CCQM-K154.a and CCQM-K154.a.1

Key Comparison Study – Organic Solvent Calibration Solution

Gravimetric preparation and value assignment of *trans*-zearalenone (*trans*-ZEN) in acetonitrile (ACN)

Final Report

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SUMMARY

The CCQM-K154.a and subsequent CCQM-K154.a.1 comparisons were coordinated by the BIPM and NIM on behalf of the CCQM Organic Analysis Working Group (OAWG) for National Measurement Institutes (NMIs) and Designated Institutes (DIs) which provide measurement services in organic analysis under the 'Comité International des Poids et Mesures' Mutual Recognition Arrangement (CIPM MRA) and/or have participated in the BIPM's Mycotoxin Metrology Capacity Building and Knowledge Transfer (MMCBKT) project as part of its "Metrology for Safe Food and Feed in Developing Economies" Capacity Building Programme. Gravimetrically-prepared solutions having an assigned mass fraction of specified organic analytes are routinely used to calibrate measurement processes for the quantification of the same analytes in matrix samples. Appropriate assignments of the property value and associated uncertainty of calibration solutions thus underpin the traceability of routine analysis and are critical for accurate measurements. Evidence of successful participation in relevant international comparisons is needed to document calibration and measurement capability claims (CMCs) made by national metrology institutes and designated institutes. In total, eleven NMIs/DIs participated in the Track C, Model II, Key Comparison CCQM-K154.a and the Subsequent Comparison CCQM-K154.a.1 [Gravimetric preparation and value assignment of trans-zearalenone (*trans*-ZEN) 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 *trans*-zearalenone (*trans*-ZEN) 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.a and CCQM-K154.a.1 for MMCBKT participants was intended to demonstrate measurement capabilities for preparation and value assignment of zearalenone 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 non-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
DI	Designated Institute
DoE	Degree of equivalence
ESI	Electrospray ionization
GLS	Generalized Least Squares regression analysis
KCRV	Key Comparison Reference Value
LC-DAD-MS/MS	Liquid chromatography with (UV) diode array and tandem mass spectrometric detection
MMCBKT	Mycotoxin Metrology Capacity Building and Knowledge Transfer
NMI	National Metrology Institute
NMR	Nuclear magnetic resonance spectroscopy
OAWG	Organic Analysis Working Group
*pK*ow	Negative log base 10 of the octanol-water partition coefficient
qNMR	Quantitative nuclear magnetic resonance spectroscopy
SRM	Selected reaction monitoring
ZAN	Zearalanone
ZEN	Zearalenone

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.a and CCQM-K154.a.1 comparisons, agreed by the CCQM, were organized to support National Metrology Institutes (NMIs) or Designated Institutes (DIs) that have developed capabilities to prepare and value assign mycotoxin calibration solutions to benchmark and demonstrate the comparability of their measurement services.

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

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

The CCQM-K154.a and CCQM-K154.a.1 comparisons on the gravimetric preparation and value assignment of the *Fusarium* mycotoxin zearalenone (ZEN) in acetonitrile (ACN) allowed NMIs/DIs that have participated in the MMCBKT project to demonstrate the compatibility of the capabilities and services they have established in their laboratories [2]. The comparison 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 non-polar organic analytes in organic solutions. This study involved a comparison at the BIPM of a suite of ZEN calibration solutions prepared by each of the participating laboratories. Seven laboratories took part in the framework of the MMCBKT while four laboratories participated in order to demonstrate their in-house calibration solution production capabilities. The calibration solutions sent to the BIPM where an LC-DAD(-MS/MS) method was used to compare the value assignments of the mass fraction content of ZEN in the solutions provided by each participant.

ZEN is an estrogenic mycotoxin produced by several molds of the genus *Fusarium* which frequently contaminates crops as maize, wheat, rice and soybeans. ZEN is heat stable, can enter the food chain through contaminated food and feed and is a suspected carcinogen. It is also an

endocrine disruptor that can provoke hormonal imbalance and reproductive disorders in humans [3, 4, 5].

MEASURAND, QUANTITIES AND UNITS

The measurand was the mass fraction of *trans*-zearalenone [(*E*)-ZEN] present in solution acetonitrile (ACN), with the assigned value expressed in mg/kg (or one of its multiples μg/g, mg/g or ng/g).

PARTICIPANTS AND SCHEDULE

This study involved a simultaneous comparison of a suite of nine calibration solutions of (*E*)-ZEN in ACN gravimetrically prepared and value assigned by each of the participating laboratories.
Seven laboratories (INMETRO, INTI, KEBS, NIM, NIMT and UME) took part in the CCQM-K154.a comparison within the framework of the MMCBKT, using a value assigned stock solution of ZEN in ACN supplied by the BIPM. Four laboratories (EXHM, NIM, NMISA and NRC) took part in CCQM-K154.a using their own stock solution of ZEN. NIM participated in CCQM-K154.a using both their own calibration solution and the solution supplied by the BIPM within the framework of the MMCBKT. INTI participated in both CCQM-K154.a and the CCQM-K154.a.1 subsequent comparison.
The study schedule for CCQM-K154.a is given in Table 1. The subsequent comparison CCQM-K154.a.1 was launched at the April 2019 OAWG meeting in order to repeat measurements of INTI samples. The measurements for the subsequent comparison were performed in January 2020 at the BIPM and the results were included in the present report.

Table 1: CCQM-K154.a Timetable

Action	Date
Initial discussion	April 2017 MMCBKT and OAWG meetings
Study Proposal and draft protocol	September 2017 OAWG meeting
Approval of study protocol and confirmation	April 2018 OAWG meeting
Stock solution distribution	until September 2018 (MMCBKT participants)
Call for participation	June 1st, 2018
Final date to register	October 1st, 2018
Samples and data due to coordinator	November 30th, 2018
Initial report and discussion of results	April 2019 OAWG meeting
Draft B report	May 2020
Final report to OAWG Chair	June 2020

ZEN PRIMARY CALIBRATOR STOCK SOLUTION

The BIPM provided the MMCBKT participants with a stock solution of ZEN in acetonitrile (OGP.025) that was to be used for the preparation of ZEN calibration solution batches submitted for comparisons CCQM-K154.a and CCQM-K154.a.1.
The *trans*-ZEN mass fraction and associated expanded uncertainty ($k = 2$) of the ZEN stock solution OGP.025 was 130.1 ± 2.2 mg/kg. The uncertainty corresponding to the gravimetric value assignment and the homogeneity 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.

ZEN purity characterization

An essential requirement of the MMCBKT project was to obtain and characterize a primary reference material for ZEN that could be used subsequently to establish a calibration hierarchy to underpin the metrological traceability of results linked through calibration to this material [6]. The characterization and purity assignment studies to assess identity and purity of a primary reference material for ZEN used to deliver the BIPM MMCBKT program are described in detail in the Purity Evaluation Guideline: Zearalenone [7]. 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 ZEN 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 ZEN it contained. Due to the relatively complex

structure of ZEN, the assignment by qNMR only provides in the first instance an estimate of the total ZEN and related structure impurity mass fraction. This initial value needed to be corrected for the relevant related structure impurity mass fraction as assigned separately by LC-MS/MS and LC-DAD methods to give the final value for the true ZEN 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.
In the case of the ZEN material the initial value of the purity of the uncorrected total ZEN mass fraction and its expanded uncertainty was 998.0 ± 1.8 mg/g (k = 2). This was the mean of sixteen qNMR assignments (four samples each analyzed in quadruplicate) using freshly prepared sample quantified against a signal for one hydrogen atom at 7.0 ppm.

Table 2: Impurity assignments

Impurity	Mass fraction (mg/g)	u (mg/g)	Assignment
7-dehydro-ZEN	1.03	0.027	LC-MS/MS and LC-DAD
Zearalanone (ZAN)	1.23	0.007	LC-MS/MS and LC-DAD
cis-ZEN	0.29	0.002	LC-MS/MS
Total residual solvent	1.93	0.115	NMR

Using estimates of the mass fraction of the impurity components in the material listed in Table 2 the assignment of true ZEN mass fraction was obtained after correction of the initial qNMR value for 7-dehydro-ZEN and *cis*-ZEN (or ZEN isomer). ZAN does not contribute to the NMR signal at 7.0 ppm and was not included. The mass fraction and corresponding expanded uncertainty of *trans*-ZEN in the material (BIPM reference OGO.178a) assigned in this manner was 996.7 ± 1.8 mg/g (k = 2).

Gravimetric preparation of ZEN stock solution

The ZEN stock solution (OGP.025) was prepared gravimetrically by dissolving an accurately weighed sample of about 100 mg of ZEN powder material (OGO.178) in 1 L of acetonitrile. Mettler Toledo MX5 and $XP_1$0002S balances were used for the weighing of the ZEN powder and the final solution mass, respectively. Table 3 demonstrates the preparation of the stock solution and the mass fraction assignment, calculated according to Equation 1.

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

	Weighed mass (m)	Buoyancy (*b*)	m x b
ZEN powder (mg)	101.605	1.000872	101.694
Solution (g)	778.11	1.001386	779.189
Purity ± U (mg/g)	996.7 ± 1.8		
Final mass fraction (µg/g)	130.1		

$$w_{stock} = \frac{m_p \cdot b_p \cdot w_p}{m_{sol} \cdot b_{sol}} \qquad \text{Equation 1}$$

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

The uncertainties from input quantities in Equation 1 were combined (Equation 2) and the final combined standard uncertainty was calculated as depicted in Table 4. A minor uncertainty component, u(V), was included to account for the potential solvent loss due to evaporation during sample preparation and weighing. The buoyancy mass correction and its uncertainty were calculated as described by the Calibrant Assessment Guideline: Zearalenone [8].

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

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

Uncertainty source	Value (%)
$\frac{u(m_p)}{m_p}$	0.0033
$\frac{u(b_p)}{b_p}$	0.0031
$\frac{u(w_p)}{w_p}$	0.09
$\frac{u(m_{sol})}{m_{sol}}$	0.0028
$\frac{u(b_{sol})}{b_{sol}}$	0.0012
$\frac{u(V)}{V}$	0.005
u_{rel} (%)	0.091
u(w_{stock}) µg/g	0.118
U(w_{stock}) µg/g (k = 2)	0.236

Filling of ZEN stock solution

The 1 L flask containing the stock solution was agitated thoroughly and about 100 mL were transferred to prepare a calibration solution. The rest of the stock solution was stored at 4 °C until ampouling, which took place within 24 h of the preparation.

A 500 mL bottle and 1-10 mL bottle-top dispenser (Dispensette, Brand GmbH) were rinsed twice with the ZEN 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.025, one at a time, to minimize the impact of evaporation of acetonitrile. A refrigerant (Jelt Refroidisseur 5320) was sprayed onto the lower part of the ampoule before being placed in the ampouling carousel to further reduce the ignition risk. After flame sealing, ampoules were allowed to adjust to room temperature in an upright position.

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

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

Homogeneity studies of ZEN stock solution

The BIPM investigated the levels of within and between ampoule homogeneity of the main component and selected significant minor components, and identified a minimum sample size which reduces to an acceptable level the effect of between bottle inhomogeneity of both the main component and the minor components. The homogeneity of the ZEN stock solution was studied using an LC-DAD-MS/MS method that allowed for the quantitative determination of ZEN by UV and of the 7'dehydroZEN, ZAN and the ZEN isomer by MS/MS detection.
The results of the ANOVA are summarised in Table 5.

Table 5: Homogeneity results of the ZEN stock solution (OGP.025)

	ZEN	7'DehydroZEN	ZAN	Isomer ZEN
N	30	30	30	30
s_{wb} (%)	0.50	4.79	4.02	4.13
s_{bb} (%)	0.83	1.75	2.37	2.45
u^*_{bb} (%)	0.16	1.56	1.31	1.34
u_{bb}[(1)] (%)	**0.83**	**1.75**	**2.37**	**2.45**
F	9.43	1.40	2.04	2.06
F_{crit}	2.39	2.39	2.39	2.39

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

No differences in the within- and between-sample variances could be detected by the F-tests at the 95 % confidence level for the three impurities (7'dehydroZEN, ZAN and the ZEN isomer). The material could be regarded as homogeneous. Differences in the within- and between sample variances were detected by the F-test at the 95 % confidence level due to the high precision of the LC-DAD method. However, the material was regarded to be homogeneous since the u_{bb} of 0.83 % is very small compared with the target uncertainties of < 2 % and in agreement with typical u_{bb} of 0.92 % for similar materials [15].
Representative normalized results due to the analysis and filling sequences are presented for the main component ZEN (Figure 1) and a minor impurity, the ZEN isomer (Figure 2). The first, second and third replicates are represented by circles, grey filled circles and dots respectively.

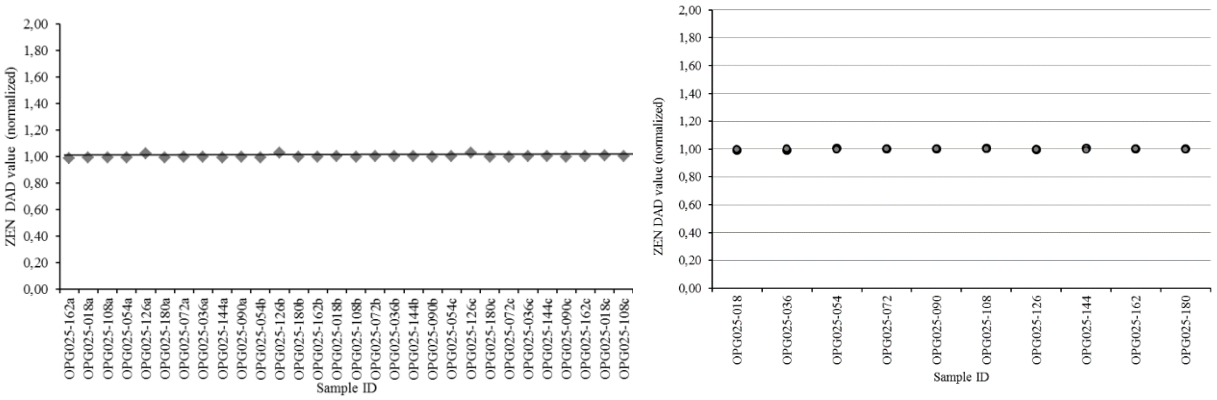

Figure 1: Homogeneity of ZEN by LC-DAD at 274 nm – Main component - Injection and filling sequence

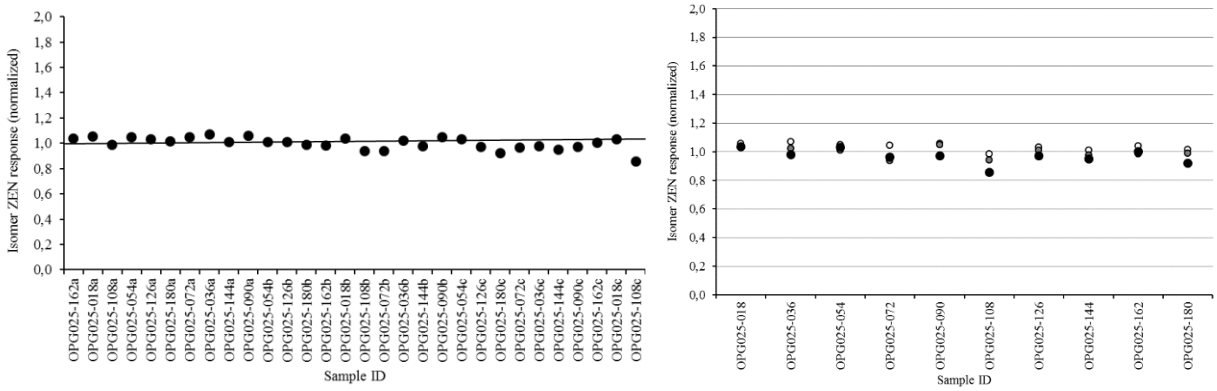

Figure 2: Homogeneity of ZEN isomer by LC-MS/MS – Representative minor impurity - Injection and filling sequence

The s_{bb} of 0.83 % was adopted as the upper limit estimate for the uncertainty contribution due to potential inhomogeneity for ZEN (main component). The s_{bb} of 1.75 %, 2.37 % and 2.45 % were adopted as the upper limit estimates for the uncertainty contribution due to potential inhomogeneity for 7'dehydrozearalenone, ZAN and *cis*-ZEN, respectively.

