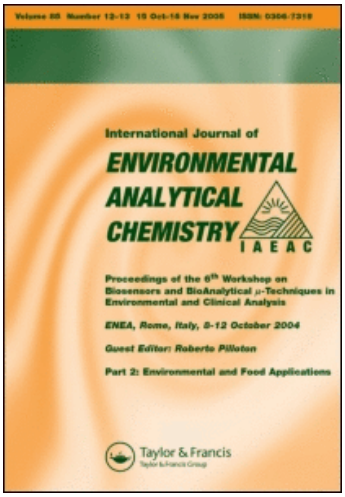

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METHODOLOGY FOR BIOASSAY-DIRECTED FRACTIONATION STUDIES OF AIR PARTICULATE MATERIAL AND OTHER COMPLEX ENVIRONMENTAL MATRICES

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A normal phase HPLC methodology using a semi-preparative polyaminocyano column in conjunction with a selection of short-term genotoxicity assays has been developed for bioassay-directed fractionation studies of complex environmental mixtures. To illustrate the effectiveness of this methodology, an organic extract prepared from respirable air particulate samples collected in Hamilton, Canada was separated into a non-polar aromatic fraction and a polar aromatic fraction using a combination of alumina and Sephadex LH20 chromatography. These fractions were evaluated for their genotoxic potential using the *Salmonella*/microsome (Ames) assay with six different strains of *Salmonella*.

The non-polar aromatic fraction was analyzed by normal phase HPLC and the eluent was collected in one-minute subfractions; these subfractions were bioassayed in three different *Salmonella* strains (YG1021 -S9, YG1024 -S9 and YG1029 +S9) to afford three different mutation profiles of this sample. Some subfractions which exhibited high mutagenic responses were subjected to further chemical analyses using GC/MS in order to identify those compounds responsible for the genotoxic responses. The nitroarene compounds 2-nitrofluoranthene, 1-nitropyrene and 2-nitropyrene and higher molecular weight polycyclic aromatic hydrocarbons such as benzo[a]pyrene and indeno[1,2,3-cd]pyrene were identified and quantified in some of the biologically active subfractions. The normal phase gradient conditions afforded very reproducible retention times for a series of polycyclic aromatic standards with a broad range of compound polarities. In addition, polycyclic aromatic hydrocarbons (PAH) were observed to elute from the normal phase HPLC column in a series of peaks; successive peaks contained PAH of increasing molecular weight while any individual peak was shown to contain PAH of the same molecular weight.

KEY WORDS: Urban air particulate, polycyclic aromatic compounds, nitroarenes, genotoxicity, *Salmonella typhimurium*, bioassay-directed fractionation.

INTRODUCTION

Polycyclic aromatic compounds (PAC), which include polycyclic aromatic hydrocarbons (PAH), are commonly determined pollutants because of their ubiquitous presence in the environment and their demonstrated mutagenic and carcinogenic properties[1–4]. The mutagenic potencies of extracts of respirable air particulate sampled from urban environments have been ascribed to elevated levels of select PAC[5–7]. A powerful methodology known as bioassay-directed fractionation, which utilizes short-term

biological assays in conjunction with analytical chemistry techniques, has been used successfully in the identification of a variety of mutagenic PAC in complex mixtures[8], including compounds isolated from organic solvent extracts of urban air particulate[5,6,9,10].

We have previously described a bioassay-directed fractionation approach for the determination of mutagenic PAC in organic solvent extracts of inhalable (PM10) air particulate material from Hamilton, Ontario, Canada[11] and particulate material collected in a steel foundry in Hamilton[12]. These samples were extracted sequentially with dichloromethane then with methanol using a Soxhlet apparatus. The combined extracts were then subjected to open column alumina chromatography and Sephadex LH20 gel chromatography. Semi-preparative normal phase high-performance liquid chromatography (HPLC) using a polyaminocyano column (Whatman PAC column) was employed to separate the non-polar aromatic fraction into compound classes such as PAH, nitro-PAH, keto-PAC and aza-PAC. Using this methodology, we have examined extracts of Hamilton air particulate material and have identified both direct-acting mutagenic PAC such as 2-nitrofluoranthene and indirect-acting mutagens, such as PAH[11,13].

Bioassay-directed fractionation methods have also been useful for the determination of mutagens in extracts prepared from bottom sediments, suspended sediments and freshwater mussel tissues[14–16]. In these matrices and in air particulate material, the higher molecular weight PAH such as benzo[a]pyrene and indeno[1,2,3-cd]pyrene were determined to be significant contributors to the overall mutagenic activities of the extracts; these compounds require oxidative enzymatic transformations to exhibit their effects. In conclusion, the extracts of many complex environmental mixtures contain mutagenic PAC of widely varying molecular weights and polarities. These results illustrate the need and utility of a general methodology for sample extraction, clean-up and fractionation that is applicable to a variety of complex environmental mixtures, ranging from air particulate material to soils and sediments to biological samples.

In this paper, we present a modification of our previously reported[16] normal phase HPLC method that has been developed for the separation, collection and identification of mutagenic compounds and compound classes in complex organic solvent extracts. The modified method has been applied to the examination of extracts of Hamilton air particulate to illustrate the effectiveness of the technique. Air particulate material was chosen for this study because of the wide variety of compound classes that are present in extracts prepared from this type of sample matrix. The non-polar aromatic fractions of extracts of Hamilton air particulate were separated using a polyaminocyano HPLC column and one minute subfractions were collected and subjected to bioassay analysis with selected *Salmonella typhimurium* strains. Subfractions exhibiting significant amounts of biological activity were the focus of analyses by GC-MS to identify the biologically active compound(s).

EXPERIMENTAL

Instrumentation

Normal-phase HPLC was performed on a Hewlett-Packard Model 1090 liquid chromatograph with built-in diode-array detector (Hewlett-Packard Co., Mississauga, Ontario). A Beckman Model 110A HPLC pump equipped with a Beckman Model 153 UV detector (Beckman Instruments, Fullerton, CA) was used in the Sephadex LH20

clean-up procedure. Gas chromatography-mass spectrometry (GC-MS) was performed on a Hewlett-Packard model 5890 series II gas chromatograph equipped with an HP model 5971A mass-selective detector and an on-column injector.

Materials

An Anderson PM10 hi-vol air sampler (General Metal Works, Cleveland, OH) was used to collect a total of 38 respirable airborne particulate samples. Each sample represented the particulate collected from 1630 m^3 of air over a 24 hour period. Respirable particulate material was collected on 8 × 10 inch Teflon-coated glass fibre filters (Pallflex TX40M120WW). Air sampling was carried out between May 1990 and June 1991 at an air quality monitoring station in downtown Hamilton, operated by the Ontario Ministry of Environment and Energy, and at the Canada Centre for Inland Waters (CCIW), Burlington, Ontario. A total of twenty filters was collected at the downtown Hamilton site, representing the particulate material (1298 mg) obtained from 32620 m^3 of air. A further eighteen filters, collected at the CCIW site, afforded an additional 679 mg of particulate material obtained from the sampling of 30580 m^3 of air.

