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QUANTITATIVE ANALYSIS OF POLYCYCLIC AROMATIC COMPOUNDS IN DIESEL EXHAUST PARTICULATE EXTRACTS BY COMBINED CHROMATOGRAPHIC TECHNIQUES

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SUMMARY

High resolution gas chromatography–flame ionization detection (GC–FID) with on-column injection was used for quantitative analysis of oxy-polycyclic aromatic hydrocarbons (oxy-PAH), nitro-PAH, and phthalate esters in the extracts of three different diesel exhaust particulate samples. Various compounds in the extracts were sorted into different compound classes by high-performance liquid chromatography fractionation and then, components were tentatively identified by GC–mass spectrometry and retention indices. Combined with the quantitative results obtained in previous studies, the comparative quantitative bar diagrams with more than one hundred polycyclic aromatic compounds were constructed for these three diesel particulate extracts. Some results of the quantitative analysis can be related to responses in a toxicity test of the original extracts.

INTRODUCTION

An increasing number of diesel engines are expected to be used in the next 25 years[1]. Diesel passenger cars emit more particulate matter than conventional automobiles. Furthermore, some compounds identified in diesel exhaust particulate are mutagenic[2]. Consequently, there have been increasing investigations on the chemical characterization and mutageneity associated with diesel exhaust particulate[3–9]. Different analytical techniques, combined with various cleanup procedures, have been used in the analysis of organic compounds in the extract of diesel exhaust particulate. A number of components have been identified and tentatively identified[3,7,9,10]. Although a small number of individual compounds have been quantified, quantitative information on most polycyclic aromatic compounds (PAC) found in diesel exhaust particulate has not yet been reported.

There are two major reasons for the lack of quantitative information. First, there is the need for a separation method to effectively and quantitatively isolate

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various kinds of compounds from each other, consequently eliminating the interferences among the numerous components. Secondly, compared with the number of components found in diesel exhaust particulate matter, the number of available standard compounds for accurate quantitation of individual components is inadequate in most laboratories.

After the separation of organic compounds in a complex mixture into different classes by high-performance liquid chromatography (HPLC), recent studies showed that it is feasible to quantify multiple components in certain classes by gas chromatography (GC) with flame ionization detection (FID) using average response factors[11]. This suggested procedure has been used to accurately determine the amount of polycyclic aromatic hydrocarbons (PAH) in diesel exhaust particulate extract[12]. However, more critical evaluation of the effect of pollutants of diesel exhaust particulate on human health dictates the more comprehensive quantitation of compounds found in these extracts.

A number of short term tests indirectly evaluating the toxicity of chemicals or pollutants on human health have been developed[13]. The most widely used method is the Ames test which uses mutants of *Salmonella typhimium* bacteria[14]. This test has been used in studying the extract of diesel exhaust particulate[6,10]. Mutagens which require activation by a mammalian metabolic system to be mutagenic to strains of *Salmonella typhimuirium*, as well as direct-acting mutagens which require no activation, are both present on diesel exhaust particulates.

Recently, a short term toxicity test using human blood leukocytes and high-resolution two-dimensional electrophoresis has been reported[15]. The principle of this method involves the observation of the effect of pollutants on the synthesis of proteins normally produced by human blood leukocytes. Human leukocytes from freshly drawn blood will synthesize about 2000 proteins when incubated with a mixture of nutrients. The synthesized proteins can be separated by high-resolution two-dimensional electrophoresis and, when previously grown in a radioactive medium, can be visualized on autoradiograph film. The addition of pollutants can affect the ability of leukocytes to synthesize proteins. Three effects may be seen. Firstly, synthesis of some proteins is quite resistant to the presence of a pollutant and there will be no change in protein pattern. Secondly, pollutants may cause suppression of the synthesis of certain proteins as a function of the concentration of pollutant. Thirdly, pollutants will induce the synthesis of new proteins. The observation of these effects can provide a new approach to directly evaluating the toxic, genotoxic and mutagenic behaviour of pollutants on human health.

In this study, a number of oxygenated PAH (oxy-PAH) and some phthalate esters have been quantified in the extracts of three different diesel exhaust particulate samples using the HPLC–GC–FID procedure. The test for human response to toxicity was applied to all three raw extracts with strong positive responses as the result. Combined with the results obtained in previous studies[12,16], the contents of PAH, sulfur-PAH (S-PAH), oxy-PAH, nitropyrene, and phthalate esters were quantitatively compared among these three extracts and were related to the results of toxicity test.

EXPERIMENTAL

Solvents and chemicals

All solvents used were distilled in glass, UV grade from Caledon Labs. (Georgetown, Canada). Most standards were purchased from Aldrich (Montreal, P.Q., Canada) or Chem Service (West Chester, PA, U.S.A.), all with purities of 95–99%. A 1-nitropyrene and a deuterated (d_9) 1-nitropyrene standard were received from Ford Motor Company (Dearborn, MI, U.S.A.).

All glassware used was cleaned by ultrasonic agitation with detergent, then rinsed with deionized water and dried at 250°C for 3 h. Immediately before its use, it was rinsed several times with dichloromethane and benzene.

Sample collection and extraction

Three dichloromethane extracts of diesel particulate matter collected from in-use diesel automobiles (Volkswagen) were received solvent free from the New York State Department of Environmental Conservation. The details of these three samples, designed as VW-1, VW-2 and VW-3, have been reported previously[12].

HPLC preseparation

The instrument used for the HPLC separation was a Spectra-Physics SP-8000 HPLC equipped with an SP-8400 UV–VIS detector and an SP-4100 integrator. The monitoring wavelength was 254 nm. A 10-μm, semi-preparative Spherisorb silica column (25 cm × 9.4 mm I.D., Terochem, Toronto, Canada) was employed with a 140-μl sample loop.

The gradient elution program used in this study has been described previously[12]. It consisted of 100% *n*-hexane for 20 min; programmed to 100% dichloromethane over 30 min, held for 20 min; programmed to 100% acetonitrile over 10 min, held for 1 min; programmed back to 100% dichloromethane in 5 min and finally to 100% *n*-hexane in another 5 min. During this gradient program, six separate fractions were collected at elution times of 0 to start of first peak, 20, 40, 50, 70 and 91 min. They were designated as fraction 1 to fraction 6. The flow-rate was 5 ml/min.

Sample preparation for the HPLC separation and the concentration procedure for HPLC fractions was described in a previous publication[12].

The sample VW-3 was subjected to duplicate HPLC fractionation procedure, each consisting of four injections of the extract. Fractions were used subsequently to test for the quantitative reproducibility.

Fractions 3, 4 and 5 collected from this HPLC separation procedure were subjected to detailed analysis in this study.

After careful cleaning of the chromatograph, a blank was run to test for impurities inherent in the HPLC separation procedure[12].

Gas chromatographic–mass spectrometric analysis

The GC–MS analyses were performed on two GC–MS–DS systems. One was a Hewlett-Packard HP5992 GC–MS–DS system equipped with glass capillary effluent splitter interface and an on-column injector. The other was a Hewlett-Packard HP5987A GC–MS system with an HP1000 data system and an HP7914 Winchester disk drive. An open split GC–MS interface and cool on-column injector were used

for the HP5987A GC–MS system in this study. Electron impact ionization with 70 eV was used in both GC–MS–DS systems.

