



Institute for Reference
Materials and Measurements



European Reference Materials

CERTIFICATION REPORT

**Certification of the mass fraction of
aflatoxin B1 in acetonitrile (ERM[®]-AC057)
aflatoxin B2 in acetonitrile (ERM[®]-AC058)
aflatoxins G1 in acetonitrile (ERM[®]-AC059)
and of aflatoxin G2 in acetonitrile (ERM[®]-AC060)**

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Joint Research Centre
Institute for Reference Materials and Measurements

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aflatoxin B2 in acetonitrile (ERM[®]-AC058)
aflatoxins G1 in acetonitrile (ERM[®]-AC059)
and of aflatoxin G2 in acetonitrile (ERM[®]-AC060)**

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Summary

This report describes the preparation of aflatoxin B₁, B₂, G₁ and G₂ calibrants (ERM-AC057, ERM-AC058, ERM-AC059 and ERM-AC060) and the certification of their content (mass fraction) in the solutions.

The preparation of the calibrants, the homogeneity and stability studies and the characterisation with a discussion of the results is described hereafter. Uncertainties were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [2] and include uncertainties due to the processing, purity assessment and possible instability. The certified values are listed below:

CRM Code	Certified value ¹⁾	Uncertainty ²⁾
ERM-AC057	3.79 µg/g	0.11 µg/g
ERM-AC058	3.80 µg/g	0.08 µg/g
ERM-AC059	3.78 µg/g	0.13 µg/g
ERM-AC060	3.80 µg/g	0.07 µg/g

- 1) These values are the mass fractions based on weighed amounts and purity. The values were confirmed experimentally by two laboratories
- 2) The uncertainties are the expanded uncertainties ($k = 2$) of the values defined in 1).

The certified mass fractions correspond to following mass concentrations (not certified) at 20 °C and a density for acetonitrile of 0.783 kg/dm³ [1]:

AC057	2.97 ± 0.09 µg/mL
AC058	2.98 ± 0.06 µg/mL
AC059	2.96 ± 0.10 µg/mL
AC060	2.98 ± 0.06 µg/mL

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Glossary

Δ	Difference between two measurement results
Δ_m	Difference between measured and certified value
δ	Chemical shift
ACN	Acetonitrile
AU	Arbitrary units for UV-absorbance
B1	Aflatoxin B ₁
B2	Aflatoxin B ₂
CRM	Certified reference material
DAD	Diode array detector
DSC	Differential scanning calorimeter
FAO	Food and Agriculture Organization of the United Nations
G1	Aflatoxin G ₁
G2	Aflatoxin G ₂
GUM	Guide to the Expression of Uncertainty in Measurement
HPLC-DAD	High performance liquid chromatography with diode array detection
HPLC-FLD	High performance liquid chromatography with fluorescence detection
HPLC-MS/MS	High performance liquid chromatography coupled with tandem mass spectrometer
MW	Molar mass
n	Number of replicates
NIST	National Institute of Standards and Technology (USA)
NMR	Nuclear magnetic resonance spectrometry
s	Standard deviation
SI	International Systems of Units
U	Expanded uncertainty
u	Standard uncertainty
u_{Δ}	Combined uncertainty of certified value and measured value
u_{bb}^*	Uncertainty due to the inhomogeneity that can be hidden by the method repeatability
u_{CRM}	Combined uncertainty of certified value
u_{lts}	Uncertainty of long-term stability
u_m	Uncertainty of measurement result
u_{prep}	Uncertainty deriving from preparation
u_{pur}	Uncertainty of purity assessment
UV	Ultra violet

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 and welfare. Contamination of food and feed can appear at two stages: on the field and/or during storage. Moulds infecting food on the field are different from moulds affecting food during storage and produce a different spectrum of mycotoxins.

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 2005 annual report of the rapid alert system for food and feed of the European Union [3] also shows that 40 % of the information notifications were due to mycotoxin contaminations. Around 95 % of these notifications concerned aflatoxins.

Maximum levels for certain mycotoxins have been introduced in the European Union since 1998 [4].

Aflatoxin B₁ (B1, Figure 1) [2, 3, 6 α , 9 α -Tetrahydro-4-methoxycyclopenta [c] furo [3', 2':4, 5] furo [2, 3-*h*] [l] benzopyran-1, 11-dione, CAS Nr. 1162-65-8], Aflatoxin B₂ (B2, Figure 1) [2, 3, 6 α , 8, 9, 9 α -Hexahydro-4-methoxycyclopenta [c] furo [3', 2':4, 5] furo [2, 3-*h*] [l] benzopyran-1, 11-dione, CAS Nr. 7220-81-7], Aflatoxin G₁ (G1, Figure 1) [3, 4, 7 α , 10 α -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 G₂ (G2, Figure 1) [3, 4, 7 α , 9, 10, 10 α -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].

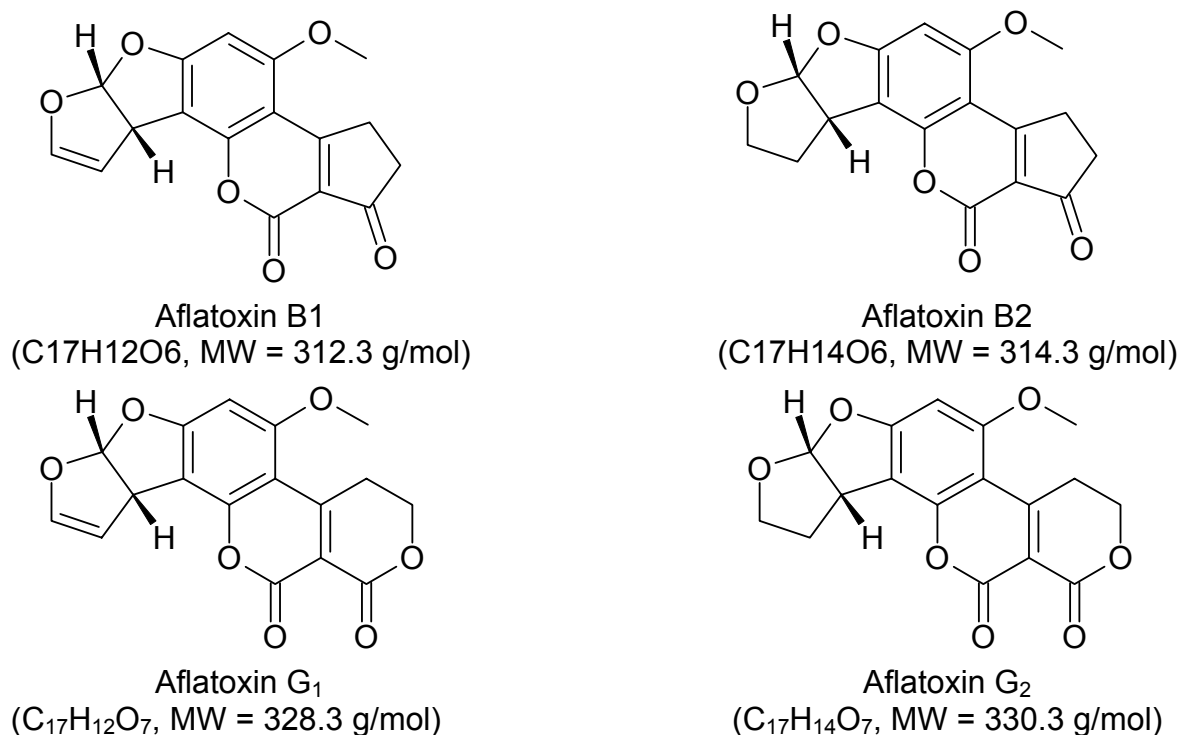


Figure 1 Molecular structure of aflatoxins

2 Participants

Project management, processing and evaluation:

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

Purity determination:

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

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

Homogeneity and stability measurements:

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

Confirmation analysis:

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

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

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

Table 1. Schedule of the project

Study	Schedule
Purity and identity	April 2003 - February 2004
Processing	March 2004 - September 2004
Homogeneity study	June 2005
Stability studies	January 2005 - May 2006
Confirmation measurements	March 2006 - May 2006

3 Production of the calibrants

3.1 Purity assessment of the starting materials

Neat crystalline aflatoxins were purchased from Sigma-Aldrich (Gillingham, United Kingdom). Sigma supplied certificates of analysis stating a purity of 99 % for B2, G1, G2 and of 98 % for B1 based on thin layer chromatography.

