CCQM-K154.b.1

Subsequent Bilateral Key Comparison Study

Organic Solvent Calibration Solution

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

Final Report

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SUMMARY

The CCQM-K154 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 NMIs/DIs. In total, eleven NMIs/DIs participated in the original 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. INRAP, Tunisia repeated the study in a subsequent bilateral comparison CCQM-K154.b.1 with the BIPM.

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 original CCQM-K154 comparisons, agreed by the CCQM, were 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 original 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 [6]. 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.

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 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 [6]. 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}. The mechanism of formation of AfB_{2a} by acid induced addition of water to the Af_{B1} is reported in literature [7, 8].
INRAP, Tunisia repeated the study in a subsequent bilateral comparison CCQM-K154.b.1 with the BIPM as described in the following.

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 [9, 10, 11]. 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 [12]. 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 [13].

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 is a subsequent bilateral key comparison CCQM-K154.b.1 of a solution of AfB_1 in ACN gravimetrically prepared and value assigned by INRAP, Tunisia that took part within the framework of the MMCBKT, using a value assigned stock solution of AfB_1 in ACN supplied by the BIPM. The study schedule for CCQM-K154.b.1 is given in Table 1.

Table 1: CCQM-K154.b.1 Timetable

Action	Date
Stock solution distribution	until November 2019 (MMCBKT participants)
Study Proposal and draft protocol	October 2019 OAWG meeting
Approval of study	December 2022 OAWG meeting
Samples to coordinator	November 2022
Study number/registration	January 2023
Data to coordinator	January 2023
Comparison analysis at the BIPM	September 2023
Draft A report	December 2023
Draft B report	January 2024
Final report	February 2024

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 both comparisons CCQM-K154.b and CCQM-K154.b.1.

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 described in the final report of the original key comparison CCQM-K154.b [6] and in the corresponding Purity Evaluation Guideline: Aflatoxin B_1 [14] and Calibrant Assessment Guideline: Aflatoxin B_1 [15].

Six units of the AfB_1 stock solution, each containing a minimum of 4 mL of material, were distributed to INRAP, Tunisia on 29th November 2019 by express mail service in an insulated box equipped with temperature indicator within the course of the original study CCQM-K154.b. 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 ºC during shipping.

The samples were received on 3rd December 2019 by INRAP, Tunisia (only 4 days in transit) in good condition.

STUDY MATERIALS

The participant was required to gravimetrically prepare and ampoule their own (about 4 mL per ampoule) standard solution of aflatoxin B_1 (AfB_1) in acetonitrile and to send it to the BIPM for comparison measurements. The mass fraction value targeted (in the range 2 mg/kg to 50 mg/kg) is 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 as outlined in the original key comparison CCQM-K154.b [6].

STUDY GUIDELINE

INRAP 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. The participant was required to provide the estimate of the mass fraction of AfB_1 in the solution and its corresponding uncertainty based on the gravimetric preparation corrected for purity. INRAP provided results using the reporting sheet provided with the samples. The results were sent via e-mail to the study coordinator. Submitted results were considered final and no corrections or adjustments of analytical data were accepted.
INRAP submitted the samples on 24th October 2022 and returned the filled in data submission forms to the BIPM on 17th January 2023. There was a delay in delivery of only 2 days and ampoules were received in good condition for comparison.

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 2 for both CCQM-K154.b.1 and the original CCQM-K154.b [6]. The details of the gravimetric preparation, calculation of the AfB_1 mass fraction values and assessment of corresponding expanded uncertainties are described in the Annex A for CCQM-K154.b.1 for INRAP, Tunisia. The details for each NMI/DI that participated in the original key comparison CCQM-K154.b are described in the Final Report [6].

VALUE ASSIGNMENT PROCEDURE OF THE COORDINATING LABORATORY

The AfB_1 mass fraction assigned solutions provided by the INRAP, Tunisia was 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 1.5 µg/g to 6 µg/g of AfB_1.
A two-point calibration with external bracketing using an AfB_1 standard assigned at the BIPM was used for quantification and comparison.

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) [6]. The gravimetric preparation of the stock solution was performed in the same way as described in detail in the chapter ‘Gravimetric preparation and filling of AfB_1 stock solutions’ [6] and the mass fraction and its corresponding standard uncertainty was 99.14 ± 0.79 mg/g (k = *1*). 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 [15] and the mass fractions and their corresponding standard uncertainties were 2.60 ± 0.02 mg/kg (k = *1*) and 4.44 ± 0.04 mg/kg (k = *1*), respectively.

