CCQM-K154.d

Key Comparison Study – Organic Solvent Calibration Solution

Gravimetric preparation and value assignment of patulin (PAT) in acetonitrile (ACN) with 0.1 % formic acid (FA)

Final Report

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SUMMARY

The CCQM-K154.d comparison was 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 Mycotoxin Metrology Capacity Building and Knowledge Transfer (MMCBKT) project of the 'Bureau International des Poids et Mesures' (BIPM) 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, twelve NMIs/DIs participated in the Track C, Model II, Key Comparison CCQM-K154.d [Gravimetric preparation and value assignment of patulin (PAT) 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 patulin (PAT) 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.d for MMCBKT participants was intended to demonstrate measurement capabilities for preparation and value assignment of patulin (PAT) calibration solutions in the mass fraction range of 10 mg/kg to 100 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 polar analytes in the mass fraction range of 10 mg/kg to 100 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
ANOVA	Analysis of variance
CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	Calibration and Measurement Capability
CRM	Certified reference material
DI	Designated Institute
DoE	Degree of equivalence
ESI	Electrospray ionization
FA	Formic acid
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
PAT	Patulin
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.d 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) [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) [6, 7, 8] and the CCQM-K154.c comparison on the gravimetric preparation and value assignment of the *Fusarium* mycotoxin deoxynivalenol (DON) in acetonitrile (ACN) were the initial 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 [9, 10, 11]. The CCQM-K154.d comparison on the gravimetric preparation and value assignment of the *Penicillium/Aspergillus* mycotoxin patulin (PAT) 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 PAT calibration solutions prepared by each of the participating laboratories. Eight laboratories took part in the framework of the MMCBKT, two laboratories used reference materials for preparing their own solutions while five 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 PAT in the solutions provided by each participant.

Patulin (PAT), a polyketide and unsaturated heterocyclic lactone, is a mycotoxin produced by a variety of moulds, particularly by fungi of the genus *Aspergillus* and *Penicillium*. Often found in rotting apples and apple products, PAT can also occur in various other moldy fruits, grains and other foods. Major human dietary sources of PAT are apples and apple juice made from affected fruit [12]. The acute symptoms in animals include liver, spleen and kidney damage and toxicity to the immune system. For humans, nausea, gastrointestinal disturbances and vomiting have been reported. PAT is considered to be genotoxic however a carcinogenic potential has not been demonstrated yet [13]. PAT is relatively stable when given a short-acting heat-treatment (pasteurization), especially in an acidic environment [14]. The importance of monitoring PAT content in primary products and derived foodstuffs is reflected in the existence of regulations controlling the maximum limits for PAT in about 48 countries. A typical minimum residue level is 50 μg/kg in food and juices [15]. Levels below this were established in the European Union with regard to solid apple products of 25 μg/kg and for in apple-based baby food/juices of 10 μg/kg [16, 17]. The analytical difficulty and the importance of controlling PAT in baby food and beverages support the need for solution and matrix certified reference materials. They are invaluable tools to ensure comparability and traceability in PAT measurements and are very useful for the implementation of written standards, legislation/regulations and laboratory accreditation.

MEASURAND, QUANTITIES AND UNITS

The measurand was the mass fraction of patulin [PAT] present in solution acetonitrile (ACN) with 0.1 % formic acid (FA), 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 thirteen calibration solutions of PAT in ACN gravimetrically prepared and value assigned by each of the participating laboratories.
Eight laboratories (INM, INMETRO, INRAP, INTI, LATU, NIM, NIMT and UME) took part in the CCQM-K154.d comparison within the framework of the MMCBKT, using a value assigned stock solution of PAT in ACN supplied by the BIPM. Two laboratories (NIS and SASO) took part in CCQM-K154.d using other non-proprietary reference materials for the preparation of their own PAT solution. Three laboratories (EXHM, NIM and NMISA) took part in CCQM-K154.d using their own value assigned stock solution of PAT. NIM participated in CCQM-K154.d 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.d is given in Table 1.

Table 1: CCQM-K154.d Timetable

Action	Date
Initial discussion	April and October 2021 MMCBKT and OAWG meetings
Study Proposal and draft protocol	March 2022 OAWG meeting
Approval of study protocol and confirmation	April 2022 OAWG meeting
Stock solution distribution	beginning March 2021 (MMCBKT participants)
Call for participation	April 20th, 2022
Final date to register	August 1st, 2022
Samples and data due to coordinator	October 31st, 2022
Initial presentation and discussion of results	April 2023 OAWG meeting
Draft A report	
Draft B report	July 2023
Final report to OAWG Chair	September 2023

PAT PRIMARY CALIBRATOR STOCK SOLUTION

The BIPM provided the MMCBKT participants with a stock solution of PAT in ACN with 0.1 % (v/v) FA. (OGP.035) that was to be used for the preparation of PAT calibration solution batches submitted for comparison CCQM-K154.d.
The PAT mass fraction and associated expanded uncertainty ($k = 2$) of the PAT stock solution OGP.035 was (261.2 ± 5.6) 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.

PAT purity characterization

An essential requirement of the MMCBKT project was to obtain and characterize a primary reference material for PAT that could be used subsequently to establish a calibration hierarchy to underpin the metrological traceability of results linked through calibration to this material [18]. The characterization and purity assignment studies to assess the identity and purity of a primary reference material for PAT used to deliver the BIPM MMCBKT program are described in detail in the Purity Evaluation Guideline: PAT [19]. 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 PAT 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 PAT it contained. In addition, an LC-UV-MS/MS method has been developed and applied in case the initial value obtained by NMR needed to be corrected for relevant related structure impurity mass fractions to give the final value for the true PAT mass fraction of the material. 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 assigned PAT mass fraction value and its corresponding expanded uncertainty of (995.1 +3.9/-4.2) mg/g was obtained by combining two independently obtained qNMR values (995.9 ± 1.6) mg/g and (993.7 ± 2.1) mg/g using a Hierarchical Bayes random effects model. A combined standard uncertainty of 2.6 mg/g with a dark uncertainty contribution of 1.9 mg/g was obtained applying the Bayesian method [20].

The only significant impurity identified in the PAT material was residual organic solvent as listed in Table 2. The amounts of dichloromethane and ethanol present assigned by qNMR are consistent with the assigned content of the primary component, PAT. No significant amounts of related structure impurities or water were quantified by LC-UV-MS/MS or KFT, respectively.

The final value for the PAT material (OGO.180) analyzed at the BIPM and used subsequently in the CCQM-K154.d comparison was (995.1 +3.9/ 4.2) mg/g [21].

Table 2: Impurity assignments

Impurity	Mass fraction (mg/g)	U (mg/g)	Assignment
PAT related impurities	-	-	LC-UV (and LC-MS/MS)
VOCs	4.8	0.22	qNMR
Water	-	-	KFT (and TGA)

Gravimetric preparation of PAT stock solution

The PAT stock solution (OGP.035) was prepared gravimetrically by dissolving an accurately weighed sample of about 200 mg of PAT powder material (OGO.180) in 1 L of ACN with 0.1 % (v/v) FA. Mettler Toledo MX5 and XP10002S balances were used for the weighing of the PAT powder and the final solution, 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 PAT stock solution and the calculation using Eq. 1 of its PAT mass fraction.

	Weighed mass (m)	Buoyancy (*b*)	m x b
PAT powder (mg)	204.967	1.000635	205.079
Solution (g)	780.310	1.001386	781.392
Purity ± U (mg/g)	995.1 +3.9/-4.2		
Final mass fraction (µg/g)	261.19		

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

where:
m_p: weighed mass of PAT powder
b_p: buoyancy correction for powder weighing
w_p: purity of PAT powder
m_{sol}: weighed mass of stock solution
b_{sol}: buoyancy correction for solution 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: PAT [21].

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

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

Uncertainty source	Value (%)
$\frac{u(m_p)}{m_p}$	0.0023
$\frac{u(b_p)}{b_p}$	0.0016
$\frac{u(w_p)}{w_p}$	0.26
$\frac{u(m_{sol})}{m_{sol}}$	0.0028
$\frac{u(b_{sol})}{b_{sol}}$	0.0012
$\frac{u(V)}{V}$	0.0050
u_{rel} (%)	0.26
u(w_{stock}) µg/g	0.68
U(w_{stock}) µg/g (k = 2)	1.36

Filling of PAT stock solution

The 1 L flask containing the stock solution was agitated thoroughly and about 125 mL were transferred to prepare a calibration solution. The rest of the stock solution was stored at -20 °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 PAT 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.035, one at a time, to minimize the impact of evaporation of solvent. 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 (Heraeus) 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 [21].

Homogeneity studies of PAT stock solution

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

Table 5: Homogeneity results of the PAT stock solution (OGP.035)

	PAT (276 nm)
N	30
s_{wb} (%)	0.35
s_{bb} (%)	0.13
u^*_{bb} (%)	0.11
$\mathbf{u_{bb}}^{(1)}$ (%)	0.13
F	1.39
F_{crit}	2.39

(1) Higher value (u^*_{bb} or s_{bb}) was taken as uncertainty estimate for potential inhomogeneity

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 0.13 % for PAT (Table 5). The material was regarded to be homogeneous since the u_{bb} of 0.13 % is very small compared with the target uncertainties of < 2 % and in agreement with typical u_{bb} for similar mycotoxin materials [22].
Representative normalized results due to the analysis and filling sequences are presented for the main component PAT in Figure 1a and 1b, respectively. The first, second and third replicates are represented by circles, grey filled circles and dots, respectively (Figure 1b).

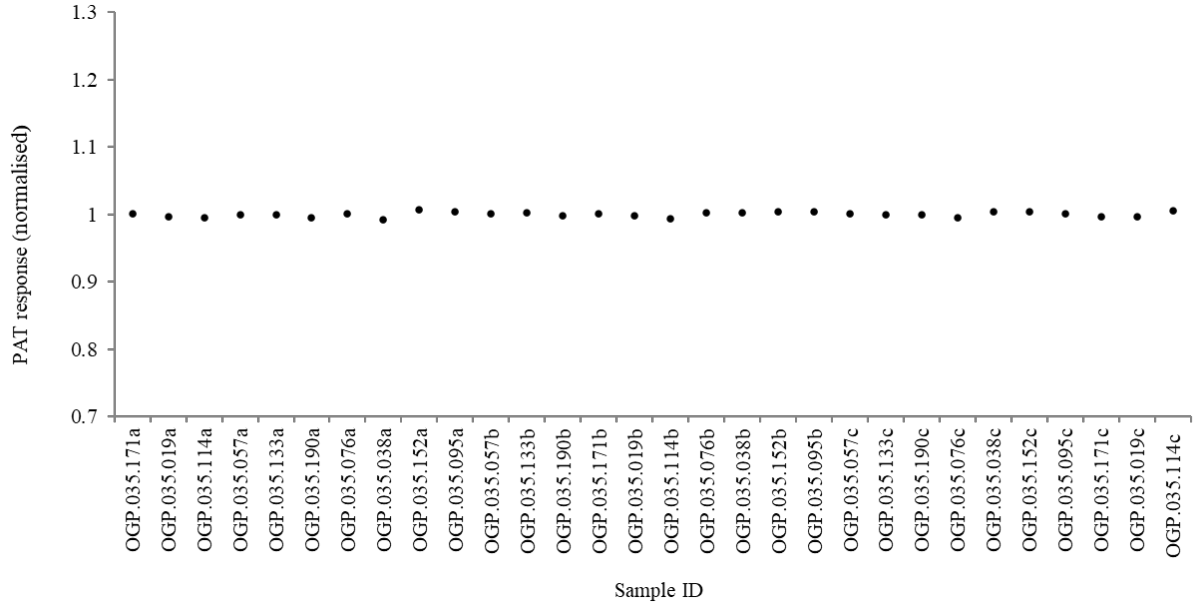

Figure 1a: Homogeneity of PAT by LC-DAD at 276 nm – Main component - Injection sequence

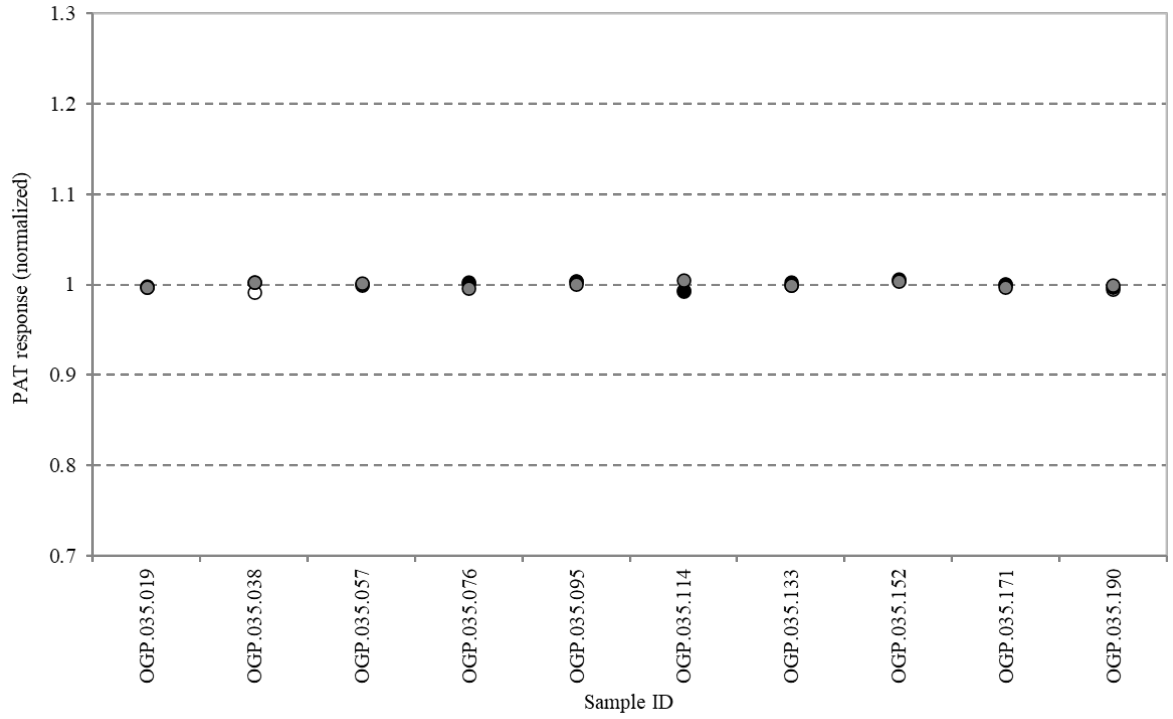

Figure 1b: Homogeneity of PAT by LC-DAD at 276 nm – Main component - Filling sequence

Stability studies of PAT stock solution

Preliminary studies demonstrated that PAT is not stable in solution in acetonitrile unless stabilized with a weak organic acid. These findings are supported by literature [14]. 0.1 % (v/v) Formic acid has been used for the preparation of the 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 samples were measured by LC-DAD-MS/MS. For the main component PAT, no calibration was performed as peak areas (LC-DAD) were directly normalized to the PAT peak area (276 nm) of the reference samples. Data were evaluated as a function of the storage time at each of the studied temperatures. LC-MS/MS were used for confirmation only.
The acidified stock solution was found to be stable over the entire study period of 8 weeks at storage temperatures of 4 °C, 22 °C and 40 °C as shown in Figure 2a, 2b and 2c, respectively.
It was concluded that the PAT stock solution (OGP.035) was suitably stable for short-term transport provided it was not exposed to light and to temperatures significantly in excess of 40 °C for more than four weeks. To minimize the potential for changes in the material composition, long-term storage is recommended at -20 °C in the dark.
A conservative stability uncertainty contribution of 2.65 µg/g (1.02 %) was estimated based on the 4 °C stability data taking in consideration storage to cover the comparison period.

