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Table I. Fitting of Pb in 50 μ L of Tridistilled Water^a

entered Y	entered X	fitted X	% error
68.0000	10.0000	9.3259	6.7405
156.0000	20.0000	20.6303	-3.1517
301.0000	40.0000	39.2569	1.8575
397.0000	50.0000	51.5889	-3.1779
573.0000	75.0000	74.1977	1.0696

$$X = (5.90722 \times 10^{-1}) + (1.28459 \times 10^{-1})Y$$

residual variance	1.524 095 54
correlation for fit	0.999 127 04

^a X is the Pb concentration (ng/mL).

Table II. Fitting of Pb in 200 μ L of Tap Water by the Method of Known Standard Additions with Sr as Internal Standard^a

entered Y	entered X	fitted X	% error
0.0020	0.0000	2.1486	
0.0032	10.0000	9.5447	4.5520
0.0048	20.0000	19.4063	2.9683
0.0062	30.0000	28.0351	6.5494
0.0097	50.0000	49.6073	0.7854
0.0141	75.0000	76.7264	-2.3019
0.0178	100.0000	99.5312	0.4687

$$X = -(1.011782 \times 10) + (6.16345 \times 10^3)Y$$

residual variance	2.478 317 24
correlation for fit	0.999 217 53

^a X is the added Pb concentration (ng/mL).

with infinitely thin samples excited under a 45° incident beam presents much lower matrix effects than the total reflection method.

In addition, with our method, inhomogeneous sample dispositions with or without internal standards do not affect the results of the analysis. A flexibility of the excitation is possible

by changing the anode material of the X-ray tube. Low cost automation of the whole procedure is possible.

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New Approach to Phase and Modulation Resolved Spectra

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Time domain fluorescence spectrometry offers a versatile and powerful approach to the analysis of heterogeneous emitting systems. In this paper we describe a new approach, based on software, to the acquisition of phase and modulation resolved spectra. Mixtures of fluorophores with different lifetimes can be analyzed in real time to give the individual excitation or emission spectra. Examples of two- and three-component mixtures are given and comparisons are made with the commercially available hardware approach.

Fluorescence spectrometry is presently being applied to increasingly complex systems in the physical, chemical, and

biological sciences. The degree of sample heterogeneity in such systems is often a fundamental consideration. Information on the nature and extent of heterogeneity is important whether the fluorophore is used to probe its molecular environment or whether a compositional description is sought for analytical purposes.

An extensive research effort has been expended on the methodologies of analysis of heterogeneous emissions. The application of fluorescence spectrometry to the identification of petroleum oils and similar materials began with the work of Parker and Barnes (1). The original matrix analysis approach of Weber (2) has been greatly extended and developed into a powerful rank analysis method, the excitation-emission matrix or EEM (3). The EEM approach and the related

synchronous luminescence techniques (4) have been useful in the analysis of many complex mixtures such as the quantitation of polynuclear aromatics in air particulate extracts (5) or coal-derived extracts (6, 7) and in oil identification procedures (8–10). Sophisticated computer file searching techniques have also been developed to aid in interpreting emission spectra (11).

The procedures mentioned above all rely upon differences in the excitation or emission spectra. Many fluorophores, however, exhibit very similar spectra and are not always amenable to the aforementioned techniques. Time domain data offer a powerful alternative or supplemental means for examining and quantitating heterogeneous emitting systems. The recent appearance of true multifrequency cross-correlation phase and modulation fluorometry (12) has greatly extended the scope and power of the harmonic response approach to time domain measurements. Operational principles of this technique and its application to the analysis of heterogeneous emissions have been recently reviewed (13, 14). A complete heterogeneity analysis as a function of wavelength will yield the component lifetimes and fractional contributions to the intensities from a mixture of fluorophores; such global analyses have been accomplished by using both impulse response and harmonic response techniques (13–17). Such analyses permit reconstitution of the spectra of the individual components from the amplitude data. Although the global analysis offers the most complete approach to the time domain data, a more rapid alternative to the resolution of spectra may be available in the form of phase resolved spectra.

