EURAMET Supplementary Comparison EURAMET.QM-S15
Measurement of PAHs in Protein Matrix

Supplementary Comparison

Study Protocol
July 2022

Proposed dates
09/2022 to 09/2023

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are a class of chemicals, consisting of hydrocarbons with at least two connected aromatic ring systems. They may be produced during the processing of foodstuffs, such as smoking. They may, however, also find their way into foodstuffs unintentionally, e.g. from mineral oils. Because they may have negative effects on health – they are associated with cancer and other diseases – threshold values have been set for PAHs. Accurate analyses regarding these chemicals are therefore called for.

There were other comparisons previously conducted for the determination of PAHs. CCQM conducted key comparisons with final reports published in 2018 (CCQM-K95.1: Polycyclic Aromatic Hydrocarbons (PAHs) in Tea) and 2020 (CCQM-K146: Benzo[a]pyrene in Olive Oil). So far, however, no comparison of PAHs in a protein-rich matrix has been carried out.

In this comparison the target PAHs are benz(a)anthracene (BaA), benzo(a)pyrene (BaP), benzo(b)fluoranthene (BbF), and chrysene (Chr) in protein-rich matrix.

Successful participation in this supplementary comparison will demonstrate the measurement capabilities in determining mass fractions of low polarity organic compounds with molecular masses of about 150 - 500 g/mol in the mass fraction range from 0.1 µg/kg to 100 µg/kg (as received) in a protein-rich matrix.

TIMELINE

Table 1 lists the time schedule for the proposed study.

Table 1: Proposed study schedule

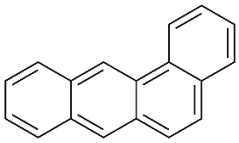
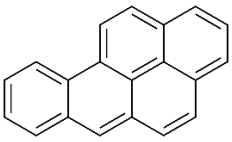
Date	**Action**
December 2020	Sample preparation
From March to May 2022	Homogeneity and stability testing
September 2022	Call for participation
November 2022	Sample distribution
April 2023	Deadline for submission of results
June 2023	Preliminary discussion of results

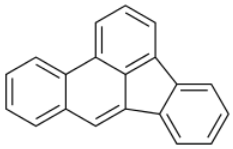
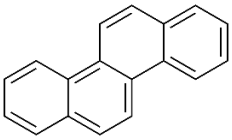
MEASURANDS

The measurands are the mass fractions (as received) of BaA, BaP, BbF and Chr in high-protein powder with the assigned value expressed in µg/kg. For each measurand, the indicative value for the mass fraction is 0.1 µg/kg to 50 µg/kg.

Table 2 below contains information about these compounds.

Table 2: Information of PAHs investigated in this supplementary comparison

	Benz(a)anthracene (BaA)	**Benzo(a)pyrene (BaP)**
CAS	56-55-3	50-32-8
Molecular formula	$C_{18}H_{12}$	$C_{20}H_{12}$
Molecular weight	228.3	252.3
Structure		

	Benzo(b)fluoranthene (BbF)	**Chrysene (Chr)**
CAS	205-99-2	218-01-9
Molecular formula	$C_{20}H_{12}$	$C_{18}H_{12}$
Molecular weight	252.3	228.3
Structure		

STUDY MATERIAL

The study material is a high-protein powder, spiked with the aforementioned PAHs. The material was produced by spiking fresh liquid protein concentrate with a solution of PAHs in acetonitrile. After mixing, the liquid protein concentrate was subjected to spray drying. A powder with about 80 % of protein was obtained. The powder was then bottled into pre-cleaned glass-amber bottles, each containing 30 g of material.

Each participant will receive one bottle containing approximately 30 g of material. Measurement results were to be reported on an as-received basis.

Recommended Minimum Sample Amount

The recommended minimum sample amount for analysis is at least 1.0 g.

