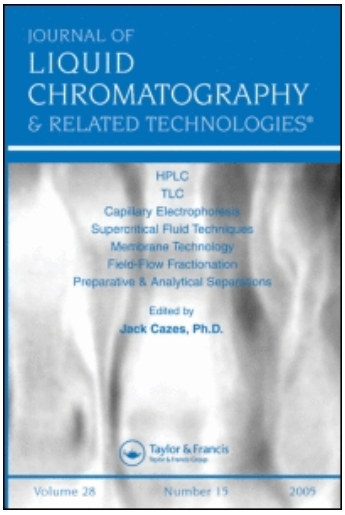

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Optimisation of Fluorescence Detection for Polyaromatic Hydrocarbon Determination by Using High Performance Liquid Chromatography

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OPTIMISATION OF FLUORESCENCE DETECTION FOR POLYAROMATIC HYDROCARBON DETERMINATION BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The procedures needed for the optimisation of programmable fluorescence detectors have been investigated. We found that wavelength programs had to be developed from data obtained from the particular detector for which the program was to be used - programs reported in the literature or developed from data obtained on other instruments gave substantially lower sensitivities. The wavelength program we developed was a compromise between: chromatographic separation, abundance and relative sensitivity of each PAH; the ease with which a timed event could be included in the elution profile and; the relative importance of each PAH to the analyst. The success of programmable fluorescence detection was dependant on the reproducibility with which the detector was able to reset specified wavelengths, with corresponding detector response variability of up to 20% for one new detector.

INTRODUCTION

Polyaromatic hydrocarbon determination is often one of the requirements in site evaluations and chemical audit studies, particularly where the site usage is to change

from industrial to recreational or domestic housing. It is also a general parameter included in air particulate studies[1] and water quality evaluations[2] in the urban environment, where PAHs accumulate as a result of vehicle and industrial emissions. Baseline studies are also carried out to gauge the input of PAHs to the environment from natural events such as forest fires[3]. The ubiquity of PAHs and concerns over their participation in health related problems[4] has led to the current worldwide activity in PAH determinations.

A number of different methods tend to be popular for PAH studies, such as high performance liquid chromatography (HPLC) with UV and/or fluorescence detection[5-7] and capillary gas chromatography with mass spectrometric detection[8,9]. A number of other techniques have also been employed, such as supercritical fluid chromatography[10], but are used to a lesser extent. PAHs are a unique group of compounds to study by HPLC-fluorescence detection due to their strong native fluorescence. Their different optimal emission and excitation wavelengths means that programmed fluorescence detection (PFD) can be gainfully used to provide a 'best set' of conditions for their determination, since a single condition will most probably only give adequate determination for a few of the 16 USEPA priority PAH[11] which are usually of interest. The low background concentrations of most PAHs also means that sensitive detection procedures, with some degree of specificity to suppress response to interfering components, should be employed.

GC-MS and HPLC-PFD tend to be the most reliable methods of analysis due to their sensitivity and selectivity. This paper will not address the former technique, but rather will concentrate on the use of HPLC-PFD. The reason for this is that in a recent Australian inter laboratory study it was found that most contract laboratories used HPLC, rather than GC-MS for determination of PAHs[12]. Further it was found that programmed fluorescence detection was not uniformly used by this group. Hence it was of interest to evaluate the sorts of difficulties which may result from inappropriate or improper use of the technique.

In reviewing the literature, we found that the wavelength programs used for PFD varied substantially between different groups. Risner and Conner[13] developed a method for the quantification of PAHs in the particulate matter of indoor air samples using reversed phase HPLC with PFD. However due to considerations such as spectral properties, chromatographic resolution, biological activity and generally low sample concentrations their method analysed for only five PAHs. Each one being analysed at its optimum excitation and emission wavelengths. This proved to be unsatisfactory for our

studies as a broader molecular weight range needed to be screened to successfully use PAHs as indicators of other organic pollutants in the environment. Other researchers, such as Smith et al.[14], used stepwise fluorescence detection but used compromise conditions for several groups of compounds and as a result, only a few changes in wavelengths had to be made. This procedure is practical where detectors can accommodate only a limited number of possible wavelength changes in each program. Likewise, a study of the Great Barrier Reef[15] off the east coast of Australia used only two excitation and emission changes for the determination of seven PAHs.

The studies reported here were part of a larger study directed towards an evaluation of the total input and distribution of PAHs in a modern urban environment. PAHs were chosen because their transport and persistence may be used in the first approximation to gauge the distribution of other organic pollutants in this system.

