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Bioassay-directed fractionation of Polycyclic aromatic hydrocarbons of Molecular mass 302 in coal tar contaminated sediment.

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NWRI Contribution No. 98-266

MANAGEMENT PERSPECTIVE

- **Title:** Bioassay-directed fractionation of polycyclic aromatic hydrocarbons of molecular mass 302 in coal tar contaminated sediment.

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- **NWRI Publication #:** 98-266
- **Citation:**

- EC Priority/Issue: This work was done as part of the Great Lakes 2000 Program and the Great Lakes University Research Fund, to aid in the assessment and remediation of contaminated sediments in areas of coal tar contamination such as Sydney Harbour and Hamilton Harbour. This work supports the EC priority of implementing ecosystem initiatives focused on ecosystems of national priority including AOCs in the Great Lakes and Atlantic Coastal Areas.

- Current Status: This work is an investigation of the genotoxicological potential of high molecular mass polycyclic aromatic hydrocarbons; these compounds fall outside of the scope of conventional analyses for POPs. This work parallels research at the National Institute of Standards and Technology (USA) that has determined these compounds to be prevalent in a wide variety of environmental matrices. A combination of multi-dimensional chromatographic techniques and the Ames *Salmonella*/microsome assay was used to asses the contribution of the molecular mass 302 benzologue class and some individual compounds to the total genotoxic burden of coal-tar contaminated sediment. This work underscores the significant contribution of these compounds to the biological activity of complex environmental

mixtures; their presence must be taken into consideration when planning remediation of PAH-contaminated sediments.

- Next Steps: Publish in a scientific journal and communicate to RAP processes. Any continuation of this work is contingent upon funding.

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Bioassay-directed fractionation of PAH of molecular mass 302 in coal tar-contaminated sediment

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Abstract

Bioassay-directed fractionation was used to characterise genotoxic polycyclic aromatic hydrocarbons (PAH) of molecular mass 302 amu in organic solvent extracts of coal tar-contaminated sediment from Sydney Harbour, Nova Scotia. A normal phase HPLC technique was employed to separate PAH-rich solvent extracts into fractions containing PAH of single molecular mass classes. The 302 amu molecular mass fraction was isolated and further separated using reversed phase HPLC; subfractions were collected every 30 s and subjected to bioassay analyses with *Salmonella typhimurium* strain YG1025 with the addition of oxidative metabolism (4% S9). Compounds eluting in the most active subfractions included naphtho[2,1-*a*]pyrene and naphtho[2,3-*a*]pyrene. The results of this study underscore the significant contribution that molecular mass 302 PAH make to the biological activity of complex environmental mixtures. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Bioassay-directed fractionation; Coal tar; *Salmonella typhimurium*; Sediment; Polycyclic aromatic hydrocarbons; Genotoxicity

1. Introduction

Determination of polycyclic aromatic hydrocarbons (PAH) in complex environmental mixtures is almost always restricted to designated priority pollutant compounds. However, some studies have shown priority pollutant PAH may constitute only a portion of the mutagenic and carcinogenic PAH-related activity in some complex environmental mixtures (Grimmer et al., 1991; Marvin et al., 1993, 1994, 1995; Durrant et al., 1998; Hannigan et al., 1998). We have previously used a bioassay-directed fractionation methodology to

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show that almost all of the mutagenic activity detected in extracts of coal tar-contaminated sediments to be attributable to PAH with molecular masses equal to or exceeding 252 atomic mass units (amu). Benzo[*a*]pyrene, indeno[1,2,3-*cd*]pyrene, dibenz[*a,h*]anthracene and PAH of molecular mass 302 amu were identified in mutagenic fractions collected from reversed phase HPLC analysis of extracts of coal tar-contaminated sediments from Hamilton Harbour, Lake Ontario (Marvin et al., 1993) and Sydney Harbour, Nova Scotia (Marvin et al., 1994, 1995). These fractions exhibited significant responses in the Ames *Salmonella typhimurium* microsome assay using a strain that detects base-pair substitutions (a TA100-like strain) with the addition of oxidative metabolism (Aroclor 1254-induced rat liver supernatant S9). Recently, a human lymphoblast mutagenicity assay was used to identify a number of 302 amu PAH as potent mutagens in urban air extracts (Durrant et al., 1998; Hannigan et al., 1998)