Homogeneity Assessment of Study Material

For the homogeneity assessment, 10 bottles covering the whole bottling range were randomly selected. Three independent test portions of each bottle were analyzed. The measurements were performed under repeatability conditions, using a validated method and according to a random sequence to prevent possible trends in analytical sequence and filling order. According to ISO Guide 35:2017 [1], the assessment of the homogeneity was carried out by a one-way analysis of variance (ANOVA). For all four PAHs, the observed F-values ($MS_{between\ bottles}/MS_{within\ bottles}$) were lower than the critical F-values, indicating that the variances of the measured values within and between the bottles do not differ significantly at a 95 % confidence level. No evidence of statistically significant inhomogeneity was therefore observed. The results of the

homogeneity study and the estimated uncertainties for potential inhomogeneity (u_{bb}) are given in table 3 and figure 1.

Table 3: Results of the homogeneity assessment

PAH	s_{wb} (%)	s_{bb} (%)	u^*_{bb} (%)	u_{bb} (%)[a)]	F_{obs}	F_{crit}
BaA	2.7	0.5	0.9	0.9	1.099	2.393
BaP	4.3	n/a	1.4	1.4	0.147	2.393
BbF	3.2	n/a	1.1	1.1	0.222	2.393
Chr	3.2	0.4	1.1	1.1	1.051	2.393

a) For the estimation of u_{bb} the higher value of u^*_{bb} and s_{bb} was taken. u^*_{bb} and s_{bb} were calculated according to [2].