Neutral alumina (Brockman activity I, 80–200 mesh) was obtained from Fisher Scientific (Fairlawn, NJ) and was activated by heating at 170°C for a minimum of forty-eight hours. Sephadex LH-20 gel (25–100 μm) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Soxhlet extraction

Air filters were extracted individually using a Soxhlet apparatus with 180 mL of dichloromethane for 24 h, followed by methanol (180 mL) for 24 h. The dichloromethane and methanol extracts were combined and adsorbed to neutral alumina (1 g) by solvent evaporation under reduced pressure. The alumina containing the adsorbed sample was poured on top of 2 g of cooled, freshly activated alumina which had been dry-packed in a glass column (1 cm diameter). Following elution with 20 mL of hexane to remove aliphatic material, the non-polar PAC were obtained by sequential elution with 20 mL of benzene then with 25 mL of dichloromethane/ethanol (99:1, v/v). The polar PAC were removed from the alumina column by sequential elution with 20 mL of methanol followed by 20 mL of methanol/water (4:1, v/v). The non-polar PAC fraction was chromatographed on a Sephadex LH20 column[16] (mobile phase: hexane/methanol/dichloromethane, 6:4:3, v/v) to remove any remaining aliphatic components. Column dimensions: 10 cm × 0.9 cm i.d.; flow rate: 0.4 mL/min. All compounds eluting before the start of the naphthalene standard were discarded; all compounds eluting after the start of the naphthalene peak were collected as the non-polar aromatic fraction.

HPLC operating conditions

The HPLC operating conditions were as follows: diode array UV absorption detection over the wavelength range from 211 nm to 400 nm; column temperature: 40°C. Normal phase chromatography was performed using an amino precolumn (Brownlee Labs, Santa Clara, CA, 1.5 cm × 3.2 mm i.d.) and a 10 micron Whatman Partisil M9 PAC semi-

preparative column (Whatman, Clifton, NJ, 25 cm × 9.4 mm i.d.). A 100 µL sample injection loop and a mobile phase flow rate of 4.2 mL/min were used in conjunction with two linear gradient elution programs. Methods A and B used the same hexane/dichloromethane gradient profile; however, in Method A the column was eluted with acetonitrile at the end of the gradient in order to elute residual polar compounds such as quinolines, acridines and other polar compounds. Method A was used to produce the mutation chromatogram shown in Figure 3A.

Method A: (elapsed time, composition of mobile phase): initial, 95% hexane and 5% dichloromethane; 10 min., 95% hexane and 5% dichloromethane; 35 min., 70% hexane and 30% dichloromethane; 55 min., 30% hexane and 70% dichloromethane; 65 min., 100% dichloromethane; 70 min., 100% dichloromethane; 75 min., 100% acetonitrile; 80 min., 100% acetonitrile; 85 min., 100% dichloromethane; 95 min., 100% dichloromethane; 100 min., 95% hexane, 5% dichloromethane; 130 min., 95% hexane, 5% dichloromethane.

Method B is a modification of Method A with the following changes: 70 min., 100% dichloromethane; 75 min., 95% hexane, 5% dichloromethane; 110 min., 95% hexane, 5% dichloromethane. In cases which required a series of repetitive injections to prepare sufficient material, we have used Method B for all of the injections except the last injection which was made using Method A. Thus, all of the polar material from the previous injections elutes in the acetonitrile wash at the end of Method A.

GC-MS operating conditions

The GC-MS operating conditions were as follows: transfer line temperature: 300°C; helium carrier gas velocity: 30 cm/sec. For standard PAC analyses the following temperature program was used: 100° to 160°C at 20°C/min, 160° to 290° at 3°C/min, hold for 10 min at 290°C. For the determination of retention index values the following temperature program was used: hold at 40°C for 2 minutes, then 40°C to 300°C at 4°C/min.; hold for 20 min. at 300°C. The column used was a 30 m × 0.25 mm i.d. DB-5 with a 0.25 µm stationary phase film coating (J&W Scientific, Folsom, CA). An internal standard method was used for quantitation (benzo[a]anthracene-d_{12} for PAH analyses and 1-nitropyrene-d_9 for nitro-PAH analyses).

Bioassays

The *Salmonella typhimurium* bacterial strains used in this study were strains YG1020, YG1021, YG1024, YG1025, YG1026 and YG1029[17–19]. Strains YG1020, YG1021 and YG1024 are TA98 type strains that are auxotrophic for histidine and contain plasmid pKM101. Strain YG1020 is equivalent to strain TA98, while strains YG1021 and YG1024 are variants of strain YG1020 which have been modified by the addition of plasmid pBR322; this multi-copy plasmid was engineered to contain either the gene for the enzyme nitroreductase (about 50 copies per bacterium, YG1021) or the gene for the enzyme O-acetyltransferase (YG1024). Strains YG1025, YG1026 and YG1029 are the TA100 type counterparts of strains YG1020, YG1021 and YG1024, respectively.

The protocol used for the bioassays was modified from Maron and Ames[20]. Bacteria were grown for a period of 10 hours at 37°C in Oxoid Nutrient Broth #2 (15 mL) with ampicillin (50 µg/mL) and tetracycline (6.25 µg/mL). A dose range of five concentrations of organic extracts (dissolved in 50 µL DMSO) was tested in duplicate.

Some assay conditions included the addition of a preparation of rat liver microsomes (S9) as a source of metabolic activation (4% Aroclor 1254-induced rat liver S9). After a 48-hour incubation period at 37°C (or 72 hours in the case of strain YG1021), the number of revertant colonies (histidine independent) was determined using a Biotran II colony counter (New Brunswick Scientific, Edison, NJ). Mutagenic potency was calculated from the linear segments of the dose response curves at lower doses. For the mutation chromatograms individual assays of the one minute subfractions of HPLC eluent were performed either in the absence (YG1021 and YG1024) or in the presence (YG1029) of 4% S9.

RESULTS AND DISCUSSION

Hamilton, an industrialized city with a population of approximately 300,000, is the site of the two largest steel mills in Canada. Chemical analyses of Hamilton air particulate extracts[21,22] have resulted in the determination of elevated PAH concentrations compared to some cities in Canada and the United States. Coking operations at the iron and steel facilities and mobile sources (particularly diesel engines) are significant contributors to these elevated PAH levels[23].

A total of thirty-eight individual air particulate filters, collected at two sampling sites in the Hamilton area, contained 1.98 grams of respirable air particulate material and represented the collection from approximately 63,000 m^3 of air. The individual filters were extracted using a Soxhlet apparatus, first with dichloromethane then with methanol; the extracts from each extraction were combined and a portion of each combined extract was weighed. The average mass of the 38 individual organic extracts represented 36% (±13% S.D.) of the mass of particulate material collected on the corresponding filter.

