CCQM-K146.1 Subsequent Comparison

Low-Polarity Analyte in high fat food: Benzo[a]pyrene in Olive Oil

Draft B Report

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ACRONYMS

CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	Calibration and Measurement Capability
CRM	certified reference material
CV	coefficient of variation, expressed in %: CV = 100·s/$\bar{x}$
DI	designated institute
DoE	degrees of equivalence
GCxGC-TOF MS	Comprehensive gas chromatography with time-of-flight mass spectrometry detection
GC-MS/MS	gas chromatography with tandem mass spectrometry detection
GC-TOFMS	gas chromatography with time-of-flight mass spectrometry detection
HPLC-FLD	high pressure liquid chromatography with fluorescence detection
IDMS	isotope dilution mass spectrometry
KC	Key Comparison
KCRV	Key Comparison Reference Value
MADe	median absolute deviation from the median (MAD)-based estimate of s: MADe = 1.4826·MAD, where MAD = median($\lvert x_i$-median(x_i)$\rvert$)
MRM	multiple reaction monitoring
NMI	national metrology institute
NMR	nuclear magnetic resonance spectroscopy
OAWG	Organic Analysis Working Group
qNMR	quantitative nuclear magnetic resonance spectroscopy
SPE	solid phase extraction

SYMBOLS

d_i	degree of equivalence: x_i - KCRV
$\%d_i$	percent relative degree of equivalence: $100 \cdot d_i/\text{KCRV}$
k	coverage factor: $U(x) = k \cdot u(x)$
n	number of quantity values in a series of quantity values
s	standard deviation of a series of quantity values: $s = \sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2/(n-1)}$
$u(x_i)$	standard uncertainty of quantity value x_i
$\bar{u}(x)$	pooled uncertainty: $\bar{u}(x) = \sqrt{\sum_{i=1}^{n} u^2(x_i)/n}$
$U(x)$	expanded uncertainty
$U_{95}(x)$	expanded uncertainty defined such that $x \pm U_{95}(x)$ is asserted to include the true value of the quantity with an approximate 95 % level of confidence
$U_{k=2}(x)$	expanded uncertainty defined as $U_{k=2}(x) = 2 \cdot u(x)$
x	a quantity value
x_i	the i^{th} member of a series of quantity values
$\bar{x}$	mean of a series of quantity values: $\bar{x} = \sum_{i=1}^{n} x_i/n$

Background

In 2017, the CCQM conducted a key comparison (CCQM-K146) for the determination of benzo[a]pyrene in edible oil with participation of 15 NMIs. Some labs who do not perform well request a subsequent bilateral comparison. So, CCQM organized such a study for those NMIs or DIs had not participated in K146 or wished to improve their results from K146. As NIM had coordinated K146 and results also agree well, it served as the anchor point to which the other participants in the subsequent study could be compared.

Summary of CCQM K146

A Track A key comparison on the determination of benzo[a]pyrene in edible oil organized by the CCQM was carried out in 2017. The demonstration of competency and equivalence for the assessment of levels of contaminants and nutrients in primary foodstuffs is a priority within the 10-year strategy for the OAWG Track A core comparisons. The measurements are core challenges for reference material producers and providers of calibration services. This key comparison related to low polarity analytes in a high fat, low protein, low carbohydrate food matrix and Benzo[a]pyrene in edible oil was the model system selected to align with this class within the OAWG strategy. Evidence of successful participation in formal, relevant international comparisons is needed to document measurement capability claims (CMCs) made by national metrology institutes (NMIs) and designated institutes (DIs).

Participants in K146 included 16 participant: Bundesanstalt für Materialforschung und -prüfung (BAM) [Germany], Federal Office of Consumer Protection and Food Safety (BVL) [Germany], Designated Reference Institute for Chemical Measurements (Bangladesh) [DRiCM], General State Chemical Laboratory (EXHM/GCSL)[Greece], Government Laboratory, Hong Kong Special Administrative Region (GLHK)[Hong Kong, China], Health Sciences Authority (HSA) [Singapore], Organic Analysis Laboratory (INMETRO) [Brazil], Korea Research Institute of Standards and Science (KRISS) [Rep of Korea], Laboratory of the Institute for Oliveculture [Slovenia], Laboratory of the Government Chemist (LGC) [UK], National Institute of Metrology, (NIM) [China], National Institute of Metrology, Thailand (NIMT) [Thailand], National Institute of Standards and Technology (NIST)[USA], National Metrology Institute of Japan (NMIJ)[Japan], National Metrology Institute of Turkey (UME) [Turkey], D.I. Mendeleyev Research Institute for Metrology (VNIIM) [Russia]. Two olive oil samples were sent to each of the participants for the determination of benzo[a]pyrene with HPLC-FLD, HPLC-MS/MS, and GC-MS or GC-MS/MS methods. Agreement of results among the participants was generally good, with a few exceptions. Based upon these results, it is clear that experience with this measurement is critical to the achievement of high accuracy and precision.

In conclusion, Successful participation in CCQM-K146 demonstrates the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 100 g/mol to 500 g/mol, having low polarity pKow < -2, in mass fraction range from 0.1 µg/kg to 1000 µg/kg in a in a high fat, low protein, low carbohydrate food matrix.

Conduct of Subsequent Comparison CCQM-K146.1

Timeline

Table 1: Timeline for CCQM-K146.1

Date	Action
March 2019	EXHM propose a bilateral comparison
April 2019	OAWG authorized CCQM-K146.1 as a subsequent CCQM-K146 Comparison; protocol approved
May 2019	Call for participation to OAWG members
May 2019	Study samples shipped to participants.
August 2019	Results submit to coordinating laboratory
September 2019	Draft A report distributed to OAWG
November 2020	Draft B report distributed to OAWG

Measurand

CCQM-K146.1 relates to "Low-Polarity Analyte in high fat food: Benzo[a]pyrene in Olive Oil". The measurand was the mass fraction of BaP in the olive oil material in µg/kg. Information on CAS number, molecular formula, molecular mass, polarity and chemical structure are shown in Table 2. Each participating NMI/DI was provided with two vials of comparison materials.

Table 2: Information of BaP

Benzo(a)pyrene	
CAS	50-32-8
Molecular formula	$C_{20}H_{12}$
M_W	252.31

pK_{ow}	-6.35
Structure	

Participants

5 National Metrology Institutions participated in the subsequent Comparison CCQM-K146.1.

Table 3 Information on participating NMIs/DIs, contacts and sample receipt

No.	Institute	Country	Contact Person	Result submission
1	EXHM/GCSL-EIM	Greece	Dr Elias Kakoulides	2019.07.30
2	NIM	China	Xiaomin Li	2019.07.30
3	NMISA	South Africa	Laura Quinn	2019.08.05
4	Laboratory of the Institute for Oliveculture, Science and Research Centre Koper	Slovenia	Ms Milena Bucar-Miklavcic	2019.07.28
5	TUBITAK UME	Turkey	Dr. Taner Gokcen	2019.08.07

Results

Calibration Materials

The details of the source and purity assessment of the calibration materials used by each participant are provided in Table 4.

Table 4: Calibration materials used by participants

NMI/DI	Source(s)	Purities/Concentration and Uncertainties (95% CI)	Purity techniques	Evidence of Competence
EXHM	BaP (Sigma-Aldrich and NIST SRM 2260a)	60.48 ng/g ± 0,82 ng/g(k=2), (0.68%)	in-house calibrant prepared by BaP provided by Sigma-Aldrich purity checked by qNMR (971.0 mg/g ± 3.1 mg/g) 60.48 ng/g ± 0,82 ng/g verified by IDMS against NIST SRM 2260a	CCQM-K55 series
NIM	Benzo(a)pyrene GBW(E)080476	4.52µg/mL U=2.8%(k=2), 1.4%	The certified value of pure benzo[a]pyrene was determined using mass balance approach.	The capability is underpinned by participating in CCQM-K131
NMISA	NIST SRM 1647f and SRM 2260a	6.22±0.11 mg/kg (k=2), 0.88%; 4.71±0.17 mg/kg (k=2) 1.8%		N/A
Oliveculture/ Slovenia	Supelco EPA 610-N PAH Kit, cat. No. 47351 (lot LC26415V		pure compound, Supelco EPA 610-N PAH Kit, cat. No. 47351 (lot LC26415V), purity assigned by qNMR, performed by LGC	N/A
UME	NIST SRM 1647f	6.22±0.11 mg/kg (k=2)		N/A

Table 5 Information on Internal standards

NMI/DI	Source(s)	Chemical (CP) and Isotopic Purities (IP)
EXHM	benzo[a]pyrene-d12, Chem Service	N/A
NIM	D12-Benzo(a)pyrene (Dr.Ehrenstorfer)	CP:99%, IP: 98.5%
NMISA	NIST SRM 2270 Perdeuterated PAH-II solution in hexane/ toluene	N/A
Oliveculture/Slovenia	Benzo(b)chrisene	N/A
UME	(Benzo[a]pyrene-d12) from NIST SRM 2270	N/A

NIM, NMISA, UME utilized the existing pure or solution CRMs as the calibrating materials. EXHM was opting to make the value assignments of the commercial BaP materials based on qNMR approaches (Table 4), their capability is underpinned by participating in CCQM-K55 series. For institute of Oliveculture/Slovenia, purity of commercial BaP materials was assigned by qNMR, performed by LGC. The standard relative uncertainty of the pure reference material is 1.79%, and that of standard solutions are from 0.88% to 1.8%.

Table 6 Standard uncertainties of the calibrants

	Standard uncertainty of calibrants	
NMI	Purity calibrants	Solution calibrants
EXHM	/	1.8%
NIM	/	1.4%
NMISA	/	0.88% SRM 1647f 1.8% SRM 2260a
Oliveculture	1.79%	/
UME	/	0.88%

Internal standards used by participants are listed in Table 5, three labs used isotopically labelled internal standards from a variety of sources, one utilized benzo(b)chrisene to check the recovery.

Methods Used by Participants

A summary of the sample intake, pre-treatment, clean-up and IS spiking and equilibration times are given in Tables 7 & 8 with full details in Appendix E-1.

Acetone/acetonitrile has been applied in the extraction procedure by EXHM and NMISA. NIM adopted saponification before the extraction. Laboratory of the Institute for Oliveculture and UME did not have any extraction process, they used SPE directly for the pre-treatment. C18 cartridges, florisil cartridges, amino cartridges, Z-Sep/C18, EZ-POP NP, LC-NH2, SUPELCLEAN LC-SI, were the main choice for the clean-up process.

Laboratory of the Institute for Oliveculture used HPLC-FLD technique, NMISA used GC-TOFMS and GCxGC-TOF MS, and NIM, EXHM and UME used GC-MS/MS. Details are given in Appendix E-2.

