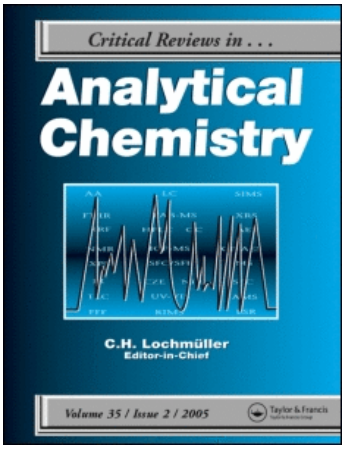

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PHASE-RESOLVED FLUORESCENCE IN CHEMICAL ANALYSIS

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I. INTRODUCTION

The phenomenon of fluorescence emission offers a variety of parameters that can be exploited to achieve highly selective chemical analysis.[1] Only a small fraction of chemical species are fluorescent, providing inherent selectivity. Limited applicability, a drawback of the selectivity, can often be overcome by using derivatization or indirect methods based on effects such as quenching, complexation, enzymatic catalysis, and immunochemical equilibria.

Dimensions of information that can be used for fluorimetric analysis include excitation and emission spectra, polarization, quantum yield, and fluorescence lifetime (τ). The actual experimental parameters associated with the fluorescence lifetime dimension depend upon the instrumental technique that is used to incorporate fluorescence lifetime information into the analysis. The two basic approaches to the exploitation of fluorescence lifetimes are the use of pulsed sources to produce decay curves directly and phase-modulation fluorescence in which an amplitude-modulated source is employed. Phase-resolved fluorimetry, the topic of this review, is based on the phase-modulation technique. The term "phase-resolved luminescence spectroscopy" was proposed to refer to the phase resolution technique for both fluorescence and phosphorescence.[2] For fluorescence alone, the use of "phase-resolved" has been favored by some investigators while others prefer the term "phase-sensitive". The two terms have been used synonomously in the literature. In this article, the present authors will use the term "phase-resolved" to refer to the technique and to spectra, and the term "phase-sensitive" will refer to the detection used to accomplish phase resolution.

A number of texts and articles have reviewed various aspects of the history, theory, and instrumentation of phase-modulation fluorimetry[3-8] and phase-resolved fluorimetry.[5,8-11] The phase technique was first described and demonstrated by Gaviola.[12] In the following years, the theory of phase fluorimetry was further developed[13] and improvements were made on the type of instrumentation used by Gaviola.[14] The determination of fluorescence lifetimes from both phase and modulation measurements was introduced by Birks and Little in 1953.[15] Numerous developments in instrumentation have since occurred, including the use of cross-correlation techniques[15-18] and the construction of instruments with continuously variable excitation modulation frequencies.[7,19-22]

The basis of the phase-modulation technique is the use of an excitation beam that is modulated at a high frequency. In the following discussion, and throughout the review, the present authors will generally assume that the source is continuous and that the modulation is sinusoidal, although other waveforms can also be used. The time-dependent excitation function, E(t), has the form

$$E(t) = A(1 + m_{ex}\sin\omega t) \quad (1)$$

where A is the steady-state (DC) wavelength-dependent excitation function, m_{ex} is the modulation depth (i.e., the ratio of the AC amplitude to the DC intensity), and ω is the angular modulation frequency (equal to $2\pi f$, where f is the linear modulation frequency). The time-dependent fluorescence response, F(t), from a single emitting species is phase-delayed (phase-shifted) and demodulated relative to the excitation beam:

$$F(t) = A'[1 + m_{ex}m\sin(\omega t - \phi)] \quad (2)$$

where A' is the steady-state (DC) wavelength-dependent emission function incorporating spectral and instrumental factors, ϕ is the phase delay, and m is the demodulation factor (i.e., the ratio of the modulation depth of the emission signal to that of the excitation, m_{ex}, also equal to $\cos\phi$). The excitation and emission functions are shown in Figure 1. Fluorescence lifetimes can be calculated from the phase-delay:

$$\tau_p = (1/\omega)\tan\phi \quad (3)$$

or from the demodulation:

$$\tau_m = (1/\omega)[(1/m^2) - 1]^{1/2} \quad (4)$$

The excitation phase (zero, by definition) and modulation (m_{ex}, less than or equal to unity) are calibrated by the use of a reference solution, which can be either a scattering solution ($\tau = 0$) or a fluorophore with a known fluorescence lifetime.[23,24] Factors affecting the calibration have been discussed.[25]

In phase-resolved fluorescence spectroscopy, the AC portion of the emission function F(t) is multiplied by a periodic function P(t) and integrated over time. The function P(t) alternates between two values, each maintained for one half-cycle. The relative position of the integration interval with respect to F(t), referred to as the detector phase angle, ϕ_D, is continuously variable between 0 and 360°. The resulting integrated signal is a time-independent phase-resolved intensity that is a function of the fluorescence spectral and lifetime characteristics and concentration(s) of the emitter(s):

$$F(\phi_D) = A'm_{ex}m\cos(\phi_D - \phi) \quad (5)$$

If ϕ_D equals the phase of F(t), maximum phase-resolved intensity is observed (Figure 2a). If ϕ_D is 90° out-of-phase with F(t), the emission is nulled (suppressed) and the phase-resolved intensity is zero (Figure 2b). Detector phase angle settings between these two extremes result in intermediate phase-resolved intensities (Figure 2c). For a mixture of emitters at a given ϕ_D, the total phase-resolved intensity is the sum of the individual contributions. This additivity property is the key to multicomponent phase-resolved fluorimetric determinations and can be used in a manner analogous to the use of Beer's Law additivity in absorptiometric analysis or fluorescence intensity additivity in conventional fluorimetric analysis. The advantage of phase-resolved fluorimetry is that

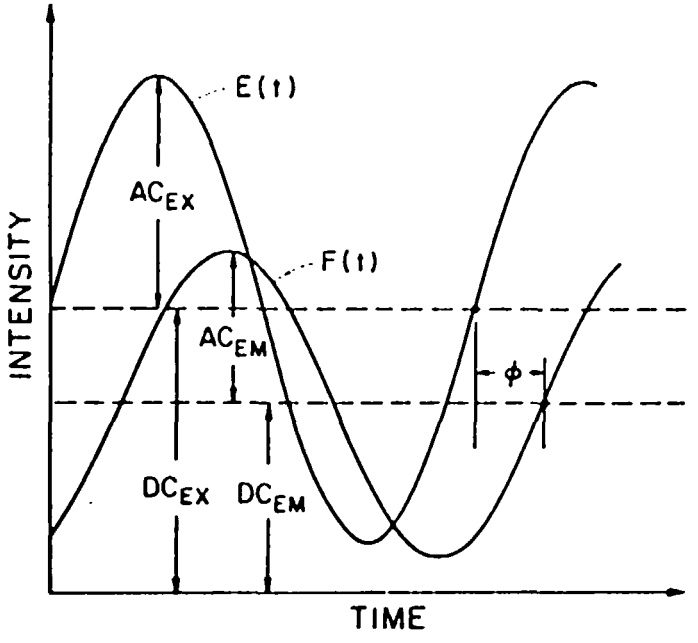


FIGURE 1. Schematic representation of the excitation E(t) and fluorescence F(t) waveforms. (From Gratton, E., Jameson, D. M., and Hall, R. D., *Annu. Rev. Biophys. Bioeng.*, 13, 105, 1984. With permission.)

multiple detector phase angles can be used instead of or in addition to multiple wavelengths to generate sufficient equations for the determinations of multiple unknown concentrations. Even components with identical spectra can be simultaneously determined by the use of different detector phase angles, provided they have sufficiently different fluorescence lifetimes. Therefore, analyses that could not be performed with conventional fluorimetry based on wavelength selectivity alone may be possible with phase-resolved fluorimetry.

II. PHASE-MODULATION HETEROGENEITY ANALYSIS

Heterogeneous, nonexponential decay of fluorescence emission from systems with more than one ground-state component is evidenced in phase-modulation fluorescence by the fluorescence lifetime calculated from the observed phase-delay being shorter than the lifetime calculated from the observed demodulation. The analysis of such heterogeneous systems can be accomplished by the use of measurements of phase-delay and demodulation at multiple excitation modulation frequencies to generate independent equations which are solved for the fluorescence lifetimes and fractional intensity contributions of each of the emitting species. Exact solutions to the equations for two- and three-component systems, requiring measurement of phase-delay and demodulation at two and three modulation frequencies, respectively, have been given by Weber.[26] Nonlinear least-squares methods for phase-modulation heterogeneity analysis have also been described,[7,27,28] and the relationship between the analysis of heterogeneous systems by using pulse data and phase-modulation data has been discussed.[29] A brief discussion of the use of heterogeneity analysis is included here because of its close relationship to phase-resolved fluorimetry.

Phase-modulation heterogeneity analysis has been applied to the study of a number

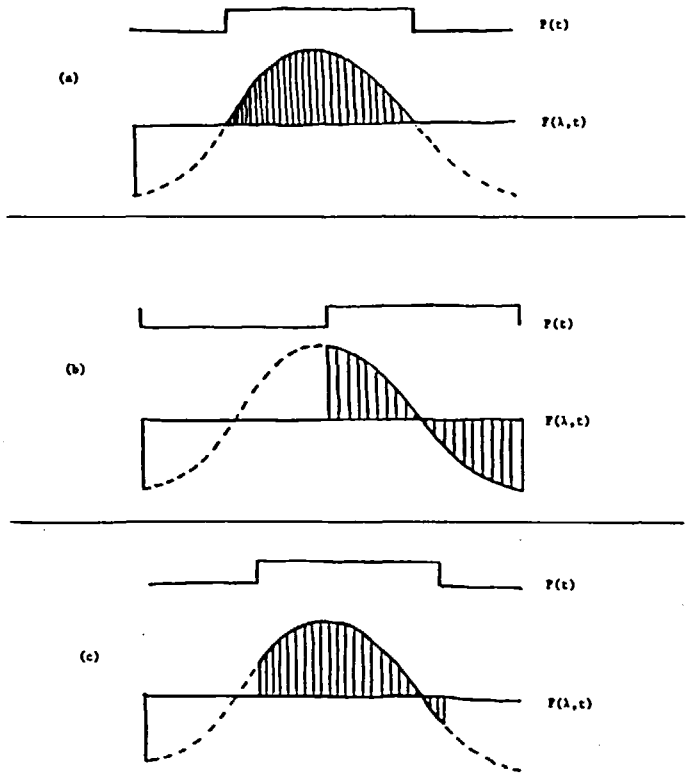


FIGURE 2. Depiction of the periodic integration interval relative to F(t). Phase-sensitive detection (a) in phase with F(t), (b) 90° out of phase with F(t), and (c) at an intermediate phase relative to F(t). (From McGown, L. B. and Bright, F. V., *Anal. Chem.*, 56, 1400A, 1984. With permission.)

of chemical systems. For example, Jameson and Weber resolved the fluorescence emission of tryptophan into its zwitterion (τ = 3.1 nsec) and anion (τ = 8.7 nsec) components in the pH range of 8 to 10.[30] The best results were obtained by using 6 and 30 MHz as the two excitation modulation frequencies to yield resolved lifetimes within ±0.4 nsec of the true values and fractional intensity contributions within 10 to 20% of the expected values. Barrow and Lentz used heterogeneity analysis to study the emission decay of a fluorescent probe in lipid bilayer vesicles as a model system for lipid bilayer structural domains in biological membranes.[31] Brand and co-workers resolved the heterogeneous fluorescence decay of mixtures of anthracene and 9-cyanoanthracene, as well as that of horse liver alcohol dehydrogenase.[28] Recent work has focused on the development of multifrequency phase-modulation fluorimetry using instruments with continuously variable excitation modulation frequencies and least-squares data analysis methods, which are superior to the use of exact solutions for the multifrequency data.[7]

Phase-modulation heterogeneity analysis has proved to be useful when the fluorescence lifetimes and fractional intensity contributions of a heterogeneous two- or three-component system is required. Quantitative determinations require the additional steps of measuring the DC intensities of the solutions and of standards (to find the molar intensities) at each excitation modulation frequency. The number of modulation frequencies used must equal or exceed the number of homogeneous-decay components in the system. If the fluorescence lifetimes of the components are known, the number of

modulation frequencies required can be reduced, but any errors in the assumed lifetime values will cause systematic errors in the determination results.

Compared with phase-modulation heterogeneity analysis, phase-resolved fluorimetry is generally better suited to multicomponent determinations. Fluorescence lifetime differences provide the basis of the selectivity derived from the use of different detector phase angles, but the lifetime values themselves need never be determined. It is the use of multiple detector phase angles at a single excitation modulation frequency rather than multiple modulation frequencies that generates the series of analytical equations, so that the instrumental readjustment required for each modulation frequency is eliminated. As discussed in a later section, the use of several modulation frequencies in phase-resolved determinations has been described but is not a requirement of the technique. Finally, since phase-resolved fluorimetry measures concentration-dependent fluorescence intensities directly, the generated equations can be solved directly for component concentrations. Whereas phase-modulation heterogeneity analysis has, to date, been described only for two- and three-component systems, as many as six components have been resolved by using phase-resolved fluorimetry.[32] It should also be noted that quantitative determinations of component concentrations using heterogeneity analysis have never been described; results have only been reported in terms of fractional intensities, although conversion of fractional intensities to concentrations would only require the measurement of steady-state (DC) intensities of standard solutions of each of the components.

III. INSTRUMENTATION

Numerous instruments have been designed for phase-modulation fluorescence lifetime measurements, from the first instrument used by Gaviola[12] to the modern multifrequency instruments. Details of phase-modulation instrumentation have been discussed elsewhere and will not be covered here. Various lamps and lasers have been used as light sources. Gaviola's instrument employed a Kerr cell for excitation modulation and a second Kerr cell as a phase-sensitive detector.[12] The Debye-Sears acousto-optic ultrasonic modulator, which can achieve a single fundamental modulation frequency and several harmonic overtones, and the Pockels cell electro-optic modulator capable of an essentially continuous range of modulation frequencies, are among the modulation devices that have been used in modern phase-modulation fluorometers. Performance has been greatly enhanced by the use of cross correlation[15-17] in which the photomultiplier gain is modulated at frequency $\omega + \Delta\omega$ to produce a cross-correlation signal of $\Delta\omega$ which is isolated with a low pass filter.

The first phase-resolved studies by Veselova et al.[33] were performed with a phase fluorometer described by Bonch-Bruevich et al.,[34] which incorporated an optical diffraction modulator operating at 11.2 MHz, a light dividing system photomultiplier tube (PMT) detection, and a phase meter unit which measured the phase difference between the dual inputs from the sample and a scattering solution (Figure 3). Phase-resolved spectra were resolved by using either an optical or electrical phase shifter to adjust the detector phase to be out of phase with the component to be nulled.

The phase-resolved phosphorimeter described by Mousa and Winefordner (Figure 4)[2] used a lock-in amplifier with a variable-frequency control, a calibrated adjustable phase shifter, and a phase quadrant switch to shift the phase of the internal reference by 90, 180, or 270°. The reference signal from the lock-in amplifier was used to drive the modulation system for the lamp.[35] If the phase controls on the lock-in amplifier are adjusted to the phase angle at quadrature, i.e., the point at which the rectified signal passes through zero for the excitation signal and for the luminescence signal, the

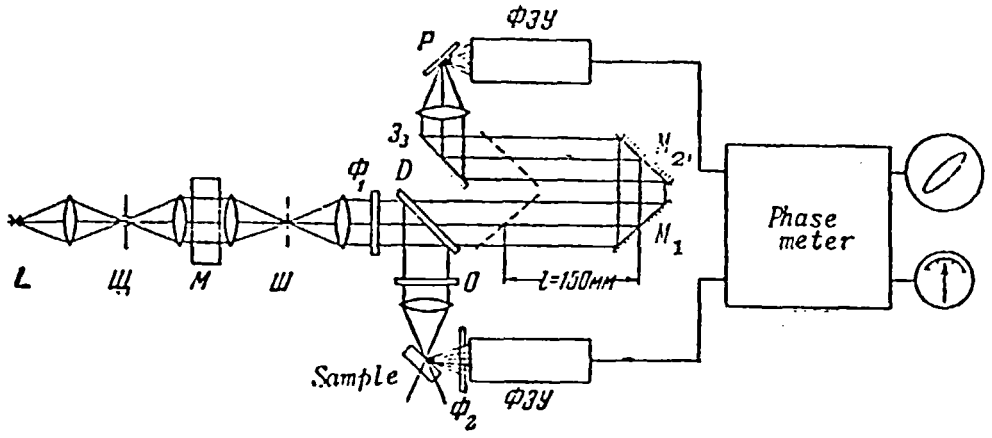
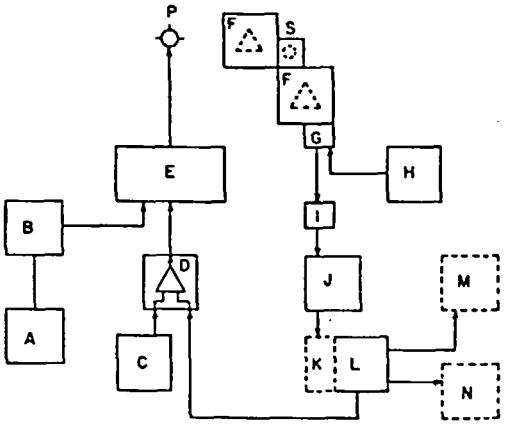


FIGURE 3. Diagram of the phase fluorometer described by Bonch-Bruevich et al. L = light source; ω_1, M, and ω = components of the optical modulator; ϕ_1 and ϕ_2 = light filters; D = light dividing plate (beam splitter); O = neutral weakener; P = scatterer; M1 and M2 = system of moveable mirrors; ϕ3y = photomultipliers. (From Bonch-Bruevich, A. M., Molchanov, V. A., and Shirokov, V. I., *Bull. Acad. Sci. U.S.S.R. Phys. Ser.*, 20, 541, 1956. With permission.)



A = 0-40V, 0-30A DC power supply (Harrison Model 6268A)
B = starter circuit
C = 0-100V, 0-0.2A DC power supply (Harrison Model 6116A)
D = summing operational amplifier and current booster (Heath Model EUW-19)
E = modulation circuit
F = excitation and emission monochromators
G = photomultiplier tube and housing
H = high voltage power supply
I = load resistors
J = differential amplifier (Tektronix Model 1A7A)
K = amplifier (optional) (PAR Model 211)
L = lock-in amplifier
M = strip-chart recorder (optional)
N = X-Y recorder (optional)
P = xenon arc lamp
S = sample compartment

FIGURE 4. Block diagram of the phase-resolved spectrometer described by Mousa and Winefordner. (From Mousa, J. J. and Winefordner.

