Journal of Chromatography, 508 (1990) 37–49
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 358

Solid-surface room-temperature phosphorescence detection for high-performance liquid chromatography

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(First received December 15th, 1989; revised manuscript received February 16th, 1990)

SUMMARY

In the present study, we have optimized the use of a two nebulizer system for solid-surface room-temperature phosphorescence (SSRTP) as a selective, permanent record detector for high-performance liquid chromatography (HPLC). The chromatographic parameters and the analytical figures of merit of five well known phosphorescent compounds were compared to those obtained by ultraviolet detection. Calibration curves with satisfactory linear dynamic ranges and limits of detection in the nanogram and subnanogram level showed the feasibility of the SSRTP detector for HPLC. In addition, overlapped compounds were individually identified demonstrating that the selectivity of the proposed detector can be a useful feature in case of incomplete chromatographic separations of complex mixtures.

INTRODUCTION

Solid-surface room-temperature phosphorescence (SSRTP) has been developed into a sensitive, selective and simple analytical tool for the determination of compounds of diversified interest[1–5]. Among the solid supports investigated for SSRTP[6–9], cellulose, and particularly filter paper, appears to be the most suitable substrate for the technique. The sensitivity of SSRTP can be improved by using appropriate concentrations of heavy-atom salts. Thallium(I), silver(I), and lead(II) cations and iodide are among the most common heavy-atom perturbers succesfully used to perform qualitative analysis at the nanogram and subnanogram level[3,9,10]. The reproducibility of the technique ranges from 5 to 15% and depends on the kind of substrate, analyte, experimental conditions and experimentator's expertise[10,11]. Since cellulose is susceptible to moisture, special attention must be taken to avoid humidity on the substrate[12]. Quenching of the analyte signal by water will certainly affect the precision and the sensitivity of the method. As a consequence, a drying step prior to detection is usually performed by either placing the sample under an infrared lamp or

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0021-9673/90/$03.50

in an oven at high temperature. Quenching effects can also be caused by oxygen. Its presence is usually avoided by performing the RTP measurements under a flow of dry nitrogen.

The selectivity of the technique allows the qualitative and quantitative analysis of multicomponent mixtures without previous separation[12]. When the desired compound is the predominant phosphor of the mixture, the excitation and/or the emission spectrum of the sample can be easily used for detection purposes[13,14]. When the sample contains several compounds with comparable phosphorescent intensity signal, the similarity and/or overlapping of phosphorescence bands usually interfere in the characterization process. Many alternatives have been proposed to overcome this problem including synchronous scanning[15], selective external heavy-atom perturbation[10,16], and the combination of substrate and heavy-atom effects to preferentially enhance the phosphorescent signal of the target compound[16].

When dealing with complex matrices in various applications, previous separation is usually necessary to eliminate possible interferences and therefore facilitate the detection procedure. Several workers have proposed paper and thin-layer chromatography as separation techniques for SSRTP detection[5,17,18]. The unique possibility of complete analysis of complex mixtures using the same substrate turns this combination into a relatively simple and useful tool for analytical purposes.

SSRTP has also been associated with high-performance liquid chromatography (HPLC). Ford and Hurtubise[19] used silica chromatoplates as solid substrates to identify benzo[*f*]quinoline and phenanthridine in shale oil samples. The RTP spectra of the HPLC fractions corresponding to the retention times of those two compounds were compared to those obtained with the pure analytes. Filter paper discs were utilized by Vo-Dinh[12] to quantitatively determine benzo[*a*]pyrene and benzo[*e*]pyrene in coal liquid sales. In both cases[12,19], the HPLC fractions were manually collected and spotted onto the substrates for SSRTP detection.

In two previous studies[20,21], we reported the development of a two nebulizer automatic system which eliminated the manual steps usually involved in SSRTP analysis. In this report, the system is optimized as a selective detector for HPLC. To our knowledge, this is the first time that SSRTP is employed as a detection technique for HPLC analysis in a continuous automated mode. The chromatographic parameters and the analytical figures of merit (AFOMs) of five well known phosphorescent compounds of environmental interest were compared to the ones obtained by UV detection. The versatility and selectivity of the SSRTP detector is demonstrated by showing that overlapped compounds can be identified either from the SSRTP chromatogram or from their RTP spectral characteristics. Quantitative determination can be done by selecting the appropriate excitation and emission wavelengths of measurement. A permanent record of the analyzed sample can then be obtained by storing the substrate under proper conditions.

