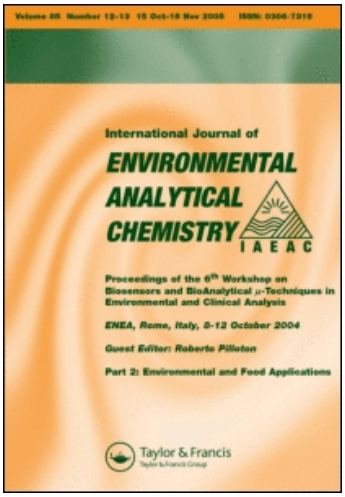

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Estimation of Total Aromatic Hydrocarbons in Environmental Samples by High Pressure Liquid Chromatography

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Estimation of Total Aromatic Hydrocarbons in Environmental Samples by High Pressure Liquid Chromatography

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Many of the harmful effects of oil pollution can be attributed to the release of toxic aromatic hydrocarbons. This paper describes a rapid, high pressure liquid chromatographic (HPLC) method for the estimation of total aromatic hydrocarbons in environmental samples.

The method employs an amine column to separate mixtures of aromatics into groups of compounds with a similar spectrophotometric response. An estimate for total aromatics is then derived by comparing the response of each group with the response of suitable reference compounds. Since results must be expressed in terms of these reference compounds, the method is semi-quantitative. However, values derived for total aromatics in refinery effluents, extracts of the mussel *Mytilus edulis*, and two petroleum oils agreed well with those given by more cumbersome traditional methods.

KEY WORDS: Aromatic hydrocarbons, HPLC, mussels, refinery effluents.

INTRODUCTION

Crude oil and other petroleum products represent a significant form of water pollution, both as acute spillages and as chronic inputs. The

various classes of compounds in oil have toxic effects of different types and magnitude. However it is generally accepted that the aromatic hydrocarbons are particularly significant because of their persistence and toxicity.[1,2]

Analysis of trace petroleum hydrocarbons as a whole is most commonly undertaken by using gas chromatography. This method has several advantages: it responds fairly equally to all classes of hydrocarbons present (when a flame ionisation detector is used), it is reasonably sensitive and it often provides qualitative as well as quantitative information, at least on relatively undegraded oil. However, disadvantages include the need for careful clean-up of samples, particularly those of biological origin, and a lack of discrimination between, for example, aromatic and alicyclic components. Thus in estimating aromatics as a sub-class of "petroleum hydrocarbons" using gas chromatography, reliance must be placed on a previous separation of aromatics from other constituents.[3]

Certain individual polycyclic aromatic hydrocarbons (PAH's) receive attention because of their carcinogenic and mutagenic properties. However, to date, little attempt has been made to measure total aromatics as a class despite recognition of the environmental significance of such a characteristic.[4] Besides separation and gas chromatographic estimation, analytical methods for aromatics include UV absorbance and fluorescence spectrophotometry. However, aromatic mixtures of fossil origin are extremely complex and there are orders of magnitude differences in the response to different classes of aromatics. This renders such methods barely semi-quantitative, unless the exact nature of the contaminant is known. Thus Zsolnay[5] reported no correlation between spectrophotometric and gas chromatographic indicators of petroleum pollution in shellfish.

The non-quantitative nature of spectrophotometric methods is principally caused by the very large differences in response to the single, double and multi-ring aromatics. Since the response within each ring class is much less variable, an improved spectrophotometric analysis would be obtained following chromatographic separation of the ring classes.

Recent reports[6,7,8] describe the use of High Pressure Liquid Chromatography (HPLC) with polar bonded phases eluted with

non-polar solvents (i.e. normal phase) for the ring number separation of aromatic hydrocarbons. These systems, exemplified by the amine column with hexane eluent used by Wise et al.[6] have advantages over the classical alumina or silica columns. They are not deactivated by traces of water and give a more distinct separation by ring number, that is, retention is less affected by the presence of alkyl substituents. This latter property is important since fossil hydrocarbon mixtures show a strong dominance of alkyl over parent aromatic forms.[9] Chmielowiec and George[7] have tested various polar bonded phases for their ability to separate aromatics by ring number. Grizzle and Thompson[8] have also compared an amine bonded column with alumina and a charge transfer complex column.

The experimental work described here is an assessment of the feasibility of using HPLC with fixed wavelength detection for the semi-quantitative determination of total aromatics in contaminated shellfish and in refinery effluents. Identification of individual aromatic hydrocarbons was not attempted, but it was borne in mind that the various ring classes separated during normal phase chromatography could be collected and subjected to reverse phase HPLC to identify particular compounds (separated at this stage by substituent effects). Also the eluate prior to the appearance of aromatics could be collected and analysed by gas chromatography for saturated hydrocarbons.

In developing the method, emphasis was placed on Gippsland crude oil, the source oil for most of the local pollution, since it has not been well characterised previously.

MATERIALS AND METHODS

High pressure liquid chromatography. (HPLC)

The HPLC equipment used was a Waters Associates modular instrument consisting of M6000 and M45 pumps, U6K injector, M600 solvent programmer and M440 dual channel absorbance detector fitted with 254 and 280 nm filters. Data was collected by a Varian A-25 chart recorder and Shimadzu E1A data processor.

Determination of aromatic hydrocarbon retention indices and the fractionation of Gippsland crude oil were carried out on a 25 cm by 0.4 cm column packed with μ-Bondapak-NH_2 (Waters Assoc.) eluted

with hexane at 1 ml/min. Other work employed a 25 cm by 0.7 cm semi-preparative column packed with the same material and eluted at 3 ml/min. with hexane. For reverse-phase chromatography a 10 cm by 0.5 cm radially compressed μ-Bondapak-C_{18} column was eluted either isocratically with 70% acetonitrile/water (retention index calculations), or with a linear gradient of 60 to 100% acetonitrile/water over 20 minutes. The flow rate was 2 ml/min.

All solvents were liquid chromatography grade filtered and degassed before use.

Gas chromatography (GC)

GC analyses were performed on a Hewlett–Packard 5830 gas chromatograph fitted with a 4 m × 3 mm I.D column packed with 3% OV-101 on 80–100 mesh gas-chrom Q. The carrier gas was nitrogen at 12 ml/min and a single flame ionisation detector was used. Oven temperature was programmed from 100 to 300°C followed by a hold at the final temperature for 20 mins. Calibration of GC analyses was accomplished by reference to an external standard containing *n*-alkanes of carbon number 14 to 32.

Gas chromatography/mass spectrometry (GC/MS)

Two types of instrument were used for GC/MS work:

i) Finnigan 3200/9500/6100

ii) Hewlett–Packard 5985A MS/computer interfaced with a HP 5840A Gas Chromatograph.

The operating parameters used for each instrument are shown in Table I.

