CHROMSYMP. 2268

On-line coupling of liquid chromatography to thin-layer chromatography for the identification of polycyclic aromatic hydrocarbons in marine sediment by fluorescence excitation and emission spectroscopy

R. J. VAN DE NESSE*, G. J. M. HOOGLAND, J. J. M. DE MOEL, C. GOOIJER, U. A. Th. BRINK-MAN and N. H. VELTHORST

Department of General and Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)

ABSTRACT

Storage of the effluent from a reversed-phase column liquid chromatography separation on a thin-layer chromatography plate offers two advantages: (1) detection principles that are not applicable on-line with flow systems can be used; (2) the thin-layer chromatography plate itself allows a second separation to be carried out. Both advantages are demonstrated for the separation and identification of polycyclic aromatic hydrocarbons in a marine sediment sample. The effluent from a microbore liquid chromatography column was deposited on a linearly moving thin-layer chromatography plate through a spray jet assembly interface. As with isocratic liquid chromatography, not all polycyclic aromatic hydrocarbons present in the sample could be separated; a second separation of the immobilized chromatogram was applied by means of thin-layer chromatography. The overlapping peaks were then resolved completely. Eleven polycyclic aromatic hydrocarbons were identified by measuring their fluorescence excitation and emission spectra on the thin-layer chromatography plate with a conventional spectrometer.

INTRODUCTION

The on-line coupling of column liquid chromatography (LC) to thin-layer chromatography (TLC), accomplished by depositing the column effluent from an LC column on a TLC plate moving at constant speed, can be used to combine the separation power of LC with the detection potential of TLC. Since the LC chromatogram is stored on the TLC plate as a continuous trace, in principle a broad variety of time-consuming spectroscopic methods capable of yielding structural information from the immobilized analytes can be applied, *e.g.* two-dimensional absorption [1], fluorescence excitation and emission [2], Fourier transform infrared (FT-IR) [3], surface-enhanced Raman (SERS) [4,5] and fluorescence line narrowing (FLN) spectroscopy [2,6].

An important condition for a successful coupling is the conservation of the resolution of the LC chromatogram during the immobilization on the TLC plate. In

previous papers on this subject we have shown that optimization of both the construction and the characteristics of the interface between the LC and TLC system results in a deposition without serious additional band broadening [2,7,8]. the use of fluorescence excitation and emission spectroscopy and of FLN spectroscopy as off-line detection methods has been demonstrated for some model compounds [2].

Until now, in our work the TLC plate served only to store the LC chromatogram for the application of off-line detection methods, while its chromatographic properties were not exploited. Two-dimensional chromatography can be realized by subjecting the immobilized LC chromatogram to a second separation by means of TLC in a direction perpendicular to the deposited trace. In this way, the separation efficiency can be enhanced when different types of chromatographic systems are used in the LC and TLC mode. In 1987, Jänchen [1] described the (on-line) coupling of reversed-phase LC and normal-phase TLC for the separation of 56 pesticides.

In this study the on-line coupling of LC to TLC was directed to the identification of polycyclic aromatic hydrocarbons (PAHs) present in a marine sediment. The same sediment has been analyzed by the Tidal Waters Division (Groningen, The Netherlands) using reversed-phase LC with gradient elution and on-line fluorescence detection; in that case, identification of the PAHs was based only on the retention times of the eluting compounds. In the present study isocratic reversed-phase LC was used, and identification was accomplished by recording fluorescence excitation and emission spectra of the spots in the immobilized chromatogram. The unresolved spots were further separated by developing the stored chromatogram by TLC, thus performing two-dimensional chromatography.