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 3 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 2: AfB_1 mass fraction values and corresponding uncertainties submitted by the NMIs/DIs CCQM-K154.b and **CCQM-K154.b.1**

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	CCQM-K154.b	own	10.052	0.143	2	0.287
INM, Colombia	CCQM-K154.b	CBKT	3.99	0.050	1.97	0.10
INMETRO, Brazil	CCQM-K154.b	CBKT	8.24	0.154	2	0.31
INRAP, Tunisia	CCQM-K154.b	CBKT	6.15	0.05	2	0.10
INTI, Argentina	CCQM-K154.b	CBKT	15.006	0.148	2	0.296
KEBS, Kenya	CCQM-K154.b	CBKT	12.20	0.1	2	0.2
LATU, Uruguay	CCQM-K154.b	CBKT	14.861	0.127	2	0.254
NIM, China	CCQM-K154.b	own	6.44	0.062	2	0.13
	CCQM-K154.b	CBKT	6.45	0.080	2	0.16
NIMT, Thailand	CCQM-K154.b	CBKT	2.053	0.029	2	0.058
NMISA, South Africa	CCQM-K154.b	own	31.69	0.38	2	0.76
UME, Turkey	CCQM-K154.b	CBKT	5.11	0.07	2	0.14
INRAP, Tunisia	**CCQM-K154.b.1**	**CBKT**	**3.5106**	**0.0335**	**2**	**0.067**

Table 3: 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 INRAP, Tunisia were each measured in triplicate by LC-DAD-MS/MS. The CCQM-K154.b comparison measurements were undertaken in one batch according to the target mass fraction range of about 2.6-4.4 mg/kg to achieve narrow and linear calibrations and a short injection sequence to minimize instrument drift.

About 300 µL of the INRAP 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 sequence. 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 sequence for the CCQM-K154.b.1 is given in Table 4.

Table 4: Detailed injection sequences for the different calibrations of CCQM-K154.b.1

Injection	Calibration (2.6-4.4 mg/kg)
1	Blank
2	Low-1
3	BIPM-A-1
4	High-1
5	Blank
6	Low-2
7	INRAP-X-1
8	High-2
9	Blank
10	Low-3
11	INRAP-Y-1
12	High-3
13	Blank
14	Low-4
15	BIPM-A-2
16	High-4
17	Blank
18	Low-5
19	INRAP-X-2
20	High-5
21	Blank
22	Low-6
23	INRAP-Y-2
24	BIPM-A-3
25	High-6
26	Blank
27	Low-7
28	BIPM-A-4
29	High-7
30	Blank
31	Low-8
32	INRAP-X-3
33	BIPM-A-5
34	High-8
35	Blank
36	Low-9
37	INRAP-Y-3
38	High-9
39	Blank
40	Low-10
41	BIPM-A-6
42	High-10
43	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 [16, 17]. 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) [18, 19] 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 [20]. Statistical approaches including GLS are discussed in detail by Duewer *et al*. [21].

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 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 4). The AfB_1 mass fraction and associated standard uncertainty of the INRAP 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 INRAP solutions (w_{BIPM}), corresponding standard u(w_{BIPM}) and expanded uncertainties U(w_{BIPM}) are listed in Table 5. The bracketing calibration with the values, standard uncertainties and UV peak area responses for the solutions submitted by INRAP, low and high mass fraction level calibrants and internal control samples for CCQM-K154.b.1 are depicted in Figure 1.

Additional inspection of the LC-MS/MS data of the INRAP samples of CCQM-K154.b.1 did not show the significant formation of AfB_{2a} that occurred in the INRAP samples of the original comparison CCQM-K154.b [6].

Table 5: AfB_1 mass fraction values and absolute corresponding and expanded uncertainties measured by the BIPM for CCQM-K154.b.1 INRAP ampoules

NMI/DI	w_{BIPM} *(mg/kg)*	$u(w_{BIPM})$ *(mg/kg)*	$U(w_{BIPM})$ *(mg/kg)*	*Quantification range (mg/kg)*
INRAP (CBKT) A	3.54	0.09	0.18	2.6-4.4
INRAP (CBKT) B	3.43	0.05	0.10	2.6-4.4

