CCQM-K154.b

Key Comparison Study – Organic Solvent Calibration Solution

Gravimetric preparation and value assignment of aflatoxin B_1 (AfB_1) in acetonitrile (ACN)

Final Report

February 2022

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SUMMARY

The CCQM-K154.b comparisons were coordinated by the BIPM and NIM on behalf of the CCQM Organic Analysis Working Group (OAWG) for National Measurement Institutes (NMIs) and Designated Institutes (DIs) which provide measurement services in organic analysis under the 'Comité International des Poids et Mesures' Mutual Recognition Arrangement (CIPM MRA) and/or have participated in the BIPM's Mycotoxin Metrology Capacity Building and Knowledge Transfer (MMCBKT) project as part of its "Metrology for Safe Food and Feed in Developing Economies" Capacity Building Programme. Gravimetrically prepared solutions having an assigned mass fraction of specified organic analytes are routinely used to calibrate measurement processes for the quantification of the same analytes in matrix samples. Appropriate assignments of the property value and associated uncertainty of calibration solutions thus underpin the traceability of routine analysis and are critical for accurate measurements. Evidence of successful participation in relevant international comparisons is needed to document calibration and measurement capability claims (CMCs) made by national metrology institutes and designated institutes. In total, eleven NMIs/DIs participated in the Track C, Model II, Key Comparison CCQM-K154.b [Gravimetric preparation and value assignment of aflatoxin B_1 (AfB_1) in acetonitrile (ACN)] for emerging areas of global interest and innovation. Participants were requested to gravimetrically prepare calibration solutions and value assign the mass fractions, expressed in mg/kg, of aflatoxin B_1 (AfB_1) in the acetonitrile (ACN) solution. Study samples, with assigned values and associated uncertainties were prepared by the comparison participants and sent to the coordinating laboratory for comparison. The Key Comparison Reference Values (KCRVs) were assigned of all participant values that agreed within their expanded uncertainty with the values measured by the coordinating laboratory based on calibrations obtained from independent gravimetrically prepared calibrant solutions.

Successful participation in CCQM‑K154.b for MMCBKT participants was intended to demonstrate measurement capabilities for preparation and value assignment of aflatoxin B_1 (AfB_1) calibration solutions in the mass fraction range of 2 mg/kg to 50 mg/kg, prepared from a mycotoxin stock solution of pre-assigned content or solid of known purity. Successful participation for other participants, having value assigned their pure Primary Reference Materials, was intended to demonstrate measurement capabilities for the purity value assignment capabilities of organic materials with molar mass in the range 100 g/mol to 500 g/mol and polarity (*pKow*) > -2, with relative uncertainties at or above the relative uncertainty achieved in the comparison for calibration solutions as well as for the preparation and value assignment of single component organic calibration solutions with non-polar analytes in the mass fraction range of 2 mg/kg to 50 mg/kg, polarity (*pKow*) > -2, with molar mass in the range of 100 g/mol to 500 g/mol.

TABLE OF CONTENT

ACRONYMS

ACN	Acetonitrile
AfB_1	Aflatoxin B_1
ANOVA	Analysis of variance
CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	Calibration and Measurement Capability
DI	Designated Institute
DoE	Degree of equivalence
ESI	Electrospray ionization
GLS	Generalized Least Squares regression analysis
KCRV	Key Comparison Reference Value
LC-DAD-MS/MS	Liquid chromatography with (UV) diode array and tandem mass spectrometric detection
MMCBKT	Mycotoxin Metrology Capacity Building and Knowledge Transfer
NMI	National Metrology Institute
NMR	Nuclear magnetic resonance spectroscopy
OAWG	Organic Analysis Working Group
pKow	Negative log base 10 of the octanol-water partition coefficient
qNMR	Quantitative nuclear magnetic resonance spectroscopy
SRM	Selected reaction monitoring

SYMBOLS

D_i	Degree of equivalence
$D_{rel,\,i}$	Percent relative degree of equivalence
k	Coverage factor
n	Number of quantity values in a series of quantity values
$u(x_i)$	Standard uncertainty of quantity value x_i
$U(x_i)$	Expanded uncertainty of quantity value x_i
$U_{95}(x_i)$	Expanded uncertainty defined such that $x_i \pm U_{95}(x_i)$ is asserted to include the true value of the quantity with an approximate 95 % level of confidence
x	A quantity value
x_i	i^{th} member of a series of quantity values
w_i	Mass fraction of organic analyte in kg/kg or subunits thereof in a given matrix

INTRODUCTION

The CCQM-K154.b comparison, agreed by the CCQM, was organized to support National Metrology Institutes (NMIs) or Designated Institutes (DIs) that have developed capabilities to prepare and value assign mycotoxin calibration solutions to benchmark and demonstrate the comparability of their measurement services.

Calibration solutions prepared from well characterized, high purity compounds are the source of metrological traceability of most routine organic analysis results. The preparation and characterization of these solutions is therefore essential within the measurement infrastructure that supports the delivery of reliable results. It is particularly challenging in the case of the provision of standards to underpin mycotoxin testing in developing economies due to stringent export / import regulations, challenging logistics and high costs.

A number of NMIs/DIs have participated in the BIPM's Mycotoxin Metrology Capacity Building and Knowledge Transfer (MMCBKT) project as part of its "Metrology for Safe Food and Feed in Developing Economies" Capacity Building Programme. The project was designed to allow NMIs/DIs to work together to strengthen mycotoxin metrology infrastructure; provide knowledge transfer to scientists developing capabilities in this area, including periods as visiting scientists at the BIPM; and enable NMIs to provide mycotoxin calibrant and matrix reference materials and proficiency test materials to support mycotoxin testing laboratories within their countries [1].

The CCQM-K154.a and CCQM-K154.a.1 comparisons on the gravimetric preparation and value assignment of the *Fusarium* mycotoxin zearalenone (ZEN) in acetonitrile (ACN) were the first comparisons of a series of comparisons that allowed NMIs/DIs that have participated in the MMCBKT project to demonstrate the compatibility of the capabilities and services they have established in their laboratories [2, 3, 4, 5]. The CCQM-K154.b comparison on the gravimetric preparation and value assignment of the *Aspergillus* mycotoxin aflatoxin B_1 (AfB_1) in acetonitrile (ACN) tests core skills and competencies required in gravimetric preparation and value assignment of organic solvent-based calibration solutions of mycotoxins. It is considered as a Track C, Model II comparison. Track C comparisons are for an emerging area of global interest and innovation. The aim of Track C key comparisons is to underpin future CMCs. Model II signifies that study samples are sent to the coordinator for comparison under repeatability conditions. In addition, the comparison is used to demonstrate the compatibility of laboratory capabilities to assign the mass fraction of single polar organic analytes in organic solutions. This study involved a comparison at the BIPM of a suite of AfB_1 calibration solutions prepared by each of the participating laboratories. Nine laboratories took part in the framework of the MMCBKT while three laboratories participated to demonstrate their in-house calibration solution production capabilities. The calibration solutions have been sent to the BIPM where an LC-DAD(-MS/MS) method was used to compare the value assignments of the mass fraction content of AfB_1 in the solutions provided by each participant.

Aflatoxins are a class of mycotoxins generally produced by fungi of the genus *Aspergillus* that have access either pre- or post-harvest to grain and nut crops in environmental conditions of relatively high temperatures and humidity. Frequently contaminated food products include dried figs, hazelnuts, groundnuts, chili peppers, pistachio and almond. AfB_1, among the four major types of aflatoxins, is the most toxic and the most potent carcinogen in humans and animals. Chronic dietary exposure to aflatoxins, mostly occurring in developing countries, results in hepatotoxicity, genotoxicity, immune suppression and malnutrition [6, 7, 8]. The importance of monitoring aflatoxin content in primary products and derived foodstuffs is reflected in the existence of regulations controlling the maximum limits for total aflatoxins and, in particular, of AfB_1 in about sixty countries. A typical minimum residue level is 2 μg/kg in food [9]. The Rapid Alert System for Food and Feed of the European Union (EU) provides evidence that mycotoxin contamination of food is a global trade issue. Contamination with mycotoxins, especially aflatoxins, constitute the lion's share of notifications and border rejections (419 in 2016) for imports of food from nonmember countries to the EU [10].

MEASURAND, QUANTITIES AND UNITS

The measurand was the mass fraction of aflatoxin B_1 [AfB_1] present in solution acetonitrile (ACN), with the assigned value expressed in mg/kg (or one of its multiples μg/g, mg/g or ng/g).

PARTICIPANTS AND SCHEDULE

This study involved a simultaneous comparison of a suite of twelve calibration solutions of AfB_1 in ACN gravimetrically prepared and value assigned by each of the participating laboratories.
Nine laboratories (INM, INMETRO, INRAP, INTI, KEBS, LATU, NIM, NIMT and UME) took part in the CCQM-K154.b comparison within the framework of the MMCBKT, using a value assigned stock solution of AfB_1 in ACN supplied by the BIPM. Three laboratories (EXHM, NIM, and NMISA) took part in CCQM-K154.b using their own stock solution of AfB_1. NIM participated in CCQM-K154.b using both their own calibration solution and the solution supplied by the BIPM within the framework of the MMCBKT.
The study schedule for CCQM-K154.b is given in Table 1. Two submission deadlines have been offered to NMIs/DIs to be able to cope with laboratory closures and shipping issues caused by the Coronavirus pandemic.

Table 1: CCQM-K154.b Timetable

Action	Date
Initial discussion	April 2019 MMCBKT and OAWG meetings
Study Proposal and draft protocol	October 2019 OAWG meeting
Approval of study protocol and confirmation	April 2020 OAWG meeting
Stock solution distribution	until November 2019 (MMCBKT participants)
Call for participation	July 1st, 2020
Final date to register	August 1st, 2020
Samples and data due to coordinator	October 30th, 2020 and January 31st, 2021
Initial report and discussion of results	May 2021 OAWG meeting
Draft B report	October 2021
Final report to OAWG Chair	February 2022

AFB_1 PRIMARY CALIBRATOR STOCK SOLUTION

The BIPM provided the MMCBKT participants with a stock solution of AfB_1 in acetonitrile (OGP.030) that was to be used for the preparation of AfB_1 calibration solution batches submitted for comparison CCQM-K154.b.

The AfB_1 mass fraction and associated expanded uncertainty ($k = 2$) of the AfB_1 stock solution OGP.030 was 129.0 ± 2.1 mg/kg. The uncertainty corresponding to the gravimetric value assignment the homogeneity and stability uncertainty contribution were combined to calculate the combined standard uncertainty of the stock solution mass fraction assignment. The details of the purity assessment, gravimetric preparation, homogeneity and stability studies and corresponding uncertainty evaluations are briefly described below.

AfB_1 purity characterization

An essential requirement of the MMCBKT project was to obtain and characterize a primary reference material for AfB_1 that could be used subsequently to establish a calibration hierarchy to underpin the metrological traceability of results linked through calibration to this material [11]. The characterization and purity assignment studies to assess identity and purity of a primary reference material for AfB_1 used to deliver the BIPM MMCBKT program are described in detail in the Purity Evaluation Guideline: Aflatoxin B_1 [12]. The guideline is also intended to be of use to other NMIs/DIs and reference measurement service providers needing to characterize their own source material for AfB_1 analysis. Particular reliance was placed on nuclear magnetic resonance spectroscopy (NMR) studies both to confirm the qualitative identity of the main component of the material and to assign the mass fraction of AfB_1 it contained. Due to the relatively complex

structure of AfB_1, the assignment by qNMR only provides in the first instance an estimate of the total AfB_1 and related structure impurity mass fraction. This initial value needed to be corrected for the relevant related structure impurity mass fraction as assigned separately by an LC-MS/MS method to give the final value for the true AfB_1 mass fraction of the material. LC-UV and LC-CAD methods were used for verification. Additional analyses for the assessment of other potential impurities were undertaken to support and confirm the value assigned through combination of the qNMR and LC data.

The initial qNMR value for the AfB_1 mass fraction and its expanded uncertainty in the material had been estimated by qNMR at 981.3 ± 2.3 mg/g (k = 2). This value was corrected for the total related structure impurity contributions (1.68 ± 0.13 mg/g) determined by LC-MS/MS (Table 2) to give the final assigned value for the "true" AfB_1 mass fraction and corresponding expanded uncertainty of AfB_1 in the material (BIPM reference OGO.193a) of 979.6 ± 2.3 mg/g (k = 2).

Table 2: Impurity assignments

Impurity	Mass fraction (mg/g)	u (mg/g)	Assignment
AfB_2	1.16	0.12	LC-MS/MS (and LC-UV)
AfB_{2a}	0.52	0.02	LC-MS/MS (and LC-UV)
VOC (CH_2Cl_2, EtOH)	-*		qNMR
Unidentified aliphatic impurities (lipid, etc)	18.7	2.4	qNMR by difference (LC-CAD)

* VOC mass fractions could not be quantified and are not reported.

The unidentified impurity visible in the upfield region of the NMR spectrum of the material in the area 0.8 – 1.0 ppm appears to be primarily aliphatic in nature and is probably lipidic residue from the harvesting and purification of the AfB_1 from its biological source material. The assigned value was obtained by difference from the upper purity limit of 1000 mg/g and the combined mass fraction of AfB_1 and related structure impurities as determined by qNMR, given that there was no evidence of other significant impurities present in the material.

Gravimetric preparation of AfB_1 stock solution

The AfB_1 stock solution (OGP.030) was prepared gravimetrically by dissolving an accurately weighed sample of about 100 mg of AfB_1 powder material (OGO.193a) in 1 L of acetonitrile. Mettler Toledo MX5 and XP_10002S balances were used for the weighing of the AfB_1 powder and the final solution mass, respectively. Table 3 demonstrates the preparation of the stock solution and the mass fraction assignment, calculated according to Equation 1.

Table 3: Experimental data used for the preparation of the AfB_1 stock solution and the calculation using Eqn. 1 of its AfB_1 mass fraction.

	Weighed mass (m)	Buoyancy (*b*)	m x b
AfB_1 powder (mg)	102.630	1.000596	102.691
Solution (g)	779.060	1.001386	779.140
Purity ± U (mg/g)	979.6 ± 2.3		
Final mass fraction (µg/g)	128.95		

$$w_{stock} = \frac{m_p \cdot b_p \cdot w_p}{m_{sol} \cdot b_{sol}} \quad \text{Eq. 1}$$

where:
m_p: weighed mass of AfB_1 powder
b_p: buoyancy correction for powder weighing
w_p: purity of AfB_1 powder
m_{sol}: weighed mass of stock solution
b_{sol}: buoyancy correction for solution (ACN) weighing

The uncertainties from input quantities in Equation 1 were combined (Equation 2) and the final combined standard uncertainty was calculated as depicted in Table 4. A minor uncertainty component, u(V), was included to account for the potential solvent loss due to evaporation during sample preparation and weighing. The buoyancy mass correction and its uncertainty were calculated as described by the Calibrant Assessment Guideline: Aflatoxin B_1 [13].

$$u(w_{stock}) = w_{stock} \cdot \sqrt{\left[\frac{u(m_p)}{m_p}\right]^2 + \left[\frac{u(b_p)}{b_p}\right]^2 + \left[\frac{u(w_p)}{w_p}\right]^2 + \left[\frac{u(m_{sol})}{m_{sol}}\right]^2 + \left[\frac{u(b_{sol})}{b_{sol}}\right]^2 + \left[\frac{u(V)}{V}\right]^2} \quad \text{Eq. 2}$$

Table 4: Individual uncertainty components contributing to the combined uncertainty of the AfB_1 stock solution mass fraction

Uncertainty source	Value (%)
$\frac{u(m_p)}{m_p}$	0.0037
$\frac{u(b_p)}{b_p}$	0.0017
$\frac{u(w_p)}{w_p}$	0.23
$\frac{u(m_{sol})}{m_{sol}}$	0.0058
$\frac{u(b_{sol})}{b_{sol}}$	0.0012
$\frac{u(V)}{V}$	0.005
u_{rel} (%)	0.235
$u(w_{stock})$ µg/g	0.303
$U(w_{stock})$ µg/g (k = 2)	0.61

Filling of AfB_1 stock solution

The 1 L flask containing the stock solution was agitated thoroughly and about 50 mL were transferred to prepare a calibration solution. The rest of the stock solution was stored at 4 °C until ampouling, which took place within 24 h of the preparation.

A 500 mL bottle and 1-10 mL bottle-top dispenser (Dispensette, Brand GmbH) were rinsed twice with the AfB_1 stock solution and a stainless-steel flat tip syringe needle was fitted at the outlet of the dispenser to ensure that all solution is discharged at the bottom of the ampoule.

10 mL glass ampoules were selected for a filling volume of 4 mL to ensure that sufficient head space remains above the liquid and therefore minimize the risk of accidental ignition of the solvent during the sealing process. An Ampoulmatic (Bioscience Inc) system connected to propane and oxygen cylinders was used to ampoule the batch. The flow of both gases was adjusted to produce a bright blue flame at the neck of the ampoules.

