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QUALITATIVE IDENTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY AND FLUORESCENCE SCANNING DENSITOMETRY

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SUMMARY

A procedure is described for the accurate measurement of emission response ratios for polycyclic aromatic hydrocarbons using fluorescence scanning densitometry and reversed-phase high-performance thin-layer chromatography. By optimizing instrumental conditions and using perylene as a reference standard, the emission response ratios can be reproduced to better than 3% relative standard deviation on a day-to-day basis. The emission response ratios enable the optimum detection conditions for sensitivity or selectivity to be easily identified. The normalized emission response ratios can be used to improve the confidence in the identification of a particular compound made by coincidence of retention in the chromatographic system. The method has been applied to the identification of polycyclic aromatic hydrocarbons in samples of laboratory dust and a diesel engine exhaust particulate sample.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants often present as a complex mixture. As several of the PAHs are known or suspect mutagens and carcinogens, methods are required for their environmental monitoring. Capillary column gas chromatography (GC) or high-performance liquid chromatography (HPLC) with fluorescence detection are the methods most frequently used[1]. Most of these methods require extensive sample cleanup resulting in fairly lengthy analysis times. Also, being serial sample methods, the sample throughput is low. The ability of thin-layer chromatography (TLC), to simultaneously separate multiple samples has been one of the primary reasons for adopting this technique for screening environmental samples for PAHs[2–4]. Furthermore, unlike column systems, the TLC plate is a disposable item and, therefore, relatively crude sample extracts can be analyzed without problems caused by non-eluted organic or inorganic components which are troublesome in HPLC and GC. However, TLC lacks the separating power of either GC or HPLC and quantitation can also be more problematical.

Recent improvements in the materials, practice, and quantitation methods used in TLC, generally known as high-performance thin-layer chromatography

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(HPTLC), have done much to regenerate interest in this technique[5-8]. Increased separating power, shorter analysis time, and greater sensitivity are the advantages that HPTLC has over TLC while maintaining the traditional advantages of TLC, namely, methodological simplicity, simultaneous sample analysis, and static sample detection. Static detection is important as it facilitates selection of optimum detection conditions for all sample components and allows qualitative identification of sample components by substance-specific response ratios[9,10]. In this paper sequential wavelength scanning is used to develop substance-specific emission response ratios suitable for the qualitative identification of individual PAHs. The emission response ratios are also useful for identifying conditions where wavelength discrimination can be used to quantify PAHs in spots containing unseparated components.

EXPERIMENTAL

Anthracene (Ant), benzo[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*e*]pyrene (BeP), benzo[*g,h,i*]perylene (BPer), chrysene (Chr), coronene (Cor), dibenzo[*a,h*]anthracene, (DBahAnt), fluoranthene (Flt), fluorene (Flu), perylene (Per), phenanthrene (Phen), pyrene (Pyr) and triphenylene (Tri) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Benzo[*b*]fluoranthene (BbFlt), benzo[*j*]fluoranthene (BjFlt), benzo[*k*]fluoranthene (BkFlt) and indeno[1,2,3-*c,d*]pyrene (IncdPyr) were obtained from the Community Bureau of Reference (Brussels, Belgium) and dibenzo[*a,i*]pyrene (DBaiPyr) from Sigma (St. Louis, MO, U.S.A.). All solvents were HPLC grade (Burdick & Jackson, Muskegon, MI, U.S.A.). Separations were carried out on 10 cm × 10 cm KC18 reversed-phase HPTLC plates from Whatman (Clifton, NJ, U.S.A.).

Sample volumes of 200 nl were applied to the plates using fixed volume Pt-Ir dosimeters (Applied Analytical Industries, Wilmington, NC, U.S.A.) in conjunction with a Nanomat HPTLC spotter (Camag, Muttenz, Switzerland). The plates were developed in a short-bed continuous development chamber (Regis, Morton Grove, IL, U.S.A.).

In situ sample detection was performed with a Shimadzu CS-910 scanning densitometer (Shimadzu, Columbia, MD, U.S.A.) using a 100-watt high-pressure mercury source for excitation, a medium bandpass monochromator to select the excitation wavelength, and interference filters to isolate the fluorescence emission wavelengths from the excitation background. The maximum transmittance of the 400, 450, 500, 550 and 600 nm interference filters is 15, 33, 32, 42 and 43%, respectively. A broad-band filter (UV-D2) having a transmittance envelope from 300 to 400 nm centered at 350 nm with a peak transmittance of 78% was also used. Since the densitometer has no provision for reproducibly setting the gain of the PMT detector in the fluorescence mode the reference plate calibration method was used previously in this laboratory[11]. This procedure has been abandoned in favor of setting the PMT voltage directly. This requires that the auto feedback control for the PMT voltage be disabled and the PMT voltage measured on a digital multimeter by tapping into the PMT voltage supply via a diagnostic socket at the rear of the instrument. The "H.V." control can then be used to set the PMT voltage to any desired value. Peak profiles were recorded on a Shimadzu U-135 strip chart recorder and peak areas with either a Spectra Physics minigrator or SP4100 computing integrator (Spectra Physics, Santa Clara, U.S.A).