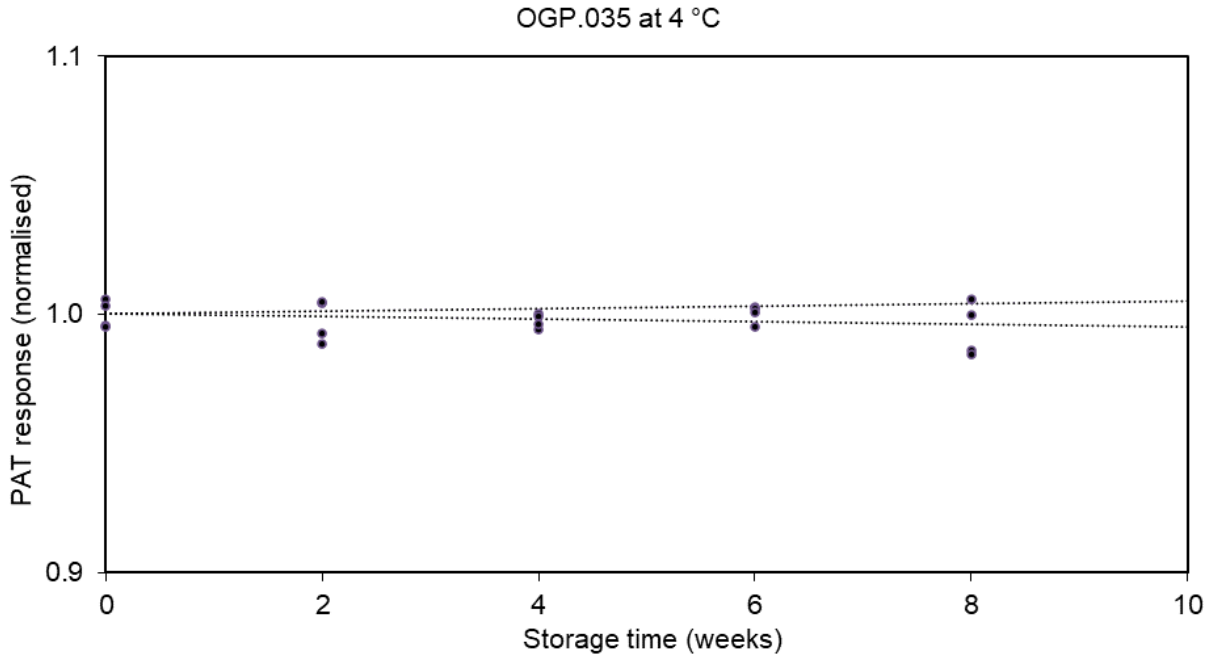

Figure 2a: Stability study of PAT by LC-DAD (276 nm) at 4 °C

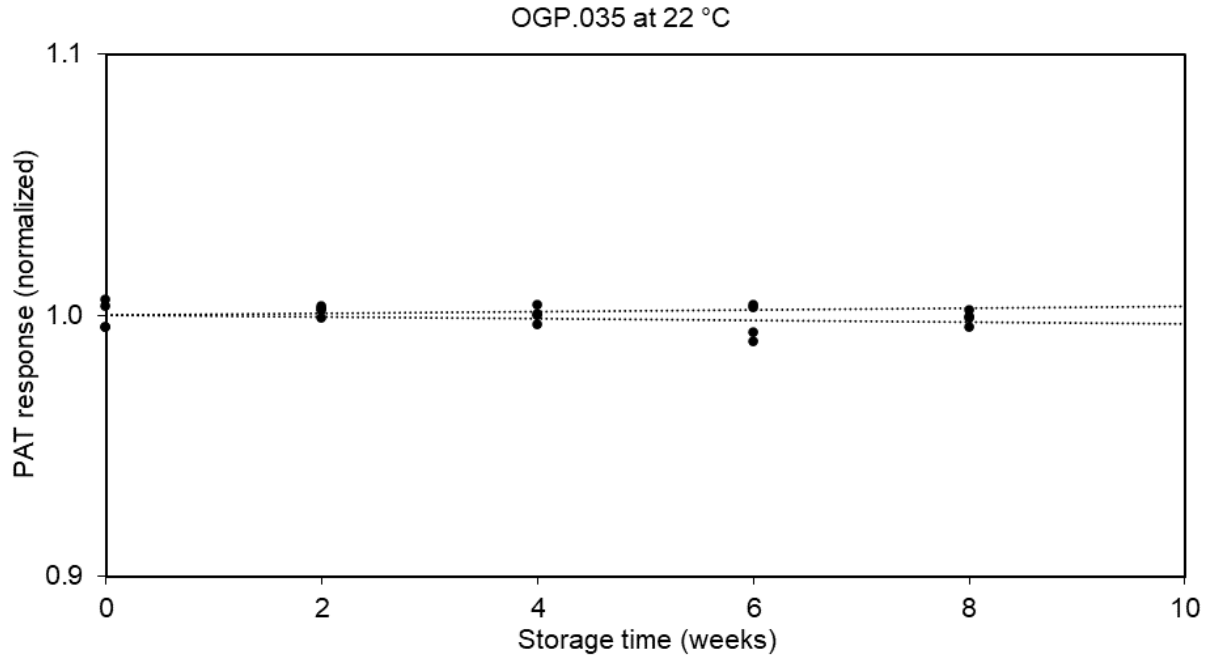

Figure 2b: Stability study of PAT by LC-DAD (276 nm) at 22 °C

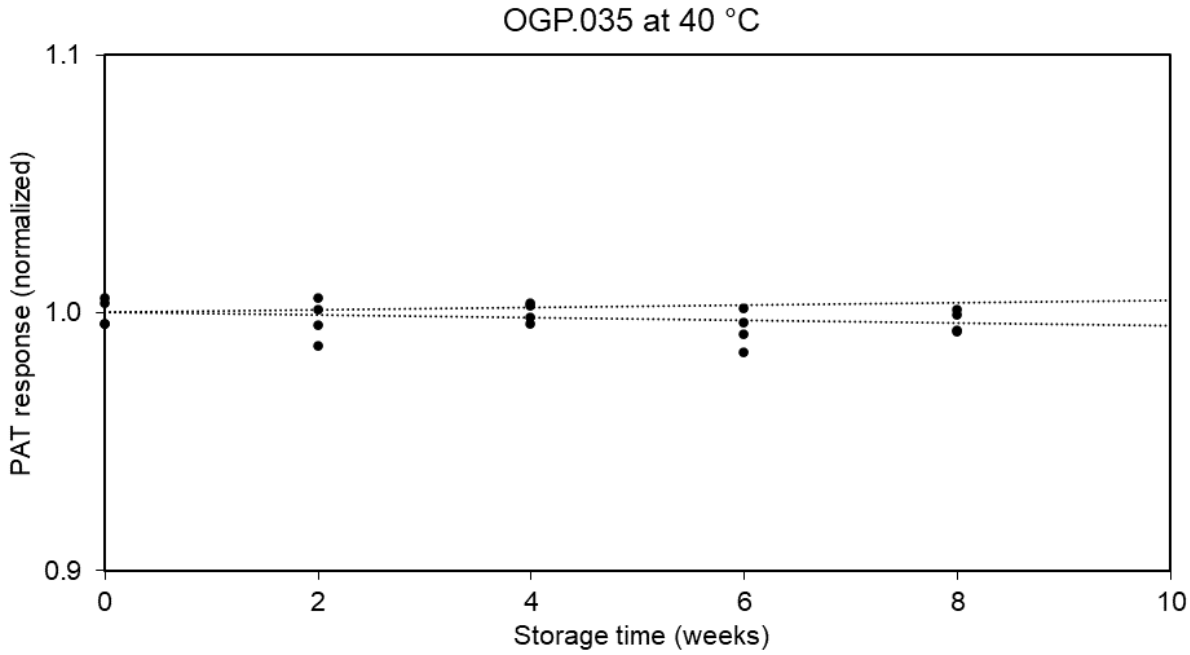

Figure 2b: Stability study of PAT by LC-DAD (276 nm) at 40 °C

PAT stock solution and corresponding uncertainty

A mass fraction of 261.2 µg/g was calculated for the PAT 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 5.6 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 PAT 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 PAT stock solution

PAT stock solution	
$u_{rel}(w_{stock})$ (%)	0.26
$u_{bb,rel}$ (%)	0.13
$u_{lts,rel}$ (%)	1.02
$u_{c,rel}$ (%)	1.06
w_{stock} (µg/g)	261.2
u_c (µg/g)	2.8
U (µg/g), $k = 2$	5.6

Sample distribution of PAT stock solution

Six units of the PAT 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 ampoules or if temperatures has exceeded 40 ºC during shipping. The shipping details are listed in Table 7. There was a delay in delivery (22 days) of the ampoules sent to INTI, Argentina. It has been decided that the ampoules were fit for purpose as they have been received in good condition without exceeding 40 ºC temperature during shipping. The ampoules sent to NIMT, Thailand have reached about 40 ºC temperature but the ampoules arrived at destination within 2 days only. It was decided that the NIMT ampoules were fit for purpose as well. Unfortunately, shipping to KEBS, Kenya was repeatedly rejected even though all international air freight standards (IATA) have been met.
All other samples were delivered to the NMIs/DIs without incident.

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

NMI/DI	Shipping date	Date of receipt	In transit (days)	Comments
INMETRO	23.03.2021	24.03.2021	1	-
INM	23.03.2021	24.03.2021	1	Two ampoules broken
INRAP	23.03.2021	25.03.2021	2	-
INTI	23.03.2021	14.04.2021	22	Samples were retained at customs
KEBS	23.03.2021	Shipping rejected	-	2nd Shipping on 07.05.2021 also rejected
LATU	23.03.2021	26.03.2021	3	One ampoule leaking
NIM[#]	23.03.2021	25.03.2021	2	-
NIMT	23.03.2021	25.03.2021	2	Exposure to 40 ºC
NMISA*	23.03.2021	31.03.2021	8	One ampoule missing
UME	23.03.2021	25.03.2021	2	-

[#] For their participation in the study NIM chose to submit PAT calibrator solutions prepared using both in-house value-assigned materials and based on the MMCBKT PAT stock solution OGP.035.

* As an MMCBKT participant NIMSA was supplied with the PAT stock solution OGP.035. However, they chose to submit a PAT 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 PAT in ACN and to send these to the BIPM for comparison measurements. The mass fraction values targeted (in the range 10 mg/kg to 100 mg/kg) are intended to be representative of the mass fraction content of PAT in a standard solution provided as a reference standard used for calibrations in PAT 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 PAT 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 PAT 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 (PAT in ACN). 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 PAT 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.d comparison was undertaken end of 2022. The deadline to submit the samples and to return the filled in data submission forms to the BIPM for participation in the comparison was 31st October 2022.

The details of the shipping of the comparison solutions from the NMIs/DIs to the BIPM are listed in Table 8. LATU, Uruguay and NIS, Egypt had difficulties to meet the deadline as they had issues with shipping companies to deliver the ampoules to the BIPM. NIM, China had also difficulties to ship the samples in time and one ampoule was found to be empty on arrival at the BIPM. The other three ampoules from NIM were received in good condition. All ampoules of other NMIs/DIs were delivered before the deadline, within four weeks and were received in good condition for comparison at the BIPM.

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

NMI/DI	Shipping date	Date of receipt	In transit (days)	Comments
INTI	22.09.2022	27.09.2022	5	-
INMETRO	20.09.2022	21.10.2022	31	-
NIM	29.10.2022	03.11.2022	5	One ampoule empty
INM	03.09.2022	08.09.2022	5	-
NIS	16.10.2022	14.11.2022	29	-
EXHM	27.10.2022	28.10.2022	1	-
SASO	16.10.2022	27.10.2022	11	-
NMISA	10.10.2022	20.10.2022	10	-
NIMT	19.10.2022	25.10.2022	6	-
INRAP	24.10.2022	26.10.2022	2	-
UME	14.09.2022	16.09.2022	2	-
LATU	21.11.2022	30.11.2022	9	-

REPORTED MASS FRACTIONS OF PAT AND IMPURITIES

The values reported by participating NMIs/DIs for the PAT mass fractions of their PAT 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 PAT 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.
NMISA reported the detection of two minor unidentified structurally related impurities in their material. The NMISA material was directly purity assigned by using quantitative NMR providing for the mass fraction and corresponding uncertainties of structurally related impurities.

