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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATION OF AROMATIC HYDROCARBON METABOLITES FROM LIVERS OF FISH EXPOSED TO FUEL OIL

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SUMMARY

Metabolites of several two- and three-ring aromatic hydrocarbons (AHs) have been found in livers of English sole exposed to No. 2 fuel oil. Four metabolites of the C_2H_5-naphthalenes, six of the C_3H_7-naphthalenes and one each of fluorene, phenanthrene and anthracene have been partially characterized and their concentrations, which ranged from 50 to 1100 ng/g, were determined. Metabolites were separated from the liver matrix using an automated extractor/concentrator. The resulting extract was then purified by high-performance liquid chromatography, and the metabolites were characterized and quantitated by gas chromatography–mass spectrometry.

INTRODUCTION

In many areas, marine waters and sediments contain hundreds of chemicals[1–3], many of which are petroleum-related aromatic hydrocarbons (AHs). Marine organisms accumulate AHs and metabolize them[4–7] to products which may be more deleterious than the parent compounds[8,9]. In order to assess the effects of exposing organisms to a multitude of xenobiotics, techniques are needed for determining the complex mixtures of these chemicals and their metabolites in animal tissues.

Although analyses for parent AHs in tissue samples from aquatic organisms are performed routinely[10–12], the determination of AH metabolites is a more difficult task. The polar metabolites often are non-volatile or labile and, thus, must be determined with special gas chromatographic (GC) columns or procedures. High-performance liquid chromatography (HPLC) can be used to analyze for individual compounds in simple mixtures of polar compounds; however, the resolution is usually insufficient to separate a mixture of metabolites as complex as those resulting from the exposure of organisms to petroleum.

Nevertheless, progress has been made in analyzing for some mixtures of metabolic products and other polar compounds. For example, 2,6-dimethylnaphthalene metabolites can be separated from biological matrices using an automated extractor/concentrator[13], and metabolites in the extracts can be determined by reversed-phase

HPLC with fluorescence detection[14,15]. Alternatively, a method combining silica gel column chromatography, preparative normal-phase HPLC and gas chromatography–mass spectrometry (GC–MS) has been used to analyze for polar compounds in tissue extracts from marine organisms[16].

In this study, we have combined several of these techniques to determine metabolic products resulting from the exposure of English sole (*Parophyrs vetulus*) to No. 2 fuel oil. We used an extractor/concentrator to isolate, HPLC to purify and GC–MS to identify and quantitate AH metabolites from livers of fish exposed to the oil.

EXPERIMENTAL

*Chemicals**

2-Methyl-1,4-naphthoquinone, 9-phenanthrol, α-methyl-2-naphthalenemethanol and 9-hydroxyfluorene were obtained from Aldrich (Milwaukee, WI, U.S.A.), and 4-methyl-1-naphthol, 1-naphthaldehyde and 2,2-dihydroxybiphenyl from K & K Labs (Plainview, NY, U.S.A.). 1-Naphthol was purchased from Sigma (St. Louis, MO, U.S.A.) and 2,6-dimethyl-3-naphthol, 6-methyl-2-naphthalenemethanol and *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene were prepared in our laboratories[7]. Ultrex-grade acetic acid and HPLC-grade water were purchased from J. T. Baker Chemical (Phillipsburg, NJ, U.S.A.). Distilled-in-glass methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

GC–MS standard

A GC–MS standard of the following reference compounds (ng/μl), dissolved in methanol, was prepared: 1-naphthaldehyde (30.8), 2,2′-dihydroxybiphenyl (26.5), α-methyl-2-naphthalenemethanol (26.5), 9-hydroxyfluorene (26.2), 4-methyl-1-naphthol (25.0), 9-phenanthrol (24.2), 2-methyl-1,4-naphthoquinone (26.2), 1-naphthol (34.8), 2,6-dimethyl-3-naphthol (14.9), 6-methyl-2-naphthalenemethanol (7.88), *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene (8.65) and hexamethylbenzene (4.10) (internal standard).

