On-the-Fly Fluorescence Lifetime Detection in Liquid Chromatography with Data Collected Simultaneously at Multiple Emission Wavelengths

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A novel liquid chromatography (LC) fluorescence detector simultaneously generates fluorescence decay curves at multiple emission wavelengths. The fourth harmonic (256 nm) excitation light from a pulsed Nd:YAG laser is focused into a standard LC flow cell. A small spectrograph disperses the fluorescence. Fiber-optic delay lines positioned in the exit focal plane time the arrival of photons at the photomultiplier tube detector. Four wavelength channels with 50 ns channel spacing were used in this study. The intensity-decay time waveform is averaged with a digital storage oscilloscope. Temporal overlap between adjacent wavelength channels is removed during the post-processing of the data. System performance was characterized for chromatographic elution of polycyclic aromatic hydrocarbons (PAHs). Detection limits are 1.6 ppb for naphtalene and 0.14 ppb for fluorene. Resolution of co-eluting 1,4-dimethyl-naphthalene and anthracene components on the basis of differences in their lifetimes and spectral distributions is demonstrated.

Index Headings: Liquid chromatography; Laser-induced fluorescence; PAHs; Fluorescence lifetime; Fiber optic.

INTRODUCTION

Versatility and simplicity make liquid chromatography (LC) a cornerstone of analytical chemistry. Typically the chromatographer will tailor the choice of stationary phase, column diameter and length, and mobile phase composition per the application. However, the very complex mixtures encountered during drug metabolism studies and environmental analyses often challenge the abilities of conventional LC detection schemes. A major difficulty is that peaks of interest may be poorly resolved from interfering peaks. Incremental improvements in column materials and fabrication are likely, but new detection schemes may offer better prospects for a fundamental breakthrough. Hyphenated techniques such as LC-MS have proliferated in recent years. The pharmaceutical industry's combination of on-line high field NMR and time-of-flight mass spectrometry with LC probably represents the height of detector sophistication.

LC detectors generally provide either a single value (e.g., a voltage reading) or a one-dimensional array of numbers; such data are referred to here as zeroth order and first order, respectively. Conductivity and refractive index detectors provide zeroth-order data. Many absorbance or fluorescence detectors, including those with programmed wavelength changes during the elution, yield zeroth order. The first-order data from diode array detectors and mass spectrometers improve selectivity and specificity.

The literature contains a few examples of spectral techniques that produce first-order LC fluorescence data acquired with an optical multichannel analyzer or a charge-coupled device (CCD) camera. Polychromator approaches involving multiple photomultiplier tubes (PMTs), each monitoring a preset wavelength, have also been described. Time-resolved fluorescence spectroscopy can potentially enhance LC selectivity. An advantage of lifetime measurements over spectral techniques is that a priori knowledge (e.g., a spectral database) is not needed to assess peak homogeneity (purity). McGown and her students have acquired fluorescence lifetimes on the fly with a commercial multiharmonic Fourier transform lifetime fluorometer (MHTF) that incorporates a modulated continuous-wave laser excitation source. The lifetime can be extracted from either the phase shift or demodulation of the fluorescence signal.

An alternative lifetime technique presented by our group relies on the identification of the entire fluorescence decay profile with a pulsed laser excitation source, PMT, and digital storage oscilloscope (DSO). It extends previous methods in which the intensity ratio of two points on the decay curve is measured. In our earlier work, the wavelength interval over which the emission is monitored was selected with a filter. This paper introduces an innovation that lets us collect complete fluorescence decay profiles at several wavelengths simultaneously while retaining the simplicity of one conventional PMT detector and one DSO channel. The advancement to second-order lifetime and spectral data does not sacrifice sensitivity in comparison to methods that are limited to either spectral properties or lifetime discrimination.

The innovation recognized that much of the DSO record length capability was unused in our single-wavelength collection scheme. Scopes that digitize at 1-2 gigasamples per second (corresponding digitization interval of 0.5-1 ns) are readily available. Even for a short record length of 500 points, one can follow the fluorescence decay over many lifetime intervals. The new technique averages and stores several fluorescence decay curves in a single DSO channel by arranging their placement in the overall record with delay lines. An elaboration of the conventional polychromator strategy would be to employ multiple PMTs and delay the arrival of the respective signals at the scope input with graded lengths of coaxial cable. We choose instead to optically delay the arrival of fluorescence light at a single PMT detector.