Phase resolved detection (also referred to as phase sensitive detection), a standard methodology in fluorometry for many years (18, 19), was first applied by Veselova et al. to the resolution of individual intensity components of heterogeneous emissions from simple fluorescence solutions (20, 21). The recent availability of commercial phase fluorometers led to a renewed interest in this technique and in its application to systems of chemical and biochemical interest (22–27). All of the recent work, however, has utilized the same approach to the data acquisition methodology, specifically a hardware-based approach. We present here a different approach to this general problem, which we term phase and modulation resolved spectra (PMRS), based upon a software analysis that offers striking advantages over the traditional hardware approach. We should note at the outset that the new approach gives, in real time, the component spectra as the wavelengths are scanned.

PRINCIPLES OF PHASE RESOLVED SPECTRA

The principles of phase fluorometry and phase resolved fluorometry have been reviewed recently (13, 27) and we shall only summarize the important considerations. The operational principle of the technique is as follows. If a population of fluorophores composed of species which emit with characteristically different spectra and lifetimes (hence different phase angles) is excited by a source with an intensity modulation sinusoidally, then spectral resolution of the different components can be effected, in principle, using a detector sensitive to the phase angle. The total intensity, $S(\lambda, t)$, of the fluorescence emission obtained by a sinusoidally modulated excitation is given as the sum of the intensity components of the individual species, each with its characteristic phase angle, ϕ_i , and relative modulation, M_i . Thus

$$S(\lambda, t) = \sum_i I_i(\lambda) f_i(\lambda) M_i \sin(\omega t - \phi_i) \quad (1)$$

where $I_i(\lambda)$ describes the unit intensity contribution of the i th species as a function of the wavelength and $f_i(\lambda)$ the contribution of the i th species to the total intensity relative to the other contributing species. By definition, $\sum_i f_i = 1$.

The phase sensitive detected signal (PSD), applied in our cases to the acquisition of phase resolved spectra, is obtained by multiplying the $S(\lambda, t)$ function by a periodic function $P(t)$ and then integrating the product over a period T .

$$\text{PSD} = \int_0^T S(\lambda, t) P(t) dt \quad (2)$$

where

$$P(t) = \begin{cases} = 0 & \text{from } \phi \text{ to } \phi_D \\ = 1 & \text{from } \phi_D \text{ to } \phi_D + T/2 \\ = 0 & \text{from } \phi_D + T/2 \text{ to } T \end{cases} \quad (3)$$

Integration of eq 2 gives

$$\text{PSD} = \sum_i I_i(\lambda) f_i(\lambda) M_i \cos(\phi_D - \phi_i) \quad (4)$$

When $\phi_D - \phi_i = 90^\circ$ or 270° , the intensity contribution of the i th component to the signal PSD is zero and the observed intensity will be proportional to the contribution of the remaining species.

Selection of the appropriate detector angle for nulling a specific component of the emission requires that the lifetime of that component be known or that an appropriate standard compound is available. In some cases one can determine the detector angle which nulls the emission signal at wavelengths where only one component contributes to the signal. An alternative to the direct nulling approach is indirect nulling in which case standards and samples are measured at a series of detector phase angles covering the range 0° to 360° . This approach has permitted the determination of three and four components in some systems (27); in these cases the aim was quantitative analysis as opposed to simple spectral acquisition. Hardware-based approaches do not at present exist for the acquisition of modulation resolved spectra.

MATHEMATICAL APPROACH TO SOFTWARE BASED PHASE AND MODULATION RESOLVED SPECTRA

Given a circular frequency, ω_r , where $\omega_r = 2\pi f_r$ (f_r represents the linear modulating frequency and r is an index covering the frequency range utilized), one can measure a phase shift, ϕ_r , and relative modulation, M_r ; these values may be expressed as

$$\begin{aligned} \phi_r &= \tan^{-1}(S_r/G_r) \\ M_r &= (S^2 + G^2)^{1/2} \end{aligned} \quad (5)$$

where G and S can be directly related to the lifetimes and fractional intensities of the individual components by the general equations (28)

$$\begin{aligned} G_r &= \sum_i \frac{f_i}{1 + \omega_r^2 \tau_i^2} \\ S_r &= \sum_i f_i \frac{\omega_r \tau_i}{1 + \omega_r^2 \tau_i^2} \end{aligned} \quad (6)$$

The essence of the software analysis is to obtain the values of f_i as a function of wavelength which yields a lifetime resolved spectra. At each wavelength one measures the angle, ϕ_r , and relative modulation, M_r . With these data the G_r and S_r functions can be readily calculated using the inverted forms of equations (5).