A multitude of methods were used to assess further the purity and the identity of the aflatoxins. Purity and identity were evaluated using ^1H and ^{13}C NMR, elemental analysis, DSC, HPLC-DAD, HPLC-MS/MS. Individual findings are summarized in brief:

- The ^1H NMR spectra of B1, B2 and G2 were in agreement with literature data. Data did not show any impurities distinguishable to signal noise. The ^1H NMR spectra of G1 agreed with literature data but showed an impurity with $\delta = 0.88$ and 1.26 ppm. This impurity could be confirmed in a ^{13}C NMR ($\delta = 29.6$ and 30.8 ppm). These signals correspond well with the signals of grease [6]. This impurity is most likely a polymeric hydrocarbon grease (e. g. Apiezon L, MW ~ 1300 g/mol). Taking the high molecular mass and the structure of this impurity into account the purity can be roughly estimated as 98.2 %.
- Results of elemental analysis were in agreement with the elemental composition of the aflatoxins.
- DSC of B2 gave a purity of 98.88 mol % ($\Delta = 0.05$ mol %, $n = 2$). DSC of G2 gave a purity of 99.10 mol % ($\Delta = 0.02$ mol %, $n = 2$). Purity assessment of B1 and G1 was not possible due to the decomposition of the samples.
- No significant impurities could be detected by HPLC-DAD and HPLC-MS/MS.

The purity of acetonitrile was assessed by HPLC-DAD. No impurity could be detected. The associated uncertainty is assumed to be negligible and was therefore not considered in any uncertainty budgets. The acetonitrile was also used for blank measurements in the purity assessments.

Conclusions:

The identity of aflatoxins was confirmed by data from NMR, elemental analysis and HPLC-MS/MS.

The impurity of B1 was estimated to be maximal 2 % as only with TLC impurities could be detected. The impurity of B2 was estimated to be around 1 % in agreement with the TLC and DSC data. The impurity of G1 was estimated to be around 2 % in agreement with the TLC and NMR data. The impurity for G2 was estimated to be around 1 % in agreement with TLC and DSC data.

The purity of B1 was assessed to 98.5 %, B2 and G2 to 99 % and G1 to 98 %. To cover the range of purity a tolerance of ± 0.5 % was chosen. The conservative assumption of a rectangular distribution was made. Dividing the tolerance by the square root of three results in an uncertainty of $u_{\text{pur}} = 0.3$ % for the purity.

The purities and their relative uncertainties are summarised in Table 2.

Table 2. Purities and their uncertainties

Toxin	purity [%]	u_{pur} [%]
B1	98.5	0.3
B2	99.0	0.3
G1	98.0	0.3
G2	99.0	0.3

3.2 Preparation and filling of the calibrants

The crystalline toxins were weighed into weighing boats and transferred with the weighing boats into volumetric flasks. In a second step the solvent was weighed. These operations were carried out at 22 ± 2 °C. The balances used were calibrated by Deutscher Kalibrierdienst (Göttingen, Germany) accredited by the Physikalisch-technischen Bundesanstalt (Braunschweig, Germany). Calibration guarantees the traceability to national standards. The masses and their uncertainties are summarized in Table 3. The uncertainties of these gravimetric preparations are obtained by combining the uncertainty of each weighing step. Therefore the square root of the sum of the squares of the relative uncertainty of each weighing step was calculated. This resulted in uncertainties of $u_{\text{prep}} = 0.07$ % for all calibrants.

Table 3. Gravimetric preparation of calibrants

	Aflatoxin	Mass [g]	u [g]	Solvent	Mass [g]	u [g]
AC057	B1	0.01500	0.00001	ACN	3895	1
AC058	B2	0.01497	0.00001	ACN	3895	1
AC059	G1	0.01502	0.00001	ACN	3896	1
AC060	G2	0.01497	0.00001	ACN	3895	1

4 mL of each calibrant were filled in amber glass ampoules flushed with inert gas. The ampoules were flame sealed and checked for leaks. Individual masses of filled ampoules were recorded and printed on the label. About 1200 ampoules were filled for each material.

3.3 Homogeneity studies

For the homogeneity study, 20 samples (~ 1.7 % of the total batch) of each calibrant were chosen using a random stratified sample picking scheme. HPLC-FLD was selected as analytical technique.

Samples were measured in a random order to allow distinction between an analytical trend and a trend in the filling sequence.

Results (Annex A Homogeneity data) were evaluated using SoftCRM [7] to detect any trends regarding filling or analysis sequence and to estimate the uncertainty contribution from possible heterogeneity. The distribution of ampoule averages was checked employing normal probability plots for normal distribution and histograms for unimodal distribution. One outlier was detected for AC057 at a 95 % level of confidence and one outlier was detected for AC059 at a 99% level of confidence using the Grubbs test. Outliers were not excluded as no technical reason was found to do so.

Conclusions:

No significant trends regarding filling sequence were detected. Ampoule averages were not always normal-distributed but followed a unimodal distribution in each case. The two outlying ampoule means were scrutinised but no technical reason for exclusion was found. A possible heterogeneity is hidden by the method repeatability. Therefore a maximum possible heterogeneity was calculated as $u_{\text{bb}}^* = 0.9$ % for AC057, $u_{\text{bb}}^* = 1.0$ % for AC058, $u_{\text{bb}}^* = 0.6$ % for AC059 and $u_{\text{bb}}^* = 0.4$ % for AC060 as described by Linsinger et al. [8].

It was concluded that the between unit heterogeneity of the four materials is negligible as the aflatoxins in acetonitrile are a true solution and it is well established that true solutions can be perfectly homogeneous.

The minimum sample intake is 100 μL . All calibrants are true solutions and the stability and homogeneity studies were performed using 100 μL of calibrant solution proved that the individual samples are homogenous at least to this level.

3.4 Stability studies

Two stability studies were performed, one 4 weeks isochronous study to evaluate stability of the materials during transport and one 12 months isochronous study to evaluate stability during storage.

For the short-term studies samples were stored in the dark at 4 °C, 18 °C, 60 °C and for reference at -30 °C. For the long-term studies samples were stored in the dark at 4 °C, -20 °C and for reference at -30 °C. Two ampoules were stored at each temperature for 0, 1, 2 and 4 weeks (January 2005 - February 2005) for the short-term study and 0, 3, 6, and 12 months (January 2005 – January 2006) for the long-term study. After the indicated periods the samples were transferred to storage at -30 °C until analysis. The reference temperature of -30 °C was chosen to avoid freezing of the acetonitrile and thermal stress to the glass ampoules.

After the final time all samples were measured together under repeatability conditions in a random order with HPLC-FLD. Samples were quantified using an external calibration and the peak area.

SoftCRM [7] was employed to test results (Annex B) for significant trends (degradation, enrichment) due to the storage conditions. The resulting slopes were tested for their significance at 95 % and 99 % level of confidence.

Uncertainties due to storage were also calculated using SoftCRM [7]. One outlier was detected for AC057 in the 18 °C short-term study and one in the -20 °C long-term study at a 99 % level of confidence. For AC058 in the 60 °C short-term study and in the 4 °C long-term study a double outlier was detected (level of confidence 95 %). One double outlier was also detected in the 60 °C short-term study of AC059 and one outlier in the 18 °C short-term study (level of confidence 95 %). One outlier was detected for AC060 in the 18 °C short-term study and one in the 4 °C short-term study at a 95 % level of confidence. Grubbs outlier test was employed. But these outliers were not excluded as after scrutinising the data no technical reason was found to do so.

Conclusions:

No significant slope at 95 % level of confidence was detected for any calibrant, 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 calibrants were stable in both long-term studies therefore 4 °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 12 months at 4 °C. u_{Its} for the calibrants are summarised in Table 4.

Table 4. Uncertainty contribution due to storage

Calibrant	u_{Its} [%]
AC057	1.4
AC058	1.0
AC059	1.7
AC060	0.9

4 Certification

The concentration of the calibrants is certified on the basis of the gravimetric preparation and the estimated purity of the base products.

Concentrations were confirmed by measuring the UV absorbance at the wavelength maximum around 355 nm and calculating the concentration employing the molar absorbance coefficient published in [9]. Therefore two laboratories measured three ampoules of each calibrant in triplicate (Annex C). The photometers employed were calibrated for wavelength and absorbance accuracy using certified filters or certified solutions.

The uncertainty of the CRM (u_{CRM}) comprises contributions from purity assessment (u_{pur}), long-term stability (u_{Its}) and the gravimetric preparation (u_{prep}). Contributions of shipment to the overall uncertainty were found to be negligible and are therefore not included in the uncertainty budget.

Conclusions:

The certified mass fractions are based on the gravimetric preparation corrected for the purity of the materials. These concentrations could be verified in independent measurements. The concentrations measured by the two laboratories were not significantly different to the concentration of the CRMs employing the formula described below (chapter 6.3). Therefore it was concluded that the certified concentration is confirmed.

