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Certification of mass fractions of aflatoxin B_1, B_2, G_1 and G_2 in peanut meal BCR-263R

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Institute for Reference Materials and Measurements

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REFERENCE MATERIALS

Certification of mass fractions of aflatoxin B_1, B_2, G_1 and G_2 in peanut meal

BCR-263R

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Summary

This report describes the preparation of peanut meal (BCR-263R) matrix reference material and the certification of its aflatoxins B_1, B_2, G_1 and G_2 content (mass fraction).

The preparation of the material, the homogeneity and stability studies and the characterisation are described hereafter and the results are discussed. Uncertainties were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [1] and include uncertainties due to possible heterogeneity and instability. The certified values are listed below:

BCR-263R	Certified value [1)]	Uncertainty [2)]	Number of accepted sets of results (p)
Aflatoxin B_1	17.1 µg/kg	2.4 µg/kg	5
Aflatoxin B_2	3.0 µg/kg	0.4 µg/kg	4
Aflatoxin G_1	3.0 µg/kg	0.5 µg/kg	7

1) These values are the mass fractions based on the unweighted mean of accepted results.
2) The uncertainties are the expanded uncertainties (k = 2) of the values defined in 1).

Additionally the following indicative values have been assigned:

BCR-263R	Indicative value [1)]	Uncertainty [2)]	Number of accepted sets of results (p)
Aflatoxin G_2	0.62 µg/kg	0.21 µg/kg	5
Sum of Aflatoxin B_1, B_2, G_1, G_2	23.7 µg/kg	2.5 µg/kg	-

1) These values are the mass fractions based on the unweighted mean of accepted results.
2) The uncertainties are the expanded uncertainties (k = 2) of the values defined in 1).
3) The uncertainty for the sum of aflatoxins B_1, B_2, G_1, G_2 is calculated from the individual absolute standard uncertainties as $U_{sum} = 2 \cdot \sqrt{u_{B_1}^2 + u_{B_2}^2 + u_{G_1}^2 + u_{G_2}^2}$.

The assigned values and their uncertainties are based on a minimum sample intake of 10 g.

Table of contents

1 Glossary

a_w ..... Water activity
ANOVA ..... Analysis of variances
ACN ..... Acetonitrile
CRM ..... Certified reference material
FAO ..... Food and Agriculture Organization of the United Nations
GUM ..... Guide to the Expression of Uncertainty in Measurement [1]
HPLC-FLD ..... High performance liquid chromatography with fluorescence detection
IAC ..... Immunoaffinity column
$MS_{between}$ ..... 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
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 standard deviation
s_{wb} ..... Within-bottle standard deviation
SI ..... International Systems of Units
U ..... Expanded uncertainty
u ..... Standard uncertainty
u_{B1} ..... Uncertainty of the mass fraction of aflatoxin B_1
u_{B2} ..... Uncertainty of the mass fraction of aflatoxin B_2
u_{G1} ..... Uncertainty of the mass fraction of aflatoxin G_1
u_{G2} ..... Uncertainty of the mass fraction of aflatoxin G_2
u_{Δ} ..... Combined 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 characterisation exercise
u_{CRM} ..... Combined, relative 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} ..... Uncertainty of measurement result
U_{sum} ..... Expanded uncertainty of the mass fraction of the sum of aflatoxins B_1, B_2, G_1 and G_2
u_{sts} ..... Relative uncertainty of short-term stability
x ..... Pre-defined shelf life
$\overline{x}$ ..... Average of all time points in an isochronous stability study
x_i ..... Time point i in an isochronous stability study
$\overline{y}$ ..... Average of all results of a homogeneity study
Δ ..... Difference between two measurement results
Δ_m ..... Difference between measured and certified value
$v_{MSwithin}$ ..... Degrees of freedom of MS_{within}

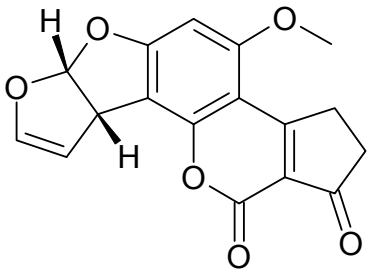
2 Introduction

Mycotoxins are secondary metabolites of moulds. These toxic metabolites occur as contaminants 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: on the field and/or during storage. Moulds infecting food on the field produce different mycotoxins compared to those moulds infecting food during storage.

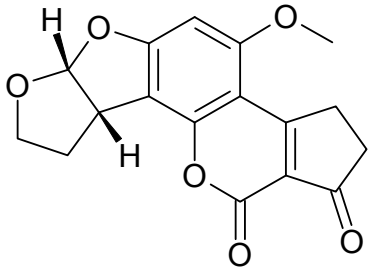
The impact of mycotoxins on agricultural production is massive. The Food and Agriculture Organization of the United Nations (FAO) estimates that 25 % of the world-wide production is affected. The 2006 annual report of the rapid alert system for food and feed of the European Union [2] also shows that 30 % of the notifications were due to mycotoxin contaminations. Around 90 % of these notifications concerned aflatoxins and a third of these concerned peanuts and peanut products.

Maximum levels for certain mycotoxins have been introduced in the European Union since 1998 [3]. The maximum level for aflatoxin B_1 in peanuts for direct human consumption is 2.0 µg/kg and for the sum of aflatoxin B_1, B_2, G_1 and G_2 4.0 µg/kg [4].

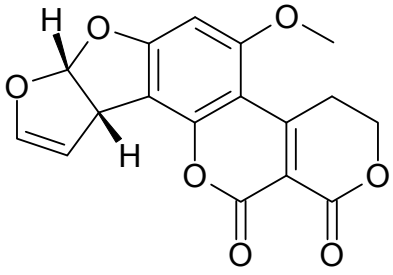
Aflatoxin B_1 (Figure 1)[2, 3, 6aα, 9aα-Tetrahydro-4-methoxycyclopenta [*c*] furo [3´, 2´:4, 5] furo [2, 3-*h*] [*l*] benzopyran-1, 11-dione, CAS Nr. 1162-65-8], Aflatoxin B_2 (Figure 1) [2, 3, 6aα, 8, 9, 9aα-Hexahydro-4-methoxycyclopenta [*c*] furo [3´, 2´:4, 5] furo [2, 3-*h*] [*l*] benzopyran-1, 11-dione, CAS Nr. 7220-81-7], Aflatoxin G_1 (Figure 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, CAS Nr. 1165-39-5] and Aflatoxin G_2 (Figure 1) [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, CAS Nr. 7241-98-7] are potent liver carcinogens. The aflatoxins are classified as group 1 carcinogens [5].



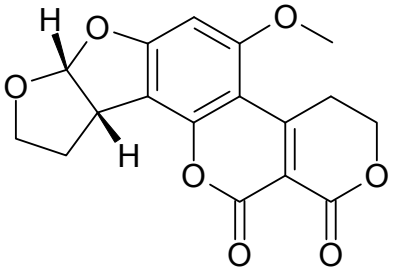
Aflatoxin B_1
($C_{17}H_{12}O_6$, MW = 312.3 g/mol)



Aflatoxin B_2
($C_{17}H_{14}O_6$, MW = 314.3 g/mol)



Aflatoxin G1
($C_{17}H_{12}O_7$, MW = 328.3 g/mol)



Aflatoxin G_2
($C_{17}H_{14}O_7$, MW = 330.3 g/mol)

Figure 1 Molecular structure of aflatoxins

3 Participants

Project management and evaluation:

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

Processing:

Wiertz-Eggert-Joerissen, DE
(accredited to ISO 17025 for measurement of aflatoxins in food and feed; DAP-PL-1453.99)

Homogeneity and stability measurements:

Central Science Laboratory, GB
(accredited to ISO 17025 for measurement of aflatoxins in food; UKAS 1642)

Nederlandse Organisatie voor Toegepast-Natuurwetenschappelijk Onderzoek (TNO), NL
(accredited to ISO 17025 for measurement of aflatoxins in food and feed; RvA L027)

University for Natural Resources and Applied Life Sciences, Department of Agrobiotechnology IFA-Tulln, Environmental Biotechnology, AT

Wiertz-Eggert-Joerissen, DE
(accredited to ISO 17025 for measurement of aflatoxins in food and feed; DAP-PL-1453.99)

Characterisation analysis:

Central Science Laboratory, GB
(accredited to ISO 17025 for measurement of aflatoxins in food; UKAS 1642)

Finnish Customs Laboratory, FI
(accredited to ISO 17025 for measurement of aflatoxins in food and feed; FINAS T006)

Instituto Nacional de Engenharia e Tecnologia (INETI-LIA), PT
(accredited to ISO 17025 for measurement of aflatoxin B_1 in feed; IPAC L0094)

Laboratorio Normativo de Salud Pública de Bilbao, ES
(accredited to ISO 17025 for measurement of aflatoxins in food; ENAC 132/LE326)

LGC Ltd., GB
(accredited to ISO 17025 for measurement of aflatoxins in food and feed; UKAS 0003)

Nederlandse Organisatie voor Toegepast-Natuurwetenschappelijk Onderzoek (TNO), NL
(accredited to ISO 17025 for measurement of aflatoxins in food and feed; RvA L027)

PhytoLab GmbH & Co. KG, DE
(accredited to ISO 17025 for measurement of contaminants by high performance liquid chromatography; SAL-BY-G037-01-05)

Wiertz-Eggert-Joerissen, DE
(accredited to ISO 17025 for measurement of aflatoxins in food and feed; DAP-PL-1453.99)

The timelines for major project tasks are summarized in Table 1.