VALUE ASSIGNMENT PROCEDURE OF THE COORDINATING LABORATORY

The PAT 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 PAT. 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 9 µg/g to 68 µg/g of PAT.
Two-point calibrations with external bracketing using PAT standards assigned at the BIPM were used for quantification and comparison. It was decided to split the CCQM-K154.d comparison in seven groups (A, B, C, D, E, F and G) with separate calibrations for each of the 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 PAT bracketing standards were prepared immediately before use as solutions in ACN (Hipersolv HPLC grade, VWR, France) with 0.1 % (v/v) FA of the pure BIPM PAT material (OGO.180) having a PAT mass fraction of (995.1 +3.9/-4.2) mg/g (k = 2) as outlined in the chapter ‘PAT 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 PAT 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: PAT [21]. The PAT 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 Shiseido Capcell PAK C18 MG S-5 column (250 mm × 4.6 mm, 5 µm from AIT (Houilles, France) maintained at 25 °C. The mobile phases consisted of (A) ACN/water (5/95, v/v) with 0.1 % FA and (B) ACN with 0.1 % FA. The separation was performed by use of a gradient program with a constant flow rate of 800 µL/min. The gradient started with 100 % A and holding for 20 min. The column was then washed by decreasing to 5 % A in 2 min, holding at 5 % A for 2 min and returning to starting conditions (100 % A) in 2 min. The column was re-equilibrated for a further 14 min at 100 % A. The total run time was 40 min and the injection volume was 5 µL. The detection wavelength of the UV diode array detector (DAD) was 276 nm. The wavelengths of 254 nm and 267 nm were 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 positive electrospray ionization mode. A capillary voltage of 4500 V with source temperature of 550 ºC was employed for the positive ESI mode. 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 40 psi. The collision gas (CAD) was set at ‘Mid’. Table 11 lists MS/MS transitions of PAT 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: PAT mass fraction values and corresponding uncertainties submitted by the NMIs/DIs CCQM-K154.d

Participant	Primary calibrator used	PAT Mass fraction (µg/g)	PAT Combined standard uncertainty (µg/g)	Coverage factor (*k*)	Expanded uncertainty (µg/g)
INTI, Argentina	CBKT	22.84	0.26	2	0.52
INMETRO, Brazil	CBKT	19.24	0.22	2	0.45
NIM, China	CBKT	26.12	0.35	2	0.70
	own	26.21	0.24	2	0.47
INM, Colombia	CBKT	10.00	0.13	2	0.26
NIS, Egypt	CRM	14.5	0.25	2	0.5
EXHM, Greece	own	10.00	0.154	2	0.31
SASO, Saudi Arabia	CRM	27.16	0.67	2	1.34
NMISA, South Africa	own	63.8	0.69	2	1.4
NIMT, Thailand	CBKT	12.60	0.152	2	0.31
INRAP, Tunisia	CBKT	15.9157	0.2387	2	0.4774
UME, Turkey	CBKT	14.65	0.21	2	0.41
LATU, Uruguay	CBKT	20.08	0.28	2	0.57

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

Calibration	Mass fraction range	PAT Stock solution		PAT High level calibration solution		PAT Low level calibration solution	
		w (mg/kg)	u (mg/kg)	*w* (mg/kg)	u (mg/kg)	*w* (mg/kg)	u (mg/kg)
A	9-11 mg/kg	100.42	1.11	10.49	0.12	9.56	0.11
B	11-13 mg/kg	100.42	1.11	13.33	0.15	11.50	0.13
C	13-17 mg/kg	100.42	1.11	16.88	0.19	13.60	0.15
D	18-21 mg/kg	100.42	1.11	21.29	0.24	18.20	0.20
E	21-24 mg/kg	100.42	1.11	24.09	0.35	21.49	0.31
F	24-29 mg/kg	100.42	1.11	28.61	0.42	24.88	0.36
G	60-68 mg/kg	100.42	1.11	67.51	0.98	60.65	0.88

Table 11: Summary of selected precursor and product ions, optimized time, DP, CE, EP and CXP settings for the detection of PAT 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)
Patulin (PAT)	153.0	109.0	50	-60	-17	-8.5	-16
Ascladiol (ASC)	155.0	125.0	50	-60	-20	-8.5	-16
Desoxypatulinic acid (DPA)	155.0	111.0	50	-60	-15	-8.5	-16
		63.0	50	-60	-30	-8.5	-16

Samples, sequence preparation and measurement order

Two ampoules supplied by each participant were each measured in triplicate by LC-DAD-MS/MS. CCQM-K154.d with measurements performed from end of 2022 until begin of 2023. The comparison measurements were undertaken in seven batches (A, B, C, D, E, F and G) on different days involving one to three participants according to their target mass fraction ranges. 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.d participant samples is listed in Table 12.

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

			Calibration			
A 9-11 mg/kg	B 11-13 mg/kg	C 13-17 mg/kg	D 18-21 mg/kg	E 21-24 mg/kg	F 24-29 mg/kg	G 60-68 mg/kg)
INM (CBKT)	NIMT (CBKT)	NIS (CRM)	INMETRO (CBKT)	INTI (CBKT)	NIM (own)	NMISA (own)
EXHM (own)		INRAP (CBKT) UME (CBKT)	LATU (CBKT)		NIM (CBKT) SASO (CRM)	

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 acetonitrile with 0.1 % FA (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 the CCQM-K154.d comparison (A, B, C, D, E, F and G) are given in Table 13.

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

Injection	Calibration A (9-11 mg/kg)	B (11-13 mg/kg)	C (13-17 mg/kg)	D (18-21 mg/kg)	E (21-24 mg/kg)	F (24-29 mg/kg)	G (60-68 mg/kg)
1	Blank	Low-1	Blank	Low-1	Blank	Blank	Blank
2	Low-1	High-1	Low-1	BIPM-D-1	Low-1	Low-1 (inj. error)	Low-1
3	INM-X-1	Blank	High-1	INMETRO-X-1	BIPM-E-1	BIPM-F-1	BIPM-G-1
4	EXHM-X-1	Low-2	Blank	High-1	High-1	High-1	High-1
5	High-1	NIMT-X-1	Low-2	Blank	Blank	Blank	Blank
6	Blank	High-2	BIPM-C-1	Low-2	Low-2	Low-2	Low-2
7	Low-2	Blank	UME-X-1	LATU-X-1	INTI-X-1	NIM-CBKT-X-1	NMISA-X-1
8	BIPM-A-1	Low-3	INRAP-X-1	INMETRO-Y-1	High-2	SASO-X-1	High-2
9	EXHM-Y-1	BIPM-B-1	High-2	High-2	Blank	High-2	Blank
10	High-2	NIMT-Y-1	Blank	Blank	Low-3	Blank	Low-3
11	Blank	High-3	Low-3	Low-3	INTI-Y-1	Low-3	NMISA-Y-1
12	Low-3	Blank	NIS-X-1	BIPM-D-2	High-3	NIM-OWN-X-1	High-3
13	BIPM-A-2	Low-4	UME-Y-1	LATU-Y-1	Blank	NIM-CBKT-Y-1	Blank
14	INM-Y-1	BIPM-B-2	NIS-Y-1	High-3	Low-4	NIM-OWN-Y-1	Low-4
15	High-3	High-4	High-3	Blank	BIPM-E-2	High-3	BIPM-G-2
16	Blank	Blank	Blank	Low-4	High-4	Blank	High-4
17	Low-4	Low-5	Low-4	INMETRO-X-2	Blank	Low-4	Blank
18	EXHM-X-2	NIMT-X-2	BIPM-C-2	BIPM-D-3	Low-5	BIPM-F-2	Low-5
19	BIPM-A-3	High-5	INRAP-Y-1	High-4	INTI-X-2	SASO-Y-1	NMISA-X-2
20	High-4	Blank	NIS-X-2	Blank	High-5	NIM-OWN-X-2	High-5
21	Blank	Low-6	High-4	Low-5	Blank	High-4	Blank
22	Low-5	NIMT-Y-2	Blank	LATU-X-2	Low-6	Blank	Low-6
23	INM-X-2	BIPM-B-3	Low-5	INMETRO-Y-2	INTI-Y-2	Low-5	NMISA-Y-2
24	EXHM-Y-2	High-6	UME-X-2	High-5	BIPM-E-3	NIM-CBKT-X-2	BIPM-G-3
25	High-5	Blank	INRAP-X-2	Blank	High-6	SASO-X-2	High-6
26	Blank	Low-7	BIPM-C-3	Low-6	Blank	BIPM-F-3	Blank
27	Low-6	BIPM-B-4	High-5	BIPM-D-4	Low-7	High-5	Low-7
28	BIPM-A-4	High-7	Blank	LATU-Y-2	BIPM-E-4	Blank	BIPM-G-4
29	INM-Y-2	Blank	Low-6	High-6	High-7	Low-6	High-7
30	High-6	Low-8	UME-Y-2	Blank	Blank	NIM-CBKT-Y-2	Blank
31	Blank	NIMT-X-3	INRAP-Y-2	Low-7	Low-8	SASO-Y-2	Low-8
32	Low-7	BIPM-B-5	NIS-Y-2	BIPM-D-5	INTI-X-3	NIM-OWN-Y-2	NMISA-X-3
33	BIPM-A-5	High-8	High-6	INMETRO-X-3	BIPM-E-5	High-6	BIPM-G-5
34	EXHM-X-3	Blank	Blank	High-7	High-8	Blank	High-8
35	High-7	Low-9	Low-7	Blank	Blank	Low-7	Blank
36	Blank	NIMT-Y-3	BIPM-C-4	Low-8	Low-9	BIPM-F-4	Low-9
37	Low-8	High-9	INRAP-X-3	LATU-X-3	INTI-Y-3	SASO-X-3	NMISA-Y-3
38	INM-X-3	Blank	UME-X-3	INMETRO-Y-3	High-9	NIM-CBKT-X-3	High-9
39	EXHM-Y-3	Low-10	High-7	High-8	Blank	High-7	Blank
40	High-8	BIPM-B-6	Blank	Blank	Low-10	Blank	Low-10
41	Blank	High-10	Low-8	Low-9	BIPM-E-6	Low-8	BIPM-G-6
42	Low-9	Blank	BIPM-C-5	LATU-Y-3	High-10	BIPM-F-5	High-10
43	INM-Y-3	Low-1	NIS-X-3	BIPM-D-6	Blank	NIM-OWN-X-3	Blank
44	BIPM-A-6		INRAP-Y-3	High-9		SASO-Y-3	
45	High-9		High-8	Blank		High-8	
46	Blank		Blank	Low-10		Blank	
47	Low-10		Low-9	High-10		Low-9	
48	High-10		UME-Y-3	Blank		NIM-CBKT-Y-3	
49	Blank		NIS-Y-3	Low-1		NIM-OWN-Y-3	
50			BIPM-C-6			BIPM-F-6	
51			High-9			High-9	
52			Blank			Blank	
53			Low-10			Low-10	
54			High-10			High-10	
55			Blank			Blank	
56						Low-1 (repeat)	
57						Blank	

Measurements and results

Subsequent to the LC-DAD-MS/MS analyses the UV absorption peak areas of PAT at 276 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 [23, 24]. 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) [25, 26] 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 [27]. Statistical approaches including GLS are discussed in detail by Duewer *et al*. [28].

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 PAT 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 PAT 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.d A, B, C, D, E, F and G are depicted in Figures 3-9, respectively.

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

NMI/DI	Calibration	w_{BIPM} (mg/kg)	$u(w_{BIPM})$ (mg/kg)	$U(w_{BIPM})$ (mg/kg)	*Quantification range* (mg/kg)
INM (CBKT) A	A	9.84	0.13	0.25	9-11
INM (CBKT) B	A	9.72	0.10	0.20	9-11
EXHM (own) A	A	10.21	0.12	0.24	9-11
EXHM (own) B	A	10.29	0.20	0.40	9-11
NIMT (CBKT) A	B	12.41	0.14	0.27	11-13
NIMT (CBKT) B	B	12.41	0.12	0.24	11-13
NIS (CRM) A	C	14.15	0.18	0.36	13-17
NIS (CRM) B	C	14.18	0.17	0.33	13-17
INRAP (CBKT) A	C	15.73	0.23	0.47	13-17
INRAP (CBKT) B	C	15.73	0.19	0.39	13-17
UME (CBKT) A	C	14.72	0.23	0.45	13-17
UME (CBKT) B	C	14.69	0.20	0.40	13-17
INMETRO (CBKT) A	D	19.02	0.52	1.03	18-21
INMETRO (CBKT) B	D	18.95	0.24	0.48	18-21
LATU (CBKT) A	D	20.33	0.34	0.67	18-21
LATU (CBKT) B	D	20.18	0.21	0.42	18-21
INTI (CBKT) A	E	22.45	0.27	0.55	21-24
INTI (CBKT) B	E	22.60	0.26	0.52	21-24
NIM (own) A	F	25.69	0.42	0.83	24-29
NIM (own) B	F	25.75	0.42	0.85	24-29
NIM (CBKT) A	F	25.85	0.40	0.80	24-29
NIM (CBKT) B	F	25.92	0.41	0.82	24-29
SASO (CRM) A	F	26.19	0.39	0.78	24-29
SASO (CRM) B	F	26.16	0.37	0.74	24-29
NMISA (own) A	G	63.40	0.86	1.72	60-68
NMISA (own) B	G	62.92	0.88	1.77	60-68

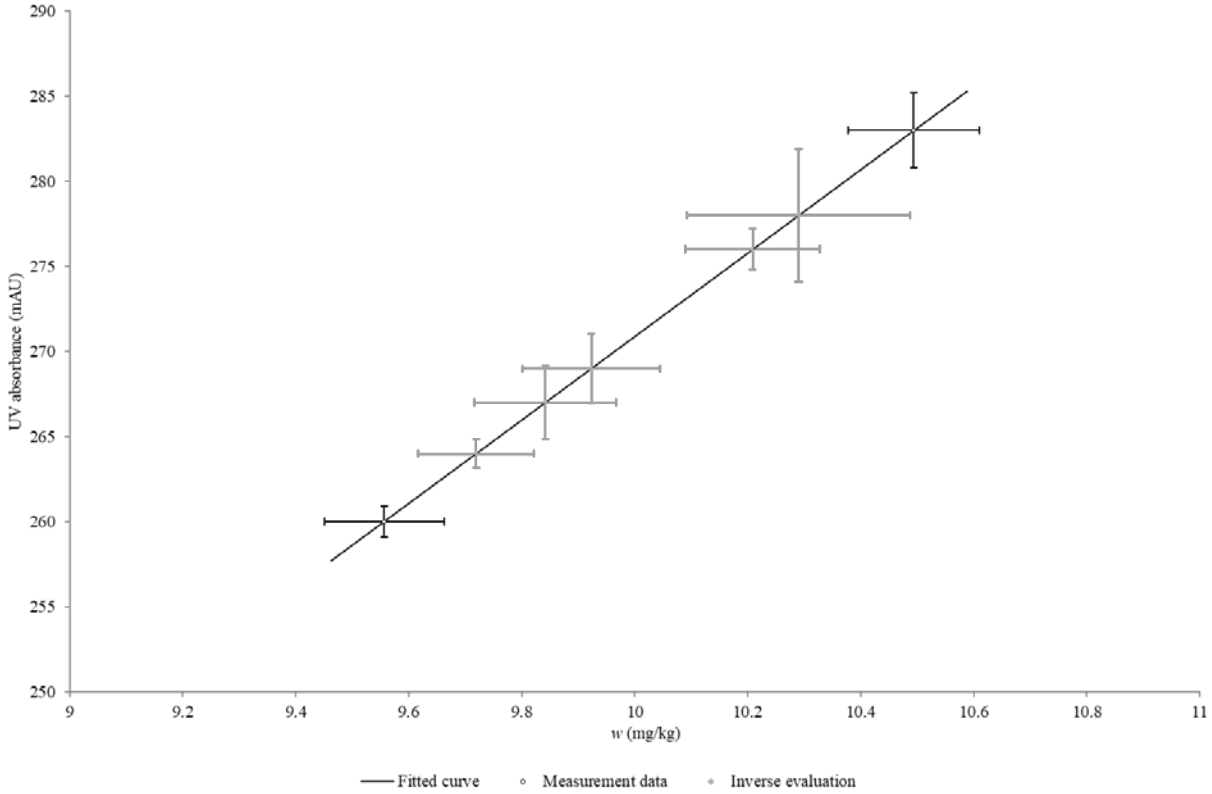

Figure 3: CCQM-K154.d - Calibration A - Bracketing calibration for the PAT mass fraction quantification range of 9-11 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as black circles at the upper and lower end of the calibration line. Inverse evaluation data of INM (CBKT), EXHM (own) and internal control sample are depicted as grey dots.