The ampoules were filled with 4 mL of OGP.030, one at a time, to minimize the impact of evaporation of acetonitrile. A refrigerant (Jelt Refroidisseur 5320) was sprayed onto the lower part of the ampoule before being placed in the ampouling carousel to further reduce the ignition risk. After flame sealing, ampoules were allowed to adjust to room temperature in an upright position.

To test for possible leaks, ampoules were placed into a vacuum drying oven (Haraeus) in an upright position and vacuum (about 50 mbar) was applied for at least 4 hours. The ampoules then remained in the sealed oven overnight, after which they were visually inspected for changes in the solution

levels. Leaking ampoules were recorded and discarded while the rest of the batch was stored at -20 °C.

Homogeneity studies of AfB_1 stock solution

The BIPM investigated the levels of within and between ampoule homogeneity of the main component and selected significant minor components and identified a minimum sample size which reduces to an acceptable level the effect of between bottle inhomogeneity of both the main component and the minor components. The homogeneity of the AfB_1 stock solution was studied using an LC-DAD-MS/MS method that allowed for the quantitative determination of AfB_1 by UV and of the other AfB_1 related impurities by MS/MS detection.
The results of the ANOVA are summarised in Table 5.

Table 5: Homogeneity results of the AfB_1 stock solution (OGP.030)

	AfB_1	AfB_2	AfB_{2a}	Af_{DIOL}	AfQ_1	AfP_1
N	30	30	30	30	30	30
s_{wb} (%)	0.62	8.08	8.23	11.15	6.96	6.90
s_{bb} (%)	0.237	-[2]	4.68	6.70	11.82	2.65
u^*_{bb} (%)	0.20	2.62	2.67	3.62	2.26	2.24
$\mathbf{u_{bb}}$[1] (%)	0.237	2.62	4.68	6.70	11.82	2.65
F	1.44	0.84	1.97	2.08	9.66	1.44
F_{crit}	2.39	2.39	2.39	2.39	2.39	2.39

[1] Higher value (u^*_{bb} or s_{bb}) was taken as uncertainty estimate for potential inhomogeneity
[2] Not calculable because $MS_{bb} < MS_{wb}$

Homogeneity evaluation was performed by single factor ANOVA, allowing for the separation of the variation associated with the method (s_{wb}) from the actual variation between ampoules (s_{bb}), which is an estimate of the uncertainty associated to batch heterogeneity. This uncertainty was 0.24 %, 2.62 % and 4.68 % for AfB_1 and the two major impurities AfB_2 and B_{2a}, respectively (Table 5). The other impurities showed larger associated uncertainties due to being present at mass fractions near the limit of detection of the method. The material was regarded to be homogeneous since the u_{bb} of 0.237 % is very small compared with the target uncertainties of < 2 % and in agreement with typical u_{bb} for similar materials [14].
Representative normalized results due to the analysis and filling sequences are presented for the main component AfB_1 (Figure 1) and an impurity, the AfB_{2a} (Figure 2). The first, second and third replicates are represented by circles, grey filled circles and dots respectively.

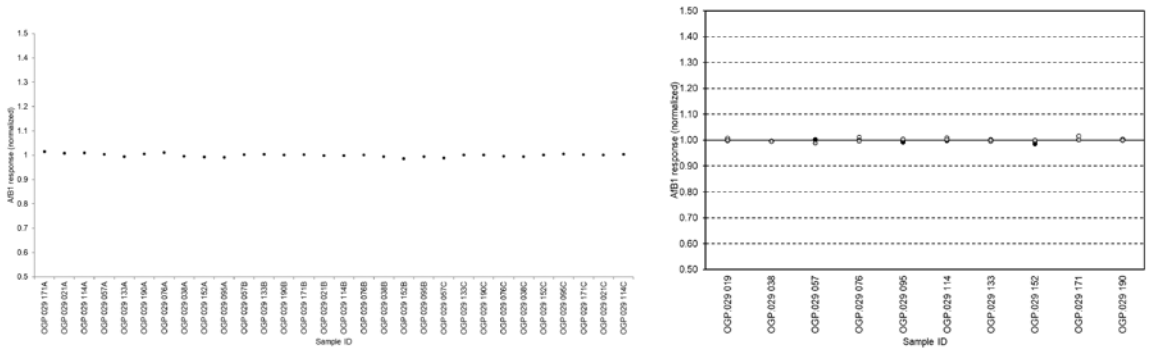

Figure 1: Homogeneity of AfB_1 by LC-DAD at 362 nm – Main component - Injection and filling sequence

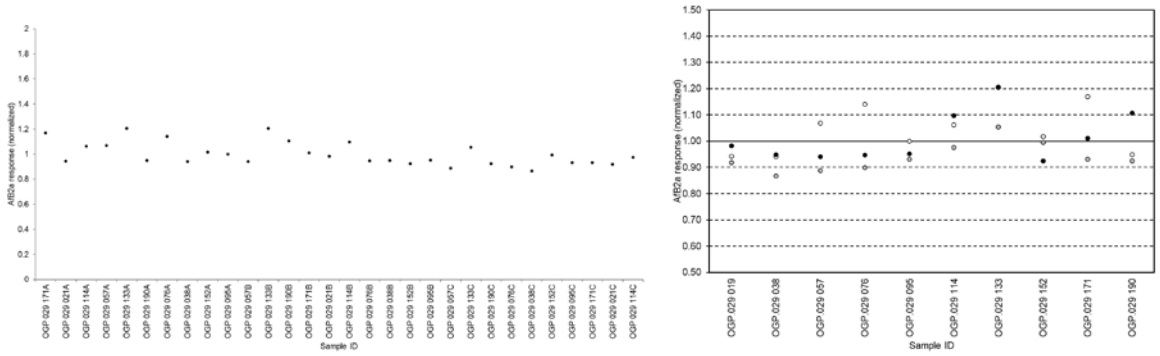

Figure 2: Homogeneity of AfB_{2a} by LC-MS/MS – Representative impurity - Injection and filling sequence

Stability studies of AfB1 stock solution

Isochronous stability studies were performed using a reference storage temperature of -20 °C and test temperatures of 4 °C, 22 °C and 40 °C. A set of units from the production batch were stored at each selected temperature over 8 weeks, with units transferred to reference temperature storage at 2 week intervals. The detected AfB_1 related impurities in the stock solution were AfB_2, AfB_{2a}, AfP_1, AfQ_1 and Af_{DIOL}. They were measured in the tested ampoules by LC-MS/MS whereas the main component AfB_1 was measured by LC-DAD. Original impurity standards were used for external calibration of the LC-MS/MS method and the calculated mass fractions were normalized to the reference samples (stored at -20°C). For the main component AfB_1, no calibration was performed as absorbance values were directly normalized to the main peak absorbance of the reference samples. Data were evaluated as a function of the storage time at each of the studied temperatures.

The AfB_1 mass fraction of the material was stable on storage at 4 °C and 22 °C but did decrease significantly after storage beyond 4 weeks at 40 °C as shown in Figure 3. The AfB_2 impurity mass fractions of the material were stable on storage at 4 °C, 22 °C and 40 °C. The AfB_{2a} (representative impurity for AfP_1, AfQ_1 and Af_{DIOL}) mass fraction of the material was stable on storage at 4 °C but did decrease significantly after storage beyond 2 weeks at both 22 °C and 40 °C as shown in Figure 4. It was concluded that the stock solution (OGP.030) should be stored at maximum 4 °C in the dark for long-term storage and can be shipped at maximum 22 °C in the dark. A conservative stability uncertainty contribution of 0.93 µg/g (0.72 %) was estimated based on the 22 °C stability data taking in consideration storage to cover the comparison period (20 months).

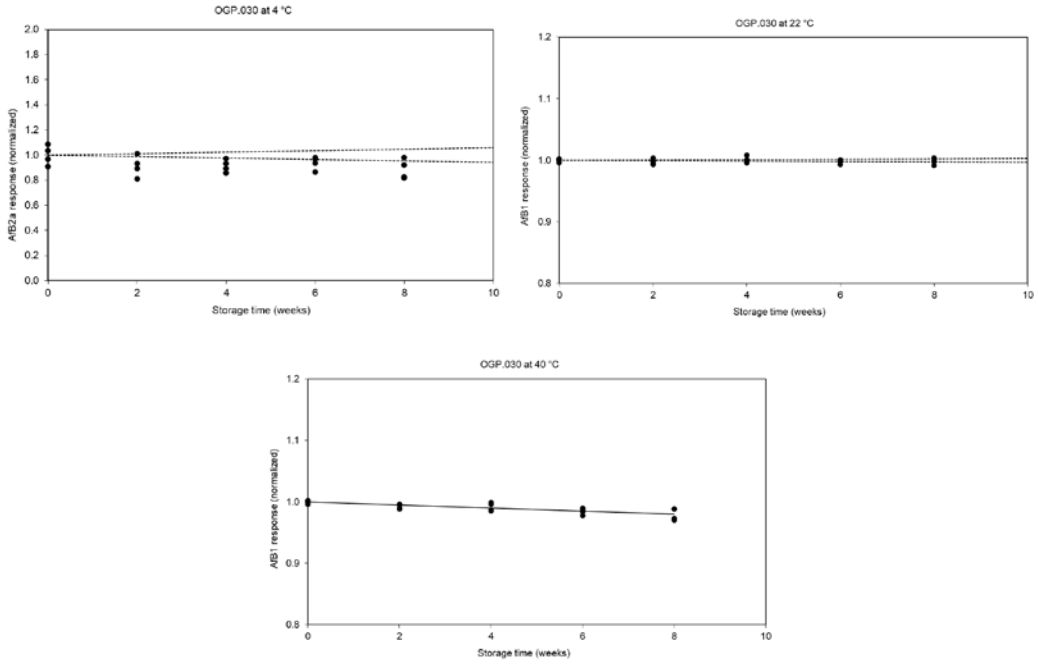

Figure 3: Stability study of AfB_1 by LC-DAD

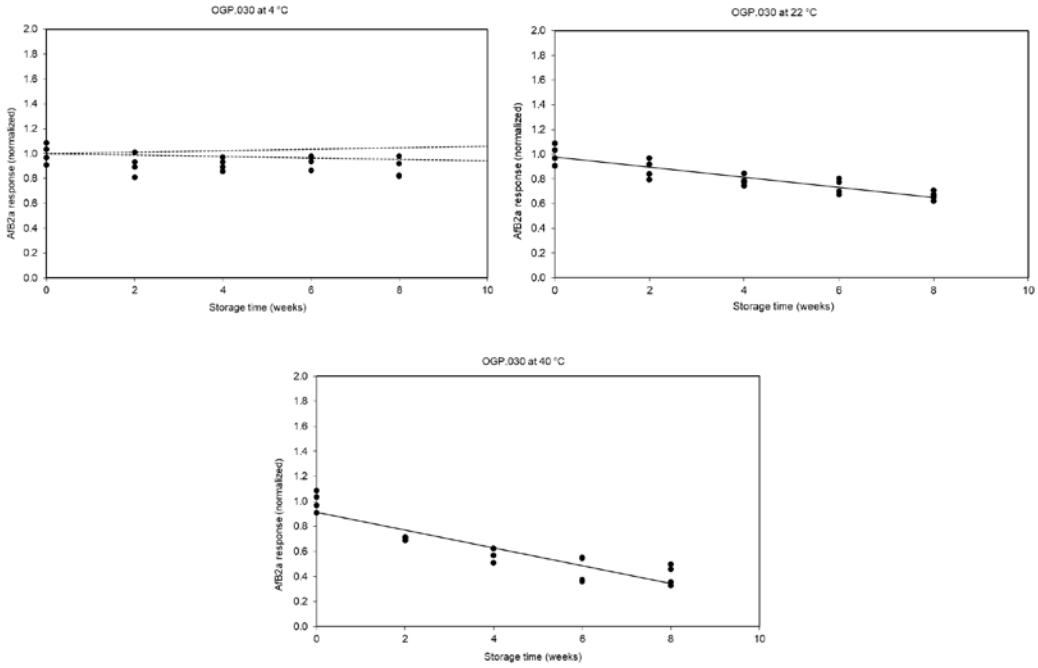

Figure 4: Stability study of AfB_{2a} by LC-MS/MS

AfB_1 stock solution and corresponding uncertainty

A mass fraction of 129.0 µg/g was calculated for the AfB_1 stock solution based on the gravimetric preparation. The expanded uncertainty *(U)* with a coverage factor of $k = 2$, corresponding to a level of confidence of approximately 95 %, was estimated to be 2.1 µg/g. Uncertainty contributions arising from the gravimetric preparation, $u(w_{stock})$, as well as from homogeneity, u_{bb} and stability assessment, u_{lts} were taken into consideration. Details of the uncertainty contributions, mass fraction (w_{stock}) and corresponding combined (u_c) and expanded uncertainty *(U)* of the AfB_1 stock solution are summarized in Table 6.

Table 6: Uncertainty contributions, mass fraction *(w_{stock})* and corresponding combined *(u_c)* and expanded uncertainty *(U)* of the AfB_1 stock solution

AfB_1 stock solution	
$u_{rel}(w_{stock})$ (%)	0.24
$u_{bb,rel}$ (%)	0.24
$u_{lts,rel}$ (%)	0.72
$u_{c,rel}$ (%)	0.79
w_{stock} (µg/g)	129.0
u_c (µg/g)	1.03
U (µg/g), $k = 2$	2.1

Sample distribution of AfB1 stock solution

Six units of the AfB_1 stock solution, each containing a minimum of 4 mL of material, were distributed to each MMCBKT participant by express mail service in insulated boxes equipped with temperature indicators. Participants were asked to acknowledge receipt of the samples and to advise the coordinator if any obvious damage had occurred to the vials or if temperatures has exceeded 40 °C during shipping. The shipping details are listed in Table 7. All samples were delivered to the comparison participants in good condition.

Table 7: Shipping details for the AfB_1 stock solution from the BIPM to MMCBKT participants

NMI/DI	Shipping date	Date of receipt	In transit (days)	Comments
INMETRO	29.11.2019	03.12.2019	4	-
INM	29.11.2019	06.12.2019	7	-
INRAP	29.11.2019	03.12.2019	4	-
INTI	29.11.2019	12.12.2019	13	-
KEBS	29.11.2019	06.12.2019	7	-
LATU	29.11.2019	12.12.2019	13	-
NIM[#]	29.11.2019	04.12.2019	4	-
NIMT	29.11.2019	06.12.2019	7	-
NMISA*	29.11.2019	09.12.2019	10	-
UME	29.11.2019	11.12.2019	12	-

[#] For their participation in the study NIM chose to submit AfB_1 calibrator solutions prepared using both in-house value-assigned materials and based on the MMCBKT AfB_1 stock solution OGP.030.

* As an MMCBKT participant NIMSA was supplied with the AfB_1 stock solution OGP.030. However, they chose to submit a AfB_1 calibrator solution prepared using in-house value-assigned materials for their participation in the study.

STUDY MATERIALS

The participants were required to gravimetrically prepare and ampoule their own (about 4 mL per ampoule) standard solutions of aflatoxin B_1 (AfB_1) in acetonitrile and to send these to the BIPM for comparison measurements. The mass fraction values targeted (in the range 2 mg/kg to 50 mg/kg) are intended to be representative of the mass fraction content of AfB_1 in a standard solution provided as a reference standard used for calibrations in AfB_1 analyses.
Prior to sending samples to the BIPM, participants should have demonstrated that the levels of within and between vial inhomogeneity of the mass fraction of AfB_1 in acetonitrile were sufficiently small so as to not influence the validity of the comparison. Isochronous stability studies should have been completed to confirm that the material was sufficiently stable within the proposed time scale of the study. Participants should also have ensured that AfB_1 was stable in acetonitrile in the ampoule in the dark and under controlled temperature conditions. Appropriate conditions for storage, transport and handling of the solution should have been established by the participants.

STUDY GUIDELINE

Each participant provided the BIPM at least four ampoules with each ampoule containing at least 4 mL of solution (AfB_1 in acetonitrile). Two ampoules were required by the BIPM for analysis to obtain the comparison results and the additional ampoules were available as a reserve. The ampoules were stored at -20 °C in the dark until use. Participants were required to provide their estimate of the mass fraction of AfB_1 in the solution and its corresponding uncertainty based on the gravimetric preparation corrected for purity. Each participant provided results using the reporting sheet provided with the samples. The results were sent via e-mail to the study coordinator prior to the result submission deadline. Submitted results were considered final and no corrections or adjustments of analytical data were accepted.
It was proposed by the coordinator and decided by the CCQM OAWG that the CCQM-K154.b comparison was divided into two rounds of comparisons because NMIs/DIs had difficulties to assure participation at certain dates because of coronavirus pandemic restrictions. 30^{th} October 2020 or 31^{st} January 2021 were the deadlines to submit the samples and to return the filled in data submission forms to the BIPM for participation in the first and second comparison round, respectively.
The details of the shipping of the comparison solutions from the NMIs/Dis to the BIPM are listed in Table 8. There was a prolonged delay in delivery (19 days) of the ampoules sent from INMETRO, Brazil to the BIPM because of delayed flights. One ampoule (no. 37) was broken and has been removed and was not used for comparison. LATU, Uruguay had difficulties to ship the samples before the deadline of 31.01.2021 because of the coronavirus pandemic restrictions. The

shipping could be arranged in the begin of March 2021 and samples were received within 6 days and in good condition. All samples of other NMIs/DIs were delivered within less than 14 days to the BIPM and were received in good condition for comparison.