Standards

Benzene and alkyl benzene standards, except for 1,2,3-trimethylbenzene, were obtained from Polyscience Corporation (Niles U.S.A.). Other aromatic hydrocarbons were supplied by Fluka A. G. Chemicals (Switzerland) through various local suppliers. Stock solutions were 200–1000 μg/ml in hexane. For reverse-phase HPLC

TABLE I
GC/MS operating parameters

Parameter	Finnigan 3200	Hewlett–Packard 5985A
Column	15 m × 0.5 mm SCOT SP-2100	20 m × 0.2 mm WCOT SE-54
Carrier gas	He. 2 ml/min	H_2. 2 ml/min
Split ratio	Splitless	1:10
Program	Ambient to 100°C rapid 4°C/min to 250°C	120–250°C at 4°C/min
Electron mult. Voltage	1600 V	2200 V
Masses scanned	50–350	20–400
Integrate time	3 ms	2.5 ms
Secs/scan	1	1
Scan start delay	Until solvent elutes	1 minute

work, separate standards 2–10 μg/ml in acetonitrile were prepared. All of the above solutions were stored in the dark when not in use.

Samples

A sample of Gippsland crude oil was supplied by Shell (Australia) Pty. Ltd. No. 2 fuel oil (a distillate oil distributed by the American Petroleum Institute for the purpose of chemical and biological testing) was obtained from the United States Environment Protection Agency quality assurance branch, (Cincinatti, Ohio, U.S.A.). Both oils were diluted in hexane to approximately 0.1 g/ml, centrifuged and filtered through a 0.5 μm filter before use. This removed particulate matter and any compounds insoluble in hexane.

Mussels (*Mytilus edulis*) were shucked and the tissues were extracted according to the methods of Burns and Smith,[10] except that benzene was omitted from the extraction solvent. All samples were from areas of Port Phillip Bay (Australia), found by Smith and Burns[11] to be subject to various levels of chronic oil pollution.

Effluent samples (2000 ml) were collected in solvent-washed glass bottles from the main discharge point of three local refineries. Although each of these refineries processes mainly Gippsland crude oil, their effluents are quite dissimilar because of differences in the treatment given to each before discharge, and the influence of other refinery activities such as base chemical synthesis. The three effluents were named RE-1, RE-2 and RE-3.

Chromatography of model compounds

Initially a series of model aromatic hydrocarbons (Table II) were chromatographed on the amine column (normal-phase) and also, for comparative purposes, on the C-18 column (reverse-phase). Retention indices for each column and compound were calculated in the manner described by Popl *et al.*[12] and compared with the response observed for each compound at the fixed detection wavelengths. These responses were documented as "weight response factors" (R_{254} and R_{280}) expressed in terms of integrator area counts per microgram (a.c/μg) of each compound injected. The ratio of the response factors at 254 and 280 nm, i.e. the absorption ratio, was calculated as an aid to the identification of unknown compounds.

Chromatography of oils

Chromatography of oil samples on the amine column was performed for two reasons: Firstly to test the behaviour of the column towards complex mixtures of aromatics typically found in the marine environment as pollutants, and secondly, as one means of assessing the accuracy of semi-quantitative measurement of total aromatics by HPLC. For Gippsland crude oil, HPLC fractions tentatively assigned to ring classes were collected and subjected to further analysis by gas chromatography/mass spectrometry and reverse-phase HPLC. After selection of suitable reference compounds, HPLC was used to estimate total aromatic hydrocarbons in samples of Gippsland crude and No. 2 fuel oil. These estimates were then compared with results obtained by other methods.

Chromatography of mussel extracts

In order to compare semi-quantitative HPLC and gas chromatographic indicators of aromatic hydrocarbons in shellfish, twelve mussel extracts were subjected to open column chromatography on silica/alumina columns, as described in reference 10. The aromatics isolated in this way were then quantified by the HPLC method and by gas chromatography. Prior isolation of aromatics was not a pre-requisite for HPLC since it does not respond to saturates, but by analysing samples which had already been prepared for gas chromatography, sources of variation other

than those inherent to the two measurement techniques could be eliminated. To confirm that the mussel extracts could be analysed by HPLC without prior column chromatography, some extracts were given a simplified clean-up (to remove very polar compounds and pigments) before injection directly onto the HPLC. This treatment consisted of shaking the extract with activated florisil in 50:50 hexane/dichloromethane over 30 mins, removing the solvent and redissolving the extract in hexane. Recovery tests showed that this procedure did not remove aromatics in the boiling range encountered in mussels up to fluoranthene. Higher polycyclic aromatics were partially removed. HPLC chromatograms of extracts treated in this way resembled closely those obtained after column chromatography.

Chromatography of refinery effluent extracts

Refinery effluent samples were extracted with 1×100 and 2×50 ml of hexane at pH 12. The first 100 ml of hexane was used to rinse the sample bottle. Aliquots of 50 to 250 μl were injected onto the HPLC and, if a sufficiently large signal resulted, aromatics including volatile compounds were calculated from the chromatogram. If the trace was too small for accurate measurement the extract was carefully concentrated by rotary evaporation, followed by evaporation to 2 ml in a stream of nitrogen. It was then analysed for non-volatile aromatics only. When sulphur interference occurred it was removed by allowing the extract to stand overnight in a vial containing a ball of acid washed copper wool.

Non-volatile aromatics in the effluent extracts were also quantified by open-column chromatography/gas chromatography so that the results could be compared with those given by HPLC. The procedure used for this comparison was the same as that described for the mussel extracts except that it was not necessary to florisil treat effluent extracts before direct injection.

RESULTS AND DISCUSSION

Model compounds

Table II lists retention index and response data for a series of model aromatic hydrocarbons and Figure 1 shows a chromatogram of the

TABLE II

Retention index (I) and response data for model aromatic hydrocarbons.

Compound	No. of Π electrons	log I (NH_2 column) This work	log I (NH_2 column) Ref. 6	log I (C_{18} column) This work	log I (C_{18} column) Ref. 6	Weight response[a] factor 254 nm	Weight response[a] factor 280 nm	Absorbance ratio (254/280)
benzene[b]	6	1.00	1.00	1.00	1.00	1.91	ND	ND
toluene	6	0.96	1.00	1.66	1.51	3.38	0.085	39.8
ethylbenzene	6	0.94	—	2.15	—	2.85	ND	ND
n-pentylbenzene	6	0.91	0.71	3.78	3.80	2.05	ND	ND
n-hexylbenzene	6	0.89	—	4.30	—	1.88	ND	ND
n-octylbenzene	6	0.90	—	>5	—	1.59	ND	ND
n-decylbenzene	6	0.85	—	>5	—	1.45	ND	ND
o-xylene	6	1.00	—	2.15	—	2.57	0.27	9.69
m-xylene	6	0.93	1.00	2.30	2.16	2.14	0.38	5.68
p-xylene	6	0.92	—	2.29	—	2.80	1.15	2.43
1,3,5-trimethylbenzene	6	0.88	—	2.84	—	1.25	0.37	3.37
1,2,3-trimethylbenzene	6	1.10	—	2.66	—	1.74	0.19	9.01
naphthalene[b]	10	2.00	2.00	2.00	2.00	43.3	50.3	0.86
2-methylnaphthalene	10	1.95	1.96	2.58	2.59	39.7	48.4	0.82
2,3-dimethylnaphthalene	10	2.01	2.08	3.10	3.47	29.2	39.5	0.74
2,6-dimethylnaphthalene	10	1.92	1.94	3.28	3.15	36.8	40.0	0.92
fluorene	12	2.48	2.61	2.73	2.78	166	53.4	3.10
biphenyl	12	2.16	2.25	2.55	2.57	164	11.8	13.6
anthracene	14	2.91	2.95	3.12	3.02	1662	3.61	461
phenanthrene[b]	14	3.00	3.00	3.00	3.00	602	108	5.59
pyrene	16	3.30	3.68	3.56	3.51	112	55	2.02
fluoranthene	16	3.45	3.39	3.41	3.42	158	205	0.77
benz(a)anthracene[b]	18	4.00	4.00	4.00	4.00	—	—	—
dibenz(a, h)anthracene[b]	22	5.00	5.00	5.00	5.00	—	—	—

[a]Expressed as integrator area counts/μg/10,000.
[b]Reference compounds for retention index calculations.
ND Not determined due to low response at 280 nm.