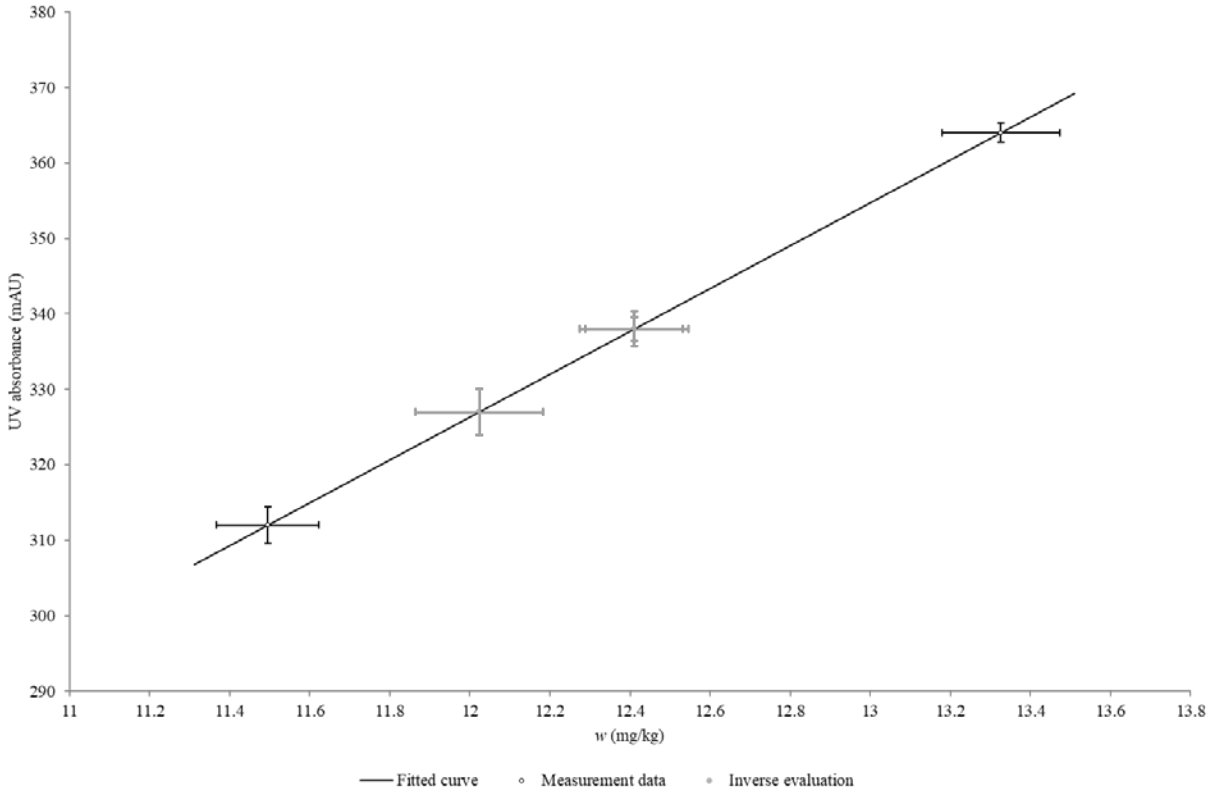

Figure 4: CCQM-K154.d - Calibration B - Bracketing calibration for the PAT mass fraction quantification range of 11-13 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as black circles at the upper and lower end of the calibration line. Inverse evaluation data of NIMT (CBKT) and internal control sample are depicted as grey dots.

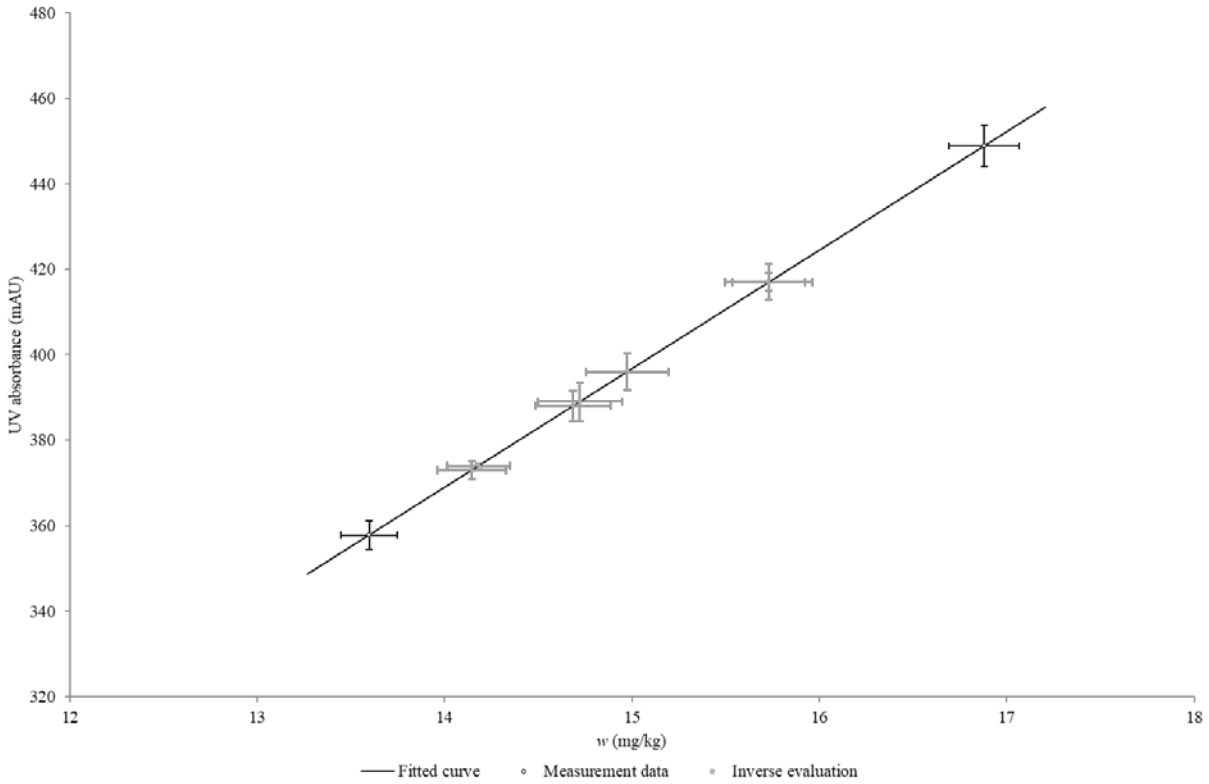

Figure 5: CCQM-K154.d - Calibration C - Bracketing calibration for the PAT mass fraction quantification range of 13-17 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as black circles at the upper and lower end of the calibration line. Inverse evaluation data of NIS (CRM), UME (CBKT), INRAP (CBKT) and internal control sample are depicted as grey dots.

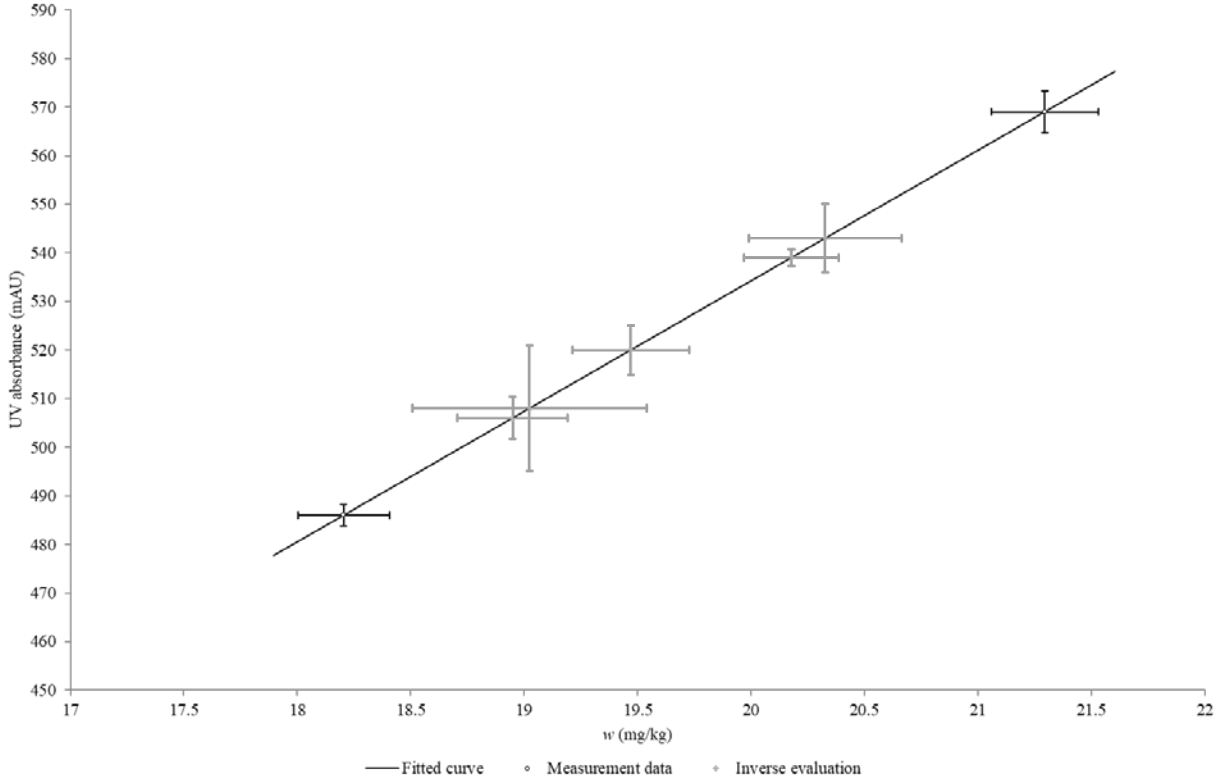

Figure 6: CCQM-K154.d - Calibration D - Bracketing calibration for the PAT mass fraction quantification range of 18-21 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as black circles at the upper and lower end of the calibration line. Inverse evaluation data of INMETRO (CBKT), LATU (CBKT) and internal control sample are depicted as grey dots.

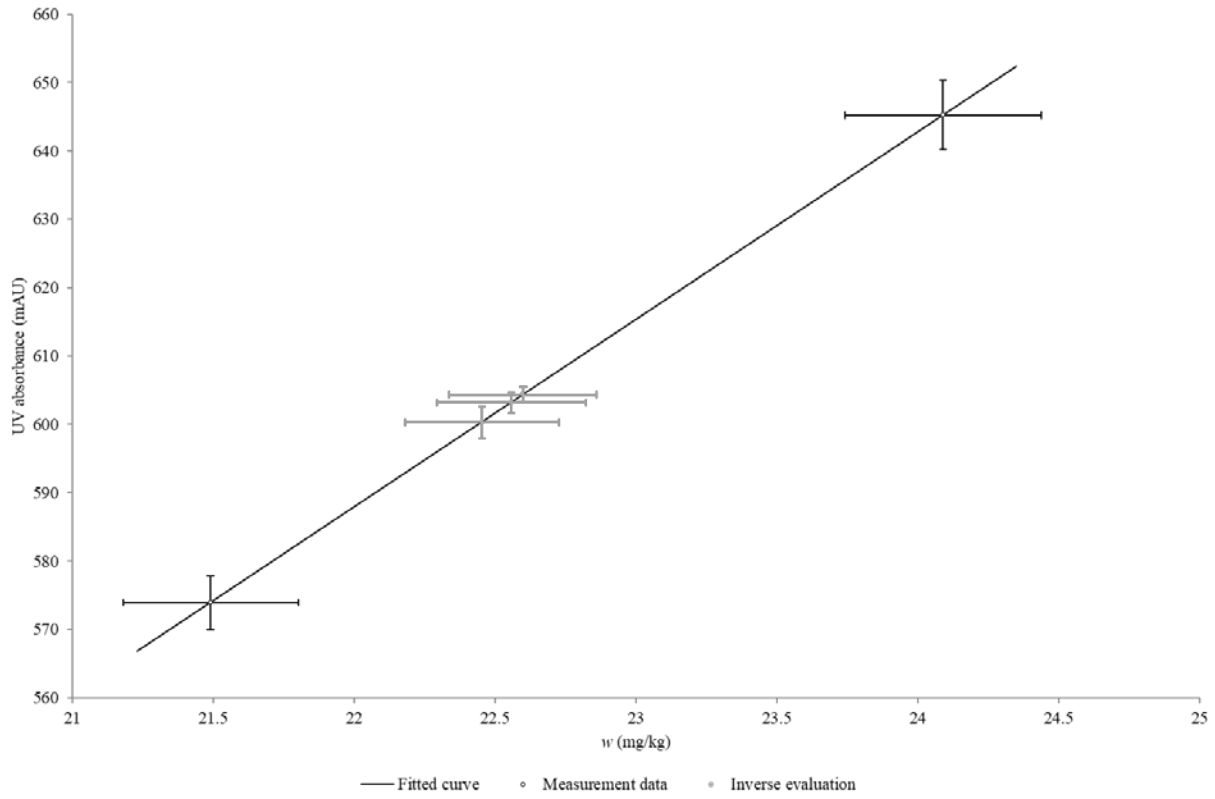

Figure 7: CCQM-K154.d - Calibration E - Bracketing calibration for the PAT mass fraction quantification range of 21-24 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as black circles at the upper and lower end of the calibration line. Inverse evaluation data of INTI (CBKT) and internal control sample are depicted as grey dots.