TABLE II

EMISSION RESPONSE RATIOS AT 266 nm EXCITATION

Compound	*Emission wavelength (nm)*					
	*350**	*400*	*450*	*500*	*550*	*600*
Per	–	2	100	41	7	–
Flt	–	1	12	12	6	1
Pyr	222	18	3	–	–	–
BaA	133	25	12	1	–	–
Tri	409	6	–	–	–	–
Chr	584	21	3	–	–	–
BkFlt	–	43	100	27	4	–
BbFlt	–	16	76	52	19	3
BaP	–	66	71	10	–	–
BeP	51	8	2	–	–	–
BjFlt	–	–	–	4	4	2
BPer	–	9	14	2	–	–
Cor	–	–	2	–	–	–

* UV-D2 filter.

TABLE III

EMISSION RESPONSE RATIOS AT 313 nm EXCITATION

Compound	*Emission wavelength (nm)*				
	400	*450*	*500*	*550*	*600*
Per	–	100	42	8	–
Flu	–	–	–	–	–
Phen	1	–	–	–	–
Ant	47	20	–	–	–
Flt	8	94	95	41	–
Pyr	273	45	4	–	–
BaA	91	43	4	–	–
Tri	–	–	–	–	–
Chr	69	9	–	–	–
BkFlt	800	1847	493	82	6
BbFlt	54	261	180	66	11
BaP	92	101	15	1	–
BeP	112	30	2	–	–
BjFlt	–	3	108	137	51
BPer	54	108	18	4	–
IncdPyr	–	36	522	433	121
DBahAnt	398	209	24	–	–
Cor	5	192	42	4	–
DBaiPyr	79	924	523	103	–

TABLE IV

EMISSION RESPONSE RATIOS AT 365 nm EXCITATION

Compound	*Emission wavelength (nm)*				
	400	*450*	*500*	*550*	*600*
Per	3	100	42	8	–
Flu	–	–	–	–	–
Phen	–	–	–	–	–
Ant	15	7	2	–	–
Flt	2	20	21	9	2
Pyr	1	–	–	–	–
BaA	11	5	–	–	–
Tri	–	–	–	–	–
Chr	–	–	–	–	–
BkFlt	30	76	20	3	–
BbFlt	12	49	33	12	2
BaP	92	101	15	1	–
BeP	–	–	–	–	–
BjFlt	–	–	6	7	3
BPer	29	54	9	1	–
IncdPyr	–	–	54	45	13
DBahAnt	–	–	–	–	–
Cor	–	–	–	–	–
DBaiPyr	5	126	71	12	1

The emission response ratio values measured on reversed-phase plates at 254, 266, 313 and 365 nm excitation are given in Tables I–IV. For visual comparison the raw data are plotted in three-dimensional form in Figs. 1–4. This allows the optimal emission wavelength for detection at any particular excitation wavelength to be easily found. It also provides a convenient means of selecting combinations of excitation and emission wavelengths to maximize discrimination in the detection of specific PAHs in unresolved spots. The product obtained by dividing any combination of the emission response ratios by each other at a fixed excitation wavelength provides a substance-specific ratio suitable for qualitative identification.

A blank value in Tables I–IV indicates that no signal was observed for a 10-ng sample. A larger sample size may or may not lead to a signal being detected. Virtually all the PAHs can be detected at all combinations of excitation and emission wavelengths if their concentration is very high. The sample size of 10 ng was selected to minimize this effect as far as possible and to provide the widest characteristic response range consistant with remaining within the linear response range of the densitometer.

The complete separation of the PAHs given in Table I can not be achieved by reversed-phase TLC using conventional development. The multiple development technique, which takes advantage of the spot reconcentration mechanism, must be used to improve sample resolution[10,17–19]. The substance-characteristic R_F value has no meaning in multiple development and is replaced by the absolute migration distance of the spot. The reproducibility of the spot migration distance is generally inadequate for identification purposes unless samples and standards are separated simultaneously. In this way variations in the experimental conditions are adequately

Emission response ratios

To determine the emission response ratios, nominal sample amounts of 10 ng were spotted 1.0 cm apart and 0.5 cm from the bottom of the plate. The samples were developed for 5 min at position 4 in a short-bed continuous development chamber using methanol–dichloromethane–water (20:3:3) as the mobile phase. After development the plates were dried with a gentle stream of nitrogen. Prior to measurement the mercury arc source was allowed to stabilize for 45 min. The slit height and slit width were 6.0 mm and 0.50 mm, respectively. The PMT voltage was set to -600 volts and the scan rate was 24 mm min^{-1}. In order to eliminate variations in setting the excitation wavelength, the compounds were first scanned at all emission wavelengths. The next excitation wavelength was then set and the process repeated until all combinations were recorded.