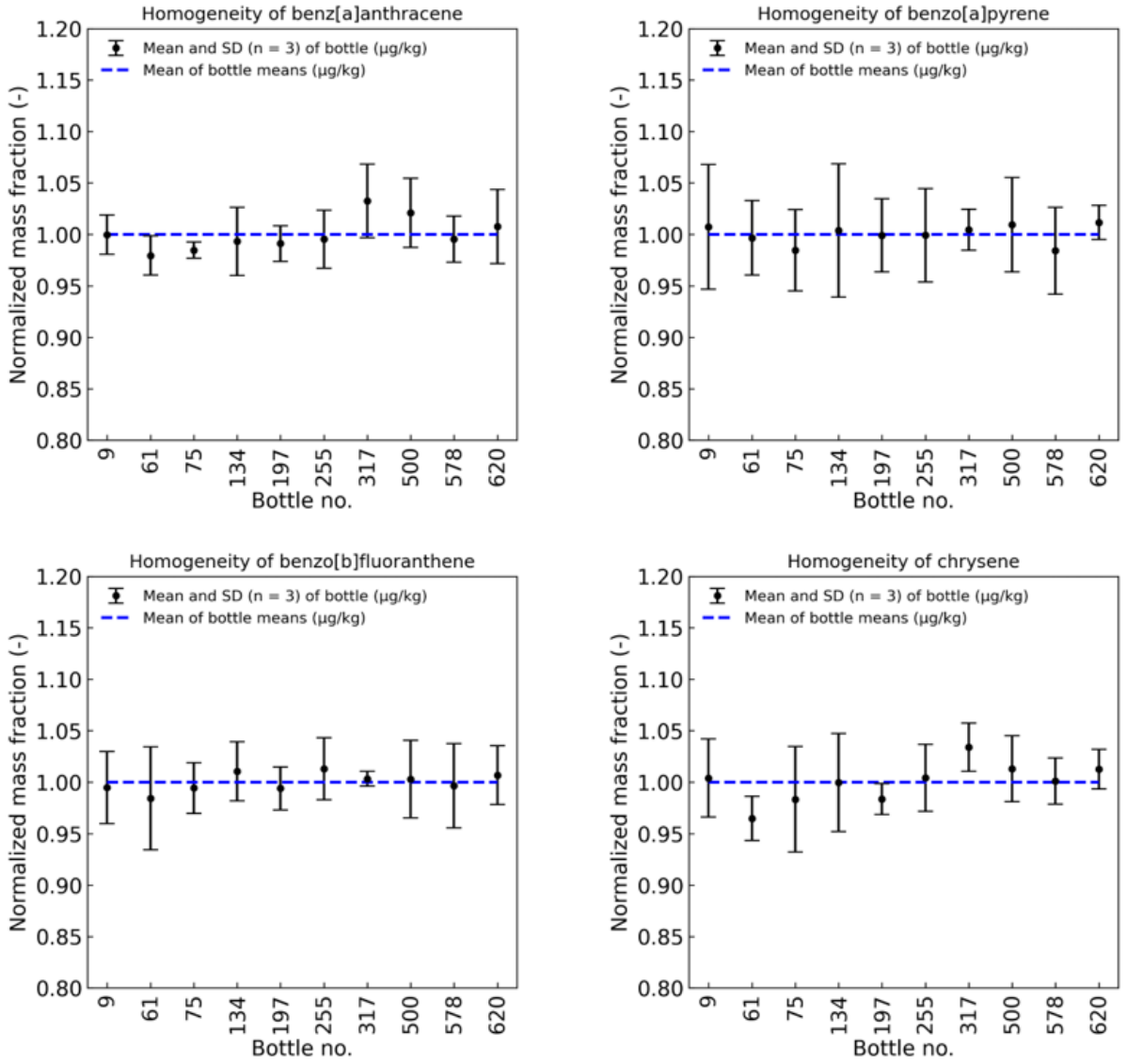


Figure 1: Homogeneity study of the four PAHs BaA, BaP, BbF and Chr: mean values of 10 selected bottles with their corresponding standard deviations (n = 3).

Stability Assessment of Study Material

For the stability assessment, an isochronous approach was used. The investigated bottles (one for each stability point) were stored for 1.5, 3, 6 and 12 months at different temperatures: -20 °C (reference temperature), 4 °C, room temperature (ca. 20 °C) and 45 °C (up to 3 months only). After the storage time was reached for a certain stability point, the corresponding bottle was stored at the reference temperature before it was analyzed three times. For t = 0, data from the homogeneity study were used. According to ISO Guide 35:2017 [1] and Linsinger et al. [3], the stability was assessed by applying a linear regression model. The slope b_1 and intercept b_0 were fit to the stability data. Using a two-tailed t-test, $t_{b1} = |b_1|/s(b_1)$, it could be shown that the slopes for all PAHs at all investigated temperatures do not differ significantly from 0 at a 95 % confidence level. Therefore, since no evidence of statistically significant instability was found at the various temperatures during the investigated storage times, b_1 was set to 0 for further calculations.

For the estimation of the uncertainties for potential long-term instability (u_{lts}) at the storage temperature of -20 °C the extrapolation model $u_{lts} = u(b_1=0) \cdot t$, with t = 24 months, was used [1, 3]. The results are given in table 4 and figure 2. The long-term stability, including 2 years and 4 years stability points, will be further investigated. For storage temperatures at 4 °C and room temperature (ca. 20 °C) similar uncertainty estimates were obtained as for -20 °C. This indicates that the long-term stability is also given at temperatures up to room temperature.

Table 4: Results of the long-term stability assessment at a storage temperature of -20 °C

PAH	**b_1 (months^{-1})**	**b_0 (-)**	**$s(b_1)$ (-)**	**t_{b1}**	**t_{crit}**	**$s(b_1=0)$ (-)**	**u_{lts} (%)**
BaA	-0.001275	1.005737	0.001948	0.654	2.160	0.001908	4.6
BaP	-0.001408	1.006334	0.002029	0.680	2.160	0.002029	4.9
BbF	0.001560	0.992980	0.001963	0.795	2.160	0.001937	4.7
Chr	-0.002210	1.009946	0.002151	1.027	2.160	0.002156	5.2

