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Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile

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Abstract

In fish, metabolites of polycyclic aromatic compounds (PACs) in gall bladder bile can be used as biomarkers for recent environmental exposure to PACs. These metabolites in the bile result from hepatic biotransformation processes whereby the lipophilic parent PACs are transformed in two steps (hydroxylation and subsequent conjugation) to more soluble forms and then passed to the gall bladder for elimination from the organism. As a biomarker of exposure, the determination of PAC metabolites in bile has several advantages over other assessment techniques. Several bile PACs are strong fluorophores and can thus be measured semi-quantitatively and very easily by means of straightforward fluorescence detection techniques. For example, fixed fluorescence detection and synchronous fluorescence scanning can be used for this purpose. These techniques are excellent for rapid screening of overall PAC exposure levels, but less suitable for the determination of individual compounds. The next level of resolution is reached with a high-performance liquid chromatographic (HPLC) separation of the conjugated bile metabolites prior to the fluorescence detection, so that individual metabolites and their patterns are discernable. Furthermore, PAC metabolites in bile can be enzymatically hydrolyzed to allow detection of free hydroxy PACs. After a centrifugation step the sample can be measured directly by HPLC/fluorescence (F). For analysis by gas chromatography/mass spectrometry (GC/MS) an extraction procedure is normally used to separate the hydroxy PACs from the bile matrix; derivatization can be used in order to increase separation and sensitivity. The latter set of methodological approaches can be used to determine individual metabolites, and a large number of hydroxy PACs are available as standards for accurate quantitation. In general, GC/MS methods are optimal for smaller compounds with 2–3 rings due to their better selectivity, while HPLC/F often provides better detection limits for larger metabolites with 4–5 rings. In the present review, the state-of-the-art for the various alternative methods is presented. Aspects of analytical quality control, interlaboratory comparability of data and the use of certified reference materials are also discussed. The advantages and limitations of each approach are discussed with respect to the use of PAC metabolites in bile as biomarkers of environmental PAC exposure in fish.

Keywords: PAC pollution, fish, environmental monitoring, bile metabolites, biomarker, method review.

PREFACE

Analyzing fish bile for metabolites of polycyclic aromatic compounds (PACs) is an increasingly popular approach to assess recent environmental exposure to PACs. This publication discusses advantages/disadvantages of the various existing methods, rather than providing standard operating procedures (SOPs) for any given technique. One of the main reasons is a matter of principle: in modern analytical chemistry, prescribing strict protocols is regarded as an ineffective strategy, discouraging scientists to improve their methods and forcing them to carry out a procedure that will become more and more outdated, possibly even requiring instruments or parts that may no longer be on the market in a few years from now. Furthermore, with strict protocols followed in all laboratories there is also the risk of everyone making the same systematic error. Instead, we recommend that analytical procedures should be used that produce quantitative data (traceable to SI units), and that are verifiable with an independent technique or with a certified reference material (CRM). Agreement on which parameters to measure seems to be more important than standardizing the analytical technique.

Summarizing some of the main aspects of this review, we recommend the following:

- For exploratory studies one can use a rapid screening method for non-hydrolyzed bile (discussed in Section 2), especially if large numbers of samples are to be analyzed in the same laboratory. The intensities are directly related to the total PAC burden, and the relative intensities at different excitation/emission wavelengths can give some insight into the predominant PAC source. However, with these methods instrument-dependent sum parameters are measured, and therefore this approach is not recommended if interlaboratory comparability is important.
- For long-term, international monitoring programs specific metabolites should be determined, requiring more elaborate chemical analysis. Enzymatic hydrolysis with β-glucuronidase/arylsulfatase should be applied, and the resulting hydroxylated metabolites are to be quantified. Suggested metabolites for which standards are available: 1-hydroxypyrene, 1-hydroxyphenanthrene, 3-hydroxybenzo[a]pyrene, and 1-hydroxy naphthalene. There should be an option in the database to add other compounds as well. Concentrations should be expressed in ppm: microgram of the deconjugated compound per gram bile (wet wt).
- GC/MS is recommended in case of exposure to petroleum PAHs.
- HPLC-Flu is recommended in cases where the PAH burden is mostly due to combustion.
- For GC/MS and HPLC/F several suitable procedures can be found in the literature, as listed in Tables 1 and 2, respectively. Whatever analytical method is selected, proper validation and the use of CRMs should be part of the QA procedure. Only in that case may the resulting data be entered into the ICES databases.
- Bile accumulation in non-feeding fish may lead to relatively high metabolites as compared to feeding fish exposed to the same environment. However, most attempts to correct for this bias on the basis of protein or biliverdin levels have failed to produce lower standard deviations, often only adding extra measurement uncertainty. Correcting is therefore not recommended.
- Biological and environmental factors such as (but not limited to) fish species, feeding status, fishing method, season, water temperature, etc. should be clearly documented.

1 INTRODUCTION

Polycyclic aromatic compounds (PACs) comprise a diverse class of organic molecules that are ubiquitous environmental contaminants (Harvey, 1997). Sources of PACs are both natural (e.g., oil seeps, forest fires, and volcanoes) and anthropogenic (e.g., combustion of fossil fuels, vehicle emissions, petroleum spills, and oil well blow-outs) (Robertson, 1998). The most significant sources of petrogenic PACs in the marine environment are petroleum spills and "produced" water released from offshore drilling installations (Røe, 1998). Furthermore, the primary source of pyrogenic PACs is combustion of fossil fuels for heat and transportation (Robertson, 1998). In contrast, natural sources make only a minor contribution to the total PAC burden in the environment (Neff, 1979, 1985).

PACs have been shown to be toxic to marine life (Heintz *el al.*, 2000; Malins *et al.*, 1987; Myers *et al.*, 1991; Varanasi *et al.*, 1992). For example, certain PACs [e.g., benz[*a*]anthracene and benzo[*a*]pyrene (BaP)] are metabolically activated in vertebrates to form carcinogens (Dipple *et al.*, 1984). In addition, strong causal links between environmental PAC concentrations and the incidence of liver neoplasms and liver tumors in fish have been demonstrated in several studies (Baumann and Harshbarger, 1995; Malins *et al.*, 1988; Reichert *et al.*, 1998; Varanasi *et al.*, 1987; Vethaak *et al.*, 1996). Furthermore, Heintz *et al.* (2000) reported delayed growth and reduced marine survival of pink salmon (*Onchorhynchus gorbuscha*) following exposure to PACs from oil as embryos.

Levels of PACs in the marine environment have been monitored in many areas from the time that these compounds were recognized as a serious environmental concern (Robertson, 1998). For example, higher molecular weight PACs deposited in sediment are resistant to bacterial degradation (Cerniglia, 1984) and determining their sediment concentrations yields valuable information about the PAC load. Furthermore, the feasibility of measuring tissue concentrations of PACs in marine species to monitor for exposure depends on rates of uptake, biotransformation, and excretion. Although the ability of invertebrates to take up and eliminate PACs has been well documented (James, 1989; Neff, 1979), biotransformation rates in invertebrates are highly species dependent. However, these rates are considered to be low in many invertebrates, e.g., molluscs (James, 1989). In contrast, fish and other marine vertebrates rapidly biotransform PACs to form epoxy and hydroxy derivatives during phase I metabolism. Subsequently, during phase II metabolism, the phase I metabolites are converted into highly water-soluble conjugates (e.g., glucuronides or sulfates) that accumulate in the bile (Lech and Vodicnik, 1985; Lee *et al.*, 1972; Varanasi and Gmur, 1981; Varanasi *et al.*, 1985). Therefore, measuring PAC metabolites in bile provides a means for determining exposure (Krahn *et al.*, 1986).

Analysis for metabolites in fish bile has been used to monitor for PAC exposure in a number of studies (Krahn *et al.*, 1986; McDonald *et al.*, 1995; Oikari, 1986; Statham *et al.*, 1976; van der Oost *et al.*, 1994). Other studies have shown that PAC metabolite concentrations in bile correlated well with the exposure to PACs (Aas *et al.*, 2001; Collier and Varanasi, 1991; Krahn *et al.*, 1984; Krahn *et al.*, 1992). The complex nature of PAC metabolites in bile of fish exposed to oil or other environmental pollution makes analysis challenging. As a result, selecting the best analytical method will depend upon the questions that need to be answered.

This paper will present an overview of the various techniques used to characterize metabolites of PACs in fish bile. It is not meant to be a collection of standard operating procedures, but instead to provide enough detail about the methods—including the advantages and limitations of each technique—so that a scientist or resource manager can decide whether the procedure would be useful in answering specific questions. The individual can then access the method in the original literature to obtain the details necessary for their application. The paper will also demonstrate the need for strong quality control when making measurements of PAC metabolites

in bile to allow intercomparability among laboratories. Finally, the process used to develop two certified reference materials (CRMs) for PAC metabolites in fish bile is explained. The sections in which to find detailed information about each topic follow.

Various strategies for PAC metabolite determination are illustrated in Figure 1: (1) rapid screening of non-hydrolyzed fish bile; or (2) compound-specific analysis of individual metabolites. Both fixed wavelength fluorescence (FF; Section 2.2) and synchronous fluorescence spectrometry (SFS; f 2.3) of bile are sensitive and cost-effective analytical tools capable of discriminating between exposed and non-exposed individual fish. Although both these methods are rapid, specific metabolites are neither identified nor quantified. However, by selecting excitation and emission wavelength pairs carefully, a limited degree of compound class specificity can be achieved (Sections 2.2 and 2.3). Furthermore, additional information can be gained about possible PAC sources through use of metabolite patterns from HPLC/F screening of non-hydrolyzed bile (Section 2.4).

Identification and determination of individual PAC metabolites requires labor-intensive methods for sample preparation and analysis. Sample preparation usually includes an enzymatic treatment to produce free hydroxy PACs, followed by extraction or centrifugation and possibly derivatization of the hydroxy groups (Section 3.1). HPLC/F or gas chromatography (GC) with mass spectrometry (MS) detection are frequently used for identifying and quantifying individual PAC metabolites (Section 3.2). Furthermore, combining HPLC with MS detection is an alternative technique for determining oxygenated PACs (Section 3.2.4), but HPLC/MS has not yet been used in routine monitoring for PAC exposure. A brief overview of other less frequently used techniques is given in Section 3.2.5.

When applying these analytical techniques in monitoring studies, reproducibility and accuracy of the data are crucial, so quality control measures need to be implemented. Long-term reproducibility is essential when monitoring studies stretch out over several years. Quality requirements will be most stringent for studies involving several institutes or even different methods, so that there is no significant interlaboratory bias (systematic error). The various options for assuring the quality of PAC metabolite measurements are given in Section 4. Among the options to check for systematic errors of an analytical procedure, one of the best is to analyze a certified reference material (CRM) and check the results against the certified value. Until recently, such a material did not exist for PAC metabolites in fish bile, hampering the quality control and especially the intercomparability of data. The development of two bile CRMs is discussed in Section 5. Conclusions and a discussion on whether to use rapid screening or more elaborate compound-specific methods are presented in Section 6. The paper focuses on PAC metabolite analysis for monitoring purposes, although several of the methods described here will also be suitable for more specific fish metabolism studies in a laboratory or mesocosm setting.

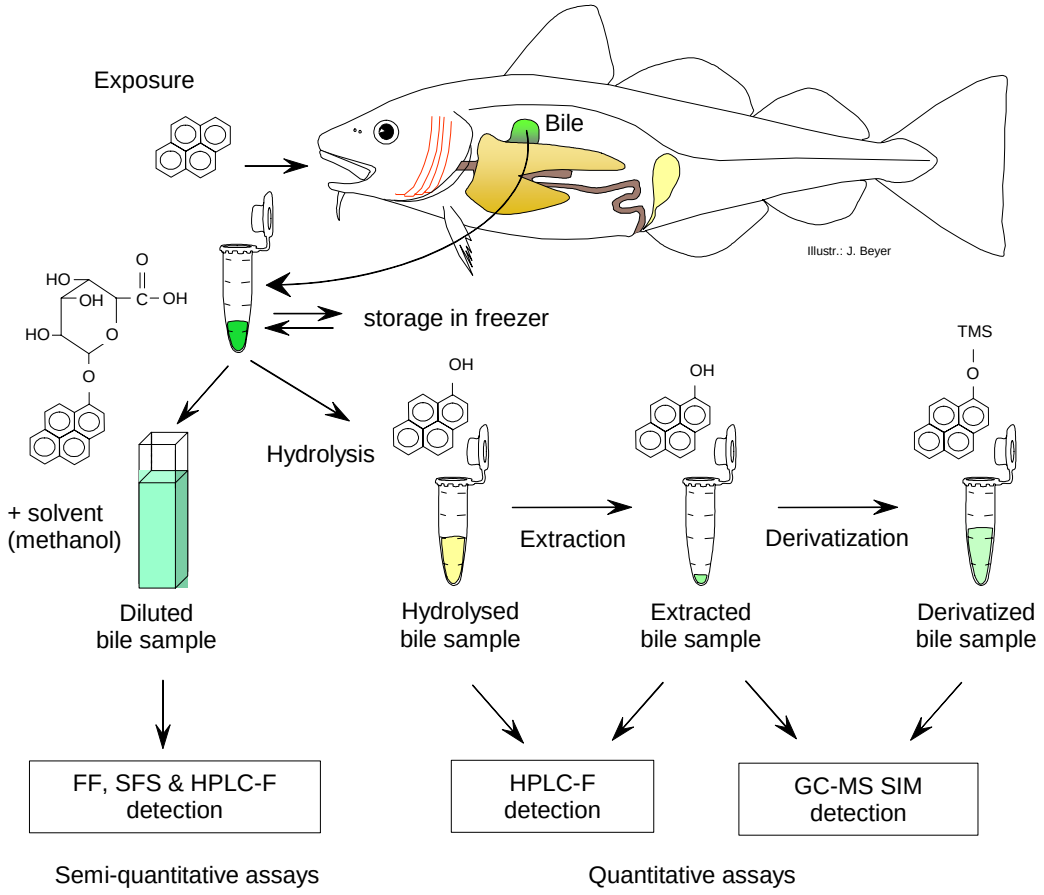

Figure 1. Overview of method alternatives for detection of PACs in fish bile, using pyrene as an example contaminant.