The extracts of the filters were chromatographed individually on a neutral alumina column. After removal of the aliphatics by elution with hexane, the non-polar PAC were eluted from the alumina with benzene followed by dichloromethane/ethanol (99:1). The non-polar aromatic fraction accounted for only 3% (±2% S.D.) of the mass of the crude extracts. The polar PAC which were eluted from the alumina column by methanol followed by methanol:water (4:1) accounted for 58% (±19% S.D.) of the mass of the crude extracts. The non-polar PAC fractions were then chromatographed on Sephadex LH20 to remove the residual aliphatic components in the samples. About 40% of the mass of the crude extract remained adsorbed to the alumina or was removed as aliphatic material.

The non-polar aromatic fractions were combined to afford a composite non-polar aromatic fraction; in a similar fashion a composite polar aromatic fraction was prepared. The composite non-polar aromatic fraction was analyzed by GC/MS both in the selected ion monitoring mode and in the full scan mode. The reconstructed ion chromatogram of the selected ion monitoring analysis is shown in Figure 1 and the concentrations of selected PAC (in ng/m^3) are listed in Table 1.

The *Salmonella* strains used in this study were selected for their increased sensitivity to products of fossil fuel combustion compared to the responses observed in the conventional TA98 and TA100 strains[17-19]. The O-acetyltransferase-rich strains (YG1024 and YG1029) and the nitroreductase-rich strains (YG1021 and YG1026) were engineered to contain multiple copies of the genes that encode for proteins that are responsible for the metabolism and biological activation of some urban air pollutants.

The TA98 type strains (YG1021 and YG1024) are particularly sensitive to compounds which induce frameshift mutations (e.g., nitroarenes) while the TA100 type

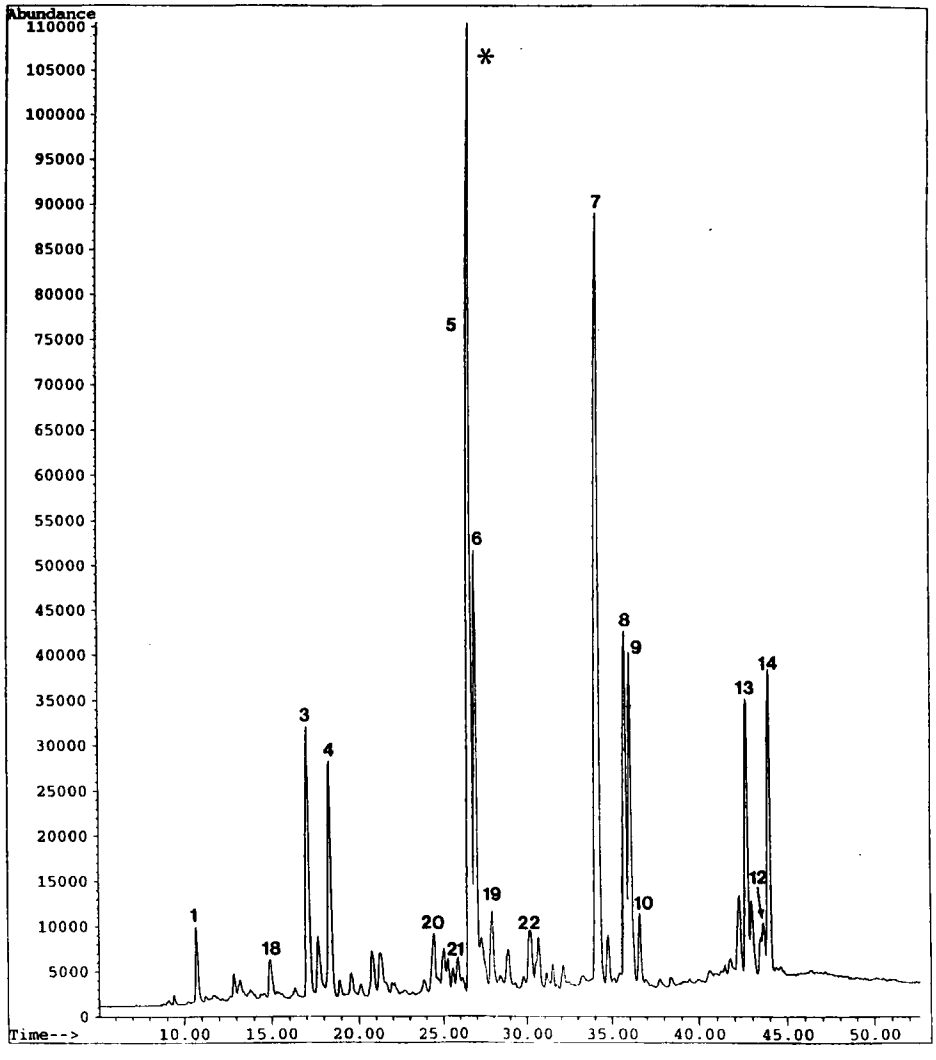

Figure 1 Reconstructed GC-MS ion chromatogram of the pooled non-polar aromatic fraction. Column: J&W 25 m DB5. Temperature program: 100°–160° at 20°C/min., 160°–290° at 3°C/min and held at 290° for 10 minutes. Selected ions monitored (m/z): 178, 184, 192, 198, 202, 208, 212, 216, 217, 228, 230, 234, 240, 248, 252, 254, 258, 276, 278, 280. Benzo[a]anthracene-d_{12} (m/z 240) was used as an internal standard and is labelled with an asterisk. For peak identifications refer to Table I.

strains, YG1026 and YG 1029, are much more sensitive to compounds which cause base-pair substitution type mutations (e.g., PAH). Compounds which exhibit their mutagenic effects in the absence of oxidative metabolism are termed "direct-acting" mutagens while some classes of compounds require the presence of oxidative enzymes to exhibit their mutagenic effects; these latter compounds are called "indirect-acting" mutagens. The oxidative metabolic capability needed for these compounds to exhibit their mutagenic behaviour is usually added to the assay mixture as a microsomal suspension prepared from rat liver called "S9".

Table 1 Concentrations of polycyclic aromatic compounds determined using GC-MS analysis of the non-polar aromatic fraction prepared from respirable air particulate material collected in Hamilton, Ontario.

*Peak no.**	*Polycyclic aromatic compound*	*Concentration (ng/m^3)*
1	phenanthrene	0.19
2	anthracene	ND
3	fluoranthene	0.68
4	pyrene	0.63
5	benzo[a]anthracene	1.01
6	chrysene/triphenylene	1.71
7	benzo[b,j&k]fluoranthenes	4.06
8	benzo[e]pyrene	1.69
9	benzo[a]pyrene	1.15
10	perylene	0.32
11	dibenzo[ac&ah]anthracenes	0.10
12	benzo[b]chrysene	0.14
13	indeno[cd]pyrene	2.72
14	benzo[ghi]perylene	2.67
15	benzo[b]naphtho[2,1-d]thiophene	0.11
16	benzo[b]naphtho[1,2-d]thiophene	0.02
17	benzo[b]naphtho[2,3-d]thiophene	0.05
18	anthraquinone	0.14
19	benzanthrone	0.39
20	11H-benzo[a]fluoren-11-one	0.32
21	7H-benzo[c]fluoren-7-one	0.27
22	benzo[a]anthracene-7,12-dione	0.26
23	2-nitrofluoranthene	0.006
24	1-nitropyrene	0.012
25	2-nitropyrene	0.004

ND=not detected
detection limit for PAH, thiaPAH and oxyPAH: 0.0003 ng/m^3
detection limit for nitroPAH: 0.001 ng/m^3

* Peak numbers refer to chromatographic peaks identified in Figures 1 and 4.