Table 1. Schedule of the project

Study	Schedule
Processing	January 2002 - September 2002
Homogeneity study	April 2003
Stability studies	March 2003 -March 2007
Certification measurements	March 2006 - August 2006

4 Processing of BCR-263R peanut meal (medium level)

A high and low contaminated lots of peanut press pellets from oil refinement were broken in a RAS Mill from Romerlabs (Tulln, AT). The material originated from China.
This material was homogenised using a ploughshare mixer from Lödige (Paderborn, DE). The material was milled to a particle size < 0.5 mm with an impact mill from Bauermeister (Hamburg, DE).
Higher and lower contaminated batches have been mixed and homogenised using a ploughshare mixer from Lödige (Paderborn, DE) to an average B1 contamination of 20 µg/kg.
After assessment of the bulk homogeneity, portions of ~ 100 g were filled in aluminium coated plastic bags. The bags were sealed with a vacuum packaging machine from Komet (Plochingen, DE), where the bags were nitrogen flushed before evacuation and sealing.

5 First material characterisation measurements

5.1 Water content and water activity

The water content and water activity of the peanut material were measured by Karl Fischer titration [6] and the dew point technique [7], respectively. 15 bottles were chosen using a random stratified sample picking scheme and analysed in duplicate. The results are summarized in Table 2.

Table 2. Water content and water activity

Material	Water [g/kg]	Water activity (a_W)
BCR-263R	54.4 ± 1.3	0.254 ± 0.004

5.2 Particle size measurements

Five particles size analyses have been carried out for the final peanut meal material. The measurements were carried out with an accredited in-house method employing a laser diffraction measuring device from Sympatec (Clausthal, DE). The materials were dispersed in methanol and the particle size distribution measured over a range of 0.5 to 875 µm. During the 10 s of measuring time, the sample was stirred with a magnetic stirring bar at 1200 rpm.
A representative graph of the particle size distribution for BCR-263R is shown in Figure 2.

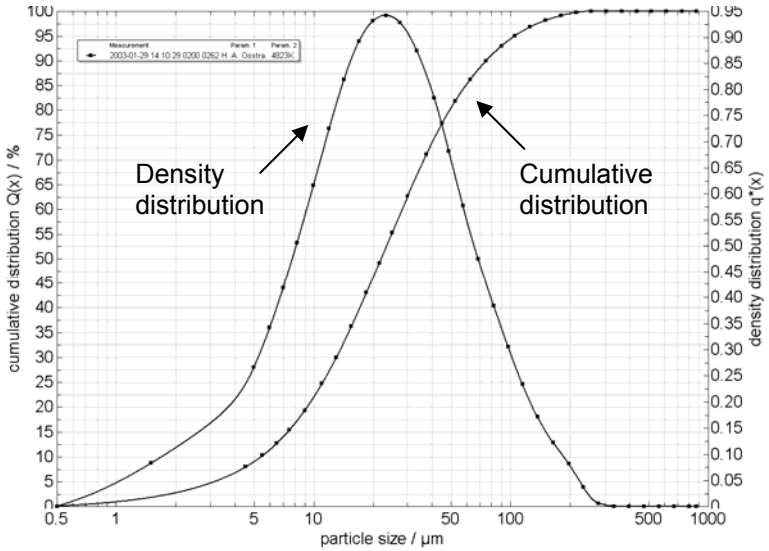


Figure 2. Particle size distribution of BCR-263R

6 Homogeneity studies

For the homogeneity study, 31 samples (~ 2.0 % of the total batch) of BCR-263R were chosen using a random stratified sample picking scheme and analysed for their aflatoxin content in triplicate.
Samples were measured in a random order to allow distinction between an analytical trend and a trend in the filling sequence. Measurements were performed on five different days. In order to exclude the influence of day-to-day variability the results were normalized to the mean of the results on this day. The normalized results on these five days (Annex A) were combined and evaluated to detect any trends regarding filling or analysis sequence and to estimate the uncertainty contribution from possible heterogeneity. Therefore the results were evaluated by a one-way analysis of variance (ANOVA). From the results of the ANOVA calculation, the following figures were calculated:

Method repeatability (s_{wb}) expressed as a relative standard deviation is given as follows:

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

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:

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

$\nu_{MSwithin}$: degrees of freedom of MS_{within}

The larger value of s_{bb} or u^*_{bb} was used as uncertainty contribution for homogeneity, 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 %. Outliers were scrutinised but not excluded as no technical reason was found to do so.

Conclusions:
No significant trend regarding filling sequence was detected. Sample averages were not always normally-distributed but followed a unimodal distribution in each case, allowing the use of ANOVA for heterogeneity determination. The following outliers were found: one single outlier (level of confidence 95 %) for aflatoxin B_1, one single outlier (level of confidence 99 %) for aflatoxin B_2, one double outlier (level of confidence 99 %) for aflatoxin G_1 and one double outlier (level of confidence 95 %) for aflatoxin G_2. The outlying means were scrutinised but no technical reason for exclusion was found. The individual contributions of heterogeneity to the uncertainty budget are summarized in Table 3.

Table 3. Results of homogeneity study

BCR-263R	Mean (normalised)	*RSD* [%]	s_{wb} [%]	s_{bb} [%]	u^*_{bb} [%]	u_{bb} [1] [%]
Aflatoxin						
B_1	1.00	3.6	8.1	- [2]	2.0	**2.0**
B_2	1.00	3.6	7.9	- [2]	1.9	**1.9**
G_1	1.00	3.8	7.1	- [2]	1.7	**1.7**
G_2	1.00	4.1	8.2	- [2]	2.0	**2.0**

[1] higher value, u^*_{bb} or s_{bb} is taken as contribution of heterogeneity
[2] cannot be calculated as $MS_{within} > MS_{between}$

The minimum sample intake is 10 g. The stability and homogeneity studies were performed using 10 g of material proving that the individual samples are homogeneous at least to this level.

7 Stability studies

Three stability studies were performed, one isochronous study over 4 weeks to evaluate stability of the materials during transport and one isochronous study over 18 months and 36 months, respectively to evaluate stability during storage.

For the short-term study, samples were stored in the dark at 18 °C, 40 °C and for reference at -70 °C. For the 18 months long-term studies, samples were stored in the dark at 4 °C, -20 °C and for reference at -70 °C and for the 36 months long-term studies, samples were stored in the dark at -20 °C and for reference at -70 °C. Two units were stored at each temperature for 0, 1, 2 and 4 weeks (March 2003 - April 2003) for the short-term study and 0, 6, 12 and 18 months (March 2003 – September 2004) and 0, 12, 24 and 36 months (March 2003 – March 2006), respectively for the long-term studies. After the indicated periods, the samples were transferred to storage at -70 °C until analysis.

After the final time of each isochronous sample storage scheme, the samples were measured together under repeatability conditions in a random order in duplicate. The laboratories employed their in-house HPLC-FLD methods. Documentation of the method employed is kept in the quality system of the respective laboratory. 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, enrichment) due to the storage conditions. Therefore the data points were plotted against time and the regression line calculated.

The uncertainty of stability u_{lts} of the materials was then calculated for the pre-defined 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 16 individual results of the relevant stability study, x_i being the time point for each replicate, $\overline{x}$, being the average of all time points and x being the pre-defined shelf life (36 month 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:

No significant slope at 95 % level of confidence was detected for any material, neither in the short-term study nor in the long-term study.

As no degradation could be observed under any conditions neither, in the short-term nor in the long-term study, it was concluded that no special precautions regarding temperature control during shipment are necessary. The uncertainty of the short-term stability (u_{sts}) is assumed to be negligible since no degradation is expected to happen during this short time.

The materials were stable in both long-term studies. Nevertheless, -20 °C was chosen as storage temperature. Using the data from the 36 months 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 materials are summarised in Table 4.

Table 4. Uncertainty contributions due to storage for BCR-263R

Aflatoxin	u_{lts} [%]
B_1	3.8
B_2	3.5
G_1	7.2
G_2	8.9

8 Characterisation

8.1 Design of the study

The certification exercise was performed in 2006. Eight laboratories were carefully selected to perform the analytical measurements. The laboratories had to prove their measurement capabilities and had to demonstrate previous experience in the analytical field concerned.
Each laboratory was provided with the following samples:

- 3 units of *"Peanut meal (medium level aflatoxins)"* BCR-263R
- 6 units of *"Aflatoxin B_1 in defatted peanut meal"* BCR-262
- 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 had to be performed on three different days. On each day one unit of BCR-263R and BCR-262 had to be analysed in duplicate. Additionally individual recovery experiments had to be carried out on each day with the sets of BCR-262.
In order to determine the recovery rates of the participant's analytical procedures in the concentration range of the naturally contaminated candidate reference material, the participants had to spike the "Aflatoxin B1 in defatted peanut meal (very low level)", BCR-262 with a solution of the common calibrants to a mass fraction of each aflatoxins (B1, B2, G1, G2) of 3.0 µg/kg. The laboratories were asked to use their in-house spiking procedure.
Analyses of the 'blank', spiked and, naturally contaminated peanut materials had to be performed together on each day, since the certification results are corrected by the daily recovery factor. External calibration was 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 BCR-263R in duplicate	1 unit of BCR-263R in duplicate	1 unit of BCR-263R in duplicate
1 unit of BCR-262 in duplicate	1 unit of BCR-262 in duplicate	1 unit of BCR-262 in duplicate
1 unit of BCR-262 spiked in triplicate	1 unit of BCR-262 spiked in triplicate	1 unit of BCR-262 spiked in triplicate

8.2 Results and technical evaluation

All laboratories used their in-house methods based on immunoaffinity column (IAC) clean up and reversed phase high performance liquid chromatography with post column derivatisation and fluorescence detection. The individual methods used are summarized in Table 6. Laboratory 6 did not submit any results for technical reasons.