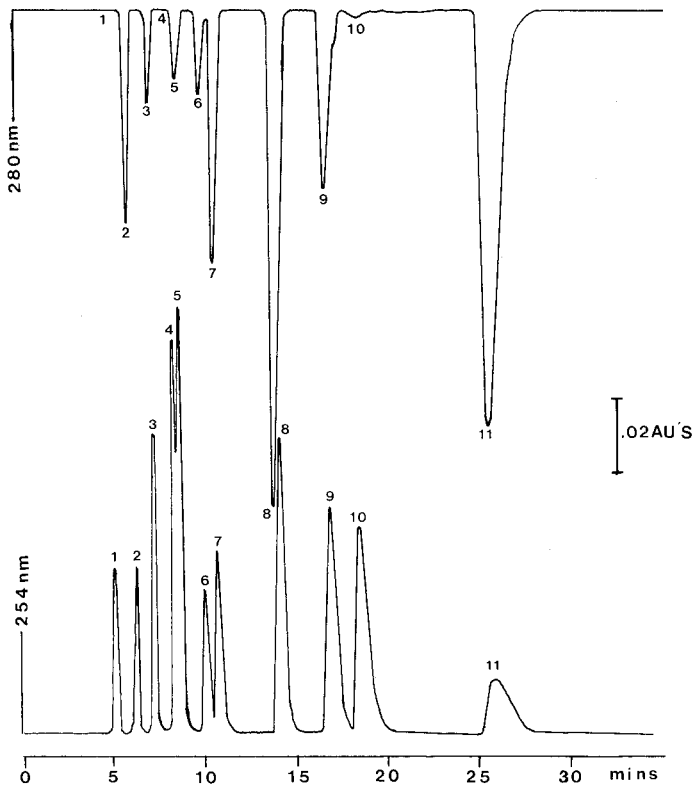

FIGURE 1 Amine column chromatogram of parent aromatic hydrocarbons: 1—benzene, 2—naphthalene, 3—fluorene, 4—anthracene, 5—phenanthrene, 6—pyrene, 7—fluoranthene, 8—benzo(a)anthracene, 9—benzo(a)pyrene, 10—perylene, 11—dibenzo(a, h)anthracene.

unalkylated compounds with detection at 254 and 280 nm. The data in Table II show that retention on the amine column is only slightly affected by alkylation of the aromatic ring and the dominant factor in determining retention is the number of PI electrons (or equivalently, the number of aromatic carbon atoms) in the condensed ring system. The retention indices listed in Table II are generally similar to those reported by Wise *et al.*,[6] but fluoranthene eluted after rather than before pyrene. In the case of the higher ring number aromatics, data were collected for the parent compounds

only. The reasons for this were firstly the difficulty in obtaining the alkylated compounds, secondly these were expected to comprise only a small part of the total aromatics in samples and thirdly, as the size of the ring system increased, alkylation was expected to have a diminishing effect on the weight response factor.

For the purposes of this work, the important feature of the amine column's behaviour is its ability to group together compounds with a similar response at the fixed detection wavelengths, so that a single representative standard can be used to quantify each group. Table II shows that response varies much more between ring groups than within them. At 254 nm the relative response of benzene, naphthalene and phenanthrene is about 1:20:300. Retention on the amine column and absorbance of UV-radiation both primarily depend on the number of PI system electrons in a particular compound, so intrinsically the amine column separates compounds into groups of similar UV response. Figure 2 depicts the relation between the logarithm of the weight response factor at 254nm and retention index on the amine bonded column. For compounds having from six to fourteen PI electrons (e.g. benzene to phenanthrene) the logarithm of the weight response factor (log R_{254}) was found to be directly proportional to the logarithm of the retention index (log I). Because of the absence of data for the alkyl homologs of the higher ring aromatics we cannot define this relationship further at this stage, but a least squares fit to the available data yields:

$$\log R_{254} = 1.31 \log I - 0.92$$

In contrast to the normal-phase system, the data for the reverse-phase system show that compounds having very different weight response factors elute in the same region of the chromatogram. This would not be of concern were it possible to resolve, identify and obtain pure standards for the bulk of the aromatics likely to be present in environmental samples, but where petroleum is the contaminant this is unlikely to be the case. Thus even though reverse-phase HPLC can be of use in identifying specific aromatic hydrocarbons it is not considered suitable for the estimation of total aromatics.

The relative abundance of aromatic classes in petroleum is monoaromatics > diaromatics > triaromatics.[13] Experience with

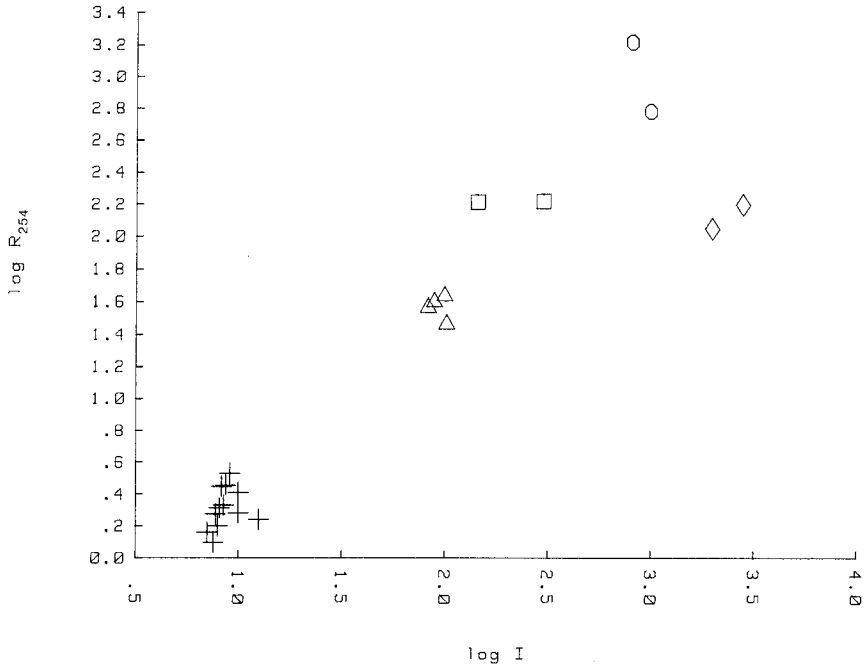

FIGURE 2 The relationship between retention on the amine column (log I) and response at 254 nm (log R_{254}). Symbols distinguish groups of compounds with a given number of PI system electrons: Crosses—6, Triangles—10, Squares—12, Circles—14, Diamonds—16.

mussel and water extracts has convinced us that this is also the order of abundance in environmental samples. For the standards analysed, the monoaromatics showed the greatest intra-group variation in response: 3.38 area counts/μg for toluene to 1.25 area counts/μg for 1,3,5-trimethylbenzene. A single alkyl substituent on the benzene ring causes the whole absorption spectrum to shift to the red,[14] resulting in an increase in absorptivity at 254 nm. Little further change in the spectrum occurs as the length of the substituent increases, but the weight response factor decreases as the non-absorbing side chain becomes a larger proportion of the molecule. Multiple alkyl substitution of the benzene ring causes a further shift to the red so that absorption at 280 nm becomes significant. Of the multi-substituted monoaromatics studied, 1,3,5-

trimethylbenzene gave the lowest, and *p*-xylene the highest response at 254 nm.