At the outset it was important to evaluate the relative responses of the various *Salmonella* strains to both the non-polar aromatic fraction and the polar aromatic fraction prepared from the composite air particulate extract and it was important to determine the relative contribution of each fraction to the total mutagenic burden. The slope of the least squares regression line over five dosages (in duplicate) was used to calculate the mutagenic potency of each extract, expressed in units of net *Salmonella* revertants per cubic metre of air sampled. Figure 2 shows the dose response data and the linear regression lines of best fit for three TA98-like strains (strains YG1020, YG1021 and YG1024) in the absence of S9. The data for all strains tested, both with and without S9, are listed in Table 2. The aliphatic fractions from the alumina and the Sephadex LH20 chromatographic steps showed no response in any bioassay.

The *Salmonella* strains containing extra copies of the nitroreductase gene (YG1021 and YG1026) or extra copies of the O-acetyltransferase gene (YG1024 and YG1029) showed marked increases in their mutagenic responses compared to the standard tester strains YG1020 and YG1025, respectively (Table 2). In the absence of S9 the increases in response in both the TA98 and TA100 type strains varied between 7-fold and 10-fold.

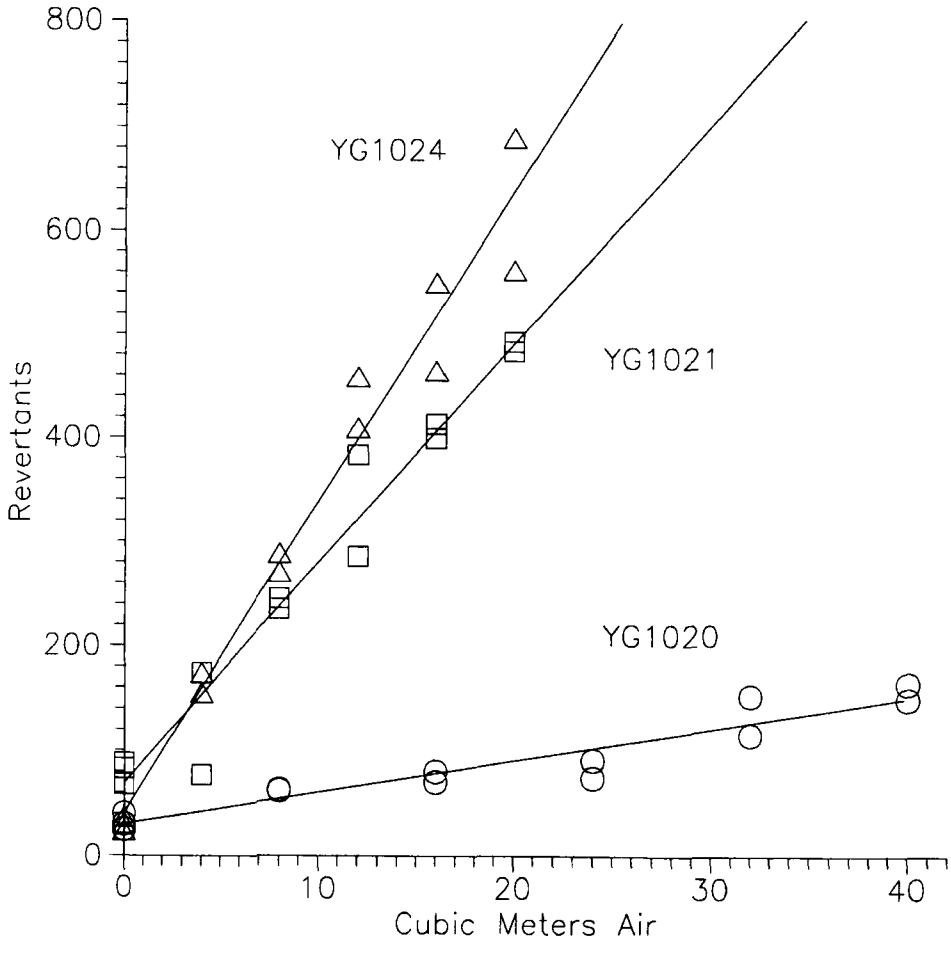

Figure 2 Dose-response curves obtained from assays of the non-polar aromatic fraction prepared from extracts from Hamilton air particulate using *Salmonella* strains YG1020, YG1021 and YG1024 in the absence of S9. Mutagenic potencies were determined from the linear portions of the dose-response data.

The increased sensitivities of these strains to mutagens in air particulate extracts is both dramatic and very practical; about 7-fold to 10-fold less extract is consumed in each set of bioassay analyses using these strains. In spite of this increase in mutagenic response the bioassay procedures still consume far more of a sample than the chromatographic analyses. In the presence of S9 the increase in response was less dramatic (only 2.5-fold to 3-fold).

The contribution of the non-polar aromatic fraction toward the total mutagenic activity observed in the organic extract averaged 77% (±15% S.D.) in all of the strains and conditions tested (Table 2). The non-polar aromatic fraction accounted for 69% (±19% S.D.) of the total mutagenic burden in the absence of S9 (i.e., direct-acting mutagens) and for 84% (±3% S.D.) of the total mutagenic burden in the presence of S9 (i.e., indirect-acting mutagens, Table 2). Furthermore, the non-polar aromatic fraction which accounted for 77% of the mutagenic burden of the air particulate extract represented only 3% of the mass of the crude extract. The polar aromatic fraction was

Table 2 Mutagenic responses (expressed in revertants/cubic metre air) found in the nonpolar aromatic and polar aromatic fractions of the composite air particulate extract in various strains of *S. typhimurium*.

Salmonella strain		*Non-polar aromatic fraction*	*Polar aromatic fraction*		*Percent total activity in non-polar aromatic fraction*
YG1020	–S9	3 rev/m^3	1 rev/m^3		75%
	+S9	10	2		83
YG1021	–S9	21	4		84
	+S9	32	5		86
YG1024	–S9	30	8		79
	+S9	24	5		83
YG1025	–S9	1	2		33
	+S9	13	2		87
YG1026	–S9	11	3		78
	+S9	38	5		88
YG1029	–S9	12	6		67
	+S9	29	7		80
			average (± S.D.)	–S9	69% (± 19%)
				+S9	84% (± 3%)
			overall average (± S.D.)		77% (± 15%)

Standard deviation of the dose-reponse corves is +/–15% absolute standard deviation.

not found to be amenable to analysis by GC-MS and, since its contribution to the extract's total mutagenicity was relatively low, this fraction was not subjected to further analyses at this time.

