i being the time point for each replicate, $\bar{x}$ being the average of all time points and x being the set shelf life (36 months in this case).

Data were checked for single and double outliers employing the Grubbs' test at a level of confidence of 95 % and 99 %. Outliers were scrutinised but not excluded as no technical reason was found to do so.

Conclusions:

At 60 °C a significant slope at 99 % level of confidence was detected for aflatoxin B_1 and B_2 in the short-term stability study, leading to the conclusion that special precautions regarding temperature control during shipment are necessary. The material has to be shipped under conditions preventing the temperature to rise above 18 °C.

The material was stable at both temperatures of the long-term stability study, therefore -20 °C was chosen as storage temperature. Using the data from the long-term study, the uncertainty due to possible degradation was calculated for a storage time of 36 months at -20 °C. u_{lts} for the material is summarised in Table 4.

Table 4. Uncertainty contributions due to storage

ERM-BE375	u_{lts} [%]
aflatoxin B_1	2.4
aflatoxin B_2	5.2
aflatoxin G_1	6.4

6 Characterisation

6.1 Design of the study

The characterisation exercise was performed in 2008. Nine laboratories were carefully selected to carry out the measurements. The laboratories had to prove their measurement capabilities and had to demonstrate experience in aflatoxin analysis.

Each laboratory was provided with the following samples:

- 3 units of *"Compound feedingstuff (high level)"* ERM-BE376
- 6 units of *"Compound feedingstuff (low level)"* ERM-BE375
- 1 unit of *"Compound animal feed (low level)"* BCR-376
- 3 ampoules of the common calibrant aflatoxin B_1 in acetonitrile, ERM-AC057
- 3 ampoules of the common calibrant aflatoxin B_2 in acetonitrile, ERM-AC058
- 3 ampoules of the common calibrant aflatoxin G_1 in acetonitrile, ERM-AC059
- 3 ampoules of the common calibrant aflatoxin G_2 in acetonitrile, ERM-AC060

The measurements were performed on three different days. On each day one unit of ERM-BE376 and ERM-BE375 were analysed in duplicate. Additionally recovery experiments had to be carried out on each day with ERM-BE375.

In order to estimate the recovery rates of the participants' analytical procedures, the participants were required to spike the *"Compound feedingstuff (low level)"*, ERM-BE375 with a solution of the common calibrants to a mass fraction 15 µg/kg for aflatoxin B_1, 1 µg/kg for aflatoxin B_2, 5 µg/kg for aflatoxin G_1 and 0.5 µg/kg for aflatoxin G_2. No strict procedure was prescribed on how to perform the spiking. The laboratories used their in-house procedures for spiking.

In addition, the laboratories were required to measure one unit of BCR-376 (certified for its aflatoxin B_1 content) in duplicate on the second day to assess the trueness of results of the laboratory.

Analyses of the 'low level', spiked and, naturally contaminated materials were required to be performed together on each day, since the certification results are corrected by the daily recovery factor. Calibration solutions were based on dilutions of the provided common calibrants. A new calibration had to be performed on each day. The measurement program is visualised in Table 5.

Table 5. Measurement program

Day 1	Day 2	Day 3
Calibration	Calibration	Calibration
1 unit of BE376 in duplicate	1 unit of BE376 in duplicate	1 unit of BE376 in duplicate
1 unit of BE375 in duplicate	1 unit of BE375 in duplicate	1 unit of BE375 in duplicate
1 unit of BE375 spiked in triplicate	1 unit of BE375 spiked in triplicate	1 unit of BE375 spiked in triplicate
	1 unit of BCR-376 in duplicate	

6.2 Results and technical evaluation

All laboratories except one used their in-house methods based on immunoaffinity column (IAC) clean-up and reversed phase HPLC with post column derivatisation and fluorescence detection (HPLC-FLD). Laboratory 4 used a reversed phase HPLC with mass spectrometric detection and no clean-up. The individual methods used are summarized in Table 6. Laboratory 7 did not submit any results for technical reasons.