2 FLUOROMETRIC TECHNIQUES FOR ESTIMATING TOTAL METABOLITES (SCREENING)

2.1 General introduction to fluorometry

PACs and many other aromatic compounds are known to be relatively strong fluorophores. The fluorescent properties of PAC molecules are due to the rigid, aromatic structure with delocalized π-electrons that are distributed throughout the molecule. When a PAC molecule absorbs a photon with energy $h\nu_{ex}$, one of its π-electrons is brought to a higher energy level, and the molecule is said to be in an excited singlet state. As this electron returns to the ground state, a photon with energy $h\nu_{ex}$ is emitted and this process is known as fluorescence emission (photoluminescence). The wavelengths at which the molecule can be excited and the wavelengths at which it emits are characteristic for the compound and can be used for identification. Several textbooks exist on fluorescence and fluorometric applications, for example Sharma and Schulman (1999).

The relevant energy levels of a fluorescent molecule (electronic states with superpositioned vibrational levels) are shown in Figure 2. The energy difference between the lowest vibrational

level of the ground state S0 and the lowest level of the first excited state S_1 is called the 0-0 energy. For excitation the photon energy must be equal to 0-0 or higher. After excitation the molecule rapidly returns to the lowest level of S_1 (losing excess energy to the solvent in the form of heat), from where it can return to the ground state by emitting a fluorescence photon. From this diagram it follows that the energy of the emitted light is always lower than the excitation energy, with one point of overlap (at the 0-0 energy). In fact, if we look at the process more closely, in the liquid state the 0-0 energy in emission is even a bit lower (longer wavelength) than the 0-0 energy in excitation, due to solvent reorientation effects (see Figure 2B). This difference in energy is called the Stokes' shift, which in synchronous fluorometric assays (see Section 2.3) is often taken as delta lambda ($\Delta\lambda$) or the excitation/emission wavelength interval.

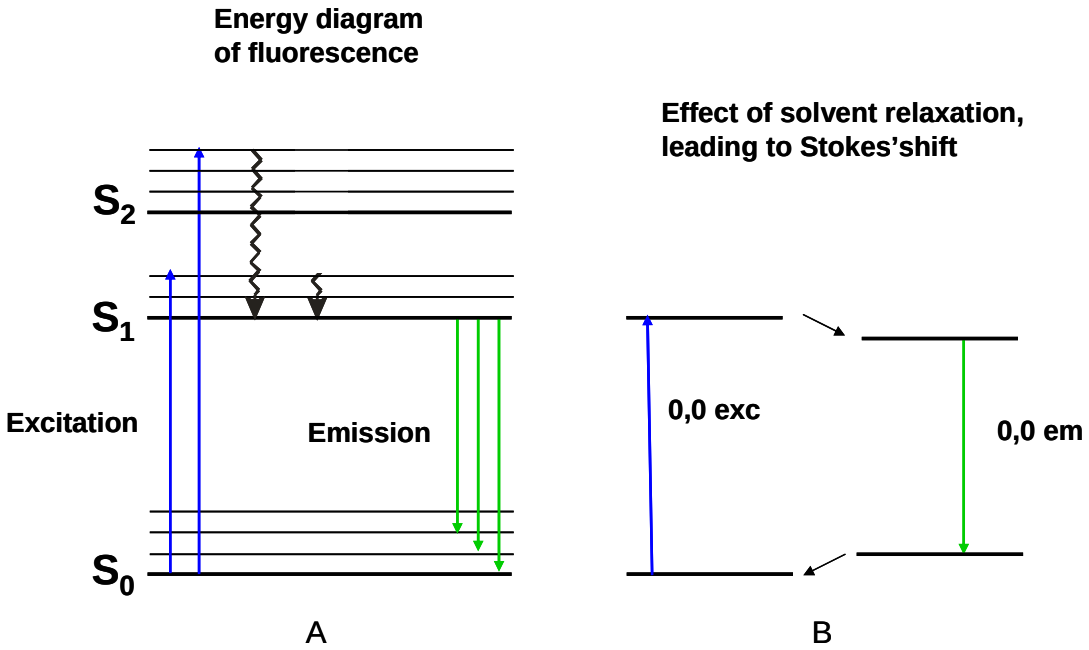

Figure 2. A: Jablonski diagram showing molecular energy levels and the processes of excitation, radiation-less decay and fluorescence emission. B: Origin of the Stokes's shift in liquids: solvent reorientation, in which the solvent adapts to the new electron configuration, results in a lowering of the energy of the 0,0 emission transition, compared to the 0,0 in excitation.

Each PAC compound displays different fluorometric characteristics. In the early seventies, Lloyd (1971) suggested exploiting these characteristics for the semi-quantitative screening of PACs in complex mixtures (such as automobile engine oil) without a chromatographic separation into individual components. Certain PACs or classes of compounds could be discerned in the spectrum of the mixture, even though the spectra partially overlapped. Later, the possibility of applying similar principles for screening the PAC content in biological samples was investigated (Statham *et al.*, 1976). Fluorometric detection of PAC metabolites in bile of fish from polluted sites was reported by Krahn *et al.* (1984), who described an HPLC/F based screening method for BaP, naphthalene and their metabolites. Chemical structures of four representative hydroxy PACs and their abbreviations used in this paper are given in Figure 3. Ariese *et al.* (1993a) described a synchronous fluorescence spectrometry assay of 1-OH pyrene (1-OH Pyr) in fish bile for use as a rapid screening technique to monitor PAC exposure in fish. Subsequently, fixed wavelength fluorescence (FF) of fish bile was proposed as a even easier means of screening for PAC exposure in fish (Aas *et al.*, 2000; Lin *et al.*, 1996). In general, these three bile screening methods all represent tools for easy, rapid, and semi-quantitative assessment of PACs in fish. A brief review of each method is provided below.

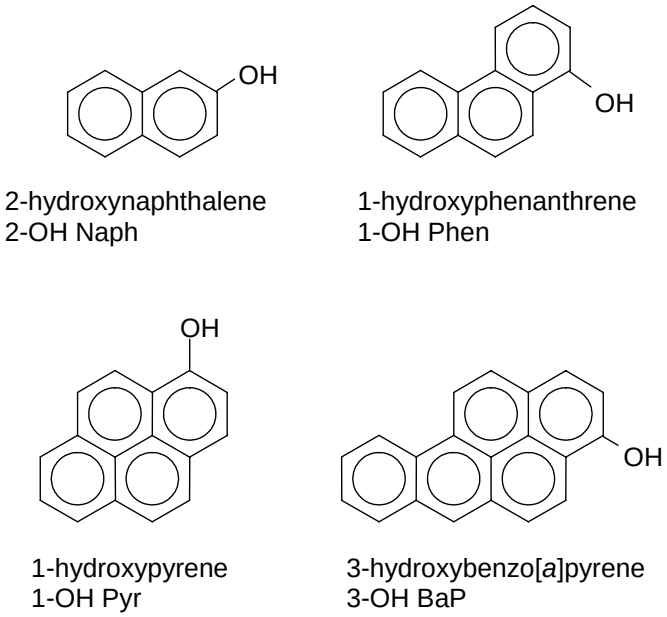

Figure 3. Structures of four PAC metabolites and their abbreviations. These compounds were selected as target analytes for certification (Section 5).

Most of the larger PAC molecules in the bile of PAC-exposed fish are present in the form of hydrophilic conjugates such as glucuronides (see Figure 1). One of the attractive features of these rapid techniques is that the time-consuming hydrolysis step can be left out and the conjugated species can be measured directly by fluorescence. However, for quantitation one should be aware of the fact that their fluorescence intensities are usually enhanced relative to the hydroxy PACs and the optimal excitation and emission wavelengths are slightly shifted to shorter wavelengths (i.e., higher energy levels). Therefore, when the conjugated species is not available, as is most often the case, the corresponding free hydroxy PAC cannot be used for calibration without caution (Ariese *et al.*, 1993a).

2.2 Fixed wavelength fluorescence

In FF, PAC metabolite levels are determined semi-quantitatively in diluted bile from the fluorescence intensities detected at specific excitation and emission wavelengths (Aas *et al.*, 2000; Lin *et al.*, 1996). In general, small PAC metabolite molecules with few fused benzene rings (e.g., naphthalene- and phenanthrene-derived metabolites) require a higher excitation energy than do larger PACs (e.g., BaP metabolites). By measuring the fluorescence of hydroxy PAC standard solutions and diverse PAC mixtures in bile at different wavelength pairs, various PAC metabolites (or classes of these) can be identified. These characteristics form the basis of the FF screening method. However, because PAC fluorescence spectra under conventional, room temperature conditions are relatively broad, they are often not specific enough to distinguish between closely related isomers.

In brief, the procedure can be described as follows. A bile sample is mixed well (preferably sonicated) and centrifuged. Because bile constituents other than PAC metabolites are able to absorb incoming light, it is important to dilute the bile sample sufficiently to avoid significant loss of signal due to this "inner filter" effect. Also, fluorescence calibration curves deviate from linearity at high concentrations; as a rule of thumb, the total absorbance of the sample at the wavelength of interest should not be higher than 0.05. Thus, the bile is diluted with solvent

(50:50 methanol:water is best, but similar ethanol:water mixtures may also be used). A dilution factor at 1,500–2,000 times is most often enough to avoid the problem, but a simple dilution effect study is recommended when optimizing the FF method or when some samples in a set are much more concentrated than the rest (Aas *et al.*, 2000). In bile samples with very high PAC metabolite concentrations, very high dilution factors (>20,000 times) have sometimes been needed to keep the detector signal on scale or within the linear range of the assay (Lin *et al.*, 1996).

The diluted bile sample is analyzed in a quartz cuvette using a fluorescence spectrometer (Aas *et al.*, 2000; Lin *et al.*, 1996). The selection of spectrometer slit widths is a compromise of signal strength and specificity. Although some researchers use different slit widths for excitation and emission, widths of 2.5 nm for both excitation and emission have, in most cases, been found suitable for FF detection of bile PAC mixtures. Standard solutions of hydroxy PACs and bile samples from fish exposed to single compounds of PACs have both been used to establish optimal FF wavelength pairs for different classes of PAC metabolites. The latter approach is preferred because the optimal excitation/emission wavelengths of free hydroxy PACs will not be exactly the same as those of the conjugated species that are found in real bile samples. Recommendations of specific excitation/emission wavelengths for detection of certain PACs may vary slightly among published papers. In general, the wavelength pairs 290/335, 341/383, and 380/430 nm have been found suitable for the screening of 2/3-ring PACs (e.g., naphthalene and phenanthrene metabolites), 4-ring PACs (e.g., pyrene metabolites), and 5/6-ring PACs (e.g., BaP metabolites), respectively (Aas *et al.*, 2000; Krahn *et al.*, 1984; Krahn *et al.*, 1987; Lin *et al.*, 1996).

The signal intensity ratios at the three wavelength pairs 290/335, 341/383, and 380/430 nm may also provide information about the dominant PAC source: a complex mixture of non-alkylated and alkylated naphthalene metabolites is typically found in fish exposed to petrogenic contamination (crude oil or marine fuel related), leading to stronger FF fluorescence intensity at 290/335 nm, whereas combustion-type sources (traffic, domestic heating) yield a stronger FF signal at 341/383 and 380/430 nm due to the presence of 4- to 5-ring PACs (Aas *et al.*, 2000; Lin *et al.*, 1996). Because of a lack of glucuronidated hydroxy PAC standards, FF data are often expressed in arbitrary fluorescence units, which may be considered as acceptable within the framework of a screening tool (e.g., in discriminating exposed samples from non-exposed), as long as all samples are analyzed with the same instrument under identical conditions. The FF signal may also be expressed as equivalents of a PAC standard, such as pyrene (Aas *et al.*, 2000). Whenever an analytical sum parameter is determined and a single compound (whether one of the analytes or a different standard) is used for calibration, instrumental settings (in the case of FF: wavelengths, spectral slit widths, lamp and detector type) need to be specified if data are to be compared among different laboratories. In addition, bile reference materials (e.g., BCR 720 and 721; see Section 4.8) should be used by the participating laboratories to determine interlaboratory comparability.

2.3 Synchronous fluorescence spectrometry

In synchronous fluorescence spectrometry (SFS), the excitation/emission light detection conditions are varied simultaneously, with a fixed wavelength interval ($\Delta\lambda$), and over a specific wavelength range (Aas *et al.*, 2000; Ariese *et al.*, 1993a; Lin *et al.*, 1994). With diluted fish bile (e.g., the same diluted bile samples as for the FF), the SFS assays often yield very characteristic fluorescence spectra, depending on the PAC metabolites present in the assay mixture. Through SFS analyses of bile from fish exposed to single PAC compounds, the different fluorescence characteristics of PAC metabolites classes have been demonstrated (Figure 4). Similar spectral trends can be demonstrated with bile samples from fish exposed to PACs from petrogenic and pyrogenic sources.

Through variation of the wavelength interval (Δλ), different scans can be produced. When the target compound has a strong S0-S1 absorption (see Figure 2), a very small Δλ value (typically 5 nm) can be chosen, corresponding to the Stokes' shift. In principle, it would be more correct to apply a constant energy difference, because the Stokes' shift is not constant in wavelength units, but that approach has not found widespread use. If, on the other hand, excitation into the S2 state is much more efficient, a larger Δλ will be preferred, corresponding to the difference between S0-S2 in excitation and 0,0 in emission. Indeed, optimization of Δλ towards specific PAC mixtures should be conducted whenever necessary. Experience has shown, however, that a Δλ of 42 nm is suitable for many PAC metabolites and discriminates to a reasonable degree between bile PACs of different size categories (Figure 4).

