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This Research Contribution is in Commemoration of the Life and Science of I. M. Kolthoff (1894-1993).

On-the-Fly Fluorescence Lifetime Detection in HPLC Using a Multiharmonic Fourier Transform Phase-Modulation Spectrofluorometer

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Frequency-domain fluorescence lifetime detection has been demonstrated recently as a technique for detecting and resolving overlapping peaks on-the-fly in reversed-phase high-performance liquid chromatography (RP-HPLC). However, instrument limitations necessitate multiple injections of the same sample for peak resolution. The introduction of a commercially available multiharmonic Fourier transform spectrofluorometer (MHF) eliminates this problem and offers other advantages as well. The MHF data acquired on-the-fly during a single chromatographic run contain all of the multifrequency information needed to determine lifetimes and to indicate and resolve overlapping peaks at intervals as short as several milliseconds. This allows essentially continuous monitoring of both lifetime and intensity during chromatographic elution. This approach to the addition of lifetime in fluorescence detection increases the information content of chromatographic-based analysis without increasing the time per analysis. This paper presents the first results obtained using MHF detection, including measurements of single components and simple mixtures. Optimization of instrumental and experimental conditions is discussed, including the nature of the raw data and the procedures that were developed to extract and process the necessary lifetime and intensity information.

INTRODUCTION

Sensitive and selective detection in HPLC can be achieved using fluorescence techniques, which are applicable to both intrinsically fluorescent and fluorescent-derivatized compounds. However, traditional steady-state approaches based on measurements of intensity at one or more wavelengths have several limitations. Detection at only one or a few wavelengths may be inadequate to resolve overlapping peaks, while the use of array detection to collect an entire spectrum can decrease sensitivity as a result of dispersion. Furthermore, the techniques may not be readily able to detect the presence of minor components or matrix effects. Therefore, it is desirable to explore new approaches to fluorescence detection that are capable of identifying and resolving overlapping chromatographic peaks as well as indicating the presence of impurities and matrix effects. Moreover, the techniques should minimize assumptions regarding chromatographic peak shape and the identity, properties, or spectral characteristics of the analytes.

This paper describes an approach that moves toward these goals through the addition of fluorescence lifetime to spectral information to increase the dimensionality and information content of the chromatographic data. Fluorescence lifetime detection has been explored previously using both time-domain and frequency-domain techniques for analysis of polycyclic aromatic hydrocarbon (PAH) compounds. In an early time-domain study, fluorescence intensity chromatograms were measured using various delay times (0–40 ns) following the excitation pulse from an N_2 laser-pumped dye laser.[1] In such a system, a compromise exists in choice of the optimal delay time, which should be long enough for adequate reduction of prompt background signals but short enough to obtain sufficient analyte signal, which itself decays according to the fluorescence lifetime of the analyte. Collection of several chromatograms, each at a different delay time, was used in order to discriminate among different analytes on the basis of differences among their fluorescence lifetimes. However, the lifetime resolution in the study was limited by the pulse width of 10 ns, which was sufficient only to distinguish between groups of shorter lived and longer lived signals but could not accomplish resolution of individual components within the groups, nor were actual lifetimes of individual components determined. A later study used the same approach but with a subnanosecond dye laser system which improved the time resolution to the nanosecond range but still required one chromatogram per delay time.[2] Measurements at multiple delay times in a single chromatogram were achieved in a later study[3] by continuous monitoring of the signal resulting from pulsed excitation at a repetition rate of 20–30 Hz as the compounds were eluted. The chromatogram for a given delay time could then be constructed from the appropriate point following each pulse in the original chromatogram in order to obtain decay curves for the various chromatographic peaks. However, the signal-to-noise ratio was not adequate for resolution of coeluting peaks or recovery of the individual lifetimes of coeluting analytes from multiexponential curves.

Another approach was taken to time-domain lifetime detection with pulsed laser excitation, in which the PMT anode current was split in half and one of the halves was

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delayed by 10 ns before being sent to its channel of a two-channel oscilloscope.[4] Two intensity chromatograms are thus obtained representing two different delay times that are 10 ns apart. A ratio of the two chromatograms provides a "ratiogram" in which each lifetime is identified by a unique, concentration-independent ratio. A changing ratio across a peak is indicative of coelution. Indication and resolution of severely overlapped peaks was problematic and could only sometimes be solved by the use of detection at a second wavelength.

On-the-fly frequency-domain lifetime detection has been highly successful in indicating and resolving overlapping peaks in HPLC of synthetic mixtures of PAHs.[5-8] Previous to the present work, however, the technique was hindered by limitations of the frequency-domain spectrofluorometers, which could only collect data at one frequency per chromatogram and therefore required multiple injections of the sample. The resulting chromatograms had to be perfectly overlaid and the data combined at each point along the chromatographic peaks.

With the recent introduction of a commercially available multiharmonic Fourier transform spectrofluorometer (MHF), all of the frequency information is collected on-the-fly during a single chromatographic run, and multiple injections are no longer needed. This paper describes the first use of the MHF for on-the-fly fluorescence lifetime detection and evaluates its performance for reversed-phase HPLC of one- and two-component systems of PAHs. Unlike previous detection in both the time domain and frequency domain, MHF detection is sufficiently information-rich to provide on-the-fly lifetime determination, indication of peak overlap, and resolution of coeluting peaks during a single chromatographic run.