The HP5992 GC–MS system used has the limited special libraries and a terminal phonelinked to Cornell University, with which mass spectra search, probability based matching (PBM), and self-training interpretive retrieval system (STIRS), can be used for compound identification. In the mode of GC–MS with selected ion monitoring (GC–MS–SIM), six selected ions were monitored simultaneously.

The HP5987 GC–MS system has a PBM and STIRS library system based on over 70,000 reference compounds. In GC–MS–SIM analysis, five groups of 20 selected ions may be monitored at different times.

Three fused-silica capillary columns, a 50 m × 0.32 mm I.D. SE-54 and two 30 m × 0.32 mm I.D. DB-5 (J & W Scientific, Rancho Cardova, CA, U.S.A.) were used in this study.

Details of the GC–MS analysis were described previously[12].

High-resolution gas chromatographic analysis

GC analysis was performed on a Hewlett-Packard HP5880A gas chromatograph equipped with a flame ionization detector and cool on-column injector. A microcomputer data system with a cartridge tape allows storage of chromatographic information for future calculations.

A user-developed software for calculation of retention index of PAC based on Lee's work[17], was stored on an HP5880A terminal and used to automatically calculate the retention indices of PAC after a sample run was completed[18].

A 30 m × 0.32 mm I.D. DB-5 capillary column (J & W Scientific) was used for GC analysis. The GC conditions and details of the GC analysis were described earlier[12].

Qualitative analysis

Compound identification was primarily based on GC–MS data obtained from fractions 3 to 5 of each sample. This was achieved by matching the mass spectra of sample components to those of references. The data for reference mass spectra were obtained from the standard compounds injected, atlas of mass spectra[19,20], publications[3,9,10,21,22] and by computer library search of the two GC–MS systems.

Occasionally, the PAH retention index data obtained from GC analysis was also used to facilitate identification of compounds. Highly precise retention index data using the PAH reference systems have been published for more than 200 standards of PAC[17,23]. In this study GC retention index data used for qualitative analysis were based on duplicate injections.

Quantitative analysis

Integrated peak areas from gas-chromatograms were used for component quantification. Since similar column and chromatographic conditions were used in both GC and GC–MS analyses, the GC–FID trace and total ion current (TIC) trace of each sample in both analyses were qualitatively similar, and the corresponding peaks in each trace were easily located. Duplicate injections were made for each fraction and the averaged peak area was used for quantification. Some representative standard compounds were multi-injected to determine their GC–FID response factor.

A modified injection technique called "four segment injection" was used for on-column injection of small sample volumes on the wall-coated open-tubular column at room temperature in both GC and GC–MS analyses[16].

Toxicity test using human blood leukocytes and two-dimensional electrophoresis

The toxicity test of the three extracts were done at the Institute of Clinical Biochemistry, University of Oslo, Norway. The equipment for high-resolution two-dimensional electrophoresis was obtained from Electro-Nucleonics, Oak Ridge, TN, U.S.A. This apparatus, based on the ISO-DALT system, allows isoelectric focussing of 20 samples at a time[24,25]. The second dimension gels were 8–18% linear gradient polyacrylamide slab gels. The technique used and the interpretation of results has been previously reported[15].

Each dry sample of diesel particulate extract was dissolved in dimethylsulphoxide to give a concentration of 10 μg of extract per μl dimethylsulphoxide. Volumes of 2 and 10 μl of this dimethylsulphoxide solution of each extract were used for the high-resolution two-dimensional electrophoresis test. Volumes of 2 and 5 μl of dimethylsulphoxide solution containing 0.1 μg per μl of 1-nitropyrene standard were also used for this test.

RESULTS AND DISCUSSION

The HPLC separation procedure described in the experimental section was designed to fractionate the organic components in diesel exhaust particulate extract into different classes according to their relative polarities on a silica column[10,26]. This procedure was established by experiments with a standard mixture containing some representative standard compounds on HPLC. Six separate HPLC fractions were collected for diesel particulate extract. The major components in each fraction are listed in Table I. A large amount of aliphatic hydrocarbons covering a wide range of boiling points were found in HPLC fraction 1. After HPLC fractionation of the diesel extract, a small amount of aliphatic hydrocarbons remained in other fractions but did not seriously interfere with the qualitative and quantitative analyses of other

TABLE I

THE TYPES OF MAJOR COMPOUNDS IN HPLC FRACTIONS

Fraction number	*Type of major compound*
1	Aliphatic hydrocarbons
2	Polycyclic aromatic hydrocarbons (PAH)
	Sulfur containing PAH (S-PAH)
3	Carboxaldehyde derivative of PAH (oxy-PAH)
	Ketone derivative of PAH (oxy-PAH)
	Quinone derivative of PAH (oxy-PAH)
	Nitrogen containing PAH (N-PAH)
	Nitrated PAH (nitro-PAH)
4 and 5	Ketone derivative of PAH (small amount)
	Quinone derivative of PAH (small amount)
	Phthalate
6	Oil-like compound and polar compound

TABLE II

COMPOUNDS IDENTIFIED AND TENTATIVELY IDENTIFIED IN HPLC FRACTION 2 OF DIESEL PARTICULATE EXTRACT

a, Identified by sample mass spectra; b, identified by retention index published in refs. 17 and 23; c, identified by standard injected on GC–MS and GC; d, can be found in refs. 3, 9, 10 and 21.

No.	*Compound*	*MW*	*Retention time (min)*	*Identification method*
1	Acenaphthylene	152	17.40	a, b, d
2	Trimethylnaphthalene	170	20.30	a
2	Trimethylnaphthalene	170	20.60	a
4	Trimethylnaphthalene	170	21.30	a, b
5	Trimethylnaphthalene	170	21.90	a, b
6	Fluorene	166	22.46	a, b, c, d
7	Dimethylbiphenyl	182	23.63	a, b
8	C_4-Naphthalene	184	25.90	a
9	C_4-Naphthalene	184	26.80	a
10	C_4-Naphthalene	184	27.08	a
11	Trimethylbiphenyl	196	28.30	a
12	Dibenzothiophene	184	28.65	a, b, c, d
13	Phenanthrene	178	29.75	a, b, c, d
14	Anthracene	178	29.99	a, b, c, d
15	Methyldibenzothiophene	198	32.24	a, d
16	Methyldibenzothiophene	198	32.62	a, d
17	Methyldibenzothiophene	198	32.97	a, d
18	3-Methylphenanthrene	192	33.75	a, b, d
19	2-Methylphenanthrene	192	33.92	a, b, d
20	2-Methylanthracene	192	34.41	a, b, c, d
21	4H-Cyclopenta[*def*]phenanthrene	190	34.72	a, b, d
22	Ethyldibenzothiophene	212	35.73	a, d
23	2-Phenylnaphthalene	204	36.40	a, b, d
24	9 or 2-Ethylphenanthrene/anthracene	206	37.49	a, b, d
25	Dimethylphenanthrene	206	37.81	a, b, d
26	Dimethylphenanthrene	206	37.90	a, b, d
27	Dimethyl(phenanthrene/anthracene)	206	38.60	a, d
28	Dimethyl(phenanthrene/anthracene)	206	38.85	a
29	Fluoranthene	202	39.25	a, b, c, d
30	Benz[*def*]dibenzothiophene	208	39.53	a, d
31	Benzacenaphthylene	202	39.91	a, d
32	Pyrene	202	40.87	a, b, c, d
33	Ethylemethyl(phenanthene/anthracene)	220	41.97	a, d
34	Ethylmethyl(phenanthrene/anthracene)	220	42.16	a, b, d
35	Methyl(fluoranthene/pyrene)	216	42.93	a, d
36	Methyl(fluoranthene/pyrene)	216	43.51	a
37	Methyl(pyrene/fluoranthene)	216	43.63	a
38	Methyl(pyrene/fluoranthene)	216	43.77	a, d
39	Benzo[*a*]fluorene	216	44.39	a, b, c, d
40	Benzo[*b*]fluorene	216	45.12	a, b, d
41	1-Methylpyrene	216	45.32	a, b, d
42	Methyl substituted PAH	242	47.30	a
43	Benzo[*b*]naphtho[2,1-*d*]thiophene	234	48.50	a, b, d
44	Cyclopentapyrene	226	48.80	a, d
45	Benzo[*ghi*]fluoranthene	226	48.87	a, b, d
46	Benzonaphthiophene	234	49.70	a, d
47	Benzo[*a*]anthracene	228	50.54	a, b, d

