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Report on the inter-laboratory comparison organised by the European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons for the validation of a method for quantifying the four EU marker PAHs in Food

Method based on pressurised liquid extraction,
size exclusion chromatography and solid phase extraction clean-up
with gas-chromatography coupled with mass spectrometric detection / isotope dilution

Donata Lerda, Patricia Lopez Sanchez, Szilard Szilagyi,
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European Union Reference Laboratory
Polycyclic Aromatic Hydrocarbons

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1 Summary

The European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons (EU-RL PAHs) operated by the Institute for Reference Materials and Methods (IRMM) of the Joint Research Centre (JRC), a Directorate General of the European Commission, organised a method validation study by inter-laboratory comparison (ILC-MVS) to evaluate the precision characteristics of a method for the determination of benz[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*b*]fluoranthene (BbF) and chrysene (CHR) in various foodstuffs. These four PAHs were recently identified by the European Food Safety Authority (EFSA) as the four marker PAHs for future legislative provisions [1].

The method is based on pressurised liquid extraction, size exclusion chromatography (SEC) plus solid phase extraction (SPE) on silica as purification steps, followed by quantification with gas-chromatography coupled with mass spectrometry (GC-MS) applying isotope dilution.

The matrices included in this study cover the food categories listed in Regulation (EC) No 1881/2006 [2]: oil (edible olive oil), meat (sausage minced and cooked meat), fish (smoked fish cooked and minced in pate format), infant formula (milk powder), mussels (homogenised mussels tissue), and cereals (wheat flour and extruded wheat flour). No blank sample was included in the study as recovery could be estimated from labelled standards added to samples at the beginning of the analytical procedure, prior to the extraction, assuming that labelled compounds behave as the corresponding native compounds.

Samples and consumables were sent to 18 laboratories from 11 EU Member States.

For oil, mussels, cereals and infant formula, participants were provided with blind duplicate samples, whilst for both meat and fish they received a single aliquot to be analysed in duplicate.

The relative standard deviations for reproducibility (RSD_R) ranged from 7 to 54 % and the relative standard deviations for repeatability (RSD_r) from 2 to 17 %. Calculated $HorRat_R$ and $HorRat_r$ were compliant with the legislative requirements as set in Commission Regulation (EC) No 333/2007 [3] for all matrices and analytes but for BaP in mussel's homogenate.

Commission Regulation (EC) No 333/2007 [3] lays down performance criteria that must be met by a method to determine BaP in food: $HorRat_r$ <2, $HorRat_R$ <2 for precision, and recovery (intended as yield of the method) has to range from 50 to 120 %. For the purpose of this study and in view of legislation which shall apply from 1st September 2012 [4, 5], these criteria were extended to the other three marker PAHs, BaA, BbF, and CHR.

Values obtained from the analytical procedure are to be considered as corrected for recovery due to the application of isotope dilution. However, as part of the quality control procedures, the recovery (yield) of the method is also calculated. The mean values of the yields obtained from participants ranged from 62 to 86%, depending on the matrix and on the analyte; therefore recoveries met the legislative criteria [3].

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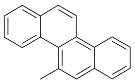
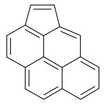
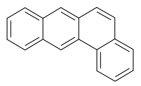
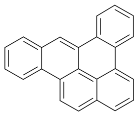
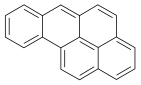
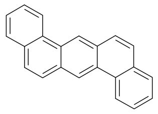
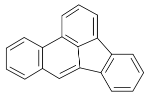
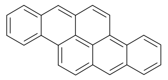
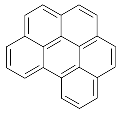
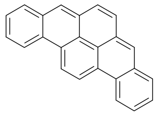
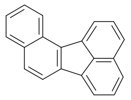
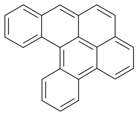
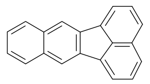
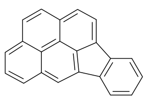
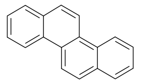
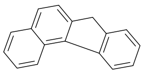
2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of organic substances. Their chemical structure consists of two or more fused aromatic rings. PAHs may be formed during the incomplete combustion of organic compounds and can be found in the environment and in other matrices, food included. In food, PAHs may be formed during processing and domestic food preparation, such as smoking, drying, roasting, baking, frying, or grilling.

In 2002 the European Commission's Scientific Committee on Food identified 15 individual PAHs as being of major concern for human health. These 15 EU priority PAHs should be monitored in food to enable long-term exposure assessments and to verify the validity of the use of the concentrations of benzo[*a*]pyrene (BaP) as a marker for a "total-PAH content" [6]. The toxicological importance of these compounds was confirmed in October 2005 by the International Agency for Research on Cancer (IARC), which classified BaP as carcinogen to human beings (IARC group 1), cyclopenta[*cd*]pyrene (CPP), dibenzo[*a,h*]anthracene, and dibenzo[*a,l*]pyrene as probably carcinogenic to human beings (group 2a), and nine other EU priority PAHs as possibly carcinogenic to human beings [7].

As a consequence, the European Commission (EC) issued Commission Regulation (EC) No 1881/2006 [2] setting maximum levels of BaP in food, Commission Regulation (EC) No 333/2007 [3] laying down sampling methods and performance criteria for methods of analysis for the official control of BaP levels in foodstuffs, and Commission Recommendation 2005/108/EC on the further investigation into the levels of PAHs in certain foods [8]. In order to distinguish this set of PAHs from a set of PAHs that has been addressed by the US Environmental Protection Agency, known as the 16 EPA PAHs, the terminology 15+1 EU priority PAHs was chosen. The monitoring of benzo[*c*]fluorene (BcL), which corresponds to the "+1" on the EU priority PAH list, had been recommended in 2006 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [9]. They are listed in Table 4.

Table 4: Names and structures of 15+1 EU priority PAHs

1	5-Methylchrysene (5MC)		9	Cyclopenta[*cd*]pyrene (CPP)	
2	**Benz[*a*]anthracene (BaA)**		10	Dibenzo[*a,e*]pyrene (DeP)	
3	**Benzo[*a*]pyrene (BaP)**		11	Dibenz[*a,h*]anthracene (DhA)	
4	**Benzo[*b*]fluoranthene (BbF)**		12	Dibenzo[*a,h*]pyrene (DhP)	
5	Benzo[*ghi*]perylene (BgP)		13	Dibenzo[*a,i*]pyrene (DiP)	
6	Benzo[*j*]fluoranthene (BjF)		14	Dibenzo[*a,l*]pyrene (DlP)	
7	Benzo[*k*]fluoranthene (BkF)		15	Indeno[1,2,3-*cd*]pyrene (IcP)	
8	**Chrysene (CHR)**		+ 1	Benzo[*c*]fluorene (BcL)	

(the four marker PAHs are in bold characters)

To evaluate the suitability of BaP as a marker for occurrence and toxicity of PAHs in food, the European Commission asked the European Food Safety Authority (EFSA) to review the previous risk assessment on PAHs carried out by the Scientific Committee on Food (SCF).

The scientific opinion on PAHs in food was published by EFSA's Panel on Contaminants in the Food Chain in June 2008 [10]. The Contaminants Panel concluded that benzo[*a*]pyrene on its own was not a suitable indicator for the occurrence of PAHs in food and that, based on the currently available data on occurrence and toxicity, four (PAH4) or eight substances (PAH8) would be the most suitable indicators of PAHs in food, with PAH8 not providing much added value compared to PAH4. Following these conclusions, it was agreed that maximum levels should be set for the sum of the four PAHs (PAH4 – BaA, BaP, BbF, and CHR) and they were included in Regulation (EC) No 835/2011 [4] amending the amendment of Commission Regulation (EC) No 1881/2006. In addition, maximum levels for BaP were maintained to ensure comparability of data. In the following the PAH4 will be also indicated as "the four marker PAHs".

As a follow-up of these last updates, and as part of its duties [11, 12], the EU-RL PAHs developed and validated, first in-house and then by an inter-laboratory comparison, a method for the quantitation of the four marker PAHs in the food matrices listed in legislation, with a possible extension to the other 12 PAHs (all materials were contaminated with most of the 15+1 EU priority PAHs and participants were asked to report as many of them as feasible). This study was part of the annual work-programme of the EU-RL PAHs as agreed with the Directorate General for Health and Consumers (DG SANCO).

The analytical procedure to be evaluated was based on pressurised liquid extraction, size exclusion chromatography (SEC) plus solid phase extraction (SPE) on silica as purification steps, followed by quantification with gas-chromatography coupled with mass spectrometry (GC-MS) applying isotope dilution. As the same sample extract preparation is applied to different foodstuffs, this method can be considered as a horizontal method and fulfils the respective requirements expressed in Regulation (EC) No 882/2004 [12] at Annex III note 3.

Due to the complexity of the method (see standard operating procedure - SOP in ANNEX 1), the study was organised so to enable participants to familiarise with it prior to the analysis of the blind samples. Therefore, the study was divided in three main steps. During STEP 1 participants were asked to analyse two samples, one oil and one fish (in the following indicated as training samples), with undisclosed contents and to report their results, together with the main deviations applied to the SOP, if any, and their comments/corrections on the SOP.
STEP 2 mainly consisted in the evaluation of data from STEP 1 to decide whether and when participants were ready to join the study. As the materials sent out to participants were also used in the past as PT materials, they were sufficiently homogeneous and the PAHs contents were known; therefore results received during this step could be evaluated for bias. In addition, as replicate results were reported, also precision was assessed. During this step, continuous feed-back was provided regarding possibly allowed deviations from SOP, analytical problems, consumables supply, etc. No statistical evaluation for outlier detection was carried out as the scope of the familiarisation steps was to maintain on board as many laboratories as possible and to harmonise the application of the method. An additional outcome for STEP 2 was the final SOP obtained modifying the draft SOP upon relevant comments from participants (this draft SOP is available as additional information upon request).
After conclusion of STEP 2, participants were asked to start with STEP 3 which consisted in analysing the blind samples, reporting (see ANNEX 2) to the organiser the results obtained, recoveries included (yields, calculated from the amount of labelled standard found in the purified extract in comparison with the amount added before the extraction step), and answering to a questionnaire (see ANNEX 3) The results reported in STEP 3 constituted the base for the evaluation of method performance.

3 Scope

This inter-laboratory comparison study aimed to evaluate the precision and to estimate recovery (yield of the respective labelled standards) of an analytical method, based on pressurised liquid/Soxhlet extraction (for solid samples only), clean-up by SEC and SPE in sequence, gas-chromatography coupled with mass-spectrometry detection (GC-MS) and quantification by isotope dilution of the four marker PAHs, BaA, BaP, BbF, and CHR, in several food matrices (see Table 6).
The validation was intended to cover, by using appropriate materials for each foodstuff, the respective maximum levels as set in Commission Regulation (EC) No 1881/2006 [2].

4 Participating Laboratories

Some of the National Reference Laboratories for PAHs of the EU Member States, some public and private food control laboratories, and other institutions, like universities and public research institutes joined the study.

Table 5: List of participants to the ILC for the validation of a method on PAHs in food

Institute	Country
National Food Administration, Region West, Århus	Denmark
Finnish Customs Laboratory	Finland
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL)	Germany
Eurofins WEJ Contaminants GmbH	Germany
Central Agricultural Office, Food and Feed Safety Directorate (NRL Food)	Hungary
Central Agricultural Office Food and Feed Safety Directorate (NRL Feed)	Hungary
Dublin Public Analyst Laboratory	Ireland
Chemical Controls srl	Italy
Consorzio I.N.C.A.	Italy
Public Health Research Centre of Valencia	Spain
Centro National de Alimentación – Spanish Food Safety and Nutrition Agency	Spain
Swiss Quality Testing Services (SQTS)	Switzerland
RIKILT Institute of Food Safety	The Netherlands

Eighteen laboratories subscribed to the exercise, of which 13 reported results (listed in Table 5). One of them, highlighted in grey, reported results for the first step (STEP 1, familiarisation with the method) only. One of the participants applied a method different from the proposed SOP. As a result, 11 sets of results were retained for the evaluation of method parameters.

5 Design of the study

5.1 Time frame

The study was announced via the EU-RL PAHs web-page and via CEN TC275/WG 13 on 5th and 12th of August 2010 respectively (ANNEX 4). Thirty-one laboratories expressed their interest in participating in the study and all of them were invited to subscribe with the aim of having the maximum number of participants, and of having different laboratory typologies, with different kinds of experience (e.g. control analysis, research, etc.), and different countries included.

The subscription PDF form was sent out on 1st of October with a deadline set on 15th of October 2010.

Together with the subscription form, participants also received the outline of the study and the draft SOP (which is available upon request, as mentioned in the previous paragraph) (ANNEX 5). Registered laboratories were asked to send back to the organiser comments and amendments to the draft SOP if necessary. Eighteen laboratories decided to join the study.

Parcels were dispatched on 9th of November 2010 (ANNEX 6) and the day after participants received the amended SOP, the instructions (ANNEX 7), the outline and the reporting forms for the two training samples by e-mail. Deadline for reporting results of STEP 1 was set on 3rd of December 2010; however, the last results were reported only on 7th of February 2011 due to the need of repeated feed-back and corrective action to get to a harmonised application of the SOP.

Laboratories having to perform additional analysis to correct the bias received additional ampoules of standards when necessary.

STEP 2 was considered concluded when all participants demonstrated, by reporting acceptable values for the training samples and by accepting to reduce as much as feasible deviations from the SOP (ANNEX 8), that they could carry-out the analysis on the blind samples applying correctly the SOP. STEP 3 started, upon conclusion of STEP 2, on 26th of January 2011 with deadline for reporting the results for blind samples set on 14th of March 2011. Participants received the reporting FORMS for all the samples, the questionnaire, and the specification sheets for the two training samples used for the familiarisation step (STEP1).

The last set of reported results was sent back to the organiser on 15th of April 2011 and some additional results for the mussels homogenate sample on the 5th of May 2011.

5.2 Materials and documents

Each participant received:

a. One inter-laboratory comparison sample receipt form to be sent back to the organiser upon reception of the parcel
b. The ILC-MVS outline
c. The instructions regarding materials storage, samples treatment, requirements, study STEPs, deadlines.
d. The final version of the SOP of the method
e. The participation code (LAB ID)
f. One gas-chromatographic column Select PAHs™ - 15 m lenght, 0.15 mm i.d., 0.10 µm df
g. The Mixed PAHs stock solution (in cyclohexane) to be used for calibration
h. The Mixed labelled PAH process solution (in toluene) to be used for spiking, calibration and recovery estimation
i. The Injection standard solution (in toluene) to be used for the calculation of the response factor and recovery estimation
j. 4 training samples (2+2) with undisclosed content of the four target PAHs (about 50 grams each)
k. 16 coded blind test materials (quantities varied from about 6 to about 50 g per aliquot)
l. Safety sheets for the solvents and for some of the PAHs
m. Two PDF FORMs for reporting the results for the training samples

In addition, upon STEP 3 starting, they received:

n. The specification sheets for the two training samples
o. Nine PDF Forms for reporting the results for the blind samples

p. A PDF Form with a questionnaire regarding general information on the participating laboratory, on their opinion on the design of the study, and on the deviations from the SOP they applied at their laboratory, if any.
q. The instructions for the use of the additional ampoules (ANNEX 9) of the mixed labelled PAH process solution

5.3 Organisation

Taking into account participants' comments and amendments, the SOP was changed, whenever it was considered appropriate, prior to the study.
Participants were given the option to choose between different techniques described in the SOP; these options were proven to be equivalent via an adequate set of experiments carried-out at the organiser's laboratories before carrying-out the ILC-MVS. They could choose:

1. between *n*-hexane and cyclohexane as the extraction solvent (see chapter 5 and paragraph 7.3 of the SOP),
2. between pressurised liquid extraction (PLE) and Soxhlet extraction (SE) (see chapter 6 and paragraph 7.3 of the SOP)
3. between PTV injection and split-splitless injection (see chapter 6 and paragraph 8.1 of the SOP)

Secret codes were attributed to each participant and each material aliquot, used for both dispatching and correct attribution of reported results.
Participants received for most of the samples an amount allowing one analysis only. In case the analytical process should fail, they could ask the organiser for receiving an additional aliquot of the sample. For fish and meat they received a higher amount, allowing multiple analyses, but they had to report duplicate results from the same sample (open duplicate).

In addition participants had to fill in a questionnaire (ANNEX 3), where they were asked, in particular, to report any deviation from the SOP they might have applied at their laboratory. This information was used to identify non compliances and outliers detected from the statistical tests.

6 Test Materials

6.1 Description

Different materials were obtained either from external suppliers, like the mussel homogenate, wheat flour based materials, meat, and fish, or obtained by spiking blank materials at IRMM premises, like oil and infant formula. These materials were selected with the scope of covering:

1. the food categories included in legislation [2]
2. the concentration levels of interest (below, above and around the maximum levels set for BaP [2]). These levels may differ from matrix to matrix but span from 1.0 µg/kg for infant formula and processed cereals (wheat flour and extruded wheat flour) to 10.0 µg/kg for bivalve molluscs (mussel homogenate).

It was decided to use the mussel homogenate reference material obtained from the International Atomic Energy Agency (IAEA), despite content levels for BaP, did not fulfil the criterion 2 (recommended value for BaP content was 0.9 µg/kg, about 1/10 of the maximum level of 10 µg/kg). This material was chosen, despite this draw-back, to allow evaluating method trueness, considering that it was the only reference material available for this matrix when the study was organised. When evaluating the method trueness, it should be also taken into account that the values for the analytes of interest were not certified but only recommended.

Table 6: Test samples and training samples *(contents reported were either estimated from in-house experiments or recommended by supplier)*

Sample description	**Sample code**	**Supplier**	**BaA µg/kg**	**BaP* µg/kg**	**BbF µg/kg**	**CHR µg/kg**	**Design**
Extruded wheat flour	EXWFLOUR	ICT, Prague	0.8	0.4 (1.0)	1.0	1.5	2 blind replicates
Smoked fish minced and cooked in a pate'-wise format	FISH_B	MRI, Kulmbach	3.5	9.6 (5.0)	4.6	5.6	2 open replicates
Infant formula – milk powder characterised by a low BaP content	IF_2010	IRMM (PT 2010)	2.1	0.9 (1.0)	4.0	1.4	2 blind replicates
Infant formula – milk powder characterised by a high BaP content	IF_2011	IRMM	6.0	6.0 (1.0)	5.0	3.9	2 blind replicates
Meat minced and cooked in a pate'-wise format	MEAT_A	MRI, Kulmbach	2.9	2.2	2.3	3.2	2 open replicates
Mussel homogenate – IAEA 432 – obtained according to the instructions***	MUSS_DRY	IAEA	3.8**	0.9** (10.0)	4.8**	5.5**	2 blind replicates
Olive oil characterised by a medium BaP content	OIL_1	IRMM	4.1	5.0 (2.0)	10.4	6.3	2 blind replicates
Olive oil characterised by a high BaP content	OIL_2	IRMM	8.1	12.4 (2.0)	5.2	7.8	2 blind replicates
Wheat flour	WHFLOUR	ICT, Prague	1.2	0.6 (1.0)	1.7	2.5	2 blind replicates
Smoked fish minced and cooked in a pate'-wise format	*Training sample FISH*	*MRI, Kulmbach*	*2.4*	*3.0*	*5.4*	*3.4*	*Open replicates*
Olive oil	*Training sample OIL*	*IRMM*	*8.9*	*2.9*	*9.2*	*8.1*	*Open replicates*

Marked in blue are the rows corresponding to the training samples (results reported for these samples were not used for the assessment of method parameters).

* among parenthesis the corresponding maximum level for the matrix as laid down in Commission Regulation (EC) No 1881/2006. As regards meat, a maximum level is set for smoked meat only.

** recommended value.

*** Participants were asked to determine the humidity of the sample by applying the procedure recommended in the Reference Sheet of the material (UUhttp://nucleus.iaea.org/rpst/Documents/rs_iaea-432.pdf). Two sets of results were reported for each of the two aliquots: one obtained without correction for humidity and one calculated on dry weight basis (MUSS_DRY). However, according to what recommended in the Reference Sheet of the material, only the second set of results was considered as valid and included in this study.

6.2 Preparation and verification

Test samples

The test materials were used as obtained from the supplier or from the in-house preparation.

In addition to the materials received, participants were required to prepare the dry mussel homogenate, by applying the procedure described in the instructions (see ANNEX 7 and http://nucleus.iaea.org/rpst/Documents/rs_iaea-432.pdf), and to report results also on dry weight basis.

The PAHs contents of the materials listed in Table 6 were measured by applying the method protocol which constituted the SOP for this ILC-MVS.

Test solutions

A common calibrant was distributed for the preparation of the calibration curve. It was obtained from Dr. Ehrenstorfer (PAH-mix 183) and the concentration level was 10 µg/ml for all 15+1 EU priority PAHs.

The Mixed labelled PAH process solution and the Injection standard solution were prepared at IRMM. The dilutions were performed gravimetrically.

As far as the injection standard is concerned, 9-fluorobenzo[*k*]fluoranthene was supplied by Chiron A as a 0.1 µg/ml solution in toluene; participants received 4 ml ampoules of a 436 ng/ml solution in toluene.

The labelled PAHs were obtained from Cambridge International Laboratories, CIL, as 100 µg/ml solutions in toluene; participants received 8 ml ampoules of a solution with the contents reported in Table 7. A second preparation was necessary before STEP 3, due to running out of the first ampoules supplied (repetition of analysis during STEP1).

Table 7: Mixed labelled PAHs process solutions composition

Labelled compound	Content solution 09/11/2010 ng/ml	Content solution 07/02/2011 ng/ml
5-Methylchrysene methyl D_3	40.0	39.0
Benz[*a*]anthracene $^{13}C_6$	41.0	42.5
Benzo[*a*]pyrene $^{13}C_4$	41.0	41.4
Benzo[*b*]fluoranthene $^{13}C_6$	41.0	39.0
Pyrene $^{13}C_3$	41.1	43.4
Benzo[*ghi*]perylene $^{13}C_{12}$	40.9	41.5
Benzo[*k*]fluoranthene $^{13}C_6$	41.0	42.7
Chrysene $^{13}C_6$	40.9	43.3
Dibenzo[*a,e*]pyrene $^{13}C_6$	41.0	41.9
Dibenzo[*a,h*]anthracene $^{13}C_6$	41.2	41.4
Dibenzo[*a,i*]pyrene $^{13}C_{12}$	33.7	39.1
Indeno[1,2,3-*cd*]pyrene $^{13}C_6$	41.0	41.3

6.3 Homogeneity

In the standards regarding ILC-MVSs, homogeneity of materials is considered as an important requirement to avoid having a significant contribution to the reproducibility standard deviation stemming from material heterogeneity. However neither in ISO standards [18, 19], nor in IUPAC/ AOAC protocol [16], a guide to homogeneity check criteria and respective design of experiments is included.

It was decided to assess homogeneity of the test samples according to the IUPAC Harmonised Protocol for Proficiency Testing [13]. The criterion to consider the material sufficiently homogeneous is given in Equation 1.

Equation 1 $\sigma^2_{sam} \le 0.07C$

Where

σ^2_{sam}: sampling variance

C: content of analyte in the sample as estimated by the organiser prior to materials dispatch

0.07 =0.3X0.22 *(0.22C is the reproducibility standard deviation defined in the Horwitz equation as modified by Thompson for concentrations below 120 ppb [14])*

For the materials tested at IRMM, ten samples were chosen at regular intervals along the packing order of each test material to check for possible trends in composition. All test materials were rated sufficiently homogeneous (and no trend was observed). Details of the homogeneity tests are given in Annex 10.

The two oil samples (OIL_1 and OIL_2) were not tested for homogeneity due to the fact that, being liquid, they were taken as homogeneous.

For the sample MUSS_DRY, in the certificate of the material IAEA 432 it is stated that the homogeneity of the material was checked by determining the concentration of some compounds (chlorinated pesticides and petroleum hydrocarbons) in 10 replicate analyses taken randomly in the bulk of the powder and that a one-way variance analysis indicated that the material could be considered homogeneous [15].

As regards the sample MEAT_A, it was tested for homogeneity when produced in 2006, at MRI, Kulmbach, and in that case 5 samples were analysed in duplicate (data were made available to IRMM and are included in ANNEX 10).

7 Evaluation of results

7.1 General

The study was designed and evaluated according to the IUPAC Harmonised Protocol (16, 17). Statistical analysis was performed along the lines of ISO 5725 [18, 19].

Values obtained for the precision and recovery for the four target analytes were verified against the method performance criteria set in Regulation (EC) No 333/2007 [3] for BaP: HorRat_R [20] values of less than 2 and recovery between 50% and 120%.

Questionnaire

Each participant was asked to answer several questions regarding the organisation of the study, the method, and the analytical process as carried out at their laboratory.

Apart from the subscription FORM, were they were asked to choose between options for extraction solvent, extraction apparatus, and injection system, participants were asked to report possible deviations from the methods at different stages of the analysis: at STEP 1, when first applying the method for the analysis of the training samples, and at STEP 3 when analysing the blind samples.

In case of reported relevant deviations from the SOP, results were excluded from the statistical evaluation.

Results and recoveries

For each of the 9 test samples analysed in duplicate (a total of 18 expected results, plus corresponding recoveries from each laboratory; two additional results were reported for the mussel homogenate without correction for humidity but they were not included in the study), the set of results and recoveries were evaluated as reported by participants.

All reported data are listed in ANNEXES 11 and 12 for analytes contents and recoveries respectively.

For the identification of outliers plus the calculation of means and of precision parameters the Excel template CLSTD.XLT version 3.6- 2/12/98, CSL, Food Science Laboratory, NORWICH, UK was applied. Statistical evaluation was carried out as specified in ISO-5725 [18, 19] for both the target analytes contents and the respective recoveries.

For the contents, in addition to the parameters calculated with the above mentioned Excel template, the Horwitz ratio for reproducibility, HorRat_R [20] (see Equation 2), was obtained with an in-house developed Excel sheet. The Horwitz equation as modified by Thompson for the concentrations below 120 ppb [14] was applied.

Equation 2 $$HorRat_R = \frac{RSD_R}{PRSD_R}$$

Where

RSD_R: relative standard deviation obtained from the study (reproducibility conditions)

$PRSD_R$: relative standard deviation as predicted by the Horwitz equation as modified by Thompson for concentrations below 120 ppb [21])

As required by the protocols applied [16, 17, 18, 19], received data were scrutinised for consistency and outliers. The graphical consistency technique (Mandel's *h* and *k* statistics) was applied globally for each analyte and all materials to obtain a first picture of possible inconsistent data sets. Then, for each material/analyte combination, the following was carried-out:

1. Removal of incomplete sets (one of the two duplicate results missing or below LOD/LOQ): they were considered as non-compliant and were removed prior the evaluation
2. Checking of sets of data for non-compliances and outliers; those last were identified applying Cochran's (C) and single and double Grubbs' tests (GS, GD) in this sequence.
3. Participants were contacted upon outlier identification to ask about possible causes, when not evident from the questionnaire
4. Exclusion of all statistical outliers from calculation of the mean values for contents of BaA, BaP, BbF, CHR and for the respective recoveries (yields)
5. Calculation of precision parameters (within-laboratory and between-laboratory variability) of contents and recoveries by applying one-way analysis of variance (ANOVA).

Non compliances are reported in the tables in ANNEXES 11 and 12 as grey cells. Outliers are reported in detail in paragraph 7.2 below.

Trueness

For the material MUSS_DRY, beside precision and recovery of the method, also its trueness was estimated based on the recommended values included in the specifications of the material (IAEA 432). It is not possible to numerically express trueness; however it is inversely related to systematic measurement error which may be estimated by measurement bias. Bias was calculated based on Equation 3.

Equation 3 $$Bias = | \bar{x} - Rx |$$

Where

$\bar{x}$: mean value obtained from reported data after outliers rejection

Rx: recommended value as reported in the material Reference Sheet (see Table 6 and bibliographic reference 15)

The trueness of the method for these matrices was considered as acceptable if the bias was smaller than the expanded uncertainty obtained from the combined uncertainty of the two values, where 2 was the coverage factor applied. In such a case the bias could be considered as not significantly different from 0.

Equation 4 $$\Delta < 2 * u_{\Delta}$$

Where

Δ: bias
2: coverage factor for 95% confidence interval
u_Δ: combined uncertainty of the bias

Equation 5 $$u_\Delta = \sqrt{u_{\bar{x}}^2 + u_{rec}^2}$$

Where
$u_{\bar{x}}$: standard uncertainty of the mean value obtained from this study
u_{rec}: standard uncertainty of the recommended value as reported in the material Reference Sheet

For calculating the two standard uncertainties to be entered in Equation 5, Equation 6 and 7 were respectively applied:

Equation 6 $$u_{\bar{x}}^2 = \frac{s_{R\bar{x}}^2}{n\bar{x}}$$

Where
$u_{\bar{x}}$: standard uncertainty of the mean value obtained from this study
$S_{R\bar{x}}$: reproducibility standard deviation obtained from this study
$n_{\bar{x}}$: number of accepted reported data for this study

Equation 7 $$u_{rec}^2 = \frac{s_{Rrec}^2}{n_{rec}}$$

Where
u_{rec}: standard uncertainty of the recommended value
S_{Rrec}: reproducibility standard deviation from the Reference Sheet of the material
n_{rec}: number of accepted reported data for the recommended value

Calculated u_Δ were checked for each analyte against the uncertainty function (Uf) as defined in the Commission Regulation (EC) No 333/2007 [3] (see Equation 8) to verify the appropriateness of trueness assessment.

Equation 8 $$Uf = \sqrt{(\mathrm{LOD}/2)^2 + (\alpha \mathrm{C})^2}$$

In Table 8 the outcome of trueness evaluation is summarised.

Where
Uf: maximum tolerated standard measurement uncertainty
LOD: required limit of detection (for the scope of this trueness assessment is set equal to 0.30 µg/kg)
α: numeric factor depending on the concentration C (for concentrations below 50 µg/kg is set equal to 0.2)

For the four PAHs, Uf was calculated for both $\bar{x}$ and the recommended value. The two Uf values were used to calculate a "combined Uf" (see Equation 9)

Equation 9 $$Uf_{\Delta} = \sqrt{Uf_{\bar{x}}^{2} + Uf_{rec}^{2}}$$

Where

Uf_{Δ}: maximum tolerated measurement uncertainty for the bias

Uf_{x} maximum tolerated standard uncertainty for the mean value obtained from this study

Uf_{rec} maximum tolerated standard uncertainty for the recommended value

In case the combined uncertainty of the bias should exceed the maximum tolerated uncertainty for the bias, the trueness requirements (Equation 4) would be considered inappropriate (too generous) to state method trueness.

Table 8 — Evaluation of trueness for the reference material IAEA 432 (MUSS_DRY)

Analyte	u_{rec}^{2}	$u_{\bar{x}}^{2}$	Δ	$2*u_{\Delta}$	$\Delta < 2*u_{\Delta}$	Uf_{Δ}	$u_{\Delta} < Uf_{\Delta}$
Benz[*a*]anthracene (BaA)	0.40	0.09	1.1	1.4	YES	1.9	YES
Benzo[*a*]pyrene (BaP)	0.01	0.03	0.02	0.4	YES	0.7	YES
Benzo[*b*]fluoranthene (BbF)	0.29	0.07	0.4	1.2	YES	2.6	YES
Chrysene (CHR)	1.32	0.27	0.8	2.5	YES	2.9	YES

As the combined uncertainty of the bias was for the four analytes below the respective maximum tolerated uncertainty, the trueness requirements were considered to be appropriate. As bias between the mean values obtained from the ILC-MVS data and the recommended values was not significant for all four target analytes, it can be concluded that the trueness of the method is verified.

7.2 Evaluation of questionnaire – deviations from the SOP

All answers regarding the method option selected, deviations from the SOP, and problems/anomalies observed by the participants during the analysis of blind samples were compiled in the tables in the ANNEXES 13, 14, and 15 respectively. Responses obtained for other questions included in the questionnaires distributed, after having verified that they did not imply any significant influence on the reported results, were not reported in the ANNEXES and are available as additional information upon request.

Scrutinizing the results, after rejection of outliers, with regards of the method options did not evidence any significant influence of the choice made by participants on the data reported for all four analytes and for all materials with the sole exception of extraction apparatus for BaA and CHR in mussel homogenate material MUSS_DRY: results reported by the three laboratories using Soxhlet extraction were significantly lower than those reported by the eight using PLE. Laboratory 6658 reported that less than 30 extraction cycles were carried-out with Soxhlet apparatus; this could be considered as a deviation from the SOP (recommending at least 7 hours and about 6 cycles/hour), however no outlier was statistically identified among results reported by 6658.

Critical points considered for possible non compliance were *significant* deviations from the SOP and problems/abnormalities reported by the participants.
This was the case for laboratory 7524: its results were excluded from the evaluation due to application of a method different from the one described in the SOP (in particular the solvent used for the PLE extraction, the SPE purification, and the GC-MS method are different from those described in the SOP).
In no other case reported deviations from the SOP were considered to be relevant for rejecting the whole set of results from a participant.
Problems reported by participants were evaluated in relation with the rejection of statistically identified outliers.

7.3 Evaluation of results

All data reported by participants are listed in ANNEXES 11 and 12 respectively for analyte contents and for recovery.
The method parameters, obtained from the reported data as described in 7.1, are listed in Tables 9 to 16 in the following. The splitting of method parameters in two tables for each of the four target analytes was done for an improved readability, but the method should be evaluated on the basis of the performances obtained for all the materials. In those Tables the evaluation of trueness for the mussel homogenate material is included and when the requirement set in Equation 4 is fulfilled "Compliant" is read in the corresponding cell (the trueness of the method was proven). For the estimation of recoveries, in some cases the number of valid data was not sufficient for the evaluation: in the corresponding cells in Tables 9 to 12 it is read "Not calculated".

