# Applications of Multidimensional Absorption and Luminescence Spectroscopies in Analytical Chemistry

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## Contents



## I. Introduction

The ability to analyze complex multicomponent mixtures without resorting to tedious separation procedures is extremely useful for routine analysis. Fluorescence spectroscopy is one example of an analytical technique suitable for multicomponent analysis due to its inherent sensitivity, selectivity, and versatility. In addition, the low limits of detection makes molecular fluorescence the basis of many analytical methods. However, single-wavelength measurements can be limited for the analysis of complicated multicomponent samples, or even a simple mixture which contains severely overlapping emission and/or excitation spectra. This is because conventional fluorescence spectra reveal fluorescence of a sample within one spectral region, and do not provide nearly enough data to distinguish between two or more closely related molecules.

The disadvantages of fluorescence can sometimes be overcome by extending the dimensionality of the luminescence measurement. For example, Knorr and Harris[1] have demonstrated that the fluorescence emission from mixtures can be resolved by use of the characteristic decay time or the chromatographic retention time. An increase in the dimensionality of the measurement improves not only the capability for resolution of overlapped fluorescence spectra, but will often also decrease the time of analysis. Furthermore, the selectivity is markedly enhanced by simultaneously measuring several fluorescence properties of the analyte in the same experiment. Thus, in the analysis of a mixture of fluorophores, the extent of spectral overlap can be determined by obtaining the total spectrofluorimetric information available in the luminescence measurement. Several techniques have been examined to exploit the inherent selectivity of fluorescence. Examples of these approaches include synchronous luminescence, phase-resolved luminescence, and multidimensional luminescence.

Synchronous fluorescence alone[2,3] or in combination with derivative techniques[4,5] has been used for the analysis of mixtures of fluorescent compounds in solutions which have overlapping fluorescence bands. Synchronous fluorimetry involves the simultaneous scanning of both the excitation and emission monochromator wavelengths, while the wavelength interval ($\Delta\lambda = \lambda_{EM} - \lambda_{EX}$) between them is maintained constant. The simplification of the spectral profile together with the reduction of bandwidth are its main characteristics.[3] The combination of synchronous fluorimetry and derivative techniques is advantageous when compared with differentiation of conventional emission spectra, in terms of sensitivity, because the amplitude of the derivative signal is inversely proportional to the bandwidth of the original spectrum.[5,6] In addition, the selectivity of the analysis is greatly enhanced by using the two techniques in combination.[6] The main disadvantage of synchronous scanning is that the optimum value for $\Delta\lambda$ must be previously determined. Also, in some multicomponent samples, several different values for $\Delta\lambda$ might be necessary for a complete identification. The best value or values for $\Delta\lambda$ can be determined from a three-dimensional (3-D) plot of interests as a function of excitation and emission wavelengths. This determines whether it would be possible, with a synchronous scan, to suppress the fluorescence contributions of some compounds and analyze only selected components in the mixture.

Phase-resolved fluorescence spectroscopy (PRFS) is an alternative multidimensional approach. The PRFS method is based upon the phase-modulation approach

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to fluorescence lifetime measurements. In this method, the excitation source is modulated at a high frequency and combined with phase-sensitive detection, which results in phase-resolved fluorescence intensities. The PRFS approach has proved to be a useful technique for the resolution of overlapping fluorescence spectra and for the suppression of scattered light in complex systems. This approach is important when highly scattering samples are used, such as protein-containing biological samples, micellar systems, environmental samples, and microbiological samples because important information can be obscured by the intense scattered light peaks.[7] Demas and Keller[8] have demonstrated the utility of PRFS for the suppression of background luminescence in Raman experiments and for the suppression of Raman scattered light contributions to fluorescence emission spectra. Phase-resolved suppression of the scattered light results in increased sensitivity and selectivity for the method.

Total luminescence spectroscopy (TLS) involves the simultaneous acquisition of multiple excitation and emission wavelengths in order to increase the selectivity of the measurement. The resulting emission–excitation data matrix (EEM) provides a total intensity profile of the sample over the range of excitation and emission wavelengths scanned. This technique is used to obtain additional information about the samples and has many applications in analytical chemistry. The TLS approach has been used for the identification and quantification of polyaromatic hydrocarbons (PAHs) in environmental samples,[9] the identification of oil spills by analysis of the fluorescent PAHs in the samples,[10] the identification of oil and fuel samples in forensic studies,[11] the analysis and characterization of pharmaceutical compounds,[12] the observation of luminescence properties of rocks and minerals,[13] bacterial identification,[14,15] and the study of marine phytoplankton.[16–18]

The isometric projection of the EEM can also be represented as contour levels, which are more informative.[19] In the contour plots, the data set is viewed from a point vertically above the ($\lambda_{EX}$, $\lambda_{EM}$) plane; the *fluorescence levels are represented by contours in this* plane. The contour representation of the 3-D data matrix is also more convenient than pseudoisometric plots since all the information is displayed in a single plane. The small fluorescence peaks are not hidden by larger foreground peaks, as is true for isometric projections. The contour representation of the fluorescence profile of the mixture indicates the most suitable trajectory in the EEM which allows for the complete resolution of overlapping component peaks when employing synchronous scan. Parallel diagonal lines superimposed on the contour plots represent the scan paths through the EEM for a given $\Delta\lambda$. The optimum path that passes near the maxima of the compounds investigated will allow optimal determination in terms of selectivity.

Modern instrumentation capable of very fast scans allows the use of different experimental strategies for monitoring time-dependent phenomena, such as enzymatic processes, reaction kinetics, flow-injection analysis, and high-performance liquid chromatographyy (HPLC).[20] In these cases, spectral data are captured and stored, facilitating numerous possibilities for data processing and graphical presentation. Therefore, various techniques to convolute overlapping spectra can be employed. The use of optoelectronic imaging devices capable of acquiring fluorescence spectral data simultaneously over a large wavelength range necessitates this approach. Examples of multichannel detectors used in multidimensional analysis include vidicons[21,22] and linear photodiode arrays.[23,24] In addition, Glick et al.[25] have developed a modular multiwavelength fluorescence detector based on the use of the multiple sensitive photomultiplier tube (PMT). The system provided high selectivity, rivaling that of array-based detectors. Fell et al.[26] reported the use of low noise charge couple devices (CCD) to add a new dimension to detection

systems for analytical and luminescence measurements. In particular, the CCD provides significant potential for enhanced detection capabilities for use with HPLC. Recently, Denton et al.[27] compared the sensitivity of a CCD for measuring extremely low light levels to that of a photon-counting PMT. This suggests that the emergence of new analytical instruments and techniques will require the processing of data with increased dimensionality. This gives rise to a great need for multidimensional filtering techniques and other data processing methods. For example, Vicsek et al.[28] have demonstrated the usefulness of time-domain filtering for enhancing the information of two-dimensional fluorescence.

This review is concerned with the applications of multidimensional luminescence spectroscopy in analytical chemistry. The review will focus mainly on luminescence measurements and is limited to work reported in the last 10 years. The utility and applications of multidimensional spectroscopy using luminescence detectors will be examined. Various techniques in which multidimensional luminescence measurements are employed and the advantages of these techniques are presented. First, we give a brief overview of multidimensional synchronous fluorimetry as an optimization technique for the resolution of overlapping spectra of mixtures of compounds. A complete and exhausting review of this technique is beyond the scope of this paper. However, for some industrial developments and applications of multidimensional techniques, the reader is referred to a recent review by Crummett et al.[29] Also, the reader interested in the applications of two-dimensional nuclear magnetic resonance may consult a review by Perrin and Dwyer.[30] The theory and data manipulation methods have been described in detail elsewhere.[22,31-33]

## II. Multidimensional Fluorimetry as an Optimization Technique for the Resolution of Overlapping Spectra of Mixtures of Compounds

### A. Synchronous Scanning Fluorescence

Acquisition of an entire EEM is often more tedious than is practical for many analytical applications. For routine analysis, the synchronous scan method is a subset of the data and can often be used for analysis. The synchronous approach is accomplished by first optimizing the synchronous path with use of multidimensional information available from the EEM. In this regard, several interesting applications have been accomplished by using this approach.

Muñoz de la Peña et al.[34] interfaced a microcomputer to a spectrofluorimeter in order to acquire, store, and analyze multidimensional data. Using this approach, they have proposed first derivative synchronous fluorimetry for the simultaneous determination of salicylic and salicyluric acids in urine.[35] The method has been extended and modified to allow the determination of binary and/or ternary mixtures of salicylic acid and its two main urinary metabolites, salicyluric and gentisic acids.[36] Figure 1 shows the 2-D representation of the data as contour plots of the fluorescence spectra of salicylic acid, gentisic acid, and a mixture of these compounds. Optimum scanning paths ($\Delta\lambda$) of 90 and 115 nm were found for salicylic–salicyluric and salicylic–gentisic mixtures, respectively. The results obtained show that the combined use of multidimensional fluorimetry for synchronous scan optimization, and derivative synchronous fluorimetry, leads to a rapid and straightforward method in spectrally resolving the ternary mixture, obviating prior separation procedures.

