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**BCR information**
REFERENCE MATERIALS

# Certification of mass fractions of aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$ in peanut butter

# BCR-385R and BCR-401R

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# Summary

This report describes the preparation of peanut butter (BCR-385R and BCR-401R) matrix reference materials and the certification of their content (mass fraction) of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$.

The preparation of the materials, 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], including uncertainties due to possible heterogeneity and instability. The certified values are listed below:

| BCR-385R | Certified value [1)] | Uncertainty [2)] | Number of accepted sets of results |
|---|---|---|---|
| Aflatoxin $B_1$ | 1.77 µg/kg | 0.30 µg/kg | 7 |
| Aflatoxin $B_2$ | 0.48 µg/kg | 0.08 µg/kg | 6 |
| Aflatoxin $G_1$ | 0.9 µg/kg | 0.4 µg/kg | 6 |
| Aflatoxin $G_2$ | 0.30 µg/kg | 0.12 µg/kg | 4 |
| Sum of Aflatoxin $B_1$, $B_2$, $G_1$, $G_2$ | 3.5 µg/kg | 0.5 µg/kg[3)] | |

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

| BCR-401R | Certified value | Number of accepted sets of results |
|---|---|---|
| Aflatoxin $B_1$ | < 0.2 µg/kg [1)] | 7 |
| Aflatoxin $B_2$ | < 0.2 µg/kg [2)] | 6 |
| Aflatoxin $G_1$ | < 0.2 µg/kg [2)] | 6 |
| Aflatoxin $G_2$ | < 0.2 µg/kg [2)] | 4 |

1) This value is the mass fractions based on the limits of quantification of the methods used and the highest found level of accepted results, with a 80% probability the measurand content is below this level.
2) These values are the mass fractions based on the limits of quantification of the methods used and the highest found level of accepted results, with a 95% probability the measurand content is below this level.

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# 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 |
| 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 (in)homogeneity 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 $G_2$ |
| $u_\Delta$ | 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 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 |
| $\bar{x}$ | 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 |
| $\Delta$ | Difference between two measurement results |
| $\Delta_m$ | Difference between measured and certified value |
| $v_{MSwithin}$ | Degrees of freedom of $MS_{within}$ |

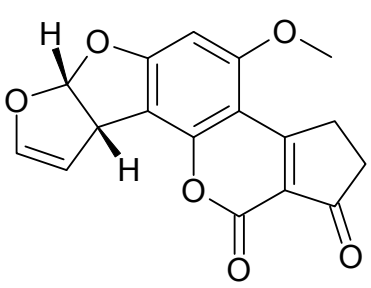
# 1 Introduction

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.

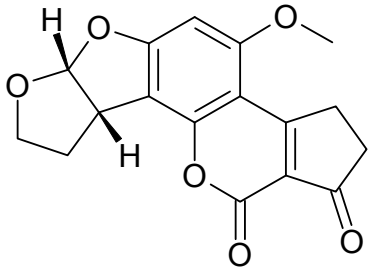
The impact of mycotoxins on agricultural production is massive. The Food and Agriculture Organization of the United Nations (FAO) estimates that 25 % of the world-wide production is affected. The 2006 annual report of the rapid alert system for food and feed of the European Union [2] also shows that 30 % of the notifications were due to mycotoxin contaminations. Around 90 % of these notifications concerned aflatoxins and a quarter 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 aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ 4.0 µg/kg [4].

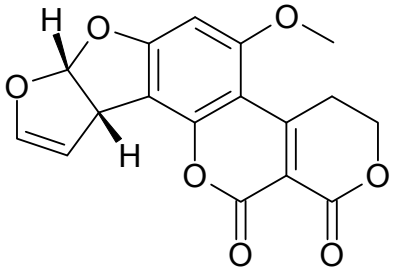
Aflatoxin $B_1$ (Figure 1)[2, 3, 6aα, 9aα-Tetrahydro-4-methoxycyclopenta [*c*] furo [3´, 2´:4, 5] furo [2, 3-*h*] [*l*] benzopyran-1, 11-dione, CAS Nr. 1162-65-8], aflatoxin $B_2$ (Figure 1) [2, 3, 6aα, 8, 9, 9aα-Hexahydro-4-methoxycyclopenta [*c*] furo [3´, 2´:4, 5] furo [2, 3-*h*] [*l*] benzopyran-1, 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].



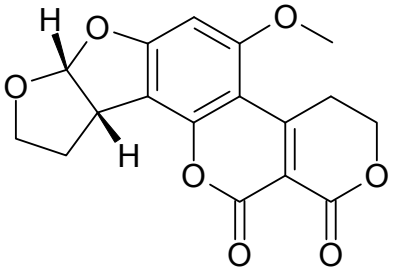
Aflatoxin $B_1$
($C_{17}H_{12}O_6$, MW = 312.3 g/mol)



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



Aflatoxin $G_1$
($C_{17}H_{12}O_7$, MW = 328.3 g/mol)



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

**Figure 1. Molecular structures of aflatoxins**

# 2 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)

**Certification 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 |

# 3 Processing of the peanut materials

## *3.1 BCR-401R peanut butter (very low level)*

250 kg of peanut butter from Lorenz Bahlsen Snack-World (Neuenburg, DE) were homogenised for one hour with 1.25 kg Lecithin EMULFLUID E from Lucas Meyer (Hamburg, DE) in a horizontal kneader from Döpke (Norden, DE).
After assessment of the bulk homogeneity, portions of more than 100 g were filled in aluminium cans. The paste was homogenised once more by using a drill and colour mixer before filling into the filling machine (Hamba, Wuppertal, DE). The aluminium cans were flushed with nitrogen and the lids heat sealed.

## *3.2 BCR-385R peanut butter (low level)*

400 kg of Argentinean roasted peanut kernels were processed to a paste by a coronamill from Probst & Class (Rastatt, DE). The paste was mixed with 2 kg Lecithin EMULFLUID E from Lucas Meyer (Hamburg, DE).
35 kg of contaminated Chinese peanut kernels were roasted at 120 °C in a drying cabinet and processed to a paste by a coronamill from Probst & Class (Rastatt, DE).
170 kg of the Argentinean paste were pre-mixed with 35 kg of the Chinese paste and subsequently homogenised in a horizontal kneader from Döpke (Norden, DE).
After assessment of the bulk homogeneity, portions of more than 100 g were filled in aluminium cans. The paste was homogenised once more by using a drill and colour mixer before filling into the filling machine (Hamba, Wuppertal, DE). The aluminium cans were flushed with nitrogen and the lids heat sealed.

## *3.3 Additional characterisation measurements*

### 3.3.1 Water content and water activity

The water content and water activity of the two peanut materials 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-401R | 8.0 ± 0.2 | 0.174 ± 0.004 |
| BCR-385R | 16.0 ± 0.9 | 0.2023 ± 0.0019 |

### 3.3.2 Particle size measurements

Three particles size analyses have been carried out for each peanut butter material. The measurements were carried out employing a laser diffraction measuring device from Sympatec (Clausthal, DE). The materials were dispersed in methanol and the particle size distributions of the particles in the resulting suspension, were measured over the range from 0.5 to 875 µm. During the 10 s of measuring time, the samples were stirred with a magnetic stirring bar at 1200 rpm.
Representative graphs of the particle size distributions are shown in Figure 2 and Figure 3 for BCR-385R and BCR-401R, respectively.

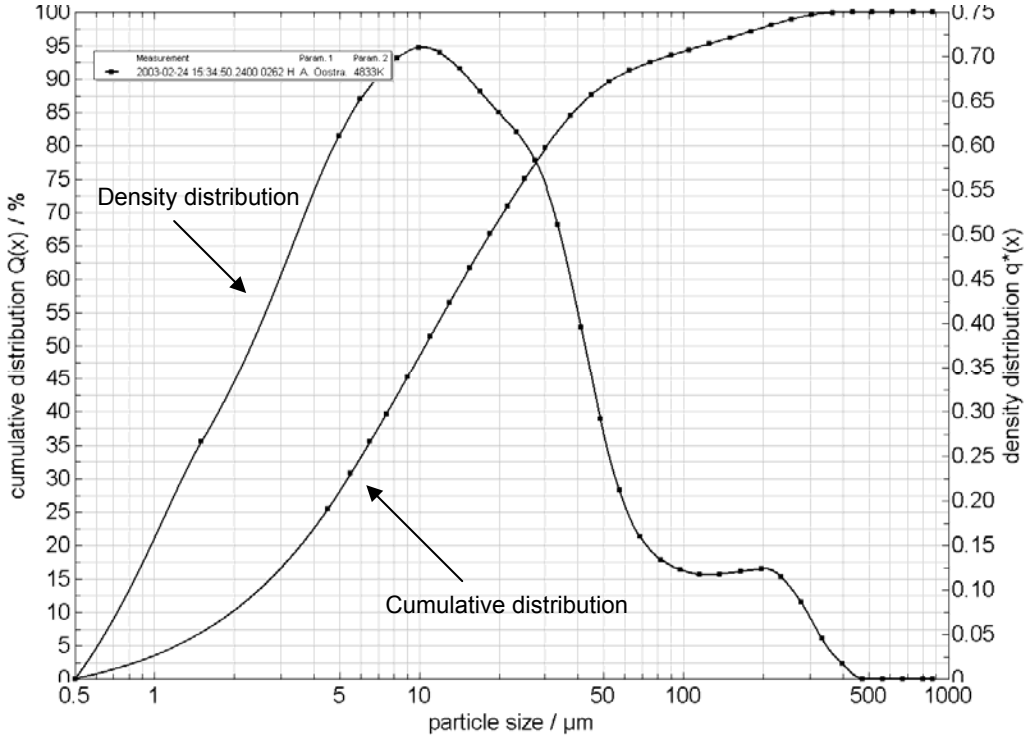



**Figure 2. Particle size distribution of BCR-385R**

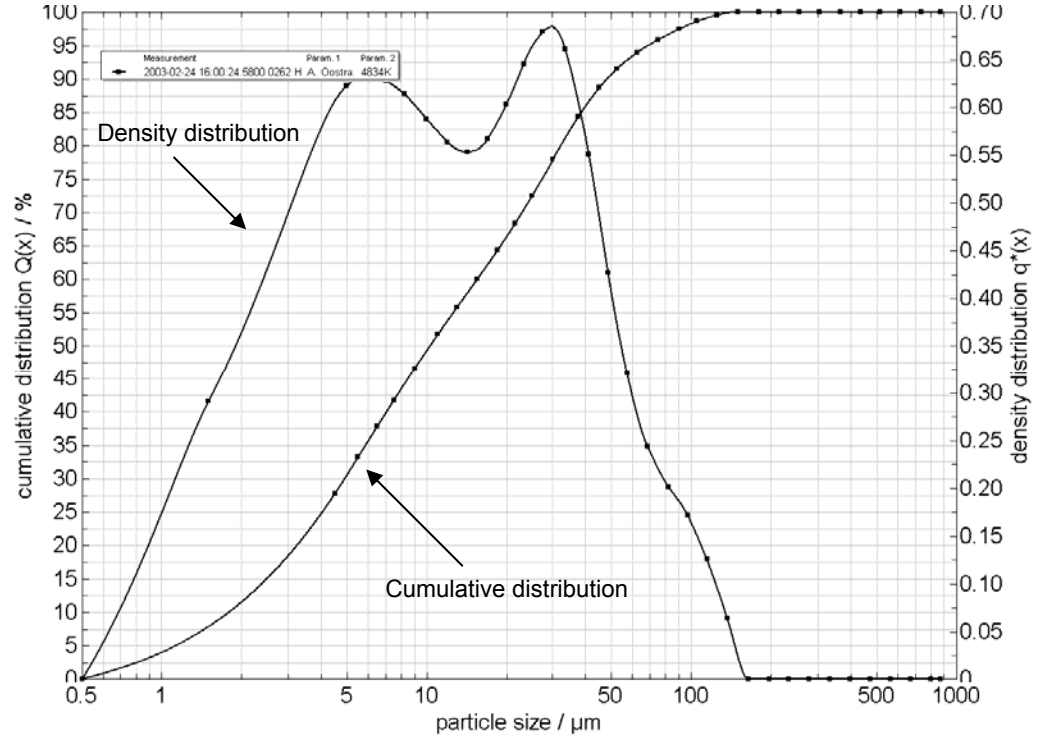



**Figure 3. Particle size distribution of BCR-401R**

# 4 Homogeneity studies

For the homogeneity study, 31 samples (about 2.0 % of the total batch) of BCR-385R were chosen using a random stratified sample picking scheme and analysed for their aflatoxin content in triplicate. Additionally 31 samples (about 2.0 % of the total batch) of BCR-385R and BCR-401R were chosen using a random stratified sample picking scheme and analysed for total nitrogen content according to Kjeldahl in duplicate.
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 variance 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 $u^*_{bb}$ were 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 trends regarding filling sequence were detected. Sample averages were not always normal-distributed but followed a unimodal distribution in each case, allowing the use of ANOVA for heterogeneity determination. The following outliers were found for BCR-385R: one single outlier (level of confidence 99%) for aflatoxin $B_1$, $G_1$ and $G_2$. 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 and Table 4.