Table 8: Shipping details of the CCQM-154.b AfB_1 comparison solutions from NMIs/DIs to the BIPM

NMI/DI	Comparison round	Shipping date	Date of receipt	In transit (days)	Comments
EXHM	2	28.01.2021	29.01.2021	1	-
INM	2	15.12.2020	29.12.2020	14	-
INMETRO	2	06.02.2021	25.02.2021	19	One ampoule broken (no. 37)
INRAP	2	20.01.2021	27.01.2021	7	-
INTI	2	22.12.2021	04.01.2021	13	-
KEBS	2	31.01.2021	09.02.2021	9	-
LATU	2	09.03.2021	15.03.2021	6	-
NIM	1	28.10.2020	05.11.2020	8	-
NIMT	2	30.01.2021	04.02.2021	5	-
NMISA	1	07.10.2020	14.10.2020	7	-
UME	2	26.01.2021	01.02.2021	6	-

REPORTED MASS FRACTIONS OF AFB_1 AND IMPURITIES

The values reported by participating NMIs/DIs for the AfB_1 mass fractions of their AfB_1 comparison solutions and their corresponding uncertainties based on the gravimetric preparation (corrected for purity for non-MMCBKT participants) are given in Table 9. The details of the gravimetric preparation, calculation of the AfB_1 mass fraction values and assessment of corresponding expanded uncertainties are described in Annex A for each participating NMI/DI. If the uncertainty includes contributions deriving from other sources (for example, homogeneity and/or stability testing) details are also provided in Annex A.
In addition to the compulsory AfB_1 mass fraction of their AfB_1 comparison solution and its corresponding uncertainties the EXHM reported the presence of one unknown impurity at a mass fraction of 1.8 µg/g, two unknown impurities at 0.2 µg/g and two unknown impurities of less than 0.1 µg/g without providing corresponding uncertainties.

VALUE ASSIGNMENT PROCEDURE OF THE COORDINATING LABORATORY

The AfB_1 mass fraction assigned solutions provided by the NMIs/DIs were measured and compared at the BIPM under repeatability conditions by an in-house developed and validated LC-DAD-MS/MS method. UV detection was used for the quantification of AfB_1. MS/MS detection served as a verification tool for the determination of potential related structure impurities. Preliminary experiments demonstrated that the UV response was linear over the mass fraction range of about 5 µg/g to 35 µg/g of AfB_1.
Two-point calibrations with external bracketing using AfB_1 standards assigned at the BIPM were used for quantification and comparison. It was decided to split the CCQM-K154.b comparison in four groups (A, B, C and D) with separate calibrations for each of the two comparison rounds to allow working at narrow and linear mass fraction ranges. Thus, injection sequences were short in order to minimize the extent of instrument drift.

Materials and calibrants

The AfB_1 bracketing standards were prepared immediately before use as solutions in acetonitrile (Hipersolv HPLC grade, VWR, France) of the pure BIPM AfB_1 material (OGO.193a) having a AfB_1 mass fraction of 979.6 ± 2.3 mg/g ($k = 2$) as outlined in the chapter ‘AfB_1 purity characterization’. The gravimetric preparation of the stock solutions were performed in the same way as described in detail in the chapter ‘Gravimetric preparation and filling of AfB_1 stock solutions’. Low and high level calibration solutions were gravimetrically prepared from the stock solutions according to the procedure described in detail in the Calibrant Assessment Guideline:

Aflatoxin B_1 [13]. The AfB_1 mass fractions and corresponding standard uncertainties for the stock and calibration solutions are listed in Table 10.

LC-DAD-MS/MS method

Liquid chromatographic (LC) separation and UV diode array detection (DAD)

An LC 1100 system (Agilent, Les Ulis, France) consisting of an 1100 Series G1312A binary pump, 1100 Series G1329A autosampler, 1100 temperature-controlled column compartment with cooling and 1200 diode-array detector was employed for LC-DAD analysis.
LC separation was performed on a Kinetex EVO C18 100 A column (250 mm × 4.6 mm, 2.6 µm from Phenomenex (Le Pecq, France) maintained at 25 °C. The mobile phases consisted of (A) acetonitrile/methanol (50:50, v/v) and (B) purified water. The separation was performed by use of a gradient program with a constant flow rate of 600 µL/min. The gradient started with 30 % A and was increased to 90 % A in 30 min. The column was then washed by increasing to 100 % A in 1 min, holding at 100 % A for 1 min and returning to starting conditions (30 % A) in 2 min. The column was re-equilibrated for a further 6 min at 30 % A. The total run time was 40 min and the injection volume was 10 µL. The detection wavelength of the UV diode array detector (DAD) was 362 nm. The wavelength of 265 nm was recorded for verification.

Mass spectrometric detection (MS/MS)

Mass spectrometric detection was performed for verification purposes of potentially occurring impurities. A SCIEX QTRAP 4000 tandem mass spectrometer (Sciex, Villebon sur Yvette, France) fitted with an electrospray ionization (ESI) source was used. The MS parameters were optimized in negative/positive switching electrospray ionization mode. A capillary voltage of 5500 V with source temperature of 600 ºC and a capillary voltage of -4500 V with source temperature of 550 ºC was employed for the positive and negative ESI mode, respectively. Nitrogen was used as the ion source gas, curtain gas and collision gas. The Gas 1 and Gas 2 of the ion source were set at 55 psi and 60 psi, respectively. The curtain gas (CUR) was set at 15 psi. The collision gas (CAD) was set at Mid. Table 11 lists MS/MS transitions of AfB_1 and potential impurities with optimized dwell time, declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) settings.

Table 9: AfB_1 mass fraction values and corresponding uncertainties submitted by the NMIs/DIs CCQM-K154.b

Participant	Comparison round	Primary calibrator used	AfB_1 Mass fraction (µg/g)	Combined standard uncertainty (µg/g)	Coverage factor (*k*)	Expanded uncertainty (µg/g)
EXHM, Greece	2	own	10.052	0.143	2	0.287
INM, Colombia	2	CBKT	3.99	0.050	1.97	0.10
INMETRO, Brazil	2	CBKT	8.24	0.154	2	0.31
INRAP, Tunisia	2	CBKT	6.15	0.05	2	0.10
INTI, Argentina	2	CBKT	15.006	0.148	2	0.296
KEBS, Kenya	2	CBKT	12.20	0.1	2	0.2
LATU, Uruguay	2	CBKT	14.861	0.127	2	0.254
NIM, China	1	own	6.44	0.062	2	0.13
	1	CBKT	6.45	0.080	2	0.16
NIMT, Thailand	2	CBKT	2.053	0.029	2	0.058
NMISA, South Africa	1	own	31.69	0.38	2	0.76
UME, Turkey	2	CBKT	5.11	0.07	2	0.14

Table 10: Details of the gravimetric preparation of the BIPM bracketing calibration standards for AfB_1

Comparison round	Calibration	Mass fraction range	AfB_1 Stock solution		High level calibration solution		Low level calibration solution	
			w (mg/kg)	u (mg/kg)	*w* (mg/kg)	u (mg/kg)	*w* (mg/kg)	u (mg/kg)
1	A	4-10 mg/kg	100.83	0.80	10.04	0.08	3.97	0.04
1	B	29-35 mg/kg	103.83	0.80	34.96	0.28	28.97	0.23
2	C	1-7 mg/kg	94.91	0.76	6.58	0.05	1.61	0.02
2	D	6-16 mg/kg	94.91	0.76	15.76	0.13	5.99	0.05

Table 11: Summary of selected precursor and product ions, optimized time, DP, CE, EP and CXP settings for the detection of AfB_1 and potential related structure impurities by electrospray ionization MS/MS

Compounds	Precursor ion Q1 (*m/z*)	Product ion Q3 (*m/z*)	Optimized parameters				
			Time (ms)	DP (V)	CE (V)	EP (V)	CXP (V)
AfB_1	311.3	296.0*	50	-50	-25	10	10
		283.0	50	-50	-25	10	10
AfB_2	315.4	287.2*	50	70	38	10	10
		259.1	50	70	38	10	10
AfG_1	327.2	283.0*	50	-50	-25	10	10
		268.0	50	-50	-25	10	10
AfG2	329.2	285.0*	50	-50	-25	10	10
		242.0	50	-50	-25	10	10
AfM_1	327.4	312.1*	50	-50	-30	10	10
		299.2	50	-50	-30	10	10
AfM_2	329.3	314.1*	50	-50	-30	10	10
		301.1	50	-50	-30	10	10
AfB_{2a}	329.2	258.1*	50	-50	-30	10	10
		243.2	50	-50	-30	10	10
AfQ_1	327.4	312.2*	50	-50	-25	10	10
		299.1	50	-50	-25	10	10
AfP_1	299.4	271.2*	50	70	40	10	10
		229.2	50	70	40	10	10
Af_{DIOL}	345.2	283.2*	50	-50	-25	10	10
		327.2	50	-50	-25	10	10

Samples, sequence preparation and measurement order

Two ampoules supplied by each participant were each measured in triplicate by LC-DAD-MS/MS. CCQM-K154.b was grouped in comparison round 1 and 2 with measurements performed end of 2020 and begin of 2021, respectively. Comparison round 1 measurements were undertaken in two batches (A and B) on different days involving two and one participants according to their target mass fraction ranges of about 4-10 mg/kg and 29-35 mg/kg, respectively. Comparison round 2 measurements were also performed in a two batches (C and D) on different days involving four and five participants with a target mass fraction range of about 1-7 mg/kg and 6-16 mg/kg, respectively. Grouping in different mass fraction ranges provided for narrow and linear calibrations. Thus, injection sequences were short to minimize instrument drift. The grouping of CCQM-K154.b participant samples is listed in Table 12.

Table 12: Grouping of CCQM-K154.b participant samples

Comparison round 1		Comparison round 2	
Calibration A (4-10 mg/kg)	Calibration B (29-35 mg/kg)	Calibration C (1-7 mg/kg)	Calibration D (6-16 mg/kg)
NIM (CBKT)	NMISA (own)	INM (CBKT)	EXHM (own)
NIM (own)		INRAP (CBKT)	INMETRO (CBKT)
		NIMT (CBKT)	INTI (CBKT)
		UME (CBKT)	KEBS (CBKT)
			LATU (CBKT)

About 300 µL of the NMI/DI samples, low and high mass fraction level calibrant solutions and control samples (BIPM) were transferred in LC vials and injected separately. Calibrants (Low and High), control samples (BIPM) and pure acetonitrile (Blank) vial were distributed and injected over the sequences. The results for blanks and control samples served to identify potential carry-over and instrument drifts, respectively. Neither carry-over nor significant instrument drifts were observed. The detailed injection sequences for CCQM-K154.b comparison round 1 (A and B) and round 2 (C and D) are given in Table 13.

Table 13: Detailed injection sequences for the different calibrations of CCQM-K154.b

	Comparison round 1		Comparison round 2	
Injection	Calibration A (4-10 mg/kg)	Calibration B (29-35 mg/kg)	Calibration C (1-7 mg/kg)	Calibration D (6-16 mg/kg)
1	Blank	Blank	Blank	Blank
2	Low-1	Low-1	Low-1	Low-1
3	High-1	High-1	NMIT-CBKT-A-1	EXHM-OWN-A-1
4	Blank	Blank	INM-CBKT-A-1	INMETRO-CBKT-A-1
5	Low-2	Low-2	INRAP-CBKT-A-1	INTI-CBKT-A-1
6	BIPM-C-1	NMISA-OWN-X-1	High-1	KEBS-CBKT-A-1
7	NIM-CBKT-X-1	High-2	Blank	High-1
8	High-2	Blank	Low-2	Blank
9	Blank	Low-3	BIPM-C-1	Low-2
10	Low-3	Control-A-1	UME-CBKT-A-1	BIPM-C-1
11	NIM-OWN-Y-1	NMISA-OWN-Y-1	INM-CBKT-B-1	LATU-CBKT-A-1
12	BIPM-C-2	High-3	High-2	INMETRO-CBKT-B-1
13	High-3	Blank	Blank	KEBS-CBKT-B-1
14	Blank	Low-4	Low-3	High-2
15	Low-4	Control-A-2	NMIT-CBKT-B-1	Blank
16	NIM-OWN-X-1	High-4	BIPM-C-2	Low-3
17	NIM-CBKT-Y-1	Blank	UME-CBKT-B-1	BIPM-C-2
18	High-4	Low-5	High-3	LATU-CBKT-B-1
19	Blank	NMISA-OWN-X-2	Blank	EXHM-OWN-B-1
20	Low-5	High-5	Low-4	INTI-CBKT-B-1
21	BIPM-C-3	Blank	INRAP-CBKT-B-1	High-3
22	NIM-OWN-X-2	Low-6	NMIT-CBKT-A-2	Blank
23	NIM-CBKT-X-2	NMISA-OWN-Y-2	INM-CBKT-A-2	Low-4
24	High-5	Control-A-3	High-4	LATU-CBKT-A-2
25	Blank	High-6	Blank	INMETRO-CBKT-A-2
26	Low-6	Blank	Low-5	BIPM-C-3
27	BIPM-C-4	Low-7	UME-CBKT-A-2	INTI-CBKT-A-2
28	NIM-OWN-Y-2	Control-A-4	INRAP-CBKT-A-2	High-4
29	High-6	High-7	BIPM-C-3	Blank
30	Blank	Blank	High-5	Low-5
31	Low-7	Low-8	Blank	EXHM-OWN-A-2
32	NIM-CBKT-Y-2	NMISA-OWN-X-3	Low-6	KEBS-CBKT-A-2
33	High-7	Control-A-5	INM-CBKT-B-2	LATU-CBKT-B-2
34	Blank	High-8	INRAP-CBKT-B-2	INMETRO-CBKT-B-2
35	Low-8	Blank	UME-CBKT-B-2	High-5
36	BIPM-C-5	Low-9	High-6	Blank
37	NIM-CBKT-X-3	NMISA-OWN-Y-3	Blank	Low-6
38	NIM-OWN-X-3	High-9	Low-7	BIPM-C-4
39	High-8	Blank	NMIT-CBKT-B-2	KEBS-CBKT-B-2
40	Blank	Low-10	BIPM-C-4	INTI-CBKT-B-2
41	Low-9	Control-A-6	INRAP-CBKT-A-3	EXHM-OWN-B-2
42	NIM-OWN-Y-3	High-10	High-7	High-6
43	NIM-CBKT-Y-3	Blank	Blank	Blank
44	BIPM-C-6		Low-8	Low-7
45	High-9		INM-CBKT-A-3	KEBS-CBKT-A-3
46	Blank		UME-CBKT-A-3	LATU-CBKT-A-3
47	Low-10		BIPM-C-5	INTI-CBKT-A-3
48	High-10		High-8	BIPM-C-5
49	Blank		Blank	High-7
50			Low-9	Blank
51			NMIT-CBKT-A-3	Low-8
52			INRAP-CBKT-B-3	INMETRO-CBKT-A-3
53			UME-CBKT-B-3	EXHM-OWN-A-3
54			High-9	LATU-CBKT-B-3
55			Blank	KEBS-CBKT-B-3
56			Low-10	High-8
57			INM-CBKT-B-3	Blank
58			NMIT-CBKT-B-3	Low-9
59			BIPM-C-6	INTI-CBKT-B-3
60			High-10	EXHM-OWN-B-3
61			Blank	BIPM-C-6
62				INMETRO-CBKT-B-3
63				High-9
64				Blank
65				Low-10
66				High-10
67				Blank

Measurements and results

Subsequent to the LC-DAD-MS/MS analyses the UV absorption peak areas of AfB_1 at 362 nm were automatically integrated, manually verified and refined using the Analyst software (Sciex, Villebon sur Yvette, France).

XLGENLINEv1.1 (National Physics Laboratory, United Kingdom) an Excel-based software program was used for the further treatment of the data. It allows the undertaking of a Generalized Least Squares (GLS) regression analysis that is fully compliant with the International Standard ISO 6143 [15, 16]. This approach is fully implemented and widely used for very similar applications in the field of gas mixture standards analysis and related Model II key comparisons of the CCQM Gas Analysis Working Group (GAWG) [17, 18] where typically mass fractions and corresponding uncertainties are of the same order of magnitude. Model II comparisons of the CCQM OAWG are until now mainly applied for the comparison of CRMs with mass concentrations that span several orders of magnitude [19]. Statistical approaches including GLS are discussed in detail by Duewer *et al*. [20].

