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Institute for Reference Materials and Measurements

The certification of the mass fractions of PAH metabolites (1-hydroxyphenanthrene, 1-hydroxypyrene and 3-hydroxybenzo[a]pyrene) in two fish bile materials

BCR-720 (sediment-exposed flounder bile)
BCR-721 (oil-exposed plaice bile)

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Report EUR 21068

The mission of IRMM is to promote a common European measurement system in support of EU policies, especially health and consumer protection, environment, agriculture, internal market and industrial standards.

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REFERENCE MATERIALS

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BCR-720 (sediment-exposed flounder bile)
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ABSTRACT

This report describes the preparation, homogeneity study, stability study and certification of two fish bile materials: BCR-720, sediment-exposed flounder bile and BCR-721, oil-exposed plaice bile. In BCR-720 three metabolites of polycyclic aromatic hydrocarbons (PAHs) are certified: 1-hydroxy phenanthrene, 1-hydroxy pyrene and 3-hydroxy benzo[*a*]pyrene. 1-hydroxy pyrene is certified in BCR-721. A description of the analytical procedures used in the homogeneity and stability studies as well as in the certification study is given. All relevant data from the homogeneity and stability study and certification measurements are presented. Major conclusions from earlier feasibility studies, the results obtained for 2-hydroxy naphthalene (not certified), and implications for the optimal use of the materials are also discussed.

The certified values are:

Substance	***BCR-720***		***BCR-721***	
	Certified value (mg/kg wet mass basis)	*Uncertainty* (mg/kg wet mass basis)*	*Certified value (mg/kg wet mass basis)*	*Uncertainty* (mg/kg wet mass basis)*
1-hydroxy phenanthrene	0.21	0.07	Not certified	
1-hydroxy pyrene	52	9	2.1	0.4
3-hydroxy benzo[a]pyrene	0.063	0.026	Not certified	

* expanded uncertainty according to the GUM with a coverage factor $k = 2$

LIST OF ABBREVIATIONS AND SYMBOLS

1-OH phen	1-hydroxyphenanthrene
1-OH pyr	1-hydroxypyrene
2-OH naph	2-hydroxy naphthalene
3-OH BaP	3-hydroxybenzo[*a*]pyrene
ANOVA	analysis of variance
BaP	benzo[*a*]pyrene
BCR	Bureau Communauté de Référence
BHT	butylhydroxytoluene
BSTFA	N,O-Bis(trimethyl)trifluoro acetamide
CI	confidence interval
CRM	certified reference material
CV	coefficient of variation (relative standard deviation in %)
DMSO	dimethylsulfoxide
Flu	fluorescence (detection)
GC	gas chromatography
HPLC	high-performance liquid chromatography
HW	half-width
ICES	International Council for Exploration of the Seas
i.p.	intra peritoneal
IRMM	Institute for Reference Materials and Measurements
IS	internal standard
ISO	International Organisation for Standardisation
k	number of spot samp les
LC	liquid chromatography
MeOH	methanol
MS	mass spectrometry
$MS_{between}$	mean square between the bottles
MS_{within}	mean square within the bottles
n	number of replicates
n.r.	not reported
OSPARCOM	Oslo and Paris Commissions
PAH	polycyclic aromatic hydrocarbon
RP	reversed phase
rpm	rotations per minute
RSD	relative standard deviation
s_{bb}	between-bottle standard deviation
SD	standard deviation
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
U_{CRM}	Expanded uncertainty of the certified value
u_{bb}	Uncertainty contribution due to possible inhomogeneity
u^*_{bb}	Upper limit of inhomogeneity detectable by the method chosen
u_{char}	Uncertainty contribution due to batch characterisation exercise
u_{lts}	Uncertainty contribution due to long-term stability

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1. INTRODUCTION

1.1 Background: need for a CRM

Polycyclic aromatic hydrocarbons (PAHs) are hazardous substances (EU priority environmental pollutants) that are formed during incomplete combustion and are also a natural constituent of petroleum products. They can enter the aquatic environment via several routes, such as atmospheric deposition, run-off, leaching from tarred/creosoted structures, or oil spills. Numerous studies, mainly carried out in North America and Europe, have shown a link between PAH levels in the aquatic environment and liver tumour incidence in fish populations [1,2].

A major fraction of the PAHs absorbed by fish is rapidly metabolised to hydroxylated derivatives (phase-1 metabolism), then conjugated to polar groups (phase-2 metabolism), and stored in the gall bladder to be excreted. The analysis of PAH metabolite levels in fish bile can be used to assess the actual PAH uptake [3-5]. This approach is gaining ground as a useful biomarker of PAH exposure in the aquatic environment, and its implementation has been recommended by international bodies such as ICES and OSPARCOM [6]. Fish bile typically contains a very large number of compounds, and quantitation is difficult due to chromatographic overlap and a limited availability of calibration standards. For monitoring purposes, one would have to select a number of target compounds which can be measured reliably and for which calibration standards can be obtained. Unfortunately, so far there were no certified reference materials (CRMs) for method development and quality control.

1.2 Choice of the material and the properties to be certified

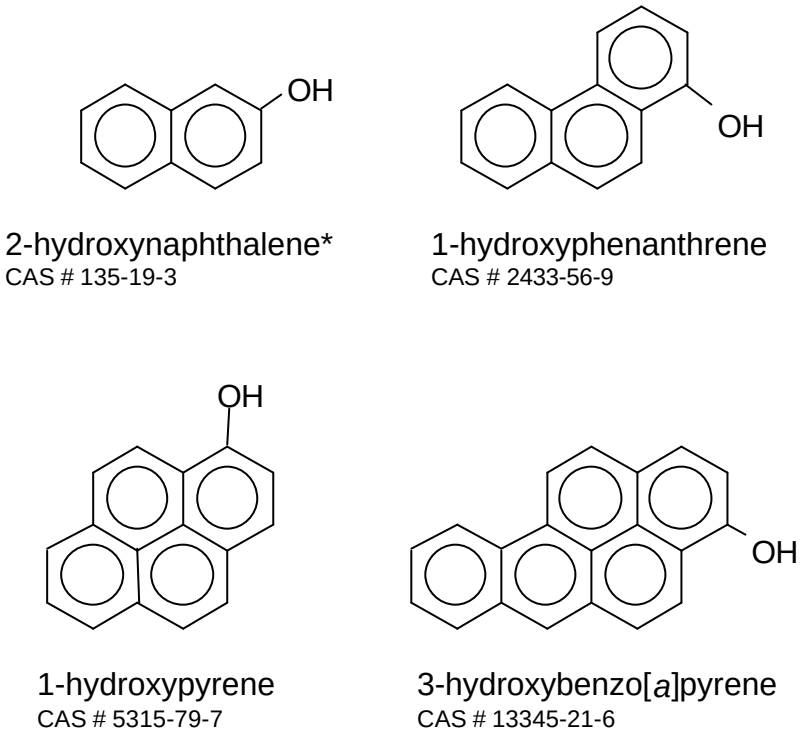
In a polluted environment fish are typically exposed to dozens of different PAHs, each of which can be biotransformed into one or more metabolites. Depending on the source of PAH pollution the metabolite profiles can be very different. Petrochemical pollution (e.g., oil spills) typically contains many lighter PAHs (2-3 rings), often with one or more alkyl substituents. PAHs formed during incomplete combustion processes (e.g., urban run-off, emissions from chimneys and car exhausts) tend to contain larger aromatic systems (3-7 rings), often without alkyl substituents. Both types of pollution can be encountered in aquatic ecosystems, and would result in very different PAH metabolite profiles in fish bile. In order to accommodate for the differences in average molecular weight and chromatographic complexity, it was decided that two different matrix materials should be developed. Rather than producing a spiked material, large groups of fish were exposed in mesocosms to crude oil or to sediment from a heavily industrialised harbour, resulting in realistic levels of PAH metabolites and realistic levels of interferences in the bile. The fish species selected were the estuarine flatfish flounder for the sediment exposure (*Platichthys flesus*; BCR-720) and the marine flatfish plaice for the oil exposure (*Pleuronectes platessa*; BCR-721). However, the CRMs will also be useful for the quality control of PAH metabolite measurements in bile from other fish species.

1.3 Principles and design of the studies

A 40-months program was started in 1998 to prepare two batches of fish bile to serve as matrix reference materials. BCR-720 would contain predominantly metabolites from combustion-type PAHs, while BCR-721 would reflect exposure to petrochemical PAHs. In a prestudy (Section 3) the practical feasibility of exposing fish in mesocosms to crude oil or polluted sediment was tested, and the stability of fish bile materials under different storage conditions was determined. Two intercomparison exercises were conducted to assess the level of analytical intercomparability and to check the certification potential of a preliminary list of target compounds. Bile samples from fish exposed to a single PAH through injections were

made available for method optimisation. Standard solutions of the PAH metabolites were also dispatched to each laboratory to verify their calibration procedures. The details of the feasibility studies are given in section 3.

After optimisation of the exposure conditions, larger groups of flatfish were exposed to crude oil or polluted sediment for the preparation of the two candidate CRMs (Section 4). Homogeneity tests and 13-month stability tests were carried out for both materials (Section 5). Finally, for the certification a number of target compounds, narrowed down during the feasibility phase to four monohydroxylated PAHs (see Fig. 1), was to be determined in both materials. The methods used for the certification (Section 6) and the technical and statistical evaluation (Sections 7 & 8) are presented in this report.



2-hydroxynaphthalene*
CAS # 135-19-3

1-hydroxyphenanthrene
CAS # 2433-56-9

1-hydroxypyrene
CAS # 5315-79-7

3-hydroxybenzo[*a*]pyrene
CAS # 13345-21-6

Figure 1 - Structures of the target compounds selected for certification (not certified)*

2. PARTICIPANTS

Preparation of the materials

Sediment exposures

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Oil exposures

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3. FEASIBILITY STUDY

3.1 General

Before starting the certification of the two reference materials an extensive feasibility study was performed, both on material preparation and material stability. Two intercomparison exercises were organised to verify the ability of the participating laboratories to measure the target analytes.

3.2 Preparation of sediment-exposed test bile

At the field station of the Netherlands Institute for Coastal and Marine Management/ RIKZ at Jacobahaven, flounder (*Platichthys flesus*), collected from a relatively unpolluted estuarine site in Oosterschelde, were exposed in three large mesocosms to a 6 cm layer of polluted harbour sediment from Wemeldinge, the Netherlands. HPLC-fluorescence measurements showed that the sediment used for the test exposure had a typical pyrogenic PAH profile, with concentrations of the major PAHs in the 200-600 ng/g range. The latter was much lower than anticipated. The exposure lasted for 14 days, during which time the fish were not fed. The fish were killed by a blow to the head. The bile was collected from the gall bladder by means of disposable syringes, immediately cooled on an ice bath in 10 ml centrifuge tubes, and centrifuged. The total volume was 50 ml; the test batch was stored at – 60 ^{o}C.

3.3 Preparation of oil-exposed test bile

Plaice (*Pleuronectes platessa*, mean weight 483 g) were caught in the vicinity of the Ølberg beach, 20 km south of Stavanger, Norway. This location is open towards the North Sea and relatively far from any significant sources of pollution. The specimens were kept alive on board ship in a seawater tank and were transported by car the following day to the Akvamiljø A/S research station at Mekjarvik, Stavanger. The fish were in very good shape with virtually no injuries from catch, captivity or transport. During a few weeks of acclimation the fish were fed carefully with thawed frozen shrimp until one week before the exposure start. No lethality occurred during acclimation and exposure. Feeding was stopped one week before the start of the exposure study. The fish were exposed in a mesocosm to petrochemical PAHs by mixing crude North Sea oil as micro-droplets with seawater (1-2 ppm) using a high-pressure mixing valve. Oil and water flows and temperature were constantly monitored. Oil droplet distributions were determined using a Coulter Multisizer II counter, the major fraction was in the 3-30 µm size range.

After two weeks of exposure, the plaice were killed by a sharp blow to the head. The weight and length of the fish were measured. The abdominal cavity was then opened. This was done with a pair of scissors cutting from the anterior left side and in a 45^{o} posterior direction across the spine and into the anterior part of the cavity. The organs were then exposed by bending the head region of the fish in the right direction. The gall bladder was secured by means of an artery clamp and cut free from the liver and intestine. The bile was drained into a 15 ml batch tube standing all the time in ice. The weight of the bile from each fish was measured; the amount of bile obtained was typically between 0.2 - 0.3 % of total body weight. When reaching a 10-12 ml volume, the bile sub-batch was frozen to -80 ^{o}C. A total of six sample vials was obtained from the oil exposed plaice (later pooled to a total volume of 68 ml), whereas one batch sample vial was obtained from the seven controls.

3.4 Homogenisation and bottling of the test materials

The test materials were transported on dry ice to the Institute for Reference Materials and

Measurements (IRMM) in Geel (Belgium), although later stability studies (*vide infra*) showed the fish bile samples to be much more stable than previously assumed. Both bile materials were centrifuged at 3000 rpm for 5 min, decanted, and homogenised by slow propellor stirring. Using an automated ampoulation machine, 300 µl aliquots were sealed under argon/helium in amber glass ampoules. A trace of helium was added for leak detection immediately after sealing of the ampoules. Bile samples were stored at –30 °C; for reference purposes some ampoules were stored at -70 °C.

3.5 Preparation of calibrants and test solutions

At the onset of the project, a separate set of bile samples was prepared for method optimisation. Flounder and Atlantic cod (*Gadus morhua*) were injected i.p. with single PAHs (typical dose 1-5 mg/kg bodyweight). The resulting bile samples thus contained a limited number of compounds (only metabolites from a single PAH) at relatively high levels. These samples were distributed to all participants and could be used for the optimisation of hydrolysis, separation and detection conditions.

At the inception meeting the participating expert laboratories selected a set of five monohydroxylated PAH metabolites as preliminary target compounds: 2-OH naphthalene, 1-OH phenanthrene, 1-OH pyrene, 1-OH chrysene, and 3-OH benzo[*a*]pyrene. This selection was based on the following criteria: expected concentrations in fish bile, availability of standards, and ecotoxicological relevance. The selection of metabolites represents a range of 2-5 ring parent PAHs.

At subsequent technical meetings the list of PAH metabolites was modified in light of the experiences gained during the feasibility studies. 1-OH-chrysene was withdrawn because of the very low levels found in both test materials. It was replaced by chrysene-1,2,-diol for one exercise. However, this compound was withdrawn at a later stage due to the difficulty in the analysis, the apparent instability in the calibration solutions and the very low concentrations observed in the exposed fish. Thus, the set of target compounds that were finally selected for the certification exercise were: 2-OH-naphthalene, 1-OH-phenanthrene, 1-OH-pyrene and 3-OH-benzo[*a*]pyrene (see Fig. 1 for structures).

For the preliminary intercomparison exercises calibration solutions and standard test solutions with undeclared concentrations were prepared in ethanol/water 80/20 with 0.5 % of ascorbic acid added as anti-oxidant. This worked well for laboratories using HPLC, but the solutions were not directly compatible with typical GC solvents. Calibration solutions with butyl hydroxy toluene (BHT) were also used as an alternative anti-oxidant in the second intercomparison, but this was found to be unsuitable due to coelution problems. It was concluded that for the final certification exercise the participants should be provided with pure crystals of each calibrant, allowing each laboratory to choose its own solvents and anti-oxidants.

