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Fluorescence detection in liquid chromatography with an intensified diode-array detector

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ABSTRACT

The capabilities of full fluorescence spectral detection for the analysis of polyaromatic hydrocarbons after a liquid chromatographic separation were evaluated. The limits of detection and the linear dynamic range were determined for three test compounds, benzo[*a*]pyrene, perylene and anthracene. With the instrument used, detection limits in the micromolar concentration range (*ca.* 100 ng injected), and a linear dynamic range of at least three orders of magnitude were observed. In addition, the pair of isomers 1- and 2-aminoanthracene, which are incompletely resolved both spectroscopically and chromatographically, were studied. Finally, a commercially available mixture of polyaromatic hydrocarbons was characterized, and it was possible to resolve the chromatographically unresolved pair of isomers benzo[*a*]anthracene and chrysene. The data analysis methods used included Kalman filter-based methods for adaptive subtraction of background responses, shift correction and linear regression analysis of overlapped responses. The results indicate that full fluorescence spectral detection in liquid chromatography should be an increasingly useful technique.

INTRODUCTION

Fluorescence spectroscopy is one of the most sensitive and inherently selective methods available for liquid chromatographic (LC) detection. Two of the most important applications of fluorescence detection include biomedical and environmental separations [1–4]. In environmental studies, the greatest attention has been paid to polycyclic aromatic hydrocarbons (PAHs), which are on the US Environmental Protection Agency's priority pollutant list [5]. The PAHs fluoresce strongly and are relatively non-volatile, therefore LC with fluorescence detection is one of the best quantitative separation methods available for their analysis.

Most of the work concerned with the fluorescence detection of LC effluents has been conducted using first-order fluorescence detectors, *i.e.*, that supply first-order data (data with one independent variable, *e.g.*, retention time). First-order fluorescence is usually done at a fixed excitation wavelength and either the total fluorescence intensity or a selected emission wavelength is monitored. Although first-order detectors are simple, reliable and inexpensive, they can suffer from problems due to variable retention times and overlapped peaks. Both of these problems can be minimized, but rarely eliminated, with first-order detectors.

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Second-order detectors can alleviate both problems and provide much more information about a mixture [6]. Second-order detectors produce data as a function of two independent variables, in this instance elution time and emission wavelength. Because of the advantages of using second-order detectors, much interest and attention has been devoted to them in the past 10 years. Some of the better known second-order systems include LC–UV–VIS with photodiode-array-based detection [7,8], LC–mass spectrometry [9] and LC–Fourier transform infrared spectrometry [10]. Second-order fluorescence detectors were developed in the 1970s and 1980s but the data analysis methods required to analyze the large amounts of data were not well developed [11,12]. Since then, little work concerning second-order fluorescence detectors has been conducted, but in the same period array detector technology and data analysis methods have advanced significantly. Therefore, second-order fluorescence detection is now a viable technique and should recieve more attention. Recently, papers have appeared that reflect this resurgence of interest [13,14]. The second-order detector examined in this work is based on a commercial spectrofluorimeter with modified optics and an intensified photodiode array (PDA) replacing the photomultiplier tube. A more detailed description of the modifications to the spectrofluorimeter is given below.

Fluorescence detection of LC effluents has several problems at the chemical level that must be addressed in order for this technique to become more widely accepted. These problems include fluorescence intensity fluctuations, spectral distortions, spectral response shifts, scattered light intereferences, variable background contributions and the qualitative and quantitative analysis of overlapped spectral and chromatographic data. These problems are examined here because they seem to represent the limitations to the acceptance of photodiode-array fluorescence detectors for routine analyses. We show that our detector is capable of reasonable detection limits and a good linear dynamic range. Further, methods for the analysis of chromatograms and the component fluorescence spectra that should be applicable to any second-order fluorescence detection method are examined. Finally, some of the advantages of the second-order fluorescence detection method are discussed.

EXPERIMENTAL

Chromatography

The chromatographic portion of the apparatus consisted of two Rainin (Woburn, MA, USA) HP pumps, each equipped with a 5-ml pump head. A Rainin pressure monitor and zero-dead-volume solvent-mixing chamber were also used. Injection was accomplished with a Rheodyne six-port injection valve with a 20-μl sample loop and an injection event marker. The columns used included two 15 cm × 1 cm I.D. Microsorb ODS C_{18} columns and a Spherisorb 10 cm × 1 cm I.D. ODS2 C_{18} column. All columns had 5-μm packing material.