Laboratory of the Institute for Oliveculture used external calibration method, and the others used IDMS methods. Details in are given in Appendix E-3.

The participants' approaches to estimating uncertainty are provided in Appendix F.

NMISA use two methods for the detection and calibration, and the results were using the combined values of the different methods.

Table 7 Summary of sample pre-treatment

NMI/DI	Sample (g)	extraction solvent	extraction time total, temperature	repeat	others
EXHM	2.5	acetone/acetonitrile 40/60,10mL	N/A	three times	N/A
NIM	0.5	1 mol/L KOH ethanol-water solution (90:10, v/v) water+ n-hexane	at 75 ℃, 2H, vortex 2 min, Centrifuged 3min	twice	N_2 flow, 40 °C, reconstituted with cyclohexane

NMISA	1	10 mL acetonitrile/ acetone mixture (6:4)	at room temperature in an ultrasonic bath for 15 min	twice	The supernatant was evaporated using a centrifugal evaporation system at room temperature to final volume of 2 mL
Oliveculture	0.200188	no extraction	N/A	N/A	N/A
UME	0.5	no extraction	N/A	N/A	N/A

Table 8 Summary of clean-up methods and internal standard spiking

NMI/DI	SPE	Recon. time, temperature	IS Spikes Before or after reconstitution	IS, Equilibration, time, temp	Final Solvent
EXHM	a - C18 cartridges b - florisil cartridges c - amino cartridges	N/A	Before	equilibrated overnight	N/A
NIM	LC-Florisil® (upper layer) and Z-Sep/C18 (lower layer)	N_2 flow 40 °C	before	12h	0.2mL of isopropanol
NMISA	EZ-POP NP (SupelcleanTM,12 mL). Followed by a LC-NH2 (SupelcleanTM,6 mL)	EZ-POP NP using acetonitrile for elution; LC-NH2 using a mixture of hexane and toluene (7:3)	Before	equilibrate for 30 min on an orbital shaker at room temperature	Acetonitrile
Oliveculture	SUPELCLEAN LC-SI	eluted with hexane/dichloromethane (70:30 v/v)	Before	N/A	N/A
UME	SUPELCO Supelclean EZ-POP NP	elute with 15 mL of acetonitrile	Before	one hour	N/A

Participants' results

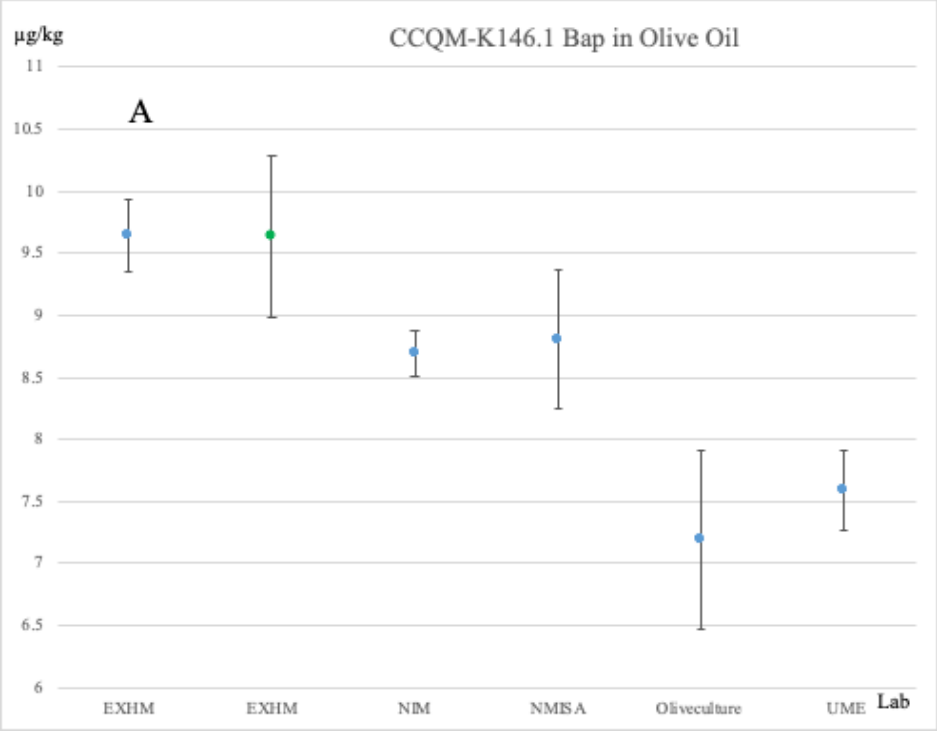
The results for CCQM-K146.1 for the determination of BaP are detailed in Table 9 and presented graphically in Figure 1.

Table 9: Reported Results for BaP (CCQM K146.1) (µg/kg)

NMI/DI	x	$u(x)$	k	$U(x)$
Greece (EXHM)	9.64	0.29	2	0.58
Greece (EXHM)	9.64	0.65*	2	1.29*
China (NIM)	8.69	0.18	2	0.36
South Africa (NMISA)	8.8	0.56	2	1.1
Slovenia (Lab of the Institute for Oliveculture)	7.19	0.72	2	1.44
Turkey (UME)	7.59	0.32	2	0.64
n	5			
$\bar{x}$	8.38	Median	8.69	
s	0.99	MADe	1.41	
CV	12%			

n = number of results included in summary statistics; $\bar{x}$ = mean; s = standard deviation; CV = $100 \cdot s/\bar{x}$

*Green represents the revised value reported by EXHM after further analysis



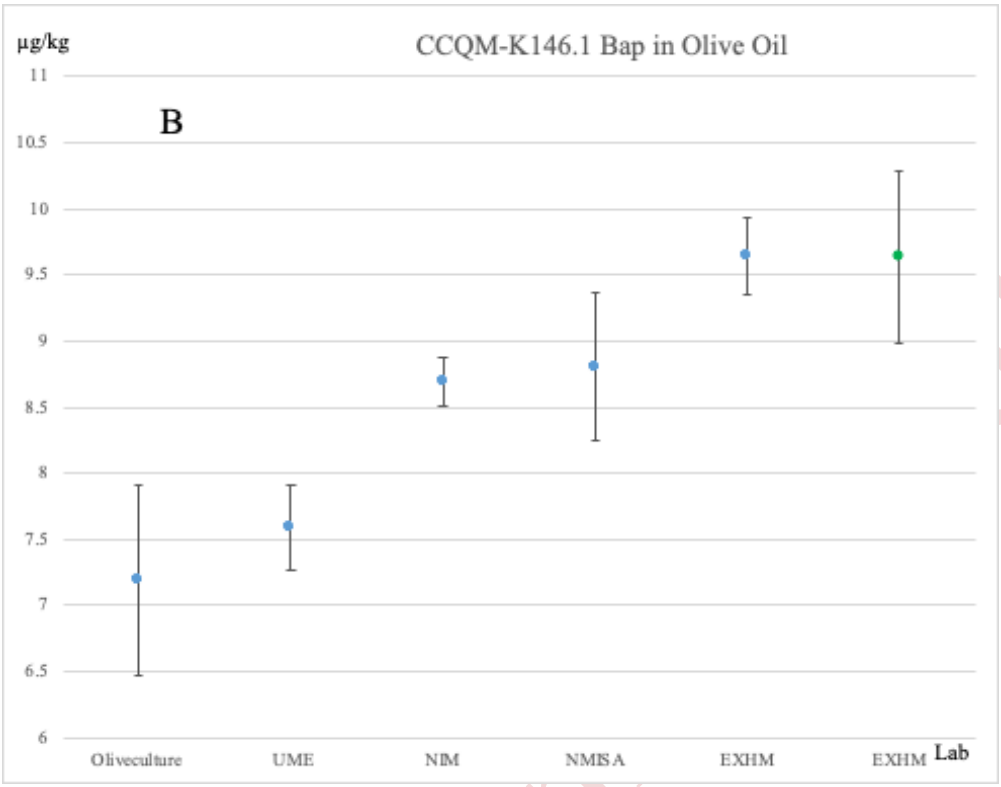


Figure 1: Dot-and-Bar Display of Reported Results for BaP, µg/kg

Panels A and B display the reported results for the measurand; panel A displays the results sorted alphabetically by NMI acronym; panel B displays results sorted by increasing reported value. Dots represent the reported values, *x*; bars represent their standard uncertainties, *u*(*x*). The thin horizontal gridlines are provided for visual guidance. *Green dot represents the revised value reported by EXHM after further analysis

Key Comparison Reference Values (KCRV)

As agreed in the OAWG meetings in April 2019, the KCRV and *u*(KCRV) will be the result reported by NIM. This result is a justifiable anchor for CCQM-K146.1 because NIM was a participant of K146 that produced a result equivalent with the CCQM-K146 KCRV and demonstrated acceptable performance in determining the respective measurand. The u(KCRV) is an evidence-based inference of the certainty in the central NIM value used as an anchor for CCQM-K146.1. Table 10 lists the KCRV value, standard uncertainty, $u_{(x)}$, and 95% expanded uncertainty, $U_{95}(x)$

Table 10: Candidate Key Comparison Reference Value for Measurand BaP in Olive Oil (µg/kg)

KCRV	$u_{(x)}$	$U_{95}(x)$
8.69	0.18	0.36

The results from NMISA agreed with the NIM result, while the result of EXHM was lower, and results from Lab of the Institute for Oliveculture, and UME were biased high. The uncertainties for the results from the subsequent study for 4 participants were calculated by taking into account the relative standard uncertainties in the KCRV values, the NIM results from K146, the NIM results from the subsequent study, the participant's results from the subsequent study, and the relative standard deviations in the differences between NIM and the KCRVs and between NIM and the participant.