To facilitate identification of genotoxic PAH, we developed a normal phase (NP)-HPLC method to separate PAH-rich organic solvent extracts into fractions containing PAH of a single benzologue class (Marvin et al., 1995). This preliminary separation of PAH into molecular mass classes assisted in identification of PAH molecular mass classes, including 302 amu PAH, as mutagens. Each of the PAH fractions were further separated by reversed phase (RP)-HPLC and subfractions were collected and subjected to the *Salmonella*/microsome assay; the identity of bioactive PAH in these subfractions was confirmed by GC-MS analysis (Legzdins et al., 1995; Marvin et al., 1995). Approximately 20% of the total mutagenic activity exhibited by an extract of coal-tar-contaminated sediment from Sydney Harbour, Nova Scotia, was attributed to 302 amu PAH (Marvin et al., 1995).

In this paper, we have further investigated the mutagenic potency of 302 amu PAH using multidimensional liquid chromatography and the *Salmonella typhimurium*/microsome assay. The use of a semi-preparative polyaminocyano HPLC column resulted in fractionation of an extract of coal tar-contaminated sediment into isomeric molecular mass PAH classes. The molecular mass 302 PAH fraction was then fractionated by RP-HPLC using a Vydac 201TP column and subfractions of the column effluent were collected. Tentative identifications based on UV-visible spectra were assigned to 302 amu PAH present in the mutagenic subfractions.

2. Methods

2.1. Instrumentation

Extraction was performed using a 300-W Fisher Sonic Dismembrator Model 300 with a 0.75-inch diameter titanium horn (Fisher Scientific, Fairlawn, NJ, USA). Reversed and normal phase HPLC were performed on a Hewlett-Packard Model 1090 liquid chromatograph with a diode array detector (Hewlett-Packard Co., Mississauga, ON, Canada).

2.2. Extraction and clean-up

Organic solvent soluble material was prepared using an extraction and fractionation procedure previously reported (Marvin et al., 1992). Dry sediment (5 g) was suspended in 50 ml of dichloromethane in a glass beaker and eight consecutive ultrasonic pulses (15-s duration each) were applied at full power. The suspension was filtered and the procedure was repeated with 50 ml of fresh dichloromethane, followed by a final sonication with 50 ml of methanol.

The dichloromethane/methanol extract was adsorbed onto alumina (3 g, Brockman activity 1, 80–200 mesh) by solvent evaporation under reduced pressure and applied to the top of fresh alumina (6 g dried at 170°C for 48 h) contained in a glass column. Organic components were eluted using solvents of increasing polarity. Hexane (60 ml) afforded an aliphatic fraction. Non-polar PAC were eluted by sequential addition of benzene (50 ml) and dichloromethane/ethanol (70 ml, 99:1 v/v), which were combined to afford a single fraction. Methanol (50 ml) and methanol/water (50 ml, 3:1 v/v) were collected and combined to

afford a polar PAC fraction. The non-polar PAC fraction was then subjected to a Sephadex LH20 (Pharmacia Fine Chemicals, Uppsala, Sweden) column clean-up step (mobile phase, hexane/methanol/dichloromethane (6:4:3 v/v), flow rate 3 ml/min) to remove aliphatic compounds.

2.3. Normal phase HPLC fractionation

The non-polar PAC fraction from the alumina-Sephadex LH20 clean-up was separated into PAH molecular weight classes using NP-HPLC (Fig. 2); this procedure has been described in detail elsewhere (Marvin et al., 1995). A normal phase 10-μm 25 cm × 9.4 mm i.d. Whatman Partisil M9 PAC (polyaminocyano) semi-preparative HPLC column (Whatman, Clifton, NJ, USA) was used with a 100 μl sample loop and a mobile phase flow rate of 4.2 ml/min with the following linear gradient elution program (elapsed time, composition of mobile phase): initial, 95% hexane and 5% dichloromethane; 10 min, 95% hexane and 5% dichloromethane; 30 min, 75% hexane and 25% dichloromethane; 45 min, 45% hexane and 55% dichloromethane; 60 min, 100% dichloromethane; 65 min, 100% acetonitrile; 70 min, 100% acetonitrile; 75 min, 100% dichloromethane; 80 min, 95% hexane, 5% dichloromethane. The column temperature was 40°C.