Fish exposures

English sole were caught near Meadow Point, WA, and maintained in flow-through seawater aquaria. The water temperature was 9.5 ± 1°C and the salinity was 24 ± 2‰. Fish were exposed to polychlorinated biphenyls (PCBs, Aroclor 1254) and to fuel oil in an LC_{50} study[17]. Eight days prior to fuel oil exposure, fish (13.8 ± 0.6 cm, 21.5 ± 2.3 g) were injected intraperitoneally with 25 μg/g of Aroclor 1254 in *ca.* 100 μl of salmon oil. Control fish received a single i.p. injection of 100 μl of salmon oil carrier. Then, fish in flow-through aquaria were exposed to 50 mg/l of fuel oil for 5 days. At the end of the LC_{50} study, the fish were killed and their livers were removed and frozen until needed for analysis. A water sample (1 l) was collected from the exposure tank at the end of the experiment, extracted, concentrated to 1 ml and analyzed by GC for total hydrocarbon content by comparing the total peak areas of hydrocarbons from the water extract with those from a No. 2 fuel oil standard.

* Reference to a company or product does not imply endorsement by the U.S. Department of Commerce to the exclusion of others that may be suitable.

Metabolite extraction

Sample preparation. Livers were thawed (seven experimental, four unexposed controls), pooled within groups and then homogenized in 4 parts of 1.15% potassium chloride solution (described previously[13]).

Extraction. Homogenized liver samples were extracted with a Prep I automated extractor/concentrator (DuPont, Wilmington, DE, U.S.A.), which used glass microbeads and Type W extraction cartridges packed with a styrene–divinylbenzene copolymer resin (DuPont)[13,18]. Aliquots (500 and 750 μl, respectively) of liver homogenates (5.0 ml experimental, 1.5 ml control) were into cleaned cartridges, diluted with 1 ml of buffer (1.0 *M* citric acid, 0.2 *M* disodium hydrogen orthophosphate heptahydrate, pH 2.5), and loaded into the extractor/concentrator. The samples were washed with water (10 ml) and extracted twice with 10 ml of acetone–methanol (1:1). The extraction sequence was repeated twice more with 10 ml of a second solvent [methylene chloride–2-propanol–water (75:25:2)]. After the extraction steps had been completed, liver extracts from the experimental fish (1.0 g of liver) were combined and the volume was reduced, at 30°C under a flow of nitrogen, to 1.0 ml. Similarly, liver extracts from control fish (0.3 g of liver) were combined and reduced to a volume of 0.2 ml.

Reversed-phase HPLC

Instrumentation. Separations were performed with a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 8000B high-performance liquid chromatograph. A Perkin-Elmer (Norwalk, CT, U.S.A.) Model MPF-44A fluorescence spectrometer was used for detection (excitation at 290 nm and measurement at 335 nm). Samples were injected using a Waters (Milford, MA, U.S.A.) Model 710A variable-volume automatic sampler.

Columns. The analytical column was a 25 × 0.26 cm I.D. reversed-phase HC-ODS column (Perkin-Elmer). A 5 × 0.21 cm I.D. stainless-steel guard column dry-packed with Vydak 37-μm reversed-phase packing (Separations Group, Hesperia, CA, U.S.A.) was also used.

Gradient elution conditions and fraction collection. Acetic acid–water (0.5:100; solvent A) and methanol (solvent B) were used to separate non-conjugated metabolites from conjugated metabolites and parent compounds in a linear gradient as follows: 100% solvent A to 100% solvent B in 5 min; 10 min at 100% B; 2 min to return to 100% A; and 8 min equilibration at 100%. The flow-rate was 1.5 ml/min, except during equilibration, when it was increased to 2.5 ml/min. The oven temperature was 50°C. Liver extract (200 μl) ($n = 3$ for exposed organisms, $n = 1$ for control) was injected into the HPLC system, and the non-conjugated fraction was collected during an elution interval of 8.7–11.2 min. The conjugated fraction was not collected because low volatilities of conjugated metabolites precluded their determination by GC.