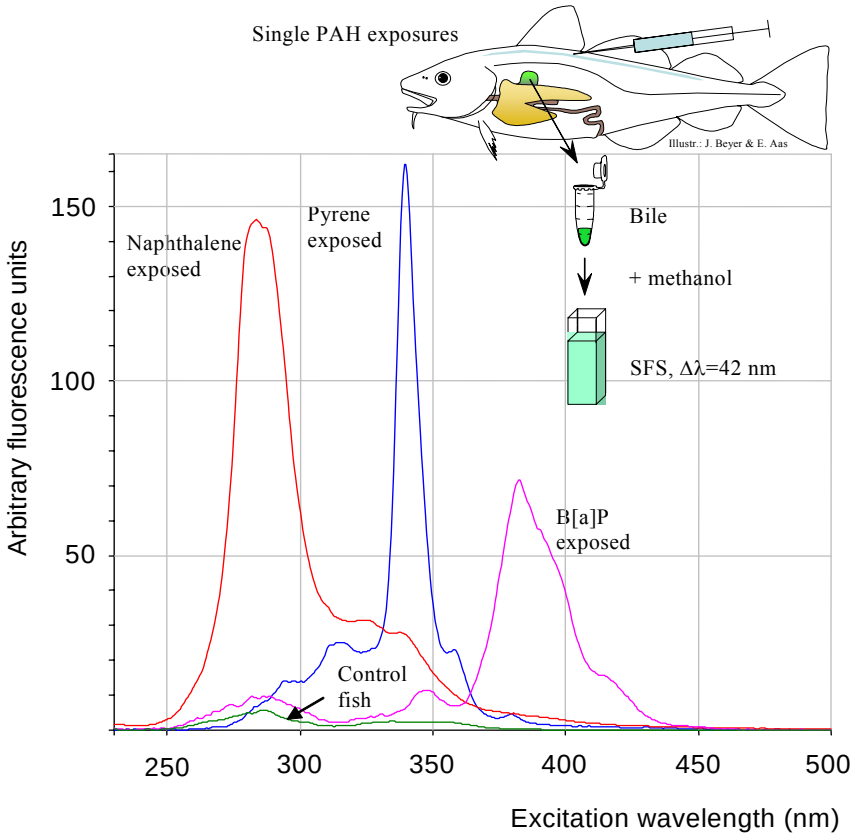

Figure 4. PAC metabolite specificity in SFS screening assays. Fluorescent metabolites of three separate parent PACs are discriminated to a reasonable degree by SFS analysis of diluted bile. The wavelength interval used in all scans shown above is 42 nm. SFS scan data obtained from Aas *et al.* (2000) with permission.

Similar to FF, the SFS analysis of non-hydrolyzed bile samples is a very rapid technique that normally takes only a few minutes. In screening studies of fish bile samples, SFS is often conducted as a supplement to FF measurements. Both non-hydrolyzed and hydrolyzed bile samples can be used. However, in order to save time, and because the conjugated PAC metabolites typically display enhanced fluorescence compared to their hydrolyzed counterparts, it is generally recommended that non-hydrolyzed samples be used for PAC screening purposes. As with FF (Section 2.2), SFS is used to measure a sum parameter rather than individual compounds, although concentrations of predominant metabolites such as conjugated 1-OH Pyr can be determined with reasonable accuracy (Ariese *et al.*, 1993a). Usually only a single compound is used as the calibrant and the results are expressed in "equivalents." The method is primarily meant for studies in which all samples are analyzed by the same laboratory with the same method. Whenever data obtained in different laboratories need to be comparable, the analytical protocol, instrument settings, and calibration method need to be very clearly defined and reference materials (RMs) need to be exchanged to check the comparability (see Section 4.8).

Other types of fluorescence scanning techniques, such as excitation or emission spectroscopy, can also be utilized for determination of biliary PAC metabolites. For example, as demonstrated by Hawkins *et al.* (2002), metabolite emission spectra collected at a fixed excitation wavelength of 254 nm can be used for screening detection of phenanthrene metabolites in bile and other sample matrices.

2.4 HPLC/fluorescence screening

In the HPLC/F screening method, bile metabolites are separated by reverse-phase HPLC prior to fluorescence detection. Non-hydrolyzed bile (1–5µL) is injected directly onto a reverse-phase C-18 HPLC column and eluted with a linear gradient from 100% water (containing a small amount of an acid, e.g., acetic acid) to 100% methanol or acetonitrile as described by Escartin and Porte (1999a; 1999b), Krahn *et al.* (1984; 1986), Leonard and Hellou (2001), and McDonald *et al.* (1995). Chromatograms can be recorded at one or more excitation/emission wavelength pairs where the parent compounds and metabolites fluoresce. A number of different wavelengths can be used for detecting particular PAC metabolites (see Section 2.2).

A semi-quantitative estimate of metabolite concentrations are made using either a parent PAC calibrant (e.g., naphthalene, BaP) (Escartin and Porte, 1999a, 1999b; Krahn *et al.*, 1986; Leonard and Hellou, 2001) or a phenolic calibrant, such as 1-OH Pyr or 3-OH BaP (Krahn *et al.*, 1986). Integrated areas of peaks eluting after about 7 minutes (determined for each chromatographic system) are summed and the sum is expressed as the amount of the chosen standard ("equivalents") that would be present if the area were attributable only to that compound. Because a single calibrant is normally used to measure a large number of metabolites that fluoresce at each selected wavelength, accurate quantitation is not possible. Thus, the results provide only relative concentrations of total metabolites that fluoresce at a particular set of wavelengths. However, judicious choice of the quantitation standard can improve accuracy when screening results are compared to detailed methods for quantitation of metabolites. For example, when a metabolite (e.g., 1-OH Pyr) was used as the quantitation standard at 380/430 nm rather than a parent hydrocarbon (e.g., BaP), the accuracy of the screening determination improved compared to results obtained by GC/MS (Krahn *et al.*, 1987). Furthermore, HPLC/F can be used to accurately measure individual analytes that are well-resolved from coeluting compounds (e.g., 1-OH Pyr; see Section 3.2.3). As discussed for FF and SFS (Sections 2.2 and 2.3), when data analyzed in different laboratories need to be compared, analytical and calibration methods, as well as instrument settings, need to be clearly defined. In addition, RMs need to be exchanged to check the comparability (see Section 4.8).

HPLC/F screening provides more information about the metabolites present than is obtainable by the other screening methods. Metabolite patterns are readily discernable and these patterns can assist in establishing the source of pollution (Krahn *et al.*, 1993). For example, the chromatographic pattern from bile of a rock sole (*Lepidopsetta bilineata*) captured from Prince William Sound after the Exxon Valdez spill was distinct and was very dissimilar from that of an English sole (*Parophrys vetulus*) exposed to contaminants from an urban site (Figure 5). Bile from the fish collected from the oiled site shows many more metabolites, most likely because crude oil contains an array of alkylated PACs whereas urban areas often contain a greater proportion of the non-alkylated pyrogenic PACs (Krahn *et al.*, 1993).

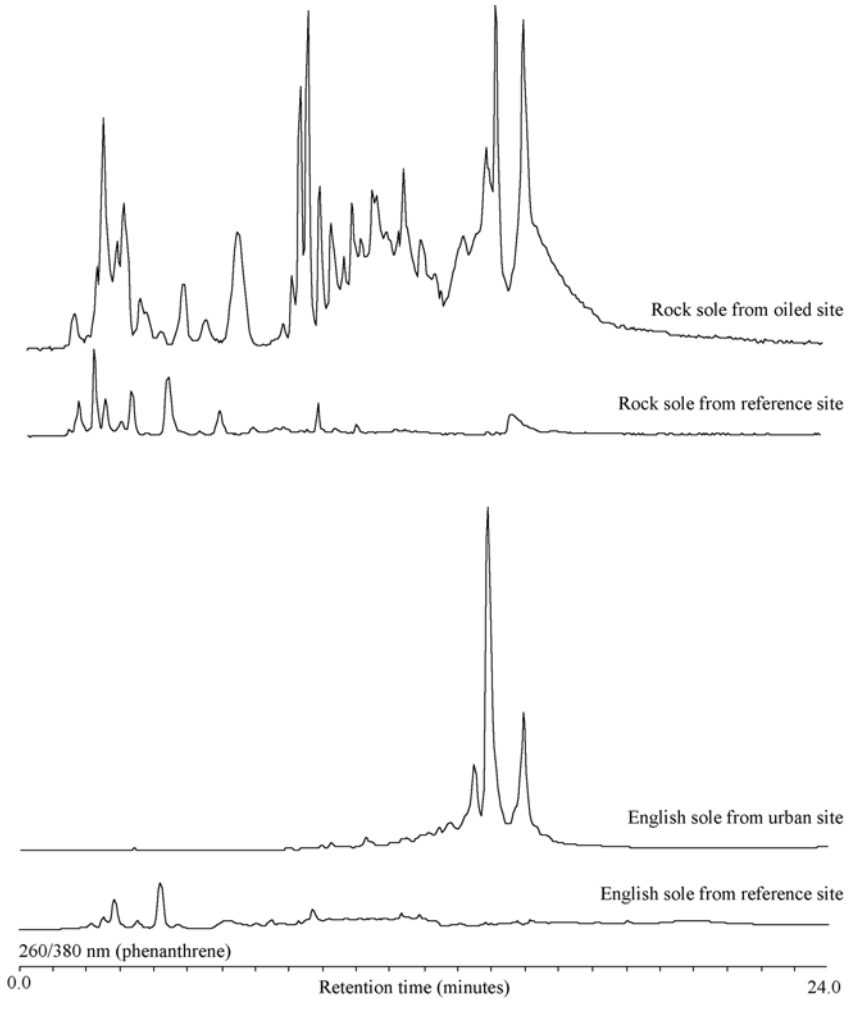

Figure 5. Chromatograms from HPLC/F screening (260/380 nm; phenanthrene wavelengths) of PAC metabolites in bile of rock sole from oiled (*Exxon Valdez* crude) and reference (clean) sites and in bile of English sole from urban and reference sites.

Bile screening by HPLC/F has proven to be a powerful tool for estimating exposure of fish and other marine vertebrates to PACs, including nitrogen and sulfur homologs. These rapid screening methods have been used routinely in monitoring for chronic pollution and following pollution events, such as oil spills (Escartin and Porte, 1999a; Escartin and Porte, 1999b, 1999c; Krahn *et al.*, 1987, 1992; McDonald *et al.*, 1995). In addition, screening methods have been used in laboratory exposure studies to measure dose- and time-related changes in bile metabolite concentrations (Collier and Varanasi, 1991; Stroomberg *et al.*, 2003). Finally, estimated concentrations of bile metabolites that fluoresce at 380/430 nm have been statistically correlated with the prevalence of certain hepatic lesions, including neoplasms, in English sole from Puget Sound, WA, USA (Krahn *et al.*, 1984; Krahn *et al.*, 1986).

3 TECHNIQUES FOR DETERMINING CONCENTRATIONS OF INDIVIDUAL METABOLITES

3.1 Preparation of samples

Two tables have been prepared to provide a reference guide to the preparation and analysis for PAC metabolites in bile. Methods for sample preparation and GC/MS analysis are given in Table 1 and those for sample preparation and HPLC/F analysis are found in Table 2.

3.1.1 Enzymatic hydrolysis

Samples taken from the freezer are thawed and briefly homogenized in a sonication bath. Typical sample amounts range from 5–100 µl. Because metabolites of PACs exist mainly as conjugates (e.g., sulfates and glucuronides) in bile and few standards of the conjugated metabolites are available, bile samples are first hydrolyzed to give free hydroxy metabolites. As described by Hellou and Payne (1987), Jonsson *et al.* (2003), Kennedy *et al.* (1989), Krahn *et al.* (1987), Steward *et al.* (1990), and van Schanke *et al.* (2001), glucuronides and sulfates can be simultaneously hydrolyzed by adding ß-glucuronidase containing arylsulfatase activity and pH 4.8–5.0 acetate buffer, followed by incubation for one to 24 hours at 37–40 °C. In order to provide information about the relative concentrations of different conjugates a successive deconjugation can be performed. Accordingly, Kennedy *et al.* (1989), Steward *et al.* (1990), and Willett *et al.* (2000) separated bile metabolites into groups of free hydroxy compounds, glucuronides, and sulfates. To prevent oxidation of the hydrolyzed metabolites, Ariese *et al.* (2000) and Richardson *et al.* (2001) added 0.5 and 4% ascorbic acid, respectively, to a mixture of the bile and the deconjugation enzymes. An internal standard is usually also added prior to hydrolysis. The use of an internal standard is more critical for GC methods (because of a more complex procedure and more volatile solvents) than for HPLC methods. Internal standards are discussed in more detail in Section 4.5.

The majority of publications about PAC metabolite analysis in fish bile focus on the determination of glucuronides and sulfates, but a significant portion of PAC conjugated metabolites can be present as glutathione conjugates (Varanasi *et al.*, 1987). However, unlike glucuronides and sulfates these conjugates are more likely to be retained in the liver (Plummer *et al.*, 1980; Varanasi *et al.*, 1987). Glutathione conjugates in fish bile can be hydrolyzed at ambient temperature by acidic hydrolysis (pH 0–1) as described by van Schanke *et al.* (2001) and Yu *et al.* (1995), or by addition of γ-glutamyltranspeptidase followed by incubation at 37 °C for six hours (Willett *et al.*, 2000).

Table 1. Sample preparation and GC/MS analysis of hydrolyzed PAC metabolites.