The simultaneous collection of fluorescence decay curves at several wavelengths has applications beyond...
FIG. 1. The experimental layout of the pulsed excitation and detection system. Four fiber-optic strands with successive length differences of 10 m transport the fluorescence at different wavelengths to a single photomultiplier tube detector.

LC. For example, it forms the basis of fingerprinting petroleum contamination in soil at depths greater than 100 feet below ground surface without the need to return material to the ground surface. However, LC and capillary electrophoresis (CE) likely offer the largest domain of potential applications, with LC as the focus of the work presented here.

EXPERIMENTAL

A schematic presentation of the experimental configuration, which provides time and spectral domain information simultaneously during a chromatographic run, is shown in Fig. 1. The excitation source is a CFR-200 Nd:YAG laser (Big Sky Laser Technologies, Inc.), whose relatively high repetition frequency (50 pulses/second) is advantageous for signal averaging purposes. The CFR-200 is rated at 50 mJ/pulse at 532 nm, and ultraviolet pulse energies of several millijoules, far in excess of what can be used productively in this experiment, are available after frequency doubling to the fourth harmonic. The 266.0 nm output from the KDP doubling crystal is sequentially isolated with a fused-silica Pellin–Broca prism, attenuated to a few hundred microjoules, and focused with a biconvex lens (75 mm focal length) into a high-purity fused-silica flow cell (NSG Precision Cells, 1 mm square bore). The fluorescence emitted from the bore of the flow cell is imaged onto the entrance slit of a small spectrograph (Acton Spectra Pro, 150 mm focal length, 1200 lines/mm grating) by an f/2 focusing lens located equidistant between the flow cell and the spectrograph entrance slit. The cone of light matches the f/4 numerical aperture of the spectrograph. A plate mounted at the exit focal plane of the spectrograph was drilled with holes to accept 600 μm optical fibers. The holes are positioned at a nominal 20 nm wavelength spacing.

In the experiments discussed below, the light impinging at positions corresponding to center wavelengths of 320, 340, 360, and 380 nm is collected and transmitted to the PMT via fibers of length 2, 12, 22, and 32 m, respectively. Note that in recognition of the attenuation properties of the fiber, the lengths are chosen so that the shortest delay fiber is paired with the shortest emission wavelength. The light emerging from the bundled fibers at the distal end is converted to a current at the PMT (Hamamatsu R928, wired for fast response, biased at −950 V), and the current is fed directly to the DSO (Tektronix 620). The fibers introduce nominal delays of 10, 60, 110, and 160 ns between the spectograph exit slit and the PMT. A new decay curve is obtained every 5.6 s, which is the time required to average data from 256 laser shots and download the waveform to memory. A more detailed approach and discussion of this general approach can be found elsewhere. Isocratic reversed-phase HPLC is performed with a Shimadzu LC-10AS pump and SPD-10AV UV-VIS detector as described previously. Briefly, the samples are loaded with a 10 μL loop attached to a Rheodyne multiport injection valve and introduced into a 150 × 4.6 mm Rainbow Microsorb MV chromatographic column with 5 μm C18 packing. The fluorescence flow cell is placed in series after the absorbance detector.

Naphthalene, fluorene, phenanthrene, anthracene, and 1,4-dimethylanthanthene (98% pure, Chem Service), and 2-methylnaphthalene (Eastman Chemical) were used as received to prepare stock solutions. The mobile phase (70% ACN in water) was prepared from HPLC-grade acetonitrile (EM Science) and deionized water. Stock solutions of the polycyclic hydrocarbons (PAHs) (10⁻¹⁰ M) were prepared in 100% acetonitrile, stored in amber bottles with Teflon-lined caps (National Scientific Company), and refrigerated prior to use to minimize decomposition. Less concentrated solutions were prepared by serial dilution.