Table 6. Overview of analytical methods used for certification

Lab code	Extraction solvent	Extraction technique	Defatting	IAC producer	Chromatography	Derivatisation
1	Methanol + water	Blender	Hexane	R-biopharm rhône	Isocratic	PBPB[1]
2	Methanol + water	Shaker	Hexane	Vicam	Isocratic	PBPB
3	Methanol + water	Blender	Hexane	Vicam	Isocratic	Kobra cell[2]
4	Chloroform + water	Shaker	Hexane	R-biopharm rhône	Isocratic	Kobra cell
5	Acetonitrile + water	Blender		R-biopharm rhône	Isocratic	PBPB
7	Acetonitrile + water	Blender		R-biopharm rhône	Isocratic	Kobra cell
8	Acetonitrile + water	Ultrasonic bath		Vicam	Gradient	Kobra cell
9	Methanol + water	Blender	Hexane	R-biopharm rhône	Isocratic	PBPB

[1] bromination with pyridinium hydrobromide perbromide
[2] electrochemical bromination with potassium bromide

After reception of the data sets, the results were subject to technical evaluation. Results not fulfilling the criteria laid down in Commission Regulation 401/2006 [8] regarding recovery rates or below the limit of quantification were eliminated. Satisfactory recovery rates for the spiking level of 3 µg/kg are between 70 % and 110 % [8]. Satisfactory within-laboratory reproducibility is below 38 % based on the Horwitz equation, which all laboratories fulfilled. 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 not satisfactory recovery rates:

- Lab 1: the results of one day for aflatoxin G_2 were rejected
- Lab 2: the results of all three days for aflatoxin B_1 were rejected as well as the results of one day each for aflatoxin B_2, G_1 and G_2
- Lab 3: the results of one day for aflatoxin G_1 was rejected
- Lab 4: the results of one day for aflatoxin G_1 and G_2 each were rejected
- Lab 5: no data was rejected
- Lab 7: all results were rejected except the result of two days for aflatoxin G_1
- Lab 8: the results of all three days for aflatoxin B_1 and G_2 were rejected as well as the results of one day for aflatoxin G_1
- Lab 9: the results of all three days for aflatoxin G_2 were rejected

In total 30 values of aflatoxin B_1 from 5 laboratories, 40 values of aflatoxin B_2 from 7 laboratories, 40 values of aflatoxin G_1 from 8 laboratories and 24 values for aflatoxin G_2 from 5 laboratories were accepted after technical scrutiny for further statistical data assessment.

The accepted sets of results 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 t-test to detect outlying lab means
- Grubb's test to detect single and double outliers
- Cochran test to check for outlying lab variances
- Bartlett test to check for homogeneity of lab variances
- ANOVA to assess between lab and within lab variances and test their significance employing the SNEDECOR F-test
- Skewness and Kurtosis test to assess the normality of the lab means distribution. The later tests are only used if seven or more datasets have been accepted; otherwise normal probability plots have been used.

First, the datasets have been subjected to the Cochran test to check for outlying lab variances. Datasets with outlying variances have been rejected on the basis that all laboratories used a similar method and an outlying variance indicates poor repeatability and therefore a suboptimal control over method performance. The following datasets have been rejected:

- Lab 2 data of aflatoxin B_2 and G_1
- Lab 5 data of aflatoxin B_2
- Lab 9 data of aflatoxin B_2

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 BCR-263R are summarized in Table 7.

Table 7. Summary of statistical evaluation for BCR-263R

BCR-263R				
Aflatoxin	B_1	B_2	G_1	G_2
Number of data sets	5	4	7	5
Number of replicate measurements	30	24	36	24
Mean of means [µg/kg]	17.06	3.01	3.04	0.62
Relative standard deviation of mean of means [%]	11.6	12.4	8.2	30.8
Relative standard error of mean of means [%]	5.2	6.2	3.1	13.8
All data sets compatible two by two? (Scheffe's test)	no	no	no	no
Outlying means? (Dixon test, Nalimov t-test, Grubbs test)	Lab 5 Nalimov (p=0.05)	no	L1 (p=0.05) all tests, Nalimov (p=0.01)	no
Outlying lab variances? (Cochran test)	no	no	no	no
Lab variances homogeneous? (Bartlett test)	no	no	no	no
Distribution of means normal? (Skewness & kurtosis, normal probability plot)	yes	yes	yes (p=0.01)	yes
Variances between labs significantly different? (SNEDECOR)	yes	yes	no (p=0.01)	yes

The individual results are corrected by the daily recovery. The uncertainty of the daily recovery does not contribute 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.
The outlying mean of B1 for BCR-263R lies within two standard deviations of the mean of means and is therefore not significantly different to the mean value. Contrary, the outlying mean of G1 for BCR-263R lies outside two standard deviations of the mean of means and has therefore to be scrutinized further. The confidence interval (p=0.05) of the distribution of individual laboratory means around the mean of means (3.04 µg/kg) is 0.61 µg/kg. The mean of laboratory 1 (2.52 µg/kg) lies within this interval and can therefore be considered as not significantly different from the mean of means.

8.3 Certified values and their uncertainties

The certified values for BCR-263R 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 mass fractions of the aflatoxins. The standard error is calculated as the standard deviation divided by the square root of accepted data sets.

The combined uncertainty of the certified value includes contributions from the between bottle heterogeneity, long-term storage, the characterisation study and the contribution of the common calibrant. The uncertainty of the mass fraction (aflatoxin in acetonitrile) of the common calibrants propagates in the calibrations and can therefore not be neglected.

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 values are summarised in Table 8.

Table 8. Certified values and their uncertainties for BCR-263R

BCR-263R			
Aflatoxin	B_1	B_2	G_1
Certified value [µg/kg]	**17.1**	**3.0**	**3.0**
u_{lts} [%]	3.8	3.5	7.2
u_{bb} [%]	2.0	1.9	1.7
u_{char} [%]	5.2	6.2	3.1
u_{cal} [%]	1.4	1.0	1.7
u_{CRM} [%]	6.9	7.4	8.2
U_{CRM} (k=2) [%]	13.8	14.9	16.4
$U_{CRM,\ abs}$ (k=2) [µg/kg]	**2.4**	**0.4**	**0.5**

Additionally to the values given in Table 8, the following indicative values have been assigned to BCR-263R:

- Aflatoxin G2 0.62 ± 0.21 µg/kg
- Sum of aflatoxins B_1, B_2, G_1 and G_2 23.7 ± 2.5 µg/kg.

The expanded uncertainty for the sum of aflatoxins B_1, B_2, G_1 and G_2 is calculated from the individual absolute standard uncertainties according to:

$$U_{sum} = 2 \cdot \sqrt{u_{B_1}^2 + u_{B_2}^2 + u_{G_1}^2 + u_{G_2}^2}$$

9 Metrological traceability

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. As three different solvents and extraction techniques have been used independence from the extraction method can be assumed.
The certified values for the mass fractions of aflatoxins are traceable via the common calibrants used. The mass fractions of the common calibrants are certified for aflatoxins in acetonitrile. The certified value of the calibrants is traceable to SI due to the gravimetric preparation employed. Therefore the mass fractions of aflatoxins in the CRM are traceable to the SI.

BCR-263R is prepared from naturally contaminated material. Therefore there is no reason to assume that BCR-263R would behave differently from natural samples with similar particle size.

10 Instructions for use

10.1 Storage conditions

The materials should be stored at or below -20 °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.

10.2 Safety precautions

The usual laboratory safety precautions apply.

10.3 Use of the material

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

10.4 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 by Linsinger [9]. The procedure is described here in brief:

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

11 Acknowledgements

The authors would like to thank Jacob de Boer (Free University Amsterdam, NL), Paul Finglas (Institute of Food Research, GB), Petra Gowik (Federal Office of Consumer Protection and Food Safety, DE), Gert Roebben (IRMM, BE) and James Snell (IRMM, BE) for the review of this report. Additionally the authors would like to thank Thomas Linsinger (IRMM, BE) for his technical advice.