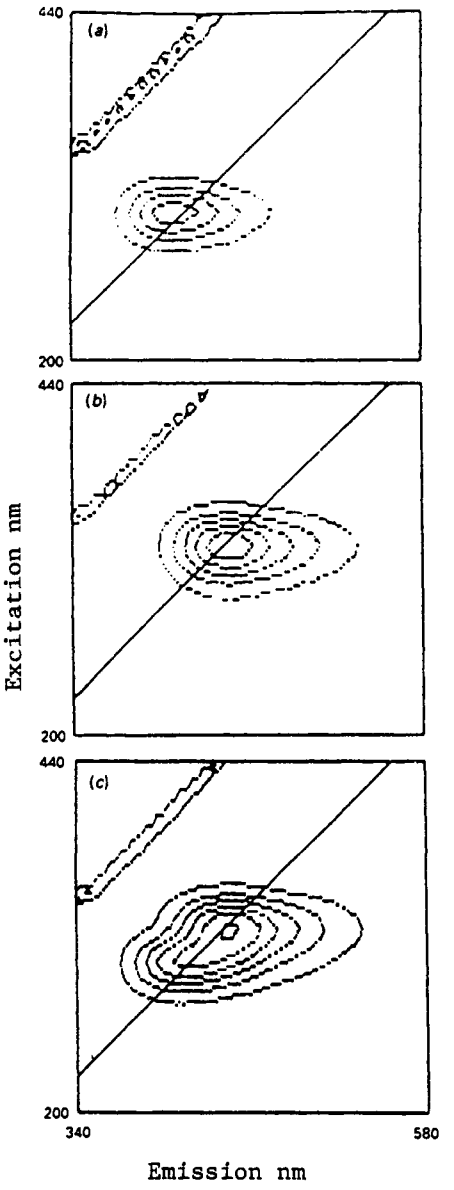


**Figure 1.** Two-dimensional representation, as contour plots, of total fluorescence spectra of (a) salicylic acid, (b) gentisic acid, and (c) a mixture of both compounds. The synchronous fluorescence path (shown by solid line) slices the data matrix at $\Delta\lambda = \lambda_{EM} - \lambda_{EX} = 115$ nm (reprinted from ref 37, copyright 1989 the Royal Society of Chemistry).

In the field of inorganic analysis, Salinas et al.[37] have used first-derivative synchronous fluorimetry in the simultaneous determination of molybdenum and tungsten mixtures. Their method is based on the formation of a fluorescent complex with carminic acid at pH 5.1. The optimum constant wavelength difference ($\Delta\lambda$) as determined by multidimensional fluorimetry was found to be 50 nm. Multidimensional fluorimetry was also employed for the optimization of the analysis of mixtures of doxycycline and oxytetracycline in pharmaceutical preparations through the formation of their chelates with aluminum ion.[38] In this case, the analysis of the multidimensional data demonstrates that

the best resolution can be accomplished by an emission scan. This could not be found with any of the possible synchronous scans. The mixtures of both compounds were determined by first-derivative fluorimetry.

First-derivative synchronous fluorimetry has also been applied to the simultaneous resolution of propranolol and hydralazine in pharmaceutical preparations.[39] Propranolol, a β-adrenergic blocking agent, is often prescribed in combination with hydralazine for the treatment of hypertension. Thus, the simultaneous determination of these drugs in pharmaceutical preparations is of considerable interest. The determination was performed in concentrated sulfuric acid, heating the samples at 70 °C for 15 min and scanning the synchronous spectra while maintaining a constant Δλ (48 nm). The combination of synchronous scanning and derivatives techniques[40] has also been used for the determination of oxytetracycline and riboflavine mixtures. The method is based on the formation of the fluorescent complex between aluminum ion and tetracycline and on the native fluorescence of riboflavine. The experiment is performed at pH 5 and the optimum scanning wavelength difference was found to be at Δλ = 60 nm. Multidimensional synchronous fluorimetry offers an elegant approach to the problem of resolving spectral overlap.

## B. Variable-Angle Synchronous Scanning Fluorescence

Variable-angle synchronous fluorimetry is a modified approach of synchronous fluorimetry. However, variable-angle synchronous scanning fluorimetry offers greater flexibility.[41] In this technique, the separation between the excitation and emission wavelengths is continuously varied through the scan. In certain cases, this can enhance selectivity.[19]

There are three different instrumental configurations for performing a variable-angle synchronous scan. First, the speed of the monochromators can be manipulated by two independent motors and the motors are scanned at different rates. Miller[19] has modified a commercial analog instrument in order to allow for this possibility. Only linear scan paths can be produced by this approach. A second approach consists of acquiring the EEM and storing the data on the interfaced microcomputer, and the desired angle (linear or nonlinear trajectory) is determined by using software.[42] Recently, Garcia Sanchez et al.[43,44] have modified a commercial digital instrument to generate the variable-angle synchronous scan directly from the spectrofluorimeter output. In this approach, a few minutes will suffice to obtain the spectra from the samples by following the path previously selected by inspecting the contour lines.

In the previous two approaches, the information available in the multidimensional EEM may be used to optimize the path that will produce the best variable-angle scanning spectra (i.e. give the highest signal value, smallest band width at half-maximum intensity and interference free bands). Such an approach was employed to determine oxytetracycline in the presence of the additives vitamin C, thiamine, nicotinamide, and riboflavine mixtures and mixtures of chlorpromazine and its principal degradation product, chlorpromazine sulfoxide.[45] Contour plots representing riboflavin and a standard mixture of oxytetracycline and riboflavine

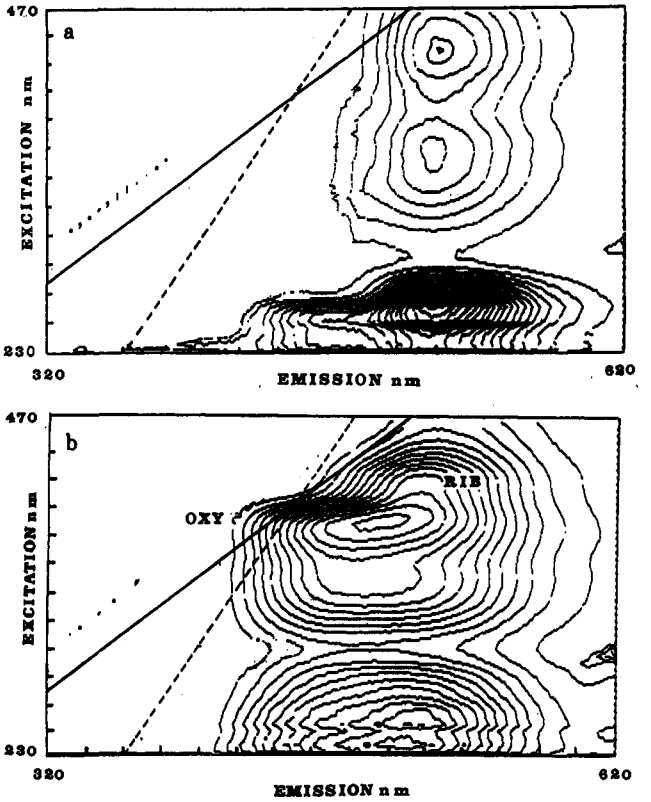


**Figure 2.** Contour plots of (a) riboflavine fluorescence and (b) oxytetracycline and riboflavine fluorescence. Lines indicate the following: (—) the conventional synchronous luminescence path slicing the data matrix at 45° (Δλ = 40 nm); (- - -) the variable-angle synchronous scanning path at 63.4° grating from ($\lambda_{EX}$, $\lambda_{EM}$)) = (230, 340 nm) (from ref 45, copyright 1985 Elsevier Science Publishers).

are shown in Figure 2. The best attainable spectral resolution of oxytetracycline and riboflavine was obtained by slicing the matrix at 63.4°. These data were obtained in the presence of aluminum such that the measured species is the fluorescent oxytetracycline aluminum chelate. This technique allowed for the quantification of oxytetracycline independent of the riboflavine concentration. The quantification of riboflavine was not possible in the presence of oxytetracycline. However, riboflavine could be readily assayed by measurement in the absence of aluminum ion, where the oxytetracycline contribution is very minimal. This approach permitted the light-scattering peaks to be avoided. The chlorpromazine hydrochloride emission peak was similarly resolved from the interfering sulfoxide by slicing the excitation matrix at a "reversed" angle of 135°, with the excitation wavelength values decreasing with increasing emission wavelengths.[45] An alternative method for the resolution of oxytetracycline and riboflavine is by synchronous derivative fluorimetry, allowing the determination to be performed in only one scan.[40]

Garcia Sanchez et al.[46] have applied variable-angle scanning fluorimetry to the determination of closely overlapped pesticide mixtures. The technique proved to be effective for the determination of the components of ternary mixtures of carbaryl, fuberdazol, and warfarin, all of which are pesticides with intrinsic fluorescence and closely overlapping profiles. The optimum scanning route was determined by inspecting both the isometric projection spectra and the corresponding two