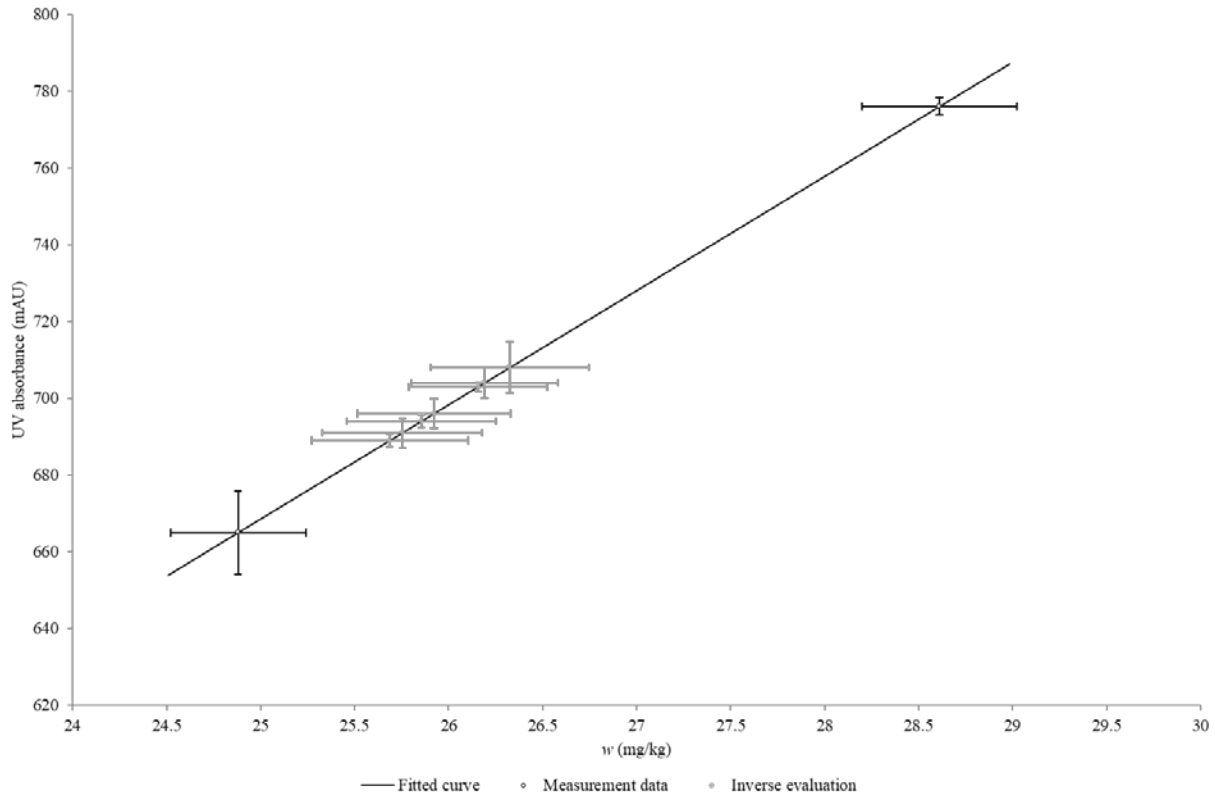

Figure 8: CCQM-K154.d - Calibration F - Bracketing calibration for the PAT mass fraction quantification range of 24-29 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as black circles at the upper and lower end of the calibration line. Inverse evaluation data of NIM (own), NIM (CBKT), SASO (CRM) and internal control sample are depicted as grey dots.

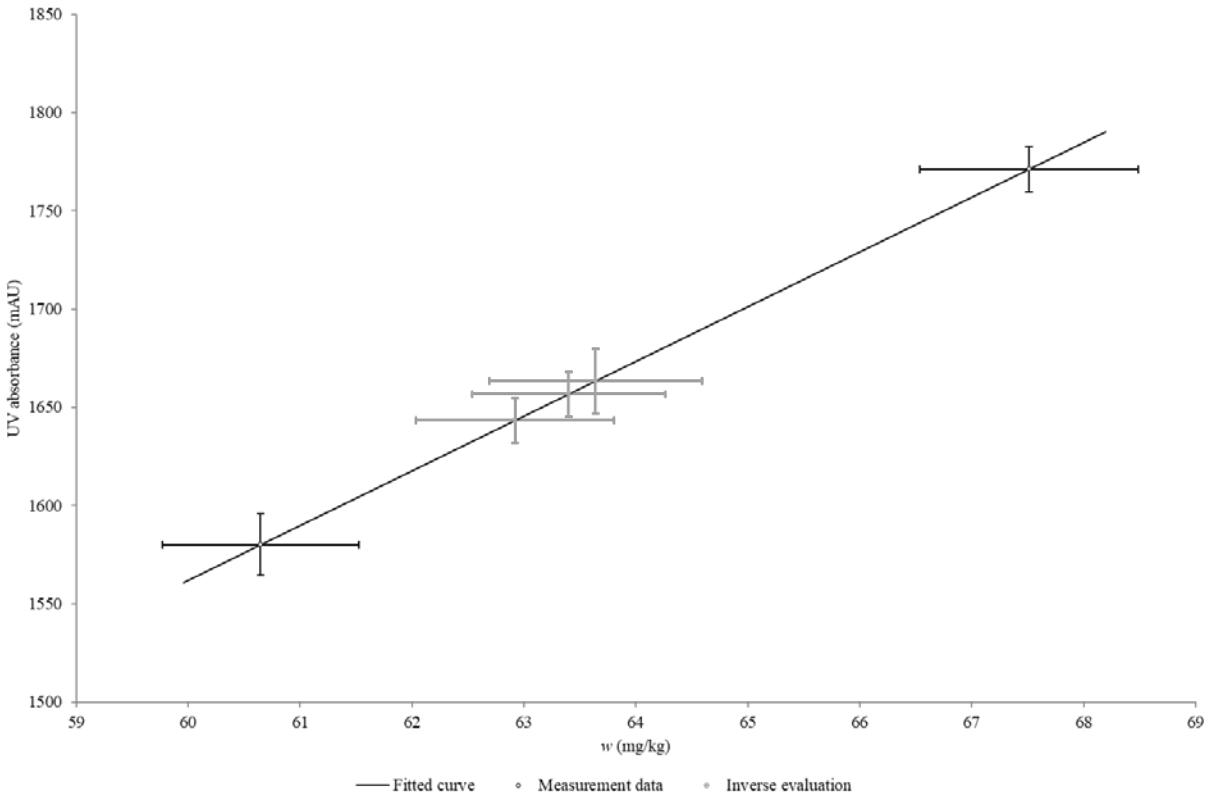

Figure 9: CCQM-K154.d - Calibration G - Bracketing calibration for the PAT mass fraction quantification range of 60-68 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as black circles at the upper and lower end of the calibration line. Inverse evaluation data of NMISA (own) and internal control sample are depicted as grey dots.

KEY COMPARISON REFERENCE VALUES FOR CCQM-K154.d

The PAT mass fraction values used to establish the Key Comparison Reference Values (KCRVs) for CCQM-K154.d 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 PAT mass fraction (w_{KCRV}) and its corresponding uncertainty ($u(w_{KCRV})$). All NMIs/DIs (i) participating in CCQM-K154.d were required to submit estimates for the PAT 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 Equation 3.

$$D_i = w_i - w_{KCRV} \qquad \text{Eq. 3}$$

The expanded uncertainty U_i at a confidence level of about 95 % associated with the D_i was calculated by Equation 4.

$$U(D_i) = 2 \cdot \sqrt{u(w_i)^2 + u(w_{KCRV})^2} \qquad \text{Eq. 4}$$

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 by Equation 5.

$$D_{rel,i} = 100 - \left(\frac{100 \cdot w_{KCRV}}{w_i}\right) \qquad \text{Eq. 5}$$

The expanded uncertainty $U_{rel}(D_{rel,i})$ at a confidence level of about 95 % associated with the ($D_{rel,i}$) was calculated by Equation 6.

$$U_{rel}\left(D_{rel,i}\right) = 2 \cdot \sqrt{u_{rel}(w_i)^2 + u_{rel}(w_{KCRV})^2} \qquad \text{Eq. 6}$$

The PAT mass fractions values and associated absolute uncertainties with degree of equivalences for CCQM-K154.d 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 PAT 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: PAT mass fractions and absolute corresponding uncertainties with degree of equivalences for CCQM-K154.d

NMI/DI	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)
INM (CBKT) A	9.84	0.13	0.25	10.00	0.13	0.26	0.16	0.36	9-11
INM (CBKT) B	9.72	0.10	0.20	10.00	0.13	0.26	0.28	0.33	9-11
EXHM (own) A	10.21	0.12	0.24	10.00	0.154	0.31	-0.21	0.39	9-11
EXHM (own) B	10.29	0.20	0.40	10.00	0.154	0.31	-0.29	0.50	9-11
NIMT (CBKT) A	12.41	0.14	0.27	12.60	0.152	0.31	0.19	0.41	11-13
NIMT (CBKT) B	12.41	0.12	0.24	12.60	0.152	0.31	0.19	0.39	11-13
NIS (CRM) A	14.15	0.18	0.36	14.5	0.25	0.5	0.35	0.62	13-17
NIS (CRM) B	14.18	0.17	0.33	14.5	0.25	0.5	0.32	0.60	13-17
UME (CBKT) A	14.72	0.23	0.45	14.65	0.21	0.41	-0.07	0.62	13-17
UME (CBKT) B	14.69	0.20	0.40	14.65	0.21	0.41	-0.04	0.58	13-17
INRAP (CBKT) A	15.73	0.23	0.47	15.9157	0.2387	0.4774	0.18	0.67	13-17
INRAP (CBKT) B	15.73	0.19	0.39	15.9157	0.2387	0.4774	0.18	0.62	13-17
INMETRO (CBKT) A	19.02	0.52	1.03	19.24	0.22	0.45	0.22	1.12	18-21
INMETRO (CBKT) B	18.95	0.24	0.48	19.24	0.22	0.45	0.29	0.65	18-21
LATU (CBKT) A	20.33	0.34	0.67	20.08	0.28	0.57	-0.25	0.87	18-21
LATU (CBKT) B	20.18	0.21	0.42	20.08	0.28	0.57	-0.10	0.70	18-21
INTI (CBKT) A	22.45	0.27	0.55	22.84	0.26	0.52	0.39	0.75	21-24
INTI (CBKT) B	22.60	0.26	0.52	22.84	0.26	0.52	0.24	0.74	21-24
NIM (own) A	25.69	0.42	0.83	26.21	0.24	0.47	0.52	0.96	24-29
NIM (own) B	25.75	0.42	0.85	26.21	0.24	0.47	0.46	0.98	24-29
NIM (CBKT) A	25.85	0.40	0.80	26.12	0.35	0.70	0.27	1.06	24-29
NIM (CBKT) B	25.92	0.41	0.82	26.12	0.35	0.70	0.20	1.08	24-29
SASO (CRM) A	26.19	0.39	0.78	27.16	0.67	1.34	0.97	1.55	24-29
SASO (CRM) B	26.16	0.37	0.74	27.16	0.67	1.34	1.00	1.53	24-29
NMISA (own) A	63.40	0.86	1.72	63.8	0.69	1.4	0.40	2.21	60-68
NMISA (own) B	62.92	0.88	1.77	63.8	0.69	1.4	0.88	2.24	60-68

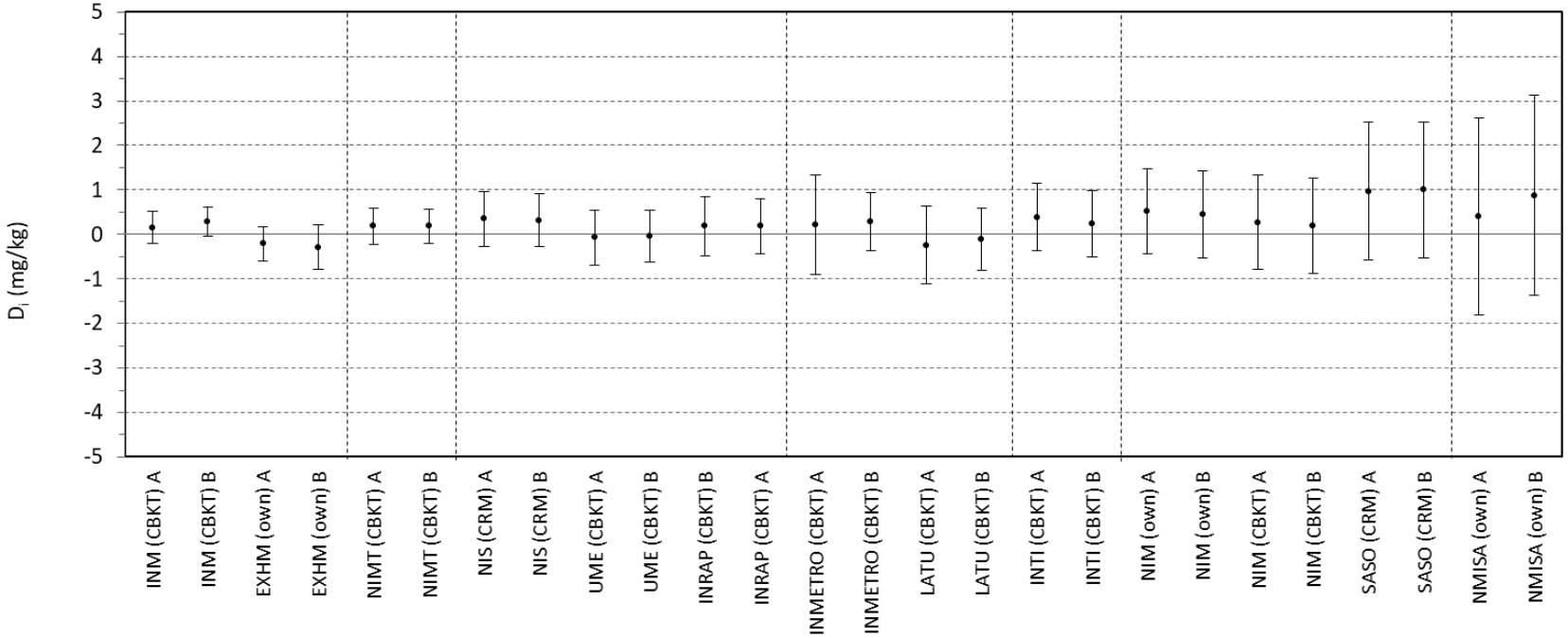

Figure 10: Absolute values for the degree of equivalence for CCQM-K154.d

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

NMI/DI	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)
INM (CBKT) A	9.84	1.28	2.55	10.00	1.30	2.60	1.58	3.64	9-11
INM (CBKT) B	9.72	1.05	2.10	10.00	1.30	2.60	2.80	3.35	9-11
EXHM (own) A	10.21	1.16	2.32	10.00	1.54	3.10	-2.09	3.86	9-11
EXHM (own) B	10.29	1.92	3.85	10.00	1.54	3.10	-2.90	4.93	9-11
NIMT (CBKT) A	12.41	1.10	2.19	12.60	1.21	2.46	1.50	3.26	11-13
NIMT (CBKT) B	12.41	0.98	1.96	12.60	1.21	2.46	1.50	3.11	11-13
NIS (CRM) A	14.15	1.29	2.58	14.5	1.72	3.45	2.44	4.30	13-17
NIS (CRM) B	14.18	1.17	2.34	14.5	1.72	3.45	2.20	4.17	13-17
UME (CBKT) A	14.72	1.53	3.07	14.65	1.43	2.80	-0.49	4.20	13-17
UME (CBKT) B	14.69	1.36	2.71	14.65	1.43	2.80	-0.25	3.95	13-17
INRAP (CBKT) A	15.73	1.48	2.97	15.9157	1.50	3.00	1.16	4.22	13-17
INRAP (CBKT) B	15.73	1.23	2.47	15.9157	1.50	3.00	1.16	3.88	13-17
INMETRO (CBKT) A	19.02	2.71	5.42	19.24	1.14	2.34	1.13	5.88	18-21
INMETRO (CBKT) B	18.95	1.28	2.55	19.24	1.14	2.34	1.51	3.43	18-21
LATU (CBKT) A	20.33	1.65	3.31	20.08	1.39	2.84	-1.23	4.33	18-21
LATU (CBKT) B	20.18	1.04	2.07	20.08	1.39	2.84	-0.49	3.47	18-21
INTI (CBKT) A	22.45	1.22	2.43	22.84	1.14	2.28	1.69	3.33	21-24
INTI (CBKT) B	22.60	1.16	2.32	22.84	1.14	2.28	1.05	3.25	21-24
NIM (own) A	25.69	1.62	3.24	26.21	0.92	1.79	2.00	3.72	24-29
NIM (own) B	25.75	1.65	3.30	26.21	0.92	1.79	1.74	3.77	24-29
NIM (CBKT) A	25.85	1.54	3.08	26.12	1.34	2.68	1.02	4.08	24-29
NIM (CBKT) B	25.92	1.58	3.15	26.12	1.34	2.68	0.76	4.14	24-29
SASO (CRM) A	26.19	1.48	2.96	27.16	2.47	4.93	3.57	5.76	24-29
SASO (CRM) B	26.16	1.41	2.82	27.16	2.47	4.93	3.69	5.68	24-29
NMISA (own) A	63.40	1.36	2.72	63.8	1.08	2.19	0.63	3.47	60-68
NMISA (own) B	62.92	1.40	2.81	63.8	1.08	2.19	1.38	3.54	60-68