Target compounds	Sample preparation					GC analysis		Reference
	Enzymatic treatment	**Incubation**	**Extraction solvent**	**Derivatization products**	**Internal standards**	**Column**	**Detection**	
Phenanthrene metabolites	β-glucuronidase sulfatase	37 °C for 10 h.	Ethylacetate	TMS-ethers	α-naphthol	SE-54 5% phenyl; 20 m × 0.33 mm	MS EI, 70 eV	(Solbakken *et al.*, 1980)
2- to 4-ring PAC-metabolites	β-glucuronidase sulfatase	40 °C for 4 h.	Methylene chloride and isopropanol	TMS-ethers	hexamethylbenzene	J & W DB5 MS 5% phenyl; 30 m × 0.25 mm	MS EI, 70 eV	(Krahn *et al.*, 1984; Krahn *et al.*, 1987)
2- to 3-ring alkylated & non-alkylated hydroxy PACs	β-glucuronidase	37 °C for 24 h.	Methylene chloride and methanol	Acetylates	*n*-pentadecane	SE-30	MS EI, 70 eV	(Hellou and Payne, 1987)
2- to 4-ring alkylated & non-alkylated hydroxy PACs	β-glucuronidase sulfatase	40 °C for 3 h.	Methylene chloride and methanol		2,6-dibromophenol	J & W DB5 MS 5% phenyl; 30 m × 0.25 mm	MS EI, 70 eV	(Krahn *et al.*, 1992)
2- to 5-ring alkylated & non-alkylated hydroxy PACs	β-glucuronidase sulfatase	40 °C for 2 h.	Methylene chloride and methanol	TMS-ethers	2,6-dibromophenol, 1-naphthol-d_8, phen-d_{10}, chry-d_{12}, flu-d_{10} and BaP-d_{12}	J & W DB5 MS 5% phenyl; 30 m × 0.25 mm	MS EI, 70 eV	(Yu *et al.*, 1995)
2- to 4-ring hydroxy PACs	β-glucuronidase sulfatase	40 °C for 2 h.	Ethylacetate		2,6-dibromophenol hexamethylbenzene	HP-5 MS 5% phenyl; 30 m × 0.25 mm	MS EI, 70 eV	(Escartin and Porte, 1999a; Escartin and Porte, 1999b)
1- to 4-ring aromatic compounds	β-glucuronidase sulfatase	37 °C overnight	Hexane : methyl-*t*-butyl ether (2:1)		octadecane	MSBP5 5% phenyl; 30 m × 0.25 mm	MS EI, 70 eV	(Stephensen *et al.*, 2000)
2- to 4-ring hydroxy PACs	β-glucuronidase sulfatase			Acetylates	decachlorobiphenyl	GC separation	MS EI, 70 eV	(Ariese *et al.*, 2000)
2- to 4-ring hydroxy PACs	β-glucuronidase sulfatase	40 °C for 2 h.	Ethylacetate	TMS-ethers	2,6-dibromophenol	CP-Sil 8 5% phenyl; 50 m × 0.25	MS EI, 70 eV	(Jonsson *et al.*, 2003)
Chrysene metabolites	β-glucuronidase sulfatase	40 °C for 2 h.	Ethylacetate	TMS-ethers	4-Cl-naphthol perylene	CP-Sil 8 5% phenyl; 50 m × 0.25	MS EI, 70 eV	(Jonsson *et al.*, 2004)

Table 2. Sample preparation and HPLC analysis of hydrolyzed PAC metabolites.

Target compounds	**Sample preparation**				**HPLC analysis**			**Reference**
	Enzymatic treatment	**Incubation**	**Solvent**	**Internal standard**	**Column**	**Mobile phase**	**Detection**	
BaP metabolites	β-glucuronidase or sulfatase	37 °C for 6 h.	Extracted with ethylacetate and acetone		Zorbax ODS 5 µm; 4.6 mm × 250 mm	Gradient: water, acetonitrile and methanol	Scintillation counting	(Steward *et al.*, 1990)
1-OH Pyr	β-glucuronidase and sulfatase	37 °C for 2 h.	Ethanol, ≈50 or 100%		Rosil C-18 HL; 3.1 mm × 200 mm	Acetonitrile:water (70:30 v/v)	Fluorescence	(Ariese *et al.*, 1993a)
pyrene metabolites	No treatment or acid hydrolyzed	Acid hydrolysis at 80 °C for 3 h.	Methanol or methylene chloride		Marck® LiChroCART® RP-18 5 µm; 4.0 mm × 125 mm	Gradient: ammonium acetate buffer, 10 mM, and acetonitrile	LC-APCI-MS/MS	(Law *et al.*, 1994)
BaP metabolites	β-glucuronidase, sulfatase or γ-glutamyltranspeptidase	37 °C for 6 h.	Extracted with ethyl acetate and acetone	6-OH chrysene benzanthracene-7,12-dione	S80A C-18 ECQ 5 µm; 4.6 mm × 250 mm	Gradient: water and methanol	LC-APCI-MS	(Willett *et al.*, 2000)
2- to 5-ring hydroxy PACs	β-glucuronidase sulfatase		Ethanol	Anthracene-d_{10}	Xterra RP-18	Gradient: water containing 1 mg l^{-1} ascorbic acid and acetonitrile	Fluorescence	(Ariese *et al.*, 2000)
2- to 5-ring hydroxy PACs	β-glucuronidase sulfatase		Ethanol		RP silica C-18	Gradient: water and acetonitrile	Fluorescence	(Ariese *et al.*, 2000)
BaP metabolites	β-glucuronidase and sulfatase or HCl, pH 0	37 °C for 1 h. or 30 °C for 30 min.	Methanol (50%)		Vydac 201TP54 C-18; 4.6 mm × 250 mm	Gradient: water and methanol	Fluorescence	(van Schanke *et al.*, 2001)
2- to 4-ring hydroxy PACs	β-glucuronidase sulfatase	37 °C for 1 h.	Ethanol (84%)		Vydac 201TP54 C-18; 4.6 mm × 250 mm	Gradient: acidified water, pH 4, and acetonitrile	Fluorescence	(Richardson *et al.*, 2001)
3- to 5-ring PAC metabolites	β-glucuronidase sulfatase	37 °C for 45 min.	Methanol (50%)		Vydac 201TP54 C-18; 4.6 mm × 250 mm	Gradient: water and methanol	Fluorescence	(Ruddock *et al.*, 2002) (Ruddock *et al.*, 2003)
2- to 5-ring hydroxy PACs	β-glucuronidase sulfatase	40 °C for 2 h.	Acetonitrile (80%)	Triphenylamine	Vydac 201TP52 C-18 5 µm; 2.1 mm × 250 mm	Gradient: ammonium acetate buffer, pH 4, and acetonitrile	Fluorescence	(Jonsson *et al.*, 2003)
Chrysene metabolites	β-glucuronidase sulfatase	40 °C for 2 h.	Acetonitrile (70%)	Perylene	Vydac 201TP52 C-18 5 µm; 2.1 mm × 250 mm	Gradient: ammonium acetate buffer, pH 4, and acetonitrile	Fluorescence	(Jonsson *et al.*, 2004)

3.1.2 Extraction of PAC metabolites

The majority of reports on HPLC analysis of hydrolyzed bile for individual hydroxy PACs describe simple sample preparation without any extraction step involved (Jonsson *et al.*, 2003; Richardson *et al.*, 2001; van Schanke *et al.*, 2001). For example, hydrolyzed bile samples were diluted 2- to 5-fold or more with ethanol (Ariese *et al.*, 1993a; Richardson *et al.*, 2001) or methanol (Jonsson *et al.*, 2003; van Schanke *et al.*, 2001), followed by centrifugation before injection onto the HPLC column.

Alternatively, extraction of bile samples can be done as a clean-up step prior to (Leonard and Hellou, 2001; Steward *et al.*, 1990) or following deconjugation of PAC metabolites (Ariese *et al.*, 1993a; Leonard and Hellou, 2001; Willett *et al.*, 2000). For example, Steward *et al.* (1990) extracted bile with ethyl acetate:acetone (2:1 v/v) prior to enzymatic treatment in order to remove unwanted lipophilic compounds from the bile. Extraction is usually performed following enzymatic hydrolysis of bile in order to transfer hydroxy PACs into a volatile organic solvent compatible with gas chromatography analysis (Krahn *et al.*, 1987). Hydrolyzed metabolites have been successfully extracted from acidified (pH 4.8–5.0) samples using methylene chloride containing 10% methanol (Hellou and Payne, 1987; Krahn *et al.*, 1992), ethyl acetate (Escartin and Porte, 1999a; Jonsson *et al.*, 2003; Solbakken *et al.*, 1980), or a mixture of hexane and methyl-t-butyl ether (2:1) (Stephensen *et al.*, 2000). The extractions are usually repeated three to five times and the combined organic phase dried with anhydrous sodium sulfate and concentrated to 0.1–0.5 ml (Escartin and Porte, 1999a; Jonsson *et al.*, 2003), or to dryness for some derivatization procedures (Krahn *et al.*, 1992).

Law *et al.* (1994) used a chromatographic cleanup step (Bond Elut C-18 cartridge containing 500 mg sorbent) to separate hydrolyzed PAC metabolites from the bile matrix. The metabolites were eluted with 100% methanol, evaporated to dryness, and the residues dissolved in derivatizing reagent [N,O-bis(trimethylsilyl)acetamide] before injection on the GC column. Additionally, Bond Elut C-18 columns were used as an alternative to successive liquid-liquid extraction (Hellou and Payne, 1987; Willett *et al.*, 2000) to separate conjugated from deconjugated metabolites. Furthermore, Willet *et al.* (2000) successively extracted the bile sample before and after treatment with pure ß-glucuronidase and arylsulfatase, providing information about the relative amounts of deconjugated metabolites, sulfates, and glucuronides.

3.1.3 Derivatization of hydroxy metabolites

Hydroxy metabolites are not especially amenable to analysis by GC/MS, so chemically modifying the hydroxy group to a less polar form improves the chromatographic characteristics of this type of metabolite (Jacob and Grimmer, 1988). Derivatization also prevents dehydration of dihydrodiols to the two corresponding phenolic compounds in the GC (Jacob and Grimmer, 1988; Krahn *et al.*, 1984; Krahn *et al.*, 1987). Therefore, hydrolyzed bile metabolites (hydroxy PACs) are frequently converted to trimethylsilyl ethers (TMS-ethers) or acetylates before GC/MS analysis.

There are several commercially available reagents that easily form TMS-ethers with alcohols, of which bis-(trimethylsilyl)trifluoroacetamide (BSTFA) is the most widely used reagent (Evershed, 1993). Hydroxy PACs are derivatized by adding 100–200 µl BSTFA to the concentrated organic extract (Jonsson *et al.*, 2003) or to the dried residues of the metabolites as described by Krahn *et al.* (1987) and heating the mixture for two hours at 60 °C. Trimethylsilylimidazole (TMSI) in pyridine (1:4) forms TMS ethers with phenolic PACs as well as with diols and tetrols within 15 minutes at room temperature (Day *et al.*, 1991; Jonsson *et al.*, 2004). Additional silylating reagents used for derivatization of PAC metabolites are 1,1,1,3,3,3-hexamethyldisilazane (SYLON TP) (Solbakken *et al.*, 1980) and N,O-

bis(trimethylsilyl)acetamide (BSA) (Law *et al.*, 1994). Acetylated hydroxy PACs are prepared for GC/MS analysis using ethereal diazomethane as described by Hellou and Payne (1986; 1987).

3.2 Instrumental determination of individual metabolites

3.2.1 GC/MS

Following enzymatic hydrolysis, extraction, and often a derivatization step as described above, quantitation of individual PACs metabolites can be achieved by GC/MS in electron impact (EI) mode. Standard chromatographic conditions are as follows. Columns, 30 m × 0.25 mm i.d. crosslinked 5% PH ME siloxane or similar (Escartin and Porte, 1999a; Stephensen *et al.*, 2003), are generally programmed from 80 ºC to 120 ºC at 15 ºC·min^{-1} and from 120 ºC to 300 ºC at 6 ºC·min^{-1}, holding the final temperature for 5–10 min. The carrier gas is helium at 80 kPa. The injector temperature is in the range of 250–280 ºC, and the ion source and the analyzer are maintained at 200–220 ºC and 250–280 ºC, respectively. The mass spectra are obtained at 70 eV and selected ion monitoring (SIM) mode is routinely used to get higher sensitivity. A selection of typical GC/MS methods is listed in Table 1.

Metabolites (derivatized or underivatized) are identified by comparing retention time and mass spectra with those of reference standards. Due to the limited number of standards available, compound identification is often based on interpretation of the mass spectra, which are characterized by the presence of the molecular ion and specific fragment ions. The spectra of both derivatized (e.g., TMS-ethers) and underivatized metabolites contain a prominent molecular ion (either a base peak or a peak with over 40% abundance compared to the base peak), as well as some other fragment ions. For derivatized metabolites the most abundant fragment ions include [M-15]+ and [M-31]+, and a strong [M-89]+ ion [-O-Si(CH3)3] that indicates the presence of a hydroxyl group (Yu *et al.*, 1995). For underivatized metabolites a major fragment ion is [M-29] + (Escartin and Porte, 1999a; Krahn *et al.*, 1992; Stephensen *et al.*, 2003).

The quantitation of individual metabolites is based on their GC/MS response relative to that of an internal standard. The GC/MS is calibrated by injection of standards of different concentrations. The molecular ion of the PAC metabolites, together with a major fragment ion, are used for quantitation, e.g., m/z 144,115 for 1-naphthol; m/z 182, 152 for 9-fluorenol; m/z 194, 165 for 9-phenanthrol, and m/z 218, 189 for 1-pyrenol (Escartin and Porte, 1999a; Stephensen *et al.*, 2003); or alternatively, those corresponding to the trimethylsilyl derivatives, e.g., m/z 216 for 1-naphthol; m/z 254,165 for 9-fluorenol; m/z 266 for 9-phenanthrol, and m/z 290 for 1-pyrenol (Yu *et al.*, 1995). The instrumental limits of detection (LODs) of the GC/MS/EI technique for underivatized metabolites—calculated for a signal-to-noise ratio of 3:1—were at the low pg level (4–9 pg), except for 1-naphthol (95 pg) and 1-pyrenol (68 pg) (Escartin and Porte, 1999a). For underivatized 3-OH BaP, the LOD is much higher, ca. 1000 pg (Jonsson *et al.*, 2003). For underivatized metabolites, run-to-run reproducibility ranged from 2 to 4%, with the exception of 1-OH Pyr (19%). The LODs for TMS-derivatives were 1.2, 2.4, and 6 pg for 2-naphthol, 1-phenanthrol, and 1-pyrenol, respectively. For 3-OH BaP, the LOD was 50 pg when analyzed with a clean ion source, but increased as the number of analyzed samples increased; in real samples the LOD was about 3000 ng/g bile (Jonsson *et al.*, 2003). For derivatized molecules, the run-to-run reproducibility for TMS ethers was less than 5% for 1-naphthol, 2-naphthol, and 1-phenanthrol, and less than 10% for 1-pyrenol (Jonsson, unpublished results).