**Table 3. Results of homogeneity study for aflatoxins**

| **BCR-385R** | Mean (normalized) | RSD [%] | $s_{wb}$ [%] | $s_{bb}$ [%] | $u^*_{bb}$ [%] | $\mathbf{u_{bb}}$ [1] **[%]** |
|---|---|---|---|---|---|---|
| Aflatoxin | | | | | | |
| $B_1$ | 1.00 | 4.2 | 7.3 | -[2] | 1.8 | **1.8** |
| $B_2$ | 1.00 | 4.1 | 7.7 | -[2] | 1.9 | **1.9** |
| $G_1$ | 1.00 | 16.6 | 17.5 | 13.2 | 4.3 | **13.2** |
| $G_2$ | 1.00 | 17.9 | 21.5 | 12.8 | 5.3 | **12.8** |

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

**Table 4. Results of homogeneity study for nitrogen**

| | Mean | RSD [%] | $s_{wb}$ [%] | $s_{bb}$ [%] | $u^*_{bb}$ [%] | $\mathbf{u_{bb}}$ [1] **[%]** |
|---|---|---|---|---|---|---|
| **BCR-385R** | | | | | | |
| Nitrogen | 4.68 g/100 g | 0.8 | 1.4 | -[2] | 0.5 | **0.5** |
| **BCR-401R** | | | | | | |
| Nitrogen | 3.90 g/100 g | 0.9 | 1.5 | -[2] | 0.5 | **0.5** |

[1] higher value $u^*_{bb}$ or $s_{bb}$ 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 homogenous at least to this level.

# 5 Stability studies

Three stability studies were performed for BCR-385R, 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. The stability of material BCR-401R has not been assessed as its content of aflatoxins is below limits of quantification.

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 study samples were stored in the dark at 4 °C, -20 °C and for reference at -70 °C and for the 36 months long-term study, 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 -70 °C for storage until analysis. The results of both long term stability studies were combined by normalizing the individual result to the mean of the results of the zero month samples.

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 32 individual results of the relevant stability study, $x_i$ being the time point for each replicate, $\bar{x}$ 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 the 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 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 5.

**Table 5. Uncertainty contributions due to storage for BCR-385R**

| Aflatoxin | $u_{lts}$ [%] |
|---|---|
| $B_1$ | 4.8 |
| $B_2$ | 4.5 |
| $G_1$ | 7.9 |
| $G_2$ | 11.5 |

# 6 Certification

## *6.1 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 experience in the analytical field concerned.
Each laboratory was provided with the following samples:

- 3 units of *"Peanut butter (low level aflatoxins)"* BCR-385R
- 6 units of *"Peanut butter (very low level aflatoxins)"* BCR-401R
- 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-385R and BCR-401R had to be analysed in duplicate. Additionally individual recovery experiments had to be carried out on each day with the additional sets of BCR-401R.
In order to reveal the recovery rates of the participant's analytical procedures in the concentration range of the naturally contaminated candidate reference materials, the participants had to spike the "Peanut butter (very low level aflatoxins)", BCR-401R with a solution of the common calibrants to a mass fraction of each aflatoxin ($B_1$, $B_2$, $G_1$, $G_2$) of 0.5 µg/kg. No strict procedure was given on how to perform the spiking.
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 calibrations 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 6.

**Table 6. Measurement program**

| Day 1 | Day 2 | Day 3 |
|---|---|---|
| Calibration | Calibration | Calibration |
| 1 unit of BCR-385R in duplicate | 1 unit of BCR-385R in duplicate | 1 unit of BCR-385R in duplicate |
| 1 unit of BCR-401R in duplicate | 1 unit of BCR-401R in duplicate | 1 unit of BCR-401R in duplicate |
| 1 unit of BCR-401R spiked in triplicate | 1 unit of BCR-401R spiked in triplicate | 1 unit of BCR-401R spiked in triplicate |

## 6.2 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 7. Laboratory 6 did not submit any results for technical reasons.

**Table 7. 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 were eliminated. Satisfactory recovery rates for the spiking level of 0.5 µg/kg are between 50 % and 120 % [8]. Satisfactory within-laboratory reproducibility is below 50 % 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 all three days for aflatoxin $G_2$ in BCR-385R were rejected
- Lab 2: the results of one day for aflatoxin $B_1$ in BCR-385R were rejected
- Lab 3: no data was rejected
- Lab 4: the results of all three days for aflatoxins $G_1$ and $G_2$ in BCR-385R were rejected
- Lab 5: no data was rejected
- Lab 7: no data was rejected
- Lab 8: the results of all three days for aflatoxins $G_1$ and $G_2$ in BCR-385R were rejected
- Lab 9: the results of one day for aflatoxin $B_1$ in BCR-385R were rejected

In total 44 values for aflatoxin $B_1$ from 8 labs, 48 values for aflatoxin $B_2$ from 8 labs, 36 values of aflatoxins $G_1$ from 6 labs and 30 values of aflatoxins $G_2$ for from 5 labs for BCR-385R 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 plot 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 of method performance. The following datasets have been rejected:

- Lab 2 data of aflatoxins $B_2$ for BCR-385R
- Lab 5 data of aflatoxins $G_2$ for BCR-385R
- Lab 8 data of aflatoxins $B_1$ and $B_2$ for BCR-385R

**Table 8. Summary of statistical evaluation for BCR-385R**

| BCR-385R | | | | |
|---|---|---|---|---|
| Aflatoxin | $B_1$ | $B_2$ | $G_1$ | $G_2$ |
| Number of data sets | 7 | 6 | 6 | 4 |
| Number of replicate measurements | 38 | 36 | 36 | 24 |
| Mean of means [µg/kg] | 1,77 | 0,48 | 0,92 | 0,30 |
| Standard deviation [%] | 16,7 | 14,1 | 16,1 | 19,5 |
| Relative standard error [%] | 6,3 | 5,8 | 6,6 | 9,8 |
| All data sets compatible two by two? (Scheffe's test) | no | no | no | no |
| Outlying means? (Dixon test, Nalimov t-test, Grubbs test) | Lab2 Nalimov (p=0.05) | no | no | no |
| Outlying lab variances? (Cochran test) | no | no | no | no |
| Lab variances homogeneous? (Bartlett test) | yes (p=0.01) | yes | yes (p=0.01) | yes |
| Distribution of means normal? (Skewness & kurtosis, normal probability plot) | yes | yes | yes | yes |
| Variances between labs significantly different? (SNEDECOR) | yes | 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 BCR-385R are summarized in Table 8.
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 aflatoxins $B_1$ for BCR-385R lies within two standard deviations of the mean of means and is therefore not significantly different to the mean value.

Only data sets which satisfied the criteria for recovery rate, as mentioned above, were scrutinized and taken into account for the value assessment of BCR-401R (Annex C). The values of laboratory 5 were not taken into account as the limit of detection and limit of quantification were calculated on the basis that BCR-401R is a real blank and are therefore erroneous. The limits of quantification of the methods used were equal to or less than 0.2 µg/kg except for the method used by laboratory 5, where limits of quantification for aflatoxin $B_1$ and $G_1$ were higher.

Five values for aflatoxin $B_1$ were below limit of detection with the highest limit of detection of 0.09 µg/kg (Table 9). Nine values were below the limit of quantification with the highest limit of quantification of 0.2 µg/kg. Two quantified values of 0.19 and 0.16 µg/kg were found as well.

**Table 9 Results of characterisation measurements for aflatoxin $B_1$ of BCR401R**

| Aflatoxin $B_1$ mass fraction in BCR-401R [µg/kg] | | | | | |
|---|---|---|---|---|---|
| Lab code | Day 1 | Day 2 | Day 3 | LOQ [µg/kg] | LOD [µg/kg] |
| 1 | < LOQ | < LOQ | < LOD | 0.2 | 0.05 |
| 2 | < LOD | < LOD | - | 0.13 | 0.07 |
| 3 | < LOQ | 0.19 | < LOQ | 0.18 | 0.07 |
| 4 | < LOD | < LOD | < LOQ | 0.05 | 0.02 |
| 7 | < LOQ | < LOQ | < LOQ | 0.2 | 0.02 |
| 9 | - | 0.16 | < LOQ | 0.14 | 0.09 |

Although all values are below 0.2 µg/kg it can not be conclude with a confidence of 95 % that the values is below 0.2 µg/kg. The probability of the value being below 0.2 µg/kg was assessed by calculating the average and its standard deviation. The average was calculated by using the limit of detection for values below the limit of detection and the limit of quantification for values below the limit of quantification but above the limit of detection. This results in an average of 0.10 µg/kg and a standard deviation of 0.08 µg/kg, assuming normal distribution. Therefore it can be concluded that the value is below 0.2 µg/kg with a probability of 80 %.
For comparison the assumption of a rectangular distribution with range from 0 µg/kg to 0.2 µg/kg was made. The uncertainty of the rectangular distribution, expressed as half of the range divided by the square root of three (0.06 µg/kg), would result in a 90 % confidence of the level being below 0.2 µg/kg.
The assumption of a normal distribution was chosen for certification.

Values of four laboratories for aflatoxin $B_2$ are below limit of detection with the highest limit of detection at 0.03 µg/kg (Table 10). One laboratory reported values of 0.14, 0.10 and one below 0.1 µg/kg (limit of quantification). As all other laboratories report values below 0.03 µg/kg this was taken as an indication that laboratory 3 had a contamination in the analytical process. Nevertheless the two values are below 0.2 µg/kg.

**Table 10 Results of characterisation measurements for aflatoxin $B_2$ of BCR401R**

| Aflatoxin $B_2$ mass fraction in BCR-401R [µg/kg] | | | | | |
|---|---|---|---|---|---|
| Lab code | Day 1 | Day 2 | Day 3 | LOQ [µg/kg] | LOD [µg/kg] |
| 1 | < LOD | < LOD | < LOD | 0.1 | 0.03 |
| 3 | 0.10 | 0.14 | < LOQ | 0.1 | 0.03 |
| 4 | < LOD | < LOD | < LOD | 0.05 | 0.02 |
| 7 | < LOD | < LOD | < LOD | 0.2 | 0.02 |
| 9 | < LOD | < LOD | < LOD | 0.05 | 0.03 |

All values for aflatoxin $G_1$ except two were below the limits of detection (Table 11). The two values above the limit of detection were nevertheless far below the limit of quantification of 0.2 µg/kg and below the limit of detection of the other laboratories. The highest limit of detection is 0.15 µg/kg. Although one laboratory has a limit of detection above 0.2 µg/kg the value is certified as below 0.2 µg/kg based on the more sensitive methods used by the other laboratories.

**Table 11 Results of characterisation measurements for aflatoxin $G_1$ of BCR401R**

| Aflatoxin $G_1$ mass fraction in BCR-401R [µg/kg] | | | | | |
|---|---|---|---|---|---|
| Lab code | Day 1 | Day 2 | Day 3 | LOQ [µg/kg] | LOD [µg/kg] |
| 1 | < LOD | < LOD | < LOD | 0.2 | 0.05 |
| 2 | < LOD | < LOD | < LOD | 0.1 | 0.07 |
| 3 | < LOD | < LOD | < LOD | 0.18 | 0.09 |
| 7 | < LOQ | < LOQ | < LOD | 0.2 | 0.02 |
| 9 | < LOD | < LOD | < LOD | 0.24 | 0.15 |

All values for aflatoxin $G_2$ were below the limits of detection with the highest limit of detection at 0.07 µg/kg (Table 12).