The solvents used included methanol, acetonitrile and water. Methanol and acetonitrile were of Omnisolv (EM Scientific) spectroscopic grade and were used as received. Water was de-ionized, filtered through a 10-μm membrane and degassed with a helium sparge for 30 min before each run. The sample loop was rinsed with 500 μl of acetonitrile between injections. A sample volume of at least ten times the sample loop volume was injected each time.

All of the neat PAHs were obtained from Foxboro/Analabs (North Haven CT, USA) and were at least 98% pure. Aminoanthracenes were also purchased from Foxboro/Analabs and were of at least 90% purity and received no further purification. Standard solutions were made using degassed solvents. A standard mixture was purchased from Alltech (Deerfield, IL, USA) and contained anthracene (ANT), fluoranthene (FLA), phenanthrene (PHA), pyrene (PYR), chrysene (CHR) and benzo[*a*]anthracene (BAA) at about 10 ppm levels in toluene. This mixture was used as received.

Detector and optics

Our laboratory-constructed detector is similar to that described by Jadamec *et al.* [11]. The detector consists of a modified Farrand MK1 spectrofluorimeter with a 200-W Hg–Xe arc lamp as a source. The lamp housing was updated with the addition of an internal fan that stabilized the arc. The housing modifications were obtained from Optical Devices/Farrand (Elmsford, NY, USA). The fluorimeter was further modified by the replacement of the 28 000 line/in. gratings with 14 000 line/in. gratings obtained from Edmund Scientific. The excitation grating was blazed for maximum throughput in the first order at 250 nm and the emission grating was blazed at 500 nm. The lower resolution grating causes a larger portion of the first-order spectrum diffracted from the grating to be imaged on the diode array. The slits that were used on the excitation monochromator were both 20 nm, and the entrance slit to the emission polychromator was 1 nm. The cuvette holder in the sample compartment was replaced by an 8-μl flow cell purchased from Farrand. The flow cell was square and included focusing optics. In order to mount the diode array, a 1-in. diameter hole was drilled in the monochromator where the photomultiplier tube had previously been located. An adaptor plate was fabricated, mounted on the exterior of the monochromator and the diode array was attached to it. The final 90°C mirror in the Czerny–Turner monochromator was replaced with one that was wider so that a 200-nm portion of the emission spectrum would fall on the array. The intensified diode-array system was purchased from Tracor Northern and consisted of a TN-6122a intensified diode array, a TN-6600 Intelligent Interface and data acquisition and control software called ISIS. The TN-6122a is an electrothermally cooled PDA and therefore no additional cooling is necessary. The diode array can collect a spectrum every 0.1 s. Longer integration times reduce the noise and increase the signal proportionally. However, if the integration time is too long, the background signal will saturate the array. Therefore, a balance between the intensifier setting and integration time is necessary. In this instance an integration time of 1.1 s was found to be appropriate.

Control and acquisition

The computer that was used to control the LC pumps and the diode array was a PC-type 80386-based clone with a 320-megabyte hard disk, a Metrabyte Dash 16 A/D card, dual RS-422 ports and the TN-6600 interface card. All of the data collection and control programs were designed to run under the Microsoft Windows operating environment. All of the data analysis programs operate under DOS and were written using the PASCAL programming language. The program that controls the LC pumps was written with the Microsoft C compiler and the Microsoft Windows

software development kit. The Windows environment allowed both ISIS and the pump control program to run concurrently so that data could be collected as gradient control was being conducted. The injection was triggered with an injection event marker as a binary on–off switch through a parallel input on the A/D board for the gradient program and through the trigger input on the TN-6600 interface for the ISIS software. The pumps were controlled using the Rainin ASCII pump control protocol via the RS-422 ports.

