Certification Report

Certified Reference Material

BAM-A001

Polycyclic Aromatic Hydrocarbons (PAH)
in Olive Oil

Berlin, September 2023

Contact: Bundesanstalt für Materialforschung und -prüfung (BAM)
Department 1 – Analytical Chemistry; Reference Materials
12200 Berlin
Internet: https://www.bam.de

Coordination: Thomas Sommerfeld
Bundesanstalt für Materialforschung und -prüfung (BAM)
Division 1.7 – Organic Trace and Food Analysis
Richard-Willstätter-Str. 11
12489 Berlin
Phone: +49 (0)30/8104 5695
Email: thomas.sommerfeld@bam.de

Technical Support: Christian Jung, Tatjana Mauch, Juliane Riedel, Jan Lisec
Bundesanstalt für Materialforschung und -prüfung (BAM)
Division 1.7 – Organic Trace and Food Analysis
Richard-Willstätter-Str. 11
12489 Berlin

Sales: Email: sales.crm@bam.de
Internet: www.webshop.bam.de

Summary

This report describes the preparation, characterization and certification of the certified reference material (CRM) BAM-A001. BAM-A001 is available as extra virgin olive oil from a commercial product contaminated with polycyclic aromatic hydrocarbons (PAHs). This CRM is intended to be used for performance control and validation of analytical methods for the determination of PAHs in olive oils and similar vegetable edible oils.

Certified Values

Measurand [1)]	Mass fraction [2)] in µg kg^{-1}	Uncertainty [3)] in µg kg^{-1}
Benz[*a*]anthracene	1.72	0.13
Chrysene	2.87	0.31
Benzo[*b*]fluoranthene	1.30	0.14
Benzo[*a*]pyrene	1.44	0.09

1) PAH congener determined by sample preparation (extraction, clean-up) and gas chromatographic separation with mass spectrometric detection (GC-MS) using stable isotopic dilution analysis as specified in Section 3 of this certification report.

2) Unweighted mean value of 3 BAM workplace mean values (60 individual results in total).

3) Estimated expanded uncertainty U with a coverage factor of $k = 2$, corresponding to a level of confidence of approximately 95%, as defined in the Guide to the Expression of Uncertainty in Measurement (GUM), ISO/IEC Guide 98-3:2008.

Content

Page

List of abbreviations 5

1. Introduction 6

1.1 Need of reference materials for PAHs in food 6
1.2 Strategy of the certification project 6

2. Production of the candidate material 7

2.1 Procurement and preparation 7
2.2 Bottling of the candidate material 7

3. Analytical methods 7

3.1 Sample preparation 7
3.2 GC-MS analysis 8
3.3 Calibration and quantification 9

4. Homogeneity study 9

5. Stability study 10

5.1 Initial stability monitoring 10
5.2 Post-certification monitoring 12

6. Certification study 13

6.1 Design of the study 13
6.2 Results of certification 13
6.3 Uncertainty budget 14
6.4 Summary of certified values 15
6.5 Metrological traceability 15
6.6 Commutability 15

7. Information on the proper use of BAM-A001 15

7.1 Material description 15
7.2 Recommended use 15
7.3 Handling and safety instructions 16
7.4 Transport and storage 16
7.5 Analytical methods 16
7.6 Shelf-life 16

8. Information on and purchase of the CRM 16

9. References 17

10. Annexes 18

List of abbreviations

(if not explained elsewhere)

ANOVA	Analysis of Variance
BaA	Benz[*a*]anthracene
BaP	Benzo[*a*]pyrene
BbF	Benzo[*b*]fluoranthene
CCQM	Consultative Committee for Amount of Substance
Chr	Chrysene
CRM	Certified reference material
EI	Electron impact
FLD	Fluorescence detection
GC	Gas chromatography
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
ID	Inner diameter
ISO	International Organization of Standardization
ISTD	Internal standard
k	Coverage factor
L/L	Liquid/Liquid
LVI	Large volume injection
MIP	Molecularly imprinted polymer
MS	Mass spectrometry
NIST	National Institute of Standards and Technology
PAH	Polycyclic aromatic hydrocarbon
PTFE	Polytetrafluoroethylene
SEC	Size exclusion chromatography
SIDA	Stable isotope dilution analysis
SIM	Single ion monitoring
SRM	Standard reference material

1. Introduction

1.1 Need of reference materials for PAHs in food

Quality and safety are key factors for confidence in food, which is why they have become increasingly important in recent decades. Vegetable edible oils are known as healthy food used for cooking, ingredient of food products or direct consumption. Among these oils, olive oil is a traditional food product that is widely consumed throughout the world, with thousands of years of history. Particularly, extra-virgin olive oils represent a product of high nutritional quality [1]. World olive oil production has tripled in the last 60 years, reaching about 3.1 million tons in the 2021/22 crop year, with Spain being the largest producing country, accounting for 44% of world production [2].

But like all foods and vegetable oils, also olive oils may be contaminated with a wide range of lipophilic contaminants, such as polycyclic aromatic hydrocarbons (PAHs). PAHs are a large class of ubiquitous environmental and processing contaminants produced through incomplete combustion or pyrolysis of organic matter [3]. They are toxic to various organisms, including humans. Several PAHs have been proven to be genotoxic and mutagenic [4]. Olive oils are susceptible to PAHs contamination, generally due to the combined effects of several factors and processes, including environmental pollution (e.g., air pollution from automobile traffic), oil processing, and migration from food contact materials [1]. During the oil production process, PAHs are co-extracted from the olive surface due to their lipophilic polarity.

Monitoring of PAHs contamination in olive oil is therefore essential to ensure food safety and consumer protection. The first European maximum levels for four priority PAHs (PAH-4) in oils were established in 2011 and updated by Commission Regulation 2023/915 in 2023 [5]. In addition to the single maximum level for benzo[*a*]pyrene (BaP) of 2.0 µg/kg, the sum of BaP, benz[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF) and chrysene (Chr) is set to 10 µg/kg.

Although maximum levels for PAH-4 are in force since 2011, there is no certified reference material (CRM) for the determination of PAHs in olive oil available to date. Thus, a new CRM for PAHs in an extra virgin olive oil (BAM-A001) was developed at BAM. The produced CRM is intended to be used for performance control and validation of analytical methods for the determination of PAHs in olive oils and similar vegetable edible oils.

1.2 Strategy of the certification project

Since spiking of target analytes should generally be avoided for matrix CRMs whenever possible, a real-life extra virgin olive oil with PAHs contents in the range of the EU maximum level was targeted for CRM production, i.e. BaP ~ 2 µg/kg and PAH-4 ~ 10 µg/kg.

Although HPLC-FLD is still applied for routine analysis of PAHs in different samples, GC-MS combined with stable isotope dilution analysis (SIDA) using corresponding deuterated PAHs as internal standards (ISTD) is the method of choice for PAHs analysis regarding accuracy (trueness and precision). Therefore, SIDA-GC-MS was applied for the certification of the PAHs mass fractions of CRM BAM-A001.

Development of BAM-A001 was planned based on in-house certification at BAM with participation of three independent workplaces. Certification of BAM-A001 was

carried out based on ISO 17034 [6] and the relevant ISO-Guides for CRM developments [7, 8].