Stability studies of ZEN 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.

Trend analysis of the data obtained by LC-DAD-MS/MS analysis of the stability test samples under repeatability conditions indicated no significant changes in the relative composition of ZEN or of the related peptide impurities over longer time and at elevated temperatures.

The *trans*-ZEN mass fraction of the material was stable on storage at 4 °C and 22 °C but did decrease significantly after storage beyond 2 weeks at 40 °C as shown in Figure 3. The *cis*-ZEN (or ZEN isomer) mass fraction of the material was stable on storage at 4 °C but did increase significantly after storage beyond 2 weeks at both 22 °C and 40 °C as shown in Figure 4. Both 7'dehydroZEN and ZAN mass fractions of the material were stable on storage at 4 °C, 22 °C and 40 °C. There was some evidence of *cis*-ZEN formation at higher temperatures and exposure to light.

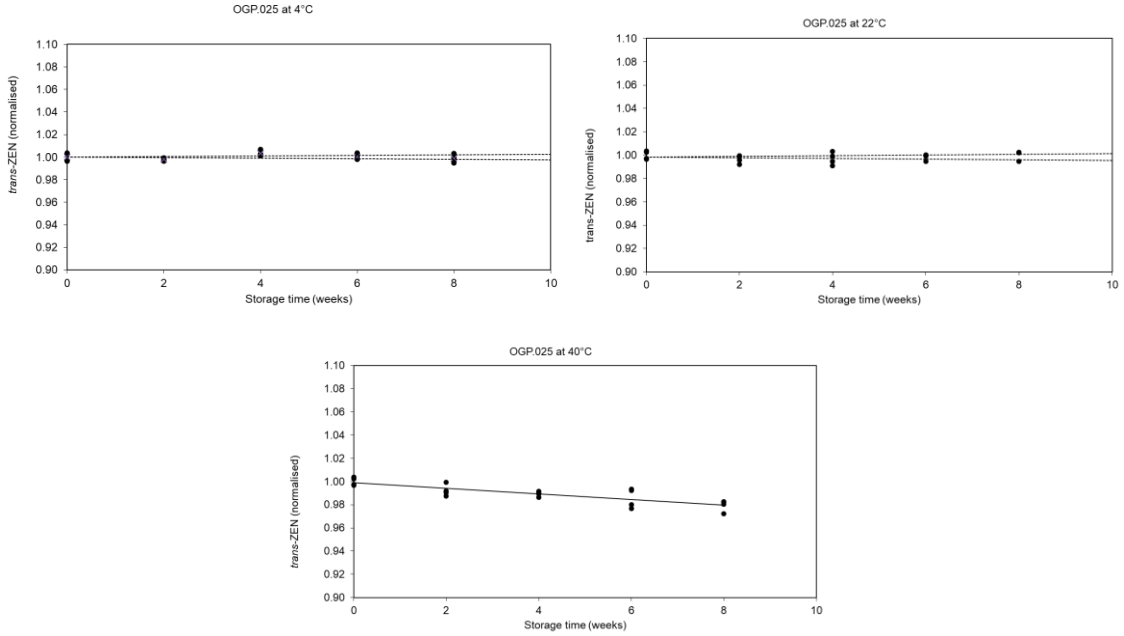

Figure 3: Stability study of *trans*-ZEN by LC-DAD

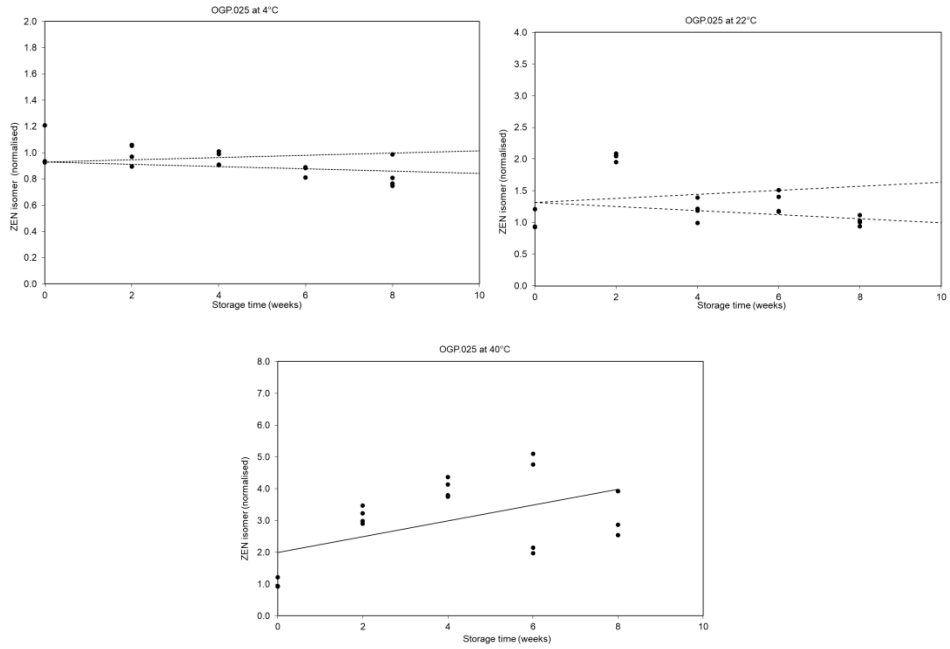

Figure 4: Stability study of the ZEN isomer (or *cis*-ZEN) by LC-MS/MS

Stability of ZEN stock solution under light exposure

It was suspected that ZEN could be light sensitive because of the occasional occurrence of the ZEN isomer (*cis*-ZEN). An accelerated stability study was performed to investigate the light sensitivity of ZEN. The stability of the ZEN stock solution was tested under three storage conditions for seven days: 4 °C in dark, 22 °C in dark and 22 °C exposed to light. The mass fraction of ZEN isomer was determined on day 0, 1, 2, 3, 4 and 7. ZEN stock solutions of 0.5 mL were transferred from storage conditions to HPLC vials and the mass fractions of ZEN isomer were determined by external calibration each day. For the stability study measurements, ZEN stock solutions were analysed in triplicate (n = 3) for their mass fraction of ZEN isomer by LC-MS/MS method under repeatability conditions.

It was demonstrated that no *cis*-ZEN was formed in the ZEN stock solution provided the solution was not exposed to light no matter if it was stored at 4 °C or 22 °C. Formation of *cis*-ZEN could be observed in the stock solution when exposed to light while stored at 22 °C. ZEN is sensitive to light and easily converts to the isomer form as shown in Figure 5. The mass fraction of the *cis*-ZEN impurity in the ZEN stock solution increased to about 7 mg/g after seven days of light exposure. It was concluded that exposure of the solution to light should be minimised and avoided as much as feasible during shipment and storage.

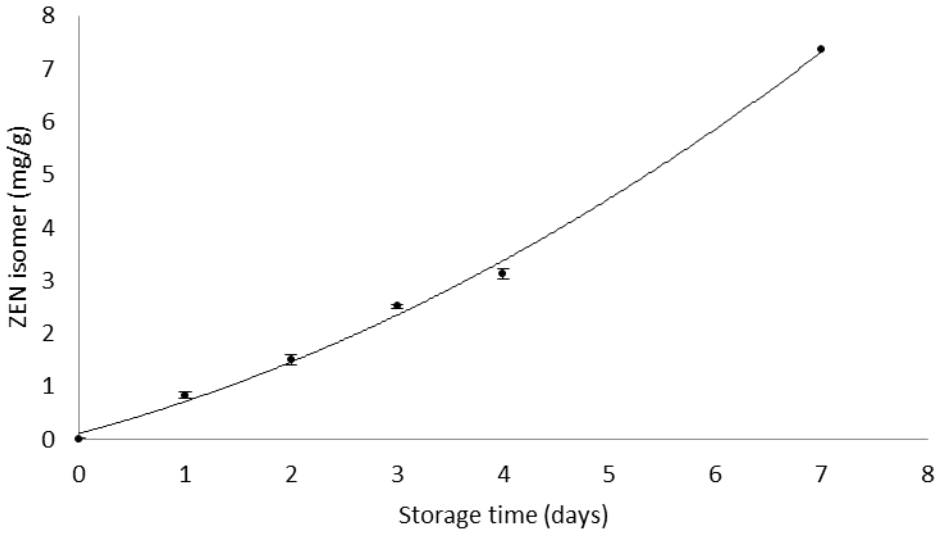

Figure 5: Formation of *cis*-ZEN in ZEN stock solution at 22 °C exposed to light

These findings were confirmed by another isochronous stability study performed using a separate diluted ZEN solution prepared from the stock solution (OGP.025) to evaluate the stability of ZEN and related impurities upon exposure to light for a total period of 4 weeks [2, 8].
On the basis of these studies it was concluded that for the purposes of the comparison the material was suitably stable for short-term transport provided it was not exposed to light and to temperatures significantly in excess of 22 °C.
To minimize the potential for changes in the material composition, participants were instructed to store the material at -20 °C.

Sample distribution of ZEN stock solution

Ten units of the ZEN stock solution, each containing a minimum of 4 mL of material, were distributed to each MMCBKT participant by express mail service in insulated boxes. Participants were asked to acknowledge receipt of the samples and to advise the coordinator if any obvious damage had occurred to the vials during shipping. The shipping details are listed in Table 6, There was a prolonged delay in delivery (24 days) of the original samples sent to Brazil (INMETRO). The samples were retained by the Health Authority at the airport but were not exposed to light. Replacement samples were not required as the original samples were still in good condition. Otherwise all samples were delivered to the comparison participants without incident.

Table 6: Details of the shipping of the ZEN stock solution from the BIPM to MMCBKT participants

NMI/DI	Shipping date	Date of receipt	In transit (days)	Comments
INMETRO	10.07.2018	03.08.2018	24	Samples retained at the airport but without exposure to light.
INTI	10.07.2018	16.07.2018	6	-
KEBS	10.07.2018	11.07.2018	1	-
NIM	18.09.2018	29.09.2018	11	-
NIMT	10.07.2018	11.07.2018	1	-
NMISA*	10.07.2018	13.07.2018	3	-
UME	10.07.2018	11.07.2018	1	-

* As an MMCBKT participant NIMSA was supplied with the ZEN stock solution OGP.025. However they chose to submit a ZEN 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 zearalenone (ZEN) in acetonitrile and to send these to the BIPM for comparison measurements. The mass fraction values targeted (in the range 10 mg/kg to 20 mg/kg) are intended to be representative of the mass fraction content of *trans*-ZEN in a standard solution provided as a reference standard used for calibrations in *trans*-ZEN 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 *trans*-ZEN 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 ZEN 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 (ZEN in acetonitrile). Two ampoules were required by the BIPM for analysis to obtain the comparison results and the additional ampoules were available as a reserve. The ampoules were stored at 4 °C in the dark prior to opening. Participants were required to provide their estimate of the mass fraction of *trans*-ZEN 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.
The details of the shipping of the comparison solutions from the NMIs/Dis to the BIPM are listed in Table 7. There was a prolonged delay in delivery (17 days) of the ampoules sent from INTI, Argentina to the BIPM. The INTI ampoules were retained at the airport in France. It is unknown if the shipping box was opened and the ampoules exposed to light. Otherwise all samples were delivered to the BIPM without incident. The INTI samples were analyzed in the CCQM-K154.a round being well aware that they may have altered during transport. The CCQM-K154.a.1 subsequent comparison was organized to reanalyze INTI samples from the original batch that were shipped to the BIPM and stored under recommended conditions.

Table 7: Details of the shipping of the ZEN comparison solutions from NMIs/DIs to the BIPM

NMI/DI	Comparison	Shipping date	Date of receipt	In transit (days)	Comments
EXHM	CCQM-154.a	27.11.2018	29.11.2018	2	-
INMETRO	CCQM-154.a	30.11.2018	04.12.2018	4	-
INTI	CCQM-154.a	20.11.2018	07.12.2018	17	Samples were retained at the customs and exposed to light.
INTI	CCQM-154.a.1	04.04.2019	05.04.2019	1	-
KEBS	CCQM-154.a	27.11.2018	30.11.2018	3	-
NIM	CCQM-154.a	15.11.2018	29.11.2018	14	-
NIMT	CCQM-154.a	28.11.2018	30.11.2018	2	-
NMISA	CCQM-154.a	19.11.2018	27.11.2018	8	-
NRC	CCQM-154.a	16.11.2018	20.11.2018	4	-
UME	CCQM-154.a	27.11.2018	30.11.2018	3	-

REPORTED MASS FRACTIONS OF *TRANS*-ZEN AND IMPURITIES

The values reported by participating NMIs/DIs for the *trans*-ZEN mass fractions of their ZEN comparison solutions and their corresponding uncertainties based on the gravimetric preparation (corrected for purity for non-MMCBKT participants) are given in Table 8. The details of the gravimetric preparation, calculation of the *trans*-ZEN mass fraction values and assessment of corresponding expanded uncertainties are described in Annex A for each participating NMI/DI. If the uncertainty includes contributions deriving from other sources (for example, homogeneity and/or stability testing) details are also provided in Annex A.
In addition to the compulsory *trans*-ZEN mass fraction of their ZEN comparison solution and its corresponding uncertainties the NRC reported the presence of ZAN at a mass fraction of 0.03 µg/g and a corresponding expanded uncertainty of 0.02 µg/g ($k = 2$) in their solution and EXHM reported traces of *n*-hexane and dichloromethane detected at mass fractions of about 0.2 % each for their own solution.

VALUE ASSIGNMENT PROCEDURE OF THE COORDINATING LABORATORY

The *trans*-ZEN 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 *trans*-ZEN. MS/MS detection served as a verification tool for the determination of potential related structure impurities. Preliminary experiments demonstrated that the UV response was linear over the mass fraction range of about 5 µg/g to 150 µg/g of *trans*-ZEN.
Two point calibrations with external bracketing using *trans*-ZEN standards assigned at the BIPM were used for quantification and comparison. It was decided to split the CCQM-K154.a comparison in three groups (A, B and C) with separate calibrations on separate days to allow working at narrow and linear mass fraction ranges. Thus injection sequences were short in order to minimize the extent of instrument drift. The CCQM-K154.a.1 comparison covered only INTI, Argentina.

Materials and calibrants

The *trans*-ZEN bracketing standards were prepared immediately before use as solutions in acetonitrile (Hipersolv HPLC grade, VWR, France) of the pure BIPM ZEN material (OGO.178a) having a *trans*-ZEN mass fraction of 996.7 ± 1.8 mg/g ($k = 2$) as outlined in the chapter ‘ZEN 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 ZEN 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: Zearalenone [8]. The *trans*-ZEN mass fractions and corresponding standard uncertainties for the stock and calibration solutions are listed in Table 9.

