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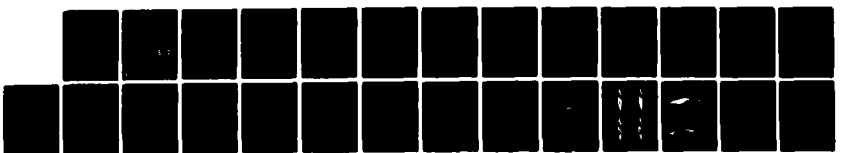

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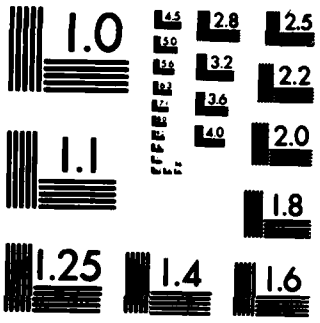

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Due to its sensitivity and selectivity, luminescence detection is often the choice in a wide variety of analytical applications. However, until recently, only one parameter of luminescence was being monitored in a single experiment. For example, emission spectrum scanned at a fixed single excitation wavelength, and decay of luminescence observed at a single wavelength of excitation and emission. In this paper we will present some of the recent developments to provide multiparametric detection of luminescence phenomena. For example, a com-
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plete intensity map of phosphorescence, simultaneously, at multiple wavelengths of excitation and emission over time. Another area involves the use of polarized radiation in effecting luminescence detection. We will discuss the instrumentation and data analysis for these kinds of experiments.

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TECHNICAL REPORT NO. 2

Multiparametric Detection in Modern Luminescence Spectrometry

by

Chu-Ngi Ho, Mae E. Rollie

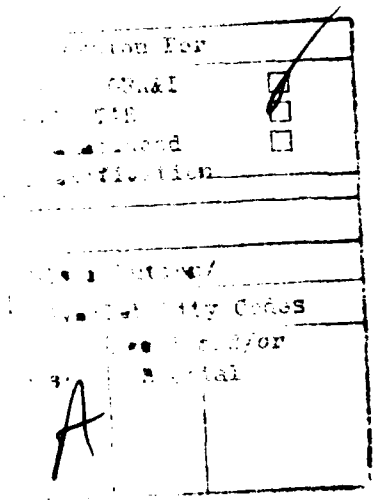
and Isiah M. Warner

Prepared for Publication

in

Optical Engineering

Department of Chemistry
Emory University
Atlanta, Georgia 30322



June 1983

Luminescence spectroscopy has been increasingly recognized as an important tool for chemical analysis and as a probe into fundamental properties of chemical systems. Its sensitivity and selectivity are the most commonly cited characteristics responsible for its widespread applications. However, for most compounds in room temperature solutions, the spectra are usually broad and extensive overlap may occur so as to greatly diminish the specificity of the analysis and minimize the sensitivity advantage in multicomponent samples. With adequate apriori knowledge of the samples, one may devise techniques to analyze an analyte in a mixture successfully with, for example, selective excitation (1), synchronous scan (2), phosphorescence combined with fluorescence (3) and other parameters to achieve greater selectivity or ultimately even greater specificity. But such apriori knowledge may be difficult to obtain. Thus, as analytical samples become more complex and the need to avoid cumbersome and time consuming separation increases, it becomes obvious that for luminescence measurements, specificity can generally be attained only if one can measure simultaneously in the same experiment many different properties associated with the luminescence from the analytes. Hence, the desirability of multiparametric measurements is evident.

Recently, technological advances in microprocessors and specialty devices have made measurements possible which at one time were deemed formidable or impractical. This progress stimulates the development of novel instrumentation. Thus we witness a resurgence of activities in areas involving properties which are less widespread and experimentally more sophisticated to measure such as polarization and short lifetimes.