EXPERIMENTAL

Instrumentation

The HPLC system consisted on an Altex pump Model 110A used with a Rheodyne valve and a 20-μl sample loop. The Altex column was 15 cm $\times$ 4.6 mm I.D., and filled with ultrasphere ODS (5 μm particle diameter). The UV detector was

an Altex Model 153 with a cell volume of 8 μl, response time constant of 1 s and fixed wavelength at 254 nm. The two nebulizer system for SSRTP detection has been described in detail elsewere[20,21]. The eluent from the chromatographic column was sprayed onto the moving filter paper strip by connecting, with a PTFE tube (0.25 mm I.D.), the exit of the UV detector cell to the analyte nebulizer of the automatic system. The PTFE tube was kept as short as possible (< 15 cm) to minimize band broadening. Prior to detection, the paper strip was irradiated by two infrared lamps to evaporate continuously the solvent of the heavy-atom solution and the mobile phase. The dry substrate was then pulled into the detection unit by means of a Technicon AutoAnalyzer continuous filter (Technicon, Tarrytown, NY, U.S.A.). The detector consisted of a Perkin-Elmer LS-5 luminescence spectrofluorimeter driven by a Perkin-Elmer 3600 data station with a laboratory-constructed filter paper guide. All the SSRTP measurements were performed under a flow of dry nitrogen with a delay time of 0.03 ms and a gate time for collection of data of 9.0 ms. The excitation and the emission slits were set to 10 nm and 5 nm, respectively.

Reagents and procedure

All chemicals were analytical-reagent grade and used as received. Carbazole was purchased from Eastman Kodak; pyrene, benzo[*e*]pyrene, fluoranthene, phenanthrene and thallium(I) acetate (TlOAc) were obtained from Aldrich. HPLC-grade methanol (Fischer Scientific) and "nanopure" demineralized water (Barnsted Sybron) were used throughout. S&S 2043-A filter paper (Schleicher and Schuell) was obtained as a 2.5 cm wide × 24 m long filter paper roll and utilized as a substrate. Methanol–water solution of different volume ratios were employed a solvents to prepare 200-μg/ml stock solutions of carbazole and phenanthrene (80:20, v/v), and pyrene and fluoranthene (70:30, v/v). The same mass of benzo[*e*]pyrene was dissolved in pure methanol. A 0.1 *M* solution of TlOAc in methanol–water (50:50, v/v) was employed as a heavy-atom enhancer and continuously sprayed onto the moving filter paper at a flow-rate adjusted to deliver approximately 10 μl of solution per cm^2 of substrate. All the RTP measurements were performed in a continuous mode with the use of a kinetic program which permitted plotting the phosphorescent intensity as a function of time. While the desired section of filter paper was passing through the sample compartment of the spectrofluorimeter, the excitation slit was opened and the RTP emission at the selected excitation and emission wavelengths was registered. The total sampling time was chosen according to the separation time in the chromatographic column and a data interval of 0.2 s was kept constant through all the experiments. For all the AFOMs, a sampling time of 800 s was employed to obtain 4000 points per chromatogram. The mobile phase drying process introduced a delay time of 330 s. This delay time in the SSRTP chromatogram corresponded to the time spent by the analyte on the substrate to "travel" from the nebulizer to the excitation slit of the spectrofluorimeter. Since the time spent for the effluent to go from the UV detector to the analyte nebulizer was negligible for all the flow-rates tested, the same delay time was observed through all the experiments. The retention times in the SSRTP chromatograms were then corrected with respect to the observed values (330 s).

RESULTS AND DISCUSSION

Five well known phosphorescent compounds, carbazole, phenanthrene, pyrene, benzo[*e*]pyrene (B*e*P) and fluoranthene, were selected to compare the UV and the SSRTP system for HPLC detection. All of them strongly absorbed at 254 nm, at which the UV measurements were performed. The 0.1 *M* TlOAc solution, chosen as a heavy-atom perturber, was shown to be particularly efficient in the enhancement signal of most of the probe analytes[9].

Fig. 1 shows a SSRTP chromatogram along with the corresponding classical UV chromatogram of a mixture containing carbazole, phenanthrene, pyrene and B*e*P. In order to simplify the measurement procedure, only two sets of detection wavelengths were employed. The measurement wavelengths selected took into consideration the relative phosphorescent intensity of the studied compounds. When necessary, the chosen wavelength pair was set closer to the wavelengths for maximum RTP signal of the weaker phosphor. The wavelength and chromatographic parameters are listed in Table I.