TABLE II (*continued*)

No.	*Compound*	*MW*	*Retention time (min)*	*Identification method*
48	Chrysene or triphenylene	228	50.86	a, b, c, d
49	Phenyl(phenanthrene/anthracene)	254	51.60	a
50	1,2-Binaphthyl	254	51.80	a, b, d
51	9-Phenylphenanthrene	254	52.20	a, b
52	Methylbenz[*a*]anthracene	242	52.80	a, b, d
53	3-Methylchrysene	242	53.97	a, b
54	1-Phenylphenanthrene	254	54.71	a, b
55	2,2-Binaphthyl	254	54.97	a, b
56	Phenyl(anthracene/phenanthrene)	254	55.47	a
57	Phenyl(anthracene/phenanthrene)	254	55.80	a
58	Unknown PAH	250	56.40	a
59	Unknown PAH*	278	57.50	a
60	Benzo[*j*]fluoranthene	252	58.71	a, b, d
61	Benzo[*b*]fluoranthene	252	58.80	a, b
62	Benzo[*k*]fluoranthene	252	59.35	a, b
63	Benzo[*e*]pyrene	252	60.43	a, b, c, d
64	Benzo[*a*]pyrene	252	60.72	a, b, c, d
65	Dibenz[*a,h*]anthracene	278	62.50	a, d
66	Unknown PAH	264	64.04	a
67	Indeno[1,2,3-*cd*]pyrene	276	66.20	a, b, d
68	Unknown PAH**	276	66.80	a
69	Unknown PAH**	276	67.37	a
70	Unknown PAH**	276	67.47	a
71	Unknown PAH**	276	67.91	a
72	Benzo[*ghi*]perylene	276	69.30	a, b, d
73	Unknown PAH***	288	72.50	a
74	Dibenzopyrene or dibenzo[*def, p*]chrysene	302	75.38	a, d
75	Dibenzopyrene or dibenzo[*def, p*]chrysene	302	76.16	a
76	Coronene	300	77.47	a, d

* Tentatively identified as benzo(b)chrysene.

** The possible compounds are: ideno[1,7-*ab*]pyrene, ideno[1,7,6,5-*cdef*]chrysene, ideno[5,6,7,1-*defg*]chrysene, benzo[*e*]cyclopenta[*jk*]pyrene, cyclopenta[*cd*]perylene, anthanthrene.

*** Tentatively identified as 1,12-methylene benzo[*ghi*]perylene in ref. 21.

components. The predominate components found in fraction 6 are oil-like compounds. The carcinogenic and mutagenic properties of numerous PAH, oxy-PAH, and nitro-PAH are well known. This paper will focus on the discussion of fractions 2, 3, 4 and 5, which contain these types of compounds.

PAH and their alkyl substituted derivatives are predominate in fraction 2. In addition, some sulfur containing PAH (S-PAH) were also found in this fraction. A detailed analysis of components in fraction 2 of three samples was reported in the previous study[12]. The results obtained are now briefly presented here for the later discussion of the toxicity test. Table II lists the compounds identified or tentatively identified in this fraction. The quantitative results for fraction 2 of the three extracts were expressed in terms of ng of component per mg of extract and are shown in the bar diagram of Fig. 1. The retention times in Fig. 1 correspond to those in Table II. In order to graphically compare it with other fractions, which will be discussed later, the full scale in Fig. 1 is designed as 2000 ng per mg extract. For components which