THEORY

The theory of phase-modulation fluorescence lifetime measurements has been treated extensively in the literature[9] and is only briefly discussed here. The sample is excited with an excitation beam that is intensity-modulated at a linear frequency in the megahertz range, producing a fluorescence signal, $F(t)$, that is modulated at the same frequency but phase-shifted by angle ϕ and demodulated by a factor m relative to the exciting light:

$$F(t) = A'(1 + m_{ex} m \sin(\omega t - \phi)) \quad (1)$$

where A' is the wavelength-dependent, steady-state (dc) emission intensity, m_{ex} is the modulation depth of the exciting light (i.e., the ratio of the ac amplitude to the dc intensity), m is the demodulation, which is equal to the modulation depth of the emitted light divided by m_{ex}, and ω is the angular modulation frequency (equal to $2\pi f$, where f is the linear frequency in hertz).

The fluorescence lifetime of the sample can be calculated independently from the observed phase shift and demodulation:

$$\tau_{p,obs} = \frac{\tan \phi}{\omega} \quad (2)$$

and

$$\tau_{m,obs} = \frac{1}{\omega}\left(\frac{1}{m^2} - 1\right)^{1/2} \quad (3)$$

If $\tau_{m,obs} = \tau_{p,obs}$, then a homogeneous system containing a single lifetime component is indicated. If $\tau_{m,obs} > \tau_{p,obs}$, then ground-state heterogeneity (i.e., the presence of more than one fluorescence lifetime component) is indicated. Heterogeneity analysis of such a system is achieved by using measurements of ϕ and m at multiple modulation frequencies to resolve the fluorescence lifetime and fractional intensity contribution of each component in the system.[10,11]

EXPERIMENTAL SECTION

Benzo[*a*]pyrene and benzo[*k*]fluoranthene (B*a*P and B*k*F, respectively; each 99%, AccuStandard), benzo[*b*]fluoranthene (B*b*F; 99%, Aldrich), and 9-anthracenecarbonitrile (9AC; 98%, Lancaster Synthesis) were used as received. Acetonitrile (HPLC grade, Burdick & Jackson) and water (HPLC grade, in laboratory Modulab Type I water purification system), which were used for sample preparation and as the HPLC mobile phase (80% CH_3CN in H_2O), were purified further by vacuum filtration as suggested by the HPLC manufacturer. Stock solutions (5×10^{-4} M) of the fluorescent compounds were prepared in mobile phase, stored in amber bottles with Teflon-lined caps, and refrigerated to prevent degradation. Aliquots were removed to prepare solutions in a concentration range of 10^{-9} –10^{-5} M. Scattering solutions were prepared by addition of kaolin (Sigma) to water.

Steady-state and dynamic fluorescence measurements were made using the MHF (SLM Instruments, Inc.) with He–Cd laser excitation at 325 nm (LiCONiX Model 4240NB). A Pockels cell was used to electrooptically modulate the excitation beam intensity for dynamic-state measurements. The emission was passed through a combination of a 399-nm long-pass filter and a 600-nm short-pass filter (Oriel) and into a PMT for detection.

Flow experiments were performed by interfacing an HPLC system (Waters) to the MHF. Reversed-phase chromatography was performed on manually injected samples by a C-18 guard column and a Vydac 201-TP-B-5 packed 10 cm x 0.3 cm glass cartridge analytical column, designed specifically for separation of PAHs. The PAH solutions and mobile phase were not degassed or deoxygenated. Compounds were isocratically eluted from the analytical column, passed through the UV absorbance detector fixed at 254 nm, and routed through a four-port valve to a 20-μL flow cell with an 8-μL observation volume (Hellma cells) located in the thermostated sample chamber of the MHF. The four-port valve allowed the acquisition of reference measurements in the stopped-flow mode. The fluorescence emission signal, from which both intensity and lifetime information is derived, was collected on-the-fly by a Dell 325D computer.

On-the-fly fluorescence lifetime detection was performed in the Kinetic Lifetimes mode of the MHF. This mode allows virtually continuous collection of phase and modulation data at as many as 50 modulation frequencies simultaneously. Lifetime determinations and heterogeneity analysis of the multifrequency phase and modulation data were performed by a commercial software package (Globals Unlimited, Urbana, IL). The software uses nonlinear least-squares (NLLS) methods to calculate τ_{obs} by fitting the data to a one-component model and to resolve multiple components into their individual lifetimes and fractional intensity contributions by fitting the data to two-component or higher order models. The software also allows global linking of data files. Goodness of fit is indicated by the reduced chi-squared (χ^2_R) value.[11]

RESULTS AND DISCUSSION

Lifetime Data Acquisition. A more detailed explanation of dynamic-state data acquisition by the MHF is warranted in order to understand the nature of on-the-fly data acqui-

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sition.[12] The exciting light intensity is electrooptically modulated by a Pockels cell at the generated harmonics of a designated base frequency, F. The instrument provides base frequencies between 4 and 7 MHz and a frequency range that extends up to approximately 250 MHz, depending upon the base frequency. This upper limit is imposed by degradation of the signal-to-noise ratio at higher frequencies.