Table 6. Overview of analytical methods used for certification

lab code	extraction solvent [v+v]	extraction technique / time [min]	IAC producer	chromatography	derivatisation	sample intake [g]
1	methanol + water 8+2	shaker / 30	Vicam	isocratic	Kobra cell[1]	20
2	acetonitrile + water 6+4	blender / 2	R-Biopharm Rhône	isocratic	Kobra cell	25
3	acetone + water 85+15	blender / 2	R-Biopharm Rhône	isocratic	PBPB[2]	20
4	acetonitrile + water 85+15	shaker / 60	no clean up	gradient	LC-MS[3]	10
5	chloroform	shaker / 30	R-Biopharm Rhône	isocratic	Kobra cell	20
6	methanol + water 8+2	shaker / 30	R-Biopharm Rhône	isocratic	Kobra cell	50
8	chloroform + water 3+1	shaker / 30	R-Biopharm Rhône	gradient	Kobra cell	10
9	acetonitrile + water 6+4	shaker / 30	R-Biopharm Rhône	isocratic	Kobra cell	20
10	methanol + water + hexane 8+2+5	shaker / 30	R-Biopharm Rhône	isocratic	PBPB	25

[1] electrochemical bromination with potassium bromide
[2] bromination with pyridinium hydrobromide perbromide
[3] mass spectrometry used for detection instead of fluorescence

After receipt of the data sets, the results were subjected to technical evaluation. The results for BCR-376 could not be taken into consideration for the trueness assessment, as the different composition of that material compared to ERM-BE375 had an influence on the extractability of the aflatoxins. The recovery for the two materials had to be considered as different.

The individual results are corrected by the daily recovery. The uncertainty of the daily recovery does not contribute separately to the overall uncertainty as the relative standard error of the mean of means is used as an estimation of the uncertainty contribution of the characterisation exercise.

Results not fulfilling the criteria laid down in Commission Regulation 401/2006 [9] regarding recovery rates were eliminated. Satisfactory recovery rates for the spiking level of 15 µg/kg for aflatoxin B_1 are between 80 % and 110 %, for the levels of 1 µg/kg and 5 µg/kg for aflatoxin B_2 and G_1, respectively, are between 70 % and 110 %, and for the level of 0.5 µg/kg for aflatoxin G_2 , are between 50 % and 120 % [9]. If the criteria for one measurand were not met on two separate days, the third day results were omitted as well.

This led to the rejection of the following data sets due to non - satisfactory recovery rates:

Lab 1: the results of all three days for aflatoxin B_1 in ERM-BE375 were rejected
Lab 3: the results of all three days for aflatoxin G_2 in ERM-BE375 were rejected
Lab 4: all results were rejected (only results for aflatoxin B_2 fulfil the recovery requirements).
Lab 5: the results of one day for aflatoxin B_2 and G_1 in ERM-BE375 were rejected
Lab 10: the results of one day for aflatoxin B_1, B_2 and G_1 in ERM-BE375 were rejected

In total 40 values for aflatoxin B_1 from 7 labs, 44 values for aflatoxin B_2 from 8 labs, 44 values of aflatoxins G_1 from 8 labs and 42 values of aflatoxins G_2 for from 7 labs for ERM-BE375 were accepted after technical scrutiny for further statistical data assessment.

The obtained results reflect the current obtainable data quality for aflatoxin analysis of expert laboratories and is in agreement with recently published data for the certification of aflatoxins in peanut materials [10].

For aflatoxin G_2 all results except one have been below the limit of detection (LOD). Therefore separate statistical considerations had to be made. The accepted sets of results for aflatoxin B_1, B_2 and G_1 were submitted to the following statistical tests:

- Scheffe's multiple t-test to check if the means of two labs are significantly different
- Dixon's test to detect outlying lab means
- Nalimov's t-test to detect outlying lab means
- Grubbs' test to detect single and double outliers and strugglers
- ANOVA to assess between lab and within lab variances and to test their significance employing Snedecor's F-test
- Skewness and kurtosis test to assess the normality of the lab means distribution.