This paper will outline the studies undertaken to optimise the PFD conditions. It will also present typical chromatograms of a soil sample extract produced under different PFD and single-wavelength fluorescence detection (herein abbreviated as SFD) conditions, and river water extract under optimised PFD conditions. The conclusion that properly optimised conditions are necessary for confident data generation will be illustrated.

EXPERIMENTAL

Chemicals

Polyaromatic hydrocarbon standards were obtained from Alltech Associates, were of analytical reagent grade, and were used as received. PAH standard solutions were prepared as 100 ppb stock solutions in acetonitrile and analytical solutions (5 - 50 ppb) were made from these stock solutions by addition of appropriate volumes into standard flasks and making them up to the mark with acetonitrile. All solvents (BDH chemicals) were of HPLC grade quality. Reagent-grade water was obtained from a Millipore Milli-Q system.

Instruments Used

Fluorescence studies on individual stock PAH solutions in acetonitrile were carried out on an Hitachi F-4010 scanning fluorescence spectrophotometer, with a stated

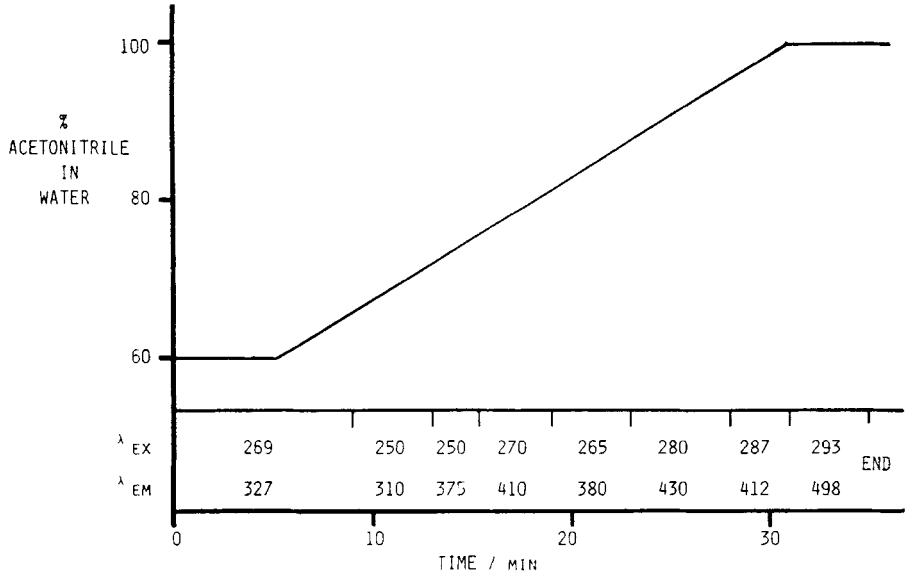

FIGURE 1. Time relationship between the modified wavelength program and the solvent gradient used for determination of PAHs.

bandwidth of 5 nm and wavelength accuracy of 2 nm. HPLC analyses were performed on two separate systems. System I was a dual pump gradient system (Waters Associates model M6000A controlled by a 660 solvent programmer). Optimised gradient conditions used an initial eluent of 60:40 acetonitrile:water, maintained for 5 min, then linearly programmed to 100 % acetonitrile over a 25 min period (Figure 1). Samples were injected with a Rheodyne single port injection valve connected to a 20μL sample loop and an injection event marker. Chromatographic separation was achieved on a 15 cm x 3.6mm id Vydac 201TP C-18 reversed-phase column, with a Phenomenex ultracarb 5 ODS (50 mm x 4.6 mm id) pre-column. Both an Hitachi model F-1050 single condition (ie non-programmable) fluorescence detector and a Shimadzu model RF-551 programmable fluorescence detector were available for detection of eluted aromatics. Chromatograms were recorded on a Shimadzu Chromatopak CR3-A recorder. System *II* was a Shimadzu LC-10A equipped with an RF-10A PFD and a SPD-10A UV-visible detector. Solvent gradient and column were as stated above.

Environmental PAH Samples

Soil samples were taken from a sewage treatment farm (Werribee, Victoria, Australia) at depths from 10 cm to 15 cm. Soils were dried, homogenised and 20-30 g lots then extracted with dichloromethane. The extract was passed through a florisil column and the collected aromatic fraction concentrated using a Kuderna-Danish apparatus. The fraction was then reconstituted with acetonitrile and analysed by HPLC. Details of this procedure will be reported in a later paper.