LC-DAD-MS/MS method

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

An EXIONLC system (Sciex, Villebon sur Yvette, France) consisting of an EXIONLC 5 channel solvent degasser, EXIONLC AD UHPLC pump, EXIONLC AD cooled autosampler, EXIONLC AC column oven with cooling and EXIONLC PDA detector was employed for LC-DAD analysis. LC separation was performed on a Kinetex EVO C18 100 A column (250 mm × 4.6 mm, 2.6 µm from Phenomenex (Le Pecq, France) maintained at 25 °C. The mobile phases consisted of (A) acetonitrile/water/formic (40:60:0.1, v/v/v) and (B) acetonitrile/formic acid (100:0.1, v/v). The separation was performed isocratically (100 % A) over 45 min with a flow rate of 600 µL/min. The column was then washed by increasing to 95 % B in 1 min, holding at 95 % B for 1 min and returning to isocratic conditions (100 % A) in 1 min. The column was re-equilibrated for a further 17 min at 100 % A. The total run time was 65 min and the injection volume was 10 µL. The detection wavelength of the UV diode array detector (DAD) was 274 nm.

Mass spectrometric detection (MS/MS)

Mass spectrometric detection was performed for verification purposes of potentially occurring impurities. A SCIEX QTRAP 6500+ tandem mass spectrometer (Sciex, Villebon sur Yvette, France) fitted with an electrospray ionization (ESI) source was used. The MS parameters were optimized in negative electrospray ionization (ESI neg) with a capillary voltage of -4500 V, source temperature of 550 ºC. 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 50 psi, respectively. The curtain gas (CUR) was set at 20 psi. The collision gas (CAD) was set at Mid. Table 10 lists MS/MS transitions of ZEN and potential impurities with optimized dwell time, declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) settings.

Table 8: *trans*-ZEN mass fraction values and corresponding uncertainties submitted by the NMIs/Dis CCQM-K154.a/K154.a.1

Participant	Comparison	Primary calibrator used	trans-ZEN Mass fraction (µg/g)	Combined standard uncertainty (µg/g)	Coverage factor (*k*)	Expanded uncertainty (µg/g)
EXHM, Greece	CCQM-K154.a	own	15.026	0.161	2	0.321
INMETRO, Brazil	CCQM-K154.a	CBKT	14.64	0.14	2	0.28
INTI, Argentina	CCQM-K154.a CCQM-K154.a.1	CBKT	14.803	0.143	2	0.285
KEBS, Kenya	CCQM-K154.a	CBKT	14.659	0.124	2	0.248
NIM, China	CCQM-K154.a	own	12.7	0.15	2	0.3
	CCQM-K154.a	CBKT	13.1	0.14	2	0.3
NIMT, Thailand	CCQM-K154.a	CBKT	14.73	0.148	2	0.30
NMISA, South Africa	CCQM-K154.a	own	12.30	0.090	2	0.18
NRC, Canada	CCQM-K154.a	own	66.5	0.5	2	1.0
UME, Turkey	CCQM-K154.a	CBKT	12.69	0.11	2	0.22

Table 9: Details of the gravimetric preparation of the BIPM bracketing calibration standards for *trans*-ZEN

Comparison	Calibration	Mass fraction range	*trans*-ZEN Stock solution		High level calibration solution		Low level calibration solution	
			w (mg/kg)	u (mg/kg)	*w* (mg/kg)	u (mg/kg)	*w* (mg/kg)	u (mg/kg)
CCQM-154.a	A	12-13 mg/kg	103.280	0.103	15.400	0.019	10.066	0.015
CCQM-154.a	B	14-16 mg/kg	103.280	0.103	20.705	0.021	10.120	0.011
CCQM-154.a	C	64-68 mg/kg	103.280	0.103	72.190	0.072	61.777	0.062
CCQM-154.a.1	D	14-16 mg/kg	100.429	0.101	20.654	0.023	10.366	0.015

Table 10: Summary of selected precursor and product ions, optimized time, DP, CE, EP and CXP settings for the detection of ZEN 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)
Zearalenone (*trans*-/*cis*-ZEN)	317.2	131.1*	50	-95	-40	-11	-10
		175.1	50	-95	-30	-11	-10
		187.0	50	-95	-27	-11	-10
Zearalanone (ZAN)	319.3	275.0*	50	-110	-30	-11	-10
		205.1	50	-110	-33	-11	-10
Zearalenol (α-/β-ZEL)	319.3	275.0*	50	-110	-30	-11	-10
		160.1	50	-110	-41	-11	-10
Zearalanol(α-/β-ZAL)	321.3	277.1*	50	-110	-33	-11	-10
		303.2	50	-110	-31	-11	-10
Dehydrozearalenone (dehydroZEN)	315.3	175.1*	50	-90	-30	-11	-10
		271.1	50	-90	-30	-11	-10

Samples, sequence preparation and measurement order

Two ampoules supplied by each participant were each measured in triplicate by LC-DAD-MS/MS. CCQM-K154.a measurements were grouped in three batches (A, B and C) undertaken on different days involving four, five and one participants according to their target mass fraction ranges of about 12-13 mg/kg, 14-16 mg/kg and 64-68 mg/kg, respectively. CCQM-K154.a.1 measurements were performed in a single batch with a target mass fraction range of about 14-16 mg/kg. 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.a and -K154.a.1 participant samples is listed in Table 11.

Table 11: Grouping of CCQM-K154.a and -K154.a.1 participant samples

Calibration A (12-13 mg/kg)	CCQM-K154.a.1 Calibration B (14-16 mg/kg)	Calibration C (64-68 mg/kg)	CCQM-K154.a.1 Calibration (14-16 mg/kg)
NIM (CBKT)	EXHM (own)	NRC (own)	INTI (CBKT)
NIM (own)	INMETRO (CBKT)		
NMISA (own)	INTI (CBKT)		
UME (CBKT)	KEBS (CBKT)		
	NIMT (CBKT)		

About 300 µL of the NMI/DI samples, low and high mass fraction level calibrant solutions and control samples (BIPM) were transferred in LC vials and injected separately. Calibrants (Low and High), control samples (BIPM) and pure acetonitrile (Blank) vial were distributed and injected over the sequences. The results for blanks and control samples served to identify potential carry-over and instrument drifts, respectively. Neither carry-over nor significant instrument drifts were observed. The detailed injection sequences for CCQM-K154.a A, B and C and CCQM-K154.a.1 are given in Table 12.

Table 12: Detailed injection sequences for the different calibrations of CCQM-K154.a/K154.a.1

Injection	Calibration A (12-13 mg/kg)	CCQM-K154.a Calibration B (14-16 mg/kg)	Calibration C (64-68 mg/kg)	CCQM-K154.a.1 Calibration (14-16 mg/kg)
1	Blank	Blank	Blank	Blank
2	Low-1	Low-1	Low-1	Low-1
3	NIM (CBKT)-A-1	EXHM-A-1	High-1	High-1
4	NMISA-A-1	INMETRO-A-1	Blank	Blank
5	UME-A-1	INTI-A-1	Low-2	Low-2
6	High-1	KEBS-A-1	High-2	INTI-C-1
7	Blank	High-1	Blank	High-2
8	Low-2	Blank	Low-3	Blank
9	BIPM-A-1	Low-2	Control-A-1	Low-3
10	NIM (own)-A-1	BIPM-A-1	NRC-A-1	BIPM-C-1
11	NMISA-B-1	NMIT-A-1	High-3	INTI-D-1
12	High-2	INMETRO-B-1	Blank	High-3
13	Blank	KEBS-B-1	Low-4	Blank
14	Low-3	High-2	NRC-B-1	Low-4
15	NIM (CBKT)-B-1	Blank	Control-A-2	BIPM-C-2
16	BIPM-B-1	Low-3	High-4	High-4
17	NIM (own)-B-1	BIPM-B-1	Blank	Blank
18	High-3	NMIT-B-1	Low-5	Low-5
19	Blank	EXHM-B-1	Control-A-3	INTI-C-2
20	Low-4	INTI-B-1	NRC-A-2	High-5
21	UME-B-1	High-3	High-5	Blank
22	NIM (CBKT)-A-2	Blank	Blank	Low-6
23	NMISA-A-2	Low-4	Low-6	INTI-D-2
24	High-4	NMIT-A-2	NRC-B-2	BIPM-C-3
25	Blank	INMETRO-A-2	Control-A-4	High-6
26	Low-5	BIPM-A-2	High-6	Blank
27	NIM (own)-A-2	INTI-A-2	Blank	Low-7
28	UME-A-2	High-4	Low-7	BIPM-C-4
29	BIPM-A-2	Blank	Control-A-5	High-7
30	High-5	Low-5	NRC-A-3	Blank
31	Blank	EXHM-A-2	High-7	Low-8
32	Low-6	KEBS-A-2	Blank	INTI-C-3
33	NMISA-B-2	NMIT-B-2	Low-8	BIPM-C-5
34	UME-B-2	INMETRO-B-2	NRC-B-3	High-8
35	NIM (own)-B-2	High-5	Control-A-6	Blank
36	High-6	Blank	High-8	Low-9
37	Blank	Low-6	Blank	INTI-D-3
38	Low-7	BIPM-B-2	Low-9	High-9
39	NIM (CBKT)-B-2	KEBS-B-2	High-9	Blank
40	BIPM-B-2	INTI-B-2	Blank	Low-10
41	UME-A-3	EXHM-B-2	Low-10	BIPM-C-6
42	High-7	High-6	High-10	High-10
43	Blank	Blank	Blank	Blank
44	Low-8	Low-7		
45	NMISA-A-3	KEBS-A-3		
46	NIM (own)-A-3	NMIT-A-3		
47	BIPM-A-3	INTI-A-3		
48	High-8	BIPM-A-3		
49	Blank	High-7		
50	Low-9	Blank		
51	NIM (CBKT)-A-3	Low-8		
52	UME-B-3	INMETRO-A-3		
53	NIM (own)-B-3	EXHM-A-3		
54	High-9	NMIT-B-3		
55	Blank	KEBS-B-3		
56	Low-10	High-8		
57	NMISA-B-3	Blank		
58	NIM (CBKT)-B-3	Low-9		
59	BIPM-B-3	INTI-B-3		
60	High-10	EXHM-B-3		
61	Blank	BIPM-B-3		
62		INMETRO-B-3		
63		High-9		
64		Blank		
65		Low-10		
66		High-10		
67		Blank		

Measurements and results

Subsequent to the LC-DAD-MS/MS analyses the UV absorption peak areas of *trans*-ZEN at 274 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 [9, 10]. 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) [11, 12] 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 [13]. Statistical approaches including GLS are discussed in detail by Duewer *et al*. [14].
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 *trans*-ZEN 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 12). The *trans*-ZEN 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 13. 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.a A, B, C and CCQM-K154.a.1 are depicted in Figures 6-9, respectively.

Table 13: *trans*-ZEN mass fraction values and absolute corresponding and expanded uncertainties measured by the BIPM for CCQM-K154.a/K154.a.1 participants' ampoules

NMI/DI	Study	w_{BIPM} (mg/kg)	$u(w_{BIPM})$ (mg/kg)	$U(w_{BIPM})$ (mg/kg)	Quantification range (mg/kg)
UME (CBKT) A	CCQM-154.a	12.80	0.09	0.18	12-13
UME (CBKT) B	CCQM-154.a	12.83	0.08	0.15	12-13
NMISA (own) A	CCQM-154.a	12.18	0.10	0.20	12-13
NMISA (own) B	CCQM-154.a	12.20	0.12	0.25	12-13
NIM (own) A	CCQM-154.a	12.88	0.18	0.36	12-13
NIM (own) B	CCQM-154.a	12.77	0.10	0.20	12-13
NIM (CBKT) A	CCQM-154.a	12.86	0.20	0.41	12-13
NIM (CBKT) B	CCQM-154.a	13.00	0.09	0.18	12-13
INTI (CBKT) A	CCQM-154.a	14.40	0.12	0.24	14-16
INTI (CBKT) B	CCQM-154.a	14.45	0.10	0.19	14-16
INMETRO (CBKT) A	CCQM-154.a	14.38	0.10	0.19	14-16
INMETRO (CBKT) B	CCQM-154.a	14.47	0.33	0.66	14-16
EXHM (own) A	CCQM-154.a	15.23	0.10	0.19	14-16
EXHM (own) B	CCQM-154.a	15.29	0.10	0.20	14-16
KEBS (CBKT) A	CCQM-154.a	14.53	0.08	0.16	14-16
KEBS (CBKT) B	CCQM-154.a	14.71	0.07	0.14	14-16
NIMT (CBKT) A	CCQM-154.a	14.45	0.13	0.27	14-16
NIMT (CBKT) B	CCQM-154.a	14.51	0.10	0.19	14-16
NRC (own) A	CCQM-154.a	66.90	0.49	0.98	64-68
NRC (own) B	CCQM-154.a	66.68	0.48	0.96	64-68
INTI (CBKT) C	CCQM-154.a.1	14.92	0.07	0.13	14-16
INTI (CBKT) D	CCQM-154.a.1	14.92	0.10	0.19	14-16

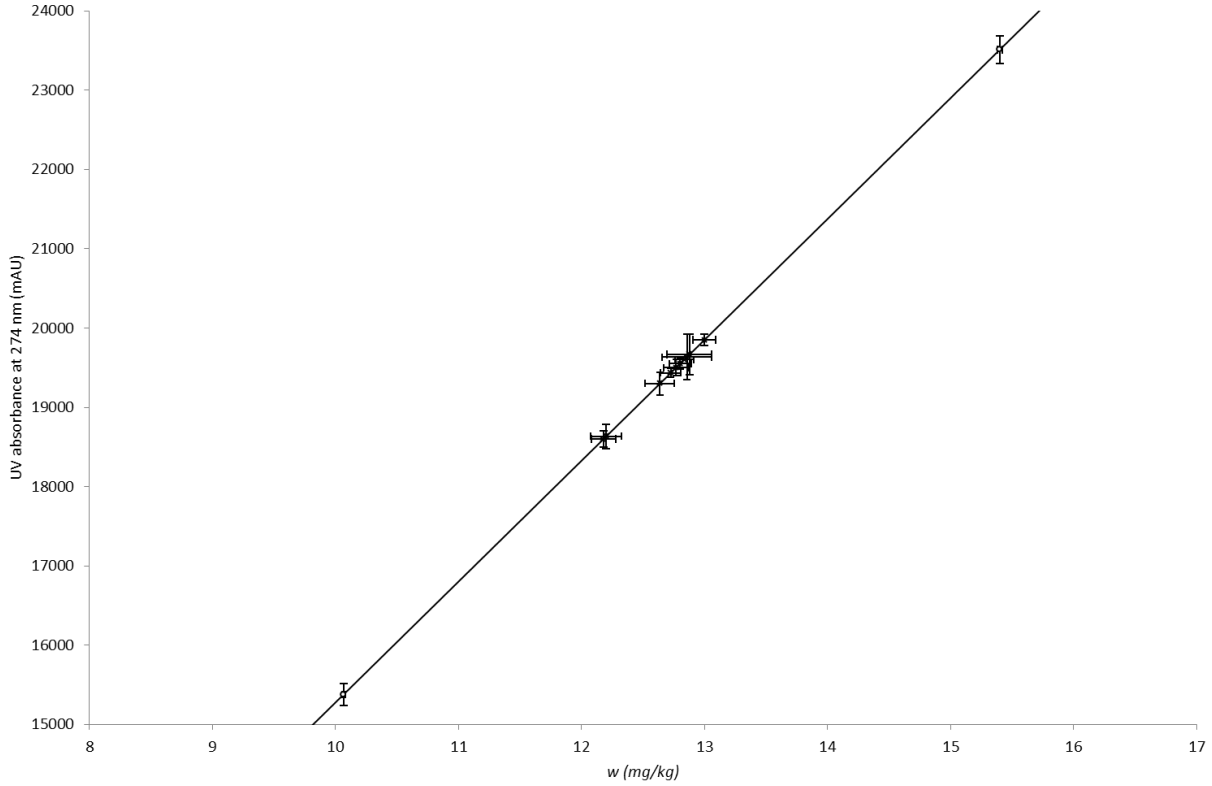

Figure 6: CCQM-K154.a - Calibration A - Bracketing calibration for the ZEN mass fraction quantification range of 12-13 mg/kg. UV absorbance values (mAU) and corresponding mass fractions (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at the upper and lower end of the calibration line. Inverse evaluation data of NMISA (own), NIM (own), NIM (CBKT), UME (CBKT) and internal control sample are depicted as crosses.