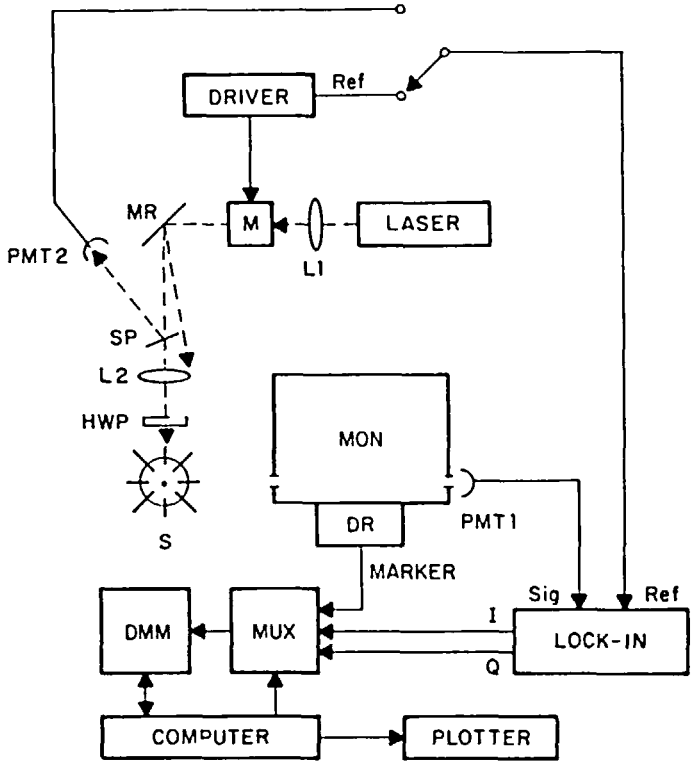


FIGURE 5. Schematic representation of the phase-resolved spectrometer described by Demas and Keller. LASER = ionized argon laser; PMT 1 = signal phototube; PMT 2 = reference phototube; L1 = 10-cm focusing lens for acousto-optic modulator; L2 = defocusing lens or neutral density filters; DRIVER = modulator oscillator/driver; M = acousto-optic or electro-optic modulator; HWP = half-wave plate for electro-optic modulator; MON = monochromator; DR = Compuscan monochromator wavelength drive; LOCK-IN = lock-in amplifier; MR = mirror; SP = microscope slide beam splitter; MUX = analog multiplexer; S = sample; DMM = digital multimeter. (From Demas, J. N. and Keller, R. A., *Anal. Chem.*, 57, 538, 1985. With permission.)

phase-delay of the luminescence can be found. To acquire phase-resolved spectra, the intensity contribution of an emitting component is nulled by setting the detector phase angle to be in quadrature for the component, thereby allowing the acquisition of the phase-resolved spectrum of the other component(s) in the system.

Instruments developed in recent years for phase-resolved spectroscopy have been described by Demas and Keller[36] (Figure 5) and Van Hoek and Visser[37] (Figure 6) and employ argon ion laser excitation with external amplitude modulators and PMTs and commercial lock-in amplifiers for phase-resolved detection. Bright et al. described an argon-ion laser phase-resolved phosphorimeter with a fiber optic probe (Figure 7).[38] Berndt described an instrument (Figure 8) in which a silicon avalanche photodiode, which was gain modulated by adding a high frequency signal to the DC bias voltage, served as an optoelectronic cross correlator to achieve phase-sensitive detection at modulation frequencies up to 875 MHz.[39]

A commercial phase-modulation spectrofluorometer (Figure 9) is available with phase resolution capabilities.[9] *In situ* modification of the commercial instrument (one that lacked the manufacturer's phase resolution option) to provide phase resolution

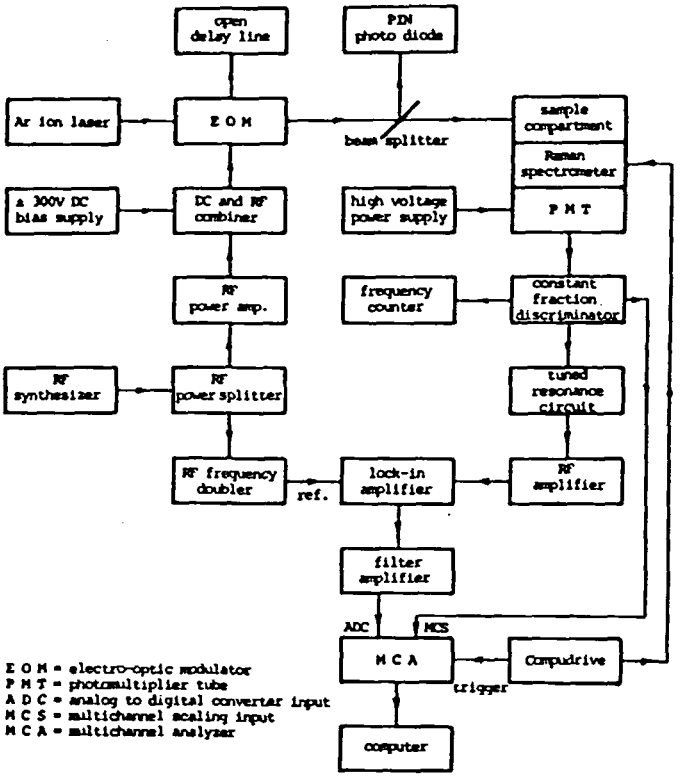


FIGURE 6. Block diagram of the experimental set-up described by Van Hoek and Visser. (From Van Hoek, A. and Visser, A. J. W. G., *Anal. Instrum.*, 14, 143, 1985. With permission.)

has also been described by Lakowicz and Cherek.[40] Both instruments incorpora cross-correlation electronics,[17] Debye-Sears acousto-optic excitation modulation at 18, and 30 MHz, and phase-sensitive PMT detection with a lock-in amplifier. T detector phase angle is continuously variable from 0 to 360°. A later commercial moc uses a Pockel's cell modulator to provide continuously variable multifrequency phas modulation and phase resolution capabilities. The use of a xenon arc lamp source these instruments enables continuous scanning of the excitation wavelength, as well synchronous scanning, in which the excitation and emission wavelengths are simult neously scanned.

Precisions in modern phase resolution instruments generally range from several p coseconds to several tenths of a nanosecond, depending upon the lifetime being dete mined and the modulation frequency used. These precisions can be achieved with bo laser and lamp excitation sources.

IV. STUDIES OF PHASE-RESOLVED SPECTRA

If a mixture of emitting components A and B is measured with the detector out phase (in quadrature) with A, the individual spectrum of B can be obtained. If ϕ_A a ϕ_B are the phase angles of A and B, then the phase-resolved intensity of B at $\phi_D = \phi_A$ 90° relative to its phase-resolved intensity at $\phi_D = \phi_B$ will be attenuated by the fact $\sin(\phi_B - \phi_A)$. The individual spectrum of A, attenuated by $\sin(\phi_A - \phi_B)$, can be simila obtained (Figure 10).[2]

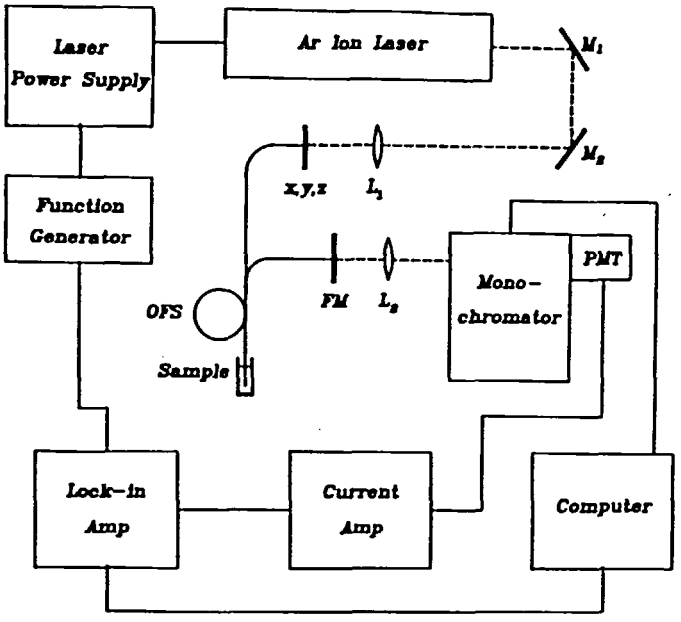


FIGURE 7. Schematic of the apparatus described by Bright et al. M_1 and M_2 = mirrors; L_1 and L_2 = lenses; x,y,z = x,y,z translation stage; OFS = optical fiber sensor; FM = fiber mount. (From Bright, F. V., Monnig, C. A., and Hieftje, G. M., *Anal. Chem.*, submitted. With permission.)

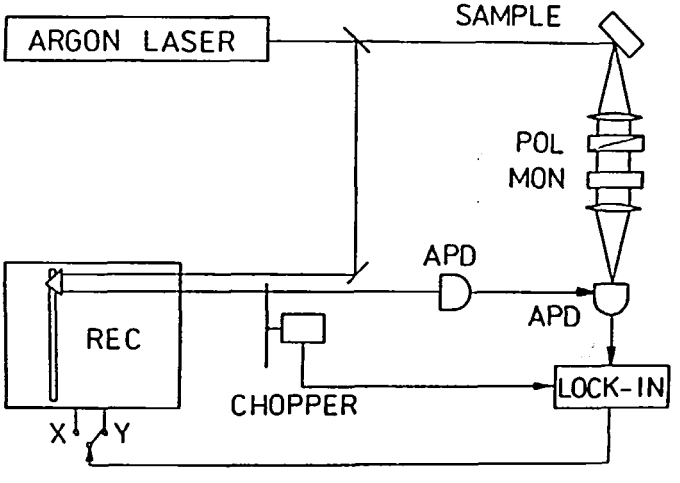


FIGURE 8. Experimental arrangement described by Berndt. APD = avalanche photodiode. (From Berndt, K., *Opt. Commun.*, 56, 30, 1985. With permission.)

Essentially all of the early work done with phase-resolved fluorescence spectroscopy (PRFS) involved the resolution of the individual spectra in two-component systems. The original use of phase resolution by Veselova et al. was for the resolution of the spectral contributions from 3-amino-*N*-methylphthalimide (3AMP) and 3,6-diacetylamino-*N*-methylphthalimide (3,6DAMP) in 1:1 mixtures of the two.[33] Fluorescence lifetimes of 2.25 nsec for 3AMP and 10.7 nsec for 3,6DAMP were found from

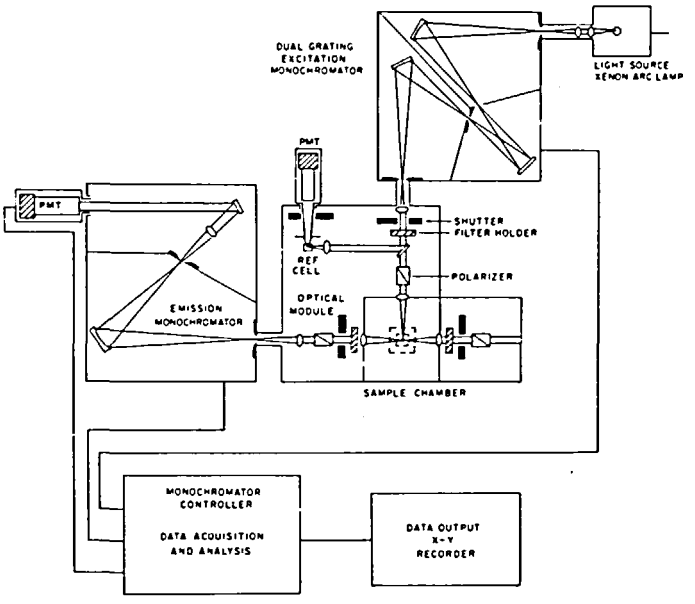


FIGURE 9. Schematic diagram of an SLM 4800S spectrofluorometer (SLM Instruments, Urbana, Ill.) (From Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983. With permission.)

the phase angle maxima ($\phi_D = \phi_{comp}$) of the components. The spectrum of each individual component was obtained at the null phase angle of the other component. In the same study, the individual spectra of anthracene in the monomeric ($\tau = 3.59$ nsec) and aggregated ($\tau = 7.56$ nsec) forms were resolved for a 2×10^{-4} *M* solution of anthracene quick frozen at −196°C (Figure 11).

In a later study, Veselova et al. used PRFS to resolve the individual spectra for 3AMP free and bound to an active solvent (acetone, pyridine, or dimethylformamide) in hexane.[41] It was demonstrated that the formation of an exciplex by 3AMP with the active solvent is a diffusion-controlled process that occurs during the lifetime of the excited state, with kinetics resembling those of excimer formation.

Two-component systems have been spectrally resolved using a commercial phase resolution spectrofluorometer, including mixtures of 1,4-*bis*(5-phenyloxazol-2-yl) benzene (POPOP) and dimethylPOPOP (Me_2POPOP) which have highly overlapping emission spectra, mixtures of perylene and anthracene, and mixtures of tyrosine and tryptophan.[10] The phase-resolved spectra of POPOP and Me_2POPOP are shown in Figure 12.

Lakowicz and co-workers have used PRFS to study a variety of chemical and biochemical systems. In their earliest work, phase resolution was used to resolve the spectra of the individual components in mixtures of 2-*p*-toluidinylnaphthalene-6-sulfonic acid (TNS) and 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN) (Figure 13) and to resolve the highly overlapping spectra of dibenzo(a,h)anthracene (DBA) and dibenzo(c,g)carbazole (DBC) in mixtures of the two (Figure 14).[40]

Lakowicz and Cherek were able to resolve the tryptophan and tyrosine emission spectra for solutions of denatured human serum albumin.[42] The phase-resolved and steady-state spectra obtained for the protein are shown in Figure 15. Tryptophan and tyrosine emission spectra were also resolved for bovine serum albumin by Mattheis et al.,[10] as shown in Figure 16.

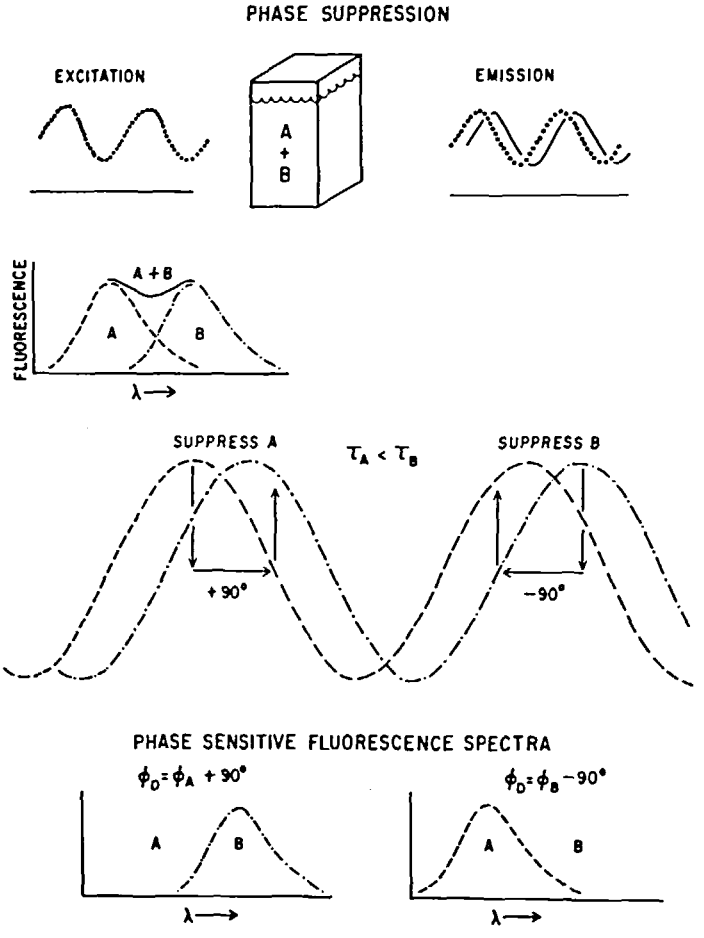


FIGURE 10. Intuitive description of phase suppression for a fluorophore mixture. (From Lakowicz, J. R. and Cherek, H., *J. Biochem. Biophys. Meth.*, 5, 19, 1981. With permission.)

Lakowicz and Balter used PRFS to study the solvent relaxation of a tryptophan derivative *N*-acetyl-L-tryptophanamide (NATA).[43] The phase-resolved emission spectrum of NATA in propylene glycol as a function of temperature is shown in Figure 17, and the data used to generate the Arrhenius plot shown in Figure 18 are summarized in Table 1. A value of 3 kcal/mol was calculated for the activation energy of the relaxation process, which was less than the value normally found for translational or rotational diffusion in propylene glycol of comparably sized molecules.[44] The activation energies for the excited state relaxation of TNS at the lipid-water interface of several phospholipid vesicles were experimentally determined in a similar manner.[45] The phase-resolved spectra of the TNS-labeled vesicles as a function of temperature are shown in Figure 19. The red and blue suppressed spectra are comparable to those expected for the unrelaxed and the relaxed emission of TNS, respectively.

In another study,[46] a new fluorescent probe called Patman (6-palmitoyl-2-[(2-[trimethylammonio]ethyl)methylamino]naphthalene chloride) was characterized and used to study the phase transitions of phospholipid bilayers. PRFS was used to record the relaxed and unrelaxed emission spectra for Patman in dimyristoyl-L-alpha-phosphatidylcholine (DMPC) vesicles at 2 and 52°C, respectively. As shown in Figure 20,

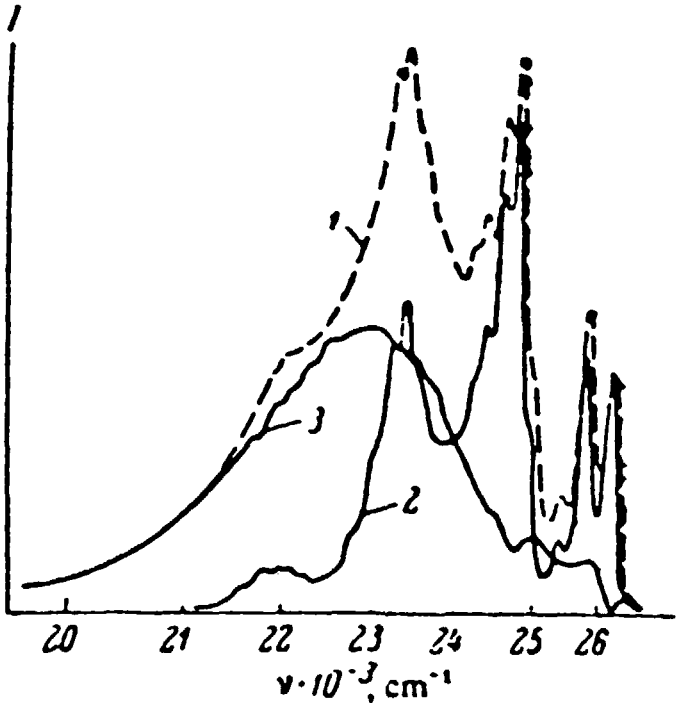


FIGURE 11. Fluorescence spectra of an anthracene solution in *n*-heptane at −196°C (concentration: 0.2 m*M*); (1) total spectrum, (2) monomer spectrum, and (3) aggregate spectrum. (From Veselova, T. V., Cherkasov, A. S., and Shirokov, V. I., *Opt. Spectrosc.*, 29, 617, 1970. With permission.)

only partial resolution was achieved at the lower temperature (the longer-lived emission giving a spectrum that retains much of the character of the shorter-lived component), indicating the complexity of the relaxation processes.

The reversible relaxation of the excited state of acridine in ammonium nitrate was studied by Lakowicz and Balter.[47] Ammonium nitrate protonates the acridine, causing a decrease in the observed fluorescence from the unprotonated acridine (τ = 1.27 nsec) and the appearance of fluorescence from the protonated form (τ = 16.72 nsec) at longer wavelengths (Figure 21). The phase-resolved emission spectrum of acridine (0.20 *M* NH_4NO_3, pH 7.3) as a function of detector phase angle is shown in Figure 22. Lakowicz and Balter also studied the excited-state proton transfer of 2-naphthol,[48] which was shown to be a completely reversible process. The steady-state and phase-resolved emission spectra of 2-naphthol at pH 2.1 and 6.7 are shown in Figure 23.