Fresh water samples were taken from a main urban river (Yarra R., Victoria, Australia). They were collected on an adsorbent material which has been found to be selective for polyaromatic hydrocarbons. The adsorbed compounds were extracted from the washed and dried material with two lots of 50 mL methanol:concentrated ammonia (50:1). The solvent was changed to acetonitrile and the extract concentrated to 1 mL as above. This solution was taken for HPLC analysis. Details of this procedure will also be reported in a later paper.

RESULTS AND DISCUSSION

Initial Wavelength Selection

Excitation and emission spectra were obtained for the 16 PAH under investigation using the Hitachi F-4010 spectrophotometer in order to determine the ideal excitation and emission wavelengths for each PAH. The peak maxima found for each compound are given in Table I.

Using only SFD with say, the ideal conditions for fluorene, clearly gave poor sensitivity for the PAHs (Figure 2a), while ideal conditions for benz[a]anthracene give little useable data on the more important larger aromatics and the baseline also drifts (Figure 2b). Likewise, more general conditions (λ_{ex} = 290 nm and λ_{em} = 380 nm) were also unsatisfactory, with low sensitivity (Figure 2c). Clearly, confidence in quantitation will be low when the PAHs give such low detector responses and elute on such large sloping baselines.

On the basis of the Hitachi F-4010 information, a timed wavelength program was devised for the PFD to automatically switch wavelengths and autozero the detector at certain defined positions in the chromatogram. Table II shows the initial program used in

TABLE I
Excitation and Emission Peak Maxima for PAHs

COMPOUND NAME	NO.	LITERATURE VALUES [11] [b]	F-4010 [a]	RF - 551 [a]
NAPHTHALENE	1	220 278, 326 337	276, 336	219 269, 327
ACENAPTHENE	2	234 296, 326 342	292, 340	277, 319
FLUORENE	3	265 296, 316	261, 315	246, 300
PHENANTHRENE	4	259 290,354 370 390	250, 366	246, 373
ANTHRACENE	5	252 360 381, 385 405 430	251, 401	249, 405 302
FLUORANTHENE	6	284 358, 460	286, 459	229 279, 455
PYRENE	7	246 276 336, 379 398	335, 397	263 330, 369 385
BENZ[a]ANTHRACENE	8	286 340, 390 412 436	287, 388	277, 382 403
CHRYSENE	9	268 318, 364 384 405	267, 382	262, 375 358 395
BENZO[b]FLUORANTHENE	10	298 352, 443	300, 440	286, 433
BENZO[k]FLUORANTHENE	11	254 308 381 399, 415 438	307, 411	296, 404 426
BENZO[a]PYRENE	12	268 296 378, 406 432 460	384, 406	258 283, 380 423
BENZO[g,h,i]PERYLENE	13	299 368 385, 412 422 434	383, 408	287 360 378, 405
DIBENZ[a,h]ANTHRACENE	14	296 350, 400 424 448	297, 396	287, 390 412 436
INDENO[1,2,3-cd]PYRENE	15	255 308 371, 478 504	302, 505	258 293 359, 473 498

NOTE: All wavelength values in nanometers. Excitation and emission values are separated by commas. The strongest band is underlined where appropriate.
[a] Spectra obtained in 100% acetonitrile. [b] Spectra obtained in 1:1 acetonitrile:water.