The remainder of the work described in this paper was focussed on the characterization of the mutagenic components in the non-polar aromatic fraction. Based on the data in Table 2, we selected three *Salmonella* strains and conditions which we felt would give the broadest range of responses to the variety of mutagens contained in the non-polar aromatic fraction prepared from the air particulate extract. These strains and conditions are: YG1021 –S9, YG1024 –S9 and YG1029 +S9.

The non-polar aromatic fraction was chromatographed under normal phase HPLC conditions with a polyaminocyano column using a modification of a method we have reported previously for the compound class fractionation of extracts of air particulate material and sediments[12,16,24] (see Figure 3A). To obtain profiles of the mutagenic activities in the non-polar aromatic fraction, a 1.3 mg sample of this fraction (corresponding to 132 mg of air particulate material collected from 4000 m^3 of air) was chromatographed in a single injection using Method A. The chromatographic eluent was collected in one-minute subfractions and each subfraction was then divided into five equal portions. Three portions were assayed individually using the three *Salmonella* tester conditions identified above based on data in Table 2; the other portions were retained for analysis by GC-MS. The net mutagenic responses of the one-minute subfractions were plotted together with the UV absorption profile at 254 nm to afford three mutation chromatograms (Figure 3).

The profiles of the mutagenic activities of the three *Salmonella* tester conditions showed similarities and differences which illustrate the utility of this NPLC/multiple

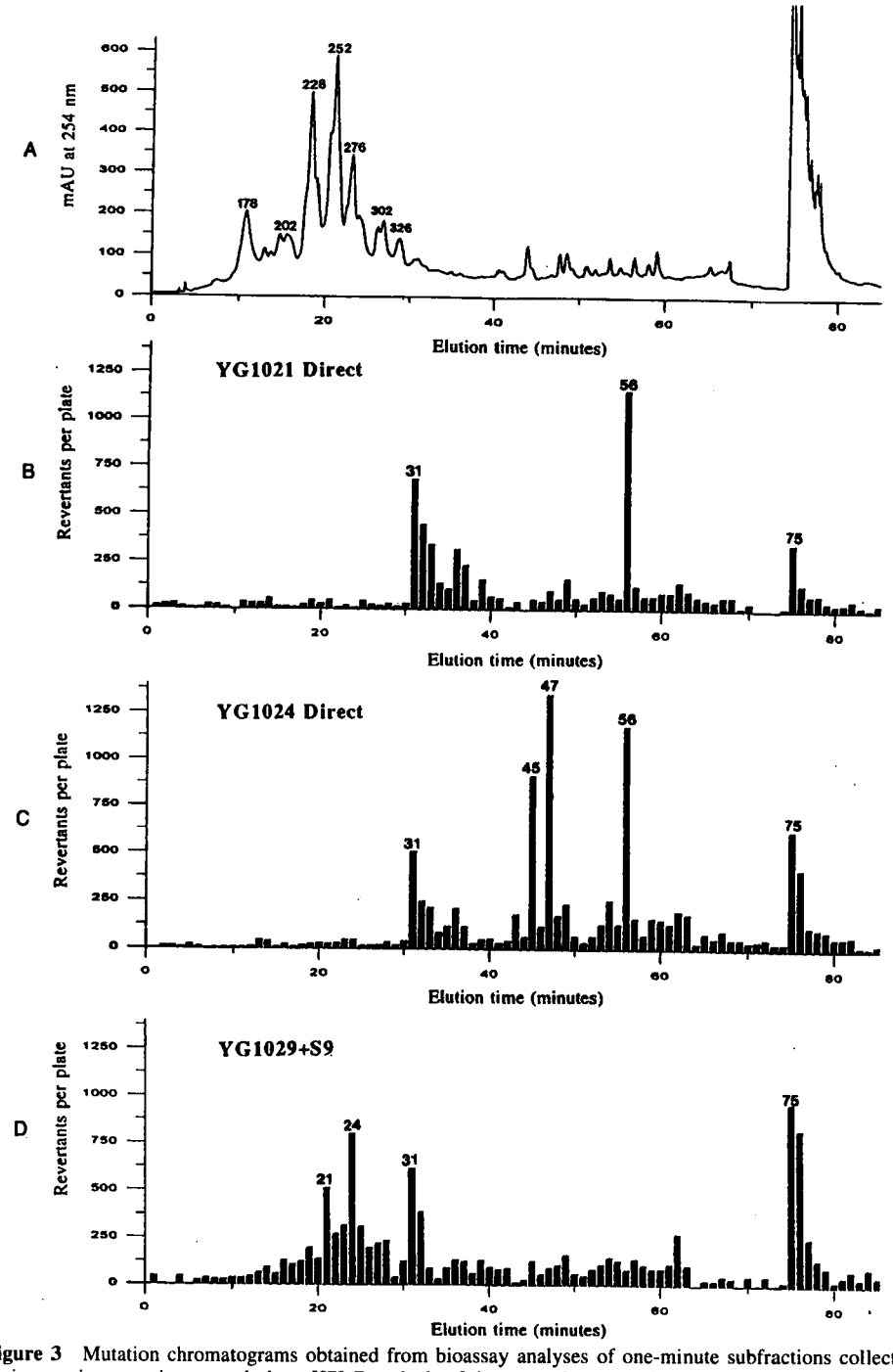

Figure 3 Mutation chromatograms obtained from bioassay analyses of one-minute subfractions collected during semi-preparative normal phase HPLC analysis of the non-polar aromatic fraction. The UV absorption profile (A) was plotted at 254 nm; labels above the chromatographic peaks show the molecular weights (in amu) of the predominant constituents, as determined by GC-MS analyses. Subfractions were bioassayed with strain YG1021 –S9 (B), strain YG1024 –S9 (C) and strain YG1029 +S9 (D). The mutagenic potencies of the subfractions have been plotted as net revertants.

bioassay approach. For example, all three *Salmonella* assay conditions showed the presence of mutagens in the 31–33 min elution range and in the 75–76 min elution range (Figures 3B–D). However, strain YG1024 –S9 (TA98 type, O-acetyltransferase) was the only bioassay condition to respond to mutagens in the 45–47 min range (Figure 3C). Similarly, strains YG1021 and YG1024 in the absence of S9 responded to a mutagen in the 56 minute range (Figures 3B and 3C) which was not detected by strain YG1029 +S9 (Figure 3D). The PAH eluted between 15 and 30 min and, as expected, showed mutagenic responses only with strain YG1029 +S9, (Figure 3D).