The following linear gradient elution program was used for secondary separation of the molecular mass 302 PAH fraction to afford eight fractions for RP-HPLC analyses (Fig. 4): initial, 88% hexane and 12% dichloromethane; 30 min, 80% hexane and 20% dichloromethane; 32 min, 88% hexane and 12% dichloromethane; 40 min, 88% hexane and 12% dichloromethane.

2.4. Reversed phase HPLC

Reversed phase HPLC was performed using a 5-μm 25 cm × 4.6 mm i.d. Vydac analytical column (201TP54, Separations Group, Hesperia, CA, USA) or a Bakerbond wide-pore octadecyl 5-μm 25 cm × 4.6 mm i.d. analytical column (7100-00, J.T. Baker Inc., Philipsburg, NJ, USA). A mobile phase flow rate of 1.0 ml/min was used with the following linear gradient elution programs (elapsed time, composition of mobile phase): Program A: Column temperature, 35°C; initial, 60% acetonitrile and 40% water; 30 min, 100% acetonitrile; 55 min, 50% acetonitrile and 50% dichloromethane; 60 min, 100% acetonitrile; 65 min, 60% acetonitrile, 40% water; Program B: Column temperature, 0°C; initial, 80% acetonitrile and 20% dichloromethane; 10 min, 80% acetonitrile and 20% dichloromethane; 40 min, 100% dichloromethane; 45 min, 80% acetonitrile and 20% dichloromethane.

2.5. Bioassays

The *Salmonella typhimurium* bacterial strain used was YG1025 which is auxotrophic for histidine (*his* G46) and contains pKM101 (Watanabe et al., 1991). Strain YG1025 is practically identical to strain TA100 and exhibits similar responses to standard mutagens, but has been modified by the addition of the plasmid pBR322.

The protocol for bioassays was adapted from Maron and Ames (1983). Bacteria were grown for 10 h at 37°C in Oxoid Nutrient Broth #2 (15 ml) with ampicillin (50 μg/ml) and tetracycline (6.25 μg/ml). Dilutions of organic extracts dissolved in 50 ml DMSO were assayed in duplicate with metabolic activation (4% Aroclor 1254-induced rat liver S9). Dose ranges of five concentrations were tested in duplicate to produce dose responses. Mutation chromatograms were produced from duplicate assays of subfractions. After a 48 h incubation period at 37°C the number of revertant colonies (histidine-independent) was determined. Biological activity values were extrapolated from linear segments of the dose–response curves. The average spontaneous reversion rate (background rate) of strain YG1025 + S9 was 190 revertants. The positive control was benzo[*a*]pyrene (1 μg/plate).

3. Results and discussion

Sediment was sampled near the outflow of Muggah Creek in Sydney Harbour, Nova Scotia, in an area heavily contaminated by coal tar

(Matheson et al., 1983; Kiely et al., 1988). Sediment was air-dried and extracted sequentially by ultrasonication with dichloromethane and methanol. The multi-dimensional chromatographic scheme used in sample preparation is illustrated in Fig. 1. The crude sample extract (8.0% by weight of dry sediment) was subjected to open column alumina chromatography affording a non-polar aromatic polycyclic aromatic compound (PAC) fraction and a polar PAC fraction. The non-polar PAC fraction (28% of weight of crude extract) was passed through a Sephadex LH20 gel column to remove aliphatic compounds. Total PAH content of the sample (sum of concentrations of 16 priority pollutant PAH) was approximately 10 000 μg/g dry sediment (Marvin et al., 1994).