2. Production of the candidate material

2.1 Procurement and preparation

In a survey of 52 commercial olive oils (extra virgin) from different European countries of origin, all products purchased from retail markets in Berlin/Germany, two olive oils were identified with relevant PAHs contents and congener pattern. Once these oils were selected as suitable candidates, 11 bottles (each containing 0.5 L) of each candidate product were procured with the same lot numbers. All 22 bottles were visually checked for particles/turbidity. Afterwards, they were combined in a flask (11 L) and thoroughly mixed. The clear mixture was colored dark green.

2.2 Bottling of the candidate material

Prior to bottling, the olive oil mixture was purged with argon for 5 hours to remove residual oxygen. Afterwards, a total number of 779 units of BAM-A001 was produced by filling a volume of 14 mL oil in 20 mL amber glass vials using a dispenser. Before filling, the empty vials were flushed with argon to exclude oxygen. The vials were sealed immediately after filling using crimp caps with butyl/PTFE inlets. The vials were labeled with general information and specific numbers according to their filling sequence. The whole batch was stored at -20 °C in a freezer.

3. Analytical methods

3.1 Sample preparation

Analyses for homogeneity, stability and certification assessment were carried out at BAM according to the accredited in-house procedure BAM-1.7-PV043 [9]. This method complies with the performance characteristics specified in Commission Regulation (EU) No 836/2011 [10]. The method is based on extraction and clean-up of PAHs from olive oil sample by means of molecularly imprinted polymers (MIP) followed by gas chromatography coupled with a single quadrupole MS (GC-MSD) using a SIDA approach.

MIP sample preparation: All steps of sample preparation were gravimetrically controlled, the volumes stated below are indicative. 0.5 mL of olive oil sample were weighed into a 2 mL glass vial with screw cap (PTFE inlet). After adding 100 µL of ISTD-solution (deuterated PAH-mix) and dilution with 400 µL of cyclohexane, the sample was extracted by MIP (SupelMIP® SPE-PAHS 50mg, volume 3 mL). The procedure contains the conditioning of the MIP column with 1 mL of cyclohexane, loading the diluted oil sample to bind the PAHs onto the MIP phase, and washing the loaded MIP column with 3 x 1 mL of cyclohexane. The PAHs are eluted by applying 3 x 1 mL of ethyl acetate. Finally, the eluate was evaporated to dryness and re-dissolved in 0.2 mL of toluene for GC-MS analysis (Table 1).

An additional sample preparation method was used for the in-house certification study based on liquid/liquid (L/L)-extraction followed by gel permeation chromatography (GPC). The GPC clean-up is comparable to the size exclusion chromatography (SEC) step applied in the standard procedure EN 16619 [11]. Both methods, MIP and L/L-extraction combined with GPC were successfully applied by BAM in the CCQM-K146 study (2018) to quantify BaP in olive oil.

L/L extraction and GPC sample preparation: An oil sample amount of 3.0 g was weighed into an 80 mL centrifuge glass tube with screw cap (PTFE inlet). After adding 10 µL of ISTD-solution (deuterated PAH-mix) and 10 mL of acetonitrile, the sample was extracted for 5 minutes using a vortex mixer followed by ultrasonication for 15 minutes. For better phase separation, the sample was centrifuged at 3800 rpm for 15 minutes. The acetonitrile extract (upper phase) was separated from the oily lower phase and transferred into a 250 mL Kuderna-Danish flask. The extraction step was repeated three more times, and the combined extract was evaporated at 45 °C and 200 mbar almost to dryness. The residue was re-dissolved in 1 mL of cyclohexane/ethyl acetate (1:1) and transferred to a 5 mL flask. After rinsing the Kuderna-Danish flask with 3x1 mL of cyclohexane/ethyl acetate (1:1) into the 5 mL flask and filling-up the flask to the mark, a volume of 4 mL of the clear yellow extract was injected into a GPC system (Separation column: 660 x 40 mm, ID = 25 mm; Bio-Beads S-X3, 200-400). The PAHs fraction was collected and transferred into a Kuderna-Danish flask. The solvent was evaporated at 45 °C and 250 mbar almost to dryness. Subsequent evaporation to dryness was completed in a gentle stream of nitrogen. The residue was re-dissolved in 1 mL of toluene and transferred to a vial for GC-MS analysis.

3.2 GC-MS analysis

All analyses for PAHs determination of BAM-A001 were performed by GC-MS using workplace-specific instrumental conditions and settings.

Tab. 1: Example of GC-MS instrumental settings used for PAH analysis of BAM-A001

Instrumental parameter	GC-MS system
GC system	6890N (Agilent)
Column	Select PAH (Agilent)
Column dimensions	30 m x 0.25 mm x 0.15 µm ID
Oven program	70°C (1 min) → (85°C/min) 180°C → (3°C/min) 230°C (7 min) → (28°C/min) 280°C (10 min) → (14°C/min) 350°C (3 min)
Carrier gas	He 5.0 (2 mL/min)
Injection	5 µL (LVI)
MS system	MSD 5975B inert XL (Agilent)
Ionization	70 eV (EI)
Acquisition mode	Single ion monitoring (SIM)

Native PAHs and deuterated ISTD were recorded in single ion monitoring (SIM) mode to increase measurement sensitivity and to reduce background noise. The MS-parameters are displayed Table 2.

Tab. 2: Example of MS-parameters for PAH measurements of BAM-A001

Compound	Sum formula	SIM (m/z)	Retention time (min)	Quantified using internal standard
Benz[*a*]anthracene	$C_{18}H_{12}$	228.1	26.6	D_{12}-Benz[*a*]anthracene
Chrysene	$C_{18}H_{12}$	228.1	27.1	D_{12}-Chrysene
Benzo[*b*]fluoranthene	$C_{20}H_{12}$	252.1	31.7	D_{12}-Benzo[*b*]fluoranthene
Benzo[*a*]pyrene	$C_{20}H_{12}$	252.1	34.2	D_{12}-Benzo[*a*]pyrene
D_{12}-Benz[*a*]anthracene	$C_{18}D_{12}$	240.2	26.4	---
D_{12}-Chrysene	$C_{18}D_{12}$	240.2	26.9	---
D_{12}-Benzo[*b*]fluoranthene	$C_{20}D_{12}$	264.2	31.6	---
D_{12}-Benzo[*a*]pyrene	$C_{20}D_{12}$	264.2	34.0	---

3.3 Calibration and quantification

PAHs were quantified using the corresponding deuterated congener as ISTD to perform SIDA-GC-MS, defined as primary ratio method. For native PAHs calibration, the certified standard SRM 2260a (NIST, Gaithersburg, USA) was used containing all relevant PAHs dissolved in toluene. The deuterated internal standards were provided via the PAH-Mix 9 (Dr. Ehrenstorfer GmbH, Germany) dissolved in toluene. At least six-point calibrations were used for the measured peak area ratios. The calibration functions used for quantification of the PAH congeners were assumed to be linear obtained by regression analysis.