Table 7. Summary of statistical evaluation for ERM-BE375

ERM-BE375			
Aflatoxin	B_1	B_2	G_1
Number of data sets	7	8	8
Number of replicate measurements	40	44	44
Mean of means [µg/kg]	2.60	0.20	0.40
Relative standard deviation [%]	14	19	25
Relative standard error [%]	5.2	6.7	8.9
All data sets compatible two by two? (Scheffe's test)	no	no	no
Outlying means? (Dixon's test, Nalimov's t-test, Grubbs' test)	yes lab 3 Nalimov p=0.05	no	yes lab 9 Nalimov p=0.05
Distribution of means normal? (Skewness & kurtosis)	yes	yes	yes
Variances between labs significantly different? (Snedecor's F-test)	yes	yes	yes

The accepted individual results after technical and statistical scrutiny are given in Annex C. The results of the statistical tests of the finally considered data for ERM-BE375 are summarized in Table 7. The two means detected by the Nalimov's test as stragglers were examined again but no significant difference could be seen after taking the measurement uncertainty into account.

The values for aflatoxin G_2 (Annex C, Table C4) were scrutinised, in particular, the one value above the limit of quantification (LOQ). LODs of the methods used varied from 0.008 µg/kg to 0.1 µg/kg. LOQs varied from 0.02 µg/kg to 0.2 µg/kg. The one value above the LOQ was quantified with 0.05 µg/kg.

6.3 Certified values and their uncertainties

The certified values for ERM-BE375 are calculated as the mean of means of the accepted datasets. The standard error of the mean of means was used as an estimation of the uncertainty contribution of the characterisation exercise to the uncertainty of the certified mass fractions of the aflatoxins. The standard error is calculated as the standard deviation divided by the square root of the number of accepted data sets.

The combined uncertainty of the certified value includes contributions from the potentially hidden between bottle heterogeneity (u_{bb}), potential instability during storage (u_{lts}), the characterisation study (u_{char}) and the contribution from the common calibrant (u_{cal}). The uncertainty of the mass fraction of the common calibrants propagates in the calibrations and can therefore not be neglected.

The common calibrants are certified for their mass fraction of aflatoxin in acetonitrile (materials ERM-AC057, ERM-AC058, ERM-AC059 and ERM-AC060). The uncertainty of the mass fraction (aflatoxin in acetonitrile) is taken from the corresponding certificate. The stated absolute, expanded uncertainty is transformed into a relative uncertainty by dividing the expanded uncertainty by the coverage factor (from the certificate) and by dividing by the certified value.

The relative combined uncertainty is calculated as the square root of the sum of squares of the relative uncertainties of the individual contributions, according to:

$$u_{CRM} = \sqrt{u_{lts}^2 + u_{bb}^2 + u_{char}^2 + u_{cal}^2}$$

The absolute, expanded uncertainty $U_{CRM,\ abs}$ is calculated by multiplying the certified value with the relative, expanded uncertainty U_{CRM}.

The certified values are summarised in
Table 8.

Table 8. Certified values and their uncertainties for ERM-BE375

	ERM-BE375		
Aflatoxin	B_1	B_2	G_1
Certified value [µg/kg]	**2.6**	**0.20**	**0.4**
u_{lts} [%]	2.4	5.2	6.4
u_{bb} [%]	0.8	2.7	4.1
u_{char} [%]	5.2	6.7	8.9
u_{cal} [%]	1.4	1.0	1.7
u_{CRM} [%]	5.9	8.9	11.8
U_{CRM} (k=2) [%]	11.8	17.9	23.7
$U_{CRM,\ abs}$ (k=2) [µg/kg]	**0.4**	**0.04**	**0.1**

The mass fraction of aflatoxin G_2 is certified based on the quantified result and the LOQs of the methods. 41 results are below the limit of detection of the respective methods. The quantified result is four times below the limit of quantification of the least sensitive method.

Therefore the certified mass fraction of aflatoxin G_2 is with a confidence of 95 % below 0.2 µg/kg, the limit of quantification of the least sensitive method.