Since the excitation beam contains multiple frequencies (the base frequency and harmonics), the emission will contain the same multiple frequencies, but the signal at each frequency will be phase-shifted and demodulated as a function of both modulation frequency and fluorescence lifetime of the sample. The phase and modulation information is determined using the cross-correlation technique, in which a second, slightly offset, frequency, $F + \Delta F$, is phase-locked to the excitation frequency. The base cross-correlation frequency, ΔF, is selected by the user and can be as high as 1560 Hz. The harmonics of the signal of $F + \Delta F$ frequency are produced by a harmonic comb generator, as are the harmonics of the base signal of frequency F, and are used to modulate the gain of the PMT. Thus, if 4 MHz is the base frequency and 10 Hz is the base cross-correlation frequency, then the 10-Hz component carries the phase and modulation response of the sample to 4-MHz excitation, the 20-Hz component carries the information for an 8-MHz excitation frequency, and so on. The frequency information is then digitized and Fourier transformed so that phase and modulation values may be determined for each frequency.

Figure 1A shows raw data collected on-the-fly in real time for an eluting chromatographic peak, while Figure 1B shows the phase and modulation information resulting from the Fourier transform of the digitized signals represented in Figure 1A. Panels C and D of Figure 1 show a 1-s portion of the data in panels A and B on an expanded scale.

The operator chooses the total number of frequencies (i.e., base frequency + harmonics) at which data are to be collected. Subsequent data analysis can use the information at all of the frequencies or at some designated subset of the frequencies.

A study was done to find the optimum base frequency and base cross-correlation frequency for detection of the polycyclic aromatic compounds. Phase and modulation data were collected for B*a*P at the various combinations of five base frequencies and 10 cross-correlation frequencies. Best reproducibility for the lifetime fits, and the most random distribution of residuals, were obtained using a base frequency of 4.1 MHz and cross-correlation frequencies of 5.208 and 10.417 Hz.

The cross-correlation frequency is important in the Kinetic Lifetimes mode since its reciprocal is the interval at which the signal is sampled (Figure 1C), and thus establishes the number of data points collected per unit time. The large range of cross-correlation frequencies available offers flexibility in the amount of averaging performed. For example, if a cross-correlation frequency of 5, 50, or 100 Hz is used, the signal is sampled every 0.2, 0.02, or 0.01 s, respectively, resulting in an average of 5, 50, or 100 measurements per 1-s window being analyzed. Unless otherwise indicated, a base cross-correlation frequency of 10.417 Hz was used in this work, resulting in measurements every 0.096 s during chromatographic elution. These measurements were then averaged to produce data points at 0.96-s intervals across the chromatographic peak. Therefore, each data point in the chromatogram is an average of 10 signal samplings.

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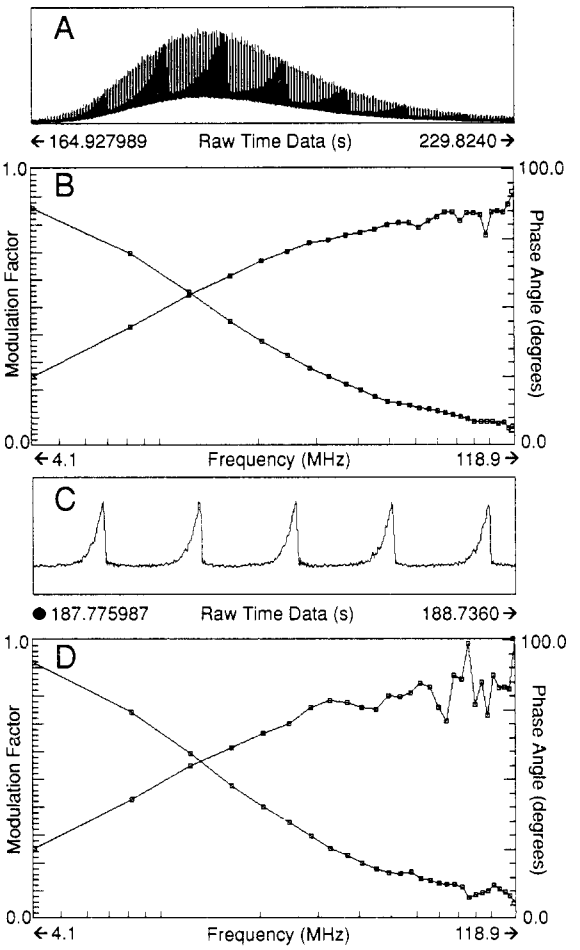

Figure 1. Raw lifetime data collected on-the-fly by the MHF for an eluting B*a*P peak: (A) total MHF signal (raw time data); (B) average phase and modulation for entire B*a*P peak shown in (A); (C) expansion of 1-s window of data in (A); (D) average phase and modulation for interval shown in (C).