3.6 Stability and homogeneity of the test materials

The stability of the test bile samples was tested isochronously. That is, at the onset of the study all samples were stored at –70 °C, and at specified time points before the end of the test period, ampoules were transferred to higher temperatures (-20; +4, +20 and +40 °C). All samples were analysed by HPLC-fluorescence at the end of the study (t = 15 months) in one continuous analytical series, thus minimizing intralaboratory variability. Samples were analysed after enzymatic hydrolysis, and 1-OH pyrene, 1-OH phenanthrene, and 2-OH naphthalene were used as target analytes (3-OH BaP being too close to the detection limit). The average concentrations found in the samples stored at –70 °C were used as reference values.

The main conclusions were that no degradation was observed for 1-OH pyrene and 1-OH phenanthrene in the samples stored at -20°C or +4°C. Even the samples stored at +20°C were found to be surprisingly stable, although a separate test had shown a significant decrease in the pyrene-1-glucuronide levels after only a few months. Apparently, at elevated temperatures hydrolysis of the conjugate can occur, but the resulting deconjugated phase-1 metabolites are not very sensitive to further degradation in these samples. With a method aimed at identifying hydroxy-metabolites after enzymatic deconjugation one would of course never notice the spontaneous hydrolysis. The 2-OH naphthalene data were first believed to reflect good stability, but later results indicated the presence of interfering compounds (see Section 5.2).

In a preliminary homogeneity test the concentration of pyrene-1-glucuronide in both test batches (single measurements in 10 ampoules each) had shown very low standard deviations: 4.2 % in the oil-exposed test bile and 4.4 % in the sediment-exposed test bile. These numbers did not differ significantly from the standard deviation of the HPLC-fluorescence method used (see the 2nd progress report, ref [7] for details). In addition, a more extensive homogeneity study was performed on the oil-exposed plaice bile. Ten ampoules that had been stored at -70°C were thawed and analysed in duplicate after enzymatic hydrolysis. 1-OH pyrene, 1-OH phenanthrene, and 2-OH naphthalene were determined (3-OH BaP was too close to the detection limit for reliable quantitation) and the resulting variances were subjected to a single-factor ANOVA tests. For 1-OH pyrene and 1-OH phenanthrene the P-values were below the critical values, thus passing the homogeneity criterion at the 95 % significance level. The 2-OH naphthalene data appeared to show good homogeneity, but later results indicated the presence of interfering compounds (see Section 5.2).

3.7 Preliminary interlaboratory studies

3.7.1 First intercomparison

For the first intercomparison exercise participants were asked to determine five compounds in two standard solutions, in an oil-exposed test bile and in a sediment-exposed test bile. Calibration solutions and bile from fish injected with a single PAH were provided. Ten (out of eleven) data sets were returned and analysed using the Quasimeme software package [8]. At a technical meeting the results were discussed and the following conclusions were reached: The results for 1-OH pyrene in the standard solutions showed a reasonable level of agreement, but there were a few outliers. It was argued that the solvents and antioxidants used to prepare the standard solutions were not directly compatible with standard GC procedures. In fish bile samples 1-OH pyrene is often easily quantitated in terms of sensitivity and absence of interferences. Indeed, most laboratories were able to determine this compound in the two real fish bile samples, large deviations could be linked to specific technical problems. Chromatograms of hydrolysed fish bile from pyrene-injected fish showed that 1-OH pyrene is the single major metabolite. In conclusion, it was decided that 1-OH pyrene should definitely be part of the final metabolite selection.

The 3-OH BaP results for the two standard solutions showed outlying results for some labs, probably due to the experimental difficulties mentioned above for 1-OH pyrene. GC-MS detection limits for TMS-derivatised 3-OH BaP were fairly poor. Only a few laboratories were able to measure the low levels of this metabolite in the sediment-exposed sample, and options for obtaining bile with higher concentrations were discussed. In view of the carcinogenic properties of benzo[a]pyrene it was considered important to include this metabolite among the target compounds. Since B[*a*]P is only a very minor constituent in crude oil, 3-OH BaP was not found in the oil-exposed test sample.

Chromatograms showed that in hydrolysed bile from chrysene-injected cod, 1-OH chrysene is actually a very minor metabolite. Although no serious difficulties were encountered with the determination of 1-OH chrysene in the standard solutions, its presence at very low levels in

natural samples led to the decision to remove 1-OH chrysene from the list of target analytes and replace it with chrysene-1,2-dihydrodiol.

For 1-OH phenanthrene the intercomparison results were very promising for the two standard solutions. In the sediment-exposed test bile the levels were very low and only few laboratories were able to determine 1-OH phenanthrene. In the oil-exposed bile the levels were much higher, but several laboratories reported interferences from coeluting compounds. It was decided to keep 1-OH phenanthrene on the list of selected target analytes. Due to the crowded chromatograms and the limited selectivity of the fluorescence detector, GC-MS methods are preferred for this compound.

The results for 2-OH naphthalene were very similar to those obtained for 1-OH phenanthrene. There was a fairly good agreement for the two standard solutions for most laboratories. The crowded chromatograms from the oil-exposed fish bile led to selectivity problems with HPLC fluorescence, calling for GC-MS based methods. It was decided to keep 2-OH naphtalene among the target species, and HPLC laboratories were encouraged to improve their separations.

3.7.2 Second intercomparison

It was decided to carry out a second intercomparison with a slightly different list of target compounds, different solvent and anti-oxidant (methanol with BHT), and higher metabolite levels in the sediment-exposed sample. A spiking experiment was also included in the protocol (to check the recoveries and to check peak assignment in a co-injection approach) and satisfactory recoveries were obtained. Twelve (out of twelve) data sets were returned, and the results were discussed at a technical project meeting.

Biplots of Z-scores obtained by the various laboratories for the two standard solutions and the recovery experiments showed that most of the results were evenly spread around the origin rather than along the diagonal, indicating that deviations were random rather than systematic. For the real samples some laboratories showed very high Z-scores, which were in most cases traceable to coelution. The statistical distribution of the data sets and the extent of overlap between laboratories was assessed using the novel statistical approach of Cofino *et al.* [9]. The recovery experiments yielded very good results, with most laboratories reporting yields over 70 %.

Several laboratories had noticed that the chrysene-1,2-diol calibrant solution produced two peaks with different ratios, indicating degradation. This compound was therefore not selected for certification. The antioxidant BHT was found to cause coelution problems with 2-OH naphthalene. In order to circumvent these problems the calibrants for the certification should be distributed as pure compounds. Participants can then decide which solvent and anti-oxidant to use. 2-OH naphthalene and 1-OH phenanthrene were difficult to resolve under HPLC conditions. A separate hydrolysis test of a pyrene-1-glucuronide solution, in which participants were asked to check the 1-OH pyrene yield after their standard incubation time, half that time and double that time, showed that in all laboratories the hydrolysis is well under control and is not a critical step.

3.8 Conclusions

The feasibility study was an essential part of the project. It allowed to optimise the fish exposure procedures, handling of calibrants and standard solutions, and gave participants the opportunity to optimise and finetune their methods prior to the actual certification of the two candidate CRMs. The following target compounds were selected for certification: 2-OH-naphthalene, 1-OH-phenanthrene, 1-OH-pyrene and 3-OH-benzo[*a*]pyrene.

4. PREPARATION OF THE MATERIALS

4.1 Preparation of sediment-exposed flounder bile for BCR-720

At the project meeting in September 1999 it had been decided that the large-scale sediment exposure should preferably be carried out with a sediment with higher PAH levels than the sediment from Wemeldinge harbour that was used for the feasibility study. The second sediment was collected in IJmuiden harbour, the Netherlands, where high PAH levels are often found due to heavy shipping (main passage to Amsterdam harbour) and the coke oven installations of a nearby steel plant. The sediment was subsampled and was analysed by means of HPLC-fluorescence at IVM-Amsterdam. Indeed, the PAH levels were approximately a factor of ten higher than in the first batch and very close to the PAH levels foreseen in the original proposal. The PAH content (US Environmental Protection Agency list of 16 priority pollutants) is listed in Table 1:

Table 1 - PAH content of the sediment used for the flounder exposure for BCR-720, in mg/kg dry wt.

Compound	*Concentration in mg/kg dry weight*
Naphthalene	2.2
Acenaphthylene	<3
Acenaphthene	<0.6
Fluorene	0.8
Phenanthrene	2.3
Anthracene	1.6
Fluoranthene	12
Pyrene	6.3
Benz[a]anthracene	3.8
Chrysene	3.9
Benzo[b]fluoranthene	3.2
Benzo[k]fluoranthene	1.6
Benzo[a]pyrene	3.0
Dibenz[a,h]anthracene	0.35
Benzo[ghi]perylene	2.2
Indeno[1,2,3-cd]pyrene	2.3

At the RIKZ field station in Jacobahaven, the Netherlands, groups of flounder (*Platichthys flesus*) were available from relatively unpolluted areas: Eastern Scheldt, Volkerak and the North Sea entrance of the Nieuwe Waterweg. After acclimation they were exposed to a layer of IJmuiden sediment in large (2 m diameter) tanks with water from the Eastern Scheldt. The exposure was carried out in several batches, each lasting typically 10-14 days. During the exposure the fish were not fed in order to stimulate the accumulation of bile in the gall bladder. After exposure, the fish were killed by a blow on the head. The bile was collected from the gall bladder by means of disposable syringes, immediately cooled on an ice bath in large, 50 ml centrifuge tubes and subsequently stored at -60 ^{o}C. The combined bile volume was 430 ml and was transported on dry ice to IRMM for the production of BCR-720.

4.2 Preparation of oil-exposed plaice bile for BCR-721

For the preparation of the bile material for BCR-721 a large-scale exposure of plaice to oil was performed in the period October-December 1999. With minor modifications the exposure was carried out as described in Section 3.2 for the test batch. The same facility was used (but with four fish tanks, each 100 x 100 x 40 cm, instead of one), the same crude oil (Frøy crude), the same dispersion technique (pressure drop), and the same exposure length (14 days). The plaice used were collected at the same field location, in the vicinity of the Ølberg beach, South of Stavanger. The fish had a weight range of 129–1090 g, and beside minor lesions on the fins of a few individuals the fish were generally in very good condition. No fish mortality was recorded during the exposures. Due to the large number of specimens the exposure was carried out in three separate batches, each of 14 days of length.

Based on the positive experience from the January 1999 exposure, it was decided to expose the fish to a dose equal to 1 ppm (based on volume) dispersed oil. The oil concentration was detected by means of a Coulter Counter and the dispersed oil droplets to be quantified were in the size range of 3-30 micrometer. During dispersion, an ultra-precise pump delivered a steady flow of 0.05 ml/min Frøy crude oil into the dispersion chamber where it was dispersed in 9 l/min of pure seawater. This 9 l/min of oil dispersion contained a nominal dose of oil of 5.56 ppm, but only a certain fraction of this oil volume was actually dispersed. Undispersed oil was continuously removed from the exposure setup since in each tank the water outflow was located at the surface. The oil-seawater dispersion was then split in four sub-flows (2.25 l/min); each was directed into one of the four exposure tanks. Additionally, the four fish tanks received each 4 l/min of pure seawater, this in order to ensure a sufficient water quality for the fish during the exposure. The concentration of dispersed oil droplets (size 3-30 µm) was measured regularly in water samples from each of the four exposure tanks. Most values detected throughout the three exposure periods were within the 0.80 – 1.10 mg/kg range (not shown), and with a min-max range of 0.737 – 1.5 mg/kg. This was considered satisfactory.

During sampling, the biological data of the fish (weight, length and sex) were recorded. A total amount of 523 g of bile was collected from a total of 547 plaice. The bile was collected in batch glass vials on ice. For approximately each thirty individuals the weight of the collected bile was measured and the bile sub-batch frozen at –80 °C in a ultrafreezer. After the end of the exposure, crack formation in some of the glass vials was discovered. The bile was therefore thawed and transferred to polypropylene tubes (Sarstedt AG) and refrozen. Subsequently, the vials were shipped by courier on dry ice to IVM to be transported by car to IRMM for the production of BCR-721.

4.3 Homogenisation and bottling

The flounder bile resulting from exposure to polluted sediment (Section 4.1) and the plaice bile resulting from exposure to dispersed crude oil (Section 4.2) were transported on dry ice to IRMM for the preparation of the two candidate reference materials. Details such as storage temperature, homogenisation conditions, prevention of oxidative damage, minimisation of losses, selection of samples for homogeneity, etc., were discussed in detail with the responsible staff at IRMM. On 25/26 September 2000 the following steps were performed:

- Thawing of the centrifuge vials to 4 ^{o}C overnight.
- Centrifugation at 3000 rpm for 5 minutes to precipitate any undesired solid material, with temperature monitoring of the centrifuge at 4 ^{o}C.
- Decantation: manually pouring the clear phase of each centrifuge vial into a clean, dry, pre-cooled 2-L glass container.

- Homogenisation under nitrogen flushing during 50 minutes, using a magnetic stirrer, with cooling of the material in the glass bottle via a coil system at 4 ^{o}C. Cooling and flushing with nitrogen of the bulk material was continued during the filling phase.
- Filling and sealing under argon/helium (90/10) of 0.3 ml aliquots in 3-ml irradiated DURAN glass ampoules at a speed of 17 units per minute. The operation took about an hour and a half per batch.
- Immediate labelling, respecting the filling order, and subsequent storage at –30 ^{o}C.

The final products were:

- BCR-720 Flounder bile, sediment exposed; 1200 units
- BCR-721 Plaice bile, oil exposed; 1320 units

The major part of these materials is currently stored at IRMM at –30 ^{o}C, with 100 units of each material stored at –70 ^{o}C for reference purposes. Samples intended for the certification, stability and homogeneity studies (150 ampoules of each material) were packed per set of three in styrofoam boxes with labels and transported by car on dry ice to IVM (Free University Amsterdam) on 19 October 2000.

5. TESTING OF THE MATERIALS

5.1 Analytical methods used for the homogeneity and stability tests

The analytical measurements for the homogeneity and stability tests were carried out by means of HPLC-fluorescence. This method was selected for its good repeatability (typical RSDs 3-5 %). In contrast to GC methods no derivatisation step is necessary, thus reducing the number of sample pretreatment steps.

5.1.1 Sample preparation

The ampoules were thawed and briefly sonicated before opening. 50 µl subsamples were taken with a pipettor and transferred to microcentrifuge tubes. To each sample, 10 µl of a β-glucuronidase/arylsulfatase enzyme solution (Merck) and 200 µl of water were added. The mixture was incubated for 2 hours in a water bath, then briefly centrifuged to remove any liquid from the lid of the microcentrifuge tubes. Then 200 µl of cold ethanol was added to precipitate the enzymes. After 5 min of centrifugation at 3000 rpm the supernatant was transferred to glass HPLC vials. Perdeuterated anthracene was added as internal standard. Throughout the procedure all amounts were determined by weighing and the dilution factors were calculated on a weight basis.