In the present case, XLGENLINEv1.1 calculates the values and uncertainties of the 'unknowns', displays a plot of the fitted regression function, and outputs the parameters of the fit. Slope and y-intercepts of the calibrations were calculated by use of the UV absorption peak area responses. Regression lines were built by use of the bracketing low and high mass fraction level calibrants prepared by the BIPM. Input AfB_1 mass fractions and standard uncertainties of the bracketing low and high mass fraction level calibrants based on the gravimetrical preparation (Table 9) are compared with the arithmetic mean and corresponding standard deviation of the UV absorption peak area responses of ten replicates each. The ten replicates of each of the bracketing low and mass fraction level calibrant were strategically placed to cover the entire injection sequence (Table 13). The AfB_1 mass fractions and associated standard uncertainties of the NMI/DI solutions were evaluated inversely based on the UV absorption peak area responses and the standard deviation of its three replicates.

The mass fraction values assigned at the BIPM using this procedure for the NMI/DI solutions (w_{BIPM}), corresponding standard u(w_{BIPM}) and expanded uncertainties U(w_{BIPM}) are listed in Table 14. The bracketing calibrations with the values, standard uncertainties and UV peak area responses for the solutions submitted by NMIs/DIs, low and high mass fraction level calibrants and internal control samples for CCQM-K154.b A, B, C and D are depicted in Figures 5-8, respectively.

Table 14: AfB_1 mass fraction values and absolute corresponding and expanded uncertainties measured by the BIPM for CCQM-K154.b participants' ampoules

NMI/DI	Comparison round	w_{BIPM} (mg/kg)	$u(w_{BIPM})$ (mg/kg)	$U(w_{BIPM})$ (mg/kg)	*Quantification range* (mg/kg)
NIM (own) A	1	6.35	0.05	0.09	4-10
NIM (own) B	1	6.32	0.09	0.18	4-10
NIM (CBKT) A	1	6.41	0.06	0.12	4-10
NIM (CBKT) B	1	6.40	0.09	0.18	4-10
NMISA (own) A	1	31.51	0.16	0.31	29-35
NMISA (own) B	1	31.57	0.11	0.22	29-35
INM (CBKT) A	2	3.95	0.05	0.10	1-7
INM (CBKT) B	2	3.94	0.06	0.12	1-7
INRAP (CBKT) A	2	4.82	0.05	0.10	1-7
INRAP (CBKT) B	2	3.96	0.05	0.09	1-7
NIMT (CBKT) A	2	2.02	0.02	0.04	1-7
NIMT (CBKT) B	2	2.02	0.02	0.04	1-7
UME (CBKT) A	2	5.06	0.05	0.09	1-7
UME (CBKT) B	2	5.09	0.05	0.09	1-7
EXHM (own) A	2	9.88	0.07	0.14	6-16
EXHM (own) B	2	9.91	0.07	0.14	6-16
INMETRO (CBKT) A	2	8.20	0.05	0.11	6-16
INMETRO (CBKT) B	2	8.20	0.05	0.11	6-16
INTI (CBKT) A	2	14.99	0.15	0.30	6-16
INTI (CBKT) B	2	14.95	0.14	0.28	6-16
KEBS (CBKT) A	2	12.19	0.10	0.20	6-16
KEBS (CBKT) B	2	12.12	0.10	0.21	6-16
LATU (CBKT) A	2	14.76	0.14	0.28	6-16
LATU (CBKT) B	2	15.16	0.14	0.29	6-16

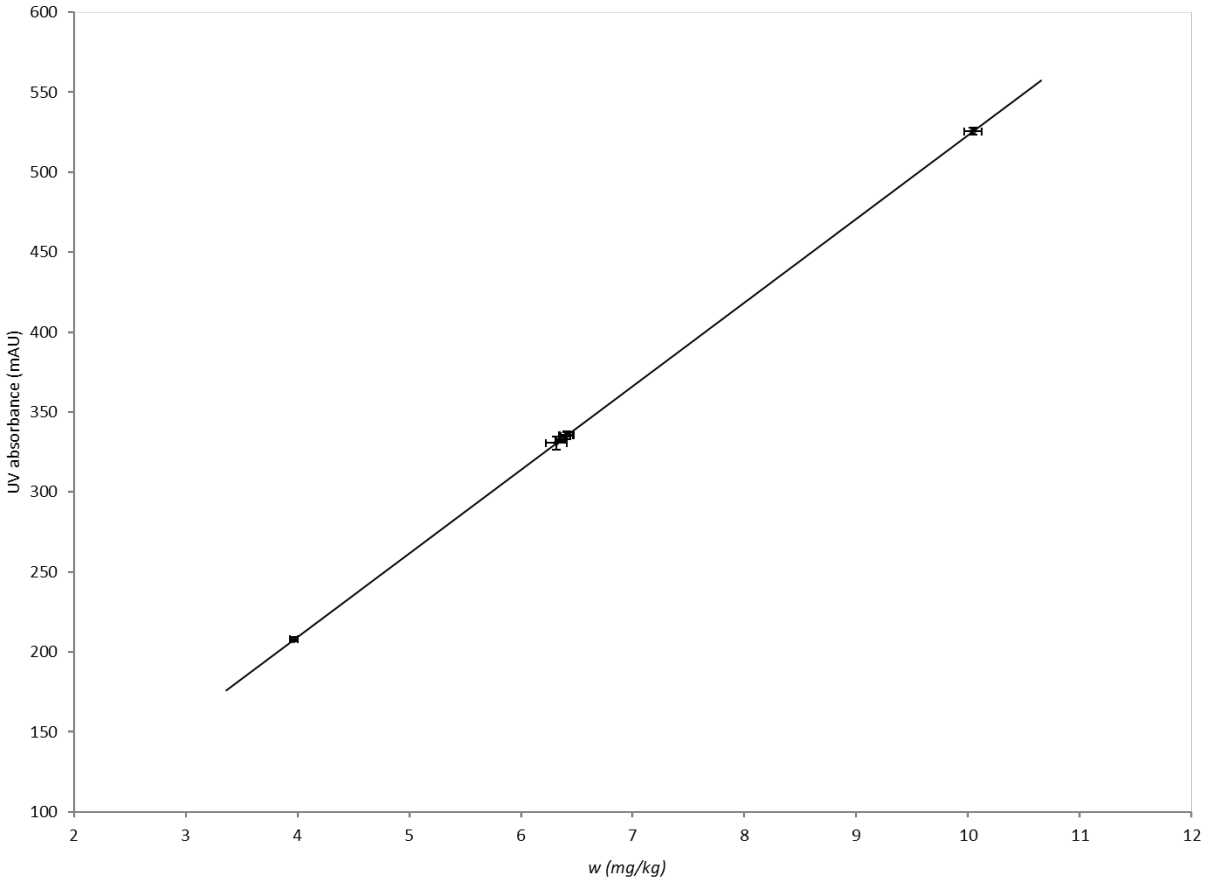

Figure 5: CCQM-K154.b – Comparison round 1 - Calibration A - Bracketing calibration for the AfB_1 mass fraction quantification range of 4-10 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at the upper and lower end of the calibration line. Inverse evaluation data of NIM (own) and NIM (CBKT) and internal control sample are depicted as dots.

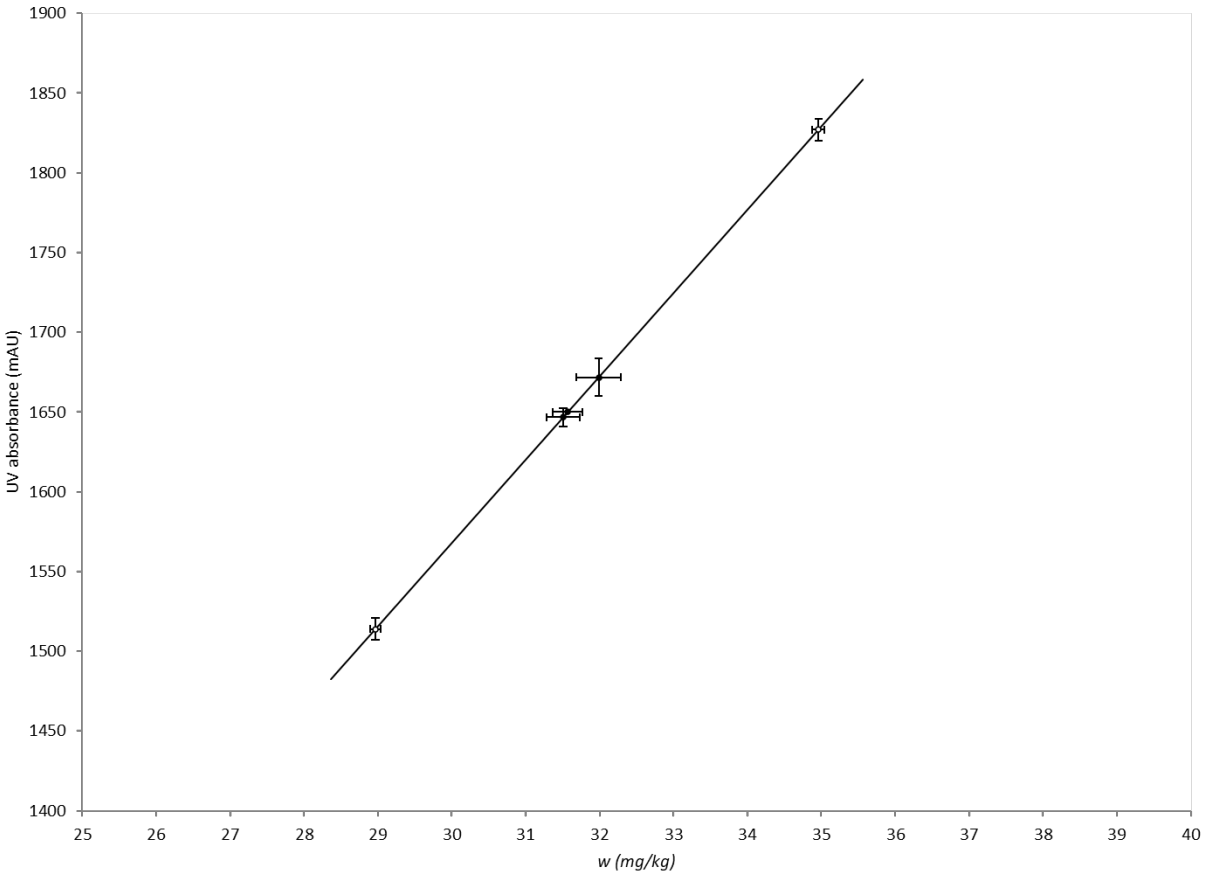

Figure 6: CCQM-K154.b - Comparison round 1 - Calibration B - Bracketing calibration for the AfB_1 mass fraction quantification range of 29-35 mg/kg. UV absorbance (mAU) and corresponding mass fraction (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at upper and lower end of the calibration line. Inverse evaluation data of NMISA (own) and internal control sample are depicted as dots.

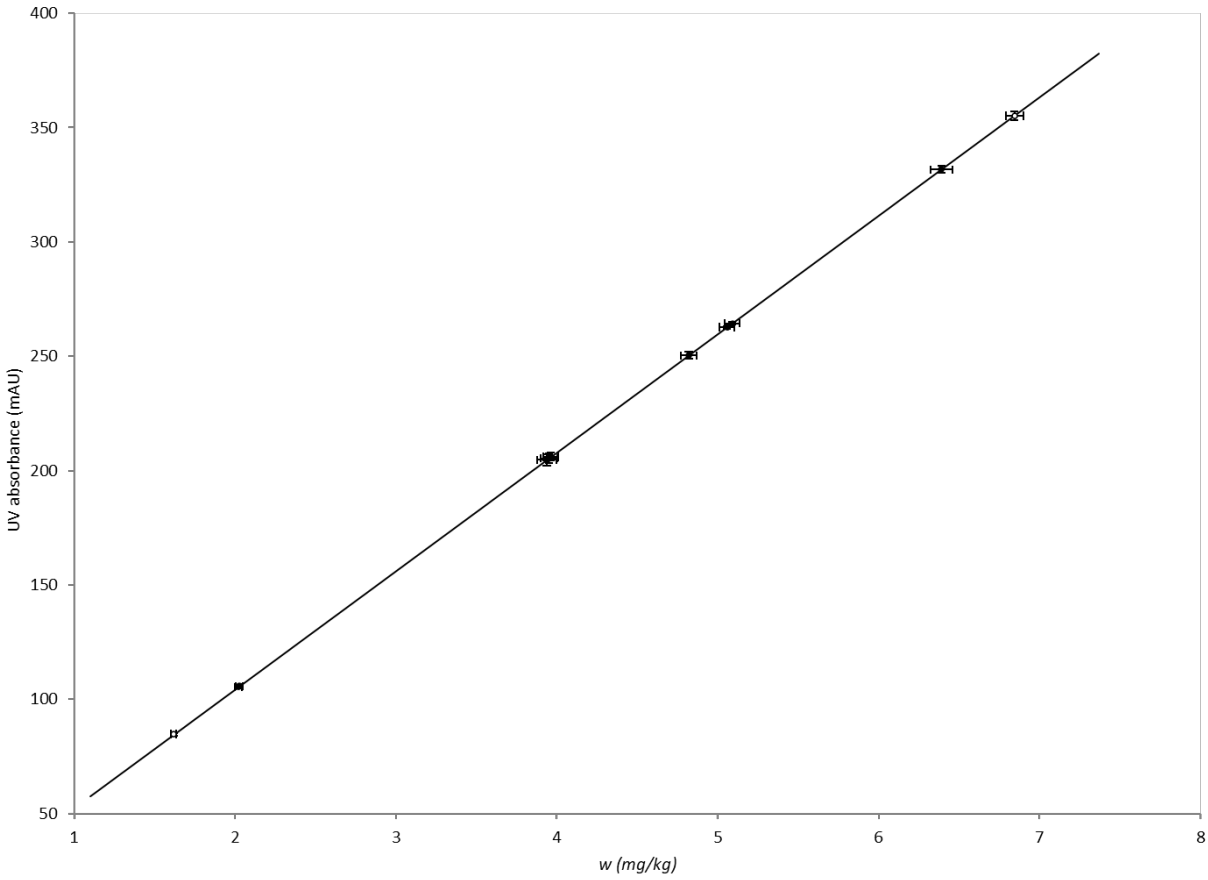

Figure 7: CCQM-K154.b - Comparison round 2 - Calibration C - Bracketing calibration for the AfB_1 mass fraction quantification range of 1-7 mg/kg. UV absorbance (mAU) and corresponding mass fraction (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at upper and lower end of the calibration line. Inverse evaluation data of INM (CBKT), INRAP (CBKT), NIMT (CBKT), UME (CBKT) and internal control sample are depicted as dots.

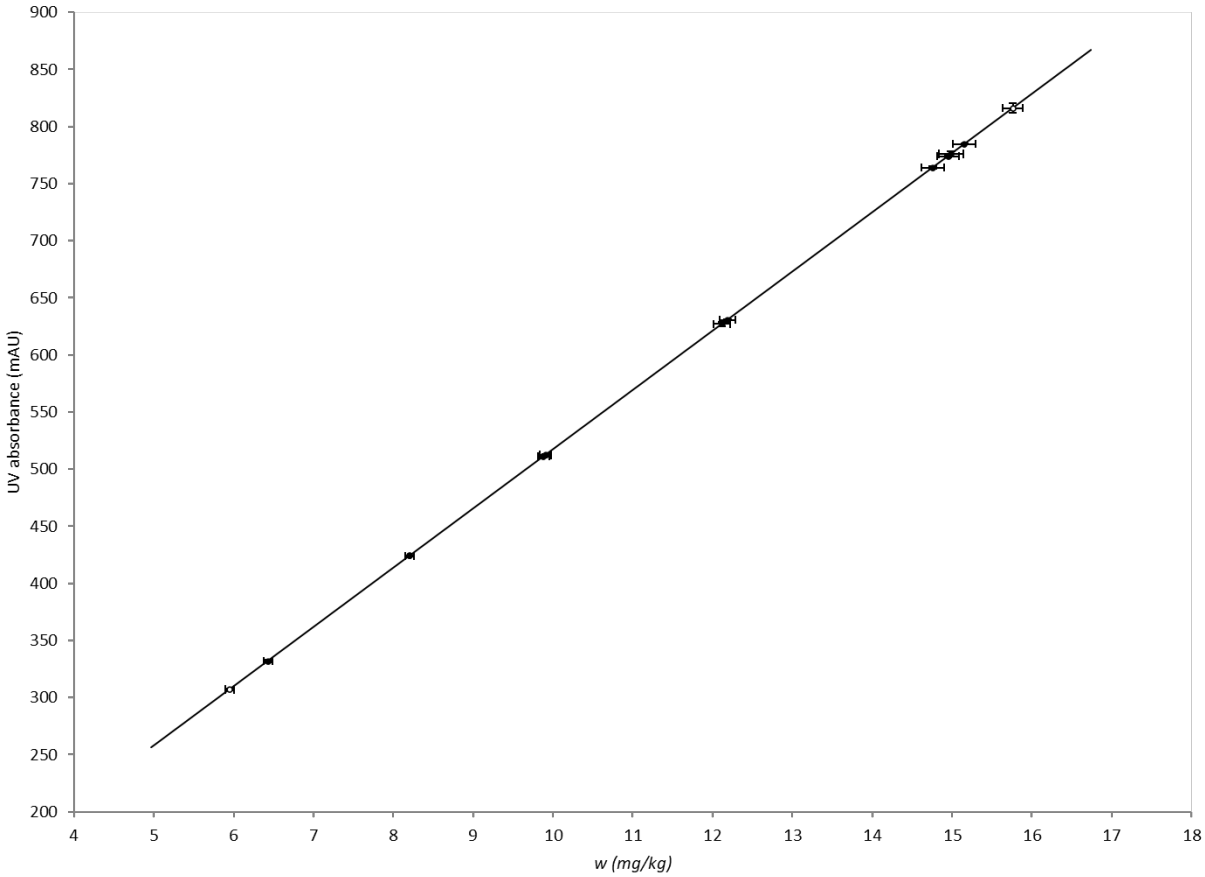

Figure 8: CCQM-K154.b - Comparison round 2 - Calibration D - Bracketing calibration for the AfB_1 mass fraction quantification range of 6-16 mg/kg. UV absorbance (mAU) and corresponding mass fraction (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at upper and lower end of the calibration line. Inverse evaluation data of EXHM (own), INMETRO (CBKT), INTI (CBKT), KEBS (CBKT), LATU (CBKT) and internal control sample are depicted as dots.