Lakowicz and Keating studied the binding of 11-(3-hexyl-1-indolyl)-undecyltrimethylammonium bromide (6-In-11) to micelles of hexadecyltrimethylammonium bromide (HDTBr).[49] The system was used as a model for the association of proteins and peptides to biomembranes. Phase-resolved spectra of the free and micelle-bound 6-In-11 are shown in Figure 24. Phase-resolved intensities measured at the two detector phase angles required to null the intensity contributions of the free and micelle-bound 6-In-11 were used to calculate the ratios of the fluorophore in each environment as a function of micelle concentration (Figure 25).

Gratton and Jameson have described a technique for the recording of individual component spectra of two- and three-component mixtures using phase-modulation measurements rather than phase-resolution hardware.[50] Phase-delay, demodulation,

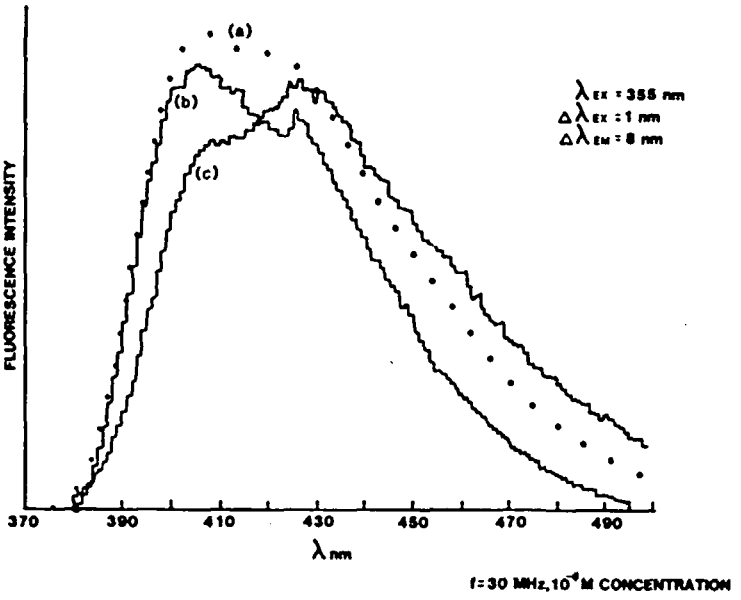


FIGURE 12. The steady-state emission spectrum of a 1:1 mixture of POPOP and Me_2POPOP in ethanol with an excitation wavelength of 355 nm (a). The phase-resolved emission spectra after suppressing the contribution of Me_2-POPOP (b) and after suppressing the contribution of POPOP (c). (From Mattheis, J. R., Mitchell, G. W., and Spencer, R. D., *New Directions in Molecular Luminescence*, American Society for Testing and Materials, Philadelphia, 1983. With permission.)

and DC intensity values are recorded as a function of wavelength at a single modulation frequency. Fluorescence lifetime values for the components (determined in separate experiments and assumed to be wavelength-independent) are used to calculate the fractional contributions of each component to the total intensity at each wavelength, yielding the spectrum of each component. Unlike the use of phase-resolution hardware to obtain component spectra as implemented to date, this approach allows the spectral resolution of three-component systems. An example of the resolution of three spectral components including perylene, POPOP, and 2,5-diethylaminonaphthalene sulfonate (DENS) is shown in Figure 26.

V. MULTICOMPONENT ANALYSIS

The applications described in the previous section involved the acquisition of phase-resolved spectra of one component at the detector phase angle required to null the contribution from the other component in two-component systems. In some cases, intensities taken from the spectra were used to evaluate the distribution of a chemical species between two different forms in solutions containing a known, constant analytical concentration of the species. Concentration determinations were not performed.

Quantitative determinations of concentrations require appropriate standardization using standard solutions of the components. Mousa and Winefordner described the first use of phase resolution for quantitative phosphorimetric analysis of binary mixtures.[2] Several binary systems were studied, including mixtures of 4-bromobiphenyl and 4-iodobiphenyl, benzophenone and 4-bromobiphenyl (for which the phase-resolved spectra are shown in Figure 27), and 4-iodobiphenyl and 4-bromobiphenyl. Analytical curves for each component in the latter system obtained with the other com-

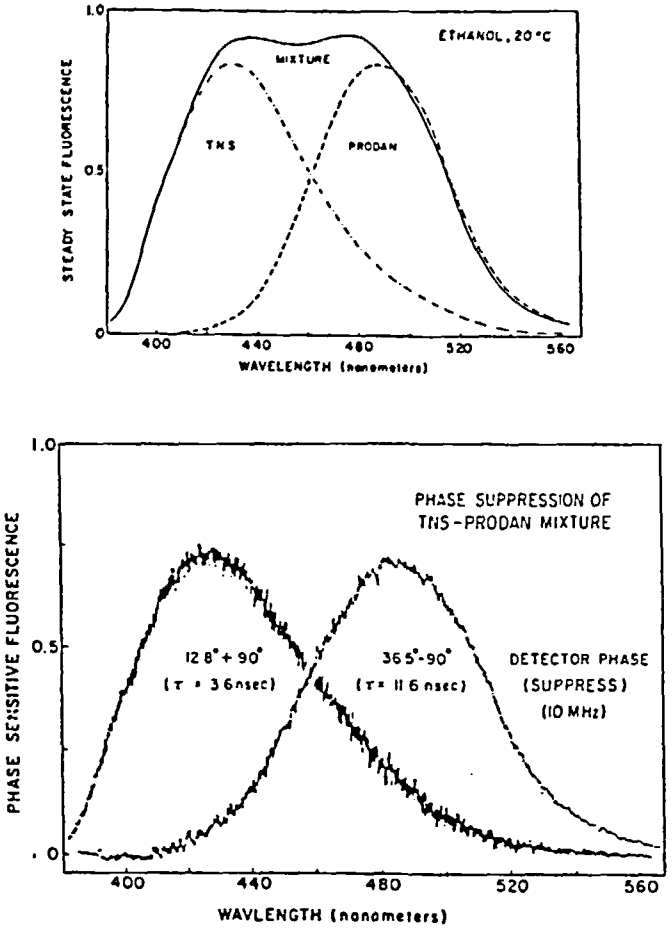


FIGURE 13. Steady-state emission spectra (upper) of TNS and PRODAN and a mixture of the two. Phase-resolved emission spectra (lower) of the mixture at the detector phase angles required to suppress the emission from each component. (From Lakowicz, J. R. and Cherek, H., *J. Biochem. Biophys. Meth.*, 5, 19, 1981. With permission.)

ponent nulled are shown in Figure 28, and results for the determination of the two components in the mixtures are shown in Table 2. The phosphorescence lifetimes for the components in these systems were in the millisecond range.

McGown used phase resolution for the simultaneous determination of fluorescein that was bound in two different ways to human serum albumin, including physical adsorption and covalent attachment ($\Delta\tau$ = 0.3 nsec), demonstrating the use of phase resolution for quantitative fluorimetric analysis.[51] Since the fluorescence lifetimes of the two components are so similar, the use of direct nulling is hampered by the difficulty of measuring the non-nulled component at a detector phase angle so close to its own null point, thereby resulting in very low intensities with large associated photon noise. Therefore, this study investigated the use of measurements at two non-nulling detector phase angles for the two-component determinations. Standardization was accomplished by using standard solutions of the individual fluorescein species to find the molar fluorescence intensities of the two forms ($\bar{I}_A$ and $\bar{I}_B$). The two independent equations

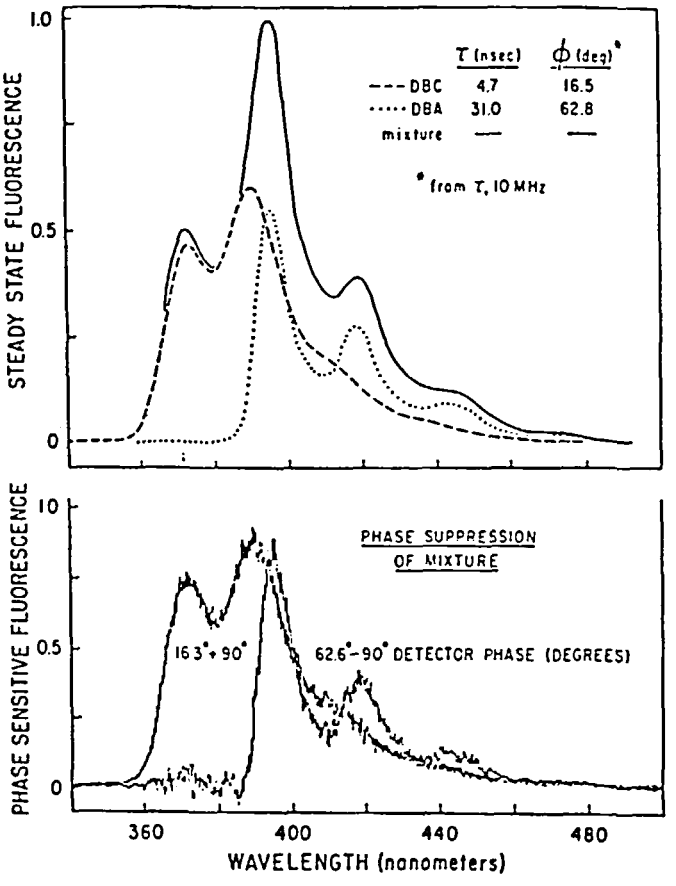


FIGURE 14. Steady-state emission spectra (upper) of DBC and DBA and a mixture of the two. Phase-resolved emission spectra (lower) of the mixture at the detector phase angles required to suppress the emission from each component. (From Lakowicz, J. R. and Cherek, H., *J. Biochem. Biophys. Meth.*, 5, 19, 1981. With permission.)

$$F(\phi_{D1}) = \bar{I}_{A,\phi D1} C_A + \bar{I}_{B,\phi D1} C_B$$
$$F(\phi_{D2}) = \bar{I}_{A,\phi D2} C_A + \bar{I}_{B,\phi D2} C_B \quad (6)$$

were then solved for the two unknown concentrations C_A and C_B. The results for determinations made at three different phase angle conditions ($\phi_{D1} = \phi_A$ and $\phi_{D2} = \phi_B$; $\phi_{D1} = \phi_A + 45°$ and $\phi_{D2} = \phi_B + 45°$; $\phi_{D1} = \phi_A + 90°$ and $\phi_{D2} = \phi_B + 90°$) are shown in Table 3. This is an example of a determination that could not be performed using conventional steady-state fluorimetry due to the complete spectral overlap of the two components in the system.

The first application of phase-resolved fluorimetry to a three-component system was described by McGown and Bright and involved the simultaneous determination of anthracene (4.07 nsec), POPOP (1.35 nsec), and Me_2POPOP (1.42 nsec).[52] The components have highly overlapping excitation spectra and emission spectra (see Figure 12). Various combinations of excitation and emission wavelengths with detector phase angles were used to generate three simultaneous equations in three unknowns (similar to Equation 6), which were then solved for the unknown concentrations. The results for

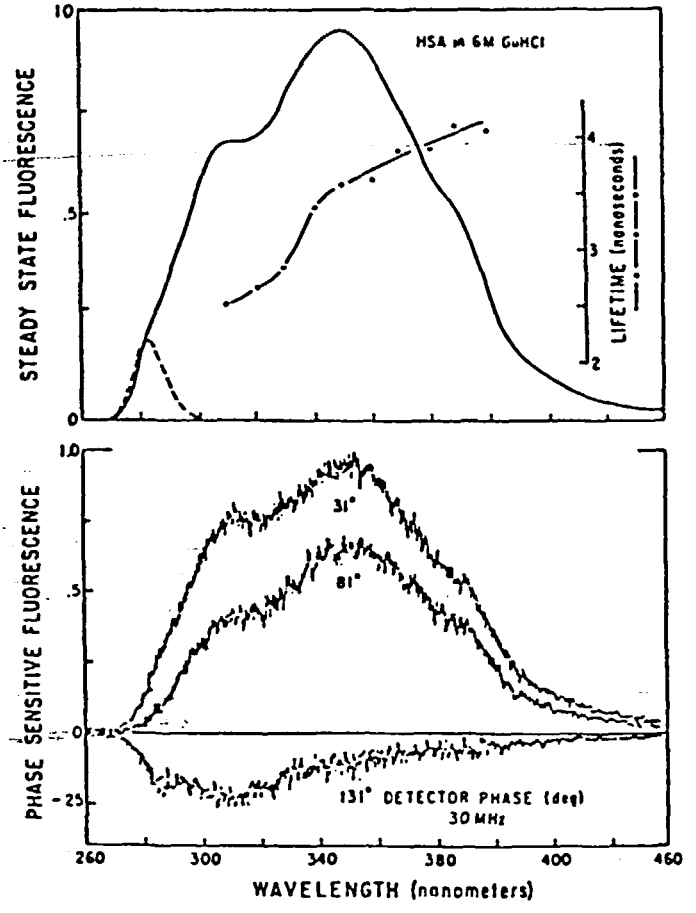


FIGURE 15. Steady-state (upper) and phase-resolved (lower) emission spectra of denatured human serum albumin. (From Lakowicz, J. R. and Cherek, H., *J. Biol. Chem.*, 256, 6348, 1981. With permission.)

determinations using five different sets of phase-resolved conditions were compared with results obtained for steady-state determinations in which the same wavelength combinations and total number of measurements were used to generate the three independent equations (Table 4).

Overdetermined matrices (m equations with n unknowns, where $m \geq n$) have also been used for phase-resolved fluorimetric determinations. This approach allows the full exploitation of the available selectivity parameters for a given system by using as many equations as required to minimize determination errors. The overdetermined matrices have the general form:

$$\begin{aligned} I_{\phi D1} &= \bar{I}_{1,\phi D1}C_1 + \bar{I}_{2,\phi D1}C_2 + \ldots + \bar{I}_{n,\phi D1}C_n \\ I_{\phi D2} &= \bar{I}_{1,\phi D2}C_1 + \bar{I}_{2,\phi D2}C_2 + \ldots + \bar{I}_{n,\phi D2}C_n \\ &\vdots \\ I_{\phi Dm} &= \bar{I}_{1,\phi Dm}C_1 + \bar{I}_{2,\phi Dm}C_2 + \ldots + \bar{I}_{n,\phi Dm}C_n \end{aligned} \quad (7)$$

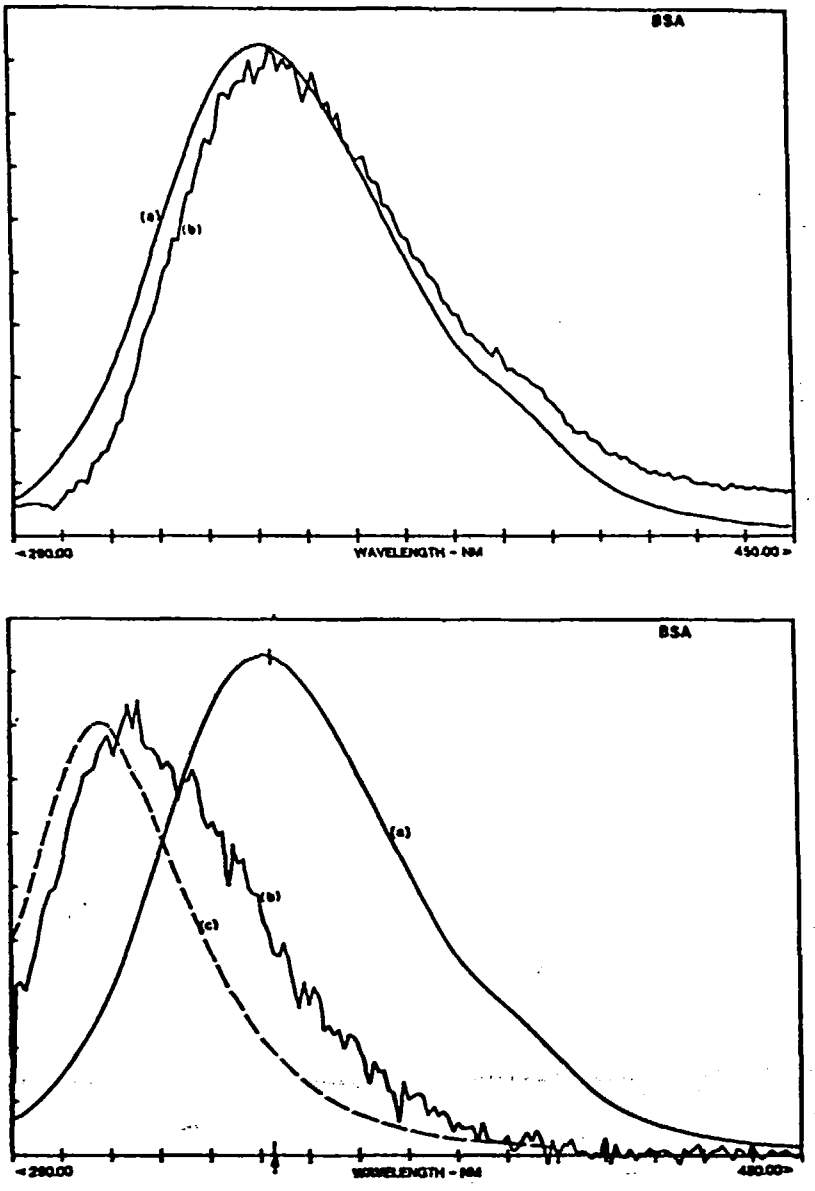


FIGURE 16. Top: (1) The steady-state emission spectrum of bovine serum albumin (BSA), excitation at 280 nm; (b) the phase-resolved spectrum after nulling the contribution of tyrosine at 300 nm. Bottom: (a) The steady-state emission spectrum of BSA; (b) the phase-resolved spectrum after nulling the contribution of tryptophan at 400 nm; (c) the steady-state emission spectrum of free tyrosine. (From Mattheis, J. R., Mitchell, G. W., and Spencer, R. D., *New Directions in Molecular Luminescence*, American Society for Testing and Materials, Philadelphia, 1983. With permission.)

and can be solved by using least-squares approaches such as the Gauss-Newton iterative method.[53] A non-negative least-squares routine[54] has also been used in more recent studies.

Overdetermined matrices were applied to phase-resolved fluorimetric determinations by McGown and Bright for the determination of mixtures of POPOP and Me_2POPOP.[55] Several different experimental schemes were used to generate the data.