Figure 1 shows some of the various parameters that can be monitored from a luminescence sample. It is clear that the luminescence technique is inherently selective as one can surely, from this host of properties, find one that is quite selective for an analyte. In this paper, we will discuss how we can exploit several of these parameters simultaneously in an experiment to achieve greater selectivity with a combination of unique instrumentation and data reduction algorithms.

The Emission-Excitation Matrix (EEM)

The selectivity of luminescence analysis can be greatly enhanced if one can simultaneously examine a series of excitation and emission spectra of a sample. We call such an array of luminescence intensity values an emission-excitation matrix (EEM). This array is a function simultaneously of multiple wavelengths of excitation and multiple wavelengths of emission. Figure 2 is an example of a contour plot of an EEM. This EEM represents a mixture of perylene and tetracene. It is instructive to note the following characteristics of an EEM: (1) every row of the EEM represents an emission spectrum of the sample excited at a given wavelength set at the row; (2) a column represents the excitation spectrum monitored at that specific wavelength of emission set at the column; (3) any diagonal is a synchronous spectrum with the fixed wavelength intervals between excitation and emission ($\Delta\lambda$'s) determined by the wavelength settings at the rows and columns of the diagonals. Thus we have simultaneously exploited three spectral parameters using the EEM. Also, familiarity with such an EEM enables one to estimate the number (lower bound) of emitting components in the sample because of the mirror image

rule (4), and the fact that the emission spectrum of a pure component under normal experimental conditions is independent of the wavelength of excitation and vice versa. These properties constrain the kind of image that an emitter may impart to the EEM. One obvious application of this is the ability to quickly determine if a so called one-component solution contains significant emitting impurities. Again, from Figure 2 we can clearly see that perylene and tetracene overlap to quite an extent in both the emission and excitation spectra. However, we will also agree that there are regions in the EEM which are uniquely, or specifically, perylene or tetracene. This characteristic of an EEM is a very useful method for finger-printing applications. This has in fact been done for identification of bacterial cells (5). Thus an EEM can, at a glance, provide the analyst with several important and useful qualitative spectral information about a given sample. Based upon this information, intelligent decisions can be made regarding subsequent experimentations.

To generate the EEMs experimentally using the conventional fluorometer with single channel detection is rather time consuming. Acquisition times of 1-hour have been quoted for computerized systems (6). The most rapid and elegant approach for EEM acquisition is via a video-fluorometer (7,8) which incorporates a unique multiple wavelength excitation scheme with a rapid scanning two-dimensional imaging detector. The video fluorometer can acquire EEMs very rapidly with acquisition times of a few tens of milliseconds to a few seconds. It is obvious at the outset that such large data matrices require efficient computational algorithms to perform data reductions and other manipulations such as data display. Hence, developments in computer algorithms for qualitative and

quantitative analyses must parallel instrumentation and application developments for the EEM.

For a pure one-component solution, an element of the EEM can be written as:

$$M_{ij} = \alpha x_i y_j \tag{1}$$

where x_i is the relative number of photons emitted at wavelength λ_i; y_j, the relative number of photons absorbed at wavelength λ_j; and α, a concentration dependent parameter.

We can represent a wavelength sequenced set of $\{x_i\}$ as a column vector $\underset{\sim}{x}$ in the λ_i space corresponding to the emission spectrum, and a similar set of $\{y_j\}$ as another column vector $\underset{\sim}{y}$ in the λ_j space representing the excitation spectrum. Then for a one-component EEM we can write

$$\underset{\sim}{N} = \alpha \underset{\sim}{x}\underset{\sim}{y}^T \tag{2}$$

where T denotes matrix transposition.

For an EEM with r emitting components, with negligible synergistic effects, the resulting EEM is a linear sum of the individual component EEMs:

$$\underset{\sim}{M} = \sum_{n=1}^{r} \underset{\sim}{N}_n \tag{3}$$

$$\underset{\sim}{M} = \sum_{n=1}^{r} \alpha_n \underset{\sim}{x}_n \underset{\sim}{y}_n^T \tag{4}$$

where n enumerates the components.