EXPERIMENTAL

Apparatus
        Fig. 1 shows a scheme of the LC–TLC coupling set-up. The various aspects are described below.
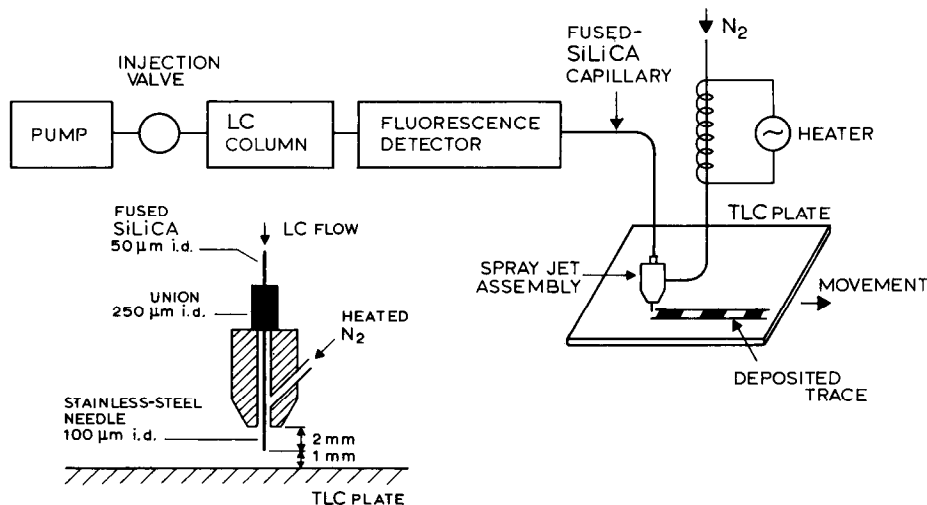

Fig. 1. Schematic of the LC–TLC system and the coupling interface.

Chromatography. A laboratory-made syringe pump and two laboratory-made injection valves were used; one valve had an internal loop of 1.9 μl, the other an external loop of 10 μl. Separations were done by isocratic elution with methanol–water (9:1, v/v) at a flow-rate of 22μl/min on a 170 mm × 1.1 mm I.D. column packed with 5-μm RoSil C_{18} (Research Separations Labs., Eke, Belgium).

For the two-dimensional chromatography experiments, the TLC plates were developed twice in a direction perpendicular to the deposition trace over a distance of *ca.* 6 cm with methanol–diethyl ether–water (6:4:1, v/v/v).

On-line (LC) detection. A Varian (Walnut Creek, CA, U.S.A.) Fluorichrom fluorescence detector was used for the on-line monitoring of the LC effluent. To prevent band broadening, the original flow cell and inlet and outlet tubing were replaced by a 40 cm × 150 μm I.D. fused-silica capillary, the appropriate part of the coating being burned off. Excitation was performed with a deuterium lamp, the light passing through a 10-mm-pathlength cuvette filled with 25% (w/v) nickel sulphate and 40% (w/v) cobalt sulphate in water and a 2-mm UG 11 (Schott, Mainz, Germany) glass band filter. In this way a spectral window ranging from 255 to 368 nm with a maximum transmittance of 32% at 318 nm was obtained. At the emission side a 4-mm Schott GG 13 glass cut-off filter rejected unwanted stray light.

Interface. From the fluorescence detector, the LC effluent was led to the spray jet assembly through a 40 cm × 50 μm I.D. fused-silica capillary. Basically the present spray jet assembly was the same as that of ref. 7; modifications were made with respect to capillary connections and the means to adjust the position of the needle. The capillary was connected by a 250 μm I.D. (1/16 in. O.D.) union to a 100 μm I.D. (475 μm O.D.) stainless-steel syringe needle with a conically shaped tip. One side of the union was glued to the needle; the other end was attached to the capillary by a fingertight connection. A nitrogen flow around the tip of the needle ensured gentle deposition of the effluent on the TLC plate and provided the partial removal of the mobile phase. For the latter purpose, an electric heater, capable of heating the nitrogen gas up to 100°C, was also used. The distance between the needle tip and the TLC plate was 1 mm; the needle protruded 2 mm from the spray jet.

The TLC plate was moved by placing it on the translation table of a Camag (Muttenz, Switzerland) Linomat III line applicator; a translation table speed of about 3.3 mm/min was used. For all coupling experiments the translation table was started 8 min after injection of the sample.

Off-line (TLC) detection. The immobilized chromatogram was scanned with a Carl Zeiss (Oberkochen, Germany) densitometer operated in the fluorescence mode. Excitation was done with a 48-W high-pressure mercury arc lamp. After passing an M4Q-III prism monochromator, the light was focused onto the TLC plate as a rectangular spot; the spatial resolution was 0.7 mm in the direction of the scan and 2.1 mm perpendicular to the scan direction. The luminescence of the compounds was distinguished from the excitation light by the use of optical filters and was detected by an RCA (Lancaster, PA, U.S.A.) 1P28 photomultiplier. The response time was 5 s.