5.1.2 HPLC analysis

The deconjugated PAH metabolites in the hydrolysed bile samples were determined by means of HPLC with fluorescence detection. The system consisted of a Varian 9100 autosampler, a Varian 9012 pump, a Vydac 201TP54 25/4.6 (Aurora, USA) column thermostated at 18 ^{o}C, and a Jasco FP920 fluorescence detector. The injection volume was 20 µl. The eluent system was an acetonitrile/water gradient starting (t_0) at 20 % acetonitrile, increasing linearly to 100 % at t = 25 min, constant (100 %) at t = 40 min. The flow rate was 1 ml/min. The excitation/emission wavelength settings of the fluorescence detector were programmed for optimal detection of each target analyte: 2-OH naphthalene, 325/358 nm; 1-OH phenanthrene, 269/380 nm; pyrene, 346/384 nm; anthracene-d_{10} (IS), 250/400 nm; 3-OH benzo[*a*]pyrene, 385/450 nm.

For calibration a dilution series of the four target analytes was prepared in ethanol/water 50:50 with 5 mg/ml ascorbic acid as antioxidant. Calibration was performed based on peak height. During the analytical run, a calibration solution (75 ng/ml level) was analysed after each 10 injections to check for possible instrument drift.

The nominal dilution factor of the sample preparation procedure was ca. 10x. The 1-OH pyrene levels in BCR-720 were determined after an additional dilution to 1000x. The 2-OH naphthalene levels in BCR-721 were determined after an additional dilution to 100x. These dilutions were made with ethanol/water 50:50 with 5 mg/ml ascorbic acid. Since relative values can be used to establish inhomogeneity or instability, the measured concentrations were not corrected for recovery.

5.2 Homogeneity study

Fifteen ampoules of each material were taken at random intervals along the ampoule filling scheme. Each ampoule was analysed in duplicate in a random order in a single analytical series. PAH metabolite concentrations were determined by HPLC-fluorescence after enzymatic hydrolysis (deconjugation) as described above. A calibration standard was injected every ten runs in order to monitor possible instrument drifts.

All measurements were performed with a sample intake of 50 µl of bile. This relatively large intake was chosen in order to improve the precision of the measurements for those compounds that were close to the detection limit. No attempt was made to test the homogeneity at lower sample volumes.

The concentration of 3-OH BaP in BCR-721 was below the detection limit, and homogeneity results cannot be reported. The HPLC determination of 2-OH naphthalene suffered from severe chromatographic overlap with an unknown compound, both in the case of BCR-720 and of BCR-721. Therefore, no homogeneity data are available for 2-OH naphthalene in either CRM. The individual data of the homogeneity tests are listed in Annex 1.

The results of the homogeneity study are summarised in Table 2. The estimation of the uncertainty component of the between-bottle homogeneity (u_{bb}) is described in section 8.3. In all cases, except for 1-OH phen, s_{bb} can be used to estimate u_{bb}. However, for 1-OH phen the value of sbb is below the minimum value as determined by the repeatability of the method and the number of replicates performed. In this case u^*_{bb} is used as an estimate of u_{bb}. For the respective formulas for s_{bb} and u^*_{bb} refer to section 8.3 and the literature cited.

Table 2 - Homogeneity study in BCR-720 and BCR-721

Analytes and units	*BCR-720*			*BCR-721*	
	1-OH phen	*1-OH pyr*	*3-OH BaP*	*1-OH phen*	*1-OH pyr*
	[ng/g wet wt]	*[ng/g wet wt]*	*[ng/g wet wt]*	*[ng/g wet wt]*	*[ng/g wet wt]*
k	15	15	15	15	15
n	2	2	2	2	2
$MS_{between}$	132.09	7194029	47.00	3243	7127
MS_{within}	259.56	3285179	15.24	1619	4050
F from ANOVA	0.509	2.19	3.08	2.00	1.76
$CV_{between}$	3.7 %	4.9 %	11.0 %	4.3 %	3.9 %
CV_{within}	5.8 %	3.7 %	7.0 %	3.3 %	3.4 %
s_{bb}	*/*	3.61 %	9.02 %	*/*	2.55 %
u^*_{bb}	3.14 %	2.00 %	3.77 %	*/*	1.76 %
Value adopted as u_{bb}	3.14 %	3.61 %	9.02 %	*/*	2.55 %

K = number of ampoules

n = number of replicates per ampoule

$MS_{between}$ = ANOVA mean square between the ampoules

MS_{within} = ANOVA mean square within the ampoules

F = $MS_{between}$ / MS_{within} (ANOVA criterion); F_{crit} = 2.42

CV = coefficient of variation (relative standard deviation, RSD) related to the grand mean (n = 30)

$CV_{between}$ = RSD of 15 average concentrations (15 ampoules, each measured in duplicate)

CV_{within} = Average of RSDs of duplicate measurements, (15 ampoules)

s_{bb} = between-bottle standard deviation

u_{bb} = uncertainty component from homogeneity

u^*_{bb} = upper limit of inhomogeneity that can be hidden by the method repeatability

As in principle, the freezing/thawing process could lead to a phase separation within the

ampoule, it is recommended that each time a frozen bile sample is thawed it should be re-homogenised by a brief sonication step. Furthermore, as the homogeneity test was carried out at an intake level of 50 µl, this is the recommended sample intake for the determination of low-level PAH metabolites.

5.3 Stability study

The stability study on the four target analytes in BCR-720 and BCR-721 was performed in November 2001 after 13 months of sample storage. Storage of sub-samples was started in October 2000 immediately after the production of the two candidate CRMs.

An isochronous measurement design [10] was applied to test possible analyte degradation under four conditions: –20 °C in a freezer, +4 °C in a refrigerator, room temperature (+20 °C) in a climate-controlled laboratory in the dark and +40 °C in a thermostated oven. At specific times prior to the end of the experiment the required number of samples was taken from the –70 °C freezer and transferred to the above mentioned temperatures. Thus, samples were investigated after 1, 2, 4, 7 and 13 months of storage at higher temperatures; for each time point and for each condition two ampoules were analysed in duplicate (34 ampoules in total for each CRM, see Table 3). The +40 °C measurements were only done for 1 and 2 months of storage. The reference temperature used for this study was –70 °C. At this temperature no relevant impact on the analyte contents over a long time period can be expected. The concentrations found in the samples stored at –70 °C throughout the experiment (averages of 6 ampoules analysed in duplicate) were set at 100 %.

At the end of the stability test, all ampoules were thawed, briefly sonicated and 50 µl samples were hydrolysed and analysed as described in Section 5.1. All analyses were carried out in a single HPLC series for maximum repeatability. For the reasons outlined above (see Section 5.2) no reliable data could be obtained for 2-OH naphthalene due to co-elution in both CRMs. The level of 3-OH BaP in BCR-721 was too low to be detected.

Table 3 - Scheme for the isochronous stability study of BCR-720 and BCR-721

		Number of ampoules analysed (in duplicate) for each CRM				
Storage temperature T in °C	- 70	6	-	-	-	-
	– 20	2	2	2	2	2
	+ 4	2	2	2	2	2
	+ 20	2	2	2	2	2
	+ 40	-	-	-	2	2
Duration of storage in months		13	7	4	2	1

The results of the isochronous stability study were analysed using the SoftCRM software package [11] and the results are shown in Figures 2-6, referring to 1-OH phenanthrene in BCR-720, 1-OH pyrene in BCR-720, 3-OH BaP in BCR-720, 1-OH phenanthrene in BCR-721, and 1-OH pyrene in BCR-721, respectively. In each graph the results are plotted as relative contents against storage time; the average level found in the reference ampoules (six duplicates), stored continuously at -70 °C is set as the 100 % reference point. For each compound under each storage condition the slope of the regression line was calculated; in a stable CRM the slope should not be significantly different from zero. The raw data are listed in Annex 2.

5.3.1 1-OH phenanthrene in BCR-720

No instability was observed regarding the 1-OH phenanthrene content of BCR-720 at any of the temperature conditions tested (see Fig. 2). None of the regression lines showed a slope significantly different from zero at the 95 % significance level. A minor, statistically not significant increase was observed after 2 months of storage at +40 °C (see Fig. 2). This puzzling increase could be due to an unknown coeluting degradation product.

5.3.2 1-OH pyrene in BCR-720

Due to a technical defect in the HPLC autosampler when analysing the diluted samples, there are no 2-months data for BCR-720 stored at +20 and +40 °C. For this compound in BCR-720 no significant change (95 % significance level) was observed over 13 months at –20, +4 and 20 °C (see Figure 3). In the sample stored at +40 °C for one month a small but significant (t-test) increase was observed, which is probably due to an unknown coeluting degradation product.

5.3.3 3-OH BaP in BCR-720

No instability was observed regarding the 3-OH BaP content of BCR-720 at any of the temperature conditions tested (see Fig. 4). None of the regression lines showed a slope significantly different from zero at the 95 % significance level.

5.3.4 1-OH phenanthrene in BCR-721

This compound showed excellent stability at –20, +4 and +20 °C. As in BCR-720, a minor, statistically not significant increase was observed after 2 months of storage at +40 °C (see Fig. 5). The latter could be due to an unknown coeluting degradation product (similar to 1-OH phenanthrene in BCR-720).

5.3.5 1-OH pyrene in BCR-721

No instability was observed regarding 1-OH pyrene in BCR-721 at any of the temperature conditions tested (see Fig. 6). None of the regression lines showed a slope significantly different from zero at the 95 % significance level.

5.4 Conclusions regarding storage and transport

In this study the stability of 1-OH phenanthrene, 1-OH pyrene and 3-OH BaP in fish bile was demonstrated, stored at -20, +4 and +20 °C. Even though no degradation was observed in the refrigerator or at ambient temperatures, storage in the freezer (-20 °C or colder) is recommended for optimal long-term stability. One argument for this extra precaution is that due to analytical uncertainty small changes in metabolite levels may not be significant after 13 months but could become noticeable after longer storage periods. However, if no significant change is observed at room temperature after 13 months, no significant degradation is expected in frozen bile over a period of several years. Also, during the feasibility phase it was found that at ambient or high temperatures conjugate hydrolysis may occur, although further degradation was found to be much slower (see Section 3.6). Given the excellent short-term stability at room temperature, shipment on dry ice will usually not be necessary, but prolonged storage at high temperatures should be avoided [7].

It should be stressed that in contrast to the good stability in untreated bile samples, several researchers have observed that PAH metabolites are significantly less stable in clean solvents. It is therefore recommended that calibrant solutions or spiking solutions be prepared fresh and that a suitable antioxidant should be added to avoid degradation. For the same reason, bile hydrolysis should be carried out immediately prior to analysis.

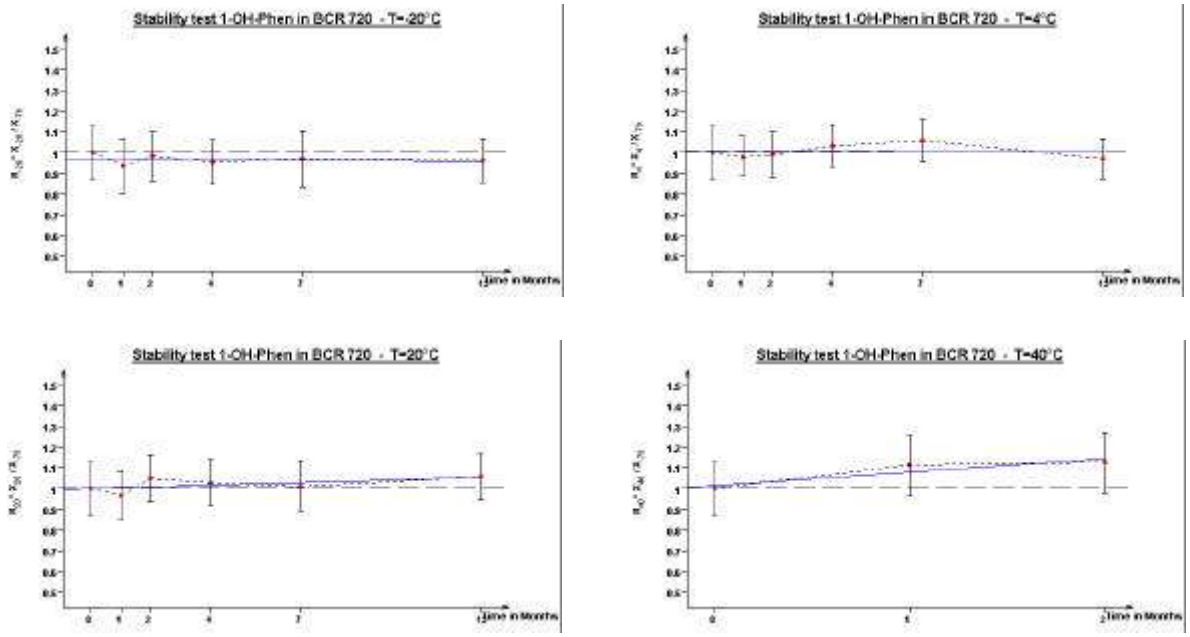

Figure 2 - Stability study of 1-OH phenanthrene in BCR-720 at –20, 4, 20 and 40 °C. Concentrations are given relative to the average found in the samples stored at -70 °C (= t_0 point).

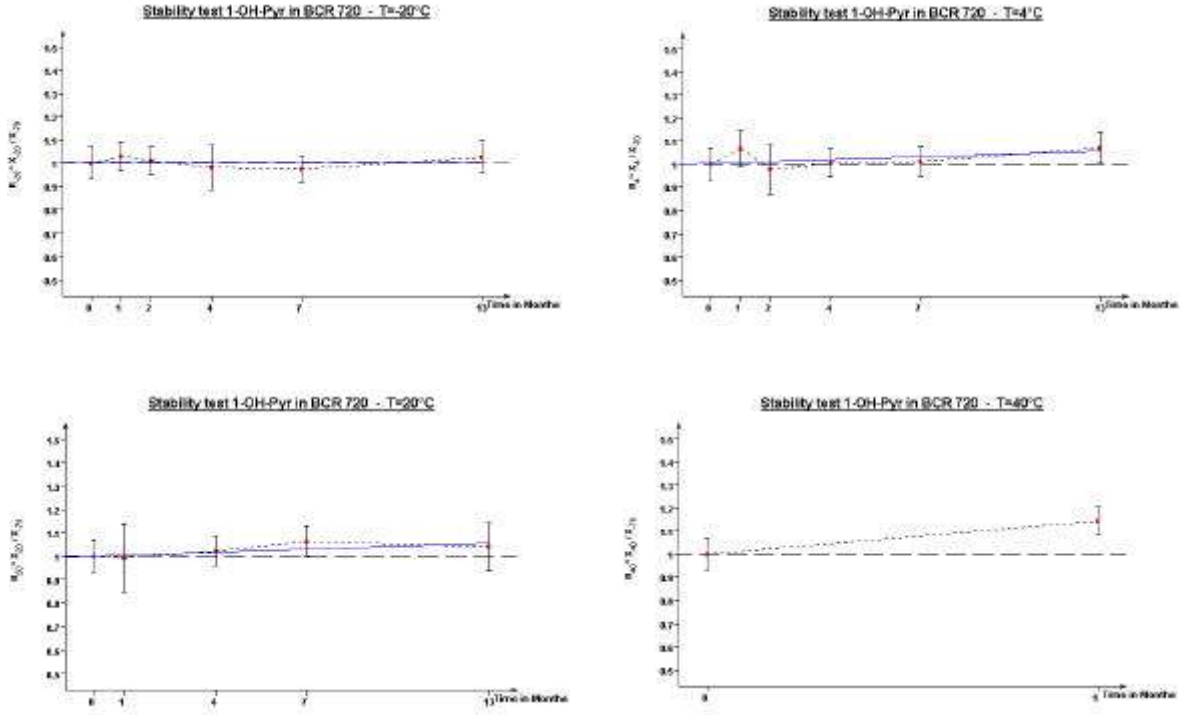

Figure 3 - Stability study of 1-OH pyrene in BCR-720 at –20, 4, 20 and 40 °C. Concentrations are given relative to the average found in the samples stored at -70 °C (= t_0 point).