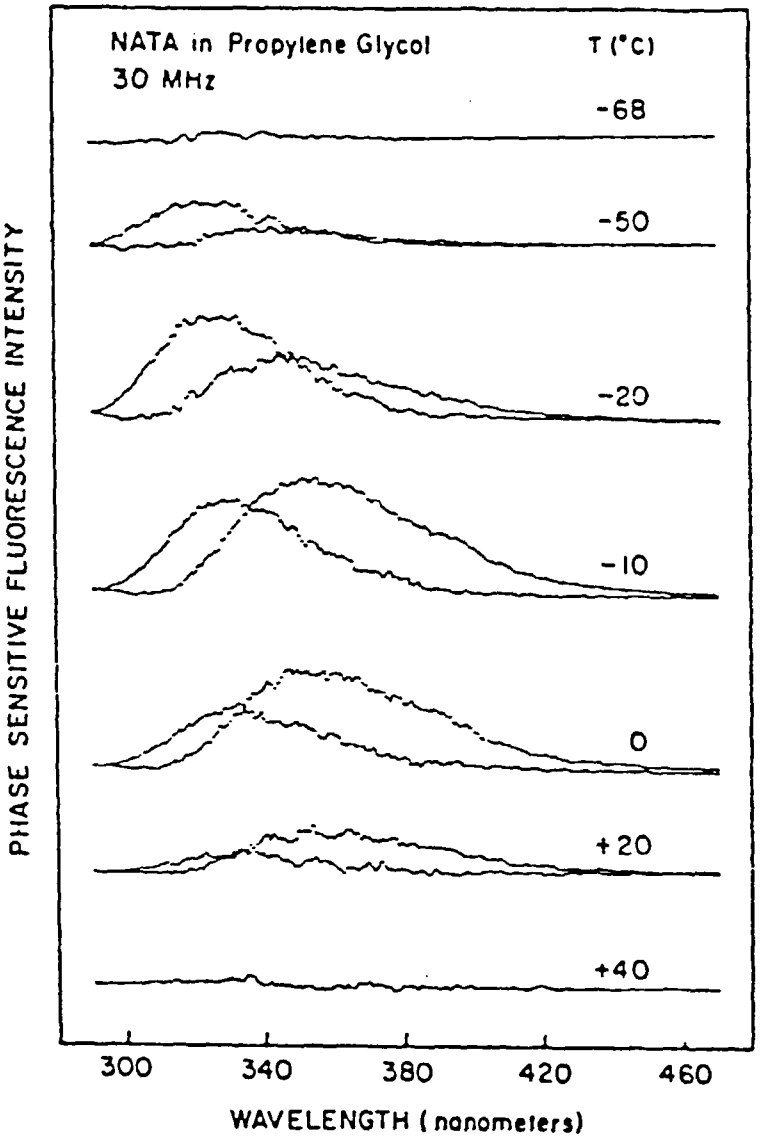


FIGURE 17. The phase-resolved emission spectrum of NATA as a function of temperature. Peaks at 330 and 350 nm are attributed to unrelaxed and solvent NATA, respectively, each measured at the phase angle required to suppress the other component. (From Lakowicz, J. R. and Balter, A., *Photochem. Photobiol.*, 36, 125, 1982. With permission.)

Phase-resolved fluorescence intensity (PRFI) measurements were made for detector phase angles at 45° intervals, from which sinusoidal curves of PRFI vs. detector phase angle were generated using a least-squares fitting routine. There were two different data analysis schemes tried. In an indirect nulling approach, the phase-resolved intensities of the mixtures and standards are used in a manner analogous to the direct nulling approach except that the values are all taken from the fitted curves. In the simultaneous equation approach, mixture and standard phase-resolved intensities are read at 45° intervals from the fitted curves and a series of overdetermined equations are solved for the concentrations of the two components. Best results were obtained for POPOP and Me-POPOP when eight simultaneous equations were used (Table 6).

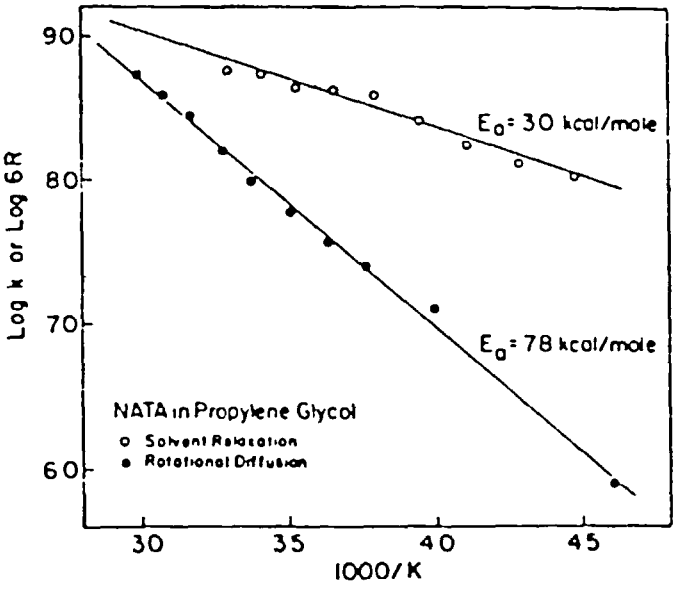


FIGURE 18. Arrhenius plot for solvent relaxation and rotational diffusion. (From Lakowicz, J. R. and Balter, A., *Photochem. Photobiol.*, 36, 125, 1982. With permission.)

Table 1
PHASE ANGLES (ϕ_F, ϕ_R) AND DEMODULATION FACTORS (M_F, M_R) FOR UNRELAXED (F) AND SOLVENT RELAXED (R) NATA IN PROPYLENE GLYCOL

o_C	ϕ_F	ϕ_λ	ϕ_R	M_F^λ	M_R^λ	I_F/I_R[a]	τ_s (nsec)[b]
−68	—	41.2	—	—	—	—	—
−60	38.6	40.0	40.8	0.78	0.76	—	—
−50	39.8	40.5	48.8	0.77	0.66	2.40	9.3
−40	39.8	42.3	49.8	0.77	0.64	1.85	7.4
−30	37.2	44.3	58.7	0.80	0.52	1.69	5.7
−20	33.3	47.1	66.3	0.84	0.40	1.43	3.9
−10	30.2	47.6	60.8	0.86	0.49	0.79	2.6
0	34.5	50.9	57.3	0.94	0.54	0.64	2.4
10	36.6	47.6	50.6	0.81	0.64	0.50	2.3
20	36.3	42.8	43.8	0.81	0.72	0.42	1.8
30	33.8	38.8	39.8	0.84	0.77	0.44	1.7
40	30.8	34.3	32.8	0.86	0.84	—	—

Note: ϕ_λ is the phase for the entire emission at the emission maximum.

[a] Phase-resolved intensity ratio measured from the phase-resolved emission maxima at either 310 or 410 nm.

[b] Calculated from $\tau_s = (I_F M_R / I_R M_F)\tau_o$, where τ_s is the solvent relaxation time estimated from the phase-resolved spectra shown in Figure 17.

[c] Calculated from $M_i = (1 + \omega^2\tau_i^2)^{-1/2}$.

From Lakowicz, J. R. and Balter, A., *Photochem. Photobiol.*, 36, 125, 1982. With permission.

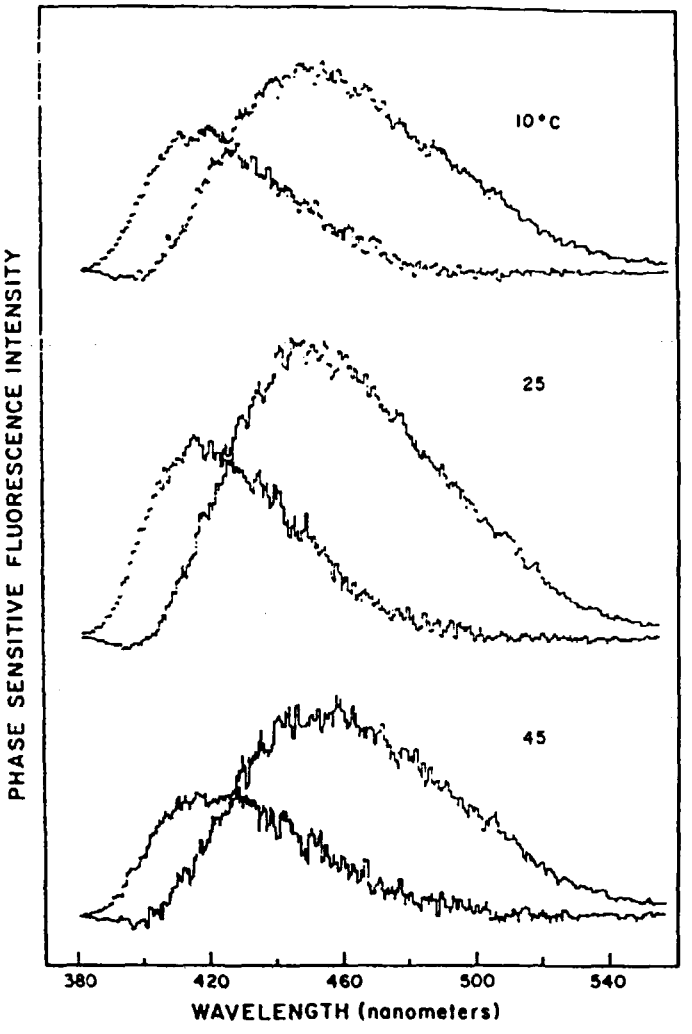


FIGURE 19. Phase-resolved emission spectra of TNS-labeled dimyristoylphosphatidylcholine vesicles, each measured at the phase angle required to suppress the other component. (From Lakowicz, J. R., Thompson, R. B., and Cherek, H., *Biochim. Biophys. Acta*, 734, 295, 1983. With permission.)

Bright and McGown analyzed a four-component system including anthracene and three chloroanthracene derivatives by using an overdetermined system with various combinations of wavelengths and detector phase angles.[56] Raw data for the phase-resolved intensities at detector phase angles taken at 45° intervals were fit with a least-squares routine to a cosine curve, and the equations for the data matrix were generated from the fitted curves at the different wavelength combinations. Relative errors of 1% or less were consistently obtained for two of the components, and errors were below 8% for the other two components in most of the mixtures analyzed.

A different four-component system of anthracene derivatives (emission spectra shown in Figure 29)* was studied by Bright and McGown, using two different excitation modulation frequencies (18 and 30 MHz) instead of a single frequency for the determinations.[57] Again, overdetermined equations were generated using wavelength and

* Figures 29 to 42 and Tables 6 to 14 follow Section VIII and start on page 278.

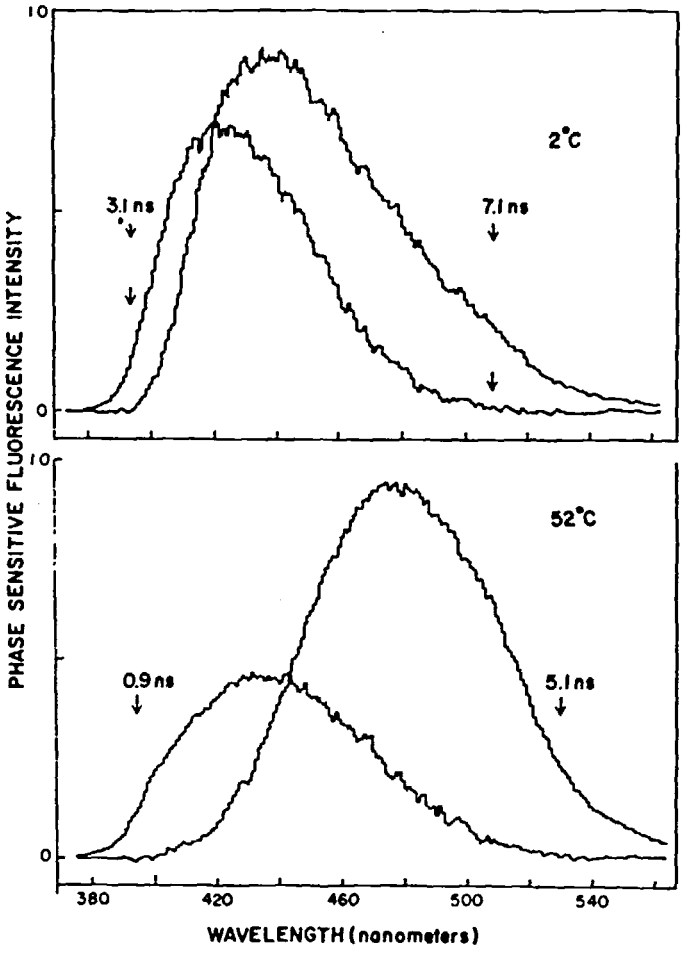


FIGURE 20. Phase-resolved fluorescence spectra of Patman-labeled DMPC vesicles at two temperatures. The arrows indicate the wavelengths used for phase suppression, and the numbers indicate the apparent lifetimes suppressed at the chosen phase angles. (From Lakowicz, J. R., Bevan, D. R., Maliwal, B., and Cherek, H., *Biochemistry*, 22, 5714, 1982. With permission.)

detector phase angle selection. Determination errors for different numbers of equations used are summarized in Table 6. Excellent results were achieved by using 24 equations.

Metals can be determined fluorimetrically by using chelating agents to form fluorescent metal chelates, and such applications have been recently reviewed.[58] Unfortunately, the chelates formed with a given chelating agent and different metals have very similar, broad fluorescence spectra. Multicomponent determinations of the metals are therefore very difficult to achieve using fluorescence spectra alone. However, the metal chelates often have significantly different fluorescence lifetimes. Vitense and McGown have achieved two-component determinations of mixtures of Al(III) and Ga(III), Ga(III) and In(III), and Zn(II) and Cd(II) by chelation with 8-hydroxyquinoline-5-sulfonic acid.[59] The fluorescence lifetimes of the complexes and determination errors for the two-component mixtures are shown in Table 7. In an earlier study, Hiraki et al. used pulsed-source time-resolved fluorimetry to perform two-component determinations using the same chelating agent and metals in the same concentration range.[60] They were able to resolve Al(III) and Ga(III) with comparable overall accuracy (al-

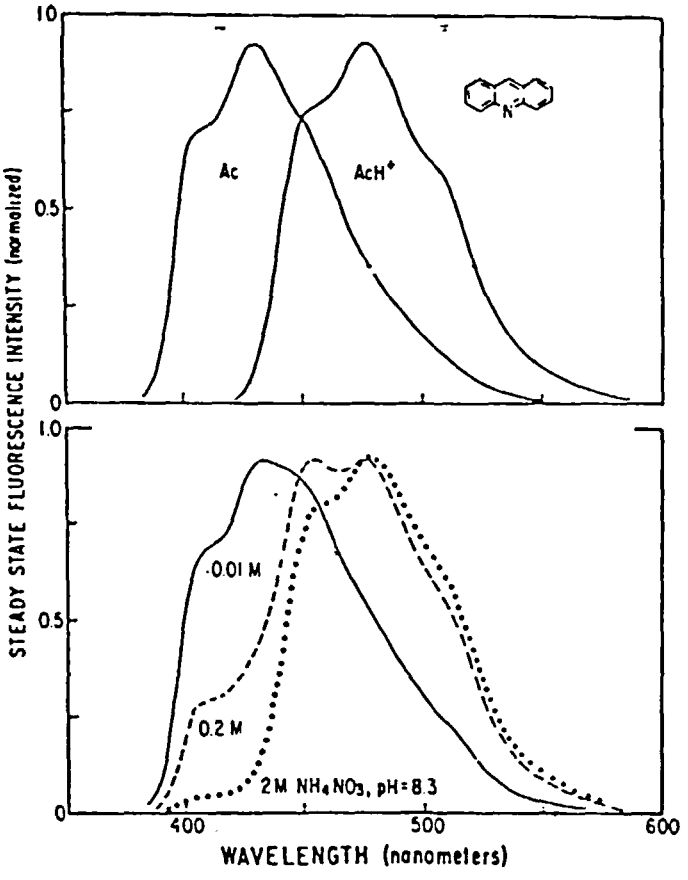


FIGURE 21. Steady-state (upper) and phase-resolved (lower) emission spectra of acridine in acid and base. (From Lakowicz, J. R. and Balter, A., *Biophys. Chem.*, 16, 117, 1982. With permission.)

though the individual error magnitudes were three times larger) but could not resolve Ga(III) and In(III) or Zn(II) and Cd(II) on their instrument.

Nithipatikom and McGown used PRFS in conjunction with synchronous scanning to generate a data format that is generalized, i.e., in which parameters are varied at constant intervals rather than using parameter conditions that are optimized for particular analyte components.[61] Two-, three-, and four-component mixtures were prepared from four PAHs, including 9-phenylanthracene (9PA, $\tau = 4.78$ nsec), 9,10-diphenylanthracene (9,10DPA, $\tau = 6.09$ nsec), benzo(k)fluoranthene (BkF, $\tau = 7.5$ nsec), and benzo(a)pyrene (BaP, $\tau = 14.60$ nsec). Multicomponent determinations were accomplished by generating data matrices for standard solutions of each PAH and for the mixtures in which PRFI is plotted as a function of synchronously scanned wavelength on one axis and detector phase angle on the other (Figure 30). Excitation modulation frequency was also varied in some cases, thereby adding another dimension to the data matrix. A comparison of phase-resolved and steady-state synchronous excitation determinations for the same mixtures using the same number of equations and total measurements showed superior overall performance by the phase-resolved technique only for the four-component mixtures (Table 8). In a later study by the same authors, five- and six-component mixtures were analyzed by using the phase-resolved synchronous excitation data format.[32] The steady-state synchronous spectra of the six PAHs used

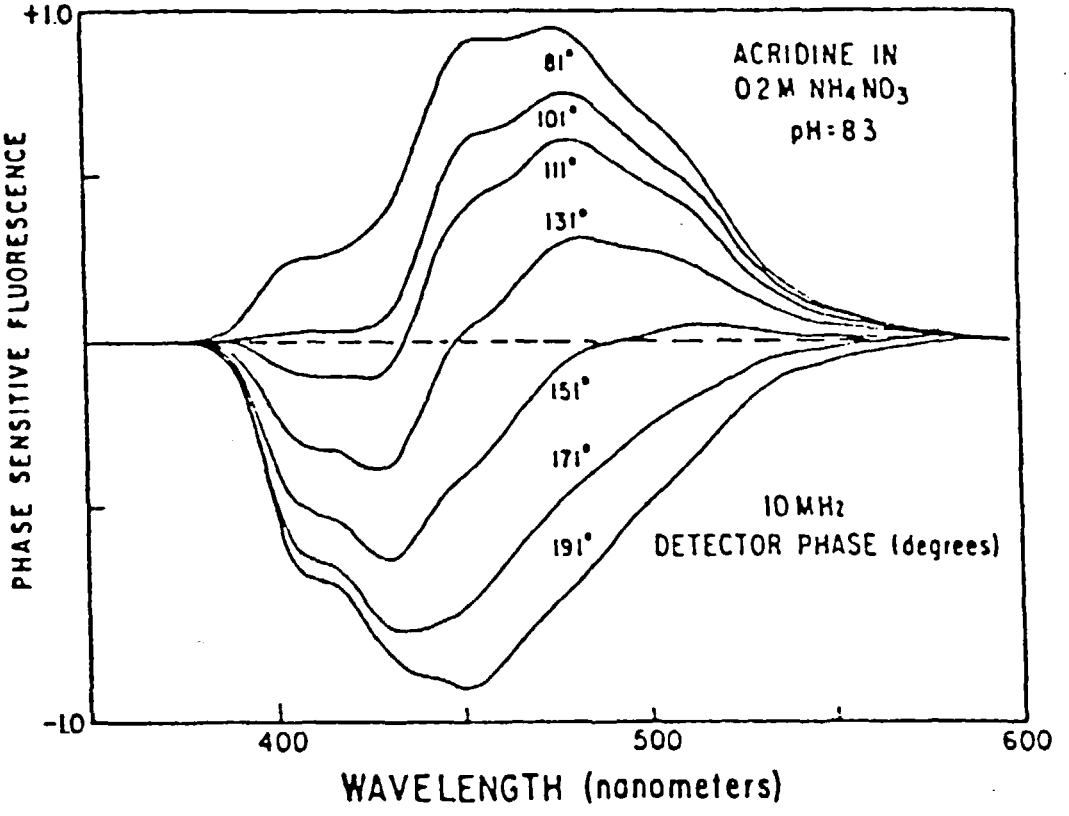


FIGURE 22. Phase-resolved emission spectrum of acridine as a function of detector phase angle. (From Lakowicz, J. R. and Balter, A., *Biophys. Chem.*, 16, 117, 1982. With permission.)

are shown in Figure 31 and the determination errors are summarized in Table 9, including those obtained for comparable steady-state synchronous excitation determinations. The development of the generalized multidimensional data format is a step towards achieving qualitative analysis capabilities for the multicomponent samples.