The GC/MS technique, although limited by the few metabolite standards available, provides detailed information about which particular metabolites are present in the hydrolyzed sample and may therefore provide information about the probable source of those PACs. Crude and refined oils are enriched in parent and alkylated naphthalenes, phenanthrenes, and dibenzothiophenes, with lesser amounts of pyrenes and other large, non-alkylated PACs. The GC/MS analyses of bile from oil-exposed fish have often evidenced this PAC metabolite pattern (Figure 6, top) (Krahn *et al.*, 1987, 1992; Yu *et al.*, 1995). In contrast, the bile of fish exposed to PACs of pyrolytic origin are characterized by the presence of a relatively high amount of 1-OH Pyr and the absence of alkylated PACs (Escartin and Porte, 1999a) (Figure 6 bottom). Additionally, the GC/MS technique allows the detection of a number of nitroaromatic compounds, namely diphenylamine and several hydroxylated metabolites, 1,2-dihydro-2,2,4-trimethylquinoline, and N-(1-methylethyl)-N9-phenyl-1,4-benzenediamine, in the bile of fish exposed to other PAC sources, such as car tire rubber (Stephensen *et al.*, 2003).

3.2.2 Derivatized vs. underivatized samples

Not carrying out a derivatization step prior to GC/MS obviously saves time, but this approach should be followed with caution, especially when large series of samples are to be analyzed. Jonsson *et al*. (2003) reported a decrease in signal intensity (measured as peak area) within 25 serial injections of a number of underivatized hydroxy PACs and the decrease was more pronounced for 1-OH Pyr (>60%) than for 2-OH Naph (25%) and 1-OH-Phn (30%). At a concentration of 1 μg g^{-1}, 3-OH BaP could barely be observed in the chromatogram if underivatized. The formation of TMS derivatives, acetylates, or methyl ethers resulted in more symmetrical and narrower peaks as compared to underivatized compounds, and therefore improved separation and detection limits (Jacob and Grimmer, 1988; Jonsson *et al*., 2003). Furthermore, the tendency of dihydrodiols to decompose in the GC is avoided when the hydroxy groups are derivatized (Jacob and Grimmer, 1988; Krahn *et al*., 1987). However, the derivatization efficiency decreases as the number of fused rings increases, resulting in lower recovery and reduced sensitivity for larger hydroxy PACs (Jonsson *et al.*, 2003).

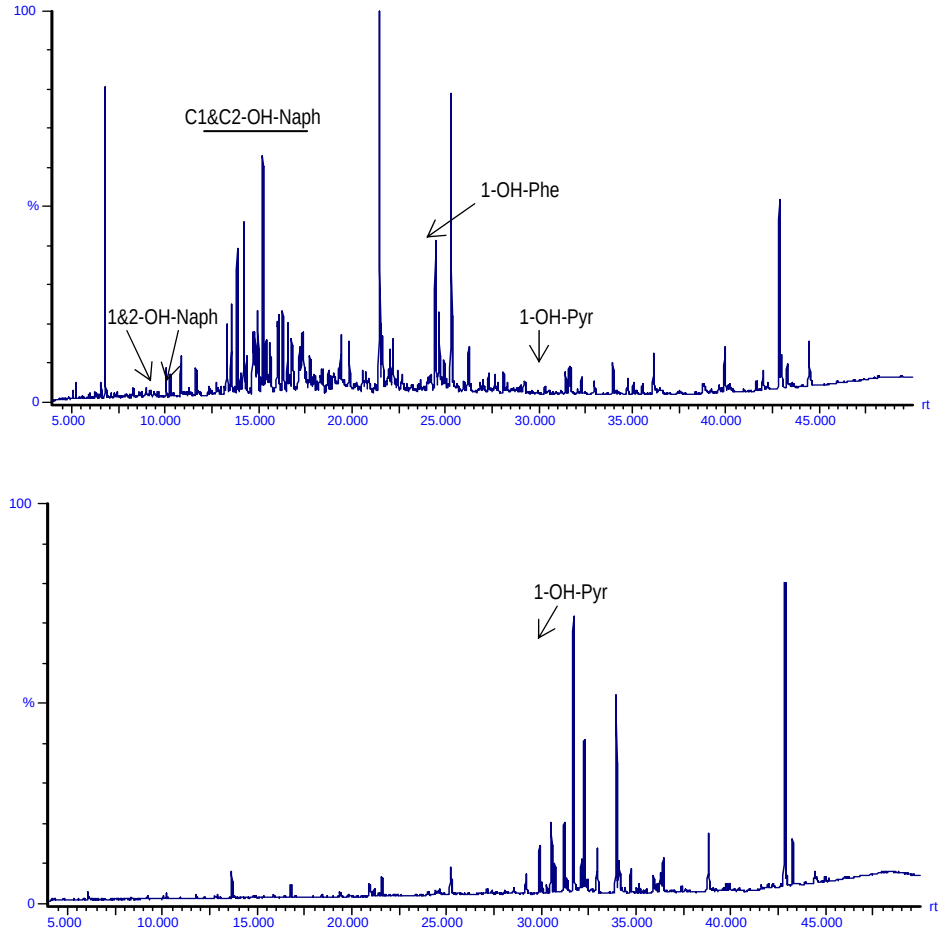

Figure 6. Chromatographic profiles (total ion current) obtained by GC/MS/EI SIM mode from a representative (underivatized) bile sample of fish exposed to PACs of petrogenic (top) and pyrolytic origin (bottom).

3.2.3 HPLC/fluorescence

As an alternative to GC/MS, the deconjugated hydroxy PACs can also be separated and quantified by means of HPLC/F. As described in Section 3.1.2 extraction is possible, but usually only a brief centrifugation step is carried out. Most laboratories apply reversed phase HPLC conditions and an acetonitrile/aqueous buffer gradient. Thermostated columns are preferred to avoid slow drifts of retention times. For the homogeneity and stability tests of the two CRMs (see Sections 5.3 and 5.4), the following typical conditions were used. The deconjugated PAC metabolites in the hydrolyzed bile samples were determined using a thermostated Vydac 201TP54 25/4.6 (Aurora, USA) column and an acetonitrile/water gradient. The fluorescence excitation/emission wavelength settings were programmed for optimal detection of each target analyte: 2-OH Naph, 325/358 nm; 1-OH Phen, 269/380 nm; 1-OH Pyr, 346/384 nm; anthracene-d10 (internal standard), 250/400 nm; 3-OH BaP, 385/450 nm. External calibration was used, based on peak heights. A calibration solution (75 ng ml^{-1} level) was analyzed after each 10 injections to check for possible instrument drift. A selection of typical HPLC approaches, using fluorescence or other detection methods, is listed in Table 2.

HPLC/F methods generally offer excellent sensitivity, especially for the larger PACs. Typical LODs for 3-OH BaP are 2–10 ng g^{-1} bile. On the other hand, the chromatographic resolution of HPLC is poorer than that of GC and the fluorescence detector does not add sufficient extra selectivity. Therefore, HPLC/F chromatograms tend to be very crowded and in some regions show considerable overlap, especially for smaller, two-ring PAC metabolites in bile from oil-exposed fish.

The specific analysis for conjugated metabolites in non-hydrolyzed bile is not a common part of monitoring studies, but could be important in special cases, such as studying the spontaneous hydrolysis of pyrene-1-glucuronide into 1-OH Pyr upon long-term storage at ambient temperature (Ariese *et al.*, 2000) (see also Section 4.2). Stroomberg and coworkers optimized the separation of conjugated PAC metabolites using ion-pair conditions (acetonitrile gradient at pH =2 with 5 mM tetrabutylammonium bromide as ion pair reagent in the buffer) for phase-2 metabolism studies (Stroomberg *et al.*, 1999).

3.2.4 HPLC/MS

The combination of HPLC separation with the selectivity of an MS or MS/MS detector constitutes a very powerful tool for quantitative and qualitative analysis of water-soluble and thermolabile compounds (Ferrer and Barcelo, 1998; Voyksner, 1994), such as conjugated metabolites (Andreoli *et al.*, 1999; Capotorti *et al.*, 2004; Law *et al.*, 1994; Simpson *et al.*, 2002) and PACs containing one or more free hydroxy groups (Galceran and Moyano, 1996; Willett *et al.*, 2000). HPLC/MS has played an important role in environmental analysis for many years, particularly for pesticide residue analysis in various sample materials (Ferrer and Barcelo, 1998; Niessen, 1999). HPLC/MS/MS combines the advantages of HPLC (simple sample preparation, suitability for polar species) with the extra selectivity of (tandem) mass spectrometry.

The use of HPLC/MS for the determination of PAC metabolites in fish bile has not yet been routinely implemented, even though this technique has many advantages, e.g., identification of different conjugates and a sensitive determination of poorly fluorescent PAC metabolites (diones) (Doerge *et al.*, 1993; Koeber *et al.*, 1997; Willett *et al.*, 2000). According to Oehme *et al.* (2002), HPLC/MS is still not optimized for trace analysis due to potential problems caused by adsorption, contamination, surface reactions, or other interferences. Using HPLC/MS, both conjugated and unconjugated PAC metabolites can be determined by various ionization techniques, such as electrospray (ESI), atmospheric pressure chemical ionization (APCI), thermospray (TSP), and particle beam (PB). Today, ESI and APCI are by far the most frequently used ionization techniques and they can both be operated in negative and positive ionization mode (Voyksner, 1994; Willoughby *et al.*, 1998).

To our knowledge, there are only a few publications available where HPLC/MS has been used to determine PAC metabolites in fish bile. For example, Law *et al.* (1994) used HPLC/MS technology to characterize metabolites in bile, as well as in urine of pyrene-exposed trout (Oncorhynchus mykiss). Conjugated and unconjugated metabolites were first separated on an HPLC reversed-phase column, followed by identification using HPLC/APCI/MS. In a later study, Willet *et al.* (2000) were able to determine 3-OH BaP, two BaP-dihydrodiols and three BaP-diones in deconjugated fish bile using HPLC/APCI/MS. Diones are not easily determined by HPLC/F due to their low fluorescence intensity (van Schanke *et al.*, 2001), which underscores the importance of HPLC/MS as an alternative analytical method for these compounds. HPLC/APCI/MS detection limits were excellent for BaP-diones (1 ng) and also very good for BaP-dihydrodiols and 3-OH BaP (2.5–25 ng) (Willett *et al.*, 2000).

Oxidized PACs have been determined in clam tissue (Simpson *et al.*, 2002), cricket excreta (He *et al.*, 1998), bovine (Ferrari *et al.*, 2002), and human urine (Andreoli *et al.*, 1999) by HPLC/ESI/MS and in fish bile (Law *et al.*, 1994; Willett *et al.*, 2000), air particulate matter (Koeber *et al.*, 1999), and atmospheric aerosol samples (Letzel *et al.*, 1999) by HPLC/APCI/MS. These publications demonstrate the potential of HPLC/MS for analysis of conjugated and unconjugated PAC metabolites in environmental monitoring.

3.2.5 Other analytical techniques

The applicability of capillary electrophoresis with fluorescence detection (CE-F) was demonstrated by Kuijt *et al*. (2001). In order to achieve the separation of neutral compounds, a cyclodextrin-modified micellar electrokinetic chromatography mode (CD-MEKC) was used. Both conventional (lamp-excited) and laser-induced fluorescence (LIF) were tested. LIF detection at 266 nm yielded excellent detection limits (2–6 ng ml^{-1}), but for complex mixtures, a tuneable laser system would be more selective. When analyzing a complex PAC metabolite mixture in a bile sample from oil-exposed plaice (*Pleuronectes platessa*) a very good separation was obtained. The elution order was very different from that of reversed-phase HPLC. The technique was used to separate conjugated metabolites of pyrene. A major advantage would be that only minute sample volumes are required (for instance for the analysis of bile from very small fish), but so far the method has not been applied to monitoring studies.

Ariese *et al*. (1993b) demonstrated that the specificity of fluorescence spectra can be dramatically increased in frozen, crystalline n-octane matrices at cryogenic temperatures (Shpol'skii spectroscopy). Under such conditions, spectral bandwidths improve from typically 10 nm to 0.1 nm, and fingerprint fluorescence spectra are obtained. For example, the Shpol'skii spectra of all 12 hydroxy BaP isomers are easily distinguished. Compounds can thus be determined in a mixture without chromatographic separation. The selectivity and sensitivity was improved even further by means of site-selective laser excitation (using a tuneable excimer laser/dye laser combination) and time-resolved detection. After enzymatic hydrolysis and extraction, hydroxy PAC metabolites were methylated using methyliodide and frozen in n-octane to 23 K. In real samples, extremely low LODs down to 0.005 ng ml^{-1} bile were obtained, and 3-OH BaP could even be detected in fish bile from control sites. A disadvantage is that the method requires a rather complex spectroscopic setup and the limited tuning range of most dye lasers limits the ability to determine different metabolite classes in the same sample. It will be most useful for the determination of compounds that are present at very low levels, or when isomer-specificity is crucial.