**Table 12 Results of characterisation measurements for aflatoxin $G_2$ of BCR401R**

| Aflatoxin $G_2$ mass fraction in BCR-401R [µg/kg] | | | | | |
|---|---|---|---|---|---|
| Lab code | Day 1 | Day 2 | Day 3 | LOQ [µg/kg] | LOD [µg/kg] |
| 2 | < LOD | < LOD | < LOD | 0.07 | 0.04 |
| 3 | < LOD | < LOD | < LOD | 0.1 | 0.03 |
| 7 | < LOD | < LOD | < LOD | 0.2 | 0.02 |
| 9 | < LOD | < LOD | < LOD | 0.12 | 0.07 |

## 6.3 Certified values and their uncertainties

The certified values for BCR-385R 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 for BCR-401R are certified as to be with a 95 % probability less than 0.2 µg/kg except for aflatoxin $B_1$, where the probability is 80 %. This is in agreement with the set of accepted results including four results with mass fractions of less than 0.2 µg/kg and the majority of results showing mass fractions below the limits of quantification or even limit of detection of the respective methods.

The certified values are summarised in Table 13 and Table 14.

**Table 13. Certified values and their uncertainties for BCR-385R**

| BCR-385R | | | | |
|---|---|---|---|---|
| Aflatoxin | $B_1$ | $B_2$ | $G_1$ | $G_2$ |
| Certified value [µg/kg] | 1,77 | 0,48 | 0,9 | 0,30 |
| $u_{lts}$ [%] | 4,8 | 4,5 | 7,9 | 11,5 |
| $u_{bb}$ [%] | 1,8 | 1,9 | 13,2 | 12,8 |
| $u_{char}$ [%] | 6,3 | 5,8 | 6,6 | 9,8 |
| $u_{cal}$ [%] | 1,4 | 1,0 | 1,7 | 1,0 |
| $u_{CRM}$ [%] | 8,2 | 7,6 | 16,8 | 19,8 |
| $U_{CRM}$ (k=2) [%] | 16,5 | 15,3 | 33,7 | 39,7 |
| $U_{CRM}$ (k=2) [µg/kg] | 0,30 | 0,08 | 0,4 | 0,12 |

Additionally to the values given in Table 13 BCR-385R is certified for the sum of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$. The sum of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ is 3.5 ± 0.5 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 uncertainties according to:

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

**Table 14. Certified values for BCR-401R**

| BCR-401R | | | | |
|---|---|---|---|---|
| Aflatoxin | $B_1$ | $B_2$ | $G_1$ | $G_2$ |
| Certified value [µg/kg] | < 0.2 [1] | < 0.2 [2] | < 0.2 [2] | < 0.2 [2] |

[1] with a 80 % probability, the measurand content is below this level
[2] with a 95 % probability, the measurand content is below this level

# 7 Metrological traceability and commutability

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 CRMs BCR-385R and BCR-401R are traceable to the SI.

BCR-385R is prepared from naturally contaminated material. Therefore there is no reason to assume that BCR-385R would behave differently from natural samples with similar particle size.

# 8 Instructions for use

## 8.1 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.

## 8.2 Safety precautions

The usual laboratory safety precautions apply.

## 8.3 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.

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

- Calculate the absolute difference between mean measured value and the certified value ($\Delta_m$).
- 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 ($U_\Delta$) from the combined uncertainty ($u_\Delta$) 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 %.

# 9 Acknowledgements

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# Annex A. Homogeneity data

**Table A1. Results of homogeneity study for BCR385R**

| BCR-385R | | | | | | |
|---|---|---|---|---|---|---|
| | means (n=3) | | | | | mean (n=2) |
| bottle | B1 [µg/kg] | B2 [µg/kg] | G1 [µg/kg] | G2 [µg/kg] | bottle | nitrogen [g/ 100 g] |
| 30 | 1.81 | 0.56 | 0.74 | 0.22 | 20 | 4.72 |
| 82 | 1.80 | 0.56 | 0.78 | 0.26 | 57 | 4.67 |
| 143 | 1.79 | 0.50 | 0.78 | 0.26 | 124 | 4.71 |
| 176 | 1.84 | 0.57 | 1.13 | 0.49 | 157 | 4.71 |
| 238 | 1.82 | 0.58 | 0.75 | 0.27 | 215 | 4.69 |
| 289 | 1.85 | 0.51 | 1.27 | 0.39 | 261 | 4.64 |
| 340 | 1.75 | 0.53 | 0.81 | 0.25 | 318 | 4.67 |
| 397 | 1.88 | 0.62 | 0.71 | 0.23 | 353 | 4.69 |
| 432 | 1.77 | 0.57 | 0.88 | 0.28 | 408 | 4.67 |
| 478 | 1.80 | 0.56 | 0.69 | 0.31 | 468 | 4.65 |
| 529 | 1.73 | 0.51 | 0.70 | 0.19 | 518 | 4.75 |
| 597 | 1.72 | 0.55 | 0.86 | 0.34 | 564 | 4.68 |
| 646 | 1.78 | 0.59 | 0.84 | 0.30 | 618 | 4.69 |
| 678 | 1.53 | 0.46 | 0.66 | 0.20 | 664 | 4.68 |
| 741 | 1.86 | 0.62 | 0.83 | 0.26 | 709 | 4.66 |
| 790 | 1.77 | 0.55 | 0.90 | 0.29 | 776 | 4.67 |
| 843 | 1.71 | 0.55 | 0.74 | 0.23 | 819 | 4.75 |
| 894 | 1.51 | 0.47 | 0.62 | 0.21 | 859 | 4.64 |
| 945 | 1.78 | 0.57 | 0.73 | 0.23 | 922 | 4.71 |
| 981 | 1.77 | 0.54 | 0.79 | 0.26 | 967 | 4.64 |
| 1036 | 1.57 | 0.47 | 0.68 | 0.22 | 1020 | 4.68 |
| 1095 | 1.80 | 0.58 | 0.94 | 0.32 | 1066 | 4.75 |
| 1136 | 1.90 | 0.58 | 0.78 | 0.35 | 1102 | 4.77 |
| 1195 | 1.56 | 0.46 | 0.73 | 0.23 | 1153 | 4.63 |
| 1242 | 1.71 | 0.54 | 0.82 | 0.28 | 1203 | 4.67 |
| 1292 | 1.80 | 0.54 | 0.76 | 0.35 | 1254 | 4.62 |
| 1329 | 1.76 | 0.57 | 0.72 | 0.23 | 1312 | 4.67 |
| 1390 | 1.67 | 0.51 | 0.69 | 0.19 | 1377 | 4.70 |
| 1439 | 1.66 | 0.47 | 0.66 | 0.18 | 1409 | 4.69 |
| 1494 | 1.81 | 0.57 | 0.90 | 0.28 | 1466 | 4.70 |
| 1524 | 1.81 | 0.56 | 0.99 | 0.31 | 1513 | 4.67 |

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

| BCR-385R | | | | |
|---|---|---|---|---|
| | means (n=3) normalized | | | |
| bottle | B1 | B2 | G1 | G2 |
| 30 | 1.002 | 0.963 | 0.898 | 0.836 |
| 82 | 0.996 | 0.997 | 0.956 | 0.999 |
| 143 | 1.070 | 1.047 | 1.102 | 1.185 |
| 176 | 1.014 | 1.017 | 1.334 | 1.329 |
| 238 | 1.026 | 1.017 | 0.931 | 0.944 |
| 289 | 1.140 | 1.062 | 1.604 | 1.608 |
| 340 | 0.999 | 0.980 | 1.019 | 1.042 |
| 397 | 1.041 | 1.087 | 0.870 | 0.864 |
| 432 | 0.982 | 0.992 | 1.064 | 1.089 |
| 478 | 0.994 | 0.994 | 0.815 | 0.847 |
| 529 | 0.987 | 0.927 | 0.890 | 0.792 |
| 597 | 0.951 | 0.986 | 1.017 | 0.934 |
| 646 | 1.006 | 1.029 | 1.050 | 1.051 |
| 678 | 0.943 | 0.955 | 0.825 | 0.820 |
| 741 | 1.031 | 1.065 | 1.002 | 0.967 |
| 790 | 0.980 | 0.963 | 1.103 | 1.094 |
| 843 | 0.976 | 1.020 | 0.927 | 0.935 |
| 894 | 0.928 | 0.978 | 0.783 | 0.853 |
| 945 | 0.986 | 0.977 | 0.891 | 0.888 |
| 981 | 0.983 | 0.956 | 0.968 | 0.975 |
| 1036 | 0.941 | 0.979 | 0.966 | 0.992 |
| 1095 | 0.999 | 1.003 | 1.145 | 1.220 |
| 1136 | 1.048 | 1.037 | 0.928 | 0.944 |
| 1195 | 0.959 | 0.940 | 0.926 | 0.938 |
| 1242 | 0.968 | 0.954 | 1.019 | 1.004 |
| 1292 | 0.992 | 0.966 | 0.906 | 0.946 |
| 1329 | 1.005 | 1.047 | 0.912 | 0.936 |
| 1390 | 1.029 | 1.065 | 0.862 | 0.781 |
| 1439 | 0.990 | 0.974 | 0.932 | 0.823 |
| 1494 | 1.000 | 0.998 | 1.104 | 1.068 |
| 1524 | 1.033 | 1.027 | 1.252 | 1.294 |