Based upon this mathematical formalism, several algorithms have been developed for qualitative and quantitative analysis of multicomponent EEMs. In the case where there are not more than 2 emitting components in the sample, one can deconvolute the EEM using eigenanalysis (9) to obtain the excitation and emission spectra of each component. With a ratioing method (10), the deconvolution of more than 2 components can be achieved. For quantitative analysis, when the number of luminescing components is known, the method of least squares (11) provides a rapid and reliable means for quantification of each component. However such qualitative information may not always be obtainable. Thus, the method of rank annihilation (12) was developed to allow the analyst to quantify a particular component of interest known to be present, without having to know the identities of other emitters in the sample. The interested readers are requested to consult the references for details and applications of the algorithms. No attempt will be made to discuss these algorithms here.

Multidimensional Phosphorimetry

The phosphorescence phenomenon has been known for a long time. Lewis and Kasha in 1944 (13) showed that phosphorescence emission is due to a transition from the excited triplet state to the ground state of a molecule. In 1959, Kier et al. (14) published a paper on the analytical applicability of phosphorimetry. Since then, Winefordner's group has been the most active in contributing to the growth of the technique by introducing new methodologies and demonstrating how phosphorimetry can be

useful for diverse applications (15).

However, phosphorimetry has not gained as widespread an acceptance as fluorimetry because of the need for cryogenic conditions and some experimental sophistication. But recent developments in room temperature phosphorescence (RTP) may change this situation. Thus, renewed interest in the RTP technique has spurred active researches into some fundamental properties of RTP in different matrices (16), development of improved instrumentation and expanding the range of applications (17).

Phosphorimetry has several attractive features. First, it is as sensitive as fluorimetry and complements it. Thus, it helps to extend the range of compounds amenable to luminescence analysis. Second, and most important of all, the lifetime of phosphorescence is easily within the reach of conventional electronic circuitry and thus time-resolved phosphorimetry can be implemented rather easily. With time-resolved phosphorimetry, compounds which are spectrally very similar can now be distinguished through differences in their lifetimes. Consequently, a new dimension is added to luminescence analysis. In 1972, Fisher and Winefordner (18) introduced the concept of pulsed-source phosphorimetry which permitted measurement of shorter lifetimes and increased the sensitivity of the technique. With further sophistication in instrumentation, including computerized control of a pulsed laser source and data acquisition, Wilson and Miller (19) achieved time and component resolved phosphorimetry. In this technique, they obtained a complete decay curve at one emission wavelength and then stepping through the wavelength setting of the monochromator to obtain a two-dimensional data matrix whose intensity values are a simultaneous functon of the emission

wavelength and decay time. Goeringer and Pardue (20) obtained the same multidimensional data by using a vidicon array detector to acquire a complete emission spectrum wthout having to step through the wavelength setting of a monochromator. Thus, the time for data acquisition was reduced immensely. They also applied sophisticated data reduction strategies to analyze room temperature phosphorescence multicomponent samples.

Recently, we have achieved an extra dimension for phosphorimetry by use of a video fluorometer (21). We can acquire a set of phosphorescence emisson-excitation matrices (PEEM) along the decay curve. This allows time resolution of an entire PEEM. With this added dimensionality, we have enhanced the capability of luminescece analysis for multicomponent samples by providing greater selectivity.

The video fluorometer acquires a 50 x 50 (emission x excitation) data matrix (PEEM) in about 0.5 seconds. When the excitation of the sample is terminated to record the phosphorescence, the intensity decays exponentially. Even if the vidicon starts scanning as soon as the excitation beam is cut off, by the end of the scan, the intensity of the phosphorescence would be diminished by an amount according to its lifetime. Thus the PEEM would be distorted because the spectral information has been convoluted with the readout process. We have overcome the problem by employing the integration capability of the vidicon. Thus, we avoid the need for extremely rapid scanning and the accompanying complicated circuitry. This technique should be useful for situations where similar instrumental contraints occur.