The four subfractions which eluted in the 30–33 min time range were analyzed by GC/MS in the selected ion monitoring mode to detect nitroarenes of molecular weight 247. Only the 31 and 32 min subfractions contained the nitroarene compounds 2-nitrofluoranthene, 1-nitropyrene and 2-nitropyrene (compounds 23, 24 and 25, respectively in Table 1 and Figure 4). Identification of these compounds was confirmed by comparison of their mass spectral ion ratios (m/z 247, 217, 201 and 189) and their retention index values with those of authentic standards under the same chromatographic conditions. These nitroarene compounds are potent mutagens and carcinogens[4] and have been shown to be contributors to the mutagenic potencies of extracts of air particulate material[7,13,28,29]. While 1-nitropyrene is known to be produced during combustion processes,[30] both 2-nitropyrene and 2-nitrofluoranthene are products of hydroxyl radical-mediated atmospheric nitration reactions involving pyrene and fluoranthene, respectively[31,32].

The YG1029 +S9 mutachromatogram (Figure 3D) exhibited mutagenic responses in the 21–28 min time range, a range which corresponds to the retention times of higher molecular weight PAH. Two fractions (at 21 min and 24 min) showed substantial responses. Comparison of these retention times with those of standards (Table 3) and comparisons of UV absorption spectra (210 nm to 400 nm) with library spectra resulted in the identification of PAH of molecular weight 252 (benzofluoranthenes and benzo[a]pyrene) in the 21 min subfraction and PAH of molecular weight 276 (indeno[1,2,3-cd]pyrene and benzo[ghi]perylene) in the 24 min subfraction.

GC-MS analyses of the individual subfractions (data not shown) from 16 min to 28 min showed that all of the 252 molecular weight PAH (peaks 7–10 in Table 1) were contained in the 21 min and 22 min subfractions, corresponding to the peak marked "252" in Figure 3A. The earlier eluting peak (18–19 min) was shown to contain only 228 amu PAH, while the later eluting peak (24 min) was shown to contain only 276 amu PAH. The relevant peaks in Figure 3A have been annotated to indicate the molecular weights of the PAH in those peaks. This chromatographic method has greatly simplified the isolation and identification of higher molecular weight PAH that previously had co-eluted or been unresolved under other normal phase and reversed phase HPLC analysis conditions.[27]

The shoulder on the front side of the "252" peak was identified as benzo[a]pyrene from its distinctive UV spectrum; all of the benzo[a]pyrene in the non-polar aromatic fraction was found in the 21 min subfraction. Of the 252 MW PAH identified in this sample (peaks 7–10 in Table 1) benzo[a]pyrene is by far the most potent mutagen; in TA100 in the presence of S9 the benzofluoranthenes[37], benzo[e]pyrene[37] and perylene[35] exhibited mutagenic potencies ranging from no response to about 4% of the mutagenic response obtained with benzo[a]pyrene[38]. Thus, we have ascribed the majority of the mutagenic response in the 21 min subfraction to benzo[a]pyrene.

The GC-MS analysis of the 24 min subfraction showed that it contained essentially two compounds, indeno[1,2,3-cd]pyrene and benzo[ghi]perylene (peaks 13 and 14, respectively, in Table 1). Neither of these compounds was detected in the GC-MS analyses of the 23 or 25 min subfractions. These two compounds which were present in

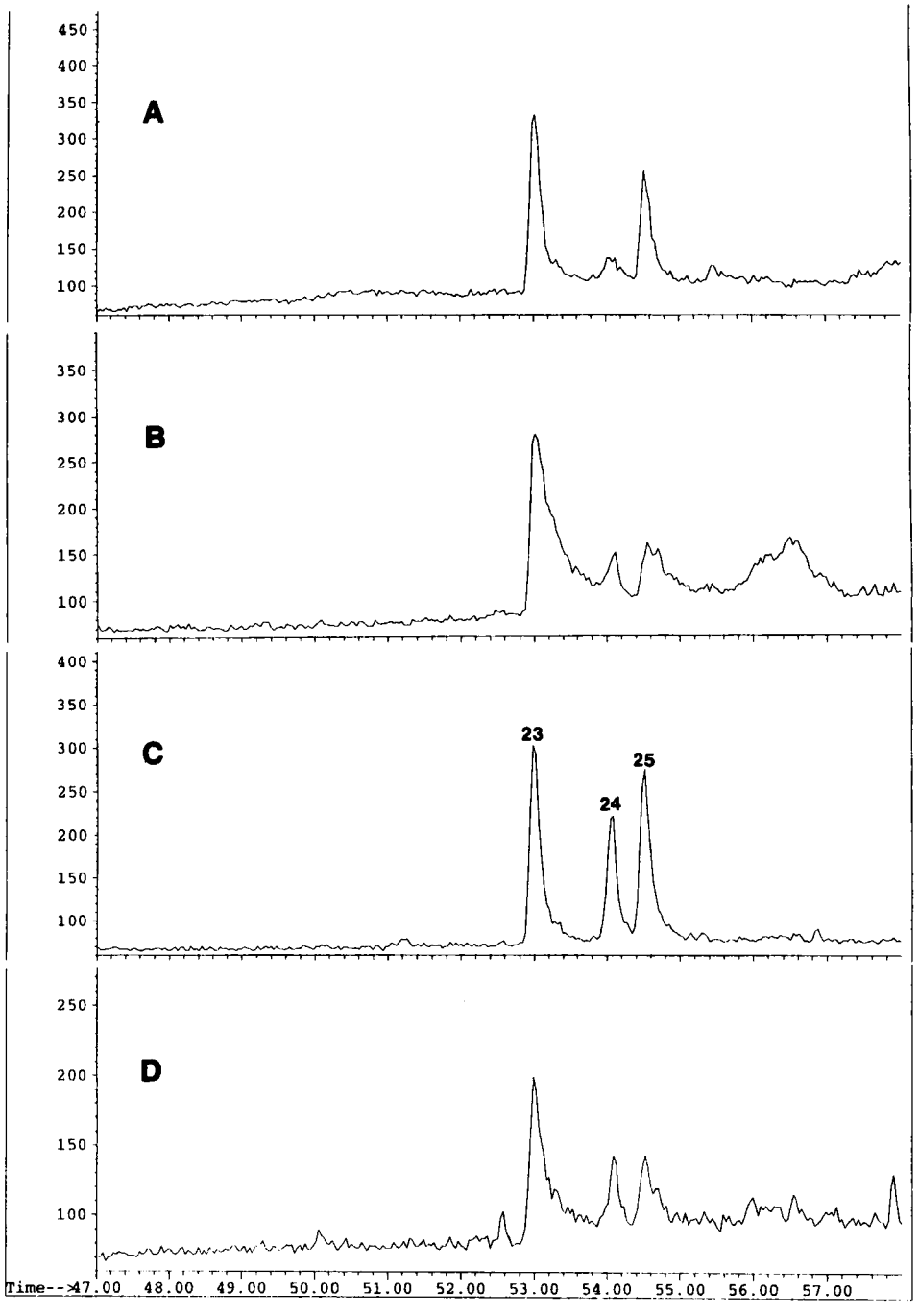

Figure 4 GC-MS selected ion monitoring chromatograms of the 31 min subfraction from the non-polar aromatic fraction. Selected ions were: (A) m/z 247, (B) m/z 217, (C) m/z 201, and (D) m/z 189. Peak identifications are: 2-nitrofluoranthene **(23)**, 1-nitropyrene **(24)** and 2-nitropyrene **(25)**. 1-Nitropyrene and 1-nitropyrene-d_9 standards were found to give mass spectral response factors that were somewhat lower than those of 2-nitrofluoranthene and 2-nitropyrene standards.

almost equal amounts in the sample (Table 1) have rather different mutagenic responses in *Salmonella*; indeno[1,2,3-cd]pyrene was about an order of magnitude more mutagenic than benzo[ghi]perylene[33,38]. Thus, the principal mutagen in the 24 min subfraction was identified as indeno[1,2,3-cd]pyrene.