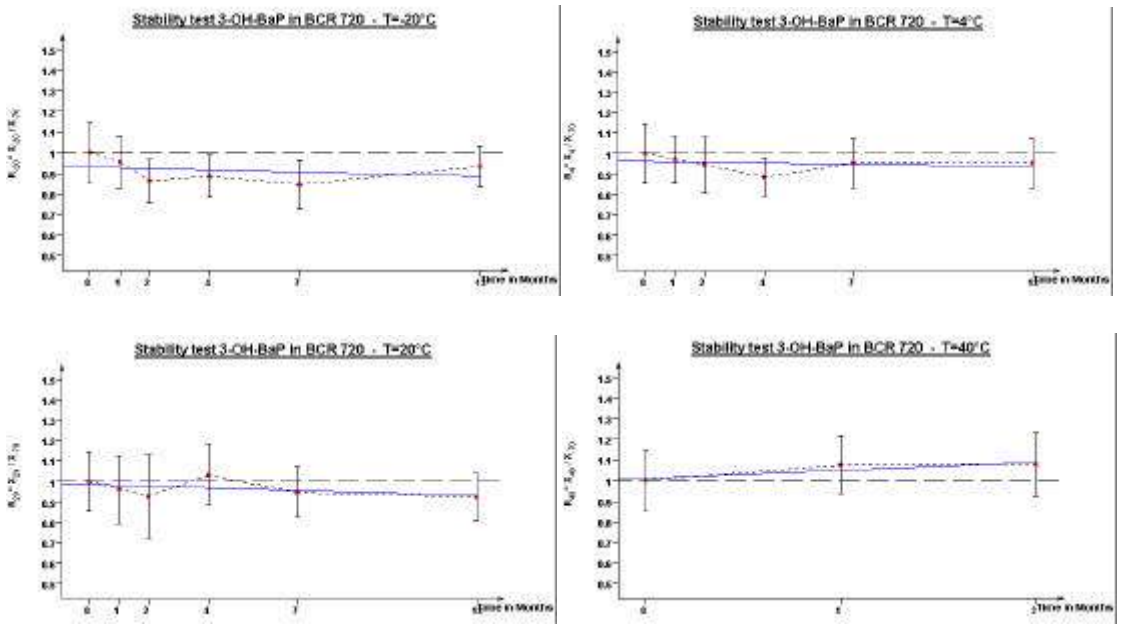

Figure 4 - Stability study of 3-OH BaP in BCR-720 at –20, 4, 20 and 40 °C. Concentrations are given relative to the average found in the samples stored at -70 °C (= t_0 point).

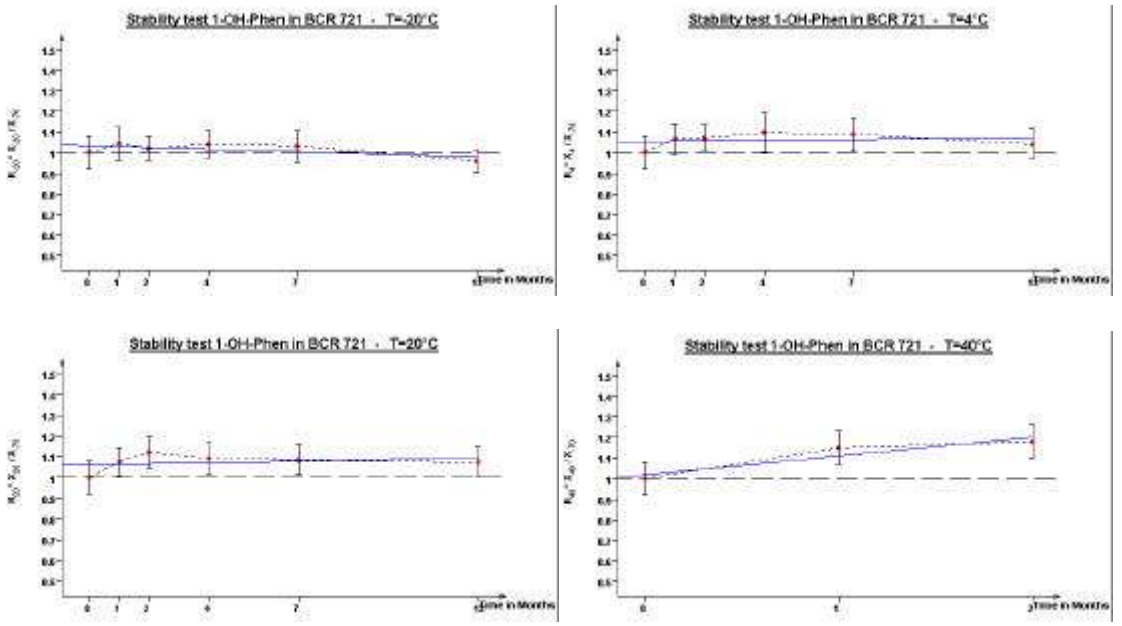

Figure 5 - Stability study of 1-OH phenanthrene in BCR-721 at –20, 4, 20 and 40 °C. Concentrations are given relative to the average found in the samples stored at -70 °C (= t_0 point). Included for information purposes only, compound NOT certified.

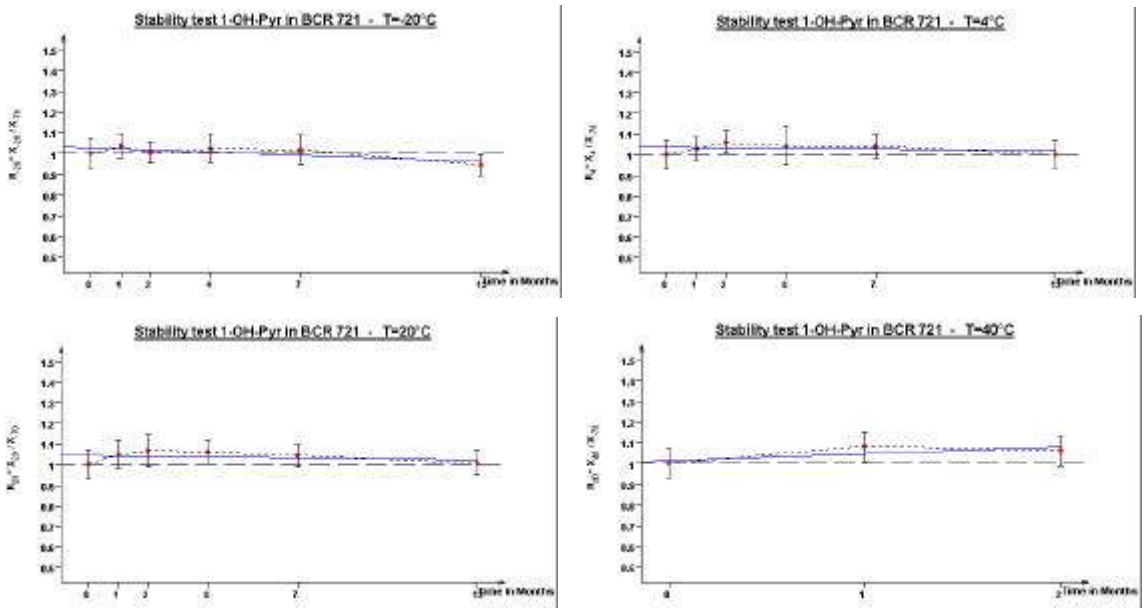

Figure 6 - Stability study of 1-OH pyrene in BCR-721 at –20, 4, 20 and 40 °C. Concentrations are given relative to the average found in the samples stored at -70 °C (= t_0 point).

6. CERTIFICATION MEASUREMENTS

Measurements for the certification exercise were performed according to the BCR Guidelines [12]. Participants were requested to carry out the following tasks:

- Six independent measurements of the 4 target metabolites had to be performed from two ampoules of BCR-720, at least on two different days.
- Six independent measurements of the 4 target metabolites had to be performed from two ampoules of BCR-721, at least on two different days.
- The recoveries of the four target compounds were to be determined based on a spiking experiment (3 blank measurements and three measurements at each of three spiking levels.

The following materials were distributed for the certification:

- 3 ampoules of approximately 0.3 ml of BCR-720;
- 3 ampoules of approximately 0.3 ml of BCR-721;
- 1 ampoule of “blank” fish bile from a control site to be used for the recovery experiment.
- A mixed spiking solution of known concentrations for the recovery experiment
- Calibration standards supplied as pure compounds

The analytical methods used for the determination are described in detail in section 6.5. Each laboratory used its own optimised procedure for sample preparation, hydrolysis, extraction, clean up, derivatisation (where appropriate), method of injection, chromatographic separation and detection. These methods were optimised after the feasibility study. All procedures and measurements were recorded and reported by each laboratory according to a given protocol using the automated Quasimeme database provided.

Remark

Twelve laboratories participated in this project, and all delivered results in the framework of the certification exercise. One laboratory delivered two independent data sets using different methods.

6.1 Calibration and internal standards

Each laboratory prepared separate calibration solutions according to its own laboratory procedures. These solutions were used for calibrating the relevant detector within its working range. The calibrants were supplied as pure solids; the corresponding purities as stated by the manufacturer are listed in Table 4.

The calibrants were supplied as solid compounds. Participants were not required to use the calibrants supplied; instead, they could use their own calibrants, provided these were of sufficiently high and known purity. Participants were advised to protect their calibrant solutions from oxidative damage by adding an appropriate amount of anti-oxidant, such as ascorbic acid (HPLC users) or BHT (GC users).

At least one internal standard had to be used, to be added to the final solution; the choice of

internal standards was made by each participating laboratory. The main purpose of adding an internal standard was to check for variability in injection volume and/or solvent evaporation. The internal standards applied for these certification methods are listed in Tables 6A and 6B.

Table 4 - Purity of PAH metabolite calibrants

Compound	*Supplier*	*Stated purity (%)*
2-OH naphthalene	Sigma-Aldrich	>99
1- OH phenanthrene	Promochem	>99
1-OH pyrene	Sigma-Aldrich	>98
3-OH benzo[a]pyrene	NCI-Midwest Res. Inst.	>99

All standards and samples were prepared on a mass basis using calibrated balances. Working solutions were prepared by mass controlled dilution. The calibrant solutions had to be measured together with the fish bile samples on the same day using the same analytical procedure and in the same analytical series.

The working range of the detector was established for each analyte individually from calibration solutions of different concentrations. The working range of the detector is defined as the range where the detector response per mass unit versus the mass injected shows a linear response. At least 4 calibration points had to be used.

The calibration of the detector was verified at least once in a sequence of injections on each measurement day.

6.2 Analytical blanks

Analytical blanks were performed at each day of analysis. All solvents used were of highest purity grade. The metabolite levels in the “blank” fish bile were measured in triplicate. Since the “blank” fish bile sample was collected from flounder from a relatively unpolluted area, low background levels of the PAH metabolites were anticipated; especially 1-OH pyrene was found to be present in measurable amounts. The recoveries of the analytes found after spiking to the blank bile were corrected for these values.

6.3 Analytical methods used for the certification

The analytical procedures used for the determination of the analyte contents in BCR-720 and 721 consisted of three or four distinct steps of analytical work:

1) sample preparation and conjugate hydrolysis;
2) extraction and derivatisation (not necessary for HPLC);
3) chromatographic separation partly by HPLC (9 labs) and partly by GC (4 labs);
4) calibration and detection.

For the GC analysis of PAH metabolites, three out of four labs used a derivatisation step before chromatography to improve the chromatographic separation, peak shape and to enhance the sensitivity of detection.

6.3.1 Sample preparation and conjugate hydrolysis

PAH metabolites in fish bile exist predominantly as conjugates (glucuronides or sulfates). Deconjugation is necessary for the following reasons: very few conjugate standards exist for calibration, and conjugates are not amenable to GC separation. Also, from an exposure monitoring point of view, the Phase-1 metabolite levels are considered more informative than the phase-2 conjugate levels. The analysis therefore starts with a deconjugation step; all participating labs used enzymatic hydrolysis with beta-glucuronidase/arylsulfatase rather than heat or chemical methods. The efficiency of the hydrolysis step had been tested and optimised in the feasibility study. It was found to be very efficient and not very sensitive to experimental conditions such as incubation time.

Table 5 - Sample preparation and analytical methods

Lab #	*Sample preparation*	*Extraction/derivatisation method*	*Analytical method*
1	Enzymatic hydrolysis Addition of ethanol; centrifugation	none	HPLC with fluorescence detection
2	Enzymatic hydrolysis Addition of ethanol; centrifugation	none	HPLC with fluorescence detection
3	Enzymatic hydrolysis Addition of ethanol; centrifugation	none	HPLC with fluorescence detection
4	Enzymatic hydrolysis Addition of methanol; centrifugation	none	HPLC with fluorescence detection
5	Enzymatic hydrolysis	Extraction, conversion to acetyl derivatives	GC-with MS detection
6	Enzymatic hydrolysis Addition of methanol; centrifugation	none	HPLC with fluorescence detection
7	Enzymatic hydrolysis Addition of methanol; centrifugation	none	HPLC with fluorescence detection
8	Enzymatic hydrolysis Addition of methanol; centrifugation	none	HPLC with fluorescence detection
9	Enzymatic hydrolysis in acetate buffer	Extraction with ethylacetate 4 times; BSTFA derivatisation	GC-with MS detection
10	Enzymatic hydrolysis in acetate buffer	Extraction with methylene chloride (twice). Derivatisation with BSTFA / 1 % TMCS	GC-with MS detection
11	Enzymatic hydrolysis	Extraction with ethyl acetate, no derivatisation	GC-with MS detection
12	Enzymatic hydrolysis Addition of ethanol; centrifugation	none	HPLC with fluorescence detection
13	Enzymatic hydrolysis Addition of ethanol; centrifugation	none	HPLC with fluorescence detection

Following hydrolysis, laboratories using HPLC would apply a simple centrifugation step after addition of ethanol or methanol to denature and precipitate the enzymes. The supernatant is

then directly injected in to the HPLC system. Some laboratories would mount a guard column prior to the actual separation column.