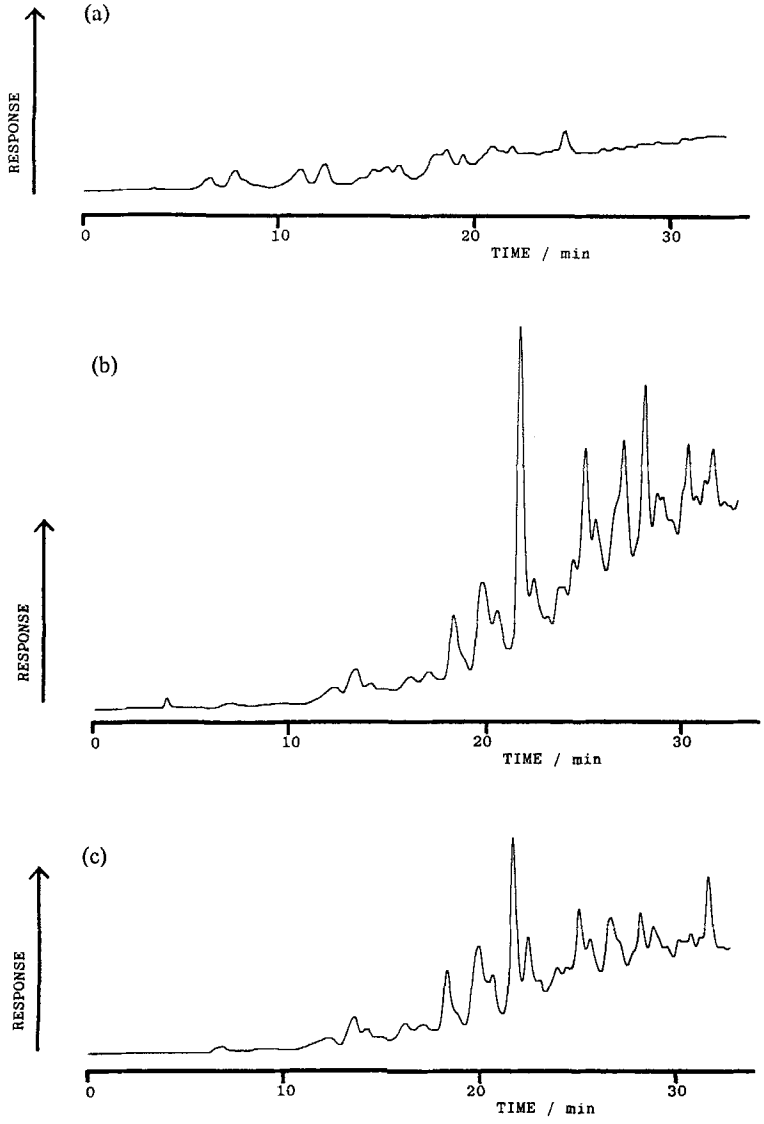

FIGURE 2. Chromatograms of a PAH extract of a soil sample using HPLC System I and the RF-551 detector at attenuation 4 and under the following single wavelength conditions:

(a) λ_{ex} = 260nm and λ_{em} = 315nm,
(b) λ_{ex} = 287nm and λ_{em} = 388nm and
(c) λ_{ex} = 290nm and λ_{em} = 380nm.

TABLE II
Initial and Modified Wavelength Programmes

PAH NUMBER	GROUP	WAVELENGTH PROGRAM (nm) INITIAL		MODIFIED		SENSITIVITY CHANGE (%)
		Ex	Em	Ex	Em	
1	A	276	336	269	327	+19
2	B	270	330	250	310	+65
3						+65
4	C	250	383	250	375	+73
5						+23
6	D	270	410	270	410	-
7						-
8	E	268	385	265	380	-18
9						+57
10	F	300	435	280	430	+55
11						-17
12						+174
13	G	298	422	287	412	+185
14						+40
15	H	308	504	293	498	+70

this study and the groups of compounds which we have chosen to analyse under common compromise conditions. The following criteria were used to decide these conditions:

(a) the elution characteristics of each PAH,

(b) the likely abundance of each PAH in an environmental sample,

(c) the relative detection sensitivity of each PAH compound, and

(d) the ease with which a timed event can be precisely included within the elution profile of typical samples.

Ideally, for the 16 components, we would use the 16 ideal excitation and emission wavelengths determined using the F-4010 spectrophotometer (actually 15, since acenaphthylene is only very weakly fluorescent). However, some PFDs do not allow this many timed events, with some restricted to 6 or 8 (some include autozero). We initially thought this restriction would apply to the RF-551, but control of the detector by an external computer allows more changes than permitted by the detector alone; a program was written for this purpose (but was eventually not required). Criteria (a) and (d) determine the number of programmed changes which can be accommodated during the chromatogram. Components need to be satisfactorily resolved in order to allow precise and reproducible changes at the same position in each chromatogram. For instance, if two components are resolved with a resolution of 1.5 (ie just baseline resolved) then the ability to quantitate the peaks will be affected by retention time variability. It is not uncommon in premixed solvent systems, as used in our work, that variation in mixture composition from day to day will lead to retention time variation. Temperature fluctuations can also lead to retention time changes, of the order of 10-15 sec, larger than the allowable tolerance for a timed change between some peaks. If increased resolution of peaks is not possible, then it is necessary to use the one set of excitation and emission conditions for that peak group. Criteria (b) and (c) above need to be considered when selecting the compromise conditions for a group. If one of the peaks is of large abundance, and has good fluorescence sensitivity, but the other component is of low abundance, then fluorescence conditions could be chosen so as to favour the low abundance component. Another factor to consider is the relative importance of each group component to the end user of the analytical data. A compilation of approximate relative sensitivities and abundances taken from a number of literature references, is given in Table III.