Chromatographic band broadening

The peak efficiencies were obtained assuming Gaussian peaks and are listed in Table I. The low efficiency obtained for carbazole (N_{UV} = 400, N_{RTP} = 370) was not

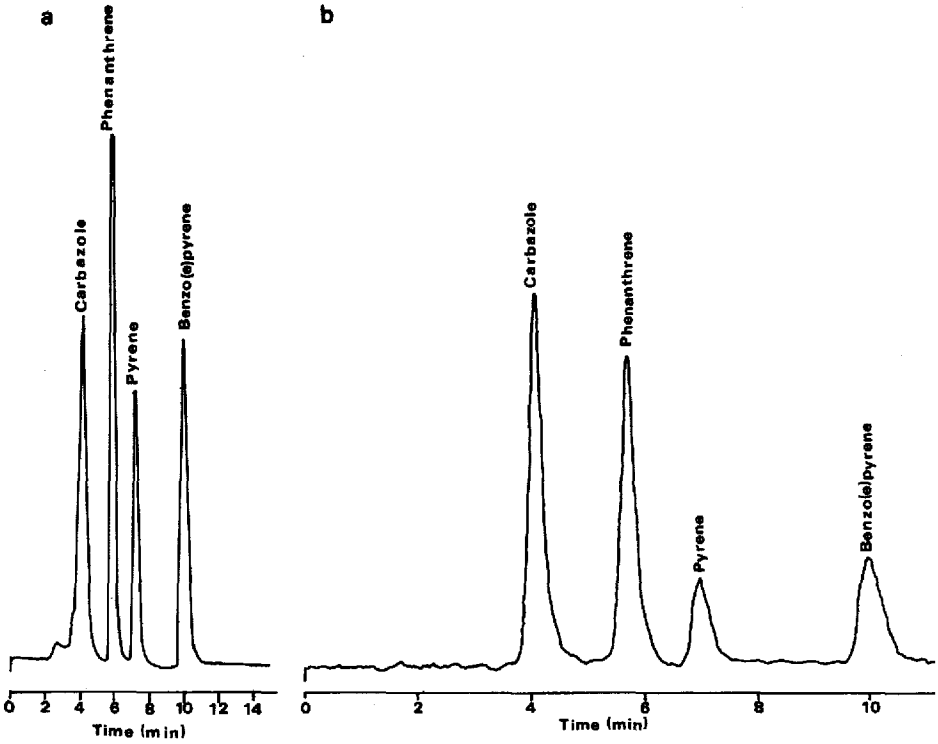

Fig. 1. UV (a) and SSRTP (b) chromatogram showing the separation of a mixture containing 200 pg of phenanthrene and 500 pg of carbazole, pyrene and benzo[*e*]pyrene. Mobile phase: methanol; flow-rate: 0.443 ml/min; sample volume: 20 μl.

TABLE I

WAVELENGTH SETTINGS AND CHROMATOGRAPHIC PARAMETERS

Retention volumes (V_R) obtained with a flow-rate of 0.443 ml/min. Capacity factors (k') calculated for a dead volume (V_0) of 0.97 ml. Efficiencies calculated using $N=4(V_R/W_{0.6H})^2$ where $W_{0.6H}$ is the peak width at 60% of the peak height expressed in volume units and N is the plate number; accuracy 20%.

Solute	*Excitation wavelength (nm)*[a]	*Emission wavelength (nm)*[a]	V_R *(ml)*	k'	*Efficiency*	
					UV plates	*RTP plates*
Carbazole	290 (296)	470 (440)	1.95	1.00	400	370
Phenanthrene	290 (285)	470 (505)	2.66	1.73	1400	1300
Pyrene	343 (343)	585 (595)	3.10	2.18	3000	2400
Benzo[*e*]pyrene	343 (335)	585 (543)	4.34	3.45	3200	2800
Fluoranthene	343 (365)	585 (545)	2.92	2.00	3000	2400

[a] The values in parenthesis correspond to the wavelengths for maximum emission. The others are the selected wavelengths for the SSRTP chromatogram.

surprising. Carbazole, or dibenzopyrrole, is an amino-containing basic compound. The –NH amino group of the pyrrole ring has a high affinity for surface silanols of the silica stationary phase. This affinity induces a peak tailing and low efficiency. The relatively low efficiency measured for phenanthrene ($N_{UV} = 1400$, $N_{RTP} = 1300$) may be due to extra column band broadening effects apart from the detectors. We point out that the chromatographic system was not optimized for maximum efficiency. However, it is interesting to estimate the band broadening induced by the SSRTP detector. A 15% efficiency loss was observed comparing the UV absorption to the SSRTP values (Table I). The band broadening produced by the connecting tubing, nebulizer and moving paper strip can be roughly quantified through variance estimation using the equation $\sigma^2 = V_R^2/N$. Defining σ_{SSRTP} and σ_{UV} from the SSRTP and the UV absorption chromatograms, the difference $\sigma^2_{SSRTP} - \sigma^2_{UV}$ corresponds to the band broadening due to the SSRTP detector because variances are additive. The estimated value was found to be approximately $900 \pm 300\ \mu l^2$ which corresponded to a dead volume of $30 \pm 15\ \mu l$ for the SSRTP detector. It is important to note that, although the moving filter paper strip induced a 330-s response delay for drying and reaching the observation window, it did not produce excessive band broadening. Since the mobile phase and the solvent of the heavy atom solution were evaporated by infrared lamps, the solutes were trapped into the cellulose fibers of the paper which restrained their mobilities. By reducing the UV–SSRTP connecting tubing (16 μl) and using a micronebulizer, it should be possible to minimize the band broadening even more.