Laboratories applying GC would in most cases derivatise the phenolic compounds to improve separation and detection sensitivity. Lab 11 applied GC without derivatisation. The sample preparation conditions are listed in Table 5.

6.3.2 High-performance liquid chromatography

Reversed-phase HPLC was applied by nine laboratories for the determination of the PAH metabolites. All laboratories used acetonitrile gradients and fluorescence detection, in most cases with programmed wavelength detection optimised for each target analyte. The analytes were identified on the basis of retention time, the internal standard was used to monitor any instrument drifts in detector response or retention times. The identity of the peaks was also confirmed by standard addition in the recovery experiments. Quantification was based on peak area or peak height, using internal standards and external calibration as described in section 6.3. Details on separation conditions, detection, and internal standards used are given in Table 6.

Table 6 - HPLC-based separation conditions, detection and calibration

Lab #	*Separation*	*Detection*	*Calibration*
1	HPLC gradient acetonitrile/water with 1 mg/l ascorbic acid Xterrra RP18 with guard column	Fluorescence detection; wavelength programmed	External calibration, calibrants supplied by coordinator IS= anthracene-d_{10}
2	HPLC gradient acetonitrile/water	Fluorescence detection; wavelength programmed	External calibration, calibrants supplied by coordinator; diluted in ethanol/water 80:20 with ascorbic acid
3	HPLC gradient acetonitrile/water with 1 mg/l ascorbic acid RP silica C18	Fluorescence detection; wavelength programmed	External calibration, calibrants supplied by coordinator IS= perylene
4	HPLC gradient acetonitrile/water with RP18 PAH column	Fluorescence detection; wavelength programmed	External calibration, calibrants supplied by coordinator IS= triphenylamine
6	HPLC gradient acetonitrile/water	Fluorescence detection; wavelength programmed	External calibration, calibrants supplied by coordinator
7	HPLC gradient acetonitrile/water with ammonium acetate buffer	Fluorescence detection; fixed excitation/emission wavelength at 260/380 nm	External calibration, calibrants supplied by coordinator IS= anthracene
8	HPLC gradient acetonitrile/water with ammonium acetate buffer; Vydac 201TP52 column	Fluorescence detection; wavelength programmed	External calibration at 7 levels, calibrants supplied by coordinator IS= triphenylamine
12	HPLC gradient acetonitrile/water Column Nucleosil C18 + guard	Fluorescence detection; wavelength programmed	External calibration, calibrants supplied by coordinator
13	HPLC gradient acetonitrile/water; column RP silica C18	Fluorescence detection; wavelength programmed	External calibration, calibrants supplied by coordinator IS= anthracene-d_{10}

6.3.3 Gas chromatography-mass spectrometry (GC-MS)

Capillary gas chromatography was applied by four laboratories for the determination of the PAH metabolites. All analytes were identified on the basis of retention times relative to

standards, and ion masses in MS. The identity of the peaks was also confirmed by standard addition in the recovery experiments. Quantification was based on peak area or peak height of the selected quantitation ion(s), using internal standards and external calibration as described in sections 6.2 and 6.3. Details on separation conditions, detection, and internal standards used are given in Table 7.

Table 7 - GC-based separation conditions, detection and calibration

Lab #	*Separation*	*Detection*	*Calibration*
5	GC separation	MS detection	External calibration, using calibrants supplied by coordinator after acetylation IS= decachlorobiphenyl
9	GC separation; column CP-Sil-8 0.25 µm film	MS detection, identification based on 3 ions	External calibration using authentic calibrants (2-OH naph from Fluka, 1-OH phen from Promochem, 1-OH pyr from Acros and 3-OH BaP from Chiron) in ethylacetate at 10 levels, derivatised in same way. Surrogate std. dibromophenol IS = triphenylamine
10	GC separation; splitless, no retention gap Column: CP-Sil 5; 0.3 µm film	MS detection in selected ion monitoring mode (4 ions)	Used calibrants supplied by coordinator. Calibration based on quadratic regression of the quantitation ion relative to the surrogate std. 4-choro-1-hydroxy naphthalene
11	GC separation; splitless, column HP 5MS 0.25 µm film	MS detection	Calibration using calibrants supplied by coordinator IS = hexamethylbenzene

6.4 Determination of the recovery

The recoveries of the four target analytes during the complete analysis of the hydrolysed fish bile samples were determined on the basis of a spiking experiment at three levels. A mixed solution containing the four analytes at known concentrations was supplied with the candidate CRMs. According to a detailed protocol, participants were instructed to add 0, 5, 10 or 20 µl of the mixed solution to 10 µl of "blank" bile.

The recovery at each spiking level was measured in triplicate and corrected for the average (n = 3) metabolite level found in the blank bile (zero-spike). The recovery was then calculated as the average of the nine spiking experiments; the results are listed in Table 8. No recovery correction was applied in situations when the recoveries are automatically corrected for (e.g., in standard addition approaches), or when the recovery was so close to 100 % (within one standard deviation) that correcting would only add to the overall uncertainty. Numbers in bold refer to recovery percentages that were actually used to correct data accepted for certification.

Table 8 - Recovery percentages, averaged over 9 independent spiking experiments. Numbers in bold refer to recovery percentages that were actually used to correct accepted certification data.

Lab #	*2-OH naph* *average ± SD*	*1-OH phen* *average ± SD*	*1-OH pyr* *average ± SD*	*3-OH BaP* *AVERAGE ± SD*
1	n.r. [a]	n.r. [a]	n.r. [a]	n.r. [a]
2	84 ± 3 [a]	93 ± 3 [a]	99 ± 4 [c]	91 ± 6 [a]
3	89 ± 5 [a]	57 ± 4 [a]	**56 ± 6**	90 ± 13 [c]
4	80 ± 10 [a]	99 ± 11 [a]	**86 ± 13**	62 ± 4 [a]
5	81 ± 9 [a]	91 ± 8 [a]	**92 ± 6**	48 ± 4 [a]
6	111 ± 15 [a]	225 ± 28 [a]	149 ± 16 [a]	62 ± 26 [a]
7	105 ± 2 [a]	**82 ± 2**	**77 ± 2**	n.r. [a]
8	90 ± 6 [a]	67 ± 1 [a]	**94 ± 4**	**64 ± 13**
9	80 ± 9 [a]	**63 ± 6**	**63 ± 3**	n.r. [a]
10	66 ± 37 [a]	81 ± 17 [b]	78 ± 5 [b]	26 ± 9 [b]
11	106 ± 16 [a]	97 ± 37 [a]	121 ± 24 [c]	n.r. [a]
12	76 ± 12 [a]	107 ± 8 [a]	105 ± 13 [c]	105 ± 23 [c]
13	89 ± 10 [a]	**75 ± 6**	**77 ± 5**	**45 ± 5**

[a] For information only, data not retained for certification.

[b] Recovery automatically corrected for by calibration procedure

[c] Recovery not significantly different from 100 %; no correction applied

7. TECHNICAL DISCUSSION

The results of the certification study were discussed in an evaluation meeting of all participants on 6-7 December 2001 in Montpellier, France. The representative of Laboratory 5 could not attend the meeting, but was available for comments via telephone/fax and e-mail. All steps of the procedure were reviewed in detail. In particular all chromatograms were thoroughly checked regarding the peak identity and separation.

7.1 Certification results for BCR-720

7.1.1 2-OH naphthalene in BCR-720

2-OH naphthalene is a minor constituent in this sample, and only six laboratories submitted data for this compound. As a general remark it was noted that for small PAH metabolites such as 2-OH naphthalene the sensitivity of GC-MS is excellent, and due to its better selectivity GC-MS is often preferable over HPLC-Flu, especially in complex samples. Indeed, Laboratory 13 decided to withdraw their HPLC-fluorescence data because of indications of coelution. Laboratory 11 had observed a calibration problem for this compound and the data were withdrawn. Laboratory 10 remarked that the GC-MS system was less stable on the second day of measurement, which is why for this compound only three results were submitted. Laboratory 6 had reported results for 2-OH naphthalene, but during discussions on other compounds and from checking the chromatograms it became clear that all results in real samples were much too low. On the other hand, this laboratory had reported reasonable recovery numbers for most compounds. It was concluded that incomplete hydrolysis was most likely to blame for the low results in the real samples, and subsequently all certification results from laboratory 6 were withdrawn.

Data from three laboratories: 5, 9, and 10 (all using GC-MS) were accepted; the average concentration found was 46 ± 11 ng/g. Because of the limited number of independent data sets, the large uncertainty within two of the data sets, and the inability to demonstrate homogeneity and stability for this compound (see Section 5), certification was NOT recommended.

7.1.2 1-OH phenanthrene in BCR-720

Seven laboratories submitted results for 1-OH phenanthrene in BCR-720. The results of Laboratory 6 were withdrawn for the reasons mentioned earlier. Low recovery results and subsequent overcorrection could be the reason for the higher results found by Laboratory 3 and the data were withdrawn. The HPLC-Flu chromatograms of Laboratory 8 showed an interference with a coeluting compound, leading to an overestimation of the true value. The results were withdrawn, but most scientists present at the meeting agreed that careful integration of the chromatogram would have resulted in a concentration of ca. 200 ng/g, in agreement with the data submitted by the other laboratories.

The results from four laboratories: 7, 9, 10, and 13 were accepted; the resulting level of agreement is shown in the bar graph (see Annex 3). The data were obtained by HPLC-Flu (2x) and GC-MS (2x). Certification was recommended.

7.1.3 1-OH pyrene in BCR-720

1-OH pyrene is a major compound in most fish bile samples, and all laboratories were able to determine this compound in BCR-720. Thirteen data sets were submitted. The results of Laboratory 6 were withdrawn for the reasons mentioned above. Laboratory 1 remarked that

the measured 1-OH pyrene levels depended non-linearly on the HPLC injection volume, although a linear relationship was observed for the internal standard. No explanation could be given for this phenomenon, but since the samples and calibration solutions had been measured with different injection volumes the results were withdrawn. Due to instrumental problems at Laboratory 11 only four results were submitted.

The results from eleven laboratories: 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, and 13 were accepted; the resulting level of agreement is shown in the bar graph (see Annex 3). The data was obtained by HPLC-Flu (7x) and GC-MS (4x). Certification was recommended.

7.1.4 3-OH benzo[a]pyrene in BCR-720

3-OH BaP is a minor metabolite in most fish bile samples, and only six laboratories submitted certification data for this compound in BCR-720. Laboratory 10 remarked that their GC-MS system was less stable on the second day of measurement, which is why for this compound only three values were submitted. The results of Laboratory 6 were withdrawn for the reasons mentioned earlier.

Data from five laboratories: 3, 8, 10, 12, and 13 were accepted; the resulting level of agreement is shown in the bar graph (see Annex 3). The data were obtained by HPLC-Flu (4x) and GC-MS (1x). Certification was recommended.

7.2 Certification results for BCR-721

7.2.1 2-OH naphthalene in BCR-721

Although the 2-OH naphthalene concentration was higher in the oil-exposed fish bile than in BCR-720, many laboratories employing HPLC-Flu had difficulty determining this compound because of the crowdedness of the chromatograms and the limited selectivity of the fluorescence detector. Seven data sets were submitted. Laboratory 13 remarked that during later measurements on the same sample slightly better chromatographic conditions were used and it was found that instead of a major peak only a minor shoulder had the retention time of 2-OH naphthalene. The shoulder could not be integrated accurately and no results were submitted, but the estimated levels of ca. 500 ng/g confirmed the concentrations found by the GC-MS laboratories. Laboratory 12 reported very high levels and had not observed any sign of coelution, but based on the evidence provided by Laboratory 13 and the much lower GC-MS data it was concluded that also in this case coelution may have caused major interference and the results were withdrawn. The results of Laboratory 6 were withdrawn for the reasons mentioned earlier. Laboratory 10 remarked that the GC-MS system was less stable on the second day of measurement, which is why for this compound only three results were submitted. Only four reliable chromatograms were obtained by Laboratory 11.

Data from four laboratories (5, 9, 10, and 11, all using GC-MS) were accepted; the average concentration found was 420 ± 170 ng/g. Because of the limited number of independent data sets, some discrepancy between the data submitted by Laboratory 11 and the other GC-MS laboratories, and the inability to demonstrate homogeneity and stability for this compound (see Section 5), certification was NOT recommended.

7.2.2 1-OH phenanthrene in BCR-721

Seven laboratories submitted results for 1-OH phenanthrene in BCR-721. The results of Laboratory 6 were withdrawn for the reasons mentioned above. Laboratory 2 found the chromatograms difficult to integrate due to coelution and the results were withdrawn. There was poor overlap between the data sets, with results ranging from 303 to 2049 ng/g. Coelution

may have been the cause of the relatively high levels found by Laboratories 13 and 3. However, there was also no agreement between the GC-MS results from laboratories 9 and 10. The statistical model of Cofino *et al.* [9] revealed a multi-mode structure of the data sets. In conclusion, certification was NOT recommended and at this point an indicative value cannot be given.

7.2.3 1-OH pyrene in BCR-721

1-OH pyrene is a major compound in bile samples, and most laboratories were able to determine this compound in BCR-721. Twelve data sets were submitted. Laboratory 1 remarked that the 1-OH pyrene calibration curve was not straight, and that especially at low levels the use of a calibration line through zero may have caused a significant error. The results were withdrawn. The results of Laboratory 6 were withdrawn for the reasons mentioned above. The chromatograms of Laboratory 7 (using HPLC-fluorescence with less selective short-wavelength excitation) showed interference from a coeluting peak which hampered integration; the results were withdrawn.

The results from nine laboratories: 2, 3, 4, 5, 8, 9, 10, 12 and 13 were accepted; the resulting level of agreement is shown in the bar graph (see Annex 3). The data were obtained by HPLC-Flu (6x) and GC-MS (3x). Certification was recommended.

7.2.4 3-OH benzo[a]pyrene in BCR-721

Since BaP is a very minor component in crude oil, the 3-OH BaP levels were expected to be even lower than the levels in BCR-720. Only two laboratories submitted data for 3-OH BaP in this sample. Laboratory 10 remarked that the levels observed were not very different from the blank levels and were probably the result of an artefact; the results were withdrawn. The results of Laboratory 6 were also withdrawn, for the reasons mentioned above for 1-OH phenanthrene in BCR-720. It was concluded that the 3-OH BaP level in BCR-721 is below the detection limit of the employed methods (typically 2-10 ng/g).

7.3 Conclusion of technical discussion

To summarise the discussions, it was decided to recommend certification of 1-OH phenanthrene, 1-OH pyrene, and 3-OH benzo[*a*]pyrene in BCR-720 and 1-OH pyrene in BCR-721. The experts agreed that the obtained overall uncertainties reflect the current state of the art for PAH metabolite determination in fish bile samples. 2-OH naphthalene would not be certified in either CRM (mainly as a result of lacking homogeneity and stability data), but the results obtained are discussed in Sections 7.1 and 7.2. Lack of agreement between the various data sets precluded the certification of 1-OH phenanthrene in BCR-721. The concentration of 3-OH BaP in BCR-721 was below the detection limit.