RESULTS AND DISCUSSION

Single Component Elution — Linearity and Sensitivity. The detection scheme sensitivity was first tested for injections of single-component samples. Plugs arranged in order of increasing concentration were injected at intervals of about 150 s. The fluorene raw data shown in Fig. 2 were obtained over the concentration range 0.5 to 100 ppb.

The signal associated with the light presented to the PMT over a particular delay fiber is referred to as a channel. Contributions from all four wavelength channels are contained within the total intensity-decay time waveform at a given elution time. In Fig. 2 the 320 nm channel (up to about 60 ns) is the most intense. The fluorene fluorescence lifetime is short enough (~7 ns) relative to the 50 ns channel spacing to ensure that tailing of the 320 nm emission into the adjacent 340 nm channel is negligible. The integrated intensity in the 340 nm channel (spanning from 60 to 110 ns) is about half that in the 320 nm channel.

Almost all the intensity in the longest wavelength channel (380 nm, spanning from 160 to 210 ns delay time) is background signal. The background signal can also be discerned in the three shorter wavelength channels prior to elution of the 0.5 ppb fluorene plug beginning at about profile number 60. In our previous publi-
Fig. 2. Three-dimensional view of six consecutive fluorescein injections recorded by the multilambda detection system. The concentrations of the fluorescein injections are 0.5, 5, 10, 25, 50, and 100 ppb.

ation we attributed the mobile phase as the source of the background, which others have also observed.

The data can be assembled into four independent conventional chromatograms by summing (i.e., time-integrating) the signal within each channel at each point along the elution profile. The process is mathematically straightforward if the signals for the different channels do not overlap in time within the waveforms. Equivalently, the relative intensities in the channels represent a crude four-point emission spectrum at each step along the elution profile.

The bold trace in Fig. 3a is the chromatogram assembled from the data in Fig. 2 by integrating over all four channels. The background signal, obtained by averaging the time-integrated waveforms before elution profile 50, has been subtracted. Figure 3a also shows the intensity integrated over only the 320 nm channel (320 nm chromatogram, light trace), which gives the best signal-to-noise ratio. The dotted trace is an expansion (×10) of the 320 nm chromatogram through elution of the two lowest concentration plugs. The 0.5 ppb injection is easily distinguished in the expanded trace. The full widths of the chromatogram features are the same whether the integration is over one or all four channels and constant over the range of concentrations studied here.

Calibration curves based on peak height vs. concentration are shown in Fig. 3b for the 320 nm channel and the signal integrated over all four channels. The calibration curve is linear in each case. The limits of detection calculated according to the method of Boumans are 0.46 ppb for all channels and 0.14 ppb for the 320 nm channel.

Similar experiments for naphthalene gave a limit of detection of 2.0 ppb from the overall chromatogram and 1.6 ppb based on integration between 320 nm and 360 nm, the wavelength range in which naphthalene has the greatest signal-to-noise ratio.

Waveforms with Temporally Overlapping Channels. Figure 4, which is reproduced from our previous publication, illustrates the identification of band homogeneity via a simple phase-plane lifetime analysis as anthracene and 1,4-dimethylnaphthalene co-elute. The phase plane method is fast, computationally convenient, and accurate for single exponential decays. The lifetime values remain constant over the peak profile of a single component until the signal becomes too weak for a reliable lifetime value. Anthracene, whose fluorescence decays faster than that of 1,4-naphthalene, elutes slightly later in time, which accounts for the progressive decline in the apparent lifetime across the band in Fig. 4. The phase-plane lifetime for multiple emitting components is

1,4 Dimethylnaphthalene & Anthracene Overlap

Fig. 3. Fluoresence chromatograms at all wavelengths (narrow solid line) and at 320 nm (thick solid line) are depicted. The dotted line is an expansion (×10) for the two lower concentration plugs. Calibration curves are shown at the right.
an average value whose relationship to the number of components or their individual lifetimes is not well defined. Thus, for completely co-eluting species, a constant lifetime value across the "peak" would also be recorded. Under such conditions, a multieponential fit would give more accurate lifetime values.