Supercritical fluid chromatography in combination with APCI/MS has been used for the characterization of hydroxy PACs. These compounds were separated on a reversed-phase column and mass spectra were obtained in both positive and negative ionization modes. Proton addition and proton abstraction were the common routes of ionization and losses of CO and H2O occurred. Transformation to quinone structure was observed for some hydroxy PACs. Run-to-run reproducibility was good (2 to 12% RSD), but detection limits were at the ng level (Moyano *et al.*, 1997).

GC/MS is usually not a favourable analytical technique for larger metabolized PACs, such as BaP, due to thermal decomposition and poor sensitivity (Jacob and Grimmer, 1988; Jonsson *et al.*, 2003). However, diols and tetrols of several four- and five-ring PACs have been successfully determined as polymethyl- (Simpson *et al.*, 2000), trimethylsilyl- (Day *et al.*, 1991), or pentafluorobenzylbromide (Väänänen *et al.*, 2003) derivatives when ionized in negative chemical ionization mode.

Tyrpień (1996) used thin-layer chromatography (TLC) to determine hydroxy PACs in airborne particulate matter. The TLC spots could be detected under UV illumination at λ 254 and 365 nm in low ng concentration after visualization with a colouring reagent. TLC is a relatively simple analytical technique that can be optimized for analyses of hydroxy PACs by careful selection of the stationary phase and the mobile phase composition (Coman *et al*., 1997).

4 QUALITY CONTROL OF ANALYSES FOR PAC METABOLITES IN FISH BILE

In the previous sections several analytical techniques have been discussed that have proven suitable for PAC metabolite analysis in fish bile. When applying such techniques for monitoring studies, reproducibility (precision) and accuracy of the data are crucial, and certain quality control measures need to be implemented.

The required level of analytical quality depends, of course, on the purpose of the study. So far, most monitoring studies involving PAC metabolites in fish bile samples were carried out by a single institute using a single analytical method and an in-house analytical protocol. Obviously, if the purpose of such studies is to map the environmental distribution of PAC pollution by studying relative differences in PAC metabolite levels between samples obtained at different sites, short-term reproducibility (minimization of random errors) will be most important. Long-term reproducibility is essential when such monitoring studies are conducted over several years. Quality requirements will be most stringent for studies involving several institutes or different methods—in those instances it is also essential that there is no significant interlaboratory bias (systematic error). In the following section we will discuss the various options for quality control for PAC metabolite analysis.

4.1 General considerations of quality control

Fish bile typically contains a very large number of compounds, and quantitation of individual compounds is often difficult due to chromatographic overlap. Compounds absorbing/emitting in the same wavelength region may cause interference in the case of HPLC/F, whereas isomers or fragments with identical masses may cause interference in the case of MS detection. Other potential analytical problems can be related to a poor signal-to-noise ratio (e.g., for some less abundant compounds or for samples from unpolluted sites), instability of the sample, instability of the calibration solutions, incomplete hydrolysis, incomplete derivatization (for GC), random errors from weighing and/or diluting, solvent evaporation, matrix effects, random variations in injection volume, non-linearity of detector response, poor recoveries, or the difficulty of accurately determining recovery correction factors. Many of these potential error sources occur in other types of chemical analyses as well, and many of the standard quality control measures that can be found in analytical chemistry textbooks can be applied to PAC metabolite measurements. Therefore, in this section these measures will only be briefly discussed, with emphasis on aspects that are specific to PAC metabolite determination. Although issues related to sampling protocols and experimental design are beyond the scope of this paper, we would like to stress that when designing a monitoring campaign one should always take into account the biological variability and the analytical error. The latter should not contribute significantly to the total variability. If the total number of analyses is limited due to analytical capacity constraints, analyzing more individual samples may be more useful than carrying out several replicate analyses per sample.

4.2 Sample stability and storage

Sample stability during sampling, shipment, and storage has traditionally been a matter of great concern, especially when cooling facilities are limited in the field. However, in our experience, PAC metabolites in fish bile samples are reasonably stable, even at ambient temperatures. For the preparation of the two CRMs (see also Section 5) the stability of PAC metabolites in fish bile was tested at four different temperatures for a period of 13 months and the results were very encouraging (Ariese *et al*., 2004). The main conclusions (samples were measured after enzymatic hydrolysis) were that no degradation was observed 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 with non-hydrolyzed samples showed a significant decrease in the pyrene-1-glucuronide levels after a few months at room temperature. Apparently, at ambient temperatures hydrolysis of the conjugate can occur, but the resulting deconjugated phase-1 metabolites are not very sensitive to further degradation in such samples. When the analytical method is aimed at hydroxy metabolites after enzymatic deconjugation, such spontaneous hydrolysis would never be noticed. The long-term stability has not been specifically tested for all possible PAC metabolites, but all experts taking part in the recent fish bile certification project (see Section 5) agreed that PAC metabolites do not seem to be very sensitive to degradation as long as they are in the fish bile matrix. The latter probably contains large amounts of anti-oxidants. For long-term stability and for the storage of reference materials, −20 °C or colder is preferred, but brief exposure to ambient temperatures during sample preparation or shipment is not likely to cause any significant degradation (Ariese *et al*., 2004). Especially when sending samples by express mail, the use of frozen icepacks is an attractive alternative to shipment on dry ice.

It should be stressed that PAC metabolites in clean solvents are much more prone to degradation than the same compounds in the fish bile matrix. Calibrant solutions should therefore be prepared fresh, and a suitable antioxidant (e.g., 0.5% ascorbic acid in water/methanol, or 1.5% butylhydroxytoluene (BHT) for nonpolar GC-solvents) should be added for protection. Matching the vial size with the solution volume is also recommended in order to reduce the amount of air (oxygen) above the sample. To save time and reduce the weighing error, a larger batch of calibrant solution could be prepared and subdivided over many vials before freezer storage.

4.3 Incomplete hydrolysis

As described above, most analytical methods aimed at quantifying individual metabolites will start with a deconjugation step to hydrolyze the glucuronides or sulfates (i.e., to liberate the hydroxylated phase-1 metabolites). In most laboratories, enzymatic hydrolysis is done using β-glucuronidase with arylsulfatase activity. An obvious potential source of error could be the incompleteness of this step, so a test was made to determine how critical this step was. Twelve participating laboratories received a test solution of pyrene-1-glucuronide to be spiked to "blank" bile, and were asked to check the 1-OH Pyr yield after different incubation times (their normal incubation time, half that long, and twice that long). The results showed no indication of incomplete hydrolysis or losses upon extended incubation, indicating that in all laboratories the hydrolysis is well under control and is not a critical step (Ariese *et al*., 2000).

4.4 Incomplete derivatization

As described above in Section 3.1.3, for most GC methods a derivatization step (e.g., with BSTFA) will be performed in order to increase the volatility (especially for the heavier PAC metabolites) and improve the chromatographic peak shape. Such derivatization reactions may not have a 100% yield, and if the losses are significant, a correction should be made. This bias will be partly compensated for if the calibrants are also derivatized with the same reagent under

similar conditions. Nevertheless, it should be realized that the yield of such derivatization reactions may not be the same in real samples and in clean solutions and a recovery check should be carried out, for example, by spiking a "blank" bile with known amounts of PAC metabolites. Alternatively, a hydroxylated compound that does not normally occur in fish bile samples could be used as the internal standard, assuming its yield in the derivatization step matches that of the analytes. As a rule, even if losses are in principle corrected for, one should always strive for maximum yield in order to reduce the impact of random errors. Derivatization is not needed for HPLC.

4.5 Internal standards

In many analytical schemes it has become common practice to add a known amount of a suitable internal standard to the sample at the onset of the sample preparation step. In some cases a second internal standard is added just prior to the final separation/detection step. Ideally, an internal standard should resemble the analyte(s) as closely as possible and thus all random or systematic errors that may occur during the analytical procedure (e.g., weighing/dilution errors, solvent evaporation, incomplete derivatization, variations in injection volume or detector response) will affect the internal standard to the same extent as the analyte. Furthermore, the internal standard should normally not be present in real samples, and should not interfere with the analyte determination.

As described by Escartin and Porte (1999b), Jonsson *et al*. (2003), and Krahn *et al*. (1987), an internal standard can be added before the hydrolysis step for quantitative determination of PAC metabolites. The selected internal standard depends on the analytical method to be used and the metabolites of interest. For GC/MS quantitation, α-naphthol (Solbakken *et al*., 1980), 2,6-dibromophenol (Krahn *et al*., 1987), n-pentadecane (Hellou and Payne, 1987), octadecane (Stephensen *et al*., 2000), and 4-chloro-1-naphthol (Jonsson *et al*., 2004) have been utilized. Furthermore, Yu *et al*. (1995) used both deuterated hydroxy PACs and PACs in addition to 2,6-dibromophenol as internal standards for GC/MS determination of a number of bile metabolites. Other internal standards are listed in Table 1.

For HPLC/F determination of hydroxy PACs, some common error sources (e.g., incomplete derivatization, extraction, solvent evaporation) play no role, and therefore internal standards appear to be used less frequently (Richardson *et al*., 2001; Ruddock *et al*., 2002; van Schanke *et al*., 2001) compared to GC/MS analysis. Willett *et al*. (2000) used 6-OH chrysene as an internal standard for the determination of BaP metabolites and Jonsson *et al*. (2003; 2004) used perylene and triphenylamine for quantitation of chrysene metabolites and two- to five-ring metabolites, respectively. Other possible internal standards are listed in Table 2.

It should be realized that the use of an internal standard does not in all cases reduce the total error. That is because the addition of a known amount of internal standard and its determination implies an extra measurement and will in itself carry a certain error. In the absence of major error sources the total error after correction for the internal standard may even become larger. In one particular case, we noticed that when using anthracene-d10 as the internal standard in HPLC/F, the internal standard peak was a little sharper than those of the analytes and during a long series of chromatographic runs, column deterioration affected the peak width and height of the internal standard more than that of the analytes (IVM-Amsterdam, unpublished results). Such cases can only be detected when the samples or calibrant solutions are analyzed more than once. We recommend that an internal standard should always be included, but based on expert judgment, the correction may not always be applied.

4.6 Peak purity and interference

Fish bile samples from the field may contain hundreds of PAC metabolites, so chromatographic separation of the target analytes may not always be possible. The MS detector offers the possibility of checking the peak purity based on the mass spectrum and sometimes tandem MS can be used for extra selectivity. Interferences are a larger problem in the case of HPLC/F, due to poorer chromatographic resolution and the limited selectivity of the fluorescence detector. A simple but useful control step is a coelution experiment: add the tentatively identified analytes to the sample and check to see if the target peak coincides exactly with that of the spike. For HPLC, thermostated columns are strongly recommended to keep the retention times as constant as possible.

4.7 Matrix effects and calibration

The matrix constituents of a fish bile sample may influence the quantitative determination of PAC metabolites in various ways. For the rapid fluorescence screening methods based on SFS or FF, there may be negative bias due to the "inner filter" effect (absorption of excitation and/or emission light) if the samples have not been sufficiently diluted. Furthermore, the yield of derivatization reactions may be lower for real samples than for clean solutions. Chromatographic peak shapes and the detector response may also be affected by the matrix. There are several approaches that correct for these effects, depending on the type of calibration used.

In the case of external calibration, a calibration series is measured at the beginning of an analytical run. In addition, it is recommended that one calibration solution be measured repeatedly (e.g., after every ten injections) in order to check for slow instrument drift, column deterioration, etc. Each sample is measured only once and the result (often after ratioing with the internal standard) is compared to the external calibration curve. If the calibration curve is made in clean solvent, it may be necessary to investigate possible matrix effects in detail and correct the final data for incomplete recovery. As an alternative, it is also possible to prepare the calibrant solution in a blank matrix, although in practice it will not be easy to obtain a true blank that does not contain measurable levels of PAC metabolites and has exactly the same level of interferences as the sample. Both in the case of recovery correction and in the case of calibrant solutions in a fish bile matrix, the matrix chosen should be as close as possible to the matrix in real samples. That may not always be realistically possible, especially because fish bile samples from the field will show a broad range of accumulated PAC levels. In such cases the matrix correction can only serve as an approximation. Optimizing the analytical conditions in such a way that matrix effects become insignificant and need no correction is, of course, always the better option.

An option to deal with matrix effects—albeit labor-intensive—is to use a standard addition approach for calibration. Each sample is measured first as is and then after addition of a known amount of analyte, preferably at two or three levels. The spiked amounts should be similar to the amount in the sample. The detector sensitivity in the presence of matrix can be derived from the increase in detector response as a function of the spiked amount. Standard addition is an excellent approach to correct for matrix effects, but a major drawback is the fact that each sample needs to be measured 3–4 times. Understandably, external calibration is preferred by most laboratories, in particular for large series of samples.

4.8 Laboratory reference materials and control charts

Laboratory reference materials (LRMs; often shortened to "reference materials"—RMs; also known as "control materials") play an important role in every quality control scheme. They can be used for method optimization, for statistical purposes, e.g., long-term reproducibility checks, or for interlaboratory comparisons. RMs can be pure compounds, calibrant solutions, extracts, spiked samples, or real samples, each with different purposes. Here, we will mainly focus on the latter category.

At the initial stage of method optimization, standard solutions could be used but this should be done with care. For instance, in HPLC/F a short excitation wavelength may give the best sensitivity for simple solutions, but for real samples it is often better to use a longer, more selective wavelength. One should therefore not wait too long before changing to more complex samples for further optimization. One very useful set of RMs was developed at the onset of the certification of BCR 720 and BCR 721 (see Section 5.2). A few Atlantic cod (Gadus morhua) and flounder (Platichthys flesus) from a relatively unpolluted area were dosed with a single PAC via intraperitoneal injection, typically at the 1 mg kg^{-1} level. A few days after injection, bile samples were obtained with natural levels of matrix interferences, but relatively high levels of the metabolites of a single PAC. Once a method has been fully optimized, quality control measurements should preferably not be performed on clean calibrant solutions, because such samples will generally be much too easy to handle in terms of interferences and therefore may give an overly optimistic impression of the method's performance. Instead, samples (RMs) should be used that have as similar a matrix to real samples as possible.