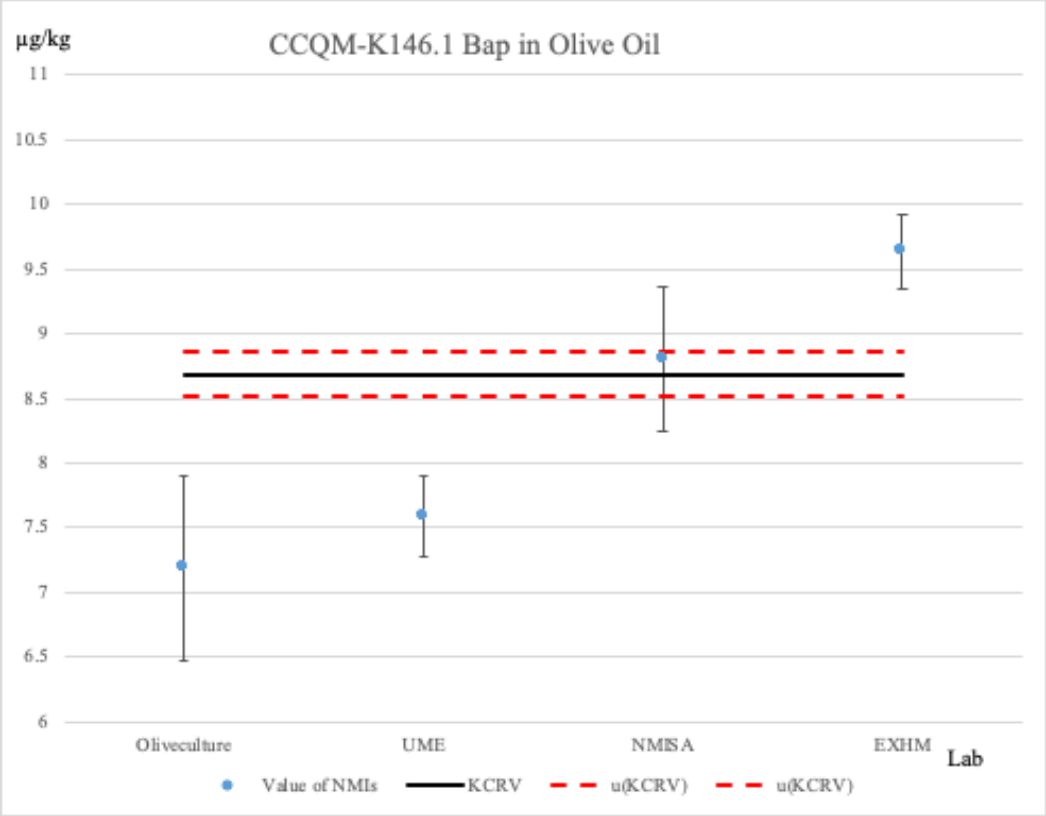


Figure 2: Key Comparison Reference Values for BaP in olive oil

Figure 2 display the KCRV for BaP. In all panels, the results are sorted by increasing reported value. Dots represent the reported mean values, x; bars represent their standard uncertainties, $u(x)$. The black horizontal line denotes the candidate KCRV. The bracketing red lines denote the standard uncertainty of the candidate KCRV.

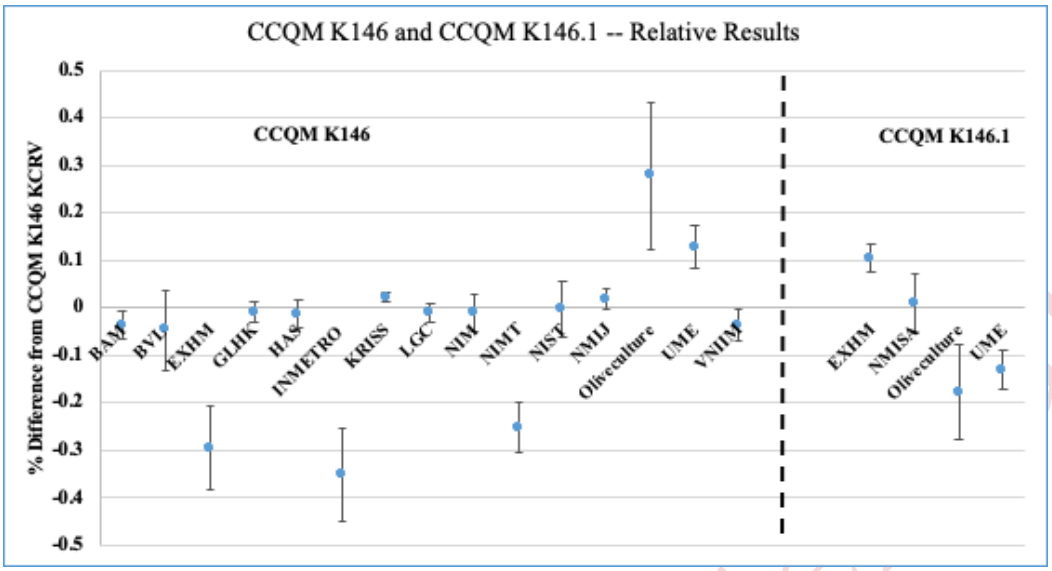


Figure 3. Extrapolated percentage differences of mean results from the subsequent study plotted with percentage differences from the KCRV values.

Discussion of Results

EXHM, Lab of Oliveculture and UME participated in both CCQM-K146 and CCQM K146.1, while NMISA only participated in CCQM-K146.1. It is clearly shown in Figures 2 and 3 that EXHM, Lab of Oliveculture and UME showed different trends of deviation in CCQM-K146 and CCQM-K146.1. Results that were above the KCRV in CCQM-K146 were below the KCRV in CCQM-K146.1 and vice versa. This seems to indicate that the laboratories had over corrected for the observed biases in CCQM-K146 during their measurement for CCQM-K146.1. The result of NMISA in CCQM-K146.1 is in good agreement with the KCRV of CCQM-K146.1.

After the discussion in the OAWG meeting in October 2019， EXHM carried out further investigation into why their results appeared biased from the consensus.

EXHM used both comparison samples from CCQM-K146 & CCQM-K146.1 as candidates to carry out their experiments. They repeated the BaP measurements on the sample supplied for CCQM-K146 and determined 2.71 ± 0.25 µg/kg , a value in accordance with the KCRV of the initial comparison (2.74 µg/kg). This value is 3.55 times lower than the value reported by EXHM for CCQM-K146.1 (9.64 ± 0.58 µg/kg). EXHM employed a sample mass of 2.5 g to perform the analysis in both comparisons. During the measurements for CCQM-K146.1, and in order to verify the validity of EXHM's determinations, they also used a mass 3.55 times less than 2.5 g (i.e. 0.7050 g) of the CCQM-K146.1 material, in order to analyze a sample amount that would potentially contain the same BaP mass found in 2.5 g of the CCQM-K146 material. From this approach EXHM obtained a value of 2.73 ± 0.24 µg/kg (comparable to the CCQM-K146 KCRV). The determination was performed in triplicate.

EXHM also performed triplicate measurements on 2.5 g of a 1:1 mixture of the CCQM-K146 (1.25 g) and CCQM-K146.1 (1.25 g) materials. This gave a result of 6.20 ± 0.37 µg/kg (theoretical value 6.18 µg/kg). By applying standard additions [3 levels: (2.5 g CCQM-146 + 0.0 g CCQM-146.1), (1.25 g CCQM-146 + 1.25 g CCQM-146.1) & (0.0 g CCQM-146 + 2.5 g CCQM 146-1)] EXHM determined a value of 9.65 ng/g for the CCQM-K146.1 material with a linear correlation coefficient r^2~1, which corroborated their result.

NIM also carried out the measurement on 2.5 g of a 1:1 mixture of 1.25 g CCQM-146 and 1.25 g CCQM-146.1. The 1:1 mixture gave a value of 5.65 ± 0.28 µg/kg (theoretical value 5.74 µg/kg). Based on these 3 levels, 2.5 g CCQM-146 (value 2.71 µg/kg), 1.25 g CCQM-146 + 1.25 g CCQM-146.1 (value 5.65 µg/kg) & 2.5 g CCQM 146-1 (value 8.69 µg/kg). The three samples gave a linear correlation coefficient R^2~0.9999.

The linearity results of EXHM and NIM are shown in Figure 4. Each data point was calculated by single-point IDMS. The slope and intercept of EXHM are 3.47 and -0.7533, those of NIM are 2.99 and -0.2933. It reveals that the results of single-point quantification will be affected by the slope and intercept of the linearity.

EXHM measured 0.7050 g of the CCQM-K146.1 material and obtained a value of 2.73 ± 0.24 ng/g. Different sample size of pretreatment and recovery of different levels may affect the result of 0.7050 g for CCQM-K146.1 and 2.5 g of CCQM-K146. However, this difference is unlikely to fully explain the large differences in the initial and revised EXHM results.

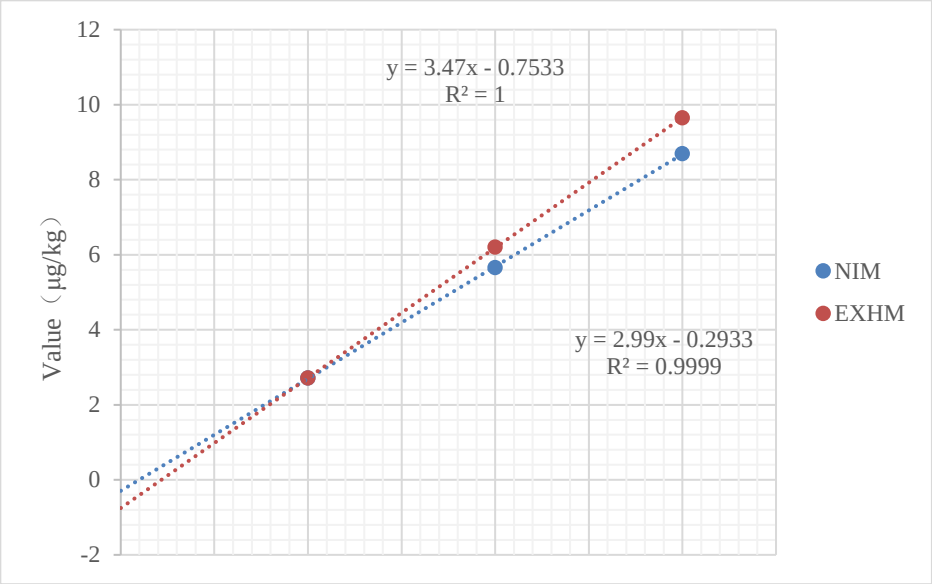


Figure 4. The results of EXHM and NIM

After the initial discussion of the results, EXHM reported that the uncertainty of their measurement had been underestimated, since the mean square of the repeatability measurements was erroneously divided by $\sqrt{18}$ instead of $\sqrt{6}$, which was the number of the independent experiments performed. They also noted that the difference observed between EXHM's value and the CCQM-K146.1 candidate KCRV may be potentially attributed to enhanced extraction efficiency of BaP from the olive oil matrix, owing to the extraction procedure they employed. This can be incorporated in the uncertainty budget of the determination, by treating the enhanced extraction as an uncertainty component for recovery with a rectangular distribution amounting to 6.35% RSU (11% higher relative recovery divided by square root of 3). Taking these into account, a new uncertainty budget was provided as given in Figure 5.

uncertainty component	value	sensitivity coefficient	standrard uncertainty	relative uncertainty	C_i x u_i	$(C_i \times u_i)^2$
method precision	9,64	1,000	0,20	0,0208	0,2000	0,0400
mass fraction of B[a]P in the calibration solution, (*µg/kg*)	60,48	0,159	0,41	0,0068	0,0655	0,0043
recovery (%)	100,00	-0,096	6,35	0,0635	-0,6120	0,3745
mass of B[a]P-d_{12} solution added to sample blend, (*g*)	0,35618	27,058	0,00007	0,0002	0,0019	0,0000
mass of test material in sample blend, (*g*)	2,48720	-3,875	0,00009	0,0000	-0,0003	0,0000
mass of B[a]P solution added to calibration blend, (*g*)	0,10023	96,152	0,00003	0,0003	0,0029	0,0000
mass of B[a]P-d_{12} solution added to calibration blend, (*g*)	0,10020	-96,181	0,00003	0,0003	-0,0029	0,0000
measured peak area ratio of the selected ions in the sample blend	1,430	6,740	considered to be included in the			
measured peak area ratio of the selected ions in the calibration blend	1,285	-7,497	estimation of method precision			

result (ng/g)	9,64
combined standard uncertainty (ng/g)	0,65
relative standard uncertainty (%)	6,72
effective degrees of freedom	869
coverage factor	2,00
expanded uncertainty (ng/g)	1,29

Figure 5 Revised uncertainty evaluation of EXHM

Degrees of Equivalence (DoE)

The absolute degrees of equivalence for the participants in CCQM-K146.1 are estimated as the signed difference between the reported result and the KCRV: $D_i = x_i - X_{KCRV}$.