Additional inspection of the LC-MS/MS data clearly indicated that significant formation of AfB_{2a} occurred in the INRAP samples received for CCQM-K154.b. Shipping of these samples from INRAP to the BIPM took only about one week and the vials were received in good condition (Table 8). No degradation or formation of impurities could be expected based on the outcome of the stability studies. However, the formation of AfB_{2a} by acid induced addition of water to the AfB_1 is a well reported mechanism [21, 22]. The remains of the INRAP solutions provided to the BIPM were pooled and pH was measured to be about 5.4. It is suspected that reagents or contact materials were contaminated with acid causing the partial conversion of AfB_1 into AfB_{2a} for the INRAP samples. A representative LC-UV chromatogram of a CCQM-K154.b INRAP sample is

Representative LC-MS/MS chromatograms of both a CCQM-K154.b INRAP sample and BIPM control sample are presented in Figure 9 confirming significant formation of AfB_{2a}. The chromatogram also shows the presence of minor AfB_2 impurity originating from the AfB_1 stock solution.

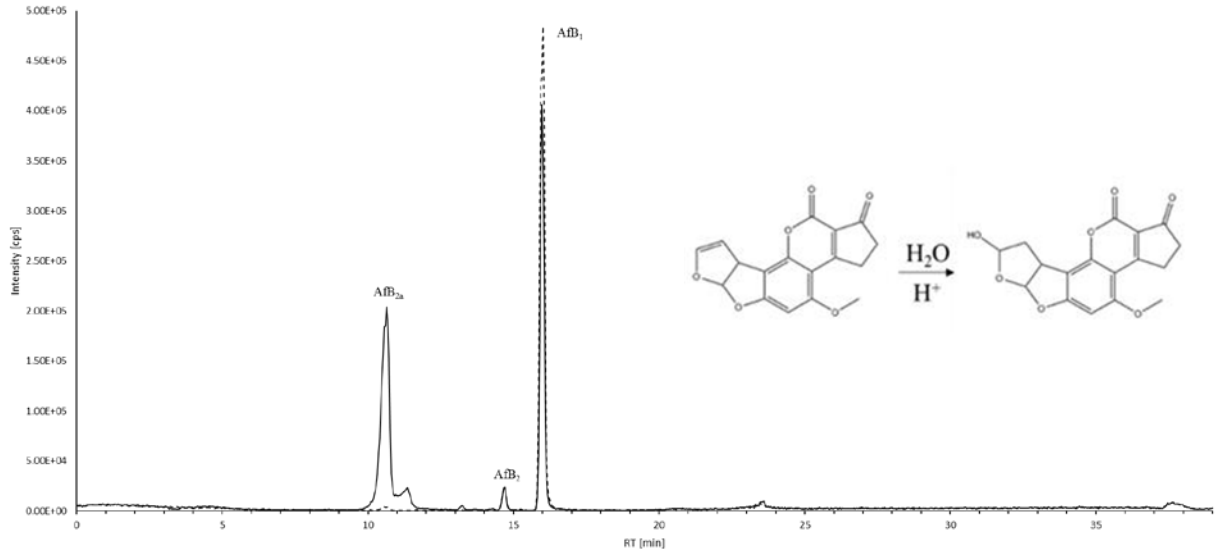

Figure 9: Representative LC-MS/MS chromatograms of both CCQM-K154.b comparison sample from INRAP (solid line) and BIPM control (dotted line).

KEY COMPARISON REFERENCE VALUES FOR CCQM-K154.b

The AfB_1 mass fraction values used to establish the Key Comparison Reference Values (KCRVs) for both rounds of CCQM-K154.b were assigned by the BIPM following the above mentioned calibration procedure ($w_{BIPM} = w_{KCRV}$). For each ampoule, the Key Comparison Reference Value is the AfB_1 mass fraction (w_{KCRV}) and its corresponding uncertainty ($u(w_{KCRV})$). All NMIs/DIs (i) participating in both rounds of CCQM-K154.b were required to submit estimates for the AfB_1 mass fraction w_i and its corresponding uncertainty $u(w_i)$ for their set of ampoules.
The degree of equivalence (D_i) of a participant's submitted value w_i with w_{KCRV} is given by:

$$D_i = w_i - w_{KCRV}$$

The expanded uncertainty U_i at a confidence level of about 95 % associated with the D_i was calculated as:

$$U(D_i) = 2 \cdot \sqrt{u(w_i)^2 + u(w_{KCRV})^2}$$

The relative degree of equivalence ($D_{rel,\,i}$) of a participant's submitted value w_i with the w_{KCRV} was calculated as participants worked at different mass fraction levels:

$$D_{rel,i} = 100 - \left(\frac{100 \cdot w_{KCRV}}{w_i}\right)$$

The expanded uncertainty $U_{rel}(D_{rel,\,i})$ at a confidence level of about 95 % associated with the ($D_{rel,\,i}$) was calculated as:

$$U_{rel}\left(D_{rel,i}\right) = 2 \cdot \sqrt{u_{rel}(w_i)^2 + u_{rel}(w_{KCRV})^2}$$

The AfB_1 mass fractions values and associated absolute uncertainties with degree of equivalences for both rounds of CCQM-K154.b are listed in Table 15. Figure 10 indicates the degree of equivalence (D_i) of each key comparison participant's result with the w_{KCRV}.
The AfB_1 mass fractions values and associated relative uncertainties with relative degree of equivalences are listed in Table 16. Figure 11 indicates the relative degree of equivalence ($D_{rel,\,i}$) of each key comparison participant's result with the w_{KCRV}.

Table 15: AfB_1 mass fractions and absolute corresponding uncertainties with degree of equivalences for CCQM-K154.b

NMI/DI	Comparison round	w_{KCRV} (mg/kg)	$u(w_{KCRV})$ (mg/kg)	$U(w_{KCRV})$ (mg/kg)	w_i (mg/kg)	$u(w_i)$ (mg/kg)	$U(w_i)$ (mg/kg)	D_i	$U(D_i)$	Quantification range (mg/kg)
NIM (own) A	1	6.354	0.046	0.091	6.44	0.062	0.13	0.09	0.16	4-10
NIM (own) B	1	6.316	0.091	0.182	6.44	0.062	0.13	0.12	0.22	4-10
NIM (CBKT) A	1	6.405	0.060	0.120	6.45	0.080	0.16	0.04	0.20	4-10
NIM (CBKT) B	1	6.339	0.091	0.182	6.45	0.080	0.16	0.05	0.24	4-10
NMISA (own) A	1	31.51	0.16	0.31	31.69	0.38	0.76	0.18	0.82	29-35
NMISA (own) B	1	31.57	0.11	0.22	31.69	0.38	0.76	0.12	0.79	29-35
INM (CBKT) A	2	3.950	0.051	0.102	3.99	0.050	0.10	0.04	0.14	1-7
INM (CBKT) B	2	3.937	0.058	0.115	3.99	0.050	0.10	0.05	0.15	1-7
INRAP (CBKT) A	2	4.819	0.050	0.101	6.15	0.05	0.10	1.33	0.14	1-7
INRAP (CBKT) B	2	3.963	0.046	0.092	6.15	0.05	0.10	2.19	0.14	1-7
NIMT (CBKT) A	2	2.018	0.022	0.044	2.053	0.029	0.058	0.03	0.07	1-7
NIMT (CBKT) B	2	2.025	0.022	0.044	2.053	0.029	0.058	0.03	0.07	1-7
UME (CBKT) A	2	5.057	0.045	0.090	5.11	0.07	0.14	0.05	0.17	1-7
UME (CBKT) B	2	5.090	0.045	0.090	5.11	0.07	0.14	0.02	0.17	1-7
EXHM (own) A	2	9.882	0.071	0.142	10.052	0.143	0.287	0.17	0.32	6-16
EXHM (own) B	2	9.907	0.069	0.139	10.052	0.143	0.287	0.14	0.32	6-16
INMETRO (CBKT) A	2	8.205	0.053	0.105	8.24	0.154	0.31	0.04	0.33	6-16
INMETRO (CBKT) B	2	8.205	0.053	0.105	8.24	0.154	0.31	0.04	0.33	6-16
INTI (CBKT) A	2	14.989	0.149	0.298	15.006	0.148	0.296	0.02	0.42	6-16
INTI (CBKT) B	2	14.951	0.141	0.282	15.006	0.148	0.296	0.06	0.41	6-16
KEBS (CBKT) A	2	12.188	0.099	0.198	12.20	0.10	0.20	0.01	0.28	6-16
KEBS (CBKT) B	2	12.117	0.103	0.206	12.20	0.10	0.20	0.08	0.29	6-16
LATU (CBKT) A	2	14.758	0.138	0.276	14.861	0.127	0.254	0.10	0.37	6-16
LATU (CBKT) B	2	15.156	0.143	0.286	14.861	0.127	0.254	-0.30	0.38	6-16

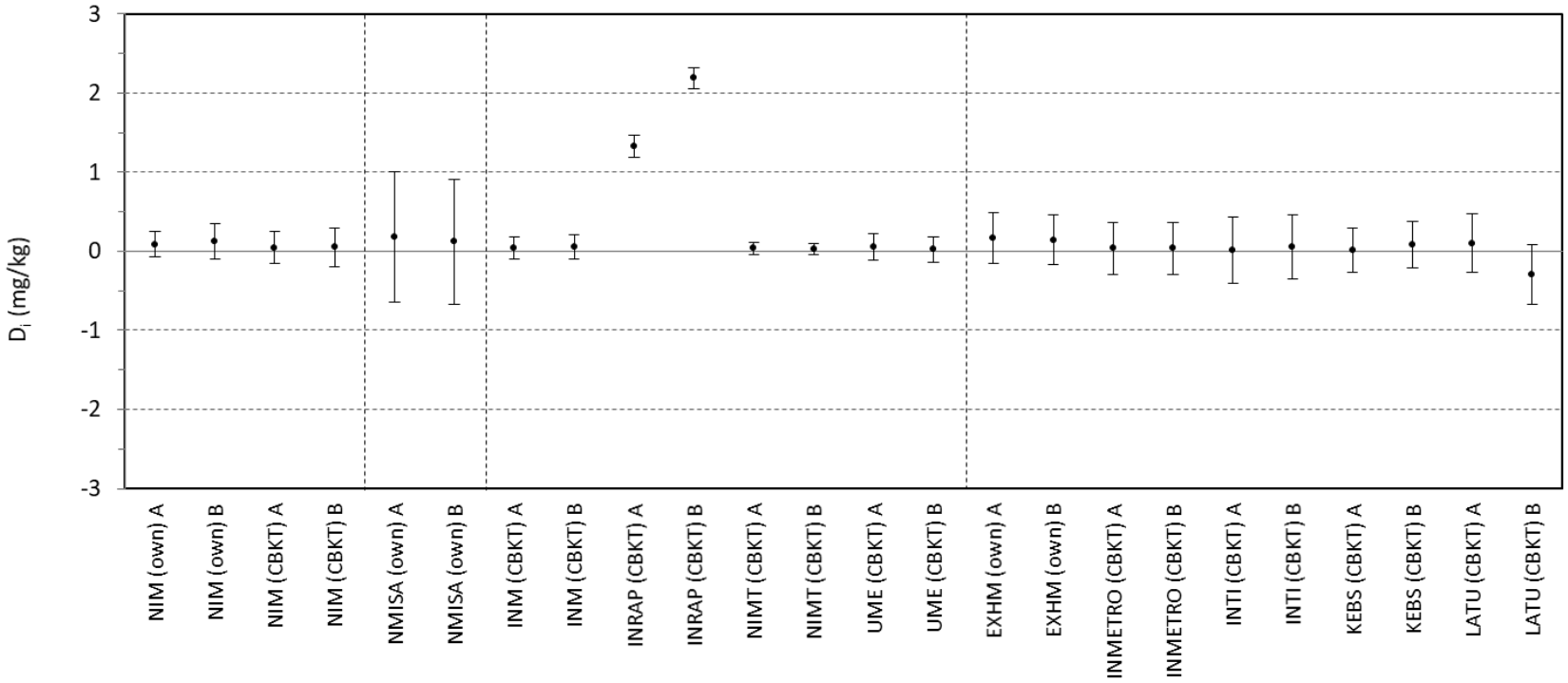

Figure 10: Absolute values for the degree of equivalence for CCQM-K154.b

Table 16: AfB_1 mass fractions and relative corresponding uncertainties with relative degree of equivalences for CCQM-K154.b

NMI/DI	Comparison round	w_{KCRV} (mg/kg)	$u_{rel}(w_{KCRV})$ (%)	$U_{rel}(w_{KCRV})$ (%)	w_i (mg/kg)	$u_{rel}(w_i)$ (%)	$U_{rel}(w_i)$ (%)	$D_{rel,\,i}$	$U_{rel}(D_{rel,\,i})$	Quantification range (mg/kg)
NIM (own) A	1	6.354	0.72	1.43	6.44	1.01	2.02	1.33	2.48	4-10
NIM (own) B	1	6.316	1.44	2.88	6.44	1.01	2.02	1.92	3.52	4-10
NIM (CBKT) A	1	6.405	0.93	1.87	6.45	1.24	2.48	0.69	3.11	4-10
NIM (CBKT) B	1	6.339	1.42	2.84	6.45	1.24	2.48	0.79	3.77	4-10
NMISA (own) A	1	31.51	0.49	0.99	31.69	1.20	2.40	0.57	2.59	29-35
NMISA (own) B	1	31.57	0.34	0.69	31.69	1.20	2.40	0.37	2.50	29-35
INM (CBKT) A	2	3.950	1.29	2.58	3.99	1.24	2.48	1.00	3.58	1-7
INM (CBKT) B	2	3.937	1.46	2.93	3.99	1.24	2.48	1.33	3.84	1-7
INRAP (CBKT) A	2	4.819	1.05	2.09	6.15	0.81	1.63	21.64	2.65	1-7
INRAP (CBKT) B	2	3.963	1.16	2.31	6.15	0.81	1.63	35.56	2.83	1-7
NIMT (CBKT) A	2	2.018	1.09	2.17	2.053	1.41	2.83	1.69	3.56	1-7
NIMT (CBKT) B	2	2.025	1.08	2.16	2.053	1.41	2.83	1.37	3.56	1-7
UME (CBKT) A	2	5.057	0.89	1.78	5.11	1.37	2.74	1.03	3.27	1-7
UME (CBKT) B	2	5.090	0.89	1.78	5.11	1.37	2.74	0.40	3.27	1-7
EXHM (own) A	2	9.882	0.72	1.43	10.052	1.42	2.85	1.69	3.19	6-16
EXHM (own) B	2	9.907	0.70	1.40	10.052	1.42	2.85	1.44	3.17	6-16
INMETRO (CBKT) A	2	8.205	0.64	1.28	8.24	1.87	3.74	0.43	3.95	6-16
INMETRO (CBKT) B	2	8.205	0.64	1.28	8.24	1.87	3.74	0.43	3.95	6-16
INTI (CBKT) A	2	14.989	0.99	1.99	15.006	0.99	1.97	0.11	2.80	6-16
INTI (CBKT) B	2	14.951	0.94	1.88	15.006	0.99	1.97	0.37	2.73	6-16
KEBS (CBKT) A	2	12.188	0.81	1.63	12.20	0.82	1.64	0.10	2.31	6-16
KEBS (CBKT) B	2	12.117	0.85	1.70	12.20	0.82	1.64	0.68	2.36	6-16
LATU (CBKT) A	2	14.758	0.93	1.87	14.861	0.85	1.71	0.69	2.53	6-16
LATU (CBKT) B	2	15.156	0.94	1.89	14.861	0.85	1.71	-1.99	2.55	6-16