Flow-rate effect

When a constant ratio between the HPLC flow-rate and the strip chart recorder speed was maintained, we observed little variations of UV peak areas with the flow-rate for fixed sample weight. Important variations of SSRTP peak areas were observed with flow-rate changes. The phosphorescent intensity was very dependent on the flow-rate of the mobile phase. Fig. 2 shows the chromatographic SSRTP response

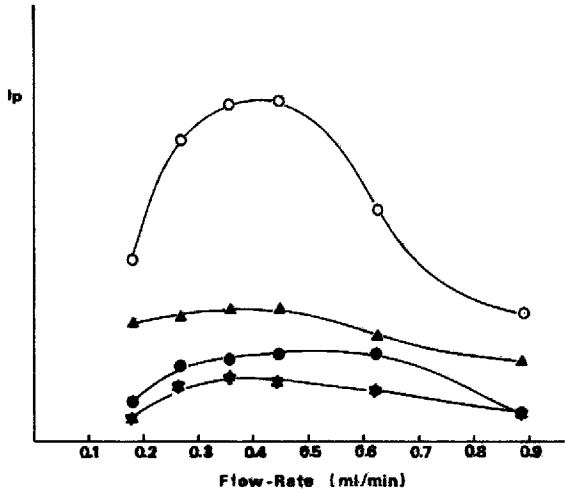

Fig. 2. SSRTP peak area (I_p) *versus* mobile phase flow-rate variations of carbazole's (▲), phenanthrene's (○), pyrene's (●) and benzo[*e*]pyrene's (★) signal with flow-rate variation of mobile phase (methanol). Sample size: 200 pg of phenanthrene, 500 pg of others, injected volume 20 μl.

(SSRTP peak area) *versus* flow-rate. It can be noticed that there is an optimum flow-rate for maximum RTP signal and therefore for highest sensitivity. Several parameters related to the flow-rate variation could be responsible for the observed phenomenon. By keeping constant the filter paper speed, the amount of analyte deposited per surface area of substrate increased with the flow-rate. If a peak has a base width of 1 min, the corresponding paper length on which the analyte would be sprayed was 21 cm; if the peak has a base width of 10 s, the same amount of analyte would be sprayed on 3.5 cm and so on. Since the analyte was deposited on a shorter length of substrate, it would be reasonable to expect higher phosphorescent signals at higher flow-rates. However, by increasing the flow-rate of the mobile phase, a larger volume of solvent per substrate length was also delivered. Since the filter paper speed was kept constant, the drying time remained the same and was insufficient for complete drying of the solvent. If this was the case, moisture could be responsible to some extent, for quenching the phosphorescent signal. In addition, the increase in the flow-rate was followed by an increase in the width of the sprayed area. Since the excitation slit of the spectrofluorimeter was kept constant, a possible diffusion of the analyte to the edges of the filter paper could also be responsible for the decrease observed in the phosphorescence intensities. With the exception of pyrene, all the other compounds showed the strongest phosphorescent signals between 0.354 and 0.443 ml/min. Considering the advantage of short analysis time, 0.443 ml/min was chosen as the optimum mobile phase flow-rate resulting in a total analysis time of approximately 17 min per SSRTP chromatogram.

AFOMs

Table II compares the AFOMs obtained with the UV absorption and the SSRTP system for HPLC detection. It is important to mention that both detectors were compared under experimental conditions for maximum SSRTP sensitivity and

TABLE II

ANALYTICAL FIGURES OF MERIT OBTAINED FOR HPLC USING SSRTP AND UV ABSORPTION DETECTION

All AFOMs were obtained under experimental conditions for maximum SSRTP sensitivity and reproducibility. The values listed in parenthesis correspond to the AFOMs obtained with the UV detector. Linear dynamic range (LDR) was estimated by dividing the upper linear concentration by the limit of detection. The slope was calculated from the curve log phosphorescence intensity (absorbance) *versus* log concentration. Signal-to-noise ratio = 3 and volumes of 20 μl (RTP) and 8 μl (UV) were used to estimate the limits of detection (LOD).