7 Metrological traceability and commutability

The certified values for the mass fractions of aflatoxins are traceable via the common, certified calibrants used (ERM-AC057, ERM-AC058, ERM-AC059 and ERM-AC060). The mass fractions of the common, certified calibrants are certified for aflatoxins in acetonitrile. The certified values of the calibrants are traceable to the SI, as stated on the respective certificate. Therefore, the mass fraction values of aflatoxins in the ERM-BE375 are traceable to the SI.

As all methods employed used reversed phase liquid chromatography with post column bromination, fluorescence detection and immunoaffinity clean-up method independence can not be guaranteed. As at least four different extraction solvents and techniques have been used independency to the extraction method is given. Therefore the aflatoxins mass fractions as stated are defined by the employed reversed phase liquid chromatography methods with post column bromination, fluorescence detection and immunoaffinity clean-up.

ERM-BE375 is prepared from naturally contaminated material. Therefore there is no reason to assume that ERM-BE375 would behave differently from compound feedingstuff with similar composition and sieved through to a particle size below 500 µm.

8 Instructions for use

8.1 Storage conditions

The materials should be stored at or below -20 °C ± 5 °C. However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of open samples.

8.2 Safety precautions

The usual laboratory safety precautions apply. The mass fractions of aflatoxins in ERM-BE375 are well below the maximum allowed levels for animal consumption within the European Union.

8.3 Use of the material

This material is intended to be used for performance control and validation purposes. Samples should be allowed to equilibrate to ambient temperature (e.g. overnight) before opening to avoid water condensation. The content of the bottle used should be thoroughly mixed before sub-samples of at least 10 g are taken. The compound feedingstuff should be weighed out immediately after opening the bottle and the mass fractions of the aflatoxins calculated based on this mass.

8.4 Use of the certified value

For assessing the method performance, the measured values of the CRMs are compared with the certified values following a procedure described below [11]:

- Calculate the absolute difference between mean measured value and the certified value (Δ_m).
- Combine measurement uncertainty ($u_{meas, abs}$) with the uncertainty of the certified value ($u_{CRM, abs}$): $u_{\Delta,abs} = \sqrt{u^2_{meas,abs} + u^2_{CRM,abs}}$
- Calculate the expanded uncertainty ($U_{\Delta, abs}$) from the combined uncertainty ($u_{\Delta, abs}$) using a coverage factor of two ($k = 2$), corresponding to a confidence interval of approximately 95 %
- If $\Delta_m \leq U_{\Delta, abs}$ then there is no significant difference between the measurement result and the certified value, at a confidence level of about 95 %.

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Annex A Homogeneity study data

Table A1. Results of homogeneity study for ERM-BE375:

Unit	Aflatoxin B_1 uncorrected for recovery [µg/kg]		
132/1	2.58	2.63	2.67
132/2	2.60	2.43	2.63
264/1	2.57	2.45	2.59
264/2	2.46	2.51	2.73
268/1	2.50	2.53	2.81
268/2	2.55	2.51	2.55
398/1	2.43	2.50	2.66
398/2	2.56	2.46	2.72
417/1	2.59	2.53	2.50
417/2	2.48	2.66	2.71
548/1	2.54	2.60	2.71
548/2	2.59	2.48	2.59
694/1	2.40	2.52	2.65
694/2	2.53	2.48	2.54
704/1	2.57	2.50	2.55
704/2	2.48	2.47	2.46
839/1	2.53	2.53	2.64
839/2	2.35	2.48	2.56
858/1	2.51	2.60	2.67
858/2	2.39	2.58	2.67

Unit	Aflatoxin B_2 uncorrected for recovery [µg/kg]		
132/1	0.21	0.23	0.21
132/2	0.19	0.19	0.24
264/1	0.22	0.18	0.20
264/2	0.20	0.19	0.22
268/1	0.20	0.22	0.21
268/2	0.22	0.21	0.19
398/1	0.20	0.19	0.22
398/2	0.23	0.20	0.21
417/1	0.25	0.20	0.21
417/2	0.22	0.26	0.25
548/1	0.22	0.21	0.18
548/2	0.25	0.19	0.20
694/1	0.20	0.19	0.19
694/2	0.22	0.20	0.20
704/1	0.20	0.19	0.20
704/2	0.19	0.19	0.20
839/1	0.26	0.22	0.21
839/2	0.20	0.20	0.18
858/1	0.19	0.20	0.22
858/2	0.17	0.26	0.20

