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DETERMINATION OF POLYCYCLIC AROMATIC COMPOUNDS IN FISH TISSUE

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SUMMARY

A method is presented for the analysis of polycyclic aromatic hydrocarbons (PAHs), polycyclic aromatic sulfur heterocycles (PASHs), and basic polycyclic aromatic nitrogen heterocycles (PANHs) in fish. The analytical procedure includes Soxhlet extraction of prepared fish tissue with methylene chloride followed by gel permeation chromatography (GPC) using Bio-beads SX-3. For PAHs/PASHs, further cleanup is performed using adsorption chromatography on Florisil (5% water deactivated) and elution with hexane. For basic PANHs further cleanup of the fish extracts after GPC is achieved using liquid–liquid partioning with 6 *M* hydrochloric acid and chloroform and then basifying the aqueous phase and extracting it with chloroform. Analysis of fortified fish samples was performed using capillary gas chromatography with flame ionization detection and capillary gas chromatography–mass spectrometry. Good agreement was observed for both methods of analysis when applied to fish samples fortified with PAHs, PASHs and basic PANHs at 0.1 to 1 μg/g, suggesting that the method is effective at removing interfering biogenic compounds prior to analysis. Average recovery of PAHs/PASHs from fortified fish tissue was 87% and 70% for fish tissue fortified at 0.24–1.1 and 0.024–0.11 μg/g, respectively. Average recovery for basic PANHs was 97% for fish fortified at 1.2–1.4 μg/g.

INTRODUCTION

Current emphasis on the development of alternate energy sources has stimulated production of synthetic fuels derived from oil shale, tar sands and coal. Although technology for producing liquid and solid fuels from these feed stocks has been available since the early 1900's (Rubin *et al.*[1]), the chemical characterization of these products has recently received increased attention. It is now clearly necessary to

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identify and eliminate specific toxic and carcinogenic compounds in order to reduce environmental and occupational health hazards associated with the production and the combustion of these materials.

As part of a study of the uptake and elimination of toxic components isolated from thermally cracked heavy oil (coker distillate fractions) by fish, the need arose to develop a method to determine the presence of polycyclic aromatic compounds in fish tissue. Of particular interest were the polycyclic aromatic hydrocarbons (PAHs), polycyclic aromatic sulfur heterocycles (PASHs), and the basic polycyclic aromatic nitrogen heterocycles (PANHs). The accumulation and metabolism of toxic PAH by fish is well documented (Varanasi and Malins[2], Vandermuelen[3], Sinkkonen[4], and Krahn and Malins[5], and the accumulation of PASHs from petroleum sources by fish has also been well studied (Ogata *et al.*[6], Ogata and Miyake[7], and Paasivirta *et al.*[8]). High levels of PAHs and PASHs have been reported in the tissue of brown bullhead catfish taken from the contaminated Black River in Ohio (Lee *et al.*[9] and Vassilaros *et al.*[10]). Upon examination, this fish was found to have several cholangiomas (bile duct tumors) (Vassilaros *et al.*[10]). A number of pathological conditions have been observed in fish from polluted coastal waters and estuaries. Hepatic neoplasia have been linked to the presence of aromatic hydrocarbons in bottom sediments (Malins *et al.*[11,12]).

Recently, interest has focused on the study of PANHs in environmental samples. Of particular interest are the basic PANHs (which are primarily azaarenes and primary aromatic amines); these compounds are highly mutagenic as determined by the Ames test (Pelroy and Petersen[13], Guerin *et al.*[14], Wilson *et al.*[15] and Hsie *et al.*[16]). Because many carcinogenic chemicals are also mutagenic (McCann *et al.*[17]), the Ames test has been used to predict risks to human health. The presence of basic PANHs in the environment is of concern because many of these compounds are known mutagens and/or carcinogens (Dipple[18]). For example, quinoline and all of its monomethyl isomers were found to be mutagens in the Ames salmonella/microsomal assay (Dong *et al.*[19]). Recently, it has been shown that basic PANHs such as acridine and quinoline are readily taken up by fish (Southworth *et al.*[20] and Bean *et al.*[21]), and hepatic neoplasms and other hepatic lesions in English sole may be correlated to the presence of basic PANHs in sediment (Malins *et al.*[11]). There is ample reason then to analyze fish, taken from the environment for PAHs, PASHs and PANHs in order to ascertain their bioconcentration and the effects these chemicals are having on the environment.