The MHF software does not provide direct measurement of steady-state (dc) fluorescence intensity in the Kinetic Lifetimes mode. The dc information provides the fluorescence intensity chromatogram and is needed for retention information and resolution of heterogeneous peaks. Fortunately, since the MHF acquires and saves all raw time data in the Kinetic Lifetimes mode, it was possible to obtain the steady-state intensity information by writing software to integrate the raw time data. Thus, phase, modulation, and steady-state intensity information are all acquired from the same MHF signal collected on-the-fly during a single chromatographic run.

Experimental Considerations. Steady-state experiments were performed to investigate possible background contributions from the flow cell and solvents. Emission spectra of H_2O, CH_3CN, mobile phase, and the empty flow cell were collected using monochromator wavelength selection. Distinct Raman peaks at 361 and 367 nm were observed for CH_3CN and H_2O, respectively. The empty flow cell showed no significant fluorescence signal.[5]

During lifetime measurements, a small portion of the modulated excitation beam is split from the main beam and directed to a reference PMT, which monitors its phase and modulation in order to normalize the instrument response during reference and sample measurements. The remainder of the modulated excitation beam is directed into the thermostated sample chamber. In normal operation, the sample and lifetime reference solutions are located on a rotating turret and alternately measured. The lifetime of

the reference solution must be predetermined and entered in the instrumental setup parameters in order to calibrate the excitation phase (defined as 0°) and modulation (defined as 1). Alternating measurements of the sample and reference are made, and the phase and modulation lifetimes of the sample are calculated according to the following relationships:

$$\tau_{p,obs} = \frac{1}{\omega}[\tan \phi_s - \phi_r + \tan^{-1}(\omega\tau_r)] \quad (4)$$

and

$$\tau_{m,obs} = \frac{1}{\omega}\left[(1 + \omega^2\tau_r^2)\left(\frac{m_r}{m_s}\right)^2 - 1\right]^{1/2} \quad (5)$$

where τ_r is the predetermined reference lifetime (if the reference is a scattering solution, then τ_r is zero by definition and eqs 4 and 5 reduce to eqs 2 and 3, respectively), ϕ_r and ϕ_s are the observed phase shifts of the reference and sample solutions, and m_r and m_s are the observed demodulation factors of the reference and sample solutions.[8,13] 9AC was chosen as the reference fluorophore for this work. Its lifetime was determined to be 13.3 ± 0.2 ns versus a light scattering solution.

For chromatographic detection, a flow cell is placed in the sample holder, which precludes rotation of the turret. Therefore, the reference must be injected and detected separately in the flow scheme, instead of rapidly alternating the sample and reference measurements as is done in batch experiments. It was found that the most consistent and accurate results were obtained when the reference was measured under stopped-flow conditions. Therefore, all reference measurements were made in the stopped-flow mode.

In previous work on the single-frequency instrument,[8] it was found to be important to match the intensity of the reference to that of the sample at each point. The lifetime at each data point of the sample peak was determined by using the lifetime data from a point of identical intensity on the reference peak. Fortunately, intensity matching was found to be unnecessary for detection with the MHF instrument. Therefore, the reference-phase and modulation measurements were made under stopped-flow conditions at the apex of the peak of the reference compound.

Initial Studies of On-the-Fly Measurements. Fluorescence intensity and lifetime information were acquired on-the-fly for B*a*P in an attempt to reproduce previous work[1] using the new instrumental approach. A cross-correlation frequency of 5.208 Hz was used for this experiment. $\tau_{m,obs}$ and $\tau_{p,obs}$ were calculated at 0.96-s intervals across the B*a*P peak and should be equal since the peak corresponds to a single fluorescent component. However, fitting the data to a one-component model indicated $\tau_{m,obs} > \tau_{p,obs}$, or ground-state heterogeneity. Better fits were obtained when the data were analyzed with a two-component model. The second component was identified as scattered light from its negligible lifetime. The scattered light was attributed to edge effects since the widths of the laser beam and the flow cell window are the same (1 mm). Even slight misalignment would prevent a portion of the laser beam from entering the flow cell, resulting in the production of scattered light at the edge and loss of both modulation depth and light intensity. In order to alleviate these effects, a converging lens was placed in the light path just prior to the flow cell to further focus the beam into an area smaller than the flow cell window. This not only made proper alignment much easier to achieve, but also allowed for a decrease in the emission channel PMT high voltage, an indication that intensity was no longer being lost. The experiment was repeated with improved alignment and resulted in good agreement of $\tau_{m,obs}$ and $\tau_{p,obs}$ across the peak, evidence that scattered light was now negligible.

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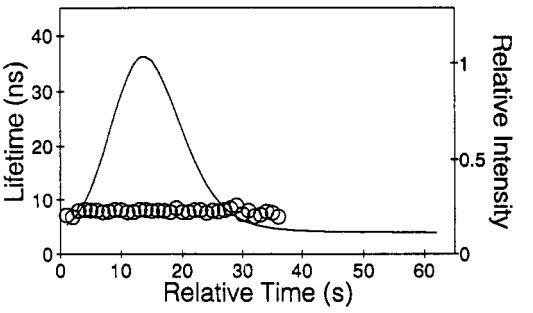

Figure 2. Fluorescence intensity (—) and lifetime (O) recovered from raw MHF data collected on-the-fly for B*k*F. The lifetime data were fit to a one-component model.