Although only a few examples of alkylated naphthalenes were available for this study, it is apparent that the effect of alkylation on response is less than that observed for the monoaromatics. This is to be expected in view of the greater contribution of the ring system to the mass of the molecule.

Anthracene and phenanthrene, the two three-ring aromatics studied, respond quite differently at the two detection wavelengths, but are only partially resolved on the amine column. This should not complicate the analysis of oil contaminated samples because the phenanthrenes are by far the most abundant three ring isomers in petroleum.[15] Furthermore, the extent to which anthracenes contribute to the three ring aromatics can be estimated from the absorption ratio.

Chromatography of oils

Chromatograms of the two oils on the amine column are illustrated in Figures 3(a) and (b). Complete resolution of ring groups was not achieved. However, on the basis of the results for the model aromatic hydrocarbons it is possible to interpret the chromatograms in terms of the distribution of aromatic types present. Thus on the basis of retention times and absorbance ratios the major groups marked in Figure 3 were identified as follows: 2—benzenoid, 3—naphthalenoid, 4—fluorenoid, 5—phenanthroid, 6—pyrenoid. Region 1 contained saturated hydrocarbons. The fraction termed "pyrenoid" in the Gippsland crude oil chromatogram contained all compounds eluting after phenanthrene, that is, compounds with four or more rings. Gas chromatographic examination of fractions 1 and 2 of the Gippsland crude oil indicated that all of the aliphatic compounds eluted in fraction 1 with none present in fraction 2.

Table III gives the results of GC/MS analysis of fractions 3, 4 and 5 of the Gippsland oil. The total ion chromatograms of these fractions appear in Figures 4(a), (b) and (c) respectively. Fraction 2 was not analysed by GC/MS because many of the major components of this fraction were expected to be lost during reconcentration after the HPLC separation.

It is evident from Table III that the names applied to the regions

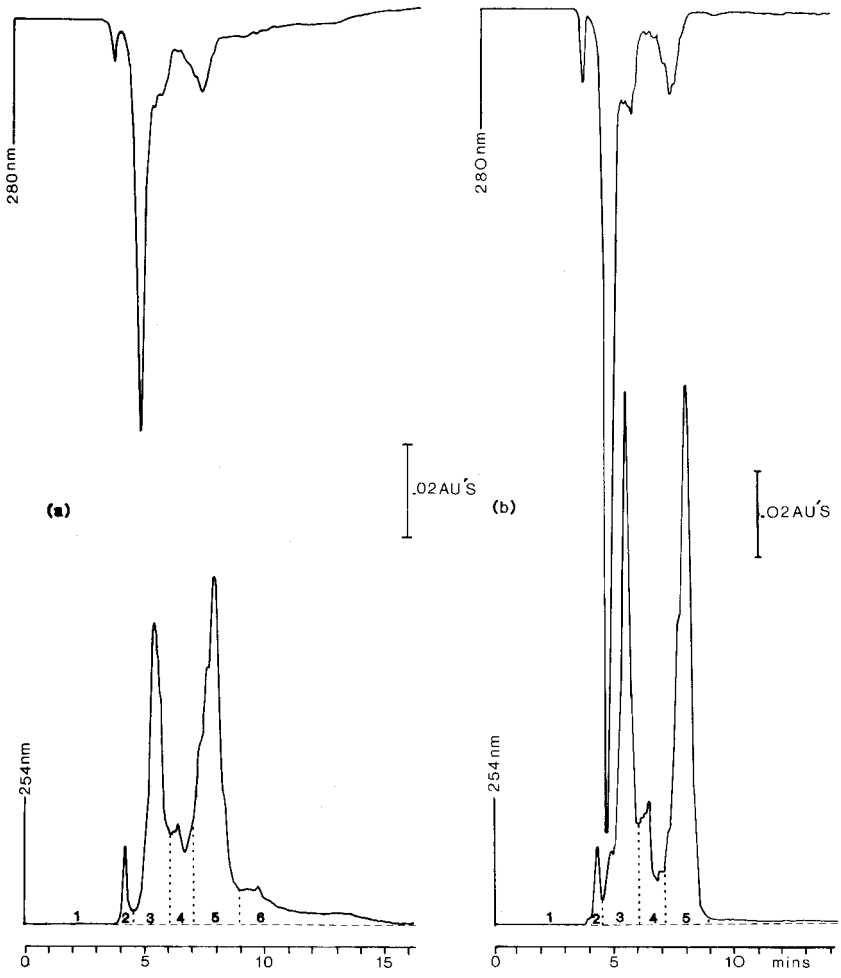

FIGURE 3 Amine column chromatogram of petroleum oils: (a) Gippsland crude oil, (b) No. 2 fuel oil. Numbered regions represent major aromatic classes as described in the text.

marked 3, 4 and 5 in Figure 3 are a valid description of the materials eluting in those regions. For each of the ring groups examined by GC/MS, the parent ring, methyl, dimethyl and trimethyl isomers were the most abundant compounds found. By inference, this is probably also the distribution of types within the

TABLE III

Aromatic hydrocarbons identified by GC/MS in HPLC fractions of Gippsland crude oil.

	Peak no.	Mol. wt.	Assignment
Fraction 3			
	1	128	Naphthalene
	2	142	Methylnaphthalene
	3	142	Methylnaphthalene
	4	154	Biphenyl
	5	156	Dimethylnaphthalene
	6	156	Dimethylnaphthalene
	7	156	Dimethylnaphthalene
	8	156	Dimethylnaphthalene
	9	156	Dimethylnaphthalene
	10	168	Diphenylmethane
	11	170	C_3-Naphthalene
	12	170	C_3-Naphthalene
	13	170	C_3-Naphthalene
	14	170	C_3-Naphthalene
	15	170	C_3-Naphthalene
	16	170	C_3-Naphthalene
Fraction 4			
	1	168	Diphenylmethane/Methylbiphenyl
	2	168	Dibenzofuran
	3	182	Dimethylbiphenyl
	4	182	Dimethylbiphenyl
	5	166	Fluorene
	6	180	Methylfluorene
	7	180	Methylfluorene
	8	180	Methylfluorene
	9	180	Methylfluorene
	10	194	Dimethylfluorene
	11	194	Unknown
	12	194	Dimethylfluorene
	13	194	Dimethylfluorene
	14	194	Dimethylfluorene
	15	194	Unknown (possibly a Dimethylfluorene)
	16	198	Methyldibenzothiophene
	17	208	C_3-Fluorene
	18	208	C_3-Fluorene
	19	208	C_3-Fluorene
	20	212	C_2-Dibenzothiophene
	21	208	C_3-Fluorene
	22	212	C_2-Dibenzothiophene

Tentative Assignment. No Matches Found

TABLE III (continued)