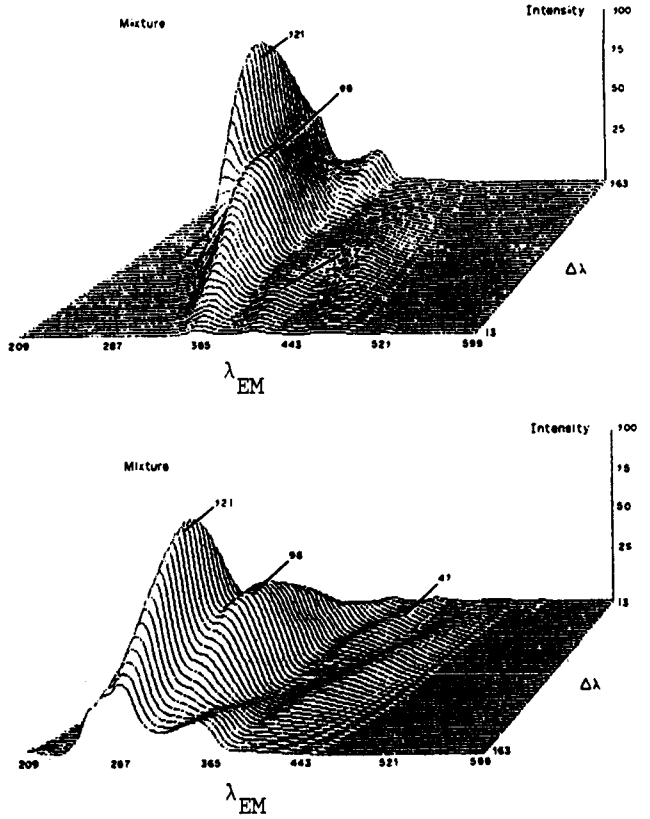


**Figure 3.** Three-dimensional synchronous spectra (ESM) of three-component mixture of the alkaloids. Optimum Δλ of 47, 98, 121 nm were used to determine berberine, luguine, and sanguinarine, respectively: 50 scans, excitation wavelength increment 3 nm (reprinted from ref 47, copyright 1990 Pergamon Press PLC).

dimensional (2-D) contour plots. The scan was selected to transverse those parts of the 3-D data matrix with the least overlap. Interference-free signals of the three components were obtained from the chosen paths, although loss of sensitivity occurred when no maximum peaks were traversed. Recently, the technique was further employed to determine three structurally related alkaloids (berberine, luguine, and sanguinarine). The isometric spectra in Figure 3 show the appropriate Δλ values for the determination of berberine, luguine, and sanguinarine. These alkaloids show a serious overlap of their excitation spectra, precluding the determination of the individual components in the mixtures by normal spectrofluorimetry.[47]

Fluorescein and dichlorofluorescein are included in the Association of Official Analytical Chemists (AOAC) list of synthetic coloring allowed in foods and drugs. These coloring compounds cannot be determined simultaneously by conventional fluorimetric techniques because of their highly overlapped excitation and emission spectra. Recently, Oms et al.[48] described a method for the analysis of mixtures of these compounds. The method is based on the use of variable-angle fluorescence spectra with further treatment of the data by multilinear regression analysis. The optimal variable-angle scan route was designed from the corresponding contour plots. For maximum sensitivity, the path was traced through the maximum peaks of the individual spectra, and two different angles of 64.5° and 116° were employed for the complete resolution of the mixture.

## C. Constant-Energy Synchronous Scanning Fluorescence

Constant-energy synchronous fluorimetry (CESF) is a modified approach of luminescence techniques with improved selectivity over conventional synchronous fluorimetry.[49] In this technique, a constant energy difference ($\Delta\nu$) is maintained between the excitation and emission wavelengths. This is achieved by stepping the emission monochromator at a constant speed, while at the same time varying the excitation monochromator. For example, in the analysis of PAHs, the constant energy difference is chosen to equal the vibrational energy separation obtained from the PAHs fluorescence spectra.[50] Furthermore, the energy difference is characteristic of a group of compounds and is independent of the spectral region. Most compounds containing aromatic rings (e.g. PAHs) have a vibrational spacing of 1400 $cm^{-1}$ and, therefore, they are suitable "candidates" for CESF measurements because of their natural characteristic vibrational mode.[51,52] Files et al.[53] applied CESF to the analysis of PAHs contained in gasoline and crude oil samples. The analysis was performed at both ambient temperatures and 77 K by using filter papers and quartz tubes, respectively. This has allowed for the identification of certain PAHs in gasoline and crude oil samples. The use of rapid scanning CESF has also been applied to evaluate various flow cell designs and constant energy interval choice.[54] The ability to simultaneously scan the excitation and emission spectra while keeping a constant-energy difference between the monochromator wavelengths, should allow CESF to be used as a sensitive detector for HPLC.

Total luminescence measurements using CESF offer an improved spectral selectivity for the trace analysis of analytes and multicomponent samples. Inman and Winefordner[49] have demonstrated the utility of multidimensional CESF for the analysis of tripelennamine hydrochloride. Figure 4 part a shows the contour plot of the total fluorescence of tripelennamine hydrochloride in water. It is interesting to note the effect of Raman scatter in Figure 4 part b. In Figure 4 part c the Raman scatter is markedly reduced when the CESF method is employed. Analytically, this dramatic suppression of solvent Raman scatter holds promise for the analysis of weakly fluorescent compounds.[49] The authors also suggested that measurements based on CESF should provide increase precision and reduce the detection limits.

Multidimensional CESF has also been applied to the analysis of PAHs mixtures.[55] A contour plot representing a mixture of anthracene, naphthalene, and perylene is shown in Figure 5. The selected $\Delta\nu$ value ($0.2 \times 10^3$ $cm^{-1}$) used for the determination of anthracene was also applicable for the determination of naphthalene and perylene. This method shows improved selectivity for multicomponent analysis. In contrast, when conventional synchronous fluorimetry was used, no single wavelength difference ($\Delta\lambda$) was found to be appropriate for the determination of a mixture of anthracene, naphthalene, and perylene. Clearly, the results suggest that multidimensional CESF gives better selectivity for multicomponent analysis over constant wavelength synchronous fluorimetry. In addition, the use of low-temperature CESF spectrometry improved

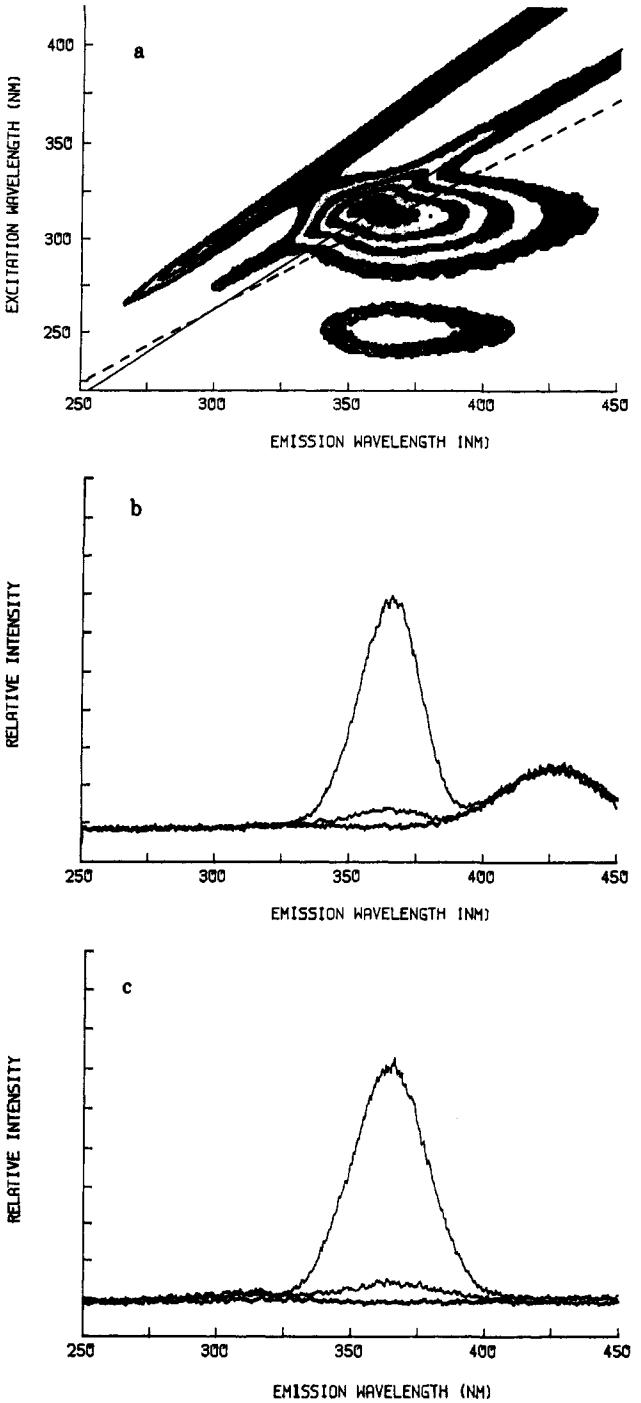


**Figure 4.** (a) Contour plot of the total fluorescence for a 1.0 $\mu$g mL$^{-1}$ solution of tripelennamine hydrochloride in water. The solid line through the spectrum is the conventional synchronous scan path for $\Delta\lambda$ = 54 nm. The broken line through the spectrum represents the CESF scan path for $\Delta\nu$ = 4730 cm$^{-1}$. (b) Conventional synchronous fluorescence spectra for 0, 0.1, and 1.0 $\mu$g mL$^{-1}$ tripelennamine hydrochloride in water; $\Delta\lambda$ = 54 nm. (c) CESF spectra for the same solutions as in (b); $\Delta\nu$ = 4730 cm$^{-1}$ (from ref 49, copyright 1982 Elsevier Science Publishers).

the spectral resolution of PAH mixtures,[51] by the reduction of their spectral bandwidths.