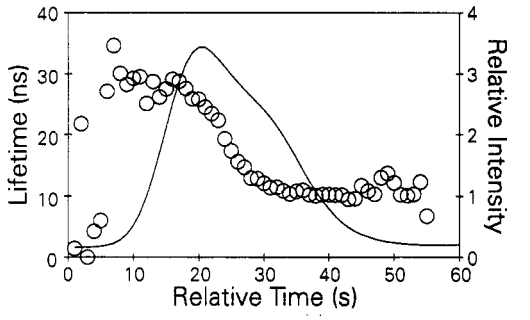

Figure 3. Fluorescence intensity (—) and lifetime (O) recovered from raw MHF data collected on-the-fly for overlapping B*b*F and B*k*F peaks. The lifetime data were fit to a one-component model. The change in recovered lifetime across the chromatogram indicates heterogeneity, i.e., peak overlap.

Analysis of On-the-Fly Data. Heterogeneity analysis and peak resolution were achieved through NLLS analysis of the lifetime data and mathematical treatment of the fluorescence intensity data. Template files were written to translate the MHF Kinetic Lifetimes data files into a format readable by the Globals Unlimited software. These files were designed to perform floating lifetime, linked lifetime, and fixed lifetime analyses, as described below, for one-, two-, and three-component models. Although files for the analysis of more than three components may be created, this is generally unnecessary for reasonable chromatographic resolution, in which it is unlikely to have more than three components at a given point in the chromatogram (i.e., more than three components coeluting).

In the floating lifetime NLLS analyses, the lifetimes and fractional intensities of the individual components are allowed to float until a global minimum is reached. This results in somewhat different lifetimes for the components at each point across the coeluting peaks. In the linked lifetime analyses, the lifetimes and fractional intensities are again allowed to float, but the lifetime of each component is linked across all chromatographic points so that a unique lifetime is recovered for each component of the coeluting peaks. In the fixed lifetime analyses, the lifetimes are fixed to some appropriate values (see below) and the fractional intensities are varied until a global minimum has been reached.

This paper describes results obtained using one-component floating lifetime fits (abbreviated as float1) and two-component fixed lifetime fits (abbreviated as fix2). Since a float1 analysis determines the best lifetime for each measurement point, it was used for heterogeneity indication. More specifically, if a single component is present, the lifetime determined for a particular measurement point is the observed lifetime of the component; for multiple components, the determined lifetime is a weighted average of the lifetimes for the

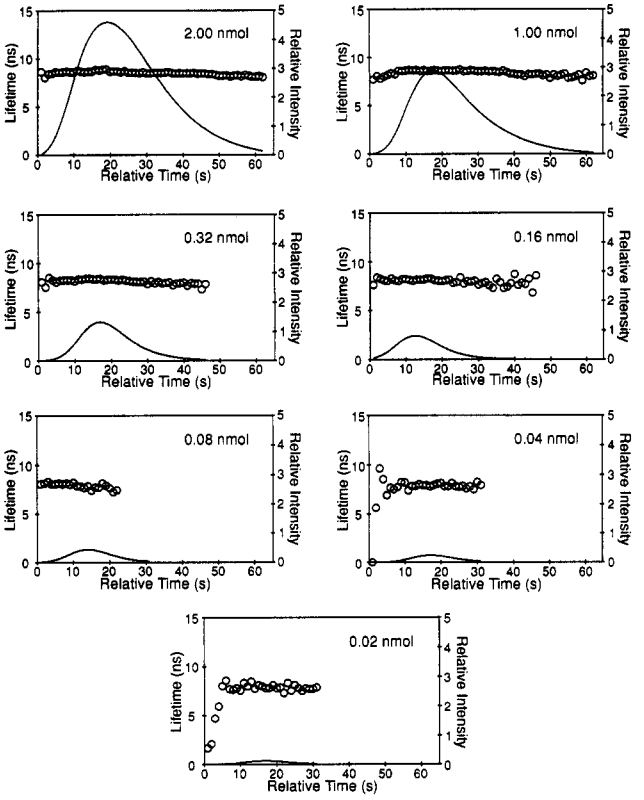

Figure 4. Fluorescence intensity (—) and lifetime (O) recovered from raw MHF data collected on-the-fly for various injection amounts of B*k*F. The lifetime data were fit to a one-component model.

components present. Figures 2 and 3 show the float1 results for single-component and multicomponent chromatographic peaks.

When heterogeneity is indicated by the float1 results, as in Figure 3, a fix2 analysis is performed. The lifetimes used for the fix2 analysis may be obtained either from the float1 results in regions of the coeluting peaks that are clearly homogeneous, generally at the peak peripheries, or from separate injections of the components, if the components are known. The fix2 analysis provides a fractional intensity for each lifetime component at each point along the chromatogram. The fractional intensity is multiplied by the steady-state fluorescence intensity at that point in order to construct the resolved peak for each component.