In order to check the long-term reproducibility of the method, Shewhart-type control charts and RMs are used in many laboratories. In order to prepare a suitable RM, a relatively large sample is collected, thoroughly homogenized, and analyzed a number of times to establish the mean x and standard deviation σ. Typically, warning limits are set at $x \pm 2\sigma$ levels, and action limits at $x \pm 3\sigma$ levels. The sample is then stored under conditions of optimal stability (for fish bile: –20ºC or colder, preferably distributed in separate vials or ampoules). At regular intervals or with each analytical series a subsample of the RM is analyzed; a result outside the action limits is an indication that perhaps the whole series measured on that particular day needs to be repeated. Smaller, but repeated deviations from the average may point at a slow drift in performance (for instance a slow decrease in the activity of the enzyme used for hydrolysis) and also calls for further investigation.

For an RM to be suitable for long-term quality control, it needs to meet a number of requirements. First of all, the RM needs to be representative in terms of matrix interferences, analyte pattern and content, physical status, and analyte-matrix interactions. Furthermore, the RM needs to be highly homogeneous (at least at the level of the typical sample intake) and long-term stability is crucial, because using the same RM for several years is preferable. The analyte content should not be very close to the detection limit, because at that level the random detection error becomes very large and may obscure all other possible sources of error.

In principle, certified reference materials (CRMs) me*et al*l these requirements and they could be used for this purpose, but they are often too expensive for regular use. Luckily, it is relatively easy to produce a non-certified fish bile RM for PAC metabolite analysis and many laboratories have done so themselves. Homogenization is not a major problem for liquid materials and no special treatment of the bile material is necessary in order to stabilize the analytes. The RM should be comparable to the type of samples that the laboratory most often encounters. For instance, if a laboratory is frequently involved in monitoring the impact of marine fuel spills, an RM should be produced from fish bile with the metabolite patterns that result from exposure to marine fuel in the field or in the laboratory. By pooling and homogenizing a number of bile

samples a sufficiently large volume should be available to last as RM for several years. For example, an interesting source of bile was used by researchers at NOAA following the 1989 Exxon Valdez oil spill in Alaska. Thousands of bile samples needed to be analyzed to assess exposure of fish and marine mammals to the crude oil. When a number of marine mammals that had been oiled died in a rehabilitation facility, bile was collected and shipped to the NOAA laboratory. The bile from one harbor seal (more than 0.5 l) was carefully homogenized and portions (approximately 0.5 ml) were sealed in ampoules for use as the laboratory CM for several years.

Of course it should be realized that quality control using a non-certified RM can be very useful to monitor precision and long-term reproducibility, but systematic errors may remain unnoticed. In case such a systematic inaccuracy is part of a strict analytical protocol, it is possible that all values in the control chart and the reference values will show the same deviation from the true value. Accuracy problems can only be identified by taking part in interlaboratory comparisons or by using a CRM.

4.9 Intercomparison exercises

One way to check the accuracy of a method is to take part in laboratory proficiency tests or intercomparison exercises. A central laboratory prepares one or several thoroughly homogenized samples and distributes these, together with the necessary measurement instructions, to the participating laboratories. The participants analyze the sample a number of times and report the data to the coordinator by a given deadline. The results are then statistically analyzed, and the participants can check how their results compare to the assigned reference values. These reference values are only known in the case of standard solutions, otherwise the results from an expert laboratory or the mean of all participants after technical scrutiny can be used as the assigned value. In case the results show a bimodal or multimodal distribution, the statistical model of Cofino *et al.* (2000) can be used to determine the assigned value. This method calculates the point of maximum overlap between submitted datasets, without the necessity of rejecting outliers. In the case of a bimodal distribution of datasets, the method will produce two possible maxima, rather than an average between the maxima. Unless all participants use exactly the same analytical protocol (and apply it correctly) any significant systematic error in one of the methods will become apparent. Preferably the exercise ends with a technical meeting, where possible error sources are openly discussed, and analysts can obtain useful advice from their colleagues. Sometimes a series of intercomparisons is organized, starting with relatively easy samples and gradually moving to more complex ones. For example, intercomparison exercises are routinely sponsored by NOAA's National Institute of Standards and Technology (NIST) each year in which participating laboratories measure organochlorine contaminants in fish and marine mammal tissues, as well as PACs in invertebrate tissues and sediments (Parris *et al.*, 2002; Rowles *et al.*, 2002). The QUASIMEME office in Aberdeen (UK) has organized several proficiency tests for PAC metabolites in fish bile; information can be found at http://www.quasimeme.marlab.ac.uk/.

5 CERTIFIED REFERENCE MATERIALS FOR PAC METABOLITE ANALYSIS IN FISH BILE

5.1 Need for certified reference materials

As discussed in the previous section, there are only a few options available to check for systematic errors in an analytical procedure. One of the best approaches is to analyze a CRM and compare the outcome to the certified value. Until recently, such a material did not exist for

PAC metabolites in fish bile, hampering the quality control and especially the intercomparability of data obtained in different laboratories.

For non-certified RMs, the material should be as close as possible to typical real samples in terms of matrix constituents, metabolite patterns and levels. Fish in aquatic environments dominated by combustion-related anthropogenic sources (deposition of soot particles, urban run-off) will be exposed to relatively high levels of larger, non-alkylated PACs, whereas petrochemical pollution will contain more of the lighter (2–3 rings), alkylated PACs. These different sources will be reflected in the PAC metabolite pattern in the bile. A single CRM would not be able to mimic these two very different types of bile in terms of relative PAC metabolite levels and levels of interferences. Within the EU "Standards, Measurements and Testing" program, a project was started in 1998 to carry out a series of experiments (feasibility phase), produce a pyrogenic and a petrogenic fish bile CRM, and certify a number of individual hydroxy PAC metabolites in these CRMs. A group of twelve institutes was assembled to take part in a series of intercomparisons and to ultimately participate in the certification campaign. A selection of target analytes—based on expected concentrations of these compounds in fish bile, availability of standards, and ecotoxicological relevance—was narrowed down during the feasibility phase to the four compounds shown in Figure 3.

5.2 Preparation of the two candidate CRMs

For a matrix CRM to be as useful as possible for analytical quality control, its constituents (analytes and interferences) should preferably be very similar to those encountered in real samples. It was therefore decided not to prepare a bile material spiked with a number of hydroxy PACs, or a fish bile material from fish exposed to a limited number of parent PACs. Instead, large groups of fish were exposed in large mesocosms to "normal" PAC mixtures both from a polluted harbor sediment and from crude oil.

For the preparation of BCR 720 (sediment-exposed flounder bile), a sediment was collected in IJmuiden harbor, the Netherlands. This harbor contains fairly high PAC levels due to heavy shipping (entrance to Amsterdam port area) and nearby coke oven installations. The sediment was subsampled and was analyzed by means of HPLC/F. As expected, the sample contained mostly non-alkylated PACs typical of combustion sources. Groups of flounder (P. flesus) from relatively unpolluted areas in the southwestern part of the Netherlands were exposed to a 6-cm layer of this sediment in 2-m diameter tanks. The exposure was carried out in several batches, each lasting typically 10–14 days. The fish were not fed during the exposure in order to stimulate the accumulation of bile. The flounder were killed by a blow to the head and bile (total amount 430 g) was collected from the gall bladders with a syringe, immediately cooled on ice and then stored at –60 °C.

For the preparation of BCR 721 (oil-exposed plaice bile) plaice (Pleuronectes platessa) were used from a relatively unpolluted area south of Stavanger, Norway. During several weeks of acclimation the fish were fed thawed frozen shrimp until one week before the exposure start. The fish were exposed in four large tanks (each measuring 100 × 100 × 40 cm) to crude North Sea oil (Frøy) added in the form of micro-droplets mixed with seawater (1 ppm) using a high-pressure mixing valve. Oil droplet distributions were determined using a Coulter Multisizer II counter, the major droplet size was in the 3–30 μm size range. The concentration of dispersed oil droplets was usually within the 0.80–1.10 mg kg^{-1} range, with a min-max range of 0.737–1.5 mg kg^{-1}. After two weeks of exposure, the plaice were killed by a blow to the head and the bile (total amount 523 g) was collected, immediately stored on ice and then frozen to −80 °C. The two fish bile materials were transported on dry ice to the Institute for Reference Materials and Measurements (IRMM; Geel, Belgium) for the preparation of the two candidate reference materials according to the following scheme:

1. Vials containing bile were thawed to 4 °C overnight.
2. The bile was centrifuged at 3000 rpm for 5 minutes to precipitate any undesired solid material, with temperature monitoring of the centrifuge at 4 °C.
3. Bile was decanted and homogenized under nitrogen flushing for 50 minutes at 4 °C, using a magnetic stirrer.
4. DURAN glass ampoules (3-ml irradiated) were filled with bile and sealed under argon in 0.3 ml aliquots (cooling and nitrogen flushing was continued during this phase).
5. Vials were labeled and subsequently stored at –30 °C.
6. The final products were 1200 units of BCR 720 and 1320 units of BCR 721. A random sub-selection was used for the certification, stability, and homogeneity tests.

5.3 Homogeneity test

An analytical method (see Section 3.2.3) consisting of enzymatic hydrolysis, centrifugation, and HPLC/F was used for the homogeneity and stability tests. All pipetted amounts were checked with a balance and the actual dilution factors were determined by weight rather than by volume. Fifteen ampoules of each CRM were analyzed in duplicate in a single analytical series (that is, under optimum repeatability conditions). A standard calibrant solution was analyzed after each 10th injection to check for possible instrument drift. The CRMs were considered homogeneous if the between-bottle variations were not significantly larger than the within-bottle variations. The results showed that 1-OH Pyr and 1-OH Phen were homogeneous in both materials and 3-OH BaP in BCR 720 (Ariese *et al*., 2004). The concentration of 3-OH BaP in BCR 721 was below the detection limit. The HPLC determination of 2-OH Naph suffered from severe chromatographic overlap, both in BCR 720 and of BCR 721. Therefore, no homogeneity data were obtained for this compound, but inhomogeneity problems are in fact not expected because the two CRMs are liquid-state samples. Nevertheless, it is recommended that each time a frozen bile sample is thawed it should be re-homogenized by a brief sonication step.

5.4 Stability test

In order to carry out the stability test under optimum repeatability conditions, an isochronous strategy (Lamberty *et al*., 1998) was applied so that all samples were analyzed at the end of the test in a single analytical series. Four temperature conditions were tested: –20 °C (freezer), +4 °C (refrigerator), room temperature (+20 °C, in the dark), and +40 °C (thermostated oven). At specific time points (1, 2, 4, 7, and 13 months) prior to the end of the experiment samples were taken from the –70 °C freezer and transferred to the above-mentioned temperatures. The +40 °C measurements were only done for 1 and 2 months of storage. The average (n = 6) concentrations found in the samples stored continuously at –70 °C were taken as a reference and set at 100%.

At the end of the 13-month stability test, all samples were analyzed by HPLC/F as described above. The samples were found to be remarkably stable: 1-OH Pyr, 1-OH Phen, and 3-OH BaP showed no sign of degradation at –20 °C, +4 °C, and even at +20 °C. Again, no reliable data could be obtained for 2-OH Naph due to coelution in both CRMs, and the level of 3-OH BaP in BCR 721 was below the LOD. The long-term stability will continue to be monitored. Since slow degradation could become noticeable for longer storage times, we recommend that RMs and CRMs be stored in the freezer (−20 °C or colder) as a precaution.

5.5 Certification measurements

After taking part in a series of intercomparison exercises to check and optimize their methods, a group of twelve expert laboratories was selected for the certification campaign. No analytical protocol was supplied to the participants; instead each laboratory was encouraged to use its own established procedures, thus reducing the risk that systematic errors would lead to a bias in the certified value. All procedures and measurements were reported and openly discussed.

The following materials and tasks were given to each participant:

- Three ampoules of BCR 720 for six independent measurements of the four target metabolites in two ampoules, measurements to be performed on at least two different days.
- Three ampoules of BCR 721 for six independent measurements of the four target metabolites in two ampoules, measurements to be performed on at least two different days.
- A mixed spiking solution of known concentrations and a "blank" bile sample (from a control site) for the recovery experiments and the blank controls.
- Calibrants (as pure compounds): 2-OH Naph (Sigma-Aldrich, purity >99%); 1-OH Phen (Promochem, purity >99%); 1-OH Pyr (Sigma-Aldrich, purity >98%); 3-OH BaP (NCI-Midwest Research Institute, purity >99%).

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. All standards and samples had to be prepared on a mass basis. The calibrant solutions were to be measured together with the fish bile samples in the same analytical sequence. The working range of the detector had to be established for each analyte from calibration solutions of different concentrations (at least 4 calibration points). The calibration was verified at least once in a sequence of injections on each measurement day. Peak identification was confirmed by means of standard addition experiments.

Blanks were analyzed each day. Because the "blank" fish bile sample was collected from flounder caught in a relatively unpolluted area, low levels of some PAC metabolites (especially 1-OH Pyr) were observed. The measured recoveries found after spiking the blank bile (three levels in triplicate) were corrected for these values. The recovery was calculated as the average of the nine spiking experiments and used to correct the final reported concentrations. No correction was applied in situations where the recoveries are automatically corrected for (e.g., in standard addition approaches), or when the recovery was close to 100%, in order to avoid a situation where correcting would mainly increase the total uncertainty. After hydrolysis, the laboratories using HPLC/F applied a simple centrifugation step followed by direct injection of the supernatant. Three out of four GC laboratories derivatized the hydroxy PACs to improve separation and sensitivity.

The results of the certification study were entered into the automated QUASIMEME database. The outcome was first assessed by the certification organizers and then discussed at a technical meeting with all participants. All steps of the procedures were reviewed in detail and chromatograms were thoroughly checked regarding peak identity and chromatographic resolution. During the meeting some datasets were withdrawn, but this was done only on the basis of the technical discussion, never on purely statistical grounds. The complete set of results is given in the certification report (Ariese *et al.*, 2004).