The combined uncertainty (u_{CRM}) was calculated as the square root of the sum of the squares of each contribution. u_{CRM} was multiplied with a coverage factor of two ($k = 2$) to express the expanded uncertainty (U_{CRM}). U_{CRM} corresponds to a confidence interval of approximately 95 %. Certified values and contributions to the uncertainty budget are summarized in Table 5.

Table 5. Certified values and uncertainties of AC057, AC058, AC059 and AC060

	AC057	AC058	AC059	AC060
nominal value [$\mu\text{g/g}$]	3.85	3.84	3.86	3.84
purity [mass %]	98.5	99.0	98.0	99.0
certified mass fraction [$\mu\text{g/g}$]	3.79	3.80	3.78	3.80
u_{Its} [%]	1.4	1.0	1.7	0.9
u_{pur} [%]	0.3	0.3	0.3	0.3
u_{prep} [%]	0.07	0.07	0.07	0.07
u_{CRM} [%]	1.4	1.0	1.7	1.0
$U_{\text{CRM}} (k = 2)$ [%]	2.9	2.1	3.5	1.9
$U_{\text{CRM}} (k = 2)$ [$\mu\text{g/g}$]	0.11	0.08	0.13	0.07

The certified mass fractions correspond to following mass concentrations (not certified) at 20 °C and a density for acetonitrile of 0.783 kg/dm³ [1]:

AC057	$2.97 \pm 0.09 \mu\text{g/mL}$
AC058	$2.98 \pm 0.06 \mu\text{g/mL}$
AC059	$2.96 \pm 0.10 \mu\text{g/mL}$
AC060	$2.98 \pm 0.06 \mu\text{g/mL}$

5 Metrological traceability and commutability

The identities of B1, B2, G1 and G2 have been confirmed by ¹H NMR and underlined by elemental analysis and HPLC-MS/MS. Therefore the identities of B1, B2, G1 and G2 are confirmed beyond reasonable doubt.

All dilutions were performed gravimetrically on calibrated balances, and the gravimetric values traceable to SI were confirmed by UV spectrometric measurements.

Aflatoxin calibrants are normally prepared by dissolving the solid substance in acetonitrile. Therefore there is no reason to assume that AC057, AC058, AC059 and AC060 would behave differently from other commercially available calibrants or from preparations in the individual laboratory. AC057, AC058, AC059 and AC060 are interchangeable to commercially available calibrants and to preparations in the individual laboratory.

6 Instructions for use

6.1 Storage conditions

The materials should be stored at or below 4 °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.

6.2 Safety precautions

The main hazard in this formulation derives from the solvent, acetonitrile; for this the following R and S - clauses apply:

R11	Highly Flammable
R20/21/22	Harmful by inhalation, in contact with skin and if swallowed
R36	Irritating to the eyes
S16	Keep away from sources of ignition - No smoking
S36/37	Wear suitable protective clothing and gloves

6.3 Use of the certified value

The main purpose of this material is for instrument calibration (e. g. external calibration, standard addition). This material can also be used to assess own calibrants.

For assessing own calibrants, the measured values of the CRMs are compared with the certified values following a procedure described by T. P. J. Linsinger [10]. 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_m) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_m^2 + u_{CRM}^2}$
- 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 %.

7 Acknowledgements

The authors would like to thank Jacob de Boer (Institute for Environmental Studies, Vrije Universiteit Amsterdam, NL), Petra Gowik (Federal Office of Consumer Protection and Food Safety, DE) and Erik Nordkvist (National Veterinary Institute, SVA, SE) for the review of this report.

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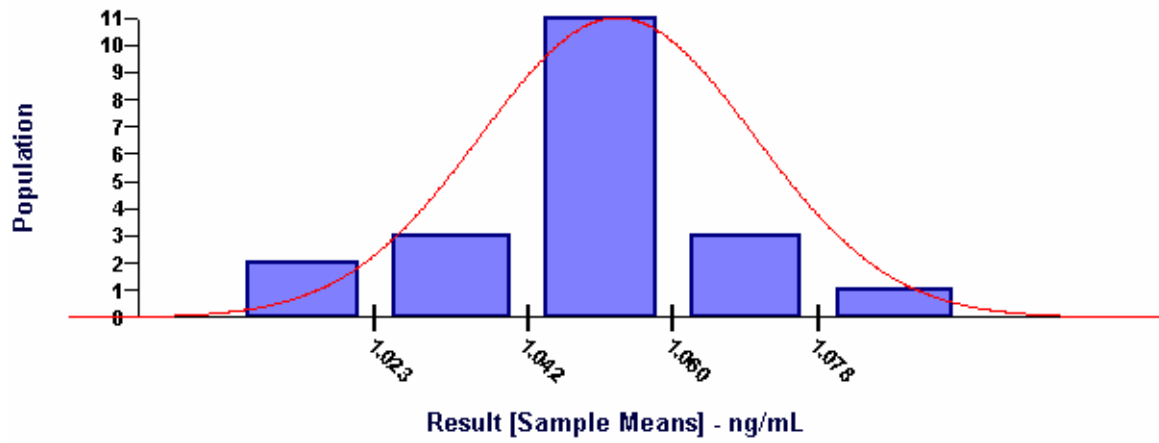
Annex A Homogeneity data

AC057:

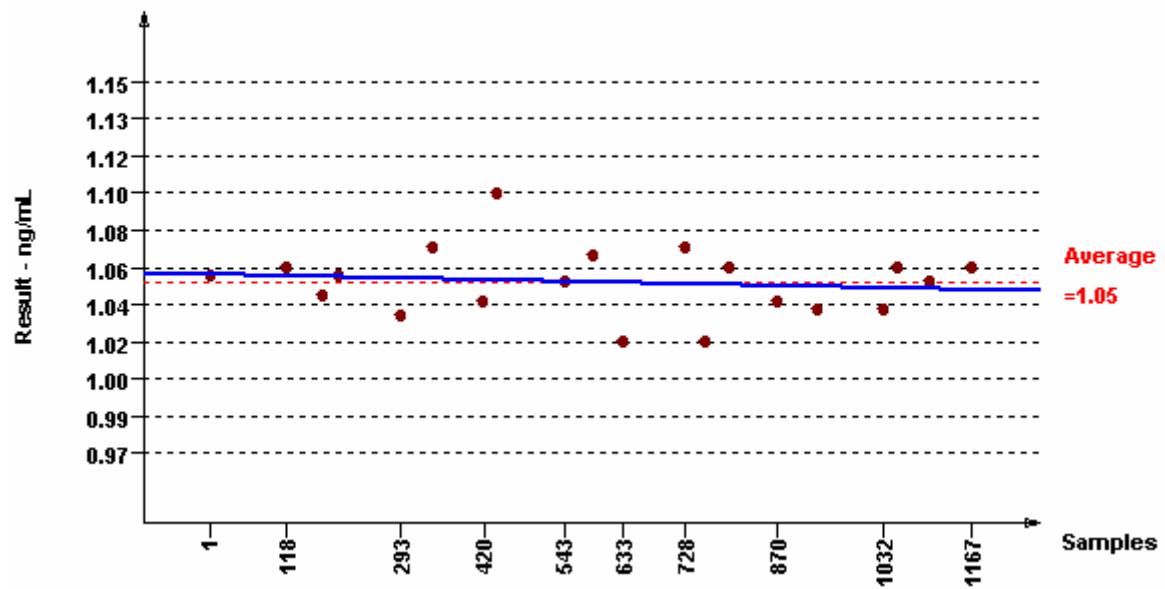
AC057 dilution			
Bottle	Replicate 1 [ng/mL]	Replicate 2 [ng/mL]	Replicate 3 [ng/mL]
1	1.01	1.08	1.08
118	1.03	1.08	1.07
174	0.99	1.07	1.08
198	1.03	1.09	1.05
293	1.00	1.05	1.06
343	1.06	1.08	1.07
420	0.98	1.08	1.07
441	1.06	1.10	1.13
543	1.00	1.09	1.07
587	1.04	1.08	1.08
633	0.97	1.07	1.03
728	1.06	1.06	1.09
760	0.99	1.05	1.03
795	1.05	1.05	1.08
870	1.00	1.07	1.06
932	1.05	1.01	1.06
1032	1.01	1.06	1.05
1053	1.03	1.09	1.06
1101	1.01	1.06	1.09

AC057			
Bottle	Replicate 1 [µg/mL]	Replicate 2 [µg/mL]	Replicate 3 [µg/mL]
1	2.52	2.69	2.70
118	2.57	2.69	2.68
174	2.47	2.67	2.70
198	2.58	2.71	2.63
293	2.50	2.63	2.64
343	2.65	2.70	2.66
420	2.45	2.70	2.66
441	2.64	2.74	2.82
543	2.49	2.72	2.67
587	2.60	2.71	2.70
633	2.43	2.66	2.58
728	2.64	2.64	2.71
760	2.47	2.63	2.57
795	2.62	2.63	2.71
870	2.50	2.68	2.66
932	2.62	2.52	2.64
1032	2.52	2.64	2.63
1053	2.59	2.73	2.66
1101	2.52	2.64	2.74
1167	2.50	2.73	2.72