We can show that even for a multicomponent mixture, the integrated

PEEM preserves the integrity of the PEEM. Hence if we write

$$I_t = I_o e^{-kt} \tag{5}$$

where I_o is the initial intensity at time $t = 0$, I_t the intensity at any time t and k is the first order rate constant, which is inversely related to the lifetime of the specie (i.e., $k = 1/\tau$).

The integrated intensity for the time interval of t_1, t_2 ($t_1 < t_2$) for a component with rate constant k is given by

$$I_{t_1,t_2} = \int_{t_1}^{t_2} I_o e^{-kt} dt$$

$$= \frac{I_o}{k_1} \left(e^{-k_1 t_1} - e^{-k_1 t_2}\right) \tag{6}$$

For an ideal r-component mixture, we can derive

$$I_{t_1,t_2} = \sum_{n=1}^{r} \frac{I_o}{k_n} \left(e^{-k_n t_1} - e^{-k_n t_2}\right) \tag{7}$$

Thus, the integrated PEEM is

$$I\ (\text{PEEM}) = \sum_{n=1}^{r} \alpha_n \frac{I_o}{k_n} \left(e^{-k_n t_1} - e^{-k_n t_2}\right) \underline{x}_n \underline{y}_n^T \tag{8}$$

Consequently, Equation 8 is in a form useful for ratio-deconvolution (10). Using Equation 2, and the same notation, we can define a standard EEM or the nth component

$$N_n = \alpha_{no}\, \underline{x}_n \underline{y}_n^T \tag{9}$$

for the mixture matrix

$$\underset{\sim}{M}_l = \sum_{n=1}^{r} \alpha'_{nl} \underset{\sim}{N}_n \tag{10}$$

where

$$\alpha'_{nl} = \alpha_{nl}/\alpha_{no} \tag{11}$$

For the ratio deconvolution of an r-component mixture, one needs a set of r EEMs, i.e. $\underset{\sim}{M}_l$ for l = 0, 1, 2, ..., r-1 to obtain a set of r matrices, each of which contains a maximum of r emitters. With the set of r equations in r unknowns, one gets

$$\underset{\sim}{M}^* = \underset{\sim}{A}\ \underset{\sim}{N}^* \tag{12}$$

where $\underset{\sim}{M}^*$ and $\underset{\sim}{N}^*$ are a series of mixture ($\underset{\sim}{M}_l$) and standard (N_n) matrices, respectively. The $\underset{\sim}{A}$ matrix is an r x r array of α'_{nl}. If $\underset{\sim}{A}$ is invertible, then we can solve Equation 12 using

$$\underset{\sim}{A}^{-1}\ \underset{\sim}{M}^* = \underset{\sim}{N}^* \tag{13}$$

The practical problem is to generate the set of $\underset{\sim}{M}_l$ experimentally. There are several ways to achieve this (10). For phosphorimetry, according to Equation 7, one can effectively vary the apparent concentration of the components relative to each other in the mixture by varying the integration periods. This can be effected very conveniently as shown by our deconvolution of a synthetic two-component mixture of

coronene and phenanthrene.

Figure 3 shows a series of PEEM taken by the video fluorometer with different time delays after the termination of excitation and where the actual integration begins. From this figure, one notices that phenanthrene decays faster than coronene. This difference allows time resolution. The deconvoluted spectra of the two components are shown in Figure 4.

A similar technique involving the use of quenchers has also been applied to achieve ratio deconvolution of fluorescence data (10). In this case, selectivity is greatly increased by using a mixture of complementary quenchers for a given multicomponent sample. However, care must be taken to insure that inner-filter effect and quenching are clearly differentiated and taken into account (22).