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Annex A. Homogeneity data

Table A1. Results of homogeneity study

BCR-263R				
	means (n=3)			
bottle	B1 [µg/kg]	B2 [µg/kg]	G1 [µg/kg]	G2 [µg/kg]
26	18.78	3.86	2.35	0.44
92	16.34	3.25	2.02	0.61
114	17.64	3.20	2.30	0.46
156	17.74	3.35	2.27	0.51
212	16.50	3.18	2.05	0.44
304	19.51	3.70	2.46	0.51
354	17.13	3.32	2.22	0.50
380	16.58	3.24	2.10	0.59
460	17.76	3.68	2.28	0.41
477	17.53	3.29	2.26	0.51
544	17.84	3.42	2.26	0.52
575	17.97	3.46	2.34	0.55
669	17.19	3.27	2.09	0.45
687	19.43	3.74	2.51	0.53
727	18.38	3.85	2.31	0.44
805	17.28	3.32	2.16	0.58
840	16.90	3.25	2.24	0.53
887	18.62	3.67	2.41	0.49
938	16.85	3.44	2.10	0.46
1029	17.67	3.39	2.16	0.57
1053	18.11	3.49	2.29	0.44
1118	18.51	3.75	2.40	0.48
1172	18.08	3.45	2.34	0.54
1226	16.82	3.17	2.11	0.44
1257	18.97	3.57	2.15	0.47
1325	16.71	3.26	2.06	0.50
1354	17.26	3.32	2.26	0.49
1413	18.91	3.59	2.61	0.58
1511	17.66	3.30	2.19	0.51
1529	17.18	3.30	2.14	0.58
1575	17.26	3.27	2.27	0.54

Table A2. Results of homogeneity study normalized by the mean of each measurement day

BCR-263R				
	means (n=3) normalized			
bottle	B1	B2	G1	G2
26	1.025	1.016	1.015	1.024
92	1.009	1.019	1.011	1.029
114	0.973	0.968	0.988	0.983
156	1.011	0.995	1.015	0.976
212	0.959	0.978	0.982	0.997
304	1.045	1.036	1.010	0.983
354	0.987	0.999	0.985	0.969
380	1.024	1.017	1.049	1.010
460	0.970	0.968	0.988	0.973
477	0.999	0.980	1.011	0.992
544	1.028	1.030	1.004	0.998
575	1.024	1.028	1.046	1.060
669	0.998	1.006	1.004	0.998
687	1.040	1.049	1.027	1.016
727	1.003	1.013	0.997	1.019
805	0.890	0.889	0.911	0.913
840	0.956	0.953	0.984	1.006
887	0.990	0.986	0.998	1.004
938	0.985	0.991	0.978	0.976
1029	1.041	1.043	1.010	1.021
1053	1.028	1.037	1.030	1.038
1118	1.016	1.013	1.022	1.008
1172	0.998	0.996	1.032	1.050
1226	0.916	0.911	0.895	0.895
1257	1.043	1.015	1.014	1.005
1325	0.968	1.000	0.896	0.922
1354	1.028	1.033	1.014	1.021
1413	1.009	1.018	1.070	1.102
1511	0.999	0.994	0.982	0.979
1529	1.036	1.032	1.018	1.026
1575	1.002	0.985	1.012	1.006

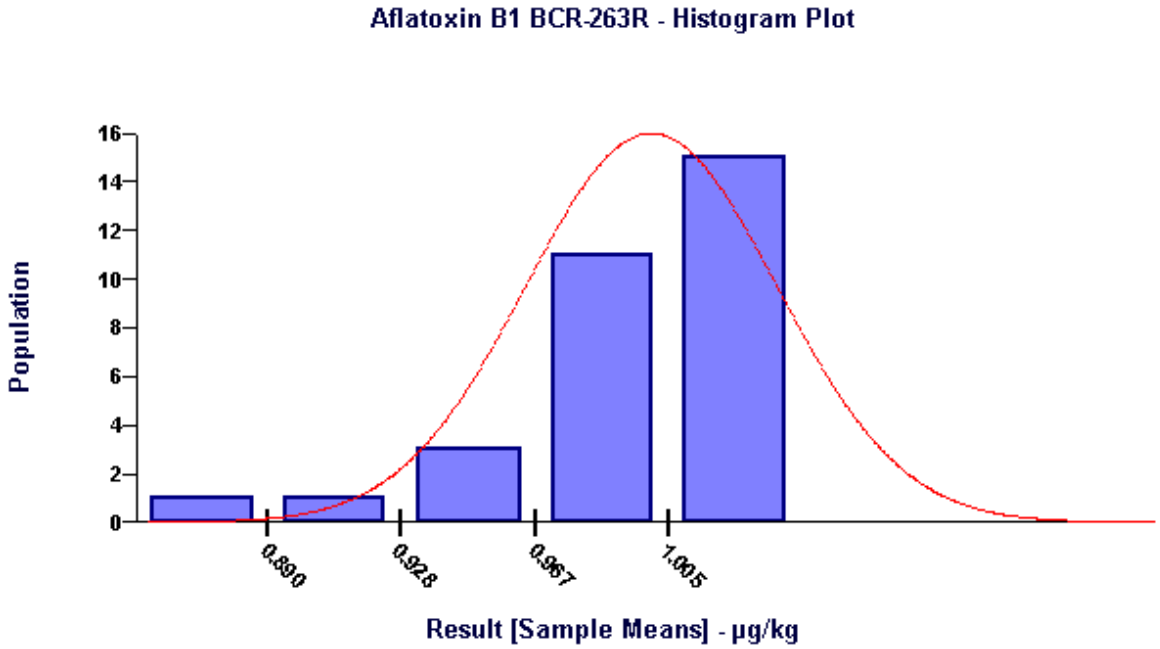

Figure A1. Histogram of homogeneity results for aflatoxin B_1

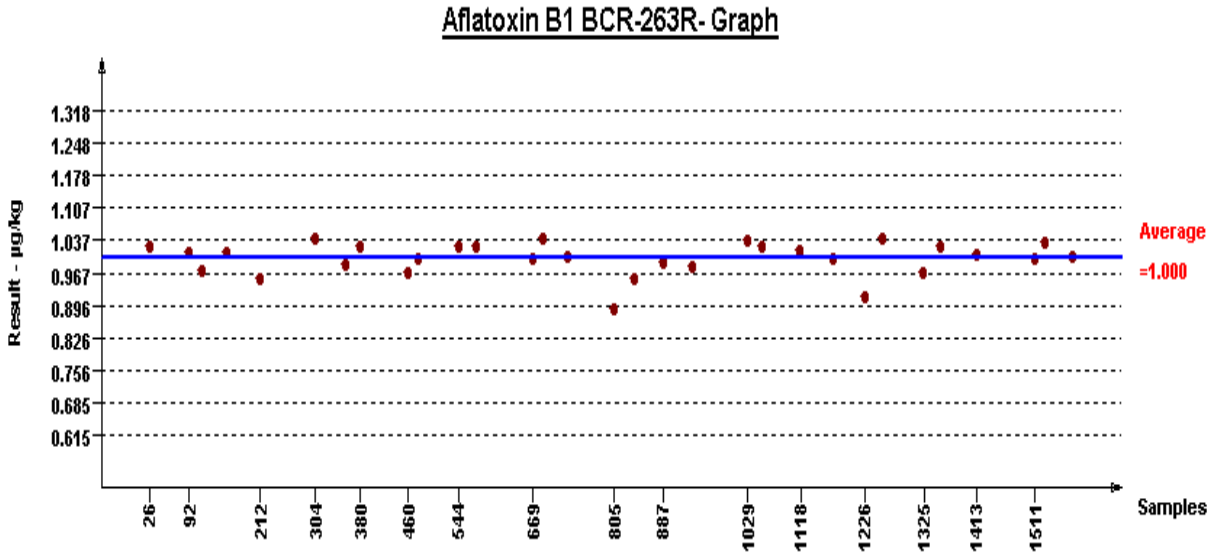

Figure A2. Results of homogeneity study for aflatoxin B_1 sorted by the filling sequence