Homogeneity - Histogram Plot



Homogeneity- Graph

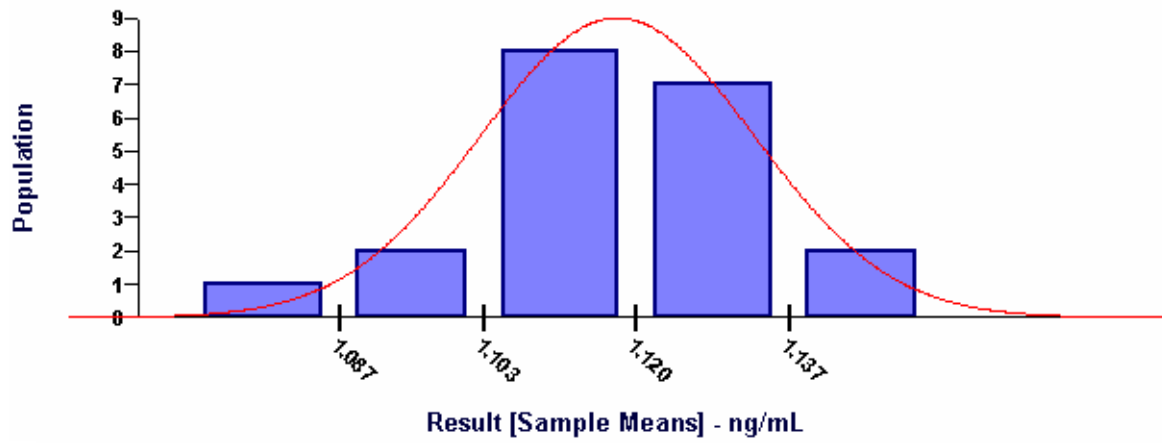


AC058:

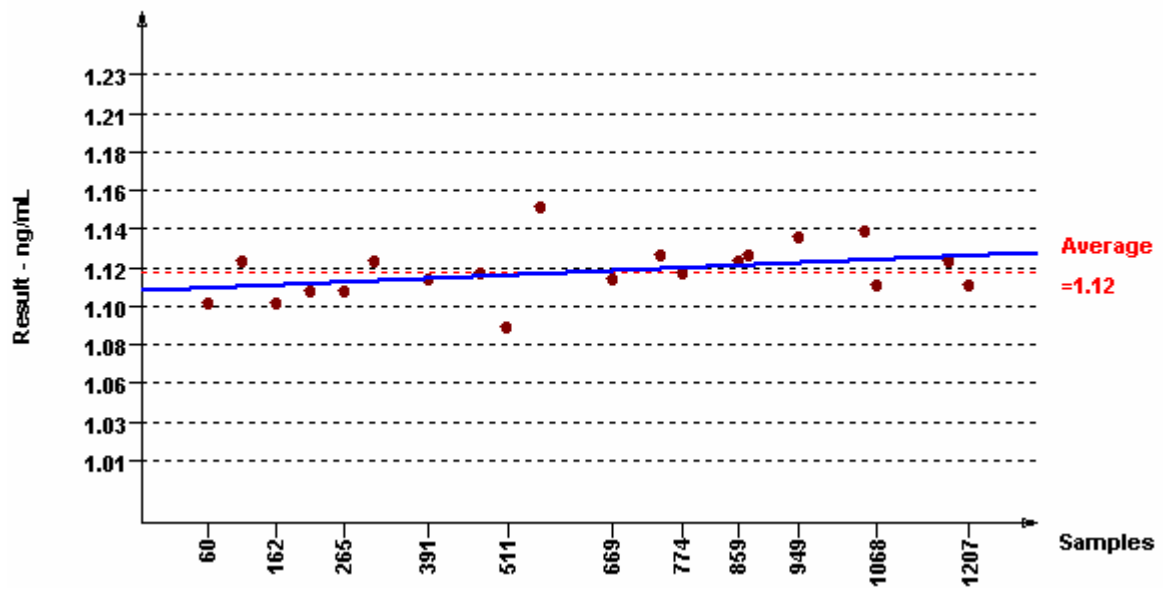
AC058 dilution			
Bottle	Replicate 1 [ng/mL]	Replicate 2 [ng/mL]	Replicate 3 [ng/mL]
60	1.08	1.13	1.09
113	1.08	1.12	1.17
162	1.07	1.12	1.11
215	1.07	1.11	1.14
265	1.08	1.13	1.11
312	1.07	1.14	1.16
391	1.08	1.13	1.13
470	1.06	1.13	1.16
511	1.04	1.11	1.11
560	1.08	1.17	1.21
669	1.10	1.14	1.10
742	1.11	1.11	1.16
774	1.10	1.12	1.13
859	1.07	1.12	1.18
874	1.12	1.15	1.11
949	1.08	1.11	1.22
1051	1.13	1.14	1.15
1068	1.09	1.09	1.15
1175	1.11	1.12	1.14
1207	1.07	1.11	1.15

AC058			
Bottle	Replicate 1 [µg/mL]	Replicate 2 [µg/mL]	Replicate 3 [µg/mL]
60	2.71	2.81	2.74
113	2.71	2.81	2.92
162	2.68	2.80	2.77
215	2.67	2.78	2.86
265	2.70	2.83	2.78
312	2.68	2.84	2.90
391	2.70	2.82	2.83
470	2.64	2.83	2.90
511	2.60	2.78	2.76
560	2.70	2.93	3.02
669	2.75	2.84	2.74
742	2.78	2.77	2.90
774	2.76	2.81	2.83
859	2.67	2.81	2.94
874	2.81	2.87	2.78
949	2.69	2.76	3.05
1051	2.82	2.85	2.86
1068	2.72	2.73	2.87
1175	2.77	2.79	2.85
1207	2.67	2.78	2.86

Homogeneity - Histogram Plot



Homogeneity- Graph

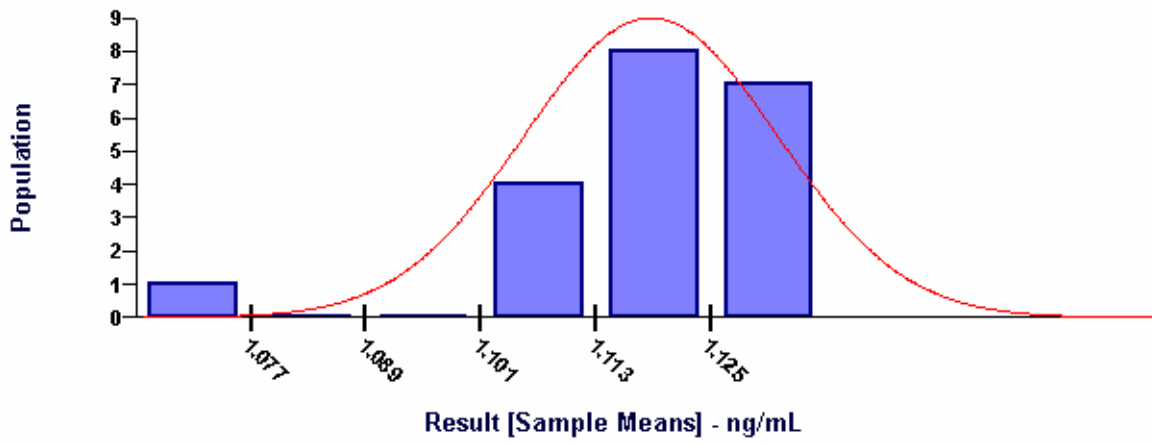


AC059:

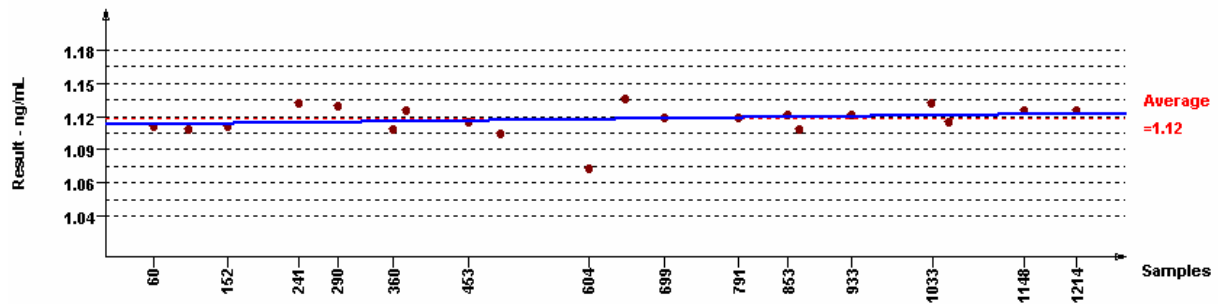
AC059 dilution			
Bottle	Replicate 1 [ng/mL]	Replicate 2 [ng/mL]	Replicate 3 [ng/mL]
60	1.12	1.14	1.08
104	1.07	1.13	1.13
152	1.12	1.14	1.08
241	1.10	1.18	1.12
290	1.13	1.14	1.12
360	1.09	1.13	1.11
376	1.14	1.12	1.12
453	1.10	1.12	1.13
494	1.14	1.11	1.07
604	1.07	1.12	1.04
650	1.15	1.14	1.12
699	1.09	1.14	1.13
791	1.14	1.12	1.10
853	1.10	1.14	1.13
868	1.12	1.11	1.10
933	1.11	1.16	1.10
1033	1.15	1.12	1.13
1054	1.10	1.13	1.12
1148	1.15	1.14	1.09
1214	1.12	1.12	1.14

AC059			
Bottle	Replicate 1 [µg/mL]	Replicate 2 [µg/mL]	Replicate 3 [µg/mL]
60	2.80	2.85	2.70
104	2.68	2.82	2.82
152	2.81	2.86	2.70
241	2.76	2.96	2.81
290	2.82	2.85	2.80
360	2.72	2.84	2.77
376	2.85	2.79	2.80
453	2.74	2.80	2.82
494	2.86	2.77	2.66
604	2.68	2.79	2.60
650	2.88	2.84	2.81
699	2.73	2.84	2.82
791	2.85	2.79	2.75
853	2.75	2.84	2.82
868	2.80	2.78	2.74
933	2.77	2.90	2.74
1033	2.88	2.80	2.83
1054	2.74	2.83	2.80
1148	2.87	2.86	2.74
1214	2.79	2.81	2.84

Homogeneity - Histogram Plot



Homogeneity- Graph

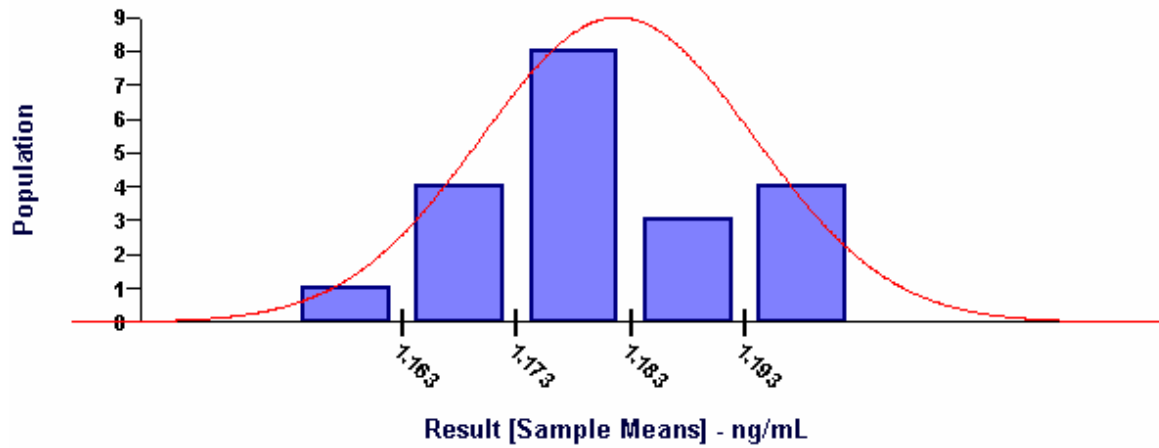


AC060:

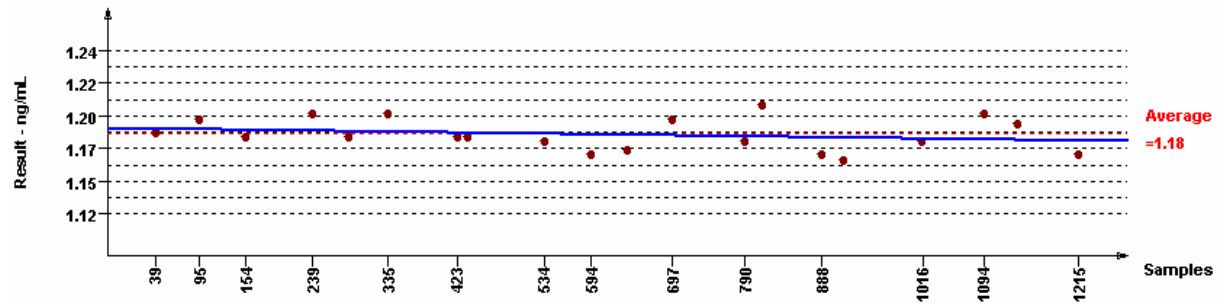
AC060 dilution			
Bottle	Replicate 1 [ng/mL]	Replicate 2 [ng/mL]	Replicate 3 [ng/mL]
39	1.19	1.19	1.17
95	1.18	1.22	1.18
154	1.17	1.18	1.19
239	1.18	1.23	1.18
285	1.18	1.18	1.18
335	1.18	1.22	1.19
423	1.18	1.19	1.17
436	1.18	1.18	1.18
534	1.16	1.19	1.18
594	1.18	1.14	1.18
640	1.17	1.18	1.16
697	1.20	1.23	1.15
790	1.17	1.19	1.17
812	1.20	1.21	1.20
888	1.17	1.18	1.15
916	1.16	1.18	1.15
1016	1.18	1.20	1.15
1094	1.17	1.19	1.23
1138	1.19	1.20	1.18
1215	1.16	1.18	1.16

AC060			
Bottle	Replicate 1 [µg/mL]	Replicate 2 [µg/mL]	Replicate 3 [µg/mL]
39	2.98	2.96	2.93
95	2.95	3.04	2.94
154	2.92	2.94	2.98
239	2.96	3.06	2.96
285	2.95	2.94	2.96
335	2.96	3.04	2.97
423	2.95	2.99	2.91
436	2.94	2.95	2.94
534	2.89	2.98	2.96
594	2.95	2.86	2.94
640	2.93	2.94	2.91
697	2.99	3.07	2.87
790	2.93	2.97	2.92
812	3.00	3.01	3.00
888	2.93	2.94	2.87
916	2.91	2.95	2.87
1016	2.95	3.01	2.88
1094	2.93	2.98	3.07
1138	2.97	3.01	2.96
1215	2.89	2.95	2.91

Homogeneity - Histogram Plot



Homogeneity- Graph



Annex B Stability data

Short-term stability study (results of dilutions)

AC057 dilution			
time [week]	4 °C [ng/mL]	18 °C [ng/mL]	60 °C [ng/mL]
0	1.17	1.17	1.17
0	1.11	1.11	1.11
0	1.06	1.06	1.06
0	1.05	1.05	1.05
1	1.09	1.05	1.05
1	1.13	1.05	1.07
1	1.10	1.04	1.05
1	1.06	1.04	1.14
2	1.05	1.08	1.03
2	1.07	1.12	1.07
2	1.04	1.06	1.08
2	1.04	1.06	1.13
4	1.08	1.05	1.02
4	1.06	1.06	1.06
4	1.11	1.36	1.04
4	1.13	1.13	1.09

AC058 dilution			
time [week]	4 °C [ng/mL]	18 °C [ng/mL]	60 °C [ng/mL]
0	1.13	1.13	1.13
0	1.15	1.15	1.15
0	1.14	1.14	1.14
0	1.14	1.14	1.14
1	1.14	1.15	1.14
1	1.13	1.14	1.14
1	1.12	1.13	1.13
1	1.14	1.14	1.17
2	1.15	1.15	1.14
2	1.14	1.16	1.17
2	1.15	1.14	1.08
2	1.14	1.14	1.11
4	1.14	1.13	1.14
4	1.13	1.12	1.13
4	1.12	1.11	1.10
4	1.14	1.16	1.14

AC059 dilution			
time [week]	4 °C [ng/mL]	18 °C [ng/mL]	60 °C [ng/mL]
0	1.05	1.05	1.05
0	1.12	1.12	1.12
0	1.13	1.13	1.13
0	1.10	1.10	1.10
1	1.09	1.11	1.10
1	1.12	1.14	1.15
1	1.15	1.13	1.15
1	1.15	1.12	1.09
2	1.09	1.11	1.10
2	1.12	1.11	1.10
2	1.12	1.12	1.04
2	1.20	1.11	1.11
4	1.08	1.11	1.10
4	1.09	1.14	1.11
4	1.07	1.07	1.10
4	1.13	1.13	1.09