Fluorescence emission and excitation spectra were taken *in situ* with a Perkin–Elmer (Norwalk, CT, U.S.A.) MPF-44A fluorescence spectrometer equipped with a special TLC accessory (Hitachi, Tokyo, Japan). A DCSU-2 differential corrected spectra unit was used to substract blanks. The spectral resolution of the scanning monochromator was 5 nm; for the fixed monochromator it was 10 nm. For all spectra, the response time was 3 s.

Materials

The model compounds anthracene (ANT; Fluka, Buchs, Switzerland), fluoranthene (FLT; Aldrich, Milwaukee, WI, U.S.A.), pyrene (PYR; EGA-Chemie, Steinheim, Germany), benz[*a*]anthracene (B[a]A; Rütgerswerke, Castrop, Germany), chrysene (CHR; Materials, Englewood Cliffs, NJ, U.S.A.), perylene (PER; Metron, Allamuchy, NJ, U.S.A.), benzo[*e*]pyrene (B[e]P), benzo[*b*]fluoranthene (B[b]F), benzo[*k*]fluoranthene (B[k]F), benzo[*a*]pyrene (B[a]P), indeno [1,2,3-*cd*]pyrene (INDP) and benzo[*ghi*]perylene (BPER; all from Radiant Dyes, Wermelskirchen, Germany) were used as received. Methanol [high-performance liquid chromatography (HPLC) quality] and diethyl ether were obtained from Baker (Deventer, The Netherlands).

Two types of TLC plates were applied: high-performance quality C_{18} modified silica plates from Merck (Darmstadt, Germany) and 30% acetylated cellulose plates from Macherey–Nagel (Düren, Germany). Both types of plates were cleaned by continuous elution with methanol. The acetylated plates were further purified by elution with diethyl ether.

Sample pretreatment

The marine sediment was collected from the Waddenzee (The Netherlands). The cleaned-up sample was received from the Tidal Waters Division as a solution in methanol. A volume of 0.317 ml methanol solution corresponded to 1.00 g of dry sediment. Analysis by the Tidal Waters Division, using reversed-phase LC with methanol–water gradient elution, revealed the presence of the PAHs mentioned as model compounds in the previous section, except for PER. They were all present in concentrations ranging from 120 to 860 ng/ml (*i.e.* $0.7 \cdot 10^{-6}$ to $5 \cdot 10^{-6}$ *M*); ANT 120 ng/ml, FLT 860 ng/ml, PYR 600 ng/ml, B[a] 360 ng/ml, CHR 445 ng/ml, B[e]P 450 ng/ml, B[b]F 650 ng/ml, B[k]F 295 ng/ml, B[a]P 375 ng/ml, INDP 555 ng/ml, BPER 475 ng/ml.

RESULTS AND DISCUSSION

The stored LC chromatogram

In this study, two aspects were mainly explored, *viz.* (1) the quality of the stored chromatogram and the information to be derived from it, and (2) the application of the on-line LC–TLC method to real-sample analysis.

A standard mixture of the PAHs known to be present in the marine sediment sample was used to compare the on-line LC chromatogram recorded by conventional fluorescence detection with the stored chromatogram recorded by fluorescence scanning of the deposited trace on the TLC plate with a densitometer. The concentrations in this mixture $3.5 \cdot 10^{-5}$ to $6 \cdot 10^{-7}$ *M* (the injected amounts range from 16 ng to 290 pg), were chosen to be about fivefold higher than in the sediment sample. In Fig. 2 the on-line chromatogram (a) and the corresponding stored chromatogram (b) are shown for the system C_{18}-modified silica LC–C_{18}-modified silica high-performance thin-layer chromatography (HPTLC). The width of the deposited trace on the TLC was about 1.5 mm.