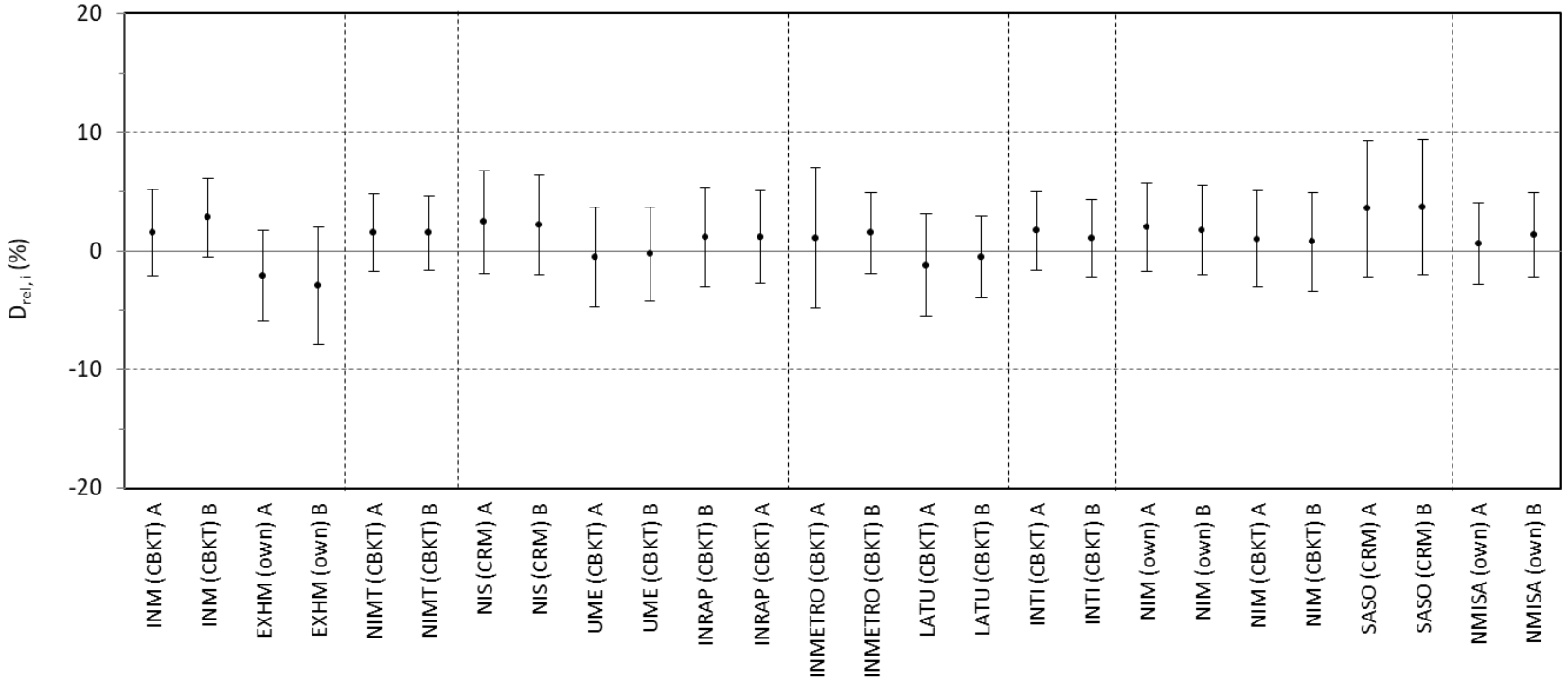

Figure 11: Relative values for the degree of equivalence for CCQM-K154.d

CONCLUSIONS

Patulin (PAT) was selected to be representative of polar *Penicillium/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 10 mg/kg to 100 mg/kg of mycotoxins with broadly similar structural characteristics.

Nine participants of the MMCBKT programme were provided with a stock solution having a known PAT mass fraction and expanded uncertainty to use to gravimetrically prepare and value assign a calibration solution. Two NMIs/DIs, namely NIS, Egypt and SASO, Saudi Arabia, participated using other non-proprietary reference materials to prepare their calibration solutions. 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 different groups.

It was decided to propose separate KCRVs for each of the two ampoules provided by the participating NMIs/DIs based on the PAT 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 PAT mass fraction.

The majority of the PAT mass fraction KCRVs (w_{KCRV}) for CCQM-K154.d spanned a mass fraction range of 9.72 mg/kg to 63.40 mg/kg. The relative expanded uncertainties $U(w_{KCRV})$ ranged from about 2.0 % to 5.4 %.

Inspection of the degree of equivalence plots (Figures 10 and 11) for the PAT mass fraction assignments in CCQM-K154.d indicated that there was an excellent agreement of results.

HOW FAR THE LIGHT SHINES STATEMENT (HFTLS)

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

a) Preparation and value assignment of patulin calibration solutions in the mass fraction range of 10 mg/kg to 100 mg/kg, prepared from a mycotoxin stock solution or solid of known purity.

Successful participation in CCQM-K154.d 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;
b) preparation and value assignment of single component organic calibration solutions in the mass fraction range of 10 mg/kg to 100 mg/kg, polarity (*pKow*) > -2, with molar mass in the range of 100 g/mol to 500 g/mol.

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[https://www.bipm.org/utils/common/pdf/rapportBIPM/RapportBIPM-2020-02.pdf]

ANNEX A – ADDITIONAL ANALYTICAL INFORMATION

Instituto Nacional de Tecnología Industrial (INTI), Argentina

Solution preparation procedure

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.035 standard PAT solution provided by BIPM

Patulin in acetonitrile with 0.1 % of formic acid stock solution. BIPM Reference: OGP.035.
Purity of main component and uncertainty: 261.2 ± 5.6 µg/g (k = 2).
Combination of PAT gravimetric preparation 261.2 µg/g with u_{char} of 0.68 ug/g (0.26 %), homogeneity contribution of ubb of 0.33 µg/g (0.13 %) and stability contribution of u_{lts} of 2.65 µg/g (1.02 %).
Used ampoules: OGP.035.134, OGP.035.068, OGP.035.029, OGP.035.105, OGP.035.180

Amount of primary calibrator used for analysis

14.47051 g

Gravimetry

Type of balance (make, model and resolution)

Make: Mettler Toledo
Model: XP 205DR
Resolution: 0.01 mg

Balance repeatability

30 µg

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 approximately 165 grams with acetonitrile with 0,1% of formic acid. The exact final weight was considered. 71 clear glass ampoules containing 3 ml of calibrant solution were produced. Batch was called 2022-PAT-DAI temporally.

Homogeneity and/or stability testing

Homogeneity study was carried out considering 10 ampoules from the batch 2022-PAT-DAI. Homogeneity was assessed by HPLC-DAD at 276 nm, 267 nm and 254 nm. The result obtained by HPLC-DAD at 267 nm was considered as inhomogeneity of batch 2022-PAT-DAI (u*bb = 0.39%). Stability study was carried out following an isochronous experiment design. Three different conditions were considered: 4 °C, 20 °C and 37 °C (all conditions in dark) during 2, 4, 6 and 8 weeks. The reference condition was -20 °C in dark. Two ampoules were measured for each temperature and each period of time. HPLC-DAD was used to evaluate the stability. The results of the stability studies of 2022-PAT-DAI did not show decreasing trends in any condition. All samples were analysed directly without dilution in a stratified random order. ISO Guide 35 (2017) was considered for all studies.

Optional: Analytical check method

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

HPLC-DAD Conditions:
Column: Kromasil 100-5-C18 (4.6 x 250mm) (Batch/Serial: 0000139143/E173118)
Mobile Phase: (Water + ACN) (75 + 25) with 0.1 % of formic acid
Flow rate: 0.8 ml/min
Run Time: 15 minutes
Retention time of PAT: 5-6 min
Oven temperature: 30 °C
Injection Volume: 10 µl
Detector Wavelengths: 276 nm, 267 nm and 254 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 PAT gravimetric preparation uncertainty (u_{char}) of 0.25 µg/g (1.07 %) and the homogeneity contribution of u_{bb} of 0.09 µg/g (0.39 %).

Mass fraction assignment

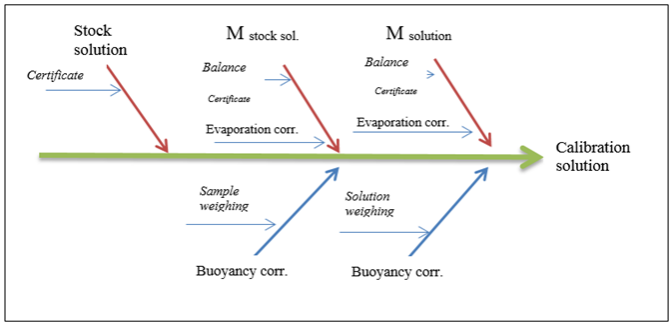
$$w_{PAT\ calib\ solution} = \frac{w_{PAT\ stock} \cdot m_{bal\ stock} \cdot b_{METTLER\ TOLEDO\ XPE205DR} \cdot Ev}{m_{bal\ Sol} \cdot b_{METTLER\ TOLEDO\ XPE205DR} \cdot Ev}$$

Calibrant Solution Preparation	
Stock Solution (mg) ($m_{bal\ stock}$) (mg)	14470.51
Whole Solution ($m_{bal\ sol}$) (mg)	165523.51
Stock Solution Concentration ($W_{PAT\ stock\ solution}$) (µg/g)	**261.2**
Buoyancy Sartorius LA230P ($b_{METTLER\ TOLEDO\ XP205DR}$)	1.00139
Evaporation Correction (Ev)	1
Calibrant Solution Concentration ($W_{PAT\ calibration\ solution}$) (µg/g)	**22.835**

Uncertainty assignment (Gravimetric Procedure)

$$u_{c,rel}(W_{PAT-calib\ sol}) = \sqrt{\left\{\frac{u_c(w_{PAT\ stock})}{w_{PAT\ stock}}\right\}^2 + \left\{\frac{u_c(m_{bal\ stock\ sol})}{m_{bal\ stock\ sol}}\right\}^2 + \left\{\frac{u_c(b_{METTLER\ TOLEDO\ XP205DR})}{b_{SMETTLER\ TOLEDO\ XP205DR}}\right\}^2 + \left\{\frac{u_c(m_{bal\ sol})}{m_{bal\ sol}}\right\}^2 + \left\{\frac{u_c(b_{METTLER\ TOLEDO\ XP205DR})}{b_{METTLER\ TOLEDO\ XP205DR}}\right\}^2 + \left\{\frac{u_c(E_v)}{E_v}\right\}^2 + \left\{\frac{u_c(E_v)}{E_v}\right\}^2}$$

Resource of Uncertainty	Value
$\frac{u_c(w_{PAT\ stock})}{w_{PAT\ stock}}$	2.800
$\frac{u(m_{bal\ stock\ sol})}{m_{bal\ stock\ sol}}$	0.00097
$\frac{u_c(m_{bal\ sol})}{m_{bal\ sol}}$	0.01104
$\frac{u_c(b_{METTLER\ TOLEDO\ XP205DR})}{b_{METTLER\ TOLEDO\ XP205DR}}$	0.0000117
$\frac{u(Ev)}{Ev}$	0.00005
$u_{c,rel}(w_{PAT-calib\ sol})$	0.0012208
$U_{Crel}(w_{PAT-calib\ sol})$(k=2)	0.0024416
$u_c(w_{PAT-calib\ sol})$ µg/g	0.245
$U_c(w_{PAT-calib\ sol})$ µg/g(k=2)	0.490
U_c (%)	**2.1441**



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

Solution preparation procedure

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.035 standard PAT solution provided by BIPM

OGP.035 standard PAT solution provided by BIPM: (261.2 ± 5.6) µg/g ; k = 2, 95 %.

Amount of primary calibrator used for analysis

14.3 g

Gravimetry

Type of balance (make, model and resolution)

Balance 1: Mettler Toledo, model XS205, resolution 0.0001g (used for OGP.035.037 / OGP.035.059 /OGP.035.126 / OGP.035.137 /OGP.035.167); Balance 2: Mettler Toledo, model PR1203, resolution 0.001g (used for whole solution).

Balance repeatability

300 µg for model XS205 and 1000 µg for model PR 1203

Solution preparation procedure

The amount of five ampoules from OGP.035 were used for gravimetric preparation in acidified acetonitrile (0.1 % formic acid). Solution was measured directly without further dilution on homogeneity and stability studies.

Homogeneity and/or stability testing

Homogeneity study was performed with 10 ampoules randomly selected. The heterogeneity contribution of 0,31 % was included on uncertainty of assigned value. The short-term stability study showed that samples can be transported up to 50°C during 28 days, without changes on certified value, and its uncertainty contribution was also included on uncertainty of assigned value. Both studies were performed using HPLC-DAD (l = 276 nm) to measure the main compound (PAT).

Optional: Analytical check method

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

UPLC-DAD-MS/MS (ESI-), XEVO TQ Waters
Column: Phenomenex Luna C18, (250 x 4.6 mm) 100 A
Mobile phase: A = H_2O; B = Acetonitrile. Isocratic: 15 % A / 85 % B.
Flow rate: 0.5 mL/min ; injection volume: 5 µL; run time: 10 min
Detector: DAD 276 nm; Resolution: 4.8 nm.

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

Bracketing. Two calibration solutions (high and low mass fraction) was used to check the gravimetric preparation of PAT batch. Both solutions were produced from BIPM OGP.035.

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

BIPM OGP.035 (PAT in acetonitrile): (261.2 ± 5.6) µg/g ; k = 2, 95 %.

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

MRM with ESI (-) and transitions m/z = 153 >109 and m/z = 153 > 81 was used to monitoring DAD analysis.

Additional Comments or Observations

The value assigned of Patulin (PAT) is the gravimetric preparation value with relative combination of uncertainties from characterization (gravimetric preparation), homogeneity and short-term stability.
The assigned uncertainty is a combination of PAT gravimetric preparation uncertainty (u_{char}) of 0.25 µg/g (1.07 %) and the homogeneity contribution of u_{bb} of 0.09 µg/g (0.39 %).