In the case of two components, knowledge of either the phase angle or modulation ratio (together with the total intensity) is sufficient to calculate the relative fractional contributions if the lifetimes of the two components are known. The explicit expressions for these fractional contributions, calculated from either phase or modulation data, have been

previously reported (13) and are repeated below for convenience

$$R_P = \frac{\tau_2 - \tau_P}{\tau_P - \tau_1} \frac{1 + \omega^2 \tau_1^2}{1 + \omega^2 \tau_2^2} \quad (7)$$

$$R_m = \frac{\omega \tau_1 - [(1 + \omega^2 \tau_1^2)(1 + \omega^2 \tau_2^2)/(1 + (\omega \tau_m)^2) - 1]^{1/2}}{[(1 + \omega^2 \tau_1^2)(1 + \omega^2 \tau_2^2)/(1 + (\omega \tau_m)^2) - 1]^{1/2} - \omega \tau_2} \quad (8)$$

where $R = f_1/f_2$ (the subscripts P and m refer to data obtained using either phase (P) or modulation (m) values) and τ_P and τ_m are the measured phase and modulation lifetimes of the mixture, respectively.

In the case of three components the explicit equations for the G and S functions at a given frequency, ω , are given in eq 9. Hence, if the three lifetime values are known, then the

$$G = \frac{f_1}{1 + \omega^2 \tau_1^2} + \frac{f_2}{1 + \omega^2 \tau_2^2} + \frac{f_3}{1 + \omega^2 \tau_3^2}$$

$$S = \frac{f_1 \omega \tau_1}{1 + \omega^2 \tau_1^2} + \frac{f_2 \omega \tau_2}{1 + \omega^2 \tau_2^2} + \frac{f_3 \omega \tau_3}{1 + \omega^2 \tau_3^2} \quad (9)$$

$$1 = f_1 + f_2 + f_3$$

fractional contributions can be calculated given both phase and modulation data at a single modulation frequency and the total intensity, by simple inversion of eq 9.

EXPERIMENTAL SECTION

Software resolved phase and modulation spectra were obtained with the multifrequency cross-correlation instrument described by Gratton and Limkeman (12) equipped with an ISS01 interface (from ISS, Inc., Urbana, IL) for an Apple II computer. The interface can simultaneously digitize the ac and dc components of both sample and reference channels as well as the phase of the sample signal relative to the reference. Software for data acquisition and analysis is currently available from ISS, Inc. Sample excitation was accomplished by using the 364-nm line of a Spectra Physics Model 164/5 argon ion laser and emission was observed through a $1/4$ m Jarrell-Ash monochromator using 4-nm slit widths. The detector was a Hamamatsu R928 photomultiplier which has been shown to have negligible color error (12, 29).

Perylene and 9-aminoacridine (9-AA) from Aldrich Chemical Co. and Pfaltz and Bauer, Inc., respectively, were used without further purification. 6-(Diethylamino)naphthalene-1-sulfonate (DENS) was synthesized in the laboratory of G. Weber at the University of Illinois. *p*-Bis[2-(5-phenyloxazolyl)]benzene (POPOP) was from Eastman Laboratory.

Phase, modulation, and intensity data were obtained at 2-nm intervals over the spectral range utilized. Steady-state emission spectra were obtained on a home-built spectrofluorometer (30) in G. Weber's laboratory. Solution absorbances, measured on a Beckman Model MVI spectrophotometer, were maintained below 0.1 at the exciting wavelength and over the entire emission spectral range utilized. Solutions, in ethanol, were not degassed for these studies.