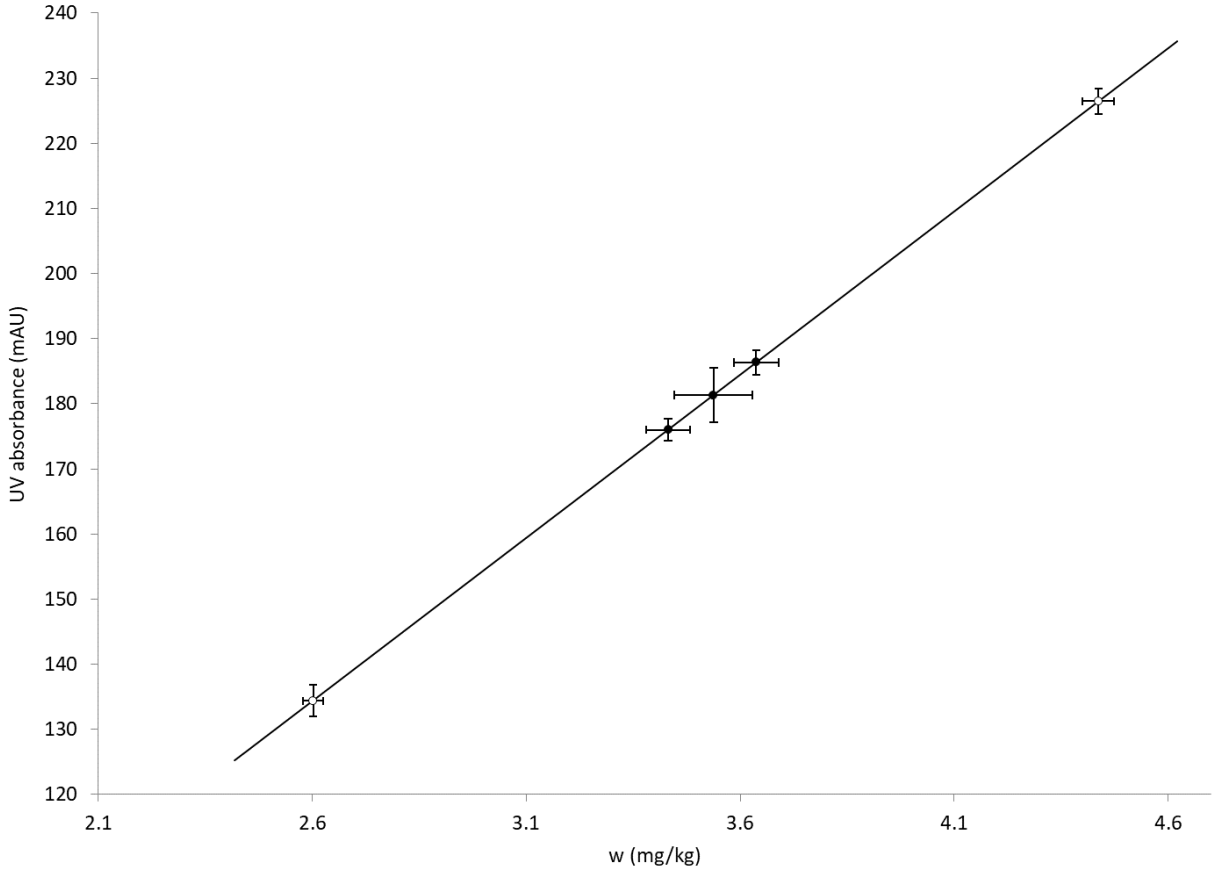

Figure 1: CCQM-K154.b.1 - Bracketing calibration for the AfB_1 mass fraction quantification range of 2.6-4.4 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at the upper and lower end of the calibration line. Inverse evaluation data of INRAP (CBKT) and internal control sample are depicted as dots.

KEY COMPARISON REFERENCE VALUES FOR CCQM-K154.b .1

The AfB_1 mass fraction values used to establish the Key Comparison Reference Values (KCRVs) for CCQM-K154.b.1 were assigned by the BIPM following the above mentioned calibration procedure ($w_{BIPM} = w_{KCRV}$). The same approach was used for the original comparison CCQM-K154.b [6] that is provided once more to facilitate assessment. For each ampoule, the KCRV is the AfB_1 mass fraction (w_{KCRV}) and its corresponding uncertainty ($u(w_{KCRV})$). All NMIs/DIs (i) participating in both CCQM-K154.b.1 and 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:

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

The AfB_1 mass fractions values and associated absolute uncertainties with degree of equivalences for both CCQM-K154.b.1 and CCQM-K154.b are listed in Table 6. Figure 2 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 7. Figure 3 indicates the relative degree of equivalence ($D_{rel,i}$) of each key comparison participant's result with the w_{KCRV}.