Extraction of environmental samples

The diesel exhaust particulate sample (Illinois Institute of Technology Research Institute, Chicago, IL, U.S.A.) was collected and extracted according to a standard protocol described elsewhere[12]. The extract contained 70.5 mg ml^{-1} of the organic soluble fraction from the particulate phase dissolved in hexane. A laboratory dust sample was collected from the particle filter used to purify the air supplied to the laboratory. The dust sample (11.9605 g) was extracted with 100 ml of toluene in a Soxhlet extractor for 24 h at a rate of *ca.* 5 cycles h^{-1}. The extract was reduced to dryness on a rotary evaporator and redissolved in 2 ml of *n*-hexane. Both particulate extracts were further fractionated by partition with dimethyl sulfoxide as described by Natusch and Tomkins[13]. The final extracts were dissolved in 1.0 ml of acetone, and 200 nl of this solution were spotted onto the HPTLC plates. A standard mixture of PAHs was spotted alongside the extract to facilitate the measurement of migration distances.

RESULTS AND DISCUSSION

The scanning densitometer used in these studies employs a medium bandpass monochromator to isolate the excitation wavelength and series of interference filters to separate the emission signal from the source of excitation. Play in the monochromator drive prevents accurate resetting of the excitation wavelength on a day-to-day basis. This does not affect the emission response ratios but excludes the use of excitation response ratios for sample identification. Emission response ratios can be reproduced to better than 3% relative standard deviation (R.S.D.) on a day-to-day basis while excitation response ratios showed a general variation greater than 50% R.S.D. To reproduce the emission response ratios it is important that the densitometer is tuned to constant sensitivity as described elsewhere[11,14,15]. Also, the emission response ratios are not independent of the stationary phase. On silica gel plates the relative intensity and position of wavelength maxima may differ substantially from those presented here for reversed-phase plates[16]. Batch-to-batch variations in the properties of reversed-phase plates did not have a significant affect on the reproducibility of the emission response ratios.

The method proposed for calculating emission response ratios uses a reference standard to account for minor changes in instrumental parameters on a day-to-day

basis. Also, provided that the amount of standard and sample remain within their linear response ranges, the emission response ratios are independent of sample amount. This range usually extends from the detection limit of 1–50 pg to the upper limit of the linear calibration curve (50–200 ng). Perylene was selected as the reference standard as it is readily available in high purity, does not decompose on the plate, and provides a suitable emission signal at all excitation wavelengths.

The emission intensity of perylene per nanogram at 450 nm was assigned a value of 100 at each excitation wavelength to provide a scale for comparison. This allows the emission response ratio to be formally defined by eqn. 1

$$\mathrm{ERR(ex, em)} = \frac{[\mathrm{PAH(ex, em)}]/[\mathrm{ng}]}{[\mathrm{Per(ex, em)}]/[\mathrm{ng}']} \times 100 \quad (1)$$

where

ERR(ex, em) = emission response ratio at a given excitation and emission wavelength

PAH(ex, em) = peak area of the test compound at the given excitation and emission wavelength

Per(ex, em) = peak area for perylene at the given excitation and reference emission wavelength