4. Homogeneity study

The analytical method employed for this study was used as described in Section 3 (MIP sample preparation). The sample intake for each analysis was 0.5 mL and is therefore recommended as the minimum sample intake. Ten units were selected equidistantly from the produced batch of the 779 units numbered in the order of bottling. The selected units were analyzed in triplicate each. All 10 units were extracted under repeatability conditions on one single day (i.e. 10 x 3 = 30 extractions). All extracts were analyzed in randomized manner under repeatability conditions in such a way that all 30 extracts were quantified against one calibration. The measurements showed no trend, neither regarding the filling/bottling order nor regarding the GC-MS measurement sequence order. All measurement results of the homogeneity study are summarized in Annex A. All measurement data which were used for statistical evaluation can be obtained as an RData-file from https://doi.org/10.5281/zenodo.8380870 and opened/viewed using the free online tool *eCerto* [12] at www.bam.de/eCerto.
Table 3 contains the results of the homogeneity study, which was evaluated by using *eCerto* performing an analysis of variance (ANOVA) on a significance level (α) of $\alpha = 0.05$. Since the calculated P-values of all four PAHs were higher than $\alpha = 0.05$, the null hypothesis of the ANOVA cannot be rejected. This means, there is no statistical evidence to conclude that there are significant differences between the units, conversely, the PAH contents in the vials are not significantly different.

Tab. 3: Analysis of variance (ANOVA) and estimates for uncertainty contribution due to homogeneity for candidate material BAM-A001

PAH congener	Mean µg kg^{-1}	*n*	*N*	$M_{between}$ µg^2 kg^{-2}	M_{within} µg^2 kg^{-2}	P	$s_{bb,r}$	$s_{bb,min,r}$
Benz[*a*]anthracene	1.888	3	10	0.0050	0.0067	0.6700	---	0.0141
Chrysene	3.158	3	10	0.0050	0.0072	0.7101	---	0.0087
Benzo[*b*]fluoranthene	1.595	3	10	0.0049	0.0078	0.7559	---	0.0180
Benzo[*a*]pyrene	1.389	3	10	0.0097	0.0086	0.3933	0.0134	0.0217

Mean Mean of the homogeneity study (= mean of vial means)
n Number of replicate measurements of each vial
N Number of selected bottles for homogeneity study
$M_{between}$ Mean of squared deviation between units (from 1-way ANOVA)
M_{within} Mean of squared deviation within units (from 1-way ANOVA)
P P-value of ANOVA
$s_{bb,r}$ Relative standard uncertainty between the units: Estimate of inhomogeneity contribution according to equation 1 (s_{bb} / mean of homogeneity study)
$s_{bb,min,r}$ Relative standard uncertainty between the units: Estimate of inhomogeneity contribution according to equation 2 ($s_{bb,min}$ / mean of homogeneity study)

While the estimate of the inhomogeneity contribution s_{bb} was calculated based on Equation 1 in accordance with ISO Guide 35 [8], the inhomogeneity contribution $s_{bb,min}$ was calculated according to Equation 2, which is given in Annex C (informative, examples) of ISO Guide 35.

$$s_{bb} = \sqrt{\frac{M_{between} - M_{within}}{n}} \qquad \textbf{Eq. 1}$$

$$s_{bb,min} = \sqrt{\frac{M_{within}}{n}} \cdot \sqrt[4]{\frac{2}{N(n-1)}} \qquad \textbf{Eq. 2}$$

The larger of the two values s_{bb} and $s_{bb,min}$ is used as contribution due to inhomogeneity (u_{bb}) for the uncertainty budget of BAM-A001 in section 6.3, also recommended by Linsinger et al. [13].

5. Stability study

5.1 Initial stability monitoring

Experience with temperature-driven deterioration of PAHs exist for various matrices when PAHs are adsorbed on the particle surface of soils, sediment and wood or bound in elastomer matrices such as toys/consumer products. There are no experiences regarding stability of PAHs dissolved in a matrix such as olive oil. Selected units of the candidate material were submitted to an isochronous accelerated ageing [14] at temperatures between +4 °C and +60 °C over periods of 1 week to 12 months as shown in Table 4. After the respective periods of time individual units were stored at -20 °C. All units were analyzed for PAHs under repeatability conditions together with reference samples which had been kept at -20 °C since bottling. For PAH quantification the method described in Section 3 (MIP sample preparation) was employed.

Tab. 4: Accelerated ageing of BAM-A001: Exposition temperatures and time periods are displayed for selected units (bottle numbers)

Ageing time	+4 °C	+23 °C	+40 °C	+60 °C	Remark
1 week	---	060	030	090	*Initial short-term study*
2 weeks	---	240	210	270	
3 weeks	---	420	390	450	
4 weeks	---	600	570	630	
3 months	120	150	180	---	*Initial long-term study*
6 months	300	330	360	---	
9 months	480	510	540	---	
12 months	690	720	750	---	
12 months	660 / 778				*Reference samples (-20 °C)*

The PAHs values obtained for samples stored at elevated temperatures over time were referenced to the PAHs values from reference samples stored at -20 °C for 12 months (= recovery). For all PAH congeners recoveries of approximately 100% were calculated for each storage temperature (+4, +23, +40 and +60 °C). A trend analysis according to [8] did not show significant PAH degradation indicating a sufficient stability over a desirable minimum shelf-life of 5 years. Because of very low uncertainty contributions from long-term (in)stability (u_{stab}), u_{stab} was not considered for the uncertainty budget (s. section 6.3). Figure 1 illustrates the high stability of BAM-A001 using BbF as an example.

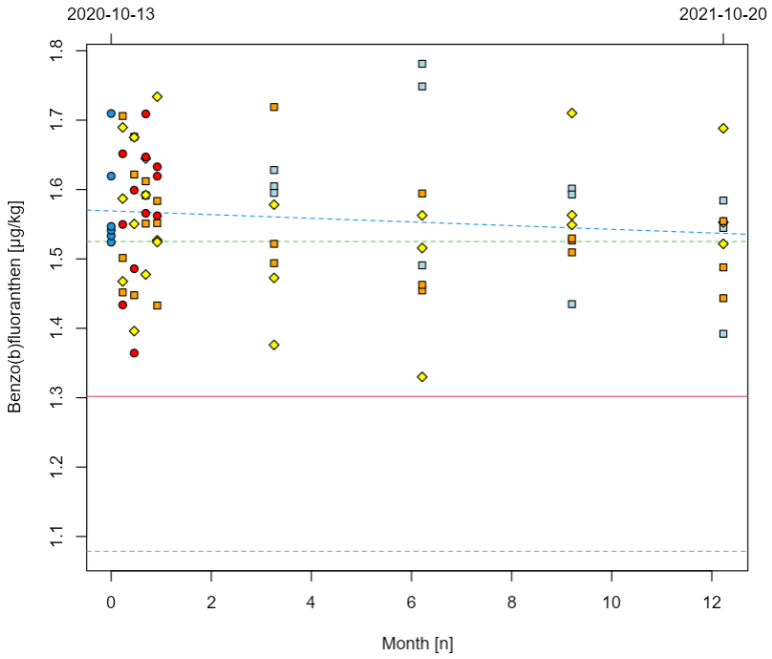

Fig. 1: BAM-A001 - Stability study for Benzo[*b*]fluoranthene (BbF) over 12 months. Horizontal lines show mean (red) and standard deviation (green) as determined in the in-house study. The blue dashed line represents the regression line of a linear model for the data obtained in the stability study. Symbol color indicates storage temperature (dark blue: -20°C, light blue: 4°C, yellow: 23°C, orange: 40°C, red: 60°C). Slope of the regression line is not significantly different from zero irrespective of the temperatures included in the model. Data points from all temperatures are shown for completeness.