In the following, for sake of shortness, the sample codes will be used (Table 6):

Table 9 — Precision data BaA from the ILC-MVS – first set of materials

Sample	**EXWFLOUR**	**FISH_B**	**IF_2010**	**IF_2011**	**MEAT_A**
Number of laboratories	11	11	11	11	11
Number of laboratories considered as non compliant	0	0	1	0	2
Number of outliers (laboratories)	1	0	1	1	0
Number of accepted results	10	11	9	10	9
Mean value, $\overline{x}$, µg/kg	0.6	3.5	1.3	5.0	2.8
Bias (Δ)	n.a.	n.a.	n.a.	n.a.	n.a.
$\Delta<2^*u_\Delta$ (see Equation 3)	n.a.	n.a.	n.a.	n.a.	n.a.
Repeatability standard deviation s_r, µg/kg	0.04	0.3	0.1	0.2	0.1
Repeatability relative standard deviation, RSD_r, %	7	8	9	4	5
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	0.1	0.8	0.3	0.6	0.4
Reproducibility standard deviation s_R, µg/kg	0.1	0.5	0.2	0.4	0.4
Reproducibility relative standard deviation, RSD_R, %	20	13	16	9	13
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	0.3	1.3	0.6	1.2	1.0
Recovery, %	66	67	66	65	71
HorRat value	0.92	0.59	0.73	0.40	0.60

n.a.: not applicable

Table 10 — Precision data BaA from the ILC-MVS – second set of materials

Sample	**MUSS_DRY**	**OIL_1**	**OIL_2**	**WHFLOUR**
Number of laboratories	10	11	11	11
Number of laboratories considered as non compliant	0	0	0	0
Number of outliers (laboratories)	0	1	0	1
Number of accepted results	10	10	11	10
Mean value, $\overline{x}$, µg/kg	2.7	4.1	7.9	1.0
Bias (Δ)	1.1	n.a.	n.a.	n.a.
$\Delta<2*u_{\Delta}$ (see Equation 3)	YES	n.a.	n.a.	n.a.
Repeatability standard deviation s_r, µg/kg	0.2	0.3	0.9	0.1
Repeatability relative standard deviation, RSD_r, %	9	8	12	8
Repeatability limit r [$r = 2,8 \times s_r$], µg/kg	0.7	1.0	2.6	0.2
Reproducibility standard deviation s_R, µg/kg	0.9	0.4	1.1	0.1
Reproducibility relative standard deviation, RSD_R, %	35	9	14	14
Reproducibility limit R [$R = 2,8 \times s_R$], µg/kg	2.6	1.0	3.2	0.4
Recovery, %	Not calculated	73	74	66
HorRat value	1.57	0.40	0.66	0.66

n.a.: not applicable

Table 11 — Precision data BaP from the ILC-MVS – first set of materials

Sample	**EXWFLOUR**	**FISH_B**	**IF_2010**	**IF_2011**	**MEAT_A**
Number of laboratories	11	11	11	11	11
Number of laboratories considered as non compliant	0	0	2	0	2
Number of outliers (laboratories)	2	2	1	1	0
Number of accepted results	9	9	8	10	9
Mean value, $\bar{x}$, µg/kg	0.5	9.2	0.6	4.8	2.2
Bias (Δ)	n.a.	n.a.	n.a.	n.a.	n.a.
$\Delta<2*u_{\Delta}$ (see Equation 3)	n.a.	n.a.	n.a.	n.a.	n.a.
Repeatability standard deviation s_r, µg/kg	0.1	0.3	0.1	0.2	0.1
Repeatability relative standard deviation, $RSD_{r,}$ %	12	3	15	5	4
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	0.2	0.7	0.3	0.7	0.2
Reproducibility standard deviation s_R, µg/kg	0.1	0.9	0.2	0.5	0.3
Reproducibility relative standard deviation, RSD_R, %	24	9	30	11	12
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	0.4	2.4	0.5	1.4	0.7
Recovery, %	79	78	72	75	76
HorRat value	1.09	0.43	1.37	0.49	0.56

n.a.: not applicable

Table 12 — Precision data BaP from the ILC-MVS – second set of materials

Sample	**MUSS_DRY**	**OIL_1**	**OIL_2**	**WHFLOUR**
Number of laboratories	10	11	11	11
Number of laboratories considered as non compliant	1	0	0	0
Number of outliers (laboratories)	1	1	2	2
Number of accepted results	8	10	9	9
Mean value, $\bar{x}$, µg/kg	0.9	4.7	11.9	0.7
Bias (Δ)	0.02	n.a.	n.a.	n.a.
$\Delta<2^*u_\Delta$ (see Equation 3)	YES	n.a.	n.a.	n.a.
Repeatability standard deviation s_r, µg/kg	0.1	0.2	0.3	0.1
Repeatability relative standard deviation, RSD_r, %	8	5	2	13
Repeatability limit r [$r = 2,8 \times s_r$], µg/kg	0.2	0.7	0.7	0.2
Reproducibility standard deviation s_R, µg/kg	0.5	0.5	0.8	0.1
Reproducibility relative standard deviation, RSD_R, %	54	10	7	18
Reproducibility limit R [$R = 2,8 \times s_R$], µg/kg	1.3	1.3	2.4	0.3
Recovery, %	Not calculated	80	78	78
HorRat value	2.46	0.44	0.32	0.84

n.a.: not applicable

Table 13 — Precision data BbF from the ILC-MVS – first set of materials

Sample	**EXWFLOUR**	**FISH_B**	**IF_2010**	**IF_2011**	**MEAT_A**
Number of laboratories	11	11	11	11	11
Number of laboratories considered as non compliant	1	1	2	1	2
Number of outliers (laboratories)	2	1	0	0	0
Number of accepted results	8	9	9	10	9
Mean value, $\bar{x}$, µg/kg	0.8	4.7	2.7	4.2	2.2
Bias (Δ)	n.a.	n.a.	n.a.	n.a.	n.a.
$\Delta<2^*u_\Delta$ (see Equation 3)	n.a.	n.a.	n.a.	n.a.	n.a.
Repeatability standard deviation s_r, µg/kg	0.04	0.3	0.4	0.2	0.1
Repeatability relative standard deviation, RSD_r, %	5	5	14	6	4
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	0.1	0.7	1.0	0.7	0.2
Reproducibility standard deviation s_R, µg/kg	0.2	0.5	0.5	0.5	0.3
Reproducibility relative standard deviation, RSD_R, %	22	10	18	12	13
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	0.5	1.4	1.4	1.4	0.8
Recovery, %	77	71	74	75	72
HorRat value	0.99	0.47	0.81	0.53	0.60

n.a.: not applicable

Table 14 — Precision data BbF from the ILC-MVS – second set of materials

Sample	**MUSS_DRY**	**OIL_1**	**OIL_2**	**WHFLOUR**
Number of laboratories	10	11	11	11
Number of laboratories considered as non compliant	1	1	1	1
Number of outliers (laboratories)	1	1	1	0
Number of accepted results	8	9	9	10
Mean value, $\bar{x}$, µg/kg	4.4	10.4	5.3	1.6
Bias (Δ)	0.4	n.a.	n.a.	n.a.
$\Delta<2^*u_\Delta$ (see Equation 3)	YES	n.a.	n.a.	n.a.
Repeatability standard deviation s_r, µg/kg	0.4	0.2	0.2	0.3
Repeatability relative standard deviation, RSD_r, %	8	2	4	17
Repeatability limit r [r = 2,8 × s_r], µg/kg	1.0	0.7	0.6	0.8
Reproducibility standard deviation s_R, µg/kg	0.7	0.9	0.5	0.4
Reproducibility relative standard deviation, RSD_R, %	16	8	10	25
Reproducibility limit R [R = 2,8 × s_R], µg/kg	2.0	2.4	1.5	1.1
Recovery, %	Not calculated	79	79	74
HorRat value	0.75	0.38	0.45	1.15

n.a.: not applicable

Table 15 — Precision data CHR from the ILC-MVS – first set of materials

Sample	**EXWFLOUR**	**FISH_B**	**IF_2010**	**IF_2011**	**MEAT_A**
Number of laboratories	11	11	11	11	11
Number of laboratories considered as non compliant	0	0	1	0	2
Number of outliers (laboratories)	2	1	0	2	0
Number of accepted results	9	10	10	9	9
Mean value, $\bar{x}$, µg/kg	0.8	5.4	0.9	3.3	3.1
Bias (Δ)	n.a.	n.a.	n.a.	n.a.	n.a.
$\Delta<2^*u_\Delta$ (see Equation 3)	n.a.	n.a.	n.a.	n.a.	n.a.
Repeatability standard deviation s_r, µg/kg	0.0	0.3	0.2	0.1	0.2
Repeatability relative standard deviation, $RSD_{r,}$ %	5	5	17	4	8
Repeatability limit r [r = 2,8 × s_r], µg/kg	0.1	0.8	0.4	0.4	0.7
Reproducibility standard deviation s_R, µg/kg	0.1	0.6	0.3	0.3	0.4
Reproducibility relative standard deviation, RSD_R, %	18	11	32	10	14
Reproducibility limit R [R = 2,8 × s_R], µg/kg	0.4	1.6	0.8	0.9	1.3
Recovery, %	65	60	70	62	65
HorRat value	0.81	0.48	1.43	0.47	0.65

n.a.: not applicable

Table 16 — Precision data CHR from the ILC-MVS – second set of materials

Sample	MUSS_DRY	OIL_1	OIL_2	WHFLOUR
Number of laboratories	10	11	11	11
Number of laboratories considered as non compliant	0	0	0	0
Number of outliers (laboratories)	0	2	1	0
Number of accepted results	10	9	10	11
Mean value, $\bar{x}$, µg/kg	4.7	6.2	7.7	1.6
Bias (Δ)	0.8	n.a.	n.a.	n.a.
$\Delta<2^*u_\Delta$ (see Equation 3)	YES	n.a.	n.a.	n.a.
Repeatability standard deviation s_r, µg/kg	0.4	0.2	0.7	0.2
Repeatability relative standard deviation, RSD_r, %	9	3	9	12
Repeatability limit r [r = 2,8 × s_r], µg/kg	1.2	0.5	2.0	0.6
Reproducibility standard deviation s_R, µg/kg	1.6	0.7	1.1	0.5
Reproducibility relative standard deviation, RSD_R, %	35	11	14	29
Reproducibility limit R [R = 2,8 × s_R], µg/kg	4.6	1.9	3.0	1.3
Recovery, %	Not calculated	73	72	64
HorRat value	1.59	0.49	0.64	1.34

n.a.: not applicable

Details on the identification of outliers and the graphic representation of reported data as Youden plots and as distribution of within laboratory average results are available as additional information upon request. The summary of outliers classified by participant is reported below.

Laboratory 3063 reported recovery values for BaP, BbF, and CHR in FISH B which were identified as outliers and rejected. No specific problem/abnormality was reported by the participant for the analytical process; deviations from the SOP were negligible and not considered as possible causes for the outliers.

Laboratory 6032 reported for BaA, BaP, BbF and CHR contents in EXWFLOUR, for BaA content in MEAT_A, and for BaA and BaP contents in WHFLOUR, values detected as outliers and rejected. In the questionnaire the participant reported that for one of the two aliquots of this material <the final solution was "dirty" (the peak shapes were wrong and the resolutions were bad)>. They also reported a poor resolution BbF/BkF and BkF/BjF for some other samples, however only for the two blind replicates of EXWFLOUR the corresponding values for BbF were detected as outliers and rejected.

Laboratory 6426 did not report any data for MUSS_DRY.

Laboratory 6482 reported recovery values for BaP in FISH_B which were identified as outliers and rejected. No specific problem/abnormality was reported by the participant for the analytical process; deviations from the SOP were not considered as possible causes for the outliers.

Laboratory 6584 reported for BaA content in IF_2010, for CHR content in IF_2011 for BbF content in MUSS_DRY, and for BaA content in OIL_1, values which were detected as outliers and rejected.
Recovery values reported for BaP in OIL_2, for BbF in IF_2010, OIL_2 and WHFLOUR, and for CHR in WHFLOUR were identified as outliers and rejected. No problem/abnormality was reported by the participant for the analytical process; deviations from the SOP (SEC conditions and PTV settings slightly changed) were not considered as possible causes for the outliers.

Laboratory 6595 reported constantly very high values for BbF for all materials. The problem was followed-up, however the participant could not identify a cause for such a biased series of data. In the questionnaire they reported <BbF Results for all samples exceed the working range, and the result have been estimated by extrapolating the calibration curve>. The corresponding values were considered as not compliant upon consultation with the participant.
They also reported for BaA in IF_2011 and for BaP in several materials (EXWFLOUR, FISH_B, IF_2010, OIL_1, OIL_2, WHFLOUR) values which were detected as outliers and rejected. No problem/abnormality was reported by the participant for the analysis of these samples; deviations from the SOP (SEC eluent and MS source temperature) were not considered as possible causes for the outliers.

Laboratory 7253 reported recovery values for CHR in OIL_1 which were identified as outliers and rejected. No specific problem/abnormality was reported by the participant for the analytical process for this sample; deviations from the SOP were negligible and not considered as possible causes for the outliers.

Laboratory 7283 reported for BbF and CHR contents in EXWFLOUR, for BaP, BbF, and CHR contents in FISH_B, for CHR content in IF_2011 and OIL_1, and for BbF and CHR contents in OIL_2, values which were detected as outliers and rejected.
Recovery values reported for BaA and CHR in IF_2011 and for BaA and CHR in OIL_1 were detected as outliers and rejected. No specific problem/abnormality was reported by the participant for the analytical process and deviations from the SOP (oven temperature program was reported as "slightly changed") were not considered as possible causes for the outliers (oven temperature program might influence peak resolution, in particular for fluoranthenes; in this case resolution of fluoranthenes was reported as compliant with requirements).

Laboratory 7669 reported for BaP content in MUSS_DRY, for BaP content in MUSSELS, for BbF and CHR contents in OIL_1, and for BaP content in OIL_2, values which were detected as outliers and rejected. No specific problem/abnormality was reported by the participant for the analytical process and they did not deviate from the SOP.

Reproducibility and repeatability values obtained from this study comply with legislative requirements [3] set for BaP and extended by analogy for the scope of this method to the other three marker PAHs (BaA, BbF, and CHR): HorRat values are in all cases below 2 but for BaP in MUSS_DRY.

For the evaluation of recoveries, all data statistically identified as outliers were rejected (see Tables in ANNEX 3).

Recoveries obtained are compliant with legislative requirements [3] being always in the range 50-120%. In the case of MUSS_DRY material, the number of reported data was not sufficient for a statistical evaluation.

The method is to be considered as validated by this study in the following concentration ranges, as defined from the four target PAH contents of the materials used:

- For BaA from 0.6 to 7.9 µg/kg.
- For BaP from 0.5 to 11.9 µg/kg (MUSS-DRY excluded)
- For BbF from 0.8 to 10.4 µg/kg
- For CHR from 0.8 to 7.7 µg/kg

8 Conclusions

Of the 18 invited participants, 11 reported compliant results for the STEP 3 of the study. The study can be considered as successful as performance of the method showed to be satisfactory for all materials of interest to confirm the scope of the method.

However, as regards legislative requirements for precision and recovery the HorRat value found for the combination BaP in MUSS_DRY was found to be not compliant (>2).

The applicability of Soxhlet extraction for the matrix MUSS_DRY was considered to be critical as significantly lower values were obtained than with PLE for CHR and BaA. Fulfilment of the requirements set in the procedure (at least 7 hours and about 6 cycles/hour) is considered as crucial for method applicability.

The JRC will submit this fully validated method to CEN TC 275/WG 13 and suggest it for formal standardisation.

The validation study included all 15+1 EU priority PAHs; however, priority was given to the four marker PAHs. For the 12 non-marker PAHs, an assessment of method performances based on the data reported by the laboratories joining this study might be envisaged.

9 Acknowledgements

We acknowledge that without the positive co-operation of all participants, this study would not have been possible.

We also thank Jean Charoud-Got and Håkan Emteborg from IRMM, Geel, Belgium for their support in the ampouling of test solutions, the former colleague José Ángel Gómez-Ruiz for his contribution to the development of the method, and Beatriz de la Calle and Franz Ulberth for their accurate revision of this report.

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10 ANNEXES

ANNEX 1 – Standard Operating Procedure (SOP) applied for the study

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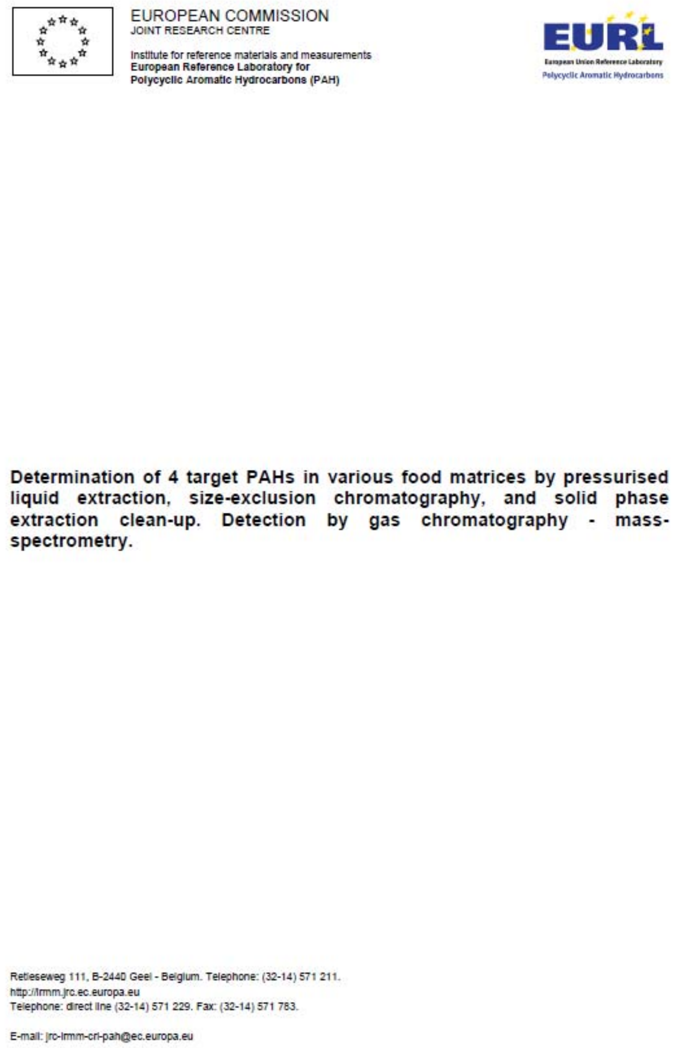
ANNEX 1 – Standard Operating Procedure (SOP)

The following document is the SOP obtained after revision from participants and is the SOP applied for the analysis of blind samples (STEP 3 of the study)



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Polycyclic Aromatic Hydrocarbons (PAH)

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Polycyclic Aromatic Hydrocarbons

Determination of 4 target PAHs in various food matrices by pressurised liquid extraction, size-exclusion chromatography, and solid phase extraction clean-up. Detection by gas chromatography - mass-spectrometry.

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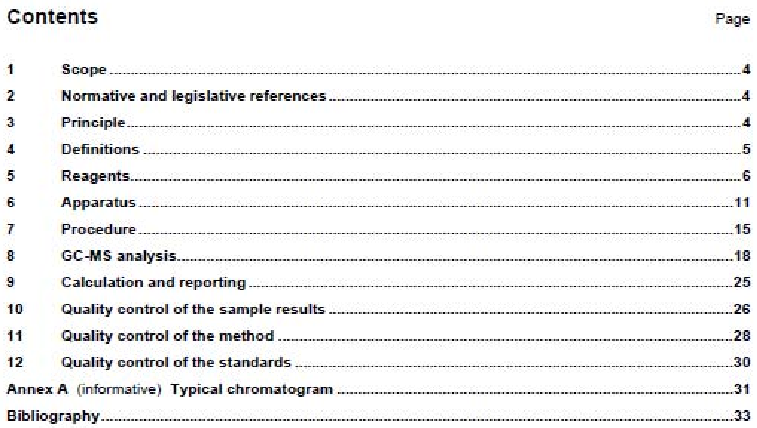


Contents Page

Foreword

THIS IS A STUDY FOR THE EVALUATION OF THE METHOD, NOT FOR ASSESSING THE PERFORMANCE OF THE LABORATORY. THE METHOD MUST BE FOLLOWED AS CLOSELY AS PRACTICABLE, AND ANY DEVIATIONS FROM THE METHOD AS DESCRIBED, NO MATTER HOW TRIVIAL THEY MAY SEEM, MUST BE NOTED ON THE REPORT FORM.

WARNING— the use of this protocol involves hazardous materials, operations and equipment. This protocol does not pretend to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This Standard Operating Procedure (SOP) specifies a method based on isotope dilution gas chromatography – mass spectrometry (GC-MS) for the determination of benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[*b*]fluoranthene (BbF), and chrysene (CHR) in food [1]

The method allows to quantify these four target PAHs in the presence of the other 12 PAHs that are part of the set of 15+1 EU priority PAHs: benzo[*c*]fluorene (BcL), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), cyclopenta[*cd*]pyrene (CPP), dibenz[*a,h*]anthracene (DhA), dibenzo[*a,e*]pyrene (DeP), benzo[*ghi*]perylene (BgP), dibenzo[*a,h*]pyrene (DhP), dibenzo[*a,i*]pyrene (DiP), dibenzo[*a,l*]pyrene (DlP), indeno[1,2,3-*cd*]pyrene (IcP), 5-methylchrysene (5MC) and of other possible interferences (e.g. Triphenylene - TRP) in various food matrices like edible oil, meat, smoked fish, bivalve molluscs, cereals and infant formula, most of them being included in Commission Regulation (EC) No 1881/2006:.

The extraction of PAHs from solid samples is performed by pressurised liquid extraction (PLE). Soxhlet extraction may be applied as an alternative to PLE. The sample is cleaned up using size exclusion chromatography (SEC) and solid phase extraction (SPE) in the given sequence. Analyte detection is achieved by gas-chromatography mass-spectrometry (GC-MS) in electron impact mode (EI).

The method is applicable for each of the four target PAHs in the content range between 0.5 µg/kg and 20 µg/kg.

2 Normative and legislative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 1042/1998, *Laboratory glassware -- One-mark volumetric flasks.*

Commission Regulation (EC) No 333/2007, *of 28 March 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 (Text with EEA relevance)[2]*

3 Principle

The test sample is homogenised, a test portion is mixed with desiccant, sand and the labelled internal standard mixture. It is then extracted with *n*-hexane or, alternatively, by cyclohexane, by pressurised liquid extraction. Soxhlet extraction has proven to give equivalent results compared to PLE, provided that a sufficient number of extraction cycles are performed (at least 7 hours of extraction with about 6 cycles/h)[I].

Co-extracted water is separated from the organic phase of the extract; then the organic extract is evaporated to small volume, filtered and purified by SEC, using a mixture of ethyl acetate and cyclohexane as eluent.

The extraction step is skipped for edible oils. With this kind of matrix a portion of the sample is diluted with a mixture of ethyl acetate and cyclohexane. Then the labelled internal standard mixture is added and the sample is directly processed by SEC.

HHHHHHHHH[I] Soxhlet extraction was tested only with *n*-hexane

After SEC, 200 µL of toluene is added as a keeper to the collected SEC fraction. The SEC fraction is then evaporated to about 200 µL, and further cleaned up by SPE on silica, using cyclohexane as eluent. The cleaned-up sample extract is evaporated again to 200 µL. Finally an injection standard is added to the sample prior to measurement by GC-MS.

The final extract is preferably injected into a programmable temperature vaporizer (PTV) inlet. However split/splitless injection may be applied alternatively. The chromatographic separation is achieved on a specific capillary column which allows the separation of the four target PAHs from the other EU priority PAHs and from triphenylene. The analytes are ionised by electron ionization (EI) at 70 eV. The target PAHs are recorded in Single Ion Monitoring (SIM) mode, and quantified by comparison with the labelled analogues.

4 Definitions

Laboratory sample: sample as prepared for sending to the laboratory and intended for inspection or testing [3] (i.e. the sample or subsample(s) received by the laboratory).

Test sample: sample prepared from the laboratory sample and from which test portions will be taken [3] (i.e. for this study it coincides with the laboratory sample).

Test portion: the quantity of material drawn from the test sample and on which the test or observation is actually carried out [3] (i.e. for this study the test portion is of 5 g for solid samples and 1 g for oil samples).

Final extract: solution containing the analytes; obtained after the last evaporation step and reconstitution of the extract.

Resolution (R_s): Ability of a column to separate chromatographic peaks; $R_s \equiv (t_{R2} - t_{R1})/[(w_{b1} + w_{b2})/2]$, where t_{R2} and t_{R1} are the retention times of the two peaks and w_b is the baseline width of the peaks. It usually is expressed in terms of the separation of two peaks (A value of 1.5 is considered sufficient for baseline resolution for two peaks of equal height.) [4].

Base peak (BP): The peak in a mass spectrum corresponding to the separated ion beam which has the greatest intensity [5].

Injection standard: Compound added before the GC-MS analysis to check the recovery of the labelled standards.

Labelled standard: deuterated or ^{13}C-labelled analogue of native PAH. The labelled standards are used to correct the losses of native PAHs during analysis. They are added to the test portion prior to the sample preparation.

Quantifier ion (Q_1): ion monitored in the mass spectra to quantify the PAH (normally it coincides with the base peak).

Qualifier ion (Q_2): ion monitored in the mass spectra for identification purpose.

Procedural blank: a blank sample made up of all reagents foreseen for the preparation of a test portion and processed in all respects as a test portion [6]. This kind of blank, tests the purity of the reagents but also other possible sources of contamination, like the glassware and the analytical instrument. **For this SOP:**

- UUUFor solid samples: 5 g of polyacrilic acid (5.5), 15 g of sand (5.6), 200 µl of mixed labelled PAHs process solution (5.20).
- For liquid sample (oil): 5 ml of the SEC eluent (5.12), 200 µl of mixed labelled PAHs process solution (5.20)

5 Reagents

5.1 General

Use only reagents of recognized analytical quality unless otherwise specified. Commercially available preparations with equivalent properties to the reagents listed may be used.

All chemicals and commercial preparations shall be stored according to the recommendations given by the supplier. Evaporation of solvents of any kind of solution shall be kept to a minimum during handling and storage. PAH standard solutions and sample extracts shall be kept in amber glassware in order to prevent UV light induced degradation of the analytes.

An analytical balance (6.3) is used for the gravimetrical preparation of both native and labelled PAH standard solutions listed in this SOP. In this way all concentrations are expressed as weight/weight. If necessary, the concentrations can be expressed as weight/volume by applying the density equation (Equation 1). All solutions and chemicals shall be used at 20°C ± 3 °C.

Equation 1

$$\rho = \frac{m}{v}$$

where

ρ	density
m	measured weight of the substance (g)
v	volume of the solution (ml)

Density of toluene (5.10) at 20°C is 0.8669 g/ml.

Density of cyclohexane (5.9) at 20°C is 0.7785 g/ml.

Density of the SEC eluent (5.12) at 20ºC is 0.8318 g/ml

WARNING 1 — Dispose of waste solvents according to applicable environmental rules and regulations.

5.2 Helium purified compressed gas (purity equivalent to 99.995% or better)

5.3 Nitrogen purified compressed gas (purity equivalent to 99.995% or better)

5.4 Disodium sulphate, (Na_2SO_4), anhydrous, granular

5.5 Poly(acrylic acid), partial sodium salt-graft-poly(ethylene oxide), granular, 90-850 µm particle size

5.6 Sand, 50 – 70 mesh particle size

5.7 *n*-Hexane

5.8 Acetone

5.9 Cyclohexane

5.10 Toluene

5.11 Ethyl acetate

5.12 SEC eluent and storage solvent for the SEC column

Mix 1 part per volume of cyclohexane (5.9) with 1 part per volume of ethyl acetate (5.11).

5.13 Glass wool, for laboratory use

5.14 SPE column

For the solid phase extraction clean-up, a silica column is used. Either commercial cartridges of 500 mg - 4ml - 50 µm particle size or custom-made with activated silica (water content should not exceed 1%) are used. The silica surface area should be around 500 m^2/g.

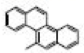
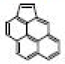
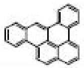
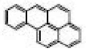
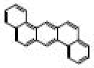
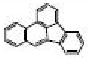
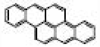
5.15 Native reference standards

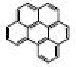
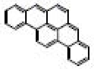
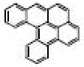
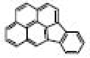
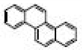
A mixed PAH stock solution will be provided for this study (see paragraph 5.19).

The list of native substances analysed with this method is reported in Table 1.

WARNING 2 — PAHs are degraded by UV-light. Protect PAHs containing solutions from light (keep in the dark, use aluminium foil or amber glassware).

Table 1 — Names and structures of the PAHs determinable by this method

Name*	CAS #	structure	Name*	CAS #	structure
5-Methylchrysene (5MC)	3697-24-3		Cyclopenta[*cd*]pyrene (CPP)	27208-37-3	
Benzo[*a*]anthracene (BaA)	56-55-3		Dibenzo[a,*e*]pyrene (DeP)	192-65-4	
Benzo[*a*]pyrene (BaP)	50-32-8		Dibenzo[a,*h*]anthracene (DhA)	53-70-3	
Benzo[*b*]fluoranthene (BbF)	205-99-2		Dibenzo[a,*h*]pyrene (DhP)	189-64-0	

Name*	CAS #	structure	Name*	CAS #	structure
Benzo[*ghi*]perylene (BgP)	191-24-2		Dibenzo[a,*i*]pyrene (DiP)	189-55-9	
Benzo[*j*]fluoranthene (BjF)	205-99-2		Dibenzo[a,*l*]pyrene (DlP)	191-30-0	
Benzo[*k*]fluoranthene (BkF)	207-08-9		Indeno[1,2,3-*cd*] pyrene (IcP)	193-39-5	
Chrysene (CHR)	218-01-9		Benzo[*c*]fluorene (BcL)	205-12-9	

*In parenthesis the short name is reported.

The four target PAHs which are taken in consideration for future legislation are in bold characters

5.16 Labelled reference standards

The labelled standards, applied for the quantification of the native PAHs included in the scope of this SOP and reported in Table 1, are listed in Table 2.

Alternatively to ^{13}C labelled standards, the deuterated analogues to the native PAHs included in this SOP could be applied.

For the method validation study a mixed labelled PAH process solution ready to use will be supplied (see paragraph 5.20).

Table 2 — List of the labelled PAHs

Labelled PAHs
5-Methylchrysene methyl D_3
Benzo[*a*]anthracene $^{13}C_6$
Benzo[*a*]pyrene $^{13}C_4$
Benzo[*b*]fluoranthene $^{13}C_6$
Pyrene $^{13}C_3$
Benzo[*ghi*]perylene $^{13}C_{12}$
Benzo[*k*]fluoranthene $^{13}C_6$
Chrysene $^{13}C_6$
Dibenzo[a,e]pyrene $^{13}C_6$
Dibenzo[a,*h*]anthracene $^{13}C_6$
Dibenzo[a,*i*]pyrene $^{13}C_{12}$
Indeno[1,2,3-*cd*]pyrene $^{13}C_6$

The labelled standards corresponding to the four target PAHs which are taken in consideration for future legislation are in bold characters

Some of the labelled standards are used for the quantification of more than one native PAH. Also the recovery of the method is calculated on the labelled standards. Table 3 lists the native PAHs with the corresponding labelled standards used for quantification..

Table 3 — Correspondence between labelled PAHs and native PAHs

Labelled PAH	Native PAH*
5-Methylchrysene methyl D_3	5-Methylchrysene (5MC)
Benz[*a*]anthracene $^{13}C6$	**Benz[*a*]anthracene (BaA)**
Benzo[*a*]pyrene $^{13}C_4$	**Benzo[*a*]pyrene (BaP)**
Benzo[*b*]fluoranthene $^{13}C_6$	**Benzo[*b*]fluoranthene (BbF)**
Pyrene $^{13}C_3$	Benzo[*c*]fluorene (BcL)
Benzo[*ghi*]perylene $^{13}C_{12}$	Benzo[*ghi*]perylene (BgP)
Benzo[*k*]fluoranthene $^{13}C_6$	Benzo[*j*]fluoranthene (BjF)
	Benzo[*k*]fluoranthene (BkF)
Chrysene $^{13}C_6$	**Chrysene (CHR)**
	Cyclopenta[*cd*]pyrene (CPP)
Dibenzo[a,*e*]pyrene $^{13}C_6$	Dibenzo[a,*e*]pyrene (DeP)
Dibenz[a,*h*]anthracene $^{13}C_6$	Dibenz[a,*h*]anthracene (DhA)
Dibenzo[a,*i*]pyrene $^{13}C_{12}$	Dibenzo[a,*h*]pyrene (DhP)
	Dibenzo[a,*i*]pyrene (DiP)
Dibenzo[a,*e*]pyrene $^{13}C_6$	Dibenzo[a,*l*]pyrene (DlP)
Indeno[1,2,3-*cd*]pyrene $^{13}C_6$	Indeno[1,2,3-*cd*]pyrene (IcP)

*In parenthesis the short name is reported.

The four target PAHs which are taken in consideration for future legislation and the corresponding labelled PAHs are in bold characters

5.17 9-fluorobenzo[*k*]fluoranthene

<u>A ready to use injection standard solution will be supplied for the method validation study (see paragraph 5.18).</u>

9-fluorobenzo[*k*]fluoranthene (FBkF) is used as injection standard (see 5.18).

5.18 Injection standard solution

The composition of the solution supplied for this study is around 436 ng/ml (500 ng/g) of FBkF in toluene.

This solution will be used for spiking of the samples prior to the measurement by GC-MS (see 7.9) to calculate the response factors and, from these, the recovery of the labelled standards (see Equation 5).

Store this solution in the dark and at a temperature below 10 °C. A solution stored in this way is stable for at least six months.

NOTE 1: The concentration will be expressed as ng/ml for all calculations concerning samples, as the addition of the injection standard is made by volume. It will be expressed as ng/g for all calculations concerning calibration solutions, as the addition of the injection standard is made by weighing.

5.19 Mixed PAH stock solution

A commercial solution in cyclohexane (10 µg/ml) is provided for this study.

Prepare, from the commercial solution of the native PAHs, a solution in toluene (5.10) with a concentration of approximately 0,4 µg/g. For this purpose, both the commercial solution and the toluene (5.10) are weighed with an analytical balance (6.3). E.g. 650 mg of the commercial solution and 20 g of toluene could be weighed in a 25 ml amber volumetric flask (6.1) (or in an amber vial - 6.2).

This solution will be used for the preparation of the mixed PAH intermediate solutions (5.21) and, finally, of the calibration standards (see 8.2). Store this solution in the dark and at a temperature below 10 °C. A solution stored in this way is stable for at least six months.

See the attached certificate for exact concentrations and the storage conditions of the commercial solution.

5.20 Mixed labelled PAH process solution

<u>A ready to use solution is supplied for this study. The composition of the solution is given in</u> Table 4.

In case deuterated PAHs should be used instead of the supplied ^{13}C labelled standards, it is recommended to prepare a mixed labelled PAH process solution with a nominal concentration of 40 ng/ml.

Table 4 — Composition of the mixed labelled PAHs process solution

Labelled PAHs	Concentration (ng/ml)	Concentration (ng/g)
5-Methylchrysene methyl D_3	40,0	46,2
Benzo[a]anthracene $^{13}C_6$	41,0	47,3
Benzo[a]pyrene $^{13}C_4$	41,0	47,3
Benzo[*b*]fluoranthene $^{13}C_6$	41,0	47,3
Pyrene $^{13}C_3$	41,1	47,4
Benzo[*ghi*]perylene $^{13}C_{12}$	40,9	47,2
Benzo[k]fluoranthene $^{13}C_6$	41,0	47,3
Chrysene $^{13}C_6$	40,9	47,2
Dibenzo[a,*e*]pyrene $^{13}C_6$	41,0	47,3
Dibenzo[a,*h*]anthracene $^{13}C_6$	41,2	47,5
Dibenzo[a,*i*]pyrene $^{13}C_{12}$	33,7	38,9
Indeno[1,2,3-*cd*]pyrene $^{13}C_6$	41,0	47,3

NOTE 2: The concentration will be expressed as ng/ml for all calculations concerning samples, as the addition of the labelled standards is made by volume. It will be expressed as ng/g for all calculations concerning calibration solutions, as the addition of the labelled standards is made by weighing.