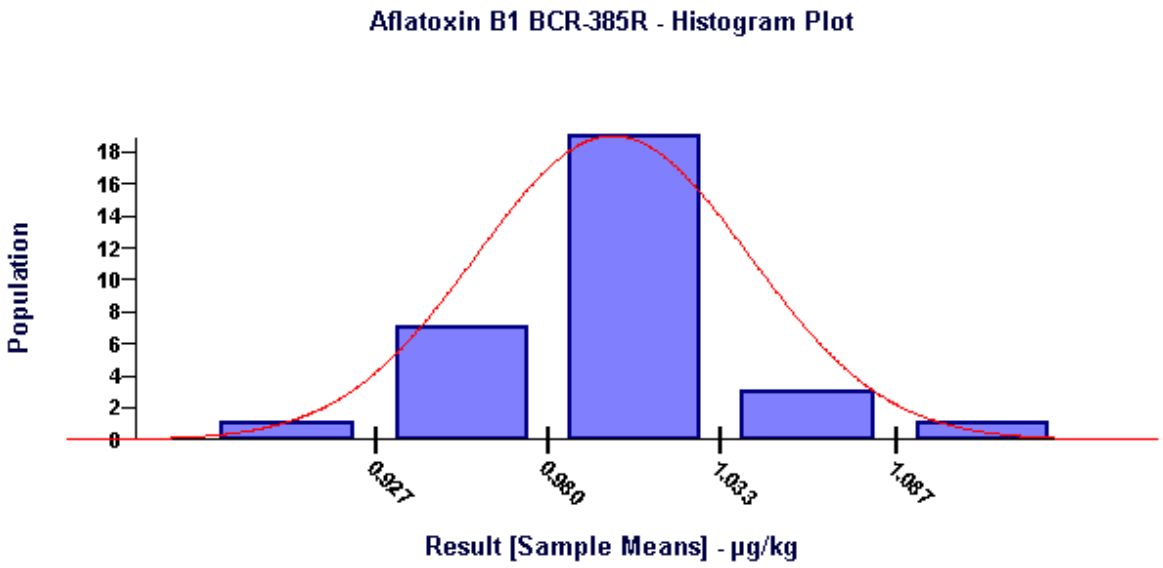


**Figure A1. Histogram of homogeneity results for aflatoxin $B_1$**

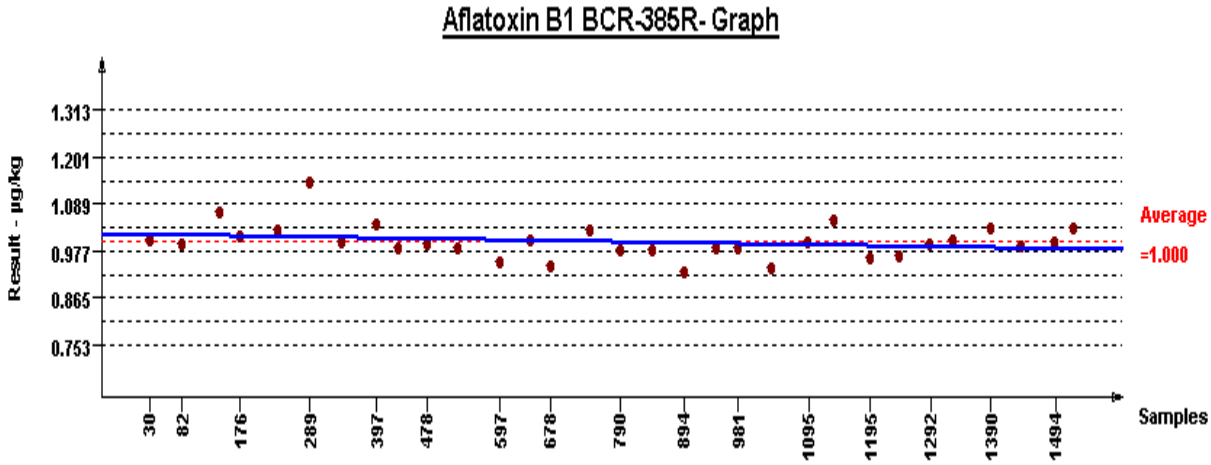


**Figure A2. Results of homogeneity study for aflatoxin $B_1$ sorted by the filling sequence**

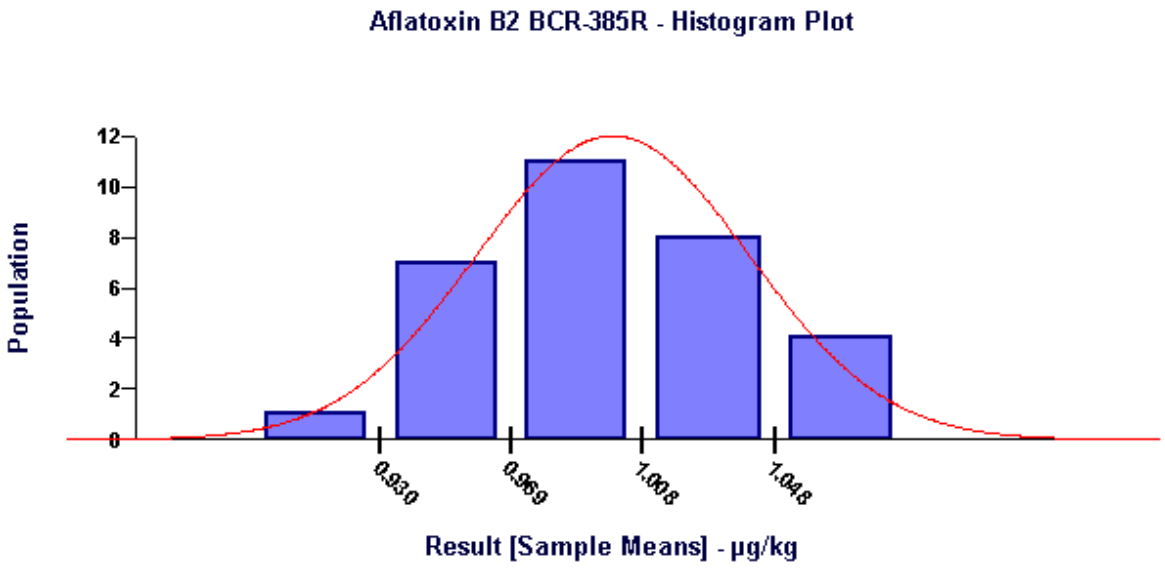


**Figure A3. Histogram of homogeneity results for aflatoxin $B_2$**

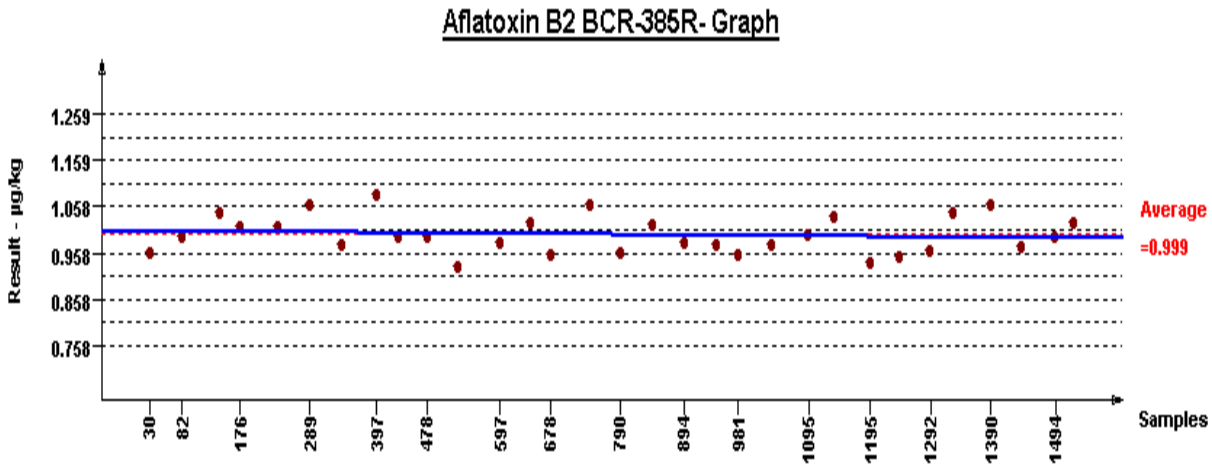


**Figure A4. Results of homogeneity study for aflatoxin $B_2$ sorted by the filling sequence**

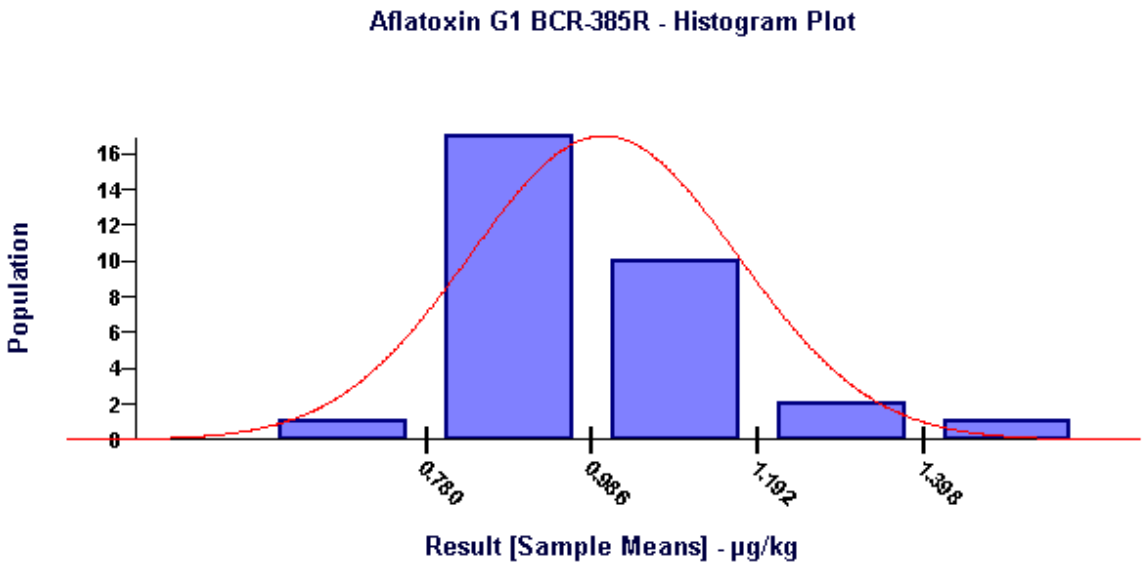


**Figure A5. Histogram of homogeneity results for aflatoxin $G_1$**

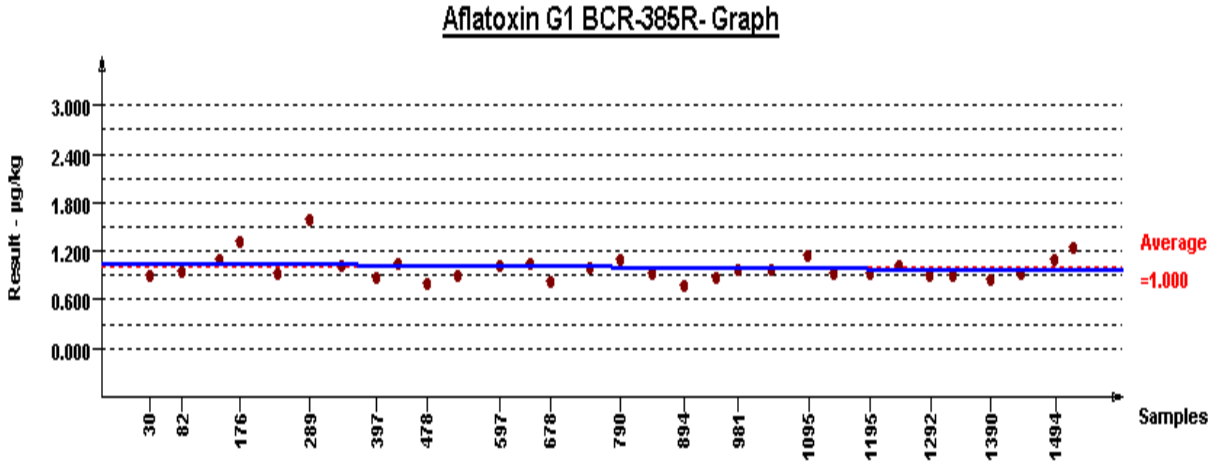


**Figure A6. Results of homogeneity study for aflatoxin $G_1$ sorted by the filling sequence**

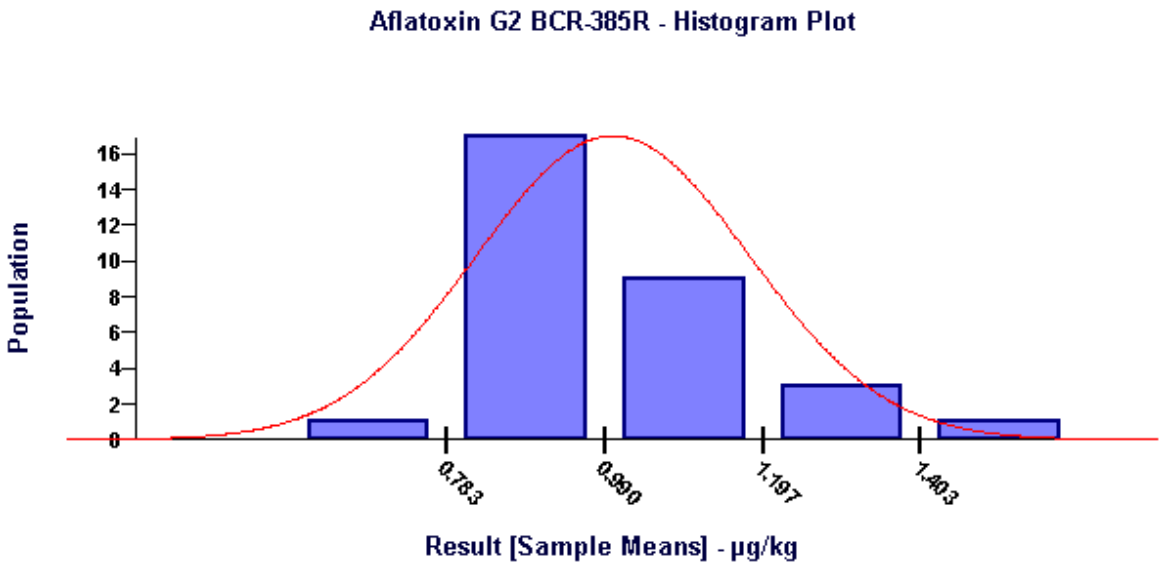


**Figure A7. Histogram of homogeneity results for aflatoxin $G_2$**

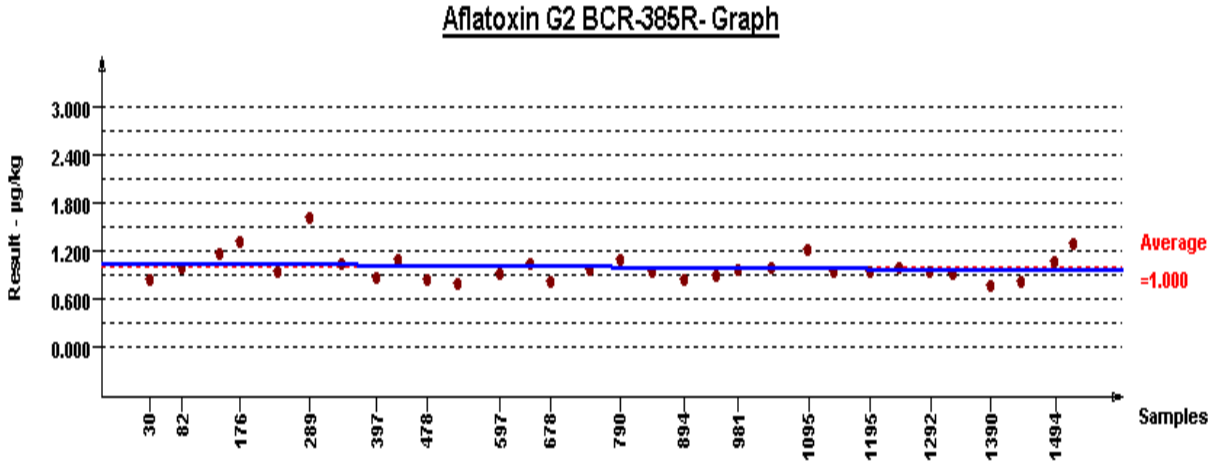


**Figure A8. Results of homogeneity study for aflatoxin $G_2$ sorted by the filling sequence**

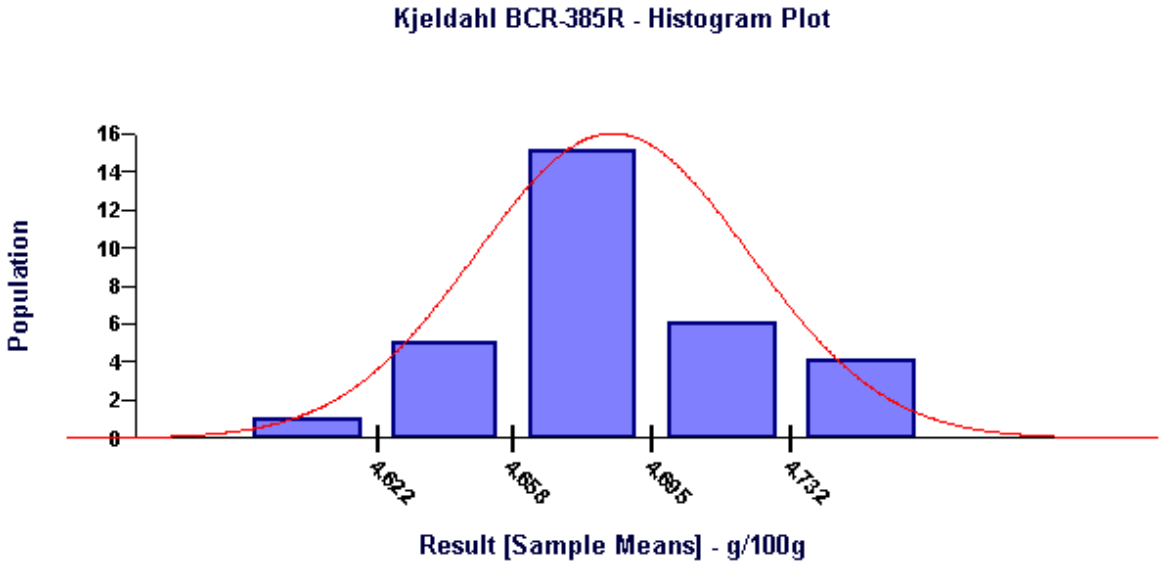


**Figure A9. Histogram of homogeneity results for nitrogen**

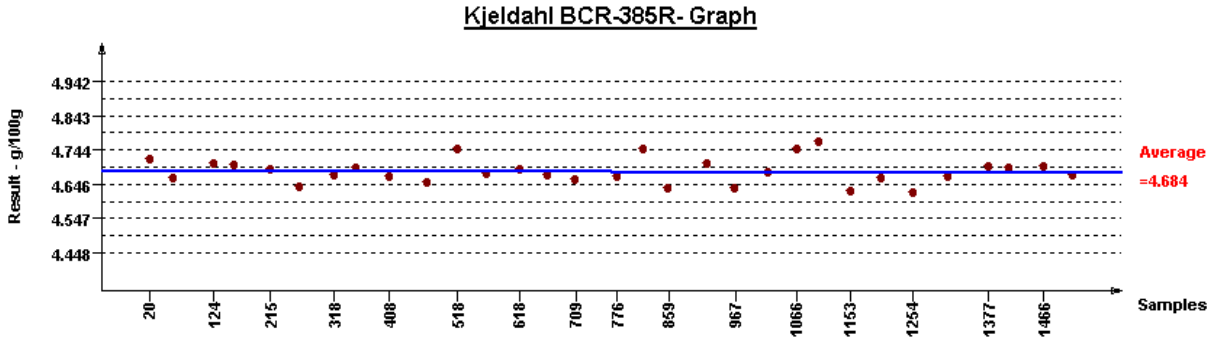


**Figure A10. Results of homogeneity study for nitrogen sorted by the filling sequence**

**Table A3. Results of homogeneity study for BCR401R**

| BCR-401R | |
|---|---|
| | mean (n=2) |
| bottle | nitrogen [g/ 100 g] |
| 4 | 3.91 |
| 74 | 3.83 |
| 146 | 3.91 |
| 191 | 3.92 |
| 236 | 3.90 |
| 275 | 3.84 |
| 309 | 3.91 |
| 355 | 3.96 |
| 422 | 3.97 |
| 469 | 3.92 |
| 507 | 3.95 |
| 584 | 3.85 |
| 669 | 3.88 |
| 692 | 3.91 |
| 737 | 3.95 |
| 834 | 3.93 |
| 904 | 3.90 |
| 975 | 3.86 |
| 1008 | 3.92 |
| 1062 | 3.87 |
| 1123 | 3.87 |
| 1175 | 3.89 |
| 1249 | 3.92 |
| 1317 | 3.88 |
| 1370 | 3.95 |
| 1432 | 3.86 |
| 1495 | 3.90 |
| 1507 | 3.87 |
| 1549 | 3.83 |
| 1570 | 3.88 |
| 1591 | 3.91 |

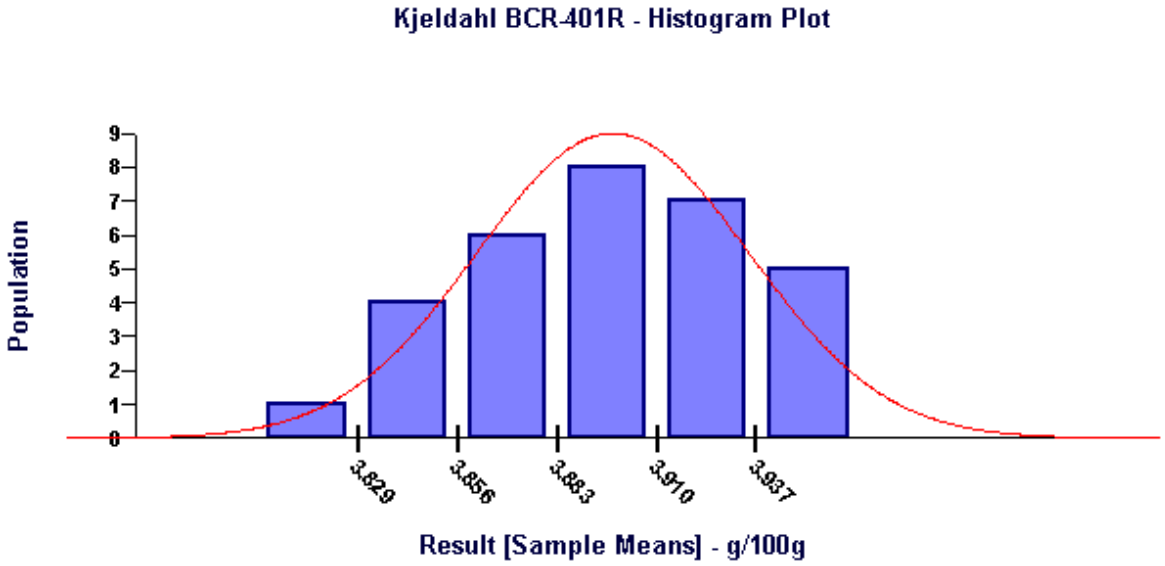