As an example of the strategy used for selection of compromise conditions, consider first phenanthrene and anthracene (group C, Table II). With phenanthrene having low sensitivity and anthracene high, some of the sensitivity of the anthracene component was

TABLE III
Comparison of Approximate Abundances and Sensitivities of PAHs

COMPOUND	RELATIVE ABUNDANCE[a]	RELATIVE SENSITIVITY[b]	LITERATURE DATA[14,c] diesel	St Louis air
Naphthalene	med	low		
Acenaphthene	low	high		
& Fluorene				
Phenanthrene	med	low	high	med
Anthracene	low	high	low	low
Fluoranthene	high	low	high	high
Pyrene	high	low	high	high
Benz[a]anthracene	med	med	low	med
Chrysene	med	med	high	high
Benzo[b]fluoranthene	med	low		
Benzo[k]fluoranthene	low	med	low	med
Benzo[a]pyrene	med	high	low	med
Dibenz[a,h]anthracene	med	med		
Benzo[g,h,i]perylene	med	low	med	
Indeno[1,2,3-cd]pyrene	med	low	med	med

a Typical soil abundances found in this study.
b Sensitivities measured at peak maxima wavelengths.
c Approximate abundances.

sacrificed by choosing a compromise condition that favoured phenanthrene. This approach was still practical even though the abundance of anthracene in samples tended to be lower than that of phenanthrene (by about a factor of 10). The initial compromise conditions chosen were λ_{ex} = 250 nm and λ_{em} = 383 nm, with phenanthrene giving about 90 % of the response obtained under its ideal conditions (λ_{ex} = 246 nm and λ_{em} = 373 nm) whereas anthracene gave only 80 % of the response obtained using its ideal conditions (λ_{ex} = 249 nm and λ_{em} = 405 nm). For benz[a]anthracene and chrysene (group E), both components have similar sensitivities and abundances in our samples. Given that the excitation and emission values for the two were similar, we opted to select wavelengths approximately midway between each so that the responses of both compounds were compromised to a similar extent. Finally, group F, which consisted of benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene. The high sensitivity of benzo[k]fluoranthene often resulted in it being the largest peak in this group. Even choosing optimum conditions for one of the other compounds still resulted in a predominance of this peak. The low sensitivity of benzo[b]fluoranthene meant that optimum group conditions were chosen which closely corresponded to those of benzo[b]fluoranthene. If there was a specific need to analyse benzo[a]pyrene with maximum sensitivity, then the program could have been adjusted accordingly. Under the compromise conditions for this group benzo[b]fluoranthene response was 98 % of its ideal conditions response, with benzo[k]fluoranthene 57% of its ideal, and that for benzo[a]pyrene almost 70%.

Figure 3 shows a typical chromatogram of a PAH standard mixture. Whilst acenaphthene and fluorene coelute, it can be seen that fluoranthene and pyrene also eluted closely together. This was also the case for benz[a]anthracene and chrysene. Therefore it was impractical to impose wavelength changes between these components.

Modified Wavelength Program

Given that the initial conditions outlined above were selected on the basis of the Hitachi F-4010 data, and that most of the compounds were detected under compromise conditions, it was of interest to determine by how much their responses changed when the excitation and emission wavelength settings on the RF-551 detector were systematically varied. Accordingly, individual standard solutions of each PAH (10 ppb) were run under the ideal settings determined with the Hitachi F-4010 and then under the compromise