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

<u>Solution preparation procedure</u>

Calibrator

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

The PAT pure material was obtained from First Standard company. The purity value of PAT material was assigned by NIM with mass balance method and qNMR method. The main component of PAT material was assigned directly by qNMR method. HPLC-MS/MS and HPLC-DAD methods were applied to measure the content of organic impurites of PAT material; the moisture and inorganic impurities were determined by Karl Fischer and ICP-MS methods. VOC content was detected by GC-ECD. Finally, a purity of 996.8 mg/g with an uncertainty of 2.9 mg/g (k = 2) was attributed to the PAT pure material.

Amount of primary calibrator used for analysis

0.0051857 g

Gravimetry

Type of balance (make, model and resolution)

Sartorius	SE2	0.0001 mg
Sartorius	ME614S	0.1 mg

Balance repeatability

0.6 µg	SE2
0.2 mg	ME614S

Solution preparation procedure

5.1857 mg of the PAT pure material was weighed and transferred to a 250 mL flask. The flask was diluted to the mark with acetonitrile (0.1 % formic acid) and weighed to 197.1924 g (250 mL at 20 °C). The solution with a final concentration of 26.42 µg/g PAT in acetonitrile was obtained. A volume of 2 mL of the calibration solution was dispensed into 5 mL amber glass ampoules using a manual dispenser. The ampules were sealed with an ampoule sealer. A total of 113 ampoules were produced, labelled and stored in a freezer at -20 °C.

Homogeneity and/or stability testing

Homogeneity of the PAT solutions was tested by selecting 10 ampules of the 113 ampoules. Two aliquots (1 mL) per ampoule were transferred into two HPLC vials and were analysed directly without dilution in stratified order by HPLC-DAD method. The results of homogeneity test were subject to an analysis of variance (ANOVA). The results from homogeneity test of PAT solutions were summarised in Table 1 as follows.
The isochronous stability study of PAT solutions was tested at 20 °C for 0, 1, 3, 6, 10 days (2 ampoules at each study time point). Two aliquots (1 mL) per ampoule were transferred into two HPLC vials and were analysed directly without dilution in stratified order by HPLC-DAD method. The results of stability test were evaluated using trend analysis. The results from stability test of PAT solutions were summarised in Table 2 as follows.

Table 1

Homogeneity-NIM	**PAT in acetonitrile**
Mean	25.98 µg/g
SD	0.03 µg/g
N	20
F	1.55
*F*crit	3.02
Significance? (*F*>*F*crit)	No
s_{wb}	0.03 µg/g
s_{bb}	0.01 µg/g
u'_{bb}	0.01 µg/g
u_{bb}	0.01 µg/g
u_{bb}	0.05%

Table 2

Stability 20 °C	**PAT in acetonitrile**
β_1	-0.0009
β_0	25.95
F	2.71
*F*crit	3.05
Significance? (*F*>*F*crit)	No
s(β1)	0.003
T	0.26
*T*crit	3.18
Significance? (*T*>*T*crit)	No
u_{st} (10 days)	0.03 µg/g
u_{st} (10 days)	0.13%

Optional: Analytical check method

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

Shimadzu LC-20A
Column: Agilent ZORBAX Eclipse plus C18（4.6 mm × 250 mm, 5 μm)
Wavelength: 276 nm
Mobile phase: Isocratic elution with 15 % methanol and 85 % water
Flow rate: 1 mL/min
Injection volume: 5 μL

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

Single-point method was applied to assign the mass fraction value of comparison sample. Based on the BIPM OGP.035 PAT stock solution, another calibration solution of 26.06 μg/g was gravimetrically prepared as the calibrator.

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

OGP.035 PAT stock solution provided by BIPM, 261.2 ± 5.6 μg/g

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

None.

Additional Comments or Observations

None. Uncertainty budget:

Uncertainty budget			
Source of uncertainty	Parameter x	Uncertainty	
		u(x)	u(x)/(x)
Purity of PAT material (mg/g)	996.8	1.5	0.15%
mPAT (mg)	5.1857	0.001	0.0182%
mPAT+ACN (g)	197.1924	0.001	0.0004%
Inhomogeneity (μg/g)	26.21	0.013	0.05%
Instability (μg/g)	26.21	0.034	0.13%
Bias caused by filliing (μg/g)	26.21	0.23	0.88%
Combined standard uncertainty (μg/g)	26.21	0.24	0.90%
Expanded uncertainty (μg/g) (k=2)	26.21	0.47	1.80%

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.035 standard PAT solution provided by BIPM

OGP.035 standard PAT solution provided by BIPM, 261.2 ± 5.6 µg/g.

Amount of primary calibrator used for analysis

6.2055 g

Gravimetry

Type of balance (make, model and resolution)

Sartorius ME235S 0.01 mg

Balance repeatability

40 µg ME235S

Solution preparation procedure

A 6.2055 g portion of the 261.2 ± 5.6 µg/g stock solution (about 8 mL at 21 °C) was transferred to a 250 mL flask. Then about 72 mL of acetonitrile with 0.1 % formic acid was transferred to the same flask, and the mixed solution (both stock solution and acetonitrile) was weighted to 62.0510 g. The solution with a final concentration of 26.12 µg/g PAT in acetonitrile was obtained. A volume of 2 mL of the calibration solution was dispensed into 5 mL amber glass ampoules using a manual dispenser. The ampoules were sealed with an ampoule sealer. A total of 40 ampoules were produced, labelled and stored in a freezer at -20 °C .

Homogeneity and/or stability testing

Homogeneity of the PAT solutions was tested by selecting 10 ampoules of the 40 ampoules. Two aliquots (1 mL) per ampule were transferred into two HPLC vials and were analysed directly without dilution in stratified order by HPLC-DAD method. The results of homogeneity test were subject to an analysis of variance (ANOVA). The results from homogeneity test of PAT solutions were summarised in Table 1 as follows.

The isochronous stability study of PAT solutions was tested at 20 °C for 0, 1, 3, 6, 10 days (2 ampoules at each study time point). Two aliquots (1 mL) per ampoule were transferred into two HPLC vials and were analysed directly without dilution in stratified order by HPLC-DAD method. The results of stability test were evaluated using trend analysis. The results from stability test of PAT solutions were summarised in Table 2 as follows.

Table 1

Homogeneity-CBKT	**PAT in acetonitrile**
Mean	26.03 µg/g
SD	0.03 µg/g
N	20
F	2.60
*F*crit	3.02
Significance? (*F*>*F*crit)	No
s_{wb}	0.02 µg/g
s_{bb}	0.02 µg/g
u'_{bb}	0.01 µg/g
u_{bb}	0.02 µg/g
u_{bb}	0.08%

Table 2

Stability 20 °C	**PAT in acetonitrile**
β_1	-0.0052
β_0	26.04
F	2.85
*F*crit	3.06
Significance? (*F*>*F*crit)	No
s(β1)	0.0020
T	2.60
*T*crit	3.18
Significance? (*T*>*T*crit)	No
u_{st} (10 days)	0.02 µg/g
u_{st} (10 days)	0.08%

Optional: Analytical check method

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

Shimadzu LC-20A
Column: Agilent ZORBAX Eclipse plus C18（4.6 mm × 250 mm, 5 μm)
Wavelength: 276 nm
Mobile phase: Isocratic elution with 15 % methanol and 85 % water
Flow rate: 1 mL/min
Injection volume: 5 μL

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

Single-point method was applied to assign the mass fraction value of comparison sample. Based on the BIPM OGP.035 PAT stock solution, another calibration solution of 26.06 μg/g was gravimetrically prepared as the calibrator.

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

OGP.035 PAT stock solution provided by BIPM, 261.2 ± 5.6 μg/g

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

None.

Additional Comments or Observations

None. Uncertainty budget:

Uncertainty budget			
Source of uncertainty	Parameter x	Uncertainty	
		u(x)	u(x)/(x)
Stock solution (μg/g)	261.2	2.8	1.07%
mPAT (mg)	6205.5	0.06	0.0009%
mPAT+ACN (mg)	62051.0	0.06	0.0001%
Inhomogeneity (μg/g)	26.12	0.02	0.08%
Instability (μg/g)	26.12	0.02	0.08%
Bias caused by filliing (μg/g)	26.12	0.21	0.80%
Combined standard uncertainty (μg/g)	26.12	0.35	1.34%
Expanded uncertainty (μg/g) (k=2)	26.12	0.70	2.69%

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.035 standard PAT solution provided by BIPM

OGP.035 PAT, stock solution (Provided by BIPM)
Metrological traceability: through PAT, OGO.180a (BIPM)
Mass fraction: 261.2 ug/g ± 5.6 ug/g (k = 2)

Amount of primary calibrator used for analysis

1.6746 g stock solution, 1.3478 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, 8.1640 g of the OGP.035 solution was weighed and acetonitrile (LC-MS grade) was used as the solvent. 213.1997 g of the solution was obtained with a mass fraction of 10.00 ug/g ± 0.11 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. Seven replicates by the unit were measured under repeatability conditions through LC-DAD.

Short term stability: The short term stability was evaluated using an isochronous and accelerated design. The material was exposed to 40 °C for two weeks (the reference temperature was -20 °C), one ampoule per time were taken, for a total of five points. The PAT determination was realized through LC-DAD, seven 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:	ACN (A)	Water (B)
Programm (Isocratic)	10	90
Column temperature (°C)	40	ThermoScientific Acclaim C18 100 mm x 2.1 mm 2.2 um
Injection volume (ul)	5	
Flow (ml/min)	0.25	
Run time (min)	4	

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

Bracketing.

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

OGP 035 Stock solution
261.2 ug/g ± 5.6 ug/g (k = 2)
Metrological traceability: through PAT OGO.180a (BIPM)

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

-/-

Additional Comments or Observations

The value obtained throught LC-DAD was 9.99 ug/g ± 0.34 ug/g (k = 1.97). This uncertainty does not include the homogeneity uncertainty or short term stability uncertainty.

National Institute of Standards (NIS), Egypt

Solution preparation procedure

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.035 standard PAT solution provided by BIPM

NMISA-QC-PAT220506

Amount of primary calibrator used for analysis

3 g

Gravimetry

Type of balance (make, model and resolution)

Make: Sartorius micro electronic balance (five digits)
Model: ME235S
Resolution: 0.01 mg

Balance repeatability

40 μg

Solution preparation procedure

Gravimetric preparation.

Homogeneity and/or stability testing

HPLC.

Optional: Analytical check method

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

Mobile phase consists of 95 % per volume of water with 5 % per volume of acetonitrile and 0.095 % per volume of perchloric acid. Patulin was separated by reversed phase C18 column with 1.0 mL/min flow rate and UV detection at 276 nm.

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

Bracketing external calibration.

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

NMISA-QC-PAT220506

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

-/-

Additional Comments or Observations

-/-

Hellenic Metrology Institute (EXHM), Greece

<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.035 standard PAT solution provided by BIPM

Powder patulin was purchased from Fermentek. Purity was determined with qNMR in acetone-d6 using 3,5 BTFMBA NMIJ CRM 4601-a as the internal standard. HSQC, COSY, ^{1}H, ^{13}C 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 Fermentek material.

Amount of primary calibrator used for analysis

3 mg

Gravimetry

Type of balance (make, model and resolution)

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

Balance repeatability

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

Solution preparation procedure

An intermediate solution was initially prepared by dissolving patulin (0.0091268 g) in acetonitrile (3.06612 g) in an amber glass vial. For the preparation of the final solution, 0.41220 g of the intermediate solution were transferred in a bottle containing acetonitrile (121.49560 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 vials.

Homogeneity and/or stability testing

For the homogeneity test: 10 vials were analyzed in duplicate with LC-DAD (at 276, 267 and 254 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 and four weeks). For each combination condition (time and temperature) two samples were 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 276, 267 and 254 nm). The mobile phase was constituted of: A = 95 % H2O + 5 % ACN + 0.1 % FA, B = acetonitrile + 0.1% FA in the following gradient mode: A = 100 %, for t = 0 - 2min, then the composition of B was increased to B = 95 % in the next 20 min and held for 2 min. The composition returned to A = 100 % in the next 2 min and the column was equilibrated for 14 min (total run time 40 min). For the chromatographic separation the column Inertsil ODS-3, 250 mm x 2.1mm, 5µm 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 bisphenol A as the internal standard.

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

The calibrants used for the verification were: reference material: N'TOX patulin in acetonitrile with concentration 100 ± 6 µg/mL. It was also verified with a different stock solution (295 mg/kg) made from the Frementek material. The verification was carried out both by bracketing and also at exact matching concentrations.

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} = 0.14 %), 2) homogeneity (u_{homo} = 1.05 %), 3) stability (u_{stab} =1.02 %) and characterization (u_{char} = 0.47 %).

The measurement equation is:

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

where
w_{pat} = mass fraction of the analyte (patulin) in the sample, (mg/kg)
P_{pat} = purity of patulin, (mg/g)
m_{pat} = the mass of patulin 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:

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

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

Purity was calculated with qNMR via the following equation by :

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

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 patulin
n_s: number of protons integrated for the quantification of each compound
Is: integral area of the respective quantification peak of patulin
P_{pat}: mass fraction of compound analysed

Finally, the combined standard uncertainty of the concentration of patulin 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:

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

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).