ng = nanograms of PAH

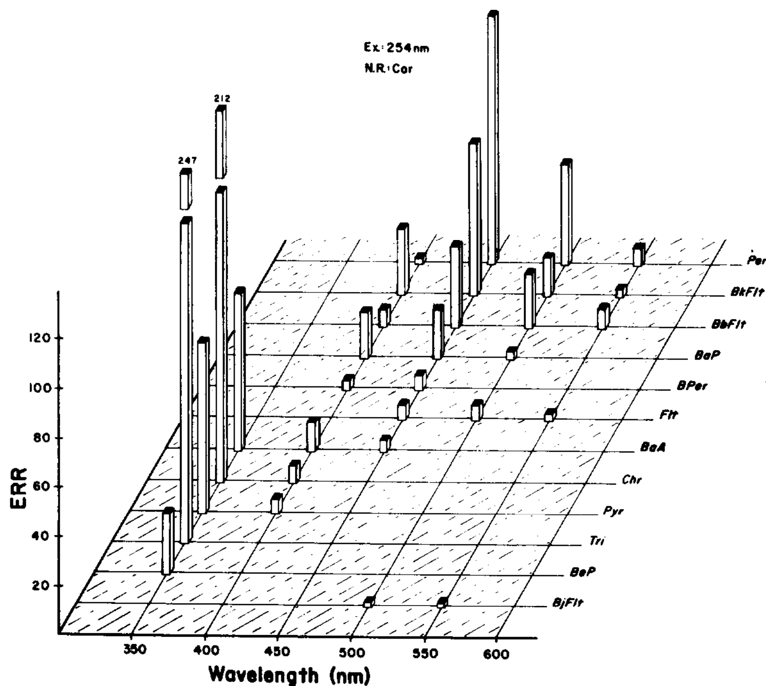
ng′ = nanograms of perylene

TABLE I

EMISSION RESPONSE RATIOS AT 254 nm EXCITATION

Compound	*Emission wavelength (nm)*					
	*350**	*400*	*450*	*500*	*550*	*600*
Per	–	2	100	41	7	–
Flu	119	–	–	–	–	–
Phen	56	4	–	–	–	–
Ant	84	57	9	–	–	–
Flt	–	–	6	6	3	–
Pyr	69	6	1	–	–	–
BaA	64	12	5	–	–	–
Tri	247	–	–	–	–	–
Chr	212	7	–	–	–	–
BkFlt	–	27	62	16	3	–
BbFlt	–	7	33	22	8	1
BaP	–	19	20	3	–	–
BeP	25	–	–	–	–	–
BjFlt	–	–	–	2	2	–
BPer	–	4	6	1	–	–
IncdPyr	–	–	–	19	15	4
DBahAnt	–	4	2	–	–	–
Cor	–	–	–	–	–	–
DBaiPyr	–	1	12	6	–	–

* UV-D2 filter.



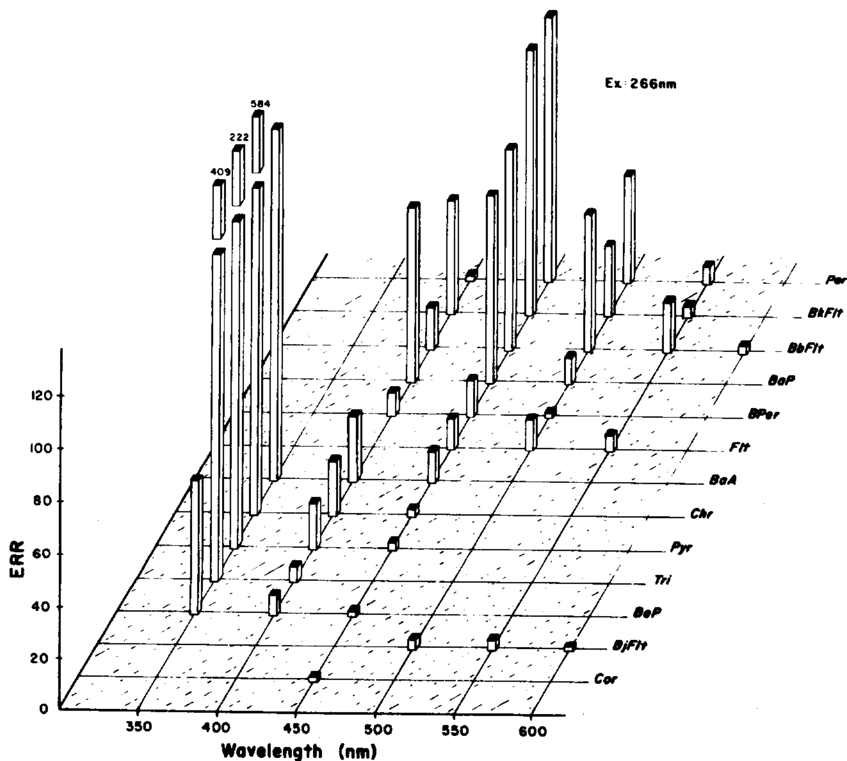


Fig. 1. Three-dimensional plot of emission response ratios at 254 nm excitation.

Fig. 2. Three-dimensional plot of emission response ratios at 266 nm excitation.