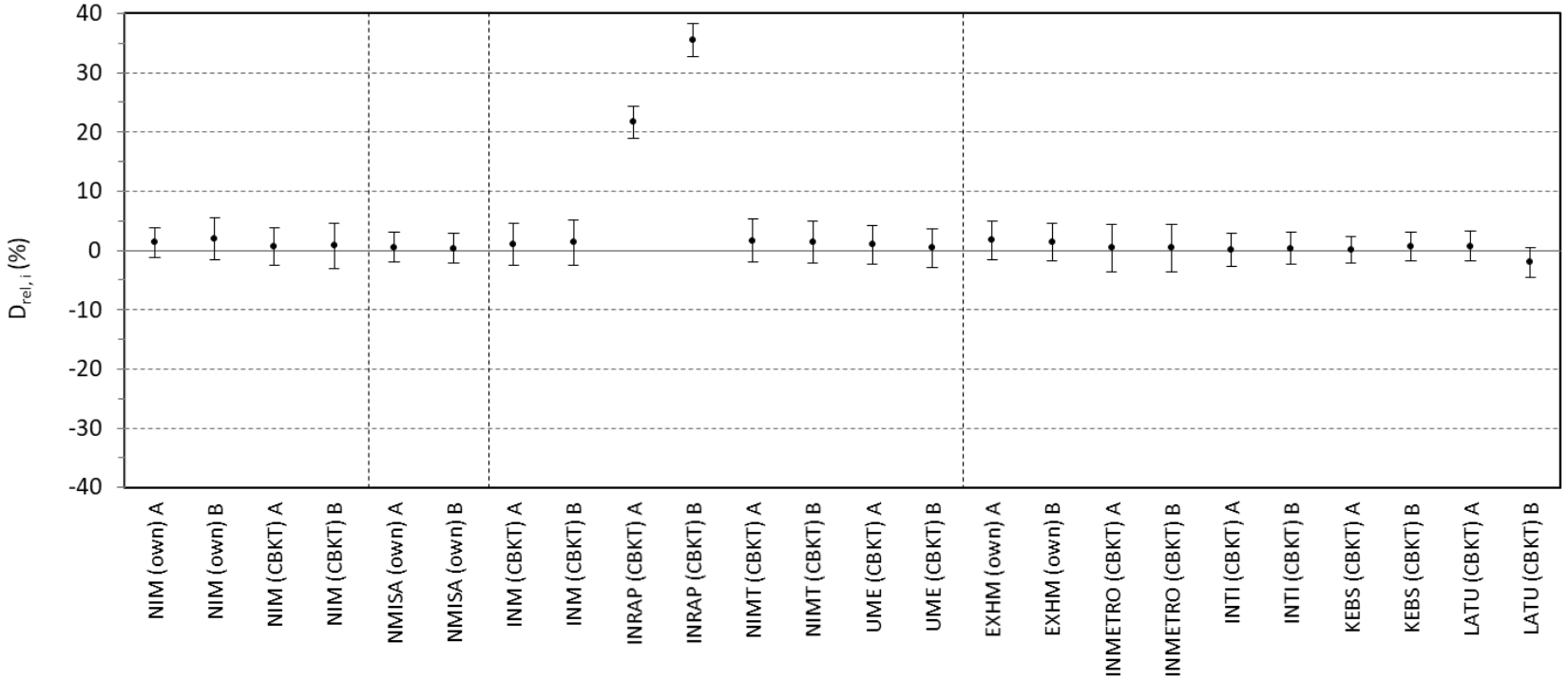

Figure 11: Relative values for the degree of equivalence for CCQM-K154.b

CONCLUSIONS

AfB_1 was selected to be representative of polar *Aspergillus* mycotoxins. It was anticipated to provide a challenge representative for the gravimetrical preparation and value assignment of calibration solutions in the mass fraction range of 2 mg/kg to 50 mg/kg of mycotoxins with broadly similar structural characteristics.

Nine participants of the MMCBKT programme were provided with a stock solution having a known AfB_1 mass fraction and expanded uncertainty to use to gravimetrically prepare and value assign a calibration solution. Three NMIs/DIs also participated using their own calibration solutions. The use of in-house solutions required an additional capacity to undertake a fit-for-purpose purity assessment. NIM was the only NMI participating using both the MMCBKT based and their own in-house assigned solutions in order to connect the two different groups.

It was decided to propose separate KCRVs for each of the two ampoules provided by the participating NMIs/DIs based on the AfB_1 mass fraction. This allowed participants to demonstrate the efficacy of their implementation of the approaches used to gravimetrically prepare calibrations solutions and to assess the AfB_1 mass fraction.

The majority of the AfB_1 mass fraction KCRVs (w_{KCRV}) for CCQM-K154.b spanned a mass fraction range of 2.02 mg/kg to 31.57 mg/kg. The relative expanded uncertainties $U(w_{KCRV})$ ranged from 0.69 % to 2.93 %.

Inspection of the degree of equivalence plots (Figures 10 and 11) for the AfB_1 mass fraction assignments in CCQM-K154.b indicated that there was an excellent agreement of results. Solely, the AfB_1 mass fraction assignments of INRAP, Tunisia did not agree with the KCRVs. As discussed earlier it was found that the samples were altered as a result of an acid contamination that must have provoked a partly conversion of AfB_1 into AfB_{2a}.

HOW FAR THE LIGHT SHINES STATEMENT (HFTLS)

Successful participation in CCQM-K154.b for MMCBKT participants will support CMCs for:

a) Preparation and value assignment of Aflatoxin B1 calibration solutions in the mass fraction range of 2 mg/kg to 50 mg/kg, prepared from a mycotoxin stock solution or solid of known purity.

Successful participation in CCQM-K154.b for other participants (having value assigned their pure Primary Reference Materials) will support CMCs for:

a) purity value assignment capabilities of organic materials with molar mass in the range 100 g/mol to 500 g/mol and polarity (*pKow*) > -2, with relative uncertainties at or above the relative uncertainty achieved in the comparison for calibration solutions;

Preparation and value assignment of single component organic calibration solutions in the mass fraction range of 2 mg/kg to 50 mg/kg, polarity (*pKow*) > -2, with molar mass in the range of 100 g/mol to 500 g/mol.

ACKNOWLEDGEMENTS

The study coordinators thank all of the participating laboratories for providing all the requested information and excellent collaboration during the course of these studies.

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ANNEX A – ADDITIONAL ANALYTICAL INFORMATION

Hellenic Metrology Institute (EXHM), Greece

Solution preparation procedure

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)

Powder aflatoxin B1 (AfB1) was purchased from n'TOX. The purity of AfB1 was determined with qNMR using of 3,5 BTFMBA NMIJ CRM 4601-a as the internal standard. HSQC, COSY, 1H, 13C were also acquired for the pure compound. Additionally, purity (as regards the related structure impurities) was also checked and confirmed by LC-UV/FLD analysis of the n'TOXmaterial.

Amount of primary calibrator used for analysis

-/-

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo UMX 5 Comparator (0.0001 mg) and Mettler Toledo 105 MS (0.01 mg) calibrated with E1 weights traceable to the Hellenic Metrology Institute (EIM).

Balance repeatability

sd = 0.25 μg and 0.04 mg respectively for the aforementioned balances, respectively

Solution preparation procedure

An intermediate solution was initially prepared by dissolving AfB1 (0.0094680 g) in acetonitrile (3.97330 g) in an amber glass vial. For the preparation of the final solution, 0.55120 g of the intermediate solution were transferred in a bottle containing acetonitrile (129.2496 g) which was then sealed and weighed. The resulting final solution was mixed, left overnight at 4 °C and then was subdivided in 4 mL CERTAN vials.

Homogeneity and/or stability testing

For the homogeneity test: 10 vials were analyzed in duplicate with LC-DAD (at 223, 263 and 360 nm) and LC-FLD (excitation at 360 nm and emission at 435 nm). The short-term stability

was examined with isochronous study at four different temperatures (-18 °C as reference, 4 °C, 20 °C and 40 °C) and at different periods of time (zero, one, two, four and eight weeks). For each combination condition (time and temperature) two samples tested and analysed in duplicate. The uncertainties of homogeneity and stability were included in the combined uncertainty.

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

The verification of the assigned value (from the gravimetric preparation) was performed with LC-DAD (at 223, 263, 360 and 254 nm). The mobile phase was constituted of: A=H2O, B=acetonitrile:methanol (50:50) in the following gradient mode: A=70 %,B=30 % for t=0 min, then the composition of B was increased to B= 90 % in the next 30 min, then to B= 100 % in the next 1 min and hold for 9 min. The composition returned to A=70 %, B=30 % in the next 2 min and the column was equilibrated for 8 min (total run time 50 min). For the chromatographic separation the column Inertsil ODS-3, 250 x 2.1 mm, 5 µm from MZ was used.

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

The verification of the assigned value was performed with bracketing (exact match) calibration using benzo[a]pyrene as internal standard.

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

The calibrants used for the verification were: 1) Powder AfB1 from ACROS ORGANICS (which its purity determined with qNMR) and solutions thereof were used as independent calibrants and 2) certified reference material aflatoxin B1 in acetonitrile with mass concentration 2.03 ± 0.05 µg/mL from Dr. Ehrenstorfer/LGC Standards. The reference material ERM-AC057 with mass fraction 3.79 +/- 0.11 µg/g from IRMM was also used for confirmation purposes.

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

-/-

Additional Comments or Observations

The combined standard uncertainty was calculated with the contribution of the uncertainty due to: 1) preparation (u_{prep}= 0.34 %), 2) homogeneity (u_{homo}= 0.31 %), 3) stability (u_{stab} = 1.33 %) and characterization (u_{char} = 0.23 %).

The measurement equation is:

$$w_{AfB1} = p_{AfB1}\frac{m_{AfB1}}{m_{AfB1}+m_{ACN,i}} \times \frac{m_{isol}}{m_{isol}+m_{ACN,F}} \times \frac{1000g}{kg}$$

where
w_{AfB1} = mass fraction of the analyte (AfB1) in the sample, (mg/kg)
p_{AfB1} = purity of AfB1, (mg/g)
m_{AfB1} = the mass of AfB1 in the intermediate solution (g)
$m_{ACN,i}$ = the mass of acetonitrile in the intermediate solution (g)
m_{isol} = mass of intermediate solution (g)
$m_{ACN,F}$ = mass of acetonitrile added to the intermediate solution (g)

The equation used to estimate standard uncertainty from preparation is:

$$u(w_{AfB1PREP}) = \sqrt{(C_P u_{PAfB1})^2 + \sum (C_i u(m_i))^2}$$

where u_{PAfB1} is the uncertainty for AfB1 purity determination, and C_i the sensitivity coefficients associated with the masses involved in the preparation stage. The purity of the AfB1 was determined by qNMR using NMIJ CRM 4601a as IS. The purity of AfB1, regarding the related structure impurities, was determined by the HPLC-DAD/FLD.

Purity was calculated with qNMR via the following equation by :

$$P_{AfB1} = \frac{I_s}{I_{Std}}\frac{n_{Std}}{n_s}\frac{M_s}{M_{std}}\frac{m_{Std}}{m}P_{std}$$

P_{std}: purity of the internal standard
m_{std}: mass of internal standard
M_{std}:molecular weight of internal standard
n_{std} :number of protons of the quantification peak of internal standard
I_{std}: integral area of quantification peak of internal standard
m_s: mass of the AfB1
n_s:number of protons integrated for the quantification of each compound
Is: integral area of the respective quantification peak of the AfB1
P_{AfB1}: mass fraction of compound analysed

Finally, the combined standard uncertainty of the concentration of AfB1 in the solutions delivered to BIPM is estimated as the sum of squares due to preparation, characterization, homogeneity and stability issues encountered in solution production:

$$u(w_{AfB1}) = \sqrt{(u_{PREP})^2 + (u_{CHAR})^2 + (u_{HMG})^2 + (u_{STAB})^2}$$

Uncertainty estimation was carried out according to JCGM 100: 2008 and the relevant components were calculated according to the procedures outlined in ISO 17034 and ISO GUIDE 35 (Reference materials -Guidance for characterization and assessment of homogeneity and stability).

qNMR uncertainty budget

uncertainty component	value	units	u_i	u_i/x_i	C_i	C_iu_i	$(C_iu_i)^2$
signal ratio Afl B1/BTFMBA	0.4540		0.00131	2.885E-03	2193.17	2.87306	8.254E+00
Aflatoxin B1 molar mass	312.280	g mol^{-1}	0.00508	1.627E-05	3.19	0.01620	2.625E-04
BTFMBA molar mass	258.117	g mol^{-1}	0.00522	2.024E-05	-3.86	-0.02015	4.059E-04
number of ^{1}H in AFL B1	2	mol/mol	0.00004	1.800E-05	-497.85	-0.01792	3.212E-04
number of ^{1}H in BFTMBA	1	mol/mol	0.00002	1.800E-05	995.70	0.01792	3.212E-04
mass of Afl B1	1.6195	mg	0.00006	3.710E-05	-614.82	-0.03695	1.365E-03
mass of BTFMBA	5.874	mg	0.00008	1.407E-05	169.51	0.01401	1.963E-04
boyancy correction factor	1.00001		0.00000	4.065E-06	995.70	0.00405	1.638E-05
BTFMBA purity	999.59	mg g^{-1}	0.26000	2.601E-04	1.00	0.25899	6.708E-02
Afl B1 purity							995.71
combined standard uncertainty							**2.89**
expanded uncertainty (k=2)							5.77

Preparation data and uncertainty

			standard	relative	sensitivity		
AfB1 - intermediate	value	units	uncertainty	uncertainty	coefficient	C_iu_i	$(C_iu_i)^2$
AfB1	0,0094680	g	0,000003	0,00032	0,24941	7,48E-07	5,60E-13
MeCN	3,97330	g	0,000046	0,00001	-0,00059	-2,72E-08	7,38E-16
AfB1 purity (qNMR)	0,99570		0,003350	0,00336	0,00238	7,96E-06	6,34E-11
concentration	2,367	mg/g					
combined std uncertainty	0,008	0,000					
relative uncertainty	0,338	(%)					
coverage factor	2						
expanded uncertainty	0,016	mg/g					
			standard	relative	sensitivity		
AfB1 FINAL	value	units	uncertainty	uncertainty	coefficient	C_iu_i	$(C_iu_i)^2$
AfB1 - intermediate	0,55120	g	0,000163	0,00030	0,01816	2,97E-06	8,81E-12
MeCN	129,24960	g	0,002145	0,00002	-0,00008	-1,66E-07	2,76E-14
concentration	2,367	g/g	0,007999	0,00338	0,00425	3,40E-05	1,15E-09
concentration	10,052	mg/kg					
combined std uncertainty	0,034	mg/kg					
relative uncertainty	0,339	(%)					
coverage factor	2						
expanded uncertainty	0,068	mg/kg					

UNCERTAINTIES (mg/kg)						
AfB1 conc (mg/kg)	u_{prep}	u_{homo}	u_{stab}	u_{char}	combined	expanded (k=2)
10,052	0,034	0,031	0,134	0,023	0,143	0,287
relative (%)	0,339	0,308	1,330	0,229	1,43	2,85

Instituto Nacional de Metrologia (INM), Colombia

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

OGP 030 AfB1, stock solution (Provided by BIPM)
Metrological traceability: through Aflatoxin B1, OGO.193a (BIPM)
Mass fraction: 129 ug/g ± 2.1 ug/g (k=2)

Amount of primary calibrator used for analysis

3.08 g stock solution, 1.03 g working solution

Gravimetry

Type of balance (make, model and resolution)

Make: Mettler Toledo
Model: XPE504
Resolution: 0.1 mg

Balance repeatability

40 µg

Solution preparation procedure

The preparation was gravimetrically realized, 11.7945 g of the OGP.030 solution was weighed and acetonitrile (LC-MS grade) was used as the solvent. 380.2230 g of the solution was obtained with a mass fraction of 4.001 ug/g ± 0.033 ug/g (gravimetrical value).

Homogeneity and/or stability testing

The material uncertainty budget includes sources as:
Homogeneity: The homogeneity was evaluated using 10 units of the material. Ten replicates by the unit were measured under repeatability conditions through UV-Vis spectrophotometry.

Short term stability: The short term stability was evaluated using an isochronous and accelerated design, The material was exposed to 40 °C for three weeks (the reference temperature was -20 °C), two ampoules per time were taken, for a total of five points. The AfB1 determination was realized through LC-DAD, five replicates by each ampoule were measured under repeatability conditions.

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

Mobile phase:	Acetonitrile (A)	Water (B)
Programm (Isocratic)	60	40
Column temperature (°C)	40	Column C18 Scherzo SM C18 150 mm*2 mm* 3um
Injection volume (ul)	5	
Flow (ml/min)	0.4	
Run time (min)	4	

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

Two points -calibration (bracketing).