5.21 Mixed PAH intermediate solutions

Prepare the intermediate solutions which will be used for calibration from the mixed PAH stock solution (5.19) by dilution in toluene (5.10).

These solutions will be used for the preparation of the calibration standards (see 8.2), together with the mixed labelled PAH process solution (5.20) and the injection standard solution (5.18).

The concentrations of PAHs in these solutions shall be approximately two times the concentrations of PAHs in the respective calibration solutions (see Table 8). The needed amounts of mixed PAH stock solution (5.19) shall be diluted in toluene (5.10) in amber volumetric flasks (6.1) of the volumes reported in Table 5. For this purpose, both the mixed PAH stock solution (5.19) and toluene (5.10) volumes indicated in Table 5 are weighed with an analytical balance (6.3).

The exact concentrations of all PAHs will be obtained taking into account the exact concentration of the mixed PAH stock solution (5.19), and the exact weights of this solution and of the toluene used for the preparation of each intermediate solution.

Table 5 — Nominal volumes of the mixed PAH stock solution (5.19) to be pipetted in order to prepare the reported volume of each of the concentration levels of the mixed PAH intermediate solutions

Mixed PAH intermediate solution	Nominal volume of mixed PAH stock solution (5.19) (ml)	Final volume of the intermediate solution (ml)	Nominal PAH* concentration in the intermediate solution (ng/g)
IS1	0,5	100	2,0
IS2	0,5	50	4,0
IS3	1,5	100	6,0
IS4	0,4	20	8,0
IS5	0,4	10	16,0
IS6	0,6	10	24,0
IS7	0,5	5	40,0
IS8	1,2	5	100,0
IS9	2,0	5	160,0
IS10	2,5	5	200,0
IS11	3,5	5	280,0
IS12	4,5	5	360,0

*the concentration level refers to the 15+1 EU priority PAHs contained in the commercial solution

Store these solutions in the dark and at a temperature below 10 °C. A solution stored in this way is stable for at least six months.

6 Apparatus

WARNING 3 — All glassware must be meticulously cleaned (except disposable glassware). The glassware is first thoroughly washed with laboratory detergent and hot water. All glassware used for the preparation and storage of standards (e.g. amber volumetric flasks 6.2) is rinsed before use with toluene (5.10) and dried in the fume hood under ambient conditions. Glassware used for other purposes (e.g. PLE solvent collection bottles 6.7.6) is rinsed before use with cyclohexane and acetone (5.8) and dried either in the fume hood or in a drying cabinet.

Usual laboratory apparatus and, in particular, the following:

6.1 Amber volumetric flasks, of 5 ml, 10, ml, 20 ml, 50 ml, and 100 ml

6.2 Amber glass vials, of 5 ml to 100 ml with PTFE layered screw caps [check Table 5 for the necessary volumes]

6.3 Analytical balance, with a mass resolution of 0,0001 g

6.4 Laboratory balance, with a mass resolution of 0,01 g

6.5 Aluminium weighing boat, or an equivalent vessel

6.6 Porcelain mortar and pestle, capacity of the mortar shall be at least 200 ml.

6.7 Pressurised liquid extraction (PLE) apparatus[‖] , (alternatively Soxhlet apparatus) comprising the following

6.7.1 PLE cells, with 33 ml of volume

6.7.2 Cellulose Filters, 30 mm diameter

6.7.3 Sample carousel (optional)

6.7.4 Degasser (optional)

6.7.5 Extraction chamber

6.7.6 Solvent collection bottles

6.7.7 Pressure control device, for the inlet and releasing of the pressuring gas (5.3) in the extraction cell

6.7.8 Temperature control device

6.7.9 Instrument control and data processing system, e.g. computer based (optional)

6.8 Soxhlet apparatus (alternatively to PLE apparatus), comprising the following

6.8.1 A heating mantle

6.8.2 Round bottom flask, of 250 ml capacity

6.8.3 Extractor, of 85 ml capacity

6.8.4 Cellulose thimble, fitting to the extractor (e.g. 33 X 94 mm), wall thickness of approximately 1 mm (10.0 µm nominal particle retention)

6.8.5 A condenser, of sufficient contact surface to obtain condensation of the *n*-hexane according to the requirements reported in paragraph 7.3.2 (e.g. Dinroth condenser with thread GL 14)

[‖] Soxhlet extraction was proven to be equivalent, provided that a sufficient number of extraction cycles are performed (at least 7 hours of extraction with about 6 cycles/h)

6.9 Evaporation apparatus, rotary evaporator or other evaporation/concentration workstation capable of evaporation under controlled temperature and vacuum.

The use of an apparatus with the control of the final volume is desirable. It shall comprise the appropriate glassware, either boiling flasks or glass tubes, depending on the apparatus in use. The required volumes are: 250 ml for the evaporation of PLE extracts (approximately 100 ml) and 100 ml for evaporation of the SEC purified samples (approximately 50 ml)

6.10 Glass Pasteur capillary pipettes, 230 mm

6.11 Glass graduated test tubes, 15 ml capacity (e.g. 17 X 120 mm), 0.1 ml graduation

6.12 Glass or plastic syringe, luer tip, 10 ml capacity

6.13 PTFE® membrane filter, Ø 25 mm and 5 µm pore size

6.14 Size Exclusion Chromatography (SEC) apparatus, comprising the following

6.14.1 Injection system, capable of injecting 5 ml

6.14.2 Sample vials, minimum capacity 5 ml

6.14.3 Liquid pump, suitable for a flow rate of 4,0 ml/min

6.14.4 A SEC column, with the following characteristics: 50 g of Bio-beads S-X3® in 25 x 500 mm

NOTE 3: Whether the SEC column is obtained by a commercial supplier or packed at the laboratory, the solvent used for the elution (5.12) shall be used for its preparation and storage.

6.14.5 Amber sample collection vials, 5 ml capacity

6.14.6 Collection bottles

Optionally, the apparatus could also include fraction collector, a fraction collector detector, an evaporating device, a sample carousel, and an instrument control and data processing system, e.g. computer based (optional)

6.15 Test tube, 15 ml capacity

6.16 Sample concentration apparatus, e.g. Techne® sample concentrator, capable of evaporating small volume of samples at controlled temperature and under a stream of nitrogen (5.3)

6.17 Automated SPE Vacuum System[III]**,** Supelco Visiprep™ Extraction Manifold, or equivalent (optional, gravity is sufficient to have the elution from the SPE cartridge of the purified sample extract)

6.18 Disposable syringe barrels, to be used as reservoirs, of 20 ml capacity, luer locks and attachments to fit to the silica columns (5.14) (optional).

[III] A support for the SPE columns and a collecting basin could be used as an alternative if no vacuum is applied for the elution

6.19 Microliter syringe(s) or calibrated microliter pipette(s), with 25 µl to 5000 µl capacity

6.20 Gas chromatograph hyphenated to a mass spectrometer (GC-MS) comprising the following

6.20.1 Injection system, a programmed temperature vaporising (PTV) injector type, suitable for temperatures up to 400ºC. Split-splitless injection was proven to give equivalent results and it should be suitable for temperatures up to 300 ºC. (See paragraphs 8.1.1 and 8.1.2 for the conditions to be applied).

6.20.2 GC oven, suitable for temperatures up to 340ºC and capable of temperature programming

6.20.3 Sample carousel (optional)

6.20.4 Amber vials for the sample carousel (6.20.3), with a capacity of about 1,5 ml

6.20.5 Amber vase vials for the sample carousel (6.20.3), with low dead volume or, in alternative, insert vials (6.20.6)

6.20.6 Amber insert vials for the sample carousel (6.20.3), with 500µl volume

6.20.7 A GC capillary column, a Select™PAH column will be supplied for this study.

This column, applied with the injection and oven conditions as described in chapter 8.1, shall ensure at least the following resolutions (expressed as R_s):

- BbF/BkF $R_s \geq 0,8$
- BkF/BjF $R_s \geq 0,4$
- CHR/TRP $R_s \geq 0,6$
- *IcP/DhA* $R_s \geq 0,6$ *(this resolution target concerns separation of two non-target PAHs)*

6.20.8 A flow control device, for obtaining a constant flow in the capillary column during the analysis

6.20.9 An interface with the mass spectrometer, with a temperature control device and suitable for temperatures up to 350ºC (see 8.1.5)

6.20.10 A mass spectrometer, with the following characteristics:

- Electron Ionisation source with inert inner surface
- Ionisation energy of 70 eV
- Mass resolution: at least 1 amu
- Temperature control devices for the ion source (up to 300 °C) and the GC-MS interface (up to a 350 °C, see 8.1.5).
- Tuning stability at least of 48 h (allowing for the analysis of a sequence of samples or standards)
- Response linearity range of at least two orders of magnitude

6.20.11 Computer based instrument control system, capable of programming different acquisition modes in different time intervals.

6.20.12 Data processing system, e.g. computer based

7 Procedure

For each batch of samples, a procedural blank (prepared as described in paragraph 4) shall be run in parallel, to assess interferences deriving from the applied reagents and apparatus.

7.1 Sample treatment

The sample should be treated as foreseen by Commission Regulation (EC) No 333/2007 [2], to obtain the laboratory sample and, from this, the test sample.

As a general precaution, all of the sample material received by the laboratory shall be used for obtaining a representative and homogeneous laboratory sample without introducing secondary contamination. In all instances, if the sample has been frozen, allow it to completely thaw before homogenising and sub-sampling.

7.2 Test portion preparation for solid samples (for oil samples see paragraph 7.4)

Weigh with an analytical balance (6.3) 5 g ± 0.05 g of the homogenised test sample, into an aluminium weigh boat or into an equivalent vessel (6.5).

Transfer the test portion in the mortar (6.6), and add 5 g of polyacrylic acid (5.5) and 15 g of sand (5.6). Mix thoroughly with the pestle until the sample is finely milled and visually homogeneous. The sand and the polyacrylic acid are weighed with a laboratory balance (6.4)

Add with a calibrated pipette 200 µl (V_{LPAH-S}) of the mixed labelled process solution (5.20) and mix again.

7.3 Extraction of solid samples and preparation for SEC

7.3.1 Pressurised liquid extraction

Take care that the cellulose filter (6.7.2) is placed in the extraction cell, and that all O-rings of the extraction cells are in good condition.

Transfer the test portion, prepared as described in the paragraph 7.2, into the extraction cell of the PLE apparatus (6.7.1) Add about 5g of anhydrous Na2SO4 5.4 to each collection bottle before starting the extraction to bind co-extracted water.

The extraction takes place under following conditions:

Pressure:	1500 psi
Temperature:	100 °C
Pre-heat time:	0 minutes
Heat time:	5 min
Static time:	10 min
Flush volume:	60%
Purge time:	180 seconds
Static cycles:	2
Solvent:	*n*-hexane 100% (5.7) - alternatively cyclohexane (5.9)

7.3.2 Soxhlet extraction

Transfer the test portion, prepared as described in the paragraph 7.2, into the thimble of the Soxhlet apparatus (6.8.4). The sample has to be topped with glass wool (5.13) so to avoid sample losses during re-condensation and reflux of the extraction solvent. The thimble has to be placed in the Soxhlet extraction chamber. 200 ml of *n*-hexane (5.7) shall be added in the 250 ml flask (6.8.1).

The heating temperature and cooling have to be set so to obtain approximately 6 cycles per hour. [IV]. The extraction duration should not be less than 7 hours.

7.3.3 Evaporation of the solvent for extracts obtained with PLE

At the end of the extraction, the test portion extract is transferred, by pouring it, into the evaporation vessel of the evaporation apparatus (6.9). The Na_2SO_4 (5.4) remaining in the collection bottle is washed 2 times with 10 ml of *n*-hexane (alternatively cyclohexane) which is transferred by a Pasteur pipette to the evaporation vessel and combined with the extract. Use a Pasteur pipette (6.10) both to mix the washing solvent with the Na_2SO_4 (and to wash the collection bottle walls in case fat drops should be present), and to transfer the washing solvent in the evaporation vessel. Avoid the transfer of Na_2SO_4!

The solvent is evaporated in the evaporation apparatus to about 2 ml. Evaporation to dryness must be avoided! Further reduction of the solvent volume to less than 1 ml can be either performed in the evaporation apparatus or under a gentle nitrogen stream at 40 °C, depending on the evaporation apparatus features.

7.3.4 Evaporation of the solvent for extracts obtained with Soxhlet

At the end of the extraction, about 5 grams of anhydrous Na_2SO_4 (5.4) shall be added to the extract in the round bottom flask and mixed with the extract to bind eventually extracted water. The test portion extract is transferred, by pouring it, into the evaporation vessel of the evaporation apparatus (6.9). The Na_2SO_4 (5.4) remaining in the collection bottle is washed 2 times with 10 ml of *n*-hexane (alternatively cyclohexane) which is transferred by a Pasteur pipette to the evaporation vessel and combined with the extract. Use a Pasteur pipette (6.10) both to mix the washing solvent with the Na_2SO_4 (and to wash the collection bottle walls in case fat drops should be present), and to transfer the washing solvent in the evaporation vessel. Avoid the transfer of Na_2SO_4!

The solvent is evaporated in the evaporation apparatus to about 2 ml. Evaporation to dryness must be avoided! Further reduce the solvent to less than 1 ml under a gentle nitrogen stream at 40°C.

WARNING 4 — Some of the PAHs are volatile. In all evaporation steps of this method, evaporation to dryness shall be avoided to obtain a consistent, acceptable recovery of the analytes.

7.3.5 Preparation of the sample for SEC

The concentrated extract is transferred to a graduated test tube (6.11), the evaporation vessel is washed with two portions of 2 ml each of the SEC eluent (5.12) and these two portions are added to the concentrated sample extract in the same test tube. The final volume is adjusted to 5 ml. The 5 ml of test sample are filtered through with a PTFE® filter (6.13) by means of a syringe (6.12) into the vial used for loading the sample onto the SEC column (e.g. 6.14.2).

7.4 Test portion preparation for liquid samples (oil)

Weigh 1 g ± 0,01 g of test sample directly into the vial used for loading the sample onto the SEC column (e.g. 6.14.2). Add 200 µl of the mixed labelled PAH process solution (5.20) for the following calculations of concentrations by the isotope dilution procedure (see 9.1).This test portion will be mixed with 4 ml of the SEC eluent (5.12) to obtain the sample for the SEC cleanup (7.5). The mixed labelled process solution (5.20) shall be added with a pipette calibrated for the volume of 200 µl (V_{LPAH-S}) (6.19).

[IV] When Soxhlet extraction is applied, special caution should be given to the choice of the condensor, taking into account the high volatility of n-hexane.

7.5 SEC cleanup

The 5 ml of test sample, prepared as described in 7.4 (for liquid samples) or in 7.3.3 will be loaded onto the SEC apparatus (6.14).

NOTE 4: For the calculation of recoveries (see paragraph 10.1), possible incomplete loading of the sample should be taken into account. Therefore, weigh the sample vial before and after the loading.

The SEC takes place with the following conditions[V]:

Flow rate: 4 mL/min
Start time of collection: 35 min (2100 second)
Collection time: 47 min (2820 seconds)
Eluent: cyclohexane: ethyl acetate 1:1 (5.12)

7.6 SEC purified extract concentration

At the end of the SEC cleanup, the cleaned-up extract is evaporated to 5 ml at 40 °C, either in the evaporation device of the SEC apparatus (6.14.5) or by means of an off-line evaporation device (6.9).

7.7 SEC purified extract preparation for SPE cleanup

The concentrated SEC extract is transferred to a test tube (6.11) and added with 200 µl of toluene (5.10) as a keeper for all following evaporation steps. It is then evaporated to 200 µl in the sample concentrator (6.16) at 40 °C by applying a gentle stream of nitrogen (5.3) (the surface of the liquid should not be broken into drops). Then 800 µl of cyclohexane (5.9) are added to the 200 µl of the SEC concentrated cleaned-up test portion extract.

7.8 SPE cleanup

All the elution steps can be performed by gravity without the use of any vacuum or pressure device, obtaining a flow of about 5-6 drops/min. Only in case of blocked columns a low vacuum or pressure is applied to facilitate elution.

Condition the SPE column (5.14) with 2 ml of cyclohexane (5.9) and discard it.

Load the 1000 µl of the SEC concentrated cleaned-up test portion extract, obtained as described in 7.7, on the top of the SPE column and let it infiltrate into the sorbent. Discard the displaced conditioning solvent.

Elute the PAHs with 10 ml of cyclohexane (5.9). The first 2 ml (1 ml x 2) are first poured to the test tube to rinse possible traces of analytes and then loaded on the SPE column. The following 8 ml are either loaded in portions of 2 ml each or, if a reservoir should be available (6.18), in one step. Let it elute by gravity (about 1 ml/min) and collect the eluate till the flow stops.

NOTE 5: No pressure or vacuum should be applied to dry up the SPE column.

NOTE 6: If different silica column characteristics are used, the conditioning and elution steps may need to be adjusted.

[V] These conditions can be applied when the column is exactly the one described in 6.20.7.

7.9 Preparation of the sample for the GC-MS analysis

The eluate, already containing the keeper as described in 7.7, is collected in a test tube (6.11), evaporated to 200 µl at 40ºC by means of the sample concentrator (6.16) under a gentle nitrogen stream, and transferred into an amber vase vial (6.20.5). The test tube is rinsed twice, each time with 100 µl of toluene, which is then combined with the extract (6.20.5). Finally, 100 µl ($V_{IS\text{-}S}$) of injection standard solution (5.18) are added for the calculation of the recovery of the labelled PAHs (see 10.1).

8 GC-MS analysis

Before starting the sequence a solvent blank - toluene (5.10) - and the standard solution CS7 (see Table 8) have to be injected to verify the instrumental performances for this specific method.

The chromatogram of the blank shall be checked for peaks which could indicate the need of cleaning the system.

The chromatogram of the standard solution will be examined to check for PAHs retention times, peak tailing, resolution between critical pairs, like BbF/BkF and BkF/BjF (see 11.1). The sensitivity of the system is checked as well (see 11.2).

8.1 GC-MS operating conditions

When the gas-chromatographic column (6.20.7) and the following settings are applied, a satisfactory separation of the 16 PAHs is obtained. All the 16 PAHs elute approximately between 10 and 45 min runtime.

8.1.1 Injection conditions, for PTV injector

Mode:	solvent vent
Injection volume:	3 µl
Initial temperature:	55 ºC
Initial time:	1.0 min
First ramp:	600 ºC/min up to 400 ºC, static time 15 min
Second ramp:	15 ºC/min down to 70 ºC, static time 0 min
Cryo:	on, at 80 ºC, for 30 min
Vent time:	0.40 min
Vent flow:	100 ml/min
Vent pressure:	50 kPa
Purge flow:	30 ml/min
Purge time:	3 min
Total flow:	34,1 ml/min
Gas type:	Helium (5.2)

8.1.2 Injection conditions, for split-splitless injector

Injection volume:	1 µl
Temperature:	300 ºC
Purge flow:	30 ml/min
Purge time:	2 min
Total flow:	34,1 ml/min
Gas type:	Helium (5.2)

8.1.3 Oven conditions

Initial temperature: 70 °C
Initial time: 1 min
Equilibration time: 1 min
First ramp: 60 °C/min up to 180 °C, static time 0 min
Second ramp: 4 °C/min up to 230 °C, static time 10 min
Third ramp: 28 °C/min up to 280 °C, static time 10 min
Fourth ramp: 14 °C/min up to 340 °C, static time 5 min

8.1.4 Column conditions

Constant flow: 1 ml/min
Gas type: Helium (5.2)

8.1.5 Transfer line conditions

Temperature: 325 °C

8.1.6 Mass spectrometer conditions

MS source temperature: 300 °C
Solvent delay: 7 min
Electron Ionisation Energy: 70 eV

8.1.7 Mass spectrometer acquisition parameters

The acquisition of the analyte signals is performed in SIM mode, applying the settings specified in Table 6. For quantification purposes the peak areas of the quantifier ions are used. However positive peak identification is only achieved if the qualifier ion peak is at the same retention time as the quantifier ion peak, and if ratios between quantifier and qualifier ions are within the acceptable range according to applying Commission Decision (EC) 2002/657 [7] (see paragraph 10.2) The selected quantifier (Q1) and qualifier (Q2) ions are listed in **Table 7**. A typical GC-MS chromatogram of 15 + 1 EU PAHs at the concentration level of 100 ng/ml is shown in Annex A. The typical retention time for each compound is specified in the chromatogram. However, they might change slightly, if a new column is installed.

NOTE 7: The ratio between Q2 and Q1 could change with the content of the native PAH. The compliance to the acceptable range of the ratio according to the above mentioned Decision is to be checked against a reference value. This reference value is to be obtained from the analysis of either a calibration solution or a reference sample containing the concerned native PAH at a level close to the level in the sample under examination.

Table 6 — Acquisition program in SIM

Group (#)	Initial time (min)	Dwell time (ms)	Ions (m/z)
Group 1	10	80	103, 108
			205, 216
Group 2	14	40	114, 116
	14	80	226, 228, 234
Group 3	18,75	100	121, 215
			242, 245
Group 4	25	80	135, 126, 128, 129
			270, 252, 256, 258
Group 5	30	80	138, 139, 141, 142, 143
	30	100	276, 278, 282, 284, 288
Group 6	38	80	150, 154, 157
	38	100	302, 308, 314

Table 7 — Ions for identification and quantification of PAHs in SIM

PAH*	Indicative retention time (min)**	Quantifier ion*** Q_1 m/z	Qualifier ion*** Q_2 m/z
5-Methylchrysene (5MC)	21.14	242	215
Benz[*a*]anthracene (BaA)	16.99	228	114
Benzo[*a*]pyrene (BaP)	28.13	252	126
Benzo[*b*]fluoranthene (BbF)	26.81	252	126
Benzo[*c*]fluorene (BcL)	12.56	216	108
Benzo[*ghi*]perylene (BgP)	35.09	276	138
Benzo[*j*]fluoranthene (BjF)	26.96	252	126
Benzo[*k*]fluoranthene (BkF)	26.90	252	126
Chrysene (CHR)	14.51	228	114
Cyclopenta[*cd*]pyrene (CPP)	17.24	226	114
Dibenzo[a,e]pyrene (DeP)	41.45	302	150
Dibenz[a,*h*]anthracene (DhA)	33.30	278	139
Dibenzo[a,*h*]pyrene (DhP)	42.06	302	150
Dibenzo[a,*i*]pyrene (DiP)	41.85	302	150
Dibenzo[a,*l*]pyrene (DlP)	40.65	302	150
Indeno[1,2,3-*cd*]pyrene (IcP)	33.12	276	138
9-fluorobenzo[k]fluoranthene	26.22	270	135
5-Methylchrysene methyl D3	21.02	245	121
Benz[a]anthracene 13C6	17.00	234	116
Benzo[a]pyrene 13C4	28.13	256	128
Benzo[*b*]fluoranthene 13C6	26.80	258	129
Pyrene 13C3	10.79	205	103
Benzo[*ghi*]perylene 13C12	35.09	288	143
Benzo[*k*]fluoranthene 13C6	26.91	258	129
Chrysene 13C6	17.50	234	116
Dibenzo[*a,e*]pyrene 13C6	41.45	308	154
Dibenz[*a,h*]anthracene 13C6	33.28	284	142
Dibenzo[*a,i*]pyrene 13C12	41.85	314	157
Indeno[1,2,3-*cd*]pyrene 13C6	33.11	282	141

*For native PAHs, in parenthesis the short name is reported.

**The retention time varies with the column conditions (e.g. the exact length) and shall be verified with the quality control standard (see chapter 8)

***Referring to Commission Decision (EC) 2002/657, a tolerance of ± 10% to 50% in the value of the ratio is accepted, depending on the amount of the diagnostic ion in relation with the target ion (for this method qualifier ion and quantifier ion respectively).

8.2 Preparation of mixed PAH calibration solutions

Prepare, directly in autosampler vials (6.20.4), the calibration solutions. They are obtained from the mixed PAH intermediate solutions (5.21), the mixed labelled PAH process solution (5.20), and the injection standard solution (5.18). For each of the calibration solutions, pipette 500 µl of the corresponding intermediate solution (5.21), 400 µl of the mixed labelled PAH process solution (5.20), and 100 µl of the injection standard solution (5.18). To obtain the exact concentration levels, the vial tare and all the additions are weighed with an analytical balance (6.3).The nominal concentration levels, used for the preparation of the calibration curve, are summarised in Table 8.

These solutions will be used for the preparation of the calibration curve (see 8.3).

Table 8— Indicative compositions of the PAH calibration solutions

Mixed PAH calibration solution	Nominal PAH* concentration in the calibration solution (ng/g)**	Nominal labelled PAH concentration in the calibration solution (ng/g)**	Nominal injection standard concentration in the calibration solution (ng/g)**
CS1	1,0	16,0	50,0
CS2	2,0		
CS3	3,0		
CS4	4,0		
CS5	8,0		
CS6	12,0		
CS7	20,0		
CS8	50,0		
CS9	80,0		
CS10	100,0		
CS11	140,0		
CS12	180,0		

*the concentration level refers to the PAHs contained in the commercial mixed solution supplied for this study

** the real concentrations have to be derived from the concentrations of the individual solutions applied for the preparation of the calibration standards (mixed PAH stock solution (5.19), labelled PAH process solution (see 5.20 and Table 5), and injection standard solution (5.18) and gravimetric preparation data.

Table 9— Indicative content levels of PAHs in the samples corresponding to the indicative content in the calibration solution

Mixed PAH calibration solution	Nominal PAH* concentrations in the calibration solutions (ng/g)**	Corresponding PAH* concentrations in the solid samples (µg/kg)#	Corresponding PAH* concentrations in edible oils (µg/kg)##
CS1	1,0	0,1	0,5
CS2	2,0	0,2	1,0
CS3	3,0	0,3	1,5
CS4	4,0	0,4	2,0
CS5	8,0	0,8	4,0
CS6	12,0	1,2	6,0
CS7	20,0	2,0	10,0
CS8	50,0	5,0	25,0
CS9	80,0	8,0	40,0
CS10	100,0	10,0	50,0
CS11	140,0	14,0	70,0
CS12	180,0	18,0	90,0

*the concentration level refers to the PAHs contained in the commercial mixed solution supplied for this study

#derived from calculations as reported in 9.1 for solid samples (5 grams of sample are processed)

##derived from calculations as reported in 9.1 for oil (1 gram of sample is processed)

8.3 Calibration curve

Prepare a calibration curve by injecting 3 µl (for PTV injection) or 1 µl (for split-splitless injection) of the calibration solutions listed in Table 1 at the beginning of every sequence of analysis. All calibration solutions shall be freshly prepared for each sequence. The injection of the standards shall be performed from the lower to the higher concentration so to reduce the risk of cross-contamination.

The calibration curve is obtained by plotting the peak area ratios of the quantifier ion of the native PAH and of the corresponding labelled standard versus the ratio of the respective concentrations of the native PAH and labelled PAHs in the calibration solutions.

Before performing the measurements of the test samples, the calibration curve shall be checked for linearity applying a suitable test such as Mandel's test, or lack-of-fit test.

If the linearity test is passed, a linear function can be applied for calculation of the results. Linear regression may be applied for the determination of the calibration function. The calibration curve covers the range from 0,1 µg/kg to 18,0 µg/kg for solid samples, which corresponds, for oil samples, due to different dilution factors, to a range from from 0,5 µg/kg to 90,0 µg/kg for oil samples. This is valid for all the analytes under the conditions of this protocol.

Depending on the level of PAH expected or found in the samples, it might be necessary to prepare a new calibration curve so as to have the sample concentration in the central part of the calibration range. The new calibration curve shall be demonstrated to be linear (see 8.3) before any calculations can be performed. Alternatively, a new sample extraction shall be performed adjusting the test portion weight so to obtain a final concentration of the PAHs in the extract within the calibration range (the test portion to be extracted can be reduced, in so far as the it remains representative of the laboratory sample).

If the calibration plot is linear, the calibration is considered successful and the calibration curve is calculated for each native PAH by linear regression.

The derived calibration curves can be described by Equation 2.

Equation 2

$$\frac{A_{PAH-C}}{A_{LPAH-C}} = a \cdot \frac{[PAH]_C}{[LPAH]_C} + b$$

Where:

A_{PAH-C}	peak area of the quantifier ion for the native compound in the calibration solution
A_{LPAH-C}	peak area of the quantifier ion for the labelled standard in the calibration solution
a	slope of the calibration curve
b	intercept of the calibration curve
$[PAH]_C$	concentration of native compound in the standard solution for instrument calibration **[ng/g]**
$[LPAH]_C$	concentration of the labelled standard in the standard solution for instrument calibration **[ng/g]**

8.4 Sample analysis

Before starting an analytical sequence all quality assurance procedures established at the participating laboratory (e.g. preventive maintenance, and/or operational qualification, and/or performance verification) should be performed in order to guarantee that the instrument is functioning according to the expected specifications.

In each sequence of analysis, after having checked the system (see chapter 8), and after the injection of the calibration curve (see 8.3), the procedural blank and the samples extracts shall be injected.

After each sample analysis, injection of toluene is performed to avoid carry-over between samples. Every 10 injections and at the end of the sequence the CS1 and CS7 calibration solutions (Table 8) are injected to verify the system response stability (see 11.5).

At the end of the sequence, results shall be checked to verify if any sample should be outside of the working range and, if necessary, the procedure described in 8.3 is applied.

The typical sequence of analysis shall be:

A. Instrumental performance verification
    1. Solvent blank (toluene) – injection of toluene shall be repeated until signal intensities of interfering peaks are below signal intensities corresponding to the limits of detection of the native PAHs.
    2. CS7

B. Calibration curve
    1. Solvent blank (toluene)
    2. CS1
    3. CS2

4. CS3
5. CS4
6. CS5
7. CS6
8. CS7
9. CS8
10. CS9
11. Solvent blank (toluene)

C. Sample analysis (the following is an example: any order of injection of the samples is valid insofar as carry-over is prevented)

1. Solvent blank (toluene)
2. Sample A
3. Toluene
4. Sample B
5. (etc)
6. Procedural blank
7. Toluene
8. Quality control sample
9. (etc)
10. CS1
11. CS7
12. (etc.)

D. Final verification

1. CS7

The part C of the sequence (sample analysis) should be set so to guarantee the maximum degree of randomisation of replicates from the same sample or of samples obtained from foodstuffs of the same category.

In case of particularly dirty extracts and/or very low levels of PAHs in the sample, the injection of toluene after and before, respectively, every injection is advisable.

9 Calculation and reporting

9.1 Calculation

The final concentration of the PAH in the sample is calculated applying Equation 3, where *a* (the slope of the calibration curve) and *b* (the intercept of the calibration curve) were obtained from the instrument calibration as described in 8.2 and 8.3.

Equation 3

$$[PAH]_{SAMPLE} = \left(\frac{\left(\frac{A_{PAH-S}}{A_{LPAH-S}} - b \right)}{a} \right) \times \frac{[LPAH] \times V_{LPAH-S}}{W_{SAMPLE}}$$

Where:

$[PAH]_{SAMPLE}$	concentration of the native compound in the sample [µg/Kg]
A_{PAH-S}	peak area of the quantifier ion for the native compound in the sample
A_{LPAH-S}	peak area of the quantifier ion for the labelled standard in the sample
a	slope of the calibration curve
b	intercept of the calibration curve
$[LPAH]$	concentration of the labelled PAH in the mixed labelled PAH process solution (5.20) [ng/µl]
V_{LPAH-S}	volume of the mixed labelled PAH process solution (5.20) added to the sample [µl]
W_{SAMPLE}	weight of the test portion (7.2) [g]

WARNING 5 — The same calculation should be performed for the procedural blank, considering the same sample weight as for the corresponding sample (5 g for solids and 1 g for liquids) and the found content has to be subtracted from the content found in the real sample.

9.2 Reporting

For each sample, the reported result will be obtained from the analysis of the sample and from the analysis of the procedural blank analysed in the same batch. The analysis results for the four target PAHs will be reported to the nearest 0.1 µg/kg,.

Participants will also be asked to report for the four target PAHs recovery values which are estimated based on the recovery of the labelled PAHs. The procedure for recovery estimation is detailed in paragraph 10.1.

10 Quality control of the sample results

10.1 Recovery

The recovery of the PAHs in the sample is measured on the labelled PAHs, whose chemical behaviour was proven to be of the same nature as of the native PAHs.

The recovery is calculated through the relative response factors (RRF) of the labelled PAHs corresponding to the four target PAH - benzo[a]pyrene $^{13}C_4$ (for BaP), benz[a]anthracene $^{13}C_6$ (for BaA), benzo[*b*]fluoranthene $^{13}C_6$ (for BbF), and chrysene $^{13}C_6$ (for CHR) - versus the injection standard.

A reference relative response factor (RRFm) is obtained from the calibration standard solutions injected in the sequence. It is calculated applying Equation 4 for each of the 4 target PAHs and each of the repeated analysis of the two control calibration solutions CS1 and CS7 (and / or CS4 if oil samples are analysed in the sequence).

Equation 4

$$RRF_m = \frac{1}{n} \cdot \sum_{1}^{i} \frac{A_{LPAH,i} \cdot [IS]i}{A_{IS,i} \cdot [LPAH]i}$$

Where:

RRF_m	mean RRF of the labelled PAH in the calibration solutions
$A_{IS,i}$	peak area of the quantifier ion of the injection standard in calibration solution *i*
$[LPAH]_i$	concentration of the labelled PAH in the calibration solution *i* **[ng/g]**
$A_{LPAH,i}$	peak area of the quantifier ion of the labelled PAH in calibration solution *i*
$[IS]_i$	concentration of the injection standard (FBkF) in calibration solution *i* **[ng/g]**

All concentrations are known from the calibration solutions preparation (8.2) and the peak areas are obtained from the GC-MS measurements.

The RRFs obtained from the calibration standards are checked for outliers according to Grubbs' test [8], outliers are excluded from following calculations only if a reason for anomalous results can be tracked back.

From the checked set of data, the mean RRF_m is calculated according to Equation 4, and entered into Equation 5

The recovery of the labelled PAHs, which is calculated as the percent ratio between the amount of labelled standard found in the sample and the amount expected to be present in the sample, is obtained by applying Equation 5.