Concentration of HPLC fraction. Metabolites in fractions collected by HPLC were extracted from the HPLC eluate using the extractor/concentrator. The HPLC fraction was first concentrated under a stream of nitrogen at room temperature to remove most of the methanol. The volume was reduced to *ca.* 1 ml, and 2 ml of water and 1 ml of the pH 2.5 citrate–phosphate buffer were added. Two extractor/concentrator extractions (10 ml of acetone, then 10 ml of methanol) were then carried out[13].

The combined extracts were reduced to *ca.* 200 μl in the instrument, then further evaporated under a flow of nitrogen to *ca.* 20 μl. (Note: concentrating samples to dryness should be avoided because this can result in the loss of some analytes[14].) Hexamethylbenzene (the GC internal standard) was added to each sample.

Recoveries of reference standards

Recoveries of AHs from liver homogenate by automated extraction were generally >90%[13]. Compounds in the GC–MS reference standard were processed by the same techniques used to recover metabolites from the liver extract. The GC–MS standard (200 μl, $n = 3$) was injected into the HPLC system and fractions were collected and concentrated. Recoveries were greater than 90% except for three compounds: 1-naphthaldehyde (23%), α-methyl-2-naphthalenemethanol (59%) and 4-methyl-1-naphthol (82%). These compounds have the lowest melting points of the reference standards, so losses may have resulted owing to their relatively high volatilities.

Gas chromatography and mass spectrometry

Instrumentation and chromatographic conditions. Portions (2 μl) of the concentrated fractions containing the metabolites (samples from HPLC) were each injected, without splitting, into a Hewlett-Packard Model 5840A gas chromatograph equipped with a flame-ionization detector. GC–MS was performed using an identical

TABLE I

AMOUNTS OF AROMATIC HYDROCARBONS IN NO. 2 FUEL OIL AND IN LIVERS FROM ENGLISH SOLE EXPOSED TO THE OIL

Compound	*Fuel oil (μg/g)**	*English sole livers (ng/g wet wt.)***
Naphthalene	760	320
1-Methylnaphthalene	1100	1800
2-Methylnaphthalene	1800	3600
2,6-Dimethylnaphthalene	1100	3500
Other C_2H_5-naphthalenes (7 isomers)***	4800	N[§]
Diphenyl	300	850
Acenaphthene	34	62
2,3,5-Trimethylnaphthalene	580	1400
Other C_3H_7-napthalenes (16 isomers)***	3100	N
Fluorene	120	830
Phenanthrene	220	810
1-Methylphenanthrene	135	94
Other methylphenanthrenes (3 isomers)***	400	N
3,6-Dimethylphenanthrene	200	<30
Other C_2H_5-phenanthrenes (9 isomers)***	1300	N

* GC–MS analysis of No. 2 fuel oil.

** GC analysis of livers from English sole exposed to No. 2 fuel oil. Preparation and analysis of sample by method described previously[10].

*** Amounts determined by GC–MS using response factors from immediately preceding isomeric compound.

[§] N = Not determined.

GC system interfaced with a Finnigan 3200 mass spectrometer (Finnigan MAT, Sunnyvale, CA, U.S.A.) and an Incos 2300 data system (Finnigan MAT). The fused silica capillary column was coated with DB-5 (30 m × 0.25 mm I.D.; J&W Scientific, Rancho Cordova, CA, U.S.A.). The flow-rate of the carrier gas (helium) was adjusted to a linear velocity of *ca.* 33 cm/sec at 150°C. The column temperature was programmed from 90 to 300°C at 4°C/min. Injections made at 90°C were best for methanolic solutions.

GC–MS identifications and quantitations. Metabolites were identified by comparison with reference compounds or with spectra in the expanded National Bureau of Standards/National Institutes of Health Mass Spectral Library (Finnigan Library).