A recent article by Lakowicz and co-workers describes two- and three-component fluorimetric determinations in which phase-resolved spectra of the mixtures are fit with steady-state spectra of the individual components using nonlinear least-squares analysis (NLLS).[62] The phase-resolved spectra are collected at a number of detector phase angles at a single modulation frequency. For example, two-component mixtures of 9,10DPA (τ = 5.87 nsec) and 9-methylanthracene (τ = 4.47 nsec) were analyzed by using spectra recorded at 15 detector phase angles, in addition to the steady-state spectra of the individual components. In contrast, the simultaneous equation (SE) approach described above only requires the measurement of the mixtures and standards at each set of detector phase angle conditions. For example, the simultaneous determination of POPOP and Me_2POPOP,[55] which differ by 100 psec, was performed by measuring the sample and 2 standard solutions in triplicate at 8 different detector phase angles and a single set of emission/excitation wavelengths, for a total of 72 measurements. This is much less than the 15 full spectra per sample and 2 steady-state spectra required by the NLLS technique. Another difference between the two approaches is the data analysis, i.e., NLLS fits vs. the simpler fits for the linear simultaneous equations. Finally, the component standards for the NLLS approach are measured under different conditions than the mixture being analyzed, and the fluorescence lifetimes of

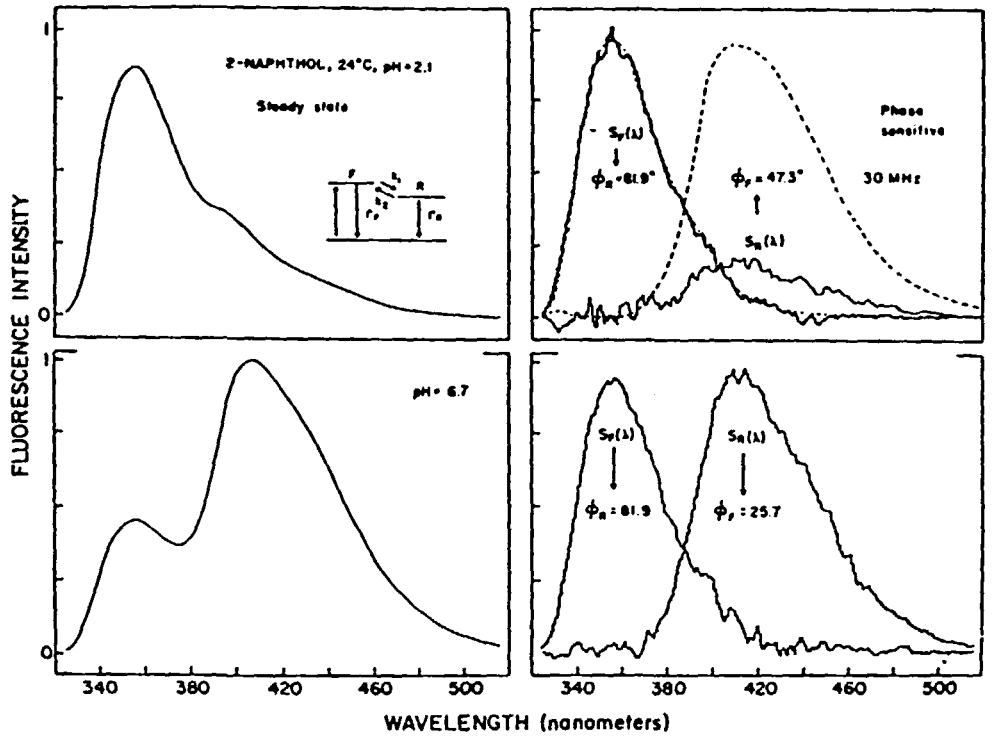


FIGURE 23. Steady-state (left) and phase-resolved (right) emission spectra of 2-naphthol. The dashed lines in the upper right panel represent the steady-state spectra of naphthol (pH = 0.5) and naphtholate (pH = 12.0). All other spectra were obtained for solutions containing 0.08 m*M* sodium acetate. (From Lakowicz, J. R. and Balter, A., *Chem. Phys. Lett.*, 92, 117, 1982. With permission.)

the components must be constant over the wavelength ranges used. In the SE approach, standards are measured along with samples under each set of conditions and the fluorescence lifetimes of the components need not be either known or wavelength-independent.

VI. PHASE-RESOLVED SUPPRESSION OF INTERFERENCES

In the broadest sense, all phase-resolved nulling experiments involve the elimination of an interfering signal in the determination of the component(s) of interest. This section addresses the elimination, via direct suppression or resolution, of general classes of interfering signals such as scattered light or fluorescence background.

Mousa and Winefordner[2] used phase resolution to suppress the fluorescence background in phosphorescence measurements. Due to the orders of magnitude difference between the lifetimes of the two types of luminescence, it should be possible to suppress essentially all of the short-lived fluorescence background by using phase resolution at the appropriate modulation frequency and detector phase. They were able to eliminate the fluorescence contribution to the luminescence spectrum of bromobiphenyl at an excitation modulation frequency of 50 Hz (Figure 32).

Suppression of fluorescence signals in the detection of scattered light is also possible due to the large lifetime difference between prompt scattering ($\tau = 0$, or picosecond scale) and fluorescence ($\tau > 0$, or nanosecond scale). Mattheis et al. described the phase-resolved suppression of the Raman scatter of water in order to record the fluo-

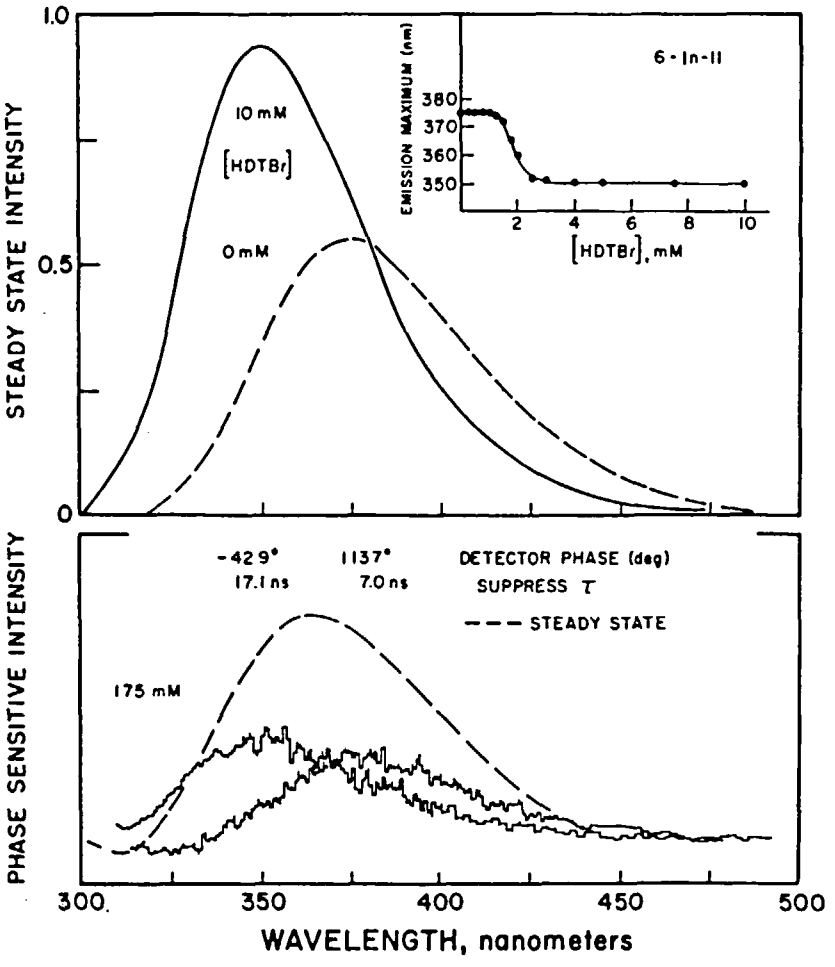


FIGURE 24. Emission spectra of 6-In-11. Top: steady-state spectra are shown for 6-In-11 in water (dashed line) and 10 m*M* HDTBr (solid line). The inset shows the fluorescence emission maxima of 6-In-11 at various concentrations of HDTBr. Bottom: phase-resolved spectra for 6-In-11 with 1.75 m*M* HDTBr. The phase angles indicate those used to suppress emission from the free and bound forms of 6-In-11. The lifetimes equivalent to those phase angles are also shown. (From Lakowicz, J. R and Keating, S., *J. Biol. Chem.*, 258, 5519, 1983. With permission.)

rescence spectrum of nanomolar quinine bisulfate (Figure 33) using a commercial instrument.[10] Genack used the apparatus shown in Figure 34 to suppress the fluorescence from 5×10^{-5} *M* fluorol in toluene ($\tau = 8.5$ nsec) in order to observe the Raman spectrum of the toluene (Figure 35).[63] The Raman spectrum (Figure 35b) was obtained by setting the emission modulator +90° to the phase of the fluorol fluorescence.

Demas and Keller used the instrument shown in Figure 5 to suppress both fluorescence interference in Raman spectra and scattered light interference in fluorescence spectra for cases in which one signal component is very weak relative to the other.[36] The spectra they worked with are shown in Figure 36 and include the Raman spectrum of water, the fluorescence spectrum of rhodamine 6G ($\tau = 3$ nsec), and the luminescence spectrum of tris(2,2'-bipyridine)ruthenium(II) ion ($[Ru(bpy)_3]^{2+}$), $\tau = 350$ nsec). The luminescence signals of rhodamine 6G and $[Ru(bpy)_3]^{2+}$ were suppressed to enhance the Raman spectrum of water (shown for rhodamine 6G in Figure 37), and the

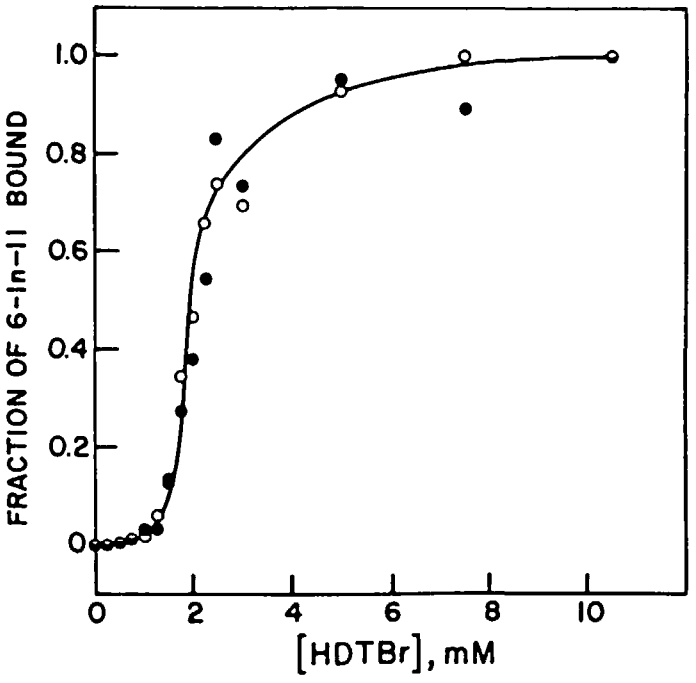


FIGURE 25. Ratio of free to HDTBr-bound 6-In-11 determined from the ratio of the phase-resolved spectra of the sample at the suppression phase angles of the free and bound forms (O) and the ratio of the experimental sample to a control containing only free (or bound) 6-In-11 at the phase angle required to suppress the bound (or free) form (•). (From Lakowicz, J. R. and Keating, S., *J. Biol. Chem.*, 258, 5519, 1983. With permission.)

Raman spectrum of water was suppressed to enhance the emission spectra of rhodamine 6G (Figure 38) and $[Ru(bpy)_3]^{2+}$. In the same paper, phase resolution is favorably compared with other background suppression techniques (Table 10) in terms of (1) being able to readily achieve both the suppression of Raman in luminescence spectra and of luminescence in Raman spectra and (2) ease of operation.

Van Hoek and Visser used phase resolution to eliminate fluorescence background from 3-methyllumiflavin solute (τ = 5 nsec) in the Raman spectrum of benzene.[37] Their instrumentation combines a conventional Raman spectrometer with a commercial high frequency lock-in amplifier (Figure 6) and allows simultaneous collection of both the conventional and fluorescence-suppressed Raman spectra. The application of a high-frequency (750 MHz) gain-modulated silicon avalanche photodiode (see Figure 8) to eliminate fluorescence background in Raman spectra has also been proposed by Berndt.[39]

Nithipatikom and McGown used phase resolution to suppress scattered light background in the fluorimetric determination of five PAHs with synchronous excitation.[64] Scattered light is particularly troublesome in determinations that require scanning with small differences between the emission and excitation wavelengths, such as the typical difference of 3 nm that was used in the study. Detection limits for the PAHs with phase-resolved nulling of the scattered light signal were better than those obtained with the steady-state measurements for three of the five PAHs studied (Table 11). The steady-state synchronous spectrum and the spectrum acquired by using phase resolution with the detector phase angle 90° out of phase with the scattered light for a mix-

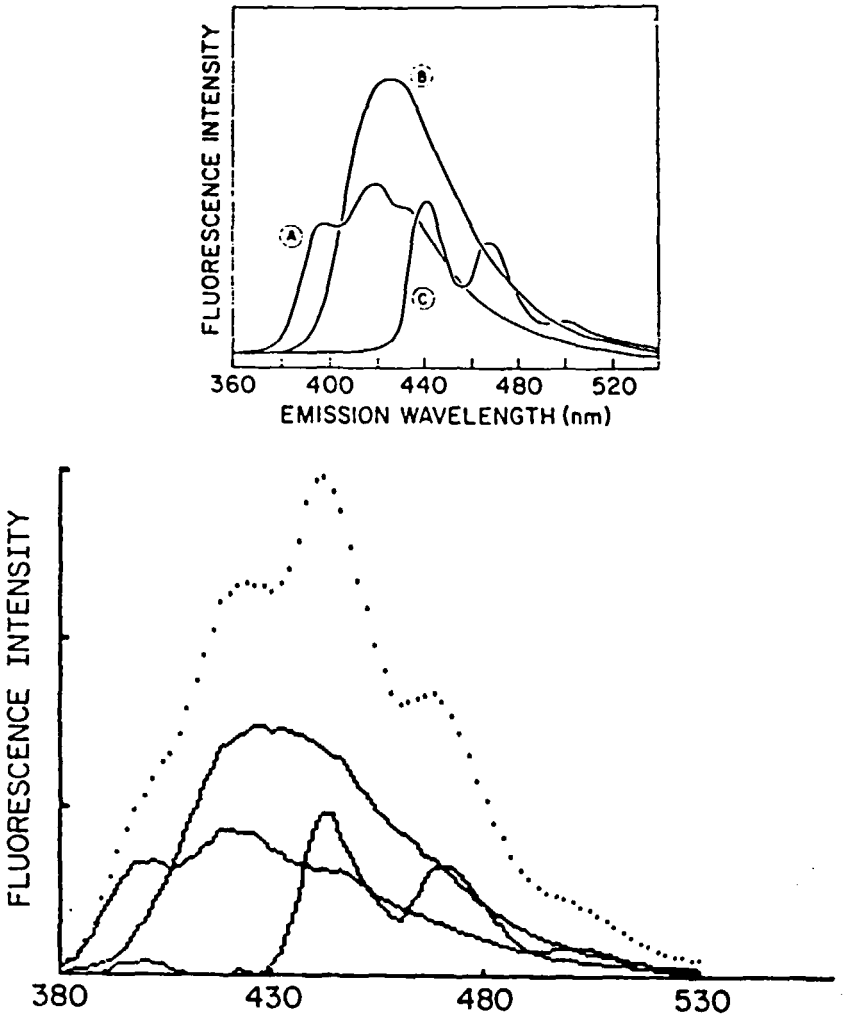


FIGURE 26. Top: steady-state emission spectra of (A) POPOP, (B) DENS, and (C) perylene in ethanol. Bottom: steady-state (dotted line) and phase-resolved (solid lines) emission spectra for a mixture of the three components. (From Gratton, E. and Jameson, D. M., *Anal. Chem.*, 57, 1695, 1985. With permission.)

Chemical species that quench luminescence constitute a type of interference that can be eliminated by the use of phase-delay and modulation amplitude, as recently demonstrated by Demas et al.[65] In this study, two methods for eliminating the quenching of uranyl by chloride were described. The first method takes advantage of the inverse relationship between luminescence lifetime and the percentage of signal modulation at a given excitation modulation frequency. If the modulation frequency is sufficiently high, the observed modulated amplitude (S) is almost independent of the extent of quenching. This is shown in Figure 40, in which the ratio of S to the unquenched modulated amplitude (S_o) at low frequency is shown as a function of A ($= 2\pi f\tau_o$), where f is the modulation frequency and τ_o is the unquenched luminescence lifetime, and of the extent of quenching ($\Phi = \tau/\tau_o$). In the second method, the modulated amplitude S and the phase-delay for the quenched sample are measured. S_o is determined from the equation:

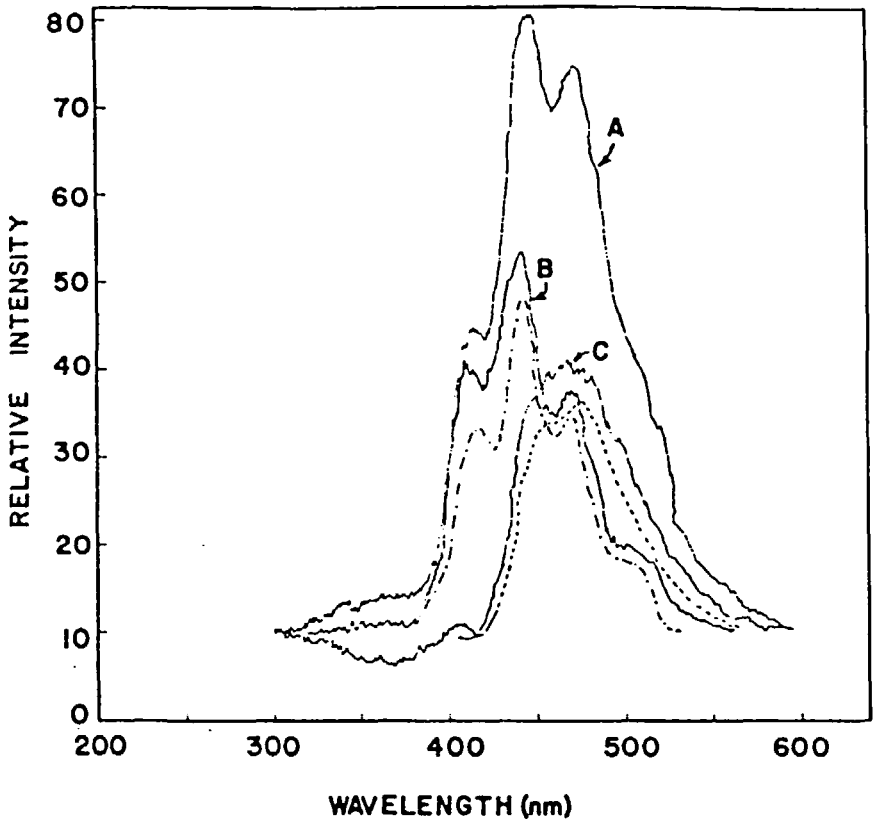


FIGURE 27. Phase-resolved emission spectrum of a mixture of 0.022 m*M* benzophenone and 0.029 m*M* 4-bromobiphenyl at 25 Hz. Excitation wavelength is 275 nm. (A) Mixture spectrum at peak phase angle (270° + 26°); (B) mixture spectrum (0° + 26°); (C) mixture spectrum (180° + 52°); (dot-dashed line) spectrum of benzophenone standard (270° + 52°); (dashed line) spectrum of 4-bromobiphenyl standard (270° + 26°). (From Mousa, J. J. and Winefordner, J. D., *Anal. Chem.*, 46, 1195, 1974. With permission.)

$$S_o = S[(1/\Phi)^2 + A^2]^{1/2} \quad (8)$$

where Φ is the ratio of τ for the quenched sample (determined from the phase-delay of the sample) to τ_o (determined separately). Results for the two methods are shown in Table 12.