Fluorescence Polarization

Up to this point, we have discussed the inherent selectivity and specificity of the fluorescence technique. Even with this inherent selectivity and specificity, some compounds will still possess very similar excitation and emission spectra such that spectroscopic resolution is not possible. Consequently, many investigators have found it useful to exploit the more selective and specific parameter of polarization.

For our purpose, we will assume that the sample excitation source is a beam of plane polarized light. Consequently, the greatest amount of absorption occurs when the plane of the electric vector of this polarized light corresponds to the direction of the transition moment in absorption. At this point, it is useful to define the "degree of polarization", p, as

$$p = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} \qquad (14)$$

where $I_{||}$ and $I_{\perp}$ are respectively the intensities of the components of the fluorescence parallel and perpendicular to the polarization plane of the excitation beam. For a solution of randomly oriented molecules, p is found to vary from +½ to -1/3 (4). Weber has indicated that maximum polarization is observed when working with a dilute solution of the fluorophore in a highly viscous solvent (23).

Many investigators have recognized the potential analytical utility of fluorescence polarization measurements when other fluorescence parameters have not proved fruitful. It is not possible to delineate all of the examples cited in the literature. However, we have selected examples which should have general analytical utility.

Conventional polarization measurements have usually employed conventional fluorometers with the addition of necessary polarization optics. Weber and Bablousian (24) improved on conventional instrumentation for measurement of polarization by using separate photomultipliers to detect the || and $\perp$ components of fluorescence. More recently, Hann (25) has developed a sensitive technique for polarization measurement using a continuous rotating polarizer and lock in amplification techniques.

Maple and Wehry (26) have recently discussed the use of photoselection techniques for distinguishing between the overlapping spectral bands of fluorophores in multicomponent samples. Bozhevol'nov et al. (27) have examined the utility of fluorescence polarization spectra

for analyzing binary mixtures of organic molecules that have practically coincident spectra. These investigators were successful in using polarization spectra for the quantitative analysis of binary mixtures of organic compounds which have significant overlapping luminescence spectra. Seitz and co-workers (28) extended this approach to the selective measurement of a component A in the presence of another component B using the equation

$$I_{\parallel T} - \frac{I_{\parallel B}}{I_{\perp B}} I_{\perp T} = \left(1 - \frac{I_{\perp A}}{I_{\parallel A}} \cdot \frac{I_{\parallel B}}{I_{\perp B}}\right) K_A C_A \qquad (15)$$

where T denotes total intensity (sum of contributions from A and B), K_A is a proportionality constant and C_A is the concentration of A. The other terms in the expression are the parallel and perpendicular components of fluorescence for A and B. This derivation assumes that synergistic effects are negligible. The applicability of this approach was demonstrated using mixtures of Rhodamine B in the presence of fluorescein.

By far, the most widely used applications of fluorescence polarization have been in the areas of biochemistry and biology. For example, fluorescence polarization is a standard technique for measuring the rotational diffusion of molecules in confined systems such as biological membranes. The technique is also useful for studying the binding of small fluorophores to proteins in solution. These applications are beyond the scope of this manuscript. However, two pertinent references (29, 30) should prove useful if additional information is desired.

Our discussions above were restricted to the use of polarized

excitation for analytical measurements. Other polarizaion parameters are available such as circularly polarized luminescence (31) and fluorescence detected circular dichroism (32). These techniques have not been extensively explored for analytical utility.

Conclusions

We can conclude from the above discussion that luminescence techniques are inherently well suited for multicomponent analysis. This arises from the many parameters which can be exploited to achieve specificity. It is interesting to note that no instrument has ever been developed to exploit all of the available luminescence parameters. Thus, we can expect a proliferation of more sophisticated fluorescence instrumentation with 1) new and improved optical methods such as improvements in polarization optics, 2) better broad band pulse sources and 3) more sophisticated detection devices.