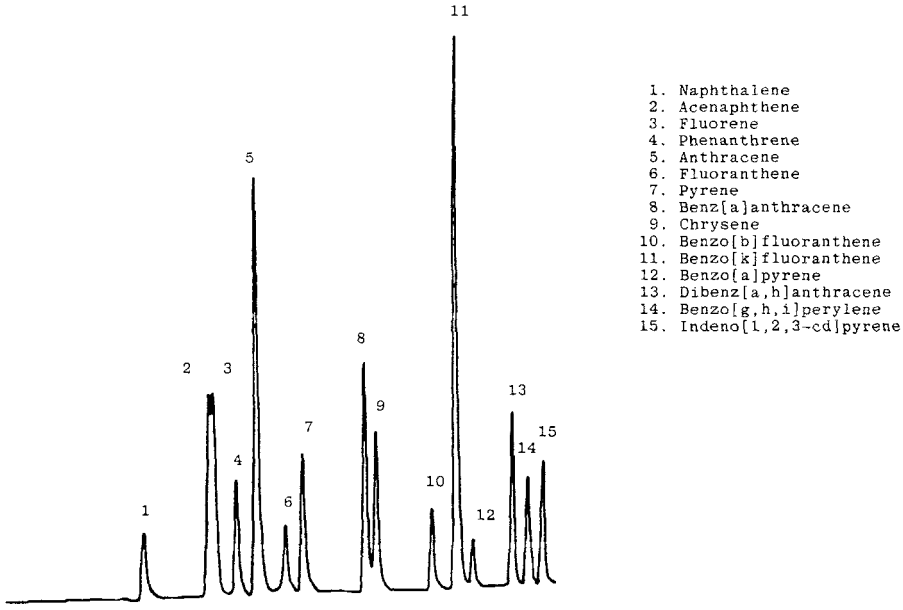

FIGURE 3. Typical chromatogram of a standard PAH mixture (50 ppb each component) obtained using HPLC System I and the initial PFD program with the RF-551 detector (Table II).

conditions. It was found in some cases that the compromise conditions in fact gave better sensitivity than the 'ideal' settings determined earlier (Table I). This meant that the Hitachi data were not directly transportable to the RF-551; this might have been due to solvent composition differences, or differences in the wavelength accuracy of the instruments, even though the wavelength calibrations for both instruments were within specifications.

The 'ideal' RF-551 wavelength settings for each PAH were determined by running excitation and emission spectra under no-flow conditions with each solute in the detector cell; these were then rechecked in separate chromatographic runs. There was no significant difference between the no-flow and chromatographic spectra, despite differences in solvent composition. However, the excitation and emission maxima obtained with the RF-551 differed from the F-4010 in wavelength settings by up to 20 nm in some cases (Table I) and the responses at the new 'ideal' settings were as much as 200% greater. This increased sensitivity allowed more flexibility in selecting compromise

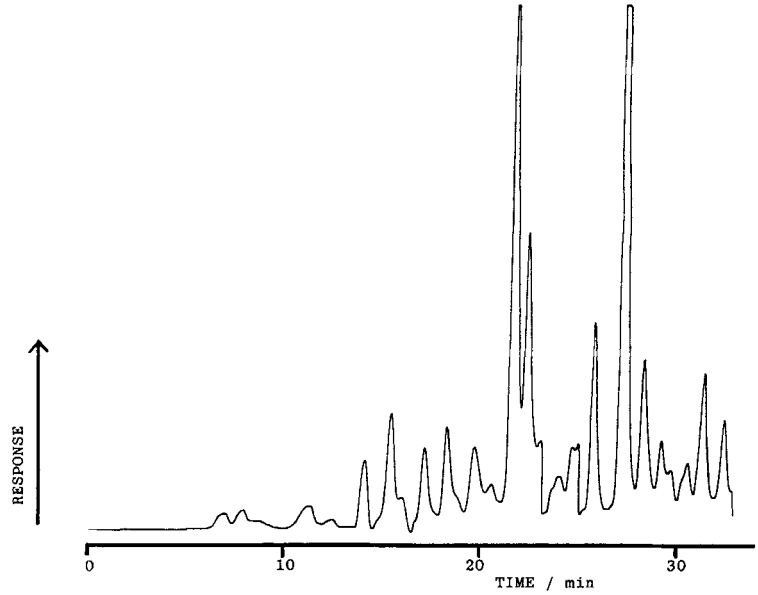

FIGURE 4. Chromatogram of the same soil extract as in Figure 2, but obtained by using HPLC System I and the modified PFD program with the RF-551 detector at attenuation 5.

wavelength settings for the PAH groups. The modified program, and the resultant changes in signal responses, are given in Table II, while Figure 1 shows the time relationship between the modified wavelength program and the solvent gradient. There was a general improvement in sensitivity for the PAHs, with the exception of benzo[k]fluoranthene (group F), which had a reduction of 17% in sensitivity, while the other two PAH in this group, benzo[b]fluoranthene and benzo[a]pyrene, had their relatively low sensitivities increased by 55 and 174% respectively (Table II). As explained earlier, these changes in sensitivity were advantageous to the determination of the two minor PAH in this group.