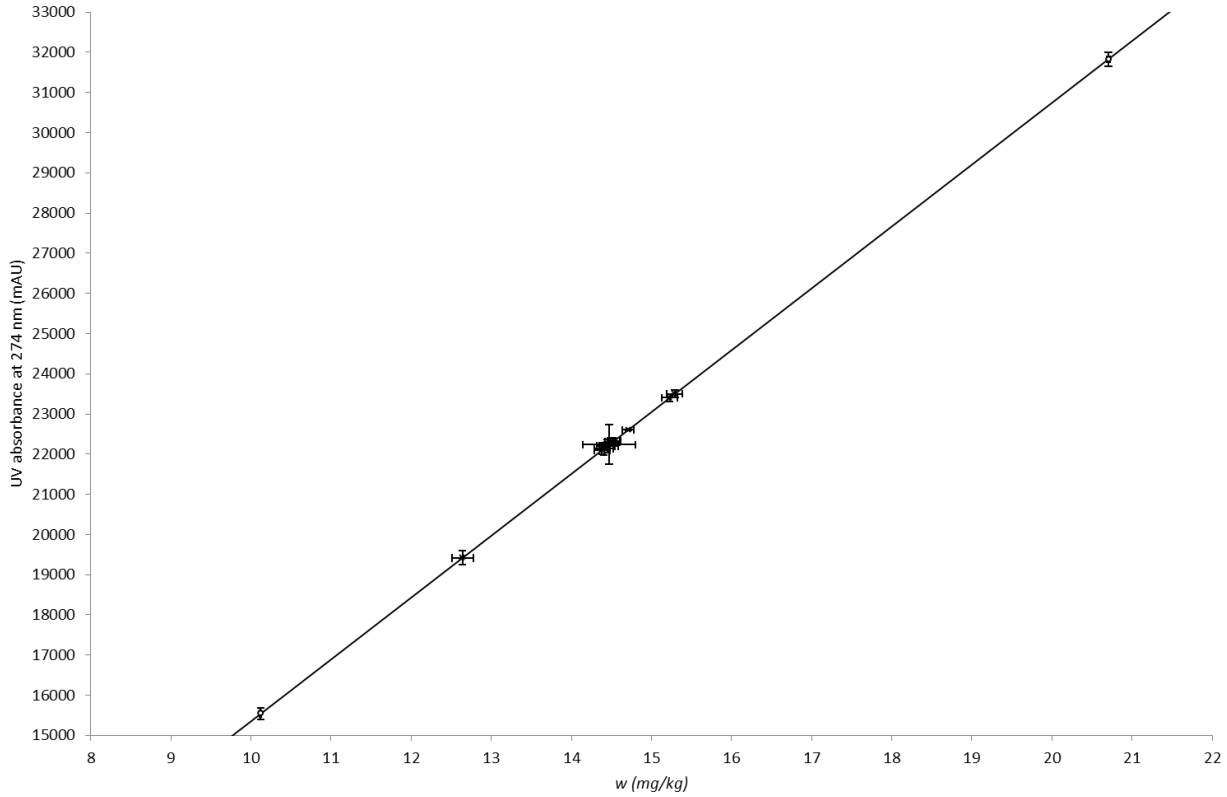

Figure 7: CCQM-K154.a - Calibration B - Bracketing calibration for the ZEN mass fraction quantification range of 14-16 mg/kg. UV absorbance (mAU) and corresponding mass fraction (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at upper and lower end of the calibration line. Inverse evaluation data of EXHM (own), INTI (CBKT), INMETRO (CBKT), KEBS (CBKT), NIMT (CBKT) and internal control sample are depicted as crosses.

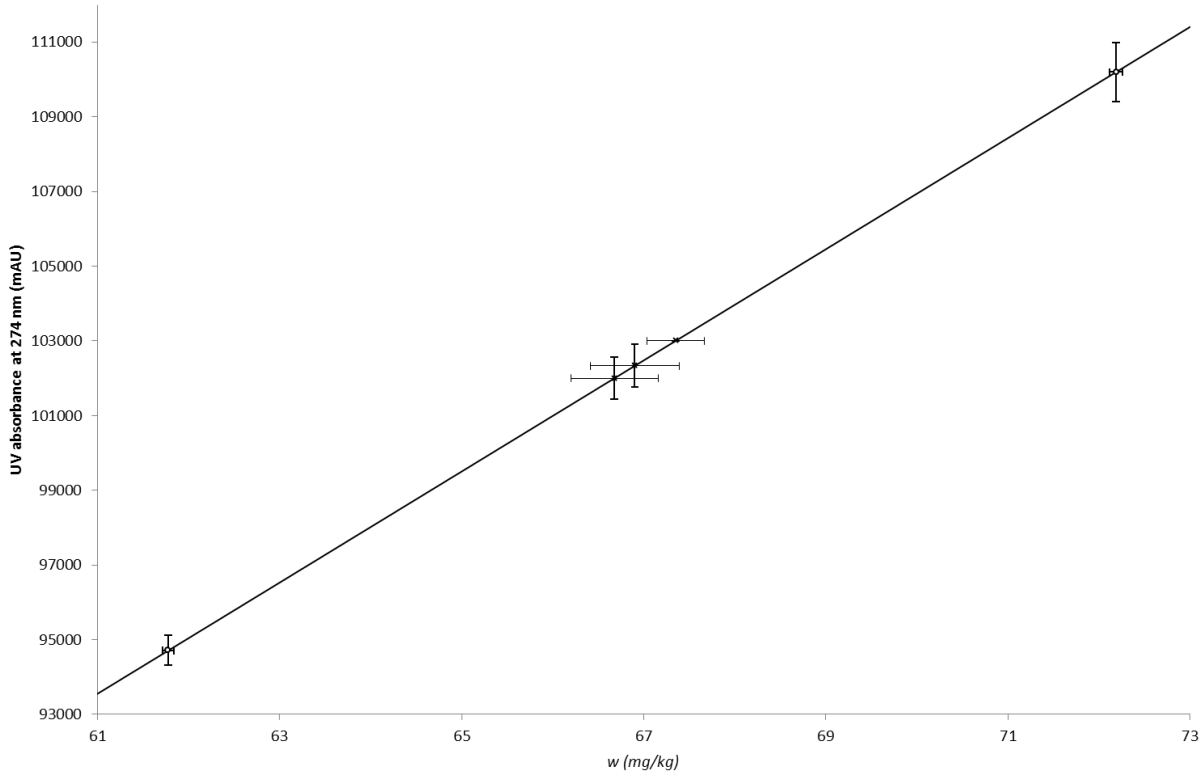

Figure 8: CCQM-K154.a - Calibration C - Bracketing calibration for the ZEN mass fraction quantification range of 64-68 mg/kg. UV absorbance (mAU) and corresponding mass fraction (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at upper and lower end of the calibration line. Inverse evaluation data of NRC (own) and internal control sample are depicted as crosses.

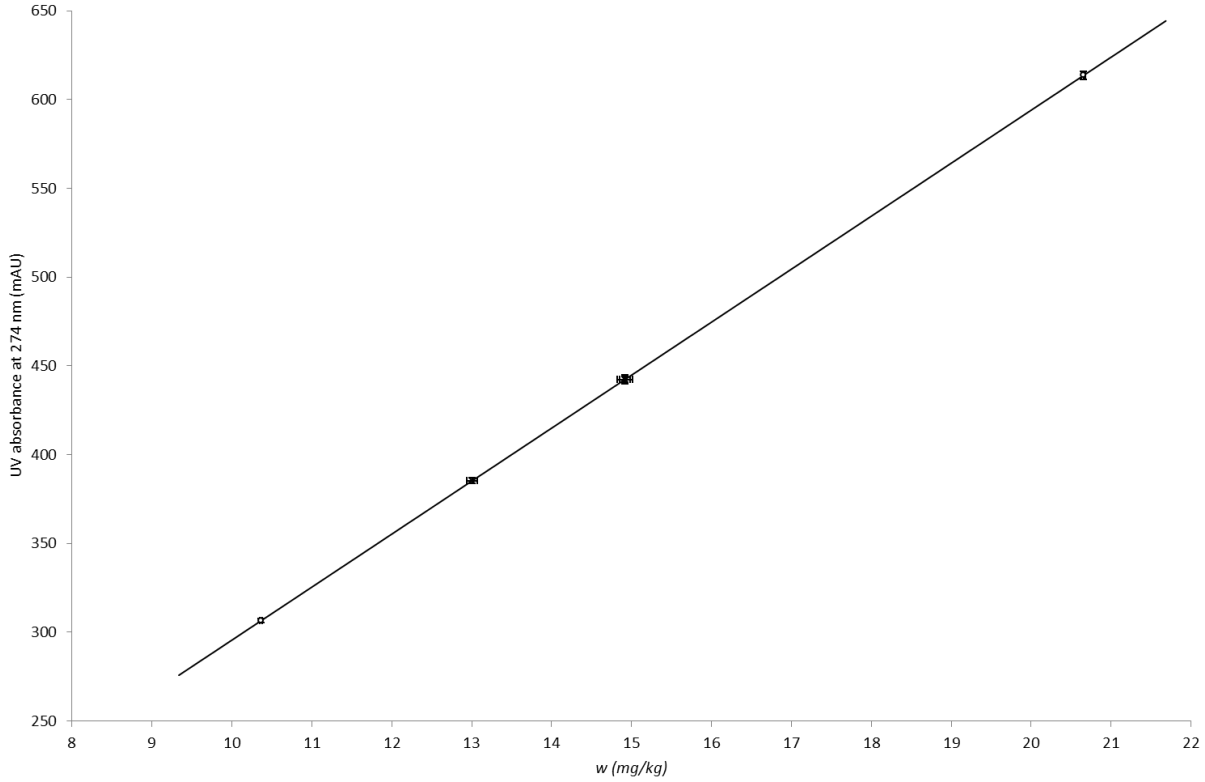

Figure 9: CCQM-K154.a.1 - Bracketing calibration for the ZEN mass fraction quantification range of 14-16 mg/kg. UV absorbance (mAU) and corresponding mass fraction (mg/kg) plotted with standard uncertainties (u). BIPM measurement data are shown as circles at upper and lower end of the calibration line. Inverse evaluation data of INTI (CBKT) and internal control sample are depicted as crosses.

Additional inspection of the LC-MS/MS data clearly indicated that formation of *cis*-ZEN occurred in the INTI samples received for CCQM-K154.a during transport. These samples were retained for about 17 days prior to customs clearance and most likely were exposed at some to light (Table 7). As demonstrated by the stability studies described earlier, exposure to light leads to isomerization of *trans*-ZEN to *cis*-ZEN [16, 17, 18]. A representative LC-DAD chromatogram of a CCQM-K154.a INTI sample is given in Figure 10. An LC-MS/MS chromatogram is presented in Figure 11 confirming significant formation of *cis*-ZEN. It also shows the presence of minor ZAN and dehydro-ZEN impurities originating from the ZEN stock solution.
The CCQM-K154.a.1 subsequent comparison was organized for INTI to repeat measurements with fresh ampoules of the same batch shipped under controlled transport conditions. The samples analyzed within CCQM-K154.a.1 did not exhibit any sign of isomerization or degradation of *trans*-ZEN.

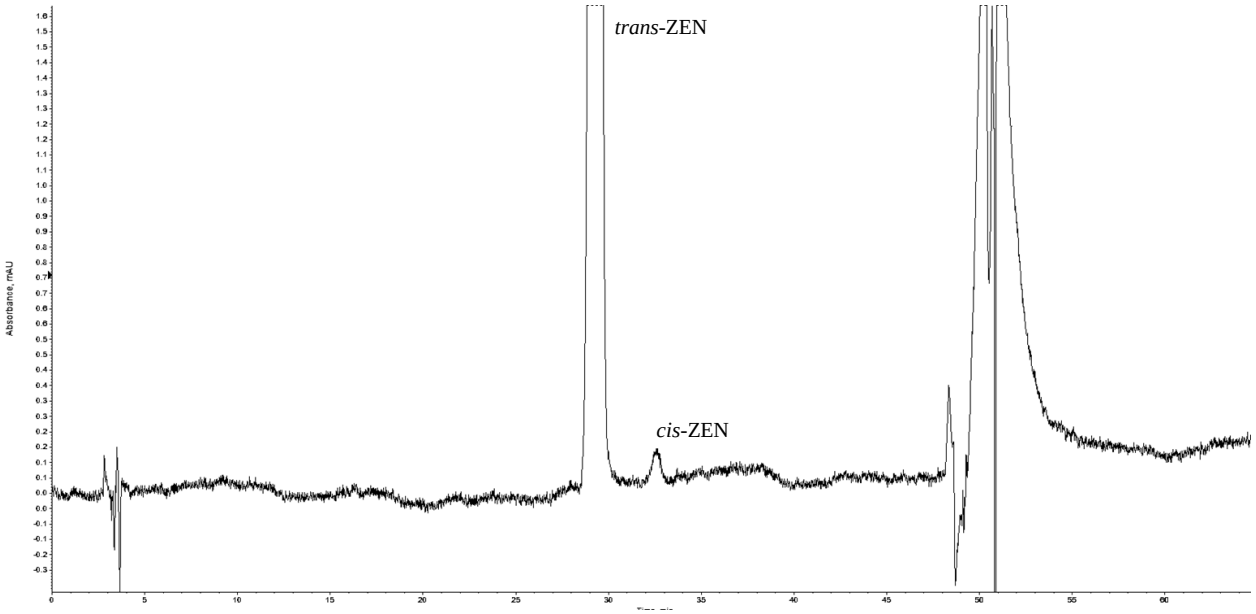

Figure 10: Enlarged representative LC-DAD chromatogram at 274 nm of a CCQM-K154.a comparison sample from INTI indicating significant formation of *cis*-ZEN