Table 6: AfB_1 mass fractions and absolute corresponding uncertainties with degree of equivalences

NMI/DI	Comparison	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	CCQM-K154.b	6.354	0.046	0.091	6.44	0.062	0.13	0.09	0.16	4-10
NIM (own) B	CCQM-K154.b	6.316	0.091	0.182	6.44	0.062	0.13	0.12	0.22	4-10
NIM (CBKT) A	CCQM-K154.b	6.405	0.060	0.120	6.45	0.080	0.16	0.04	0.20	4-10
NIM (CBKT) B	CCQM-K154.b	6.339	0.091	0.182	6.45	0.080	0.16	0.05	0.24	4-10
NMISA (own) A	CCQM-K154.b	31.51	0.16	0.31	31.69	0.38	0.76	0.18	0.82	29-35
NMISA (own) B	CCQM-K154.b	31.57	0.11	0.22	31.69	0.38	0.76	0.12	0.79	29-35
INM (CBKT) A	CCQM-K154.b	3.950	0.051	0.102	3.99	0.050	0.10	0.04	0.14	1-7
INM (CBKT) B	CCQM-K154.b	3.937	0.058	0.115	3.99	0.050	0.10	0.05	0.15	1-7
INRAP (CBKT) A	CCQM-K154.b	4.819	0.050	0.101	6.15	0.05	0.10	1.33	0.14	1-7
INRAP (CBKT) B	CCQM-K154.b	3.963	0.046	0.092	6.15	0.05	0.10	2.19	0.14	1-7
NIMT (CBKT) A	CCQM-K154.b	2.018	0.022	0.044	2.053	0.029	0.058	0.03	0.07	1-7
NIMT (CBKT) B	CCQM-K154.b	2.025	0.022	0.044	2.053	0.029	0.058	0.03	0.07	1-7
UME (CBKT) A	CCQM-K154.b	5.057	0.045	0.090	5.11	0.07	0.14	0.05	0.17	1-7
UME (CBKT) B	CCQM-K154.b	5.090	0.045	0.090	5.11	0.07	0.14	0.02	0.17	1-7
EXHM (own) A	CCQM-K154.b	9.882	0.071	0.142	10.052	0.143	0.287	0.17	0.32	6-16
EXHM (own) B	CCQM-K154.b	9.907	0.069	0.139	10.052	0.143	0.287	0.14	0.32	6-16
INMETRO (CBKT) A	CCQM-K154.b	8.205	0.053	0.105	8.24	0.154	0.31	0.04	0.33	6-16
INMETRO (CBKT) B	CCQM-K154.b	8.205	0.053	0.105	8.24	0.154	0.31	0.04	0.33	6-16
INTI (CBKT) A	CCQM-K154.b	14.989	0.149	0.298	15.006	0.148	0.296	0.02	0.42	6-16
INTI (CBKT) B	CCQM-K154.b	14.951	0.141	0.282	15.006	0.148	0.296	0.06	0.41	6-16
KEBS (CBKT) A	CCQM-K154.b	12.188	0.099	0.198	12.20	0.10	0.20	0.01	0.28	6-16
KEBS (CBKT) B	CCQM-K154.b	12.117	0.103	0.206	12.20	0.10	0.20	0.08	0.29	6-16
LATU (CBKT) A	CCQM-K154.b	14.758	0.138	0.276	14.861	0.127	0.254	0.10	0.37	6-16
LATU (CBKT) B	CCQM-K154.b	15.156	0.143	0.286	14.861	0.127	0.254	-0.30	0.38	6-16
INRAP (CBKT) A	**CCQM-K154.b.1**	**3.538**	**0.091**	**0.182**	**3.5106**	**0.0335**	**0.067**	**-0.03**	**0.19**	**2.6-4.4**
INRAP (CBKT) B	**CCQM-K154.b.1**	**3.432**	**0.051**	**0.103**	**3.5106**	**0.0335**	**0.067**	**0.08**	**0.12**	**2.6-4.4**