Commercially available reference compounds were not, in general, the same isomers present in the experimental samples. Only three of the reference compounds were found in our liver sample. Therefore, many compounds were quantitated using the response factor for an isomer (*e.g.*, the C_2H_5-naphthols were quantitated using the response factor for 2,6-dimethyl-3-naphthol). For some metabolites, no isomers were available: amounts of these compounds were determined using a response factor for the most chemically similar reference standard (*e.g.*, 2,6-dimethyl-3-naphthol was used to quantitate the C_3H_7-naphthols).

RESULTS

No. 2 fuel oil and livers of English sole exposed to the oil were analyzed for AHs by conventional analytical methods[10], and a partial listing of the compounds found is given in Table I. Existing procedures were modified to enable us to quantitate metabolites. Thirteen AH metabolites were found*, in amounts of 50–1100 ng/g wet wt. in livers of fish exposed to oil; none were found in control livers (Fig. 1 and Table II). Five other polar compounds, dibenzothiophene, fluorenemethanol, C_2H_5-fluorenone, ethoxybiphenyl and C_5H_{11}-quinoline, were tentatively identified through the Finnigan Library. Many of the metabolites in Table II were present in amounts near the lower limit of detection and quantitation, and relative standard deviations ranged from 6 to 41 %.

Estimated minimum detectable amounts of the reference compounds in GC–MS analysis are 1-naphthaldehyde 30, 2,2′-dihydroxybiphenyl 70, α-methyl-2-naphthalenemethanol 20, 9-hydroxyfluorene 15, 4-methyl-1-naphthol 50, 9-phenanthrol 150, 2-methyl-1,4-naphthoquinone 20, 1-naphthol 20, 2,6-dimethyl-3-naphthol 20, 6-methyl-2-naphthalenemethanol 50 and *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene 100 ng/g.

It was possible that the identified polar compounds were actually acquired by accumulating oxidized hydrocarbons from the fish-exposure tanks. To rule out this

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* In a preliminary study[22], analysis of a single liver sample from English sole exposed to oil showed an even wider variety of metabolites including [approximate amounts (ng/g)]: 1-naphthol (750), 1-naphthaldehyde (170), 4-methyl-1-naphthol (150), 2,6-dimethyl-3-naphthol (500), 9-hydroxyfluorene (130), 9-phenanthrol (1500), five other methylnaphthols (150–500), six other C_2H_5-naphthols (80–2000), another hydroxyfluorene (60), biphenol, naphthoic acid and five isomeric C_3H_7-naphthols.