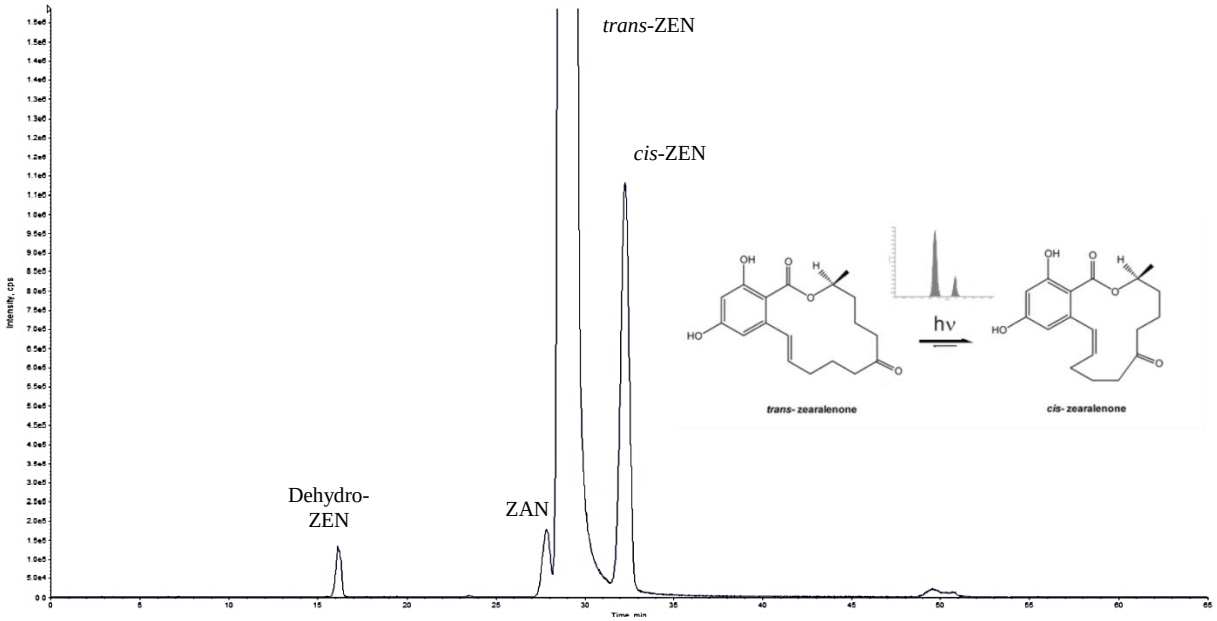

Figure 11: Enlarged representative LC-MS/MS chromatogram indicating significant formation of *cis*-ZEN and showing minor ZAN and dehydro-ZEN impurities originating from ZEN stock solution

KEY COMPARISON REFERENCE VALUES FOR CCQM-K154.a and CCQM-K154.a.1

The *trans*-ZEN mass fraction values used to establish the Key Comparison Reference Values (KCRVs) for CCQM-K154.a and CCQM-K154.a.1 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 *trans*-ZEN mass fraction (w_{KCRV}) and its corresponding uncertainty ($u(w_{KCRV})$). All NMIs/DIs (i) participating in CCQM-K154.a and CCQM-K154.a.1 were required to submit estimates for the ZEN mass fraction w_i and its corresponding uncertainty $u(w_i)$ for their set of ampoules.
The degree of equivalence (D_i) of a participant's submitted value w_i with w_{KCRV} is given by:

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

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

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

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

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

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

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

The *trans*-ZEN mass fractions values and associated absolute uncertainties with degree of equivalences for CCQM-K154.a and CCQM-K154.a.1 are listed in Table 14. Figure 12 indicates the degree of equivalence (D_i) of each key comparison participant's result with the w_{KCRV}.
The *trans*-ZEN mass fractions values and associated relative uncertainties with relative degree of equivalences are listed in Table 15. Figure 13 indicates the relative degree of equivalence ($D_{rel,\,i}$) of each key comparison participant's result with the w_{KCRV}.

Table 14: *trans*-ZEN mass fractions and absolute corresponding uncertainties with degree of equivalences for CCQM-K154.a/K154.a.1

NMI/DI	Study	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)
UME (CBKT) A	CCQM-154.a	12.80	0.09	0.18	12.69	0.11	0.22	-0.11	0.28	12-13
UME (CBKT) B	CCQM-154.a	12.83	0.08	0.15	12.69	0.11	0.22	-0.14	0.27	12-13
NMISA (own) A	CCQM-154.a	12.18	0.10	0.20	12.30	0.09	0.18	0.12	0.27	12-13
NMISA (own) B	CCQM-154.a	12.20	0.12	0.25	12.30	0.09	0.18	0.10	0.31	12-13
NIM (own) A	CCQM-154.a	12.88	0.18	0.36	12.70	0.15	0.30	-0.18	0.47	12-13
NIM (own) B	CCQM-154.a	12.77	0.10	0.20	12.70	0.15	0.30	-0.07	0.36	12-13
NIM (CBKT) A	CCQM-154.a	12.86	0.20	0.41	13.10	0.14	0.28	0.24	0.49	12-13
NIM (CBKT) B	CCQM-154.a	13.00	0.09	0.18	13.10	0.14	0.28	0.10	0.33	12-13
INTI (CBKT) A	CCQM-154.a	14.40	0.12	0.24	14.80	0.14	0.29	0.40	0.38	14-16
INTI (CBKT) B	CCQM-154.a	14.45	0.10	0.19	14.80	0.14	0.29	0.36	0.34	14-16
INMETRO (CBKT) A	CCQM-154.a	14.38	0.10	0.19	14.64	0.14	0.28	0.26	0.34	14-16
INMETRO (CBKT) B	CCQM-154.a	14.47	0.33	0.66	14.64	0.14	0.28	0.17	0.71	14-16
EXHM (own) A	CCQM-154.a	15.23	0.10	0.19	15.03	0.16	0.32	-0.20	0.38	14-16
EXHM (own) B	CCQM-154.a	15.29	0.10	0.20	15.03	0.16	0.32	-0.27	0.38	14-16
KEBS (CBKT) A	CCQM-154.a	14.53	0.08	0.16	14.66	0.12	0.25	0.12	0.30	14-16
KEBS (CBKT) B	CCQM-154.a	14.71	0.07	0.14	14.66	0.12	0.25	-0.05	0.29	14-16
NIMT (CBKT) A	CCQM-154.a	14.45	0.13	0.27	14.73	0.15	0.30	0.28	0.40	14-16
NIMT (CBKT) B	CCQM-154.a	14.51	0.10	0.19	14.73	0.15	0.30	0.22	0.35	14-16
NRC (own) A	CCQM-154.a	66.90	0.49	0.98	66.50	0.50	1.00	-0.40	1.40	64-68
NRC (own) B	CCQM-154.a	66.68	0.48	0.96	66.50	0.50	1.00	-0.18	1.38	64-68
INTI (CBKT) C	CCQM-154.a.1	14.92	0.07	0.13	14.80	0.14	0.29	-0.11	0.32	14-16
INTI (CBKT) D	CCQM-154.a.1	14.92	0.10	0.19	14.80	0.14	0.29	-0.11	0.34	14-16

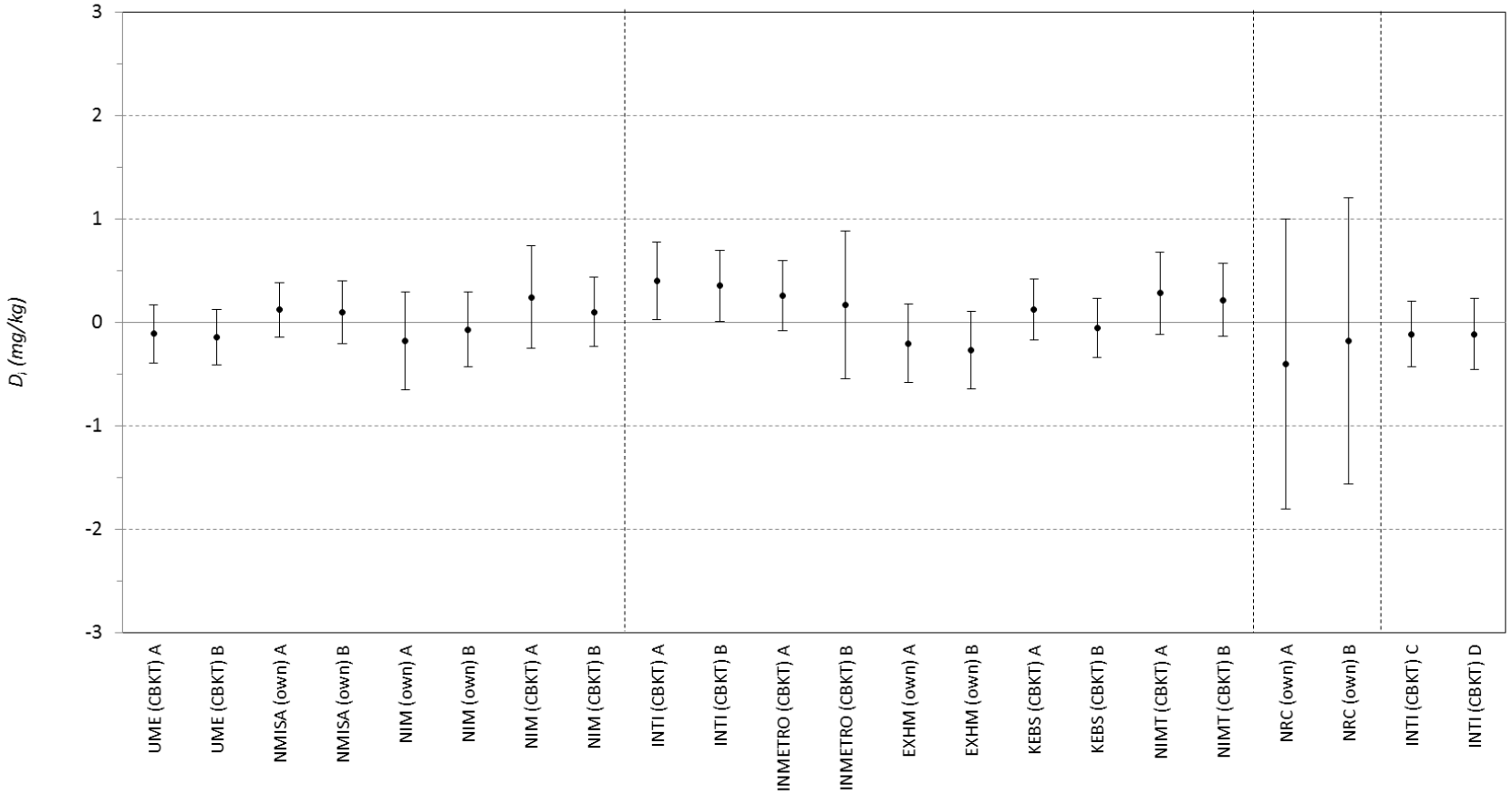

Figure 12: Absolute values for the degree of equivalence for CCQM-K154.a/K154.a.1

Table 15: *trans*-ZEN mass fractions and relative corresponding uncertainties with relative degree of equivalences for CCQM-K154.a and CCQM-K154.a.1

NMI/DI	Study	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)
UME (CBKT) A	CCQM-154.a	12.80	0.69	1.38	12.69	0.87	1.73	-0.88	2.22	12-13
UME (CBKT) B	CCQM-154.a	12.83	0.59	1.18	12.69	0.87	1.73	-1.14	2.10	12-13
NMISA (own) A	CCQM-154.a	12.18	0.81	1.61	12.30	0.73	1.46	0.99	2.18	12-13
NMISA (own) B	CCQM-154.a	12.20	1.02	2.03	12.30	0.73	1.46	0.81	2.50	12-13
NIM (own) A	CCQM-154.a	12.88	1.41	2.82	12.70	1.18	2.36	-1.41	3.68	12-13
NIM (own) B	CCQM-154.a	12.77	0.78	1.56	12.70	1.18	2.36	-0.54	2.83	12-13
NIM (CBKT) A	CCQM-154.a	12.86	1.59	3.17	13.10	1.07	2.14	1.86	3.83	12-13
NIM (CBKT) B	CCQM-154.a	13.00	0.69	1.38	13.10	1.07	2.14	0.77	2.54	12-13
INTI (CBKT) A	CCQM-154.a	14.40	0.85	1.69	14.80	0.97	1.93	2.69	2.57	14-16
INTI (CBKT) B	CCQM-154.a	14.45	0.66	1.33	14.80	0.97	1.93	2.40	2.35	14-16
INMETRO (CBKT) A	CCQM-154.a	14.38	0.67	1.33	14.64	0.96	1.91	1.76	2.33	14-16
INMETRO (CBKT) B	CCQM-154.a	14.47	2.27	4.54	14.64	0.96	1.91	1.16	4.92	14-16
EXHM (own) A	CCQM-154.a	15.23	0.64	1.28	15.03	1.07	2.14	-1.34	2.50	14-16
EXHM (own) B	CCQM-154.a	15.29	0.64	1.28	15.03	1.07	2.14	-1.77	2.49	14-16
KEBS (CBKT) A	CCQM-154.a	14.53	0.55	1.10	14.66	0.85	1.69	0.85	2.02	14-16
KEBS (CBKT) B	CCQM-154.a	14.71	0.48	0.97	14.66	0.85	1.69	-0.35	1.95	14-16
NIMT (CBKT) A	CCQM-154.a	14.45	0.92	1.84	14.73	1.00	2.01	1.91	2.72	14-16
NIMT (CBKT) B	CCQM-154.a	14.51	0.66	1.32	14.73	1.00	2.01	1.47	2.41	14-16
NRC (own) A	CCQM-154.a	66.90	0.73	1.46	66.50	0.75	1.50	-0.60	2.10	64-68
NRC (own) B	CCQM-154.a	66.68	0.72	1.44	66.50	0.75	1.50	-0.27	2.08	64-68
INTI (CBKT) C	CCQM-154.a.1	14.92	0.45	0.90	14.80	0.97	1.93	-0.77	2.13	14-16
INTI (CBKT) D	CCQM-154.a.1	14.92	0.64	1.28	14.80	0.97	1.93	-0.77	2.31	14-16

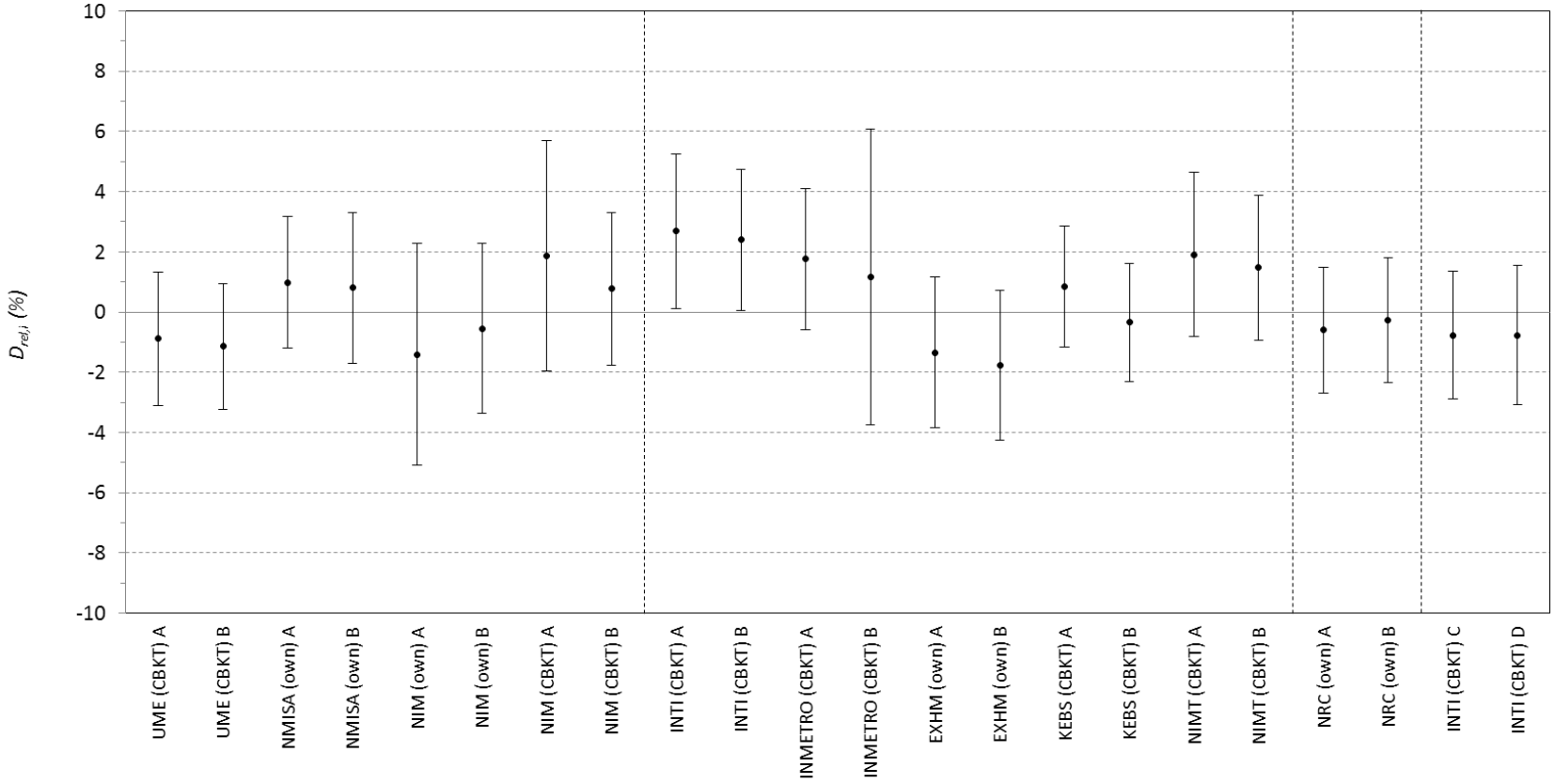

Figure 13: Relative values for the degree of equivalence for CCQM-K154.a and CCQM-K154.a.1

CONCLUSIONS

trans-ZEN was selected to be representative of non-polar *Fusarium* 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.