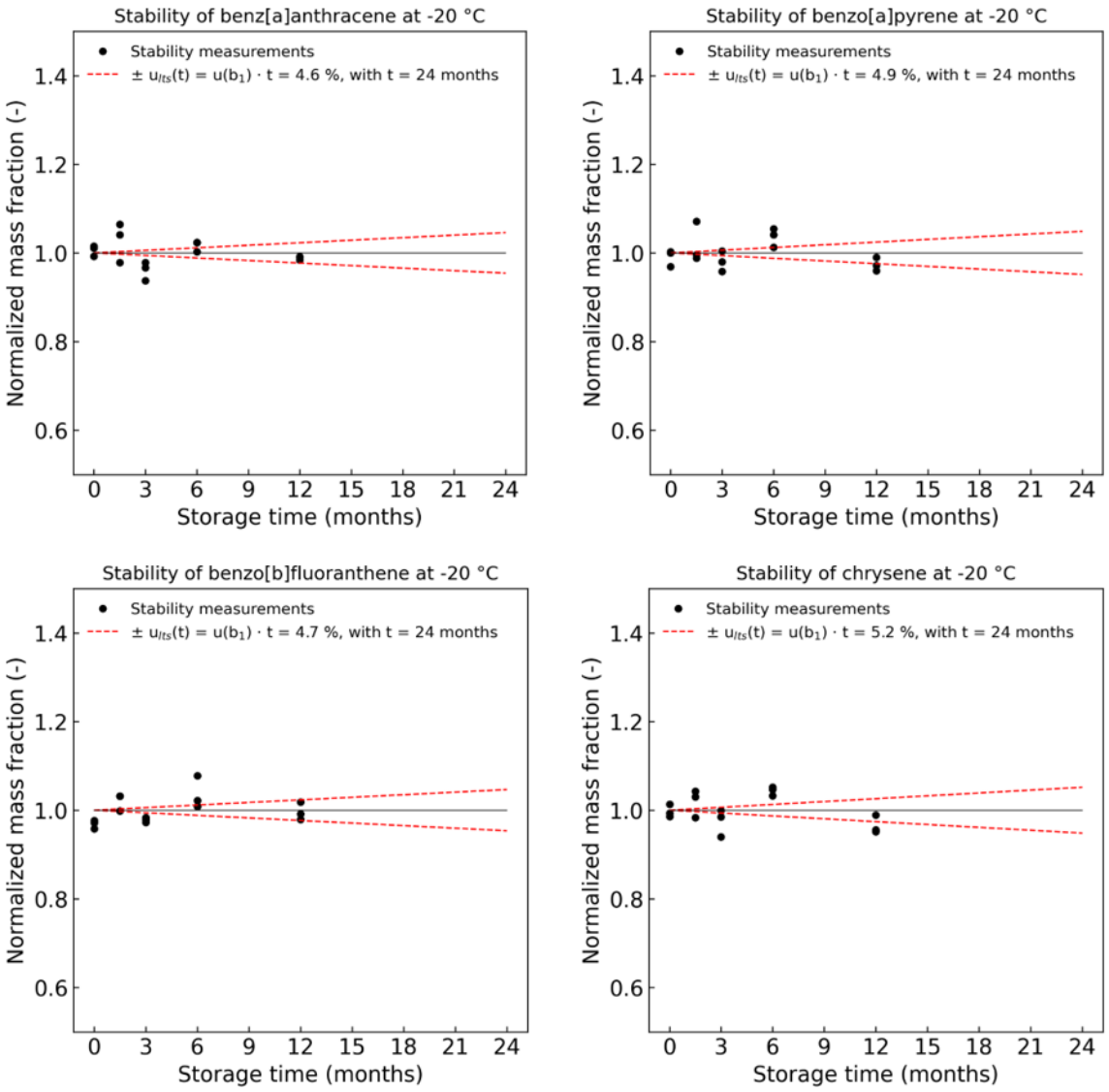


Figure 2: Long-term stability study of the four PAHs BaA, BaP, BbF and Chr with estimated uncertainty u_{lts} contributions.

The short-term stability was evaluated at a storage temperature of 45 °C. For the estimation of the uncertainty (u_{sts}) for potential instability when the material is exposed to higher temperatures during transportation a similar extrapolation model as for the long-term stability was applied. An exposure time of 2 weeks (t = 0.5 months) was used. The results are presented in table 5.