Solute	*LDR*	*Slope*	*Correlation coefficient*	*LOD (ng)*
Carbazole	$3.0 \cdot 10^3$ ($6.5 \cdot 10^2$)	1.06 (0.93)	0.983 (0.926)	0.77 (1.26)
Phenanthrene	$3.3 \cdot 10^3$ ($2.5 \cdot 10^2$)	1.04 (0.86)	0.999 (0.999)	0.96 (0.98)
Pyrene	$2.2 \cdot 10^2$ ($2.6 \cdot 10^3$)	0.95 (0.99)	0.985 (0.998)	5.24 (0.46)
Benzo[*e*]pyrene	$3.6 \cdot 10^2$ ($3.4 \cdot 10^2$)	1.00 (0.98)	0.983 (0.998)	3.16 (4.62)

reproducibility. The HPLC system was not optimized for UV-absorption detection. The limits of detection and the reproducibilities of measurements obtained with the UV detector, however, were comparable with those commonly observed when these kinds of devices are used for HPLC analysis. When measured by SSRTP, carbazole and phenanthrene showed calibration curves with larger linear dynamic ranges. Pyrene absorbed strongly in the UV and so had a low limit of detection with the UV detector; the SSRTP calibration curves for pyrene had a shorter linear dynamic range. In all cases, slopes close to unity and satisfactory correlation coefficients were obtained with both systems. The limits of detection with the UV detector were calculated from the equation $\mathrm{LOD} = 3/5(I_{p-p}/m)$ where I_{p-p} is the peak-to-peak background noise and m is the slope of the calibration curve[22]. The SSRTP values were estimated by multiplying the equation above by the factor A_i/A_t, where A_i is the irradiated area in the sample compartment and A_t is the total area of substrate on which the analyte has been sprayed. With both detection systems, carbazole, phenanthrene and BeP showed comparable limits of detection (LOD) in the nanogram range. The higher LOD value obtained for pyrene with the SSRTP detector can be attributed to the weak phosphorescence signal presented for this phoshor under the experimental conditions employed in the study. The relative standard deviations obtained with the SSRTP detector were within the reproducibility range of the technique (4–15%), and as expected, were poorer than those obtained by UV-absorption detection (2–7.6%).

Special features

Although conventional UV-absorption detectors operated at fixed wavelengths are able to discriminate between absorbing and non-absorbing species, they lack versatility and selectivity for the determination of absorbing compounds. The separation process in the chromatographic column has to be either complete or performed in such a way that compounds with minimum overlapping reach the detector. Most times this is a tedious procedure which usually involves either the optimization of a volume ratio between two solvents or the search for a convenient mobile phase. In addition, the detector wavelength does not usually coincide with the

maximum absorbance peak of the desired compound, which restricts the sensitivity of HPLC.

Commercially available UV–visible absorption detectors that consist of a scanning spectrometer or a photodetector array with grating optics offer the possibility to