BCR 720 (sediment-exposed flounder bile)

- 2-OH Naph was present at relatively low levels, and only six laboratories submitted data for this compound. Three datasets were withdrawn, mostly because of signs of coelution. Data from three laboratories (all using GC/MS) were accepted; the mean concentration was 46 ± 11 ng g^{-1}. 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 Sections 5.3 and 5.4), certification was NOT recommended.
- 1-OH Phen was measured by seven laboratories. As a result of the technical discussion, three datasets were withdrawn because of coelution or poor recovery. The results from four laboratories: HPLC/F (2 sets) and GC/MS (2 sets) were accepted and certification was recommended.
- 1-OH Pyr is a major compound and thirteen data sets were submitted. The results of 11 datasets were accepted [HPLC/F (7 sets) and GC/MS (4 sets)], and certification was recommended.
- 3-OH BaP is a minor metabolite (as in most fish bile samples), and only six laboratories submitted certification data. Data from five laboratories [HPLC/F (4 sets) and GC/MS (1 set)] were accepted; certification was recommended.

BCR 721 (oil-exposed plaice bile)

- Although the 2-OH Naph concentration was higher in the oil-exposed fish bile than in BCR 720, most HPLC/F users had difficulty determining this compound because of crowded chromatograms and the limited selectivity of the fluorescence detector. Only data from four laboratories (all using GC/MS) were accepted; the average concentration found was 420 ± 170 ng g^{-1}. Because of the reasons listed above for the same analyte in BCR 720, certification was NOT recommended.
- 1-OH Phen was determined by seven laboratories (five datasets accepted). However, there was poor concordance among the remaining data sets, with results ranging from 112 to 546 ng g^{-1}. The statistical model of Cofino *et al*. (2000) revealed a multi-mode structure of the data sets. In conclusion, certification was NOT recommended and no indicative value could be given.
- 1-OH Pyr was determined by twelve laboratories; nine datasets were accepted [HPLC/F (6 sets) and GC/MS (3 sets)]; certification was recommended.
- 3-OH BaP was below the LOD of the methods employed (typically 2–10 ng g^{-1}), in agreement with the fact that the BaP levels in crude oils are typically very low.

Summarizing, it was decided at the meeting to recommend certification of 1-OH Phen, 1-OH Pyr, and 3-OH BaP in BCR 720 and 1-OH Pyr in BCR 721. The experts agreed that the overall uncertainties obtained reflect the current state-of-the-art for PAC metabolite determination in fish bile samples.

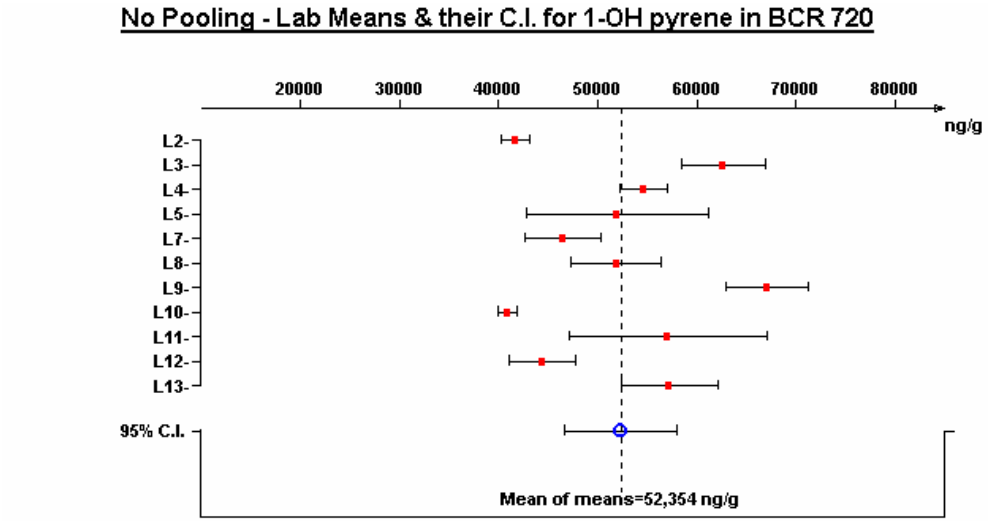

Figure 7. Certification results for 1-OH Pyr in BCR 720, showing the mean and confidence interval for each accepted dataset and the overall result.

After the technical evaluation, the accepted data sets were subjected to a series of statistical tests. Details of the statistical evaluations are given in the certification report (Ariese *et al.*, 2004). No reasons were found to reject any of the datasets. According to the BCR Guidelines (Part B, Section 9.3.1) (European Commission, 1999), the certified values of the PAC metabolites were calculated as the average of the accepted dataset averages and are listed in Table 3 after rounding off the non-significant digits. The half-width of the 95% confidence interval of the average of averages was adopted as the uncertainty of the certified value.

Table 3. Certified values and uncertainties (expressed in mg kg^{-1}, wet weight) for PAC metabolites in sediment-exposed flounder bile (BCR 720) and in oil-exposed plaice bile (BCR 721).

Substance	BCR 720		BCR 721	
	Certified value	Uncertainty*	Certified value	Uncertainty*
2-OH Naph	Not certified		Not certified	
1-OH Phen	0.21	0.07	Not certified	
1-OH Pyr	52	9	2.1	0.4
3-OH BaP	0.063	0.026	Not certified	

* half-width of the 95% confidence interval.
All analyte contents are given for the hydrolyzed species and are corrected for recovery.

5.6 Recommended use of the CRMs

For the certification, the participating institutes were encouraged to use their own methods rather than a prescribed analytical protocol. This way the risk of all laboratories making the same systematic errors was minimized. The certified levels can therefore be regarded as method-independent; the uncertainties reflect the current state-of-the-art in PAC metabolite determination.

For long-term stability the materials should be kept sealed in the amber glass ampoules and stored in the freezer (–20 °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 (for instance during shipment in summer) should be avoided. Dry ice will often not be necessary; shipment in styrofoam containers with frozen icepacks works well for trips up to three days.

After first use 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. Sample preparation should be carried out immediately before analysis because, after hydrolysis, the deconjugated PAC metabolites will be less stable than the conjugated PAC metabolites in the untreated bile sample. Calibration of equipment should be carried out using a series of solutions prepared from pure substances. Users are reminded that PAC metabolites were shown to be sensitive to oxidation in solvent; therefore calibration solutions should be prepared fresh and addition of a suitable antioxidant is recommended.

The two CRMs can be used for quality control purposes, typically with a lower frequency than an RM. When accurate values are obtained for a CRM this can be used to demonstrate a laboratory's proficiency to external partners and customers, as well as to regulatory agencies. Since the certified values are method-independent, having these materials available will also make it easier for laboratories to introduce new methods; the CRM can then be used to validate the performance. CRMs are not recommended for calibration purposes.

The experts at the technical meetings all agreed that the CRMs should be useful for PAC exposure studies with many types of fish, and not just the flatfish species used in this work. 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 (ISO, 2000). The two fish bile CRMs can be ordered from the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium (information at http://www.irmm.jrc.be/).

6 CONCLUSIONS AND RECOMMENDATIONS

Monitoring of fish bile is usually conducted to assess the PAC burden of feral fish in a particular aquatic environment. PAC pollution almost always involves a complex mixture of PACs and it may not seem important to determine individual compounds if the main goal is to quantify the overall PAC burden. Two major reasons can be given for obtaining a higher degree of specificity:

- *Source identification*. As shown in this paper, the relative intensities of certain metabolites or metabolite classes can be extremely important in deducing the major contributor(s) to the overall PAC burden (e.g., marine fuel, crude oil, creosote, or fossil fuel combustion).
- *Quality control.* When analyzing individual compounds the results can be compared to those obtained with other methods and certified values of CRMs can be used for quality control.

These points and the following aspects should be considered when deciding which of the techniques would be most appropriate for a particular application.

6.1 Screening methods

FF and SFS are both very rapid, because the preparation is simple (i.e., only dilution) and the fluorescence analysis takes just a few minutes per sample. The limited number of manual steps involved means that the repeatability of these methods is usually excellent. The short analysis time means that, in principle, a large number of samples can be analyzed, which is important because the biological variability is often substantial in field situations. Field studies have shown that FF and SFS normally are sufficiently sensitive to discriminate between control and impacted sites and that interferences from non-PAC compounds are limited (Aas *et al*., 2000). Alternatively, HPLC/F of non-hydrolyzed bile requires somewhat more time per sample than FF

and SFS, but the procedure is easily automated. Furthermore, the signal at different excitation/emission wavelengths gives a good indication of the source of PAC pollution. All three screening techniques are very suitable for monitoring a large series of samples, especially when relative differences are important. The major disadvantages of screening techniques are that individual PAC metabolites are not quantified, but instead a "sum parameter" is determined and calibrated relative to a fluorescent standard. Therefore, comparison of data with that from other laboratories is very difficult, unless identical protocols are applied and strictly followed.

6.2 Individual metabolite determinations

GC/MS has very good sensitivity and selectivity, especially for complex chromatograms, e.g., 2-ring aromatics in fish bile from oil-contaminated sites. Derivatization improves sensitivity, especially for larger PAC metabolites (smaller metabolites such as hydroxy naphthalenes can also be determined without derivatization). The disadvantages of GC/MS are that the many sample pretreatment steps are labor-intensive and also increase the risk of errors.

HPLC/F also has very good sensitivity (particularly for larger PAC metabolites) and is therefore most suitable in areas where fossil fuel combustion, coke or aluminum plants, or creosote leaching are the dominant sources of PAC pollution. Most, but not all, PAC metabolites show fluorescence. In addition, HPLC/F requires relatively simple sample pretreatment (hydrolysis, centrifugation). The disadvantages of HPLC/F are that poor chromatographic resolution hampers quantitation of, for example, hydroxy naphthalenes. The technique is therefore less appropriate for monitoring in areas where petrochemical pollution is the dominant source.

6.3 Need for quality control in measurements

When a study is conducted by a single institute using a single analytical method, short-term reproducibility (minimization of random errors) is important. For monitoring studies that are conducted over several years long-term reproducibility is also essential. Quality requirements need to be even more stringent for studies involving several institutes or different methods, because it is essential that there is no significant interlaboratory bias (systematic error). Thus, all laboratories making measurements of metabolites in bile should adopt a program that controls for quality in each step of the analysis—sampling design, as well as sample collection, storage, extraction, cleanup, and analysis. Analytical quality control is discussed in this paper, but sources of error related to sampling (e.g., influence of season or water temperature, feeding status) may be equally important and need to be considered when designing a sampling campaign. For example, clear protocols need to be provided to the sampling crew. In addition, when reporting results, all relevant details (e.g., sampling conditions, calibrants used, recovery correction factors) should be provided.

One point that deserves particular attention is how to account for differences in bile accumulation levels. During feeding, the gall bladder is emptied into the intestinal tract and then first fills up with water, followed by a slower accumulation of PAC metabolites and other hepatic breakdown products. As a result, bile from fish collected shortly after feeding tends to be relatively diluted, whereas bile collected after a period of starvation (for instance during the spawning season or when kept under stressful conditions in a mesocosm) usually shows much higher accumulation levels. Several workers have attempted to correct for these effects by using ratios of PAC metabolite levels to bile protein content or to bile absorbance at a specific wavelength (Aas *et al*., 2000; Ariese *et al*., 1997; Collier and Varanasi, 1991 and several unpublished studies). However, in most monitoring studies, PAC metabolite concentrations did not show a strong correlation with either protein or bile absorbance and no improvement in the variance was observed. Nevertheless, researchers should be aware that a bias can be introduced

when there are significant differences in feeding status, and differences in bile protein or bile absorbance can help identify such occurrences.

6.4 Use of bile reference materials

Laboratory RMs, used to check method reproducibility, are relatively easily prepared and can serve for internal quality control, but give no indication of the accuracy of the method. These materials should be prepared in sufficiently large quantities to last for several years. In contrast, CRMs can be used to check for systematic errors and are typically used less often than RMs. When accurate values are obtained for a CRM, these results can be used to demonstrate a laboratory's proficiency to external partners and customers, as well as to regulatory agencies. Results obtained from different methods can be compared directly, provided that both methods produce accurate results for the target analytes when analyzing a CRM. Because the certified values are method-independent, having these CRMs available will also make it easier for laboratories to introduce new methods, because the CRM can then be used to validate the performance. The two currently available CRMs for PAC metabolites in bile were prepared from bile from marine flatfish, but should also be suitable for quality control for monitoring studies involving other fish species.

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8 LIST OF ABBREVIATIONS

1-OH Phen	1-hydroxyphenanthrene
1-OH Pyr	1-hydroxypyrene
2-OH Naph	2-hydroxynaphthalene
3-OH BaP	3-hydroxybenzo[a]pyrene
APCI	atmospheric pressure chemical ionization
BaP	benzo[*a*]pyrene
BCR	Community Bureau of Reference
BHT	butylhydroxytoluene
BSA	bis(trimethylsilyl)acetamide
BSTFA	bis-(trimethylsilyl)trifluoroacetamide
CD-MEKC	cyclodextrin-modified micellar electrokinetic chromatography
CE	capillary electrophoresis
Chry	chrysene
CRM	certified reference material
EI	electron impact
ESI	electrospray ionization
F	fluorescence
FF	fixed-wavelength fluorescence
Flu	fluorene
GC	gas chromatography
HPLC	high-performance liquid chromatography
IRMM	Institute for Reference Materials and Measurements
LC	liquid chromatography
LIF	laser-induced fluorescence
LOD	limit of detection
LRM	laboratory reference material
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PAC	polycyclic aromatic compound
PB	particle beam
RM	reference material
RSD	relative standard deviation
SFS	synchronous fluorescence spectrometry
SIM	selected ion monitoring
TLC	thin layer chromatography
TMS	trimethylsilyl
TMSI	trimethylsilylimidazole
TS	thermospray

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