The elution of a mixture of anthracene (500 ppb) and 1,4-dimethylnaphthalene (350 ppb) has been repeated with the current multichannel apparatus under the same experimental conditions as applied for the data collected in Fig. 4. Features associated with each of the four fibers in the optical delay line are easily recognized in the representative raw waveform depicted in Fig. 5. However, in contrast to the fluorescence data in Fig. 2, the fluorescence from light carried by a given fiber has not completely decayed before the fluorescence from the next fiber arrives at the PMT. The fluorescence intensity up to the time that the 380 nm photons arrive (at about 160 ns) is entirely due to 1,4-dimethylnaphthalene. Anthracene makes the major contribution to the emission at 380 nm, but 1,4-dimethylnaphthalene also contributes.

It should be clear that, even if the decay in one channel temporally extends into the next channel, extensive signal processing is not needed to test for co-eluting components. For example, one could generate chromatograms by simply integrating the signal in a given channel up to the time that photons arrive at the PMT over the next fiber in the delay line. However, a means to resolve the temporal overlap between channels gives the greatest sensitivity for assessing band inhomogeneity. The channel cross-contamination reduces the clarity with which differences in the chromatograms can be seen.

**Stripping Analysis to Remove Temporal Overlap.**

The temporal overlap in Fig. 5a was removed by a stripping analysis performed successively from channel (short wavelength) to channel (long wavelength) across the waveform. In this particular case there is only overlap of emitting species in the final channel at 380 nm. This consideration is especially favorable in that it lets us retain the simple phase-plane lifetime analysis. The DSO waveform represents the convolution of the laser excitation profile with the molecular response function (i.e., an exponentially decaying function). If the laser excitation profile and the fluorescence decay profile are recorded with the same instrument, linear distortions introduced by the measurement system drop out during the deconvolution.

The laser profiles needed for the lifetime analysis here are extracted from p-terphenyl decay data [air saturated cyclohexane] by using the reference emitter technique. The four laser profiles thus obtained have the appropriate relative time shift at each wavelength.

Once the fluorescence lifetime for the data in the first channel has been obtained by deconvolution, continuation of the fluorescence decay data into the next channel is straightforward. The computed fluorescence intensity profile for the first channel is subtracted from the raw data, and the process is repeated. Results of the fitting procedure and the residuals are shown in Fig. 5b. One expects some accumulation in error as the stripping analysis is applied to successive channels, but the effect does not appear very significant. Our experience with the phase-plane method leads us to believe that the oscillation of the residual at the rising edges of the more intense peaks results from a slight overall time shift between the laser and data profiles.

The reconstructed chromatograms with the channel temporal overlap removed are illustrated in Fig. 6. The
chromatogram consisting of solid squares corresponds to the emission from the 340 nm fiber optic. The lesser amplitude chromatograms (solid circle, solid triangle) represent the chromatograms based on the 320 nm and 360 nm fiber-optic channels. The fourth chromatogram (hollow diamonds) corresponds to emission from the 380 nm fiber optic. The peaks from the solid chromatograms lie coincident with each other and are situated at 1745 s. The 380 nm chromatogram (hollow diamonds) is slightly offset by approximately 9 s to longer elution time. Figure 6a is re-depicted in Fig. 6b after normalization. The shifting of the 380 nm peak is readily observed in this panel.

The lifetime results shown in Fig. 6c complement the spectral data in confirming the presence of at least two species. The lifetimes in the 320, 340, and 360 nm channels, which we attribute solely to 1,4-dimethylaminonaphthalene, are 24 ± 1 ns (320 nm), 23 ± 2 ns (340 nm), and 23 ± 2 ns (360 nm); the uncertainties represent two standard deviations. The 7 ± 1 ns lifetime in the 380 nm channel is substantially shorter. The fluorescence lifetime of anthracene in the mobile phase has been previously measured at 4.22 ns.12,14 The 7 ns measurement found here results from a small contribution of 1,4-dimethylaminonaphthalene signal in the 380 nm channel. Closer investigation shows that the lifetime steadily decreases from about 8 ns to 6 ns across the 380 nm chromatogram as the contribution of 1,4-dimethylaminonaphthalene to the total intensity drops and the anthracene contribution proportionately increases.