Equation 5

$$\%REC_L = \frac{A_{LPAH-S} \times [IS]_S}{RRF_m \times A_{IS-S} \times [LPAH]_S} \times 100$$

Where:

$\%REC_L$	% recovery of the labelled standard in the sample
A_{LPAH-S}	peak area of the quantifier ion of the labelled PAH in the GC-MS sample extract
$[IS]_S$	concentration of injection standard in the sample extract calculated taking into account the volume of the internal standard solution [5.18] added to the sample extract and the final volume of the sample extract **[ng/ml]**
RRF_m	mean value of the RRF measured for the labelled PAH in the calibration standards (from Equation 4)
A_{IS-S}	peak area of the quantifier ion of the injection standard in the GC-MS sample extract
$[LPAH]_S$	concentration of the labelled PAH in the sample extract calculated taking into account the volume of the mixed labelled PAHs process solution [5.20] added to the sample extract and the final volume of the sample extract **[ng/ml]**

10.2 Peak identification

The PAHs peaks are identified by their retention times, the presence of both the quantifier and the qualifier ions and the acceptability of the value of the ratio of the peak areas corresponding to the two ions.

The PAHs are detected and quantified by monitoring of the quantifier ion (Q_1) over the chromatogram. For PAHs, Q_1 coincides with the molecular ion (M). A second ion called the qualifier ion (Q_2) is monitored for each analyte for identification purposes.

A substance eluting from the chromatographic column is identified as a target PAH only if:

- the retention time of the unknown substance coincides with the retention time of the native PAH as found in the calibration solution within 0.1 min
- both Q_1 and Q_2 are detected

- the Q_2 relative ion intensity (expressed as % of Q_1 intensity) lies within the lower and upper limits as calculated by applying Commission Decision (EC) 2002/657 [7] criteria (depending on the relative ion intensity of Q_2 a relative tolerance of ± 10% to 50% of the value of the relative ion intensity is accepted,). **See paragraph 8.1.7 and NOTE 7 at page 19.**

10.3 Peak integration

The proper integration of each analyte peak has to be checked by the analyst after automatic integration. The checked results are then used for all calculations.

11 Quality control of the method

11.1 Chromatographic resolution

Special attention has to be paid to the resolution between different PAHs and the stability of the retention times. The resolution between BbF and BkF as well as the resolution between BkF and BjF has to be monitored. The resolution is checked on the basis of chromatograms gained for the calibration solution CS7 (see Table 8). The requirements (expressed as Rs, see 6.20.7) are given in the following:

- BbF/BkF $R_s \geq 0,8$
- BkF/BjF $R_s \geq 0,4$

Retention times shall not vary by more than 0.1 min in the sequence and between samples and calibration standards.

If this resolution and retention time stability criteria are not fulfilled, the chromatographic system shall be checked and cause shall be remediated. The analytical sequence shall start only upon achievement of the target values.

In case the requirements should not be met for one of the standards injected along the sequence for checking the stability of the system, the sequence is to be re-started from the last calibration solution CS7 meeting the requirements set above. The first injections of the repeated sequence shall be those listed under letter A and B in paragraph 8.4.

11.2 Chromatographic sensitivity

The injection of calibration solutions and/or blank samples should be used to check the system sensitivity according the quality control procedures established at the participating laboratory. The analytical sequence is to be started only upon achievement of the target value(s) established at the participating laboratory.

In case requirements should not be met for one of the standards injected/blank samples injected along the sequence for checking the stability of the system, the sequence is to be re-started only upon achievement of the target values and from the last calibration solution/blank sample meeting the requirements established at the participating laboratory. The first injections of the repeated sequence shall be those listed under letter A and B in paragraph 8.4.

11.3 Ion source

The injection of calibration solutions should be used to check the system for the necessity of cleaning the ion source according the quality control procedures established at the participating laboratory. The analytical sequence is to be started only upon achievement of the target value(s) established at the participating laboratory.

In case requirements should not be met for one of the standards injected along the sequence for checking the stability of the system, the sequence is to be re-started only upon achievement of the target values and from the last calibration solution meeting the requirements established at the participating laboratory. The first injections of the repeated sequence shall be those listed under letter A and B in paragraph 8.4.

11.4 Calibration curve check

The instrument is calibrated with each sequence of samples. The linearity of the calibration curve has to be tested by Mandel test. A linear calibration function can only be applied for the calculation of results if Mandel test is passed.

11.5 Response stability

The injection of the calibration solutions CS1 and CS7 (8.2) among sample analysis allows for checking the stability of the system response, i.e. the calibration curve stability. The acceptance limits have to be defined according to the internal quality procedures of the participating laboratory (e.g. by the confidence interval of the calibration curve for the two concentration levels).

In case requirements are not met, after having checked the system according to the internal quality procedures established at the participating laboratories, the sequence is to be re-injected starting from the sample immediately after the last standard within the acceptance limits. The first injections of the repeated sequence shall be those listed under letter A and B in paragraph 8.4.

11.6 Instrument contamination check

At the beginning of each sequence a series of solvent blanks (toluene) are injected to ensure the GC/MS system is not contaminated. Solvent blanks are also injected along the sequence to check that carry-over over does not affect the results for the test samples.

The system is considered contaminated if peaks are present at the retention time of the respective analyte with a peak height exceeding 20% of the peak height of the quantifier ion peak produced for the analyte by CS4. If this requirement is not met for the second /third injection of solvent blank at the beginning of the sequence, the system shall be checked according to the internal quality procedures established at the participating laboratories. The analytical sequence is to be started only upon achievement of the target value.

In case requirements should not be met for one of the solvent blanks injected along the sequence for checking the stability of the system, the sequence is to be re-started only upon achievement of the target value and from the last solvent blank meeting the requirement set above. The first injections of the repeated sequence shall be those listed under letter A and B in paragraph 8.4.

11.7 Procedural blank

Together with every batch of samples, a procedural blank sample will be prepared and analysed to ensure an acceptable level of contamination. The whole analysis procedure is applied to the procedural blank sample, including the spiking with the mixed labelled process solution (5.20).

The level of contamination is considered as acceptable if for the procedural blank sample there are no peaks showing at the retention time of the respective analyte a peak height exceeding 30% of the peak height of the quantifier ion peak of the CS4 of the respective analyte. For acceptable contamination, the content found in the procedural blank will be subtracted as explained in paragraph 9.1.

In case the detected contamination should be higher, the results of the whole sequence will be checked for interferences. If interferences on the signals of the native PAHs are suspected, root cause analysis has to be performed and a corrective action has to be taken (e.g. by changing one of the solvents bottle or lot). The concerned sample(s) shall be re-processed and re-analysed.

12 Quality control of the standards

12.1 Integrity check

The mass of each standard preparation (storage vial plus content plus closure) has to be recorded before storing it. Before using a standard solution the mass of the standard preparation has to be determined. In comparing the actual mass with the mass recorded after preparation/previous use, potential losses of solvent can be determined. If the deviation between the actual mass and the mass before storage is larger than 0.5 % of the mass before storage, the actual concentration of the standard preparation has to be recalculated applying the density equation.

Annex A
(informative)

Typical chromatogram

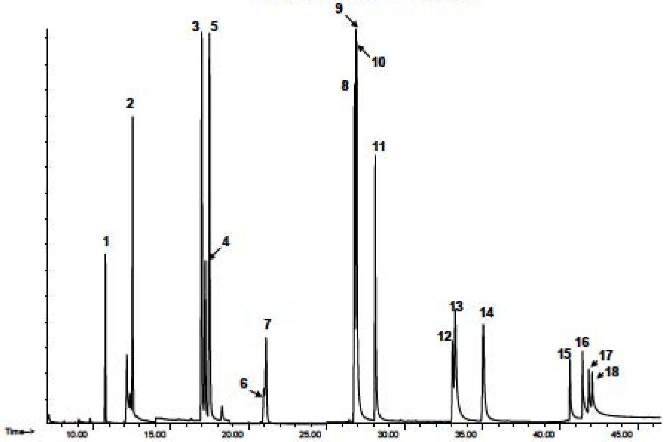


Figure A. 1— Typical GC-MS chromatogram of 15+1 EU PAHs and labelled PAHs obtained under the conditions described in chapter 8 and applying PTV injection.

Key:

x: time (min)
y: TIC (total ion current))
Peak identification: see Table A. 1

Table A. 1— Identification of chromatographic peaks

PAH*	Peak number	PAH*	Peak numbe r	PAH*	Peak number
5-Methylchrysene (5MC)	7	Cyclopenta[*cd*]pyrene (CPP)	4	Benzo[a]pyrene 13C4	11
Benz[a]anthracene (BaA)	3	Dibenzo[*a,e*]pyrene (DeP)	16	Benzo[*b*]fluoranthene 13C6	8
Benzo[a]pyrene (BaP)	11	Dibenz[*a,h*]anthracene (DhA)	13	Pyrene 13C3	1
Benzo[*b*]fluoranthene (BbF)	8	Dibenzo[*a,h*]pyrene (DhP)	18	Benzo[*ghi*]perylene 13C12	14
Benzo[*c*]fluorene (BcL)	2	Dibenzo[*a,i*]pyrene (DiP)	17	Benzo[*k*]fluoranthene 13C6	9
Benzo[*ghi*]perylene (BgP)	14	Dibenzo[*a,l*]pyrene (DlP)	15	Chrysene 13C6	5
Benzo[*j*]fluoranthene (BjF)	10	Indeno[1,2,3-*cd*]pyrene (IcP)	12	Dibenzo[a,e]pyrene 13C6	16
Benzo[*k*]fluoranthene (BkF)	9	5-Methylchrysene methyl D3	6	Dibenz[*a,h*]anthracene 13C6	13
Chrysene (CHR)	5	Benz[a]anthracene 13C6	3	Dibenzo[*a,i*]pyrene 13C12	17
				Indeno[1,2,3-*cd*]pyrene 13C6	12

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*The short name is given for native PAHs within parenthesis.

Operating conditions for Figure A.1:

Column:	Select PAHs[vi], 15 m, 0.15 mm i.d., 0.10 µm df
Initial flow rate:	1 ml/min
Carrier gas:	Helium (5.2)
Column temperature:	See oven program (8.1.3)
Injection volume:	3 µl (for PTV programme temperature injection); 1 µl (for split-splitless injection)
Detection:	m/z ions listed in Table 6 in the conditions reported in 8.1.6 and 8.1.7

[vi] Select PAHs™ is a trade name of a commercially available capillary column that allows the separation of chrysene from its potential interference triphenylene. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results

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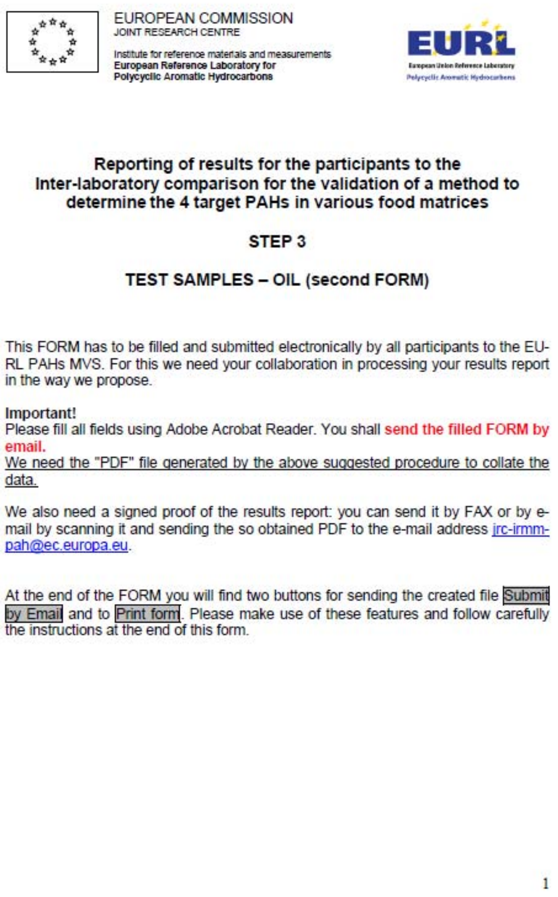
8 Grubbs E. F. (1969) Technometrics 11: 1-21

ANNEX 2 – FORM for reporting of results *(for STEP 3)*

Participants had to fill in similar FORMs for other samples. The FORM included here is intended as an example. The other FORMs are available as additional information upon request

FORM for reporting results for the blind sample OIL



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Reporting of results for the participants to the Inter-laboratory comparison for the validation of a method to determine the 4 target PAHs in various food matrices

STEP 3

TEST SAMPLES – OIL (second FORM)

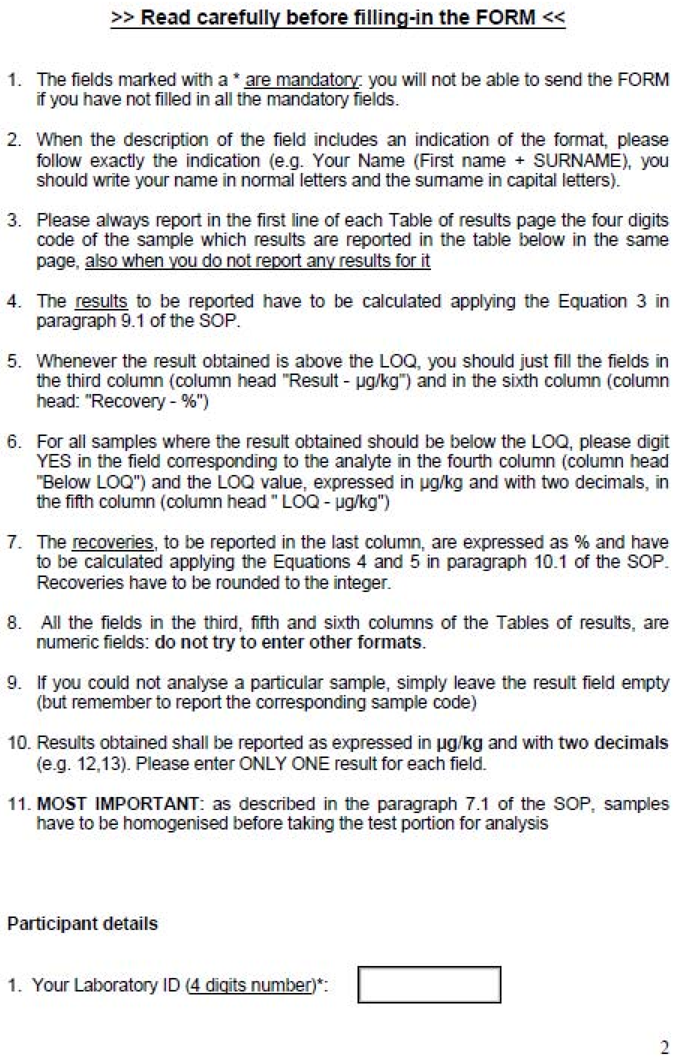
This FORM has to be filled and submitted electronically by all participants to the EU-RL PAHs MVS. For this we need your collaboration in processing your results report in the way we propose.

Important!
Please fill all fields using Adobe Acrobat Reader. You shall **send the filled FORM by email.**
We need the "PDF" file generated by the above suggested procedure to collate the data.

We also need a signed proof of the results report: you can send it by FAX or by e-mail by scanning it and sending the so obtained PDF to the e-mail address jrc-irmm-pah@ec.europa.eu.

At the end of the FORM you will find two buttons for sending the created file Submit by Email and to Print form. Please make use of these features and follow carefully the instructions at the end of this form.

1



>> Read carefully before filling-in the FORM <<

1. The fields marked with a * are mandatory: you will not be able to send the FORM if you have not filled in all the mandatory fields.
2. When the description of the field includes an indication of the format, please follow exactly the indication (e.g. Your Name (First name + SURNAME), you should write your name in normal letters and the surname in capital letters).
3. Please always report in the first line of each Table of results page the four digits code of the sample which results are reported in the table below in the same page, also when you do not report any results for it
4. The results to be reported have to be calculated applying the Equation 3 in paragraph 9.1 of the SOP.
5. Whenever the result obtained is above the LOQ, you should just fill the fields in the third column (column head "Result - µg/kg") and in the sixth column (column head: "Recovery - %")
6. For all samples where the result obtained should be below the LOQ, please digit YES in the field corresponding to the analyte in the fourth column (column head "Below LOQ") and the LOQ value, expressed in µg/kg and with two decimals, in the fifth column (column head " LOQ - µg/kg")
7. The recoveries, to be reported in the last column, are expressed as % and have to be calculated applying the Equations 4 and 5 in paragraph 10.1 of the SOP. Recoveries have to be rounded to the integer.
8. All the fields in the third, fifth and sixth columns of the Tables of results, are numeric fields: **do not try to enter other formats**.
9. If you could not analyse a particular sample, simply leave the result field empty (but remember to report the corresponding sample code)
10. Results obtained shall be reported as expressed in **µg/kg** and with **two decimals** (e.g. 12,13). Please enter ONLY ONE result for each field.
11. **MOST IMPORTANT**: as described in the paragraph 7.1 of the SOP, samples have to be homogenised before taking the test portion for analysis

Participant details

1. Your Laboratory ID (4 digits number)*: ☐

2

Results for the sample:

Sample code (written on the sample e.g. '0123'): ______

Analyte	Short name	Result	Below LOQ	LOQ	Recovery
		µg/kg	(YES)*	µg/kg	%
5-methylchrysene	5MC				
benz[*a*]anthracene	BaA				
benzo[*a*]pyrene	BaP				
benzo[*b*]fluoranthene	BbF				
benzo[*c*]fluorene	BcL				
benzo[*ghi*]perylene	BgP				
benzo[*j*]fluoranthene	BjF				
benzo[*k*]fluoranthene	BkF				
chrysene	CHR				
cyclopenta[*cd*]pyrene	CPP				
dibenzo[*a,e*]pyrene	DeP				
dibenz[*a,h*]anthracene	DhA				
dibenzo[*a,h*]pyrene	DhP				
dibenzo[*a,i*]pyrene	DiP				
dibenzo[*a,l*]pyrene	DlP				
indeno[1,2,3-*cd*]pyrene	ICP				

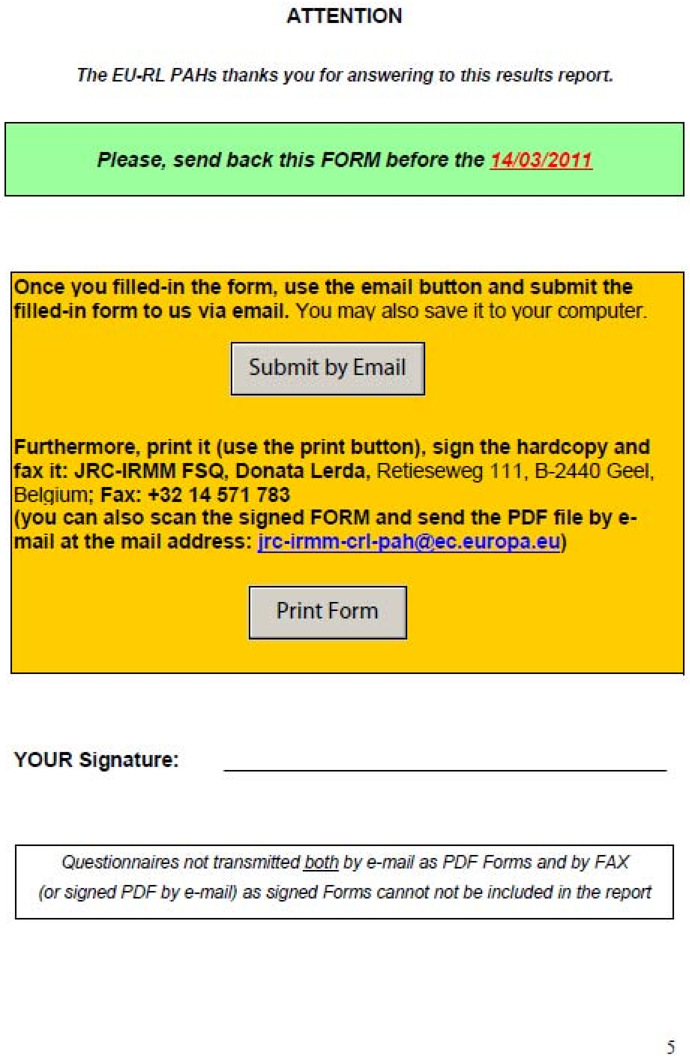
*See note 6) at page 2

Results for the sample:

Sample code (written on the sample e.g. '0123'): ______

Analyte	Short name	Result	Below LOQ	LOQ	Recovery
		µg/kg	(YES)*	µg/kg	%
5-methylchrysene	5MC				
benz[*a*]anthracene	BaA				
benzo[*a*]pyrene	BaP				
benzo[*b*]fluoranthene	BbF				
benzo[*c*]fluorene	BcL				
benzo[*ghi*]perylene	BgP				
benzo[*j*]fluoranthene	BjF				
benzo[*k*]fluoranthene	BkF				
chrysene	CHR				
cyclopenta[*cd*]pyrene	CPP				
dibenzo[*a,e*]pyrene	DeP				
dibenz[*a,h*]anthracene	DhA				
dibenzo[*a,h*]pyrene	DhP				
dibenzo[*a,i*]pyrene	DiP				
dibenzo[*a,l*]pyrene	DlP				
indeno[1,2,3-*cd*]pyrene	ICP				

*See note 6) at page 2



ATTENTION

The EU-RL PAHs thanks you for answering to this results report.

Please, send back this FORM before the 14/03/2011

Once you filled-in the form, use the email button and submit the filled-in form to us via email. You may also save it to your computer.

Submit by Email

Furthermore, print it (use the print button), sign the hardcopy and fax it: JRC-IRMM FSQ, Donata Lerda, Retieseweg 111, B-2440 Geel, Belgium; **Fax: +32 14 571 783**
(you can also scan the signed FORM and send the PDF file by e-mail at the mail address: jrc-irmm-crl-pah@ec.europa.eu)

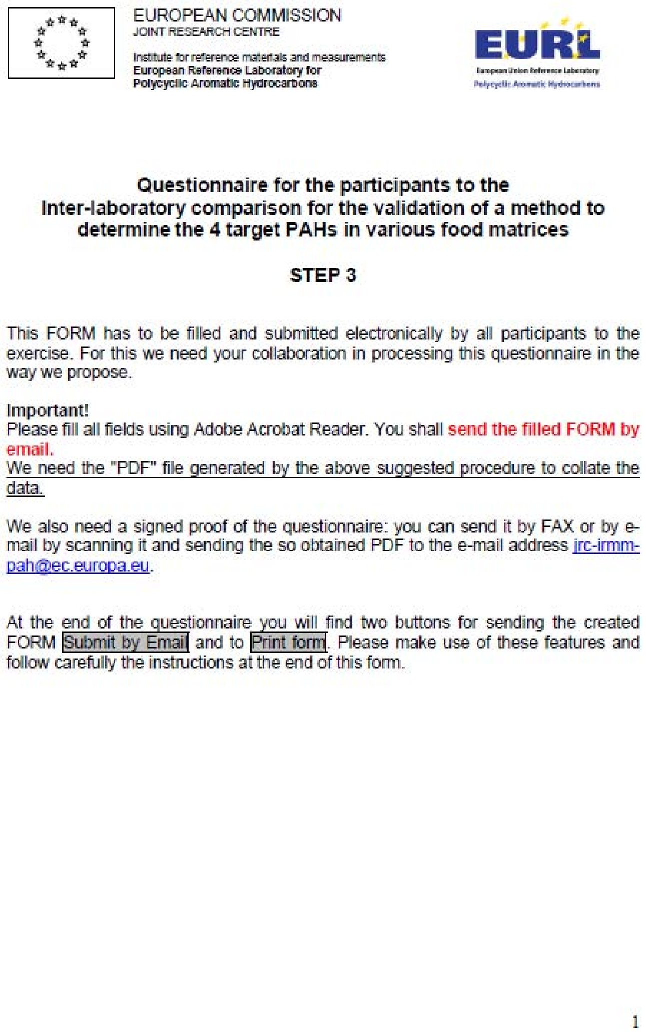
Print Form

YOUR Signature: ____________________________

Questionnaires not transmitted both by e-mail as PDF Forms and by FAX (or signed PDF by e-mail) as signed Forms cannot not be included in the report

ANNEX 3 – Questionnaire FORM *(for STEP 3)*

Participants had also to answer to a questionnaire about the application of the SOP at their laboratory at STEP1 of the study. The FORM and the compilation of answers is available as additional information upon request



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Polycyclic Aromatic Hydrocarbons

Questionnaire for the participants to the Inter-laboratory comparison for the validation of a method to determine the 4 target PAHs in various food matrices

STEP 3

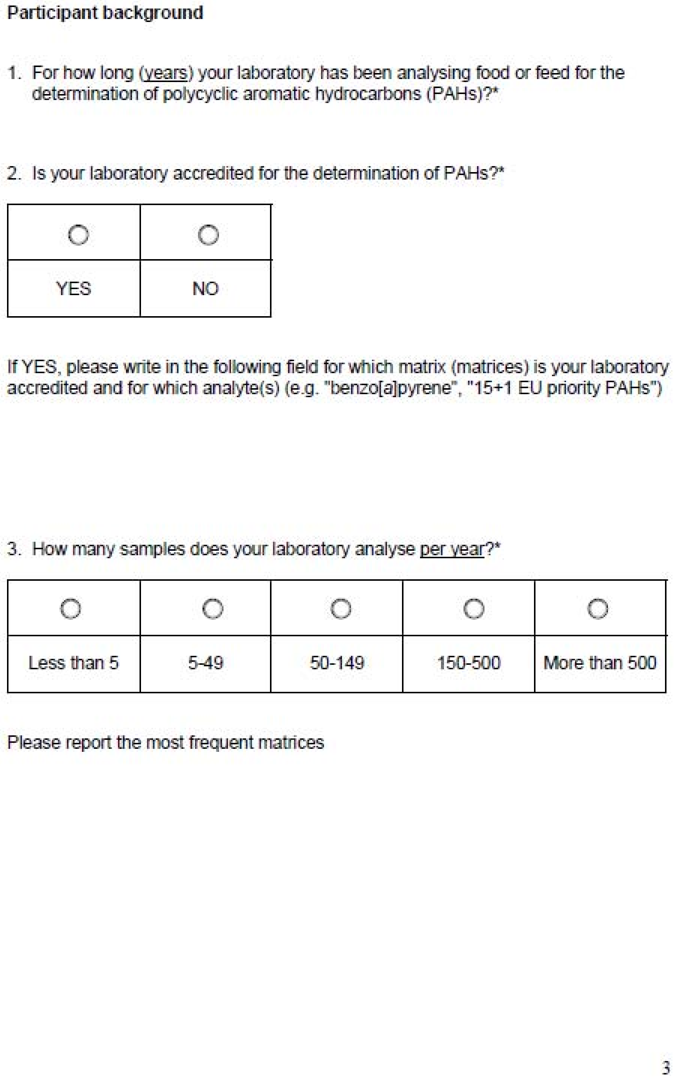
This FORM has to be filled and submitted electronically by all participants to the exercise. For this we need your collaboration in processing this questionnaire in the way we propose.

Important!
Please fill all fields using Adobe Acrobat Reader. You shall send the filled FORM by email.
We need the "PDF" file generated by the above suggested procedure to collate the data.

We also need a signed proof of the questionnaire: you can send it by FAX or by e-mail by scanning it and sending the so obtained PDF to the e-mail address jrc-irmm-pah@ec.europa.eu.

At the end of the questionnaire you will find two buttons for sending the created FORM Submit by Email and to Print form. Please make use of these features and follow carefully the instructions at the end of this form.

1



Participant background

1. For how long (years) your laboratory has been analysing food or feed for the determination of polycyclic aromatic hydrocarbons (PAHs)?*

2. Is your laboratory accredited for the determination of PAHs?*

○	○
YES	NO

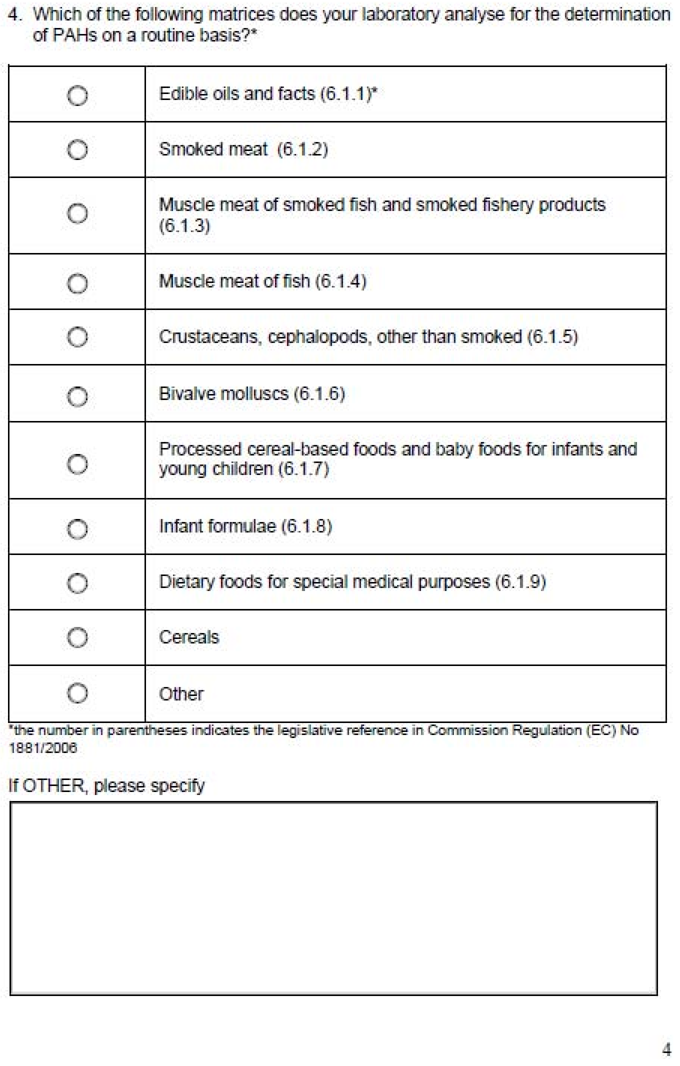
If YES, please write in the following field for which matrix (matrices) is your laboratory accredited and for which analyte(s) (e.g. "benzo[a]pyrene", "15+1 EU priority PAHs")

3. How many samples does your laboratory analyse per year?*

○	○	○	○	○
Less than 5	5-49	50-149	150-500	More than 500

Please report the most frequent matrices

3

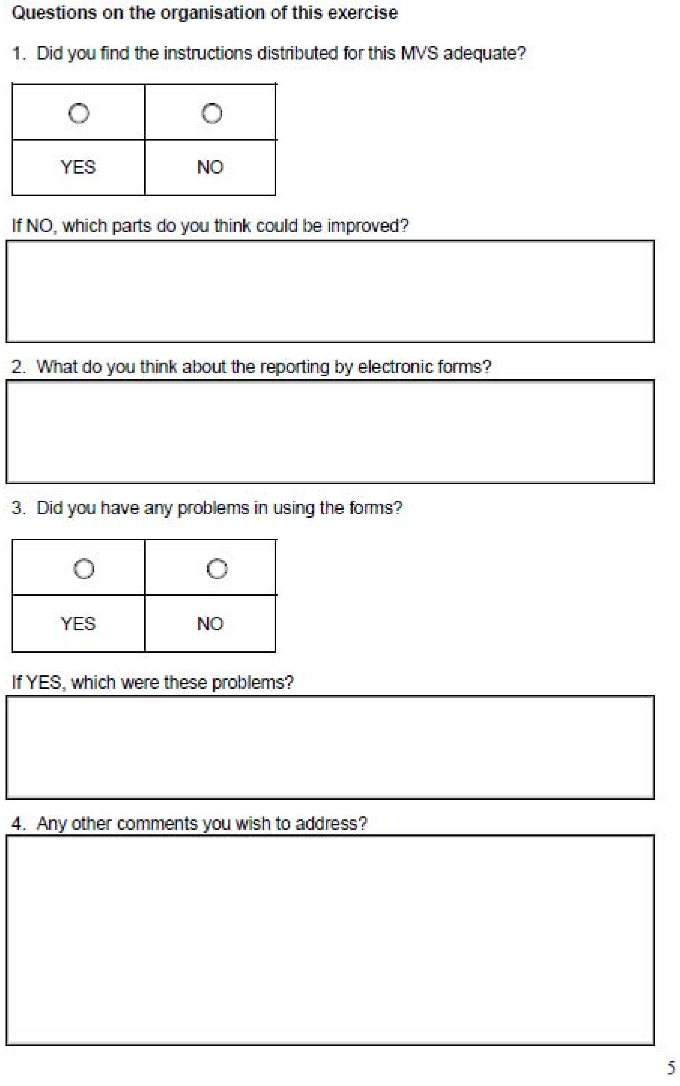


4. Which of the following matrices does your laboratory analyse for the determination of PAHs on a routine basis?*

○	Edible oils and facts (6.1.1)*
○	Smoked meat (6.1.2)
○	Muscle meat of smoked fish and smoked fishery products (6.1.3)
○	Muscle meat of fish (6.1.4)
○	Crustaceans, cephalopods, other than smoked (6.1.5)
○	Bivalve molluscs (6.1.6)
○	Processed cereal-based foods and baby foods for infants and young children (6.1.7)
○	Infant formulae (6.1.8)
○	Dietary foods for special medical purposes (6.1.9)
○	Cereals
○	Other

*the number in parentheses indicates the legislative reference in Commission Regulation (EC) No 1881/2006

If OTHER, please specify



Questions on the organisation of this exercise

1. Did you find the instructions distributed for this MVS adequate?

○	○
YES	NO

If NO, which parts do you think could be improved?

2. What do you think about the reporting by electronic forms?

3. Did you have any problems in using the forms?

○	○
YES	NO

If YES, which were these problems?

4. Any other comments you wish to address?

General questions on the method

1. Did you find the method description adequate?

○	○
YES	NO

If NO, in which part(s) could it be improved?

2. Please report below the settings you applied when options where given

Which extraction apparatus did you use?

○	○
PLE	Soxhlet or similar

Which extraction solvent did you use?

○	○
n-hexane	cyclohexane

Which injection port did you use?

○	○
PTV	Split-splitless

3. Did you follow the method in all details?*

○	○
YES	NO

If NO, are the deviations from the SOP those described in the FORM for reporting the results of the training samples?