There is also a kinetic approach (*Arrhenius* model) for stability data evaluation [15, 8], which allows to estimate analyte degradation rates for temperature dependent processes. It was successfully applied for a variety of organic compounds in food and environmental matrices in the past. However, the results of this approach have to be treated with caution if there is no degradation observed. Using the stability study data of BAM-A001 and the assumed *Arrhenius* model, it is possible for two PAH congeners (BaA, BaP) to estimate when the PAH content is likely to fall below the certified lower expanded uncertainty level. In the sense of a worst-case estimation, these calculations are carried out for the reaction rates at the upper confidence limit of the *Arrhenius* line (Fig. 2).

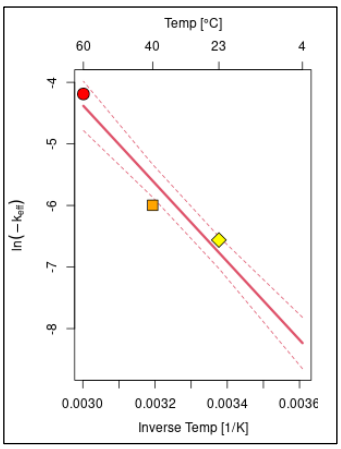

Fig. 2: Effective degradation rate (k_{eff}) of BaA vs. inverse temperature (1/T) for BAM-A001. The corresponding confidence interval (CI) for the line is also given. The data point at +4 °C could not be included in the graph because the calculated k_{eff} at +4 °C was shown to be positive over time.

Due to the high stability of the analytes in the olive oil material, the *Arrhenius* model was not applicable for Chr and BbF. However, acceptable evaluations could be done for BaA and BaP leading to a shelf-life estimation displayed in Table 5.

Tab. 5: Estimated period in months until which the certified PAH value of BAM-A001 remains within the expanded uncertainty *U* at different storage temperatures (shelf-life). In the sense of a worst-case estimation, calculations are carried out for the degradation rates at the upper confidence limit of the line as shown in fig. 2.

PAH congener	Self-life (months)		
	at +23 °C	at +4 °C	at -20 °C
Benz[*a*]anthracene	36	190	2105
Benzo[*a*]pyrene	43	160	1008

Even if no shelf-life estimations could be calculated for Chr and BbF using the *Arrhenius* model, the data for BaA and BaP indicate sufficient stability of BAM-A001 and confirm the outcome of the trend analysis. As a conclusion from the initial stability testing, storage temperatures of -20 °C and +4°C are sufficient for a desirable minimum shelf-life of 5 years. For this reason, u_{stab} was not considered for the uncertainty budget (s. section 6.3). A common user-end expiry date of two years after delivery from storage is established, provided that the sample is stored at the user's premises at a temperature of +4 °C or below. Short exposures to room temperature, e.g., during transport or handling, will not affect the stability of the reference material. Thus, BAM-A001 can be shipped unrefrigerated.

5.2 Post-certification monitoring

The initial stability estimation for BAM-A001 will be updated by periodic measurements of units stored at -20 °C and +4 °C during the availability of the reference material.

6. Certification study

6.1 Design of the study

The assignment of the certified PAHs mass fractions of BAM-A001 is based on an in-house study at BAM analyzing the candidate material at three independent workplaces (= three operators) using two different sample preparation methods and SIDA GC-MS for quantification (acc. to Section 3). For in-house certification, 15 units of the candidate reference material were randomly selected from the whole batch. Each operator analyzed 5 units applying own sample preparation, using individually prepared PAHs solutions for calibration and performed own SIDA GC-MS measurements including separate quantification and data evaluation. Four independent replicates were analyzed for each unit, resulting in 20 analyses per workplace/operator and 60 results for each PAH congener in total.

6.2 Results of certification

Table 6 displays the PAHs results of the three workplaces in the in-house certification study. All detailed measurement results are summarized in Annex B.

Tab. 6: Results of the three workplaces for the in-house certification study of BAM-A001 (values in µg kg^{-1})

PAH congener	Workplace 1		Workplace 2		Workplace 3	
	L/L & GPC		MIP		MIP	
	Mean	SD	Mean	SD	Mean	SD
Benz[*a*]anthracene	1.696	0.102	1.640	0.082	1.824	0.048
Chrysene	2.982	0.146	2.574	0.076	3.044	0.162
Benzo[*b*]fluoranthene	1.332	0.083	1.178	0.073	1.395	0.098
Benzo[*a*]pyrene	1.431	0.081	1.398	0.062	1.491	0.083

Mean Mean value of 20 results of each workplace (5 units x 4 replicates)
SD Standard deviation of 20 workplace results (combined variance of units and replicates); *informative, SD not used for further calculations*

The data sets of each workplace were examined for possible outliers, and the three workplace means were subjected to statistical tests (Grubbs, Dixon, Scheffé, Cochran). A method bias can be excluded or is very unlikely because the results of workplace 1 (GPC) were always between the results of workplace 2 and 3 (MIP). The results of the in-house certification study are summarized in Table 7.

Tab. 7: Results of the in-house certification study of BAM-A001

PAH congener	x_{char}	*SD*	*N*	$u_{char,r}$
Benz[*a*]anthracene	1.720	0.095	3	0.0317
Chrysene	2.866	0.255	3	0.0514
Benzo[*b*]fluoranthene	1.302	0.112	3	0.0495
Benzo[*a*]pyrene	1.440	0.047	3	0.0189

x_{char} Mean of workplace means (µg kg^{-1})
SD Standard deviation of workplace means (µg kg^{-1})
N Number of workplaces
$u_{char,r}$ Relative standard uncertainty of characterization (u_{char} / x_{char})

The uncertainty of characterization (u_{char}) was calculated as the standard uncertainty of the mean of workplace means according to Equation 3.

$$u_{\text{char}} = \frac{SD}{\sqrt{N}} \qquad \textbf{Eq. 3}$$

The arithmetic means of the three workplace means (x_{char}) are used as certified mass fractions (x_{cert}) of the PAHs of BAM-A001.

6.3 Uncertainty budget

The relative combined uncertainty $u_{\text{com,r}}$ for each PAH congener of BAM-A001 is calculated according to Equation 4.

$$u_{\text{com,r}} = \sqrt{u_{\text{bb,r}}^2 + u_{\text{stab,r}}^2 + u_{\text{char,r}}^2 + u_{\text{pur,r}}^2} \qquad \textbf{Eq. 4}$$

$u_{\text{bb,r}}$ Contribution due to between-bottle inhomogeneity (Section 4)

$u_{\text{stab,r}}$ Contribution of long-term (in)stability: set to zero for all PAHs (Section 5.1)

$u_{\text{char,r}}$ Uncertainty of characterization from in-house certification study (Section 6.2)

$u_{\text{pur,r}}$ Uncertainty of PAH calibration standard SRM 2260a (NIST) acc. to its certificate

Table 8 summarizes the data to calculate $u_{com,r}$ for the PAHs of BAM-A001.