Table A1. continued

Unit	Aflatoxin G_1 uncorrected for recovery [µg/kg]		
132/1	0.30	0.28	0.30
132/2	0.26	0.33	0.36
264/1	0.28	0.28	0.27
264/2	0.24	0.28	0.36
268/1	0.24	0.38	0.29
268/2	0.34	0.29	0.26
398/1	0.29	0.30	0.28
398/2	0.29	0.29	0.27
417/1	0.31	0.34	0.32
417/2	0.26	0.47	0.41
548/1	0.25	0.28	0.27
548/2	0.35	0.30	0.27
694/1	0.26	0.27	0.28
694/2	0.24	0.30	0.33
704/1	0.34	0.27	0.28
704/2	0.25	0.28	0.31
839/1	0.38	0.32	0.35
839/2	0.27	0.35	0.29
858/1	0.28	0.31	0.36
858/2	0.26	0.41	0.27

Annex B Stability study data

Table B1. Results of the isochronous studies for aflatoxin B_1

ERM-BE375 B_1 [µg/kg]				
	weeks at 4 °C			
samples	0	1	2	4
1	1.83	2.00	1.97	1.96
2	1.94	1.83	1.91	1.92
3	2.57	2.62	2.24	2.46
4	2.50	2.77	2.52	2.66
	weeks at 18 °C			
samples	0	1	2	4
1	1.83	1.84	1.82	1.83
2	1.94	1.99	1.91	1.96
3	2.57	1.82	2.00	1.84
4	2.50	1.98	1.84	1.98
	weeks at 60 °C			
samples	0	1	2	4
1	1.83	2.21	1.97	1.29
2	1.94	2.14	1.62	1.28
3	2.57	1.88	1.70	1.24
4	2.50	1.86	1.74	1.21
	months at -20 °C			
samples	0	6	12	18
1	2.66	2.77	2.78	2.66
2	2.78	2.68	2.77	2.79
3	2.54	2.65	2.69	2.59
4	2.67	2.62	2.87	2.61
5	2.64	2.87	2.84	2.80
6	2.80	2.73	2.75	2.79
7	2.67	2.69	2.70	2.68
8	2.70	2.76	2.61	2.78
9	2.66	2.66	2.70	2.74
10	2.75	2.73	2.70	2.72
	months at -30 °C			
samples	0	6	12	18
1	2.54	2.62	2.96	2.60
2	2.67	2.74	2.53	2.61
3	2.64	2.66	2.65	2.66
4	2.80	2.67	2.80	2.79
5	2.66	2.86	2.80	2.72
6	2.78	2.76	2.73	2.75
7	2.75	2.78	2.73	2.76
8	2.67	2.83	2.82	2.68
9	2.70	2.63	2.61	2.53
10	2.66	2.90	2.65	2.63

Table B2. Results of the isochronous studies for aflatoxin B_2

ERM-BE375 B_2 [µg/kg]				
	weeks at 4 °C			
samples	0	1	2	4
1	0.21	0.22	0.21	0.22
2	0.21	0.20	0.21	0.22
3	0.25	0.27	0.25	0.25
4	0.26	0.30	0.25	0.27
	weeks at 18 °C			
samples	0	1	2	4
1	0.21	0.21	0.21	0.21
2	0.21	0.21	0.20	0.21
3	0.25	0.20	0.21	0.22
4	0.26	0.21	0.21	0.22
	weeks at 60 °C			
samples	0	1	2	4
1	0.21	0.24	0.25	0.21
2	0.21	0.25	0.22	0.22
3	0.25	0.23	0.23	0.22
4	0.26	0.22	0.23	0.21
	months at -20 °C			
samples	0	6	12	18
1	0.20	0.22	0.22	0.21
2	0.22	0.21	0.24	0.21
3	0.21	0.26	0.26	0.23
4	0.19	0.21	0.23	0.22
5	0.22	0.22	0.22	0.21
6	0.22	0.22	0.22	0.23
7	0.20	0.23	0.23	0.23
8	0.22	0.22	0.23	0.24
9	0.23	0.23	0.22	0.23
10	0.23	0.22	0.23	0.23
	months at -30 °C			
samples	0	6	12	18
1	0.20	0.20	0.26	0.19
2	0.22	0.22	0.20	0.21
3	0.21	0.23	0.23	0.22
4	0.19	0.19	0.24	0.20
5	0.22	0.21	0.21	0.21
6	0.22	0.21	0.20	0.22
7	0.20	0.21	0.22	0.22
8	0.22	0.24	0.21	0.23
9	0.23	0.22	0.22	0.22
10	0.23	0.24	0.23	0.21