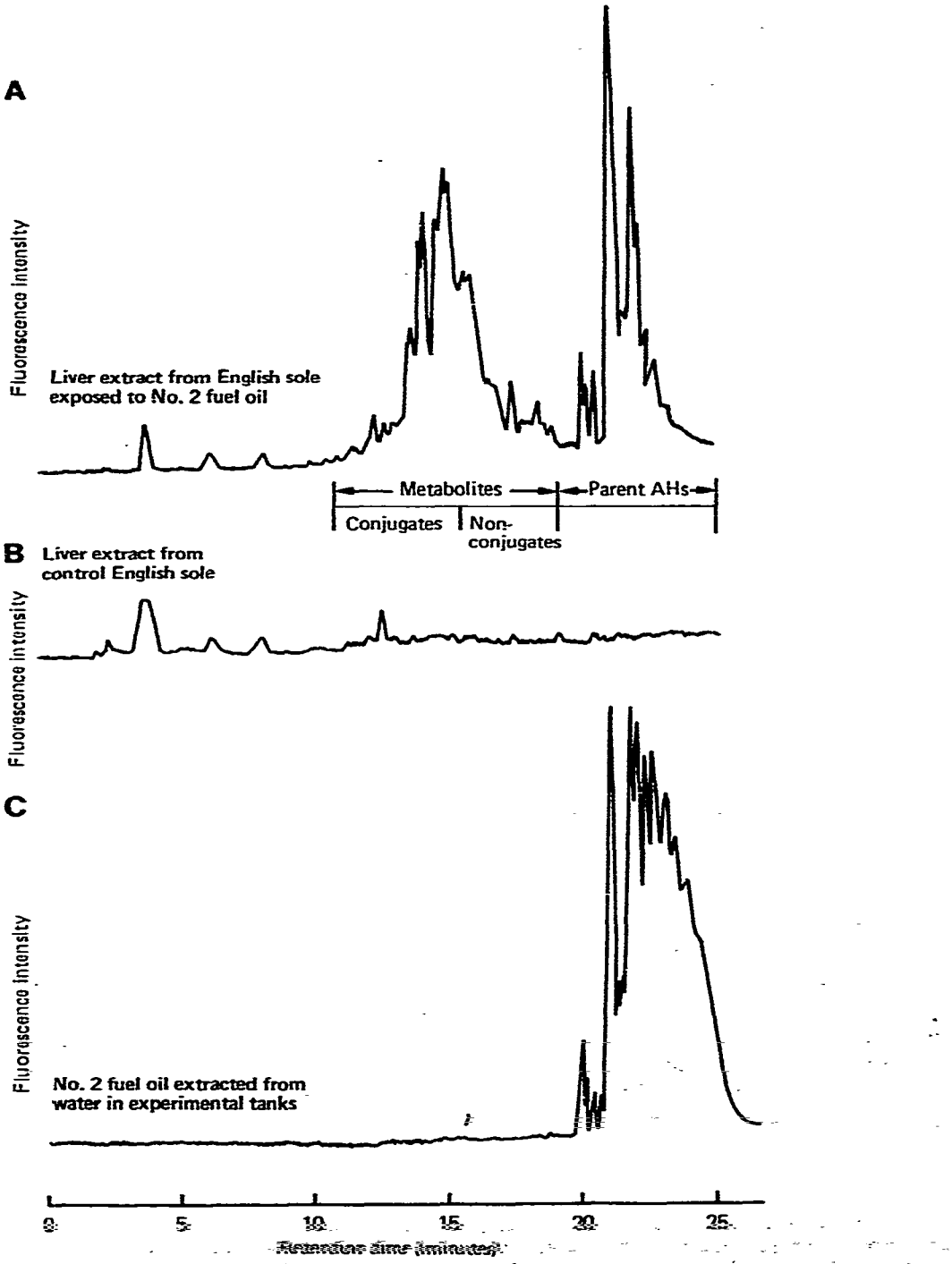

Fig. 1. Reversed-phase HPLC/fluorescence chromatograms (excitation at 290 nm, measurement at 335 nm, the optimum wavelengths for the naphthalene compounds). Gradient elution conditions have been reported previously[13]. (A) Liver extracts from fish exposed to 50 ppm of No. 2 fuel oil for 5 days. Retention times at which the naphthalene family of parent hydrocarbons and their metabolites elute are indicated. (B) Liver extracts from control fish. (C) Fuel oil was extracted from water in the experimental tank. Concentration of the oil is 10 times that of the experimental tank.

TABLE II

GC–MS QUANTITATION OF AROMATIC HYDROCARBON METABOLITES IN LIVERS OF FISH EXPOSED TO NO. 2 FUEL OIL

Scan No.	*Compound**	*Amount (ng/g wet wt)*	*R.S.D. (%)*	*Quantitation method***
1625	C_2H_5-Naphthol	160	38	2
1650	Methylnaphthalenemethanol	460	41	2
1659	6-Methyl-2-naphthalenemethanol	1000	6	1
1741	C_2H_5-Naphthol	180	28	2
1749	9-Hydroxyfluorene	49	39	1
1844	C_3H_7-Naphthol	720	19	3
1849	C_3H_7-Naphthol	440	23	3
1882	C_3H_7-Naphthol	120	12	3
1889	C_3H_7-Naphthol	750	17	3
1914	C_3H_7-Naphthol	160	22	3
1924	C_3H_7-Naphthol	150	33	3
2397	9-Phenanthrol	1100	30	1
2412	Phenanthrol or anthrone	920	25	2

* Compounds present, but not quantitated: C_5H_{11}-quinoline, dibenzothiophene, ethoxybiphenyl, fluorenemethanol, C_2H_5-fluorenone. Control livers contained no metabolites.