Clinical fluorimetric analyses of biological samples are often hampered by background signals due to native fluorescence of the samples. If the determination involves the use of fluorescent labels or tracers, it may be possible to use a label with long-lived emission that can easily be resolved from the prompt background signal, as has been demonstrated in time-resolved fluoroimmunoassays.[66] An alternative is to use phase-resolution to resolve the background signal from the desired signal. For example, Bright and McGown described the phase-resolved elimination of bilirubin (τ = 0.5 nsec) interference in the determination of fluorescein (τ = 4 nsec).[67] A high level of bilirubin, such as is found in jaundiced patients, is the major source of fluorescence background at the wavelengths used to determine fluorescein (490 nm excitation and 520 nm emission) in serum samples. Bilirubin interference in fluoroimmunoassays that use fluorescein-labeled reagents has been discussed elsewhere.[66] Using phase resolution, the determination of 1 n*M* fluorescein in ten aqueous solutions containing bili-

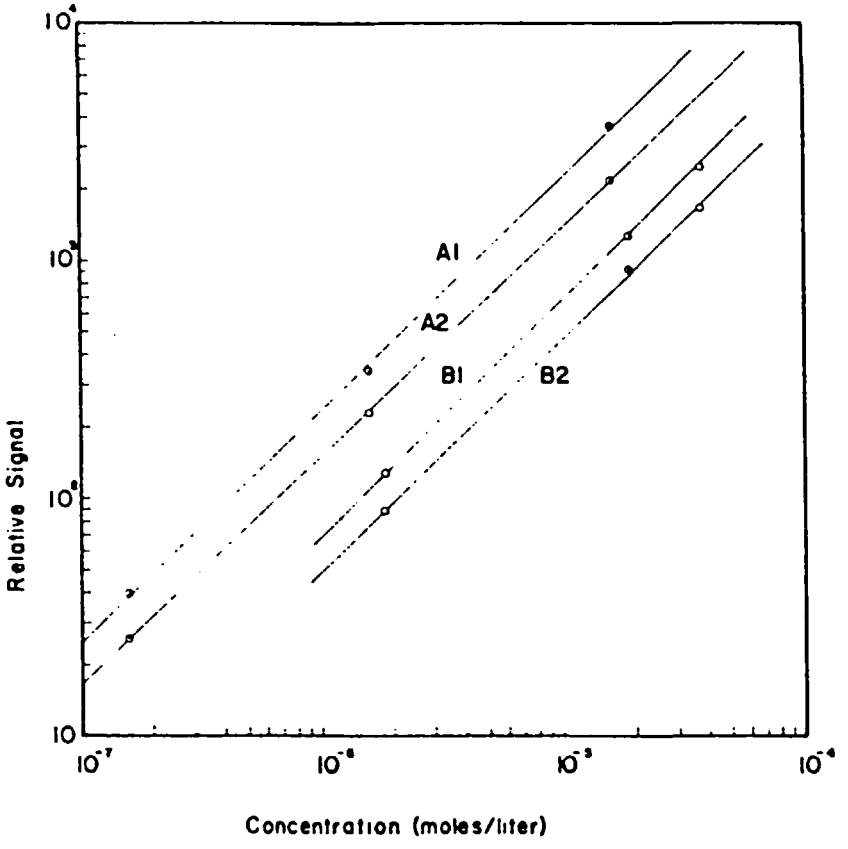


FIGURE 28. Analytical curves for 4-bromobiphenyl and 4-iodobiphenyl at the peak phase setting for each molecule and at the phase setting where one component is phased out. A1 = 4-iodobiphenyl (270° + 55°); A2 = 4-iodobiphenyl (0° + 12.5°); B1 = 4-bromobiphenyl (270° + 12.5°); B2 = 4-bromobiphenyl (180° + 55°). (From Mousa, J. J. and Winefordner, J. D., *Anal. Chem.*, 46, 1195, 1974. With permission.)

rubin in the range of 1 to 10 μM and 1 mM albumin gave an average recovery of 100% with a relative standard deviation of 13%. Steady-state determinations of the same solutions using blank subtraction to correct for the bilirubin fluorescence gave an average recovery of 90% with a standard deviation of 6%. Determinations under a variety of steady-state and phase-resolved conditions confirmed the low results obtained for the steady-state determinations, which appeared to be due to nonadditivity of the bilirubin and fluorescein intensities at bilirubin concentrations above 5 μM. The nonadditivity did not effect the phase-resolved determinations of fluorescein.

VII. OTHER APPLICATIONS

A. Phase-Resolved Determinations of Fluorescence Lifetimes and Heterogeneity Analysis

In future applications of phase-resolved fluorimetry, it may be useful to use phase-resolved intensities to determine fluorescence lifetimes for qualitative information instead of using separate experiments to measure phase-delays and demodulations. Fluorescence lifetimes can be calculated from the difference between the phase angle maxima of the sample and a scattering or fluorescent reference solution using the fitted

Table 2
PHASE RESOLUTION OF BINARY MIXTURES OF 4-IODOBIPHENYL AND 4-BROMOBIPHENYL BY THE PHASE METHOD

	Mixture	Lifetimes (msec)	Degree of peak phase angle	
A:	4-Iodobiphenyl	3.5	270 + 55.0	
B:	4-Bromobiphenyl	17.0	270 + 12.5	
	Conc. added (*M*)	Degree of phase angle setting	Conc. found (*M*)	Error (%)
A:	8.0×10^{-7}	0 + 12.5	8.3×10^{-7}	+3.8
B:	1.86×10^{-6}	180 + 55.0	1.80×10^{-6}	−3.3
A:	8.0×10^{-6}	0 + 12.5	8.0×10^{-6}	0
B:	1.86×10^{-5}	180 + 55.0	1.77×10^{-5}	−4.8
A:	1.60×10^{-5}	0 + 12.5	1.60×10^{-5}	0
B:	1.86×10^{-6}	180 + 55.0	1.20×10^{-6}	−36

Note: All measurements performed at 275 nm excitation wavelength and 475 nm emission wavelength; all measurements performed at 25 Hz; degree of source modulation 53%.

From Mousa, J. J. and Winefordner, J. D., *Anal. Chem.*, 46, 1195, 1974. With permission.

cosinusoidal curves of PRFI vs. detector phase angles for the two solutions.[68] Modulations found from the phase-resolved intensities at the phase maxima of the curves divided by the DC intensity components can be used to calculate lifetimes from demodulation. A comparison of the results of phase-resolved lifetime determinations with direct phase-modulation fluorimetric determinations is shown in Table 13.[68] In the same study, heterogeneity analyses were performed using the lifetime data from both methods for two-component mixtures, and the results are shown in Table 14.

B. Determination of Thermodynamic Binding Parameters

In a study by Bright et al., thermodynamic binding contents were determined as a function of temperature, from which entropy and enthalpy values were calculated,[69] for the binding of 4-amino-*N*-methylphthalimide (4AMP) to beta-cyclodextrin and to human and bovine serum albumins, and for the binding of PRODAN to beta-cyclodextrin. Phase resolution was used in these determinations to eliminate the significant fluorescence contribution from the free 4AMP and PRODAN, either by direct nulling or by the use of simultaneous equations to resolve the contributions of the free and bound molecular species. Plots of the formation constants obtained by using direct nulling of the free 4AMP fluorescence for the association of 4AMP with beta-cyclodextrin as a function of temperature are shown in Figure 41.

C. Phase-Resolved Fluoroimmunoassay (PRFIA)

The first homogeneous fluoroimmunoassays (those not requiring separation of free from antibody-bound labeled antigen) based on fluorescence lifetime selectivity have been accomplished by using phase-resolved fluorimetry. PRFIA methods have been described by Bright and McGown for the determination of phenobarbital (using fluorescein label)[70] and by Tahboub and McGown for the determination of human serum

Table 3
PHASE-RESOLVED FLUORIMETRIC DETERMINATIONS OF FLUORESCEIN PHYSICALLY ADSORBED TO ALBUMIN (A) AND FLUORESCEIN ISOTHIOCYANATE COVALENTLY BOUND TO ALBUMIN (B)

(λ_{ex} = 490 nm, λ_{em} = 520 nm, modulation = 18 MHz, ϕ_A = 237.0°, ϕ_B = 235.3°)

Added (n*M*)		Conc. found (n*M*) and relative error (%)[a]											
		(ϕ_A, ϕ_B)				(ϕ_A − 45°, ϕ_B − 45°)				(ϕ_A − 90°, ϕ_B − 90°)			
[A]	[B]	[A]	Error	[B]	Error	[A]	Error	[B]	Error	[A]	Error	[B]	Error
50.0	50.0	45.1	−9.8	67.4	34.8	55.0	10.0	53.1	6.2	—	—	42.2	−15.6
50.0	25.0	53.7	7.4	15.0	−40.0	54.4	8.8	24.9	−0.4	22.9	−54.2	35.9	43.6
25.0	50.0	15.8	−36.8	51.3	2.6	23.5	−6.0	45.6	−8.8	—	—	50.5	1.0
25.0	25.0	36.2	44.8	12.5	−50.0	24.8	−0.8	23.0	−8.0	—	—	5.4	−78.5
Average rel. error (%)			1.4		−13.1		3.0		−2.7		—		−12.4
SD[b]			11%					16%				2×10^3%	

[a] (−) indicates that negative concentrations were found.

[b] These are the standard deviations for the fluorescence intensity measurements. Average relative values are shown.

From McGown, L. B., *Anal. Chim. Acta*, 157, 327, 1984. With permission.

Table 4
RESULTS FOR THE TOTAL CONCENTRATION (μM IN CUVETTE) OF ANTHRACENE (A), POPOP (P), AND Me_2POPOP (M) DETERMINED IN FOUR SOLUTIONS USING VARIOUS PHASE-RESOLVED (PRFS) AND STEADY-STATE CONDITIONS

		Found						
	True value	PRFS 1	PRFS 2	PRFS 3	PRFS 4	PRFS 5	SS 1	SS 2
Total								
A	13.867	14.000	14.153	13.514	13.774	13.800	14.786	13.527
P	1.720	1.731	1.710	1.742	1.714	1.755	1.631	1.851
M	1.627	1.601	1.628	1.603	1.626	1.582	1.699	1.424
	Average % error	1.1	0.53	−0.90	−0.43	−0.43	1.9	−2.3
	Average |% error|	1.1	0.91	1.8	0.43	1.8	5.4	7.4
	Measurement precision (RSD) (%)	0.40	0.36	0.28	0.30	0.38	0.13	0.12

From McGown, L. B. and Bright, F. V., *Anal. Chem.*, 56, 2195, 1984. With permission.

Table 5
ERRORS FOR DETERMINATION OF POPOP/ Me_2POPOP MIXTURES[55]

Method	POPOP Error[a] (%)	POPOP |Error|[b] (%)	Me_2POPOP Error[a] (%)	Me_2POPOP |Error|[b] (%)
Direct nulling	45	49	−23	23
($\phi_D = \phi_{comp} \pm 90°$)				
Indirect nulling	12	15	−14	20
Simultaneous equations	3.1	8.5	5.3	8.2

[a] Average relative error for determination of component concentrations in seven solutions.
[b] Average relative error magnitudes corresponding to relative errors.[a]

small molecular (hapten) analytes and macromolecular (antigenic) analytes. In both cases, no spectral differences and very small (<10%) intensity differences were observed between the free and bound labeled analyte. Fluorescence lifetime differences were in the 100- to 200-psec range. In the PRFIA technique, an overdetermined set of SEs (corresponding to Equation 7) in two unknowns (the concentrations of the free and the antibody-bound labeled analyte) is generated as described in a previous section. The additional relationship that the sum of the concentrations of the free and bound labeled analyte is constant is also incorporated into the equation set. A PRFIA calibration curve generated for phenobarbital using five detector phase angles to generate the data matrix is shown in Figure 42.[70]

VIII. CONCLUSIONS

The earliest use of phase resolution was simply to enable the direct recording of the spectra of individual components in two-component mixtures. Since then, numerous applications have been explored, including the suppression of interfering signals such as scattered light in fluorescence detection and fluorescence emission in Raman and phosphorescence spectra, quantitative multicomponent determinations, and homogeneous fluoroimmunoassays. Phase resolution with a xenon arc lamp source allows the full exploitation of fluorescence selectivity based on emission and excitation wavelength as well as fluorescence lifetime, and phase-resolved versions of techniques such as synchronous excitation and total luminescence can be easily implemented. The use of simultaneous linear equations for fluorescence lifetime-based multicomponent analysis leads to relatively simple data analysis compared to pulsed source and phase-modulation heterogeneity analysis methods. Because of its versatility and methodological simplicity, phase-resolved spectroscopy is proving to be a fruitful and promising tool for chemical analysis.

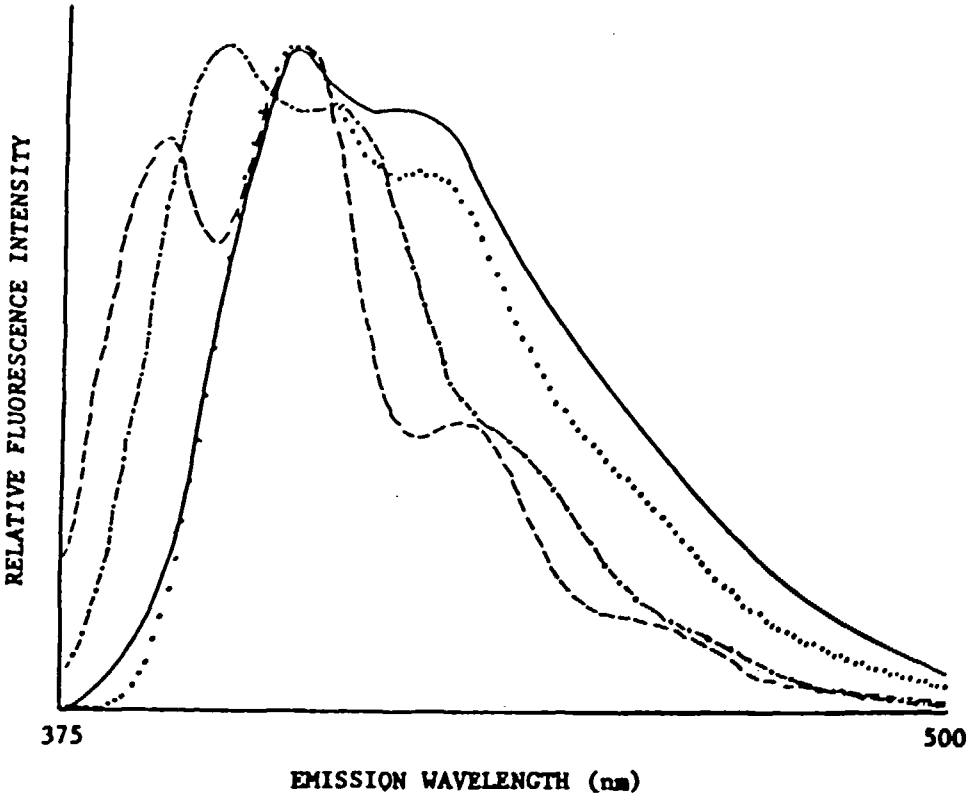


FIGURE 29. Steady-state emission spectra of 1-chloroanthracene (solid line), 9-phenylanthracene (dotted line), 9,10-diphenylanthracene (dot-dash line), and 9-vinylanthracene (dashed line), excitation at 360 nm. (From Bright, F. V. and McGown, L. B., *Anal. Chem.*, *57*, 2877, *1985*. With permission.)

Table 6
ERROR OF DETERMINATION FOR THE INDIVIDUAL COMPONENTS IN A SINGLE-FOUR COMPONENT MIXTURE AS A FUNCTION OF BOTH MODULATION FREQUENCY AND EMISSION WAVELENGTH

	Relative error (%)					
eq	1CA	9PA	9,10DPA	9VA	%E[a]	|%E|[b]
8[c]	241	−16100	−1970	366	−4370	4670
8[d]	287	−95.0	−14600	38.0	−3600	3770
12[e]	0.41	−91.0	14.5	416	84.9	130
12[f]	17.8	40.9	−11.9	247	73.4	79.4
16[g]	12.5	36.8	−9.87	59.3	24.7	29.6
24[h]	0.25	1.96	−0.19	−1.42	0.15	0.96

Note: 1CA = 1-chloroanthracene (τ = 1.48 nsec); 9PA = 9-phenylanthracene (τ = 6.12 nsec); 9,10DPA = 9,10-diphenylanthracene (τ = 5.10 nsec); 9VA = 9-vinylanthracene (τ = 7.53 nsec).

[a] Average % error.
[b] Average |% error|.
[c] 18 MHz, emission at 385 and 425 nm.
[d] 30 MHz, emission at 385 and 425 nm.
[e] 18 MHz, emission at 385, 395, and 425 nm.
[f] 30 MHz, emission at 385, 395, and 425 nm.
[g] 18 and 30 MHz, emission at 385 and 425 nm.
[h] 18 and 30 MHz, emission at 385, 395, and 425 nm.

From Bright, F. V. and McGown, L. B., *Anal. Chem.*, 57, 2877, 1985. With permission.