**Figure A11. Histogram of homogeneity results for nitrogen of BCR401R**

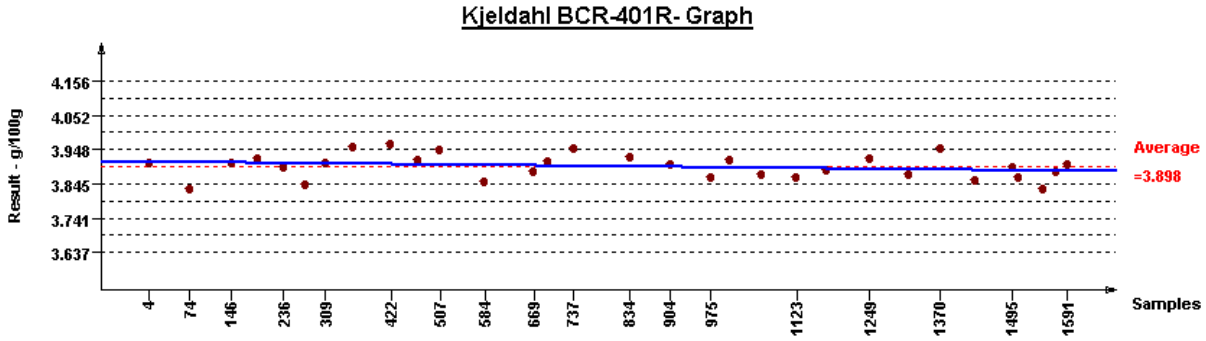


**Figure A12. Results of homogeneity study for nitrogen sorted by the filling sequence of BCR401R**

# Annex B. Stability data

**Table B1. Results of the isochronous studies for aflatoxin $B_1$**

| BCR-385R B1 [µg/kg] | | | | | | |
|---|---|---|---|---|---|---|
| | weeks at 18 °C | | | | | |
| samples | 0 | 1 | 2 | 4 | | |
| 1 | 1.71 | 1.58 | 1.72 | 1.79 | | |
| 2 | 1.59 | 1.55 | 1.30 | 1.45 | | |
| 3 | 1.59 | 1.89 | 1.69 | 1.56 | | |
| 4 | 1.57 | 1.57 | 1.37 | 1.85 | | |
| | weeks at 40 °C | | | | | |
| samples | 0 | 1 | 2 | 4 | | |
| 1 | 1.71 | 1.62 | 1.79 | 1.47 | | |
| 2 | 1.59 | 1.61 | 1.17 | 1.50 | | |
| 3 | 1.59 | 1.61 | 1.60 | 1.37 | | |
| 4 | 1.57 | 1.59 | 1.46 | 1.60 | | |
| | months at -20 °C | | | | | |
| samples | 0 | 6 | 12 | 18 | | |
| 1 | 1.68 | 1.53 | 1.54 | 1.54 | | |
| 2 | 1.54 | 1.50 | 1.63 | 1.58 | | |
| 3 | 1.55 | 1.58 | 1.15 | 1.63 | | |
| 4 | 1.97 | 1.49 | 1.55 | 1.50 | | |
| | months at 4 °C | | | | | |
| samples | 0 | 6 | 12 | 18 | | |
| 1 | | 1.33 | 1.17 | 0.84 | | |
| 2 | 1.54 | 1.25 | 1.25 | 1.46 | | |
| 3 | 1.21 | 1.01 | 1.03 | 1.5 | | |
| 4 | 1.33 | 1.39 | 1.42 | 1.39 | | |
| | months at -20 °C | | | | | |
| samples | 0 | 12 | 24 | 36 | | |
| 1 | 0.89 | 0.77 | 0.85 | 0.85 | | |
| 2 | 0.88 | 0.78 | 0.77 | 0.89 | | |
| 3 | 0.85 | 0.81 | 0.77 | 0.74 | | |
| 4 | 0.77 | 0.85 | 0.77 | 0.74 | | |
| | months at -20 °C (combined and normalised) | | | | | |
| samples | 0 | 6 | 12 | 18 | 24 | 36 |
| 1 | 1.00 | 0.91 | 0.91 | 0.91 | 1.00 | 1.00 |
| 2 | 0.91 | 0.89 | 0.97 | 0.94 | 0.91 | 1.05 |
| 3 | 0.92 | 0.94 | 0.68 | 0.97 | 0.91 | 0.87 |
| 4 | 1.17 | 0.88 | 0.92 | 0.89 | 0.91 | 0.87 |
| 5 | 1.05 | | 1.00 | | | |
| 6 | 1.04 | | 0.91 | | | |
| 7 | 1.00 | | 0.91 | | | |
| 8 | 0.91 | | 0.91 | | | |