Table B3. Results of the isochronous studies for aflatoxin G_1

ERM-BE376 G_1 [µg/kg]				
	weeks at 4 °C			
samples	0	1	2	4
1	0.26	0.27	0.30	0.30
2	0.28	0.27	0.30	0.28
3	0.29	0.29	0.35	0.40
4	0.40	0.30	0.40	0.30
	weeks at 18°C			
samples	0	1	2	4
1	0.26	0.28	0.30	0.32
2	0.28	0.30	0.27	0.30
3	0.29	0.29	0.28	0.33
4	0.40	0.31	0.30	0.29
	weeks at 60 °C			
samples	0	1	2	4
1	0.26	0.23	0.20	0.09
2	0.28	0.21	0.27	0.29
3	0.29	0.31	0.17	0.25
4	0.40	0.31	0.28	0.24
	months at -20 °C			
samples	0	6	12	18
1	0.33	0.32	0.33	0.32
2	0.36	0.34	0.46	0.32
3	0.31	0.36	0.32	0.35
4	0.35	0.31	0.34	0.34
5	0.33	0.33	0.32	0.31
6	0.36	0.32	0.36	0.33
7	0.37	0.35	0.33	0.34
8	0.34	0.32	0.34	0.35
9	0.32	0.33	0.33	0.35
10	0.32	0.31	0.34	0.32
	months at -30 °C			
samples	0	6	12	18
1	0.33	0.33	0.34	0.32
2	0.36	0.35	0.31	0.35
3	0.31	0.34	0.36	0.35
4	0.35	0.32	0.34	0.35
5	0.33	0.34	0.36	0.34
6	0.36	0.33	0.34	0.36
7	0.37	0.40	0.36	0.35
8	0.34	0.35	0.36	0.33
9	0.32	0.33	0.33	0.34
10	0.32	0.35	0.34	0.36

Annex C Characterisation measurements

Table C1. Results of characterisation measurements for aflatoxin B_1

B_1 mass fraction in ERM-BE375 [µg/kg]

Lab code	Day 1		Day 2		Day 3		Mean	s
2	3.04	2.90	3.19	3.08	2.87	3.11	3.03	0.12
3	2.29	2.42	0.73	1.24	2.58	2.53	1.97	0.78
5	2.55	2.67	2.88	3.04	2.68	2.60	2.74	0.19
6	2.44	2.36	2.42	2.48	2.43	2.45	2.43	0.04
8	2.98	2.74	2.92	2.75	2.97	2.78	2.86	0.11
9	2.81	2.80	3.13	2.76	2.61	2.31	2.74	0.27
10	d.r.	d.r.	2.11	2.56	2.60	2.40	2.42	0.22