Table 5: Results of the short-term stability assessment at a storage temperature of 45 °C

PAH	**b_1 (months^{-1})**	**b_0 (-)**	**$s(b_1)$ (-)**	**t_{b1}**	**t_{crit}**	**$s(b_1=0)$ (-)**	**u_{sts} (%)**
BaA	-0.005666	1.008499	0.007300	0.776	2.364	0.007116	0.36
BaP	-0.000824	1.001237	0.006728	0.123	2.364	0.006300	0.32
BbF	0.003833	0.994251	0.005405	0.709	2.364	0.005234	0.27
Chr	0.008454	0.987319	0.005267	1.605	2.364	0.005762	0.29

INSTRUCTIONS AND SAMPLE DISTRIBUTION

Sample distribution

Each participant will receive one bottle containing approximately 30 g of powder. The sample will be shipped dry ice cooled and should be stored at -20 °C until analysis and not exposed to intense direct light and ultraviolet radiation. 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 samples should be equilibrated to room temperature before analysis. The sample preparation should be carried out immediately after opening the bottles.

Methods

The study will require solvent extraction, separation of the target analytes from interfering matrix components (clean-up), analytical separation, and selective detection of the target analytes in high protein matrix. Participants are anticipated to perform measurements by isotope-dilution (ID) gas chromatography mass spectrometry (GC-MS). However, other techniques such as liquid chromatography (LC) may be used.

RESULTS

Reporting of results

Each participant must send the results using the reporting excel sheet provided. The results should be sent via email to the study coordinator (gisela.umbricht@metas.ch) before the submission deadline. Submitted results are considered final and no corrections or adjustments of analytical data will be accepted unless approved by EURAMET. The results must include the mass fractions of the four PAHs in protein on an as-received basis and their associated standard and expanded (95 % level of confidence) uncertainties.

Participants will be requested to report a single estimate of the mass fractions in µg/kg for BaA, BaP, BbF and Chr in protein. The reported mass fractions will be the overall mean from replicate measurements. Reporting should include the values of the individual replicates in addition to the overall mean.

In addition to the quantitative results, participants will be instructed to describe their analytical methods and their approach to uncertainty estimation.

Evaluation of results

All results of the supplementary comparison will be evaluated against the supplementary comparison reference value (SCRV). The SCRV will be determined from the results of all NMIs/DIs participating in the supplementary comparison that have used appropriately validated methods with demonstrated metrological traceability.

Available Calibration Materials

Participants may establish the metrological traceability of their results using certified reference materials (CRMs) with stated traceability and/or commercially available high purity materials for which they determined the purity.

Solution CRMs including all target PAHs are available from NIST, SRM 2260a and SRM 1647f. Other solution and solid CRMs containing only one of the target PAHs are, to the best of our knowledge, available from the suppliers given in table 6.

Table 6: Certified reference materials available for use

CRM	Provider	Analyte
SRM 2260a	NIST	Aromatic hydrocarbons in toluene including all four target PAHS
SRM 1647f	NIST	Priority pollutant polycyclic aromatic hydrocarbons in acetonitrile including all four target PAHs
4213-a	NMIJ	Benzo(a)pyrene in 2,2,4-trimethylpentane
HRM-1017A	HSA	Benzo(a)pyrene
GBW08733	NIM	Benz(a)anthracene in acetonitrile
GBW08734	NIM	Benzo(a)pyrene in acetonitrile
GBW08728	NIM	Benzo(b)fluoranthene in acetonitrile

Isotopically labeled (deuterium or carbon-13) PAHs for use as internal standards are commercially available from various sources.

USE OF THE SUPPLEMENTARY COMPARISON EURAMET.QM-S15 IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in this supplementary comparison will demonstrate the measurement capabilities in determining mass fractions of low polarity organic compounds with molecular masses of about 150 - 500 g/mol in the range from 0.1 µg/kg to 100 µg/kg in high protein matrix. Beside value assignment, the demonstration of measurement capabilities may include extraction of target analytes from the matrix, separation of target analytes from interfering matrix components (clean-up), chromatographic separation and quantification of target analytes.

Core Competency Statements and CMC support

The Core Competencies template that will be used to claim competencies by the participants in this study is given in Appendix B.