8. STATISTICAL EVALUATION, CERTIFIED VALUES AND UNCERTAINTIES

8.1 General

The certification of the selected PAH metabolites in BCR-720 and BCR-721 has been performed according to the recommendations of ISO Guide 35 [13] and in particular to its sections 8: Certification by interlaboratory testing and 9.3: Certification of a chemical composition and according to the recommendations of the BCR Guidelines [12] Part B, section 8: The Certification measurements.

The determination of PAH metabolites in fish bile requires the use of complex analytical procedures. Different analytical techniques have been used, to randomise as much as possible systematic effects of the methods and to include them into the scattering observed in the laboratory means. The agreement among these methods, together with a careful control over the performance of these methods, is the basis for this certification. As there are substantial between-laboratory effects, no pooling of data has taken place.

The final statistical treatment of measurement data was carried out after the technical assessment as described in section 7. The accepted data sets were submitted to the following statistical tests and calculations. All statistical tests were performed at the 5 % as well as at the 1 % significance level:

- the average (the unweighted arithmetic mean value), the standard deviation and the 95 % confidence interval of the average of each data set;
- the multiple t-test according to Scheffe to check whether any two sets of data could be considered as samples from the same population of data;
- the Nalimov t-test, Dixon test and Grubbs test to identify outlying data set averages;
- the Cochran test to identify outlying variances;
- the within-laboratory standard deviation and the between-laboratory standard deviation (ANOVA approach)
- the Snedecor F-test to check if the between-laboratory variance is significantly different from zero
- the Bartlett test to assess the overall consistency of the variances obtained by participating laboratories
- Kolgomorov-Smirnov-Lillefors tests to assess the conformity of the distributions of laboratory means to normal distributions
- Skewness-Kurtosis test to asses the normality of the laboratory means.
- the average of laboratory averages, its half-width of the 95 % confidence interval and of the 95 % tolerance interval.

A summary of the results of the statistical calculations and tests is given in Table 9. The individual data accepted after technical and statistical evaluation are presented in Annex 3.

8.2 Detailed statistical discussion

All data sets that were accepted for certification contained one or more pairs of data sets that were not compatible two by two according to Scheffe's multiple t-test at the 5 % significance level. These data sets were not rejected because there were no technically explainable reasons for the deviations observed.

The Dixon test did not detect any outlying data sets for any compound. Nalimov's t-test detected a single outlying data set (Lab 4 for 1-OH pyrene in BCR-721) at the 5 % significance level. The Grubbs test detected outlying highest and lowest data sets for 1-OH pyrene in BCR-720. The technical evaluation of these data sets did not detect any reasons to reject these measurement data.

Table 9 – Summary of statistical results

CERTIFIED PROPERTY	*1-OH phen BCR-720*	*1-OH pyr BCR-720*	*3-OH BaP BCR-720*	*1-OH pyr BCR-721*
Number of data sets (laboratories)	4	11	5	9
Number of individual data	24	64	27	54
All data sets compatible two by two (Scheffe's multiple t-test)	no	no	no	no
Outlying data sets (Dixon test, Nalimov t-test and Grubbs test)	no, no, no	no, no, yes	no, no, no	no,yes,no
Outlying variances? (Cochran test)	yes	yes	no	no
Average of data set averages [ng/g]	206	52354	63	2135
ANOVA: within-data sets SD [ng/g]	38	4381	10	213
ANOVA: between-data sets SD [ng/g]	53	8383	21	326
Between-data sets st.dev. significantly different from zero? (Snedecor F-test)	yes	yes	yes	yes
Variances homogeneous? (Bartlett test)	no	no	yes	no
St.dev. of data set averages [ng/g]	55	8488	21	338
Data set averages normally distributed? (Kolmogorov-Smirnov-Lilliefors test	yes	yes	yes	yes
Normality of data set means? (Skewness-Kurtosis test)	< 7 labs	normal	< 7 labs	normal
Half-width of the 95 % confidence int. of the average of averages [ng/g]	87	5702	26	260
Half-width of the 95 % tolerance int. of the average of averages [ng/g]	349	27661	106	1193

Outlying variances according to the Cochran test were detected in two cases: Lab 7 for 1-OH phenanthrene in BCR-720 (only at the 5 % significance level) and Lab 5 for 1-OH pyrene in BCR-720 (both at the 5 % and 1 % level). No technical reasons were found to reject these data sets.

According to the Snedecor F-test the between-laboratories standard deviation was detected to be significantly different from zero in the case of all analytes at the 5 % significance level. Before this, it was demonstrated that the between-bottles standard deviation is not significantly different from zero at this level (see section 5.2). This demonstrates that randomised individual bias of the different analytical procedures applied for certification, significantly contributes to the uncertainty of the certified value. It should be mentioned that significant differences were in some cases also observed between laboratories applying (practically) identical methods. These differences are therefore most probably not fundamentally related to the analytical method, but rather the result of random errors such as calibration errors or degradation of standards that affect the complete data set of a given laboratory in a systematic way.

Consistence of variances (Bartlett test) was found only in the case of 3-OH BaP in BCR-720. The variances of the data sets of 1-OH phenanthrene in BCR-720 and of 1-OH pyrene in both CRMs were found to be not homogeneous. These results do not directly influence the certified values (no pooling of individual measurement data was applied).

The laboratory averages accepted after the technical discussion formed the basis for the calculation of the certified values. The Kolgomorov-Smirnov-Lillefors test demonstrated the normal distribution of the data set averages for each of the four certified analytes. No skewness in the data set averages was observed for 1-OH pyrene in either CRM (Skewness-Kurtosis test). This test was not applied to 1-OH phenanthrene and 3-OH BaP because of the small number of data sets.

8.3 Certified values and uncertainties

The evaluation of the certified uncertainties described hereafter is based on the GUM-compliant [14] concept described by Pauwels *et al.* [15 and literature cited] and uses available data discussed in the previous chapters.

8.3.1 Conceptual considerations

Based on the findings obtained in the stability and homogeneity studies as well as the scattering of results in the batch characterisation estimates for u_{bb} (homogeneity), u_{lts} (long-term-stability) and u_{char} (batch characterisation) were obtained and combined according the following equation:

$$U_{CRM} = 2 \cdot \sqrt{u_{bb}^2 + u_{lts}^2 + u_{char}^2}$$

Due to the selected transport conditions used for dispatch, the uncertainty constituent for short-term stability (u_{sts}) is negligible and consequently not included in the overall uncertainty. The estimation of the other uncertainty sources is described below.

8.3.2 Uncertainty source "homogeneity"

The homogeneity study described in section 5.1 is based in an one-way ANOVA. From these data, an estimation of u_{bb} was derived as described by Linsinger *et al* [16].

According to this approach, s_{bb} (being the standard deviation between units) or u^*_{bb} (being the upper limit of inhomogeneity that can be hidden by the method repeatability) are used as estimates of u_{bb}. Values for s_{bb} and u^*_{bb} were calculated accordingly:

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}}$$

and

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} \cdot \sqrt[4]{\frac{2}{\nu_{MSwithin}}},$$

where n is the number of replicates per unit, MS_{within} and $MS_{between}$ the respective mean-of-squares from the ANOVA and $\nu_{MSwithin}$ the degrees of freedom of MS_{within}. If the value of s_{bb} is below the minimum value as determined by the repeatability of the method and the number of replicates performed, u^*_{bb} is used to estimate u_{bb}. The results of these calculations are shown in Table 10 and 15, respectively.

8.3.3 Uncertainty source "stability"

Similarly, a quantitative estimate of the uncertainty related to the long-term stability of the CRM, u_{lts}, was obtained plotting the results of the stability study (isochronous measurement scheme) shelf-life as described elsewhere [16]. The uncertainty was estimated for a shelf-life of 24 months. The derived expiry dates (see certificates) can be prolonged, if further stability data are obtained, e.g. by additional isochronous experiments. The obtained shelf-life plots and the derived values of u_{lts} for the compounds being certified are shown in the figures below.

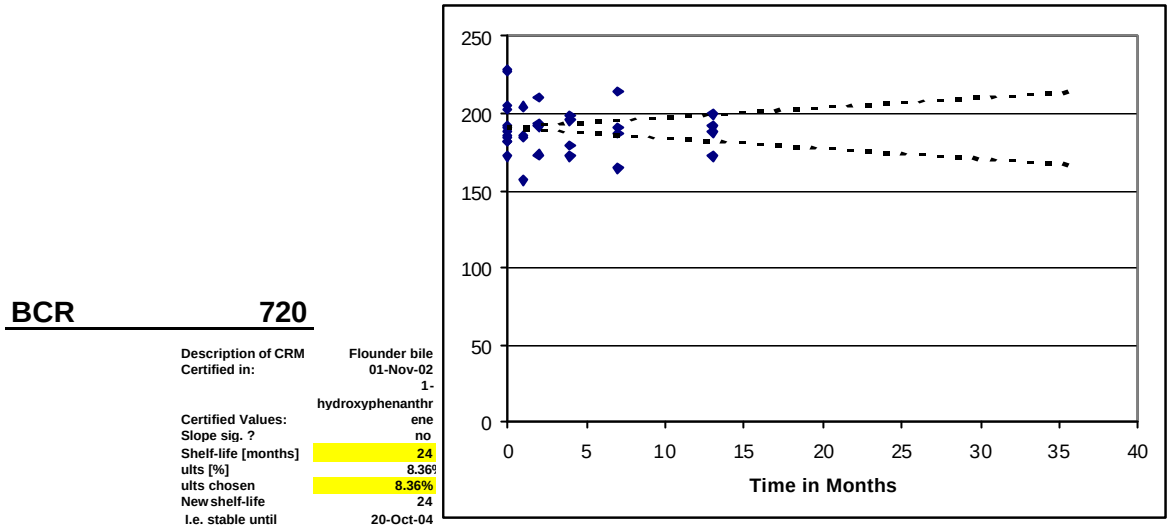

Figure 7 - Shelf-life plot for BCR-720, 1-hydroxyphenanthrene

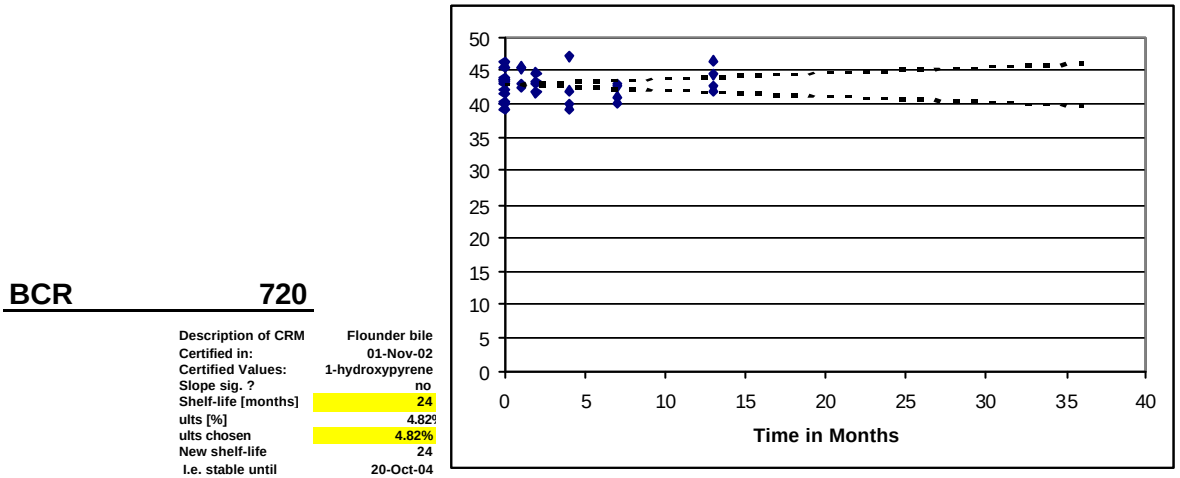

Figure 8 - Shelf-life plot for BCR-720, 1-hydroxypyrene

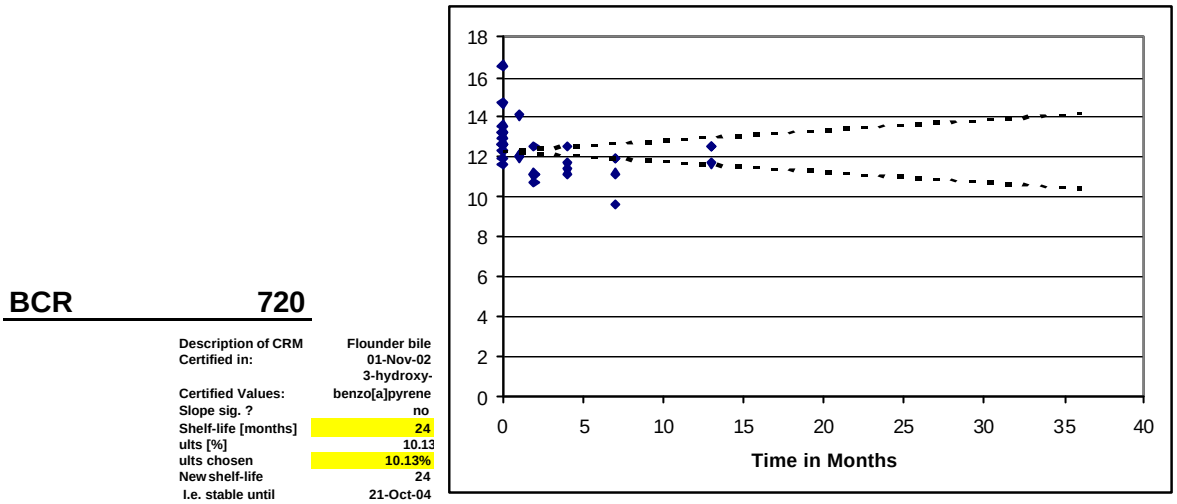

Figure 9 - Shelf-life plot for BCR-720, 3-hydroxybenzo[a]pyrene

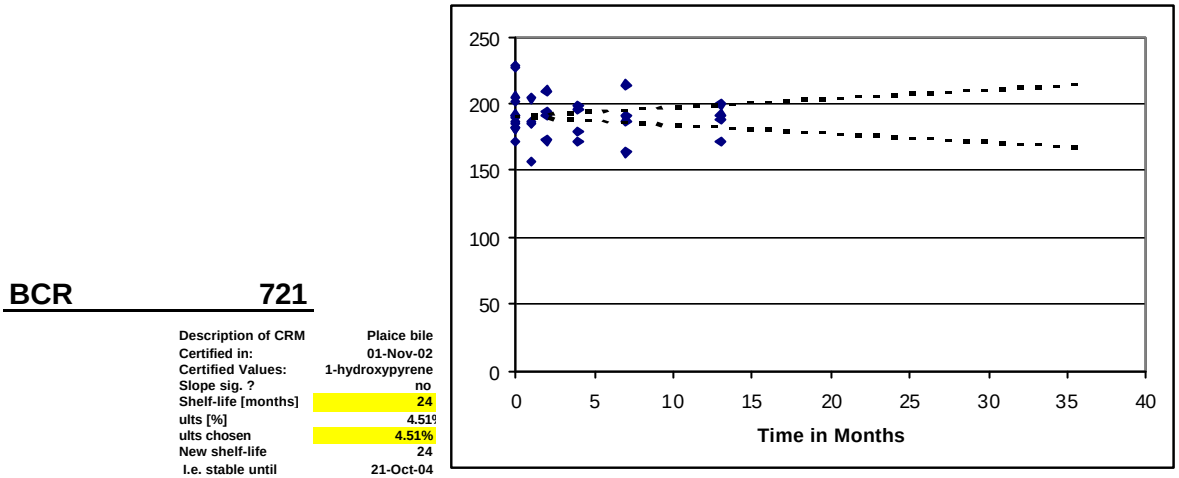

Figure 10 - Shelf-life plot for BCR-721, 1-hydroxypyrene

8.3.4 Uncertainty source "batch characterisation"

An estimate for u_{char} was derived from the standard error obtained on the mean of laboratories means.