The uncertainty budgets for patulin are shown in the following

qNMR uncertainty budget

qNMR uncertainty budget							
uncertainty component	value	units	u_i	u_i/x_i	C_i	C_iu_i	$(C_iu_i)^2$
PAT/3,5BTFMBA signal ratio	1,5729		0,00181	1,153E-03	633,57	1,1489	1,320E+00
PAT molecular mass	154,129	g mol^{-1}	0,00600	3,893E-05	6,47	0,0388	1,505E-03
3,5 BTFMBA molecular mass	258,120	g mol^{-1}	0,00600	2,325E-05	-3,86	-0,0232	5,366E-04
no of protons in signal integrated for PAT	2	nucl/mol	0,00040	1,800E-05	-498,26	-0,1993	3,972E-02
no of protons in signal integrated for 3,5BTFMBA	3	nucl/mol	0,00040	1,800E-05	332,24	0,1329	1,766E-02
PAT mass	3,0731	mg	0,00100	3,254E-04	-324,27	-0,3243	1,052E-01
3,5 BTFMBA mass	2,1751	mg	0,00100	4,597E-04	458,15	0,4582	2,099E-01
boyancy correction	1,0000		0,00000	4,065E-06	996,53	0,0041	1,641E-05
3,5 BTFMBA	999,60	mg g^{-1}	0,30000	3,001E-04	1,00	0,2991	8,945E-02
PAT purity							**996,54**
combined standard uncertainty		**mg g^{-1}**					**1,34**
expanded uncertainty (k=2)		mg g^{-1}					2,67

Preparation data and uncertainty

Patulin - intermediate	value	units	standard uncertainty	relative uncertainty	sensitivity coefficient	C_iu_i	$(C_iu_i)^2$
Patulin	0,0091268	g	0,000001	0,00011	0,32309	3,23E-07	1,04E-13
MeCN/0.1%FA	3,06612	g	0,000027	0,00001	-0,00096	-2,58E-08	6,68E-16
Patulin purity	0,99654		0,001340	0,00134	0,00297	3,98E-06	1,58E-11
							1,592E-11
concentration	2,958	**mg/g**					
combined std uncertainty	0,004	0,000					
relative uncertainty	0,135	(%)					
coverage factor	2						
expanded uncertainty	0,008	mg/g					

Patulin FINAL	value	units	standard uncertainty	relative uncertainty	sensitivity coefficient	C_iu_i	$(C_iu_i)^2$
Patulin - intermediate	0,4122	g	0,000083	0,00020	0,02418	2,00E-06	3,98E-12
MeCN/0.1%FA	121,4956	g	0,000827	0,00001	-0,00008	-6,79E-08	4,60E-15
concentration	2,958	g/g	0,003990	0,00135	0,00338	1,35E-05	1,82E-10
							1,86E-10
concentration	10,000	mg/kg					
combined std uncertainty	0,014	mg/kg					
relative uncertainty	0,136	(%)					
coverage factor	2						
expanded uncertainty	0,027	mg/kg					

UNCERTAINTIES (mg/kg)							
Patulin conc (mg/kg)	u_{prep}	u_{homo}	u_{stab}	u_{char}	combined		expanded
10,000	0,014	0,105	0,103	0,047	0,154		0,309
%	0,136	1,047	1,025	0,467	1,54		3,09

Saudi Standards, Metrology and Quality Organization (SASO), Saudi Arabia

<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.035 standard PAT solution provided by BIPM

Source of primary calibrator was by gravimetry using pure substance RM with purity of 99.3 % with uncertainty 0.35 %, Metrological traceability of the measurement results was established by the use of the refence materials RM Patulin (DRE-C15896000) produced by the DI of LGC.

Amount of primary calibrator used for analysis

0.00433 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo
model XPE205
resolution 0.01mg

Balance repeatability

14 µg

Solution preparation procedure

A stock solution was prepared by weighing about 5 mg from the RM in 22.50 g of acetonitrile (0.1% Formic Acid) using the formula(1). After that 6 Calibration points (10,20,25,30,40 and 50) ppm were prepared by dilution from the stock using the formula(2). Where:

$$C = \frac{m * P}{m_t} \quad (1)$$

where:
C: Concentration of stock solution (mg/kg)
m: Mass of CRM (mg).
P: Purity/100
m_t: Total mass of solution (kg).

$$C_1 * m_1 = C_2 * m_2 \quad (2)$$

where:
C1: concentration of the CRM stock solution (mg/kg)
m1: mass taken from the CRM (mg)
C2: concentration of the target calibration solution (mg/kg)
m2: mass of calibration solution (mg)

Homogeneity and/or stability testing

For the homogeneity study, the stratified random selection was applied to select 8 ampoules out of 106 produced. The selected ampoules were tested by HPLC-UV using the simple randomized design in which a single run with all units observed, each 6 times in random order. The results obtained were statistically analyzed by ANOVA-single factor and no statistically significant differences between units was observed. The uncertainty of homogeneity was

$$\sigma_{h\,(ubb)} = \sqrt{\frac{MS_{within}}{n}} * \sqrt{\frac{2}{v\,(MS_{within})}} \quad (3)$$

For short term stability test, it was storged at room temparature and 4°C for 3 weeks. The uncertainty was calculated by formula(4):

$$u_{sts} = \frac{SD}{\sqrt{\sum(t_i - \bar{t})^2}} \quad (4)$$

Optional: Analytical check method

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

-/-

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

-/-

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 Patulin (PAT) material was sourced from Fermentek, Israel. The material was purity assigned by NMISA using quantitative NMR. QNMR of PAT purity was performed in deuterated acetone, using Fluka TraceCERT dimethylterphthalate as internal standard with traceability established in-house to NIST PS1 Benzoic acid using QNMR. The purity value determined was 993.9 ± 6.7 mg/g (k = 2).
The OGP.036 primary calibrator from BIPM was also used as 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 0033 using the BIPM calibrator was within the expanded uncertainty of the PAT solution submitted for this study.

Amount of primary calibrator used for analysis

0.015038 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo	XPR36	0.001 mg
Mettler Toledo	MS12002TS	0.01 g

Balance repeatability

2 µg

Solution preparation procedure

1. A 50 µg/mL solution (CRM0033) was prepared by weighing ~15 mg in house value assigned PAT (CRM0032) and diluting in 300 mL acidified acetonitrile.
2. The solution was ampouled (2 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.

Homogeneity and/or stability testing

Of the 119 ampoules prepared, 5 were selected at regular intervals across the batch for the homogeneity assessment. Three repeat aliquots, without dilution, from each ampoule were evaluated using LC-UV at 280 nm. The relative standard homogeneity uncertainty determined using ANOVA, was estimated as 0.23 % and is included in the combined uncertainty reported. An isochronous stability assessment was carried out over 6 weeks, where 2 ampoules were stored at 4 °C, 20 °C, 40 °C and 60 °C, for 1, 2, 4 and 6 weeks, and 2 ampoules were stored at a reference temperature of -80 °C. All samples stored in the dark. The stability of the solutions was evaluated using LC-UV, monitoring the change in concentration of PAT. No significant trends were observed at 4 °C, 20 °C or 40 °C over the 6 week period of the stability study. A significant trend was however observed at 60 °C, with an estimated relative u(Its) of 0.8 % for storage at 60 °C for 8 weeks. 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 PAT solution (CRM 0032) was injected onto a Phenomenex Synergi RP Polar column 80 Å, 150 x 4.6 mm, 4 µm (ANA0956) column. PAT and impurities were separated in a 12 min isocratic phase (0.8 mL/min) of 95:5 aqueous mobile phase:acetonitrile. The isocratic phase was followed by a gradient and column wash with high organic solvent where some minor impurities were detected at 280 nm. The 280 nm wavelength trace was used for homogeneity and stability assessment. The samples were simultaneously analysed by MS/MS to tentatively identify potential structurally related impurities to verify the QNMR purity value assignment.

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

The OBP.036 primary calibrator from BIPM was also used as 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 0033 using the BIPM calibrator was within the expanded uncertainty of the NMISA PAT CRM 00033 Lot 220506 solution submitted for this study.
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 PAT CRM0032 purity 993.9 mg/g with expanded uncertainty 6.7 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/MS. Some of the MS/MS transitions in ESI negative mode that were used to detect potential Patulin impurities were:
deoxypatulinic Acid (DPA) 155 >111; 155 >63 ascladiol (ASC) 155>125
Patulin (PAT) 153>81; 153 >109 5-hydroxymethylfurfural (HMF) 125>95

Additional Comments or Observations

In the original NMISA PAT crystalline material (CRM0032), 2 unidentified structurally related impurities were detected in the crystalline material.

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.035 standard PAT solution provided by BIPM

OGP.035.033,OGP.035.047,OGP.035.079 BIPM stock solution

Amount of primary calibrator used for analysis

9.1977 g

Gravimetry

Type of balance (make, model and resolution)

Mettler-Toledo XPR2004SC, 0.0001 g Resolution

Balance repeatability

105.6 μg

Solution preparation procedure

Patulin (PAT) solution was gravimetrically prepared. Three ampoules, BIPM OGP.035.033, OGP.035.047, and OGP.035.079 stock solutions, were combined. The combined stock solution was then diluted with acetonitrile to the final volume of 250 mL. An aliquot of 3 mL was dispensed to each ampoule. A total of 80 ampoules were obtained.

Homogeneity and/or stability testing

Homogeneity testing: Homogeneity was tested based on the results from High-performance liquid chromatography–photodiode array (HPLC–PDA). Ten randomly selected ampoules were analyzed in triplicate. The data set was then statistically analyzed using One-way ANOVA.
Stability testing: The data from HPLC-PDA was used for stability testing. Twenty six ampoules, stored at a reference temperature of -20 °C, were randomly selected and each ampoule was analyzed in triplicate. Stability testing was carried out using isochronous scheme. At each time point (1, 2, 3, and 4 weeks), two ampoules were placed at each storage temperature (4 °C, 22 °C and 40 °C). At the end of the studies, all samples at different storage temperatures and two samples from the reference temperature of -20 °C were analyzed. Trend analysis was

performed to statistically test short-term stability. 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: water:acetonotrile of 95:5 (isocratic elution)
Total flow rate: 0.5 mL/min
Column temperature: 30 °C
Injection volume: 5 µL

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

Single point bracketing 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. A pure certified reference material (CRM) PAT in acetonitrile obtained from NMISA was used as calibration solution. Details of PAT

	Certified value (µg/g)	Expanded Uncertainty (k=2) (µg/g)
Source: NMISA	63.8	1.4

*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 evaluated from gravimetric preparation, homogeneity and stabiltiy 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.035), µg/g
m_z	mass of the stock solution (OGP.035) added (g)
m_{total}	mass of the total solution (g)

Expanded Meaurement 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.035), µg/g
m_z	mass of the stock solution (OGP.035) 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

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

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.035)
$u(m_z), u(m_{total})$	standard uncertainties of masses estimaetd from the bias and the precision of balance
$u(F_{homo})$	standard uncertainty due to homogeneity factor, estimated from one-way 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				
PAT stock	261.2	2.80E+00	1.07E-02	1.15E-04
mass PAT stock (g)	9.19775	1.52E-04	1.65E-05	2.73E-10
mass total (g)	190.656725	3.13E-04	1.64E-06	2.69E-12
combined gravimetric		1.35E-01	1.07E-02	1.15E-04
Additional source				
Homogeneity	1	2.09E-03	2.09E-03	4.35E-06
Stability @4 °C	1	5.27E-03	5.27E-03	2.78E-05
wx	12.60093		sum (uxi/xi)^2	0.000147
			(uxi/xi)	0.012125
			u(cx) /Cx	0.012125
			ux	0.152790
			U (*k* =2)	0.305580
			U(%)	2.425060

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.035 standard PAT solution provided by BIPM

OGP.035 standard PAT solution provided by BIPM.

Amount of primary calibrator used for analysis

11.7477 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo, XPR504S, 0.01 g

Balance repeatability

100 µg

Solution preparation procedure

-/-

Homogeneity and/or stability testing

According to the ISO GUIDE 35, 10 ampoules was selected from the batch, analysed by HPLC-UV at the repeatability condition, for the 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)

Column: Agilent eclipse C15 (250 × 4,6) 5µm
Mobile phase: Phase A = H_2O+Acetonitrile (95:5 v/v) Phase B = Acetonitrile
Gradient mode: t 0-20 A:100 %, 20-22 A: 5 %, 22-24 A:5 % 24-26 A:100 % 26-40 A: 100 %
Analysis time: 40 min
Temperature: 30 °C
Injection volume: 5 µL .

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

External calibration, bracketing.

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

Calibration from NMISA QC, 63.8 ± 1.4 µg/g.

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

-/-

Additional Comments or Observations

-/-

National Metrology Institute of Turkey (UME), Turkey

Solution preparation procedure

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.035 standard PAT solution provided by BIPM

BIPM OGP.035

Amount of primary calibrator used for analysis

5.6567 g

Gravimetry

Type of balance (make, model and resolution)

Sartorius MSA524S-100-DA 0.1 mg

Balance repeatability

60 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 (5.6567 g) then capped and tared. Solution of 0.1 % formic acid in acetonitrile (93.9658 g) added into the flask, capped and weighed. The solution was mixed and chilled. Solution filled into 5 mL amber glass ampules as 2 mL by ampulmatic, purged with nitrogen then sealed.

Homogeneity and/or stability testing

Totally 50 ampoules were filled and stored at -20 ˚C after preparation. Totally 6 ampoules were selected randomly by TRANS program for homogeneity testing. Isochronous stability testing was performed at 25 ˚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 0.75 % uncertainty reported

for homogeneity. Stability results were evaluated by significance test on slope and 0.33 % uncertainty was reported for stability at 25˚C.

Optional: Analytical check method

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

Thermo UltiMate 3000 HPLC with UV detector used at 276 nm. Phenomenex Luna 5u C18 150 mm x 4.6 mm 5 µm column used at 30 ˚C for separation. Gradient program with A (Acetonitrile), B (water) at flow of 1.00 mL/min and program is given below. 2 µL sample injected into column.

Retention time (min)	Flow (mL/min)	A%	B%
0	1.00	95	05
11	1.00	95	05
12	1.00	00	100
17	1.00	00	100
18	1.00	95	05
20	1.00	95	05

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

Six-point external calibration used (5-50 µg/g).

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

BIPM OGP.035

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

-/-

Additional Comments or Observations

Uncertainty Budget			
		Uncertainty	
Source of uncertainty	Parameter (x)	u(x)	u(x)/x
BIPM Stock Purity	261.2	2.8	0.010719755
m_{stock} *(g)*	5.6567	0.000320	0.0000566
m_{ACN} (g)	93.9658	0.000320	0.0000034
Repeatability	14.65	0.054	0.0037
Homogeneity	14.65	0.111	0.0075
Stability	14.65	0.048	0.0033
Combined Standard Measurement Uncertainty, %			1.40
Expanded Measurement Uncertainty, % (*k=2*)			2.80
Concentration Value (µg/g)			**14.65**
Standard uncertainty, *u* (µg/g)			0.21
Expanded Uncertainty, U (µg/g)			0.41
Expanded Uncertainty reported, U (µg/g)			**0.41**

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{bb}^2 + u_{sts}^2}$$

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.035 standard PAT solution provided by BIPM

The following ampoules were used to prepare the gravimetric dilution:
OGP.035, ampoules #156, #141, #025, #109

Amount of primary calibrator used for analysis

11.56944 g

Gravimetry

Type of balance (make, model and resolution)

Shimadzu
Model AP 225 WD
Resolution 220 g: 0.1mg
Resolution 102 g: 0.01mg

Balance repeatability

100 μg at 220 g
50 μg at 102 g

Solution preparation procedure

1. Balance performance check, tare, 2. weighing empty flask, 3. transfer stock solution, 4. weighing stock solution, 5. ACN LC-MS grade acidified 0,1% formic acid addition, 6. weighing final mass of calibration solution, 7. transfer to CERTAN (R) vials.

Homogeneity and/or stability testing

Homogeneity testing done by HPLC DAD analysis.
Stability testing pending.

Optional: Analytical check method

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

HPLC DAD, oven temperature 30ºC, extracted chromatogram 276 nm
Phenomenex Gemini 5 u C18 110 A, 250 x 4,6 mm column
Mobile phase: A: ACN 5: H_2O 95; B: ACN, both 0.1 % formic acid
Flow: 0.8 mL/min, Rt = 15 min
Injection volume: 10 µL .

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 = 1.07 %) and the homogeneity contribution (u*bb = 0.92 %). Stability contribution estimated on a 8 week basis is not included in `RESULTS´tab.