## D. Phase-Resolved Fluorescence

Mousa and Winefordner[56] have described the application of PRFS for the analysis of binary mixtures. Several phosphorescence binary mixtures with overlapping spectra were quantitatively analyzed in their study. Bright and McGown[57] have demonstrated the

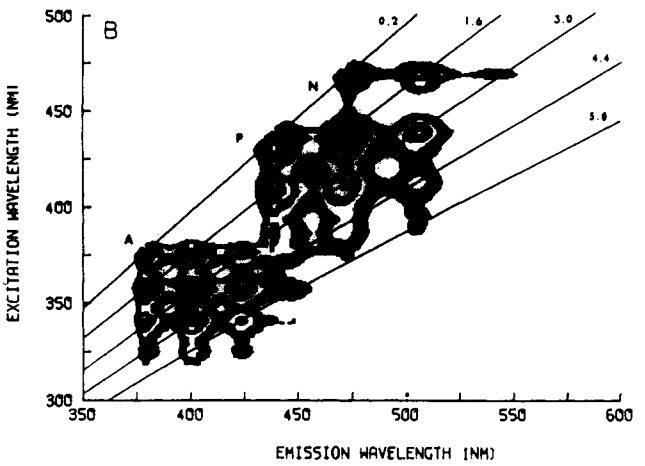


**Figure 5.** Total fluorescence spectrum of anthracene, perylene, and naphthacene in *n*-heptane (A = anthracene, P = perylene, and N = naphthalene). The numbers correspond to $\Delta\nu$ values selected to obtain the CESF scan path drawn through the spectrum (reprinted from ref 55, copyright 1982 American Chemical Society).

utility of PRFS for the simultaneous determination of a three-component system of anthracene, 1,4-bis(5-phenyloxazol-2-yl)benzene and 1,4-bis(methyl-5-phenyloxazol-2-yl)benzene. The combination of selectivity-based fluorescence lifetimes with wavelength selectivity showed an improved accuracy for the simultaneous quantification of spectrally overlapping species in multicomponent mixtures. The only drawback of this approach is that three independent measurements for the determination of three unknowns is necessary.

Scattered-light signals pose a significant problem in fluorescence spectroscopy. The application of PRFS provides a means for the direct suppression of the scattered-light signal. In the factor analysis of EEM, the scattered light may appear as several components, adding to the complexity of the data analysis and interpretation. Consequently, the spectral fingerprinting of these samples may be hampered by the loss of information near the scattered-light peaks. Nithipatikom and McGown[58] have shown an improved detection limit for synchronous excitation determinations of some PAHs. This was achieved via a substantial reduction of the blank signal by elimination of the scattered contribution by using PRFS. The PRFS method proved to be superior in these cases because it completely eliminated the scatter signal, regardless of its magnitude, imprecisions, and sources. The technique was also used to resolve mixtures of fluorescein physically bound to albumin and fluorescein isocyanate covalently bound to albumin.[59] The results show that in both binding and fluoroimmunoassay PRFS can be used for quantitative fluorimetric analysis without requiring any previous separation procedures. It is interesting to note that even very small differences in fluorescence lifetimes of species with virtually identical spectral characteristics can be successfully exploited by using the simultaneous equation approach. The utility of PRFS was extended to suppress the light contributions in total luminescence spectra of crude oil and human serum, even in the case of highly scattering samples. This direct suppression of the scattered light should be useful for analysis of multicomponent samples and for sample characterization by spectral fingerprinting.

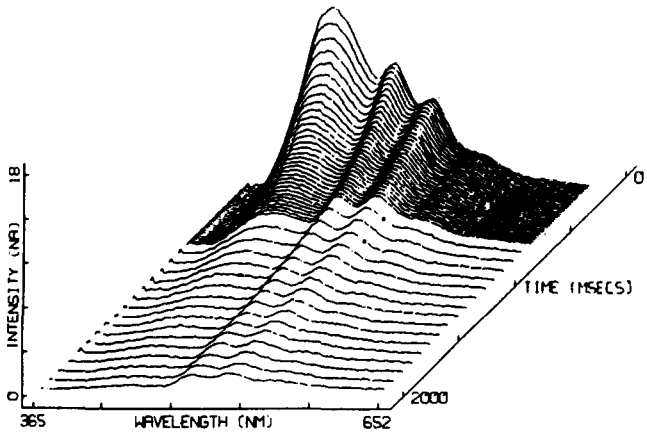


**Figure 6.** Typical time-resolved emission–time matrix (ETM) of 2-naphthoic acid and the sodium salt of *p*-aminobenzoic acid (reprinted from ref 63, copyright 1979 American Chemical Society).

## *III. Multidimensional Fluorescence Lifetime Measurements*

### A. Time-Resolved Fluorescence

The utility of time-resolved fluorescence spectroscopy for studying the structure and dynamics of macromolecules, particularly in studies of time-dependent processes, is well established.[60] Fluorescence lifetimes can be important in spectral resolution, depending upon the degree of spectral overlap between the components and their fluorescence lifetime differences. The use of laser excitation has enhanced the use of multidimensional lifetime measurements for rapid data acquisition in biochemical applications.[61] In analytical procedures, a multiple linear regression program is used to project the intensity values at selected wavelengths back to zero time. The analyte concentration is computed from ratios of initial intensity for the analyte to the initial intensity for an internal standard. Fluorescence decay information acquired at various emission wavelengths is used to resolve mixtures of fluorophores, without a priori information about the number or identities of the components.[62] A typical isometric projection representing the fluorescence decay of a mixture of 2-naphthoic acid and the sodium salt of *p*-aminobenzoic acid is shown in Figure 6.[63]

Laws and Brand[64] applied multidimensional spectroscopy for the study of excited-state proton transfer of 2-naphthol as a function of time. The nanosecond time-resolved emission spectrum was not distorted by the convolution artifact. The problem of the multiple fluorescence of 1,1′-binaphthyl in rigid solvents was resolved by using multidimensional time-resolved spectroscopy.[65] The study revealed that more than two fluorescent components are present; this supports the results obtained by using semiempirical quantum mechanical calculations. Time-resolved fluorescence detection can also be used to discriminate against Raman scattering and short-lived fluorescence from an eluting solvent in HPLC.[66]

### B. Phase-Resolved Fluorescence Lifetime

The PRFS method provides a simple and convenient means for the incorporation of fluorescence lifetime into fluorimetric analysis. Millican and McGown[67] have combined the experimental parameters of modulation frequency and the detector phase angle to provide fluorescence lifetime selectivity in multifrequency PRFS. In this method, different types of fluorescence lifetime filters corresponding to bandpass, longpass, and shortpass effects are presumably achieved. In contrast, pulsed-excitation, time-resolved experiments are restricted to longpass lifetime filters. Consequently, PRFS can be utilized to selectively enhance or reduce the fluorescence contributions of sample components as a function of their fluorescence lifetime. Therefore, PRFS has the ability to selectively enhance both shorter-lived and longer-lived fluorescence contributions in mixtures.

It has been shown that the PRFS approach is superior to steady-state measurements for the extraction of the component spectra for benzo[*b*]fluoranthene and benzo[*k*]fluoranthene mixtures in which the intensity contributions from the two components are unequal.[68] The relative contribution of benzo[*b*]fluoranthene is enhanced at 6 MHz, and that of benzo[*k*]fluoranthene is enhanced at 30 MHz, providing higher selectivity for each component than could be obtained with the steady-state EEM. In addition, pulsed-excitation and the time-resolved techniques are limited to the relative enhancement of longer-lived components. This limitation will make it impossible for pulsed-excitation time-resolved method to enhance benzo[*k*]fluoranthene relative to benzo[*b*]fluoranthene in the time domain. Figure 7 shows contour plots of the phase-resolved total luminescence spectra of benzo[*f*]fluoranthene and benzo[*b*]fluoranthene collected under different modulation frequencies[69,70] and are resolved at 30, 18, and 6 MHz, respectively. These studies demonstrate the ability of multifrequency PRFS to selectively enhance fluorescence as a function of fluorescence lifetime.