The study Coordinators may refer to CCQM/13-22 Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence, 11-Apr-2013 [1].

The nominal k=2 expanded uncertainty on the d_i, $U_{k=2}(d_i)$, is estimated according to the equation below:

$$U_{k=2}(D_i) = 2\sqrt{u^2(x_i) + u^2(\text{KCRV}) \mp \tau^2_{K146}},$$

Where the value of τ (i.e., "dark uncertainty") relative to X_{KCRV} is 1.44 %, the same as that calculated for the K146 BaP measurand, and tau of K146 normalized to K146.1 measurand level (0.0144*8.69). Preservation of this relative between-lab variability in the K146.1 $u(D_i)$ sustains a consistent expectation of laboratory performance, with respect to the X_{KCRV}, for K146 and K146.1 BaP measurands. To enable comparison with the degrees of equivalence estimates from other studies, it is convenient to express the d_i and $U_{k=2}(d_i)$ as percentages relative to the KCRV: $\%d_i = 100 \cdot d_i/\text{KCRV}$ and $U_{k=2}(\%d_i) = 100 \cdot U_{k=2}(d_i)/\text{KCRV}$.

Table 12 below lists the numeric values of d_i, $U_{95}(d_i)$, $\%d_i$, and $U_{95}(\%d_i)$ for all participants in CCQM-K146.1 for BaP.

Figure 6 Graphically illustrates both the absolute and relative DoEs for BaP.

Table 11: Degrees of Equivalence for BaP

NMI/DI	D µg/kg	$U_{k=2}(D)$	$\%D$	$U_{k=2}(\%D)$
EXHM	0.95	0.73	10.9	8.4
NMISA	0.11	1.20	1.3	13.8
Oliveculture	-1.50	1.51	-17.3	17.0
UME	-1.1	0.78	-12.7	8.3

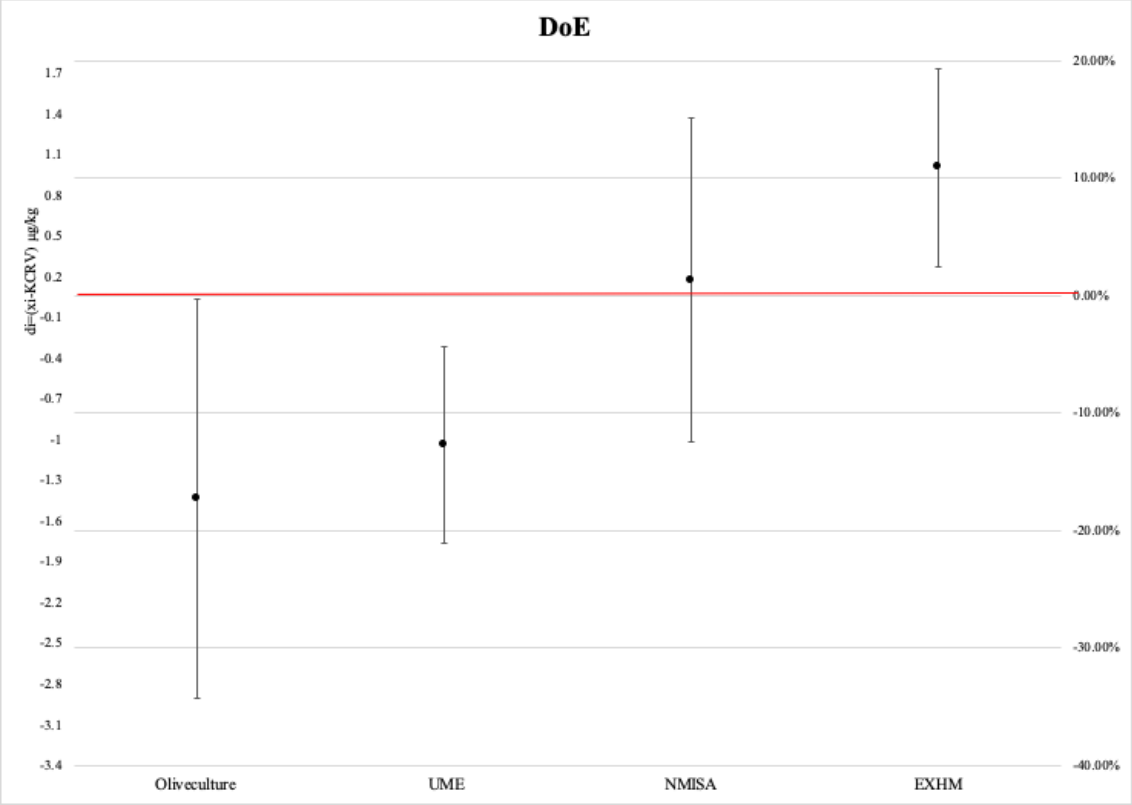


Figure 6: Degrees of Equivalence Associated with the Candidate KCRV

Figure displays the DoE for BaP. All results are sorted by increasing x. The axis to the left edge of each panel displays the absolute DoE, d, in units µg/kg. The axis to the right edge of each graph displays the relative DoE, $100 \cdot d/\text{KCRV}$, as percent. Dots represent the d, bars their approximate 95 % expanded uncertainties, $U_{95}(d)$. The thick red horizontal line denotes perfect agreement with the candidate KCRV.

USE OF CCQM-K146.1 IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K146.1 demonstrates the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 100 g/mol to 500 g/mol, having low polarity pKow < -2, in mass fraction range from 0.1 to 1000 µg/kg in a high fat, low protein, low carbohydrate food matrix.

Table 12: How Far the Light Shines

Matrix	Molecular mass (g/mol)	Analyte Polarity	Analyte Mass fraction (µg/kg)
> 60% fat* [AOAC Food Triangle Categories 1,3]	<500	pKow < -2	0.1 to 1000

This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) cleanup and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC/MS, GC-HRMS, HPLC-FLD or LC-MS.

Core Competency Statements and CMC support

Appendix G-1 to G-5 list the Core Competencies claimed by the participants in CCQM-K146.1. The information in these Tables is as provided by the participants. For any participant where their result did not agree with the KCRV and their DoE value did not cross the zero value the core competency table is flagged with a red comment at the bottom.

CONCLUSIONS

Participants in CCQM-K146.1 demonstrated their ability to identify and quantify low-polarity analytes (pKow < -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 1000 µg/kg in a high fat, low protein, low carbohydrate food matrix. NMISA demonstrated their capabilities for this analysis, whereas EXHM, UME and Institute of Oliveculture were all deviated from the KCRV. With the increased uncertainty reported by EXHM after the results were known their result did then have a DoE value that crossed zero.

Acknowledgements

The study coordinators thank the participating laboratories for providing the requested information used in this study.

References

1 CCQM/13-22 Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence, 11-Apr-2013

APPENDIX A: Protocol

CCQM-K146.1 Subsequent comparison

Low-Polarity Analyte in high fat food: Benzo[a]pyrene in Olive Oil

Key Comparison

Track A Repeat

Coordinating Laboratory: NIM

Comparison Protocol

17 May 2019

Introduction

Testing of the core competencies of laboratories that deliver measurement services of low polarity analytes and low molecule weight in high fat, low protein, low carbohydrate food material is an important area. Agreement was received in the October 2016 meeting of the OAWG to conduct the Benzo[a]pyrene (BaP) in Olive oil as a Track A matrix comparison, i.e. CCQM-K146. This comparison fitted into the 10-year strategy for the OAWG Track A comparisons, which covers a range of different types of food matrices which map against the different types of capabilities needed.

BaP is a category of polycyclic aromatic hydrocarbons (PAH) that is toxic and carcinogenic to humans. It also has some mutagenic properties as described by the World Health Organization, which make its presence in foods a health concern. BaP is one of the markers for the occurrence of PAHs in foods, for which maximum residue limits are enforced in many countries. Edible oil and fats are the main source of human PAH intake. BaP may form in edible oils by pyrolytic processes, such as incomplete combustion of organic substances. Worldwide regulatory limits of BaP in edible fats and oils are from 2.0 μg/kg to 10 μg/kg.

A subsequent comparison to CCQM-K146 will be offered to those NMIs or DIs who did not get the chance to participate in this comparison or who would like to repeat this measurement challenge.

Study Material

The matrix, olive oil, is a high fat and low protein, low carbohydrate matrix that falls within Sector 1 of the AOAC International food triangle. Olive oil was purchased from a local supermarket, spiked with BaP, and homogenized by vibro-mixing at room temperature for 24 hours. The indicative range for the mass fractions of the analyte is 0.1-100 μg/kg. Benzo(a)anthracene, benzo(b)fluoranthene, and chrysene are also spiked as interferences. The homogenized oil was separately dispensed into aluminum bottles under nitrogen atmosphere to give about 500 bottles, with content of 30 g each. Packing was in vacuum-sealed aluminum foil bags. Long term storage of the material at NIM is at about 25ºC.

Measurand

The measurand of this study is the same as for CCQM-K146, benzo(a)pyrene in olive oil.

Table 1 Information of BaP

Benzo(a)pyrene	
CAS	50-32-8
Molecular formula	$C_{20}H_{12}$
M_W	252.31
pK_{ow}	-6.35
Structure	

Methods

The study will require extraction, clean-up, analytical separation, and selective detection of the analyte in olive oil. Participants are anticipated to perform measurements by isotope-dilution gas chromatography-mass spectrometry (IDGC-MS); however, other techniques such as liquid chromatography (LC) may be used.