8.3.5 Uncertainty budget

Based on these uncertainty contributions the following uncertainty budget is established:

Table 10 - Uncertainty budget for BCR-720 and -721

	BCR-720			***BCR-721***
	1-OH phen	*1-OH pyr*	*3-OH BaP*	*1-OH pyr*
u_{bb} (s_{bb}) *[in rel. %]*	*/*	3.61	9.02	*2.55*
u^*_{bb} *[in rel. %]*	3.14	2.00	3.77	*1.76*
u_{lts} *[in rel. %]*	8.36	4.82	10.13	*4.51*
u_{char} *[in rel. %]*	13.35	4.89	14.91	*5.28*
coverage factor k	2	2	2	*2*
U_{CRM} *[in rel. %]*	32.12	15.51	40.31	*14.79*

	BCR-720			*BCR-721*
	1-OH phen	*1-OH pyr*	*3-OH BaP*	*1-OH pyr*
Mean [in µg/kg wet wt.]	**206**	**52354**	**63**	**2135**
Uncertainty [in µg/kg wet wt.]	**67**	**8123**	**26**	**316**
Rounded according to ISO 31-0 [17] and expressed in [in µg/kg wet wt.]	***210 ± 70***	***52000 ± 9000***	***63 ± 26***	***2100 ± 400***

8.4 Certified values

The certified mass concentration of 1-hydroxyphenanthrene (wet mass) for BCR-720 is:

0.21 ± 0.07 mg/kg wet mass basis

The certified mass concentration of 1-hydroxypyrene (wet mass) for BCR-720 is:

52 ± 9 mg/kg wet mass basis

The certified mass concentration of 3-hydroxybenzo[a]pyrene (wet mass) for BCR-720 is:

0.063 ± 0.026 mg/kg wet mass basis

The certified mass concentration of 1-hydroxypyrene (wet mass) for BCR-721 is:

2.1 ± 0.4 mg/kg wet mass basis

According to the recommendations of the BCR Guidelines [12], part B, section 9.3.1, the certified values of the PAH metabolites were calculated as the average of the accepted data set averages and are listed in Table 10 after rounding off the non-significant digits. All analyte contents are given for the hydrolysed species and are corrected for recovery.

Uncertainty statements are expanded uncertainty according to the GUM [14] with a covergae factor of 2.

2-OH naphthalene could not be certified; for details and levels found see Section 7.1 and 7.2

9. INSTRUCTIONS FOR USE

The following notes are a guide for the proper use of reference materials BCR-720 and 721 as analytical quality control tools for the determination of PAH metabolites in fish bile. Please consult these notes prior to opening the ampoules.

9.1 Instructions for handling of the sample

For long-term stability the materials should be kept sealed in the amber glass ampoules and stored in the freezer (-20 ^{o}C or colder) until use. Although the bile samples were found to be stable for several months at room temperature, prolonged heating at +40 °C should be avoided.

Prior to its first use, the ampoule should be thawed and briefly homogenised in an ultrasonic bath. After opening the prescored ampoules the remaining bile liquid should be transferred to a screw-capped glass vial and stored in the freezer. The vials should be closed tight to avoid a change in water content due to freeze-drying. Before each use the vial should be thawed and sonicated, since a phase separation (pure ice/concentrated bile liquid) may have occurred during freezing.

Fish bile contains a multitude of organic degradation products and careful handling of the material is recommended. Stains from spilled bile may be difficult to remove from clothing. The PAH metabolites in the bile samples are endpoints of the fishes' detoxification mechanism and are not known to constitute a carcinogenic risk to humans.

9.2 Guidelines for the use of the CRM in quality control

In this study, the homogeneity of BCR-720 and 721 was demonstrated at a sample intake of 50 µl. Sample preparation should be carried out immediately before analysis, since after hydrolysis the deconjugated PAH metabolites will be less stable than the conjugated PAH metabolites in the untreated bile sample.

9.3 Calibration

BCR-720 and 721 are **not** intended for calibration purposes. Calibration of equipment shall be carried out using a series of solutions prepared from pure substances. Users are reminded that PAH metabolites were shown to be sensitive to oxidation in clean solutions; therefore calibration solutions should be prepared fresh and addition of a suitable antioxidant is recommended.

More details on the use of this CRM for the verification or validation of an analytical procedure or the performance of a method are given in ISO Guide 33 [18].

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11. ANNEX 1 – HOMOGENEITY TESTS (RAW DATA)

Table 11 - Homogeneity test BCR-720; raw data. 15 ampoules were analysed in duplicate; all concentrations in ng/g without recovery correction

Ampoule #		*1-OH Phenanthrene*		*1-OH Pyrene*		*3-OH B[a]P*	
3-1	3-2	226	212	39401	38634	46	42
019-1	019-2	228	217	39798	41570	56	53
047-1	047-2	189	232	36393	41831	40	43
98-1	98-2	224	219	41891	39592	50	49
170-1	170-2	193	235	41807	43026	41	46
198-1	198-2	231	233	36394	34681	43	46
260-1	260-2	223	219	37355	35230	45	42
390-1	390-2	202	229	36885	36783	44	58
549-1	549-2	203	220	40249	38411	44	50
645-1	645-2	229	233	40947	34791	42	46
838-1	838-2	183	224	39196	41190	39	48
971-1	971-2	222	209	36917	38665	39	37
1003-1	1003-2	208	224	38845	39580	38	36
1104-1	1104-2	208	234	37061	36114	41	48
1202-1	1202-2	234	229	39094	37960	37	37

Table 12 - Homogeneity test BCR-721; raw data. 15 ampoules were analysed in duplicate; all concentrations in ng/g without recovery correction

Ampoule #		*1-OH Phenanthrene*		*1-OH Pyrene*	
4-1	4-2	994	1003	1591	1581
15-1	15-2	888	960	1397	1490
31-1	31-2	977	942	1547	1532
100-1	100-2	935	946	1487	1527
215-1	215-2	939	980	1512	1556
324-1	324-2	941	879	1574	1460
395-1	395-2	967	1035	1551	1693
612-1	612-2	987	986	1609	1635
781-1	781-2	852	893	1422	1484
854-1	854-2	932	959	1531	1572
891-1	891-2	927	900	1576	1487
997-1	997-2	855	986	1435	1601
1073-1	1073-2	950	957	1548	1600
1286-1	1286-2	850	950	1396	1563
1320-1	1320-2	1029	988	1674	1609

12. ANNEX 2 – STABILITY TESTS (RAW DATA)

12.1 Stability test data for BCR-720

12.1.1 Raw data of 1-hydroxyphenanthrene

Table 13 - Stability of 1-OH phenanthrene in BCR-720 – Concentration (ng/g) in reference samples stored at -70 °C

Samples	*Concentration*	*Samples*	*Concentration*
1	202	7	182
2	228	8	192
3	205	9	185
4	229	10	172
5	182	11	189
6	191	12	186

Table 14 - Stability of 1-OH phenanthrene in BCR-720 – Concentration (ng/g) vs storage time in months at –20 °C

Samples	*1*	*2*	*4*	*7*	*13*
1	186	210	172	187	188
2	157	194	196	164	192
3	185	192	179	214	172
4	204	173	198	191	199

Table 15 - Stability of 1-OH phenanthrene in BCR-720 – Concentration (ng/g) vs storage time in months at +4 °C

samples	*1*	*2*	*4*	*7*	*13*
1	200	202	192	205	181
2	184	206	206	204	190
3	193	188	197	203	199
4	190	179	211	213	188

Table 16 - Stability of 1-OH phenanthrene in BCR-720 – Concentration (ng/g) vs storage time in months at +20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	208	218	195	197	194
2	193	192	218	207	218
3	175	207	195	176	201
4	178	203	196	212	213

Table 17 - Stability of 1-OH phenanthrene in BCR-720 – - Concentration (ng/g) vs storage time in months at +40 °C

samples	*1*	*2*
1	223	200
2	236	246
3	187	211
4	223	223

12.1.2 Raw data of 1-hydroxypyrene

Table 18 - Stability of 1-OH pyrene in BCR-720 - Concentration (µg/g) in reference samples stored at -70 °C

Samples	*Concentration*	*Samples*	*Concentration*
1	46.3	7	43.1
2	40.1	8	43.4
3	43.2	9	40.4
4	45.5	10	39.3
5	42.2	11	43.4
6	43.9	12	41.4

Table 19 - Stability of 1-OH pyrene in BCR-720 - Concentration (µg/g) versus storage time in months at -20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	42.3	44.7	42.0	43.0	41.9
2	45.2	41.8	39.2	42.6	46.5
3	43.0	43.2	47.0	40.1	42.7
4	45.6	43.1	39.8	41.1	44.5

Table 20 - Stability of 1-OH pyrene in BCR-720 - Concentration (µg/g) versus storage time in months at +4 °C

samples	*1*	*2*	*4*	*7*	*13*
1	47.6	47.0	41.6	41.5	43.1
2	42.5	41.6	42.1	44.9	46.4
3	45.6	41.4	43.4	42.1	46.7
4	47.0	37.1	44.8	44.1	47.0

Table 21 - Stability of 1-OH pyrene in BCR-720 – Concentration (µg/g) versus storage time in months at +20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	39.9		44.7	43.5	50.0
2	41.6		44.5	47.7	44.1
3	37.3		41.1	44.7	41.0
4	50.8		44.7	45.7	42.8

Table 22 - Stability of 1-OH pyrene in BCR-720 - Concentration (µg/g) versus storage time in months at +40 °C

samples	*1*
1	47.1
2	50.0
3	49.1
4	49.5

12.1.3 Raw data of 3-hydroxybenzo[a]pyrene

Table 23 - Stability of 3-OH benzo[a]pyrene in BCR-720 - Concentration (ng/g) in reference samples stored at -70 °C

Samples	*Concentration*	*Samples*	*Concentration*
1	13.2	7	11.6
2	14.7	8	11.9
3	16.6	9	13.6
4	13.0	10	12.7
5	13.3	11	12.6
6	12.3	12	12.7

Table 24 - Stability of 3-OH benzo[a]pyrene in BCR-720 - Concentration (ng/g) vs storage time in months at –20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	14.1	12.5	12.5	11.9	12.5
2	12.1	10.7	11.4	9.6	12.5
3	12.1	11.2	11.7	11.9	12.5
4	12.0	11.1	11.2	11.2	11.7

Table 25 - Stability of 3-OH benzo[a]pyrene in BCR-720 - Concentration (ng/g) vs storage time in months at +4 °C

samples	*1*	*2*	*4*	*7*	*13*
1	13.0	14.0	11.3	12.3	12.3
2	11.9	13.0	12.1	11.6	11.6
3	13.7	11.9	11.3	13.7	13.7
4	12.7	11.0	12.0	12.7	12.7

Table 26 - Stability of 3-OH benzo[a]pyrene in BCR-720 - Concentration (ng/g) vs storage time in months at +20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	12.2	12.8	14.0	13.3	11.9
2	11.3	11.5	13.0	12.7	13.2
3	15.3	15.2	15.3	11.1	12.5
4	11.9	9.5	12.3	12.9	11.2

Table 27 - Stability of 3-OH benzo[a]pyrene in BCR-720 - Concentration (ng/g) vs storage time in months at +40 °C

samples	*1*	*2*
1	15.4	13.4
2	14.5	12.6
3	12.6	15.7
4	14.3	15.1

12.2 Stability test data for BCR-721

12.2.1 Raw data of 1-hydroxyphenanthrene

Table 28 - Stability of 1-OH phenanthrene in BCR-721 - Concentration (µg/g) in reference samples stored at -70 °C

Samples	*Concentration*	*Samples*	*Concentration*
1	0.93	7	0.95
2	0.96	8	0.96
3	0.93	9	0.85
4	0.90	10	0.95
5	0.86	11	1.03
6	0.99	12	0.99

Table 29 - Stability of 1-OH phenanthrene in BCR-721 - Concentration (µg/g) versus storage time in months at -20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	0.92	0.96	1.00	0.97	0.91
2	1.00	0.96	0.94	0.91	0.91
3	1.04	0.97	0.98	1.01	0.91
4	0.97	0.95	0.99	0.99	0.88

Table 30 - Stability of 1-OH phenanthrene in BCR-721 - Concentration (µg/g) versus storage time in months at +4 °C

samples	*1*	*2*	*4*	*7*	*13*
1	0.99	1.01	0.96	1.05	0.93
2	0.96	1.01	1.13	1.00	0.99
3	1.00	1.03	1.01	1.08	0.98
4	1.06	0.98	1.02	0.96	1.02

Table 31 - Stability of 1-OH phenanthrene in BCR-721 - Concentration (µg/g) versus storage time in months at +20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	0.97	1.11	1.05	0.99	1.00
2	1.04	1.04	1.08	1.00	1.00
3	1.02	1.05	0.98	1.02	0.98
4	1.02	1.01	1.00	1.07	1.06

Table 32 - Stability of 1-OH phenanthrene in BCR-721 - Concentration (µg/g) versus storage time in months at +40 °C

samples	*1*	*2*
1	1.11	1.05
2	1.11	1.13
3	1.10	1.14
4	1.01	1.12

12.2.2 Raw data of 1-hydroxypyrene

Table 33 - Stability of 1-OH pyrene in BCR-721 – Conc. (µg/g) in reference samples stored at -70 °C

Samples	*Concentration*	*Samples*	*Concentration*
1	1.53	7	1.55
2	1.57	8	1.60
3	1.58	9	1.40
4	1.49	10	1.56
5	1.43	11	1.67
6	1.60	12	1.61

Table 34 - Stability of 1-OH pyrene in BCR-721 – Conc. (µg/g) vs. storage time in months at -20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	1.59	1.57	1.67	1.56	1.50
2	1.58	1.55	1.52	1.49	1.48
3	1.67	1.58	1.61	1.66	1.44
4	1.59	1.54	1.55	1.61	1.43

Table 35 - Stability of 1-OH pyrene in BCR-721 – Conc. (µg/g) vs. storage time in months at +4 °C

samples	*1*	*2*	*4*	*7*	*13*
1	1.60	1.67	1.55	1.62	1.48
2	1.57	1.63	1.54	1.58	1.54
3	1.56	1.63	1.79	1.60	1.55
4	1.64	1.63	1.57	1.65	1.63

Table 36 - Stability of 1-OH pyrene in BCR-721 – Conc. (µg/g) vs. storage time in months at +20 °C

samples	*1*	*2*	*4*	*7*	*13*
1	1.55	1.75	1.66	1.60	1.59
2	1.63	1.67	1.69	1.61	1.54
3	1.70	1.65	1.60	1.60	1.51
4	1.63	1.55	1.62	1.66	1.61

Table 37 - Stability of 1-OH pyrene in BCR-721 – Conc. (µg/g) vs storage time in months at +40 C

samples	*1*	*2*
1	1.72	1.54
2	1.69	1.66
3	1.72	1.72
4	1.58	1.66

13. ANNEX 3 – TABLES OF INDIVIDUAL CERTIFICATION RESULTS AND GRAPHICAL PRESENTATIONS

Tables

The laboratory code is followed by an indication of the analytical method used (for details see Tables 5, 6A and 6B)

Figures

The length of the horizontal bar corresponds to the 95 % confidence interval. The vertical dotted line shows the certified value (average of laboratory averages).