○	○
YES	NO

If NO, in which part(s) did you deviate from the protocol?*

Method paragraph	Description of the deviation applied
5 – Reagents	(e.g. the labelled standards-please give the composition)
6 - Apparatus	(e.g. the SEC column)
7.2 – Test portion preparation for solid samples	
7.3 – Solid sample extraction	
7.4 - Test portion preparation for liquid samples	

Method paragraph	Description of the deviation applied
7.5 – SEC clean-up	
7-6 to 7.7	
7.8 SPE clean-up	
8.1 – GC-MS operating conditions	

Method paragraph	Description of the deviation applied
8.2 & 8.3 - Calibration	
8.4 – Sample analysis	
Others	

4. Did you encounter any problem during the analysis?*

○	○
YES	NO

If YES, what were the specific problems and to which samples do they apply?*

5. Did you notice any abnormality, which however seem to had no effect on the result (e.g. the SPE column was blocked or with very low flow)?*

○	○
YES	NO

If YES, please describe and report for which samples (codes) they occurred.*

6. Were you familiar with all the steps performed during the analysis?*

○	○
YES	NO

If NO, please describe and report for which step(s). (Refer to the respective paragraph number in the SOP*)

7. How long did it take the whole processing of the MVS test (coded) samples (from the preparation to the reporting of the results)? (hours)

8. Any other information you wish to add

Specific details on the method

1. In case you used Soxhlet extraction, how many cycles did you apply in total?*

○	○	○
Less than 30	30-45	More than 45

2. Did you need to include any "over night" stops in the analysis of the MVS samples without performing a new calibration when resuming the sequence?*

○	○
YES	NO

If YES, please state for which samples and at what stage of the analysis.*

3. Did you have any case were the sample extract evaporated to dryness?*

○	○
YES	NO

If YES, please state for which samples and at what stage of the analysis.*

4. How did you integrate the signals (automatically or manually)?*

○	○
Automatically	Manually

If AUTOMATICALLY, did you check the correctness of integration visually?*

○	○
YES	NO

If YES, for how many chromatograms was it necessary to re-integrate at least one of the target PAHs peak? (Numeric value)*

5. Which global settings did you use for automatic integration (e.g. valley-to-valley or horizontal baseline or tangential, etc.)?

6. Did you obtain the correct Q1/Q2 ratio for all the samples?*

○	○
YES	NO

If NO, please report the sample code(s) corresponding to non acceptable ratios*

Sample code	Sample code

Sample code	Sample code

7. Did you measure a resolution between BbF/BkF of at least 0.8 and between BkF/BjF of at least 0.4 for all the samples?*

○	○
YES	NO

If NO, please report the corresponding sample code(s) and values for the separation factors *

Sample code	BbF/BkF	BkF/BjF

8. Did you have any problem related with the quality control procedures (see paragraph 11 of the SOP?*

○	○
YES	NO

If YES, please describe the problem and state for which sample(s) it occurred.*

ATTENTION

The EU-RL PAHs thanks you for answering to this questionnaire.

Please, send back this FORM before the 14/03/2011

Once you filled-in the form, use the email button and submit the filled-in form to us via email. You may also save it to your computer.

Submit by Email

Furthermore, print it (use the print button), sign the hardcopy and fax it: JRC-IRMM FSQ, Donata Lerda, Retieseweg 111, B-2440 Geel, Belgium; **Fax: +32 14 571 783**
(you can also scan the signed FORM and send the PDF file by e-mail at the mail address: jrc-irmm-crl-pah@ec.europa.eu)

Print Form

YOUR Signature: ______________________________

Questionnaires not transmitted both by e-mail as PDF Forms and by FAX (or signed PDF by e-mail) as signed Forms cannot not be included in the report

ANNEX 4 – Announcement of the study

Announcement on the web

Method validation study on a GC-MS based method for PAHs analysis in food matrices

The European Union Reference Laboratory for PAHs (EU-RL PAHs) will organise in the second half of 2010 an inter-laboratory comparison for the validation of an analytical method (MVS) to determine the 4 target PAHs, according with the decision of the Standing Committee on Food Chain and Animal Health (SCFCAH), benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene, and chrysene in various foodstuffs.

The method is based on pressurised liquid extraction (PLE) (Soxhlet will be considered an equivalent alternative), a first purification step by size exclusion chromatography (SEC), and a final purification by solid phase extraction (SPE). The detection / quantitation is made by gas-chromatography coupled with mass spectrometry (GC-MS).

Design of the study

The MVS will be conducted and evaluated according to ISO 5725-2:1994.

The participants to the study will be asked to analyse a total of about 20 samples, including edible oil, meat, fish, mussel tissue, cereals, and infant formula.

The concentration levels will be between 0,5 and 20 µg/kg.

Dispatch will be pre-announced at least two week in advance and is foreseen for the second half of September.

The participants will have 6 weeks from dispatch date to report back the results.

The MVS will be open to all interested laboratories, e.g. NRLs, official food control laboratories, private food control laboratories, academia, and laboratories from industry.

If you are interested in participating to this MVS, please contact:

Donata Lerda

Institute for Reference Materials and Measurements (IRMM)

EU-RL Polycyclic Aromatic Hydrocarbons

Retieseweg 111

B-2440 Geel, Belgium

Tel.: +32-14-571 826

Fax: +32-14-571 783

E-mail: jrc-irmm-crl-pah@ec.europa.eu

Announcement by e-mail

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL PAH
Sent: Thu, 05-08-2010 11:01 AM
To:
Subject: Method validation study for the determination of PAHs in various foodstuffs
Importance: Normal

Dear Madam/ Dear Sir,

The EU-RL PAHs is organising a Method Validation Study on PAHs in various foodstuffs.
If you should be interested in participating, please read the attached invitation letter.

You can also go to the link:
http://irmm.jrc.ec.europa.eu/html/CRLs/crl_pah/interlaboratory_comparisons/PAHs_in_food_index.htm

Please let us know, by writing to this mailbox as soon as possible, whether you would like to participate.

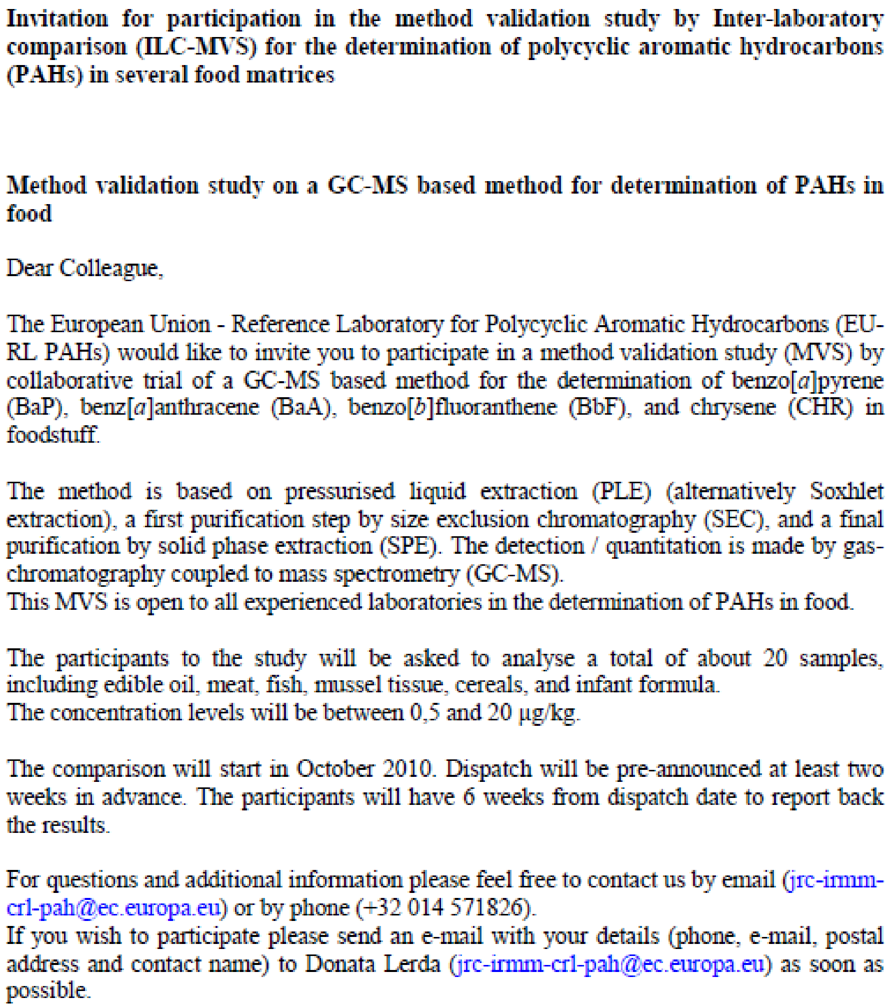
Thank you and best regards,

Donata

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: *+32 14 571 826*
Fax: *+32 14 571 783*
e-mail: donata.lerda@ec.europa.eu

Attachment to the e-mail: Invitation letter



Invitation for participation in the method validation study by Inter-laboratory comparison (ILC-MVS) for the determination of polycyclic aromatic hydrocarbons (PAHs) in several food matrices

Method validation study on a GC-MS based method for determination of PAHs in food

Dear Colleague,

The European Union - Reference Laboratory for Polycyclic Aromatic Hydrocarbons (EU-RL PAHs) would like to invite you to participate in a method validation study (MVS) by collaborative trial of a GC-MS based method for the determination of benzo[*a*]pyrene (BaP), benz[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF), and chrysene (CHR) in foodstuff.

The method is based on pressurised liquid extraction (PLE) (alternatively Soxhlet extraction), a first purification step by size exclusion chromatography (SEC), and a final purification by solid phase extraction (SPE). The detection / quantitation is made by gas-chromatography coupled to mass spectrometry (GC-MS).
This MVS is open to all experienced laboratories in the determination of PAHs in food.

The participants to the study will be asked to analyse a total of about 20 samples, including edible oil, meat, fish, mussel tissue, cereals, and infant formula.
The concentration levels will be between 0,5 and 20 µg/kg.

The comparison will start in October 2010. Dispatch will be pre-announced at least two weeks in advance. The participants will have 6 weeks from dispatch date to report back the results.

For questions and additional information please feel free to contact us by email (jrc-irmm-crl-pah@ec.europa.eu) or by phone (+32 014 571826).
If you wish to participate please send an e-mail with your details (phone, e-mail, postal address and contact name) to Donata Lerda (jrc-irmm-crl-pah@ec.europa.eu) as soon as possible.

ANNEX 5 – Subscription to the study (the draft SOP is not included in the ANNEXES)

Subscription e-mail

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL PAHs
Sent: Friday, October 01, /2010 11:04 AM
To:
Subject: JRC.DG.6/DL/bk/ARES (2010) 649538: MVS on PAHs in food
Importance: Normal

JRC.DG.6/DL/bk/ARES (2010) 649538
Dear Madame / Sir,

The European Union Reference Laboratory for PAHs announced on the 5th of August 2010 the organisation of an inter-laboratory comparison for the validation of a method to determine the four target PAHs, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, in food.
As a first step of the study we would like to ask you to send back to us your comments about the standard operating procedure (SOP) and the outline of the study which are herein attached. Please put special attention to clarity of description of the analytical procedure.

You are also asked to confirm your interest in participating in the study by filling in and signing the attached FORM (please note that some of the fields are required to be filled in before being able to send back the FORM).

Deadline for replying by sending back the FORM both via e-mail and via FAX (or e-mail if the signed form is saved as PDF) is **15 October 2010**.

FORM_Subscription
to MVS on PA...

Draft SOP PAHs in
food_MVS 201...

MVS PAHs in
food_Outline of th...

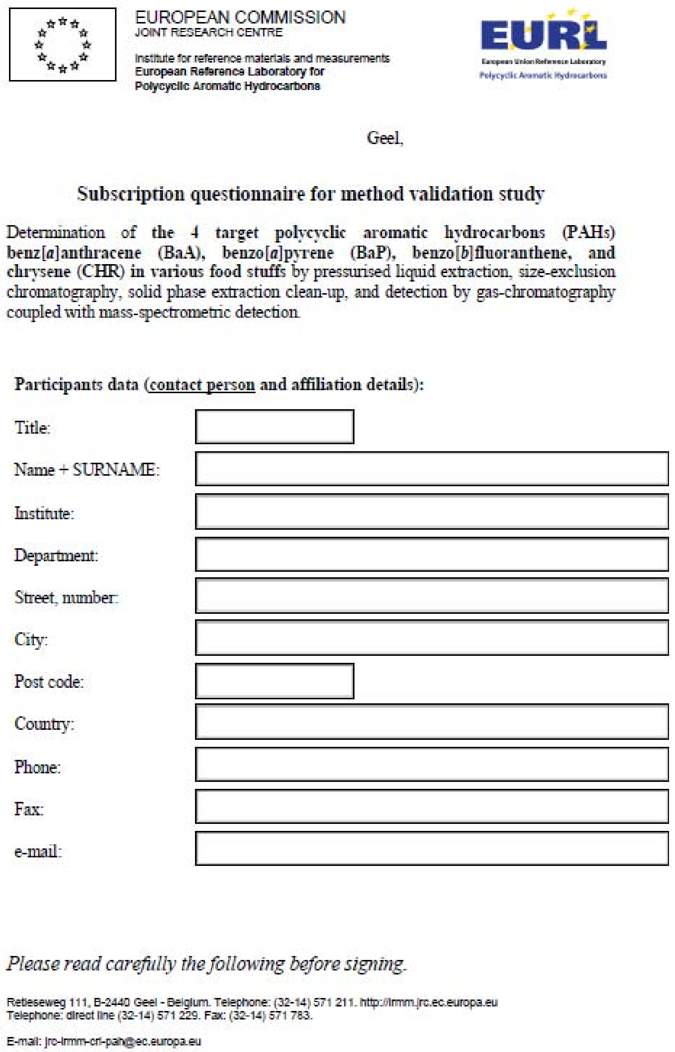
Thank you in advance for the co-operation and best regards,

Donata

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: *+32 14 571 826*
Fax: *+32 14 571 783*
e-mail: donata.lerda@ec.europa.eu

Subscription PDF Form



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JOINT RESEARCH CENTRE
Institute for reference materials and measurements
European Reference Laboratory for
Polycyclic Aromatic Hydrocarbons

EURL
European Union Reference Laboratory
Polycyclic Aromatic Hydrocarbons

Geel,

Subscription questionnaire for method validation study

Determination of the 4 target polycyclic aromatic hydrocarbons (PAHs) **benz[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*b*]fluoranthene, and chrysene (CHR) in various food stuffs** by pressurised liquid extraction, size-exclusion chromatography, solid phase extraction clean-up, and detection by gas-chromatography coupled with mass-spectrometric detection.

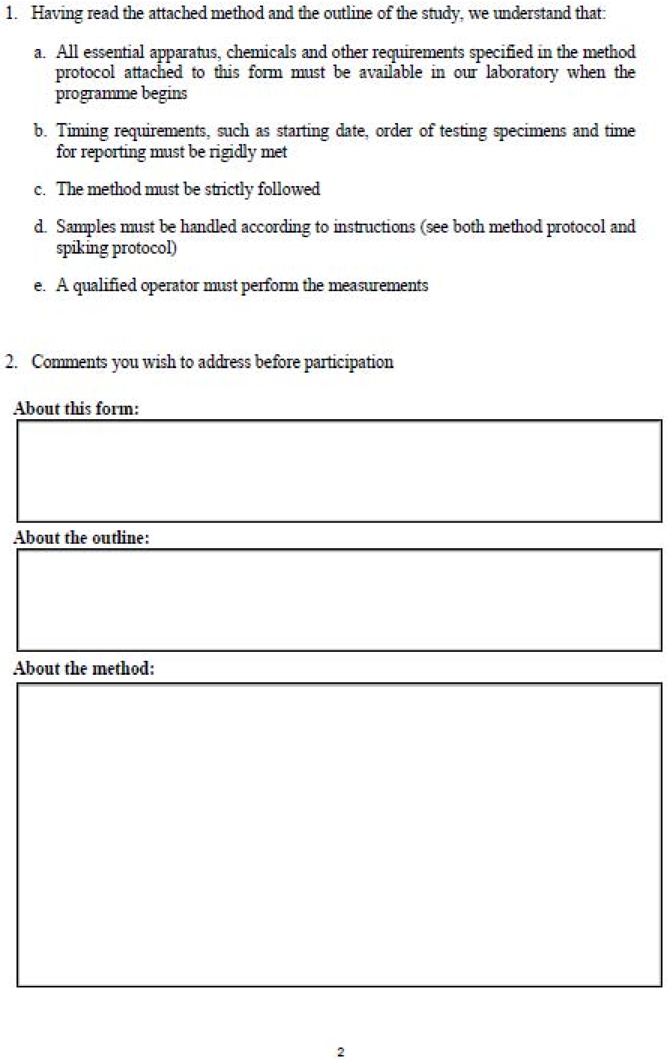
Participants data (contact person and affiliation details):

Title:
Name + SURNAME:
Institute:
Department:
Street, number:
City:
Post code:
Country:
Phone:
Fax:
e-mail:

Please read carefully the following before signing.

Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://irmm.jrc.ec.europa.eu
Telephone: direct line (32-14) 571 229. Fax: (32-14) 571 783.

E-mail: jrc-irmm-crl-pah@ec.europa.eu



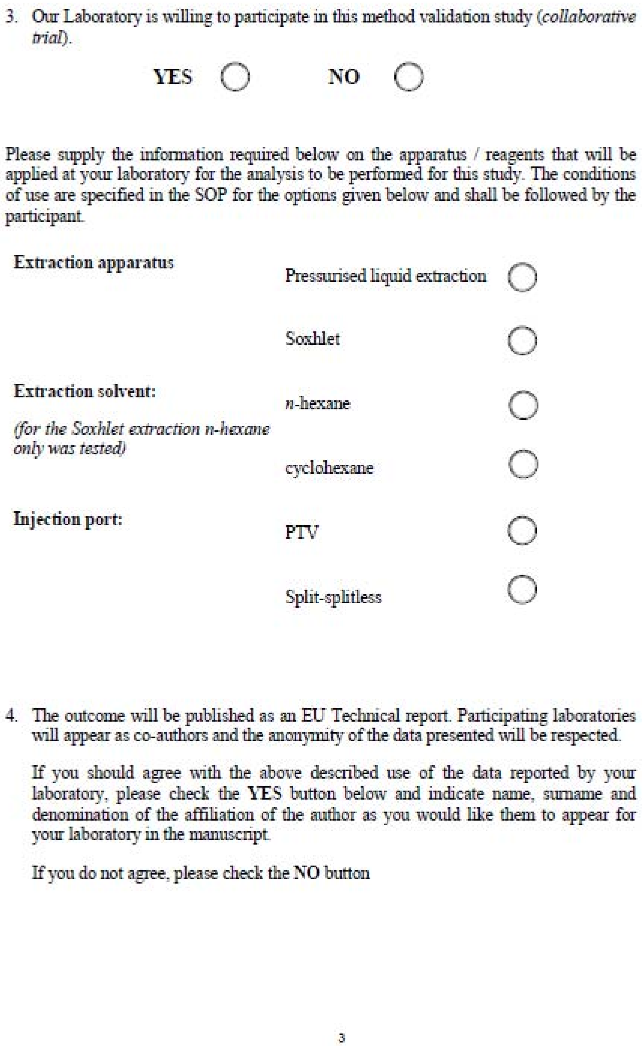
1. Having read the attached method and the outline of the study, we understand that:
    a. All essential apparatus, chemicals and other requirements specified in the method protocol attached to this form must be available in our laboratory when the programme begins
    b. Timing requirements, such as starting date, order of testing specimens and time for reporting must be rigidly met
    c. The method must be strictly followed
    d. Samples must be handled according to instructions (see both method protocol and spiking protocol)
    e. A qualified operator must perform the measurements
2. Comments you wish to address before participation

About this form:

About the outline:

About the method:

2



3. Our Laboratory is willing to participate in this method validation study (*collaborative trial*).

YES ○ **NO** ○

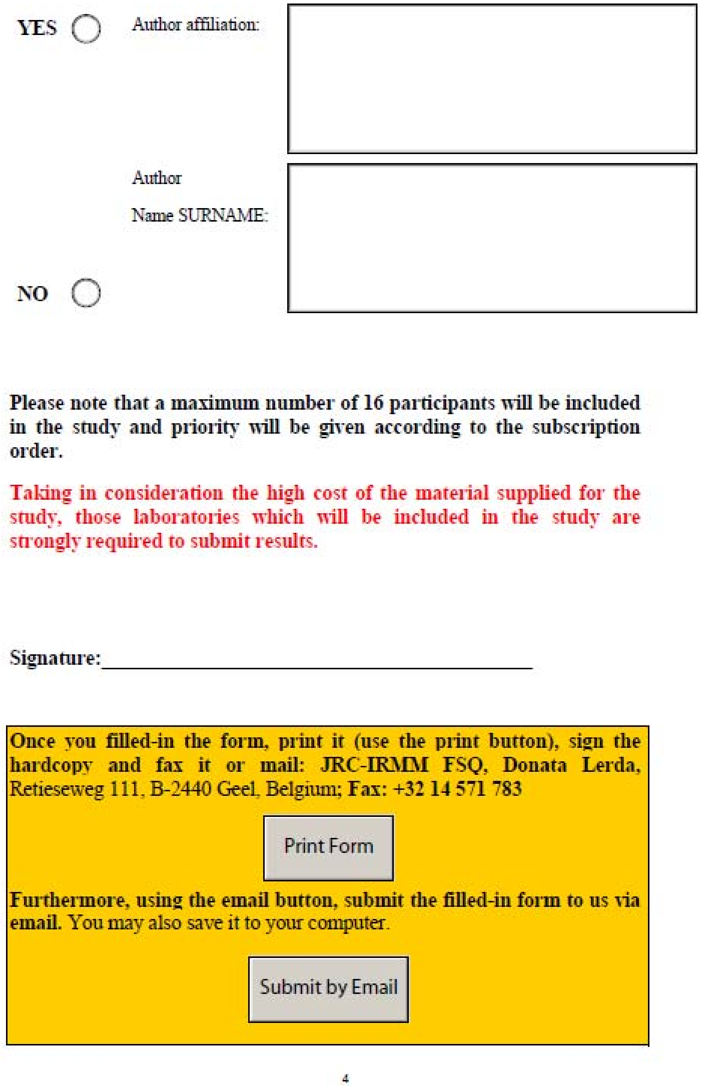
Please supply the information required below on the apparatus / reagents that will be applied at your laboratory for the analysis to be performed for this study. The conditions of use are specified in the SOP for the options given below and shall be followed by the participant.

Extraction apparatus	Pressurised liquid extraction	○
	Soxhlet	○
Extraction solvent: *(for the Soxhlet extraction n-hexane only was tested)*	*n*-hexane	○
	cyclohexane	○
Injection port:	PTV	○
	Split-splitless	○

4. The outcome will be published as an EU Technical report. Participating laboratories will appear as co-authors and the anonymity of the data presented will be respected.

   If you should agree with the above described use of the data reported by your laboratory, please check the **YES** button below and indicate name, surname and denomination of the affiliation of the author as you would like them to appear for your laboratory in the manuscript.

   If you do not agree, please check the NO button



YES ○	Author affiliation:	
	Author Name SURNAME:	
NO ○		

Please note that a maximum number of 16 participants will be included in the study and priority will be given according to the subscription order.

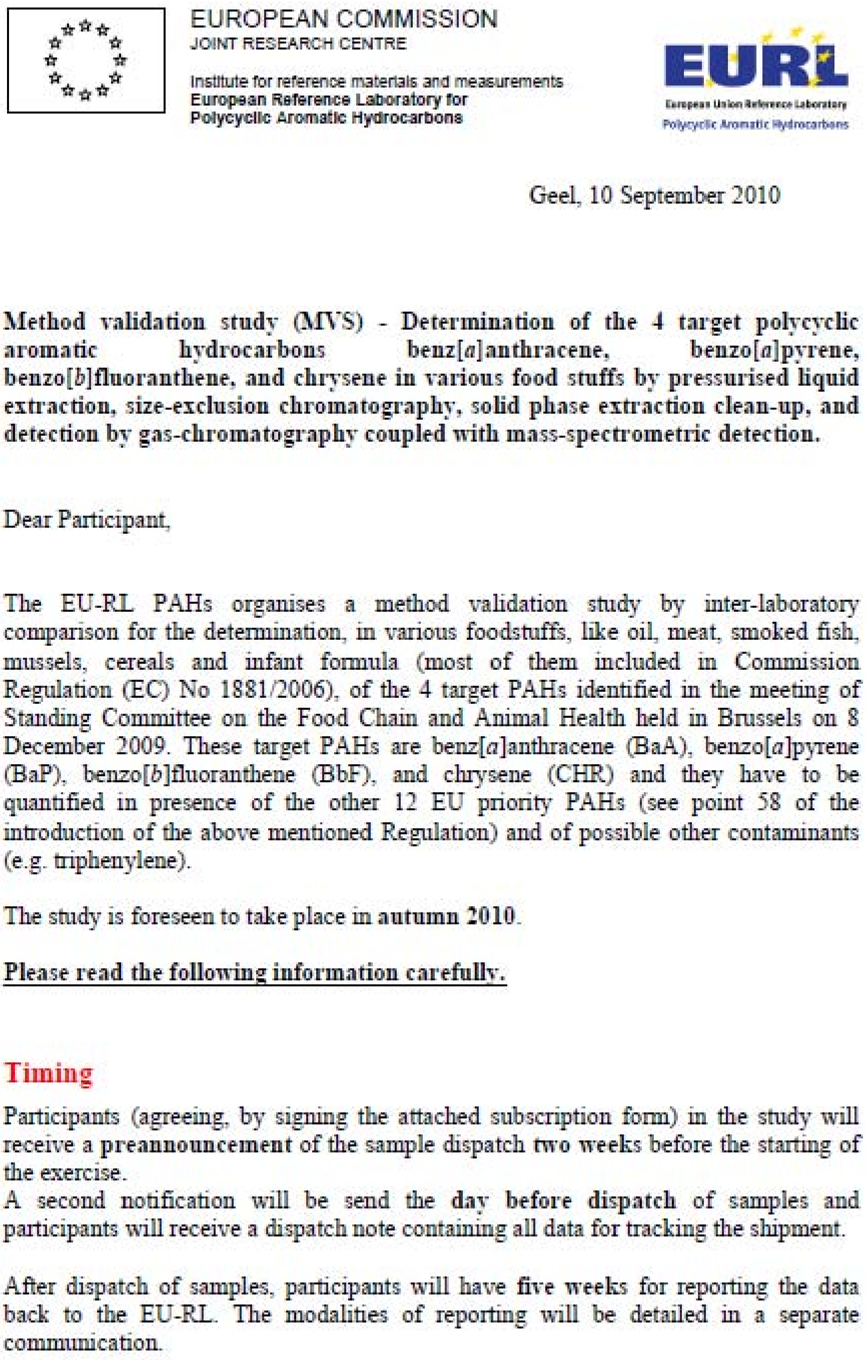
Taking in consideration the high cost of the material supplied for the study, those laboratories which will be included in the study are strongly required to submit results.

Signature:____________________________________

Once you filled-in the form, print it (use the print button), sign the hardcopy and fax it or mail: JRC-IRMM FSQ, Donata Lerda, Retieseweg 111, B-2440 Geel, Belgium; **Fax: +32 14 571 783**

Furthermore, using the email button, submit the filled-in form to us via email. You may also save it to your computer.

Outline of the study



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
European Reference Laboratory for Polycyclic Aromatic Hydrocarbons

EURL
European Union Reference Laboratory
Polycyclic Aromatic Hydrocarbons

Geel, 10 September 2010

Method validation study (MVS) - Determination of the 4 target polycyclic aromatic hydrocarbons benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, and chrysene in various food stuffs by pressurised liquid extraction, size-exclusion chromatography, solid phase extraction clean-up, and detection by gas-chromatography coupled with mass-spectrometric detection.

Dear Participant,

The EU-RL PAHs organises a method validation study by inter-laboratory comparison for the determination, in various foodstuffs, like oil, meat, smoked fish, mussels, cereals and infant formula (most of them included in Commission Regulation (EC) No 1881/2006), of the 4 target PAHs identified in the meeting of Standing Committee on the Food Chain and Animal Health held in Brussels on 8 December 2009. These target PAHs are benz[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*b*]fluoranthene (BbF), and chrysene (CHR) and they have to be quantified in presence of the other 12 EU priority PAHs (see point 58 of the introduction of the above mentioned Regulation) and of possible other contaminants (e.g. triphenylene).

The study is foreseen to take place in **autumn 2010**.

Please read the following information carefully.

Timing

Participants (agreeing, by signing the attached subscription form) in the study will receive a **preannouncement** of the sample dispatch **two weeks** before the starting of the exercise.
A second notification will be send the **day before dispatch** of samples and participants will receive a dispatch note containing all data for tracking the shipment.

After dispatch of samples, participants will have **five weeks** for reporting the data back to the EU-RL. The modalities of reporting will be detailed in a separate communication.

Materials supplied for the study

Participants will receive a parcel containing the following items:

1. Their **participant code** to be used in all following communications with the organiser (the EU-RL)
2. A **"Receipt form"**. If the material has been received damaged, the participant is asked to request immediately new material (the materials will be shipped at room temperature; storage however should be at 4° C until the analysis is performed)
3. The **Standard Operating Procedure (SOP)** to be applied for the analysis of the samples and the blank samples (for better readability, two SOPs will be distributed, one for edible oil and the other one for all other samples, but the method is considered as one)
4. The **Mixed PAHs stock solution** to be used to prepare the calibration solutions
5. The **Mixed labelled PAH process solution (PROCESS)** to be used for spiking of the samples
6. The **Injection standard solution (INJECT)** to be used for the preparation of the sample for gas-chromatographic analysis (calculation of response factors and recovery)
7. The necessary **capillary column** for gas-chromatographic analysis
8. A **set of samples**, comprising:
    a. 16 samples for single analysis with different content levels of the 4 target PAHs (the final number of samples could slightly deviate from this number) and <u>undisclosed content</u>
    b. 2 training samples, edible oil and a smoked fish, with <u>disclosed content</u> of the four target PAHs for training of the participating laboratory staff on the SOP object of this study.

Outline of the study

Samples will be supplied as a combination of known replicates (in this case a duplicate analysis for the sample is required), blind replicates and split levels (for these samples a single result has to be reported).

Participants will also receive, after dispatch of samples, a FORM for **reporting of results** and a FORM with a **questionnaire**.

Participants will be asked to **report the results** for the four target analytes and for as many of the 12 non-target PAHs as possible. Results will have to be reported for the non-training samples, with undisclosed content, only.
The requested results will have to be **reported in µg/kg** as calculated according to Equations 2 and 3 of the SOP.
Participants will be also asked to report the recoveries of the labelled standards according to Equations 4 and 5 of the SOP.

Via a **questionnaire**, participants will be asked to specify which options given in the SOP for some of the analytical steps (e.g. PTV / split-splitless injection for the GC-

MS analysis) were chosen at their laboratory for the analysis of the samples of this MVS. They will also have to report possible deviations from the SOP, which might lead to the removal of their data from the data pool.

Taking in consideration the high cost of the material supplied for the study, those laboratories subscribing and therefore included in the study, are required to submit results.

In case of questions, do not hesitate to contact us by either phone or email (see details below).

Donata Lerda
Institute for Reference Materials and Measurements (IRMM)
EU-RL PAHs
Retieseweg 111
B-2440 Geel, Belgium
Tel: +32-14-571 826
FAX: +32-14-571 783
E-mail: Jrc-irmm-crl-pah@ec.europa.eu

With kind regards,

Thomas Wenzl
(Operating Manager of the European Union Reference Laboratory for PAHs)

Cc: Donata Lerda, Almut Bitterhof, Franz Ulberth, Anne-Mette Jensen

ANNEX 6 – Materials dispatch

Announcement of dispatch e-mail

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL PAHs
Sent: Wednesday, October 20, /2010 14:52 PM
To:
Subject: Method Validation Study on PAHs in Food: pre-announcement of material dispatch
Importance: Normal

Dear Madam, dear Sir,

We are planning to dispatch the samples on week 45.
Dispatching will be done via DHL and according the international rules the content will be classified as DANGEROUS GOODS in EXCEPTED QUANTITIES.
The samples will be described as samples for laboratory use.
Parcels weight will be about 2 kg and content will be of 20 samples, 3 solutions, a GC column, and some documents.

Please let us know if you should need further description and/or a proforma invoice for an efficient and quick custom clearance of the parcel.

Thanks for the co-operation and best regards,

Donata

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

DHL tracking numbers e-mail

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL PAHs
Sent: Tuesday, November 9 /2010 16:35 PM
To:
Subject: PAHs in Food MVS samples dispatch
Importance: Normal

Dear ....,

We sent out the samples today afternoon.
To view your shipment tracking details, please click on the following link:
http://www.dhl.com/cgi-bin/tracking.pl?AWB=xxxxxxxxxx

The following lines report the description of the parcel.
WEIGHT: 3.82
PIECES: 1
CONTENTS: Dangerous Goods in excepted quantities

Please, send back to us the **sample receipt** included in the parcel as soon as you receive it.

Please, remember to store the samples and solutions at **4 °C (in the fridge)** till the use.

Best regards,

Donata

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

Sample receipt

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
European Reference Laboratory for
Polycyclic Aromatic Hydrocarbons

EURL
European Union Reference Laboratory
Polycyclic Aromatic Hydrocarbons

RECEIPT FORM

Surname of Participant	
Name of Participant	
Affiliation	
Lab ID	
Country	

YOUR SIGNATURE: ______________________________

NOTE: UPON RECEIPT STORE THE MATERIAL IN A FRIDGE (AT 4 ºC)

Please ensure that the items listed below have been received undamaged, and then check the relevant statement in the **table at next page**:

Contents of parcel

a) One inter-laboratory comparison sample receipt form (= this form)
b) A printout of the PT outline
c) A printout of the SOP of the method
d) A printout of the instructions
e) Your participation code (LAB ID)
f) A gas-chromatographic column
g) The Mixed PAHs stock solution with its specification sheet (for calibration)
h) The Mixed labelled PAH process solution (for spiking and calibration)
i) The Injection standard solution (for the calculation of the response factor and the recovery)
j) 16 coded test materials for direct analysis
k) 4 training samples (2+2) with disclosed content of the four target PAHs
l) Safety sheets for solvents and some of the PAHs

Date of the receipt of the test materials	
All items have been received undamaged	YES / NO
If NO, please list damaged items according to the letters associated at each item in the list above Please write one item per row	
Items are missing	YES / NO
If YES, please list missing items according to the letters associated at each item in the list above Please write one item per row	
Serial numbers of the samples you received	

Please fax or email (as PDF) the completed form to:

Donata Lerda
European Commission - DG Joint Research Centre
Institute for Reference Materials and Measurements
B-2440 Geel, Belgium

Fax No: 0032-14-571 783

Email: jrc-irmm-crl-pah@ec.europa.eu

ANNEX 7 – Supporting documents

In addition to the Instructions, which are in this ANNEX, participants also received the SOP (see ANNEX 1), two FORMs for reporting results for training samples (these FORMs are not included in the ANNEXES to the report; they are available as additional information upon request) and the outline (see ANNEX 5)

STEP 1 start e-mail

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL PAHs
Sent: Wednesday, November 10/2010 08:46 AM
To:
Subject: Ares (2010)758890: MVS on PAHs in food: Reporting of results for STEP 1
Importance: Normal

Ares(2010)758890

Dear Madam, dear Sir,

Parcels containing the samples to be analysed in the course of this collaborative trial were dispatched yesterday.
Starting **from now till the 03/12/2010** you can report the results for the training samples (see STEP 1 in the attached Instructions document).