Although many methods exist for the determination of PAHs in fish, relatively few procedures exist for the determination of PAHs, PASHs and basic PANHs in fish. Vassilaros *et al.*[10] presented such a method which involved alkaline hydrolysis, liquid–liquid extraction followed by alumina and gel permeation cleanup. We tried this method and found the alkaline hydrolysis method to be messy and time consuming, especially for fish with high lipid content. Furthermore, interference from biogenic compounds was observed upon analysis using capillary gas chromatography–flame ionization detection (GC–FID) suggesting that GC was not a suitable procedure for the screening of fish samples prepared by the method of Vassilaros *et al.*[10].

The purpose of this paper is to describe an analytical method for the extraction, cleanup and high-resolution gas chromatographic analysis of PAHs, PASHs and basic PANHs in fish tissues.

MATERIALS AND METHODS

Chemicals

6,7-Dimethylquinoline (6,7-DMQ) and 6,8-dimethylquinoline (6,8-DMQ) were synthesized in the University of Alberta Chemistry Department using the procedure of Manske *et al.*[22]. Purity was determined to be greater than 98% using GC–FID and GC–mass spectrometry (GC–MS). Naphthalene, benzothiophene, 1-methylnaphthalene, 2,6-dimethylnaphthalene, 2,3,5-trimethylnaphthalene, and dibenzothiophene were obtained from Aldrich and reported to be greater than 97% pure. Acenaphthene-d_{10} was obtained from Merck, Sharpe and Dome. Anhydrous sodium sulfate, celite, concentrated hydrochloric acid, glacial acetic acid, and distilled in glass dichloromethane and hexane were obtained from Fisher Scientific. Florisil (PR grade, 60–80 mesh) was purchased from Floridin. 6 *M* Hydrochloric acid was prepared from concentrated acid and purified by extraction with methylene chloride prior to use. Anhydrous sodium sulfate and celite were purified by continuous Soxhlet extraction with methylene chloride for 16 h. Following extraction the solvent was evaporated in a vacuum oven (maintained at 50°C) and the material stored in a convection oven maintained at 130°C until required. BioBeads SX-3 (Bio-Rad) were swollen with elution solvent (methylene chloride–hexane; 1:1, v/v) overnight and wet packed into a chromatographic column (19 mm I.D.) to a bed height of 50 cm. Prior to use the column was washed with several bed volumes of elution solvent. All glassware used in the analytical procedure was soaked overnight in a detergent solution (RSB-35, Pierce), rinsed with hot water followed by pesticide-grade acetone and methylene chloride, and dried in an oven maintained at 250°C for 4 h.

Preparation and extraction of fish tissue

Fish tissue samples (muscle) were prepared according to the method of Benville and Tindle[23]. This involved grinding frozen tissue with dry ice in a Waring blender until a fine flour was obtained. Ground samples were then transferred to 250 ml wide-mouth jars, covered with aluminum foil and placed in a freezer maintained at −80°C overnight in order to allow the CO_2 to sublime. Thawed subsamples (20 g) were mixed with 80 g of purified anhydrous sodium sulfate, gently packed into a glass Soxhlet extraction thimble (with extra course glass frit) containing approximately 1 in. of purified celite and extracted with approximately 300 ml of methylene chloride for 6 h in a Soxhlet extractor equipped with a Freidrich condenser. Following extraction, the extract was concentrated to approximately 5 ml with the aid of a rotary evaporator operated under reduced pressure and with the water bath temperature maintained at 35°C.

Cleanup of fish tissue extracts

Cleanup of tissue samples was performed using gel permeation chromatography (GPC). This involved diluting the extract to 10 ml with methylene chloride–hexane (1:1, v/v) and applying it to a 750 mm × 19 mm I.D. chromatographic column containing 500 mm of BioBeads SX-3 swollen with elution solvent (methylene chloride–hexane, 1:1). The column was drained to the head of the gel. The extract container was rinsed with a further 10-ml of elution solvent which was transferred to the column. Again the column was drained to the head of the gel. A 250-ml addition

funnel was then filled with 230 ml of elution solvent and attached to the chromatographic column for elution. The first 75 ml of eluate (which contains primarly lipid material) were discarded and the next 75 ml (which contains primarily xenobiotics) collected. The eluate was then concentrated to approximately 5 ml with the aid of a rotary evaporator.