There are three reasons to prefer the high-performance quality C_{18}-modified silica TLC plate to other types of TLC plates for the immobilization of the LC chromatogram. Firstly, its highly regular surface favours the attainment of low detec-
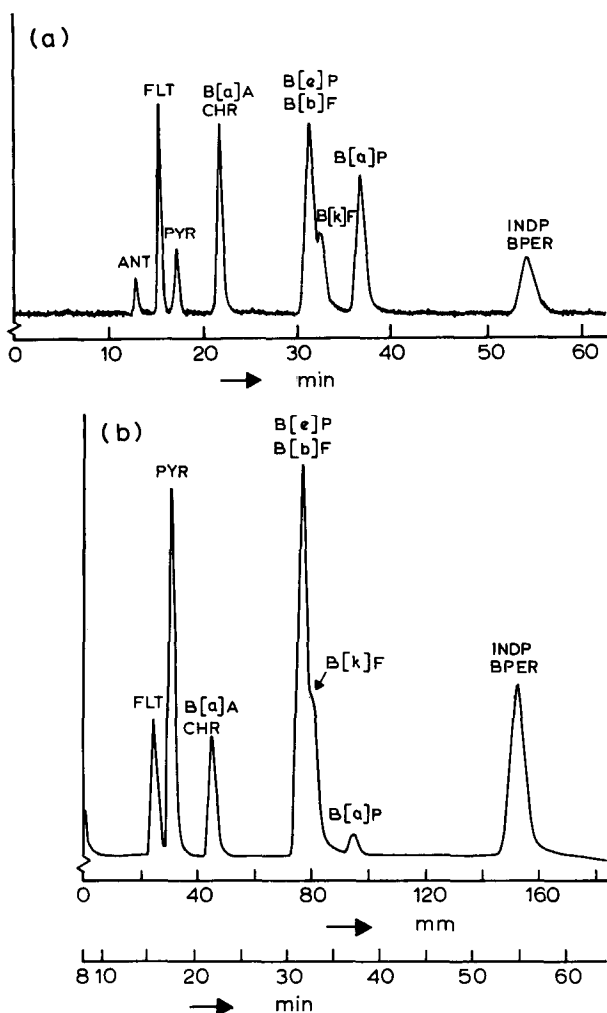
Fig. 2. LC chromatograms of a test mixture of PAHs (concentrations, $3.5 \cdot 10^{-5}$ to $6 \cdot 10^{-7}$ M; the injected amounts range from 16 ng to 290 pg). (a) On-line fluorescence detection. Excitation envelope: 225–368 nm with maximum at 318 nm. Emission wavelength: >385 nm. (b) Fluorescence detection with densitometry after deposition on a C_{18}-modified silica HPTLC plate. Translation table speed: 3.26 mm/min. Excitation wavelength: 313 nm. Emission wavelength: >390 nm. Spatial resolution: 0.7×2.3 mm. For abbreviations, see Experimental section.

tion limits in scanning densitometry. As has been pointed out in ref. 2, the detection limits are primarily determined by baseline fluctuations resulting from the surface irregularity of the TLC plate; the level of the statistical noise on the background signal is of minor importance. Secondly, the above HPTLC material has a low background luminescence, which is important when scanning of fluorescence spectra is used for the identification of analytes. As in this case the TLC plate does not move,

surface irregularity now of course plays no role. Thirdly, PAHs adsorbed to C_{18}-modified silica HPTLC plates in comparison with other types hardly suffer from (photo)decomposition [2].

All stored chromatograms were scanned under a nitrogen gas atmosphere. For some analytes this caused an enhanced signal intensity, probably because of a reduction of fluorescence and/or phosphorescence quenching by oxygen. The effect was highest with PYR; the signal increased by a factor of 2. The poor detectability of ANT on the TLC plate is at least partly the result of its relatively high volatility. Furthermore, ANT has a very low molar absorptivity at 313 nm.

Comparison of Fig. 2a and 2b reveals that the LC separation is stored on the TLC plate without substantial loss of resolution (see, for example, B[k]F shoulder). Only solutes with relatively short retention times show some additional band broadening. The resolution in the early part of the stored chromatogram can be improved by increasing the speed of the translation table. In our experiments, the table speed was restricted by the condition that all analytes in the LC chromatogram should be deposited on the 20-cm-long TLC plates. Of course higher translation table speeds also reduce the sensitivity of detection, because the analyte spots of the deposited trace become longer.

Fig. 3 shows a stored chromatogram of the sediment sample, deposited on a C_{18}-modified silica HPTLC plate. The LC separation, LC–TLC interfacing and the detection conditions were the same as in Fig. 2b. As the original LC detector flow cell was replaced by a fused-silica capillary to prevent additional band broadening, on-line fluorescence detection was not sensitive enough in this case to record the on-line chromatogram.