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

OGP 030 Stock solution
129 ug/g ± 2.1 ug/g (k=2)
Metrological traceability: through Aflatoxin B1 OGO.193a (BIPM)

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

-/-

Additional Comments or Observations

-/-

Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO), Brazil

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

OGP.030 standard AfB1 solution provided by BIPM: (129.0 ± 2.1) µg/g; k=2, 95 %.

Amount of primary calibrator used for analysis

0.5 g

Gravimetry

Type of balance (make, model and resolution)

Balance 1: Mettler Toledo, model XS205, resolution 0.0001g (used for OGP.030); Balance 2: Mettler Toledo, model PR1203, resolution 0.001g (used for whole solution).

Balance repeatability

100 µg for model XS205 and 300 µg for model PR 1203

Solution preparation procedure

Solution was measured directly without further dilution.

Homogeneity and/or stability testing

Homogeneity study was performed with 10 ampoules randomly selected (batch of 60 ampoules) using LC-DAD/MS-MS method described. The main compound (AfB1) was measured by DAD and MS, and related impurities was measured only by MS method (only for additional information). The homogeneity contribution of 0.60% was included on uncertainty of assigned value. The short-term stability study showed that samples can be transported up to 20 °C during 4 weeks, without changes on certified value.

Optional: Analytical check method

Chromatographic Conditions

(e.g., GC temperature program, LC mobile phase and gradient)

UPLC-MS/MS-PDA, XEVO TQ Waters

Column:	ACE C18 100Å, 250 x 4.6 mm, 5 µm
Mobile phase:	A = H2O ; B = Acetonitrile + Methanol (50:50 v/v). Solvent gradient: 10 % B (initial); 20 % B (5 min); 35 % B (10 min); 45 % B (20 min); 50 % B (25 min); 60 % B (27 min); 90 % B (30 min); 100 % B (31 min); 100 % B (32 min); 10 % B (34 min); 10 % B (40 min)
Flow rate:	0.6 mL/min ; injection volume: 10 µL; run time: 40 min
Detector:	DAD: 360 nm; Resolution: 4.8 nm

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

Bracketing. Two calibration solutions was diluted using BIPM OGP.030 to check the gravimetric preparation of AfB1 batch (bracketing, using ± 5 % target mass fraction).

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

BIPM OGP.030 (Aflatoxin B1 in acetonitrile): (129.0 ± 2.1) µg/g ; k=2, 95%.

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

Aflatoxin B1: 311.3>296.0 for quantification; 311.3 >283.0 for confirmation (ES-)
Aflatoxin B2: 315.4>287.2 for quantification; 315.4>259.1 for confirmation (ES+)
Aflatoxin B2a: 329.2>258.1 for quantification; 329.2>243.2 for confirmation (ES-)
Other impurities monitored, but not present in material: AfG1, AfG2, AfM1, AfM2,AfQ1, AfP1, DIOL

Additional Comments or Observations

The value assigned of Aflatoxin B1 (AfB1) is the combination of gravimetric preparation 8.24 µg/g with u_{char} of 0.146 µg/g (1.77 %) and the homogeneity contribution of u_{bb} of 0.049 µg/g (0.60 %). For additional information on homogeneity study, UV spectrophotometric analysis are also performed directly without dilution against pure acetonitrile (ACN) as reference by use of the scan method (190 - 370 nm) and by the wavelength method at 223 nm, 263 nm, 355 nm and 360 nm.

Institut National de Recherche et d'Analyse Physico-Chimique (INRAP), Tunisia

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

OGP.030 standard AfB1 solution provided by BIPM.

Amount of primary calibrator used for analysis

9.2924

Gravimetry

Type of balance (make, model and resolution)

make: sartorius
model: MC 410 S
resolution: 100 µg

Balance repeatability

54.8 µg

Solution preparation procedure

The gravimetric procedure was applied for the preparation of the calibration solution.

Homogeneity and/or stability testing

Homogeneity and stability were tested according to the calibrant Assessment Guideline: Aflatoxin B1 report BIPM-2019/07. The uncertainty of the homogeneity and the stability was not taken into account in the uncertainty value of the mass fraction.

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

Stationary Phase: Agilent Zorbax SB-C18 (250*4.6)5µm

Mobile Phase:	ACN:MeOH:H2O Milli Q (20:20:60) %
Injection Volume:	10µl
Column temperature:	25°C
λ =360-362 nm	

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

External calibration prepared from Stock solution OGP.030 standard AfB1 solution provided by BIPM.
Bracketing..

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

External calibration prepared from Stock solution OGP.030 standard AfB1 solution provided by BIPM.

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

Mass Spectrometer was not applied.

Additional Comments or Observations

-/-

Instituto Nacional de Tecnología Industrial (INTI), Argentina

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

Aflatoxin B1 (AfB1) in acetonitrile (ACN) stock solution. BIPM Reference: OGP.030. Purity of main component and uncertainty: 129.0 ± 2.1 µg/g (k = 2). Combination of AfB1 gravimetric preparation 128.95 µg/g with u_{char} of 0.30 ug/g (0.235 %), homogeneity contribution of u_{bb} of 0.31 µg/g (0.237 %) and stability contribution of u_{lts} of 0.93 µg/g (0.720 %). Used ampoules: OGP.030.006, OGP.030.046, OGP.030.075, OGP.030.155, OGP.030.194

Amount of primary calibrator used for analysis

15.4806 g

Gravimetry

Type of balance (make, model and resolution)

Analytical Balance Sartorius LA230P.
Resolution: 0.1/0.2/0.5 mg (at 60/120/230 g respectively)

Balance repeatability

100/200/500 µg (at 60/120/230 g respectively)

Solution preparation procedure

The calibrant solution preparation was carried out weighing the content of 5 ampoules of stock solution into a 250 ml plastic bottle. The plastic bottle was filled up to 133-134 grams with acetonitrile. The exact final weight was considered. 57 clear glass ampoules containing 3 ml of calibrant solution were produced. Batch was called 2020-AfB1-DAI.

Homogeneity and/or stability testing

Homogeneity study was carried out considering 10 ampoules from the batch 2020-AfB1-DAI. Homogeneity was assessed by spectrophotometry UV and HPLC-DAD at 223 nm, 264 nm and 354 nm. The result obtained by HPLC-DAD at 223 nm was considered as inhomogeneity of batch 2020-AfB1-DAI (u*bb = 0.344 %).

Stability study was carried out following an isochronous experiment design. Three different conditions were considered: 4 °C in dark, 25 °C in dark and 25 °C under light conditions during 1, 2 and 4 weeks. The reference condition was -20 °C in dark. Two ampoules were measured for each temperature and each period of time. Spectrophotometry UV and HPLC-DAD were used to evaluate the stability. The results of the stability studies of 2020-AfB1-DAI did not show decreasing trends for 4 °C in dark and 25 °C in dark as storage conditions for AfB1. However, there is a significant decreasing trend based on statistical analyses of the results at 25 °C under light as storage condition for AfB1. It was concluded that 25 °C and light conditions should be avoid during shipment and storage. All samples were analysed directly without dilution in a stratified random order. ISO Guide 35 (2017) was considered for all studies. The stability results for 4 weeks and 25 ºC in dark obtained by HPLC-DAD at 223 nm, 264 nm and 354 nm were considered as uncertainty related with stability (u_{lts} = 0.440 %). 4 weeks and 25 ºC in dark was considered as a possible condition during transport of MRC.

Optional: Analytical check method

Chromato graphic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

HPLC Conditions:
Column: Kromasil 100-5-C18 (4.6 x 250mm) (Batch/Serial: 0000139143/E173118)
Mobile Phase: (Water 0.1% ACOOH + ACN 0.1% ACOOH) (45 + 55)
Flow rate: 1 mL/min
Run Time: 12 min
Retention time of AfB1: 4.3 min
Oven temperature: 30 °C
Injection Volume: 20 µL
Detector Wavelengths: 223 nm, 264 nm and 354 nm

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

-/-

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

-/-

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

-/-

Additional Comments or Observations

The assigned uncertainty is a combination of AFB1 gravimetric preparation uncertainty (u_{char}) of 0.122 µg/g (0.813 %), the homogeneity contribution of u_{bb} of 0.052 µg/g (0.344 %) and stability contribution of u_{lts} of 0.066 ug/g (0.440 %).

Mass fraction assignment

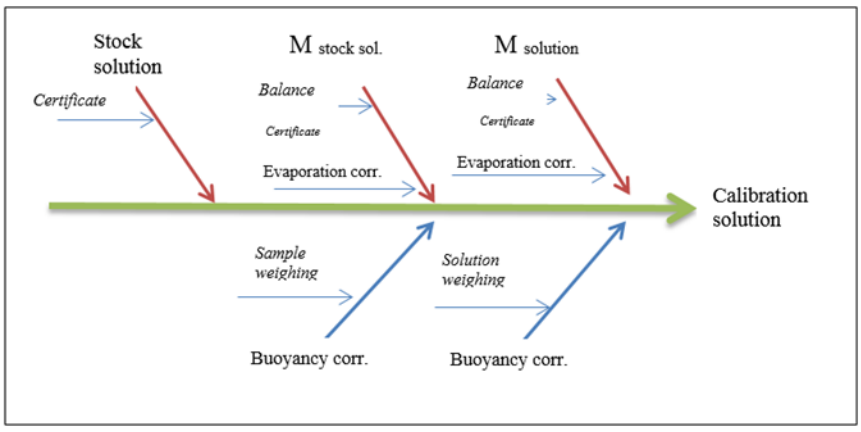
$$W_{AfB1\ calib\ solution} = \frac{w_{AfB1\ stock} \cdot m_{bal\ stock} \cdot b_{balanza} \cdot Ev}{m_{bal\ Sol} \cdot b_{balanza} \cdot Ev}$$

Calibrant Solution Preparation	
Stock Solution (mg) ($m_{bal\ stock}$) (mg)	15480.6
Whole Solution ($m_{bal\ sol}$) (mg)	133080.5
Stock Solution Concentration ($W_{AfB1\ stock\ solution}$) (	**129**
Buoyancy Sartorius LA230P ($b_{Sartorius\ LA230P}$)	1.001376
Evaporation Correction (Ev)	1
Calibrant Solution Concentration ($W_{AfB1\ calibration}$	**15.006**

Uncertainty assignment (Gravimetric Procedure)

$$u_{c,rel}(W_{AfB1-calib\ sol}) = \sqrt{\left\{\frac{u_c(w_{AfB1\ stock})}{w_{AfB1\ stock}}\right\}^2 + \left\{\frac{u_c(m_{bal\ stock\ sol})}{m_{bal\ stock\ sol}}\right\}^2 + \left\{\frac{u_c(b_{Sartorius\ LA230P})}{b_{Sartorius\ LA230P}}\right\}^2 + \left\{\frac{u_c(m_{bal\ sol})}{m_{bal\ sol}}\right\}^2 + \left\{\frac{u_c(b_{Sartorius\ LA230P})}{b_{Sartorius\ LA230P}}\right\}^2 + \left\{\frac{u_c(E_v)}{E_v}\right\}^2 + \left\{\frac{u_c(E_v)}{E_v}\right\}^2}$$

Resource of Uncertainty	Value
$\frac{u_c(w_{ZEN\ stock})}{w_{ZEN\ stock}}$	1.05
$\frac{u(m_{bal\ stock\ sol})}{m_{bal\ stock\ sol}}$	0.00065
$\frac{u_c(m_{bal\ sol})}{m_{bal\ sol}}$	0.00065
$\frac{u_c(b_{Sartorius\ LA230P})}{b_{Sartorius\ LA230P}}$	0.000012
$\frac{u_c(b_{Sartorius\ LA230P})}{b_{Sartorius\ LA230P}}$	0.0000115
$\frac{u(Ev)}{Ev}$	0.00005
$u_{c,rel}(w_{AfB1-calib\ sol})$	0.8140
$U_{C,rel}\ (w_{ZEN-calib\ sol})$(k=2)	1.6280
$u_{c,}(w_{ZEN-calib\ sol})$ µg/g	0.122
$U_{c,}(w_{ZEN-calib\ sol})$ µg/g(k=2)	0.244
U_c (%)	**1.63**



Kenya Bureau of Standards (KEBS), Kenya

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

OGP.030.

Amount of primary calibrator used for analysis

-/-

Gravimetry

Type of balance (make, model and resolution)

Sartorius ME 414 S

Balance repeatability

100 µg

Solution preparation procedure

Each ampoule broken then contents transferred to a 250 mL volumetric flask then topped with CAN. The weight of the volumetric flask was tared and weight of the solution from each ampoule taken. The final weight after topping with ACN was recorded.

Homogeneity and/or stability testing

This was not done.

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

Only mass preparation approach done.

Calibration type / details

(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

Only mass preparation approach done.

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

Only mass preparation approach done.

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

Only mass preparation approach done.

Additional Comments or Observations

-/-

Laboratorio Technologico del Uruguay (LATU), Uruguay

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

OGP.030 standard AfB1 solution provided by BIPM.

Amount of primary calibrator used for analysis

12.1959 g

Gravimetry

Type of balance (make, model and resolution)

SARTORIUS
model MSE 524S
resolution 0.0001g

Balance repeatability

100 µg

Solution preparation procedure

1. Balance performance check, tare, 2. weigh empty flask, 3. transfer stock solution, 4. weigh stock solution, 5. ACN LC MS grade addition, 6. weigh final mass of calibration solution.

Homogeneity and/or stability testing

Homogeneity and stability studies were performed in HPLC with DAD detector. UV spectrophotometer analysis was discarded because during methodology validation, was detected trending in the analysis of the data. Probably because of the instability of the lamp.

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

HPLC DAD, oven temperature 25ºC
Phenomenex Gemini 5 u C18 110 A, 250 x 4,6 mm column
Mobile phase: A: ACN: MeOH (1:1) : H2O B: ACN 95 % (30:70)
Flow: 0,6 mL/min, A:B 50:50 isocratic .

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

-/-

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

-/-

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

-/-

Additional Comments or Observations

The uncertainty shown in the `RESULTS´ tab is a combination of gravimetric preparation (u = 0.814 %) and the homogeneity contribution ($u^*_{bb}$ = 0.260 %). Stability contribution estimated on a 2 week basis is not included in `RESULTS´ tab, u_{lts} = 0.710 %
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National Institute of Metrology (NIM), China (CBKT)

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

BIPM OPG.030, 129.0 ± 2.1 µg/g

Amount of primary calibrator used for analysis

3.9228 g

Gravimetry

Type of balance (make, model and resolution)

Sartorius ME235S 0.1 mg

Balance repeatability

100 µg

Solution preparation procedure

A 3.9228 ± 0.0001 g portion of the 129.0 ± 2.1 µg/g stock solution (about 5 mL at 20 ℃) was transferred to a 100 mL flask. The flask was diluted to the mark with acetonitrile and acetonitrile is weighted 74.5617 ± 0.0001 g (100 mL at 20 ℃). The solution with a final concentration of 6.448 µg/g AFB1 in acetonitrile was obtained. A 4 mL volume of the solution was dispensed into amber glass ampules under ice conditions. The ampules were sealed with an ampule sealer. A total of 25 ampules was produced and stored in a freezer at -18 ℃.

Homogeneity and/or stability testing

Homogeneity of the AFB1 solutions was tested by selecting 5 ampules of the 25 ampules. UHPLC-DAD method was applied for homogeneity measurement. The result of the homogeneity testing were subject to an analysis of variance (ANOVA). The results from homogeneity testing of AFB1 solutions are summarised in Table 1 as follows.
The isochronous stability study of AFB1 solutions was tested under 60 °C for 7 days. UHPLC-DAD method was applied for measurement. The results of the stability testing were evaluated using trend analysis. The results from stability testing of AFB1 solutions are summarised in Table 2 as follows.

Table 1

AFB1 in acetonitrile	
Mean	6.489 µg/g
SD	0.045 µg/g
N	5
s_{wb}	0.09 %
s_{bb}	0.26 %
u^*_{bb}	0.03 %
u_{bb}	0.26 %

Table 2

AFB1 in acetonitrile	
β_1	-0.174
β_0	6.377
s	0.075
t	4.3
$\lvert\beta 1\rvert < t^* s(\beta 1)$	yes
u	0.057
u_{st}	0.89 %

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

Shimadzu LC-20AD
column: Agilent Eclipse C18 100Å,(250 x 4.6 mm, 2.6 µm)
detection wavelength: 362 nm
Mobile phase: A) actonitrile:methanol = 50:50 (v/v)
B) H2O

gradient：

Time(min)	Mobile phase A
0	30 %
30	90 %
31	100 %
32	100 %
34	30 %
40	30 %

Flow rate: 1 mL/min
Injection volume: 10 µL

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

External calibration method was applied to assign the value of comparison sample. Based on the BIPM OGP.030 stock solution, a series of calibrators were gravimetrically prepared.

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Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

BIPM OPG.030, 129.0 ± 2.1 µg/g

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

None.

Additional Comments or Observations

None.