For the analysis of PAHs/PASHs, hexane (20 ml) was added to the final 5 ml of the GPC eluate and the mixture was concentrated on a rotary evaporator to a volume of approximately 2 ml. This extract was applied to a chromatographic column which was prepared by wet packing 10 g of 5% water deactivated Florisil (w/v) into a 10 mm I.D. chromatography column. The PAHs/PASHs were eluted with 50 ml of hexane, which was concentrated to 1.0 ml using a rotary evaporator followed by nitrogen blowdown.

For basic PANHs such as dimethylquinolines, the 5-ml GPC eluate was quantitatively transferred to a 250-ml separatory by extracting three times with 2 ml chloroform. An acid–base partition was performed by adding an additional 19 ml of chloroform to the separatory funnel, thoroughly mixing the contents and extracting three times with 25 ml 6 *M* hydrochloric acid. The combined aqueous layer was cooled in an ice bath, and basified using 6 *M* sodium hydroxide to pH $>$ 11. Following extraction of the basified solution with 3 $\times$ 25 ml of chloroform, the extract was dried by passage through a column containing sodium sulfate (20 g), concentrated on a rotary evaporator to approximately 2 ml and quantitatively transferred to a calibrated 5-ml centrifuge tube with two 1-ml washings of chloroform. The ensuing extract was then concentrated to 1.0 ml with the aid of a nitrogen evaporator.

GC and GC–MS

GC was performed on a Hewlett-Packard (HP) Model 5880, or a Varian Model 3500 gas chromatograph. The HP instrument was equipped with a split/splitless injector (operated in the splitless mode), autosampler, flame ionization detector, level four data processing capability and a 30 m $\times$ 0.32 mm I.D. fused-silica, wall-coated DB-1301 capillary column (J&W Scientific). The carrier gas was helium (linear velocity was 31 cm/s at 280°C), and the temperature was increased from 40 to 280°C at 10°C/min beginning 1 min after injection. The oven temperature was maintained at 280°C for 20 min and the injector and detector temperatures were maintained at 270 and 300°C respectively. The injector was purged with helium 30 s after the injection of 2 μl of sample.

The Varian GC instrument was equipped with a split/splitless injector (operated in the splitless mode), flame ionization detector Model 600 data system and a 30 m $\times$ 0.32 mm I.D. fused-silica wall-coated DB-5 capillary column (J&W Scientific). Conditions of analysis were the same as those employed with the HP instrument except that the linear velocity was 28 cm/s at 300°C.

GC–MS was performed by interfacing a HP Model 5980 gas chromatograph to a HP Model 5970 quadrupole mass spectrometer. The GC instrument was equipped with a split/splitless injector (operated in the splitless mode), and a 12.5 m $\times$ 0.2 mm I.D. fused-silica wall-coated HP-1 capillary column (Hewlett-Packard). The carrier gas was helium (linear velocity was 36 cm/s at 300°C), and the temperature was increased from 40 to 300°C at 10°C/min beginning 1 min after injection. The oven temperature was maintained at 300°C for 8 min, and the injector, transfer line and ion source were maintained at 250, 300, and 220°C, respectively. Data was acquired 2 min

after injection using a HP Model 59970C data system. MS scans (from 35 to 350 a.m.u.) were obtained every 1.36 s.

Recovery study

Aliquots of prepared fish muscle (Rainbow trout; 20 g) were fortified with the following PAHs/PASHs: naphthalene, benzothiophene, 1-methylnaphthalene, 2,6-dimethylnaphthalene, 2,3,5-trimethylnaphthalene, and dibenzothiophene. Fish muscle was also fortified with the following basic PANHs: 6,7-dimethylquinoline and 6,8-dimethylquinoline. Concentrations of these chemicals ranged from 24 ng/g to 1.39 μg/g (Tables I–IV).

RESULTS

The results summarized in Table I were obtained from fish fortified with PAHs/PASHs. Analysis was performed using the Varian gas chromatograph and quantitation was performed using external standards (ESTD) and an internal standard, namely acenaphthene-d_{10} (ISTD). From Table I it is apparent that recovery better than 80% was obtained for the PAHs/PASHs at concentration levels of 0.24–1.1 μg/g. Precision of the method (as expressed by the relative standard deviation, R.S.D.) was satisfactory and ranged from 4 to 10% of the mean. Little gain in precision was observed by using an internal standard during quantitation in place of external standards.