Preliminary assignment of the peaks in Fig. 3 is possible by comparison with the test chromatogram of Fig. 1. Unambiguous identification is achieved by recording
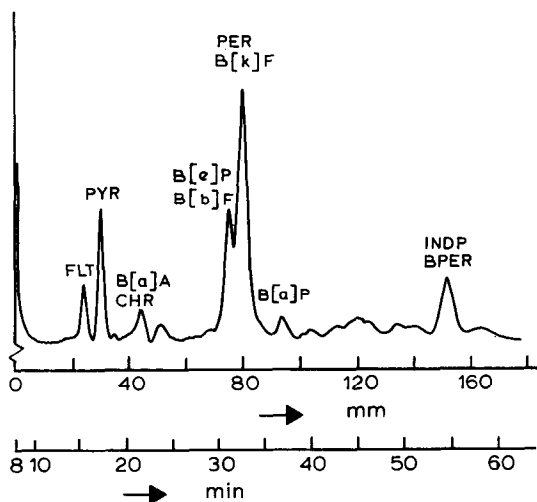

Fig. 3. Densitometer scan of a marine sediment sample (injected amount, 1.9 μl) after LC separation and deposition of LC effluent on a C_{18}-modified silica HPTLC plate. Translation table speed: 3.27 mm/min. Excitation wavelength: 313 nm. Emission wavelength: >390 nm. For abbreviations, see Experimental section.

fluorescence excitation and emission spectra *in situ* with a conventional spectrometer equipped with a TLC accessory. Identification of the constituents of the unresolved spots is difficult or even impossible since their spectra are quite similar. However, anticipating further experiments described in the text below, the peaks in Fig. 3 have already been assigned. Fig. 4 shows excitation and emission spectra of FLT, PYR and B[a]P, compounds that are completely resolved by the present LC system. These spectra are identical with those recorded in solution [9], as is shown for B[a]P. The spectra in Fig. 4 were corrected for the background contribution of the TLC plate caused by luminescence and light scattering, which are much more pronounced than
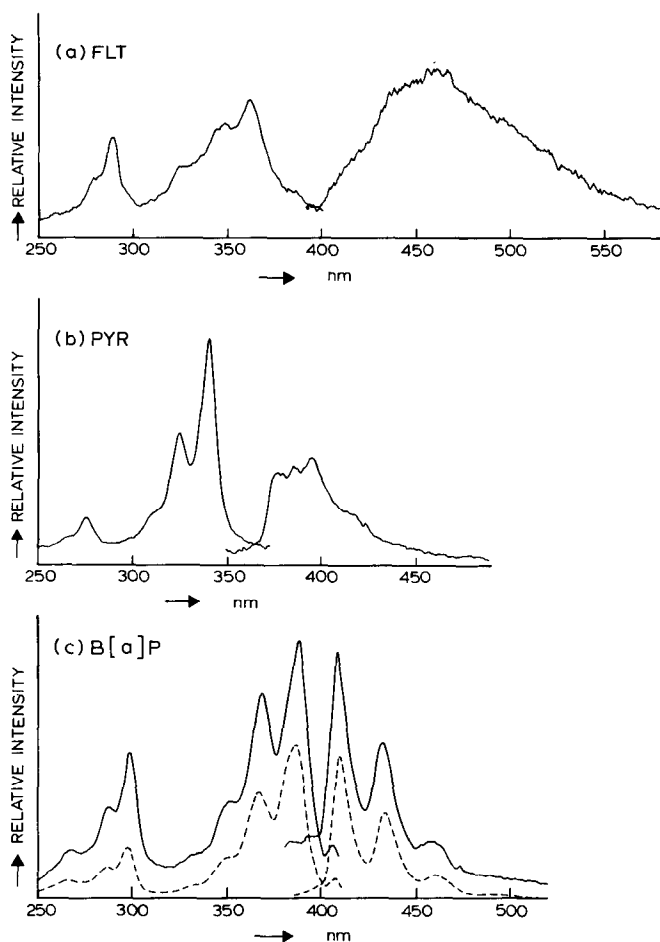

Fig. 4. *In situ* excitation and emission spectra of PAHs from the immobilized LC chromatogram of Fig. 3. (a) FLT. Excitation wavelength: 360 nm. Emission wavelength: 455 nm. (b) PYR. Excitation wavelength: 322 nm. Emission wavelength: 390 nm. (c) B[a]P. Excitation wavelength: 300 nm. Emission wavelength 430 nm. Dashed line: room temperature excitation and emission spectrum of a 10^{-6} M B[a]P solution in methanol. The illuminated area on the TLC plate was 2 × 2 mm for FLT and PYR, and 6 × 1 mm for B[a]P.

in solution. It is obvious from this figure that the spectral intensities for PYR and B[a]P are far above the level associated with the identification limit. For the worst case, FLT, identification on the basis of the excitation spectrum can still be done for about 270 pg (corresponding to a 1.9-μl injection of $7 \cdot 10^{-7}$ M), which is about six times lower than the amount associated with the spectrum in Fig. 4a.