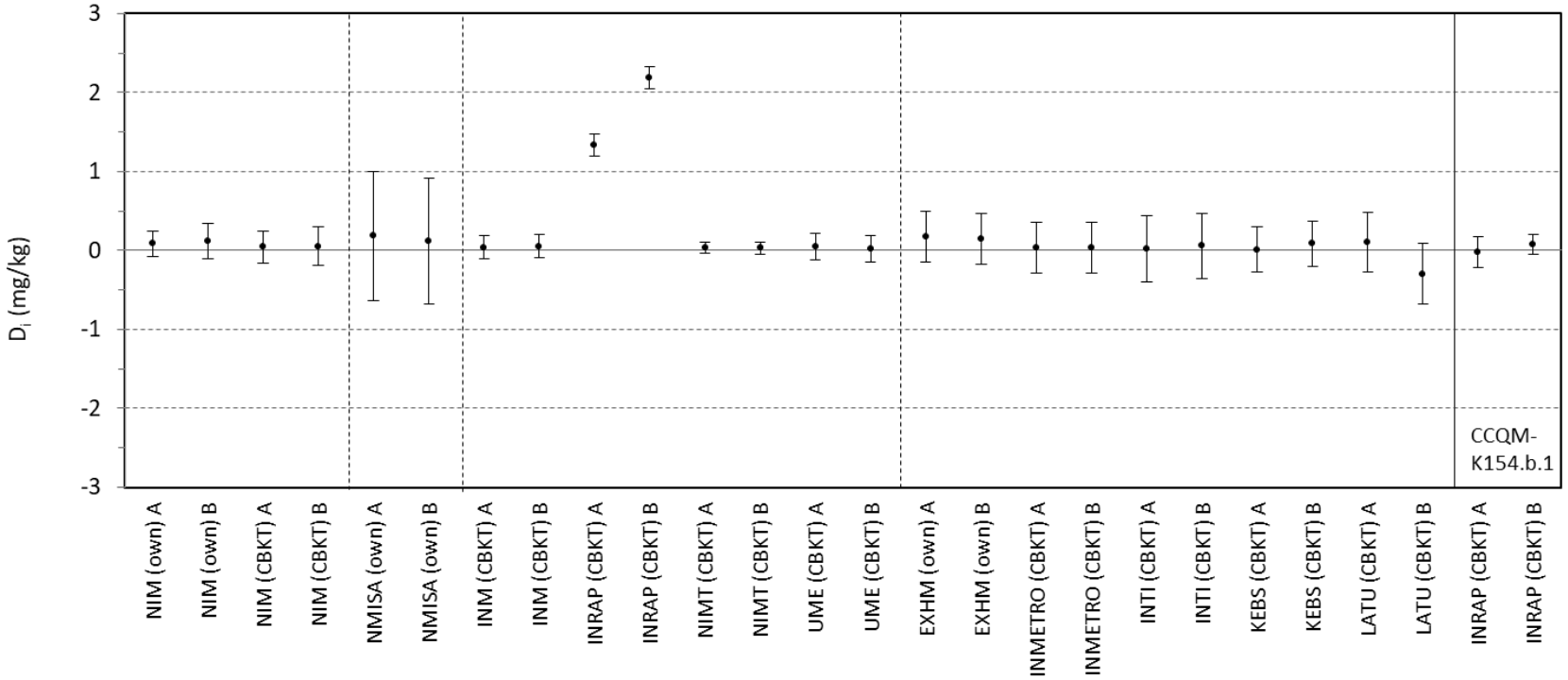

Figure 2: Absolute values for the degree of equivalence for both CCQM-K154.b and CCQM-K154.b.1

Table 7: AfB_1 mass fractions and relative corresponding uncertainties with relative degree of equivalences

NMI/DI	Comparison	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	CCQM-K154.b	6.354	0.72	1.43	6.44	1.01	2.02	1.33	2.48	4-10
NIM (own) B	CCQM-K154.b	6.316	1.44	2.88	6.44	1.01	2.02	1.92	3.52	4-10
NIM (CBKT) A	CCQM-K154.b	6.405	0.93	1.87	6.45	1.24	2.48	0.69	3.11	4-10
NIM (CBKT) B	CCQM-K154.b	6.339	1.42	2.84	6.45	1.24	2.48	0.79	3.77	4-10
NMISA (own) A	CCQM-K154.b	31.51	0.49	0.99	31.69	1.20	2.40	0.57	2.59	29-35
NMISA (own) B	CCQM-K154.b	31.57	0.34	0.69	31.69	1.20	2.40	0.37	2.50	29-35
INM (CBKT) A	CCQM-K154.b	3.950	1.29	2.58	3.99	1.24	2.48	1.00	3.58	1-7
INM (CBKT) B	CCQM-K154.b	3.937	1.46	2.93	3.99	1.24	2.48	1.33	3.84	1-7
INRAP (CBKT) A	CCQM-K154.b	4.819	1.05	2.09	6.15	0.81	1.63	21.64	2.65	1-7
INRAP (CBKT) B	CCQM-K154.b	3.963	1.16	2.31	6.15	0.81	1.63	35.56	2.83	1-7
NIMT (CBKT) A	CCQM-K154.b	2.018	1.09	2.17	2.053	1.41	2.83	1.69	3.56	1-7
NIMT (CBKT) B	CCQM-K154.b	2.025	1.08	2.16	2.053	1.41	2.83	1.37	3.56	1-7
UME (CBKT) A	CCQM-K154.b	5.057	0.89	1.78	5.11	1.37	2.74	1.03	3.27	1-7
UME (CBKT) B	CCQM-K154.b	5.090	0.89	1.78	5.11	1.37	2.74	0.40	3.27	1-7
EXHM (own) A	CCQM-K154.b	9.882	0.72	1.43	10.052	1.42	2.85	1.69	3.19	6-16
EXHM (own) B	CCQM-K154.b	9.907	0.70	1.40	10.052	1.42	2.85	1.44	3.17	6-16
INMETRO (CBKT) A	CCQM-K154.b	8.205	0.64	1.28	8.24	1.87	3.74	0.43	3.95	6-16
INMETRO (CBKT) B	CCQM-K154.b	8.205	0.64	1.28	8.24	1.87	3.74	0.43	3.95	6-16
INTI (CBKT) A	CCQM-K154.b	14.989	0.99	1.99	15.006	0.99	1.97	0.11	2.80	6-16
INTI (CBKT) B	CCQM-K154.b	14.951	0.94	1.88	15.006	0.99	1.97	0.37	2.73	6-16
KEBS (CBKT) A	CCQM-K154.b	12.188	0.81	1.63	12.20	0.82	1.64	0.10	2.31	6-16
KEBS (CBKT) B	CCQM-K154.b	12.117	0.85	1.70	12.20	0.82	1.64	0.68	2.36	6-16
LATU (CBKT) A	CCQM-K154.b	14.758	0.93	1.87	14.861	0.85	1.71	0.69	2.53	6-16
LATU (CBKT) B	CCQM-K154.b	15.156	0.94	1.89	14.861	0.85	1.71	-1.99	2.55	6-16
INRAP (CBKT) A	**CCQM-K154.b.1**	**3.538**	**2.58**	**5.16**	**3.5106**	**0.95**	**1.91**	**-0.79**	**5.50**	**2.6-4.4**
INRAP (CBKT) B	**CCQM-K154.b.1**	**3.432**	**1.50**	**3.00**	**3.5106**	**0.95**	**1.91**	**2.24**	**3.55**	**2.6-4.4**