BCR-385R Aflatoxin B1 T=18 °C

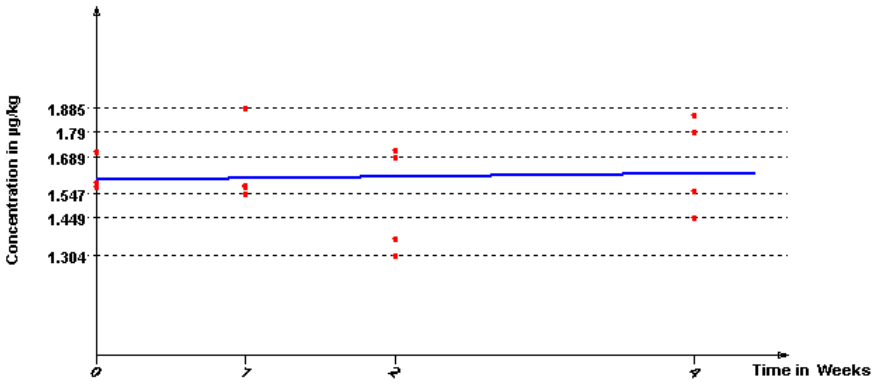


**Figure B1. Short term stability for aflatoxin $B_1$ at 18 °C**

BCR-385R Aflatoxin B1 T=40 °C

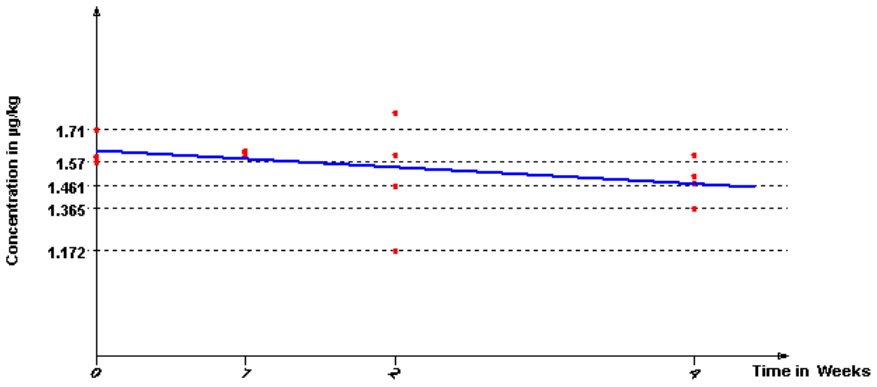


**Figure B2. Short term stability for aflatoxin $B_1$ at 40 °C**

Shelf Life and Associated Ults, T=-20 °C

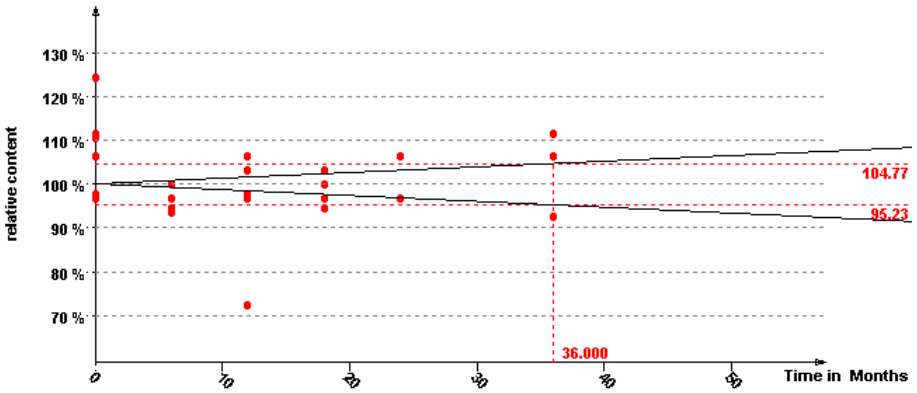


**Figure B3. Long term stability for aflatoxin $B_1$ at -20 °C with associated $u_{lts}$ for a storage period of 36 month**

**Table B2. Results of the isochronous studies for aflatoxin $B_2$**

| BCR-385R B2 [µg/kg] | | | | | | |
|---|---|---|---|---|---|---|
| | weeks at 18 °C | | | | | |
| samples | 0 | 1 | 2 | 4 | | |
| 1 | 0.49 | 0.48 | 0.53 | 0.58 | | |
| 2 | 0.43 | 0.41 | 0.43 | 0.44 | | |
| 3 | 0.47 | 0.48 | 0.51 | 0.46 | | |
| 4 | 0.44 | 0.44 | 0.45 | 0.58 | | |
| | weeks at 40 °C | | | | | |
| samples | 0 | 1 | 2 | 4 | | |
| 1 | 0.49 | 0.56 | 0.53 | 0.48 | | |
| 2 | 0.43 | 0.45 | 0.43 | 0.49 | | |
| 3 | 0.47 | 0.48 | 0.49 | 0.43 | | |
| 4 | 0.44 | 0.48 | 0.47 | 0.44 | | |
| | months at -20 °C | | | | | |
| samples | 0 | 6 | 12 | 18 | | |
| 1 | 0.45 | 0.48 | 0.53 | 0.47 | | |
| 2 | 0.45 | 0.44 | 0.47 | 0.49 | | |
| 3 | 0.46 | 0.49 | 0.42 | 0.46 | | |
| 4 | 0.59 | 0.46 | 0.47 | 0.48 | | |
| | months at 4 °C | | | | | |
| samples | 0 | 6 | 12 | 18 | | |
| 1 | | 0.24 | 0.21 | 0.14 | | |
| 2 | 0.26 | 0.3 | 0.28 | 0.28 | | |
| 3 | 0.22 | 0.12 | 0.16 | 0.29 | | |
| 4 | 0.25 | 0.24 | 0.33 | 0.29 | | |
| | months at -20 °C | | | | | |
| samples | 0 | 12 | 24 | 36 | | |
| 1 | 0.23 | 0.19 | 0.19 | 0.2 | | |
| 2 | 0.23 | 0.19 | 0.19 | 0.23 | | |
| 3 | 0.19 | 0.19 | 0.19 | 0.19 | | |
| 4 | 0.19 | 0.23 | 0.19 | 0.19 | | |
| | months at -20 °C (combined and normalised) | | | | | |
| samples | 0 | 6 | 12 | 18 | 24 | 36 |
| 1 | 0.92 | 0.99 | 1.09 | 0.96 | 0.91 | 0.95 |
| 2 | 0.92 | 0.90 | 0.96 | 1.01 | 0.91 | 1.10 |
| 3 | 0.94 | 1.01 | 0.86 | 0.94 | 0.91 | 0.91 |
| 4 | 1.21 | 0.94 | 0.96 | 0.99 | 0.91 | 0.91 |
| 5 | 1.10 | | 0.91 | | | |
| 6 | 1.10 | | 0.91 | | | |
| 7 | 0.91 | | 0.91 | | | |
| 8 | 0.91 | | 0.91 | | | |

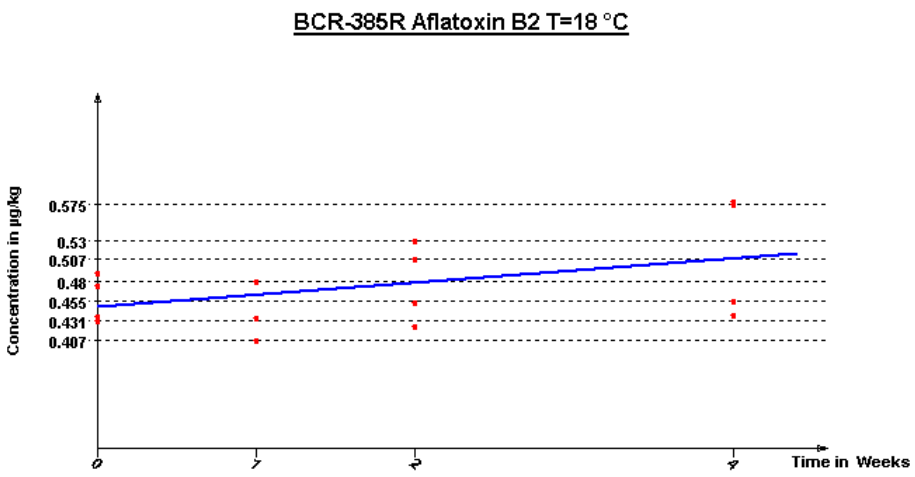


**Figure B4. Short term stability for aflatoxin $B_2$ at 18 °C**

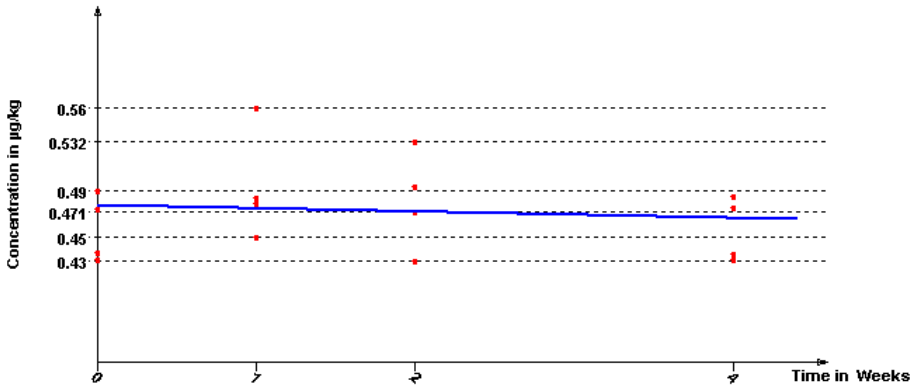


**Figure B5. Short term stability for aflatoxin $B_2$ at 40 °C**

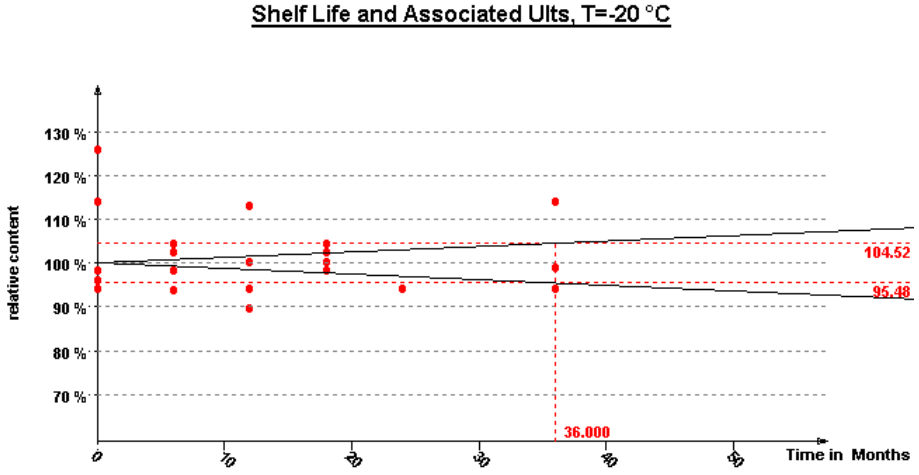


**Figure B6. Long term stability for aflatoxin $B_2$ at -20 °C with associated $u_{lts}$ for a storage period of 36 month**