Please, use the two PDF FORMs attached to this mail to send back your results to us, following carefully the procedure reported at the beginning and end of each FORM.

In case you should have any doubt or question, please do not hesitate to contact us.

Thanks again for joining the study.

Best regards,

Donata

SOP PAHs in
od_MVS 2010_fina.

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Instructions_MVS...

MVS PAHs in
food_Outline of th...

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_MVS PAHs in foo..

Results STEP
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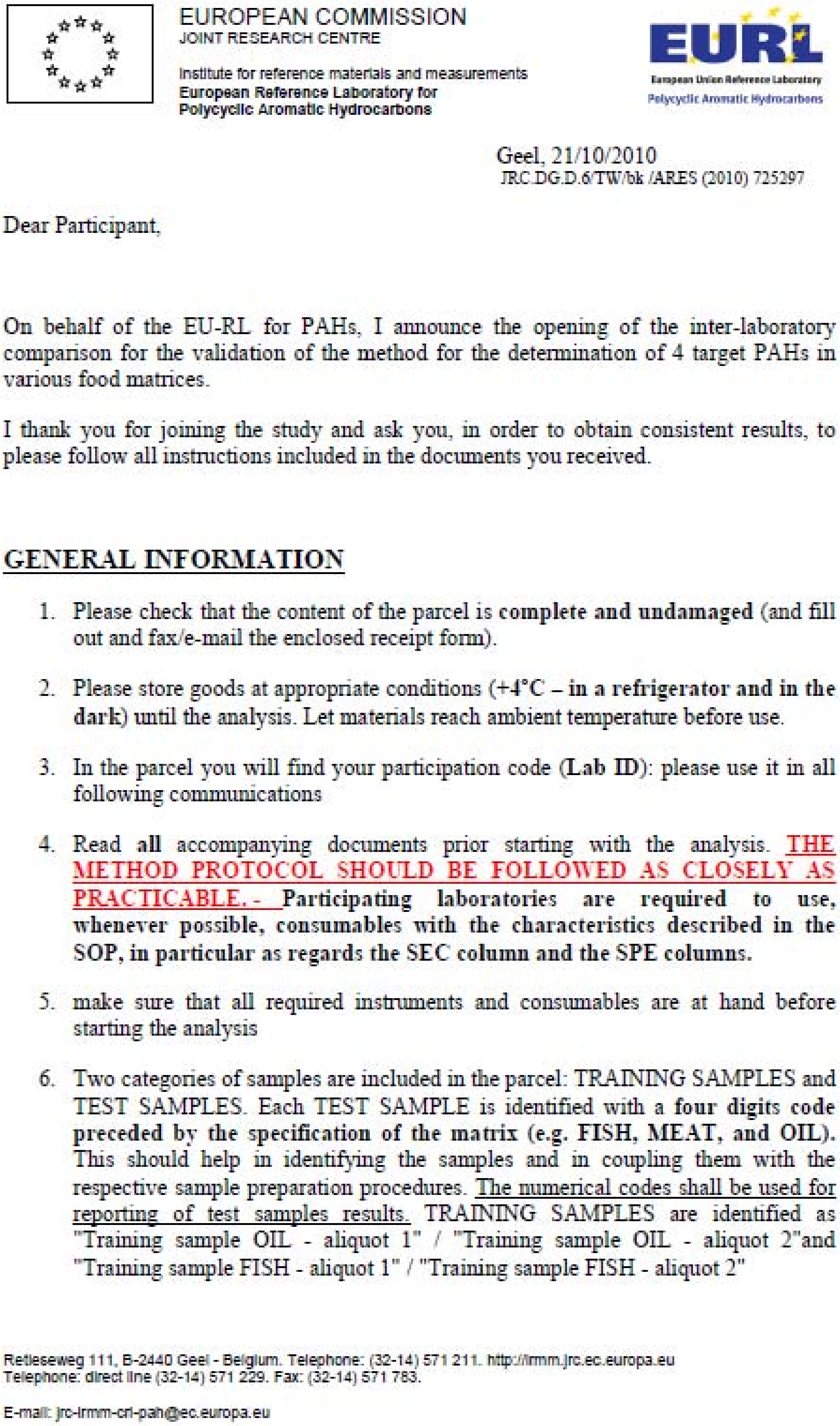
Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

Instructions of the MVS



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
European Reference Laboratory for
Polycyclic Aromatic Hydrocarbons

EURL
European Union Reference Laboratory
Polycyclic Aromatic Hydrocarbons

Geel, 21/10/2010
JRC.DG.D.6/TW/bk /ARES (2010) 725297

Dear Participant,

On behalf of the EU-RL for PAHs, I announce the opening of the inter-laboratory comparison for the validation of the method for the determination of 4 target PAHs in various food matrices.

I thank you for joining the study and ask you, in order to obtain consistent results, to please follow all instructions included in the documents you received.

GENERAL INFORMATION

1. Please check that the content of the parcel is **complete and undamaged** (and fill out and fax/e-mail the enclosed receipt form).
2. Please store goods at appropriate conditions (**+4°C – in a refrigerator and in the dark**) until the analysis. Let materials reach ambient temperature before use.
3. In the parcel you will find your participation code (**Lab ID**): please use it in all following communications
4. Read **all** accompanying documents prior starting with the analysis. THE METHOD PROTOCOL SHOULD BE FOLLOWED AS CLOSELY AS PRACTICABLE. - **Participating laboratories are required to use, whenever possible, consumables with the characteristics described in the SOP, in particular as regards the SEC column and the SPE columns.**
5. make sure that all required instruments and consumables are at hand before starting the analysis
6. Two categories of samples are included in the parcel: TRAINING SAMPLES and TEST SAMPLES. Each TEST SAMPLE is identified with a **four digits code preceded by the specification of the matrix (e.g. FISH, MEAT, and OIL).** This should help in identifying the samples and in coupling them with the respective sample preparation procedures. The numerical codes shall be used for reporting of test samples results. TRAINING SAMPLES are identified as "Training sample OIL - aliquot 1" / "Training sample OIL - aliquot 2"and "Training sample FISH - aliquot 1" / "Training sample FISH - aliquot 2"

Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://irmm.jrc.ec.europa.eu
Telephone: direct line (32-14) 571 229. Fax: (32-14) 571 783.

E-mail: jrc-irmm-crl-pah@ec.europa.eu

7. Your laboratory will be asked, for both TRAINING SAMPLES and TEST SAMPLES, to report results for as many PAHs as possible. As a matter of fact, notwithstanding the SOP scope includes only the four target PAHs, the method set up and the consumables supplied allow the determination of all 15+1 EU priority PAHs.

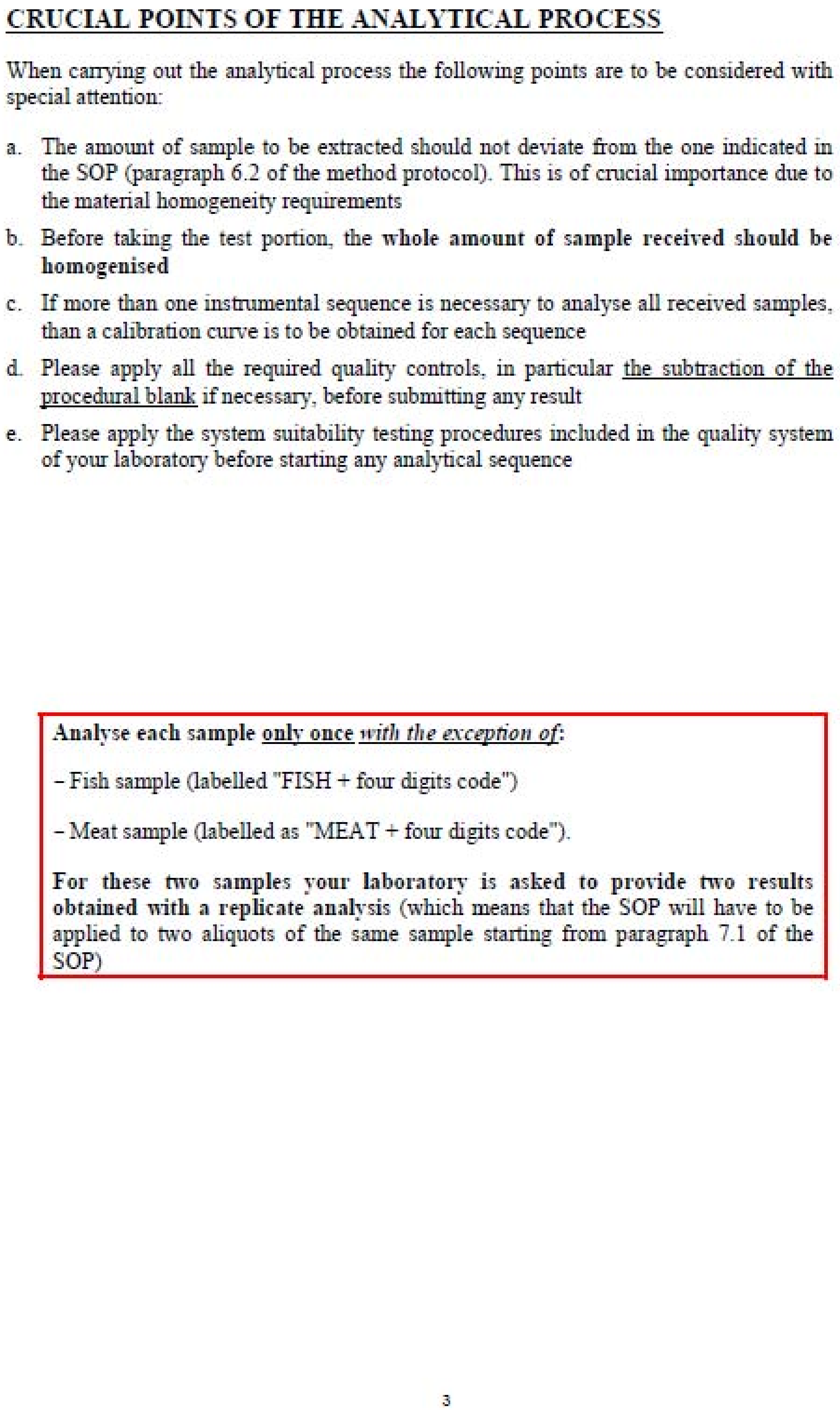
DESIGN OF THE STUDY

The study is divided in three steps:

STEP 1. FAMILIARISATION WITH THE METHOD AND EVALUATION OF THE APPLICATION OF THE METHOD AT THE PARTICIPATING LABORATORY. In the first step your laboratory is required to analyse the **first** of the two aliquots of the two training samples labelled as "Training sample OIL - aliquot 1" and "Training sample FISH - aliquot 1". Thanks to the amount of sample, you will be able to perform replicate analysis (till about 9 for FISH and till about 14 for OIL), therefore you will have the chance to familiarise with all the analytical steps described in the SOP. We also ask you to report the results to allow the organiser to verify whether the method was correctly applied. The results obtained shall be reported back to us **within three weeks** from dispatch, **by 03/12/2010** by using the "Training samples results FORM". Recoveries (from the labelled PAHs as described in paragraph 10.1 of the SOP) and native PAHs contents will have to be reported for maximum **5** replicate analyses. In the same FORM you will be also asked to describe the deviations of the method applied at your laboratory from the SOP. **In the conduction of the analysis, you are advised to pay special attention to the calibration and to the steps where your laboratory deviates from the SOP.**

STEP 2. ASSESSMENT OF THE APPLICATION OF THE SOP. In this second step the results and the recoveries reported by your laboratory will be evaluated at the EU-RL and possible reasons for unsatisfactory results will be analysed by using the information reported in the "Training samples questionnaire FORM". Joint root-cause analysis will be performed based on both the closeness to the reference values and for the precision estimation obtained from replicate analysis. You will receive this information and an indication whether further tuning of the settings applied at your laboratory should be required **within two weeks from the reporting of results for the first aliquot of the training samples**. Additional time might be necessary in case the analysis of the second aliquot of the TRANING SAMPLES is required for checking the adjustments applied to the method. At this stage, if considered necessary for the attainment of the required accuracy and precision, the EU-RL will disclose the reference values of the PAHs contents in the two training samples to the concerned laboratories.

STEP 3. METHOD VALIDATION STUDY. Your laboratory will be invited to analyse the coded samples and to report back to us the results obtained by using the "Test samples results FORM" (you will receive several FORMS for reporting to allow an appropriate collation of results) and your notes in the "Test samples questionnaire FORM"



CRUCIAL POINTS OF THE ANALYTICAL PROCESS

When carrying out the analytical process the following points are to be considered with special attention:

a. The amount of sample to be extracted should not deviate from the one indicated in the SOP (paragraph 6.2 of the method protocol). This is of crucial importance due to the material homogeneity requirements

b. Before taking the test portion, the **whole amount of sample received should be homogenised**

c. If more than one instrumental sequence is necessary to analyse all received samples, than a calibration curve is to be obtained for each sequence

d. Please apply all the required quality controls, in particular the subtraction of the procedural blank if necessary, before submitting any result

e. Please apply the system suitability testing procedures included in the quality system of your laboratory before starting any analytical sequence

Analyse each sample only once ***with the exception of*****:**

– Fish sample (labelled "FISH + four digits code")

– Meat sample (labelled as "MEAT + four digits code").

For these two samples your laboratory is asked to provide two results obtained with a replicate analysis (which means that the SOP will have to be applied to two aliquots of the same sample starting from paragraph 7.1 of the SOP)

For all samples results have to be reported on the wet weight ***with the exception of*:**

– Mussels samples (labelled "MUSSELS + four digits code")

For each of these two samples your laboratory is asked to provide both the result on total weight (in the FORM "Result [1]") and the result calculated on the dry weight (in the FORM "Result [2]").

(in the Equation 3 of the SOP "W_{SAMPLE}" is obtained by weighing of sample for "Result [1]" and by subtracting the water content (H%) as described in the following equation for "Result [2]":

$$W_{SAMPLE} = W_{MEASURED} - \frac{\%H \times W_{MEASURED}}{100}$$

Where:

W_{SAMPLE}	weight of the test portion to be entered in Equation 3 [g]
$W_{MEASURED}$	weight of the test portion measured at the beginning of the analysis (paragraph 7.2 of the SOP) [g]
$\%H$	Humidity % as calculated in the equation below

H% is obtained by drying at 105 °C an accurately weighed amount of 1,0 g of mussels sample till reaching a constant final weight

$$\%H = \frac{W_{INITIAL} - W_{FINAL}}{W_{INITIAL}} \times 100$$

Where:

$\%H$	Humidity %
$W_{INITIAL}$	weight of the test portion before drying [g]
W_{FINAL}	weight of the test portion after drying (stable weight) [g]

In case you should encounter any problem during the analysis, please contact us for a replacement of the lost sample(s)

TIMING

STEP 1: three weeks from dispatch – deadline **03/12/2010.**

STEP 2: two weeks from end of STEP 1(this timing concerns the MVS organiser) + additional timing for possible further analyses of training samples.

STEP 3: the final deadline for this collaborative trial is linked to the 3 STEPS timing. Participants will be given five weeks for reporting the results and notes from beginning of STEP 3, when the **deadline will be announced by e-mail**.

We are looking forward to hear from you and hope the method suits your needs for future use.

A detailed outline of the study was included in the MVS sample parcel together the method protocol (SOP); in addition, in this document you find further details. Anyhow we would like to encourage you to contact us, in case you seek further clarification, at the following address:

MVS coordinator

Donata LERDA (deputy in case of absence, Patricia LOPEZ)
Fax: 0032-14-571783
e-mail: jrc-irmm-crl-pah@ec.europa.eu

With kind regards,

Thomas Wenzl

(Operating Manager of the European Union Reference Laboratory for PAHs)

Cc: Donata Lerda, Almut Bitterhof, Franz Ulberth, Anne-Mette Jensen, Patricia Lopez

ANNEX 8 – Communication (conclusion of STEP 2 – beginning of STEP 3)

In addition to the e-mail below, other communications were exchanged with all participants; they are available as additional information upon request. An example for the reporting FORMs and the Questionnaire FORM are included in ANNEX 2 and 3 respectively

STEP 3 start e-mail

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL PAHs
Sent: Wednesday, January 26/2011 09:17 AM
To:
Subject: MVS on PAHs in various foodstuffs: STEP 3
Importance: Normal

Dear Madam, dear Sir,

The STEP1 and STEP2 of the study can be considered successfully concluded. Please find herein attached the PDF files of the specification sheet of the two training samples.

As from today STEP3 is started.

Please, read the instructions you received on 10/11/2010 carefully, in particular on how to carry-out the analysis for mussels samples. For each sample one analysis/one result set is required, with the exception of MEAT and FISH; for each of those two samples, after mixing the content of the can, two aliquots are to be prepared and analysed, and two results reported.

From now till the 14/03/2011 you can report the results.

Please, use the PDF FORMs attached to this mail to send back your results and questionnaire to us, following carefully the procedure reported at the beginning and end of each FORM.

The FORMs titled as "others" should be used for all samples which matrix is not clearly identified in the label (which are fish, meat, oil, and mussels). On each FORM two sets of results are to be reported and, in particular for "others" attention has to be payed to the sample codes, as it will be the only tracking code identifying the matrix when we have to proceed to data evaluation.

Those laboratories asking for additional ampoules of the standard mixture of labelled PAHs for spiking and/or of injection standard will receive soon the dispatching details. As we had to prepare a new solution, they are required to prepare the calibration curve and to spike the samples with the new ampoule of labelled PAHs they will receive. Planned dispatch date is 07/02/2011.

In case you should have any doubt or question, please do not hesitate to contact us.

Thanks again for joining the study.

Best regards,

Donata

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

Specification sheets for the two training samples

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Institute for reference materials and measurements
European Union Reference Laboratory for
Polycyclic Aromatic Hydrocarbons

Fish training sample **Product ID: TRAINING SAMPLE - fish**

Production year: 2006 Total weight: 50 g

Expiry date: *December 2010*

Test material composition:

	Product name	CAS	Conc.*	U**
			(µg/g)	± %
1	5-methylchrysene	3697-24-3	7,3	3
2	Benzo[a]anthracene	56-55-3	8,9	5
3	Benzo[a]pyrene	50-32-8	2,9	5
4	Benzo[b]fluoranthene	205-99-2	9,2	5
5	Benzo[c]fluorene	205-12-9	2,6	5
6	Benzo[ghi]perylene	191-24-2	8,5	6
7	Benzo[j]fluoranthene	205-82-3	3,7	6
8	Benzo[k]fluoranthene	207-08-9	3,6	5
9	Chrysene	218-01-9	8,1	5
10	Cyclopenta[c,d]pyrene	27208-37-3	3,1	5
11	Dibenzo[a,e]pyrene	192-65-4	4,9	5
12	Dibenzo[a,h]anthracene	53-70-3	3,6	5
13	Dibenzo[a,h]pyrene	189-64-0	4,0	5
14	Dibenzo[a,i]pyrene	189-55-9	3,8	6
15	Dibenzo[a,l]pyrene	191-30-0	3,6	7
16	Indeno[c,d]pyrene	193-39-5	7,4	9

** The concentrations were calculated taking into account the purity statements of the single products*

*** U is the expanded uncertainty calculated using the coverage factor 2 (corresponding to a confidence interval of 95%) multiplied by the combined standard uncertainty and rounding up the values so obtained. The standard uncertainty contributions were the repeatability standard deviation of the experiments for material verification, the preparation of the labelled standards and the preparation of the calibration solutions.*

Analytical method for confirmation	**Product ID: TRAINING SAMPLE - fish**

Detection: GC-MS in SIM mode (isotope dilution)

Warning	**Product ID: TRAINING SAMPLE - fish**
	Store in the dark at 4 ºC or less The European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.
Safety of the product	The material contains some teratogenic and carcinogenic substances. Check the attached material safety data sheets for information on hazard, exposure, and safe handling.

Edible oil training sample

Product ID: TRAINING SAMPLE - oil

Production year: 2009

Total volume: 15 ml

Expiry date: *December 2010*

Test material composition:

	Product name	CAS	Conc.*	U**
			(µg/g)	± %
1	5-methylchrysene	3697-24-3	1,1	6
2	Benzo[a]anthracene	56-55-3	2,4	5
3	Benzo[a]pyrene	50-32-8	3,0	5
4	Benzo[b]fluoranthene	205-99-2	5,4	5
5	Benzo[c]fluorene	205-12-9	1,8	3
6	Benzo[ghi]perylene	191-24-2	6,2	5
7	Benzo[j]fluoranthene	205-82-3	1,4	5
8	Benzo[k]fluoranthene	207-08-9	8,2	5
9	Chrysene	218-01-9	3,4	5
10	Cyclopenta[c,d]pyrene	27208-37-3	7,7	5
11	Dibenzo[a,e]pyrene	192-65-4	1,0	6
12	Dibenzo[a,h]anthracene	53-70-3	3,8	5
13	Dibenzo[a,h]pyrene	189-64-0	2,5	5
14	Dibenzo[a,i]pyrene	189-55-9	9,8	6
15	Dibenzo[a,l]pyrene	191-30-0	1,5	5
16	Indeno[c,d]pyrene	193-39-5	3,8	5

** The concentrations were calculated taking into account the purity statements of the single products*

*** U is the expanded uncertainty calculated using the coverage factor 2 (corresponding to a confidence interval of 95%) multiplied by the combined standard uncertainty. The standard uncertainty is equal to the square root of the sum of the squares of the uncertainties associated with each single operation involved in the preparation and content verification of this test sample.*

Analytical method for confirmation	**Product ID: TRAINING SAMPLE - oil**

Detection:	GC-MS in SIM mode (isotope dilution)

Warning	**Product ID: TRAINING SAMPLE - oil**
	Store in the dark at 4 ºC or less The European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.
Safety of the product	The material contains some teratogenic and carcinogenic substances. Check the attached material safety data sheets for information on hazard, exposure, and safe handling.

ANNEX 9 – Instructions for the use of the additional ampoules

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
EU reference laboratory for polycyclic aromatic hydrocarbons

EURL
European Union Reference Laboratory
Polycyclic Aromatic Hydrocarbons

Geel, 28/01/2011

Dear Participant to the method validation study for PAHs in food,
upon your request, we send you the additional ampoule(s) of standard(s).

Labelled PAHs standard solution

In case your laboratory is going to use this second ampoule for the whole analytical procedure for the coded samples, please note that:

the same labelled PAHs solution ("5.20 Mixed labelled PAH process solution" in the SOP) **has to be used for preparing the calibration curve and for spiking the sample before starting the analysis**

The composition of the solution is given in Table 1

Table 1 — Composition of the mixed labelled PAHs process solution

Labelled PAHs	**Concentration** (ng/ml)	**Concentration** (ng/g)
5-Methylchrysene methyl D_3	39.0	45.0
Benzo[*a*]anthracene $^{13}C_6$	42.5	49.0
Benzo[*a*]pyrene $^{13}C_4$	41.4	47.8
Benzo[*b*]fluoranthene $^{13}C_6$	39.0	45.0
Pyrene $^{13}C_3$	43.4	50.0
Benzo[*ghi*]perylene $^{13}C_{12}$	41.5	47.9
Benzo[*k*]fluoranthene $^{13}C_6$	42.7	49.2
Chrysene $^{13}C_6$	43.3	49.9
Dibenzo[*a,e*]pyrene $^{13}C_6$	41.9	48.3
Dibenzo[*a,h*]anthracene $^{13}C_6$	41.4	47.7
Dibenzo[*a,i*]pyrene $^{13}C_{12}$	39.1	45.0
Indeno[1,2,3-*cd*]pyrene $^{13}C_6$	41.3	47.7

Injection standard solution

The injection solution composition is the same as reported in the SOP ("5.18 Injection standard solution").

Its composition is around 436 ng/ml (500 ng/g) of FBkF in toluene.)

ANNEX 10 – Homogeneity data for the 4 target analytes

MUSSELS (IAEA – 432) was stated as homogeneous by the supplier (http://www.google.it/url?q=http://www-naweb.iaea.org/naml/files/iaea432.pdf&sa=U&ei=BolfTvXDL4KhOpnPzfIC&ved=0CBEQFjAA&usg=AFQjCNFrIcpTE8oVxHCa2GAvsoyqNEMUSA).

OIL_1 and OIL_2 were considered homogeneous due to the physical form of the sample (liquid). This conclusion was drawn from past experience with the same kind of spiked matrix used by the EU-RL PAHs as PT material in 2007, 2009 and 2010.

MEAT_A was tested for homogeneity when produced in 2006, at MRI, Kulmbach.

Samples were considered sufficiently homogeneous for the scope of the study when the sampling variance (□^{2}) was smaller than 0.3X0.22C (see Equation 1 at page 14)

Benz[*a*]anthracene

material: **EXWFLOUR**

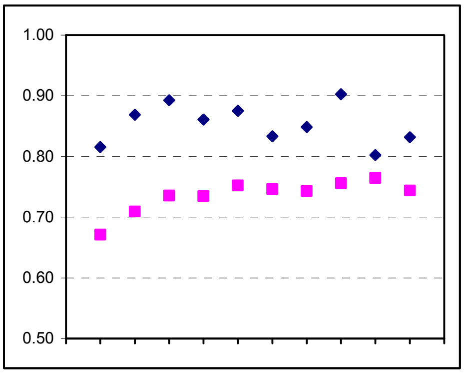
	n =	10		
	mean =	0.7943	22%	**= σ-trg(%)**
0.0005365	**s_x =**	0.0232	0.1748	**= σ-trg**
√MSW =	**s_w =**	0.0871		
	s_s =	0.0570	0.0524	**= 0,3*s**

IUPAC

(MSB-MSW)/2 -0.0033 0.0128 = F1*(0,3*s)^2+F2*MSW

passed

Bottle	Result a	Result b	diff	sum	avg
1	0.82	0.67	0.14	1.49	0.74
2	0.87	0.71	0.16	1.58	0.79
3	0.89	0.74	0.16	1.63	0.81
4	0.86	0.73	0.13	1.60	0.80
5	0.88	0.75	0.12	1.63	0.81
6	0.83	0.75	0.09	1.58	0.79
7	0.85	0.74	0.11	1.59	0.80
8	0.90	0.76	0.15	1.66	0.83
9	0.80	0.76	0.04	1.57	0.78
10	0.83	0.74	0.09	1.58	0.79



∑(diff)^2 = 0.1515629

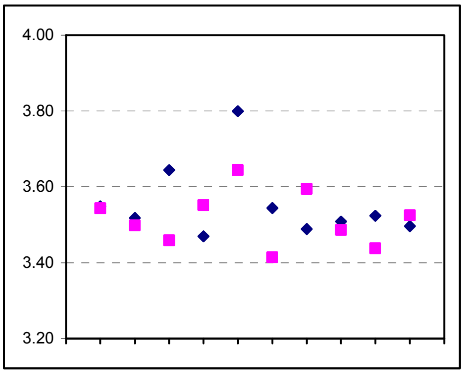
var(sum)/2 = 0.00107 =MSB

material: **FISH_B**

	n =	10		
	mean =	3.5349	22%	**= σ-trg(%)**
0.0049604	**s_x =**	0.0704	0.7777	**= σ-trg**
√MSW =	**s_w =**	0.0715		
	s_s =	0.0490	0.2333	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0024 0.1075 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	Result a	Result b	diff	sum	avg
1	3.55	3.54	0.00	7.09	3.55
2	3.52	3.50	0.02	7.02	3.51
3	3.64	3.46	0.18	7.10	3.55
4	3.47	3.55	-0.08	7.02	3.51
5	3.80	3.64	0.16	7.44	3.72
6	3.54	3.41	0.13	6.96	3.48
7	3.49	3.59	-0.11	7.08	3.54
8	3.51	3.49	0.02	7.00	3.50
9	3.52	3.44	0.09	6.96	3.48
10	3.50	3.52	-0.03	7.02	3.51



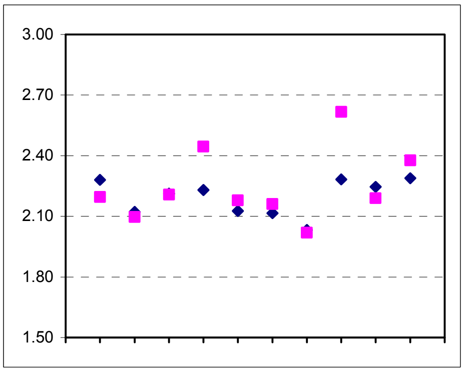
∑(diff)^2 = 0.1023562
var(sum)/2 = 0.00992 =MSB

material: **IF_2010**

	n =	10		
	mean =	2.2213	22%	**= σ-trg(%)**
0.0156578	**s_x =**	0.1251	0.4887	**= σ-trg**
√MSW =	**s_w =**	0.0955		
	s_s =	0.1053	0.1466	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0111 0.0496 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	Result a	Result b	diff	sum	avg
IF-001	2.28	2.20	0.09	4.48	2.24
IF-008	2.12	2.10	0.03	4.22	2.11
IF-016	2.21	2.21	0.00	4.42	2.21
IF-021	2.23	2.45	-0.21	4.68	2.34
IF-028	2.13	2.18	-0.05	4.31	2.15
IF-039	2.12	2.16	-0.05	4.28	2.14
IF-044	2.03	2.02	0.01	4.05	2.03
IF-053	2.28	2.62	-0.34	4.90	2.45
IF-060	2.25	2.19	0.06	4.43	2.22
IF-069	2.29	2.38	-0.09	4.67	2.33



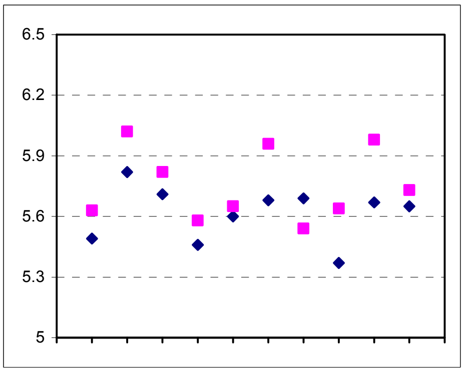
∑(diff)^2 = 0.1825512
var(sum)/2 = 0.03132 =MSB

material: **IF_2011**

	n =	10		
	mean =	5.6845	22%	**= σ-trg(%)**
0.0203581	**s_x =**	0.1427	1.2506	**= σ-trg**
√MSW =	**s_w =**	0.1351		
	s_s =	0.1060	0.3752	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0112 0.2831 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
1	5.49	5.63	-0.14	11.12	5.56
2	5.82	6.02	-0.2	11.84	5.92
3	5.71	5.82	-0.11	11.53	5.765
4	5.46	5.58	-0.12	11.04	5.52
5	5.60	5.65	-0.05	11.25	5.625
6	5.68	5.96	-0.28	11.64	5.82
7	5.69	5.54	0.15	11.23	5.615
8	5.37	5.64	-0.27	11.01	5.505
9	5.67	5.98	-0.31	11.65	5.825
10	5.65	5.73	-0.08	11.38	5.69



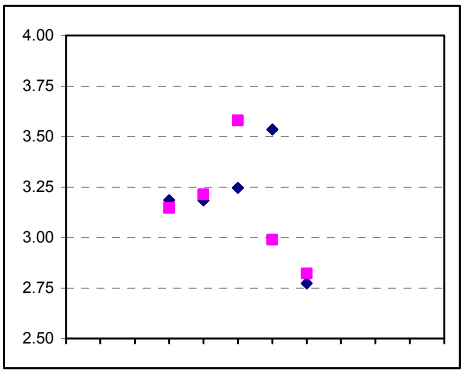
$\sum(\text{diff})^2$ = 0.3649
var(sum)/2 = 0.04072 =MSB

material: **MEAT_A**

	n =	5		
	mean =	3.1676	22%	**= σ-trg(%)**
0.0516521	**s_x =**	0.2273	0.6969	**= σ-trg**
√MSW =	**s_w =**	0.2035		
	s_s =	0.1759	0.2091	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0309 0.1240 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
3	3.18	3.15	0.04	6.33	3.17
4	3.18	3.21	-0.03	6.40	3.20
5	3.25	3.58	-0.33	6.83	3.41
6	3.53	2.99	0.55	6.52	3.26
7	2.77	2.82	-0.05	5.60	2.80



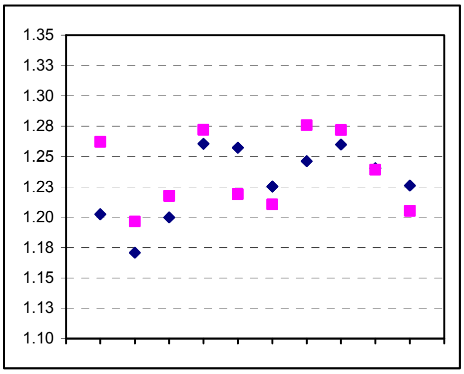
$\sum(\text{diff})^2$ = 0.4140858
var(sum)/2 = 0.10330 =MSB

material: **WHFLOUR**

	n =	10		
	mean =	1.2329	22%	**= σ-trg(%)**
0.0007336	**s_x =**	0.0271	0.2712	**= σ-trg**
√MSW =	**s_w =**	0.0198		
	s_s =	0.0232	0.0814	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0005 0.0128 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
1	1.20	1.26	-0.06	2.46	1.23
2	1.17	1.20	-0.03	2.37	1.18
3	1.20	1.22	-0.02	2.42	1.21
4	1.26	1.27	-0.01	2.53	1.27
5	1.26	1.22	0.04	2.48	1.24
6	1.23	1.21	0.01	2.44	1.22
7	1.25	1.28	-0.03	2.52	1.26
8	1.26	1.27	-0.01	2.53	1.27
9	1.24	1.24	0.00	2.48	1.24
10	1.23	1.21	0.02	2.43	1.22



∑(diff)^2 = 0.0078323
var(sum)/2 = 0.00147 =MSB

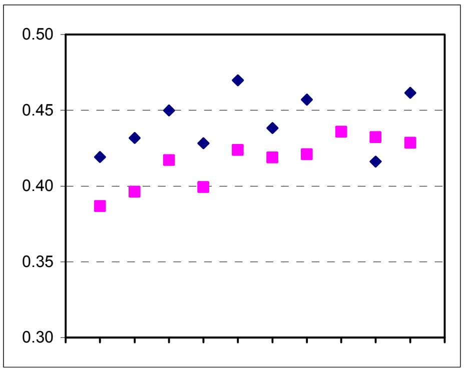
Benzo[*a*]pyrene

material: **EXWFLOUR**

	n =	10		
	mean =	0.4321	22%	**= σ-trg(%)**
0.000414	**s_x =**	0.0203	0.0951	**= σ-trg**
√MSW =	**s_w =**	0.0274		
	s_s =	0.0063	0.0285	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0000 0.0023 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	Result a	Result b	diff	sum	avg
1	0.42	0.39	0.03	0.81	0.40
2	0.43	0.40	0.04	0.83	0.41
3	0.45	0.42	0.03	0.87	0.43
4	0.43	0.40	0.03	0.83	0.41
5	0.47	0.42	0.05	0.89	0.45
6	0.44	0.42	0.02	0.86	0.43
7	0.46	0.42	0.04	0.88	0.44
8	0.51	0.44	0.08	0.95	0.47
9	0.42	0.43	-0.02	0.85	0.42
10	0.46	0.43	0.03	0.89	0.44