The advantage of PFD over SFD for the determination of a range of PAH is illustrated in Figure 4, where the modified PFD program was applied to analysing the same soil sample used earlier (Figure 2). It can be appreciated that there was greater confidence in the data obtained with better sensitivity.

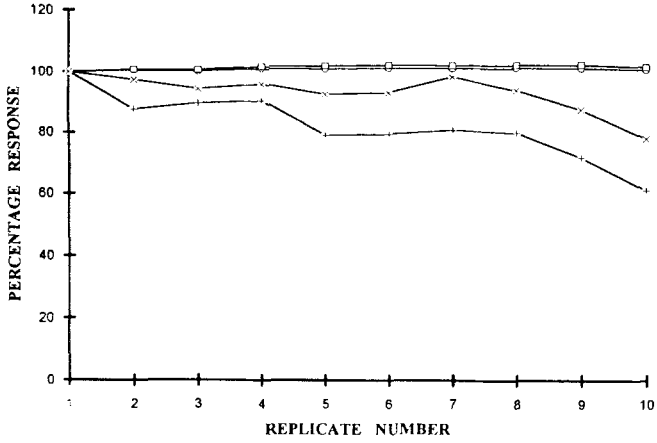

FIGURE 5. Variation in RF-551 and RF-10A detector responses with 10 replicate injections of a standard PAH mixture. The two detectors were operated in series and under the same modified program settings with HPLC System II.

dibenzo(a,h)anthracene; RF-551 ---□--- and RF-10AV ——+——
benzo(g,h,i)perylene; RF-551 ---O--- and RF-10AV ——x——

Detector Reproducibility

More recently we have used the Shimadzu LC-10A system (System II), equipped with a RF-10A fluorescence detector and the SPD-10AV UV-visible detector. The RF-10A is mechanically and optically the same as the RF-551, and we have found that their performances are comparable when running the modified wavelength program determined on the RF-551, allowing for appropriate wavelength calibrations.

In order to establish the reproducibility of this system, 10 replicate injections of a standard PAH mixture were made, with the RF-10A and SPD-10AV detectors in series. The precision for each of the PAH are listed in Table IV. Whilst the SPD-10AV gave RSDs of the order of 1-3%, the RF-10A gave poorer reproducibility, with values up to almost 20%, and with no logical trend in the variation of data. We then replaced the RF-10A with the RF-551, and the RSD figures for this detector ranged between 0-1%. (Note that these data were not corrected using internal standards.) The clear drift in RF-10A response is shown in Figure 5, where peak area data for both fluorescence detectors operated in series are plotted for dibenz[a,h]anthracene and benzo[g,h,i]perylene. The RF-

TABLE IV
Reproducibility (%RSD) of Detector Response For Ten Replicate Injections of PAH Standard Mixture

COMPOUND	DETECTOR			
	RF-551	RF-10A		SPD-10AV
		a	b	
Naphthalene	1.3	2.8	ND	1.5
Acenaphthene	ND	7.6	ND	ND
Fluorene	0.2	9.9	ND	1.2
Phenanthrene	1.0	3.6	1.8	1.2
Anthracene	0.4	5.5	0.6	1.1
Fluoranthene	1.6	9.4	1.5	2.8
Pyrene	3.3	2.2	1.8	1.3
Benz[a]anthracene	0.7	5.5	0.7	1.1
Chrysene	0.5	7.8	1.3	1.5
Benzo[b]fluoranthene	0.8	7.1	0.6	1.4
Benzo[k]fluoranthene	0.3	1.7	0.4	1.2
Benzo[a]pyrene	1.1	17.5	0.7	1.6
Dibenz[a,h]anthracene	0.5	13.1	0.7	2.4
Benzo[g,h,i]perylene	0.9	6.8	1.1	1.5
Indeno[1,2,3-cd]pyrene	ND	ND	ND	1.3

ND - Not Determined
Data for the RF-10A [a] prior to correction of fault [b] after fault remedied

551 and SPD-10AV results clearly demonstrate the excellent reproducibility of the LC-10A system. The variability in the RF-10A results was surprising given the instrument's similarity to the RF-551. We found that the poor reproducibility was caused by run-to-run variations in the wavelengths set by the detector, which only occurred when the detector was under external program control. After consultation, the manufacturers quickly traced the problem to a software error, which was readily resolved and the RF-10A now performs well (Table IV).