Uncertainty Budget		Uncertainty	
Source of uncertainty	parameter x	*u(x)*	*u(x)/x*
Stock solution	129.0	1.05	0.00814
M_{AFB1} (g)	3.9228	0.000397	0.000101
M_{ACN} (g)	74.5617	0.00385	0.0000515
Inhomogeneity	6.49	0.0195	0.0026
Instability	6.38	0.057	0.00893
Relative combined standard uncertainty(u_c)			0.0124
Relative expanded uncertainty(k=2)(U_c)			0.025
Uncertainty analysis results			
Cx	6.45	µg/g	
u_x	0.080	µg/g	
U_x(k=2)	0.16	µg/g	

National Institute of Metrology (NIM), China (own)

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)

NIM has produced gravimetrically a stock solution of 129.2 ± 1.4 µg/g by dissolving 5.138 ± 0.001 mg crystalline AFB1 (984.0 ± 9.9 mg/g) in 39.0876 g acetonitrile (50 mL at 20 °C). The purity value of AFB1 material was assigned by NIM with mass balance method and qNMR method. The main component of AFB1 material was assigned directly by qNMR method. HPLC-MS/MS method were applied to measure the content of organic impurities of AFB1 material; the moisture and inorganic impurities were determined by Karl Fischer and ICP-MS method. VOC content was detected by GC-ECD. Finally, a purity of 984.0 mg/g with a uncertainty ± 9.9 mg/g was finally attributed to the AFB1 pure material.

Amount of primary calibrator used for analysis

3.9076 g

Gravimetry

Type of balance (make, model and resolution)

Sartorius	ME235S	0.1 mg
Mettler-Toledo	XP E56Q	1 µg

Balance repeatability

100 µg	ME235S
4 µg	XP E56Q

Solution preparation procedure

A 3.9076 ± 0.0001 g portion of the 129.2 ± 1.4 µg/g stock solution (about 5 mL at 20 °C) was transferred to a 100 mL flask. The flask was diluted to the mark with acetonitrile and the acetonitrile is weighed 74.4681 ± 0.0001 g (100 mL at 20 °C). The solution with a final concentration of 6.443 µg/g AFB1 in acetonitrile was obtained. A 4 mL volume of the solution was dispensed into amber glass ampules under ice conditions. The ampules were sealed with an ampule sealer. A total of 25 ampules was produced and stored in a freezer at -18 °C.

Homogeneity and/or stability testing

Homogeneity of the AFB1 solutions was tested by selecting 5 ampules of the 25 ampules. UHPLC-DAD method was applied for homogeneity measurement. The result of the homogeneity testing were subject to an analysis of variance (ANOVA). The results from homogeneity testing of AFB1 solutions are summarised in Table 1 as follows.
The isochronous stability study of AFB1 solutions was tested under 60 °C for 7 days. UHPLC-DAD method was applied for measurement. The results of the stability testing were evaluated using trend analysis. The results from stability testing of AFB1 solutions are summarised in Table 2 as follows.

Table 1

AFB1 in acetonitrile	
Mean	6.440 µg/g
SD	0.045 µg/g
N	5
s_{wb}	0.33 %
s_{bb}	0.21 %
u^*_{bb}	0.13 %
u_{bb}	0.21 %

Table 2

AFB1 in acetonitrile	
β_1	-0.199
β_0	6.422
s	0.066
t	4.3
$\lvert\beta 1\rvert < t^* s(\beta 1)$	yes
u	0.050
u_{st}	0.78 %

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

Shimadzu LC-20AD
column: Agilent Eclipse C18 100Å,(250 x 4.6 mm, 2.6 µm)
detection wavelength: 362 nm
Mobile phase: A) actonitrile:methanol = 50:50 (v/v)
B) H2O

gradient：	Time(min)	Mobile phase A
	0	30 %
	30	90 %
	31	100 %
	32	100 %
	34	30 %
	40	30 %
Flow rate:	1 mL/min	
Injection volume:	10 µL	

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

External calibration method was applied to assign the value of comparison sample. Based on the NIM stock solution, a series of calibrators were gravimetrically prepared.

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

NIM stock solution, 129.2 ± 1.4 µg/g

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

None.

Additional Comments or Observations

None.

Uncertainty Budget		Uncertainty	
Source of uncertainty	parameter x	*u(x)*	*u(x)/x*
Stock solution	129.2	0.67	0.00519
M_{AFB1} (g)	3.9076	0.000397	0.000101
M_{ACN} (g)	74.4681	0.00385	0.0000515
Inhomogeneity	6.44	0.0135	0.0021
Instability	6.42	0.05	0.0078
Relative combined standard uncertainty(u_c)			0.0096
Relative expanded uncertainty(k=2)(U_c)			0.019
Uncertainty analysis results			
Cx	6.44	µg/g	
u_x	0.062	µg/g	
U_x(k=2)	0.13	µg/g	

National Institute of Metrology Thailand (NIMT), Thailand

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

OGP.030.097

Amount of primary calibrator used for analysis

3.1083 g

Gravimetry

Type of balance (make, model and resolution)

Mettler-Toledo XP2004S, 0.0001 g Resolution

Balance repeatability

1.54E-04 μg

Solution preparation procedure

AfB1 solution was gravimetrically prepared. An ampoule from BIPM OGP.030.097 stock solution was used. The stock solution was diluted with acetonitrile to the final volume of 250 mL.

Homogeneity and/or stability testing

Homogeneity testing: HPLC-PDA was used for homogeneity study. Ten randomly selected ampoules were analyzed in triplicates and statistically analyzed using One-way ANOVA.
Stability testing: HPLC-PDA was used for stability study. Twenty-six ampoules, stored at reference temperature of -20 °C, were randomly selected and analyzed in triplicates. Stability study was carried out using isochronous approach at 4 °C, 22 °C and 40 °C for 0,1,2,3 and 4 weeks. Trend analysis was performed to statistically test according to the conditions during the stability study. The slope was tested for its significance at 95 % confidence level.

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

LC-PDA conditions
Mobile phase: Isocratic elution with water:methanol:acetonotrile, 45:30:25 (v/v/v)
Total flow rate: 0.5 mL/min
Column temperature: 40 °C
Injection volume: 10 µL

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

One point bracketing external calibration.

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

Verification of the prepared standard solution was carried out by analyzing the prepared solution using a single point, bracketing external calibration. The pure aflatoxin B1 in acetonitrile obtained from NIM was used as calibration solution. Details of AfB1

	Certified value:	Expanded Relative Uncertainty (%, k=2)
Sorce: NIM	100 µg/mL	2

*The certified value of the standard was traceable to the SI unit of kg through gravimetric preparation and to the stated purity of the solid raw material.

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

-/-

Additional Comments or Observations

Reported value is based on gravimetric value. Measurement uncertainty was estimated from gravimetric preparation, homogeneity and stability studies.

measurement equation:

$$w(xi) = \frac{w_z * m_z}{m_{total}}$$

where;

w(xi)	mass fraction of the prepared solution, µg/g
w_z	mass fraction of the stock solution prepared from (OGP.030), µg/g
m_z	mass of the stock solution (OGP.030) added (g)
m_{total}	mass of the total solution (g)

Expanded measurement equation:

$$w(xi) = \frac{w_z * m_z}{m_{total}} . F_{stb} . F_{homo}$$

where;

w(xi)	mass fraction of the prepared solution, µg/g
w_z	mass fraction of the stock solution prepared from (OGP.030), µg/g
m_z	mass of the stock solution (OGP.030) added (g)
m_{total}	mass of the total solution (g)
F_{stb}	Stability factor, given a value of 1
F_{homo}	Homogeneity factor, given a value of 1

Combined measurement uncertainty:

$$\frac{u(w_{xi})}{w_{xi}} = \sqrt{\left(\frac{u(w_z)}{w_z}\right)^2 + \left(\frac{u(m_z)}{m_z}\right)^2 + \left(\frac{u(m_{total})}{m_{total}}\right)^2 + \left(\frac{u(F_{homo})}{F_{homo}}\right)^2 + \left(\frac{u(F_{stb})}{F_{stb}}\right)^2}$$

where;

$u(w_{xi})$	standard uncertainty of the prepared standard solution
$u(w_z)$	standard uncertainty of the stock standard solution obtained from the certificate (OGP.030)
$u(m_z), u(m_{total})$	standard uncertainties of masses estimaetd from the bias of balance and the precision of balance
$u(F_{homo})$	standard uncertainty due to homogeneity factor, estimated from ANOVA
$u(F_{stb})$	standard uncertainty due to stability testing at 4 °C, estimated from trend analysis

Uncertainty budget:

uncertainty source	Xi	uxi	uxi/xi	(uxi/xi)^2
Preparation				
AFB1 stock	129	1.05	0.008139535	6.6252E-05
mass AFB1 stock (g)	3.108275	0.00031	9.89927E-05	9.79956E-09
mass total (g)	195.297675	0.00060	3.0636E-06	9.38566E-12
combined gravimetric	2.053109311	0.016712592	0.008140137	6.62618E-05
Homogeneity	1	0.0061475	0.0061475	3.77918E-05
Stability @4°C	1	0.009737148	0.009737148	9.4812E-05
			sum (uxi/xi)^2	0.000198866
			(uxi/xi)	0.014101973
			ux	0.028952892
			U (k=2)	0.057905784
			U(%)	2.820394571

National Metrology Institute of South Africa (NMISA), South Africa

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)

Crystalline Aflatoxin B1 (AFB1) was sourced from Fermentek, Israel. The material was purity assigned by NMISA using quantitative NMR. QNMR of AFB1 purity was performed in deuterated chloroform, using Fluka TraceCERT dimethylterphthalate as internal standard with traceability established in-house to NIST PS1 Benzoic acid using QNMR. The purity value determined was 984.5 ± 8.8 mg/g (k=2).
The OBP.030 primary calibrator from BIPM was also used a calibrator to verify the NMISA value assignment. Although not used in the final value assignment of the solution, the mass concentration of NMISA CRM 0012 using the BIPM calibrator was 31.60 mg/g.

Amount of primary calibrator used for analysis

0.37536 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo	AX 26	0.002 mg
Mettler Toledo	MS12002TS	0.01 g

Balance repeatability

2 µg

Solution preparation procedure

1. A 25 µg/mL solution (CRM0012) was prepared by weighing ~37.5 mg in house value assigned AFB1 (CRM0011) and diluting in 1.5 L acetonitrile.
2. The solution was ampouled (4 mL) using the Ampulmatic 10 ampoule sealer, with the purge gas (nitrogen), and liquid filler accessories into 5 mL amber ampoules. The bulk solution was kept cool in an ice bath and continuously stirred with a magnetic stir bar during the dispensing process.
3. The ampoules were subject to leak testing overnight at 50 mbar and leaks gravimetrically identified.

Homogeneity and/or stability testing

Of the 323 ampoules prepared, 10 were selected at regular intervals across the batch for the homogeneity assessment. Two repeat aliquots, without dilution, from each ampoule were evaluated using LC-UV at 365 nm. The relative standard homogeneity uncertainty determined using ANOVA, was estimated as 0.47 % and is included in the combined uncertainty reported.
An isochronous stability assessment was carried out over 4 weeks, where 2 ampoules were stored at 4 ˚C, 20 ˚C and 60 ˚C, for 1, 2 and 4 weeks. All samples stored at 4 ˚C and 60 ˚C were stored in the dark, whilst samples stored at 20 ˚C were exposed to light (although packaged in amber ampoules) to monitor the expected degradation with exposure to light over this period. The stability of the solutions was evaluated using LC-UV, monitoring the change in concentration of AFB1. No significant trends were observed at 4 ˚C and 20 ˚C, whilst a significant trend was observed at 60 ˚C, although the relative u(Its) determined for 60 ˚C at 4 weeks was only 1.2 %.
A conservative, standard relative uncertainty of 1 % was included into the uncertainty estimate for stability of the solution.

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

A 2 µL injection of the undiluted aliquot of the AFB1 solution (CRM 0012) was injected onto a Phenomenex Synergi RP Polar column 80 Å, 150 x 4.6 mm, 4 µm (ANA0956) column. Aflatoxin B1 impurities were separated in 25 min during the isocratic phase (0.6 mL/min) of 55:45 aqueous mobile phase: acetonitrile/methanol (50/50) both containing 0.1 % formic acid. The isocratic phase was followed by a column wash with high organic solvent where some minor impurities were detected at 365 nm. UV detection was carried out at 254 nm, 274 nm and 365 nm where the 365 nm wavelength was used for homogeneity and stability assessment. The samples were simultaneous analysed by MS/MS with numerous MRM transitions (15) to tentatively identify potential structurally related impurities. Both the UV and MS detection supported the QNMR purity value assignment.

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

The concentration of the NMISA K154b solution (CRM 0012) was verified using the BIPM OGP.030 reference material and the value differed by 0.3 % (this result was not used in the value assignment and uncertainty estimate of the NMISA K154b (CRM 0012) solution).
Concentration of the impurities was determined by relative peak area percentage assuming relative response factors of 1, used to support/verify qNMR data.

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

NMISA AFB1 CRM0011 purity 984.5 mg/g with expanded uncertainty 8.8 mg/g (k = 2, 95 % level of confidence)
Mass fraction concentration traceable to SI through DMTP internal standard (Purity 997.0 mg/g with expanded uncertainty of 6.2 mg/g (k = 2, 95 % level of confidence) value assigned using QNMR internal standard NIST PS1 Benzoic acid (Purity 999.92 mg/g with expanded uncertainty -0.06 and + 0.04 mg/g (k = 2, 95 % confidence interval).

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

The identity and presence of structurally related impurities was evaluated using LC-MS and LC-MS/MS. Some of the MS/MS transitions in ESI positive that were used to detect potential Aflatoxin impurities were:
AFB1 313.1>240.9, 313.1>284.9; AFB1 8,9 epoxide 329.4>299.2; AFB2 315.1>258.9, 315.1>286.9; AFG1 329.1>199.9, 329.1>242.9; AFG2 331.1>245.0, 331.1 >312.9; AFM1 329.4>273.3, 329.4>301.1; AFM1 epoxide 347.2>329.2; Aflatoxin M2 331.3>273.3; Aflatoxin P1 299.2>271.4; Aflatoxin Q1 329.4>311.1

Additional Comments or Observations

In the original NMISA AFB1 crystalline material, 5 impurities were detected with a relative peak area percentage greater than 0.1% but less than 1 %.

National Metrology Institute of Turkey (UME), Turkey

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.030 standard AfB_1 solution provided by BIPM

BIPM OGP.030

Amount of primary calibrator used for analysis

6.1143 g

Gravimetry

Type of balance (make, model and resolution)

Sartorius MSA524S-100-DA 0.1 mg

Balance repeatability

100 µg

Solution preparation procedure

BIPM stock solution transferred from -20 ˚C to the room temperature and kept in dark condition until the solution reached to room temperature. An empty 250 mL volumetric flask placed on the balance and tared. BIPM stock added to flask (6.1143 g) then capped and tared. Acetonitrile (144.9663 g) added into the flask, capped and weighed. The solution was mixed and chilled. Solution filled into 5 mL amber glass ampules as 2.5 mL by ampulmatic, purged with argon then sealed.

Homogeneity and/or stability testing

Totally 76 ampoules were prepared and stored at -20 ˚C after preparation. Totally 13 ampoules were selected randomly by TRANS program for homogeneity testing. Isochronous stability testing was performed at 25 ˚C and 45 ˚C for 0, 1, 2 and 4 week time points. Two ampoules were selected by TRANS program randomly for each time point. Reference temperature was -20 ˚C. Homogeneity results were statistically evaluated by ANOVA and 0.93 % uncertainty reported for homogeneity. Stability results were evaluated by significance test on slope and 0.30 % uncertainty was reported for stability at 25 ˚C. At 45 ˚C stability test it was obvious that there is degradation.

.

Optional: Analytical check method

Chromatographic Conditions
(e.g., GC temperature program, LC mobile phase and gradient)

Agilent 1260 HPLC with DAD detector used at 365 nm. Fortis 150 mm x 4.6 mm 5 µm C18 column used for separation. Isocratic program with H2O:MeOH:ACN (5:4:1) at flow of 1 mL/min and at 35 ˚C column temperature used. 5 µL sample injected into column.

Calibration type / details
(e.g., single-point, bracketing /external calibration, internal standard calibration, IDMS)

Five-point external calibration used.

Calibration and/or Internal standards
(e.g., source, purity, and traceability of standards)

BIPM OGP.030

Indicate ion/MRM monitored in Mass Spectrometer (if applicable)

-/-

Additional Comments or Observations

Uncertainty Budget				
Source of uncertainty	Parameter x	Uncertainty u(x)	u(x)/x	
BIPM Stock Purity	129.0	1.05	0.0081	
mstock (g)	6.1143	0.00000233	0.00000038	
mACN (g)	144.9663	0.00005522	0.00000038	
Repeatability	5.11	0.0123	0.0024	
Homogeneity	5.11	0.0475	0.0093	
Stability	5.11	0.0147	0.0029	
Combined Standard Measurement Uncertainty, %			1.29	
Expanded Measurement Uncertainty, % (k=2)			2.58	
Concentration Value			5.11	µg/g
Standard uncertainty, u			0.07	µg/g
Expanded Uncertainty, U			0.14	µg/g