**Table B3. Results of the isochronous studies for aflatoxin $G_1$**

| BCR-385R G1 [µg/kg] | | | | | | |
|---|---|---|---|---|---|---|
| | weeks at 18 °C | | | | | |
| samples | 0 | 1 | 2 | 4 | | |
| 1 | 0.68 | 0.76 | 0.70 | 0.83 | | |
| 2 | 0.65 | 0.76 | 0.59 | 0.65 | | |
| 3 | 0.68 | 0.66 | 1.03 | 0.69 | | |
| 4 | 0.95 | 0.62 | 0.57 | 1.86 | | |
| | weeks at 40 °C | | | | | |
| samples | 0 | 1 | 2 | 4 | | |
| 1 | 0.68 | 0.63 | 0.69 | 0.70 | | |
| 2 | 0.65 | 0.68 | 0.50 | 0.67 | | |
| 3 | 0.68 | 0.64 | 0.80 | 0.59 | | |
| 4 | 0.95 | 0.76 | 0.66 | 0.65 | | |
| | months at -20 °C | | | | | |
| samples | 0 | 6 | 12 | 18 | | |
| 1 | 1.24 | 0.79 | 0.79 | 0.85 | | |
| 2 | 0.86 | 0.91 | 0.80 | 0.74 | | |
| 3 | 0.75 | 0.80 | 0.93 | 0.84 | | |
| 4 | 0.99 | 0.67 | 0.87 | 0.74 | | |
| | months at 4 °C | | | | | |
| samples | 0 | 6 | 12 | 18 | | |
| 1 | | 0.77 | 0.5 | 0.37 | | |
| 2 | 0.72 | 0.55 | 0.48 | 0.59 | | |
| 3 | 0.64 | 0.35 | 0.48 | 0.88 | | |
| 4 | 0.59 | 0.51 | 0.78 | 0.83 | | |
| | months at -20 °C | | | | | |
| samples | 0 | 12 | 24 | 36 | | |
| 1 | 0.46 | 0.5 | 0.46 | 0.54 | | |
| 2 | 0.5 | 0.52 | 0.39 | 0.54 | | |
| 3 | 0.43 | 0.39 | 0.39 | 0.5 | | |
| 4 | 0.43 | 0.43 | 0.46 | 0.46 | | |
| | months at -20 °C (combined and normalised) | | | | | |
| samples | 0 | 6 | 12 | 18 | 24 | 36 |
| 1 | 1.29 | 0.82 | 0.82 | 0.89 | 1.01 | 1.19 |
| 2 | 0.90 | 0.95 | 0.83 | 0.77 | 0.86 | 1.19 |
| 3 | 0.78 | 0.83 | 0.97 | 0.88 | 0.86 | 1.10 |
| 4 | 1.03 | 0.70 | 0.91 | 0.77 | 1.01 | 1.01 |
| 5 | 1.01 | | 1.01 | | | |
| 6 | 1.10 | | 0.86 | | | |
| 7 | 0.95 | | 0.86 | | | |
| 8 | 0.95 | | 1.01 | | | |

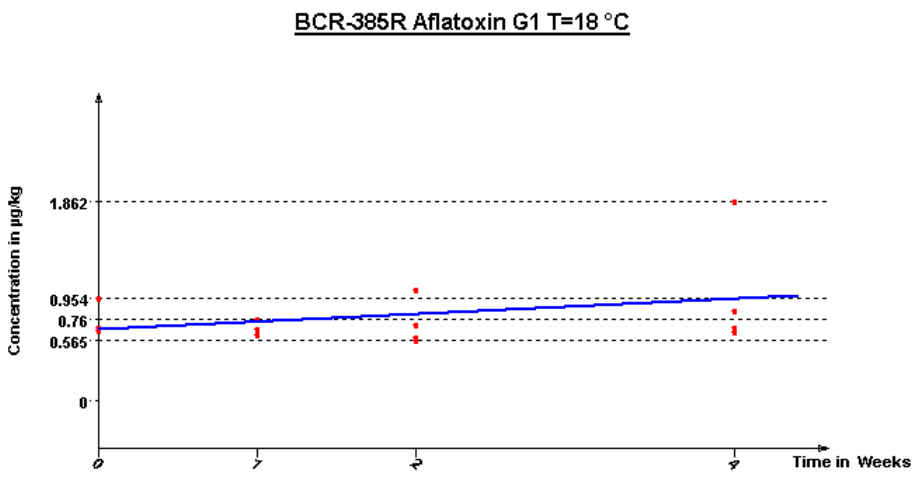


**Figure B7. Short term stability for aflatoxin $G_1$ at 18 °C**

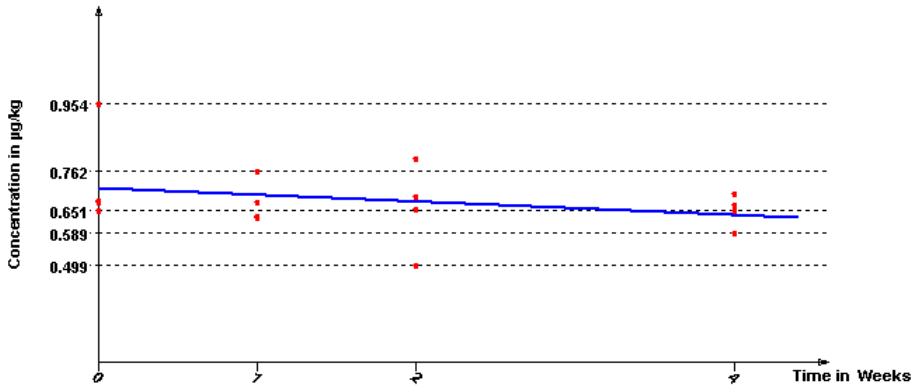


**Figure B8. Short term stability for aflatoxin $G_1$ at 40 °C**

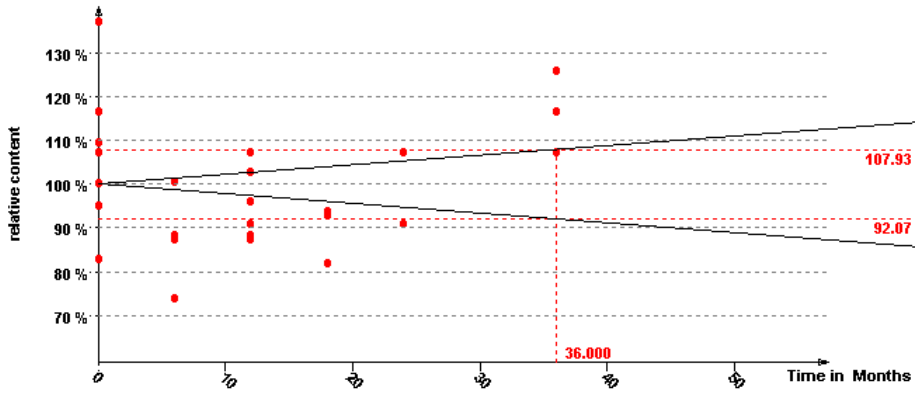


**Figure B9. Long term stability for aflatoxin $G_1$ at -20 °C with associated $u_{lts}$ for a storage period of 36 month**

**Table B4. Results of the isochronous studies for aflatoxin $G_2$**

| BCR-385R G2 [µg/kg] | | | | | | |
|---|---|---|---|---|---|---|
| | weeks at 18 °C | | | | | |
| samples | 0 | 1 | 2 | 4 | | |
| 1 | 0.24 | 0.22 | 0.19 | 0.25 | | |
| 2 | 0.20 | 0.23 | 0.19 | 0.23 | | |
| 3 | 0.21 | 0.20 | 0.36 | 0.19 | | |
| 4 | 0.18 | 0.18 | 0.22 | 0.78 | | |
| | weeks at 40 °C | | | | | |
| samples | 0 | 1 | 2 | 4 | | |
| 1 | 0.24 | 0.18 | 0.20 | 0.21 | | |
| 2 | 0.20 | 0.22 | 0.18 | 0.19 | | |
| 3 | 0.21 | 0.21 | 0.24 | 0.17 | | |
| 4 | 0.18 | 0.25 | 0.20 | 0.22 | | |
| | months at -20 °C | | | | | |
| samples | 0 | 6 | 12 | 18 | | |
| 1 | 0.44 | 0.22 | 0.23 | 0.28 | | |
| 2 | 0.26 | 0.20 | 0.25 | 0.20 | | |
| 3 | 0.18 | 0.22 | 0.31 | 0.24 | | |
| 4 | 0.37 | 0.22 | 0.25 | 0.22 | | |
| | months at 4 °C | | | | | |
| samples | 0 | 6 | 12 | 18 | | |
| 1 | | 0.17 | 0.05 | 0.03 | | |
| 2 | 0.17 | 0.08 | 0.09 | 0.12 | | |
| 3 | 0.13 | 0.07 | 0.09 | 0.25 | | |
| 4 | 0.09 | 0.07 | 0.16 | 0.23 | | |
| | months at -20 °C | | | | | |
| samples | 0 | 12 | 24 | 36 | | |
| 1 | 0.15 | 0.12 | 0.15 | 0.15 | | |
| 2 | 0.14 | 0.15 | 0.12 | 0.15 | | |
| 3 | 0.12 | 0.12 | 0.12 | 0.12 | | |
| 4 | 0.15 | 0.12 | 0.15 | 0.15 | | |
| | months at -20 °C (combined and normalised) | | | | | |
| samples | 0 | 6 | 12 | 18 | 24 | 36 |
| 1 | 1.41 | 0.70 | 0.74 | 0.90 | 1.07 | 1.07 |
| 2 | 0.83 | 0.64 | 0.80 | 0.64 | 0.86 | 1.07 |
| 3 | 0.58 | 0.70 | 0.99 | 0.77 | 0.86 | 0.86 |
| 4 | 1.18 | 0.70 | 0.80 | 0.70 | 1.07 | 1.07 |
| 5 | 1.07 | | 1.07 | | | |
| 6 | 1.00 | | 0.86 | | | |
| 7 | 0.86 | | 0.86 | | | |
| 8 | 1.07 | | 1.07 | | | |

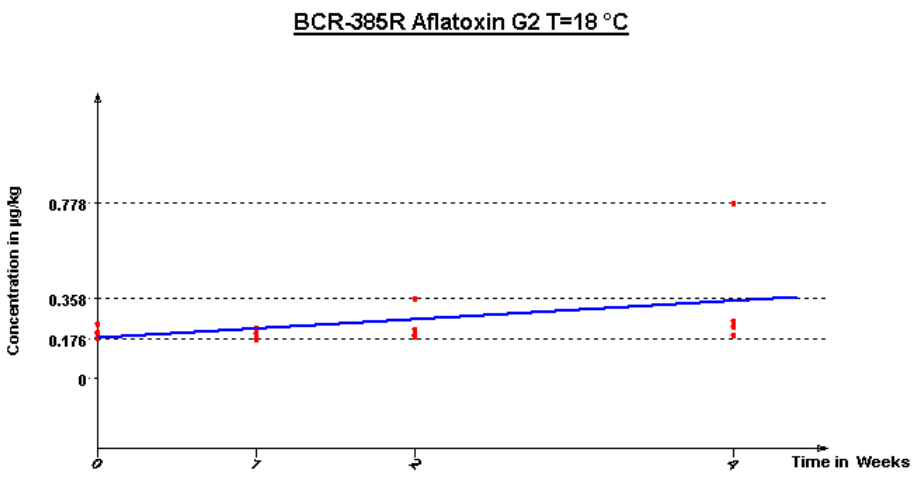


**Figure B10. Short term stability for aflatoxin $G_2$ at 18 °C**

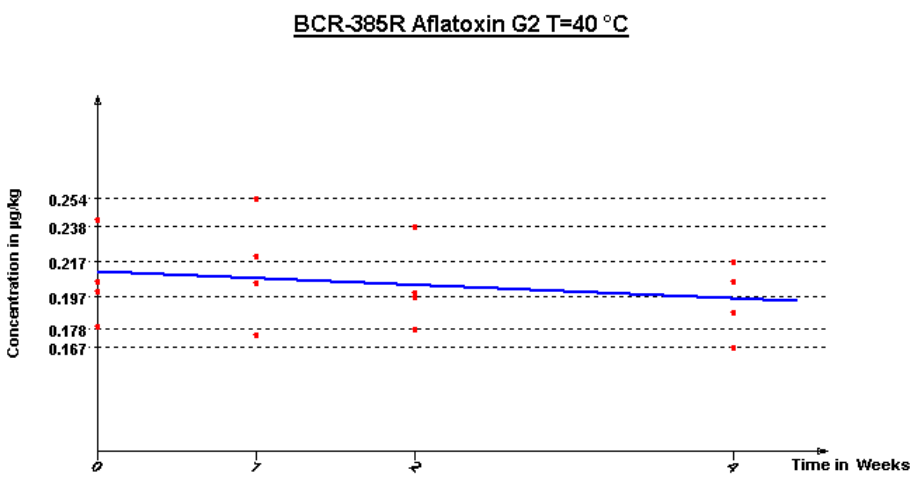


**Figure B11. Short term stability for aflatoxin $G_2$ at 40 °C**

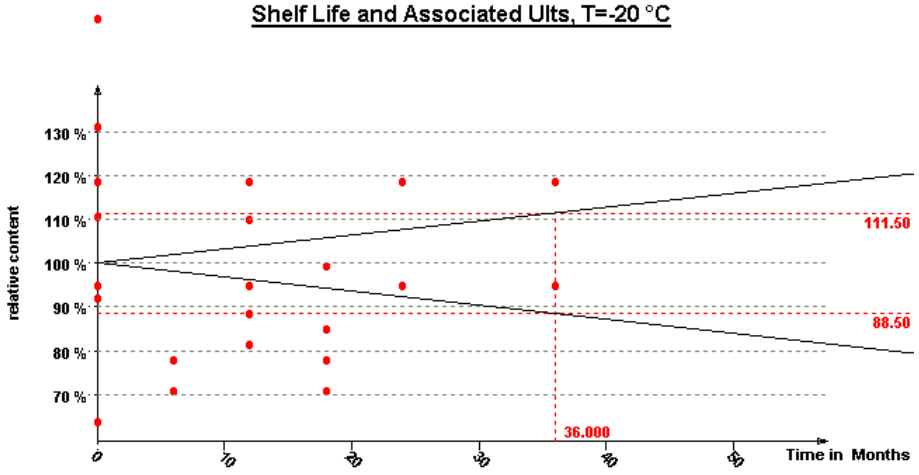


**Figure B12. Long term stability for aflatoxin $G_2$ at -20 °C with associated $u_{lts}$ for a storage period of 36 month**