Peak no.	Mol. wt.	Assignment
Fraction 5		
1	196	$C_{14}H_{28}$ Hydrocarbon—Impurity in solvent
2	222	Diethyl-o-phthalate—Contaminant from vial cap
3	184	Dibenzothiophene
4	178	Phenanthrene/Anthracene
5	198	Methyldibenzothiophene
6	192	Methylphenanthrene/Anthracene
7	192	Methylphenanthrene/Anthracene
8	192	Methylphenanthrene/Anthracene
9	192	Methylphenanthrene/Anthracene
10	204	C_2H_4-Phenanthrene/Anthracene
11	206	Dimethylphenanthrene/Anthracene
12	206	Dimethylphenanthrene/Anthracene
13	206	Dimethylphenanthrene/Anthracene
14	206	Dimethylphenanthrene/Anthracene
15	218	Unknown

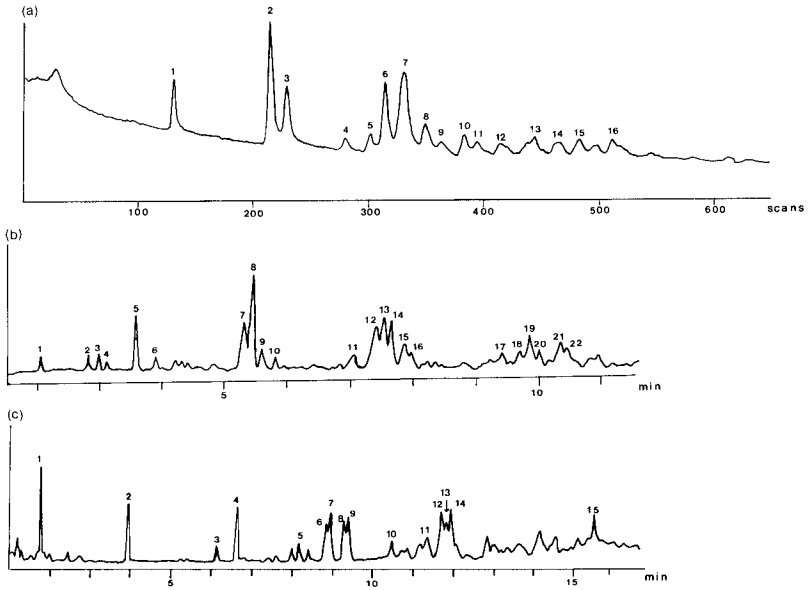

FIGURE 4 Total ion chromatograms of HPLC fractions from Gippsland crude oil: (a) Fraction 3—"Naphthalenoids", (b) Fraction 4—"Fluorenoids", (c) Fraction 5—"Phenanthroids". Numbered peaks correspond to compounds listed in Table III.

monoaromatics group. Support for this contention can be drawn from the observation that the monoaromatics in the oils give a significant response at 280 nm, indicating multiple substitution, and from knowledge of the composition of other petroleum oils. Petrakis *et al.*[16] found that the monoaromatics in the portion of two crude oils boiling above 230°C, had an average of four alkyl substituents per molecule.

GC/MS could not distinguish between the structurally isomeric anthracenes and phenanthrenes in fraction 5. Reverse-phase HPLC was applied to this fraction yielding the chromatogram shown in Figure 5. Identification of the phenanthrene and anthracene peaks was based on retention volume and absorbance ratio data but the identification of the other peaks remains tentative in the absence of authentic standards. Using the known response factors of anthracene and phenanthrene it was estimated that anthracenes are present in Gippsland oil at approximately 3% of the concentration of phenanthrenes.

Semi-quantitative analysis of aromatics in oils

In the light of the accumulated data, standards chosen for the semi-quantitative analysis of the two oils by HPLC were as follows: Benzenoids—benzene, Naphthalenoids—2,6-dimethylnaphthalene, Fluorenoids—fluorene, Phenanthroids—phenanthrene, Pyrenoid—pyrene. Benzene was chosen as the standard for estimating monoaromatics since its response at 254 nm is intermediate among those monoaromatics measured (Table II), and it is likely to be a major constituent of Gippsland oil. Alkylated compounds would have been more appropriate standards for the last three groups but none were available at the time of this study. However, for the reasons stated earlier, this deficiency was not expected to cause significant errors in the measurement of total aromatic hydrocarbons. The results of three separate analyses of No. 2 fuel oil, two by external standardization and one by a standard addition of the group reference compounds, are given in Table IV. No significant differences were observed in the results given by reference to external or added standards so the oil matrix had no discernible effect on the HPLC response of its constituent aromatics. The analysis sheet accompanying the No. 2 fuel oil sample states that

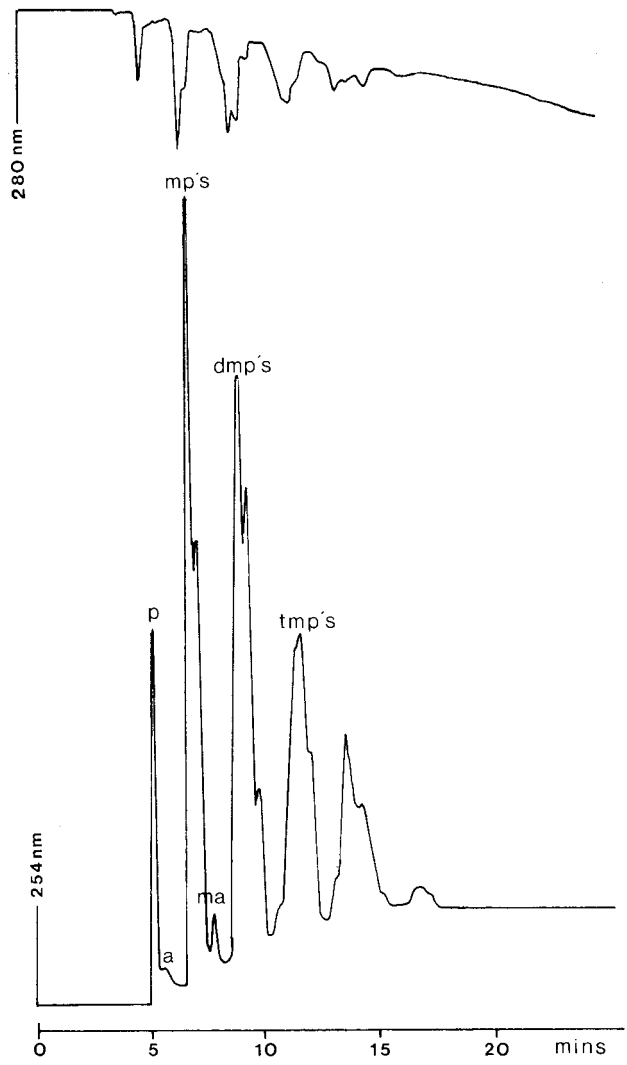

FIGURE 5 Reverse-phase HPLC chromatogram of Gippsland crude oil fraction 5 ("phenanthroids"). p—phenanthrene, mp's—methylphenanthrenes, dmp's—dimethylphenanthrenes, tmp's—trimethylphenanthrenes, a—anthracene, ma—methylanthracene.