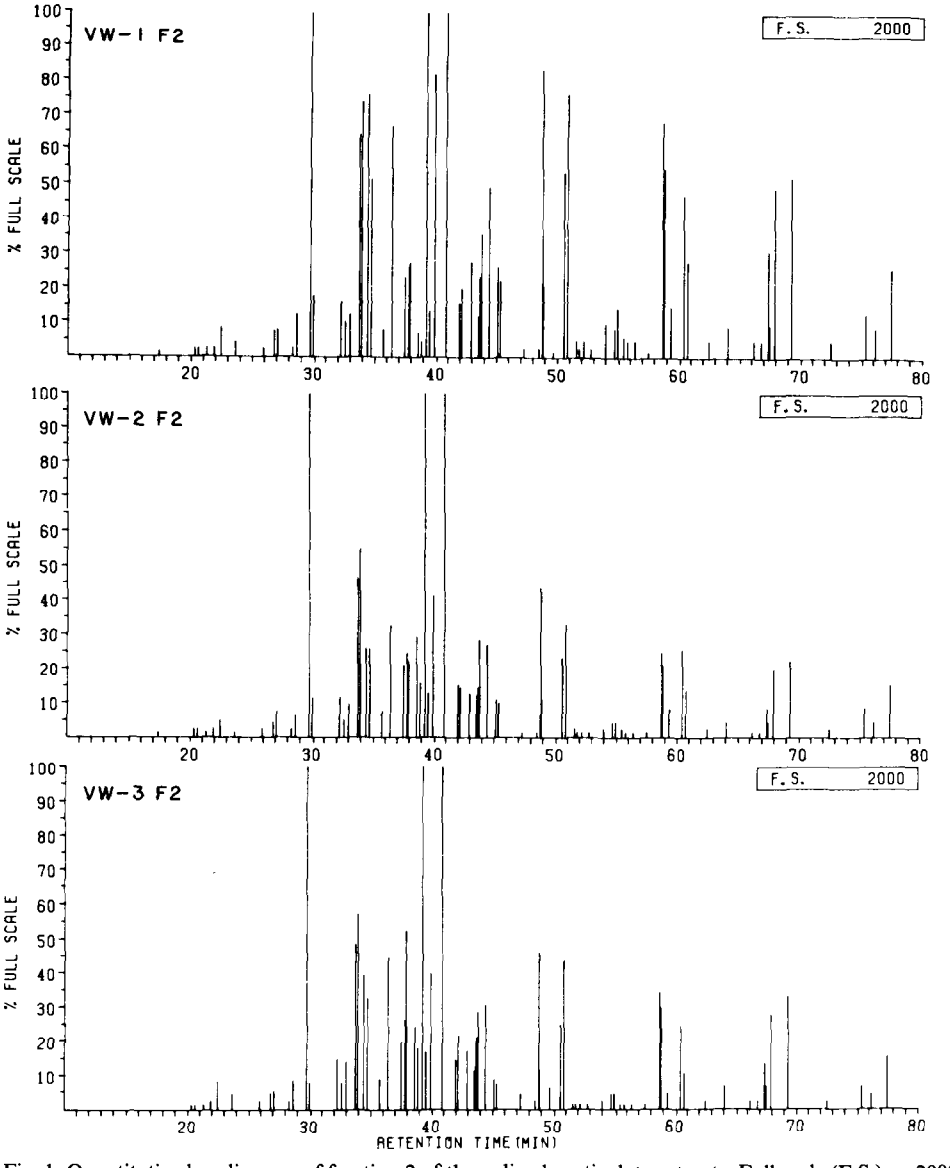

Fig. 1. Quantitative bar diagram of fraction 2 of three diesel particulate extracts. Full scale (F.S.) = 2000 ng/mg extract. For compound identification, see Table II. For overscale concentration, see Table III.

are overscale in Fig. 1, their concentrations can be found in Table III. The bar diagram was based on triplicate injections for each sample and good reproducibility was obtained[12].

The major components found in fraction 3 in each of the three diesel particulate extract are oxy-PAH. The compound identification method has been described in the experimental section and discussed in a previous publication[12]. Highly char-

TABLE III

COMPOUNDS WITH CONCENTRATION OVERSCALED IN FIG. 1

No.	*Compound*	*Retention time (min)*	*Concentration (ng/mg extract)*		
			VW-1	*VW-2*	*VW-3*
13	Phenanthrene	29.75	4883	2186	2821
29	Fluoranthene	39.25	7321	3399	3748
32	Pyrene	40.87	8002	3652	3532

acteristic ion fragments were found in the mass spectra of these compounds: high abundance of $[M - H]^+$ and $[M - COH]^+$ ions for aromatic carboxaldehydes; $[M - H]^+$, $[M - 15]^+$, $[M - COH]^+$ for methyl aromatic carboxaldehydes; $[M - CO]^+$ ions for aromatic ketone derivatives, $[M - 15]^+$, $[M - CO]^+$, for methyl aromatic ketones; and $[M - CO]^+$ and $[M - C_2O_2]^+$ ions for aromatic quinone derivatives. Fig. 2 shows some typical mass spectra of compounds found in this fraction. Table IV lists the compounds identified and tentatively identified in fraction 3 of the three samples and also lists the identification methods used. A limited number of standard compounds were used for compound identification in fraction 3. 4H-Cyclopenta-[*def*]phenanthrene-4-one was identified by an excellent match with a published spectra[22]. The presence of characteristic fragment ions containing nitrogen and $[M - NO_2]$ indicate some nitrogen containing PAH (N-PAH) and nitro-PAH as well. Nitropyene, however, was the only nitro-PAH positively identified in the extract[16]. Others have not been identified due to unavailability of standards.

Due to the shortage of auxiliary information, positive identification of the compounds in fraction 3 is more difficult than that in fraction 2. This can be seen from the identification methods listed in Table II and IV. The certainty of this identification procedure varied with the compounds identified, as discussed previously[12].

In a previous study of the response behaviour of organic compounds on GC–FID, it was indicated that, for some organic compounds classes, it is possible to quantify the different compounds in one class by GC–FID using a characteristic response factor unique to the compound class[11]. The determined FID response of eight oxy-PAH and four nitro-PAH standards are listed in Table V. The data in the table show the relative standard deviations of 7.6% and 4.7% for the average response factors of oxy-PAH and nitro-PAH respectively. Since the oxy-PAH found in those samples have similar carbon-content[11], oxy-PAH were quantified on GC–FID using the average response factor obtained for the eight oxy-PAH standards. Thioxanthone and methylthioxanthone in Table IV were quantified using the FID response factor of oxy-PAH because the response factor of S-PAH (11.84 area count/ng) is similar to that of oxy-PAH (11.90 are counts/ng)[11]. Nitro-PAH were quantified on GC–FID using their average response factor. Nitropyrene was also quantified on GC–MS–SIM using a deuterated internal standard[16]. The error generated from use of average response factor to quantify the individual compounds is smaller than many other experimental errors commonly involved in trace analysis of a complex mixture. The N-PAH were not quantified owing to the absence of standards.

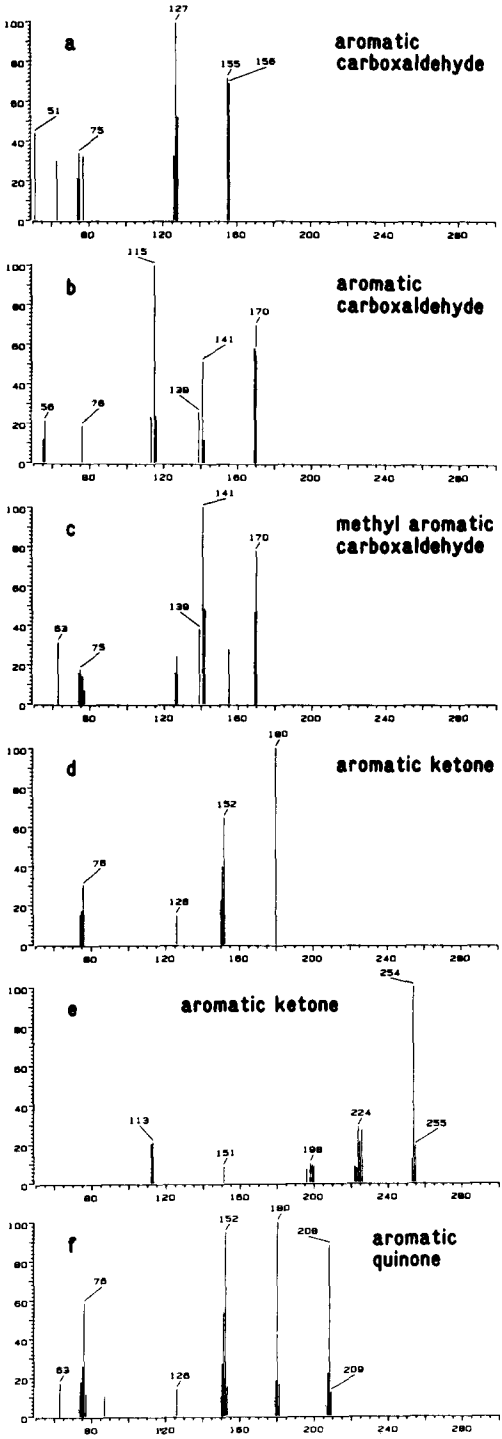

Fig. 2. Mass spectra of some typical oxy-PAH found in fraction 3 of diesel particulate extract. (a) Naphthalene carboxaldehyde; (b) tentatively identified as naphthalene acetaldehyde; (c) methylnaphthalene carboxaldehyde; (d) 9-fluorenone; (e) tentatively identified as benz[*cd* or *fg*]pyrenone; (f) anthraquinone.