Table 7
ERRORS FOR THE PHASE-RESOLVED DETERMINATION OF METALS IN TWO-COMPONENT MIXTURES AS THE 5-SULFO-8-HYDROXYQUINOLATE CHELATES AND FLUORESCENCE LIFETIMES OF THE CHELATES

System	τ_p[a]	τ_m[b]	Error[c] (%)	|Error|[d] (%)
		Zn/Cd 3?±20%02	2.1	2.6
Cd	3.31 ± 0.03	3.73 ± 0.02	−1.9	3.3
Ga/In:				
Ga	0.53 ± 0.003	1.18 ± 0.07	−4.1	4.3
In	2.23 ± 0.01	2.59 ± 0.04	−1.4	1.5
Al/Ga:				
Al	5.80 ± 0.02	9.28 ± 0.04	−0.9	1.0
Ga	0.53 ± 0.003	1.18 ± 0.07	−1.5	2.1

[a] Fluorescence lifetime in nanoseconds calculated from phase-delay, ± 1 SD (n = 5).
[b] Fluorescence lifetime in nanoseconds calculated from demodulation, ± 1 SD (n = 5).
[c] Relative error averaged for five solutions, containing 1 to 10 μM of each metal.
[d] Relative error magnitudes corresponding to average errors.[c]

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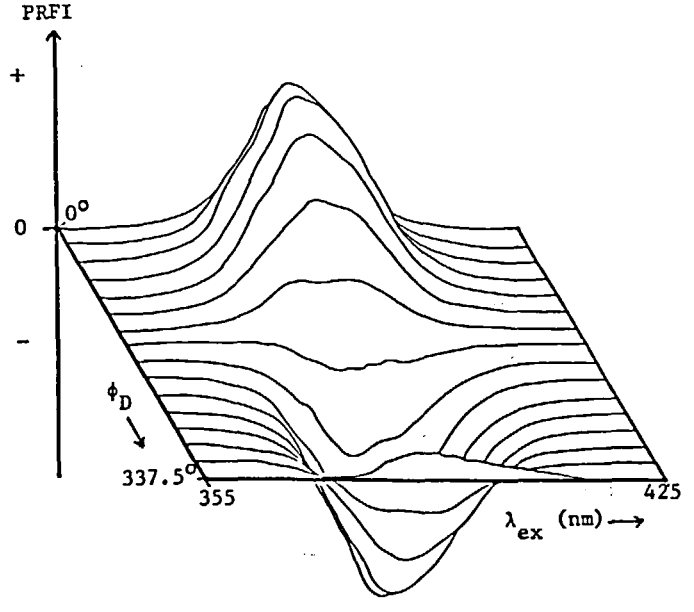

FIGURE 30. Depiction of PRFS-synchronous excitation data format for a four-component solution containing 9PA, 9,10DPA, BkF, and BaP (see Table 8), showing phase-resolved fluorescence intensity (PRFI) as a function of detector phase angle and synchronously scanned wavelength. Modulation frequency = 30 MHz, $\Delta\lambda$ = 15 nm. (From Nithipatikom, K.

Table 8
SUMMARY OF ERRORS FOR PHASE-RESOLVED (PRFS) AND STEADY-STATE (SS) SYNCHRONOUS EXCITATION DETERMINATIONS OF 9-PHENYLANTHRACENE (9PA), 9,10-DIPHENYLANTHRACENE (9,10DPA), BENZO(K)FLUORANTHENE (BkF), AND BENZO(A)PYRENE (BaP)

	Determination errors			
	9PA	9,10DPA	BkF	BaP
PRFS				
2-Component	−1.0 (1.5)	3.3 (3.3)	—	—
2-Component	—	−2.2 (2.2)	3.6 (4.1)	—
3-Component	−4.3 (4.3)	0.6 (2.9)	1.1 (1.1)	—
4-Component	−2.9 (2.9)	−3.8 (6.4)	−0.1 (2.5)	1.9 (5.9)
Average:	−2.7 (2.9)	−0.5 (3.7)	1.5 (2.6)	1.9 (5.9)
SS				
2-Component	−1.2 (2.1)	0.7 (1.3)	—	—
2-Component	—	−0.4 (1.6)	1.8 (2.1)	—
3-Component	−2.8 (2.8)	−3.7 (3.7)	1.7 (2.0)	—
4-Component	−1.6 (1.6)	5.4 (5.4)	−3.0 (3.9)	−8.8 (8.8)
Average:	−1.9 (2.2)	0.7 (2.8)	0.2 (2.7)	−8.8 (8.8)

Note: Relative errors (%) averaged for all five solutions, with average relative error magnitudes (%) in parentheses.

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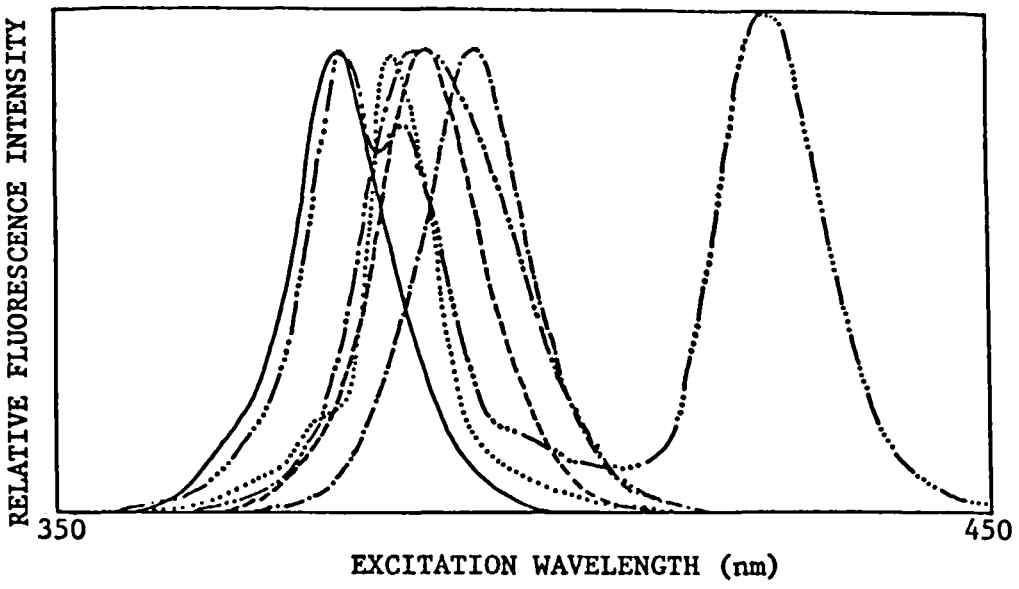

FIGURE 31. Steady-state synchronous excitation spectra ($\Delta\lambda$ = 15 nm) of six PAHs (see Table 9). 9PA = solid line; 9,10DPA = dashed line; BkF = dot-dash line; BaP = dotted line; TPP = dash-double dot line; BgP = dash-four dot line. (From Nithipatikom, K. and McGown, L. B., *Appl. Spectrosc.*, in press. With permission.)

Table 9
AVERAGE ERRORS FOR THE DETERMINATION OF PAHs IN FIVE- AND SIX-COMPONENT MIXTURES[a]

Five-Component

	9PA	9,10DPA	BkF	BaP	TPP	Average
PRFS[b]	−2.6 (4.2)	12 (15)	2.0 (4.8)	−4.3 (7.0)	−5.4 (7.5)	0.34 (7.7)
PRFS[c]	−4.0 (4.0)	3.7 (8.7)	4.4 (4.4)	4.8 (7.3)	−4.2 (4.4)	0.94 (5.8)
SS[d]	−3.1 (4.5)	13 (13)	−2.9 (7.1)	−7.2 (7.5)	−7.2 (8.1)	−1.5 (8.0)

Six-Component

	9PA	9,10DPA	BkF	BaP	TPP	BgP	Average
PRFS[b]	−7.5 (7.5)	18 (18)	12 (12)	−13 (13)	−21 (21)	34 (61)	3.8 (22)
PRFS[c]	−6.6 (8.9)	−4.4 (5.5)	−1.7 (2.7)	−1.4 (4.9)	2.0 (3.1)	−7.2 (20)	−3.2 (7.5)
SS[d]	−20 (20)	−42 (42)	[e]	−0.8 (14)	108 (108)	−26 (30)	[e]

Note: 9PA, 9,10DPA, BkF, and BaP as in Table 8. TPP = 1,3,6,8-tetraphenylpyrene; BgP = benzo(ghi)perylene.

[a] Average relative errors (%) for determination of each PAH in the five- and six-component mixtures averaged for six different solutions. Average error magnitudes (absolute values of the relative errors) are given in parentheses.

[b] PRFS determinations with 24 equations generated using 24 wavelengths and 1 detector phase angle (180°).

[c] PRFS determinations with 96 equations generated using 24 wavelengths and 4 detector phase angles (180°, 225°, 270°, and 315°).

[d] Steady-state determinations with 24 equations generated using 24 wavelengths.

[e] Error undefined due to a value of zero obtained for the concentration of the component in one or more solutions.

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Note: 9PA, 9,10DPA, BkF, and BaP as in Table 8. TPP = 1,3,6,8-tetraphenylpyrene; BgP = benzo(ghi)perylene.

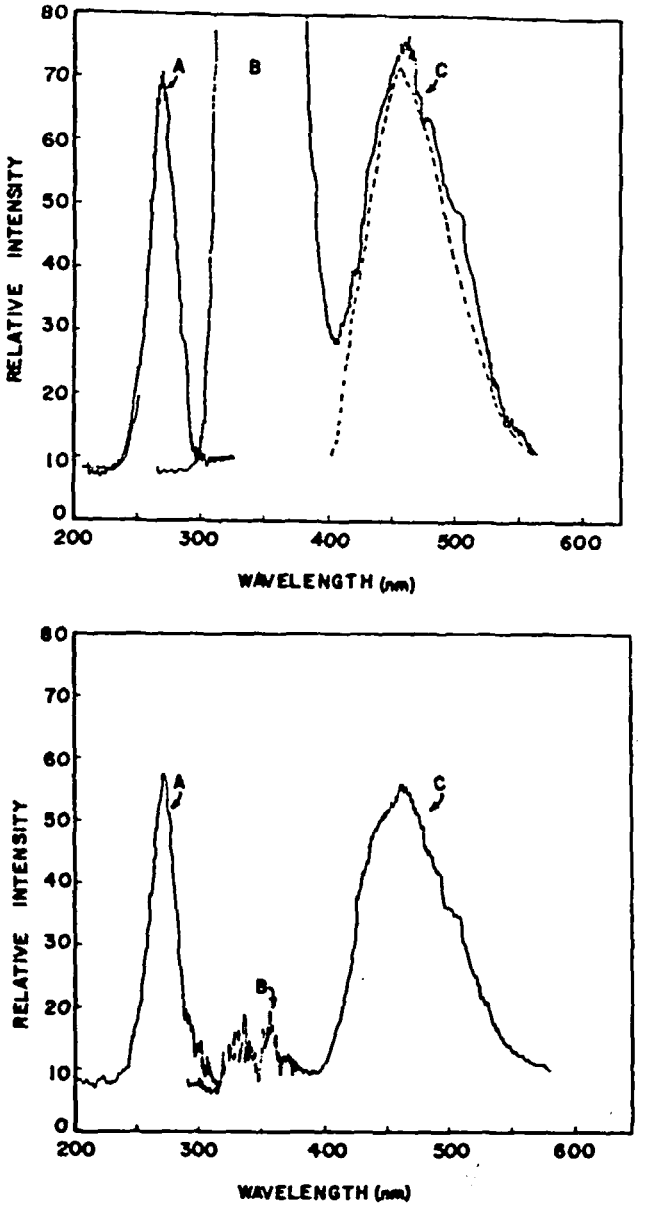

FIGURE 32. Top: emission and excitation spectra of 2.1 m*M* 2-bromobiphenyl at 50 Hz and 270° + 68°. Bottom: same as top except spectral contributions are phase-resolved at 180° + 95.3°. (A) Excitation spectrum, emission wavelength = 465 nm. (B) Fluorescence emission peak (top, excitation at 275 nm) and residual fluorescence emission (bottom, excitation at 270 nm). (C) Phosphorescence emission spectrum, excitation as in (B). Dotted line (top) is phosphorescence emission spectrum of 2-bromobiphenyl standard. (From Mousa, J. J. and Winefordner, J. D., *Anal. Chem.*, 46, 1195, 1974. With permission.)

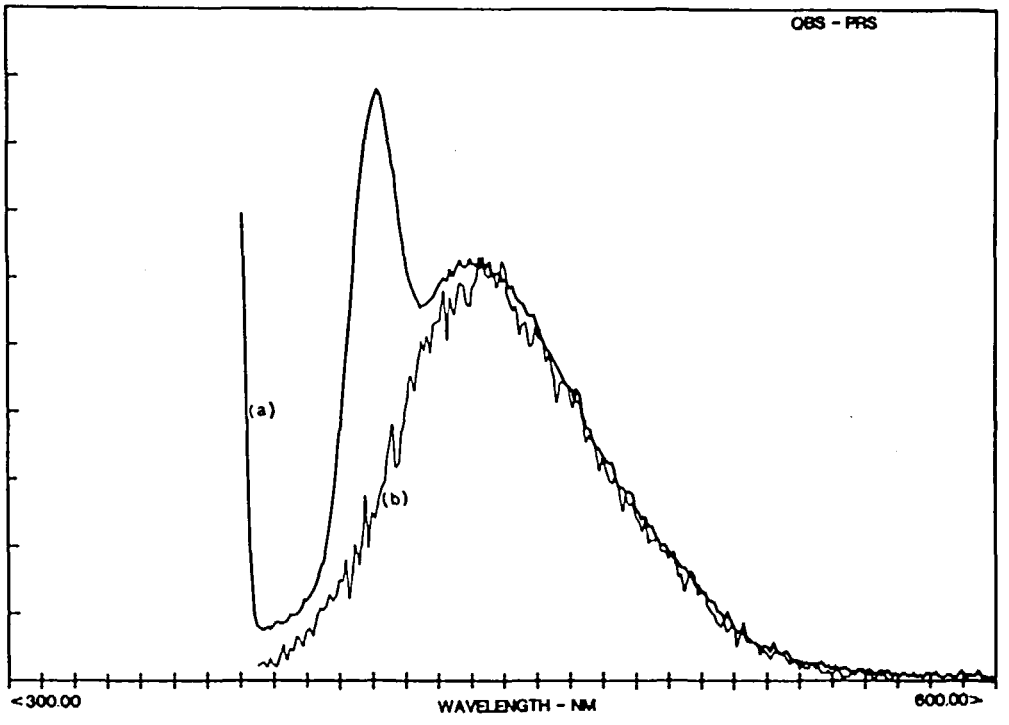

FIGURE 33. (a) Steady-state emission spectrum of quinine bisulfate fluorescence excited at 360 nm and (b) phase-resolved spectrum after nulling the fluorescence signal at 370 nm to suppress the contribution of the Raman scatter band. (From Mattheis, J. R., Mitchell, G. W., and Spencer, R. D., *New Directions in Molecular Luminescence*, American Society for Testing and Materials, Philadelphia, 1983. With permission.)

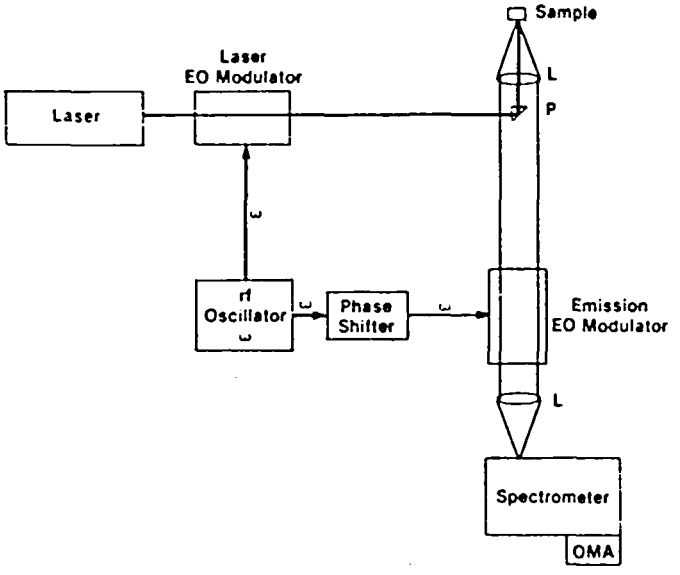

FIGURE 34. Schematic of the instrument described by Genack for electro-optic phase-sensitive detection. (From Genack, A. Z., *J. Lumin.*, 31 & 32, 696, 1984. With permission.)

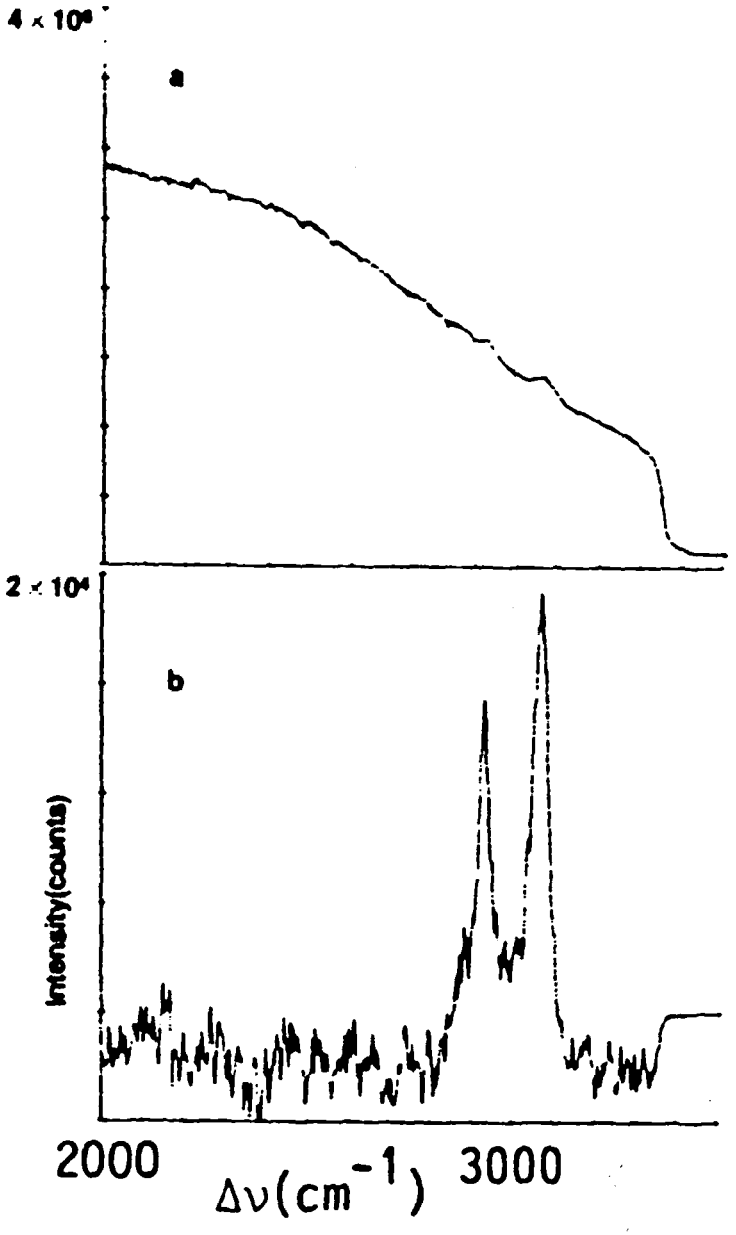

FIGURE 35. (a) Fluorescence from 0.05 m*M* fluorol 555 in toluene with modulators set in phase. (b) Difference of spectra with emission modulator set 90° out of phase with fluorescence. (From Genack, A. Z., *J. Lumin.*, 31 & 32, 696, 1984. With permission.)