It is important to recognise that without a conscious check on the performance of the detector as *part* of the LC-10A system, this problem might not have been found. This

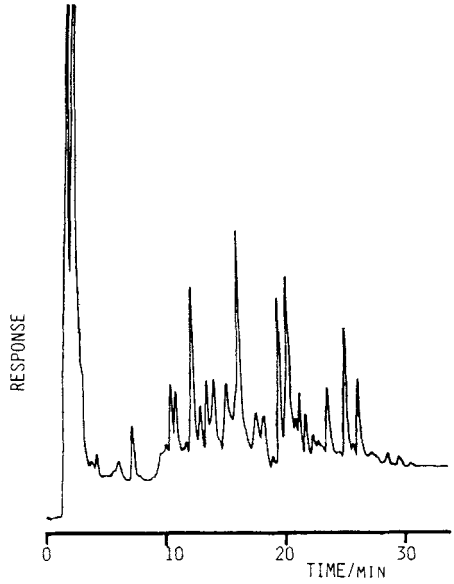

FIGURE 6. Chromatogram for a solid phase extraction of PAHs from a river water sample using HPLC System I and the modified PFD program with the RF-551 detector.

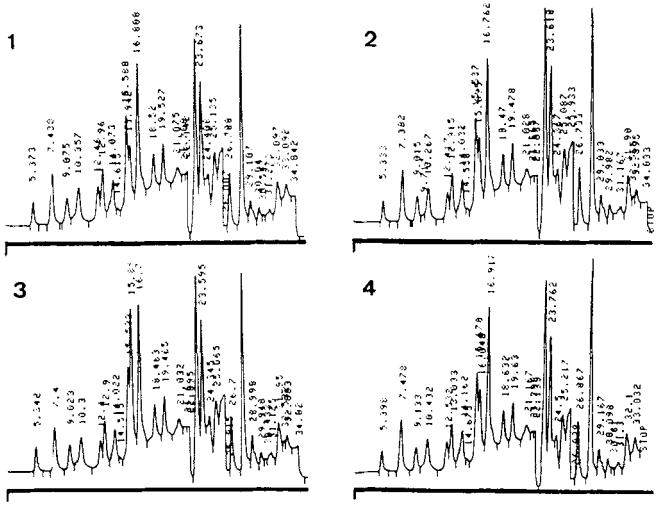

FIGURE 7. Chromatograms of four replicate extracts of a homogenised soil sample obtained using HPLC System I and the modified PFD program with the RF-551 detector.

TABLE V
Reproducibility Data for Four Extractions of a Soil Sample

PAH	MEAN [a] (ppb)	S.D. (ppb)	R.S.D (%)
1	27.5	2.5	8.9
2,3	8.1	0.9	11.0
4	38.0	2.2	5.8
5	10.6	0.8	7.3
6	179	9.7	5.4
7	80.9	3.8	4.7
8	23.7	1.7	7.3
9	22.6	0.4	1.6
10	20.1	2.8	14.1
11	9.1	0.6	6.5
12	30.6	0.7	2.4
13	18.5	2.4	13.1
14	31.4	1.2	3.8
15	33.7	0.7	2.0

[a] Mean concentration of PAH expressed as ppb dry mass

highlights the necessity of using a protocol that ensures data of the highest reliability is obtained.

In addition, compromise wavelength programs use wavelength settings that do not correspond to excitation or emission maxima, but to regions where the gradient of the spectra could be large, i.e. on shoulders of bands. Consequently, small changes in wavelength can cause large increases or decreases in sensitivity. With this in mind, the precision obtained with the programmable fluorescence detectors was noteworthy.

Applications of the Method

The application of PFD to the analysis of water and soil extracts is illustrated in Figures 6 and 7 respectively. In both cases the modified PFD conditions were able to be used to satisfactorily determine PAH concentrations. The chromatograms suggest that if PFD was not used, the quality of the data would be seriously compromised because of the

loss in sensitivity. The reproducibility of the method for soil analyses was determined by analysing four replicate sub samples of a soil. In general, RSDs were in the range 2 - 10 % (Table V).

CONCLUSIONS

Programmed fluorescence detection is a valuable tool for the determination of PAHs in environmental samples. It is necessary to optimise the detection system to fully exploit its capabilities. Wavelength settings previously reported in the literature, or measured on other equipment, might not give the best results for the suite of compounds under investigation. It might also be necessary to further modify the conditions according to the relative concentrations and sensitivities of the components in the samples. When complete resolution of components is not achieved, a compromise set of conditions must be used for these components. The ability of the HPLC system (including detector) to provide precise data must be ascertained *as a whole*. Misleading information might be obtained if system components are tested in isolation.

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