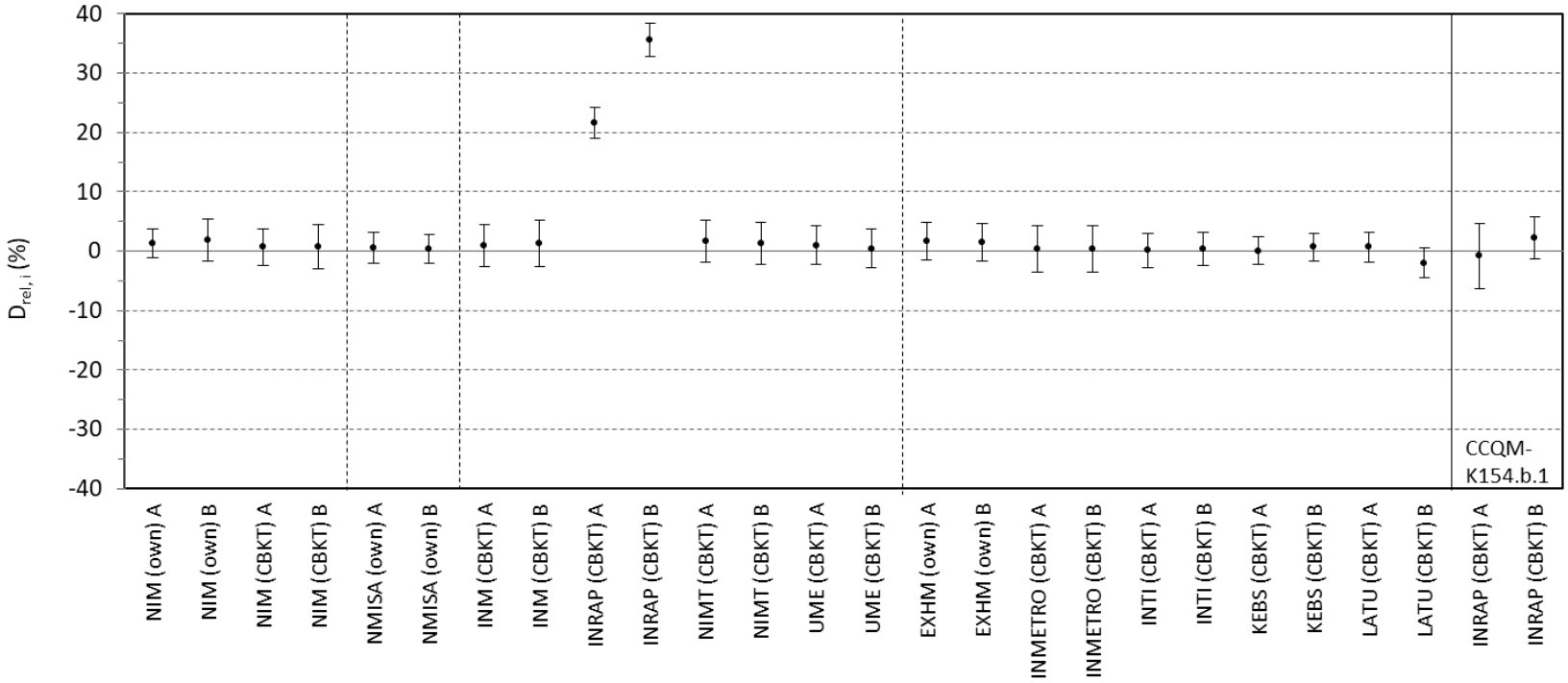

Figure 3: Relative values for the degree of equivalence for both CCQM-K154.b and CCQM-K154.b.1

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.