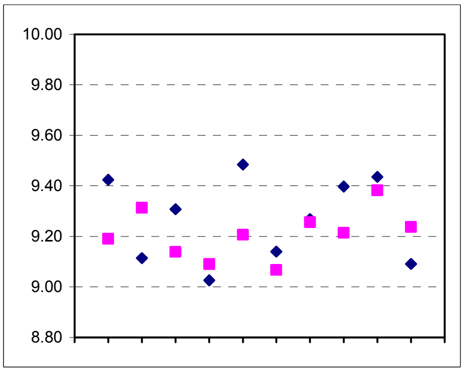
∑(diff)^2 = 0.0149954
var(sum)/2 = 0.00083 =MSB

material: **FISH_B**

	n =	10		
	mean =	9.2394	22%	**= σ-trg(%)**
0.0119545	**s_x =**	0.1093	2.0327	**= σ-trg**
√MSW =	**s_w =**	0.1156		
	s_s =	0.0726	0.6098	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0053 0.7126 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	Result a	Result b	diff	sum	avg
1	9.42	9.19	0.23	18.62	9.31
2	9.11	9.31	-0.20	18.43	9.21
3	9.31	9.14	0.17	18.45	9.22
4	9.03	9.09	-0.06	18.12	9.06
5	9.48	9.21	0.28	18.69	9.35
6	9.14	9.07	0.07	18.21	9.10
7	9.27	9.26	0.01	18.53	9.26
8	9.40	9.21	0.18	18.61	9.31
9	9.44	9.38	0.05	18.82	9.41
10	9.09	9.24	-0.15	18.33	9.16



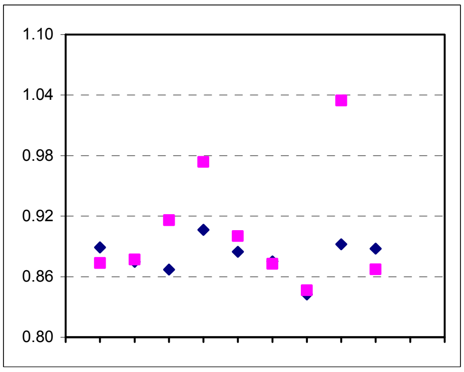
∑(diff)^2 = 0.2671398
var(sum)/2 = 0.02391 =MSB

material: **IF_2010**

	n =	10		
	mean =	0.8945	22%	**= σ-trg(%)**
0.0011855	**s_x =**	0.0344	0.1968	**= σ-trg**
√MSW =	**s_w =**	0.0381		
	s_s =	0.0215	0.0590	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0005 0.0080 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
IF-001	0.89	0.87	0.02	1.76	0.88
IF-008	0.87	0.88	0.00	1.75	0.88
IF-016	0.87	0.92	-0.05	1.78	0.89
IF-021	0.91	0.97	-0.07	1.88	0.94
IF-028	0.88	0.90	-0.02	1.78	0.89
IF-039	0.88	0.87	0.00	1.75	0.87
IF-044	0.84	0.85	0.00	1.69	0.84
IF-053	0.89	1.03	-0.14	1.93	0.96
IF-060	0.89	0.87	0.02	1.76	0.88
IF-069	0.89	0.92	-0.03	1.81	0.91



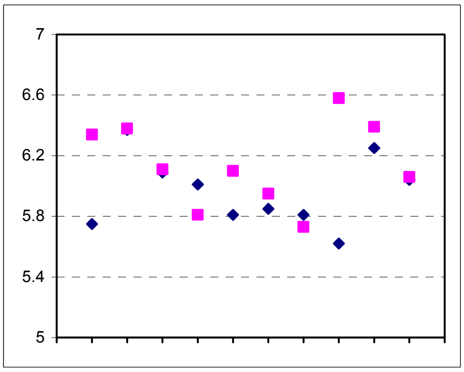
∑(diff)^2 = 0.0289788
var(sum)/2 = 0.00237 =MSB

material: **IF_2011**

	n =	10		
	mean =	6.0525	22%	**= σ-trg(%)**
0.0347792	**s_x =**	0.1865	1.3316	**= σ-trg**
√MSW =	**s_w =**	0.2675		
	s_s =	0.0314	0.3995	**= 0,3*s**

IUPAC
(MSB-MSW)/2 -0.0010 0.3722 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
1	5.75	6.34	-0.59	12.09	6.045
2	6.37	6.38	-0.01	12.75	6.375
3	6.09	6.11	-0.02	12.2	6.1
4	6.01	5.81	0.2	11.82	5.91
5	5.81	6.1	-0.29	11.91	5.955
6	5.85	5.95	-0.1	11.8	5.9
7	5.81	5.73	0.08	11.54	5.77
8	5.62	6.58	-0.96	12.2	6.1
9	6.25	6.39	-0.14	12.64	6.32
10	6.04	6.06	-0.02	12.1	6.05



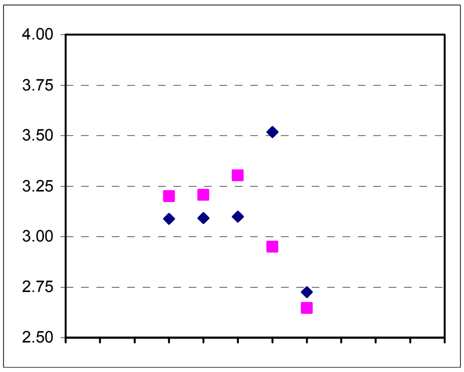
∑(diff)^2 = 1.4307
var(sum)/2 = 0.06956 =MSB

material: **MEAT_A**

	n =	5		
	mean =	3.0829	22%	**= σ-trg(%)**
0.050523	**s_x =**	0.2248	0.6782	**= σ-trg**
√MSW =	**s_w =**	0.1990		
	s_s =	0.1753	0.2035	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0307 0.1178 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
3	3.09	3.20	-0.11	6.29	3.14
4	3.09	3.21	-0.11	6.30	3.15
5	3.10	3.30	-0.20	6.40	3.20
6	3.52	2.95	0.57	6.47	3.23
7	2.73	2.65	0.08	5.37	2.69



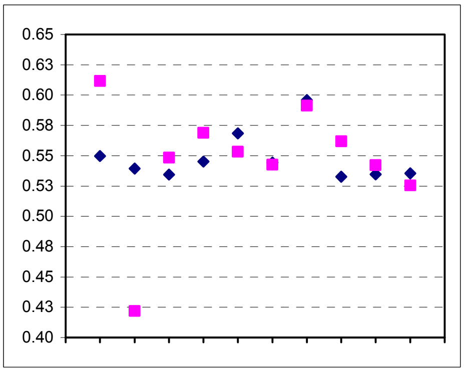
∑(diff)^2 = 0.3958195
var(sum)/2 = 0.10105 =MSB

material: **WHFLOUR**

	n =	10		
	mean =	0.5474	22%	**= σ-trg(%)**
0.0009327	**s_x =**	0.0305	0.1204	**= σ-trg**
√MSW =	**s_w =**	0.0314		
	s_s =	0.0210	0.0361	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0004 0.0034 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
1	0.55	0.61	-0.06	1.16	0.58
2	0.54	0.42	0.12	0.96	0.48
3	0.53	0.55	-0.01	1.08	0.54
4	0.55	0.57	-0.02	1.11	0.56
5	0.57	0.55	0.02	1.12	0.56
6	0.54	0.54	0.00	1.09	0.54
7	0.60	0.59	0.00	1.19	0.59
8	0.53	0.56	-0.03	1.09	0.55
9	0.53	0.54	-0.01	1.08	0.54
10	0.54	0.53	0.01	1.06	0.53



∑(diff)^2 = 0.0196784
var(sum)/2 = 0.00187 =MSB

Benzo[*b*]fluoranthene

material: **EXWFLOUR**

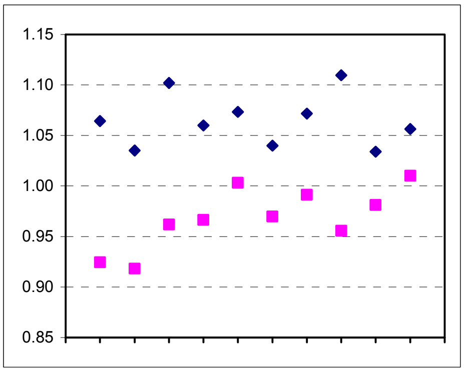
	n =	10		
	mean =	1.0165	22%	**= σ-trg(%)**
0.0004193	**s_x =**	0.0205	0.2236	**= σ-trg**
√MSW =	**s_w =**	0.0730		
	s_s =	0.0474	0.0671	**= 0,3*s**

IUPAC

(MSB-MSW)/2 -0.0022 0.0138 = F1*(0,3*s)^2+F2*MSW

passed

Bottle	Result a	Result b	diff	sum	avg
1	1.06	0.92	0.14	1.99	0.99
2	1.04	0.92	0.12	1.95	0.98
3	1.10	0.96	0.14	2.06	1.03
4	1.06	0.97	0.09	2.03	1.01
5	1.07	1.00	0.07	2.08	1.04
6	1.04	0.97	0.07	2.01	1.00
7	1.07	0.99	0.08	2.06	1.03
8	1.11	0.96	0.15	2.07	1.03
9	1.03	0.98	0.05	2.02	1.01
10	1.06	1.01	0.05	2.07	1.03



∑(diff)^2 = 0.1065934

var(sum)/2 = 0.00084 =MSB

material: **FISH_B**

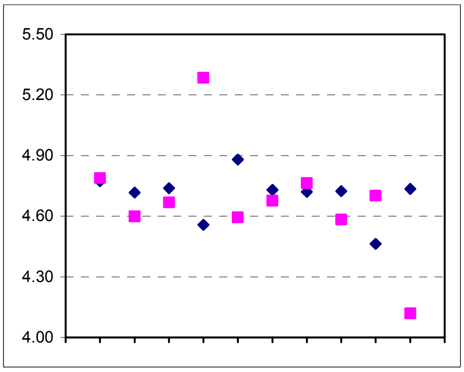
	n =	10		
	mean =	4.6910	22%	**= σ-trg(%)**
0.0166621	**s_x =**	0.1291	1.0320	**= σ-trg**
√MSW =	**s_w =**	0.2335		
	s_s =	0.1029	0.3096	**= 0,3*s**

IUPAC

(MSB-MSW)/2 -0.0106 0.2353 = F1*(0,3*s)^2+F2*MSW

passed

Bottle	Result a	Result b	diff	sum	avg
1	4.77	4.79	-0.01	9.56	4.78
2	4.72	4.60	0.12	9.32	4.66
3	4.74	4.67	0.07	9.41	4.70
4	4.56	5.28	-0.73	9.84	4.92
5	4.88	4.59	0.29	9.47	4.74
6	4.73	4.68	0.05	9.41	4.70
7	4.72	4.76	-0.04	9.48	4.74
8	4.72	4.58	0.14	9.31	4.65
9	4.46	4.70	-0.24	9.16	4.58
10	4.73	4.12	0.62	8.85	4.43



∑(diff)^2 = 1.0904019

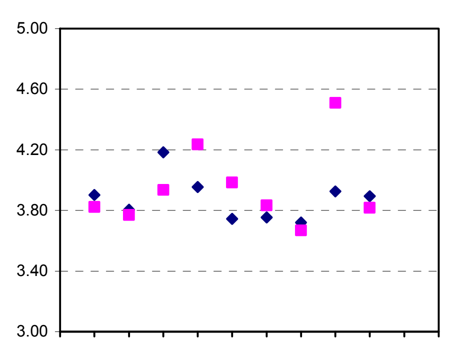
var(sum)/2 = 0.03332 =MSB

material: **IF_2010**

	n =	10		
	mean =	3.9186	22%	**= σ-trg(%)**
0.0260586	**s_x =**	0.1614	0.8621	**= σ-trg**
√MSW =	**s_w =**	0.1689		
	s_s =	0.1086	0.2586	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0118 0.1546 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	Result a	Result b	diff	sum	avg
IF-001	3.90	3.82	0.08	7.72	3.86
IF-008	3.80	3.77	0.03	7.58	3.79
IF-016	4.18	3.94	0.25	8.12	4.06
IF-021	3.96	4.24	-0.28	8.19	4.10
IF-028	3.75	3.98	-0.24	7.73	3.86
IF-039	3.75	3.83	-0.08	7.59	3.79
IF-044	3.72	3.67	0.05	7.39	3.70
IF-053	3.93	4.51	-0.58	8.44	4.22
IF-060	3.89	3.82	0.08	7.71	3.86
IF-069	3.90	4.00	-0.10	7.91	3.95



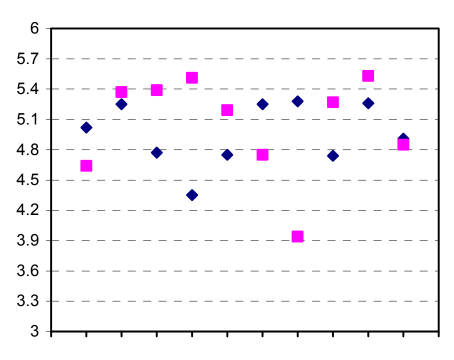
∑(diff)^2 = 0.5705113
var(sum)/2 = 0.05212 =MSB

material: **IF_2011**

	n =	10		
	mean =	5.0010	22%	**= σ-trg(%)**
0.0510822	**s_x =**	0.2260	1.1002	**= σ-trg**
√MSW =	**s_w =**	0.4736		
	s_s =	0.2471	0.3301	**= 0,3*s**

IUPAC
(MSB-MSW)/2 -0.0611 0.4313 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	Result a	Result b	diff	sum	avg
1	5.02	4.64	0.38	9.66	4.83
2	5.25	5.37	-0.12	10.62	5.31
3	4.77	5.39	-0.62	10.16	5.08
4	4.35	5.51	-1.16	9.86	4.93
5	4.75	5.19	-0.44	9.94	4.97
6	5.25	4.75	0.5	10	5
7	5.28	3.94	1.34	9.22	4.61
8	4.74	5.27	-0.53	10.01	5.005
9	5.26	5.53	-0.27	10.79	5.395
10	4.91	4.85	0.06	9.76	4.88



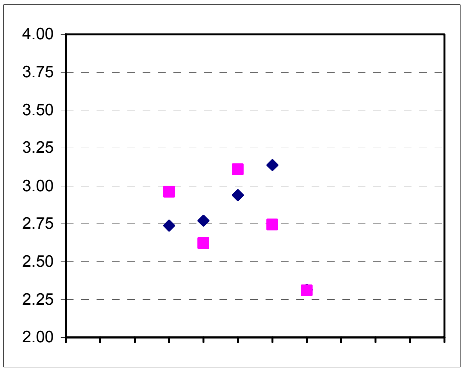
∑(diff)^2 = 4.4854
var(sum)/2 = 0.10216 =MSB

material: **MEAT_A**

n = 5
mean = 2.7647 22% **= σ-trg(%)**
0.0786344 **s_x =** 0.2804 0.6082 **= σ-trg**
√MSW = **s_w =** 0.1594
s_s = 0.2568 0.1825 **= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0659 0.0883 = F1*$(0,3*s)^2$+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
3	2.74	2.96	-0.22	5.70	2.85
4	2.77	2.62	0.15	5.39	2.70
5	2.94	3.11	-0.17	6.05	3.02
6	3.14	2.74	0.39	5.88	2.94
7	2.31	2.31	0.00	4.63	2.31



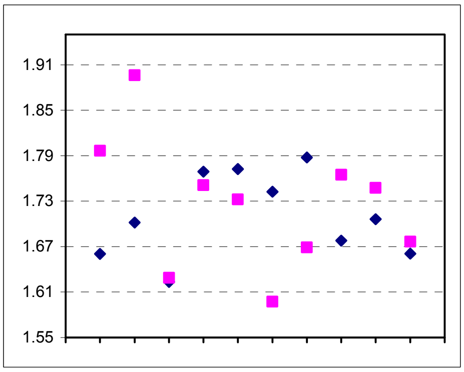
$\sum(\text{diff})^2$ = 0.2541935
var(sum)/2 = 0.15727 =MSB

material: **WHFLOUR**

n = 10
mean = 1.7182 22% **= σ-trg(%)**
0.0025547 **s_x =** 0.0505 0.3780 **= σ-trg**
√MSW = **s_w =** 0.0718
s_s = 0.0045 0.1134 **= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0000 0.0294 = F1*$(0,3*s)^2$+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
1	1.66	1.80	-0.14	3.46	1.73
2	1.70	1.90	-0.19	3.60	1.80
3	1.62	1.63	-0.01	3.25	1.63
4	1.77	1.75	0.02	3.52	1.76
5	1.77	1.73	0.04	3.50	1.75
6	1.74	1.60	0.15	3.34	1.67
7	1.79	1.67	0.12	3.46	1.73
8	1.68	1.76	-0.09	3.44	1.72
9	1.71	1.75	-0.04	3.45	1.73
10	1.66	1.68	-0.02	3.34	1.67



$\sum(\text{diff})^2$ = 0.1029981
var(sum)/2 = 0.00511 =MSB

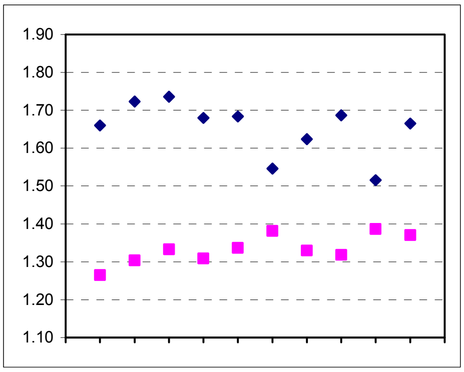
Chrysene

material: **EXWFLOUR**

	n =	10		
	mean =	1.4926	22%	**= σ-trg(%)**
0.0007736	**s_x =**	0.0278	0.3284	**= σ-trg**
√MSW =	**s_w =**	0.2350		
	s_s =	0.1639	0.0985	**= 0,3*s**

IUPAC
(MSB-MSW)/2 -0.0268 0.0740 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	Result a	Result b	diff	sum	avg
1	1.66	1.27	0.39	2.93	1.46
2	1.72	1.30	0.42	3.03	1.51
3	1.74	1.33	0.40	3.07	1.53
4	1.68	1.31	0.37	2.99	1.49
5	1.68	1.34	0.35	3.02	1.51
6	1.55	1.38	0.16	2.93	1.46
7	1.62	1.33	0.29	2.95	1.48
8	1.69	1.32	0.37	3.01	1.50
9	1.52	1.39	0.13	2.90	1.45
10	1.67	1.37	0.30	3.04	1.52



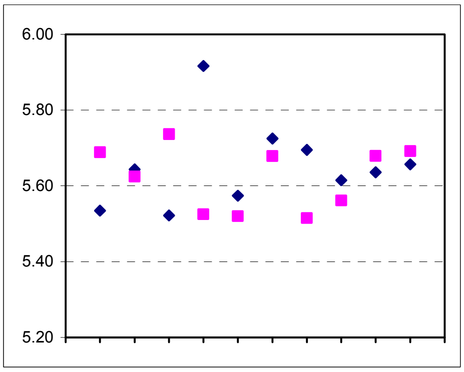
∑(diff)^2 = 1.1048385
var(sum)/2 = 0.00155 =MSB

material: **FISH_B**

	n =	10		
	mean =	5.6370	22%	**= σ-trg(%)**
0.0028076	**s_x =**	0.0530	1.2401	**= σ-trg**
√MSW =	**s_w =**	0.1154		
	s_s =	0.0621	0.3720	**= 0,3*s**

IUPAC
(MSB-MSW)/2 -0.0039 0.2737 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	Result a	Result b	diff	sum	avg
1	5.53	5.69	-0.15	11.22	5.61
2	5.64	5.62	0.02	11.27	5.63
3	5.52	5.74	-0.21	11.26	5.63
4	5.92	5.53	0.39	11.44	5.72
5	5.57	5.52	0.05	11.09	5.55
6	5.73	5.68	0.05	11.40	5.70
7	5.70	5.52	0.18	11.21	5.61
8	5.61	5.56	0.05	11.18	5.59
9	5.64	5.68	-0.04	11.31	5.66
10	5.66	5.69	-0.04	11.35	5.67



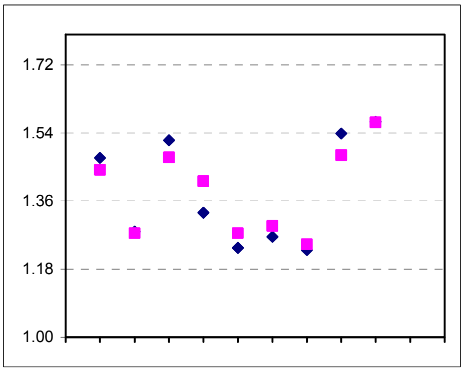
∑(diff)^2 = 0.2665272
var(sum)/2 = 0.00562 =MSB

material: **IF_2010**

	n =	9		
	mean =	1.3840	22%	**= σ-trg(%)**
0.0160937	**s_x =**	0.1269	0.3045	**= σ-trg**
√MSW =	**s_w =**	0.0297		
	s_s =	0.1251	0.0913	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0157 0.0166 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
IF-001	1.47	1.44	0.03	2.92	1.46
IF-008	1.28	1.27	0.00	2.55	1.28
IF-016	1.52	1.48	0.05	3.00	1.50
IF-021	1.33	1.41	-0.08	2.74	1.37
IF-028	1.24	1.28	-0.04	2.51	1.26
IF-039	1.27	1.29	-0.03	2.56	1.28
IF-044	1.23	1.25	-0.01	2.48	1.24
IF-053	1.54	1.48	0.06	3.02	1.51
IF-060	1.57	1.57	0.00	3.14	1.57



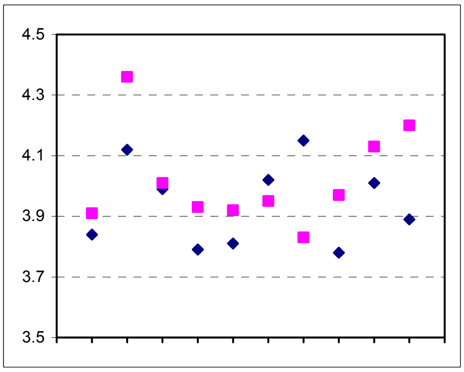
∑(diff)^2 = 0.0159254
var(sum)/2 = 0.03219 =MSB

material: **IF_2011**

	n =	10		
	mean =	3.9805	22%	**= σ-trg(%)**
0.0144581	**s_x =**	0.1202	0.8757	**= σ-trg**
√MSW =	**s_w =**	0.1320		
	s_s =	0.0758	0.2627	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0057 0.1474 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
1	3.84	3.91	-0.07	7.75	3.875
2	4.12	4.36	-0.24	8.48	4.24
3	3.99	4.01	-0.02	8	4
4	3.79	3.93	-0.14	7.72	3.86
5	3.81	3.92	-0.11	7.73	3.865
6	4.02	3.95	0.07	7.97	3.985
7	4.15	3.83	0.32	7.98	3.99
8	3.78	3.97	-0.19	7.75	3.875
9	4.01	4.13	-0.12	8.14	4.07
10	3.89	4.2	-0.31	8.09	4.045



∑(diff)^2 = 0.3485
var(sum)/2 = 0.02892 =MSB

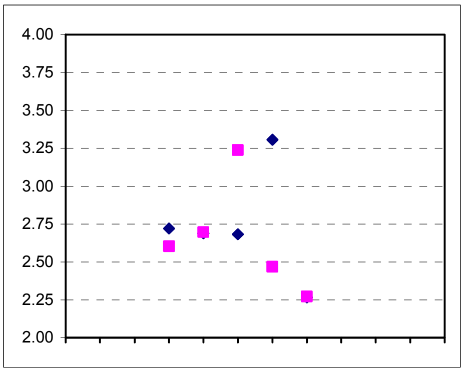
material: **MEAT_A**

	n =	5		
	mean =	2.6942	22%	**= σ-trg(%)**
0.0723585	**s_x =**	0.2690	0.5927	**= σ-trg**
√MSW =	**s_w =**	0.3204		
	s_s =	0.1450	0.1778	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0210 0.1631 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
3	2.72	2.60	0.12	5.32	2.66
4	2.69	2.70	-0.01	5.39	2.69
5	2.68	3.24	-0.56	5.92	2.96
6	3.31	2.47	0.84	5.77	2.89
7	2.27	2.27	-0.01	4.54	2.27

∑(diff)^2 = 1.0267869
var(sum)/2 = 0.14472 =MSB



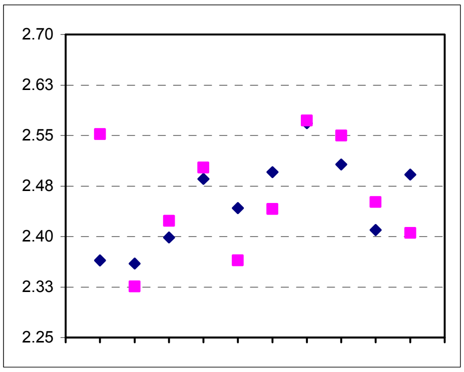
material: **WHFLOUR**

	n =	10		
	mean =	2.4555	22%	**= σ-trg(%)**
0.0042463	**s_x =**	0.0652	0.5402	**= σ-trg**
√MSW =	**s_w =**	0.0535		
	s_s =	0.0530	0.1621	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.0028 0.0523 = F1*(0,3*s)^2+F2*MSW
passed

Bottle	**Result a**	**Result b**	**diff**	**sum**	**avg**
1	2.36	2.55	-0.19	4.92	2.46
2	2.36	2.33	0.03	4.69	2.34
3	2.40	2.42	-0.02	4.82	2.41
4	2.49	2.50	-0.02	4.99	2.49
5	2.44	2.36	0.08	4.81	2.40
6	2.50	2.44	0.05	4.94	2.47
7	2.57	2.57	0.00	5.14	2.57
8	2.51	2.55	-0.04	5.06	2.53
9	2.41	2.45	-0.04	4.86	2.43
10	2.49	2.41	0.09	4.90	2.45

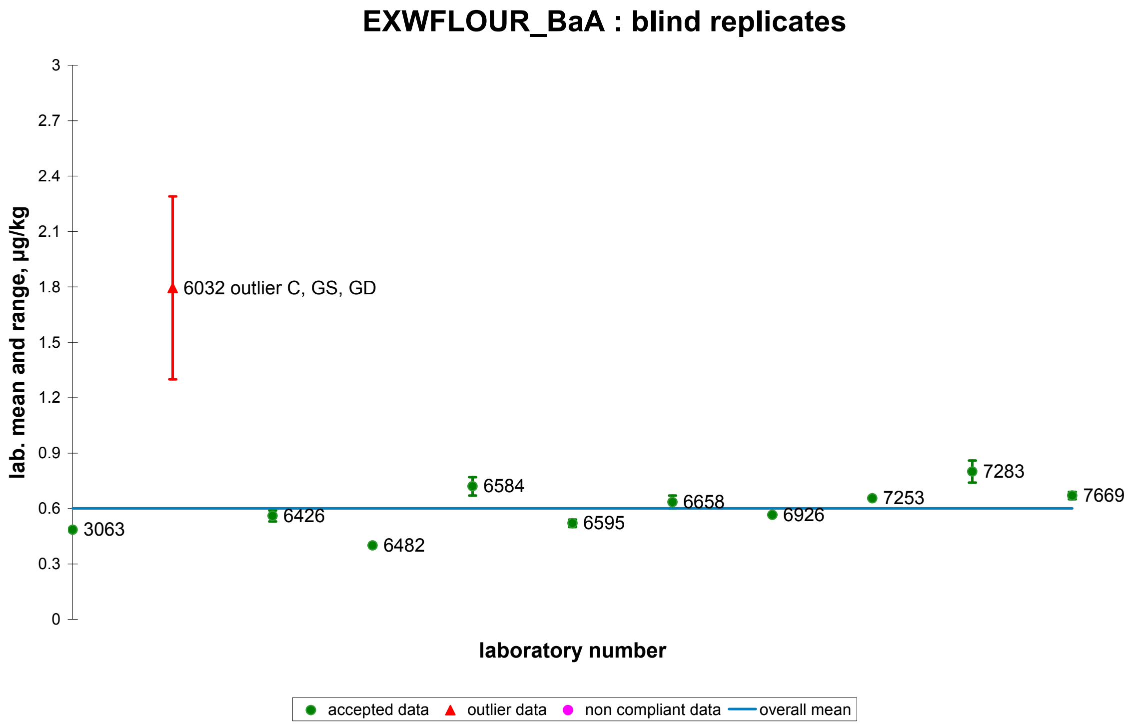
∑(diff)^2 = 0.057314
var(sum)/2 = 0.00849 =MSB



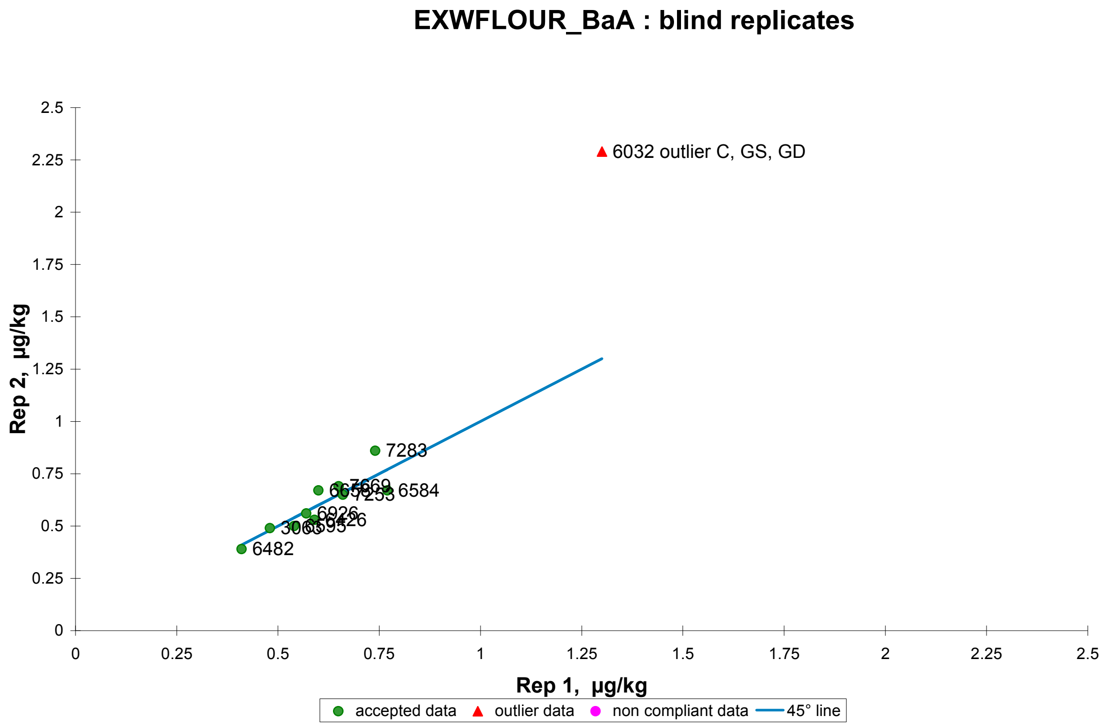
ANNEX 11 – Individual data reported by participants for analytes contents

For all combinations of analytes and materials, the data were also graphically represented as Youden plots and as distribution of individual results of replicate measurements. These graphics are available as additional information upon request. An example is reported below for BaA in Extruded wheat flour.

Distribution of individual results of replicate measurements



Youden Plot



Laboratory 7524 was excluded from statistical evaluation due to application of a method different from the one described in the SOP.

Analytical results are listed as reported by the individual participants. Values reported as <LOD are excluded from statistical treatment. Grey shaded entries in the tables refer to non compliant data. Whenever only one result was reported, the corresponding set of results (duplicate results) was considered as not compliant. Non compliant sets are highlighted in dark grey.

Table 1: individual results for BaA (expressed as µg/kg)

Material	**EXW FLOUR**		**FISH_B**		**IF_2010**		**IF_2011**		**MEAT_A**		**MUSS_DRY**		**MUSSELS**		**OIL_1**		**OIL_2**		**WH FLOUR**	
Lab ID	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**	**Rep 1**	**Rep 2**
3063	0.48	0.49	2.8	3.28	1.2	1.43	4.61	4.5	2.22	2.4	2.56	2.96	2.35	2.66	3.74	4.04	7.89	7.44	0.74	0.98
6032	1.3	2.29	3.97	4.41	1.81	1.64	4.98	5.46	3.45	2.94	3.82	3.56	3.48	3.22	4.24	3.97	7.96	8.03	1.41	2.09
6426	0.59	0.53	3.23	3.36	1.37	1.28	4.89	5.1	2.59	2.6	N.R.	N.R.	2.9	3	3.98	4.21	7.89	7.92	0.97	1.03
6482	0.41	0.39	3.38	2.98	1.19	1.04	4.59	4.36	2.5	2.48	1.97	1.65	1.79	1.6	3.73	3.68	7.24	7.1	0.79	0.75
6584	0.77	0.67	3.74	4.2	1.72	2.06	5.62	5.95	3.39	3.37	4.04	4.79	3.65	4.37	2.53	4.72	6.86	9.45	1.12	1.21
6595	0.54	0.5	2.83	2.91	1.23	1.19	9.51	4.5	2.47	N.R.	2.56	2.65	2.31	2.4	3.82	3.75	7.11	7.09	0.88	0.89
6658	0.6	0.67	3.47	3.61	1.32	1.26	4.8	4.91	2.84	2.79	1.6	1.62	1.46	1.46	4.69	4.31	7.71	7.82	1.08	1.12
6926	0.57	0.56	3.52	3.59	1.22	0.87	5.21	5.05	3.06	3.22	1.45	1.47	1.32	1.33	4.5	4.33	8.6	8.56	1.07	0.95
7253	0.66	0.65	3.5	3.4	1.36	N.R.	5.14	5.09	2.83	N.R.	2.48	2.97	2.3	2.77	4.32	3.71	8.09	7.98	1.02	1.02
7283	0.74	0.86	3.39	4.38	1.39	1.26	4.74	4.18	2.81	2.65	3.03	3.17	2.8	2.9	4.41	3.97	5.81	7.2	1.1	1.29
7669	0.65	0.69	3.37	3.19	1.28	1.35	5.26	5.2	2.67	2.63	2.95	2.82	2.83	2.69	3.78	4.98	11.72	8.59	1.03	1.03

N.R. not reported

Table 2: individual results for BaP (expressed as µg/kg)

Material	EXW FLOUR		FISH_B		IF_2010		IF_2011		MEAT_A		MUSS_DRY		MUSSELS		OIL_1		OIL_2		WH FLOUR	
Lab ID	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
3063	0.32	0.35	8.02	8.2	0.34	0.44	4.22	4.05	1.74	1.88	0.53	0.59	0.48	0.53	4.61	4.62	11.9	11.3	0.4	0.53
6032	1.04	1.42	9.52	9.41	0.99	0.9	5.06	5.4	2.34	2.41	1.37	1.25	1.25	1.13	5.05	4.9	12.5	12.15	0.98	1.51
6426	0.51	0.55	8.84	8.53	0.65	0.61	4.71	4.96	1.95	1.83	N.R.	N.R.	0.72	0.74	4.9	5.02	12.17	12.72	0.67	0.64
6482	0.41	0.45	8.87	8.33	0.49	0.52	4.4	4.13	1.94	1.98	0.46	0.32	0.42	0.31	4.46	4.52	10.58	10.38	0.58	0.55
6584	0.62	0.67	10.79	11.4	0.8	0.69	5.46	5.78	2.68	2.59	1.3	1.24	1.17	1.12	5.03	5.09	13.18	13.39	0.76	0.74
6595	1.53	1.28	1.5	1.6	1.51	0.88	0.91	1.36	1.64	N.R.	1.69	1.72	1.53	1.56	8.34	8.26	7.57	8.15	1.59	1.43
6658	0.64	0.57	8.75	9.01	0.6	0.55	4.37	4.58	2.07	2.2	0.49	0.57	0.45	0.51	4.6	4.54	11.41	11.14	0.67	0.65
6926	0.52	0.48	9.38	9.32	0.52	<LOD	4.94	4.84	2.27	2.23	<LOD	<LOD	<LOD	<LOD	5.57	5.12	12.41	12.25	0.64	0.69
7253	0.56	0.53	9.3	9.28	0.56	N.R.	4.74	4.71	2.16	N.R.	0.54	0.62	0.5	0.58	4.62	4.36	11.33	11.34	0.64	0.63
7283	0.65	0.85	8.65	11.43	0.85	0.56	4.06	4.5	2.23	2.29	0.8	0.67	0.74	0.61	4.64	5.06	11.74	12.17	0.7	0.98
7669	0.57	0.44	9.35	8.78	0.53	0.42	4.81	5.51	2.2	1.96	0.73	0.32	0.7	0.31	3.38	4.2	13.88	10.9	0.73	0.57

N.R. not reported

LOD reported by participant was 0.5 µg/kg

Laboratory 6595 reported constantly very high values for BbF for all materials. In agreement with the participant, data reported were considered as not compliant and corresponding row was marked in dark grey.