Tab. 8: Contributions to the relative combined uncertainty of PAHs congers of BAM-A001

PAH congener	$u_{\text{bb,r}}$	$u_{\text{stab,r}}$	$u_{\text{char,r}}$	$u_{\text{pur,r}}$	$u_{\text{com,r}}$
Benz[*a*]anthracene	0.0141	0	0.0317	0.0059	0.0352
Chrysene	0.0087	0	0.0514	0.0119	0.0535
Benzo[*b*]fluoranthene	0.0180	0	0.0495	0.0042	0.0528
Benzo[*a*]pyrene	0.0217	0	0.0189	0.0120	0.0312

Finally, expanded uncertainties (U) were calculated by applying a coverage factor (k) of k=2 to the relative combined uncertainties, and stating absolute U values by including the certified PAHs mass fractions x_{cert} (Eq. 5).

$$U = x_{\text{cert}} \cdot u_{\text{com,r}} \cdot k \qquad \textbf{Eq. 5}$$

The expanded uncertainty U corresponds to a level of confidence of approximately 95%, as defined in the Guide to the expression of uncertainty in measurement (GUM), ISO/IEC Guide 98-3 [16].

6.4 Summary of certified values

The certified mass fractions of four PAHs and their corresponding expanded uncertainties are summarized in Table 9.

Tab. 9: Certified mass fractions of the PAHs of BAM-A001

PAH congener	Mass fraction (µg kg^{-1})	
	Certified value	*U*
Benz[*a*]anthracene	1.72	0.13
Chrysene	2.87	0.31
Benzo[*b*]fluoranthene	1.30	0.14
Benzo[*a*]pyrene	1.44	0.09

Rounding: The certified PAH mass fractions and expanded uncertainties are rounded according to DIN 1333 [17]. Intermediate results and combined uncertainties were not rounded; rounding was done for the certified values and expanded uncertainties only.

6.5 Metrological traceability

All certified values refer to the extractable and measurable amounts of the PAH congeners from the olive oil material. Two different sample preparation methods (MIP and L/L-extraction combined with GPC) have been used to cancel out (at least partially) systematic biases. Both methods were successfully applied by BAM in the CCQM-K146 study (2018) to quantify BaP in olive oil [18]. To ensure traceability of the PAHs contents as defined above, the gravimetrically prepared certified calibration standard SRM 2260a (NIST) was employed for the in-house certification study. Traceability was further established by using stable isotope dilution analysis using isotopically labeled PAHs internal standards for GC-MS measurements.

6.6 Commutability

BAM-A001 was produced from a commercial extra virgin olive oil without changes of the matrix. Therefore, the analytical behavior is the same as for routine samples of extra virgin olive oils. For samples other than extra virgin olive oils, the commutability must be re-assessed.

7. Information on the proper use of BAM-A001

7.1 Material description

BAM-A001 is available as extra virgin olive oil from a commercial product contaminated with PAHs. Each unit of BAM-A001 contains 14 mL of olive oil in a 20 mL amber glass vial, which was prefilled with argon. After bottling, the vial was sealed with a crimp cap with butyl/PTFE inlet.

7.2 Recommended use

BAM-A001 is intended to be used for performance control and validation of analytical methods for the determination of PAHs in olive oils. This CRM may also be applicable for other similar vegetable edible oils. BAM-A001 is explicitly meant only to be used in analytical laboratories. Before taking a subsample, the vial must

have reached ambient temperature. The minimum sample intake for one determination is 0.5 mL.

7.3 Handling and safety instructions

Any use other than intended should be avoided. The personnel handling the material must be trained adequately and follow the regular safety precautions of the laboratory. It is recommended to handle and disposed of the reference material in accordance with the guidelines for analytical food samples legally in force at the site of end use and disposal. The usual laboratory safety precautions have to be applied. No hazardous effects are to be expected when the material is used under conditions usually adopted for the analysis of food samples which are low or moderately contaminated with PAHs.

7.4 Transport and storage

BAM-A001 can be shipped at ambient temperature. On receiving, the material must be stored at a temperature equal to or lower than +4 °C. The stability of the reference material is not affected by short periods of handling at ambient temperature during transport and use. However, BAM cannot be held responsible for any alteration of the material occurring during handling and storage at the customer's premises, especially of opened units.

7.5 Analytical methods

Two different methods have been used for sample preparation including extraction of PAHs from the olive oil matrix and clean-up of the sample extracts: i) Molecularly imprinted polymer (MIP) cartridges intended for PAH analysis, and ii) L/L extraction using acetonitrile followed by gel permeation chromatography (GPC). Instrumental analysis of the PAHs was performed by gas chromatography mass spectrometry (GC-MS) using the corresponding isotopically labeled PAHs internal standards.

7.6 Shelf-life

From the initial stability study, a considerably large shelf-life well above a period of 5 years at a storage temperature of +4 °C (or below) was estimated. Since the dispatch to the end user may occur at any time during this period, the certified properties will be valid for 2 years after dispatch from BAM. The validity of this information will be maintained by post-certification monitoring.

8. Information on and purchase of the CRM

The certified reference material BAM-A001 is supplied by

Bundesanstalt für Materialforschung und -prüfung (BAM)
Department 1 – Analytical Chemistry; Reference Materials
Richard-Willstätter-Str. 11, D-12489 Berlin, Germany
E-Mail: sales.crm@bam.de

Each unit of BAM-A001 will be distributed together with a certificate containing the certified PAH values and their uncertainties, a material description, information of analytical methods and traceability and instructions for use, storage and safety.

Information on CRMs can be obtained from BAM homepage https://www.bam.de and BAM-webshop www.webshop.bam.de.

9. References

[1] Bertoz V, Purcaro G, Conchione C, Moret S. A review on the occurrence and analytical determination of PAHs in olive oils. *Foods* 2021, 10:324-349.

[2] The world of olive oil. International Olive Council: https://www.internationaloliveoil.org/the-world-of-olive-oil/

[3] Lee ML, Vassilaros DL, Later DW. Capillary column gas chromatography of environmental polycyclic aromatic compounds. *Intern. J. Environ. Anal. Chem. 1982*, 11:251-262.

[4] Scientific Committee on Food (SCF). Opinion of the Scientific Committee on Food on the risks to human health of Polycyclic Aromatic Hydrocarbons in Food; SCF/CS/CNTM/PAH/29 ADD1 Final; European Commission: Brussels, Belgium, 2002.

[5] Commission Regulation (EC) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. *Off J Eur Union*, L119:103-157.

[6] International Organization for Standardization, General requirements for the competence of reference material producers. ISO 17034. Geneva: ISO; 2016.

[7] International Organization for Standardization, Reference materials - contents of certificates, labels and accompanying documentation. ISO Guide 31. Geneva: ISO; 2015.