Since GC analysis using FID is a non-specific method of analysis, a more specific method, namely, GC/MS was used to analyze one of the fortified fish samples for PAHs/PASHs and the results were compared to those obtained by GC–FID. GC–MS quantitation was performed using the general principals outlined by the United States Environmental Protection Agency[24]. Results are summarized in Table II.

It is apparent from Table II that there is close agreement between the analysis of

TABLE I

RECOVERY OF PAHs/PASHs FROM FORTIFIED FISH MUSCLE

Analyte	*Concentration (μg/g)*	*n*	*Mean recovery (%)*	*R.S.D. (%)*	*Quantitation method (ESTD/ISTD)*
Naphthlalene	0.97	4	86	5.1	ESTD
	0.97	4	85	4.8	ISTD
Benzothiophene	1.1	4	85	4.5	ESTD
	1.1	4	81	5.7	ISTD
1-Methylnaphthalene	1.0	4	88	4.3	ESTD
	1.0	4	85	5.4	ISTD
2,6-Dimethylnaphthalene	1.0	4	88	6.1	ESTD
	1.0	4	87	6.6	ISTD
2,3,5-Trimethylnaphthalene	0.24	4	93	10.1	ESTD
	0.24	4	85	9.0	ISTD
Dibenzothiophene	1.0	4	92	9.2	ESTD
	1.0	4	84	6.4	ISTD

TABLE II

ANALYSIS OF FORTIFIED FISH MUSCLE BY GC–FID AND GC–MS

Analyte	*Concentration (μg/g)*	*Recovery (%) by*	
		GC–FID	*GC–MS*
Naphthalene	0.97	84	89
Benzothiophene	1.1	81	93
1-Methylnaphthalene	1.1	88	91
2,6-Dimethylnaphthalene	1.0	90	100
2,3,5-Trimethylnaphthalene	0.24	92	95
Dibenzothiophene	1.0	88	96

fortified fish muscle using GC/FID and GC–MS analysis. The slightly higher recovery observed for the GC/MS analysis may be due to slight concentration of the extract during storage prior to the GC/MS analysis. The close agreement between the two methods of analysis is indicative of the excellent cleanup obtained using the GPC/Florisil combination. A chromatogram obtained from fish tissue fortified with PAHs/PASHs is depicted (Fig. 1). The peaks appearing before naphthalene were determined, by GC/MS, to be alkylated benzenes and were observed to be present in control fish samples. The source of these alkylated benzenes was determined to be the hexane, which was used in both the GPC and Florisil cleanup steps. The only biogenic materials found to be present in this chromatogram eluted as two significant peaks after dibenzothiophene (Fig. 1). However, these peaks were found to be present in only

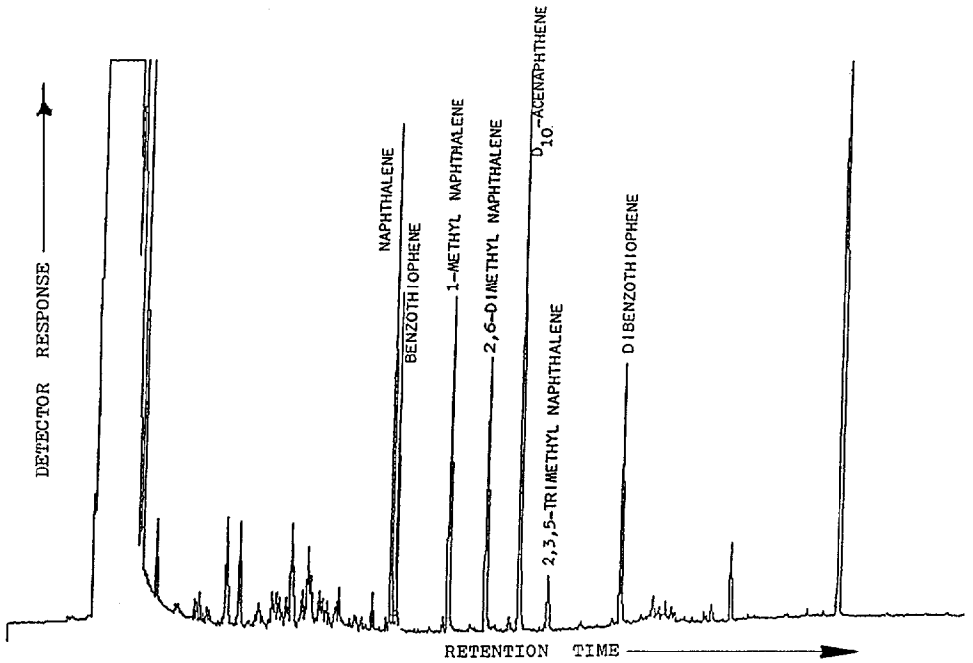

Fig. 1. Fish muscle fortified with PAH/PASHs.