Our previous work regarding on-the-fly fluorescence lifetime methods for LC has been extended to simultaneous data collection at multiple wavelengths. The fluorescence wavelength interval was isolated with bandpass filters in the earlier work, but a monochromator could have been used instead. The innovation described in this paper requires but a single modification to the apparatus, namely, a fiber-optic delay line positioned between the monochromator (in this case acting as a spectrograph) and the photomultiplier tube detector. Complete fluorescence decay curves are obtained at each of the fluorescence monitoring wavelengths and stored in a single waveform record with a digital storage oscilloscope.

We have demonstrated that the current form of the apparatus can detect polycyclic aromatic hydrocarbons at the low ppb level, which compares well with the performance of conventional LC fluorescence detectors and is sufficient for many applications in environmental analysis and pharmaceutical studies. Even lower limits of detection are expected in the future. As pointed out in our first paper on on-the-fly fluorescence lifetimes, shot-to-shot fluctuation in the excitation laser power is the dominant contributor to baseline noise. An option we plan to investigate is adding a fiber channel that monitors the laser power and stores the signal in the same waveform record as the fluorescence. This approach is aimed at reducing the effective baseline noise contribution in the overall analysis of the data.

The greater significance of our approach lies, however, in its ability to identify and resolve overlapped features from co-eluting analytes. The most widely applied multidimensional techniques currently are photodiode array absorbance detection and mass spectrometry. In each case one obtains a spectrum at each point along the elution axis. The spectra can be compared to entries in a database for verification purposes or to test for spectral homogeneity of a given band.

Integrating the signal from each wavelength channel, which is the simplest way to process the raw data our technique provides, also yields a spectrum (albeit at just four wavelengths) at each point along the elution axis. Photodiode array detectors have a few hundred points in each spectral record by comparison. The benefits of the much larger number of points in the photodiode array spectral record must be balanced against the fact that fluorescence is generally regarded as more selective and sensitive than absorption for PAHs.19 It should also be noted that the information content in the spectra is not directly proportional to the number of points in the record. The less structured the spectra, the fewer points needed to define the spectra.

Fluorescence methods may offer several advantages over the use of mass spectrometry. While advances in interfacing a liquid chromatograph to a mass spectrometer have been made, fluorescence instrumentation is still inherently simpler and easier to maintain. Fluorescence methods potentially have an advantage over mass spectrometer detectors in the analysis of isomeric PAH species. In the case of PAHs with several fused rings, there are many different species with the same molecular weight and widely different toxicities. The ability to conclusively identify a feature as benzo[a]pyrene on the basis of more than just retention time could be quite valuable.

But the additional detail that the on-the-fly fluorescence lifetime information provides is especially intriguing. The fluorescence decay curves, in conjunction with the laser excitation profile, can be fit in terms of a mathematically well-defined model (single exponential decay). As shown in our previous study, the simple and computationally fast phase-plane method allows the data to be rapidly tested for band homogeneity, which has implications for purity tests in pharmaceutical applications. Now with the extension to multiple wavelengths, even more exacting tests are envisioned. To the best of our knowledge, the work presented here represents the first time second-order data have been acquired on the fly with LC. It is also noted that the technique could be applied to process monitoring.

Future work will consider improvements in both hardware and data processing. The performance evolution of digital oscilloscopes has followed similar trends in microprocessors. Systems with 9-bit digitizers are now commercially available, compared to the 8-bit digitizers in the scope used to collect the data shown above. We have already mentioned our plans to normalize the fluorescence data for fluctuations in the laser power and to improve the collection of the fluorescence photons. We anticipate that we will be able to obtain the same voltage deflections on the digital scope with lower gain (high voltage) on the PMT; as a consequence, the photon statistics that underlie the fluorescence decay curves will be enhanced.

We have begun to implement more elaborate means to process the data. The waveform shown in Fig. 5a is not very demanding on the stripping analysis that is needed to separate temporal overlap of the signals from the different wavelength channels. The general case, in which
the temporal overlap occurs in the earlier arriving channels, will require going beyond the phase plane method. The data, which are acquired simultaneously at all four wavelengths with the same PMT and scope channel, are very amenable to global analysis. A global analysis that incorporates the full multidimensional nature of the raw data would be a very exciting test of band homogeneity.20

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