[8] International Organization for Standardization, Reference materials - guidance for characterization and assessment of homogeneity and stability. ISO Guide 35. Geneva: ISO; 2017.

[9] SOP BAM-PV043. Bestimmung von Polyzyklischen Aromatischen Kohlenwasserstoffen PAK in Olivenöl. BAM, 2021.

[10] Commission Regulation (EC) 836/2011 of 19 August 2011 amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs. *Off J Eur Union*, L215:9-16.

[11] CEN—European Committee for Standardization. Food Analysis-Determination of Benzo[a]Pyrene, Benz[a]Anthracene, Chrysene and Benzo[b]Fluoranthene in Foodstuffs by Gas Chromatography Mass Spectrometry (GC-MS); EN 16619:2015; CEN: Brussels, Belgium, 2015.

[12] eCerto Software, version 0.2.4, 2023-09-15, www.bam.de/eCerto

[13] Linsinger TPJ, Pauwels J, Van der Veen AMH, Schimmel H, Lamberty A. Homogeneity and Stability of Reference Materials. *Accred. Qual. Assur*. 2001, 6:20-25.

[14] Lamberty A, Schimmel H, Pauwels J. The study of the stability of reference materials by isochronous measurements. *Fres. J. Anal. Chem*. 1998, 360: 359–361.

[15] Bremser W, Becker R, Kipphardt H, Lehnik-Habrink P, Panne U, Töpfer A. Stability testing in an integrated scheme. *Accred. Qual. Assur*. 2006, 11: 489–495.

[16] ISO/IEC Guide 98-3 (2008), Uncertainty of measurement - Part 3: Guide to the expression of uncertainty in measurement (GUM:1995) ISO, Geneva, Switzerland.

[17] DIN 1333 (1992), Presentation of numerical data. Deutsches Institut für Normung, Berlin, 1992-02.

[18] Li XM, Li HM, Zhang QH, Lu XH, Li SQ, Koch M, et al. CCQM-K146 low-polarity analyte in high fat food: benzo a pyrene in olive oil. *Metrologia* 2020, 57 (1a):08017.

10. Annexes

All measurement data which were used for statistical evaluation can be obtained as an RData-file from https://doi.org/10.5281/zenodo.8380870 and opened/viewed using the free online tool *eCerto* [12] at www.bam.de/eCerto.

Annex A: Data of homogeneity testing for PAHs in BAM-A001

Tab. A1: Homogeneity measurements for benz[*a*]anthracene; all values in µg kg^{-1}

Vial-No.	#1	#2	#3	Mean	SD	RSD
H078	1.783	2.055	1.971	1.936	0.139	7.2%
H156	1.879	1.893	1.833	1.868	0.031	1.7%
H234	1.870	1.843	1.915	1.876	0.037	2.0%
H312	2.045	1.847	1.995	1.962	0.103	5.2%
H390	1.864	2.016	1.861	1.914	0.089	4.6%
H467	1.887	1.831	1.935	1.884	0.052	2.8%
H545	1.809	2.004	1.826	1.879	0.108	5.7%
H623	1.891	1.909	1.858	1.886	0.026	1.4%
H701	1.868	1.782	1.845	1.831	0.045	2.4%
H779	1.746	1.944	1.822	1.837	0.100	5.5%

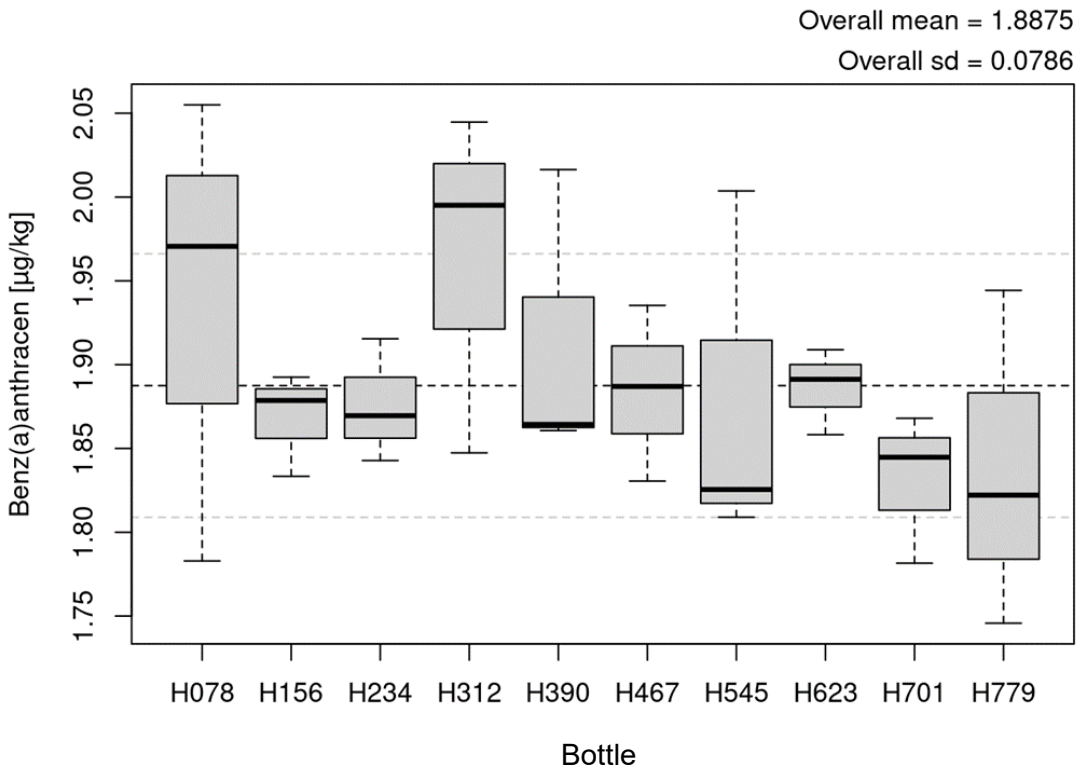

Fig. A1: Homogeneity measurements for benz[*a*]anthracene; boxes represent parameters of the measurement value distribution within each bottle (lower and upper box end: 25% (Q1) and 75% (Q3) percentile, strong line: median, whiskers: Q1 and Q3 extended by 1.5 x interquartile range); dashed horizontal lines represent mean (black) and standard deviation (grey) of bottle means; all values in µg kg^{-1}

Tab. A2: Homogeneity measurements for chrysene; all values in µg kg^{-1}

Vial-No.	#1	#2	#3	Mean	SD	RSD
H078	3.196	3.110	3.191	3.166	0.049	1.5%
H156	3.083	3.181	3.332	3.199	0.126	3.9%
H234	3.068	3.160	3.228	3.152	0.080	2.5%
H312	3.048	3.259	3.189	3.165	0.107	3.4%
H390	3.053	3.047	3.168	3.089	0.069	2.2%
H467	3.149	3.222	3.323	3.231	0.087	2.7%
H545	3.257	3.163	3.076	3.166	0.091	2.9%
H623	3.096	3.219	3.118	3.144	0.065	2.1%
H701	3.130	3.127	3.059	3.106	0.040	1.3%
H779	3.052	3.240	3.186	3.159	0.097	3.1%