d.r. data rejected

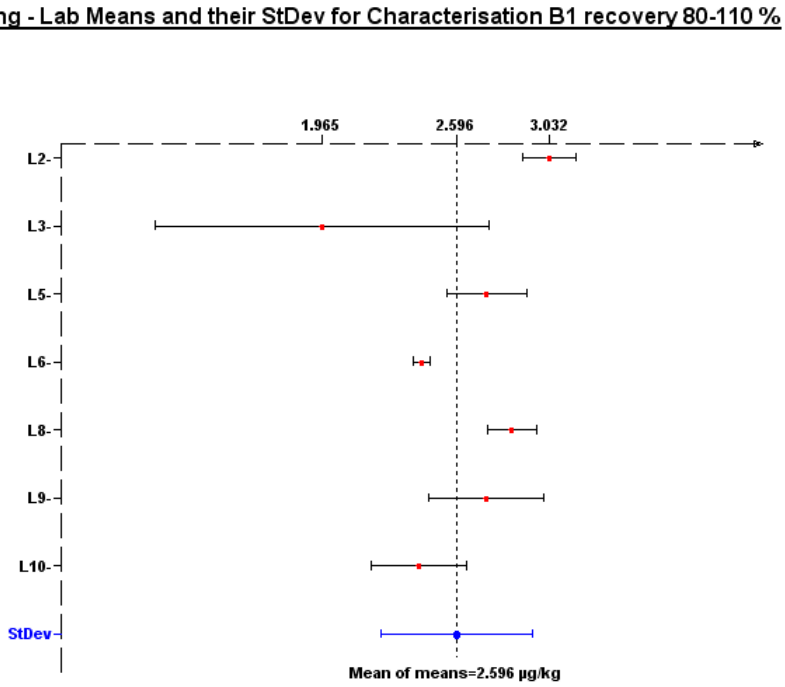

Figure C1. Laboratory means, mean of means and their standard deviation for aflatoxin B_1

Table C2. Results of characterisation measurements for aflatoxin B_2

B_2 mass fraction in ERM-BE375 [µg/kg]								
Lab code	Day 1		Day 2		Day 3		Mean	s
1	0.24	0.25	0.22	0.22	0.21	0.21	0.23	0.02
2	0.27	0.24	0.25	0.24	0.19	0.19	0.23	0.03
3	0.14	0.14	0.14	0.11	0.19	0.20	0.15	0.03
5	0.15	0.17	d.r.	d.r.	0.22	0.22	0.19	0.04
6	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.00
8	0.28	0.25	0.23	0.22	0.23	0.21	0.24	0.03
9	0.23	0.23	0.26	0.24	0.25	0.24	0.24	0.01
10	d.r.	d.r.	0.14	0.16	0.16	0.15	0.15	0.01

d.r. data rejected

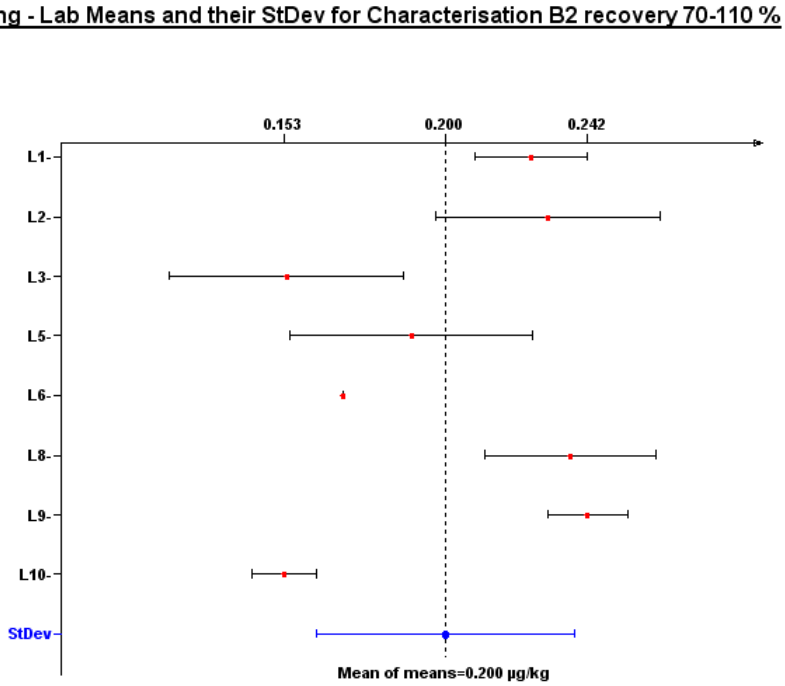

Figure C2. Laboratory means, mean of means and their standard deviation for aflatoxin B_2