## *IV. General Multidimensional Luminescence Studies*

### A. Fluorescence Measurement

Multidimensional luminescence spectra have found widespread use in analytical chemistry. Nelson et al.[71] described the effect of quenchers on component resolution of PAHs complexed with β-cyclodextrin. The addition of tertiary butanol to the β-cyclodextrin containing PAHs solution reduced the quenching dramatically. The utility of the cyclodextrin complexation scheme in the resolution of a mixture of pyrene and fluoranthene is demonstrated in Figure 8. In this figure, the EEM of a mixture of pyrene and fluoranthene in the presence of 1% tertiary butanol is shown. Wiechelman and Brunel[72] demonstrated the utility of multidimensional fluorescence for the analysis of bovine serum albumin labeled with fluorescent probe *N*-(1-pyrenyl)maleimide. The use of an isometric projection allowed for the observation of all the data points in each of the scans simultaneously, giving a more complete pictorial representation of the experimental data. Multidimensional fluorescence has also been used to examine the corrected fluorescence spectra of sulfanilic acid and its derivatives.[73] The changes in excitation peaks of the acid, showing two excitation peaks, affected the sensitivity of the method. However, the projection

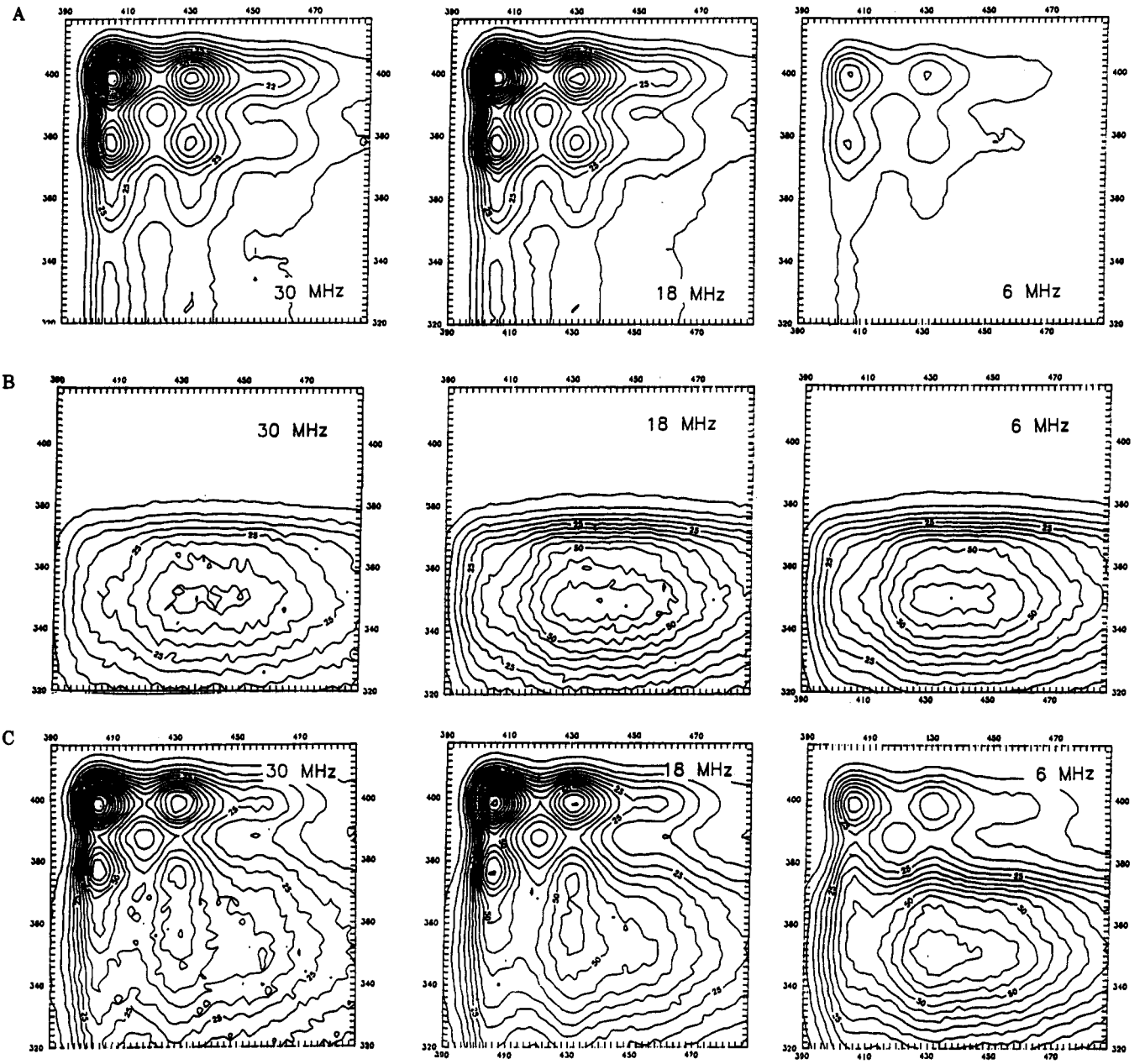


**Figure 7.** (A) Contour plots of the phase-resolved TLS of benzo[*k*]fluoranthene (BkF) at 30, 18, and 6 MHz modulation frequencies. (B) Contour plots of the phase-resolved TLS of benzo[*b*]fluoranthene (BbF) at 30, 18, and 6 MHz modulation frequencies. (C) Contour plots of the phase-resolved total luminescence spectra of a mixture of BkF and BbF at 30, 18, and 6 MHz modulation frequencies. The plots show the selectivity derived from the use of multiple modulation frequencies (adapted from ref 69, copyright 1988 Pergamon Press PLC).

display of the fluorescence spectra enabled the analysts to see these changes and correct for them. Siegel[11] has used the EEM for fingerprinting, i.e., for comparative analysis, determining and analyzing particular physical and chemical properties of compounds that have a common source. The technique was effectively applied to characterize gasoline and oils. Figure 9 shows typical EEMs of Amoco regular, lead-free, and premium lead-free gasoline[74-76] and their similarities. The method showed a definite potential as a test that allows the analyst to determine with reasonable certainty, if two species of sample have the same source. The method can be useful for both environmental and forensic applications.

Multidimensional fluorescence is well suited for the spectral fingerprinting of different classifications of algae[16,77] and in the characterization of marine algae. Such a characterization was accomplished by using EEMs as a spectral fingerprinting for marine phytoplankton population.[78] The distribution of pigments in the algae provided for sharp distinction between greens, blue-greens, and those that contain a carotenoid complex. It was also possible to differentiate between certain species within a class by visually examining their EEMs. Natural phytoplankton populations were qualitatively characterized by their in vivo fluorescence fingerprinting by using a portable multichannel fluorescence spectrometer (PMFS).[17] The versatility and potential of the PMFS is seen (1) by rapidly acquiring emission wavelength information as a function of multiple excitation wavelengths, (2) the ability to perform fluorescence measurements remote from the laboratory location, and (3) surface position. Thus, the PMFS is a potentially powerful instrument for multi-

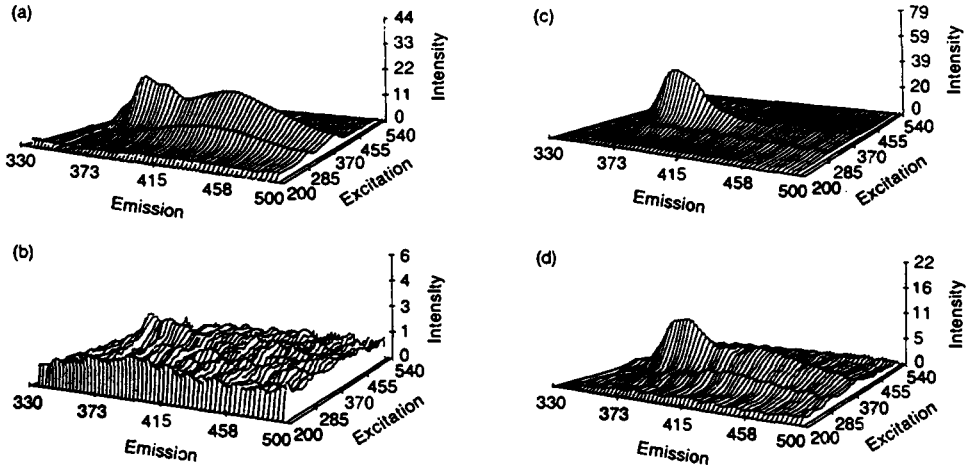


**Figure 8.** Isometric plots of fluorescence showing the EEMs of (a) the pyrene–fluoranthene mixture in 1% *tert*-butyl alcohol, (b) the pyrene–fluoranthene mixture in the presence of 1% *tert*-butyl alcohol and 100 mM iodide, (c) the pyrene–fluoranthene mixture in the presence of 1% *tert*-butyl alcohol, 100 mM iodide, and 1.5 mM β-CD, and (d) the pyrene–fluoranthene mixture in the presence of 1% *tert*-butyl alcohol, 100 mM iodide, and 3.8 mM γ-CD (reprinted from ref 71, copyright 1986 Spectroscopy Magazine/Aster Publishing Corp.).