The non-polar PAC and polar PAC fractions were bioassayed with *Salmonella typhimurium* strain YG1025 with the addition of rat liver supernatant (4% S9) to simulate mammalian metabolic mechanisms. These bioassay conditions were chosen because PAH are base-pair substitution type mutagens requiring oxidative metabolism to transform them into mutagenic products. The non-polar PAC fraction contained over 90% of the mutagenic activity exhibited by the crude sample extract. This fraction was further separated by NP-HPLC enabling collection of isomeric molecular mass PAH classes (Fig. 2). Probe EI-MS analyses were used to confirm that fractions contained PAH comprised almost exclusively of a single benzologue class. We have demonstrated this NP-HPLC methodology to be effective for isolating PAH classes and other compound classes in a variety of complex environmental samples

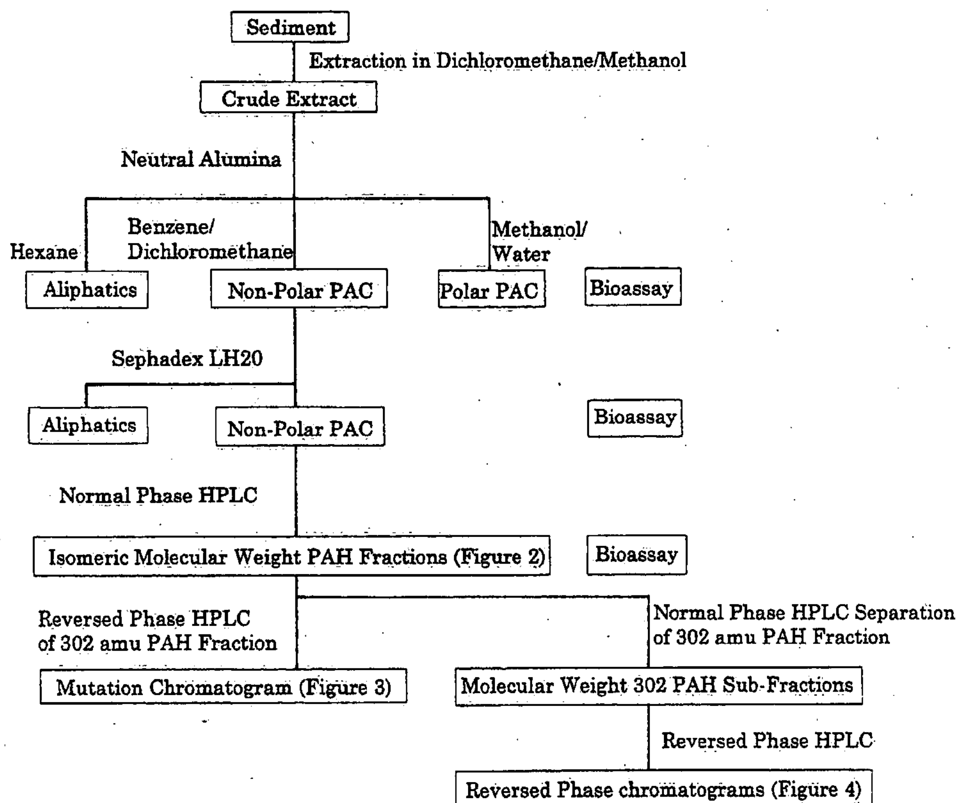

Fig. 1. Diagram of sample clean-up and fractionation scheme.

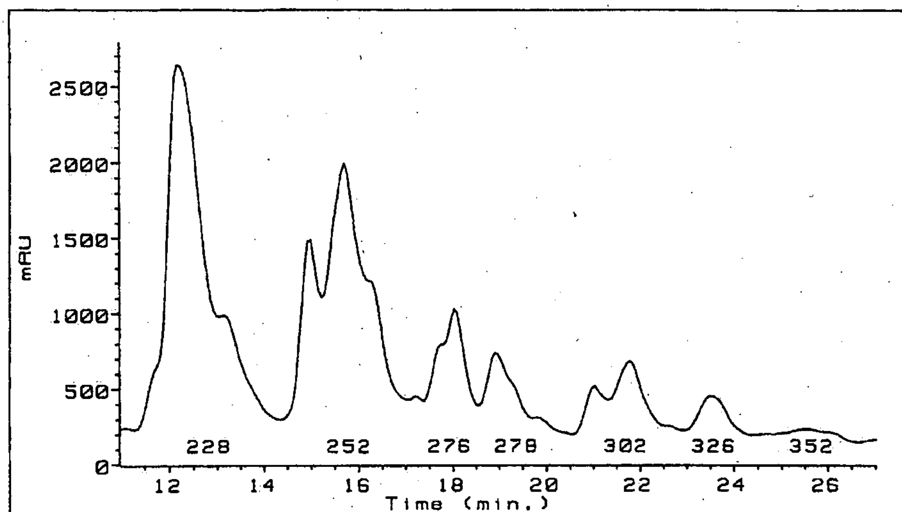

Fig. 2. Normal phase HPLC chromatogram of the non-polar polycyclic aromatic compound fraction of a coal tar-contaminated Sydney Harbour sediment extract. The numbers on the peaks correspond to masses of the principal polycyclic aromatic hydrocarbons.