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

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

The majority of the *trans*-ZEN mass fraction KCRVs (w_{KCRV}) for CCQM-K154.a and CCQM-K154.a.1 spanned a mass fraction range of 12.18 mg/kg to 15.29 mg/kg. Solely the solution of NRC exhibited a higher *trans*-ZEN mass fraction KCRVs of 66.68 mg/kg and 66.90 mg/kg. The relative expanded uncertainties U(w_{KCRV}) ranged from 0.90 % to 4.54 %.

Inspection of the degree of equivalence plots (Figures 12 and 13) for the *trans*-ZEN mass fraction assignments in CCQM-K154.a indicated that there was an excellent agreement of results. The *trans*-ZEN mass fraction assignments of INTI, Argentina were the only values that did not agree with the KCRVs. As discussed earlier it was found that the samples were altered as a result of the holdup during transport during which time light-induced isomerization of *trans*-ZEN to *cis*-ZEN must have happened. The CCQM-K154.a.1 subsequent comparison was organized for INTI to repeat measurements with new ampoules of the same batch which were provided under properly controlled transport conditions. The *trans*-ZEN mass fraction assignments of INTI within CCQM K154.a.1 were in agreement with the KCRVs. It should be pointed out the expanded uncertainties of the CCQM-K154.a.1 KCRVs had the lowest uncertainty values brought about by a much shorter sequence of analysis.

HOW FAR THE LIGHT SHINES STATEMENT (HFTLS)

Successful participation in CCQM‑K154.a or CCQM-K154.a.1 for MMCBKT participants will support CMCs for:
a) preparation and value assignment of Zearalenone 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.a or CCQM-K154.a.1 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 of non-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.

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ANNEX A – ADDITIONAL ANALYTICAL INFORMATION

Hellenic Metrology Institute (EXHM), Greece

Solution preparation procedure

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.025 standard ZEN solution provided by BIPM

Crystalline zearalenone (ZEA) powder was purchased from FERMENTEK. The purity of ZEA was determined with qNMR using of 3,5 BTFMBA NMIJ CRM 4601-a as the internal standard. HSQC, COSY, 1H, 13C were also acquired for the pure compound. Additionally, purity was also checked by a mass balance approach (LC/UV/FLD analysis of the FERMENTEK material, Karl Fisher coulometric titration for water, GC-MS and ICP-MS for the determination of volatile matter and inorganics). Purity investigation was also supplemented by LC-MS/MS in full scan mode (100-500 m/z and 315-325 m/z) - no related structure impurities were detected.

Amount of primary calibrator used for analysis

-/-

Gravimetry

Type of balance (make, model and resolution)

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

Balance repeatability

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

Solution preparation procedure

An intermediate solution was initially prepared by dissolving ZEA (0.017268 g) in acetonitrile (3.10864 g) in an amber glass vial. For the preparation of the final solution 0.34588 g of the intermediate solution were transferred in a bottle, which was then sealed and weighed. Acetonitrile (125.9456 g) were introduced in the bottle via its septum by means of a syringe. The resulting final solution was subdivided in 4 mL CERTAN vials.

Homogeneity and/or stability testing

For the homogeneity test: 10 vials were analyzed in duplicate with LC-DAD (at 236 and 274 nm) and LC-FLD (excitation at 274 nm and emission at 456 nm). The short-term stability was examined with Isochronous study at four different temperatures (-18 °C as reference, 40 °C, 20 °C and 40 °C) and at different periods of time (zero, two and four weeks). 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 (which was determined from the gravimetric preparation), the homogeneity and stability study were performed with LC-DAD (at 236, 254 and 274 nm) and LC-FLD (excitation at 274 nm and emission at 456 nm). The mobile phase was constituted of: A=H_2O, B=acetonitrile (both containing 0.1 % formic acid) in the following gradient mode: A = 60 %, B = 40 % for t = 0 min, then the composition of acetonitrile was increased to B = 80 % in the next 30 min and the column was equilibrated in A = 60 %, B = 40 % for 10 min. For the chromatographic separation the column Inertsil ODS-3, 250 x 2.1 mm, 5 μm from MZ was used.
The assigned value was also checked by means of UV spectrophotometry (at 236, 274 and 316 nm) using published data for the ZEA molar absorptivities (extinction coefficients). The instrument used was previously calibrated using NIST SRM 935a solutions. Characterization uncertainty was also included in the uncertainty budget.

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

The verification of the assigned value was performed with bracketing and benzo[a]pyrene as an internal standard.

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

Crystalline zearalenone from n'TOX (99.2%) and solutions thereof were used as independent calibrants for verification.

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}= 0.22 %), 2) homogeneity (u_{homo}= 0.26 %), 3) stability (u_{stab} = 1.01 %) and characterization (u_{char} = 0.09 %).

EQUATIONS AND UNCERTAINTY BUDGETS

For the gravimetric preparation of the ZEA solution:

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

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

The equation used to estimate standard uncertainty from preparation is:

$$u(w_{ZEA}, PREP) = \sqrt{\left(C_p u_{PZEA}\right)^2 + \sum \left(C_j u(m_i)\right)^2}$$

where u_{PZEA} is the uncertainty for ZEA purity determination, and C_j the sensitivity coefficients associated with the masses involved in the preparation stage.

Purity was determined by qNMR and checked by the mass balance approach. The respective uncertainties were calculated via the following equations:

qNMR

$$P_s = \frac{I_s}{I_{is}} \frac{N_{is}}{N_s} \frac{mw_s}{mw_{is}} \frac{m_{is}}{m_s} P_{is}$$

where

P	:	purity (mg/g)
I	:	signal intensity
N	:	number of protons
mw	:	molecular weight
m	:	mass
s	:	sample (ZEA)
is	:	internal standard (3,5 BTFMBA)

mass balance

$$P_{ZEA} = nZEA_{Area}\left(1 - \frac{w_{H_2O} + w_{vol} + w_{in}}{1000}\right)$$

where

Pzea	:	purity (g/g)
$nZEA_{area}$	:	normalized ZEA peak area in the HPLC- UV chromatogram
H_2O	:	water (mg/g)
vol	:	residual volatiles (mg/g)
in	:	inorganics and non-volatile material (mg/g)

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

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

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 detailed budgets are the following:

qNMR uncertainty budget

qNMR uncertainty budget							
uncertainty component	value	units	u_i	u_i/x_i	C_i	C_iu_i	$(C_iu_i)^2$
ZEA/3,5BTFMBA signal ratio	0,8295		0,00181	2,186E-03	1197,27	2,1711	4,714E+00
ZEA molecular mass	318,360	g mol^{-1}	0,00508	1,596E-05	3,12	0,0159	2,512E-04
3,5 BTFMBA molecular mass	258,120	g mol^{-1}	0,00522	2,023E-05	-3,85	-0,0201	4,038E-04
no of protons in signal integrated for ZEA	1	nucl/mol	0,00002	1,800E-05	-993,14	-0,0179	3,196E-04
no of protons in signal integrated for 3,5BTFMBA	1	nucl/mol	0,00002	1,800E-05	993,14	0,0179	3,196E-04
ZEA mass	3,7246	mg	0,00056	1,510E-04	-266,64	-0,1500	2,249E-02
3,5 BTFMBA mass	3,6170	mg	0,00060	1,650E-04	274,57	0,1638	2,684E-02
boyancy correction	1,0000		0,00000	4,065E-06	993,14	0,0040	1,630E-05
3,5 BTFMBA	999,60	mg g^{-1}	0,26000	2,601E-04	0,99	0,2583	6,673E-02
ZEA purity							**993,15**
combined standard uncertainty		mg g^{-1}					**2,20**
expanded uncertainty (k=2)		mg g^{-1}					4,40

Preparation data and uncertainty

ZEA - intermediate	value	units	standard uncertainty	relative uncertainty	sensitivity coefficient	C_iu_i	$(C_iu_i)^2$
ZEA	0,0172680	g	0,000003	0,00017	0,31596	9,48E-07	8,98E-13
MeCN	3,10864	g	0,000108	0,00003	-0,00176	-1,89E-07	3,57E-14
ZEA purity	0,99315		0,002198	0,00221	0,00552	1,21E-05	1,47E-10
							1,484E-10
concentration	5,486	**mg/g**					
combined std uncertainty	0,012	0,000					
relative uncertainty	0,222	(%)					
coverage factor	2						
expanded uncertainty	0,024	mg/g					

ZEA FINAL	value	units	standard uncertainty	relative uncertainty	sensitivity coefficient	C_iu_i	$(C_iu_i)^2$
ZEA - intermediate	0,34588	g	0,000021	0,00006	0,04332	9,15E-07	8,38E-13
MeCN	125,94560	g	0,003952	0,00003	-0,00012	-4,70E-07	2,21E-13
concentration	5,486	g/g	0,012180	0,00222	0,00274	3,34E-05	1,11E-09
							1,11E-09
concentration	15,026	mg/kg					
combined std uncertainty	0,033	mg/kg					
relative uncertainty	0,222	(%)					
coverage factor	2						
expanded uncertainty	0,067	mg/kg					

Total uncertainty budget

	UNCERTAINTIES (mg/kg)						
ZEA conc (mg/kg)	u_{prep}	u_{homo}	u_{stab}	u_{char}	combined	relative (%)	expanded
15,026	0,033	0,039	0,152	0,013	0,161	1,07	0,322

Mass balance uncertainty budget (for informative purposes)

Zearalenone purity by mass balance							
uncertainty component	value	units	n	uncertainty	rel uncertainty	Ci	Ci x Ui
normalization	998,63	mg	4	2,1979	7,406E-04	0,9937	2,1840
water	2,13	mg	2	0,3915	1,838E-01	-0,9986	-0,3909
volatiles	4,14	mg	2	0,0242	5,842E-03	-0,9986	-0,0242
inorganics/insolubles	0,08	mg	2	0,0310	3,875E-01	-0,9986	-0,0763
combined standard uncertainty							2,22
effective degrees of freedom							3,19
k (95% conf. level)							3,182
expanded uncertainty (95%)							7,065

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

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.025 standard ZEN solution provided by BIPM

OGP.025 standard ZEN solution provided by BIPM.

Amount of primary calibrator used for analysis

22 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo, model XS205, resolution 0.0001 g (this balance was used for weighing primary calibrator on gravimetric preparation of the batch).
Mettler Toledo, model PR 1203, resolution 0.001 g (this balance was used for weighing acetonitrile on gravimetric preparation).

Balance repeatability

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

Solution preparation procedure

The Inmetro`s calibration solution was produced by gravimetric dilution of 7 ampoules from OGP.025 stock solution (130.1 ± 2.2 µg/g, $k = 2$) in a 250 mL volumetric flask filled with pure acetonitrile. A volume of 4 mL of this calibration solution was dispensed into 10 mL amber glass ampoules using a manual dispenser. The ampoules were sealed with an ampoule sealer machine. The batch of 55 ampoules was labelled and stored in a freezer at -20 °C. The units of the calibration 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 (batch of 55 ampoules) using LC-DAD/MS-MS method described below. The main compound (trans-zearalenone) was measured by DAD, and related impurities were measured by MS method (only for additional

information). The homogeneity contribution of 0.46 % was included on uncertainty of assigned value of trans-ZEN. The short-term stability study showed that samples can be transported up to 50 °C during 4 weeks, without changes on certified value of trans-ZEN.

Optional: Analytical check method

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

System:	UPLC-MS/MS-PDA, XEVO TQ Waters
Column:	Phenomenex Luna 5 µ C18 (2) 100 Å, 250 x 4.6 mm
Mobile phase:	A = H2O + 0.1 % HCOOH
	B = ACN + 0.1 % HCOOH (isocratic mode: 50 % A and 50 % B)
Flow rate:	0.4 mL/min ; injection volume: 4 µL; run time: 50 min
Detector:	DAD: 274 nm; scan range: 210 nm - 600 nm; Resolution: 1.2 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)

BIPM OGP.025 (Zearalenone in acetonitrile): (130.1 ± 2.2) µg/g ; k = 2, 95 %.

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

Mass spectrometric analysis was performed by electro-spray ionization operating in negative mode (ESI). Multiple Reaction Monitoring (MRM) was employed for quantitative analysis. The transitions were *m/z* 317.2 > 131.1 for Trans-Zearalenone (main compound). For monitoring of related impurities, the transitions were *m/z* 315.3 > 175.1 or Dehydrozearalenone, *m/z* 319.3 > 275.0 for Zearalanone (ZAN) and *m/z* 317.2 > 131.1 for Zearalenone Isomers.

Additional Comments or Observations

The assigned value is the combination of trans-ZEN gravimetric preparation 14.64 µg/g with u(char) of 0.12 µg/g (0.85%) and the homogeneity contribution of u(bb) of 0.07 µg/g (0.46%). For additional information, UV spectrophotometric analysis are also performed directly without dilution against pure acetonitrile (ACN) as reference by use of a scan method and by a wavelength method at 235 nm, 274 nm and 314 nm.

Measurement Science and Standards - National Research Council (NRC), Canada

<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.025 standard ZEN solution provided by BIPM

The purity of the solid zearalenone source material as determined by ^{1}H-qNMR was 0.9886 ± 0.0076 g/g. The purity assignment of zearalenone is traceable to the SI from the primary calibrator, NIST benzoic acid PS-1. In brief, benzoic acid PS-1 was the primary calibrator for the qNMR certification of dimethyl terephthalate. Three independent qNMR samples were prepared and each time, approximately 18-19 mg of benzoic acid was weighed for preparation of the qNMR sample. Dimethyl terephthalate was then used as the secondary calibrator for the purity determination of zearalenone by qNMR. Three qNMR samples for zearalenone were prepared and each time, 15-16 mg of dimethyl terephthalate and 13-15 mg of zearalenone were used for the internal standard and analyte respectively.