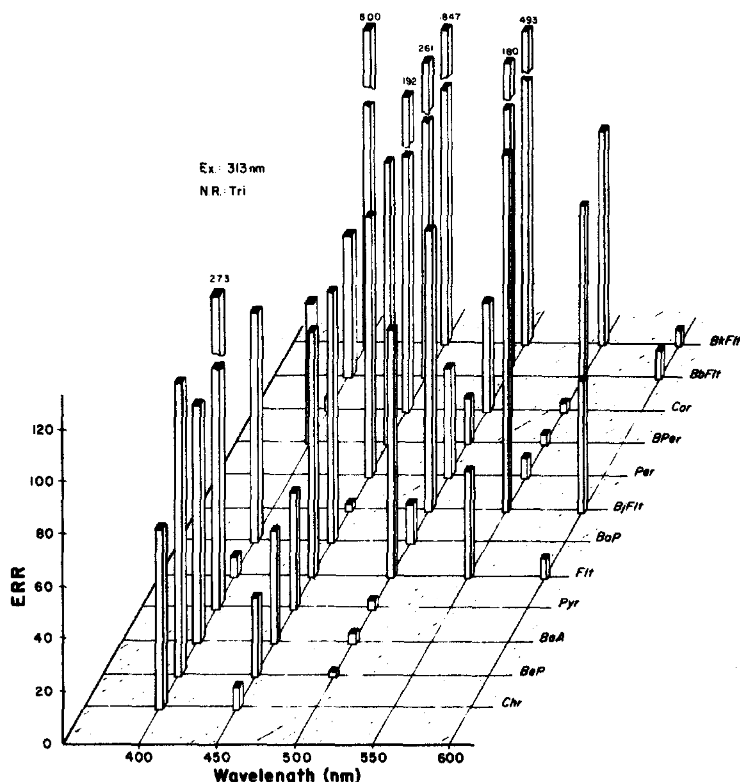


Fig. 3. Three-dimensional plot of emission response ratios at 313 nm excitation.

compensated for and reliable positional data for identification purposes obtained. A typical separation of a mixture of standards is shown in Fig. 5. Certain limitations of the separation system are obvious. Ant and Phen; IncdPyr and BPer; Per, BaP, BeP, BbFlt, BjFlt and BkFlt; and BaP and Chr are insufficiently resolved for identification purposes. Ant fluoresces at all emission wavelengths of Phen. However, Ant in the presence of Phen can be determined at 254 or 266 nm excitation and 450 nm emission where the response from Phen is very small. Likewise, IncdPyr can be determined at 313 nm excitation and 550 or 600 nm emission where the fluorescence intensity of BPer is very weak. The benzofluoranthene isomers are not separated chromatographically and can not be distinguished by wavelength selection. Owing to the overlap of emission wavelengths of Per with the benzofluoranthene isomers, there is no wavelength at which Per can be detected in the presence of significant amounts of the benzofluoranthene isomers. If the level of benzofluoranthene isomers is low compared with Per then the latter can be determined at 254 nm excitation and 550 nm emission. BeP fluoresces intensely in the UV and can be determined at 266 nm excitation and 350 (UV-D2) nm emission where interference from the benzofluoranthene isomers and Per is very low. BaP can be detected at 500 nm emission at all excitation wavelengths but will experience interference from the benzofluoranthene isomers and Per. The fluorescence intensity of Per is weak at this wavelength. It will

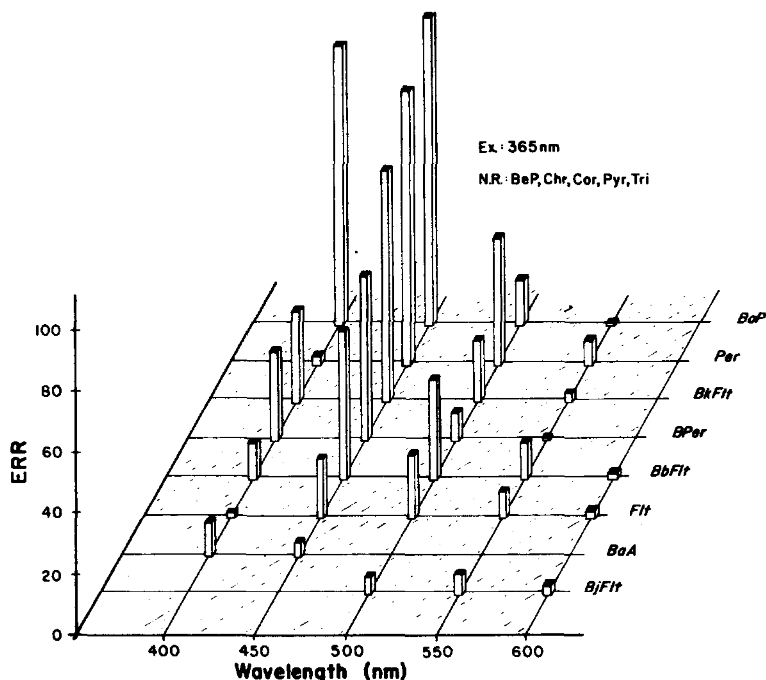


Fig. 4. Three-dimensional plot of emission response ratios at 365 nm excitation (NR = no response).

only be possible to identify BaP in samples containing relatively low concentrations of benzofluoranthene isomers. BaA can be determined at 365 nm excitation and 400 nm emission in the presence of Chr. Chr can not be determined in the presence of BaA except by difference using 266 nm excitation and 400 nm emission with subtraction of the contribution of BaA calculated from its response at 365 nm excitation and 400 nm emission.