Two-dimensional chromatography
     With the present LC system, isocratic elution does not suffice to separate all PAHs in the sediment sample. Therefore a second separation was carried out on the TLC plate. Subsequent to the storage of the LC effluent on a TLC plate, the plate was developed in a direction perpendicular to the deposited LC trace. For this, a 30% acetylated cellulose plate was preferred to a C_{18}-modified silica HPTLC plate, because elution with methanol–diethyl ether–water (6:4:1, v/v/v) allows the complete separation of all unresolved components [10]. When the second (TLC) dimension was used, the injection volume of the LC system was increased from 1.9 to 10 μl, mainly for two reasons. Firstly, the spots on the TLC plate are more easily located by viewing under UV light; this facilitates the appropriate positioning of the TLC plate in the special accessory needed for the recording of fluorescence spectra. Secondly, densitometric detection of the PAHs on the 30% acetylated cellulose TLC plate was less sensitive than on the C_{18}-modified silica HPTLC plate. The reduced detectability is a result of the more irregular surface of the former type of plate. In addition, the spots deposited on the plate after the LC separation self-evidently become somewhat larger during the TLC separation. Unfortunately, a 30% acetylated cellulose TLC plate offering a high(er) performance quality is not commercially available. The increase of the injection volume had only a minor effect on the resolution in the LC system.
     Fig. 5 shows the result of the TLC separation of a sediment sample (after previous on-line LC separation and storage). All PAHs not separated in the isocratic
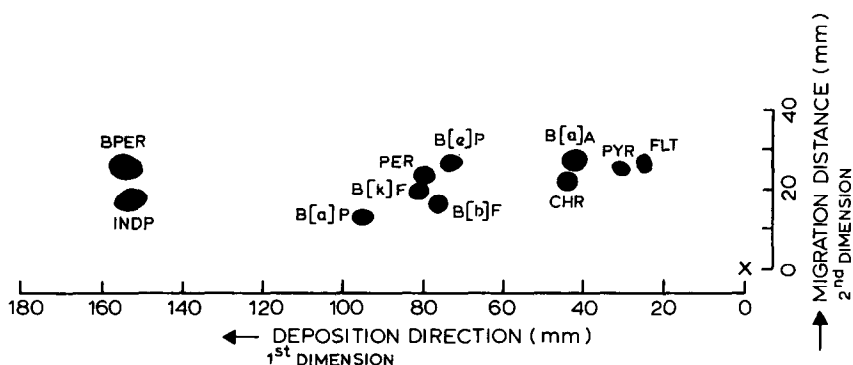

