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*Anal. Chem.*, 1993, 65 (23), 3466-3472 • DOI: 10.1021/ac00071a022

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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, instrumental 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 N2 laser-pumped dye laser. 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. Measurements at multiple delay times in a single chromatogram were achieved in a later study 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 multieponential 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. 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. 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 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 $\phi$ and demodulated by a factor $m$ relative to the exciting light:

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

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 $\omega$ 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:

$$\tau_{\text{probe}} = \frac{\tan \phi}{\omega}$$

and

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

If $\tau_{\text{m,obs}} = \tau_{\text{probe}}$, then a homogeneous system containing a single lifetime component is indicated. If $\tau_{\text{m,obs}} > \tau_{\text{probe}}$, 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 $\phi$ 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[a]fluoranthene (BAP and BAF, respectively; each 99%, AccuStandard), benzo[a]fluoranthene (BAF; 99%, Aldrich), and 9-anthracencarboxonitrile (9AC; 98%, Lancaster Synthesis) were used as received. Acetonitrile (HPLC grade, Burdick & Jackson) and water (HPLC grade, in laboratory Modubol Type I water purification system), which were used for sample preparation and as the HPLC mobile phase (80% CH$_3$CN:20% H$_2$O), were purified further by vacuum filtration as suggested by the HPLC manufacturer. Stock solutions (5 x 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$^{-4}$ - 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-$\mu$L 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 60 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 $\tau_{\text{obs}}$ 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 ($\chi^2$) 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-


\(^{10}\) SLM 49000MS Software Manual; SLM Instruments: Urbana, IL, 1991.

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, \( \Delta F \), 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 BaP 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.

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 homogeneous 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.

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

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the reference solution must be predetermined and entered in the instrumental setup parameters in order to calibrate the excitation phase (defined as $0^\circ$) 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:

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

and

$$\tau_{\text{m,obs}} = \frac{1}{\omega} \left[ (1 + \omega^2 \tau_r^2) \left( \frac{m_1}{m_2} \right)^2 - 1 \right]^{1/2}$$

where $\tau_r$ is the predetermined reference lifetime (if the reference is a scattering solution, then $\tau_r$ is zero by definition and eqs 4 and 5 reduce to eqs 2 and 3, respectively), $\phi_s$ and $\phi_r$ are the observed phase shifts of the reference and sample solutions, and $m_1$ and $m_2$ are the observed demodulation factors of the reference and sample solutions.\(^{8,13}\) SAC 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 BoP in an attempt to reproduce previous work using the new instrumental approach. A cross-correlation frequency of 5.208 Hz was used for this experiment. $\tau_{\text{m,obs}}$ and $\tau_{\text{p,obs}}$ were calculated at 0.96-s intervals across the BoP peak and should be equal since the peak corresponds to a single fluorescent component. However, fitting the data to a one-component model indicated $\tau_{\text{m,obs}} > \tau_{\text{p,obs}}$, 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 $\tau_{\text{m,obs}}$ and $\tau_{\text{p,obs}}$ across the peak, evidence that scattered light was now negligible.

**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 peaks 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

and the intensity at which an accurate lifetime was first
the resolved peak for each component.

fact that the fluorescence lifetime of a compound is concen-
injected to test for possible concentration effects (e.g., self-
peripheries.

at flow rates of 0.3, 0.5, 0.7, and 0.9 mL/min. At each flow
rate and was used in subsequent studies. The lifetime
across a chromatographic peak and well into the peak
detection technique to accurately determine fluorescence
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 concen-
tration-independent within the linear response range of the
compound.