REFERENCES

[1] ISO Guide 35:2017, Reference materials - Guidance for characterization and assessment of homogeneity and stability, International Organization (ISO), Geneva, 2017.

[2] Linsinger et al., Homogeneity and stability of reference materials, Accred. Qual. Assur., 2001, 6, 20.

[3] Linsinger et al., Estimating the uncertainty of stability for matrix CRMs, Fresenius J. Anal. Chem., 2001, 370, 183.

APPENDIX A: Reporting Form

"Participant_Information" worksheet

Reporting Form for Supplementary Comparison EURAMET.QM-S15, PAHs in Protein Matrix

Participiant Information

Please complete all pages of the reporting form and submit it by e-mail to:
gisela.umbricht@metas.ch

Reporting date (YYYY-MM-DD)	
Institute and address	
Submitted by (name)	
E-mail address	
Analyst(s)	
Bottle No.	

"Results" worksheet

Reporting Form for Supplementary Comparison EURAMET.QM-S15, PAHs in Protein Matrix

Summary of Results

Analyte	Abbreviation	Mass fraction w (µg/kg)	Combined standard uncertainty u_c (µg/kg)	Coverage factor k	Expanded uncertainty U (µg/kg)	Number of replicates n
Benz[a]anthracene	BaA					
Benzo[a]pyrene	BaP					
Benzo[b]fluoranthene	BbF					
Chrysene	Chr					

Results of each replicate

Replicate n	Mass fraction w of PAH (µg/kg)			
	BaA	BaP	BbF	Chr
1				
2				
3				
5				
6				
7				
8				
9				
Mean				
Standard deviation				

Additional comments	

"Analytical_Information" worksheet

Reporting Form for Supplementary Comparison EURAMET.QM-S15, PAHs in Protein Matrix

Information about the analytical procedure

Sample amount used for analysis (g)	
Sample pre-treatment (if applicable)	
Extraction method/conditions Briefly describe the extraction procedure (e.g. Soxhlet, ASE, Saponification, …) and the conditions (solvents, volume, time, temperature, …)	
Clean-up procedure Briefly describe the post extraction clean-up procedure (e.g. SPE, GPC, …)	
Analytical instrumentation used Specify the analytical instrument(s) you used (e.g. GC-MS/MS, LC-FLD, …) including the model (manufacturer, number, …). Please also specify the type of injector you used in case of GC-MS/MS.	
Chromatographic (pre)column(s) Specify the chromatographic pre and main columns (e.g. type, dimensions, manufacturer, …)	
Chromatographic conditions Describe the chromatographic conditions (e.g. mobile phase gradient for LC, temperature program for GC)	
Method of quantification Describe the method of quantification you used (e.g. external calibration, internal calibration, IDMS, ...)	

Calibration type Describe the type of calibration you used (e.g. single-point, bracketing, multi-point, matrix-matched, …)	
Calibration standards used Specify the standards you used for calibration (e.g. source, certified value, uncertainty, traceability, …)	
Internal standards used (if applicable) Specify the internal standards you used (e.g. compunds, source, ...)	
At which stage of the analytical process were the internal standards added? (if applicalbe)	
Purity assessment of calibrant (if applicable) If you determined the purity yourself, please describe the purity assessment (e.g. confirmation of identity, value and uncertainty assignment, analytical methods used, ...)	
MS method used Specify the MS method you used for ion detection (SIM, MRM/SRM, …)	
Ions monitored in MS Specify the ions monitored for native (calibrant) and isotopically labelled compounds (e.g. precursor and detection ions in MRM/SRM mode, collision energy, …)	
Detection methods other than MS (if applicable) Please specify your measurement procedure if you did not use an MS detector (e.g. fluorecence detection FLD).	
Additional comments and observations Please give additonal comments concerning the analytical procedure and share any special observations you made when analyzing the material.	