(Legzdins et al., 1995). The fractions were also bioassayed in strain YG1025 + S9 and mutagenic activities were calculated by regression of linear portions of the dose–response curves; the molecular mass 302 fraction contained approximately 20% of the total activity exhibited by PAH in the extract.

The molecular mass 302 PAH fraction was then separated by RP-HPLC using the Vydac column (gradient program A) and 30-s subfractions were collected. Each subfraction was subjected to duplicate bioassays using strain YG1025 + S9. Under these chromatographic conditions, compounds exhibiting significant responses were poorly resolved (data not shown). Optimal separation of these compounds was obtained by reducing the column temperature to 0°C and using an acetonitrile/dichloromethane gradient elution program (program B). The plot of mutagenic responses of the molecular mass 302 PAH subfractions and the UV absorption profile, defined as a mutation chromatogram, is shown in Fig. 3. The mutation chromatogram characterises HPLC column effluent in terms of both a conventional UV absorption profile and a mutagenic activity profile. Mutagenic activities of individual subfractions are expressed as net revertants reflecting background correction by subtraction of the spontaneous reversion rate of YG1025 + S9 (190 revertants). Subfractions exhibiting positive mutagenic activity were defined as those that exceeded 200 net revertants (390 total revertants or approximately double the spontaneous reversion rate of YG1025 + S9).

The molecular mass 302 PAH fraction mutation chromatogram exhibited a number of zones of elution containing significant mutagenic activity (Fig. 3). Tentative identifications of individual compounds (Table 1) were assigned by comparison of UV absorption spectra with spectra of authentic standards or spectral data from other studies (Fox and Staley, 1976; Peadon et al., 1980; Colmsjo and Ostman, 1988; Wise et al., 1988a,b, 1993a,b; Fetzer and Biggs, 1984, 1985; Schmidt et al., 1987). The volume of extract injected into the HPLC to produce the mutation chromatogram was equivalent to approximately 100 mg of dry sediment. Bioassays of standard solutions of dibenzo[*a,e*]pyrene, dibenzo[*a*,l]pyrene and dibenzo[*a,h*]pyrene resulted in mutagenic activities of 63 revertants per nanomole (rev/nmol), 75 rev/nmol and 25 rev/nmol, respectively. The re-