In the original study CCQM-K154.b, 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 2 and 3) 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 did not agree with the KCRVs. 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} [6].

Consequently, INRAP has produced a new batch of AfB_1 calibration solution using the AfB_1 stock solution originally provided to CBKT participants to be able 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 BIPM organized a subsequent bilateral comparison CCQM-K154.b.1 to assess the new batch of AfB_1 calibration solution of INRAP. Inspection of the degree of equivalence plots (Figures 2 and 3) for the AfB_1 mass fraction assignments indicated that there is an excellent agreement of INRAP results for CCQM-K154.b.1.

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.

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REFERENCES

[1] R. D. Josephs, Xiaomin Li, Xiuqin Li, Z. Guo, B. Garrido, I. Un, A. Daireaux, T. Choteau, G. Martos, S. Westwood, H. Li, R. I. Wielgosz: The BIPM Mycotoxin Metrology Capacity Building and Knowledge Transfer Program: Accurate Characterization of a Pure Aflatoxin B_1 Material to Avoid Calibration Errors, J. AOAC Int. 102 (2019) 6, 1740-1748.

[2] E. C. P. Rego, M. E. Simon, Xiuqin Li, Xiaomin Li, A. Daireaux, T. Choteau, S. Westwood, R. D. Josephs, R. I. Wielgosz, V. S. Cunha: Mycotoxin metrology: Gravimetric production of zearalenone calibration solution, J. Phys. Conf. Ser. 975 (2018) 012014

[3] R. D. Josephs, A. Daireaux, M. Bedu, Xiuqin Li, Xiaomin Li, Z. Guo, T. Choteau, G. Martos, S. Westwood, R. I. Wielgosz, H Li, M Simón, E. C. P. do Rego, B. C. Garrido, R. Leal, L. Carvalho, E. Guimarães, A. Bahadoor, J. Bates, J. E. Melanson, P. Giannikopoulou, C. Alexopoulos, E. Kakoulides, I. Mugenya, D. Prevoo-Franzsen, M. Fernandes-Whaley, S. Marbumrung, M. Bilsel, B. Binici, T. Gokcen: Key comparison study - organic solvent calibration solution - gravimetric preparation and value assignment of trans-zearalenone (*trans*-ZEN) in acetonitrile (ACN), Metrologia 57 (2020) Tech. Suppl., 08019.

[4] S. Westwood, R. D. Josephs, T. Choteau, X. Li, B. Garrido, I. Un, T. Yamazaki: Purity Evaluation Guideline: Zearalenone (BIPM-PEG-01), Rapport BIPM-2019/06 (2019) Bureau International des Poids et Mesures, Sèvres, France. [https://www.bipm.org/utils/common/pdf/rapportBIPM/RapportBIPM-2019-06.pdf]

[5] G. Martos, X. Li, R. D. Josephs, S. Westwood, A. Daireaux, R. Wielgosz: Calibrant Assessment Guideline: Zearalenone (BIPM-CAG-02), Rapport BIPM-2020/02 (2020) Bureau International des Poids et Mesures, Sèvres, France.

[6] R. D. Josephs, M. Bedu, A. Daireaux, Xiuqin Li, Xiaomin Li, Z. Guo, X. Li, T. Choteau, G. Martos, S. Westwood, R. Wielgosz, H. Li, M. Simón, C. Santana Smersu, M. Villarreal, T. Lopez Seal, M. Cirio, E. C. Pires do Rego, R. V. Leal, L. J. Carvalho, J. M. Rodrigues, E. de Freitas Guimarães, B. C. Garrido, L. Morales Erazo., S. Ramirez, I. Gonzalez, P. Giannikopoulou, C. Alexopoulos, E. Kakoulides, I. Mugenya, D. Prevoo-Franzsen, M. Fernandes-Whaley, S. Marbumrung, J. Nammoonnoy, K. Shearman, C. Boonyakong, H. Klich, R. Torkhani, T. Gokcen, M. Bilsel, S. Akkus Ozen, J. Cea, O. Martínez: Key comparison study - organic solvent calibration solution - gravimetric preparation and value assignment of aflatoxin B1 (AfB1) in acetonitrile (ACN), Metrologia 59 (2022) 1A, 08002.