TABLE III

GC–FID AND GC–MS ANALYSIS OF FISH TISSUE FORTIFIED AT 0.1 μg/g

Analyte	*Concentration (ng/g)*	*Recovery (%)*			
		Spike 1		*Spike 2*	
		GC–FID	*GC–MS*	*GC–FID*	*GC–MS*
Naphthalene	96.9	114	93	256	138
Benzothiophene	104	107	68	153	65
1-Methylnaphthalene	106	93	79	86	53
2,6-Dimethylnaphthalene	100	81	77	65	68
2,3,5-Trimethylnaphthalene	24		74	155	65
Dibenzothiophene	104	88	77	82	78

two samples out of 12 processed, and are of unknown origin, but were probably introduced during GPC and/or Florisil cleanup. Rigorous calibration of the GPC and Florisil chromatography would likely eliminate these compounds.

In order to test the range of concentrations which could be detected using this method, two samples of fish tissue were fortified at low levels and subjected to GC/FID and GC–MS analysis. Table III is a summary of these findings.

From Table III it can be seen that there is reasonable agreement between the GC/FID and GC/MS analysis of fish tissue samples fortified at 24 to 100 ppb*. The generally higher results obtained by GC/FID are likely due to the non-specificity of the method. Therefore, for quantitation of low levels of PAHs/PASHs in fish, GC–MS is the recommended method of choice. It is worth noting that even at 20 ppb acceptable levels of recovery were observed using GC–MS analysis. GC–MS data was obtained via scanning and extracted ion current profiles were generated for the analytes of interest and internal standard prior to integration and calculation. It is anticipated that larger signal-to-noise ratios could be obtained using selected-ion monitoring techniques and hence lower levels of detection could be realized. However, decreasing the analyte concentration could result in losses of material by adsorption onto glass surfaces etc., therefore a realistic detection limit of 10–20 ppb based on 20 g of fish and using our method is considered valid.

Table IV is a summary of the results obtained for the GC–FID analysis of basic PANHs in fortified fish muscle. Table IV reveals excellent recoveries of dimethylquinolines from fortified fish tissue using Soxhlet extraction followed by GPC and acid–base partition cleanup. Precision (defined as R.S.D.) is also acceptable and similar to that observed for the PAH/PASH analyses. Furthermore, GC–MS analysis of one fortified fish sample for 6,7-DMQ produced recoveries similar to those observed using GC–FID analysis, and indicative of effective cleanup. Reference to Fig. 2 reveals a GC–FID chromatogram obtained from fortified fish muscle and virtually free of any biogenic interfering material.

We have developed an analytical method capable of detecting accurate and precise levels of PAHs/PASHs and basic PANHs in samples of fish muscle. Good

* Throughout the article the American billion (10^9) is meant.

TABLE IV

ANALYSIS OF DIMETHYLQUINOLINES IN FORTIFIED FISH MUSCLE

Analyte	*Concentration (μg/g)*	*n*	*Mean recovery (%)*	*R.S.D. (%)*
6,8-Dimethylquinoline	1.39	4	96	8.2
6,7-Dimethylquinoline	1.15	4	98	7.0