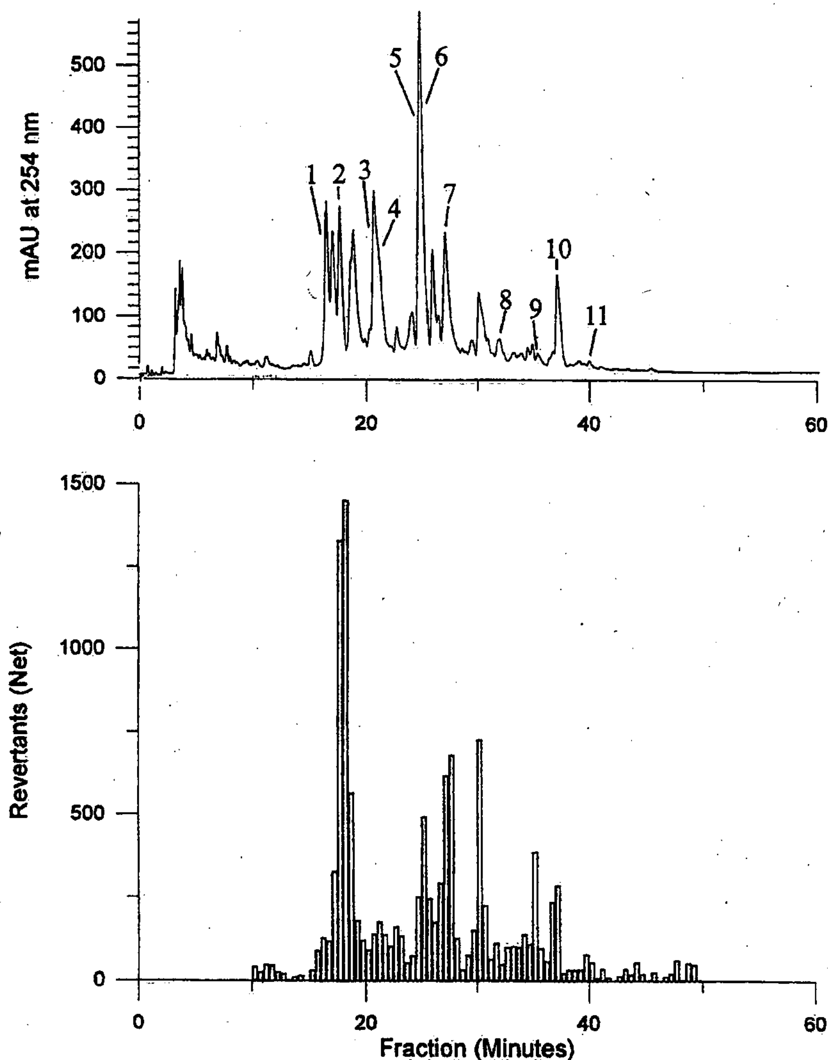

Fig. 3. Molecular mass 302 polycyclic aromatic hydrocarbon fraction mutation chromatogram. The top panel shows the UV absorption profile (254 nm) from the reversed phase HPLC separation with individual polycyclic aromatic hydrocarbons identified by number. The peak numbers correspond to the compounds listed in Table 1. The bottom panel shows the net mutagenic responses, corrected for the spontaneous reversion rate, for single doses assayed in duplicate of each of the individual 30-s subfractions assayed using strain YG1025 + S9.

sponses of these compounds in strain YG1025 + S9 were similar to that of benzo[*a*]pyrene (77 rev/nmol). Dibenzo[*a*,l]pyrene and dibenzo[*a*,*e*] pyrene have been demonstrated to be strong animal carcinogens (Lacassagne et al., 1958, 1963, 1968; DiGiovanni et al., 1983; Dipple, 1983; Dipple et al., 1984; Cavalieri et al., 1991; Gill et al., 1994) while dibenzo[*a*,*h*]pyrene has been shown to have significant SOS-inducing potency (Mersch-Sundermann et al., 1992). The zones of significant mutagenic activity in the mutation chromatogram did not correspond to elution of any dibenzopyrene isomers and the levels of dibenzo[*a*,*i*]pyrene (peak 8, Fig. 3) and

dibenzo[*a,h*]pyrene (peak 11, Fig. 3) were estimated to be lower than 3 μg/g in the sediment. We assumed the mutagenic activity of dibenzo[*a,i*]pyrene to be within the range of activities exhibited by the other dibenzopyrene isomers we assayed, based on dibenzo[*a,i*]pyrene exhibiting a similar SOS induction potential to that of dibenzo[*a,h*]pyrene (Mersch-Sundermann et al., 1992).

Tentatively identified compounds eluting within the zones of positive bioassay response in the mutation chromatogram included naphtho[2,3-*e*]pyrene (peak 2, Fig. 3), naphtho[2,1-*a*]pyrene (peak 7), naphtho[2,3-*a*]pyrene (peak 9), naphtho[2,3-*b*]fluoranthene (peak 5), dibenzo-[*b,k*]fluoranthene (peak 6) and naphtho[2,3-*k*]-fluoranthene (peak 10). All of these compounds have recently been shown to be present in urban air particulate and to be significant to modest contributors to the mutagenic potency using a human lymphoblast mutagenicity assay (Durrant et al., 1998). We have been unable to find literature evidence supporting the potential carcinogenicity of naphthofluoranthene or dibenzofluoranthene compounds but naphtho[2,1-*a*]pyrene and naphtho[2,3-*a*]pyrene have both been demonstrated to be carcinogenic (Hendricks et al., 1971; Lacassagne et al., 1960). Naphtho[2,3-*e*]pyrene has been reported to be inactive as a carcinogen (Dipple et al., 1984). Spectra of naphtho[2,1-*a*]pyrene (peak 7) and naphtho[2,3-*a*]pyrene (peak 9) were practically identical to reference spectra (Schmidt et al., 1987; Wise et al., 1988a) and the peaks exhibited a high degree of purity, indicating elution of single compounds. It is therefore highly probable that naphtho[2,1-*a*]pyrene and naphtho[2,3-*a*]pyrene were responsible for the mutagenic activities detected at 27–28 min and 35–35.5 min, respectively. Given its small peak area and relatively high mutagenic response, it is predicted that naphtho[2,3-*a*]pyrene will be a potent mutagen in the *Salmonella*/microsome assay.