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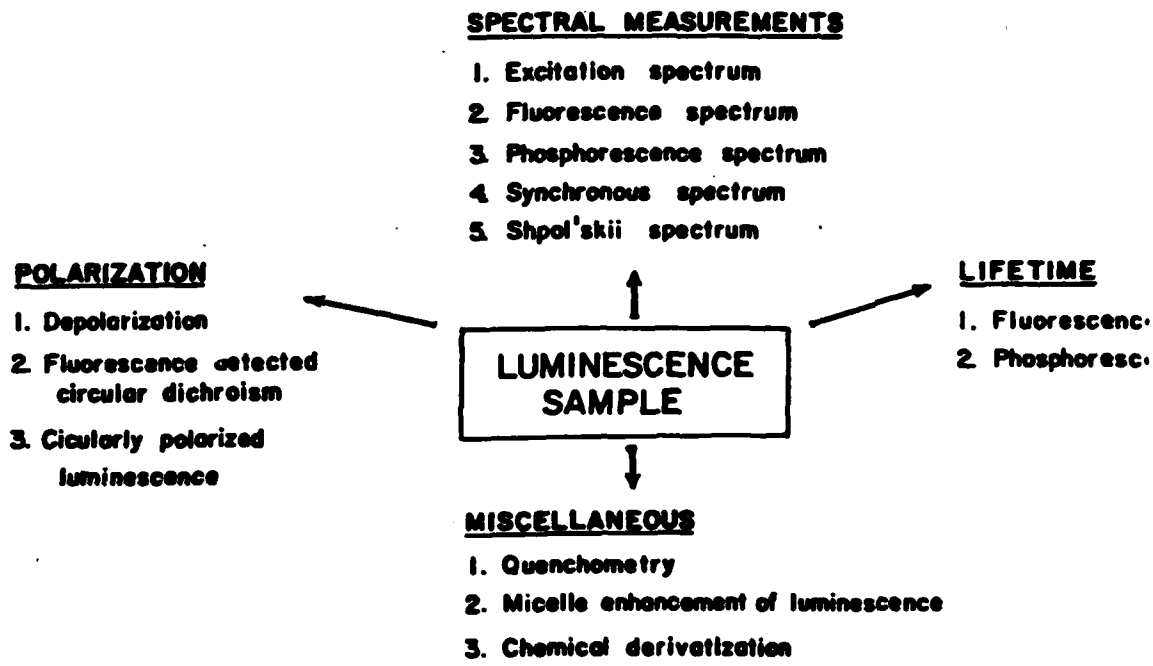
FIGURE CAPTIONS

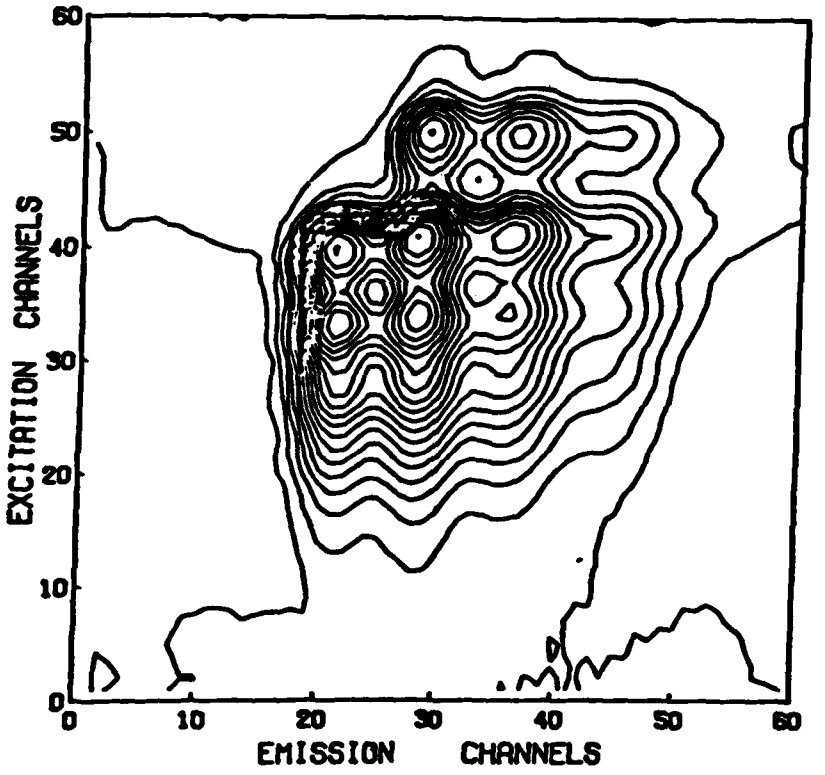
Figure 1. Parameters that can be monitored from a luminescence sample.