AC060 dilution			
time [week]	4 °C [ng/mL]	18 °C [ng/mL]	60 °C [ng/mL]
0	1.13	1.13	1.13
0	1.11	1.11	1.11
0	1.15	1.15	1.15
0	1.13	1.13	1.13
1	1.19	1.14	1.12
1	1.14	1.13	1.15
1	1.15	1.14	1.16
1	1.13	1.11	1.14
2	1.17	1.16	1.15
2	1.13	1.17	1.18
2	1.12	1.10	1.14
2	1.14	1.13	1.14
4	1.13	1.14	1.15
4	1.13	1.11	1.12
4	1.14	1.14	1.15
4	1.14	1.21	1.16

Short-term stability study

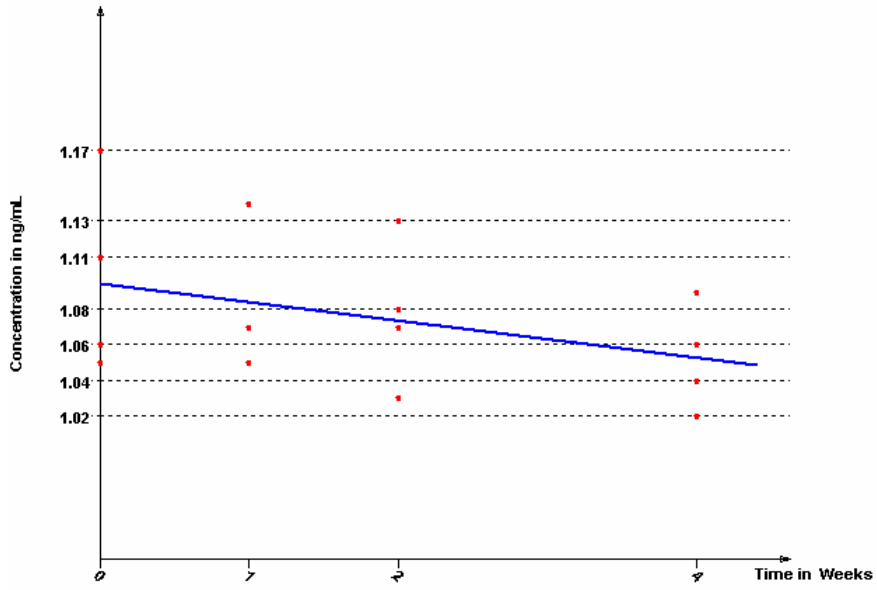
AC057			
time [week]	4 °C [µg/mL]	18 °C [µg/mL]	60 °C [µg/mL]
0	2.93	2.93	2.93
0	2.78	2.78	2.78
0	2.65	2.65	2.65
0	2.62	2.62	2.62
1	2.72	2.62	2.63
1	2.82	2.61	2.67
1	2.75	2.61	2.62
1	2.66	2.59	2.86
2	2.61	2.71	2.58
2	2.67	2.79	2.67
2	2.59	2.66	2.71
2	2.60	2.65	2.83
4	2.71	2.63	2.54
4	2.66	2.65	2.66
4	2.78	3.39	2.60
4	2.83	2.82	2.73

AC058			
time [week]	4 °C [µg/mL]	18 °C [µg/mL]	60 °C [µg/mL]
0	2.83	2.83	2.83
0	2.88	2.88	2.88
0	2.85	2.85	2.85
0	2.85	2.85	2.85
1	2.86	2.86	2.84
1	2.83	2.85	2.85
1	2.80	2.84	2.82
1	2.86	2.84	2.93
2	2.88	2.88	2.85
2	2.85	2.89	2.93
2	2.87	2.85	2.70
2	2.85	2.86	2.77
4	2.86	2.81	2.85
4	2.83	2.81	2.83
4	2.81	2.78	2.76
4	2.84	2.91	2.86

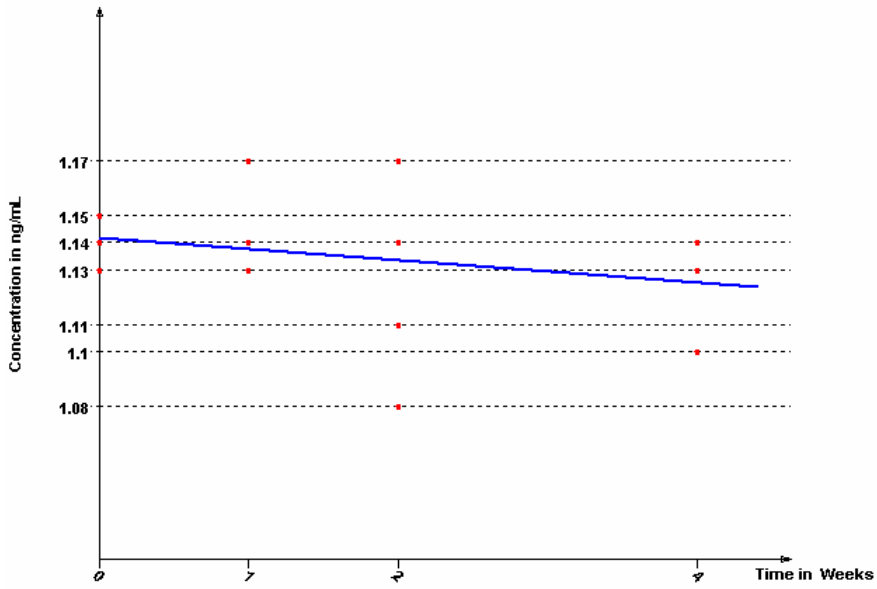
AC059			
time [week]	4 °C [µg/mL]	18 °C [µg/mL]	60 °C [µg/mL]
0	2.64	2.64	2.64
0	2.80	2.80	2.80
0	2.83	2.83	2.83
0	2.74	2.74	2.74
1	2.72	2.77	2.76
1	2.80	2.85	2.88
1	2.87	2.82	2.87
1	2.89	2.80	2.73
2	2.71	2.78	2.76
2	2.79	2.77	2.76
2	2.79	2.80	2.61
2	3.01	2.78	2.78
4	2.69	2.78	2.76
4	2.73	2.84	2.78
4	2.68	2.68	2.75
4	2.82	2.83	2.73

AC060			
time [week]	4 °C [µg/mL]	18 °C [µg/mL]	60 °C [µg/mL]
0	2.82	2.82	2.82
0	2.77	2.77	2.77
0	2.86	2.86	2.86
0	2.82	2.82	2.82
1	2.99	2.84	2.81
1	2.85	2.83	2.88
1	2.88	2.86	2.90
1	2.83	2.77	2.85
2	2.92	2.90	2.87
2	2.83	2.91	2.94
2	2.80	2.75	2.84
2	2.86	2.83	2.85
4	2.84	2.86	2.86
4	2.84	2.78	2.79
4	2.85	2.84	2.86
4	2.86	3.04	2.91