The RP-HPLC chromatographic profile belied the complexity of the 302 amu PAH fraction and

Table 1
Chromatographic peak identifications in a mutation chromatogram (Fig. 3) resulting from the analysis of the molecular mass 302 amu polycyclic aromatic hydrocarbon fraction of a coal tar-contaminated sediment sample extract

1.	Dibenzo[*b,e*]fluoranthene[a,b]
2.	Naphtho[2,3-*e*]pyrene[a,b]
3.	Naphtho[1,2-*k*]fluoranthene[a]
4.	Benzo[*b*]perylene[a]
5.	Naphtho[2,3-*b*]fluoranthene[a,b]
6.	Dibenzo[*b,k*]fluoranthene[a,b]
7.	Naphtho[2,1-*a*]pyrene[a,b]
8.	Dibenzo[*a,i*]pyrene[c]
9.	Naphtho[2,3-*a*]pyrene[a,b]
10.	Naphtho[2,3-*k*]fluoranthene[a,b]
11.	Dibenzo[*a,h*]pyrene[c]

[a]Identification based on UV spectral comparison with spectra from the literature (Fox and Staley, 1976; Peadon et al., 1980; Fetzer and Biggs, 1984, 1985; Schmidt et al., 1987; Wise et al., 1988a,b, 1993a,b; Colmsjo and Ostman, 1988).
[b]Compound elutes in retention time range that exhibited significant mutagenic activity.
[c]Identification based on UV spectral comparison with spectrum of standard.

Table 2
Chromatographic peak identifications in molecular mass 302 polycyclic aromatic hydrocarbon fractions prepared using normal phase HPLC and analysed using reversed phase HPLC (Fig. 4)[a]

1.	Dibenzo[*b,e*]fluoranthene[b]
12.	Dibenzo[*a,e*]fluoranthene[b]
13.	Dibenzo[*a,k*]fluoranthene[b]
2.	Naphtho[2,3-*e*]pyrene[b]
14.	Dibenzo[*a,e*]pyrene[c]
3.	Naphtho[1,2-*k*]fluoranthene[b]
4.	Benzo[*b*]perylene[b]
15.	Dibenzo[*e,l*]pyrene[b]
6.	Dibenzo[*b,k*]fluoranthene[b]
5.	Naphtho[2,3-*b*]fluoranthene[b]
7.	Naphtho[2,1-*a*]pyrene[b]
8.	Dibenzo[*a,i*]pyrene[b]
9.	Naphtho[2,3-*a*]pyrene[b]
10.	Naphtho[2,3-*k*]fluoranthene[b]
11.	Dibenzo[*a,h*]pyrene[c]
16.	Coronene[d]

[a]Compound numbers correspond to numbered peaks in Fig. 4.
[b]Identification based on UV spectral comparison with spectra from literature (Fox and Staley, 1976; Peadon et al., 1980; Fetzer and Biggs, 1984, 1985; Schmidt et al., 1987; Colmsjö and Ostman, 1988; Wise et al., 1988a,b, 1993a,b).
[c]Identification based on UV spectral comparison with spectrum of standard.
[d]Molecular weight 300 amu.