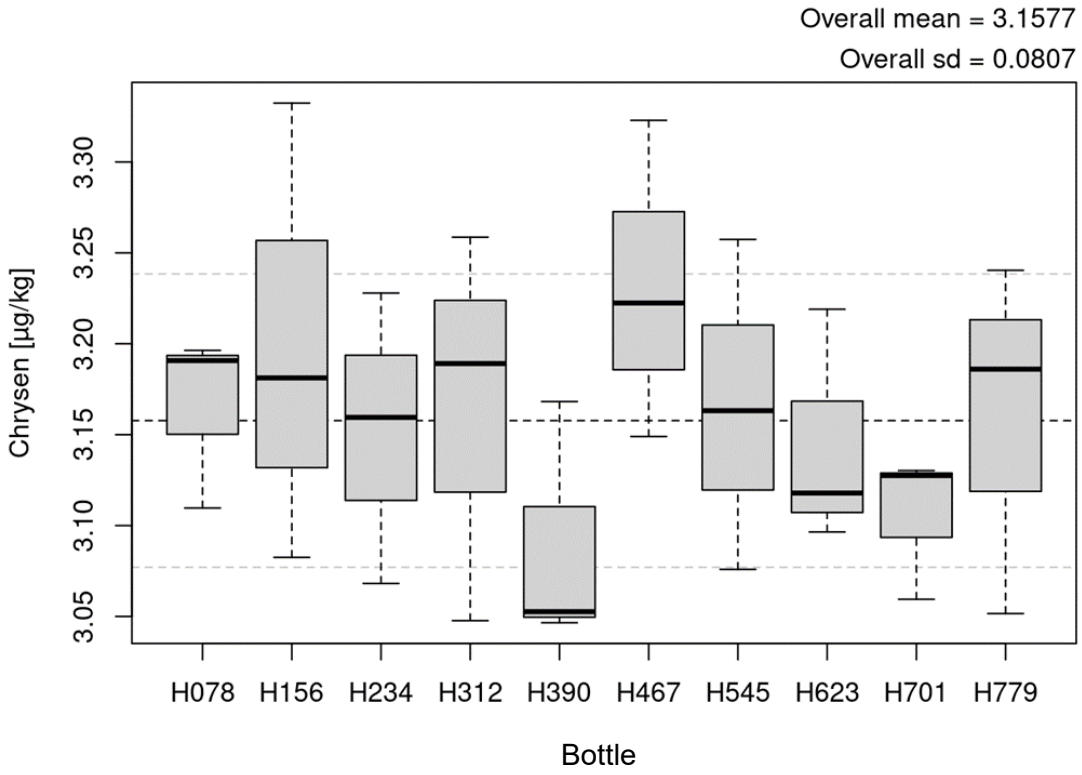

Fig. A2: Homogeneity measurements for chrysene; all values in µg kg^{-1}

Tab. A3: Homogeneity measurements for benzo[*b*]fluoranthene; all values in µg kg^{-1}

Vial-No.	#1	#2	#3	Mean	SD	RSD
H078	1.642	1.696	1.622	1.653	0.038	2.3%
H156	1.579	1.632	1.574	1.595	0.032	2.0%
H234	1.567	1.493	1.646	1.569	0.076	4.9%
H312	1.605	1.504	1.744	1.618	0.121	7.5%
H390	1.548	1.469	1.639	1.552	0.085	5.5%
H467	1.468	1.594	1.578	1.546	0.069	4.4%
H545	1.623	1.540	1.641	1.601	0.054	3.4%
H623	1.504	1.751	1.738	1.664	0.139	8.3%
H701	1.551	1.597	1.601	1.583	0.028	1.8%
H779	1.402	1.629	1.661	1.564	0.141	9.0%

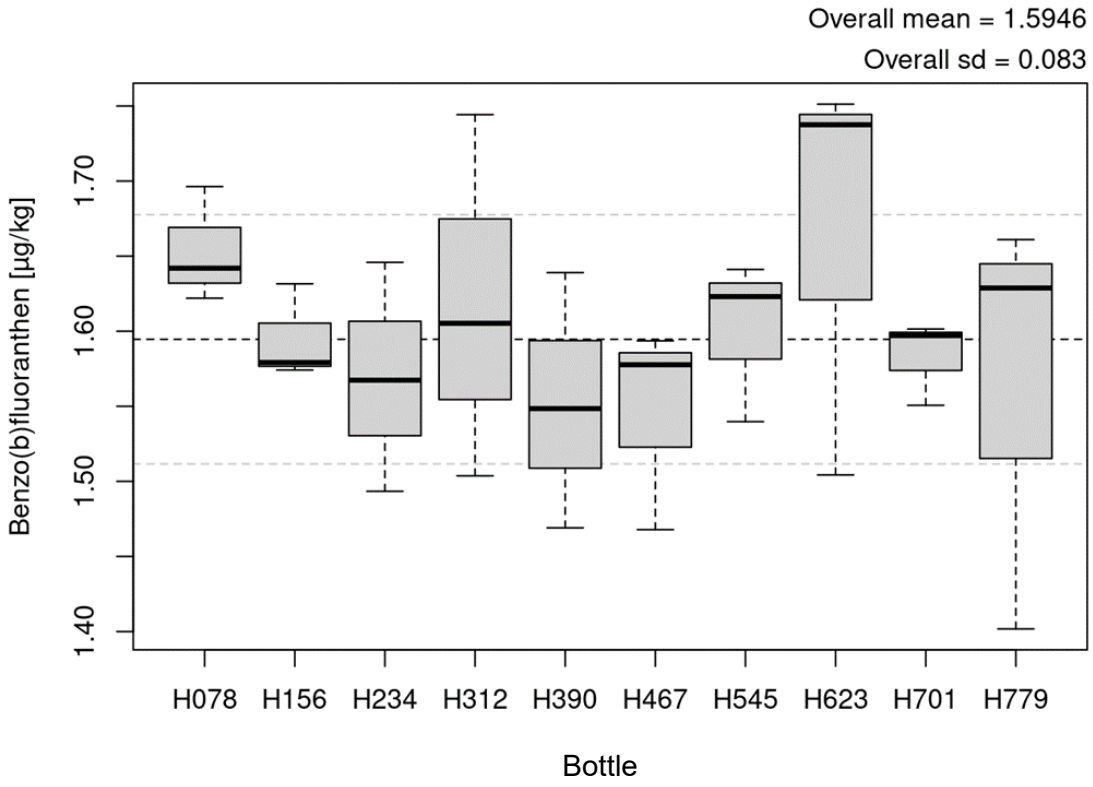

Fig. A3: Homogeneity measurements for benzo[*b*]fluoranthene; all values in µg kg^{-1}