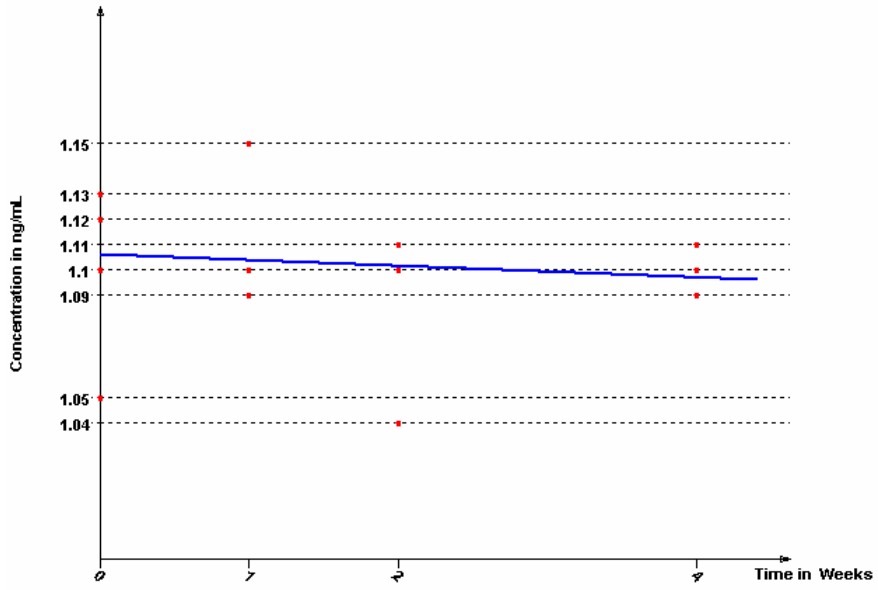
STS for ERM-AC057 at T = 60 °C



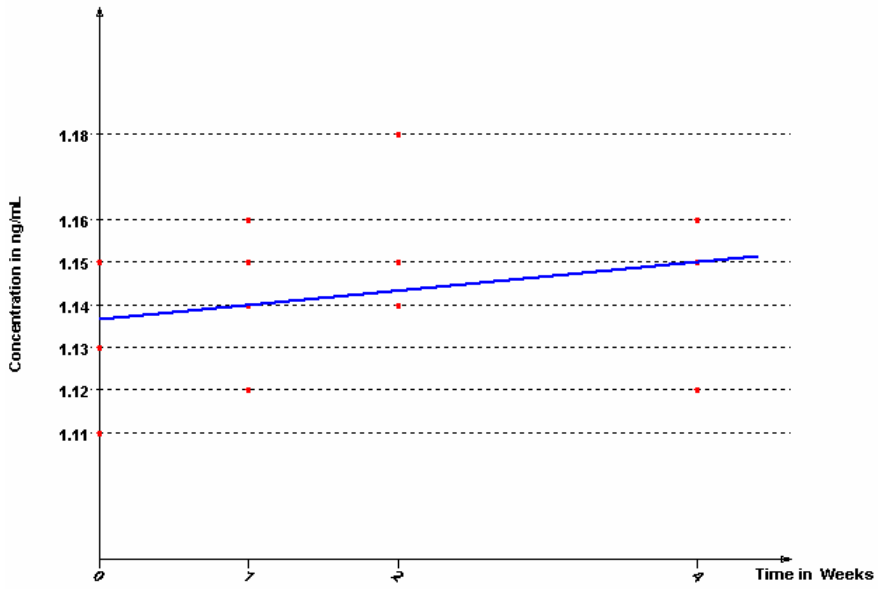
STS for ERM-AC058 at T= 60 °C



STS for ERM-AC059 at T= 60 °C



STS for ERM-AC060 at T= 60 °C



Long-term stability study (results of dilutions 1:1000)

AC057		
time [month]	4 °C [ng/mL]	-20 °C [ng/mL]
0	2.53	2.53
0	2.66	2.66
0	2.72	2.72
0	2.69	2.69
4	2.63	2.67
4	2.60	2.69
4	2.56	2.60
4	2.64	2.66
8	2.59	2.67
8	2.72	2.68
8	2.65	2.62
8	2.65	2.72
12	2.66	2.44
12	2.69	2.74
12	2.69	2.64
12	2.69	2.69

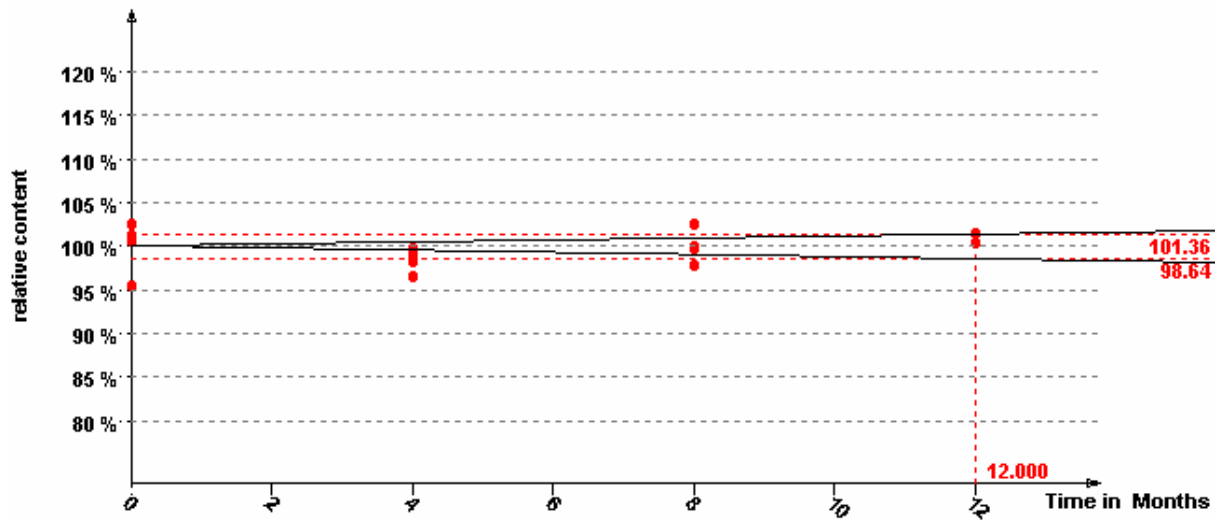
AC058		
time [month]	4 °C [ng/mL]	-20 °C [ng/mL]
0	2.39	2.39
0	2.30	2.30
0	2.32	2.32
0	2.33	2.33
4	2.32	2.34
4	2.33	2.29
4	2.26	2.36
4	2.32	2.34
8	2.35	2.28
8	2.34	2.36
8	2.33	2.30
8	2.35	2.35
12	2.33	2.37
12	2.31	2.38
12	2.38	2.37
12	2.25	2.36

AC059		
time [month]	4 °C [ng/mL]	-20 °C [ng/mL]
0	2.73	2.73
0	2.50	2.50
0	2.54	2.54
0	2.59	2.59
4	2.72	2.66
4	2.62	2.56
4	2.64	2.60
4	2.68	2.61
8	2.64	2.66
8	2.68	2.61
8	2.51	2.59
8	2.68	2.68
12	2.68	2.70
12	2.64	2.71
12	2.61	2.54
12	2.61	2.55

AC060		
time [month]	4 °C [ng/mL]	-20 °C [ng/mL]
0	2.68	2.68
0	2.71	2.71
0	2.63	2.63
0	2.69	2.69
4	2.63	2.68
4	2.72	2.67
4	2.63	2.65
4	2.70	2.66
8	2.66	2.71
8	2.66	2.72
8	2.66	2.64
8	2.69	2.65
12	2.73	2.64
12	2.62	2.71
12	2.71	2.66
12	2.64	2.64

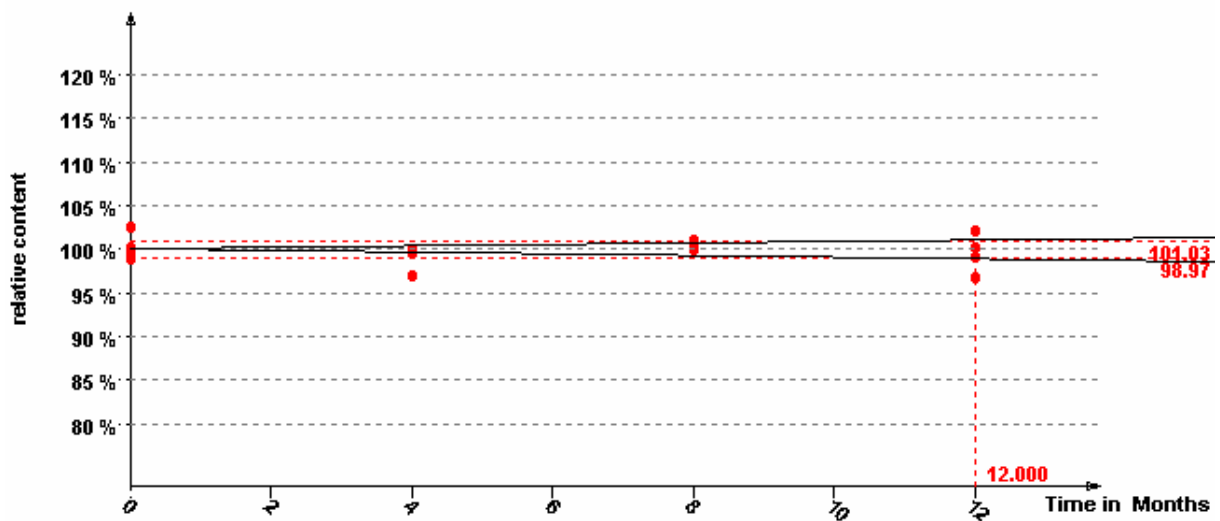
AC057:

Shelf Life and Associated ULts, T=4 °C(Ref)