Experimental conditions

The limit of detection (LOD) and linear dynamic range (LDR) calculations were based on experiments involving benzo[*a*]pyrene (B[*a*]P), perylene (PER) and anthracene (ANT). A standard solution with a concentration of 100 μM was prepared in acetonitrile for each of the PAHs. The standard solution was then diluted to the concentrations shown in Table I. An excitation wavelength of 300 nm was used for these studies. Each of the chromatograms was obtained under isocratic conditions with 100% acetonitrile at a flow-rate of 1.82 ml/min.

The second series of experiments involved 1- and 2-aminoanthracene (1-AANT and 2-AANT, respectively). A solution in acetonitrile was prepared for each of the aminoanthracenes at a concentration of 10 μM. An excitation wavelength of 300 nm was used. Five experiments were conducted. The first two involved obtaining a spectrum of each of the standard solutions of the AANTs injected directly into the flow cell. The intensifier was set so that the 2-AANT was on-scale at this concentration. The intensifier was not changed for the remainder of this series of experiments. The spectra of the pure standard solutions are called the model spectra. Then the standard solutions were injected onto the column using the following gradient conditions: flow-rate, 1.82 ml/min; linear gradient from acetonitrile–water (50:50) to 100% acetonitrile at 2%/min. The chromatograms of the standard solutions are called the model chromatograms. A 50:50 mixture of the AANT isomers was prepared and separated using the above conditions. This chromatogram is the unknown chromatogram.

The next series of experiments involved the separation and identification of a standard mixture of PAHs containing *ca.* 10 ppm of each of the PAH components. The same experimental conditions as described above were used for this analysis. Solutions of pure BAA, CHR, PYR, ANT, FLA and PHA in acetonitrile at *ca.* 50 μM were used as standards.

RESULTS AND DISCUSSION

As with any new detector design or detection method, it is necessary first to identify the capabilities of the method. One approach for instrument characterization is to determine the LDR and the LOD. Using these measures, it is possible to compare different instrument systems. Therefore, the first experiments that were conducted with the above detector included both LOD and LDR measurements. These measurements were done using B[*a*]P, PER and ANT, which were chosen for two reasons: first, they have been used extensively by workers in the fluorescence field for these types of measurements; and second, the fluorescence emission of each of the compounds falls on a different portion of the diode array, thereby allowing the LOD and LDR of the method to be determined over the entire PDA detector spectral range.

This examiniation of the PDA performance was done in order to identify any anomalous behavior over the array. The calibration data for these analytes are given in Table I. The PER data were obtained on a different day to the other two compounds. The concentrations reflect the concentration of the injected solution, not the concentration at the detector. The percentage deviation between the experimental data and the linear least-squares fit of those data is shown in Fig. 1. These residuals show no significant trends over three orders of magnitude, for a single intensifier setting. If more than one setting were used, the LDR could be increased significantly. The curvature at the upper limit of these curves comes from concentration quenching, which commonly occurs in fluorescence detection.

The calibration results for each of these fluorophores are shown in Table II. The LODs can be approximated from the standard deviations of the linear regression intercepts. This gives an estimated LOD for each of the three compounds of *ca.* 1 μM. If a different intensifier setting was used or if several replicates were run close to the detection limit, it would be possible to obtain a lower LOD. For all of these studies the intensifier setting was held constant because the changes in signal intensity brought about by changes in the intensifier setting appear to be non-linear.

TABLE I

CALIBRATION DATA FOR B[*a*]P, ANT AND PER

PAH	Concentration (μM)	Peak height (10^3 counts)
B[*a*]P[a]	100	2893
	50.0	1702
	25.0	873
	12.5	441
	6.25	250
	3.15	147
	1.56	88.0
	0.78	75.0
ANT[b]	100	3114
	50.0	1547
	25.0	858
	12.5	475
	6.25	266
	3.15	147
	1.56	99.0
	0.78	75.1
PER[c]	100	3075
	50.0	1580
	33.3	990
	10.0	347
	5.00	181
	1.00	75.1
	0.10	51.0

[a] Intensifier setting for B[*a*]P at 750.
[b] Intensifier setting for ANT at 850.
[c] Intensifier setting for PER at 800.