The separation of PAHs in the extract from the laboratory dust sample is shown in Fig. 6. The peak identities were assigned based on the coincidence of migration measurements by HPTLC. The emission response ratio values were then applied to test the reliability of these assignments. Normalized values are convenient for this purpose. For example, the ratio of the emission intensity for Ant excited at 313 nm and measured at 400 and 450 nm is 1.0:0.43. The measured normalized value for Ant in the laboratory dust extract is 1.0:0.41 indicating an acceptable correlation and a reasonable expectation of a correct assignment. For Flt at 266 nm excitation the normalized emission response ratios at 450, 500, and 550 nm are 1:1:0.5. The equivalent values measured for the dust extract are 0.8:1:0.45 thus indicating the possibility of a contribution to the fluorescence signal from a component other than Flt. At 313 nm excitation the calculated normalized emission response ratios where 1:1:0.43 and the values for the dust extract 0.9:1:0.44. The selectivity of the detection system is greater at 313 nm excitation with emission measurements at 500 or 550 nm. These conditions should be used for the reliable determination of Flt. Pyr is clearly indicated at 266 nm excitation and 400 nm emission, however, the normalized emis-

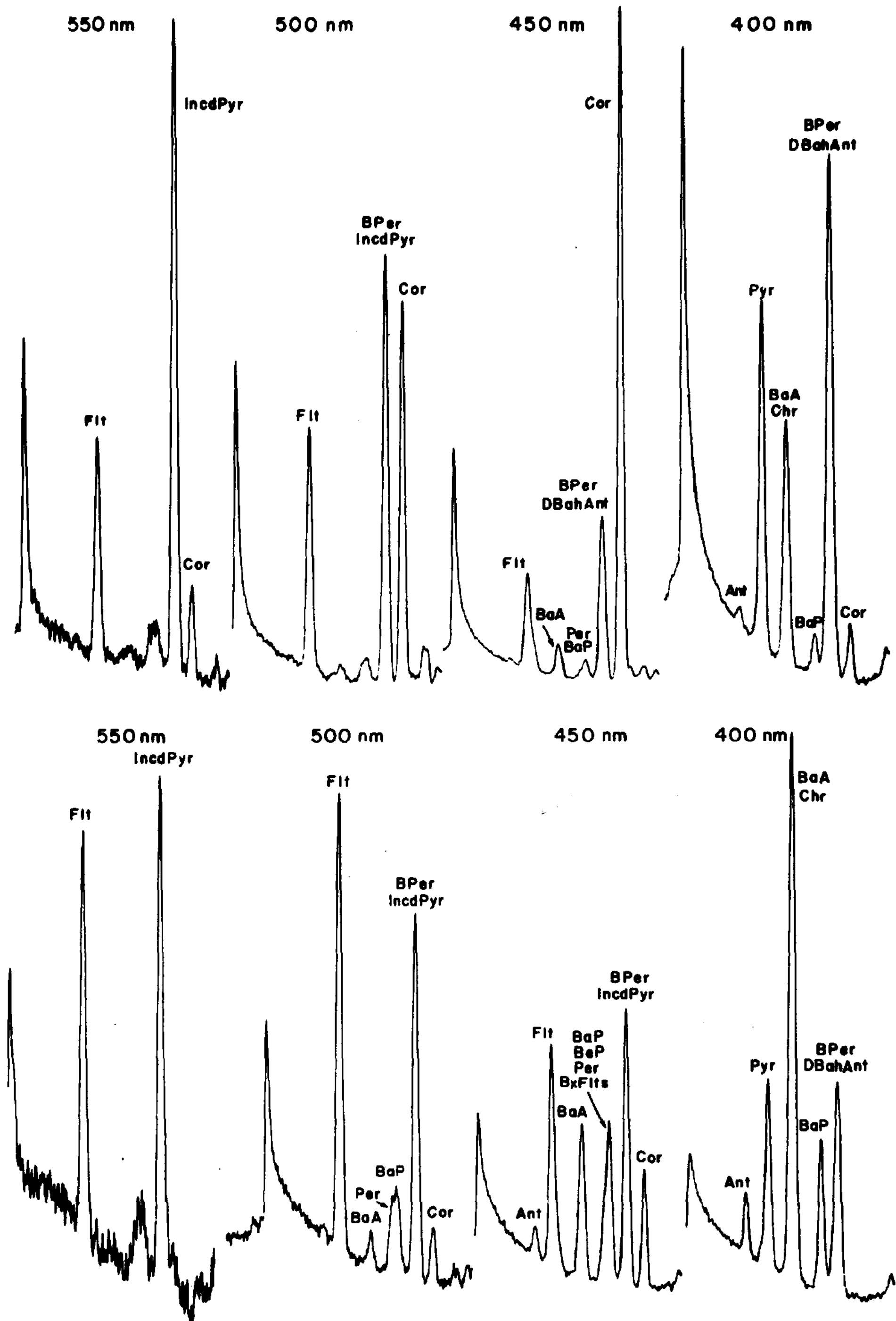


Fig. 5. Separation of a synthetic mixture of nineteen PAH standards by reversed-phase HPTLC using 10 × 10 min developments with methanol–water (20:6) as the mobile phase. The plate was scanned in the direction of development using an excitation wavelength of 313 nm (top) and 266 nm (bottom).