TABLE IV

COMPOUNDS TENTATIVELY IDENTIFIED IN HPLC FRACTION 3 OF DIESEL PARTICULATE EXTRACT

a, Identified by mass spectrum; b, identified by retention index published in refs. 17 and 23; c, identified by standard injected on GC–MS and GC; d, can be found in refs. 10 and 22. Note: concentrations of less than 53 ng/mg extract were obtained by approximate calculation.

No.	*Compound*	*MW*	*Retention time (min)*	*Identification method*	*Concentration (ng/mg extract)*		
					VW-1	*VW-2*	*VW-3*
1	2-Naphthalene carboxaldehyde	156	19.34	a, d	98	50	51
2	1-Naphthalene carboxaldehyde	156	19.48	a, d	168	68	84
3	Methylnaphthalene carboxaldehyde	170	23.33	a, d	79	50	26
4	Methylnaphthalene carboxaldehyde	170	23.46	a, d	55	50	51
5	Naphthalene acetaldehyde	170	23.67	a, d	175	111	110
6	Naphthalene acetaldehyde	170	23.89	a	83	68	65
7	Methylnaphthalene carboxaldehyde	170	24.11	a	143	113	93
8	Methylnaphthalene carboxaldehyde	170	24.44	a	141	118	124
9	Unknown nitrogen containing PAH (N-PAH)	241	25.10	a	32	130	31
10	Acenaphthene carboxaldehyde	182	26.20	a	26	12	26
11	Acenaphthene carboxaldehyde	182	26.40	a	26	12	26
12	Acenaphthene carboxaldehyde	182	27.40	a	53	50	51
13	9-Fluorenone	180	28.06	a, b, c, d	1218	758	647
14	Dimethylnaphthalene carboxaldehyde	184	28.62	a, d	84	113	123
15	Dimethylnaphthalene carboxaldehyde	184	28.79	a	153	142	150
16	Dimethylnaphthalene carboxaldehyde	184	28.90	a	26	50	51
17	Dimethylnaphthalene carboxaldehyde	184	29.75	a	147	108	133
18	Unknown nitro-PAH*	197	30.50	a	64	30	31
19	Anthrone/phenanthrone or their isomers	194	31.06	a, d	274	217	182
20	9-Xanthone	196	32.00	a, c, d	53	50	51
21	Unknown N-PAH	201	32.32	a	390	311	274
22	Anthrone/phenanthrone or their isomers	194	32.80	a, d	298	264	213
23	Methylfluorenone	194	33.28	a, d	257	178	136
24	Methylfluorenone	194	34.00	a	53	50	26
25	Methylfluorenone	194	34.60	a	53	25	51
26	Dimethylfluorenone	208	35.32	a	128	102	72
27	Anthraquinone	208	36.05	a, c, d	1010	355	658
28	Unknown N-PAH	201	37.00	a	59	61	31
29	4H-Cyclopenta[*def*]phenanthrene-4-one	204	38.37	a, d	1033	615	436
30	Methyl(phenanthrene/anthracene)quinone	222	39.55	a, c, d	79	73	51
31	Thioxanthone	212	40.10	a, d	53	50	51
32	Methyl(anthracene/phenanthrene)quinone	222	40.38	a	348	218	185
33	(Phenanthrene/anthracene)carboxaldehyde	206	40.75	a, c, d	200	251	270
34	Phenanthrene-9-carboxaldehyde	206	41.07	a, c, d	347	246	208
35	Unknown ketone derivative of PAH	218	42.30	a	26	25	54
36	Methylthioxanthone	226	43.31	a	222	168	58
37	Methyl(phenanthrene/anthracene) carboxaldehyde	220	44.60	a, d	26	50	51
38	Benzo[*a*]fluorenone	230	47.51	a, b	413	201	157
39	Benzo[*b*]fluorenone/ benzanthrone or its isomer	230	48.43	a	309	177	110
40	Benzo[*b*]fluorenone/ benzanthrone or its isomer	230	49.19	a	575	233	163
41	Benzo[*b*]fluorenone/ benzanthrone or its isomer	230	50.63	a	204	99	103

(Continued on p. 194)

TABLE IV (*continued*)

No.	*Compound*	*MW*	*Retention time (min)*	*Identification method*	*Concentration (ng/mg extract)*		
					VW-1	*VW-2*	*VW-3*
42	7H-Benz[*de*]anthrone	230	51.46	a, b, c	1281	243	378
43	Bis(2-ethylhexyl)phthalate	390	53.41	a	786	51	1834
44	7,12-Benz[*a*]anthracene quinone	258	53.96	a, c	169	75	77
45	Nitropyrene	247	54.32	a, c, d	443	119	751
46	Benz[*cd* or *fg*]pyrenone	254	57.08	a	215	102	79
47	Benz[*cd* or *fg*]pyrenone	254	57.75	a	155	76	75
48	Benz[*cd* or *fg*]pyrenone	254	58.28	a	419	194	139
49	Benz[*cd* or *fg*]pyrenone	254	60.29	a	534	25	26
50	Unknown ketone derivative of PAH**	278	66.97	a	384	173	151

* Tentatively identified as nitro-acenaphthylene.
** Tentatively identified as fluoranthone or pyrenone.

The quantitative results of oxy-PAH and nitro-PAH found in fraction 3 of three samples are listed in Table IV and shown on bar diagram in Fig. 3. These quantitative results are based on two injections for each sample using the average GC peak area. The retention time in Fig. 3 refers to that in Table IV.

Fewer compounds were found in fractions 4 and 5. Those present were found

TABLE V

FID RESPONSE FACTORS (RF) OF SOME OXY-PAH AND NITRO-PAH STANDARDS

Compound	*MW*	*RF (area counts/ng)*	*R.S.D.* (%)*
Oxy-PAH			
9-Fluorenone	180	12.97	5.2
Anthrone	194	11.64	4.1
2-Fluorenecarboxaldehyde	194	11.64	4.1
Phenanthrene-9-carboxaldehyde	206	12.03	1.7
Xanthone	196	11.88	3.5
Anthraquinone	208	12.13	2.3
Phenanthrenequinone	208	10.04	2.2
Benz[*a*]anthracene-7,12-dione	258	12.85	2.8
Average of 8 oxy-PAH standards:		11.90	7.6
Nitro-PAH			
1-Nitronaphthalene	173	10.13	4.2
2-Nitrobiphenyl	199	10.26	3.6
2-Nitrofluorene	211	9.66	1.4
9-Nitroanthracene	223	9.25	8.5
Average of 4 nitro-PAH standards:		9.83	4.7

* For oxy-PAH, based on three injections at one concentration level (100 ng); for nitro-PAH, based on total four injections at two concentration levels (two injections at 100 ng level and two injections at 20 ng level).