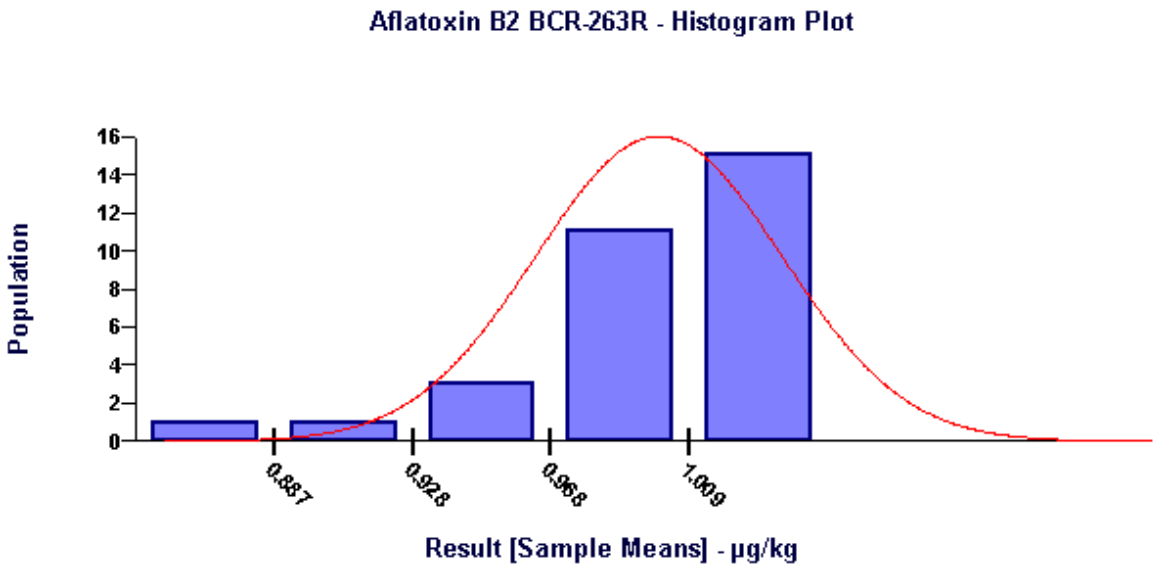

Figure A3. Histogram of homogeneity results for aflatoxin B_2

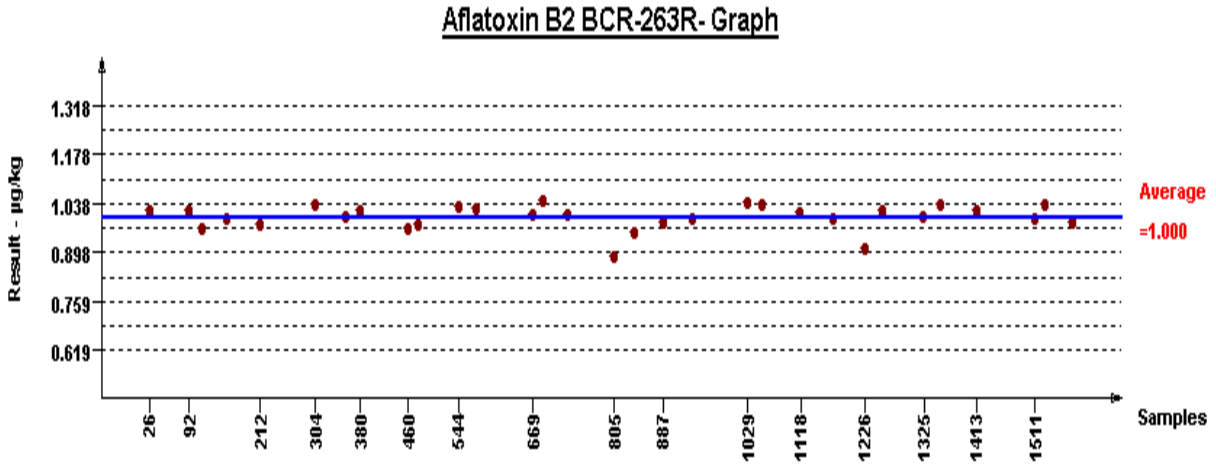

Figure A4. Results of homogeneity study for aflatoxin B_2 sorted by the filling sequence

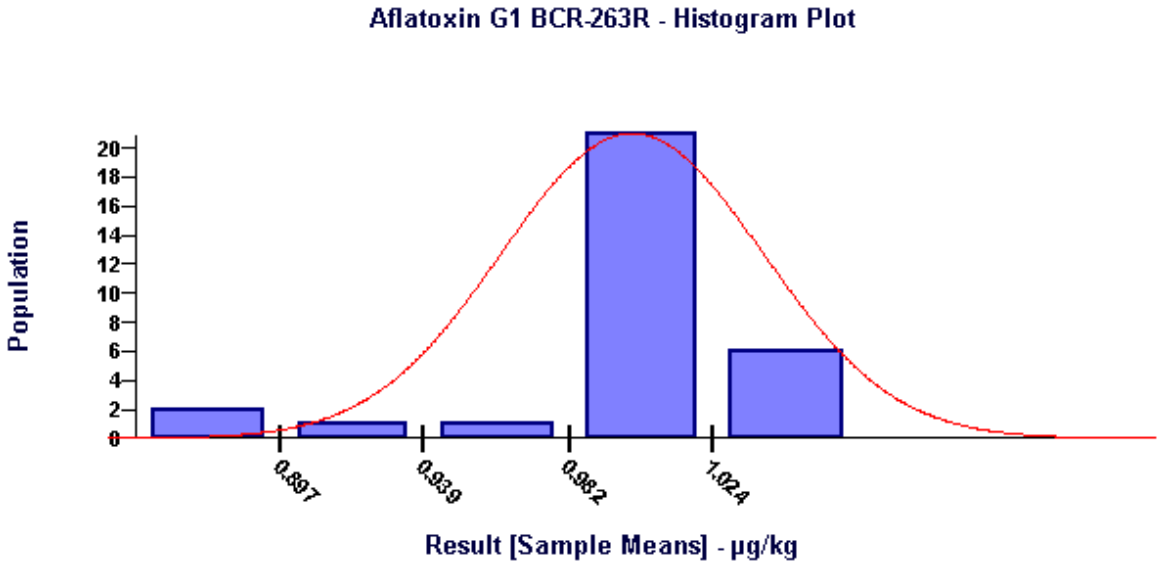

Figure A5. Histogram of homogeneity results for aflatoxin G_1

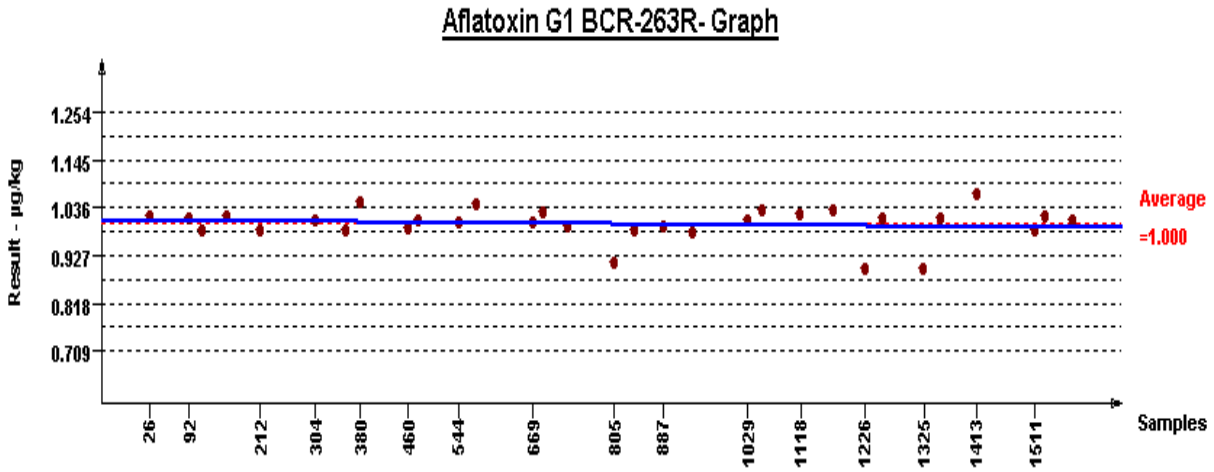

Figure A6. Results of homogeneity study for aflatoxin G_1 sorted by the filling sequence

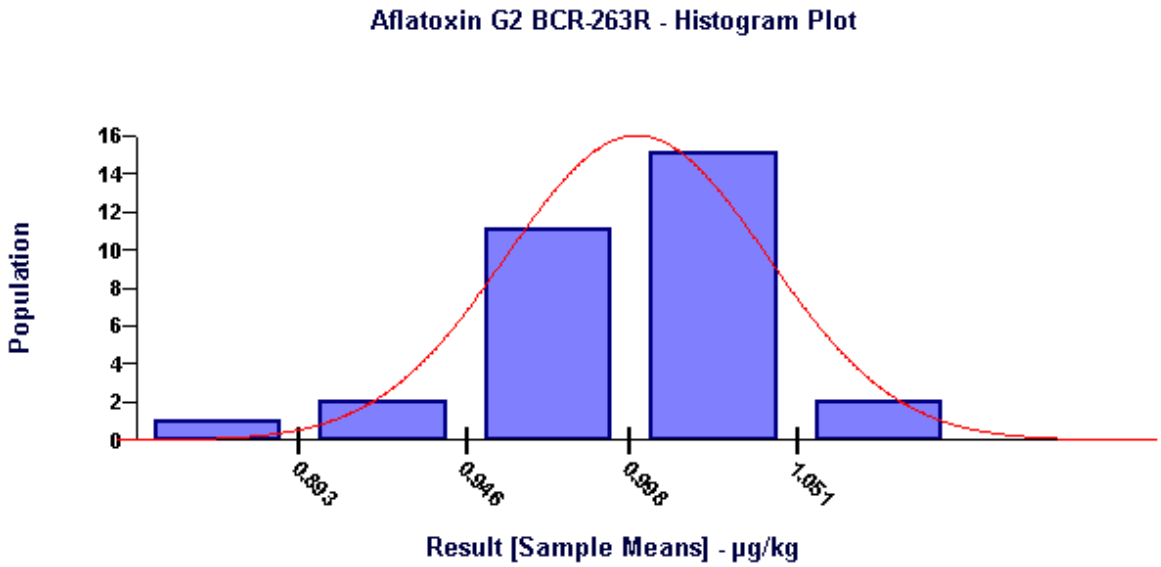

Figure A7. Histogram of homogeneity results for aflatoxin G_2

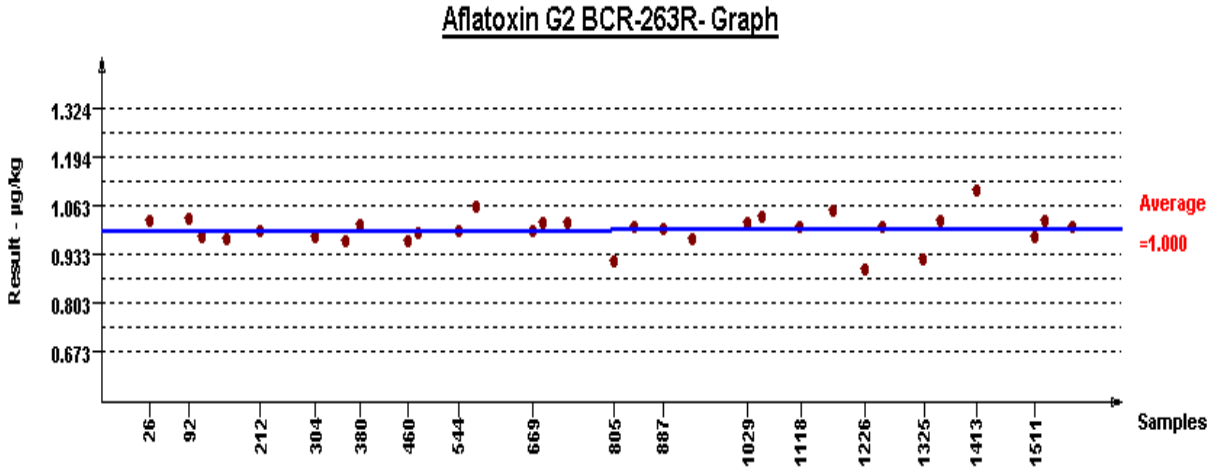

Figure A8. Results of homogeneity study for aflatoxin G_2 sorted by the filling sequence