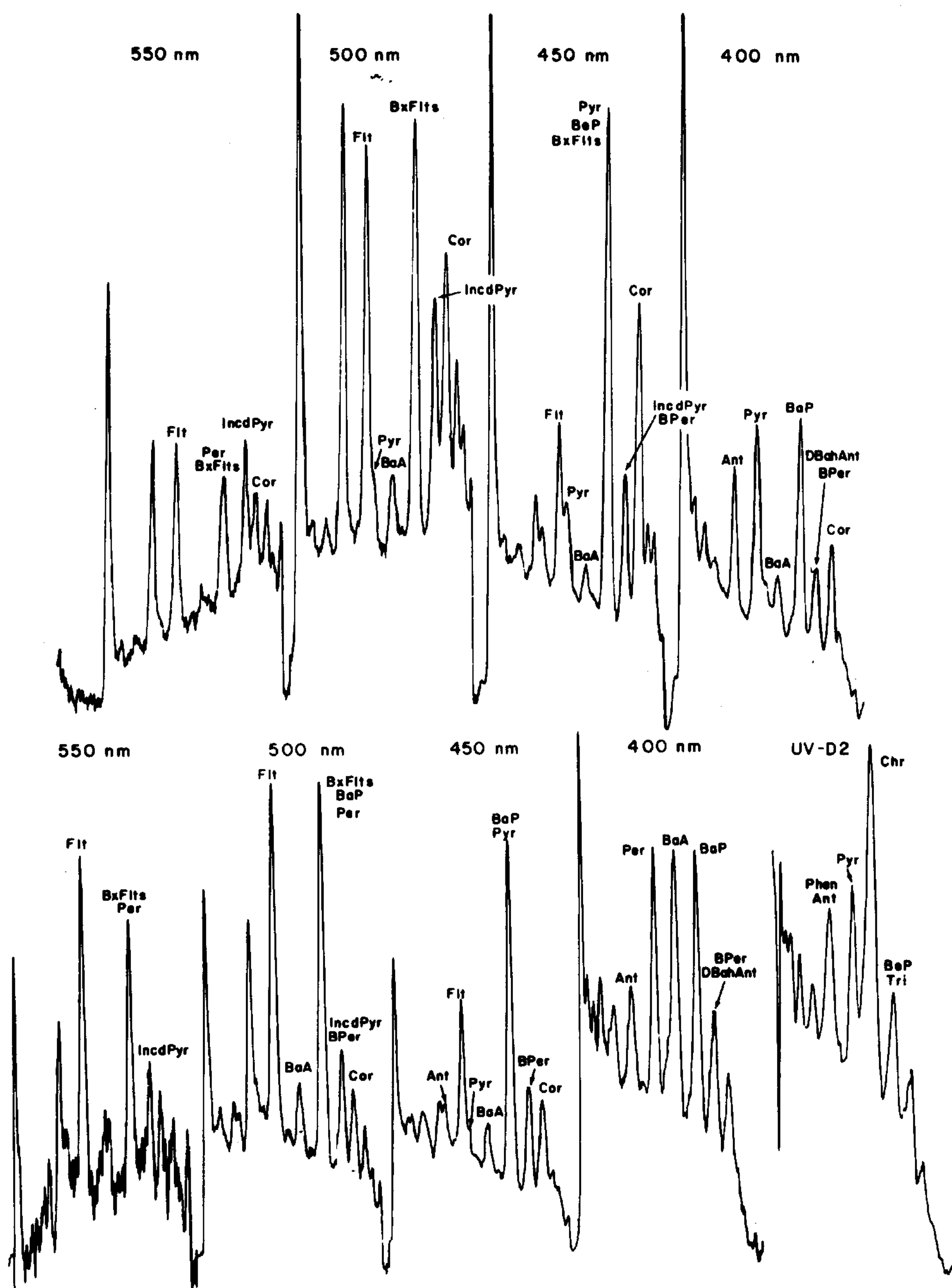


Fig. 6. Separation of PAHs in the organic soluble fraction of a laboratory dust sample. HPTLC conditions are the same as those in Fig. 5. Top, 313 nm and bottom, 266 nm excitation.