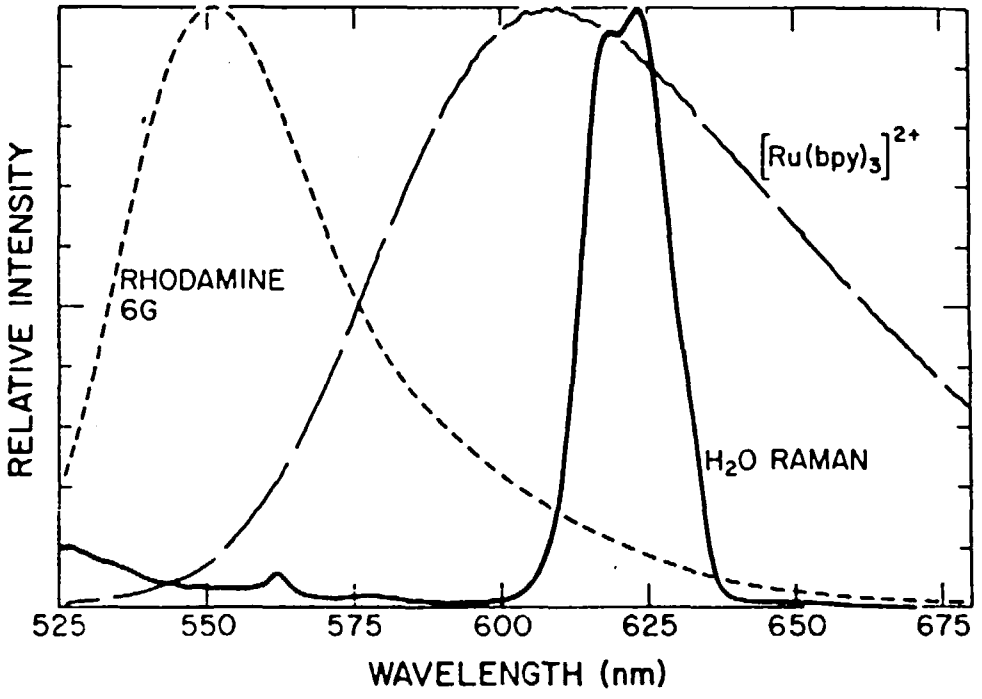

FIGURE 36. Luminescence spectrum of aqueous 400 n*M* rhodamine 6G (short dashes) and of 130 μM $[Ru(bpy)_3]^{2+}$ (long dashes). Raman spectrum of pure water (solid line). Excitation at 514.5 nm (argon ion laser). (From Demas, J. N. and Keller, R. A., *Anal. Chem.*, 57, 538, 1985. With permission.)

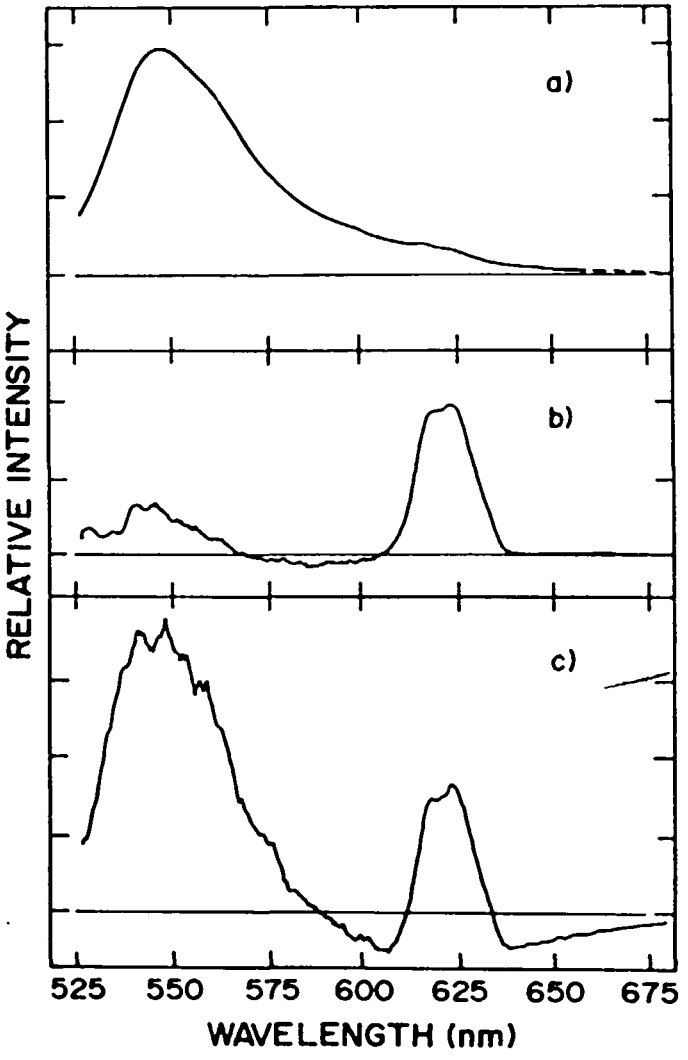

FIGURE 37. Raman water from rhodamine 6G: (a) total emission (0.6 MHz, 1-sec time constant) of an 8-n*M* rhodamine 6G solution; (b) phase-resolved spectrum (40 MHz, 1-sec time constant) of solution of (a); and (c) phase-resolved (40 MHz, 10-sec time constant) water Raman of a 40-n*M* rhodamine 6G solution. (From Demas, J. N. and Keller, R. A., *Anal. Chem.*, 57, 538, 1985. With permission.)

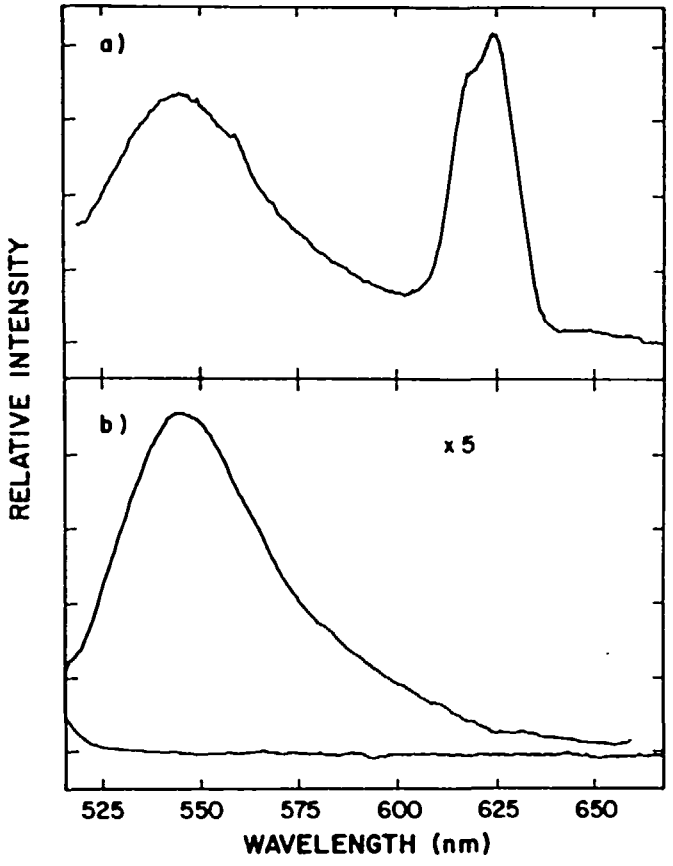

FIGURE 38. Rhodamine 6G from Raman: (a) total emission of a 0.1-n*M* rhodamine 6G solution; (b) phase-resolved (20 MHz, 10 sec) rhodamine fluorescence for the same solution. (From Demas, J. N. and Keller, R. A., *Anal. Chem.*, 57, 538, 1985. With permission.)

Table 10
COMPARISON OF BACKGROUND SUPPRESSION TECHNIQUES

Noise suppression technique	Statistic	Time-dependent background	Raman from fluorescence	Fluorescence from Raman	Duty cycle	Ease of operation	Cost
Background subtraction	−[a]	−	0	0	+	0[b]	0[b]
Frequency modulation	−	+	−	+	+	−	+
Gated detection	+	+	0	+	−	0	−
PRS	−	+	+	+	+	+	+

[a] (+) is good, (0) is medium, (−) is poor.
[b] Requires extended data handling capability for optimum results.

From Demas, J. N. and Keller, R. A., *Anal. Chem.*, 57, 538, 1985. With permission.

Table 11
LIMITS OF DETECTION CALCULATED FOR FIVE PAHs*

PAH	PRFS	Steady-state
Anthracene	27	93
BkF	5.7	9.0
BaP	28	68
Perylene	4.1	4.5
BgP	150	130

* Limits of detection expressed in nanomolar, in cuvette.

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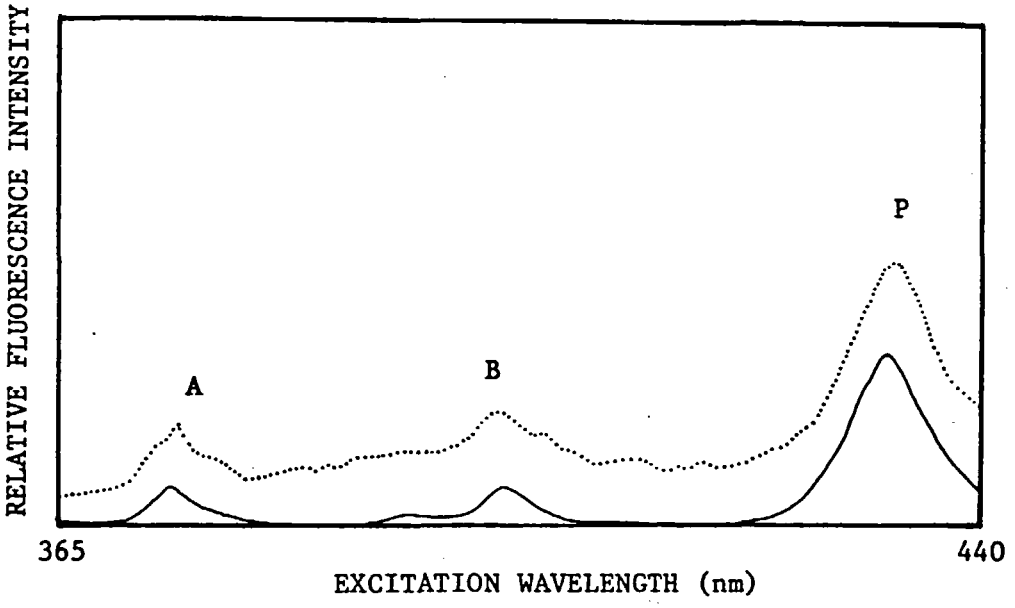

FIGURE 39. Synchronous excitation spectra ($\Delta\lambda$ = 3 nm) for a mixture of (A) anthracene (47.1 n*M*), (B) benzo(a)pyrene (50.2 n*M*), and (P) perylene (10.4 n*M*) acquired using steady-state measurements with blank correction (dotted line) and phase-resolution with scattered light suppressed (solid line). From Nithipatikom, K. and McGown, L. B., *Anal. Chem.*, 58, 3145, 1986. With permission.)

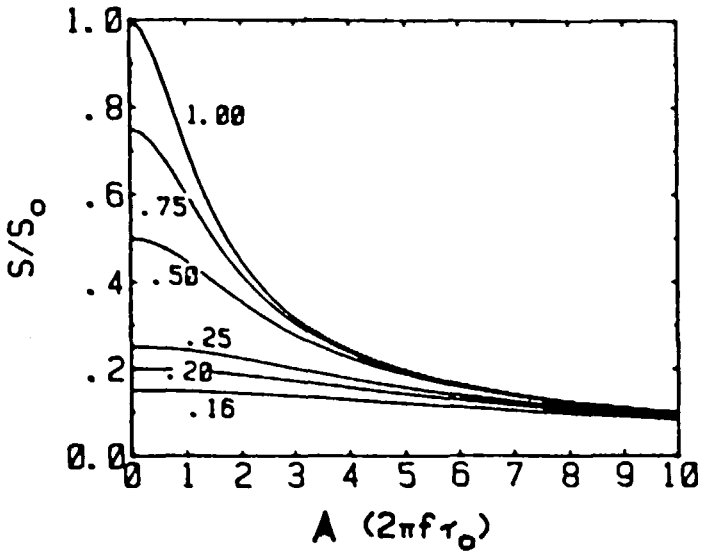

FIGURE 40. Variation of normalized signal as a function of A. The extent of quenching is indicated by each curve. (From Demas, J. N., Jones, W. M., and Keller, R. A., *Anal. Chem.*, 58, 1717, 1986. With permission.)

Table 12
EFFECT OF MODULATION FREQUENCY ON THE ELIMINATION OF QUENCHING EFFECTS

f, kHz	Method 1 $S \pm \sigma$, mV ($\sigma/\bar{S}$, %)*	Method 2 $S_o \pm \sigma$, mV ($\sigma/\bar{S}_o$, %)*
0.100	3.31 ± 2.54 (77)	8.06 ± 2.71 (34)
0.800	2.97 ± 1.87 (63)	9.46 ± 0.33 (3.5)
1.000	2.82 ± 1.65 (59)	9.37 ± 0.23 (2.5)
2.000	2.28 ± 0.93 (41)	9.61 ± 0.13 (1.4)
3.000	1.89 ± 0.56 (30)	9.69 ± 0.21 (2.2)
4.000	1.60 ± 0.36 (23)	9.67 ± 0.17 (1.8)
5.000	1.38 ± 0.24 (18)	9.70 ± 0.17 (1.8)
6.000	1.22 ± 0.17 (14)	9.57 ± 0.10 (1.0)
8.00	0.965 ± 0.091 (9)	9.63 ± 0.12 (1.3)
10.00	0.807 ± 0.052 (6)	9.64 ± 0.12 (1.2)
15.00	0.560 ± 0.019 (3.4)	9.61 ± 0.08 (0.8)
20.00	0.429 ± 0.009 (1.9)	9.67 ± 0.11 (1.1)
30.00	0.289 ± 0.002 (0.7)	9.68 ± 0.16 (1.7)

* Mean and SD are calculated for nine data points ranging from unquenched to 87% quenched. All data are at 80 ppm U in 1 *M* phosphoric acid.

From Demas, J. N., Jones, W. M., and Keller, R. A., *Anal. Chem.*, 58, 1717, 1986. With permission.

Table 13
COMPARISON OF DIRECT PHASE-MODULATION AND PRFS FLUORESCENCE LIFETIME DETERMINATIONS[a,b]

	PRODAN		9,10 DPA		1,2,5,6 DBA	
	τ_ϕ	τ_m	τ_ϕ	τ_m	τ_ϕ	τ_m
	(390/515)		(363/408)		(347/400)	
Direct	1.75 ± .08	2.63 ± .09	4.55 ± .05	4.49 ± .05	11.04 ± .19	11.95 ± .14
PRFS	1.73 ± .06	2.51 ± .12	4.50 ± .10	4.39 ± .18	10.96 ± .21	11.96 ± .19

Note: PRODAN and 9,10DPA are as defined in text; 1,2,5,6 DBA = 1,2,5,6-dibenzanthracene.

[a] Results expressed as mean ± SD; values in parentheses indicate $\lambda_{ex}/\lambda_{em}$ in nanometers, τ is in nanoseconds, and f = 30 MHz.
[b] A total of eight measurements of sample/reference at 10 average per data set, ten sets.

[1] McGown, L. B., *Anal. Instrum.*, 14, 251, 1985. With permission.

Table 14
RESULTS FOR HETEROGENEITY ANALYSIS OF THREE MIXTURES OF POPOP AND 9,10DPA[a,b]

	Mixture 1	Mixture 2	Mixture 3
True[c]	1.35 (.52)/4.3 (.48)	1.35 (.92)/ 4.3 (.08)	1.35 (.10)/ 4.3 (.90)
Direct[d]	1.85 (.71)/9.21 (.29)	1.41 (.97)/10.40 (.03)	3.21 (.66)/11.60 (.34)
PRFS[e]	0.81 (.37)/4.09 (.63)	1.31 (.94)/ 9.68 (.06)	−2.3 (.01)/ 4.49 (.99)

[a] Modulation frequencies = 30 and 18 MHz; $\lambda_{ex}/\lambda_{em}$ = 363/393 nm.
[b] Results expressed as τ_1 (α_1)/τ_2(α_2) where τ is in nanoseconds and α is the fractional contribution to the total fluorescence intensity.
[c] True values as determined by steady-state intensities of the components, $\tau_{9,10\ DPA}$ estimated from Table 1.
[d] A single set of eight sample/reference measurement pairs in 10 average mode at 30 and 18 MHz.
[e] A single set of 4 sample/reference measurement pairs in 100 average mode at 30 and 18 MHz.

From McGown, L. B., *Anal. Instrum.*, 14, 251, 1985. With permission.

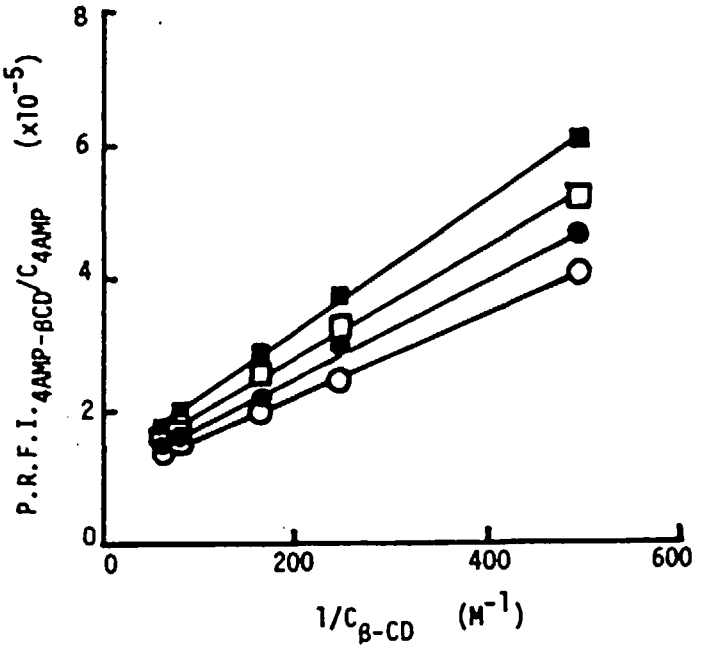

FIGURE 41. Formation constant plots for the 4AMP/beta-cyclodextrin inclusion complex at various temperatures (°C) (top to bottom): 40, 30, 20, and 10. (From Bright, F. V., Keimig, T. L., and McGown, L. B., *Anal. Chim. Acta*, 175, 189, 1985. With permission.)

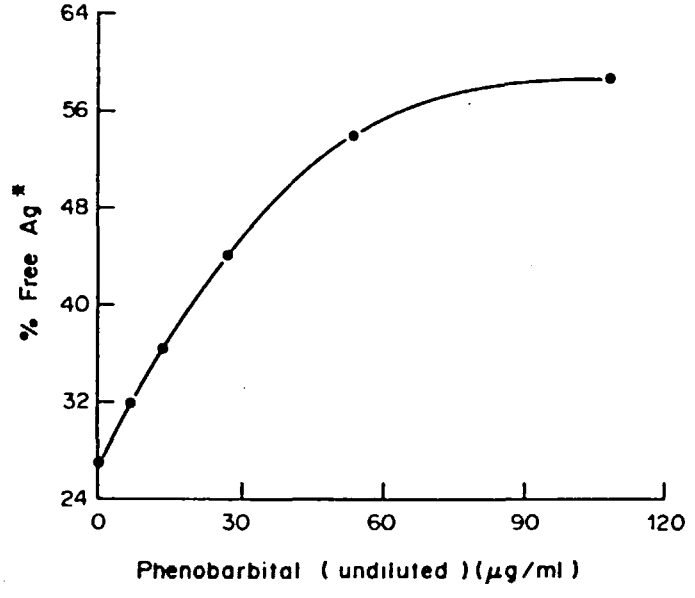

FIGURE 42. Phenobarbital calibration curves (percent of free fluorescein-labeled phenobarbital Ag* as a function of nonlabeled phenobarbital concentration in the standard solutions) obtained by using 5 detector phase angles. (From Bright, F. V. and McGown, L. B., *Talanta*, 32, 15, 1985. With permission.)

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