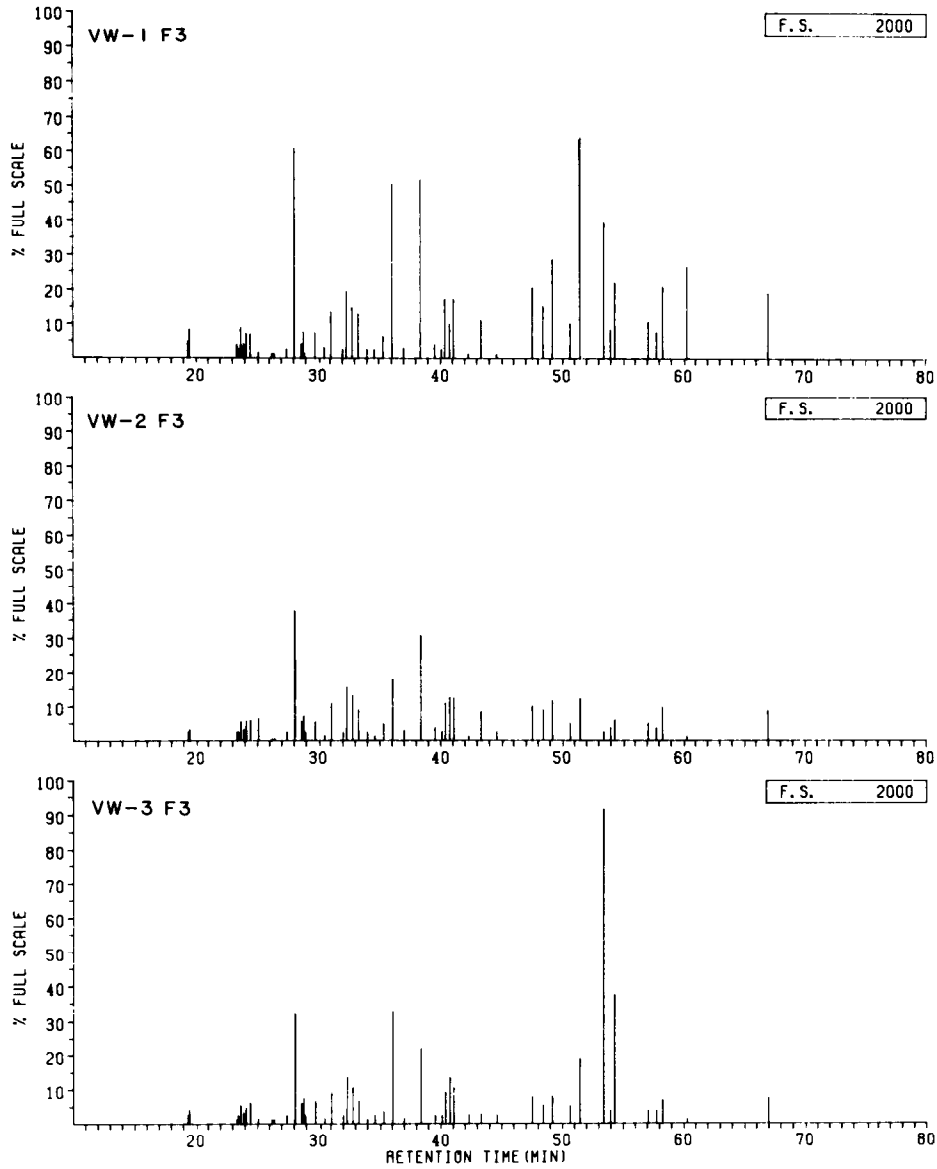

Fig. 3. Quantitative bar diagram of fraction 3 of three diesel particulate extracts. Full scale (F.S.) = 2000 ng/mg extract. For compound identification, see Table IV.

in very low concentrations with the exception of benz[*de*]anthrone, benz[*cd*]- or [*fg*]pyrenone and phthalate. Benz[*de*]anthracenone and benz[*cd*]- or [*fg*]pyrenone were also found in fraction 3. Table VI lists the compounds tentatively identified in fractions 4 and 5 of the three samples. Oxy-PAH components in these two fractions were quantified on GC–FID in the same way described above. Several phthalate standards were used to determine their FID response factors. Results are listed in

TABLE VI

COMPOUNDS TENTATIVELY IDENTIFIED IN HPLC FRACTIONS 4 AND 5 OF DIESEL PARTICULATE EXTRACT

a, Identified by mass spectrum; c, identified by standard injected on GC–MS and GC. Note: concentrations of less than 53 ng/mg extract were obtained by approximate calculation.

No.	*Compound*	*MW*	*Fraction number*	*Retention time (min)*	*Identification method*	*Concentration (ng/mg extract)*		
						VW-1	*VW-2*	*VW-3*
1	Di-isobutylphthalate	380	5	33.10	a	56	79	–
2	Unidentified phthalate	149 (bp)*	5	34.00	a	–	731	–
3	Fluorene quinone	196	4	35.60	a	26	25	–
4	Anthrone/phenanthrone	194	4, 5	36.00	a	216	155	26
5	(Anthracene/phenanthrene) quinone	208	4	36.2	a	53	74	26
6	Phenanthrene-9-carboxaldehyde	206	4	41.07	a	13	–	–
7	Unknown quinone derivative of PAH**	206	4	45.60	a	26	25	–
8	7H-Benz[*de*]anthracene-7-one	220	4	51.46	a, c	475	388	258
9	Bis(2-ethylhexyl)phthalate	390	4, 5	53.41	a	246	677	910
10	Unknown quinone derivative of PAH	258	4	56.20	a	13	25	–
11	Unknown quinone derivative of PAH	256	5	56.45	a	106	26	–
12	Benz[*cd* or *fg*]pyrenone	254	5	59.90	a	79	–	–
13	Benz[*cd* or *fg*]pyrenone	254	4	60.29	a	1813	837	754

* Base peak in mass spectrum.

** Tentatively identified as cyclopenta[*def*]phenanthrene quinone.

TABLE VII

FID RESPONSE FACTORS (RF) OF SOME PHTHALATE ESTER STANDARDS

Compound	*MW*	*Formula*	*RF (area counts/ng)*	*R.S.D.* (%)*
Dimethyl phthalate	194	$C_{10}H_{10}O_4$	7.89	2.1
Diethyl phthalate	222	$C_{12}H_{14}O_4$	8.20	2.1
Dibutyl phthalate	278	$C_{16}H_{22}O_4$	10.73	2.0
Dioctyl phthalate	390	$C_{24}H_{38}O_4$	13.32	1.8

* Based on total four injections at two concentration levels (two injections at 100 ng level and two injections at 20 ng level).

Table VII. The response factor of the dibutyl phthalate standard was used to quantify compounds 1 and 2; and the response factor of the dioctyl phthalate standards for the quantification of compound 9 in Table VI. The quantitative bar diagram of components in fractions 4 and 5 of three samples is shown in Fig. 4 and the results are also listed in Table VI.

The reproducibility of the HPLC separation and GC–FID quantitation was determined by two HPLC separations, run 1 and run 2, of sample VW-3. Duplicate and triplicate GC quantitative analysis were performed on fraction 3 of run 2 and run 1 respectively. The results for some selected compounds are listed in Table VIII. The average relative standard deviation (R.S.D.) of 9.4% shows good reproducibility.