Annex B. Stability data

Table B1. Results of the isochronous studies for aflatoxin B_1

BCR-263R B1 [µg/kg]				
	weeks at 18 °C			
samples	0	1	2	4
1	16.30	16.68	16.11	16.81
2	16.70	16.96	13.74	16.13
3	16.48	20.38	16.64	17.16
4	16.62	17.48	16.81	16.38
	weeks at 40 °C			
samples	0	1	2	4
1	16.30	16.28	16.89	16.89
2	16.70	15.35	12.86	17.10
3	16.48	12.81	16.74	16.29
4	16.62	17.17	16.33	16.23
	months at -20 °C			
samples	0	6	12	18
1	17.23	16.89	16.92	17.29
2	16.59	16.10	17.45	16.45
3	17.48	17.07	18.20	17.19
4	17.32	16.38	17.05	17.18
	months at 4 °C			
samples	0	6	12	18
1	18.82	19.80	19.07	19.66
2	18.43	19.28	19.47	18.40
3	19.32	19.83	20.01	18.23
4	19.32	19.75	19.57	18.56
	months at -20 °C			
samples	0	12	24	36
1	18.2	17.7	18.0	19.6
2	16.0	16.5	16.8	18.7
3	16.7	18.8	17.2	18.3
4	17.8	17.2	19.0	17.3

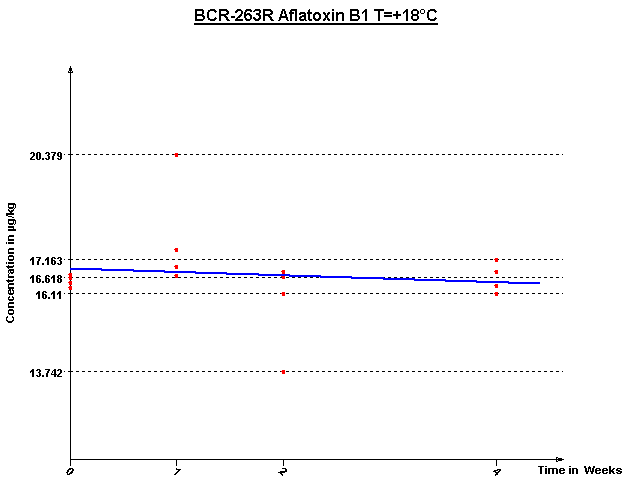

Figure B1. Short term stability for aflatoxin B_1 at 18 °C

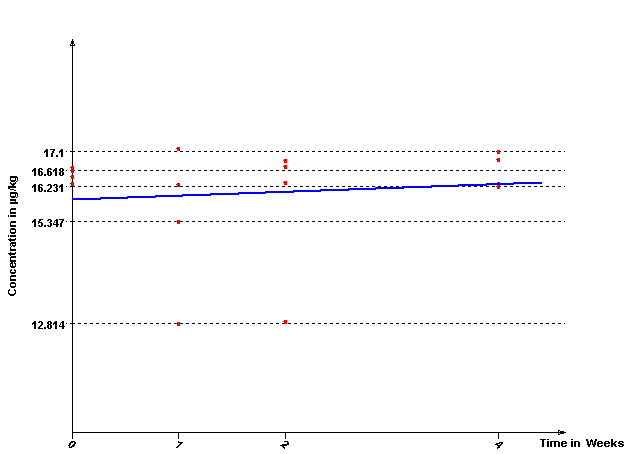

Figure B2. Short term stability for aflatoxin B_1 at 40 °C

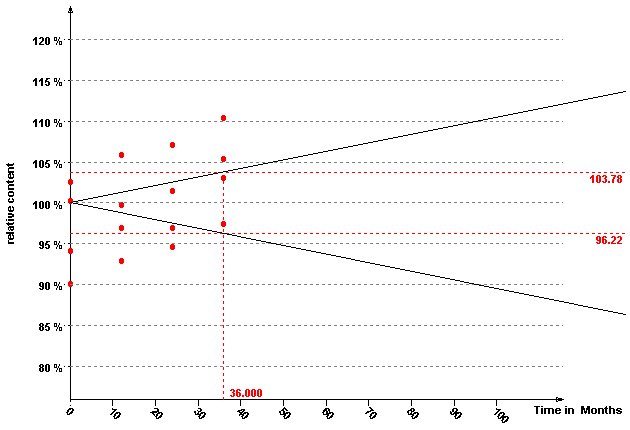

Figure B3. Long term stability for aflatoxin B_1 at -20 °C with associated u_{lts} for a storage period of 36 month

Table B2. Results of the isochronous studies for aflatoxin B_2

BCR-263R B2 [µg/kg]				
	weeks at 18 °C			
samples	0	1	2	4
1	2.85	2.98	2.91	2.92
2	2.88	2.99	2.71	2.78
3	2.96	3.69	2.91	2.91
4	2.94	3.17	2.96	2.87
	weeks at 40 °C			
samples	0	1	2	4
1	2.85	2.93	2.95	3.09
2	2.88	2.80	2.73	3.17
3	2.96	2.56	3.01	2.81
4	2.94	3.00	3.04	2.86
	months at -20 °C			
samples	0	6	12	18
1	3.05	2.98	2.92	3.02
2	2.90	2.87	3.03	2.79
3	3.06	2.94	3.21	3.02
4	2.96	2.91	2.99	2.94
	months at 4 °C			
samples	0	6	12	18
1	3.17	3.27	3.22	3.35
2	3.20	3.26	3.32	3.15
3	3.31	3.37	3.42	3.14
4	3.25	3.46	3.29	3.22
	months at -20 °C			
samples	0	12	24	36
1	3.6	3.5	3.7	3.8
2	3.2	3.5	3.4	3.6
3	3.5	3.8	3.5	3.6
4	4.0	3.5	3.8	3.6

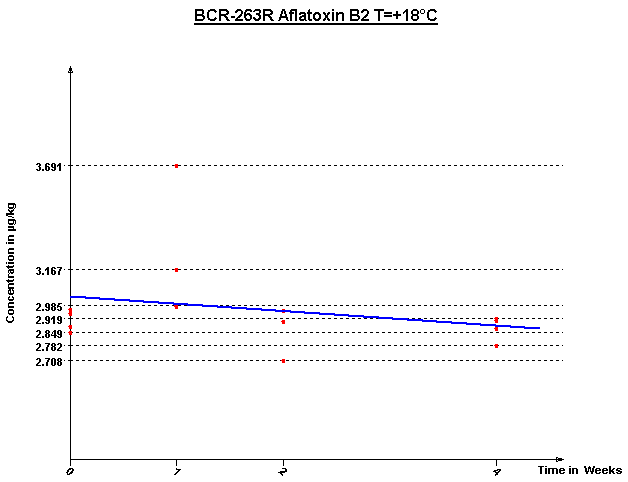

Figure B4. Short term stability for aflatoxin B_2 at 18 °C

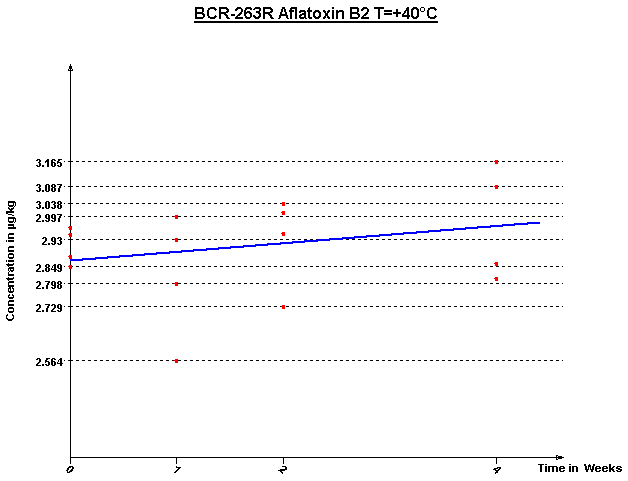

Figure B5. Short term stability for aflatoxin B_2 at 40 °C

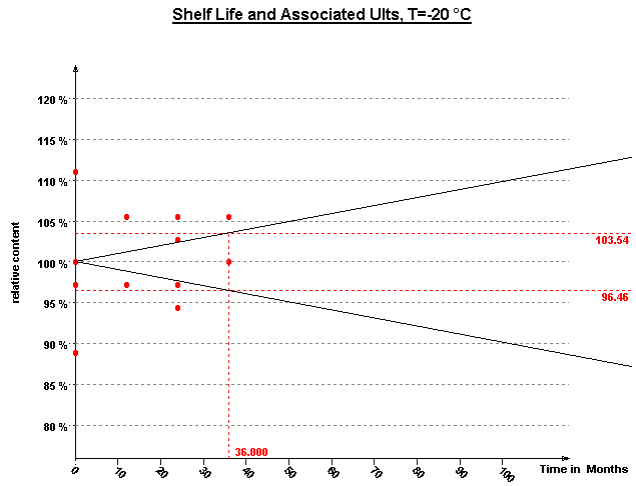

Figure B6. Long term stability for aflatoxin B_2 at -20 °C with associated u_{lts} for a storage period of 36 month