Homogeneity

All samples were kept at the storage condition of 25ºC by NIM. 15 bottles of samples were taken randomly, and analysis of triplicate sub-samples was carried out using an IDGC-MS/MS method while the absolute values were transformed relative to the mean. Results are shown in Figure 1.

The results of the homogeneity assessment indicated that the coefficient of variation (CV) was about 1.5% for the target BaP. One-way ANOVA with *F*-test in accordance with the requirements as stipulated in ISO Guide 35 was used to test whether there were significant between-packet differences in the concentration of the measurand (Table 2). The estimated between-packet standard deviations proved to be larger than within group standard deviations. The value of the relevant *F*-test ratios, *F*, is small, and *P*-value is larger than the usual critical 0.05 confidence level, which indicates that the inhomogeneity of the study material was insignificant.

Figure 1 Homogeneity of BaP

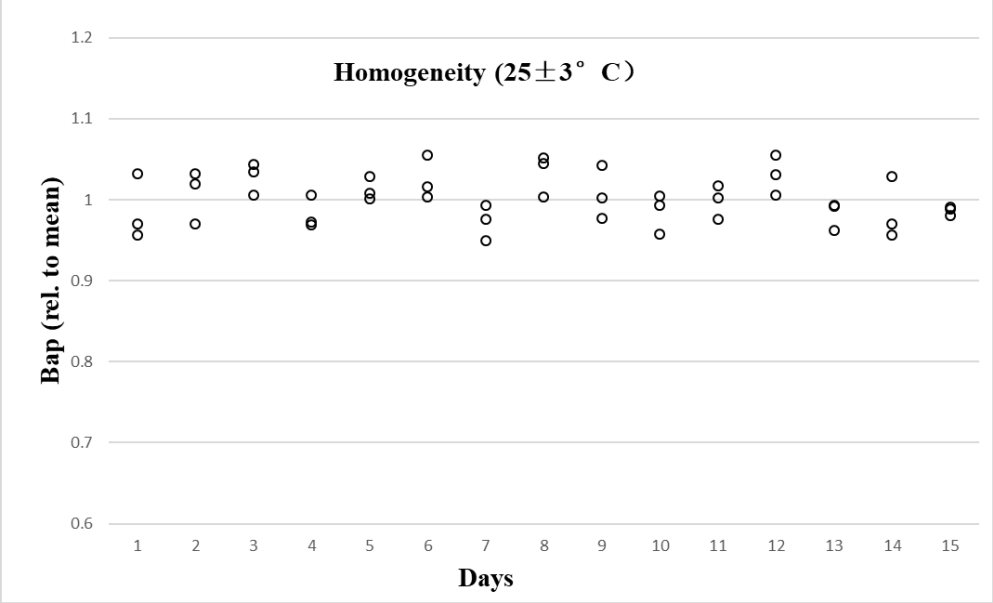


Table 2 Summary of ANOVA for homogeneity test of BaP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	F_{crit}
Between Groups	0.0175	14	0.00125	1.858	0.076	2.03742
Within Groups	0.0202	30	0.000673			

Stability

NIM has performed long-term and short-term stability testing of BaP in the olive oil samples. Samples were stored at 50ºC for 0, 5, 8, 12, 20 and 30 days for the short-term stability with two bottles being analyzed at each time point. This study was designed to test the material stability under transportation conditions. Similarly, duplicate samples were selected randomly at the storage condition of 25ºC for testing at the 0, 1, 3, 6, 12, 24, 30months time points for the long-term stability study. Duplicate sub-samples were taken from each bottle and analyzed using the IDGC-MS/MS method and the absolute values were transformed relative to the mean. The trend graphs of stability are shown in Figure 2 and 3. The results of the stability assessment indicated that the coefficient of variation (CV) was lower than 1.7% for the target BaP under both circumstances. The trend-analysis technique proposed by ISO Guide 35 was applied to assess the stability. The effect of time on the stability was evaluated using a linear approximation model by fitting linear regression lines to the data set ($Y=\beta_0-\beta_1 X$). The statistical results indicated that no significant trend at 95% confidence level was detected as the absolute values of β_1 (i.e. slope of the regression line) were smaller than the critical values of β_1 which were the uncertainty associated with the slope of the regression line for the stability times the respective Student's t-factor. Hence, the instability of the material was insignificant at the study temperature over the study period.

The stability of the study material was also evaluated through ANOVA test on the regression with results summarized in Table 3 and 4. The obtained respective p-values for both tests (all greater than 0.05) indicated that the regressions were insignificant at 95% confidence level.

Figure 2 Short-term stability of BaP

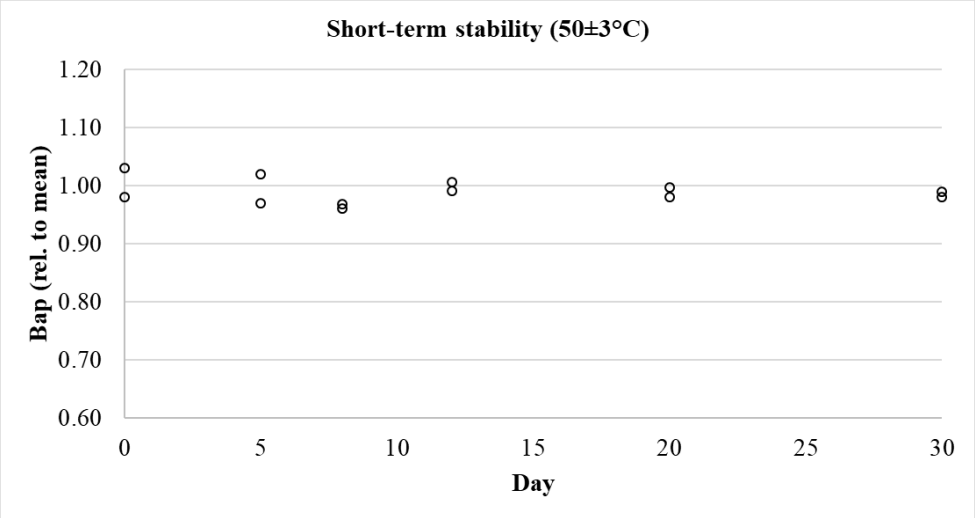


Table 3 Summary of ANOVA test for the short-term stability study of BaP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	F_{crit}
Between Groups	0.0020	5	0.000406	0.86	0.55	4.387374
Within Groups	0.0028	6	0.000471			

Figure 3 Long-term stability of BaP

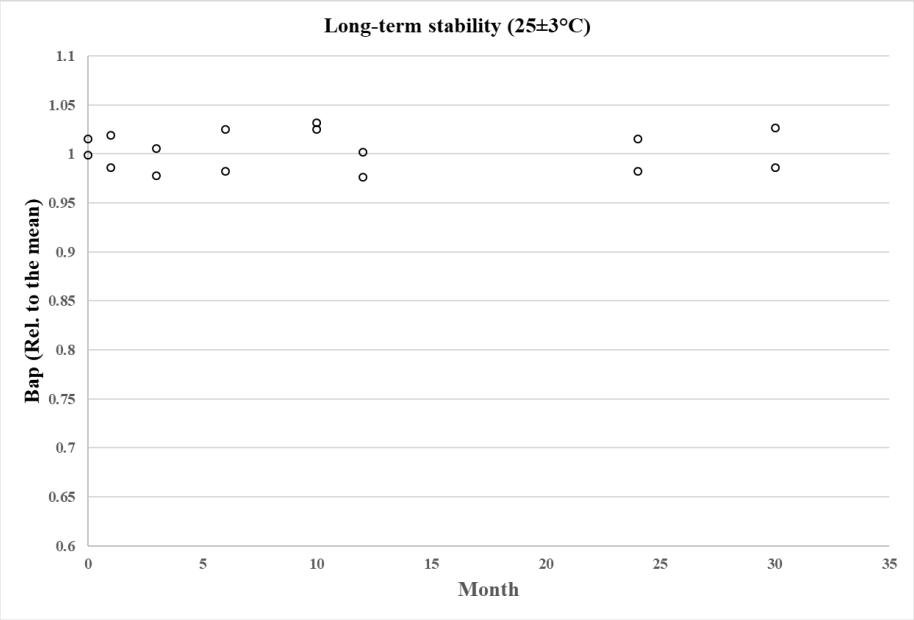


Table 4 Summary of ANOVA test for the long-term stability study of Bap

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	F_{crit}
Between Groups	0.003	7	0.000289	0.63	0.172	3.50
Within Groups	0.0037	8	0.000459			
Total	0.0057	18				

Reference Standards Available

Solution CRMs and isotopically-labeled (deuterium or carbon-13) BaP for use as internal standard are available from a number of sources including examples indicated below (Table 5 & Table 6).

Table 5 CRMs for measurand

Supplier	Solution CRM	Certified value	Expanded uncertainty
NIM	Benzo[a]pyrene in methanol (GBW(E)080476)	4.12 ug/mL	6.7% (k=2)
NMIJ	Benzo[a]pyrene in 2,2,4-trimethylpentane (CRM 4213-a)	99.2 mg/kg	3.9 mg/kg (k=2)
NIST	Priority Pollutant PAHs in Acetonitrile （SRM 1647f）	6.22 mg/kg	0.11g/kg (k=2)
NIST	Aromatic Hydrocarbons in Toluene (SRM 2260a)	4.71 mg/kg	0.17 mg/kg (k=3)

Table 6 Reference standards for isotopically-labeled BaP

Isotopically-labeled BaP		
Cambridge Isotope Laboratories	Benzo[a]pyrene D_{12}	DLM-258
Dr. Ehrenstorfer	Benzo[a]pyrene D_{12}	97688AL
Sigma-Aldrich	Benzo[a]pyrene D_{12}	451797
NIST	Benzo[a]pyrene D_{12}	SRM2270
Cambridge Isotope Laboratories	Benzo[a]pyrene $^{13}C_4$	CLM-2722

Study Guidelines

Each participant will receive 2 bottles, each containing 30 g of olive oil. One sample bottle is intended for method development and the other one is to be used for determination of the final results. Samples can be stored at room temperature. A minimum sample intake of 0.5 g is recommended. Participants may use their preferred laboratory procedures.

Reporting of Results

At the time of sample dispatch, a sample receipt form will be provided electronically to all participants and must be filled in and returned to the study coordinator on receipt of the shipments. The results reporting form and core competency template will be provided to each participant and must be completed and returned to the study coordinator before the submission deadline.

The results should be reported in the unit of µg/kg for BaP and should include standard and expanded uncertainties (95 % level of confidence) for the mean of the replicate determinations. Information on the measurement procedure (extraction, clean-up, column and conditions, quantification approach), the calibration standards, the internal standard, any quality control materials, number of replicates, the calculation of the results and the estimation of measurement uncertainty should be included.