** GC–MS determination (or estimation) of amounts using response factor of (1) compound; (2) isomer (estimated); (3) most chemically similar compound, 2,6-dimethyl-3-naphthol (estimated).

route of accumulation, tank water was analyzed by HPLC; only parent hydrocarbons were shown to be present in concentrations above the minimum detectable amount (Fig. 1C).

DISCUSSION

The variety of AHs found in livers of fish exposed to fuel oil resembled that found in the fuel oil itself (see list of selected AHs in Table I). However, each of these AHs is rapidly converted to a series of metabolic products, most often phenols, alcohols and diols[4–8,19,20]. Conjugating enzymes can then transform these metabolites into the corresponding glucuronides, sulfates or other products.

Previous studies of fish exposed to radioactive AHs have demonstrated that metabolites account for a large fraction of the total radioactivity in livers after short periods of exposure (1 day to 1 week)[4–7,19,20]. Our studies of fish exposed to fuel oil substantiate this; HPLC results of liver extracts showed that significant portions of the AHs were converted to metabolites after 5 days (Fig. 1). However, HPLC did not resolve individual metabolites in our complex mixture, so GC–MS was used to separate, identify and quantitate the metabolites.

Several comments about the methods chosen to analyze for metabolites are necessary. Metabolites were extracted from the liver matrix using an automated extractor/concentrator because that method was rapid and recoveries were good (generally >90%). Then, HPLC was used to separate the non-conjugated naphthalene-type metabolites from the conjugates and naturally occurring compounds (*e.g.*, lipids) to prepare the sample for GC–MS analysis. No attempt was made to determine metabo-

lites of all AHs found in oil; we limited the study to non-conjugated metabolites of two- and three-ring AHs. A bonded-phase GC column and methanol as solvent functioned well for the GC analysis of our polar compounds. However, two problems were encountered. Residues tended to build up in the GC injection system because many polar compounds were non-volatile, so the GC liner had to be cleaned frequently. In addition, GC columns appeared to degrade more rapidly, as measured by peak broadening and tailing, with polar samples than with samples containing predominately parent AHs.

Two points of interest were apparent from our interpretation of the mass spectral data (Table II). First, parent AHs were oxidized to the expected types of metabolites. The metabolic products detected (Table II) were primarily alcohols and phenols. Second, several metabolites were present in livers in substantial amounts (as high as 1100 ng/g). Analyses conducted previously on non-radiolabeled environmental samples had not been able to determine these xenobiotics.

Certain AH metabolites may have serious consequences to the health or behavior of organisms. For example, Varanasi *et al.*[8] have shown that the extent of binding of benzo[*a*]pyrene intermediates to hepatic DNA was comparable to values reported for mammalian tissues susceptible to AH-induced neoplasms[8]. Also, in a study on the effect of petroleum exposure on the predatory behavior of coho salmon (*Oncorhynchus kisutch*), Folmar *et al.*[21] postulated that the non-eating behavior of one group of experimental animals was probably a result of the accumulation of metabolites in the animals. However, because no method was available to determine metabolites, the postulate could not be tested. These observations, in conjunction with the results of our study, illustrate the necessity for determining both parent hydrocarbons and their metabolites in order to draw valid conclusions about the effects of xenobiotics on marine organisms and on the consumer of fishery products.

CONCLUSIONS

Tissues of fish exposed to the complex mixture of chemicals in fuel oil can now be analyzed for both parent hydrocarbons and metabolites of two- and three-ring AHs. Analyses have shown that several metabolites are present in livers of English sole in amounts from 50 to 1100 ng/g. Although metabolites may be more harmful to the organism than the parent hydrocarbons, they have previously gone undetected in environmental samples. Thus, our procedures for determining metabolites are an important first step toward routinely determining polar compounds in aquatic organisms exposed to xenobiotics in their natural environment.

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