[7] B. R. Rushing, M. I. Selim: Adduction to arginine detoxifies aflatoxin B_1 by eliminating genotoxicity and altering in vitro toxicokinetic profiles, Oncotarget 9 (2018) 4), 4559-4570.

[8] B. R. Rushing, M. I. Selim: Effect of dietary acids on the formation of aflatoxin B_{2a} as a means to detoxify aflatoxin B_1. Food Addit. Contam. Part A. 33 (2016) 9, 1456–1467.

[9] V. Betina (Ed.) Mycotoxins: Production, Isolation, Separation, and Purification, Develop. Food Sci., Vol 8 (1984) Elsevier, Amsterdam.
[10] J. E. Smith, M. O. Moss: Mycotoxins: Formation analysis, and Significance (1985) John Wiley & Sons, Chichester, United Kingdom
[11] World Health Organization International Agency for Research on Cancer (2002) IARC Monogr. Eval. Carcinog. Risks Hum. 82, 1–556.
[12] Food and Agriculture Organization (2004) Worldwide Regulations for Mycotoxins in Food and Feed in 2003, Food and Agriculture Organization of the United Nations, Rome, Italy.
[13] European Union (2017) RASFF - The Rapid Alert System for Food and Feed - 2016 Annual Report, Publications Office of the European Union, Luxembourg.
[14] S. Westwood, R. D. Josephs, G. Martos, T. Choteau, Xiuqin Li, Xiaomin Li, Zhen Guo, Xianjiang Li, B. Garrido, I. Un: Purity Evaluation Guideline: Aflatoxin B_1 (BIPM-PEG-02), Rapport BIPM-2021/01 (2021) Bureau International des Poids et Mesures, Sèvres, France.
[15] G. Martos, S. Westwood, R. D. Josephs, X. M. Li, Z. Guo, R. Wielgosz: Calibrant Assessment Guideline: Aflatoxin B_1 (BIPM-CAG-01), Rapport BIPM-2019/07 (2019) Bureau International des Poids et Mesures, Sèvres, France.
[16] M. J. T. Milton, P. M. Harris, I. M. Smith, S. A. Brown, B. A. Goody: Implementation of a generalized least-squares method for determining calibration curves from data with general uncertainty structures, Metrologia 43 (2006) S291–S298.
[17] ISO 6143:2001 Gas analysis – comparison methods for determining and checking the composition of calibration gas mixtures (2001) International Organization for Standardization, Geneva, Switzerland.
[18] R. I. Wielgosz, M. Esler, J. Viallon *et al.*: International comparison CCQM-P73: Nitrogen monoxide gas standards (30–70) μmol/mol, Metrologia 45 (2008) 08002.
[19] E. Flores, J. Viallon, T. Choteau *et al.*: International comparison CCQM-K82: Methane in air at ambient level (1800 to 2200) nmol/mol, Metrologia 52 (2015) 08001.
[20] S. Hein, R. Philipp, D. L. Duewer, *et al.*: Final report on CCQM-K79: Comparison of value-assigned CRMs and PT materials: Ethanol in aqueous matrix, Metrologia 50 (2013) 08005.
[21] D. L. Duewer, H. Gasca-Aragon, K. A. Lippa, B. Toman: Experimental design and data evaluation considerations for comparisons of reference materials, Accred. Qual. Assur. 17 (2012) 567–588.

ANNEX A – ADDITIONAL ANALYTICAL INFORMATION

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 AfB_1 solution provided by BIPM.

Amount of primary calibrator used for analysis

3.0113

Gravimetry

Type of balance (make, model and resolution)

make: Mettler Toledo
model: XP504S
resolution: 0.01 mg

Balance repeatability

100 µg

Solution preparation procedure

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

Homogeneity and/or stability testing

Homogenety test: 10 ampoules were selected from the batch, analysed by HPLC-UV at the repeatability condition and the uncertainty were calculated, the final uncertainty value includes the contribution of the homogeneity uncertainty.
Stability test: 25, 40 °C was tested (transport short term stability), an isochronous stability was established, stability long term is still progress.

Optional: Analytical check method

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

LC system:	HPLC-DAD series 1200
Stationary Phase:	Agilent eclipse C15 (250 × 4.6) 5µm
Mobile Phase:	A) H_2O, B) H_2O:Acetonitrile (50:50 v/v), gradient 0-30 A:30%-90%
Injection Volume:	20 µL
Flow rate:	0.6 mL/min
Column temperature:	25°C and λ =360 nm

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

External calibration, bracketing ±15 %

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

MRC-NMISA, 31.69 ± 0.76 µg/g (*k = 2*) and verification with the calibration solution OGP.029 from MMCBKT-2019 (6.15 µg/g)

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

-/-

Additional Comments or Observations

-/-