Evaluation of Results

All the results of the comparison will be evaluated against the reference value from NIM, as the linking laboratory to CCQM-K146. Degrees of Equivalence will be calculated in accordance with this.

How Far Does the Light Shine?

Successful participation in this Track A key comparison "Low-Polarity Analyte in high fat food: Benzo[a]pyrene in Olive Oil" will demonstrate participant's capabilities in determining the low-polarity analytes (pKow < -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 1000 µg/kg in a high fat, low protein, low carbohydrate food matrix.

This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) cleanup and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC/MS, GC-HRMS, HPLC-FLD or LC-MS.

Study schedule

The time schedule for the various stages of the Key Comparison /Pilot Study is shown as follows:

Table 7 Study schedule for CCQM K146.1

Event	Period
Preparation of sample	Oct 2015
Homogeneity testing	Nov 2015
Stability testing	From Nov 2015
Invitation to participants	17 May 2019
Deadline for registration	24 May 2019

Dispatch of samples	End May 2019
Deadline for submission of results	30 July 2019
Distribution of preliminary report	15 August 2019
Discussion of results at the CCQM OAWG	October 2019

Contact information:

For enquiries, participants may wish to make contacts as follows:

Prof. Li Hongmei, NIM, lihm@nim.ac.cn

Dr. Li Xiaomin, NIM, lixm@nim.ac.cn

APPENDIX B: Registration Form

CCQM Key Comparison/Pilot Study

BENZO[A]PYRENE IN OLIVE OIL

CCQM-K146.1

REGISTRATION FORM

My institute/laboratory would like to participate in the program "Benzo[a]pyrene in Olive Oil":

□ CCQM-K146.1

INSTITUTE / LABORATORY: _____

_____

ADDRESS: _____

_____

CONTACT PERSON: **(Prof / Dr / Mr / Ms)** _____

_____

EMAIL: _____

TELEPHONE AND FAX:

SIGNATURE: _____

DATE: _____

Grateful if you would complete the form and email to the coordinators by 24th May 2019 at: lixm@nim.ac.cn. If you do not receive an acknowledgement from us within 4 working days, please send us an email.

APPENDIX C: Reporting Form

中国计量科学研究院
National Institute of Metrology,China

CCQM Key Comparison
CCQM-K146.1
Benzo[a]pyrene in Olive Oil
Results Report Form

Please use this excel sheet for reporting.
Please submitted this report electronically to lixm@nim.ac.cn
Please fill in all blanks and use the requested units.
Please provide any extra information in the comments section or on a separate sheet if necessary.

Part I: Participant's Information

Laboratory Name:	
Submitted by:	
Reporting Date: (dd/mm/yy)	

Part II: Results:

Bottle No. used for reporting	

Analyte/ Mass Fraction	Mass fraction (µg/kg)	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
Benzo (a) pyrene					

Note: Please refer to the OAWG guidance document on significant figures when reporting values

Part III: Technical Information of Methodology Used	
Sample size used for analysis (g)	
Extraction method (Please briefly describe the extraction procedures, e.g. Liquid/Solid extraction, Soxhlet, etc., solvents, volumes, time, temperature etc.)	
Post extraction clean-up method and the transformation procedures, if any (e.g., SPE, GPC, etc) (Please briefly describe the clean-up and transformation procedures including any dilutions or concentration steps prior to analysis)	
Derivatization, if any	
Analytical instrument(s) used (e.g., LC-MS/MS, GC-MS. etc) (Please specify the model)	
Chromatographic column	
The chromatographic condition(s) (e.g., GC oven temperature programme, LC Mobile phase gradient, etc)	

Method of quantification (e.g., external calibration, Internal standard calibration, IDMS, et	
Type of calibration (e.g., single-point, bracketing, three-point calibration curve, etc.)	
Native calibration standards: source, confirmation of identity, value assignment, uncertainty and traceability	
Reference material used for calibration is in compliance with the requirements for Traceability in CIPM MRA (Document No.: CIPM 2009-24; Latest update: Revised 13 Oct. 2009):	Yes/ No
Internal standards used (Please specify the compounds, and at which stage of analysis were the internal standards added.)	

Indicate ions /MRMs measured in the mass spectrometer instrument.

Calibrant: IS:

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

Other information, observations or evidences, if any, that can further support your results.

APPENDIX D: Core Competency Tables

CCQM OAWG: Competency Template for Analyte(s) in Matrix

CCQM-K146.1	*NMI*	Low-Polarity Analyte in high fat food - Benzo[a]pyrene in Olive Oil
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate participant's capabilities in determining the low-polarity analytes (pKow < -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 1000 µg/kg in a high fat, low protein, low carbohydrate food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) cleanup and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC/MS, GC-HRMS, HPLC-FLD or LC-MS.		
Competency	**Tick, cross, or "N/A"**	**Specific Information as Provided by NMI/DI**
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		<Identity of supplier & CRM>
Identity verification of analyte in calibration material. #		<Methods used to confirm structure>
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#		<Specify>
For calibrants which are a calibration solution: Value-assignment method(s).#		<Specify>
Sample Analysis Competencies		
Identification of analyte(s) in sample		<Methods used to identify the analyte>
Extraction of analyte(s) of interest from matrix		<Specify>
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)		<Specify>
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)		<Specify>
Analytical system		<Specify>
Calibration approach for value-assignment of analyte(s) in matrix		<Specify>
Verification method(s) for value-assignment of analyte(s) in sample (if used)		<Specify>
Other		

- In the middle column place a tick, cross or say the entry is not applicable for each of the competencies listed (the first row does not require a response)
- Fill in the right hand column with the information requested in blue in each row
- Enter the details of the calibrant in the top row, then for materials which would not meet the CIPM traceability requirements the three rows with a # require entries.

APPENDIX E: Summary of Participants' Analytical Information

Table E-1: Summary of Sample Size, Extraction, and Cleanup for CCQM-K146.1

NMI/DI	Sample Size (g)	Pre-treatment
EXHM	2.5	sample preconcentrated into acetone/acetonitrile 40/60 by three successive extractions, 10 mL each, succesive solid phase extraction with a - C18 cartridges, b - fluorisil cartridges, c - amino cardridges
NIM	0.5	0.5g oil+D12-PAH (equilibrate 12 hours) 5 subsamples, Mixed with 10 mL 1 mol/L KOH ethanol-water solution (90:10, v/v) (2min) and saponificated at 75 ℃ for 2 Hours, Cool down to room temperature and added 10 mL ultrapure water, then added 15 mL n-hexane vortex 2 min, Centrifuged at 20 ℃ 4000 r/min (3min), and reextracted with hexane and combined the upper phase, concentrated under a gentle nitrogen flow at 40 ℃ to near dryness and then reconstituted with cyclohexane for analysis. Cleaned up by using Supelclean™ EZ-POP NP with acetonitrile as eluent, and reconstituted with isopropanol.
NMISA	1	Internal standard was added to the sample and allowed to equilibrate for 30 min on a orbital shaker at room temperature. Thereafter, a liquid-liquid extraction was performed on 1 g of fat using 10 mL acetonitrile/ acetone mixture (6:4). The solvent was added and the sample was extracted at room temperature in an ultrasonic bath for 15 min. The extract was centrifuged and the supernatant removed. The remaining oil was extracted again and the supernatant from both extractions combined. The supernatant was evaporated using a centrifugal evaporation system at room temperature to final volume of 2 mL. Two solid phase extraction clean-up steps were conducted after the extraction process. Firstly a EZ-POP NP (SupelcleanTM,12 mL) using acetonitrile for elution. Followed by a LC-NH2 (SupelcleanTM,6 mL) using a mixture of hexane and toluene (7:3). The final extract was evaporated to dryness and reconstitued to a final volume of 100 µL in hexane and toluene (7:3).
Laboratory of the Institute for Oliveculture	0.200188	SPE cartridge (SUPELCLEAN LC-SI, 1 g, 6 mL, cat. no. 57051) was cleaned with dichloromethane, vacuum dried and washed with hexane. Sample (0.2 g in 1 mL) and benzo(b)chrisene (10 ng in 100 µL) were loaded and eluted with hexane/dichloromethane (70:30 v/v). For standard addition, sample (0.2 ng in 1 mL), benzo(b)chrisene (10 ng in 100 µL) and standard solution of B(a)P (1.14 ng in 100 µL or 2.29 ng in 100 µL) were loaded and eluted with

		hexane/dichloromethane (70:30 v/v). The first 1 mL of eluate was discharged, next 4 mL of eluate were dried under a nitrogen stream. The residue was dissolved in 1 mL of acetonitrile and injected into the HPLC apparatus.
UME	0.5	In a vial 2 mL oil sample spiked with 200 uL IS solution, vortexed and equilibrated for one hour at room temperature. Extracted by Solid Phase Extraction with SUPELCO Supelclean EZ-POP NP (12 mL, 2.5 gram) cartridge. Cartridge conditioned with 10 mL acetone and dried by air suction under vacuum for 15 minutes. Add 0.5 g oil sample and wait for penetration with adsorbent of cartridge then elute with 15 mL of acetonitrile. 15 mL acetonitrile eluate was concentrated under nitrogen stream at 40 ºC to final volume of 200 µL.