TABLE IV
HPLC analysis of API no. 2 fuel oil

	Weight %			
Aromatic group[a]	Run 1	2	3[b]	
Benzenes	32.5	33.2	33.5	
Naphthalenes	20.5	19.7	19.6	
Fluorenes	1.2	1.5	1.4	
Phenanthrenes	2.0	1.7	1.8	Mean
Total	56.2	56.1	56.3	56.2

[a]Group names describe the major type of compounds in each group. Other compounds may be present.
[b]Standard addition method.

aromatic hydrocarbons comprise 49% of its mass, compared to a mean of 56% by our method. It is important to note that values reported for total aromatics in oils and environmental samples are often the sum of those components resolved and identified by gas chromatography. This approach neglects a significant proportion of the hydrocarbons which appear in gas chromatograms as an unresolved complex mixture. Hence Anderson *et al.*[17] report a value of 0.36% for fluorenes in a sample of No. 2 fuel oil, but they also report that only 16.6% of the oil could be accounted for by gas chromatographic analysis using peak areas only. The analysis sheet value was determined by ASTM method D-2007-73 which uses a column chromatographic separation followed by gravimetric measurement of non-aromatic components and calculation of aromatics by difference.

The results of applying the semi-quantitative HPLC method to Gippsland crude oil are shown in Table V. A search of the literature failed to reveal any previously reported data for percentage aromatics in Gippsland crude oil. However at our request a refinery laboratory used the ASTM method to derive a value of 25.2% for total aromatics in the naphtha free (>205°C) portion of this oil, compared with our value of 26.9% for the (stabilised) whole oil.

TABLE V
HPLC analysis of Gippsland crude oil

Aromatic group	Weight %
Benzenes	16.0
Naphthalenes	7.7
Fluorenes	0.8
Phenanthrenes	0.9
Pyrenes	1.4
Total	26.9

Analysis of mussel extracts

Marine environmental monitoring often utilizes analysis of the mussel *Mytilus edulis* as a convenient alternative to water analysis. Burns *et al.*[18] have explained the value of this approach. Except in cases where oil contamination has occurred recently, estimation of petroleum-derived aromatics in mussels is a difficult task. The combined effect of factors such as selective accumulation, weathering and biodegradation results in the aromatics in mussels being a complex mixture of polyalkyl and cycloalkyl substituted rings with little or no contribution from the parent compound or lower alkyl homologs. Gas chromatograms of such mixtures (Figure 6) are dominated by the unresolved envelope. Any peaks which do appear in the aromatic fraction can usually be attributed to biogenic sources. For example, the marked peaks in Figure 6 were identified by GC/MS as C-29 to C-31 olefins. (Compounds of this type have been found in *Mytilus* previously[19] and are thought to derive from dietary algae.) Mussel extracts analysed by HPLC gave chromatograms typified by Figure 7 which corresponds to the extract which gave the gas chromatogram shown in Figure 6. The absence of the simpler aromatic components in mussel extracts resulted in poorer group resolution than obtained for the oil samples. However, even basing quantitative measurements on the same simple standards as used for the analysis of oils resulted in a satisfactory estimate of total aromatic hydrocarbons. Figure 8 depicts the relationship found between semi-quantitative HPLC and GC indicators of aromatics in the twelve mussel extracts. For

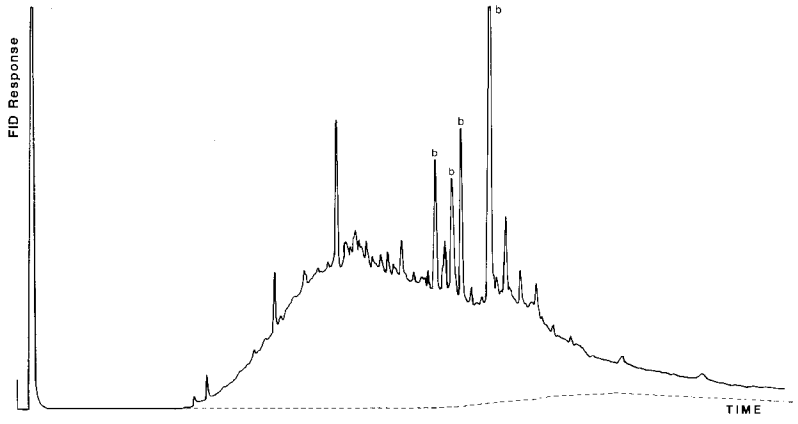

FIGURE 6 Gas chromatogram of the "aromatic hydrocarbon" fraction separated from oil contaminated mussels by column chromatography. Peaks marked "b" correspond to biogenic olefins identified by GC/MS.

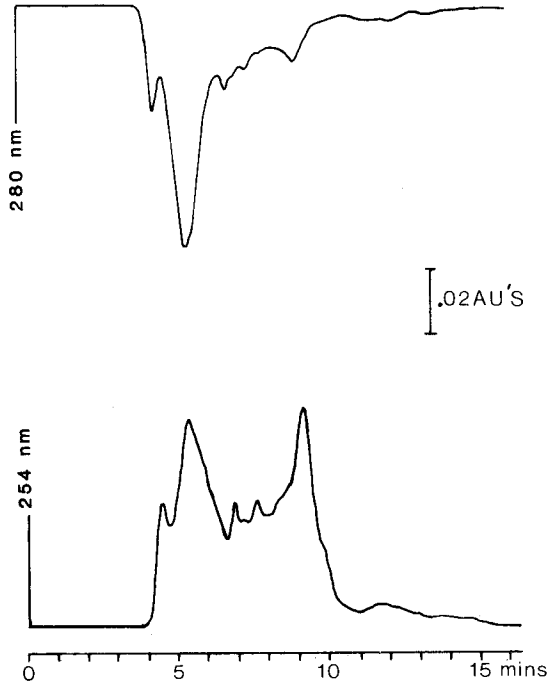

FIGURE 7 Amine column chromatogram of aromatic hydrocarbons in a mussel extract.

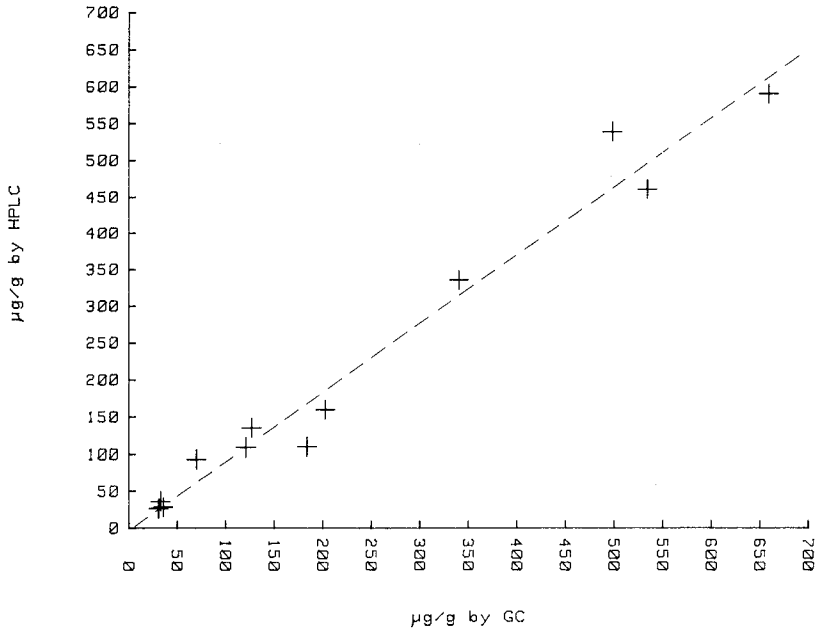

FIGURE 8 The relationship between HPLC and GC indicators of the level of aromatic hydrocarbons in mussel tissues. Values are presented as μg/g dry weight. The correlation is significant ($P < 0.001$, $r = 0.99$) and the line of best fit has a slope of 0.93 and an intercept of -1.2 μg/g.