Fig. 5. Two-dimensional LC–TLC of a marine sediment sample. First dimension: isocratic reversed-phase LC separation (methanol–water, 9:1, v/v; injected amount, 10 μl); LC effluent deposited on a 30% acetylated cellulose TLC plate; translation table speed 3.20 mm/min. Second dimension: TLC perpendicular to the deposition trace with methanol–diethyl ether–water (6:4:1, v/v/v). The cross marks the start of the translation table 8 min after injection. For abbreviations, see Experimental section.
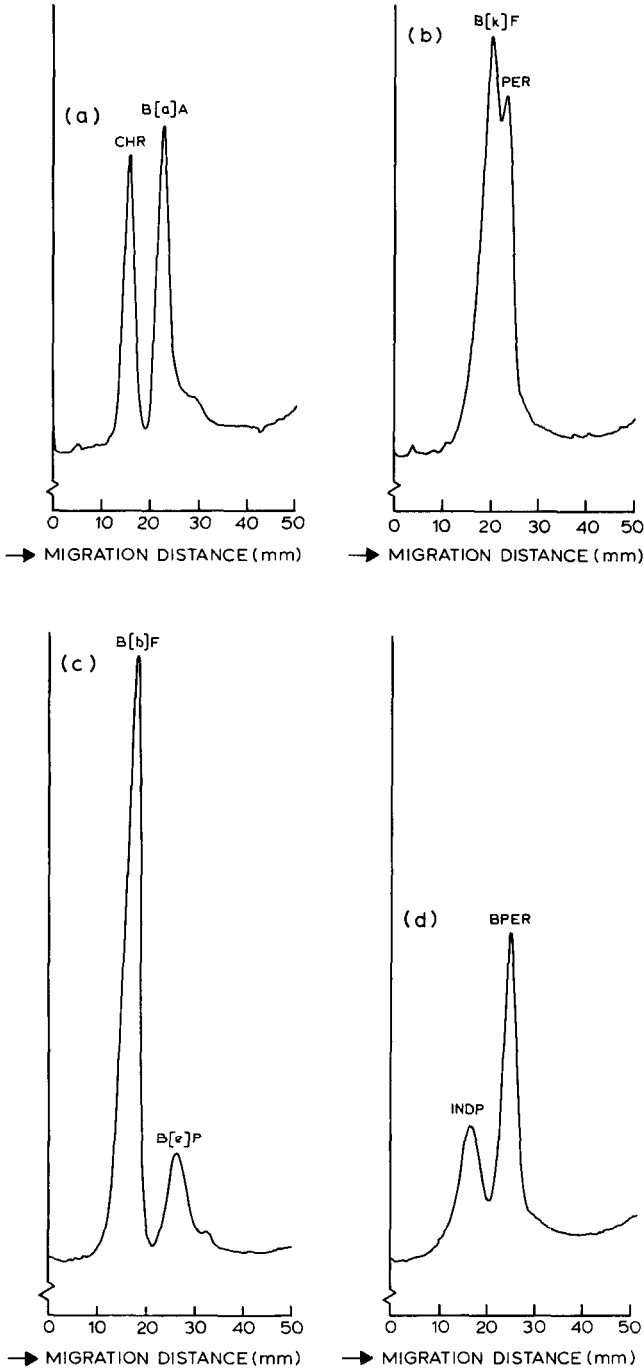
Fig. 6. Densitometer scans of the TLC plate after two-dimensional LC–TLC separation of a marine sediment sample. The scans were taken in the direction of the TLC separation at (a) 44 mm, (b) 75.5 mm, (c) 81 mm and (d) 153 mm from the starting point of the translation table in Fig. 5. (a) CHR and B[a]A. Excitation wavelength: 266 nm. Emission wavelength: >390 nm. Spatial resolution: 0.7 × 4.1 mm. (b) B[k]F and PER. Excitation wavelength: 254 nm. Emission wavelength: >430 nm. Spatial resolution: 0.7 × 2.3 mm. (c) B[b]F and B[e]P. Excitation wavelength: 266 nm. Emission wavelength: >390 nm. Spatial resolution: 0.7 × 2.3 mm. (d) INDP and BPER. Excitation wavelength: 266 nm. Emission wavelength: >390 nm. Spatial resolution: 0.7 × 4.1 mm.
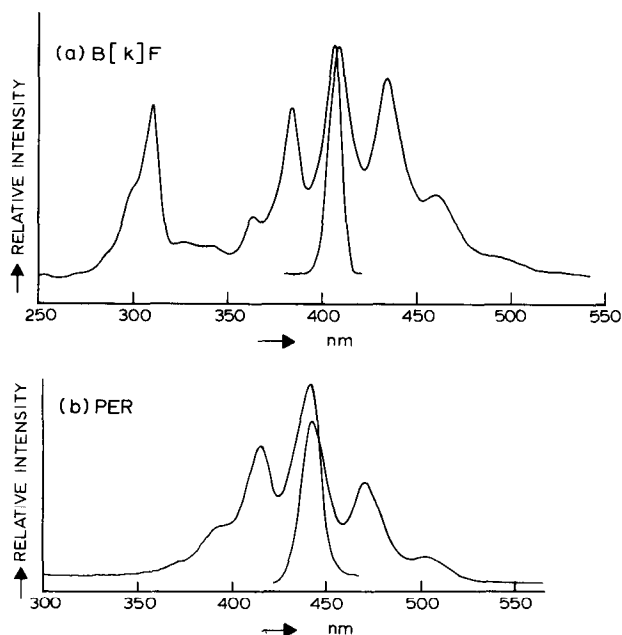
Fig. 7. *In situ* excitation and emission spectra of PAHs after two-dimensional LC–TLC of a marine sediment sample. Illuminated area on the plate: 6 × 2 mm. (a) B[k]F. Excitation wavelength: 309 nm. Emission wavelength: 430 nm. (b) PER. Excitation wavelength: 400 nm. Emission wavelength: 495 nm.