Amount of primary calibrator used for analysis

-/-

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo, XP-6U, with a readability of 0.0001 mg

Balance repeatability

0.000004 µg

Solution preparation procedure

Solid zearalenone was gravimetrically weighed, dissolved in acetonitrile and the solution sonicated for 30 min to ensure homogeneous mixing. The solution was dispensed in 1.1 mL aliquots in pre-washed and argon-filled ampules, which were immediately flame sealed.

Homogeneity and/or stability testing

Eleven boxes each containing 80 ampules were obtained. One ampule from each box was sampled to test for homogeneity and the solution was determined to be homogeneous.

With homogeneity established, an isochronous short term stability study was performed by monitoring the solution at day 7, day 14 and day 28, while stored at +37 °C, 20 °C, 4 °C, -20 °C and -40 °C. Potential degradation was assessed against samples stored at -40 °C, the reference temperature. No degradation was observed at any of the designated storage temperatures over the duration of the study.

Optional: Analytical check method

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

The homogeneity and stability studies were performed by LC-UV on an Agilent 1290 instrument fitted with an ACE-C18-PFP column (2.1 µm, 50 x 2.1 mm), heated to 40 °C. The injection volume of the samples was 2 µL, with elution proceeding at 0.3 mL/min with water and methanol, both modified with 0.1% formic acid and 5 mM ammonium acetate, as mobile phases A and B respectively. The following gradient was applied: 0-1 min (50% B), 1-8 min (50-95 %B), 8-11 min (95 %B), 11-12 min (95-50 %B) and 12-15 min (50 %B). UV absorbance was monitored at 238 and 274 nm.

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

To obtain a separate assessment of the concentration of the solution, an external calibration curve was prepared from a zearalenone certified reference material solution obtained from Aldrich. The external calibration was achieved from three different concentrations, analyzed three times within the short term stability sequence, at the beginning, in the middle and towards the end of the sequence. All other samples were analyzed in triplicate in a randomized order. According to the calibration curve, the concentration is in agreement with the calculated concentration of the zearalenone solution prepared gravimetrically from qNMR certified solid zearalenone.

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

The external calibrant used to obtain the calibration curve during the short term stability sequence was a zearalenone certified reference material obtained from Aldrich at 50.0 ± 0.3 µg/mL. The raw materials used to prepare the Aldrich certified reference material were weighed on a balance calibrated with mass standards traceable to NIST.

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

-/-

Additional Comments or Observations

Please note: (1) the external calibration points recorded during short term stability sequence also showed that measurement drift was negligible; (2) an independent external calibration curve generated by LC-UV from our qNMR certified zearalenone solid material, further confirmed a zearalenone concentration in agreement with our reported value; (3) cis- and trans-zearalenone have distinct NMR signals and the presence of cis-zearalenone in the sample was discounted after investigation by NMR and the replication of a published LC-UV method for the separation of cis- and trans-zearalenone, both failed to detect the compound.

Instituto Nacional de Tecnología Industrial (INTI), Argentina

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.025 standard ZEN solution provided by BIPM

Trans-Zearalenone (ZEN) in acetonitrile (ACN) stock solution. BIPM Reference: OGP.025. Purity of main component and uncertainty: 130.1 ± 2.2 µg/g (k = 2). Combination of ZEN gravimetric preparation 130.08 µg/g with u_{char} of 0.12 ug/g (0.091 %) and the homogeneity contribution of u_{bb} of 1.08 µg/g (0.83 %). Used ampoules OGP.025.019, OGP.025.043, OGP.025.063, OGP.025.082, OGP.025.122, OGP.025.139, OGP.025.187.

Amount of primary calibrator used for analysis

22.4369 g

Gravimetry

Type of balance (make, model and resolution)

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

Balance repeatability

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

Solution preparation procedure

The calibrant solution preparation was carried out weighing the content of 7 ampoules of stock solution into a 250 mL plastic bottle. The plastic bottle (previously marked at 250 mL) was filled up to 250 mL with acetonitrile. The final weight was considered. 52 clear glass ampoules containing 4 mL of calibrant solution were produced. Batch was called INTI-ZEN-CAL (INTI-Z-CAL).

Homogeneity and/or stability testing

Homogeneity study was carried out considering 10 ampoules from the batch INTI-ZEN-CAL. Homogeneity was assessed by spectrophotometry UV and HPLC-DAD at 235 nm, 274 nm and 314 nm. The result obtained by HPLC-DAD at 274 nm was considered as inhomogeneity of batch INTI-ZEN-CAL (u*bb = 0.46 %). Stability study was carried out following an

isochronous experiment design. Three different conditions were considered: 4 °C in dark, 22 °C in dark and 22 °C under light conditions during 1, 2 and 4 weeks. The reference condition was -20 °C in dark. Two ampoules were measured for each temperature and each period of time. Spectrophotometry UV and HPLC-DAD were used to evaluate the stability. The results of the stability studies of INTI-ZEN-CAL did not show decreasing trends for 4 °C in dark and 22 °C in dark as storage conditions for ZEN. However, there is a significant decreasing trend based on statistical analyses of the results at 22 °C under light as storage condition for ZEN. It was concluded that 22 °C and light conditions should be avoid during shipment and storage. All samples were analysed directly without dilution in a stratified random order. ISO Guide 35 was considered for all studies.

Optional: Analytical check method

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

HPLC Conditions:
Column: Kromasil 100-5-C18 (4.6x250mm) (Batch/Serial: 0000139143/E173118)
Mobile Phase: (Water 0.1% ACOOH + ACN 0.1% ACOOH) (45 + 55)
Flow rate: 1 mL/min
Run Time: 20 min
Retention time of ZEN: 8.66 min
Oven temperature: 40 °C
Injection Volume: 20 µL
Detector Wavelengths: 235 nm, 274 nm and 314 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 ZEN gravimetric preparation uncertainty (u_{char}) of 0.125 µg/g (0.845 %) and the homogeneity contribution of u_{bb} of 0.068 µg/g (0.458 %).

Kenya Bureau of Standards (KEBS), Kenya

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.025 standard ZEN solution provided by BIPM

Trans-Zearalenone (ZEN) in acetonitrile (ACN) stock solution. BIPM Reference: OGP.025.

Amount of primary calibrator used for analysis

21.883 g

Gravimetry

Type of balance (make, model and resolution)

Sartorius ME 414 S

Balance repeatability

100 µg

Solution preparation procedure

Each ampoule added to the 250 mL flask then topped with ACN.

Homogeneity and/or stability testing

Not done.

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)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.025 standard ZEN solution provided by BIPM

Crystalline zearalenone (ZEN) was sourced from the Council of Scientific and Industrial Research Biosciences, South Africa. The material was purity assigned by NMISA using quantitative NMR, with structurally related impurities confirmed through LC-MS/MS and LC-FLD. QNMR of ZEN purity was performed in deuterated acetone-d6, using Fluka TraceCERT dimtheylterphthalate as internal standard with traceability established in-house to NIST PS1 Benzoic acid using QNMR.

Amount of primary calibrator used for analysis

0.149260 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo	AX 26	0.002 mg	
Mettler Toledo	XP 205	0.01 mg (81 g)	0.1 mg (220 g)
Mettler Toledo	MS12002TS	0.01 g	

Balance repeatability

4 µg

Solution preparation procedure

1. A 100 µg/mL stock solution (CRM0005) was prepared by weighing ~150 mg in house value assigned ZEN (CRM0004) and diluting in 1.5 L acetonitrile
2. An aliquot of 20 mL of the stock solution was gravimetrically transferred into a volumetric flask and diluted to 200 mL with ACN to prepare the CCQM K154 a solution
3. The K154 a solution was ampouled (4 mL) using the Ampulmatic 10 ampoule sealer, with the purge gas (nitrogen), and liquid filler accessories
4. The ampoules were subject to leak testing overnight at 50 mbar and leaks gravimetrically identified.

Homogeneity and/or stability testing

Of the 37 ampoules prepared for the K154a study, 5 were selected at regular intervals across the batch for the homogeneity assessment. Two repeat aliquots, without dilution, from each ampoule were evaluated using LC-UV at 274 nm. The relative homogeneity uncertainty was estimated as 0.61 % and is included in the combined uncertainty reported for the NMISA K154a solution. An isochronous stability assessment was carried out over 4 weeks, where 2 ampoules were stored at 4 ˚C, 20 ˚C and 40 ˚C, for 1, 2 and four weeks. All samples were stored in the dark, as ZEN degrades when exposed to light. The stability of the solutions was evaluated using LC-FLD, monitoring the change in concentration of the ZEN and structurally related impurities.

Optional: Analytical check method

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

A 10 µL injection of the undiluted aliquot of the solution prepared for K154a was injected on the Phenomenex Kinetex EVO C18 100 Å, 250 x 4.6 mm, 2.6 µm (OGLC.65) column. The ZEN and impurities were separated in the isocratic phase of the mobile phase gradient at a flow rate of 0.6 mL/min. Impurities were separated in 36 min during the isocratic phase of 60:40 aqueous mobile phase: acetonitrile both containing 0.1 % formic acid. The isocratic phase was followed by a column wash with high organic solvent and a 10 min re-equilibration to starting conditions. UV detection at 274 nm was used for analysis of the main component whilst FLD detection (excitation 274 nm and emission 446 nm) was used to monitor the concentration of the main component as well as the structurally related impurities (for qNMR assignment verification, homogeneity and stability assessment).

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

The concentration of the NMISA K154a solution was verified using the BIPM OGP.25 reference material and the value differed by 0.77% (this result was not used in the value assignment and uncertainty estimate of the NMISA K154a solution). Concentration of the impurities was determined by relative peak area percentage assuming relative response factors of 1, used to support/verify qNMR data.

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

NMISA Zearalenone CRM0004 (Purity 988.8 mg/g with expanded uncertainty 7.8 mg/g (k = 2, 95 % level of confidence). 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 NIST PS1 Benzoic acid (Purity 999.92 mg/g with expanded uncertainty -0.06 and + 0.04 mg/g (k = 2, 95 % confidence interval).

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

The identity and presence of structurally related impurities was evaluated using LC-MS and LC-MS/MS. Some of the MS/MS transitions in ESI negative that were used to detect potential ZEN impurities were:
ZEN and iso ZEN: 317.2>131.1; 317.2>175.1; 317.2>187.0
Zearalanone (ZAN): 319.3>275.0; 319.3>205.1; 319.3>303.2
Dehydrozearalenone: 315.3>175.1; 315.3>271.1
Zearalenol: 319.3>275.0; 319.3>160.1
Zearalanol: 321.3>277.1"

Additional Comments or Observations

Only isoZEN was detected in K154a solution. In the original NMISA ZEN crystalline material; Iso-zearalenone; Zearalanone; dehydrozearalenone and an additional unidentified structurally related impurity were detected. (each below 0.1 %). In addition to the structurally related impurities, chloroform was detected.

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.025 standard ZEN solution provided by BIPM

OGP.025.021, OGP.025.026, OGP.025.050, OGP.025.070, OGP.025.087, OGP.025.100, OGP.025.111

Amount of primary calibrator used for analysis

22.023825 g

Gravimetry

Type of balance (make, model and resolution)

Mettler-Toledo XP2004S, 0.0001 g Resolution

Balance repeatability

1.09904264561041E-10 μg

Solution preparation procedure

ZEN solution was prepared by gravimetric. Seven ampoules from BIPM OGP.025 stock solution were used. Dilution of standard solution was performed in 250 mL volumetric flask using acetonitrile.

Homogeneity and/or stability testing

Homogeneity testing: Ten randomly selected ampoules were analyzed in triplicates and statistically analyzed using ANOVA.
Stability testing: Eighteen randomly selected ampoules were analyzed in triplicates. Stability study was carried out using isochronous approach at 4 °C and 40 °C for 0, 1, 2, 3 and 4 weeks. Trend analysis was performed to statistically test according to the conditions during the stability study. The slope was tested for significance on a 95 % confidence level. The reference temperature for sample storage was at -20 °C.

Optional: Analytical check method

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

LC-PDA conditions
Mobile phase: 50 % acetonitrile in water
Total flow rate: 1.0 mL/min
Column temperature: 30 °C
Injection volume: 10 µL

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

One point bracketing external calibration.

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

Verification of the prepared standard solution was carried out by analyzing the prepared solution using single point, bracketing external calibration. The pure zearalenone in acetonitrile obtained from LGC was used as calibration solution. Details of zearalenone: Source: LGC
Certified value: 101.0 µg/mL Uncertainty: 0.6 µg/mL
*Standard was traceable to the SI unit of kg through gravimetric preparation and to the stated purity of the solid raw material.

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

-/-

Additional Comments or Observations

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

CCQM-K154.a Reporting Form

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.025), µg/g
m_z	mass of the stock solution (OGP.025) added (g)
m_{total}	mass of the total solution (g)

Expanded measurement equation:

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

where;

w(xi)	mass fraction of the prepared solution, µg/g
w_z	mass fraction of the stock solution prepared from (OGP.025), µg/g
m_z	mass of the stock solution (OGP.025) added (g)
m_{total}	mass of the total solution (g)
F_{stb}	Stability factor, given a value of 1
F_{homo}	Homogeneity factor, given a value of 1

Combined measurement uncertainty:

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

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.025)
$u(m_z), u(m_{total})$	standard uncertainties of masses estimaetd from the bias of balance and the precision of balance
$u(F_{homo})$	standard uncertainty due to homogeneity factor, estimated from ANOVA
$u(F_{stb})$	standard uncertainty due to stability testing at 4 °C, estimated from trend analysis

Uncertainty budget:

Uncertainty source	xi	uxi	uxi/xi	(uxi/xi)^2
Preparation				
ZEN stock (µg/g)	130,1	1,1	0,008455	7,14876E-05
mass ZEN stock (g)	22,02383	0,000395	1,795E-05	3,22288E-10
mass total (g)	194,57540	0,002105	1,08171E-05	1,17009E-10
w(xi), (µg/g)	14,72591	0,124508	0,008455061	7,1488E-05
Homogeneity	1,0	0,002426	0,00242632	5,88703E-06
Stability @4 °C	1,0	0,004795	0,004794621	2,29884E-05
			sum (uxi/xi)^2	0,000100
			(uxi/xi)	0,010018
			ux	0,147526
			k (95% CI)	2,0
			U (k=2)	0,29505
			U(%)	2,0036

National Metrology Institute of Turkey (UME), Turkey

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.025 standard ZEN solution provided by BIPM

OGP.025.80

Amount of primary calibrator used for analysis

2.906 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo AX205 0.01 mg

Balance repeatability

40 µg

Solution preparation procedure

Before starting preparation of calibration solution, stock solution was transferred from -20 ˚C to the room temperature and kept in dark condition until the solution reached to room temperature. An empty amber bottle was placed on the balance. After measuring the weight of cap and vial, balance was tared. Acetonitrile was placed into the bottle. The weight was measured and balanced was tared. Stock solution was added onto the acetonitrile and weight was measured. The solution was mixed and filled into 20 mL transparent glass ampules as 4 mL for each.
Measurement equation:

$$C_X = \frac{C_y \times M_y}{M_{total}}$$

CX: Mass fraction of calibration solution (µg/g)
CY: Mass fraction of stock solution (µg/g)
MY: Weight of stock solution (g)
Mtotal: Weight of calibration solution (g)

Homogeneity and/or stability testing

For the assessment of homogeneity, 3 from 9 units covering the whole batch were selected with stratified sampling technique. From each selected unit, 5 parallel measurements were performed by HPLC-UV. One-way analysis of variance (ANOVA) was applied for the evaluation.
8 units were selected with stratified sampling technique for short term stability assessment. The test temperature was selected as +20 °C for time periods of 5, 9 and 13 days and the reference temperature was -20 °C. The study was performed isochronously.