Tab. A4: Homogeneity measurements for benzo[*a*]pyrene; all values in µg kg^{-1}

Vial-No.	#1	#2	#3	Mean	SD	RSD
H078	1.579	1.403	1.378	1.453	0.109	7.5%
H156	1.365	1.363	1.427	1.385	0.036	2.6%
H234	1.365	1.260	1.316	1.314	0.053	4.0%
H312	1.232	1.360	1.454	1.349	0.111	8.3%
H390	1.333	1.593	1.599	1.508	0.152	10.1%
H467	1.499	1.324	1.366	1.396	0.091	6.5%
H545	1.388	1.391	1.455	1.411	0.038	2.7%
H623	1.264	1.476	1.318	1.353	0.110	8.2%
H701	1.286	1.382	1.400	1.356	0.061	4.5%
H779	1.275	1.364	1.465	1.368	0.095	7.0%

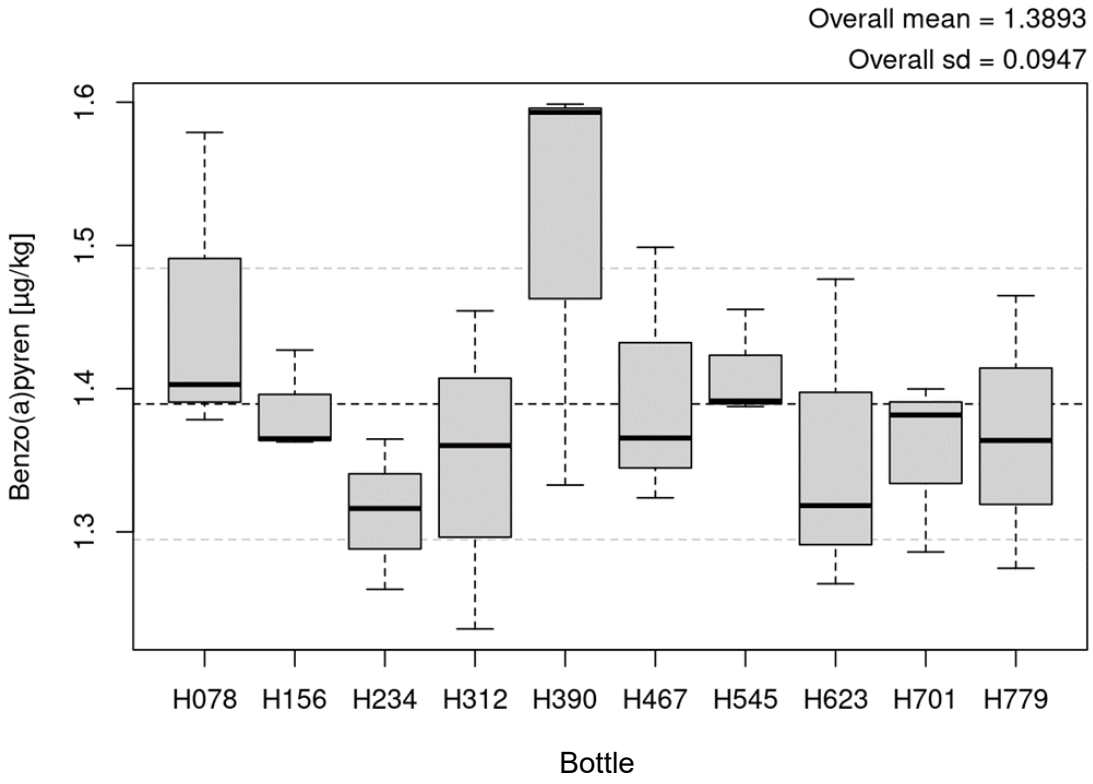

Fig. A4: Homogeneity measurements for benzo[*a*]pyrene; all values in µg kg^{-1}

Annex B: Data of in-house certification study for PAHs in BAM-A001

Tab. B1: Results of workplace 1 (**WP-1**); all values in µg kg^{-1}

Vial-No.	Replicate	BaA	Chr	BbF	BaP
WP-1/1	#1	1.650	2.947	1.311	1.377
	#2	1.755	3.193	1.312	1.568
	#3	1.897	3.273	1.581	1.689
	#4	1.686	2.987	1.340	1.399
WP-1/2	#1	1.633	2.832	1.236	1.385
	#2	1.685	3.018	1.324	1.400
	#3	1.625	2.862	1.248	1.458
	#4	1.993	3.229	1.403	1.480
WP-1/3	#1	1.676	2.975	1.343	1.402
	#2	1.649	2.891	1.318	1.386
	#3	1.633	2.925	1.297	1.396
	#4	1.769	3.047	1.407	1.440
WP-1/4	#1	1.641	2.881	1.326	1.406
	#2	1.777	3.170	1.431	1.507
	#3	1.677	2.925	1.312	1.388
	#4	1.643	2.949	1.330	1.383
WP-1/5	#1	1.711	3.025	1.378	1.454
	#2	1.664	2.994	1.310	1.407
	#3	1.595	2.771	1.206	1.352
	#4	1.564	2.737	1.230	1.339

Tab. B2: Results of workplace 2 (**WP-2**); all values in µg kg^{-1}

Vial-No.	Replicate	BaA	Chr	BbF	BaP
WP-2/1	#1	1.688	2.472	1.277	1.429
	#2	1.759	2.519	1.186	1.461
	#3	1.561	2.609	1.195	1.432
	#4	1.609	2.557	1.179	1.442
WP-2/2	#1	1.718	2.714	1.233	1.323
	#2	1.573	2.487	1.027	1.409
	#3	1.625	2.571	1.167	1.408
	#4	1.606	2.599	1.227	1.409
WP-2/3	#1	1.609	2.492	1.295	1.373
	#2	1.764	2.680	1.092	1.308
	#3	1.561	2.554	1.137	1.389
	#4	1.766	2.596	1.198	1.474
WP-2/4	#1	1.592	2.644	1.241	1.317
	#2	1.678	2.645	1.169	1.350
	#3	1.588	2.627	1.216	1.410
	#4	1.506	2.428	1.154	1.479
WP-2/5	#1	1.763	2.638	1.288	1.277
	#2	1.687	2.625	1.099	1.506
	#3	1.546	2.518	1.082	1.348
	#4	1.593	2.508	1.101	1.417

Tab. B3: Results of workplace 3 (**WP-3**); all values in µg kg^{-1}

Vial-No.	Replicate	BaA	Chr	BbF	BaP
WP-3/1	#1	1.835	2.818	1.354	1.431
	#2	1.759	3.107	1.522	1.533
	#3	1.851	3.089	1.419	1.534
	#4	1.824	3.283	1.481	1.362
WP-3/2	#1	1.874	2.917	1.228	1.578
	#2	1.744	3.074	1.477	1.484
	#3	1.850	3.053	1.487	1.554
	#4	1.792	3.255	1.324	1.427
WP-3/3	#1	1.825	2.939	1.220	1.371
	#2	1.836	2.782	1.378	1.478
	#3	1.750	3.204	1.290	1.483
	#4	1.822	3.236	1.520	1.479
WP-3/4	#1	1.867	2.873	1.381	1.459
	#2	1.897	3.200	1.347	1.387
	#3	1.866	2.893	1.548	1.574
	#4	1.860	3.223	1.470	1.691
WP-3/5	#1	1.746	3.156	1.372	1.544
	#2	1.801	2.844	1.268	1.409
	#3	1.793	3.029	1.382	1.468
	#4	1.895	2.900	1.438	1.576