Flow Rate Study. A study was performed to determine the optimum HPLC flow rate for on-the-fly data acquisition using injections of B*k*F (0.04 nmol). Lifetimes were calculated at 0.96-s intervals across the B*k*F peak from the data acquired at flow rates of 0.3, 0.5, 0.7, and 0.9 mL/min. At each flow rate, the consistency of the observed lifetime across the peak and the intensity at which an accurate lifetime was first determined were evaluated. On the basis of these considerations, 0.5 mL/min was determined to be the optimum flow rate and was used in subsequent studies. The lifetime chromatogram of B*k*F (Figure 2) obtained at a flow rate of 0.5 mL/min demonstrates the ability of this on-the-fly detection technique to accurately determine fluorescence lifetime across a chromatographic peak and well into the peak peripheries.

Concentration Effects. Various amounts of B*k*F were injected to test for possible concentration effects (e.g., self-absorption and concentration quenching) at high concentrations which could result in deviations in the observed lifetime. Such phenomena were not observed for injection amounts up to 2 nmol (Figure 4). These results reflect the fact that the fluorescence lifetime of a compound is concentration-independent within the linear response range of the compound.

Table I. Relative Standard Deviation (%) in Observed Lifetime across Various Regions of B*k*F Chromatographic Peaks

peak region	intensity threshold (%)	% RSD[a] 2.00	1.00	0.32	0.16	0.08	0.04
central[b]	>12	0.95	1.42	1.32	2.34	5.08	5.68
	>15	0.96	1.15	1.33	2.31	4.94	4.95
	>20	0.97	1.18	1.29	2.08	5.18	2.80
leading[c]	>3	0.70	1.18	3.38	4.70	7.47	5.34
	>5	0.69	1.18	1.97	3.29	7.47	5.34
	>7	0.70	1.22	1.86	3.34	4.02	5.34
	>10	0.71	1.22	1.17	2.05	3.73	5.34
	>12	0.71	1.00	1.01	2.11	3.64	5.34
	>15	0.73	0.99	1.01	2.15	3.80	5.34
	>20	0.75	0.99	1.03	1.70	3.94	2.37
trailing[d]	>3	1.06	1.53	2.40	2.40	3.56	5.87
	>5	1.06	1.53	2.07	2.40	3.56	5.87
	>7	1.06	1.53	1.61	2.40	3.56	5.87
	>10	1.06	1.59	1.51	2.47	3.56	5.87
	>12	1.06	1.57	1.52	2.36	2.20	5.87
	>15	1.06	1.22	1.53	2.30	1.99	4.41
	>20	1.06	1.26	1.50	2.28	1.94	3.09

[a] For B*k*F injections ranging from 2.00 to 0.04 nmol. [b] Region extending from center of peak (peak apex) to the points on either side at which the intensity is equal to the given threshold. [c] Region extending from peak apex to the point on the leading portion of the peak at which the intensity is equal to the given threshold. [d] Region extending from peak apex to the point on the trailing portion of the peak at which the intensity is equal to the given threshold.

The concentration independence of fluorescence lifetime offers important capabilities for chromatographic detection through the use of coinjection (i.e., addition of a suspected analyte to the chromatographic sample prior to injection). Consider a chromatographic peak with constant lifetime across the peak and a retention time that indicates a certain analyte. If the lifetime across the peak does not change upon coinjection of the suspected analyte, then the identity of the peak is confirmed. If the lifetime does change, then the peak is probably not due to the suspected analyte. Another possible use of coinjection is to determine the presence of matrix effects that would affect both the intensity and lifetime of a chromatographic peak. In this instance, the retention time of the peak indicates a certain analyte but the lifetime of the peak does not equal the expected lifetime of that analyte. If the peak lifetime does not change upon coinjection of the suspected analyte, then the peak probably is due to the suspected analyte and the inconsistency in lifetime is due to matrix effects. The ability to indicate such matrix effects is an important advantage for accurate interpretation of peak intensity.

These results also demonstrate the ability of this technique to determine accurate lifetimes well into the low concentration regions of the chromatographic peak peripheries. Uncertainties, expressed as relative standard deviation (RSD), in the detected lifetimes were examined for various windows along chromatographic peaks of B*k*F in amounts of 0.04–0.20 nmol. The RSDs are shown in Table I for "central", "leading", and "trailing" peak regions. Central regions extend out from the peak center in both directions and terminate at the point on either side at which the intensity is equal to various percentages of the maximum peak intensity, as designated in Table I. Leading and trailing regions extend to similar points but include only the leading or trailing halves of the peaks.