Optional: Analytical check method

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

HPLC-UV with the flow rate 0.6 mL/min
5 µm, 100 A, Luna C18 250x4.6 mm
Gradient program A: 0.1% Formic acid+ Water B: 0.1% Formic acid+ Acetonitrile

Minute	Solvent A (%)	Solvent B (%)
0	95	5
48	25	75
48	95	5
55	95	5

UV wavelength: 274 nm
Oven: 30 ˚C
Retention Time: 44.69

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

Multiple points external calibration.

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

OGP.025.12

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

-/-

Additional Comments or Observations

Evaluation of Measurement Uncertainty

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

Sources :

1-Preparation (u_{char})	0.85%, 0.11
1-1: Gravimetric preparation	
1-2: Purity	
1-3: Repeatability	
2-Homogenity (u_{bb})	0.09%, 0.01
3-Short Term Stability (u_{sts})	0.09%, 0.01

$k = 2$

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

Determination of the concentration of Zen stock solution by NMR

The concentration assessment of ZEN (OGP.025.056) was done by quantitative nuclear magnetic resonance (qNMR). DSS-d6 (3-(Trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt) was used as internal standard (IS) and D_2O (Merck, 99.96%) was used as NMR solvent. DSS-d6 concentration (0.0974 % ± 0.0002 k=2) was determined by UME CRM 1301 chloramphenicol with a certified value of 99.58 ± 0.15% (k=2) (TÜBİTAK UME, Gebze, TR). Five different samples were prepared from stock solution and each sample was analyzed with three repetitions.

The sample solutions of ZEN were prepared by following steps: ZEN solution (300 mg ~ 400 mL) and DSS-d6 solution (110 mg ~ 100mL) were accurately weighed, dissolved in D2O (0.2 mL), stirred with vortex for 5 seconds and 0.7 mL solution transferred to a NMR tube. All NMR measurements were carried out on a Bruker Avance NEO spectrometer operating at 400 MHz. The probe used was a Bruker's Smart Probe.

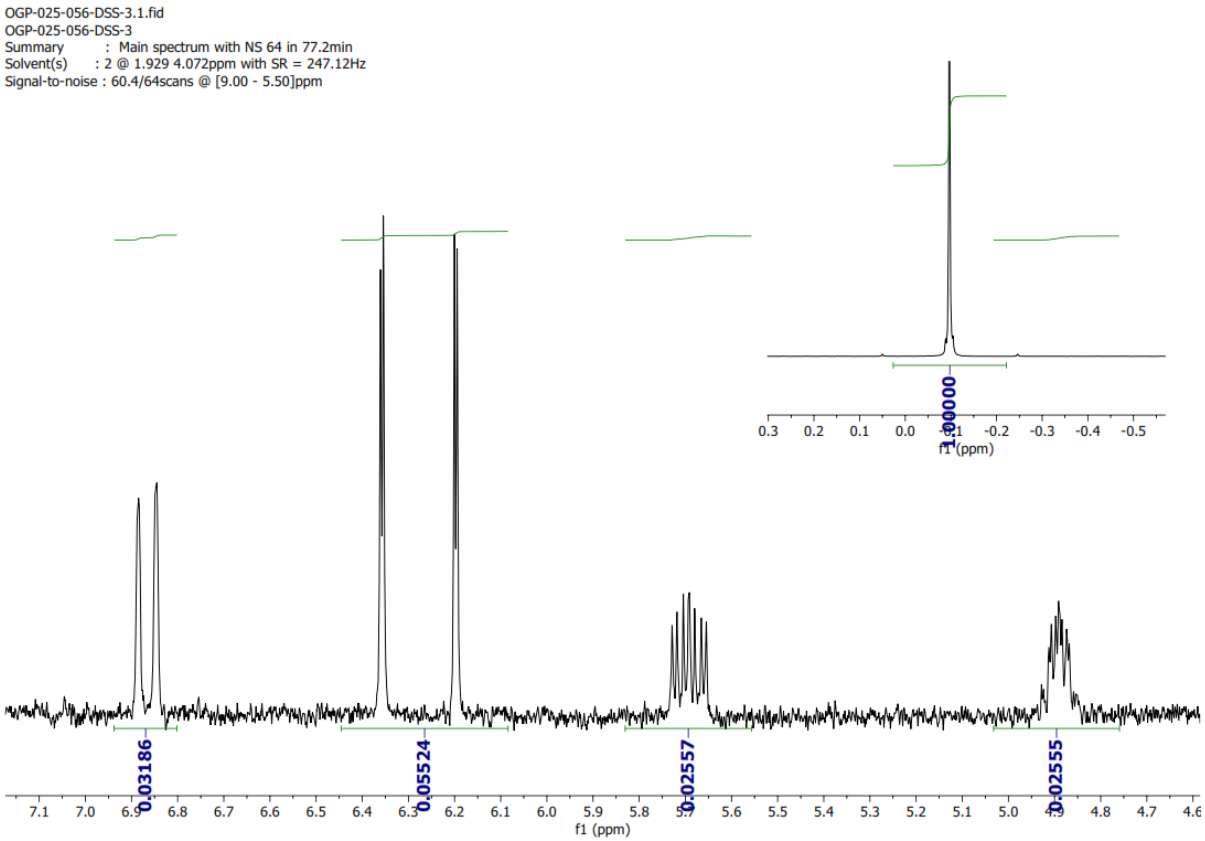

Figure 1. Representative ^{1}H-qNMR spectrum of ZEN

Table 1. Uncentainty budget of ZEN stock solution

Uncertainty Budget			
	Value (X)	**u(x)**	**u(x)/X**
Concentration of analyte (%)	0.01367	0.00029	0.020906
Concentration of IS solution (%)	0.09740	0.00010	0.001027
Molar mass of analyte	318.36428	0.00841	0.000026
Molar mass of IS	224.35874	0.00406	0.000018
Weigh of analyte (mg)	310.56000	0.00500	0.000016
Weigh of IS Solution (mg)	118.25100	0.00500	0.000042
			0.020932
Concentration of ZEN (%)	0.01367		
Combined uncertainty (%)	0.00029		
Expanded uncertainty (%)	0.00057		

All NMR spectra were processed with the software Mestrenova 12.0.3. An exponential line broadening window function of 0.5 Hz was used in the data processing. After Fourier

transformation of the free induction decays, the spectra were baseline corrected, phased, and integrated in the appropriate region. The peaks for the analyte and the internal standard were integrated inside, that is, excluding, the 13C satellites.

The calculation equation of qNMR for the concentration is as follows:

$$C_x = \frac{I_x}{I_{Std}} \frac{N_{Std}}{N_x} \frac{M_x}{M_{Std}} \frac{m_{Std}}{m_x} C_{Std}$$

I_{Std}, N_{Std}, M_{Std}, m_{Std} and C_{Std} are the peak area, number of proton, molecular weight, mass and concentration of the internal standard, respectively. I_x, N_x, M_x, m_x and P_x are the peak area, number of proton, molecular weight, mass and concentration of the sample, respectively.

The calculation equation of the relative standard uncertainty is as follows:

$$u(C_x) = C_x \sqrt{\left(\frac{u(I_x/I_{std})}{I_x/I_{std}}\right)^2 + \left(\frac{u(M_x)}{M_x}\right)^2 + \left(\frac{u(M_{std})}{M_{std}}\right)^2 + \left(\frac{u(m_x)}{m_x}\right)^2 + \left(\frac{u(m_{std})}{m_{std}}\right)^2 + \left(\frac{u(C_{std})}{C_{std}}\right)^2}$$

Table 2. Concentration result of ZEN Stock Solution

Chemical Name	**Concentration (µg/g)**	**Uncertainty (U) (µg/g)**
ZEN (Zearalenon)	136.7	5.7

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

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.025 standard ZEN solution provided by BIPM

BIPM OPG.025, 130.1 ± 2.2 µg/g

Amount of primary calibrator used for analysis

7.8296 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo XP 205 0.01 mg

Balance repeatability

100 µg

Solution preparation procedure

A 7.8296 ± 0.0001 g portion of the 130.1 ± 2.2 µg/g stock solution (about 10 mL at 20 ℃) was transferred to a 100 mL flask. The flask was diluted to the mark with acetonitrile and weighed to 77.9742 ± 0.0001 g (100 mL at 20 ℃). The solution with a final concentration of 13.06 µg/g ZEN in acetonitrile was obtained. A 4 mL volume of the solution was dispensed into amber glass ampoules under ice conditions. The ampoules were sealed with an ampoule sealer. A total of 25 ampoules was produced and stored in a freezer at -18 ℃.

Homogeneity and/or stability testing

5 ampoules of the 25 ampoules were randomly selected to test homogeneity of the ZEN solutions. UHPLC-DAD method was applied for homogeneity measurement. The result of the homogeneity testing was subject to an analysis of variance (ANOVA). The results from homogeneity testing of ZEN solutions are summarized in Table as follows. No stability testing.

ZEN in acetonitrile	
Mean	13.02 µg/g
SD	0.04 µg/g
N (m=5, n=3)	15
s_{wb}	0.19 %
s_{bb}	0.21 %
$u^{*}{}_{bb}$	0.07 %
u_{bb}	0.21 %

Optional: Analytical check method

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

UHPLC-DAD Aglient 1290 Infinity

Column:	Phenomenex Kinetex EVO C18 100 Å (250 x 4.6 mm, 2.6 µm)	
Detection wavelength:	274 nm (reference wavelength 360nm)	
Mobile phase:	A) actonitrile:H2O = 40:60 (v/v) + 0.1 % HCOOH	
	B) actonitrile (+ 0.1 % HCOOH)	
Gradient :	Time(min)	Mobile phase A
	0	100 %
	23	100 %
	24	10 %
	26	10 %
	27	100 %
	35	100 %
Flow rate:	1 mL/min	
Injection volume:	5 µL	

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

External calibration method was applied to assign the value of comparison sample. Based on the BIPM OGP.025 store solution, a series of calibrators were gravimetrically prepared.

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

BIPM OGP.025 130.1 ± 2.2 µg/g

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

Non.

Additional Comments or Observations

Non.

Uncertainty Budget				**Measurement equation:**	Y=aX+b
Source of uncertainty	Parameter x	Uncertainty			
		u(x)	u(x)/(x)		
Store solution	130,1	1,1	0,00846		
M_{ZEN} （mg）	7,8296	0,00052	0,000067		
$M_{ACN+ZEN}$ （mg）	77,9742	0,0187	0,000239		
Repeatability of testing method	13,02	0,078	0,006		
Inhomogeneity	13,02	0,027	0,0021		
Relative combined standard uncertainty (uc)			1,06%		
Relative expanded uncertainty (Uc) （*k*=2)			2,12%		
Uncertainty analysis results					
C_x	13,1	µg/g			
u_x	0,14	µg/g			
U_x (*k*=2)	0,3	µg/g			

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

<u>Solution preparation procedure</u>

Calibrator

Primary calibrator (e.g., source, purity, assignment method, establishment of traceability)
NOTE: For MMCBKT participants, the primary calibrator is the OGP.025 standard ZEN solution provided by BIPM

NIM has produced gravimetrically a stock solution of 128.7 ± 2.4 µg/g by dissolving 50.550 ± 0.001 mg crystalline ZEN (995.4 ± 4.0 mg/g) in 391.058 ± 0.001 g acetonitrile (500 mL at 20℃). The purity value of zearalenone material was assigned by NIM with mass balance method and qNMR method. The main component of ZEN material was assigned by qNMR method. HPLC-MS/MS and HPLC-DAD methods were applied to measure the content of organic impurities of ZEN material; the moisture and inorganic impurities were determined by TGA method. VOC content was detected by qNMR. Finally, a purity of 995.4 mg/g with a uncertainty ± 4.0 mg/g was attributed to the ZEN pure material.

Amount of primary calibrator used for analysis

19,3393 g

Gravimetry

Type of balance (make, model and resolution)

Mettler Toledo	XP205	0.01 mg
Mettler Toledo	XP10003S	0.001 g

Balance repeatability

100 µg	XP205
6 mg	XP10003S

Solution preparation procedure

A 19.3393 ± 0.0001 g portion of the 128.7 µg/g stock solution (about 25 mL at 20℃) was transferred to a 250 mL flask. The flask was diluted to the mark with acetonitrile and weighed to 195.697 ± 0.001 g (250 mL at 20 ℃). The solution with a final concentration of 12.72 µg/g ZEN in acetonitrile was obtained. A 4 mL volume of the solution was dispensed into amber glass ampules under ice conditions. The ampules were sealed with an ampule sealer. A total of 60 ampules was produced and stored in a freezer at -18 ℃ .

Homogeneity and/or stability testing

7 ampules of the 60 ampules were randomly selected to test homogeneity of the ZEN solutions. UHPLC-DAD method was applied for homogeneity measurement. The result of the homogeneity testing was subject to an analysis of variance (ANOVA). The results from homogeneity testing of the ZEN solutions were summarized in the Table as follows. No stability testing.

ZEN in acetonitrile	
Mean	12.63 µg/g
SD	0.08 µg/g
N (m=7, n=3)	21
s_{wb}	0.12 %
s_{bb}	0.47 %
u^{*}_{bb}	0.04 %
u_{bb}	0.47 %

Optional: Analytical check method

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

UHPLC-DAD Aglient 1290 Infinity

Column:	Phenomenex Kinetex EVO C18 100 Å (250 x 4.6 mm, 2.6 µm)	
Detection wavelength:	274 nm (reference wavelength 360nm)	
Mobile phase:	A) actonitrile:H2O = 40:60 (v/v) + 0.1 % HCOOH	
	B) actonitrile (+ 0.1 % HCOOH)	
Gradient :	Time(min)	Mobile phase A
	0	100 %
	23	100 %
	24	10 %
	26	10 %
	27	100 %
	35	100 %
Flow rate:	1 mL/min	
Injection volume:	5 µL	

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

External calibration method was applied to assign the value of comparison sample. Based on the NIM store solution (128.7 ± 2.4 µg/g), a series of calibrators were gravimetrically prepared.

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

NIM store solution 128.7 ± 2.4 µg/g

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

Non.

Additional Comments or Observations

Non.

Uncertainty Budget				**Measurement equation:**	Y=aX+b
Source of uncertainty	Parameter x	Uncertainty			
		u(x)	u(x)/(x)		
Store solution	128,7	1,2	0,00933		
M_{ZEN} （mg）	19,3393	0,00105	0,000054		
$M_{ACN+ZEN}$ （mg）	195,697	0,02101	0,000107		
Repeatability of testing method	12,63	0,0758	0,006		
Inhomogeneity	12,63	0,0594	0,0047		
Relative combined standard uncertainty (uc)			1,20%		
Relative expanded uncertainty (Uc) （*k*=2)			2,40%		
Uncertainty analysis results					
C_x	12,7	µg/g			
u_x	0,15	µg/g			
U_x (*k*=2)	0,3	µg/g			