The recovery of the HPLC fractionation step was studied with selected stan-

TABLE VIII

REPRODUCIBILITY OF QUANTITATIVE ANALYSIS OF COMPONENTS IN FRACTION 3 OF SAMPLE VW-3 USING HPLC–GC–FID

Compound	*Retention time (min)*	*Overall average result (ng/mg extract)**	*R.S.D.* (%)*
1-Naphthalene carboxaldehyde	19.48	89	6.8
Naphthalene acetaldehyde	23.67	114	3.2
Naphthalene acetaldehyde	23.89	74	14.4
Methylnaphthalene carboxaldehyde	24.44	130	5.8
9-Fluorenone	28.06	664	3.6
Dimethylnaphthalene carboxaldehyde	28.62	124	2.7
Dimethylnaphthalene carboxaldehyde	28.79	157	5.2
Anthrone/phenanthrone or their isomers	31.06	183	7.7
4H-Cyclopenta[*def*]phenanthrene-4-one	38.37	491	13.5
Methyl(anthracene/phenanthrene)quinone	40.38	224	20.7
(Phenanthrene/anthracene)carboxaldehyde	40.75	326	20.2
Benzo[*a*]fluorenone	47.51	157	6.7
7H-Benz[*de*]anthrone-7-one	51.46	343	12.3
		Average R.S.D.:	9.4%

* Based on a total of five GC injections for HPLC run 1 and HPLC run 2 (three GC injections for run 1 and two GC injections for run 2).

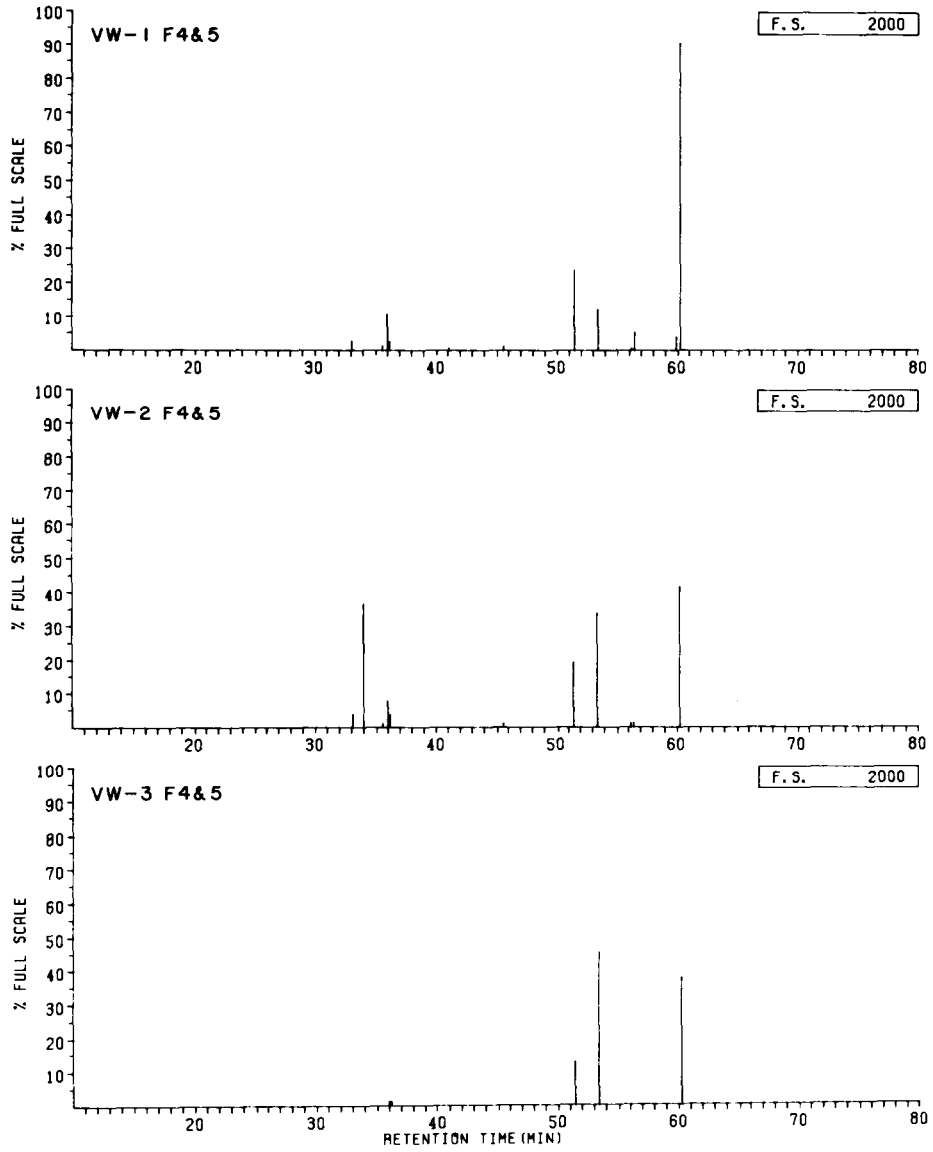

Fig. 4. Quantitative bar diagram of fractions 4 and 5 of three diesel particulate extracts. Full scale (F.S.) = 2000 ng/mg extract. For compound identification, see Table VI.

dards which underwent HPLC fractionation and quantitative GC analysis. The results shown in Table IX indicate a recovery range of 81 to 110% for selected standards.

No significant impurities were found in fractions 1–5 of the HPLC blank run.

The compounds listed in Tables II, IV and VI, and also quantified in the bar diagrams, cover most abundant components found on GC traces of fractions 2, 3,

TABLE IX

RECOVERY OF SELECTED COMPOUNDS IN HPLC FRACTIONATION STEP

Compound	*Recovery* (%)*	*Relative deviation from mean (%)*
Benzo[*e*]pyrene	89	6.7
9-Methylanthracene	88	2.2
Phenanthrene-9-carboxaldehyde	95	5.3
Anthraquinone	110	6.3
1-Nitropyrene	105	1.0
Pyrene	97	0.6
Triphenylene	101	1.6

* Averaged value from two separate HPLC runs.

4 and 5. A comparison in quantity of components can be made for the same fraction among different extract samples from the bar diagrams in one figure. Examining the bar diagrams in Figs. 1, 3 and 4 allows quantitative comparison of fractions 2, 3, 4 and 5 in each of the extracts. A few conclusions can be drawn from the data shown in these bar diagrams. First, the distribution of components in these three diesel particulate extracts is qualitatively similar, but significantly different in quantity. Secondly, among PAH, S-PAH, oxy-PAH, N-PAH, and nitro-PAH; PAH and their alkyl substituted derivatives are predominate in diesel particulate extract. Additionally, the PAH and their alkyl substituted derivative compounds with three and four rings are most abundant.

The quantitative differences in fractions 2–5 among these three diesel particulate extracts are summarized in Table X. The compounds tabulated include those which have been identified, tentatively identified, or which show very characteristic mass spectra of PAH, S-PAH, and oxy-PAH. Since a number of these compounds have been linked to direct and indirect mutagenicity and carcinogenicity, this table is used for the discussion of the toxicity test.