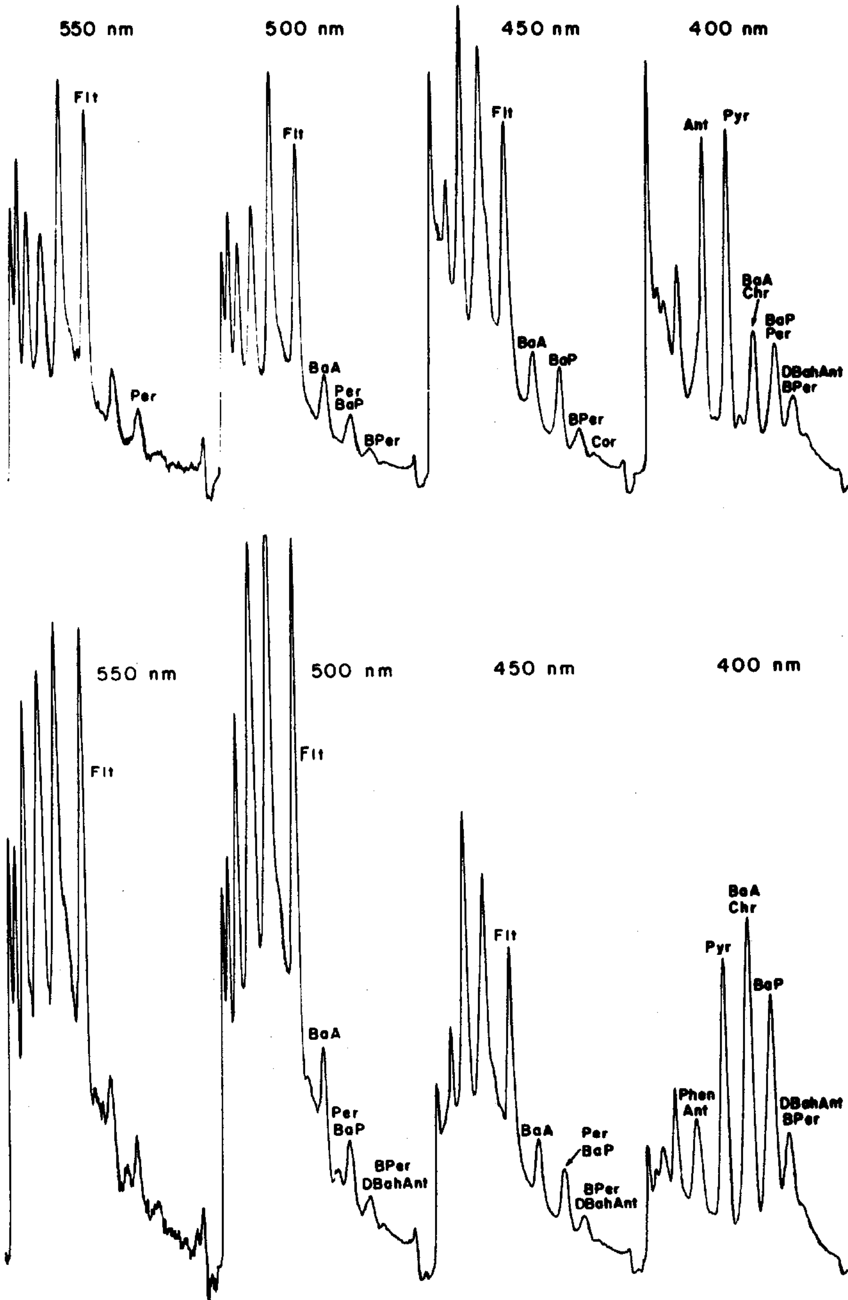


Fig. 7. Separation of PAHs in the organic soluble fraction of a diesel engine exhaust particulate sample. HPTLC conditions are the same as those given in Fig. 5. Top, 313 nm and bottom, 266 nm excitation.

sion response ratio could not be measured accurately as Pyr appears as a shoulder on the Flt peak at 450 nm emission. However, the difference in emission response is clearly outside the ratio 1:0.17 predicted for Pyr and the original assignment should be considered unreliable. The normalized emission response ratios indicate that IncdPyr determined at 313 nm excitation and 550 nm emission is reliable and interference from BPer and DBahAnt is insignificant. BPer can be reliably determined at 365 nm excitation and 400 nm emission without interference from IncdPyr or DBahAnt. DBahAnt can be determined at 313 nm excitation and 400 nm emission with only minor interference from BPer. The above assignments are thus reliable. However, the normalized emission response ratios indicate that Cor is unreliably identified and that because of the relative concentration of BaP, Per, and BxFlt (benzofluoranthene isomers) wavelength discrimination can not be applied for their identification.

The data recorded with the diesel engine particulate extract is shown in Fig. 7. Applying the same procedure as discussed above only Flt can be reliably assigned. Other assignments should be considered unreliable as their normalized emission response ratios are not in accord with the expected values. Here the method is defeated by the complexity of the sample, for although the assigned PAHs are known to be present in the sample, the degree of sample cleanup and chromatographic resolution are inadequate for reliable substance identification. This illustrates the strength of the normalized emission response values for identification purposes as the original assignments would appear reasonable on the basis of retention.

As a prelude to quantitation a method is required for substance identification and for the selection of detection conditions where interferences are minimized. The normalized emission response ratio values are ideally suited to this role in fluorescence scanning densitometry. They can be accurately determined and reproduced on a day-to-day basis. Most importantly, they can be measured with little additional effort and time by simply rescanning the plate after changing the emission filter. Each scan requires less than 2 min for completion.

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