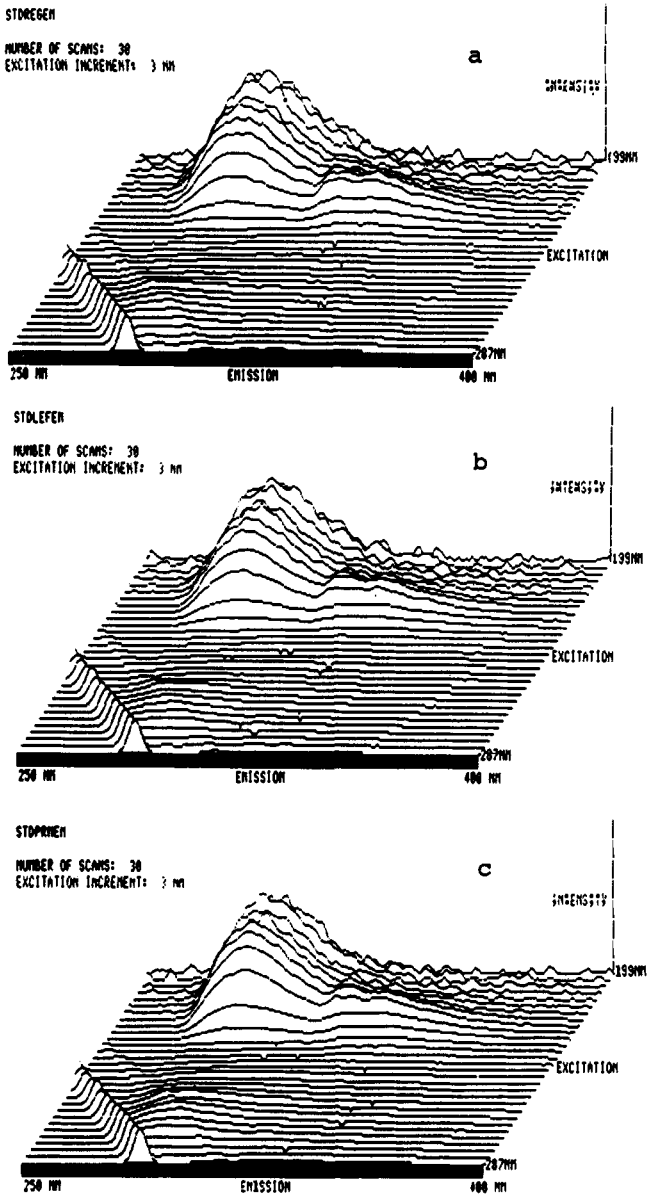


**Figure 9.** Three-dimensional spectra of Amoco gasoline: (a) regular, (b) lead-free, and (c) premium lead-free (reprinted from ref, copyright ASTM).

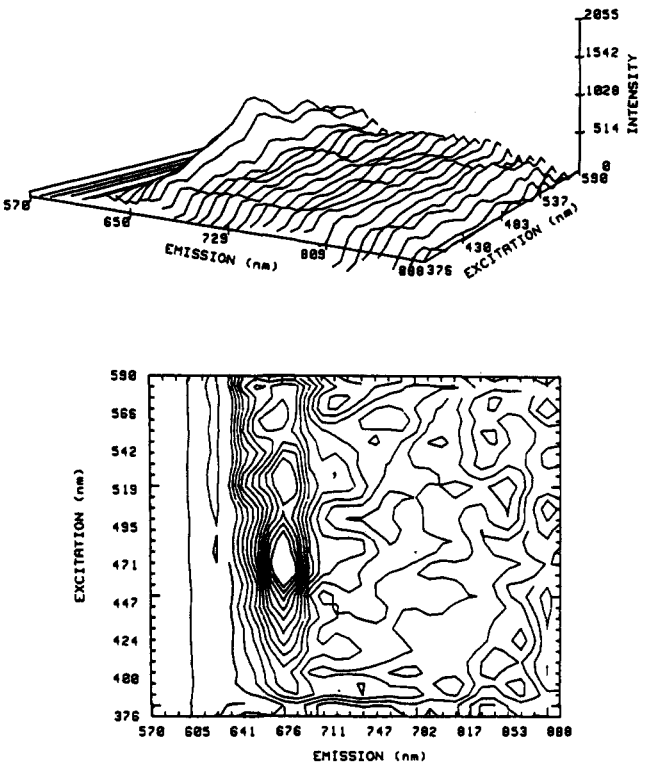


**Figure 10.** EEM of algae from the Gulf of Mexico (reprinted from ref 17, copyright 1987 Marcel Dekker Inc.).

component studies. An example of the utilization of PMFS for the analysis of seawater for algae is shown in Figure 10.

## B. Fiber Optic Based Fluorescence Measurement

Bright et al.[79] have described the use of a rapid frequency-scanned fluorimeter for remote sensing using a fiber optic probe. The instrument is capable of determining nanosecond and subnanosecond fluorescence lifetimes. Bright[80] reported the use of fiber optic based fluorescence lifetime measurements to resolve multiexponential decays of fluorescence in remotely located samples. In addition, the work also included the use of phase-resolved fluorimetric measurements using

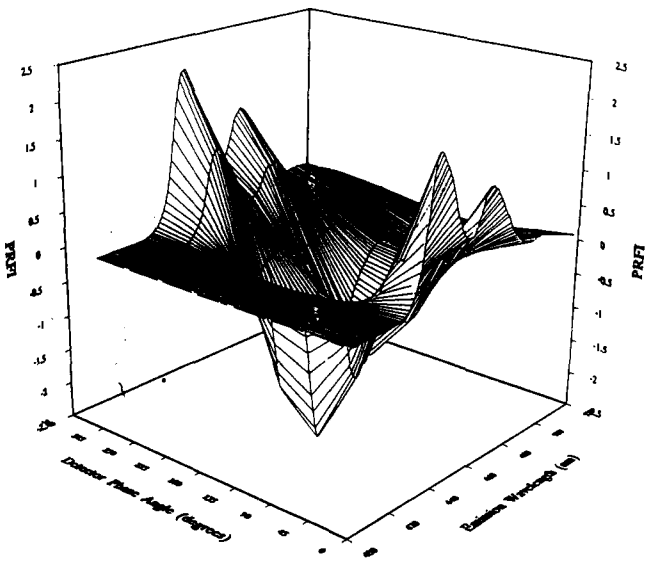

**Figure 11.** Three-dimensional plot of phase-resolved fluorescence vs detector phase angle vs emission wavelength for perylene (perylene at 30 MHz) (reprinted from ref 82, copyright 1990 Chemical Rubber Co.).

multidimensional analysis.[81] The technique was successfully applied to the elucidation of the complex decay kinetics of rhodamine 6G-impregnated Nafion films. The system was also used for the simultaneous resolution of individual spectral components in binary mixtures. In this study, the isometric plot of phase resolved fluorescence was shown as a function of emission wavelength and the detector phase angle. Figure 11 is an illustration of multidimensional data of phase-resolved fluorescence intensity as a function of the detector angle and the emission wavelength for perylene. The perylene sample was detected by using a 175-m optical fiber probe.[82]

Zung et al.[18] have demonstrated the utility of multidimensional fluorescence measurements using fiber optic sensing for the analysis of marine phytoplankton. The incorporation of optical fibers into analytical instrumentation removes the need for sometimes awkward or time-consuming sampling processes by allowing direct on-line measurements. In this approach, the fiber can be directly placed in the sampling region for measurement. The advantages of using optical fibers for sampling include geometrical flexibility, environmental versatility, small size, and in situ monitoring capability. The presence of different and characteristic fluorescent pigments such as chlorophyll and phycobilin is remarkably important for the classification of algae. Figure 12 provides typical contour plots for green and blue-green algae.

## C. Phosphorescence Measurement

Phosphorescence analysis of multicomponent samples can be markedly enhanced by the rapid acquisition of multiparametric data. The phosphorescence excitation–emission matrix (PEEM) can be depicted in the same format as the fluorescence EEM. The PEEM information should provide greater specificity and selectivity, and the time of decay for different compounds can be easily exploited. The time resolution of the PEEM of a multicomponent mixture is particularly useful for the deconvolution of the mixture into its

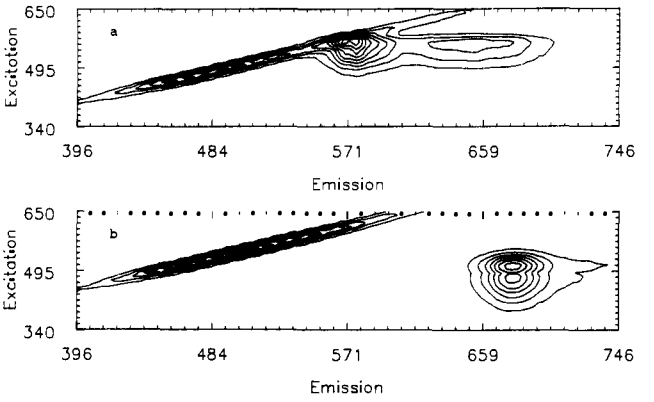


**Figure 12.** Contour plots representing (a) blue-green and (b) green algae.

constituents.[83] A ratio deconvolution may be applied to obtain a series of PEEMs for the mixture of components in which the concentrations are altered in each PEEM. The PEEM with a mixture of components of different lifetimes will provide the characteristics necessary for ratio deconvolution. The PEEM of a mixture of coronene and pentacene were obtained after two different delay times.