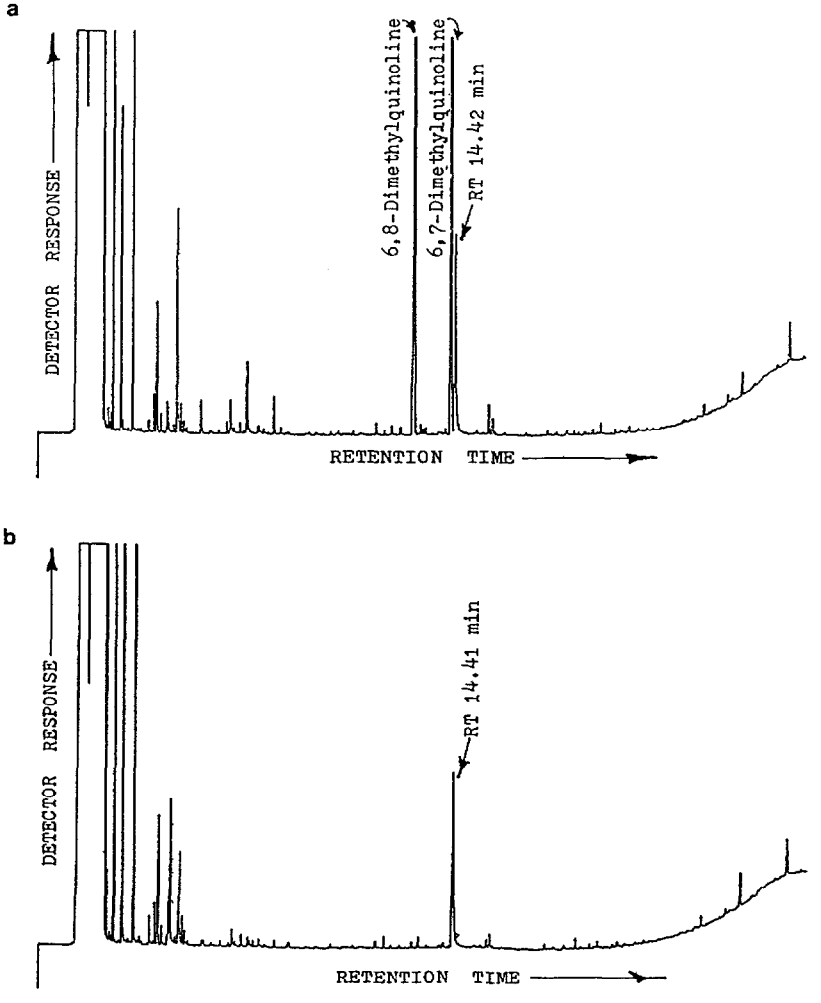

Fig. 2. (a) Fish muscle fortified with 6,7- and 6,8-dimethyl quinoline; (b) control fish muscle.

TABLE V

MUSCLE CONCENTRATION OBSERVED IN FISH EXPOSED TO PANHs AND PASHs

Analyte	*Mean exposure concentration (mg/l)*	*Exposure time (h)*	*Mean muscle concentration (μg/g)*	*Number of exposures*
6,7-Dimethylquinoline*	1.0	7.5	6.1	3
6,8-Dimethylquinoline*	1.1	8.0	14	3
Benzothiophene**	0.67	8.0	19	3

* Data taken from Birkholz *et al.*[25].
** Data obtained from Dromey[26].

agreement was observed between GC–FID and GC–MS analysis which indicates substantial removal of biogenic material during cleanup. The use of GC–FID as a screening method is desirable because of accessability by most laboratories and low cost relative to GC–MS. This method was applied to the analysis of muscle obtained from fish exposed to 6,7-DMQ, 6,8-DMQ and benzothiophene. Muscle concentrations were determined immediately after exposure and after depuration. A summary of muscle concentrations observed in fish after exposure to the three chemicals is shown in Table V. From this table it is apparent that PANHs and PASHs are bioconcentrated by fish from water.

A summary of muscle concentrations observed in fish following depuration of the three chemicals is given in Table VI. From Tables V and VI it is apparent that PANHs and PASHs have different rates of uptake and elimination. The uptake, elimination, and biotransformation of PANHs by fish is more fully described by Birkholz *et al.*[25] The uptake, elimination and biotransformation of PASHs is described by Dromey[26].

In conclusion we have developed a precise and accurate method for the determination of PAHs, PASHs and PANHs in fish muscle. The sensitivity of the method was found to be more than adequate when applied to study of the uptake and elimination of PASHs and PANHs by exposed fish.

TABLE VI

CONCENTRATION OF PANHs AND PASHs IN FISH MUSCLE FOLLOWING EXPOSURE AND DEPURATION

Analyte	*Number of exposures*	*Mean exposure concentration (μg/l)*	*Exposure time (h)*	*Depuration time (h)*	*Muscle concentration (μg/g)*
6,7-Dimethylquinoline*	3	0.97	9.5	69	0.56
6,8-Dimethylquinoline*	3	1.1	7.0	63	0.49
Benzothiophene**	3	0.67	8.0	65	2.2

* Data obtained from Birkholz *et al.*[25].
** Data obtained from Dromey[26].

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