RESULTS AND DISCUSSION

Figure 1 shows the typical graphic output of the software routine. Specifically, a mixture of perylene and 9-AA in ethanol was prepared such that each fluorophore contributed approximately equally to the emission upon excitation at 364 nm. For a two-component mixture the user inputs two lifetime values (this procedure is analogous to setting the detector phase angles in the hardware based approach whether or not this nulls the signal) and the phase or modulation resolved spectra as well as the total intensity are displayed in real time as the spectra is acquired. Figure 1 shows that the spectra obtained from either phase or modulation data are virtually identical; these data were obtained by using 20-MHz modu-

Figure 1. Intensity (dotted line) and phase and modulation resolved (solid lines) spectra for a mixture of perylene and 9-aminoacridine in ethanol (not degassed).

Figure 2. Intensity (dotted line) and phase and modulation resolved (solid lines) spectra for a mixture of DENS and perylene in ethanol (not degassed).

lation frequency (rationale for selecting the appropriate modulation frequency is given in the discussion section) and lifetime values of 12.0 ns and 4.3 ns. After the data are acquired, one can rapidly recalculate and replot the phase or modulation resolved spectra using other lifetime values; with the hardware based method, of course, completely new spectra must be taken each time a change in the suppression angle is desired.

Figure 2 shows similar results for a mixture of DENS and perylene in ethanol also excited at 364 nm using 20 MHz modulation frequency; lifetime values of 10.8 ns and 4.3 ns were utilized for both phase and modulation resolved spectra.

Figure 3 shows results from a three-component mixture. Individual solutions of perylene, POPOP, and DENS in ethanol were prepared to give similar contributions to the total emission upon excitation at 364 nm. The steady-state spectra of the individual components are shown in Figure 3A. Equal amounts of these three solutions were then mixed and the wavelength-dependent phase and modulation data were obtained (using 20-MHz modulation frequency). After the data are obtained and stored in computer memory, the three lifetime values are entered (results in Figure 3B were obtained using lifetime values of 10.8 (DENS), 4.3 (perylene), and 0.9 ns (POPOP)) and the individual resolved spectra are displayed along with the intensity.

The optimum modulation frequency to utilize in phase and modulation resolved spectrometry can be found, for the case of n components of lifetime τ_i ($i = 1, \dots, n$), by the log average of the inverse of the lifetime values

$$f_{opt} = \frac{1}{2\pi} e^{n^{-1}(\sum_i \log \tau_i^{-1})} \quad (10)$$

Figure 3. (A) Intensity spectra of individual components, POPOP (A), DENS (B), and perylene (C) in ethanol (not degassed). (B) Intensity (dotted line) and phase resolved spectra (solid lines) for the ternary mixture in A.

This expression has been obtained for the case of equal component contributions although deviations are minimal for unequal contributions. The efficacy of this approach in resolving spectral contributions depends on the error in the measured phase and modulation values. The error in the phase and modulation data strongly depends on the lifetime values but not strongly on the modulation frequency. Errors in the relative intensities of about ± 0.08 corresponding to phase and modulation data, respectively (at the optimum frequency), were found using the analysis in ref 17 with errors of $\pm 0.2^\circ$ in phase and ± 0.004 in modulation for the case of two components of equal contributions with a lifetime ratio of 1:1. These errors are typical for present day phase and modulation fluorometers.

The advantages of the software approach over the hardware approach are clear. First of all, the signal-to-noise ratio is superior in the software approach since no electronic signal suppression occurs. By the same token, the software approach does not introduce electronic noise or systematic errors due to the null detector calibration procedure. Secondly, using our approach one obtains with one run both spectra with their correct relative intensities whereas two runs are needed with the hardware approach. By placing correction factors in the computer, one could also obtain directly the corrected excitation or emission spectra in real time with the software approach. From the perspective of cost considerations the software approach is superior also since it obviates the need for relatively expensive phase sensitive electronics.

The software based approach to phase and modulation resolved spectra is, in principle, a special case of the more general global analysis. In the global approach, one would perform a complete multifrequency lifetime analysis at a number of excitation or emission wavelengths. When applicable, however, the approach described in this report has the advantage of offering a real-time analysis, i.e., time-resolved spectra can be displayed as the steady-state spectrum is scanned.

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Registry No. DENS, 96245-43-1; POPOP, 1806-34-4; 9-AA, 90-45-9; perylene, 198-55-0.

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