Ho and Warner[84] have demonstrated the applicability and usefulness of multidimensional phosphorimetry. Rapid acquisition of PEEMs is achieved by use of a time resolved scheme. The PEEM of phenanthrene and triphenylene is rather severely overlapped while coronene is quite isolated with respect to its major emission bands. Despite the strong overlap, the results of ratio deconvolution of the binary mixture of triphenylene and phenanthrene were quite successful. The PEEM combined with ratio deconvolution is able to accomplish satisfactory qualitative separation of mixtures (ternary mixture of coronene, phenanthrene and triphenylene) with components which have strong spectral overlap. In addition, the time-resolution approach is good in that short-lived phosphors do not cause significant convolution problems in the acquisition of PEEM. The method was successfully applied to the analysis of the extract of a burned oil residue.

## V. Fluorescence-Detected Circular Dichroism

Fluorescence-detected circular dichroism (FDCD) is the process whereby the fluorescence is measured from a sample when it is alternately excited with left circularly polarized light and right circularly polarized light. The fluorescence emitted is detected for these two states of polarization, and at low absorbance the difference is proportional to the circular dichroism (CD) signal. By using this method, new and fundamental information concerning solution conformation and the dynamics of base stacking can be obtained.[85] The FDCD method increases the selectivity and sensitivity of conventional transmission CD measurement for chiral fluorophores. Turner et al.[86] have reported the FDCD spectra of proteins. Their results indicated that FDCD is a sensitive and selective method for monitoring the local conformation around tryptophan. This work was extended for the investigation of the equilibrium binding of ethidium to poly(dG-Dc) and poly(Dg-m$^5$dC) by using FDCD and optical titration methods.[87] The increased sensitivity of FDCD allowed

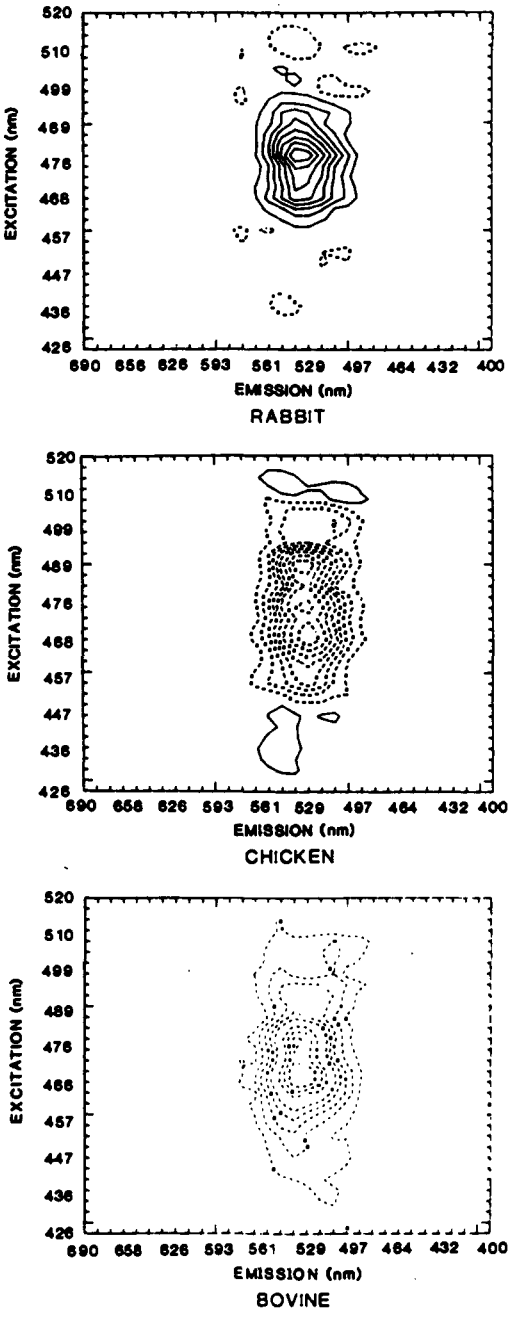


**Figure 13.** Multidimensional FDCD spectra for the complexes of bilirubin with rabbit, chicken, and bovine serum albumins (from ref 89, copyright 1987 Academic Press).

measurements below the threshold concentration for ethidium binding.

Multidimensional FDCD[88] provides additional advantages over transmission CD and conventional FDCD due to increased selectivity. Multidimensional FDCD allows the separation of components by spectral, as opposed to chemical and physical methods. Multidimensional FDCD has been used to investigate the binding of bilirubin to human serum albumin (HSA).[89] The method has also been applied in the spectral evaluation of the complexes formed when bilirubin binds to bovine (BSA), chicken (CSA), and rabbit (RSA) serum albumins (Figure 13). The FDCD matrix shows high sensitivity to conformational changes in the albumin molecule, brought about by changes in the solution pH. The utility of multidimensional FDCD measurement for biochemical analysis is demonstrated by the binding of warfarin to HSA.[90] The added selectivity of multidimensional FDCD, the relatively small analyte concentrations required for analysis, and the decrease in detection limit provided by fluorescence measurement will allow fruitful investigations of a wide range of chiral compounds.

Warner et al.[91] have exploited Stern Volmer fluorescence quenching in combination with FDCD measurement to investigate the properties of the site I and site II binding areas of human serum albumin (HSA). This demonstrated the utility of the multidimensional fluorescence technique for the investigation of binding sites of proteins. The combination of FDCD with Stern Volmer quenching to produce fluorescence-detected circular dichroism quenching (FDCDQ) is exceedingly valuable in that the FDCD signal originates only from the asymmetrically bound fluorophore. It is interesting to note that even though uncomplexed fluorophores exist in solution, the FDCD method may be used to investigate the quenching of only those fluorophores that are asymmetrically bound to HSA. In addition, probes that are bound to HSA but do not produce an asymmetric complex do not give rise to an FDCD signal. This in turn makes the multidimensional FDCD technique more selective. Therefore, multidimensional fluorescence measurement, not only detects differences among site I and site II binding areas, but also detects differences among the two site II binding areas themselves. The information obtained in this technique is valuable in the formulation of a binding model to explain the interactions at site I and site II on HSA. The FDCD quenching is a very selective chemical probe of the microenvironment of a particular chiral fluorophore or bound ligand in a complex macromolecule.

## *VI. Multidimensional Detection for Chromatography*

### A. Fluorescence Detection

The selectivity and specificity of fluorescence analysis can be especially beneficial for the identification of PAHs. For more complicated systems in which the spectra overlap, lifetime measurements may be used to identify the components. The capabilities of obtaining an EEM permit the use of data analysis techniques to resolve overlapped spectra. In chromatography, selective detection is especially important when coeluting analytes are to be identified. Fluorescence measurements can be used as a sensitive detector for HPLC separations. Gluckman et al.[92] have used a self-scanned, image-intensified photodiode array (PDA) detector, as a fluorescence detector for liquid chromatography. The detector yields high detection efficiency.

Multidimensional chromatography was successfully employed to provide a unique fingerprint for the selective identification and characterization of certain species of *Pseudomonas*.[93] Pigments of *Pseudomonas* species were detected and profiled in less than 24 h, at relatively low cell density. Blyshak et al.[94] have demonstrated the utility of EEMs to resolve overlapped spectra of several PAHs. Wegrzyn et al.[95] have also developed an unintensified PDA based multichannel fluorescence detector for use with HPLC. Figure 14 presents an isometric representation of the chromato-

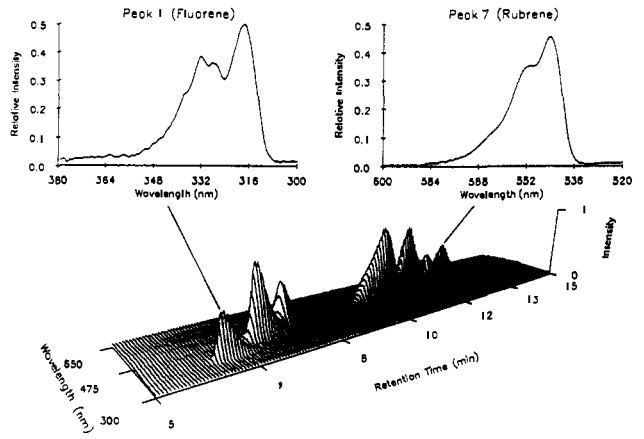


**Figure 14.** Chromatogram of a seven-component mixture of polycyclic aromatic hydrocarbons (reprinted from ref 95, copyright 1990 American Chemical Society).

gram consisting of a seven-component sample of PAHs. The linear dynamic range obtained covered 3 orders of magnitude and the detection limit for several PAHs is in the nanogram range.