Efforts to identify the mutagens responsible for the biological activities in the 45, 47, 56 and 75 min subfractions have not been as successful thusfar; analyses of these subfractions by GC-MS in the full scan mode have proven to be difficult. We are currently endeavouring to identify these compounds using reversed phase HPLC and other analytical methods, including LC-MS.

The normal phase gradient conditions were developed with a view to providing a greater separation of compound classes, thereby distributing the biological activity across the chromatographic profile. This gradient also afforded a separation of the polycyclic aromatic hydrocarbons into peaks containing isomeric PAH classes, similar to a separation of PAH derived from a coal tar sample using an amino column reported by Wise *et al*[25,26]. However, the conditions reported by Wise required a two-hour elution time to elute only PAH. In the present work, not only are the PAH separated into molecular weight classes but a range of other, more polar PAC elute from the column in a reproducible fashion (Table 3).

Table 3 Normal phase HPLC retention time data for PAH and PAH derivatives for a Whatman PAC column using two hexane/dichloromethane gradient methods. Method A exposes the column to acetonitrile at the end of the gradient whereas method B does not (see methods section for details). Bracketed values are the molecular weights of the standards.

Compound	*Method A*		*Method B*	
	Avg. retention time*	*Relative std. dev.*	*Avg*. retention time*	*Relative std. dev.*
Dibenzothiophene (184)	9.51 min	0.20 min	9.15 min	0.04 min
Anthracene (178)	10.93	0.21	10.49	0.06
Fluoranthene (202)	13.80	0.21	13.17	0.09
Pyrene (202)	15.65	0.16	15.04	0.10
Benzo[b]naphtho(2,3-d)thiophene (234)	17.00	0.13	16.45	0.10
Chrysene (228)	19.44	0.10	18.94	0.09
Benzo[a]pyrene (252)	21.52	0.08	21.04	0.08
Indeno(1,2,3-cd)pyrene (276)	24.43	0.06	23.98	0.06
9-Nitroanthracene (223)	25.43	0.06	24.99	0.08
1-Nitronaphthalene (173)	26.45	0.06	25.91	0.12
Dibenzo[ai]pyrene (302)	27.42	0.04	27.03	0.05
Dibenzo[jp]chrysene (328)	**	**	28.56	0.06
6-Nitrobenzo[a]pyrene (297)	31.58	0.03	31.22	0.07
2-Nitrofluoranthene (247)	33.08	0.03	32.66	0.11
Carbazole (167)	41.98	0.03	41.25	0.13
Benz[a]anthracene-7,12-dione (258)	41.98	0.03	41.75	0.09
9-Fluorenone (180)	45.35	0.02	44.87	0.22
Anthraquinone (208)	45.84	0.03	45.54	0.08
Naphthacene-5,12-quinone (258)	50.30	0.02	49.54	0.09
Benzanthrone (230)	54.34	0.02	53.42	0.13
Dibenzo[ai]carbazole (267)	55.59	0.03	54.39	0.18
2-Nitrodibenzopyranone (241)	59.06	0.02	57.88	0.19
Quinoline (129)	75***	**		
Acridine (179)	75***	**		

* average of three determinations

** not determined

*** eluted with 100% acetonitrile

Normal phase chromatography methods, while relatively uncommon in the analysis of extracts from complex environmental mixtures, have some distinct advantages over reversed phase approaches. Compounds elute primarily in the order of increasing polarity and, within a compound class, compounds tend to elute in the order of increasing molecular weight or benzologue number (see Table 3). Many normal phase methods have used silica columns as the stationary phase; for example, the common methods for the clean-up of samples for nitroarene analyses use silica HPLC columns to separate the PAH from the nitro-PAH.[34-36] The principal problem with normal phase columns (especially silica columns), a problem rarely addressed in the literature, is the lack of reproducibility in retention times, particularly when gradients involving polar solvents are used.[35] Substantial amounts of time may be needed for column re-equilibration in order to obtain reproducible results. The Whatman PAC column, a polyaminocyano column, used in the present work afforded extremely reproducible retention time data, provided that adequate time for re-equilibration of the column (about 30 min) is included between injections (see data in Table 3). The normal phase protocols reported here represent significant improvements over some normal phase methods we have tried, particularly those which use silica columns.

The coupling of a reproducible chromatographic method with a suite of complementary genotoxicity assays provided a series of mutation chromatograms that afforded a profile of a complex mixture that is extraordinary. By the judicious choice of the type of assay or the selection of bacterial strain used, the resulting mutation chromatograms can yield a great deal of information as to the types of chemical candidates present which may be responsible for the observed biological activity. In this paper we have shown that this methodology is viable for the isolation and detection of PAH, nitroarenes and other PAC in ambient air, at levels that are typical of average urban environments. Furthermore, we have shown the presence of other mutagens in this extract of urban air that we have yet to characterize and identify.

This normal phase method has been useful for the chemical and biological evaluation of a coal tar-contaminated sediment which contained primarily PAH and PAH derivatives[27]. The ability of this method to separate a broad range of PAC derivatives while separating the PAH into individual molecular classes has no parallel in the reversed phase liquid chromatography literature. Thus, we believe this normal phase chromatographic procedure, particularly when coupled with a suite of genotoxicity assays, provides a powerful methodology for the evaluation of extracts of complex environmental mixtures. Further analyses of normal phase fractions prepared by this method using reversed phase liquid chromatography, GC-MS and LC-MS are being explored in our laboratory and will be reported elsewhere.

CONCLUSIONS

The semi-preparative normal phase HPLC methodology reported here when coupled with a suite of genotoxicity assays is an effective approach for the separation and analysis of the nonpolar aromatic fraction prepared from extracts of air particulate material. The biologically active components were well separated, allowing for the collection and subsequent identification of some mutagenic compounds in the extract. Benzo[a]pyrene and indeno[1,2,3-cd]pyrene were identified as the principal indirect-acting mutagens in subfractions exhibiting high responses in a TA100 type strain in the presence of oxidative metabolism (S9) while 2-nitrofluoranthene, 1-nitropyrene and 2-nitropyrene were identified as principal mutagens in subfractions exhibiting high direct-

acting responses in TA98 type strains. The polycyclic aromatic hydrocarbons were eluted from the column in isomeric molecular weight classes thereby reducing the complexity of the subfractions and allowing for easier identification of individual PAH responsible for biological activity. This normal phase HPLC method, unlike other normal phase protocols, is very reproducible and can handle a broad range of compound polarities. This normal phase HPLC/multiple bioassay method should be useful for the evaluation of a range of samples derived from complex environmental mixtures.