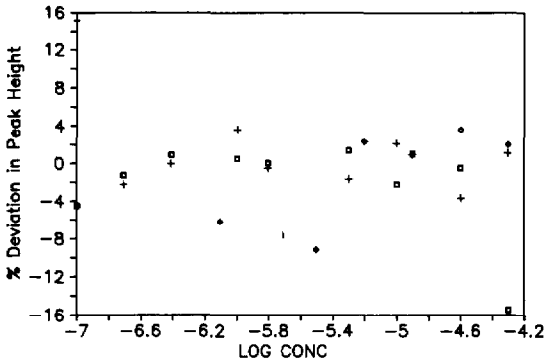

Fig. 1. Percentage deviation in peak height *versus* the log (concentration) for (□) B[*a*]P, (+) ANT and (◇) PER.

The next series of experiments involved 1- and 2-aminoanthracene (1- and 2-AANT), which were chosen because they are strong fluorophores and known carcinogens [15], and have similar spectral and chromatographic characteristics. In addition, the fluorescence efficiency of 1-AANT is an order of magnitude smaller than that of its isomer with 300 nm excitation. The emission spectra of the isomers are shown in Fig. 2. Further, it was found that both isomers undergo solvent-induced spectral response shifts. All of these factors combine to make this system very difficult to analyze using traditional approaches. The data obtained from this second-order detector were analyzed by fitting each of the spectra within the chromatographic peak envelope. The relative concentrations were summed to obtain the overall concentration estimates for each of the two isomers.

The procedure for the analysis of the 50:50 mixture of the aminoanthracene isomers was as follows. First, spectra of pure standard solutions of the aminoanthracenes in acetonitrile were obtained (Fig. 2). These spectra were background corrected using an algorithm developed previously in our laboratory [16], based on the adaptive Kalman filter fit of a measured model background of acetonitrile. In this instance, as the background was relatively featureless, the zeroth derivative was used in the fitting procedure [17]. These background-corrected spectra were used as models for the spectra obtained from a gradient run with a mixture of the isomers. A Kalman filter-based shift correction algorithm developed in our laboratory was applied to determine the spectral shifts [18,19]. The parameters used for this fit were a cut-off

TABLE II

CALIBRATION RESULTS FOR B[*a*]P, ANT AND PER

PAH	Slope (10^3 counts/μM)	Intercept (10^3 counts)
B[*a*]P	29.0 (0.9)	86 (38)
ANT	30.4 (0.3)	65 (12)
PER	30.3 (0.3)	35 (14)

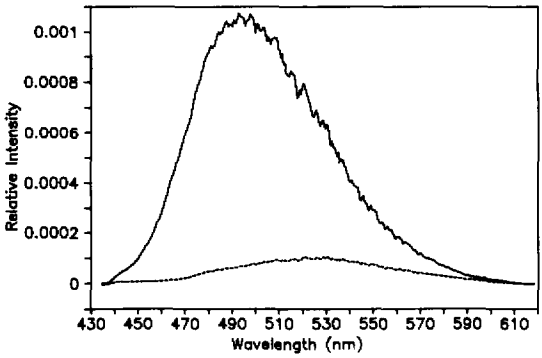

Fig. 2. Fluorescence spectra of (dashed line) 1-aminoanthracene and (solid line) 2-aminoanthracene in acetonitrile.

point of 0.5 and a measurement variance of 1×10^{-8}. Although the results were variable, especially for the weaker fluorophore, 1-AANT, they indicated a +4 nm shift for 1-AANT and a +9 nm shift for the 2-AANT, obtained from the most intense spectra in the chromatographic profile. A red shift of this magnitude is reasonable, as the model spectra were obtained in 100% acetonitrile and the aminoanthracenes elute in a solvent composition of approximately 60:40 acetonitrile–water. All spectra within the chromatographic envelope were then fitted with the regular Kalman filter to this shifted model. The extracted chromatograms are shown in Fig. 3. The chromatogram for 1-AANT shows more noise, as the model for this component had larger noise contributions. The areas under the two peaks are very similar (0.41 and 0.39 for 1- and 2-AANT, respectively). The retention times for these two peaks were identical. As the model used was based on spectra obtained from standard solutions injected directly into the flow cell, a correction factor is required to account for the number of moles flowing through the cell during the integration time. Calculations show that an average of 33.4 μl flows through the cell during this period. Correction by a factor of