Table 3: individual results for BbF (expressed as µg/kg)

Material	EXW FLOUR		FISH_B		IF_2010		IF_2011		MEAT_A		MUSS_DRY		MUSSELS		OIL_1		OIL_2		WH FLOUR	
Lab ID	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
3063	0.71	0.72	4.12	4.22	2.24	2.69	3.91	3.67	1.9	2.03	4.27	4.61	3.93	4.15	9.84	9.95	5.52	4.81	1.18	1.4
6032	1.78	1.95	5.16	5.15	2.64	3.09	4.47	4.77	2.41	2.42	5.67	5.44	5.17	4.92	10.43	10.07	5.64	5.82	1.93	2.42
6426	0.83	0.82	4.72	4.32	2.79	2.65	3.94	4.32	1.93	1.87	N.R.	N.R.	4.5	4.42	10.4	10.29	5.17	5.16	1.42	1.5
6482	0.62	0.59	4.5	4.09	2.27	2.31	3.64	3.51	1.75	1.79	3.86	3.47	3.52	3.36	9.09	9.37	4.63	4.53	1.28	1.26
6584	0.91	0.91	5.18	5.63	3.36	3.87	4.71	5.43	2.59	2.63	6.48	8.8	5.85	7.97	12.52	11.68	5.97	6.19	1.55	2.09
6595	37.8	36.89	42.54	43.17	44.3	43.19	44.37	45.08	38.65	N.R.	48.2	44.66	43.49	40.47	193.1	199.51	185.7	179.29	42.53	33.5
6658	1.02	1.1	5.35	5.23	2.81	2.9	4.55	4.73	2.07	2.11	3.67	4.32	3.34	3.9	10.29	10.26	5.26	4.93	2.24	1.9
6926	0.84	0.78	4.72	4.63	2.52	1.97	4.2	4.23	2.05	2.23	4.05	3.99	3.69	3.62	10.96	10.68	5.24	5.35	1.5	1.46
7253	0.53	0.55	4.42	4.28	2.4	N.R.	3.8	3.78	1.74	N.R.	3.62	3.91	3.36	3.65	9.8	9.6	4.56	4.6	1.12	1.09
7283	0.95	1.4	5.36	7.11	3.26	2.08	4.07	3.93	2.57	2.34	5.32	4.94	4.91	4.53	11.19	11.2	6.56	4.98	1.09	1.92
7669	0.96	0.85	4.21	4.96	2.79	2.56	4.18	4.67	2.3	2.28	5.29	4.27	5.08	4.06	8.84	12.2	5.8	5.39	1.62	1.39

N.R. not reported

Table 4: individual results for CHR (expressed as µg/kg)

Material	EXW FLOUR		FISH_B		IF_2010		IF_2011		MEAT_A		MUSS_DRY		MUSSELS		OIL_1		OIL_2		WH FLOUR	
Lab ID	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
3063	0.62	0.63	4.26	5.02	0.7	0.85	2.91	2.84	2.32	2.74	4.28	4.78	3.94	4.31	6.16	6.25	7.73	7.87	1.06	1.36
6032	1.66	2.16	6.06	6.04	1.31	1.43	3.58	4.11	3.17	3.4	6.15	5.56	5.61	5.04	6.73	6.47	8.01	8.35	2.21	2.67
6426	0.81	0.74	5.28	5.38	0.88	0.86	3.12	3.18	2.89	2.87	N.R.	N.R.	4.66	4.68	6.17	6.59	7.38	8.01	1.4	1.43
6482	0.56	0.53	5.42	4.83	0.64	0.59	3.03	2.9	2.64	2.75	3.33	2.86	3.03	2.77	5.83	6.13	6.96	6.67	1.21	1.16
6584	0.88	0.78	5.65	6.15	0.66	0.96	2.24	3.75	3.29	3.35	6.75	8.22	6.09	7.45	4.88	4.95	7.33	5.32	1.25	1.77
6595	0.68	0.67	4.59	4.7	0.79	0.74	2.95	2.99	2.7	N.R.	4.36	4.49	3.94	4.07	5.81	5.9	7.01	7.05	1.27	1.33
6658	1.03	0.96	5.81	6.28	1.04	1.25	3.39	3.43	3.57	3.23	3.1	3.04	2.82	2.74	7.42	7.2	8.09	8.12	2.1	1.98
6926	0.79	0.75	5.87	5.92	0.75	0.52	3.42	3.3	3.24	3.37	2.65	2.7	2.42	2.45	6.86	6.55	8.21	7.99	1.58	1.39
7253	0.73	0.71	5.44	5.3	0.74	N.R.	3.23	3.21	2.99	N.R.	3.98	4.6	3.69	4.3	6.3	6.02	7.04	7.62	1.3	1.3
7283	1.28	1.83	6.68	8.98	1.49	1	6.11	4.84	4.27	3.47	6.99	6.85	5.96	6.28	8.46	11.4	15.11	18.3	2.15	2.65
7669	0.85	0.79	5.2	5.13	0.75	0.75	3.7	3.52	2.85	2.93	5.05	4.79	4.85	4.56	5.98	8.03	10.98	8.6	1.63	1.58

N.R. not reported

ANNEX 12 – Individual data reported by participants for recoveries

Recoveries are listed as reported by the individual participants. No spiked material was supplied for recovery estimation. No graphic was produced, but the mean value for recovery ($\overline{R}$) and the respective relative standard deviation for reproducibility (%RSD_R) are reported at the bottom of each Table. Whenever only one result was reported, the corresponding set of results (duplicate results) was considered as not compliant. Non compliant sets are highlighted in dark grey. When the number of either the data reported or of the accepted data after outlier rejection was considered as insufficient for an appropriate statistical evaluation (e.g. < 8), the corresponding robust mean and relative standard deviation for reproducibility were not calculated

Table 5: individual recoveries for BaA (expressed as %)

Material	**EXWFLOUR**		**FISH_B**		**IF_2010**		**IF_2011**		**MEAT_A**		**MUSS_DRY**		**MUSSELS**		**OIL_1**		**OIL_2**		**WHFLOUR**	
Lab ID	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**
3063	66	72	60	82	73	63	77	73	84	85	70	67	70	67	87	82	88	79	64	73
6032	51.3	51.7	74.1	72	67.9	80.5	61.7	72.2	61.6	66.3	N.R.	N.R.	55.1	55.4	84.8	88.7	87.6	83.3	54.6	58
6426	65	63	60	66	58	60	66	58	59	71	N.R.	N.R.	56	55	67	73	62	72	61	62
6482	87.2	85.4	73.3	86.7	90.3	83.5	81.3	90.7	90.4	89.6	85.5	88.2	85.5	88.2	69.3	71.4	74.8	66.3	87.5	87.5
6584	81	78	87	87	82	78	89	112	89	89	120	93	120	93	103	115	85	112	82	78
6595	57	53	54	54	37	47	52	51	55	N.R.	N.R.	N.R.	55	54	66	66	65	66	52	53
6658	47.35	46.19	47	45	52.23	44.51	44.82	44.51	46	29	N.R.	N.R.	43	72	38.66	38.23	37.48	37.08	53.59	54
6926	78	66	79	77	75	77	57	67	63	57	N.R.	N.R.	79	80	67	71	73	72	77	70
7253	43	27	39	37	36	N.R.	40	42	32	N.R.	N.R.	N.R.	N.R.	34	46	59	48	48	39	38
7283	92	89	71	68	121	86	81	123	71	86	97	86	97	86	112	63	73	111	99	97
7669	72	72	94	60	65	65	56	63	62	79	N.R.	N.R.	64	68	77	90	85	101	61	52
$\overline{R}$ (%)	66		67		66		65		71		N.C.		72		73		74		66	
RSD_R (%)	27		25		38		29		25		N.C.		27		27		28		27	

N.R. not reported

N.C. not calculated

Table 6: individual recoveries for BaP (expressed as %)

Material	EXWFLOUR		FISH_B		IF_2010		IF_2011		MEAT_A		MUSS_DRY		MUSSELS		OIL_1		OIL_2		WHFLOUR	
Lab ID	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**
3063	55	64	57	81	64	54	70	68	73	69	70	70	70	70	79	75	72	74	57	64
6032	82	60.3	96.8	99.7	59.8	75.6	62.2	73.5	70.4	68.4	N.R.	N.R.	77.2	92.7	85.2	87	84.9	84.2	84.1	72.4
6426	74	75	79	76	63	60	71	66	67	74	N.R.	N.R.	70	70	93	81	76	82	75	78
6482	100.6	97.6	81.8	94.5	95.6	98.9	92.4	106.3	97.9	90.9	101.5	105.2	101.5	105.2	78.8	80.9	81.4	71.6	99	99
6584	88	106	100	100	85	114	104	87	98	98	70	78	70	78	95	105	77	108	88	81
6595	75	66	75	76	47	42	49	61	76	N.R.	N.R.	N.R.	69	75	37	38	37	38	70	72
6658	85.98	75.4	84	84	83.88	66.73	91.72	88.59	82	61	N.R.	N.R.	82	94	77.5	78.45	72.02	80.45	87.23	86.48
6926	86	75	75	78	85	86	63	75	64	63	N.R.	N.R.	93	93	55	72	75	75	89	82
7253	57	34	52	48	50	N.R.	42	55	43	N.R.	N.R.	N.R.	N.R.	47	68	83	69	69	51	49
7283	102	90	69	66	103	93	94	90	61	84	96	99	96	99	100	93	90	105	94	106
7669	90	95	79	76	84	64	60	85	53	87	N.R.	N.R.	108	107	89	108	105	113	78	63
$\overline{R}$ (%)	79		78		72		75		76		N.C.		86		80		78		78	
RSD_R (%)	23		19		36		24		19		N.C.		17		23		24		20	

N.R. not reported

N.C. not calculated

Table 7: individual recoveries for BbF (expressed as %)

Material	EXWFLOUR		FISH_B		IF_2010		IF_2011		MEAT_A		MUSS_DRY		MUSSELS		OIL_1		OIL_2		WHFLOUR	
Lab ID	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**
3063	64	71	55	81	67	63	72	73	73	72	69	67	69	67	78	75	73	73	64	71
6032	84.5	52.4	82.3	84	81.6	83.9	77.8	80.9	70.2	67.9	N.R.	N.R.	64.1	71	93.3	91.1	93.1	89.6	79.3	82.8
6426	76	78	72	76	68	69	76	70	71	76	N.R.	N.R.	71	72	86	84	83	84	77	77
6482	89.3	90.1	73.3	87.2	89.3	89	84.2	94.2	91.6	85.7	88.7	92.1	88.7	92.1	74	74.1	76.5	67.3	91	91
6584	99	118	105	105	91	134	109	104	129	129	72	112	72	112	73	107	84	116	129	88
6595	62	58	54	55	44	54	58	55	55	N.R.	N.R.	N.R.	57	58	55	57	57	59	55	58
6658	78.31	67.17	70	63	69.07	57.91	70.22	61.11	68	49	N.R.	N.R.	71	86	62.83	59.23	60.94	59.69	73.44	75.95
6926	88	77	79	80	86	87	65	74	68	63	N.R.	N.R.	91	91	63	74	79	76	87	82
7253	46	29	41	38	38	N.R.	42	45	34	N.R.	N.R.	N.R.	N.R.	39	67	65	56	56	41	39
7283	105	95	63	55	97	97	92	110	59	84	93	95	93	95	107	101	94	108	92	105
7669	85	89	68	67	80	59	62	81	66	80	N.R.	N.R.	76	81	82	106	115	112	75	68
$\overline{R}$ (%)	77		71		74		75		72		N.C.		77		79		79		74	
RSD$_R$ (%)	27		26		21		25		15		N.C.		17		21		24		23	

N.R. not reported

N.C. not calculated

Table 8: individual recoveries for CHR (expressed as %)

Material	EXWFLOUR		FISH_B		IF_2010		IF_2011		MEAT_A		MUSS_DRY		MUSSELS		OIL_1		OIL_2		WHFLOUR	
Lab ID	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**	**Rec 1**	**Rec 2**
3063	67	73	57	82	73	64	77	72	75	75	69	67	69	67	80	75	78	74	65	74
6032	61.1	55.2	70.9	69.1	68.6	78.9	57.9	68.6	59.6	58.1	N.R.	N.R.	51.8	53.3	77.4	78.9	78.9	75	62.2	59.2
6426	65	62	61	65	58	60	66	60	59	69	N.R.	N.R.	56	56	67	72	62	70	60	62
6482	81.2	79.8	45.2	53.4	56.5	78.2	75.2	82.6	55.5	54	78.8	92.2	78.8	92.2	64.3	65.6	68.7	60.9	81.1	81.1
6584	78	77	97	97	89	95	93	78	94	94	110	67	110	67	76	88	117	111	70	130
6595	52	49	48	49	35	47	49	47	48	N.R.	N.R.	N.R.	50	50	N.R.	54	53	53	47	48
6658	61.86	60.81	52	49	63.3	50.54	54.83	48.08	44	32	N.R.	N.R.	65	75	46.06	41.56	42.7	40.96	66.32	69.23
6926	77	63	75	77	75	76	56	65	64	60	N.R.	N.R.	74	76	62	72	73	71	76	67
7253	36	23	33	30	53	N.R.	36	35	27	N.R.	N.R.	N.R.	N.R.	29	38	81	40	39	33	31
7283	93	84	65	59	114	89	70	115	63	83	93	95	93	95	93	98	105	95	99	91
7669	69	72	56	57	71	67	51	64	60	71	N.R.	N.R.	64	68	81	82	79	92	55	51
$\overline{R}$ (%)	65		60		70		62		65		N.C.		67		73		72		64	
RSD_R (%)	25		30		26		25		25		N.C.		22		20		32		28	

N.R. not reported

N.C. not calculated

ANNEX 13 – Method options selected by participants

Questionnaire STEP 3:	*Which extraction apparatus did you use? Pressurised liquid extraction (PLE) / Soxhlet* *Which extraction solvent did you use? n-hexane(n-HEX) / cyclohexane (C-HEX)* *Which injection port did you use? PTV / Split-splitless (SS)*

LabID	Extraction apparatus	Extraction solvent	Injection port	Difference from subscription
3063	PLE	*n*-HEX	PTV	YES (SS → PTV)
6032	PLE	C-HEX	PTV	NO
6426	PLE	C-HEX	PTV	NO
6482	Soxhlet	*n*-HEX	PTV	YES (PLE → Soxhlet)
6584	PLE	C-HEX	PTV	YES (Soxhlet → PLE) (*n*-HEX → C-HEX)
6595	PLE	*n*-HEX	SS	NO
6658	Soxhlet	*n*-HEX	SS	NO
6926	Soxhlet	*n*-HEX	PTV	YES (SS → PTV)
7253	PLE	C-HEX	PTV	NO
7283	PLE	C-HEX	PTV	YES (*n*-HEX → C-HEX)
7524	PLE	other (see deviations from SOP)	SS	YES (extraction solvent)
7669	PLE	C-HEX	PTV	YES (*n*-HEX → C-HEX)

ANNEX 14 – Significant deviations (from STEP 1 plus STEP3 responses)

In the following Tables the row corresponding to the participant coded as 7524 was highlighted in grey to put in evidence the deviations from the SOP applied. These deviations caused the classification as not compliant of the whole set of reported data.
Lab 7299 was not included in the Tables as not reporting data for STEP 3.

*Did you follow the method in all details?**
*If NO, in which part(s) did you deviate from the protocol?**
5 – Reagents (e.g. the labelled standards - please give the composition)
6 - Apparatus (e.g. the SEC column)

LabID	Method followed	Reagents	Apparatus
3063	NO		
6032	NO		SEC Column: 300 mm x 8 mm (ID) SPE Column: ISOELUTE SPE 1g Si (6 ml)
6426	NO		
6482	NO		- Soxhlet Apparatus was used for extraction - SEC column: 50g Biobeads, column size 25*300; flow rate 5ml/min - automated SEC system: aliquotation step of 50% (only 5ml is put on the column from a total volume of 10 ml extract), integrated evaporation unit concentrates to 1 ml end volume, no addition of toluene as keeper possible during evaporation - no use of amber vials
6584	NO		
6595	NO		EnvironGel Column
6658	YES		Commercial Waters GPC Cleanup Column formed by 2 columns (19 x 150 mm and 19 x 300 mm, respectively) filled with styrene polyvinyl benzene.
6926	NO		SEC column: length 500 mm, width 10 mm
7253	NO		The SEC column used had 44g Bio-beads in 25x250mm.
7283	NO		
7524	NO	Native standards: mix from EURL Labelled standards: mix of deuterated PAH's containing deuterated EPA-16 PAHs + DiP-D14 (0.5 ng/µl in iso-octane) Injection standard: perylene-D12 (0.111 ng/µl for oil, 0.5 ng/µl for fish in iso-octane) SPE-sorbent: basic aluminium oxide, activity I, deactivated with 14 % H2O Extraction solvent; hexane: acetone, 1:1, v/v	SEC-column: BioBeads SX3 (200-400 mesh) in 25 x 600 mm column MS: HR-MS (resolution 10.000)
7669	YES		

7.2 – Test portion preparation for solid samples
7.3 – Solid sample extraction
7.4 - Test portion preparation for liquid samples (no answer)

LabID	Solid sample preparation	Solid sample extraction
3063		
6032		
6426		
6482		
6584	7.3.5: the final volume is adjusted to 6 ml (1g sample + 5 ml SEC eluent), because our SEC-machine has a loop of 5 ml to inject	
6595		
6658		
6926		
7253		1. At the PLE stage (7.3.1), after adding the combined sample (of extract, polyacrylic acid and sand) to the 33ml extraction vessel, each vessel was topped up with sand, leaving 1-2ml of space as per manufacturers' instructions. 2. After PLE (7.3.3), the extract and washings of Na2SO4 were filtered through GFA Whatman filter paper. These filter papers are already used in the lab for PAH analysis. Samples were then not filtered at 7.3.5 through a PTFE filter before SEC.
7283		
7524	48 µl of our mixed labelled PAH is added and solved in 15 ml SEC-eluent. Of this 12.5 ml is injected on the SEC-system.	
7669		

7.5 – SEC clean-up
7-6 to 7.7
7.8 SPE clean-up

LabID	SEC	Filtration / concentration	SPE
3063		Evaporated with 35° (not 40°C)	
6032	Column flow: 1.5 ml/min Fore Run: 900 sec Main Fraction: 1200 sec Tailing: 900 sec		Condition of the column: 4 ml cyclohexane Load sample extract from 7.7 Elution of the target compounds: 2 x 10 ml cyclohexane
6426	Injection port of SEC did now allow transferring the complete sample onto the column. The ratio of transferred/remaining solution was determined gravimetrically and taken into account when calculating recoveries.		
6482	Extract was filled up to 10 ml before SEC clean up, only 5 ml is put on SEC column (Aliquotation) Integrated evaporation unit concentrates to 1 ml end volume, no addition of toluene as keeper possible		
6584	some parameters of our SEC-machine are fix: - column 50g Bio-Beads S-X3, 25 x 320 mm - Flow: 5ml per minute => sample-fraction: 21 to 50 minutes, proved by tests with standard-substances		
6595	Dichloromethane was used as eluent for this step.		
6658	In some samples, it was not possible to obtain 5 ml after filtration. Consequently, the injected volume for SEC clean-up was taken into account for the calculations.		
6926	The sample was diluted to 4 ml and the injection volume to the SEC was 2 ml.		The final volume after SPE clean-up was 200 µl.

LabID	SEC	Filtration / concentration	SPE
7253	At the SEC step a collection time of 36 to 70 minutes was used.	In the lab we have a Buchi Syncore system for evaporating solvents. This comes with evaporation vessels that evaporate to 0.3ml, 1ml or 3ml. Therefore at 7.6 the SEC extract was evaporated to 3ml and 200ul toluene was added. The extract was then transferred to 0.3ml evaporation vessels. When the evaporation to 300ul was complete, 700ul cyclohexane was added.	At 7.9 after SPE, the eluent was evaporated to 300ul, 100ul toluene and 100ul Injection standard was added. Again this was done due to the evaporation vessels available in the lab.
7283	The sample was solved in 4 ml SEC eluent; two 1 ml aliquots were injected to the column.		
7524		evaporated to 1 ml, transferred to a test tube, 20 µl dodecane added (as keeper), evaporated to 20 µl. 0.5 ml of hexane added.	SPE-columns filled with 1 g of sorbent, conditioned with 2 ml of hexane. Loaded with the 0.5 ml extract. The test tube is rinsed 3 times with 1 ml of hexane and once with 0.5 ml of hexane and also loaded on the SPE-column. The eluate is evaporated to 20 µl (dodecane) and 180 µl of injection standard solution is added. This mixture is brought into an amber GC-vial with insert.
7669			

8.1 – GC-MS operating conditions

8.2 & 8.3 - Calibration

LabID	GC-MS	Calibration
3063		
6032	PTV injection: Temp: 60 °C hold 0.4 min 340 °C (200°C/min) hold 10min Split valve: 0 min On 80 ml/min 0.4 min Off 4 min On 80 ml/min Column flow: 0.6 ml/min constant flow Column Temp: 80 °C hold 1.5 min 220 °C (25°C/min) hold 0 min 275 °C (3°C/min) hold 0 min 300 °C (6°C/min) hold 0 min 340 °C (8°C/min) hold 10 min	Calibration solution: was prepared by volume with Hamilton syringes CS1 1 ng/250ul in toluene CS2 2 ng/250ul in toluene CS3 3 ng/250ul in toluene .... CS12 180 ng/250ul in toluene
6426	We used a Thermo DSQ GC/MS System with PTV injector. The settings for the injection port had to be modified as the conditions given in the SOP did not fit to our instrument. Injection volume 3µl was not changed.	
6482		Recommendation: As separation of triphenylene and chrysene is mentioned as criterion, triphenylene should be included in the standard solutions The injection volume for samples under 0.5µg/µl we calibrate and inject 6 µl to compensate the SEC aliquotation
6584	Varian PTV-Injector: Injection Volume: 5 ul First Ramp of Injector: 200°C per minute to 349; hold 20 minutes	
6595	MS source temperature = 290ºC (280 ºC for fish)	
6658	8.1.6.- MS source temperature : 250 ºC	
6926	The PTV and the SIM programmes were slightly changed.	
7253	Oven program started at 55°C due to injector not achieving desired temperature with oven starting at 70°C.	Quadratic function was applied to calibration curves for quantification.
7283	Column oven program: Temp Rate Hold Total (C) (C/min) (min) (min) 90 0.0 5.50 5.50 200 20. 0 0.00 11.00 325 4.0 3.75 46.00	

LabID	GC-MS	Calibration
7524	Injection: 1 µl splitless, Helium 6.0 Oven: initial temp: 70 °C initial time: 0.70 min Eq. time: 0.2 min first ramp: 85 °C/min, static 3 min second ramp: 3 °C/min static 7 min third ramp: 28 °C/min, static 10 min fourth ramp: 14 °C/min to 350 °C, static 10 min Column: constant flow 2ml/min Transfer lines: 320 °C MS Source temperature: 260 °C EI energy: 35 eV **SIM-acquisition:** **details reported by participant are available as additional information upon request**	we used 5 calibration solutions with nominal PAH-concentrations: 0.010 - 0.025 - 0.050 - 0.100 - 0.250 ng/µl (1) nominal concentration for labelled PAHs: 0.050 ng/µl nominal concentration for injection standard: 0.100 ng/µl (1) The real concentration differ slightly from the nominal one. This is accounted for in our processing method. For calibration mean RRF (relative response factor) of the 5 CS-solutions were used. We had no time for making higher concentrated CS-solutions. We used the mean RRF also for situations where the extracts were outside the CS-range.!!!!
7669		

8.4 – Sample analysis
Others

LabID	Sample analysis	Others
3063	We use automatic calculation of the calibration curve (Chem. station)	We have not changed the Dwell time, but it should be changed from 80 to 50ms for group 4 and group 5
6032		
6426		
6482		
6584		
6595		Qualifier ion for CCP has very low sensitivity.
6658	The sequence of injections was not followed as recommended in 8.4 in terms of Toluene and CS1 and CS7 injections.	
6926		
7253		
7283		
7524	order of injection: solvent blank (iso-octane) (our) CS1 (our) CS3 (our) CS5 solvent blank (iso-octane) procedure blank oil procedure blank oil oil 1 oil 2 oil 3 oil 4 oil 5 spiked procedure blank oil solvent blank (iso-octane) procedure blank fish procedure blank fish fish 1 fish 2 fish 3 fish 4 fish 5 spiked procedure blank fish (our) CS 4 (our) CS 2	our solvent for standards etc. is iso-octane in stead of toluene
7669		

ANNEX 15 – Analytical problems reported by participants (from STEP 3 responses)

In the following Tables whenever a participant indicated a sample with its numeric code, this was substituted with the corresponding sample short name, so to highlight any correspondence between the observations reported and possible outliers.

Did you encounter any problem during the analysis?
If YES, what were the specific problems and to which samples do they apply?

LabID	Problems	Description and samples
3063	YES	
6032	YES	In case of WHFLOUR the final solution was "dirty" (the peak shapes were wrong and the resolutions were bad).
6426	NO	
6482	NO	
6584	NO	
6595	YES	BbF Results for all samples exceed the working range, and the results have been estimated by extrapolating the calibration curve. There has been some problem during the injection of first replicate of the sample MEAT_A, so I cannot give results for this sample. There has been some problem in the operation of the chromatograph in one of the aliquots for MUSSELS and MUSS_DRY, and the chromatogram was interrupted after BgP, so I cannot give results for DlP, DeP, DiP and DhP
6658	YES	In some cases, interferences in the chromatograms of the qualifier ions In some cases, the ion ratio requirements were not achieved
6926	NO	
7253	YES	The MUSSELS (and MUSS_DRY) samples blocked the PLE cells, resulting in the system stopping due to over pressurisation. Only 5 to 10mls of extraction solution was collected for MUSSELS (and MUSS_DRY) samples from PLE, giving poor extraction efficiency. Subsequently two more mussel samples were supplied by the JRC. However the same problem occurred, the PLE tubes became blocked. Approximately 20mls extraction solution was obtained from one aliquot and only 5mls from the other aliquot at the PLE stage. For one of the two aliquots injection standard solution was accidentally omitted so no recovery value could be calculated. We had a problem with the GPC for one of the two MEAT_A aliquots and for one of the two IF_2010 aliquots giving unusable results. It seemed like GPC did not work properly for these samples as they were very dirty when analyzed.
7283	NO	
7524	YES	1. We noticed that in our calibration standards the response of the lockmass at the RT of BcL was lower than that at the RT of FLU-D10 (the internal standard for BcL). In the samples lockmass response was usually about the same for the RT of BcL and FLU-D10. 2. Sensitivity in the GC-HRMS was worse than usual, resulting in higher LOQ's. There was no time available for us to get a better performance of the machine before measuring.
7669	NO	

Did you notice any abnormality, which however seem to had no effect on the result (e.g. the SPE column was blocked or with very low flow)?
If YES, please describe and report for which samples (codes) they occurred.

LabID	Abnormality	Description and samples
3063	NO	
6032	YES	For samples MUSSELS and MUSS_DRY After the PLE extraction the mussels extract contained high amount of extracted materials that's why we had resolved it with high volume cyclo-hexane/ethyl-acetate and we have performed the SEC clean-up 3 step.
6426	YES	The connector between analytical column and pre- column became leaky during the sequence. After fixing the problem, the sequence was resumed without repeating the calibration. This refers to samples of FISH_B. Samples for control of recovery were measured afterwards and we did not observe any negative effect.
6482	NO	
6584	NO	
6595	NO	
6658		
6926	NO	
7253	NO	
7283	NO	
7524	NO	
7669	NO	

Did you measure a resolution between BbF/BkF of at least 0.8 and between BkF/BjF of at least 0.4 for all the samples?
If NO, please report the corresponding sample code(s) and values for the separation factors

LabID	**Rs correct**	**Samples with incorrect Rs**
3063	YES	
6032	NO	WHFLOUR: BbF/BkF=0.69, BkF/BjF=0.44
6426	YES	
6482	YES	
6584	YES	Several values were reported for both ratios, but all compliant with the requirements
6595	YES	
6658	YES	
6926	YES	
7253	YES	
7283	YES	
7524	YES	
7669	YES	

Did you obtain the correct Q1/Q2 ratio for all the samples?
If NO, please report the sample code(s) corresponding to non acceptable ratios

For these questions, answers received by participants were summarised before reporting them in the table below

LabID	Q1/Q2 correct	Samples with incorrect Q1/Q2
3063	YES	
6032	NO	MUSSELS (both aliquots), MUSS_DRY (both aliquots), WHFLOUR, EXWFLOUR, OIL_1
6426	NO	EXWFLOUR (BaP)
6482	YES	
6584	YES	
6595	YES	
6658	NO	In several cases an incorrect ratio was reported for the non-target PAHs. For some samples wrong Q1/Q2 was reported also for BaA, BbF and CHR, however corresponding sample codes were not reported.
6926	NO	EXWFLOUR (both aliquots), IF_2010
7253	YES	In several cases an incorrect ratio was reported for the non-target PAHs. MUSSELS and MUSS_DRY(BaP), WHFLOUR (BaP), IF_2010 (BaP), IF_2011 (BaP), FISH_B (BaP for both aliquots)
7283	YES	
7524	NO	EXWHFLOUR, IF_2010 (both aliquots), IF_2011, WHFLOUR
7669	YES	

European Commission

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Abstract

The European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons (EU-RL PAHs), operated by the Institute for Reference Materials and Methods (IRMM) of the Joint Research Centre (JRC), organised a method validation study by inter-laboratory comparison (ILC-MVS) for evaluating the effectiveness of a method based for the determination of the four marker PAHs (benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, and chrysene in different food commodities within the scope of Commission Regulation (EC) No 1881/2006.

The test sample is homogenised, a test portion is mixed with desiccant, sand and the labelled internal standard mixture. It is then extracted with n-hexane or, alternatively, with cyclohexane, by pressurised liquid extraction (PLE). Soxhlet extraction has proven to give equivalent results compared to PLE, provided that a sufficient number of extraction cycles are performed (at least 7 hours of extraction, with about 6 cycles/h).
Co-extracted water is separated from the organic phase of the extract; then the organic extract is evaporated to small volume, filtered and purified by size exclusion chromatography (SEC), using a mixture of ethyl acetate and cyclohexane as eluent.
The extraction step is skipped for edible oils. For this matrix a portion of the sample is diluted with a mixture of ethyl acetate and cyclohexane, then the labelled internal standard mixture is added and the sample is directly processed by SEC. After SEC, 200 µL of toluene are added as a keeper to the collected SEC fraction, which is then evaporated to about 200 µL, and further cleaned up by solid phase extraction on silica, using cyclohexane as eluent. The cleaned-up sample extract is evaporated again to 200 µL. Finally an injection standard is added to the sample prior to measurement by GC-MS.
The final extract is preferably injected into a programmable temperature vaporizer inlet. However split/splitless injection may be applied alternatively. The chromatographic separation is achieved on a specific capillary column which allows the separation of the four target PAHs from the other EU priority PAHs and from triphenylene. The analytes are ionised by electron ionization (EI) at 70 eV. The target PAHs are recorded in Single Ion Monitoring mode, and quantified by comparison with the labelled analogues.

A total of 10 materials (corresponding to 20 expected results) including edible oil, meat, smoked fish, bivalve molluscs, cereals and infant formula, containing the 15+1 EU priority PAHs (inclusive of the four marker ones) at different levels, comparable to maximum levels laid down in legislation, were sent to 18 laboratories from 9 EU Member States, and a laboratory in Switzerland. Recovery was calculated by participants from the labelled standards. All samples were sent as blinded duplicates but fish and meat which were sent as open duplicates.

12 laboratories reported results, of which 1 laboratory did not apply the SOP and was therefore excluded from the evaluation.
Relative standard deviations for reproducibility (RSD_R) ranged from 7% for BaP in oil to 55% for BaP in mussels (BaP content was 0.9 µg/kg).
The values for RSD_r values ranged from 2% for BaP and BbF in oil to 17% for BbF in wheat flour and CHR in infant formula.
Robust mean values for recoveries ranged from 60% for CHR in fish to 86% for BaP in mussels' tissue.
The Commission Regulation (EC) No 333/2007 lays down performance criteria that must be met by a method to determine BaP in food. These criteria were extended also to BaA, BbF and CHR and have been met by this method for all materials but for RSD_R for BaP in mussels.

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