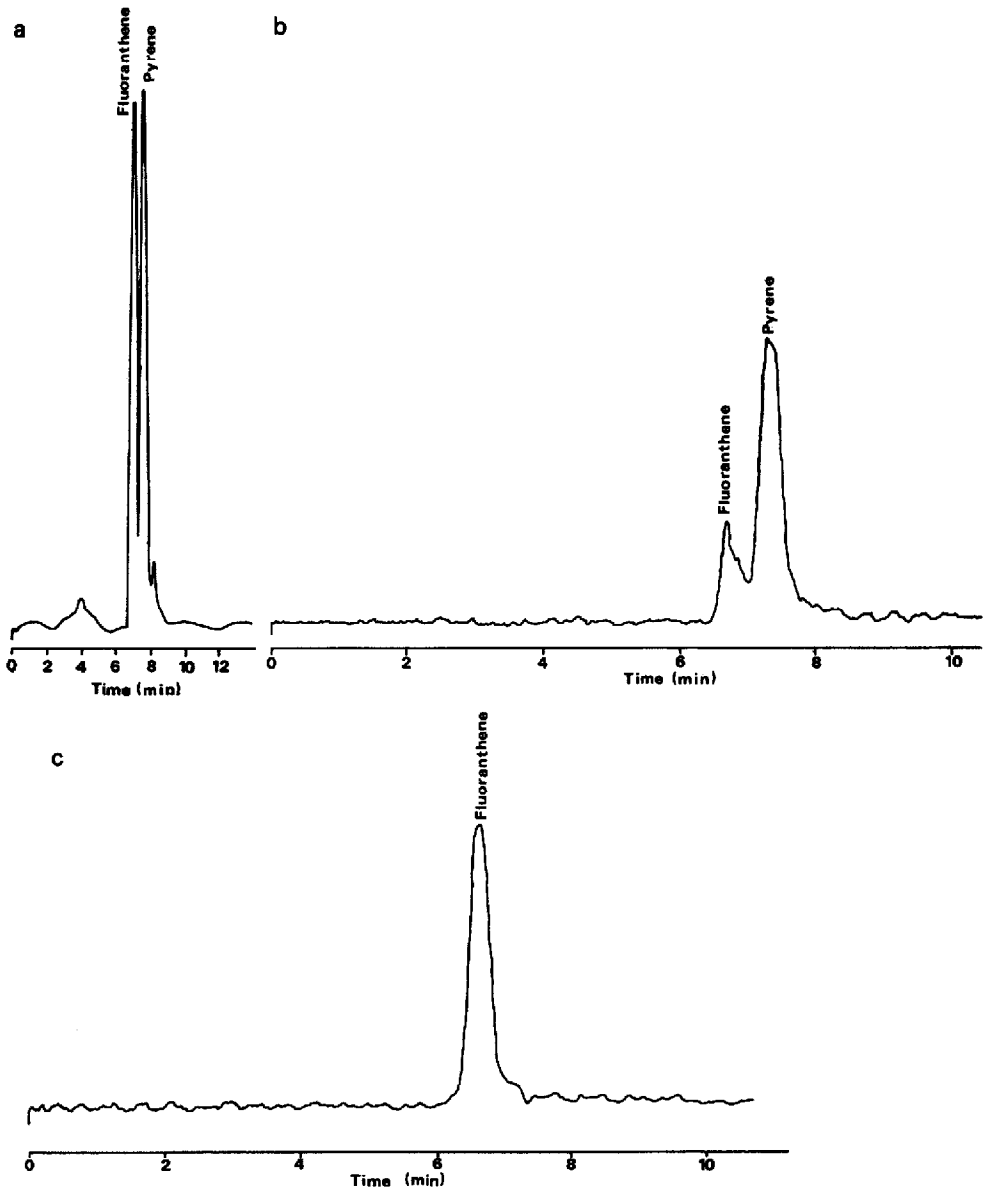

Fig. 3. Chromatograms showing the incomplete separation of fluoranthene and pyrene with methanol as a mobile phase. (a) UV absorption detection. (b) SSRTP detection using 343/585 nm as measurement wavelengths. (c) Selective SSRTP detection of fluoranthene employing 365/545 nm as measurement wavelengths. Sample size: 200 ng of each compound, injected volume 20 μl. Flow-rate: 0.443 ml/min.

select the best wavelength for each analyte. If overlapped compounds reach the detector, the selection of an appropriate detection wavelength for a single compound is often difficult due to the normally broad absorbance bands.

Even though broad molecular phosphorescence bands commonly overlap, it is unusual for different compounds to have coincident spectra in such a way that neither excitation nor emission wavelengths can be selected to measure a single component of the mixture. This unique feature of SSRTP is responsible for the high selectivity of the technique[10,12,15,16]. Fig. 3 shows the UV chromatogram of a mixture containing fluoranthene and pyrene along with the respective SSRTP chromatograms. The chromatographic parameters of fluoranthene are listed in Table I. From the UV chromatogram (Fig. 3a), it can be seen that complete resolution was not achieved by