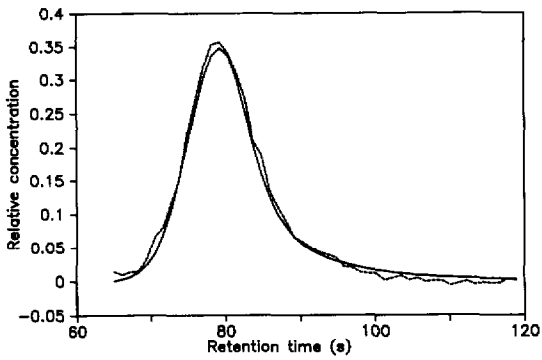

Fig. 3. Extracted chromatograms of (dashed line) 1-aminoanthracene and (solid line) 2-aminoanthracene.

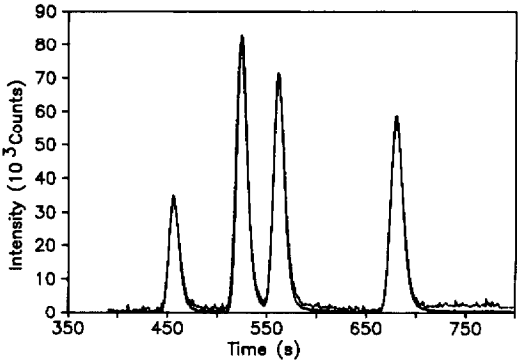

Fig. 4. (Dashed line) chromatogram of the PAH mixture and (solid line) the Kalman fit of the chromatogram. The first peak is ANT, the second is FLA, the third is PYR and the fourth contains both BAA and CHR.

33.4/20 (where 20 μl is the chromatographic injection volume) should yield corrected relative concentrations of 0.68 and 0.65 for 1-AANT and 2-AANT, respectively. The theoretical relative concentrations should be 0.5 for both isomers. Our concentration estimation errors are probably due to a change in the fluorescence efficiency in the chromatographic eluent in comparison with pure acetonitrile. We are currently investigating the reasons for this discrepancy. Note that other methods, such as rank annihilation, could not be used to analyze these data, for two main reasons: first, the spectral shifts would cause majors errors in this algorithm, and second, the two species show no chromatographic resolution, which is a requirement for successful application of the rank annihilation algorithm

Using the method outlined above, a mixture of five PAHs was separated and the concentrations were estimated. The chromatogram for the PAH mixture is shown in Fig. 4. Under these conditions, BAA and CHR co-elute. This chromatogram was fitted using pure component chromatograms for each of the five components using the shift correction technique, and the resulting fit is shown in Fig. 4. The results for the concentration estimates are given in Table III. The method used to fit the aminoanthracene spectra was also used to fit this PAH mixture. The concentration estimates obtained from fitting the spectral data are also shown in Table III. We could

TABLE III

CONCENTRATION ESTIMATES FOR PAH MIXTURE

PAH	Concentration estimate (μM)	
	Chromatographic fit	Spectral fit
ANT	7.2	7.6
FLA	3.7	5.6
PYR	5.1	14.1
BAA	5.0	6.0
CHR	6.7	11.6

not compare these results with the true values, as we found that the standard mixture was not prepared quantitatively by the manufacturer. Although the concentration estimates using the two different methods are not in complete agreement, these results help to illustrate the importance of developing careful data analysis strategies for fluorescence spectral detection in liquid chromatography. More recent studies have found that the most probable cause for this lack of agreement between the two methods is due to the irreproducibility of the gradient separation conditions.

CONCLUSIONS

This work shows that it is possible to build a relatively inexpensive second-order fluorescence detector that yields acceptable detection limits. It also shows that there is a definite need for data analysis strategies that use second-order data more effectively, especially for fluorescence spectroscopy, where several chemical and physical factors can affect the accuracy of the results. Here, a combination of methods for background subtraction, shift correction and peak resolution were used to analyze mixtures of PAHs. We can effectively correct for solvent-dependent spectral shifts and also for retention time variations in gradient methods. The chromatographic profiles of severely overlapped species can be extracted based on analysis of the emission spectral data. Using appropriate data analysis methods it should be possible to identify and quantify fluorescence signals without an extensive calibration procedure. Further work will be focused on understanding the factors affecting accurate quantification in this system.

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