Table C3. Results of characterisation measurements for aflatoxin G_1

G_1 mass fraction in ERM-BE375 [µg/kg]

Lab code	Day 1		Day 2		Day 3		Mean	s
1	0.41	0.43	0.32	0.35	0.48	0.47	0.41	0.06
2	0.50	0.50	0.41	0.40	0.37	0.41	0.43	0.06
3	0.38	0.35	0.16	0.19	0.34	0.35	0.30	0.09
5	0.32	0.38	d.r.	d.r.	0.55	0.53	0.45	0.11
6	0.39	0.37	0.42	0.42	0.38	0.38	0.39	0.02
8	0.33	0.31	0.27	0.26	0.26	0.24	0.28	0.03
9	0.59	0.51	0.65	0.62	0.64	0.53	0.59	0.06
10	d.r.	d.r.	0.28	0.37	0.35	0.32	0.33	0.04

d.r. data rejected

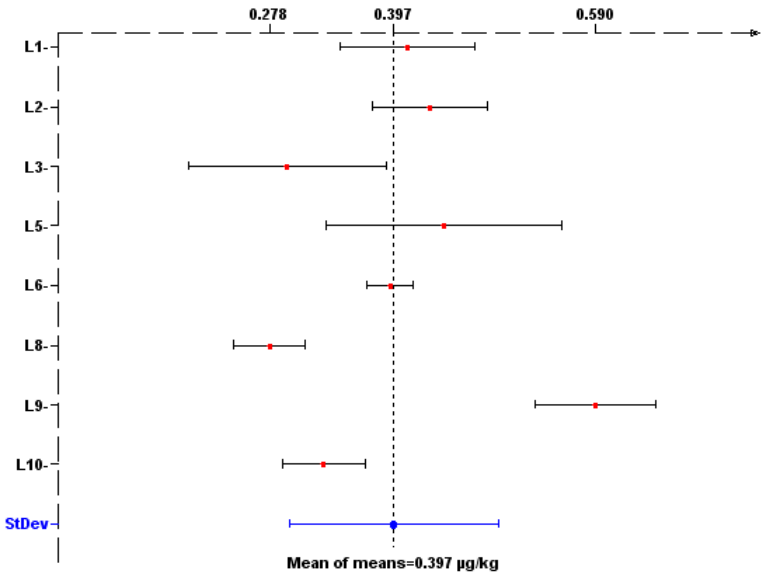

Figure C3. Laboratory means, mean of means and their standard deviation for aflatoxin G_1

Table C4. Results of characterisation measurements for aflatoxin G_2

G_2 mass fraction in ERM-BE375 [µg/kg]

Lab code	Day 1		Day 2		Day 3		LOD	LOQ
1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.01	0.04
2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.04	0.08
5	<LOD	<LOD	0.05	<LOD	<LOD	<LOD	0.01	0.02
6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.01	0.02
8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.01	0.05
9	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.1	0.2
10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.02	0.05

European Commission

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Abstract
This report describes the preparation of a compound feeding stuff (ERM-BE375) matrix reference material its characterisation for the aflatoxins B_1, B_2, G_1 and G_2 mass fractions.
The preparation of the material, between bottle homogeneity and stability studies and the characterisation with a discussion of the results is described hereafter. Uncertainties were calculated in compliance with the ISO/IEC Guide 98-3:2008 (GUM) and include contributions from possible hidden between bottle heterogeneity, long-term storage, the characterisation study and the contribution from the common calibrant. The certified values are listed below:

ERM-BE375	Certified value [1] [µg/kg]	Uncertainty [2] [µg/kg]	Number of accepted sets of results
B_1	2.6	0.4	7
B_2	0.20	0.04	8
G_1	0.4	0.1	8
G_2	< 0.2 [3]	n/a [4]	7

1) These values are the mass fractions based on the unweighted mean of p accepted sets of results.
2) The certified uncertainties are the expanded uncertainties (k = 2) of the values defined in 1).
3) The stated value is based on the limits of quantification of the methods employed, is with a 95 % level of confidence below the stated value.
4) Not applicable (see 3)

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