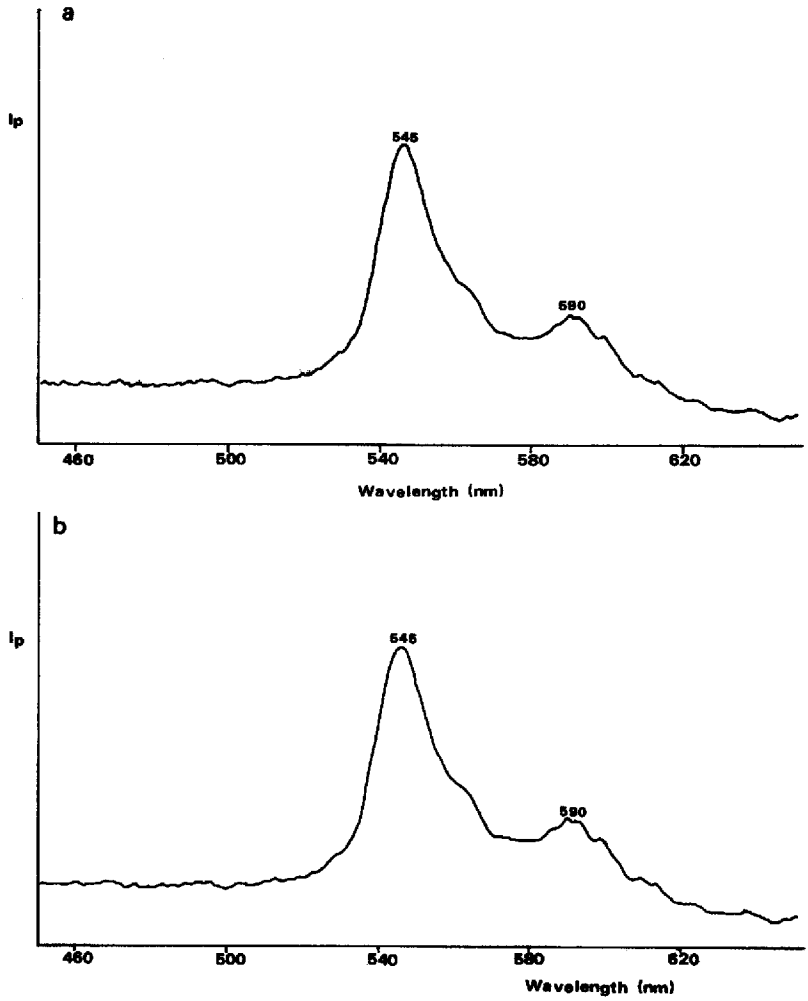

Fig. 4. (a) RTP emission (I_p) spectrum of the first eluted compound using the maximum excitation wavelength of fluoranthene (365 nm). (b) RTP emission spectrum of fluoranthene obtained under the same experimental conditions as (a).

using pure methanol as a mobile phase. Although the UV-absorption detection of either component is possible at these experimental condition, the example is useful to illustrate the selectivity of the SSRTP detector. By exciting at 343 nm and registering the phosporescent emission at 585 nm, the SSRTP chromatogram showed the presence of both solutes (Fig. 3b). When the detection wavelengths were shifted to the maximum excitation and emission of fluoranthene, the phosphorescence of pyrene was no longer registered (Fig. 3c) and the single detection of fluoranthene was possible without the presence of pyrene in the chromatogram. Further identification of fluoranthene was then performed by stopping the filter paper strip at the maximum phosphorescent intensity of the first eluted compound and comparing its emission spectrum with the corresponding spectrum of fluoranthene (see Fig. 4). The spectral identification of pyrene was done in a similar way. The substrate was stopped at the maximum phosphorescent intensity of the second eluted compound, and the emission spectrum was registered at the maximum excitation wavelength of pyrene. A spectrum with the same characteristics of the emission spectrum of pure pyrene was obtained (Fig. 5). Spectral identification was also possible when both compounds were present at the same level of concentration on the substrate. By stopping the filter paper strip at the maximum phosphorescence intensity detected between the elution of the compounds, and irradiating the substrate at the maximum excitation wavelength of pyrene, the presence of both analytes was confirmed by registering two phosphorescence bands with emission maxima at 545 nm (fluoranthene) and 592 nm (pyrene) (see Fig. 6a). When the substrate was irradiated at the maximum excitation wavelength of fluoranthene, the phosphorescence emission of pyrene was no longer detected (Fig. 6b). By comparing Figs. 5 and 6a, it can be noticed that even a weak phosphor like pyrene could be spectrally identified in the presence of similar concentrations of a strong phosphor like fluoranthene.

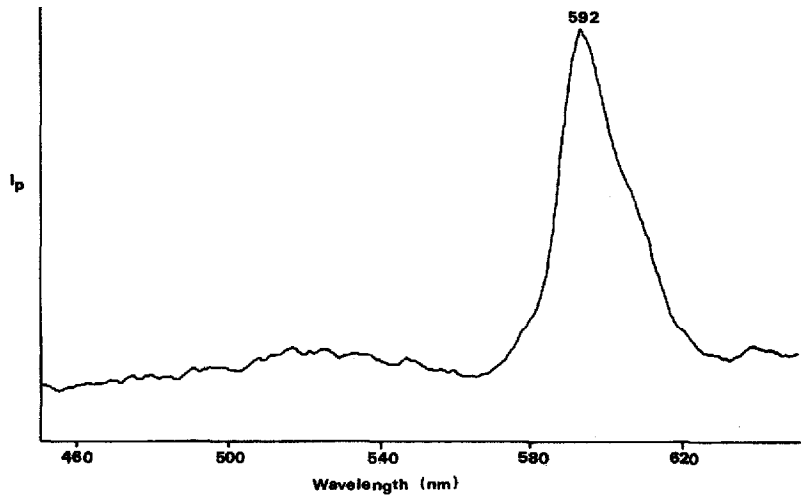

Fig. 5. RTP emission (I_p) of the second eluted compound registered at the maximum excitation wavelength of pyrene (343 nm).