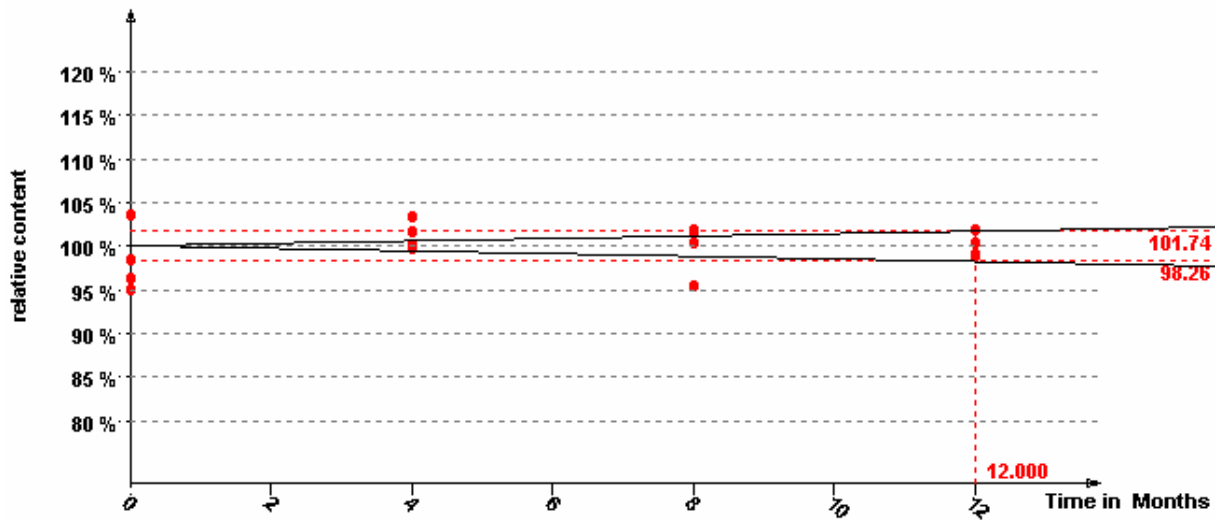
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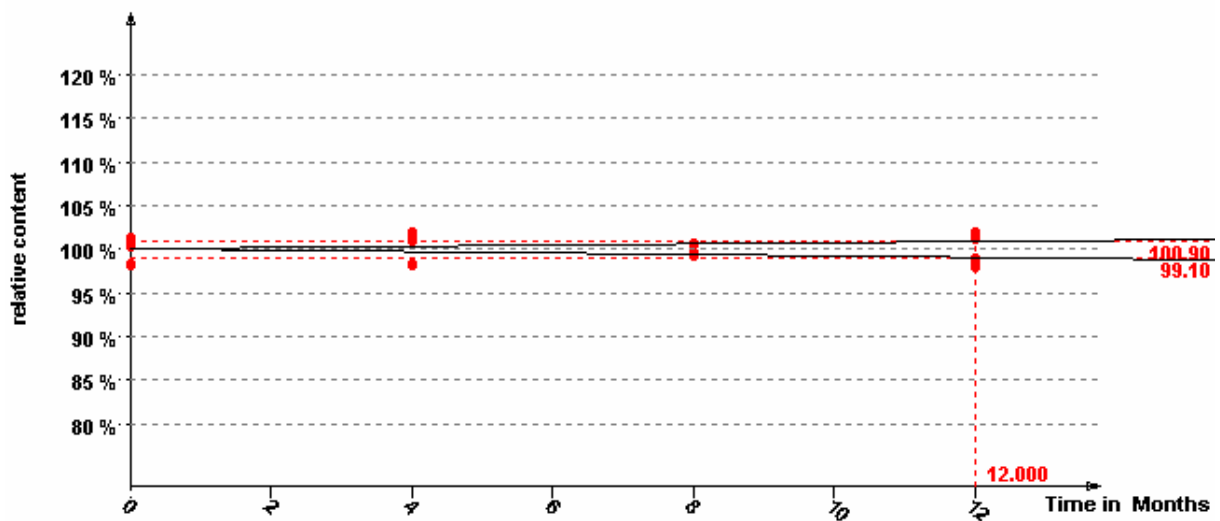
AC059:

Shelf Life and Associated ULts, T=4 °C(Ref)



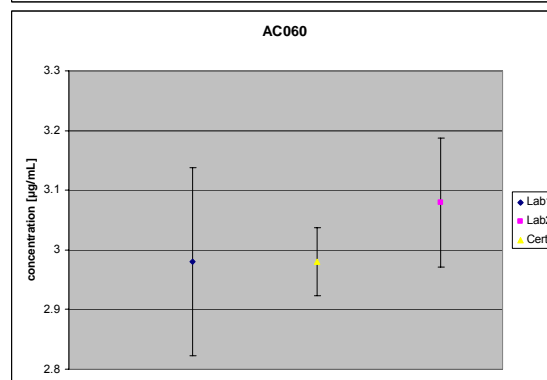
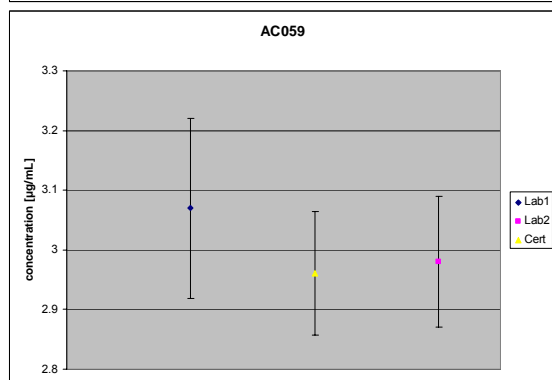
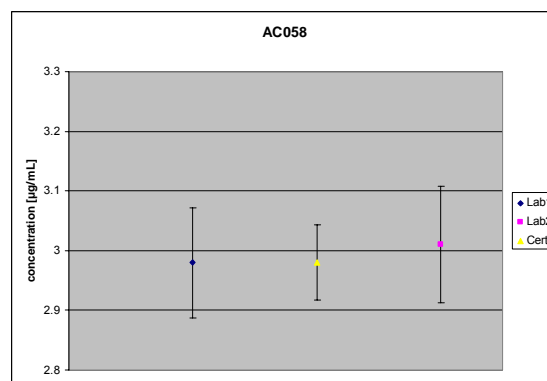
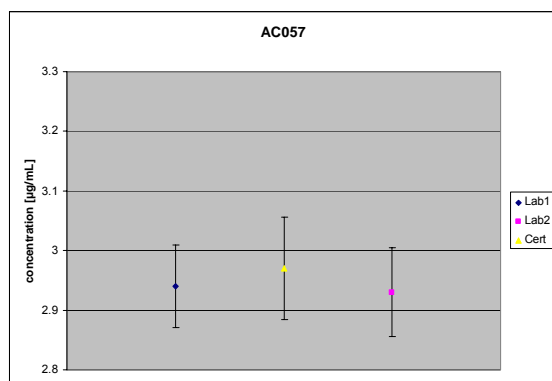
AC060:

Shelf Life and Associated ULts, T=4 °C(Ref)



Annex C Confirmation measurements

	Lab1		Lab2		gravimetric values	
	concentration [$\mu\text{g/mL}$]	U [%]	concentration [$\mu\text{g/mL}$]	U [%]	concentration [$\mu\text{g/mL}$]	U [%]
AC057	2.94	2.36%	2.93	3.00%	2.97	2.9%
AC058	2.98	3.10%	3.01	3.14%	2.98	2.1%
AC059	3.07	4.92%	2.98	3.85%	2.96	3.5%
AC060	2.98	5.29%	3.08	3.35%	2.98	1.9%



European Commission

EUR 22889 EN – Joint Research Centre – Institute for Reference Materials and Measurements

Title: Certification of the mass fraction of aflatoxin B₁ in acetonitrile (ERM[®]-AC057), aflatoxin B₂ in acetonitrile (ERM[®]-AC058), aflatoxin G₁ in acetonitrile (ERM[®]-AC059) and of aflatoxin G₂ in acetonitrile (ERM[®]-AC060)

Author(s): G. Buttinger, R. Josephs, S. Harbeck

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Abstract

This report describes the preparation of aflatoxin B₁, B₂, G₁ and G₂ calibrants (ERM-AC057, ERM-AC058, ERM-AC059 and ERM-AC060) and the certification of their content (mass fraction) in the solutions.

The preparation of the calibrants, the homogeneity and stability studies and the characterisation with a discussion of the results is described hereafter. Uncertainties were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [2] and include uncertainties due to the processing, purity assessment and possible instability. The certified values are listed below:

CRM Code	Certified value ¹⁾	Uncertainty ²⁾
ERM-AC057	3.79 µg/g	0.11 µg/g
ERM-AC058	3.80 µg/g	0.08 µg/g
ERM-AC059	3.78 µg/g	0.13 µg/g
ERM-AC060	3.80 µg/g	0.07 µg/g

- 1) These values are the mass fractions based on weighed amounts and purity. The values were confirmed experimentally by two laboratories
- 2) The uncertainties are the expanded uncertainties ($k = 2$) of the values defined in 1).

The certified mass fractions correspond to following mass concentrations (not certified) at 20 °C and a density for acetonitrile of 0.783 kg/dm³ [1]:

AC057	2.97 ± 0.09 µg/mL
AC058	2.98 ± 0.06 µg/mL
AC059	2.96 ± 0.10 µg/mL
AC060	2.98 ± 0.06 µg/mL

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