comparison Figure 9 shows the relationship between the GC result and the results which would be obtained if the mussel extracts were analysed spectrophotometrically by reference to a phenanthrene standard. It is apparent that estimation of ring groups separately results in a significant improvement in the correlation between gas chromatographic and spectrophotometric measurements of aromatic hydrocarbons in the mussel extracts. Furthermore, it is apparent from Figure 8 that, in absolute terms, HPLC and gas chromatography gave similar results. Hence, it appears that normal phase HPLC, with UV detection, is capable of providing a relatively fast and cheap alternative to gas chromatography preceded by open column chromatography. In addition, considerable resolution of aromatic classes is provided.

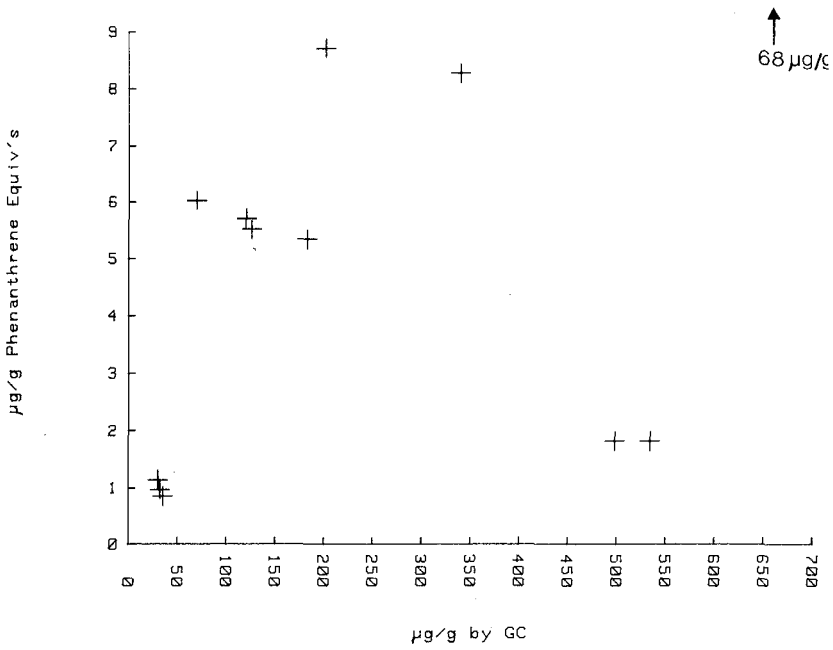

FIGURE 9 The relationship between the total HPLC trace area, as phenanthrene equivalents, and the level of aromatic hydrocarbons found in mussels by column chromatography/gas chromatography. Values expressed as µg/g dry weight. The correlation coefficient (r) is 0.60.

The scatter of the points in Figure 8 can be attributed to two sources: Imprecision and/or inaccuracy in either or both measurement methods. Duplicate analyses by HPLC never gave results differing by more than 5% and for GC, never by more than 8%, so imprecision alone cannot account for the observed scatter. Both methods are subject to some inaccuracies. In the case of gas chromatography this is principally interference from incompletely removed saturated and biogenic hydrocarbons. As noted by Zsolnay,[20] HPLC is virtually immune to such interferences because of the combined selectivity of the column and detector and during this work injection of neat squalene at the sensitivity used for aromatic hydrocarbon analysis failed to give a peak. The major sources of inaccuracy in the HPLC method are thought to be mismatching of the selected standard compounds to the poorly

defined sample constituents and overlap of the ring groups. Errors of the first type are inherent to the method and must be balanced against the advantages of speed, simplicity and discrimination it provides. Analysis of mussel extracts by HPLC required, on average, one hour (including the necessary standard runs), compared with four to five hours for a similar analysis by open column and gas chromatography. Errors due to overlap of the ring groups are most significant where poor resolution of the mono and diaromatics occurs. The separation of these groups in the mussel extract chromatograms varied from a situation of almost baseline resolution to one in which the monoaromatics appeared only as a shoulder on a much larger diaromatic peak. In the latter case the relation between the detector traces at 254 and 280 nm was of some assistance in determining the correct dividing line for the semi-quantitative calculation. Nevertheless considerable overestimation of monoaromatics probably occurred in such instances. It may be possible to improve the ring group resolution by using other types of polar bonded phases, such as the diamine phase used by Chmielowiec and George[7] but this was not attempted as part of the work reported here.

Analysis of refinery effluents

In contrast to the HPLC chromatograms of mussel extracts, those of the refinery effluent extracts showed good resolution of the ring groups. Figures 10(a) and (b) show chromatograms of extracts RE-1 and RE-2 respectively. The concentration of aromatic hydrocarbons in these effluent samples was calculated from the chromatograms using the same reference compounds as were used for the oil and mussel analyses. The results, in terms of equivalents of the reference compounds, are presented in Table VI. The values given for "volatile" and "non-volatile" compounds represent the result of analyses carried out before and after the extracts were concentrated. The differences show that the most abundant aromatics present in the fresh effluents were volatile monoaromatics (e.g. benzene, toluene, xylenes). These are the most water-soluble class of hydrocarbons and McAuliffe[21] found them to be the most abundant aromatics in water equilibrated with crude oils. Although monoaromatics were much less dominant in the non-volatile fraction, they remained the most