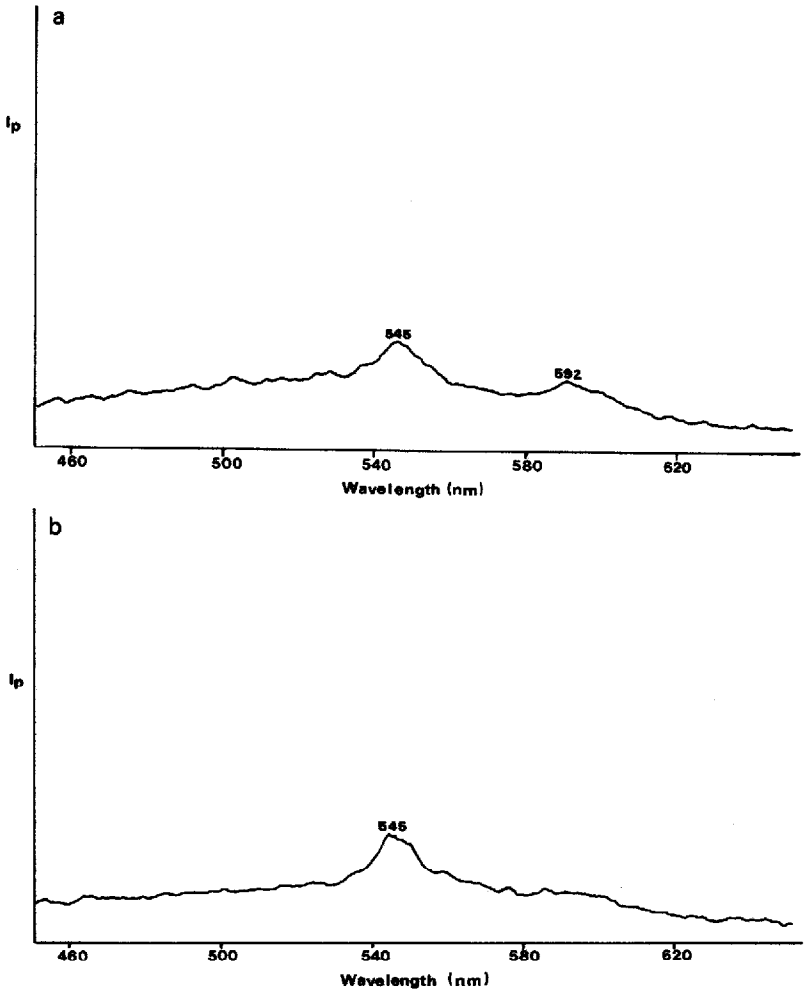

Fig. 6. RTP emission (I_p) spectra of the substrate registered at the minimum phosphorescence intensity observed between the elution of the two compounds. (a) When excited at 343 nm, two emission bands showed the presence of both compounds, fluoranthene (545 nm) and pyrene (592 nm). (b) When exited at 365 nm, only the phosphorescence emission of fluoranthene was observed.

CONCLUSIONS

In this report, we have shown the feasibility of using SSRTP as a detection technique for HPLC. By using the two nebulizer automatic system, it was possible to perform HPLC analysis in a continuous mode. The proposed detector has been evaluated by comparing its performance to the classical UV-absorption detector under the same experimental conditions. A 15% efficiency loss was observed in the SSRTP chromatograms mainly due to the UV-absorption–SSRTP detector connecting tubing (16 μl), nebulizer and moving filter paper strip. By reducing the length of the connecting tubing and using a micronebulizer, it should be possible to minimize the

observed band broadening. Comparable analytical figures of merit were obtained for both detection modes. By selecting the appropriate excitation and emission wavelengths of measurement, it was possible to characterize a single component of a mixture partially resolved by HPLC. This special feature makes the SSRTP detector a useful tool for liquid chromatographic operations. Complex systems can be separated into simpler ones, and the individual components of a mixture can be selectivily determined by choosing the appropriate set of measurement wavelengths. The versatility of the new detector permitted identification of compounds either from the retention times or from their RTP spectral characteristics, showing its potentiality for qualitative purposes. The greatest versatility, however, would result with the use of an intensified photodiode array detector. This instrument would allow phosphorescence spectra to be recorded as the peaks elute permitting "impure" probes to be resolved into individual components. We also believe that the SSRTP detector could be very useful in areas such as forensic science, where a permanent record of the analyzed samples would be extremely helpful. Compounds adsorbed on solid substrates and stored under a nitrogen atmosphere for several weeks have presented constant phosphorescence intensity[23,24]. This offers the possibility of identifying the constituents of a stored sample at any required time. By knowing the position of every compound on the filter paper strip, a new chromatogram (phosphorescence *versus* distance) can be obtained. If further identification is necessary, the substrate can then be stopped at the maximum phosphorescence intensity of every compound to run its excitation and emission spectra. Additional work, however, is necessary in the future to optimize the storing conditions for compounds of forensic interest.

ACKNOWLEDGEMENTS

A. D. C. would like to thank the "Coordenacao de Aperfeicoamento de Nivel Superior–Capes" for the grant which is making possible his stay in Gainesville and the University of Brasilia (Brazil) for permission to leave.

This work was supported by NIH ROI-GM 11 373-26.

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