References

1. R. G. Harvey, *Polycyclic Aromatic Hydrocarbons: Chemistry and Carcinogenicity* (Cambridge University Press, New York, 1991), 396 pp.
2. J. Jacob, *Sulfur Analogues of Polycyclic Aromatic Hydrocarbons (Thiaarenes)*, (Cambridge University Press, New York, 1990), 281 pp.
3. D. Warshawsky, *Environ. Carcino. & Ecotox. Revs.*, **C10**, 1–71 (1992).
4. H. Tokiwa and Y. Ohnishi, *C.R.C. Crit. Rev. Toxicol.*, **17**, 23–60 (1986).
5. M. Moller, I. Alfheim, S. Larssen and A. Mikalsen, *Environ. Sci. Technol.*, **16**, 221–225 (1982).
6. A. Greenberg, J. Lwo, T. B. Atherholt, R. Rosen, T. Hartman, J. Butler and J. Louis, *Atmos. Environ.*, **27A**, 1609–1626 (1993).
7. J. Siak, T. L. Chan, T. L. Gibson and G. T. Wolff, *Atmos. Environ.*, **19**, 369–376 (1985).
8. D. Schuetzle and J. Lewtas, *Anal. Chem.*, **58**, 1060A–1075A (1986).
9. M. Nishioka, C. C. Howard, D. A. Contos, L. M. Ball and J. Lewtas, *Environ. Sci. Technol.*, **22**, 908–915 (1988).
10. H. Pyysalo, J. Tuominen, K. Wickstrom, E. Skytta, L. Tikkanen, S. Salomaa, M. Sorsa, T. Nurmela and T. Mattila, *Atmos. Environ.*, **21**, 1167–1180 (1987).
11. D. W. Bryant, D. R. McCalla and B. E. McCarry, *Classical PAH form a minor portion of mutagens detected in Hamilton air particulates*. Interim report for the Ontario Ministry of the Environment, Project 386G.
12. D. R. McCalla, M. A. Quilliam, C. Kaiser-Farrell, C. Tashiro, K. Hoo, E. S. Gibson, N. J. Lockington, A. A. Kerr and C. Sheldrake, in: *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes* (C. M. King, L. J. Romano and D. Schuetzle, eds. Elsevier, New York, 1988) pp. 47–64.
13. A. E. Legzdins, B. E. McCarry and D. W. Bryant, *Polycyclic aromatic compounds in Hamilton air: their mutagenicity, ambient concentrations and relationships with atmospheric pollutants. Polycyclic Aromatic Compounds*, **5**, 157–165 (1994).
14. C. H. Marvin, M. Tessaro, B. E. McCarry and D. W. Bryant, *Sci. Total Environ.* **156**, 119–131 (1994).
15. C. H. Marvin, B. E. McCarry and D. W. Bryant, *J. Great Lakes Res.* (1994) **20**, 523–530.
16. C. H. Marvin, L. Allan, B. E. McCarry and D. W. Bryant, *Environ. Mol. Mutag.*, **22**, 61–70 (1993).
17. P. Einisto, M. Watanabe, M. Ishidate and T. Nohmi, *Mutat. Res.*, **259**, 95–102 (1991).
18. M. Watanabe, M. Ishidate and T. Nohmi, *Mutat. Res.*, **216**, 211–220 (1989).
19. M. Watanabe, M. Ishidate and T. Nohmi, *Mutat. Res.*, **234**, 337–348 (1990).
20. D. M. Maron and B. N. Ames, *Mutat. Res.*, **113**, 173–215 (1983).
21. M. Katz and C. Chan, *Environ. Sci. Technol.*, **14**, 838–843 (1980).
22. M. Katz, T. Sakuma and A. Ho, *Environ. Sci. Technol.*, **12**, 909–915 (1978).
23. Ontario Ministry of Environment and Energy, *1991–1992 Air Quality Data Summary*, (Queen's Printer for Ontario, Toronto, 1993), 95 pp.
24. C. H. Marvin, L. Allan, B. E. McCarry and D. W. Bryant, *Intern. J. Environ. Anal. Chem.*, **49**, 221–230 (1992).
25. S. A. Wise, L. C. Sander and W. E. May, *J. Chromatog.*, **642**, 329–349 (1993).
26. S. A. Wise, B. A. Benner, G. D. Byrd, S. N. Chesler, R. E. Rebbert and M. M. Schantz, *Anal. Chem.*, **60**, 887–894 (1988).
27. C. H. Marvin, J. A. Lundrigan, B. E. McCarry and D. W. Bryant, *Isolation, determination and genotoxicity of high molecular mass polycyclic aromatic hydrocarbons isolated from coal tar contaminated sediment*. Proceedings of "Coastal Zone Canada 94", "Cooperation in the Coastal Zone: Conference Proceedings, **3**, 1260–1272, (1994), Bedford Institute of Oceanography, Dartmouth, Nova Scotia.
28. J. N. Pitts, *Atmos. Environ.*, **21**, 2531–2547 (1987).
29. J. Arey, B. Zielinska, W. P. Harger, R. Atkinson and A. M. Winer, *Mutat. Res.*, **207**, 45–51 (1988).
30. J. Jacob, W. Karcher, J. J. Belliardo, R. Dumler and A. Boenke, *Fresenius J. Anal. Chem.*, **340**, 755–767 (1991).

31. J. Arey, B. Zielinska, R. Atkinson, A. M. Winer, T. Ramdahl and J. N. Pitts, *Atmos. Environ.*, **20**, 2339–2345 (1986).
32. R. Atkinson, J. Arey, B. Zielinska and S. M. Aschmann, *Int. J. Chem. Kinet.*, **22**, 999–1014 (1990).
33. J. E. Rice, D. T. Coleman, T. J. Hosted, E. J. LaVoie, D. J. McCaustland, J. C. Wiley, *Cancer Res.*, **45**, 5421–5435 (1985).
34. J. Arey, B. Zielinska, R. Atkinson, A. M. Winer, *Atmos. Env.*, **21**, 1437–1444 (1987).
35. E. Schneider, P. Krenmayr, K. Varmuza, *Monatsh. Chem.*, **121**, 393–401 (1990).
36. T. Nielsen, B. Seitz, T. Ramdahl, *Atmos. Env.*, **18**, 2159–2165 (1984).
37. E. Zeiger, B. Anderson, S. Haworth, T. Lawlor, K. Mortlemans, *Env. Mol. Mutagen*, **19**, supplement 21, 2–141 (1992).
38. M. Sakai, D. Yoshida, S. Mizusaki, *Mutat. Res.*, **156**, 61–67 (1985).