# Annex C. Certification measurements

**Table C1. Results of characterisation measurements for aflatoxin $B_1$ of BCR385R**

| B1 mass fraction in BCR-385R [µg/kg] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lab code | Day 1 | | Day 2 | | Day 3 | | Mean | s |
| 1 | 1.56 | 1.60 | 1.30 | 1.29 | 1.76 | 1.39 | 1.48 | 0.19 |
| 2 | 2.24 | 2.89 | 2.05 | 2.17 | - | - | 2.34 | 0.38 |
| 3 | 1.50 | 1.51 | 2.11 | 2.11 | 1.41 | 1.42 | 1.68 | 0.34 |
| 4 | 2.10 | 1.91 | 1.80 | 1.95 | 1.72 | 1.04 | 1.75 | 0.37 |
| 5 | 1.66 | 1.69 | 1.85 | 1.86 | 1.68 | 1.95 | 1.78 | 0.12 |
| 7 | 1.83 | 2.23 | 1.48 | 1.84 | 1.90 | 1.94 | 1.87 | 0.24 |
| 9 | - | - | 1.36 | 1.50 | 1.49 | 1.46 | 1.45 | 0.06 |

No Pooling - Lab Means and their StDev for Certification BCR-385R B1

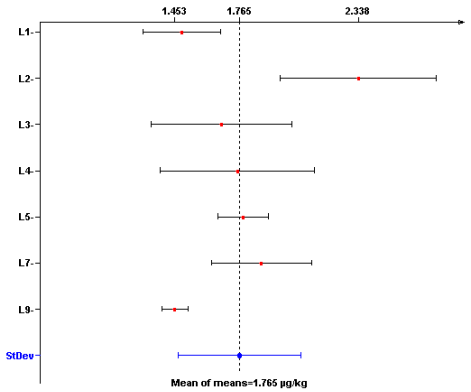


**Figure C1 Laboratory means, mean of means and their standard deviation for aflatoxin $B_1$**

**Table C2. Results of characterisation measurements for aflatoxin $B_2$ of BCR385R**

| B2 mass fraction in BCR-385R [µg/kg] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lab code | Day 1 | | Day 2 | | Day 3 | | Mean | s |
| 1 | 0.54 | 0.47 | 0.39 | 0.35 | 0.41 | 0.29 | 0.41 | 0.09 |
| 3 | 0.41 | 0.41 | 0.49 | 0.50 | 0.40 | 0.40 | 0.44 | 0.05 |
| 4 | 0.55 | 0.49 | 0.48 | 0.54 | 0.53 | 0.32 | 0.49 | 0.09 |
| 5 | 0.57 | 0.59 | 0.62 | 0.61 | 0.48 | 0.59 | 0.58 | 0.05 |
| 7 | 0.60 | 0.60 | 0.48 | 0.50 | 0.50 | 0.55 | 0.54 | 0.05 |
| 9 | 0.38 | 0.42 | 0.43 | 0.47 | 0.44 | 0.42 | 0.43 | 0.03 |

No Pooling - Lab Means and their StDev for Certification BCR-385R B2

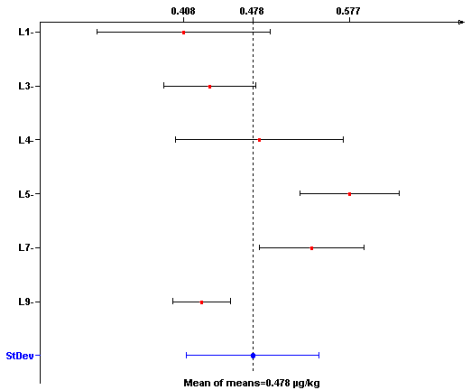


**Figure C2 Laboratory means, mean of means and their standard deviation for aflatoxin $B_2$**

**Table C3. Results of characterisation measurements for aflatoxin $G_1$ of BCR385R**

| G1 mass fraction in BCR-385R [µg/kg] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lab code | Day 1 | | Day 2 | | Day 3 | | Mean | s |
| 1 | 1.02 | 1.04 | 0.85 | 0.74 | 0.82 | 0.64 | 0.85 | 0.156 |
| 2 | 1.15 | 1.25 | 1.21 | 1.27 | 0.80 | 1.19 | 1.15 | 0.174 |
| 3 | 0.96 | 0.97 | 0.81 | 0.82 | 0.41 | 0.42 | 0.73 | 0.254 |
| 5 | 0.75 | 1.16 | 1.26 | 1.11 | 0.58 | 1.12 | 1.00 | 0.268 |
| 7 | 1.04 | 1.15 | 0.75 | 0.82 | 1.16 | 0.93 | 0.98 | 0.171 |
| 9 | 0.85 | 0.87 | 0.80 | 0.77 | 0.85 | 0.78 | 0.82 | 0.042 |

No Pooling - Lab Means and their StDev for Certification BCR-385R G1

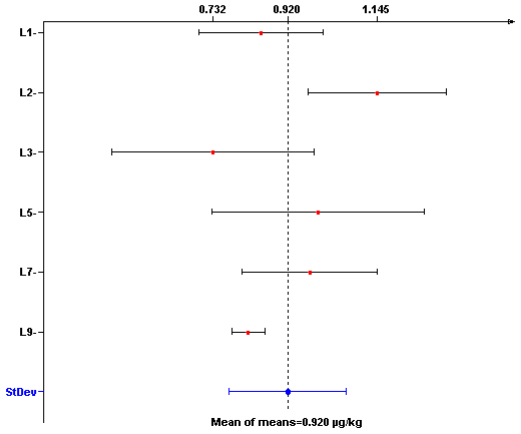


**Figure C3. Laboratory means, mean of means and their standard deviation for aflatoxin $G_1$**

**Table C4. Results of characterisation measurements for aflatoxin $G_2$ of BCR385R**

| G2 mass fraction in BCR-385R [µg/kg] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lab code | Day 1 | | Day 2 | | Day 3 | | Mean | s |
| 2 | 0.42 | 0.40 | 0.40 | 0.41 | 0.31 | 0.37 | 0.39 | 0.04 |
| 3 | 0.34 | 0.33 | 0.30 | 0.29 | 0.30 | 0.30 | 0.31 | 0.02 |
| 7 | 0.29 | 0.28 | 0.23 | 0.22 | 0.28 | 0.22 | 0.25 | 0.03 |
| 9 | 0.25 | 0.26 | 0.21 | 0.25 | 0.32 | 0.31 | 0.27 | 0.04 |

No Pooling - Lab Means and their StDev for Certification BCR-385R G2

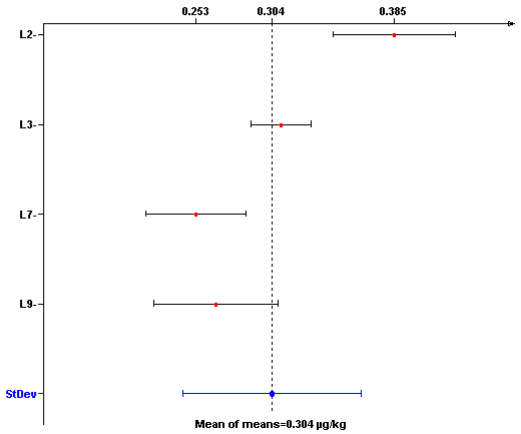


**Figure C4. Laboratory means, mean of means and their standard deviation for aflatoxin $G_2$**

**Table C5 Recovery rates for aflatoxin $B_1$**

| B1 recovery rate in spiked BCR-401R [%] | | | |
|---|---|---|---|
| Lab code | Day 1 | Day 2 | Day 3 |
| 1 | 88 | 96 | 70 |
| 2 | 100 | 113 | > 120 |
| 3 | 74 | 98 | 83 |
| 4 | 77 | 79 | 80 |
| 5 | 111 | 116 | 102 |
| 7 | 110 | 96 | 95 |
| 9 | < 50 | 114 | 108 |

**Table C6 Recovery rates for aflatoxin $B_2$**

| B2 recovery rate in spiked BCR-401R [%] | | | |
|---|---|---|---|
| Lab code | Day 1 | Day 2 | Day 3 |
| 1 | 73 | 88 | 73 |
| 3 | 88 | 105 | 87 |
| 4 | 106 | 107 | 109 |
| 5 | 88 | 98 | 104 |
| 7 | 109 | 94 | 103 |
| 9 | 102 | 93 | 92 |

**Table C7 Recovery rates for aflatoxin $G_1$**

| G1 recovery rate in spiked BCR-401R [%] | | | |
|---|---|---|---|
| Lab code | Day 1 | Day 2 | Day 3 |
| 1 | 67 | 95 | 88 |
| 2 | 83 | 95 | 103 |
| 3 | 61 | 87 | 68 |
| 5 | 82 | 66 | 109 |
| 7 | 96 | 92 | 91 |
| 9 | 103 | 94 | 102 |

**Table C8 Recovery rates for aflatoxin $G_2$**

| G2 recovery rate in spiked BCR-401R [%] | | | |
|---|---|---|---|
| Lab code | Day 1 | Day 2 | Day 3 |
| 2 | 78 | 99 | 107 |
| 3 | 78 | 95 | 70 |
| 7 | 102 | 99 | 107 |
| 9 | 110 | 93 | 50 |

European Commission

**EUR 23522 EN – Joint Research Centre – Institute for Reference Materials and Measurements**
Title: Certification of mass fractions in $B_1$, $B_2$, $G_1$, $G_2$ in peanut butter, BCR-385R and BCR-401R
Author(s): G. Buttinger, S. Harbeck, R. Josephs
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2008 – 43 pp. – 21.0 x 29.7 cm
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**Abstract**

This report describes the preparation of peanut butter (BCR-385R and BCR-401R) matrix reference materials and the certification of their content (mass fraction) of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$.

The preparation of the materials, 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], including uncertainties due to possible heterogeneity and instability. The certified values are listed below:

| BCR-385R | Certified value [1)] | Uncertainty [2)] | Number of accepted sets of results |
|---|---|---|---|
| Aflatoxin $B_1$ | 1.77 µg/kg | 0.30 µg/kg | 7 |
| Aflatoxin $B_2$ | 0.48 µg/kg | 0.08 µg/kg | 6 |
| Aflatoxin $G_1$ | 0.9 µg/kg | 0.4 µg/kg | 6 |
| Aflatoxin $G_2$ | 0.30 µg/kg | 0.12 µg/kg | 4 |
| Sum of Aflatoxin $B_1$, $B_2$, $G_1$, $G_2$ | 3.5 µg/kg | 0.5 µg/kg[3)] | |

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

| BCR-401R | Certified value | Number of accepted sets of results |
|---|---|---|
| Aflatoxin $B_1$ | < 0.2 µg/kg [1)] | 7 |
| Aflatoxin $B_2$ | < 0.2 µg/kg [2)] | 6 |
| Aflatoxin $G_1$ | < 0.2 µg/kg [2)] | 6 |
| Aflatoxin $G_2$ | < 0.2 µg/kg [2)] | 4 |

1) This value is the mass fractions based on the limits of quantification of the methods used and the highest found level of accepted results, with a 80% probability the measurand content is below this level.
2) These values are the mass fractions based on the limits of quantification of the methods used and the highest found level of accepted results, with a 95% probability the measurand content is below this level.

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