Table B3. Results of the isochronous studies for aflatoxin G_1

BCR-263R G1 [µg/kg]				
	weeks at 18 °C			
samples	0	1	2	4
1	2.66	2.78	2.63	2.79
2	2.42	2.54	2.12	2.55
3	11.70	2.76	2.71	2.60
4	2.54	2.70	2.62	2.52
	weeks at 40 °C			
samples	0	1	2	4
1	2.66	2.67	2.76	2.67
2	2.42	2.47	2.15	2.65
3	11.70	2.05	2.92	2.57
4	2.54	2.53	2.68	2.50
	months at -20 °C			
samples	0	6	12	18
1	2.72	2.68	2.89	2.83
2	2.54	2.48	2.56	2.51
3	2.78	2.65	2.92	2.64
4	2.70	2.53	2.59	2.64
	months at 4 °C			
samples	0	6	12	18
1	2.95	3.26	3.04	3.19
2	2.88	3.11	3.04	3.03
3	3.44	3.22	3.41	2.96
4	3.03	3.01	2.91	2.86
	months at -20 °C			
samples	0	12	24	36
1	2.9	2.7	2.6	2.7
2	2.2	2.5	2.9	2.8
3	2.4	2.6	2.4	2.4
4	2.4	2.2	1.9	2.6

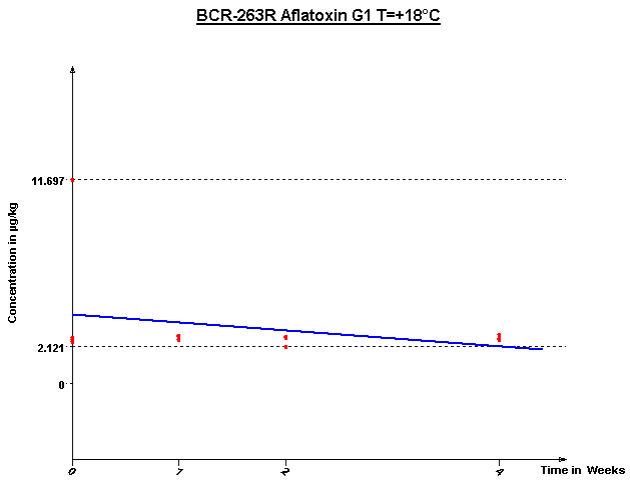

Figure B7. Short term stability for aflatoxin G_1 at 18 °C

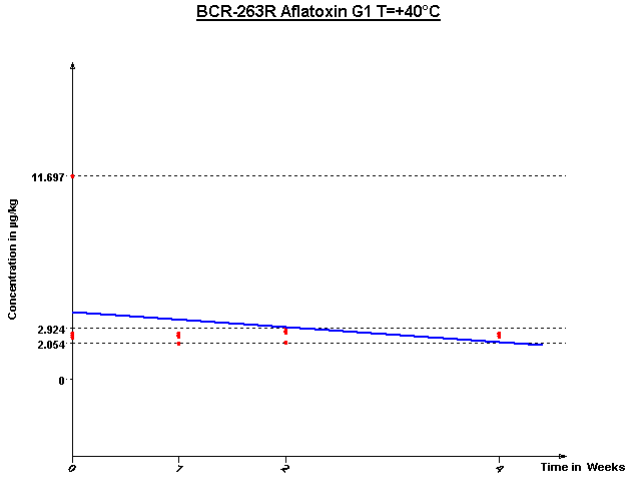

Figure B8. Short term stability for aflatoxin G_1 at 40 °C

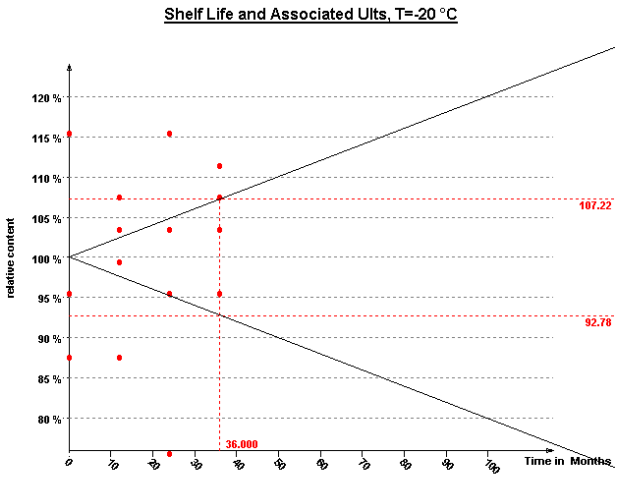

Figure B9. Long term stability for aflatoxin G_1 at -20 °C with associated u_{lts} for a storage period of 36 month

Table B4. Results of the isochronous studies for aflatoxin G_2

BCR-263R G2 [µg/kg]				
	weeks at 18 °C			
samples	0	1	2	4
1	0.40	0.44	0.39	0.40
2	0.37	0.38	0.34	0.37
3	0.41	0.41	0.40	0.37
4	0.37	0.43	0.40	0.38
	weeks at 40 °C			
samples	0	1	2	4
1	0.40	0.41	0.40	0.41
2	0.37	0.37	0.37	0.43
3	0.41	0.40	0.43	0.39
4	0.37	0.44	0.43	0.37
	months at -20 °C			
samples	0	6	12	18
1	0.27	0.27	0.28	0.28
2	0.25	0.25	0.28	0.29
3	0.33	0.26	0.32	0.26
4	0.23	0.31	0.31	0.25
	months at 4 °C			
samples	0	6	12	18
1	0.24	0.3	0.27	0.29
2	0.26	0.27	0.29	0.29
3	0.34	0.29	0.33	0.25
4	0.21	0.27	0.29	0.23
	months at -20 °C			
samples	0	12	24	36
1	0.40	0.48	0.46	0.44
2	0.44	0.47	0.44	0.51
3	0.48	0.42	0.50	0.37
4	0.37	0.36	0.34	0.34

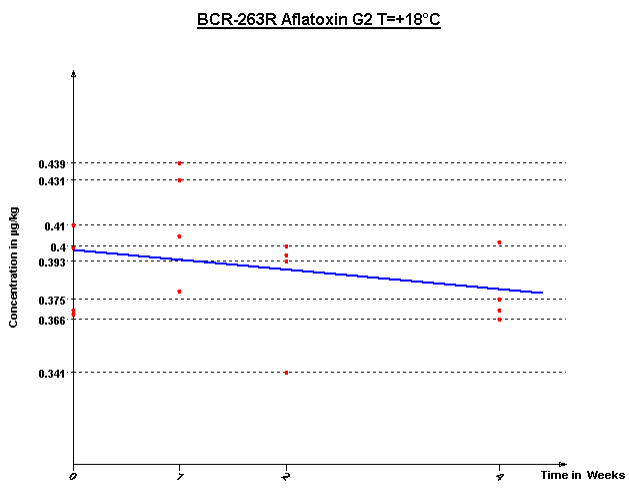

Figure B10. Short term stability for aflatoxin G_2 at 18 °C

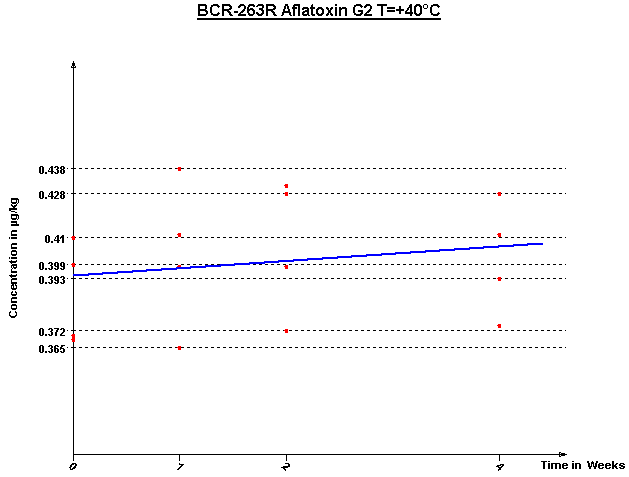

Figure B11. Short term stability for aflatoxin G_2 at 40 °C

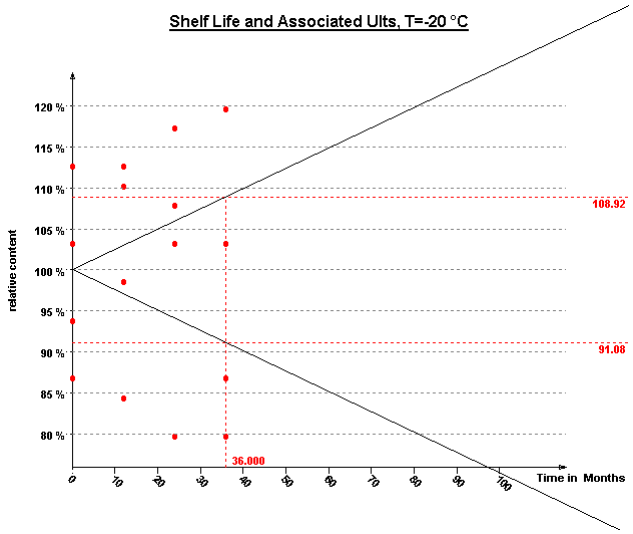

Figure B12. Long term stability for aflatoxin G_2 at -20 °C with associated u_{lts} for a storage period of 36 month