spectra of many of the chromatographic peaks indicated co-elution of multiple compounds which precluded unequivocal compound identification. To better resolve co-eluting compounds, the 302 PAH fraction was subjected to a second NP-HPLC separation using a modified gradient elution program (chromatogram not shown). This secondary NP-HPLC separation afforded eight individual fractions which were analysed by RP-HPLC using both the Vydac C-18 column and the Bakerbond C-18 column with a water/acetonitrile/dichloromethane gradient program at 35°C (program A, Fig. 4). These RP-HPLC analyses confirmed the complexity of the 302-amu PAH fraction; the frequency of co-elution of compounds was reduced which enabled identification of additional compounds. Compounds in fractions 3 and 5 were better resolved using the Bakerbond C-18 column while the Vydac C-18 column resulted in better separation of the remaining fractions. Column effluent corresponding to individual peaks was collected during the analysis of these eight fractions which resulted in collection of 90 individual compounds. Tentative identifications of individual

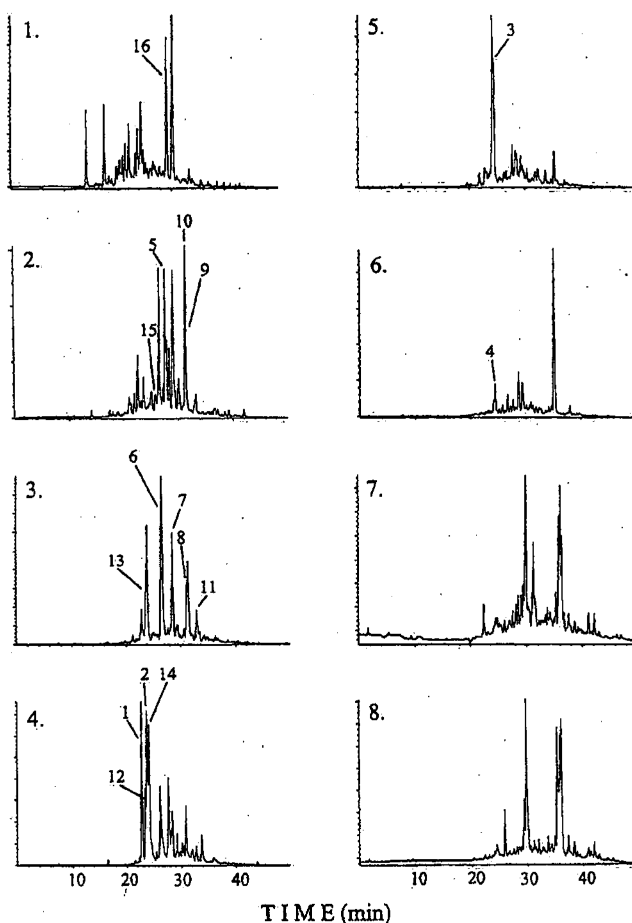

Fig. 4. Reversed phase HPLC chromatograms of the eight molecular mass 302 polycyclic aromatic hydrocarbon fractions prepared using normal phase HPLC. Fractions 1,2,4,6,7 and 8 were separated on a Vydac C-18 column; fractions 3 and 5 were separated on a Bakerbond C-18 column. The peak numbers correspond to the compounds listed in Table 2. The ultra-violet absorption profiles are the averaged responses over the wavelength range from 250 to 370 nm.

compounds are listed in Table 2 and were assigned by comparison of UV absorption spectra with spectra of standards or spectral data from other studies. Many of the compounds exhibited spectra similar to those of compounds listed in Table 1, indicating that they were alkylated 302 amu PAH derivatives. We have tentatively identified 58 of the 90 compounds as alkyl derivatives of 302 amu PAH based on probe mass spectral data. Included in the additional compounds tentatively identified was dibenzo[*a,e*]pyrene, a known carcinogen (Lacassagne et al., 1963). The relatively early elution time of dibenzo[*a,e*]pyrene under RP-HPLC conditions suggests that this may have been one of the compounds responsible for the strong bioassay responses in the 17–19-min zone of the mutation chromatogram (Fig. 3). We are currently working towards confirming the identifications of the compounds we have isolated and carrying out thorough chemical and biological characterisations.

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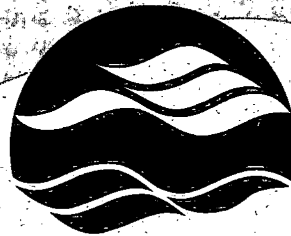

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