LC system are completely resolved, as is clear from densitometer scans (see Fig. 6). The analytes were identified by their fluorescence excitation and emission spectra. In Fig. 7 a relevant example is shown; the two-dimensional approach revealed the presence of PER in the sediment. This compound was not detected in the analysis carried out by the Tidal Waters Division; obviously it was obscured by another peak.

CONCLUSIONS

LC–TLC coupling allows the successful separation and identification – by means of fluorescence spectroscopy – of PAHs in marine sediment samples. The use of a second, TLC, separation reduces the demands on the performance of the LC separation and simple isocratic elution is sufficient. Furthermore, in the semi-on-line approach, identification of PAHs is simply accomplished by conventionally recorded fluorescence emission and excitation spectra since time constraints in detection are absent. In the on-line (on-the-fly) mode, identification at trace levels is possible, but it generally requires sophisticated instrumentation such as an intensified linear diode array detector (see, for example, refs. 11–13]. Furthermore, on-the-fly excitation spectra are not easily obtainable. In summary, the semi-on-line LC–TLC technique, although obviously more elaborate than on-line (gradient elution) LC, deserves a distinct place in chemical analysis since it offers improved chromatographic separation efficiency as well as a better detection potential.

Future work in our laboratory will focus on the combination of LC with FT–IR spectroscopy following the semi-on-line approach. The present coupling interface will be used for the storage of LC effluents on IR-transparent media such as zinc selenide.

ACKNOWLEDGEMENTS

REFERENCES

1 D. E. Jänchen, in H. Traitler, A. Studer and R. E. Kaiser (Editors), *Proceedings of the 4th International Symposium on Instrumental HPTLC*, Institute for Chromatography, Bad Dürkheim, 1987, pp. 185.
2 J. W. Hofstraat, M. Engelsma, R. J. van de Nesse, C. Gooijer, U. A. Th. Brinkman and N. H. Velthorst, *Anal. Chim. Acta*, 193 (1987) 193.
3 C. Fujimoto, T. Morita and K. Jinno, *J. Chromatogr.*, 438 (1988) 329.
4 J.-M. L. Sequaris and E. Koglin, *Anal. Chem.*, 59 (1987) 525.
5 S. A. Soper, K. L. Ratzlaff and T. Kuwana, *Anal. Chem.*, 62 (1990) 1438.
6 R. S. Cooper, R. Jankowiak, J. M. Hayes, Lu Pei-qi and G. J. Small, *Anal. Chem.*, 60 (1988) 2692.
7 J. W. Hofstraat, M. Engelsma, R. J. van de Nesse, C. Gooijer, N. H. Velthorst and U. A. Th. Brinkman, *Anal. Chim. Acta*, 186 (1986) 247.
8 J. W. Hofstraat, S. Griffioen, R. J. van de Nesse, U. A. Th. Brinkman, C. Gooijer and N. H. Velthorst, *J. Planar Chromatogr.*, 1 (1988) 220.
9 W. Karcher, R. J. Fordham, J. J. Dubois, P. G. J. M. Glaude and J. A. M. Ligthart, *Spectral Atlas of Polycyclic Aromatic Compounds*, Reidel, Dordrecht, 1983.
10 J. Kraft, A. Hartung, K. Lies and J. Schulze, in R. E. Kaiser (Editor), *Proceedings of the 2nd International Symposium on Instrumental HPTLC, Interlaken, 1982*, Institute for Chromatography, Bad Dürkheim, 1982, p. 144.
11 R. J. van de Nesse, C. Gooijer, G. Ph. Hoornweg, U. A. Th. Brinkman, N. H. Velthorst and S. J. van der Bent, *Anal. Lett.*, 23 (1990) 1235.
12 D. B. Skoropinski, J. B. Callis, J. D. S. Danielson and G. D. Christian, *Anal. Chem.*, 58 (1986) 2831.
13 J. C. Gluckman, D. C. Shelly and M. V. Novotny, *Anal. Chem.*, 57 (1985) 1546.