"Uncertainty_Budget" worksheet

Reporting Form for Supplementary Comparison EURAMET.QM-S15, PAHs in Protein Matrix

Information about the uncertainty budget

Give the complete equation(s) for the calculation of the mass fractions of each of the PAHs

Please provide details of all input quantities listed in the equation(s) and indicate how they were determined. Please give a complete description of how the estimates were obtained and combined to the overall uncertainty. The table below can be used to provide the uncertainty budget.

Benz[a]anthracene (BaA)

Input quantity	Source of uncertainty / determination	Typical value	Standard uncertainty	Unit	Type (A/B)

w(BaA)	Mass fraction of BaA		**µg/kg**	
u_c[w(BaA)]	Combined standard uncertainty		**µg/kg**	
k	Coverage factor			
u[w(BaA)]	Expanded uncertainty		**µg/kg**	

Benzo[a]pyrene (BaP)

Input quantity	Source of uncertainty / determination	Typical value	Standard uncertainty	Unit	Type (A/B)

w(BaP)	Mass fraction of BaP		**µg/kg**
u_c[w(BaP)]	Combined standard uncertainty		**µg/kg**
k	Coverage factor		
u[w(BaP)]	Expanded uncertainty		**µg/kg**

Benzo[b]fluoranthene (BbF)

Input quantity	Source of uncertainty / determination	Typical value	Standard uncertainty	Unit	Type (A/B)

w(BbF)	Mass fraction of BbF		**µg/kg**	
u_c[w(BbF)]	Combined standard uncertainty		**µg/kg**	
k	Coverage factor			
u[w(BbF)]	Expanded uncertainty		**µg/kg**	

Chrysene (Chr)

Input quantity	Source of uncertainty / determination	Typical value	Standard uncertainty	Unit	Type (A/B)

w(Chr)	Mass fraction of Chr		**µg/kg**
u_c[w(Chr)]	Combined standard uncertainty		**µg/kg**
k	Coverage factor		
u[w(Chr)]	Expanded uncertainty		**µg/kg**

APPENDIX B: Core Competency Table Form

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

EURAMET Supplementary comparison EURAMET.QM-S15	***NMI/ DI***	**Measurement of PAHs in Protein Matrix**
Scope of Measurement: Participation in this supplementary comparison would provide the opportunity to demonstrate participant's capabilities in determining low polarity organic compounds with molecular masses of about 150 – 500 g/mol in the mass fraction range from 0.1 µg/kg to 100 µg/kg in high protein matrix. Beside value assignment, the demonstration of measurement capabilities may include extraction of target analytes from the matrix, separation of target analytes from interfering matrix components (clean-up), chromatographic separation and quantification of target analytes.		
Competency	**✓,×, or N/A**	**Specific Information**
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*Indicate if you used a "pure material" or a calibration solution. Indicate its source and ID, eg CRM identifier*
Identity verification of analyte(s) in calibration material.[#]		*Indicate method(s) you used to identify analyte(s)*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).[#]		*Indicate how you established analyte mass fraction/purity (i.e., mass balance (list techniques used), qNMR, other)*
For calibrants which are a calibration solution: Value-assignment method(s).[#]		*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample		*Indicate method(s) you used to identify analyte(s) in the sample (i.e., Retention time, mass spec ion ratios, other)*
Extraction of analyte(s) of interest from matrix		*Indicate extraction technique(s) used, if any, (i.e. Liquid/liquid, Soxhlet, ASE, other)*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)		*Indicate cleanup technique(s) used, if any (i.e., SPE, LC fractionation, other)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)		*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system		*Indicate analytical system (i.e., LC-MS/MS, GC-HRMS, GC-ECD, other)*
Calibration approach for value-assignment of analyte(s) in matrix		*a) Indicate quantification mode used (i.e., IDMS, internal standard, external standard, other)* *b) Indicate calibration mode used (i.e., single-point calibration, bracketing, x-point calibration curve, other)*
Verification method(s) for value-assignment of analyte(s) in sample (if used)		*Indicate any confirmative method(s) used, if any.*
Other		*Indicate any other competencies demonstrated.*

Instructions:

- Replace "NMI/DI" with the acronym for your institution in the first cell of the middle column
- 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.