Table 38 - Summary Table for 1-OH phenanthrene in BCR-720

Lab code & method	*Mean*	*SD*	*HW CI 95 %*	*Sample #1*	*Sample #2*	*Sample #3*	*Sample #4*	*Sample #5*	*Sample #6*
07 LC-FLU	197	59	62	146	244	183	122	207	280
09 GC-MS	233	38	40	266	283	249	209	196	193
10 GC-MS	133	19	20	122	115	112	146	152	151
13 LC-FLU	260	19	20	276	237	254	265	285	242
			Range [min..max]						[112 .. 285]
			Mean of means (no pooling)						206
			95 % H.W. Confidence Interval						87
			95 % H.W. Tolerance Interval						349

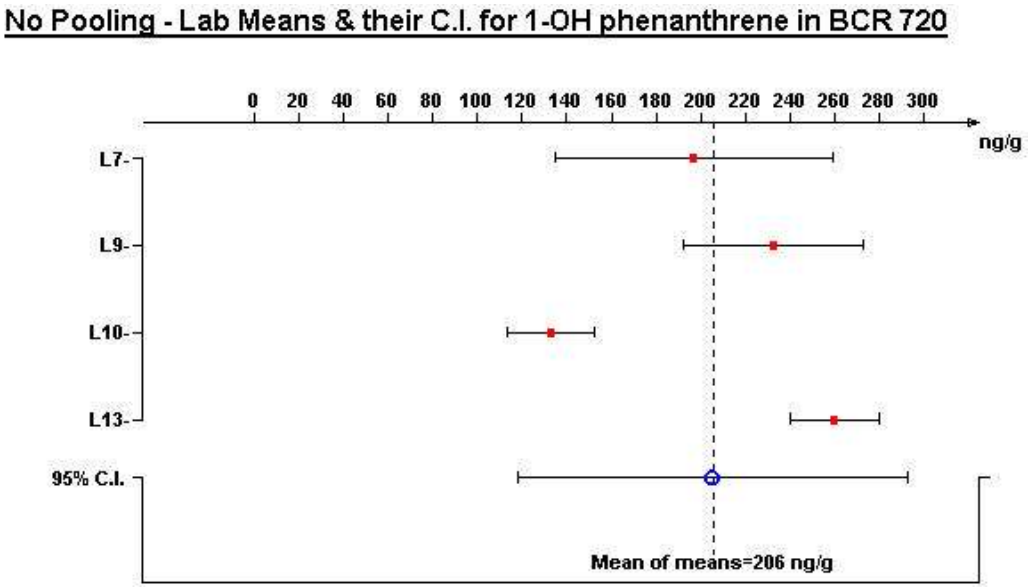

Figure 11 – Graphical presentation of certification data for BCR-720 – 1-hydroxy phenanthrene

Table 39 - Summary Table 1-OH pyrene in BCR-720

Lab code & method	*Mean*	*SD*	*HW CI 95 %*	*Sample #1*	*Sample #2*	*Sample #3*	*Sample #4*	*Sample #5*	*Sample #6*
02 LC-FLU	41,656	1,375	1,443	41,536	40,655	40,767	40,675	44,205	42,095
03 LC-FLU	62,672	4,001	4,198	67,442	66,096	65,188	58,384	59,776	59,145
04 LC-FLU	54,647	2,255	2,367	51,808	55,346	54,045	57,831	56,197	52,652
05 GC-MS	51,950	8,743	9,175	62,959	43,307	40,553	51,074	55,093	58,715
07 LC-FLU	46,482	3,657	3,837	49,820	42,480	46,100	51,940	44,370	44,180
08 LC-FLU	51,870	4,370	4,586	55,997	54,970	56,139	46,110	48,302	49,704
09 GC-MS	67,110	3,941	4,136	70,496	64,966	73,520	63,952	65,327	64,398
10 GC-MS	40,833	907	952	41,700	40,700	40,900	39,300	40,600	41,800
11 GC-MS	57,047	6,259	9,959	48,266	56,841	61,540	61,540		
12 LC-FLU	44,406	3,165	3,322	46,945	48,025	39,116	43,689	43,319	45,343
13 LC-FLU	57,222	4,656	4,886	61,344	49,245	58,452	59,006	61,011	54,273
			Range [min..max]						[39,116 .. 73,520]
			Mean of means (no pooling)						52,354
			95 % H.W. Confidence Interval						5,702
			95 % H.W. Tolerance Interval						27,661

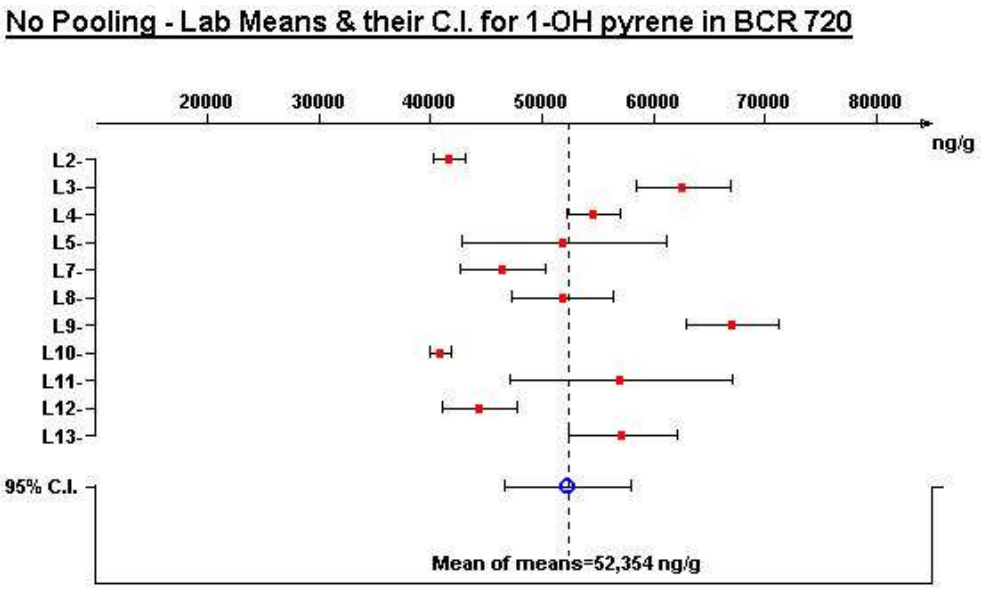

Figure 12 - Graphical presentation of certification data for BCR-720 – 1-hydroxy pyrene

Table 40 – Summary Table 3-OH BaP in BCR-720

Lab code & method	*Mean*	*SD*	*HW CI 95 %*	*Sample #1*	*Sample #2*	*Sample #3*	*Sample #4*	*Sample #5*	*Sample #6*
03 LC-FLU	85	10	11	95	86	94	91	74	72
08 LC-FLU	55	15	16	63	68	73	47	43	36
10 GC-MS	51	4	10	47	55	50			
12 LC-FLU	84	8	9	87	97	75	75	84	83
13 LC-FLU	38	6	6	37	34	31	46	45	37
			Range [min..max]						[31 .. 97]
			Mean of means (no pooling)						63
			95 % H.W. Confidence Interval						26
			95 % H.W. Tolerance Interval						106

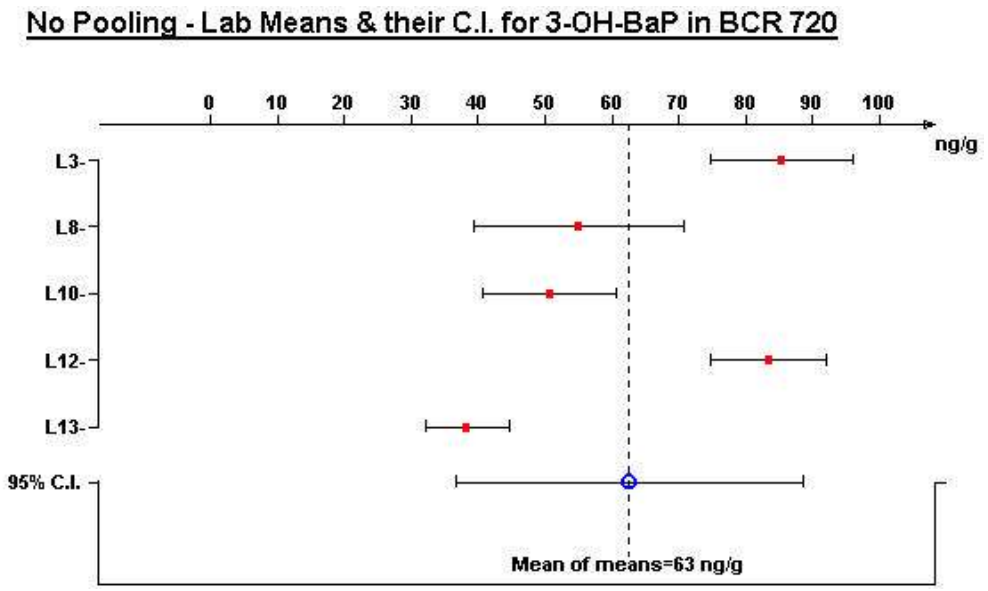

Figure 13 - Graphical presentation of certification data for BCR-720 – 3-hydroxy benzo[a]pyrene

Table 41 - Summary Table 1-OH pyrene in BCR-721

Lab code & method	*Mean*	*SD*	*HW CI 95 %*	*Sample #1*	*Sample #2*	*Sample #3*	*Sample #4*	*Sample #5*	*Sample #6*
02 LC-FLU	1,913	102	107	1,990	2,030	1,975	1,805	1,890	1,785
03 LC-FLU	1,625	181	190	1,855	1,323	1,586	1,756	1,642	1,589
04 LC-FLU	2,763	264	277	2,606	2,806	2,527	3,188	2,929	2,520
05 GC-MS	2,270	364	381	2,857	1,912	1,852	2,347	2,303	2,349
08 LC-FLU	2,031	295	309	2,306	2,349	2,237	1,800	1,753	1,741
09 GC-MS	2,442	156	163	2,430	2,554	2,137	2,541	2,509	2,478
10 GC-MS	2,095	194	204	1,950	1,890	1,940	2,160	2,300	2,330
12 LC-FLU	2,218	107	112	2,251	2,185	2,286	2,268	2,302	2,017
13 LC-FLU	1,855	50	52	1,831	1,899	1,765	1,876	1,878	1,882
			Range [min..max]						[1,323 .. 3,188]
			Mean of means (no pooling)						2,135
			95 % H.W. Confidence Interval						260
			95 % H.W. Tolerance Interval						1,193

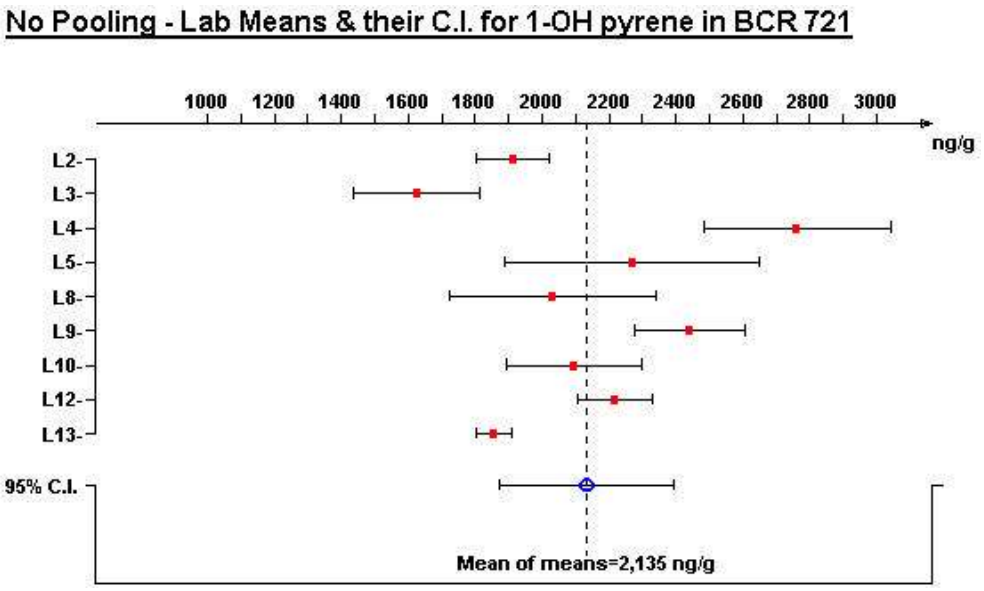

Figure 14 - Graphical presentation of certification data for BCR-721 – 1-hydroxy pyrene

European Commission

EUR 21068 – DG Joint Research Centre, Institute for Reference Materials and Measurements –
The certification of the mass fractions of PAH metabolites (1-hydroxyphenanthrene, 1-hydroxypyrene and 3-hydroxybenzo[a]pyrene) in two fish biles, BCR-720 (sediment-exposed flounder bile), BCR-721 (oil-exposed plaice bile)
Authors: F. Ariese, J. Beyer, D. Wells, E.A. Maier, B.M. Gawlik, A. Lamberty
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Abstract

This report describes the preparation, homogeneity study, stability study and certification of two fish bile materials: BCR-720, sediment-exposed flounder bile and BCR-721, oil-exposed plaice bile. In BCR-720 three metabolites of polycyclic aromatic hydrocarbons (PAHs) are certified: 1-hydroxy phenanthrene, 1-hydroxy pyrene and 3-hydroxy benzo[*a*]pyrene. 1-hydroxy pyrene is certified in BCR-721. A description of the analytical procedures used in the homogeneity and stability studies as well as in the certification study is given. All relevant data from the homogeneity and stability study and certification measurements are presented. Major conclusions from earlier feasibility studies, the results obtained for 2-hydroxy naphthalene (not certified), and implications for the optimal use of the materials are also discussed.

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