Annex C. Characterisation measurements

Table C1. Results of characterisation measurements for aflatoxin B_1

B1 mass fraction in BCR-263R [µg/kg]								
Lab code	Day 1		Day 2		Day 3		Mean	s
1	16.76	16.00	14.12	15.48	18.97	16.80	16.36	1.62
3	15.25	15.23	15.64	15.64	15.08	15.07	15.32	0.26
4	16.97	17.69	18.17	18.04	16.17	18.36	17.57	0.84
5	19.45	21.58	22.47	20.75	19.06	18.32	20.27	1.60
9	15.92	15.91	15.89	15.61	15.53	15.77	15.77	0.17

No Pooling - Lab Means and their StDev for Certification BCR-263R B1

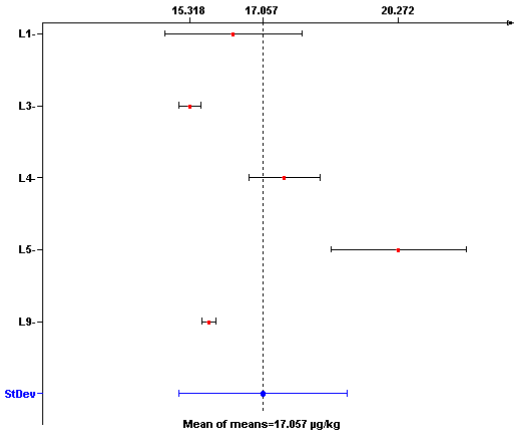

Figure C1 Laboratory means, mean of means and their standard deviation for aflatoxin B_1

Table C2. Results of characterisation measurements for aflatoxin B_2

B2 mass fraction in BCR-263R [µg/kg]								
Lab code	Day 1		Day 2		Day 3		Mean	s
1	2.57	2.46	2.17	2.39	2.86	2.60	2.51	0.23
3	3.05	3.05	3.13	3.16	3.05	3.05	3.08	0.05
4	3.00	3.04	3.06	3.14	2.88	3.18	3.05	0.11
8	3.03	3.30	3.35	3.44	3.53	3.80	3.41	0.26

No Pooling - Lab Means and their StDev for Certification BCR-263R B2

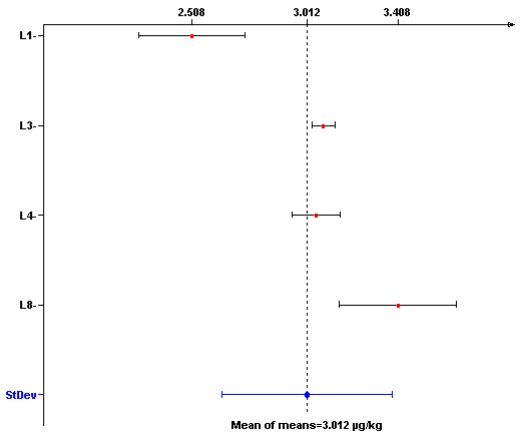

Figure C2 Laboratory means, mean of means and their standard deviation for aflatoxin B_2

Table C3. Results of characterisation measurements for aflatoxin G_1

G1 mass fraction in BCR-263R [µg/kg]								
Lab code	Day 1		Day 2		Day 3		Mean	s
1	2.44	2.44	2.14	2.36	2.98	2.74	2.52	0.30
3	-	-	3.23	3.23	3.20	3.19	3.21	0.02
4	2.95	3.08	-	-	2.93	3.25	3.05	0.15
5	2.98	3.47	3.23	2.61	3.66	3.26	3.20	0.37
7	3.36	3.35	2.37	3.42	-	-	3.13	0.50
8	2.85	3.17	3.32	3.30	3.12	3.57	3.22	0.24
9	3.61	3.41	2.60	2.49	2.81	2.84	2.96	0.45

No Pooling - Lab Means and their StDev for Certification BCR-263R G1

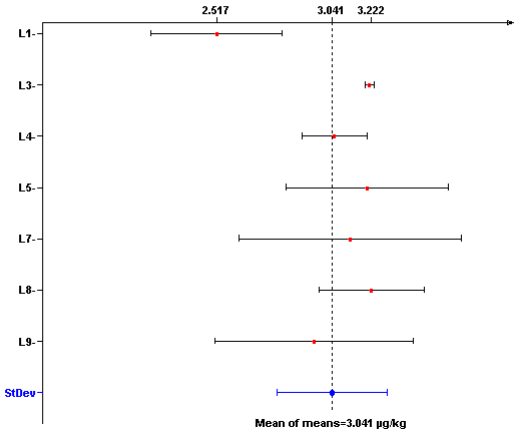

Figure C3. Laboratory means, mean of means and their standard deviation for aflatoxin G_1

Table C4. Results of characterisation measurements for aflatoxin G_2

G2 mass fraction in BCR-263R [µg/kg]								
Lab code	Day 1		Day 2		Day 3		Mean	s
1	-	-	0.33	0.41	0.33	0.35	0.36	0.04
2	-	-	0.61	0.66	0.79	0.71	0.69	0.08
3	1.14	1.14	0.71	0.71	0.59	0.60	0.82	0.26
4	0.54	0.55	-	-	0.91	0.98	0.75	0.23
5	0.40	0.45	0.50	0.52	0.59	0.48	0.49	0.06

No Pooling - Lab Means and their StDev for Certification of BCR-263R G2

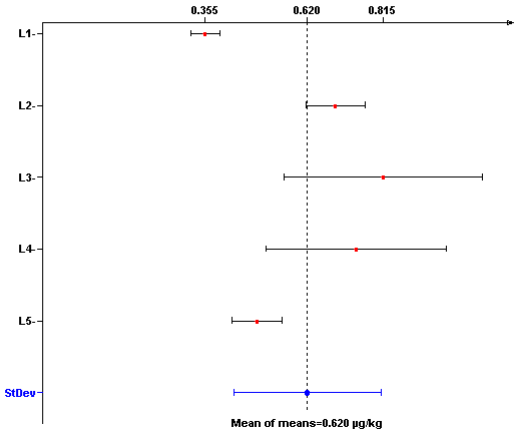

Figure C4. Laboratory means, mean of means and their standard deviation for aflatoxin G_2

European Commission

EUR 23386 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of mass fractions of aflatoxin B_1, B_2, G_1 and G_2 in peanut meal, BCR-263R
Authors: G. Buttinger, S. Harbeck, R. Josephs
Luxembourg: Office for Official Publications of the European Communities
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Abstract

This report describes the preparation of peanut meal (BCR-263R) matrix reference material and the certification of its aflatoxins B_1, B_2, G_1 and G_2 content (mass fraction).

The preparation of the material, the homogeneity and stability studies and the characterisation are described hereafter and the results are discussed. Uncertainties were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties due to possible heterogeneity and instability. The certified values are listed below:

BCR-263R	Certified value [1)]	Uncertainty [2)]	Number of accepted sets of results (p)
Aflatoxin B_1	17.1 µg/kg	2.4 µg/kg	5
Aflatoxin B_2	3.0 µg/kg	0.4 µg/kg	4
Aflatoxin G_1	3.0 µg/kg	0.5 µg/kg	7

1) These values are the mass fractions based on the unweighted mean of accepted results.
2) The uncertainties are the expanded uncertainties (k = 2) of the values defined in 1).

Additionally the following indicative values have been assigned:

BCR-263R	Indicative value [1)]	Uncertainty [2)]	Number of accepted sets of results (p)
Aflatoxin G_2	0.62 µg/kg	0.21 µg/kg	5
Sum of Aflatoxin B_1, B_2, G_1, G_2	23.7 µg/kg	2.5 µg/kg	-

1) These values are the mass fractions based on the unweighted mean of accepted results.
2) The uncertainties are the expanded uncertainties (k = 2) of the values defined in 1).
3) The uncertainty for the sum of aflatoxins B_1, B_2, G_1, G_2 is calculated from the individual absolute standard uncertainties as $U_{sum} = 2 \cdot \sqrt{u_{B_1}^2 + u_{B_2}^2 + u_{G_1}^2 + u_{G_2}^2}$.

The assigned values and their uncertainties are based on a minimum sample intake of 10 g.

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