The results in Table I demonstrate the excellent precision across the B*k*F peaks. The RSD values never exceed 8% and are generally within 2–3% except at the peak peripheries for the smallest amounts of B*k*F. The leading halves of the peaks

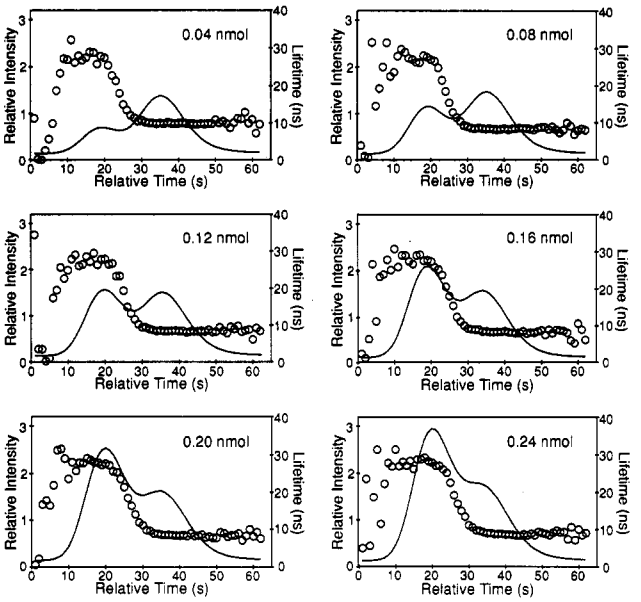

Figure 5. Fluorescence intensity (—) and lifetime (O) recovered from raw MHF data collected on-the-fly foroverlapping B*b*F and B*k*F peaks. The injection amount of B*k*F was 0.04 nmol in all cases, and the injection amount of B*b*F was varied as indicated directly on each graph. The lifetime data were fit to a one-component model.

show slightly better precision than the trailing halves at larger amounts of B*k*F; however, there is significant degradation in the precision for the trailing halves at the outermost peripheries for the smaller amounts of B*k*F. The cause of this degradation may be related to actual physical processes at the leading edge of the peak during elution, which suggests that lifetime detection may prove to be a useful tool for studying such processes.

Detection and Resolution of Overlapping Peaks. The ability to detect and resolve overlapping chromatographic peaks was examined using a mixture of B*b*F and B*k*F, each at 1×10^{-5} M. These compounds were previously found[6] to exhibit overlap under similar chromatographic conditions but exhibited minimal overlap in this experiment. Therefore, differing degrees of peak overlap were artificially achieved through appropriately timed injections of the individual compounds.

Heterogeneity indication and construction of the resolved peaks were achieved for peaks with as much as 60% overlap. This technique has not yet been applied to peaks that overlap 100%. However, such resolution is theoretically possible since this situation is analogous to determining multiple lifetime components for a heterogeneous solution under batch conditions. This is different from other approaches that require some degree of chromatographic resolution or at least varying contributions from the two components across the peak (which may be symmetric if there is no chromatographic resolution).

The reproducibility of steady-state and lifetime detection was determined for five replicate chromatographic runs in which 0.04 nmol of B*k*F was injected, followed in 30 s by 0.20 nmol of B*b*F. The resulting peaks exhibited approximately 25% overlap. The RSD of the steady-state intensity at the apexes corresponding to B*b*F and B*k*F in the overlapping peaks were 1.12% and 1.79%, respectively. The RSDs in the recovered intensity at the peak apexes for the lifetime-resolved B*b*F and B*k*F peaks were 0.53% and 3.99%, respectively.

In another set of experiments, different amounts of B*b*F were injected (0.04, 0.08, 0.12, 0.16, 0.20, and 0.24 nmol) while the injection amount of B*k*F was kept constant at 0.04 nmol. The resolved lifetimes for B*b*F and B*k*F were approximately 28 and 8 ns, respectively, in all cases (Figure 5), again illustrating the concentration independence of fluorescence

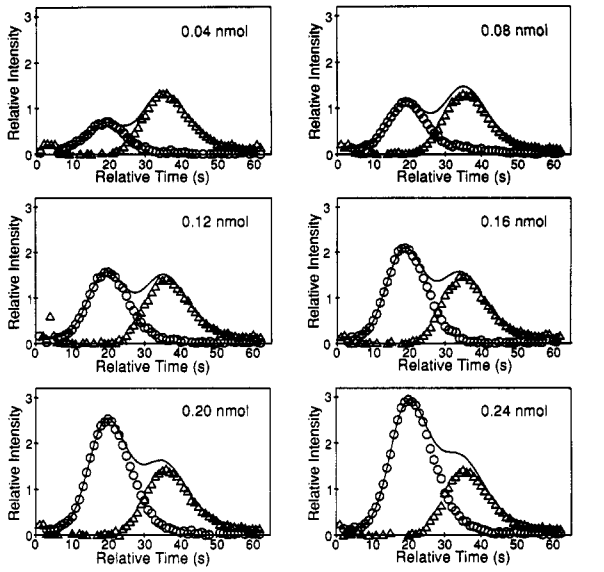

Figure 6. Lifetime-resolved peaks of overlapping B*b*F (O) and B*k*F (Δ). The lifetime data were fit to a two-component model, with the lifetimes fixed to values recovered from separate injections of B*b*F and B*k*F. (—) represents the fluorescence intensity chromatogram for overlapping components, collected on-the-fly.