The three extracts analysed above were subjected to the high-resolution two-dimensional electrophoresis toxicity test using the procedure described in the experimental section. Fig. 5 is the autoradiogram of the protein pattern formed by leukocytes incubated in the presence of increased amounts of extract and separately of the nitropyrene standard. Remarkable differences in the protein pattern among the pictures shows the strong response of those three extracts in the toxicity test. The dependence of response on the amount of extract introduced can be seen in the comparison between the control (picture o) and pictures a and b in Fig. 5. All three effects previously mentioned are observed in this comparison. The first effect is the resistance of synthesis of certain proteins to chemicals and is shown by the protein spots labelled as A and B in Fig. 5. No significant change in the intensity of spots A and B among the pictures indicates that the first effect applies to all compounds tabulated in Table X. The second effect is illustrated by observing the spots labelled in area E in Fig. 5, in which several protein spots are involved. Comparing pictures b, d and f with the control o shows that some protein spots are fainter and some spots completely disappear with increasing concentration of extracts. This demon-

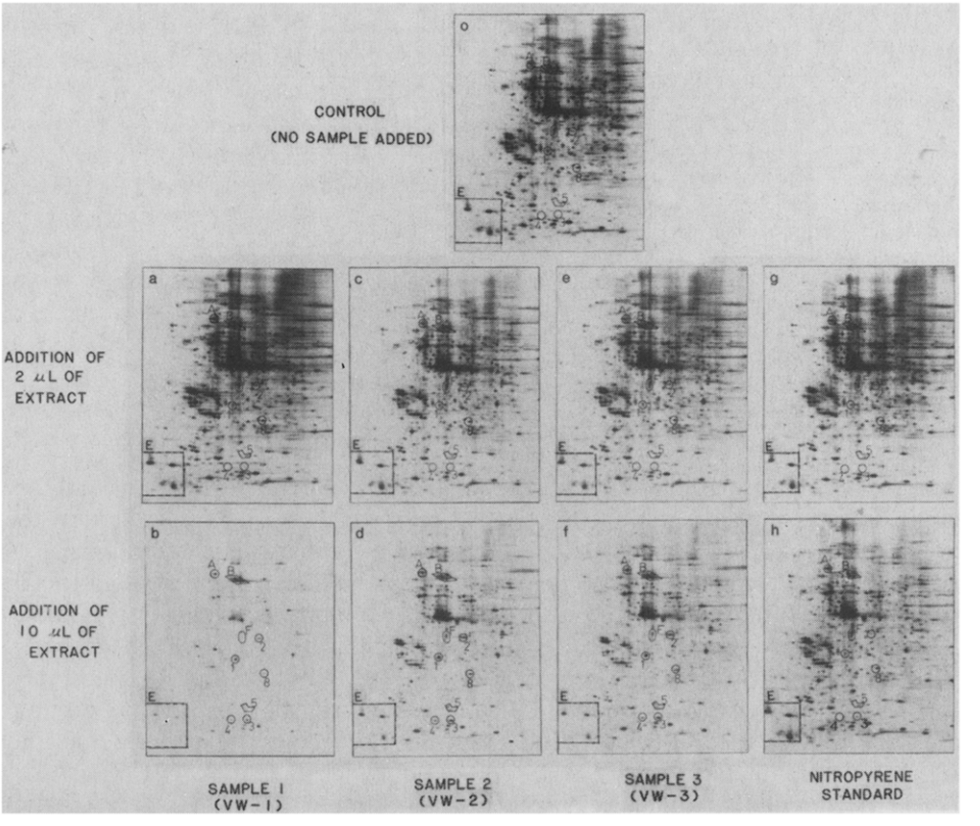

Fig. 5. 2-Dimensional protein pattern of human leukocytes incubated with increasing amounts of extract of three diesel particulate extracts and 1-nitropyrene standard. (o), control, no addition of extract; (a), (c), (d) and (g), addition of 2 μl VW-1, VW-2, VW-3 extracts and 1-nitropyrene standard respectively; (b), (d) and (f), addition of 10 μl VW-1, VW-2, and VW-3 extracts respectively; (h), addition of 5 μl 1-nitropyrene standard. Protein spot marked: (A) and (B): effect 1; (E): effect 2; (3), (4) and (5): effect 3.

strates that the synthesis of certain proteins is suppressed and others are completely blocked by compounds in the samples.

It is apparent that for mixtures as complex as these extracts, the toxicity test results cannot be related to specific compounds. However, the results may be instructive to point out correlations observed between quantities of compounds in certain

TABLE X

CONTENT (ng/mg EXTRACT) OF SOME SELECTED ORGANIC COMPOUNDS IN THREE DIESEL EXHAUST PARTICULATE EXTRACTS

Compound	*VW-1*	*VW-2*	*VW-3*
PAH and their alkyl substituted derivatives	50,769	26,325	29,799
Oxy-PAH	14,338	7741	6977
S-PAH	1522	1084	1590
Phthalate ester	1088	1538	2744
Nitropyrene	443	119	751

classes which are known to be toxic and their effects on protein synthesis. Relating the observation in the E area of pictures b, d and f to the data in Table X, sample VW-1 exhibits the strongest toxicity for the second effect and also has the highest concentration of total amount of PAH and oxy-PAH. Sample VW-2 and VW-3 show quite similar responses for the second effect and have similar concentration of total amounts of PAH and oxy-PAH. This seems to indicate that the amount of PAH and oxy-PAH has more influence on the suppression of synthesis of certain proteins.

Protein spots 3, 4 and 5 illustrate the third effect. In this effect, new proteins which were not observed in the control (o) are synthesized with increasing amounts of extracts added. Among pictures b, d and f, stronger response is observed for samples VW-2 and VW-3 than for sample VW-1. Considering the data in Table X, it appears that phthalates may contribute more to the third effect.

Nitropyrene is known as a strong direct mutagen and has a strong response in the Ames test without activation[6,10]. From pictures o, g and h, the contribution of nitropyrene to the third effect is shown by spots 3, 4 and 5. Considering that the amount of nitropyrene standard (*ca.* 500 ng) involved in obtaining picture h is much more than that (70 ng) involved to obtain picture f, the effect is not as great as would be expected from a strong mutagen. This can be seen in the comparison of the intensity of spots 3, 4 and 5 in pictures f and h.

CONCLUSION

Numerous components in an organic mixture have been quantitatively sorted into different classes by HPLC fractionation. This reduces the complexity of the mixture and thus facilitates the qualitative and quantitative analyses of components. Qualitative analysis was achieved using GC–MS, GC retention indices, the injection of standard compounds, and auxiliary information from publications. Some components were quantified by GC–FID using the average response factor which is unique to the compound class rather than the individual component. Using the combined HPLC classification–GC–MS identification–GC–FID quantitation procedure, quantitative relationships among components in a complex organic mixture, and comparisons between different mixtures, may be revealed.

The analytical procedure combined with this toxicity test is one approach useful for studying the effect of environmental pollutants found in complex mixtures. At this stage, the kind of effects demonstrated in the toxicity test are related to that of the total mixture and not to the specific compounds found. The test does provide a guide as to the types of compounds which produce toxicity effects on humans. Individual pure compounds can then be used to further confirm these results.

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