## B. UV/Vis Detection

A detailed account of the progress in the applications of multidimensional analysis in analytical chemistry using PDA detection is given elsewhere.[96-99] This section discusses some pertinent developments and applications in the field of multidimensional techniques using PDA UV/vis detection.

The broad-band absorption from a multicomponent sample results in limited specificity when single-channel detection is employed.[100] The identification of unknowns in a multicomponent sample can be performed concurrently with quantitative analysis of known species in the same chromatogram by using multichannel detection. In fact the collection of UV/vis spectral information from a single chromatogram during the elution process is markedly enhanced when the PDA detector is used. The extra dimension provided by the PDA detector permits the recording of an isometric projection in which the axes are absorbance, wavelength, and time ($A$, $\lambda$, $t$). Multiwavelength detection from PDA provide significant advantages (e.g., improved identification, ability to check the purity of chromatographic peaks, rapid selection of an optimal wavelength, and economy of time, solvents, and samples) over conventional single-wavelength spectrometers. The simultaneous detection capabilities of the PDA detector may result in an improved signal-to-noise ratio or a reduction in the observation time required for the measurement.

Overzet et al.[102] have reported a dramatic improvement of metabolic screening in excretion liquids. Multidimensional chromatography showed enhanced quantitative information with regard to butoprozine (an antianginal drug) related structures. The technique allows for a better determination of peak overlap and background interferences. Clearly, this will be very useful in the isolation and structure elucidation of potential metabolites and for those metabolic studies in which radio-labeled drug administration are limited. Monitoring and identifying relevant chromatographic peaks of vitamins in a variety of sample matrices can be well achieved using PDA UV/vis detection.[103] The vitamins are successfully identified in the presence of coextracted compounds.[104] This technique proved to be sensitive and selective for both quantitative and qualitative analysis of the fat soluble vitamins. Jinno et al.[105] have combined multidimensional chromatography with the retention prediction system for the analysis of PAHs. The system is precise and convenient for environmental analysis. The contour plot permits the identification of important chromatographic peaks. The separation of the PAHs is an important and urgent analytical problem because of the environmental hazards of this class of compounds.[106] The isometric plot and contour plots of PAHs and pharmaceutical preparations aid in the selection of optimal conditions for single-wavelength detection.[100]

Multidimensional chromatography (HPLC) has also been applied to the analysis of amino acids and peptides in food science, in agricultural chemistry, and in the pharmaceutical and biochemical sciences.[107] The method shows remarkable speed, selectivity, and sensitivity in the analysis of amino acids. The high-resolution contours confirmed the symmetry of each peak and indicates the chromatographic purity of each dipeptide examined (Figure 15). The technique is also applicable for the examination of the presence of papaverine and related alkaloids from a forensic sample of heroin.[108]

The purity of glutaraldehyde—a fixative agent used to preserve the fine structure of cells and enzyme activity—was determined by using a multichannel PDA UV detector.[109] The presence of dialdehyde, an undesirable impurity, is well characterized compared to the use of a ratio method which precludes the analysis of the individual components in the mixture. A deficiency of vitamin K results in hemorrhagic diseases of the newborn. Vitamin K is also administered to adults with fat malabsorption. Off-line multidimensional chromatography was applied for the analytical recovery of *trans*-vitamin $K_1$ from small serum sample volumes obtained from newborns.[110] Vitamins and other compounds which produce physiological effects in humans/animals are widespread. Recently, Kirk and Fell[111] demonstrated the utility of multidimensional chromatography for the quantification of $K_1$ vitamins in serum. This solved the problem of coextracted UV-absorbing contaminants (lipids). By using an isometric spectrochromatogram, the optimum wavelength of detection for vitamin $K_1$ was found to be 248 nm. The method was simple and samples could be analyzed by parallel extraction in batches of six on the Bond-Elut vacuum manifold. Less sample is required, analysis time is decreased, and sensitivity is dramatically enhanced with this technique.

Thin-layer chromatography (TLC) offers comparable simplicity and convenience. The TLC 2-D resolving power is useful in the study of complex biological and environmental samples.[112] Gianelli et al.[113] have described the use of a multichannel detector of fluorescent compounds for TLC. The system was used to analyze the separation of porphyrin mixtures. Quantification was feasible down to the picogram region. This was further extended to analyze selected components of a complex mixture.[114] The 2-D chromatograms demonstrate the advantage of quantitative analysis, even when the spots on the TLC plate are severely overlapping.

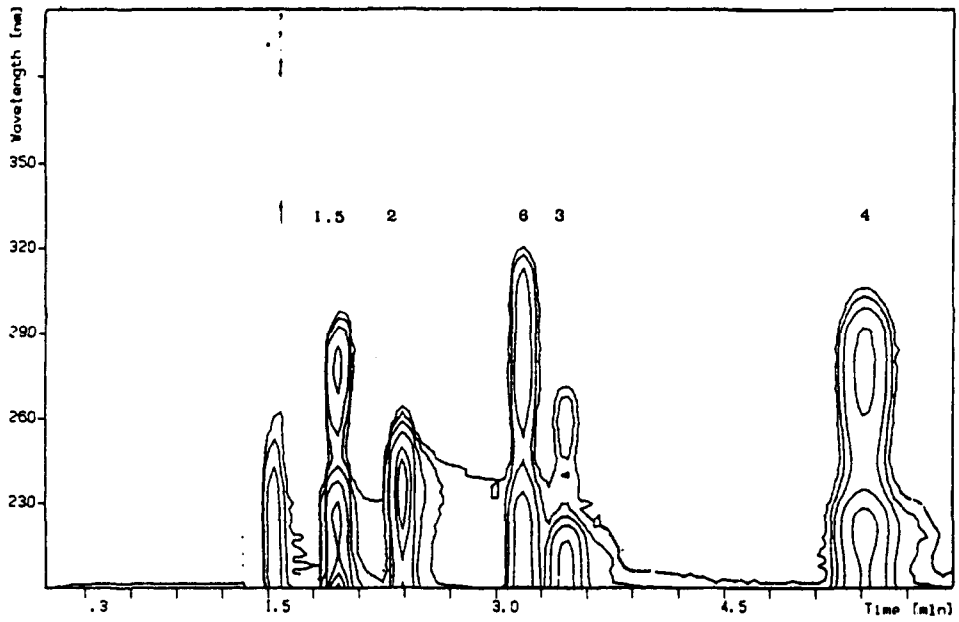


**Figure 15.** Contour map of aromatic amino acids and metabolites. Contour intervals are as follows: 1, 2, 5, 20, 100, 200, 400, 600 mAU. Peaks are as follows: 1, tyrosine; 2, internal standard; 3, phenylalanine; 4, tryptophan; 5, dopamine; 6, 5-hydroxytryptamine (from ref 107, copyright 1984 Elsevier Publishers).

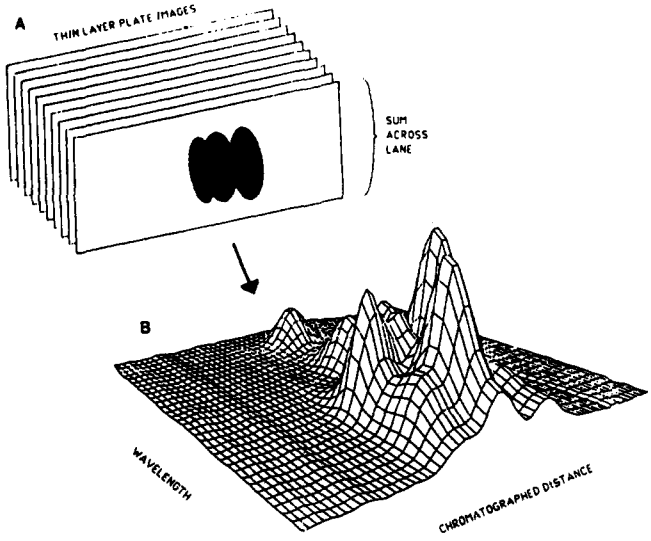


**Figure 16.** (A) Spectral images of a three component mixture which has been chromatographed in one-dimension and (B) the two-dimensional matrix obtained by summing across the chromatographic lane (from ref 116, copyright 1986 Elsevier Publishers).

The speed of acquiring absorption data for each spot enhances the versatility of this multidimensional TLC. Burns et al.[115] have demonstrated the utility of hyphenated chromatographic/spectroscopic instruments in performing quantitative least-squares analysis of the components in a complex mixture. The method is feasible even in cases where the retention time and peak vary from run to run. Clearly, in routine separation of complex mixtures multidimensional analysis is useful in resolving highly overlapping components.[116] A typical multidimensional TLC spectrum of a mixture of compounds appears in Figure 16.

## VII. Conclusion

This review primarily outlines the advantages of multidimensional absorption and luminescence spectroscopies. It is shown that these approaches provide the ability to selectively enhance spectral contributions from sample constituents/matrices and as a function of their lifetimes. The above cited studies clearly demonstrate the utility of multidimensional spectroscopy in analytical chemistry.

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