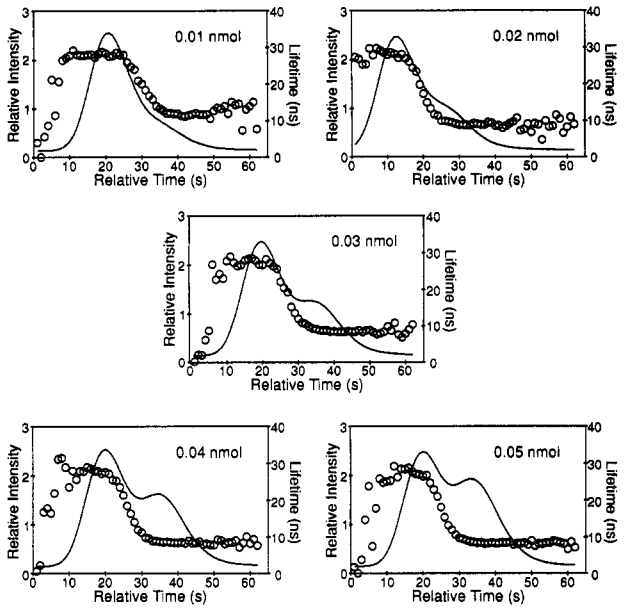

Figure 7. Fluorescence intensity (—) and lifetime (O) recovered from raw MHF data collected on-the-fly for overlapping B*b*F and B*k*F peaks. The injection amount of B*b*F was 0.20 nmol in all cases, and the injection amount of B*k*F was varied as indicated directly on each graph. The lifetime data were fit to a one-component model.

lifetime. Figure 6 shows the resolution of the individual B*b*F and B*k*F peaks. As expected, the intensity of the B*b*F peak increases while the intensity of the B*k*F component remains relatively constant, with an RSD of 4.5% in the steady-state intensity at the apex of the resolved B*k*F peak.

A similar experiment was performed in which the injection amount of B*k*F was varied (0.01, 0.02, 0.03, 0.04, and 0.05 nmol) while that of B*b*F was held constant at 0.20 nmol. Again, the resolved lifetimes were 28 and 8 ns for B*b*F and B*k*F, respectively (Figure 7), with one exception. For the lowest concentration of B*k*F (Figure 7, upper left), the observed lifetimes of B*b*F and B*k*F are 28 and 12 ns, respectively, and the B*k*F lifetime appears to actually increase along the peak tail. The cause of the deviation in the observed lifetime is apparent in the construction of the B*b*F and B*k*F contributions, shown in the corresponding plot in Figure 8 (upper

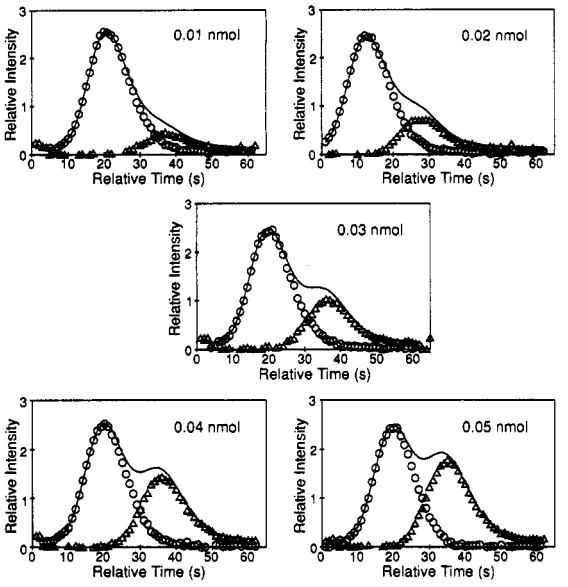

Figure 8. Lifetime-resolved peaks of overlapping B*b*F (O) and B*k*F (Δ). The lifetime data were fit to a two-component model, with the lifetimes fixed to values recovered from separate injections of B*b*F and B*k*F. (—) represents the fluorescence intensity chromatogram for the overlapping components, collected on-the-fly.

left). Due to the overlap of the small amount of B*k*F and the tailing portion of the much larger B*b*F peak, the 12-ns lifetime indicated was actually a weighted average based on the contributions of the lifetime components in this peak region. Similar deviations from an expected component lifetime value may also be caused by coelution of minor components or matrix effects since fluorescence lifetime is sensitive to the chemical microenvironment of the analyte. The RSD in the steady-state intensity at the apex of the resolved B*b*F peak was 2.0%.

CONCLUSIONS

This work demonstrates accurate lifetime detection and peak resolution for simple mixtures in HPLC. The MHF has great potential as an HPLC detector since phase, modulation, and fluorescence intensity information are acquired at multiple modulation frequencies simultaneously, on-the-fly, during chromatographic elution. This information can be used for the following: component identification; indication of coeluting peaks, matrix effects, or impurities; and resolution of overlapping peaks. Moreover, this technique makes no a priori assumptions about spectral features, chromatographic peak shape, or fluorescence lifetime. The ability to detect fluorescence lifetimes well into the low-signal regions of the peak peripheries is particularly noteworthy. These features, when coupled with the convenience of isocratic elution, offer the potential for increased sample throughput and accuracy in analysis, even in the presence of nonoptimal chromatographic resolution.

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