Table E-2: Summary of Analytical Techniques for CCQM-K146.1

NMI/DI	Analytical Technique	Chromatographic Conditions	Ion/MRM
EXHM	GC-MS/MS	GC-MS/MS(Thermo GC Ultra coupled to PolarisQ) Agilent J&W DB-35 ms (30 m x 0.25 mm ID, 0.25 µm film thickness) PTV injector - 10 µL inj vol. - inj program: initial T 85 C, split flow 100 mL/min, inj pressure 160 kPa, flow 25 mL/min evaporation temp 15 C/s to 85 C for 0,5 min, transfer temp: 15 min/s to 300 C, cleaning 14,5 C/s to 320, hold 28 minHe carrier gas - 0-15 min: 1 mL/min, with 0,1 mL/min ramp to 2 mL/min - hold for 9 min oven initial T: 80 C (stable 3 min), 25 C/min to 230, 10 C/min to 250, 3 C/min to 310, hold 5 min transfer line 280 C	Calibrant: BaP (252 - 250/246) IS: BaP-d12 (264 - 260/258)

<table>
<tr>
<td>NIM</td>
<td>GC-MS/MS</td>
<td>GC-MS (Agilent 7890A GC oven, combi PAL autosampler, Agilent 5975C MS)

Agilent DB-5 MS UI (60 m×0.25 mm×0.25 µm) Injector: splitless at 300 °C.GC oven: 70 °C for 1 min. 15 °C/min to 220 °C and kept 3min. 3°C/min to 260 °C. 30 °C/min to 300 and kept 8 min.</td>
<td>Benzo(a)pyrene: MRM 252->250

12D4- Benzo(a)pyrene: MRM 264->260</td>
</tr>
<tr>
<td>NMISA</td>
<td>GC-TOFMS and GCxGC-TOFMS</td>
<td>GC-TOFMS: Rxi-PAH (60 m x 0.25 mm x 0.1 µm) GCxGC-TOFMS: Rxi-PAH (60 m x 0.25 mm x 0.1 µm) first dimension and Rtx-200 (1 m x 0.25 mm x 0.25 µm); For 2D separation, a secondary oven temperature offset of 5°C and a modulator offset of 10°C
<table>
<tr><th>#</th><th>Rate (°C/min)</th><th>Target Temp (°C)</th><th>Duration (min)</th></tr>
<tr><td>1*</td><td>Initial</td><td>65.00</td><td>1.00</td></tr>
<tr><td>2</td><td>5.00</td><td>170.00</td><td>0.00</td></tr>
<tr><td>3</td><td>3.00</td><td>250.00</td><td>0.00</td></tr>
<tr><td>4</td><td>10.00</td><td>305.00</td><td>15.00</td></tr>
</table>
</td>
<td>Calibrant: Quantification was performed using m/z 252. Identification was confirmed using the ion ration between m/z 252 and 126

Isotope: Quantification was performed using m/z 264. Indentification was confirmed using the ion ration between m/z 264 and 132</td>
</tr>
<tr>
<td>Lab of the Institute for Oliveculture</td>
<td>HPLC-FLD</td>
<td>HPLC (Agilent 1100) Phenomenex Kinetex PAH 3,5 µm 150 × 4,6 mm (P/N 00F-4764-E0; S/N H17-339823) Flow: 1,2 mL/min; Gradient: A-water; B-ACN; 0' – 50% B – 8' – 100% B – 17.5' – 100% B – 17.75' – 50% B; equil. time: 5'</td>
<td>standard addition, three-point calibration curve</td>
</tr>
<tr>
<td>UME</td>
<td>GC-MS/MS</td>
<td>THERMO TSQ GC-Tandem Mass (triple quadrupole) Varian VF-17MS 60 m x 0.25 mm 0.25 µm. 100 ºC (1 min.); with 30 ºC/min. to 200 ºC; with 2 ºC/min. to 320 ºC (25 min.).</td>
<td>Calibrant: 252 → 250 (CE: 48V) IS: 264.2 → 260.15 (CE: 55V)</td>
</tr>
</table>

Table E-3: Summary of Calibrants and Standards for CCQM-K146.1

NMI/DI	Type of Calibration	Method of Quantification	Calibrants	Internal Standards
EXHM	single-point	IDMS at exact matching	Benzo(a)pyrene in house certified and confirmed NIST SRM 2260a	benzo[a]pyrene-d12
NIM	single-point	IDMS	Benzo(a)pyrene GBW(E)080476	Benzo[a]pyrene D12
NMISA	Multi-point calibration curve and bracketing	IDMS	NIST SRM 1647f and SRM 2260a	benzo[a]pyrene-d12
Laboratory of the Institute for Oliveculture	three-point calibration curve	Standard addition	/	/
UME	Single point	IDMS	NIST SRM 1647f	Benzo[a]pyrene-d12

APPENDIX F: Summary of Participants' Uncertainty Estimation Approaches

The following are text excerpts and/or pictures of the uncertainty-related information provided by the participants in the reporting form. Information is grouped by participant and presented in alphabetized acronym order.

EXHM

The measurement equation is:

$$w_{M,S} = w_{M,C} \frac{100}{Rechh} \times \frac{m_{is,S}}{m_{M,S}} \times \frac{m_{M,C}}{m_{is,C}} \times \frac{R_S}{R_C}$$

where $w_{M,S}$ = mass fraction of the analyte (B[a]P) in the sample, ($\mu g/kg$)

$w_{M,C}$ = mass fraction of the analyte (B[a]P) in the calibration solution, ($\mu g/kg$)

Rec = recovery (%), assessed against other independent methods

$m_{is,S}$ = mass of internal standard solution added to sample blend, (g)

$m_{M,S}$ = mass of test material in sample blend, (g)

$m_{M,C}$ = mass of the analyte (B[a]P) solution added to calibration blend, (g)

$m_{is,C}$ = mass of internal standard solution added to calibration blend, (g)

R_S = measured peak area ratio of the selected ions in the sample blend

R_C = measured peak area ratio of the selected ions in the calibration blend

The equation used to estimate standard uncertainty is:

$$u(w_{BS}) = \sqrt{\left({s_R}/{\sqrt{n}}\right)^2 + \sum\left(C_j u(m_i)\right)^2 + \sum\left(C_j u(R_i)\right)^2 + \left(C_j u(w_{MC})\right)^2 + + \left(C_j u(R)\right)^2}$$

where s_R is the standard deviation under reproducibility conditions, n the number of determinations and C_j the sensitivity coefficients associated with each uncertainty component. The uncertainty of the peak area ratios was considered to have been included in the estimation of method precision.

Uncertainty estimation was carried out according to JCGM 100: 2008. The standard uncertainties were combined as the sum of the squares of the product of the sensitivity coefficient (obtained by partial differentiation of the measurement equation) and standard uncertainty to give the square of the combined uncertainty. The square root of this value was multiplied by a coverage factor (95% confidence interval) from the t-distribution at the total effective degrees of freedom obtained from the Welch-Satterthwaite equation to give the expanded uncertainty.

The uncertainty budget for BaP is shown as follows:

Benzo[a]pyrene

uncertainty component	value	sensitivity coefficient	standrard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
method precision	9,64	1,000	0,10	0,0106	0,1020	0,0104
mass fraction of B[a]P in the calibration solution, (*µg/kg*)	60,48	0,159	0,41	0,0068	0,0655	0,0043
recovery (%)	100,00	-0,096	2,71	0,0271	-0,2612	0,0682
mass of B[a]P-d_{12} solution added to sample blend, (*g*)	0,35618	27,058	0,00007	0,0002	0,0019	0,0000
mass of test material in sample blend, (*g*)	2,48720	-3,875	0,00009	0,0000	-0,0003	0,0000
mass of B[a]P solution added to calibration blend, (*g*)	0,10023	96,152	0,00003	0,0003	0,0029	0,0000
mass of B[a]P-d_{12} solution added to calibration blend, (*g*)	0,10020	-96,181	0,00003	0,0003	-0,0029	0,0000
measured peak area ratio of the selected ions in the sample blend	1,430	6,740	considered to be included in the			
measured peak area ratio of the selected ions in the calibration blend	1,285	-7,497	estimation of method precision			
result (ng/g)	9,64					
combined standard uncertainty (ng/g)	0,29					
relative standard uncertainty (%)	2,99					
effective degrees of freedom	447					
coverage factor	2,00					
expanded uncertainty (ng/g)	0,58					

NIM

The mass fraction (µg/kg) of analytes (C_x) in the sample was calculated as follows:

The expanded measurement equation given was used to calculate the mass fraction of the measurand. The additional factors (F) in the expanded measurement equation represent aspects of the measurement procedure that may influence the measured mass fraction value. They are given a value of 1 but they add an uncertainty component to the uncertainty budget.

Expanded measurement equation:

$C_x = F_P \times (M_{IS} \times M_{AC} \times R_b)/(M_S \times M_{IC} \times R_{bc})$

Where :

Cx is the mass fraction of analytes in the sample (ng/g);

F_P is the method precision factor

M_{IS} is mass of internal standard (isotopologue) added to the sample blend (g)

M_{AC} is mass of analyte added to the calibration blend (g)

R_b is peak area ratio of analyte /isotopologue in sample blend

M_S is mass of sample (g)

M_{IC} is mass of internal standard(isotopologue) added to the calibration blend (g)

R_{bc} is peak area ratio of analyte /isotopologue in calibration blend

Source of uncertainty	Value	u(x)	u(x)/(x)
M_{IS} (g)	0.08	0.00019	0.24%
M_S (g)	0.5	0.00019	0.04%
M_{IC} (g)	0.2	0.00019	0.02%
M_{AC}(g)	0.2	0.0028	1.40%
Method Precision, F_P			1.50%
Coverage factor, *k*			2
Relative combined standard uncertainty (u_c)			2.01%

Relative expanded uncertainty (*Uc*)			4.1%
Mass Fraction (µg/kg)			8.69
Expanded uncertainty, *U* (µg/kg)			0.36

NMISA

Bracketing measurement equation:

$$W_x = W_Z \times \frac{m_z}{m_{yc}} \times \frac{m_y}{m_x} \times \frac{R'_B}{R'_{BC}}$$

W_x	Final concentration of the natural substance
W_Z	Concentration of the natural CRM
m_Z	Weight of CRM solution used in calibration blend
m_y	Weight of the isotope solution used in calibration blend
R'_B	Peak area of natural substance in sample divided by peak area of isotope
m_{yc}	Weight of isotope in sample
m_x	Weight of sample
R'_{BC}	Peak area of CRM in calibration solution divided by peak area of isotope

Linear regression measurement equation

$$Analyte\ mass = \frac{mass\ deuterated\ label\ x\ (\frac{area\ native}{area\ slope} - intercept)}{sample\ mass}$$

Example of uncertainty for linear regression:

Contributer	Description	x	u	u/x	u/x²	v	
CRM	Imported uncertainty	6.22	1.10E-01	1.77E-02	3.13E-04	1000000	inf
Mass of standard in calibration	Balance and mass uncertainty	50.00	1.07E-05	2.14E-07	4.57E-14	1000000	inf
Mass of sample used	Balance and mass uncertainty	1.24	3.76E-02	3.04E-02	9.24E-04	1000000	inf
Precision	Stdev of repeat measurements	8.68	3.01E-01	3.47E-02	1.20E-03	2	
SxO	Error of the calibration curve	8.68	3.54E-01	4.07E-02	1.66E-03	10	
					Veff	23	

Regression	
LOD (ng/g)	0.15
LOQ (ng/g)	0.51

Minimum ng/g	Maximum ng/g
-1.15	1.15

k	2.06865761	
	0.00	ng/g
	0.56	u
	1.15	U (k=2)
	13.24	Rel U

Example of uncertainty for bracketing:

Benzo[a]pyrene		x	u	u/x	u/x^2	
W_z	CRM uncertainty	4.71000	1.70E-01	3.61E-02	1.30E-03	
m_z	Weight of calibration blend (g)	50.00062	1.07E-05	2.14E-07	4.57E-14	
m_y	Weight of Isotope added to sample (g)	0.12611	1.03E-03	8.19E-03	6.71E-05	
m_{yc}	Weight of Isotope added to calibration blend (g)	0.12543	1.03E-03	8.24E-03	6.79E-05	
m_x	Weight of sampled (g)	0.99215	9.20E-03	9.27E-03	8.59E-05	
R'_B/R'_{BC}	Ratio of peaks areas of native/ labelled in the samples	0.75692	1.35E-02	1.78E-02	3.17E-04	
Repeats	ESDM of bracket calcs	9.56840	3.04E-01	3.17E-02	1.01E-03	
			Sum	0.07959	0.00285	
			V_{eff}	44.24990	**0.49948**	u
			k	2.01537	1.00665	U
					10.52052	%Rel U

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Mass fraction B[a]P(µg/kg)=$A_0 \cdot Add/(A_D - A_0)/m_S$

Where:

A_0 = Area of B(a)P without standard addition, normalised to added benzo(b)chrisene

Add = mass of standard addition of BaP (ng)

A_D = Area of B(a)P with standard addition, normalised to added benzo(b)chrisene

m_S = sample amount (g)

$u_c^2 = (u_{rep}^2 + u_{pur}^2 + u_{cal}^2)$

$U = k \cdot X \cdot u_c$

U = expanded uncertainty

u_c = combined relative uncertainty

u_{rep} = relative standard uncertainty of measurement: SD_{mean}/X_{sample} (0,0346)

u_{pur} = relative standard uncertainty of purity of BaP, determined by qNMR by LGC (0,0179)

u_{cal} = relative standard uncertainty of calibration according to EURACHEM CITAC GUIDE (0,0922)

X = mean value of B(a)P in olive oil

k = coverage factor (2)

UME

$$C_{sample} = \frac{Area_{Ratio} - a}{b} * \frac{C_{IS}}{m_{sample}}$$

C_{sample}: Concentration of sample

$Area_{Ratio}$: Area Native / Area IS

a: intercept of calibration curve

b: slope of calibration curve

C_{IS}: Concentration of IS in olive oil

m_{sample}: mass of sample used

Uncertainty Budget of Benzo[a]pyrene			
Parameters	Unit	Value (X)	u(x)	u(x)/X
Mass of sample intake	g	1.75	1.68984E-05	9.66E-06
Spiking Labelled stock solution	g	0.134	0.00000007	5.33E-07
Native stock solution	µg/kg	98.3	0.86932078	8.84E-03
Calibration		20.000	0.018	9.08E-04
Recovery		0.986	0.040	4.06E-02
Repeatability	µg/kg	7.59	0.051	6.72E-03
Relative Standard Measurement Uncertainty				0.042
Result (µg/kg)		**7.59**		
Combined Standard Measurement Uncertainty			0.32	
Expanded Uncertainty (k=2)			**0.64**	
Relative Mesurement Uncertainty (%)			8.42	

$$\frac{U_c(A)}{C_A} = \sqrt{\left(\frac{u(W_{SI})}{W_{SI}}\right)^2 + \left(\frac{u(W_{IS})}{W_{IS}}\right)^2 + \left(\frac{u(C_{NS})}{C_{NS}}\right)^2 + \left(\frac{u(c_0)}{c_0}\right)^2 + u(R_m)^2 + u(r)^2}$$

Uncertainty Sources :
1-Mass of sample intake
2-Spiking of labelled stock solution
3-Native stock solution
4-Calibration
5-Recovery
6-Repeatability

APPENDIX G: Core Competencies

Table G-1: Core Competencies Demonstrated in CCQM-K146.1 by EXHM

CCQM-K146.1	*EXHM*	Low-Polarity Analyte in high fat food - Benzo[a]pyrene in Olive Oil
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate participant's capabilities in determining the low-polarity analytes (pKow < -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 1000 µg/kg in a high fat, low protein, low carbohydrate food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) cleanup and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC/MS, GC-HRMS, HPLC-FLD or LC-MS.		
Competency	**Tick, cross, or "N/A"**	**Specific Information as Provided by NMI/DI**
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	in house BaP calibrant and calibration solution
Identity verification of analyte in calibration material. #	✓	NMR
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	✓	in-house calibrant prepared by Sigma Aldrich TraceCert BaP – 1 H NMR used to identify structure 971.0 mg/g ± 3.1 mg/g by qNMR to NMIJ CRM 4601-a
For calibrants which are a calibration solution: Value-assignment method(s).#	✓	60.48 ng/g ± 0.82 ng/g (gravimetric) verified by IDMS against NIST SRM 2260a
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	retention time, ion ratios
Extraction of analyte(s) of interest from matrix	✓	Pre-concentration and extraction of PAHs with acetone/acetonitrile
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	sequential solid phase extraction using C18, Florisil and amino cartridges
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	n/a	<none>

Analytical system	✓	GC-IT-MS
Calibration approach for value-assignment of analyte(s) in matrix	✓	IDMS at exact matching, single point
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	Pre-concentration and extraction of PAHs with acetone/acetonitrile – solid phase extraction with molecularly imprinted polymers
Other	✓	used surplus PT materials for recovery estimation

The result for EXHM was not consistent with the KCRV at the 95% confidence intervals and the DoE does not cross zero. EXHM have attributed this inconsistency to the inappropriate evaluation of the uncertainty assessment. And They did not incorporate a large enough factor for their extraction efficiency.

Table G-2: Core Competencies Demonstrated in CCQM-K146.1 by NMISA

CCQM-K146.1	NMISA	Low-Polarity Analyte in high fat food - Benzo[a]pyrene in Olive Oil
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate participant's capabilities in determining the low-polarity analytes (pKow < -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 1000 µg/kg in a high fat, low protein, low carbohydrate food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) cleanup and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC/MS, GC-HRMS, HPLC-FLD or LC-MS.		
Competency	**Tick, cross, or "N/A"**	**Specific Information as Provided by NMI/DI**
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	X	Calibration solution
Identity verification of analyte in calibration material. #	X	Verification against NIST SRM 1647 through mass spectral ion ratios and retention times using Gas Chromatography – Time-of-flight mass spectrometry (GC-TOFMS)
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	N/A	
For calibrants which are a calibration solution: Value-assignment method(s).#	N/A	CRM calibrants were used (NIST SRM 1647f and NIST SRM 2260a)
Sample Analysis Competencies		
Identification of analyte(s) in sample	X	Retention time Mass spectrometric ion ratios Full range mass spectra
Extraction of analyte(s) of interest from matrix	X	Liquid – liquid extraction
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	X	Solid phase extraction
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	

Analytical system	X	Gas Chromatography – Time-of-flight mass spectrometry (GC-TOFMS) and comprehensive Gas Chromatography – Time-of-flight mass spectrometry (GCxGC-TOFMS)
Calibration approach for value-assignment of analyte(s) in matrix	X	IDMS and bracketing
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	
Other	N/A	

Table G-3: Core Competencies Demonstrated in CCQM-K146.1 by Oliveculture

CCQM-K146.1	*Institute of Oliveculture*	Low-Polarity Analyte in high fat food - Benzo[a]pyrene in Olive Oil
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate participant's capabilities in determining the low-polarity analytes (pKow < -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 1000 µg/kg in a high fat, low protein, low carbohydrate food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) cleanup and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC/MS, GC-HRMS, HPLC-FLD or LC-MS.		
Competency	**Tick, cross, or "N/A"**	**Specific Information as Provided by NMI/DI**
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		Pure materials from Supelco
Identity verification of analyte in calibration material. #	N/A	NMR
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	✓	qNMR
For calibrants which are a calibration solution: Value-assignment method(s).#	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Chromatographic retention time
Extraction of analyte(s) of interest from matrix	N/A	
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	SPE
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	HPLC with FLD

Calibration approach for value-assignment of analyte(s) in matrix	✓	Standard addition (two levels of addition)
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	Standard addition
Other	N/A	

The result for the Institute of Oliveculture was not consistent with the KCRV at the 95% confidence intervals and the DoE does not cross zero. There was no information provided to indicate the reason for this deviation.

Table G-4: Core Competencies Demonstrated in CCQM-K146.1 by UME

CCQM-K146.1	*UME*	Low-Polarity Analyte in high fat food - Benzo[a]pyrene in Olive Oil
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate participant's capabilities in determining the low-polarity analytes (pKow < -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 1000 µg/kg in a high fat, low protein, low carbohydrate food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) cleanup and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC/MS, GC-HRMS, HPLC-FLD or LC-MS.		
Competency	**Tick, cross, or "N/A"**	**Specific Information as Provided by NMI/DI**
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	√	Calibration Solution, NIST SRM 1647f
Identity verification of analyte in calibration material. #	√	GC-MS
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	**N/A**	
For calibrants which are a calibration solution: Value-assignment method(s).#	√	Certificate
Sample Analysis Competencies		
Identification of analyte(s) in sample	√	GC-MS/MS
Extraction of analyte(s) of interest from matrix	√	SPE (SUPELCO Supelclean EZ-POP NP)
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	√	SPE (SUPELCO Supelclean EZ-POP NP)
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	√	15 mL acetonitrile eluate from SPE was concentrated under nitrogen stream to 200 µL prior to GC-MS/MS analysis.
Analytical system	√	Thermo TSQ Quantum XLS GC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix	√	5 point calibration curve, IS solution from NIST SRM 2270

Verification method(s) for value-assignment of analyte(s) in sample (if used)	**N/A**	
Other		

The result for UME was not consistent with the KCRV at the 95% confidence intervals and the DoE does not cross zero. There was no information provided to indicate the reason for this deviation.

APPENDIX H: DoE calculation

OpenBUGS code for HB REM consensus and calculation of DoE with repsect to proposed KCRV = 8.69 and u(KCRV) = 1.46 %, the same as that calculated for the K146 BaP measurand.

```
ModelBegin{
mu~dnorm(0,1.0E-6)
beta~dgamma(1.0E-5,1.0E-5)

for(I in 1:10){delta[i]~dnorm(mu,beta)
prec[i]<-1/(unc[i]*unc[i])
x[i]~dnorm(delta[i],prec[i])
pred[i]~dnorm(mu, prec[i])
DoE1[i]<-x[i]-pred[i]}
}
Inits:
list(beta=1)
Data:

x[]     unc[]
2.61    0.22
2.64    0.08
2.64    0.09
2.705   0.075
2.71    0.055
2.71    0.11
2.715   0.0611
2.73    0.16
2.79    0.062
2.799   0.026
END
}

{
mu2~dnorm(8.69, 138.42)
for(i in 1:4){preco[i]<-1/(unco[i]*unco[i])
predo[i]~dnorm(mu2, preco[i])
DoEo[i]<-xo[i]-predo[i]}
}

xo[]    unco[]
9.64    0.29
8.8     0.56
7.19    0.72
7.59    0.32
END
}
```