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 is probably 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 BkF 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 BkF peaks. The RSD values never exceed 8% and
are generally within 2–3% except at the peak peripheries for
the smallest amounts of BkF. The leading halves of the peaks

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 chromato-
gram. 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 BkF (0.04 nmol). Lifetimes were calculated
at 0.96-s intervals across the BkF 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 consid-
erations, 0.5 mL/min was determined to be the optimum flow
rate and was used in subsequent studies. The lifetime
chromatogram of BkF (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 BkF were
injected to test for possible concentration effects (e.g., self-
absorption and concentration quenching) at high concen-
trations 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

Table I. Relative Standard Deviation (%) in Observed
Lifetime across Various Regions of BkF Chromatographic
Peaks

<table>
<thead>
<tr>
<th>peak region</th>
<th>intensity threshold (%)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>central^a</td>
<td>&gt;12</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>&gt;15</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>&gt;20</td>
<td>0.97</td>
</tr>
<tr>
<td>leading^b</td>
<td>&gt;8</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>&gt;5</td>
<td>0.69</td>
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<td>&gt;7</td>
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<tr>
<td></td>
<td>&gt;12</td>
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<td>&gt;15</td>
<td>0.73</td>
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<tr>
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<td>trailing^c</td>
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<tr>
<td></td>
<td>&gt;20</td>
<td>1.06</td>
</tr>
</tbody>
</table>

^a For BkF injections ranging from 2.00 to 0.04 nmol. ^b Region extending from center of peak (peak apex) to the point 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.

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![Figure 4](image_url)
The injection amount of BkF was kept constant. The resolved lifetimes for BbF and BkF were approximately through appropriately timed injections of the individual compounds. This situation is analogous to determining multiple lifetime parameters for a heterogeneous solution under batch conditions. The Injection amount of BbF was varied as indicated directly on each graph. The lifetime data were fit to a one-component model.

Detection and Resolution of Overlapping Peaks. The ability to detect and resolve overlapping chromatographic peaks was examined using a mixture of BbF and BkF, each at $1 \times 10^{-4}$ M. These compounds were previously found 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 BkF was injected, followed in 30 s by 0.20 nmol of BbF. The resulting peaks exhibited approximately 25% overlap. The RSD of the steady-state intensity at the apexes corresponding to BbF and BkF in the overlapping peaks was 1.12% and 1.79%, respectively. The RSDs in the resolved intensity at the peak apexes for the lifetime-resolved BbF and BkF peaks were 0.53% and 3.99%, respectively.

In another set of experiments, different amounts of BbF were injected (0.01, 0.04, 0.08, 0.12, 0.16, 0.20, and 0.24 nmol) while the injection amount of BkF was kept constant at 0.04 nmol. The resolved lifetimes for BbF and BkF were approximately 28 and 8 ns, respectively, in all cases (Figure 5), again illustrating the concentration independence of fluorescence lifetime. Figure 6 shows the resolution of the individual BbF and BkF peaks. As expected, the intensity of the BbF peak increases while the intensity of the BkF component remains relatively constant, with an RSD of 4.5% in the steady-state intensity at the apex of the resolved BkF peak.

A similar experiment was performed in which the injection amount of BbF was varied (0.01, 0.02, 0.03, 0.04, and 0.05 nmol) while that of BkF was held constant at 0.20 nmol. Again, the resolved lifetimes were 28 and 8 ns for BbF and BkF, respectively (Figure 7), with one exception. For the lowest concentration of BkF (Figure 7, upper left), the observed lifetimes of BbF and BkF are 28 and 12 ns, respectively, and the BbF 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 BbF and BkF contributions, shown in the corresponding plot in Figure 8 (upper right).
Figure 8. Lifetime-resolved peaks of overlapping BbF (O) and BkF (Δ). The lifetime data were fit to a two-component model, with the lifetimes fixed to values recovered from separate injections of BbF and BkF. (—) represents the fluorescence intensity chromatogram for the overlapping components, collected on-the-fly.

left). Due to the overlap of the small amount of BkF and the tailing portion of the much larger BbF 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 BbF 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.

ACKNOWLEDGMENT

This work was supported by the Office of Exploratory Research of the Environmental Protection Agency (Grant R817127-01). Scientific Parentage of the Authors. M. B. Smalley and J. M. Shaver: Ph.D. students under L. B. McGown, Ph.D. under G. D. Christian, Ph.D. under W. Purdy, Ph.D. under D. Hume, Ph.D. under I. M. Kolthoff.

RECEIVED for review June 21, 1993. Accepted August 26, 1993.