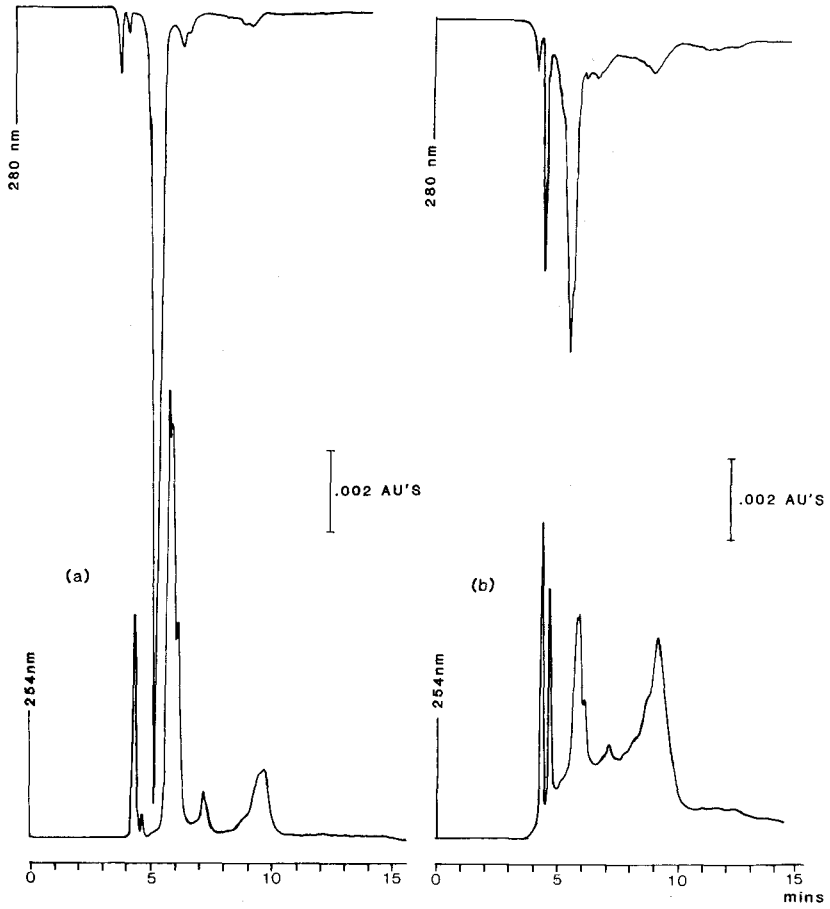

FIGURE 10 Amine column chromatogram of refinery effluent extracts: (a) RE-1 (b) RE-2.

abundant of the ring classes present. These non-volatile monoaromatics would be expected to persist for some time in the water body receiving the effluents, and their toxicity approaches that recorded for the lower naphthalene homologs.[22]

The differences, both qualitative and quantitative, between the aromatics found in RE-1 and RE-2 are related to the different origins of these two effluents. RE-1 came from a relatively simple refinery processing Gippsland crude oil only, while RE-2 came from

TABLE VI

Concentration of aromatic hydrocarbons in refinery effluent samples (mg/l).[a]

	RE-1		RE-2	
Aromatic group	Total	Non volatile	Total	Non volatile
Benzenes	4.40	1.43	29.2	5.84
Naphthalenes	1.40	0.98	6.40	5.10
Fluorenes	0.04	0.03	1.00	0.74
Phenanthrenes	0.03	0.03	0.56	0.55
Pyrenes	<0.01	<0.01	0.20	0.20
Total	5.87	2.57	37.4	12.4

[a]Results for each group represent "equivalents" of the group reference compounds.

TABLE VII

Total non-volatile aromatic hydrocarbons in refinery effluents: HPLC vs GC. (mg/l)

Effluent sample	HPLC	GC	% Diff
RE-1	4.6	3.8	17
RE-2	12	11	8
RE-3	0.32	0.37	16

a larger and more complex refinery carrying out the full range of refinery operations on more than one oil.

The sharp peak eluting between the mono and diaromatics in Figure 10(b) was identified as elemental sulphur by its retention time and absorbance ratio and by its disappearance upon treatment of the extract with copper.

Table VII compares the results of HPLC analyses of aromatics in the effluent extracts, with values derived by column chromatography/gas chromatography. At this stage we have only applied both measurement techniques to these three samples and so we cannot realistically assess the significance of the differences observed. However it is felt that the agreement is good enough to

encourage further testing of the HPLC method. To gain perspective it is worth considering some aspects of a method often used to analyse total hydrocarbons in refinery effluent samples, i.e. the infra-red spectrophotometric method for the determination of "oil and grease".[23,24] The accuracy of this method is reported to be no better than $\pm 20\%$ when a synthetic oil is used (Whittle *et al.*[25] improved this to $\pm 10\%$ using calculations not involving a synthetic oil). Larger errors occur if the sample contains high levels of aromatics, which is environmentally the most important case.

We suggest that this HPLC determination of total aromatics offers considerable advantages over existing methods for oil pollution analysis because it focusses on the most environmentally significant hydrocarbons present. If data on the saturated hydrocarbons is required, the HPLC eluate prior to the elution of the aromatics could be collected and analysed by gas chromatography.

From the point of view of chronic toxicity, many of the larger polycyclic aromatics are of concern because of their mutagenic and carcinogenic properties. The method described here does not attempt to measure these compounds specifically and they are probably best measured by reverse-phase HPLC with fluorescence detection.[26,27] However it has been shown[28] that reverse-phase analysis of complex aromatic mixtures can be considerably improved by prior amine-column chromatography.

The detection limit attainable in HPLC determination of total aromatics is limited in practical terms by factors such as the complexity of samples, the degree of degradation of the aromatics present, and the proportion of the total contributed by monoaromatics, these being the least well detected group. However, we have analysed one effluent sample containing only 0.9 mg/l total aromatics (including volatiles) and estimate that for most samples the detection limit will lie between 0.2 and 1 mg/l. By comparison, the oil and grease method is reported to be capable of detecting 0.2 mg/l total oil.[24] The recovery of volatile aromatic hydrocarbons through the extraction/analysis procedure was checked by analysing 3.5% NaCl solution which had been spiked with benzene and naphthalene at 0.5 mg/l. Three such tests gave recoveries of $87 \pm 5\%$ benzene and $94 \pm 3\%$ naphthalene.

In the case of HPLC analysis of non-volatile aromatics alone, the detection limit is determined mainly by the volume of sample

available and the purity of the extracting solvent. The sensitivity is thus best described in terms of the absolute amount of material detectable. The sensitivity of the HPLC method for non-volatile aromatics was estimated by injecting, at maximum detector gain, small volumes of the extract of effluent RE-3. This effluent passed through dissolved air flotation and aerobic lagoon treatment before discharge and so the aromatics seen in the extract are fairly degraded. Figure 11 shows the chromatogram obtained for this extract. The monoaromatics in RE-3 gave a peak height of five times the baseline noise when the amount injected was equal to 0.2 μg benzene equivalents. Depending on the sample workup adopted this would correspond to about 1 μg/l in the original effluent sample. This level of sensitivity, if realised in practice, should be more than adequate. Values reported for the acute toxicity (96 hr LC-50) of monoaromatics to aquatic life range from 0.5 to 1180 mg/l.[22]

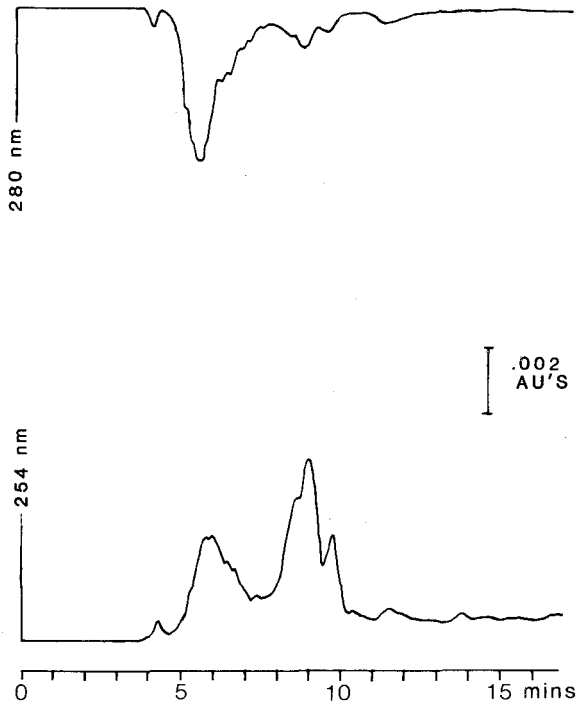

FIGURE 11 Amine column chromatogram of refinery effluent extract RE-3.

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