Figure 2. Contour plot of a mixture EEM containing perylene and tetracene.

Figure 3. Time resolved PEEMs of a binary mixture of phenathrene and coronene.

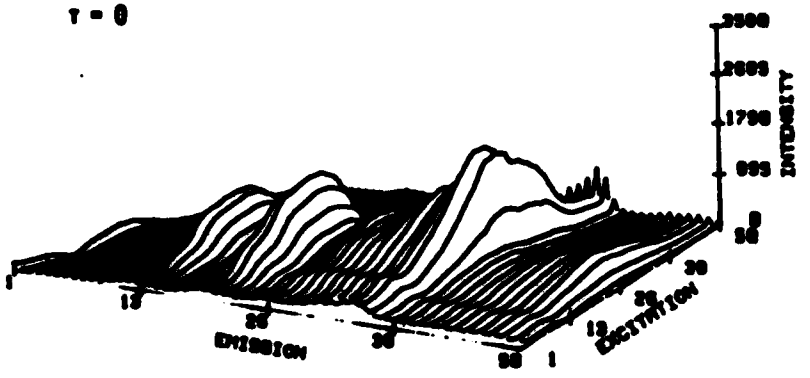
Figure 4. Deconvoluted spectra of phenanthrene and coronene.


SPECTRAL MEASUREMENTS
1. Excitation spectrum
2. Fluorescence spectrum
3. Phosphorescence spectrum
4. Synchronous spectrum
5. Shpol'skii spectrum
POLARIZATION
1. Depolarization
2. Fluorescence detected circular dichroism
3. Cicularly polarized luminescence
LUMINESCENCE SAMPLE
LIFETIME
1. Fluorescenc
2. Phosphoresc
MISCELLANEOUS
1. Quenchometry
2. Micelle enhancement of luminescence
3. Chemical derivatization


EXCITATION CHANNELS
0
10
20
30
40
50
60
EMISSION CHANNELS

2C Mixture = Coronene & Phenanthrene

T = 0



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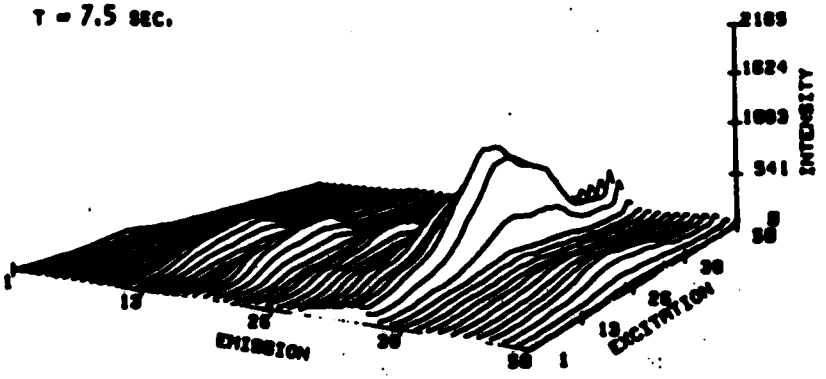

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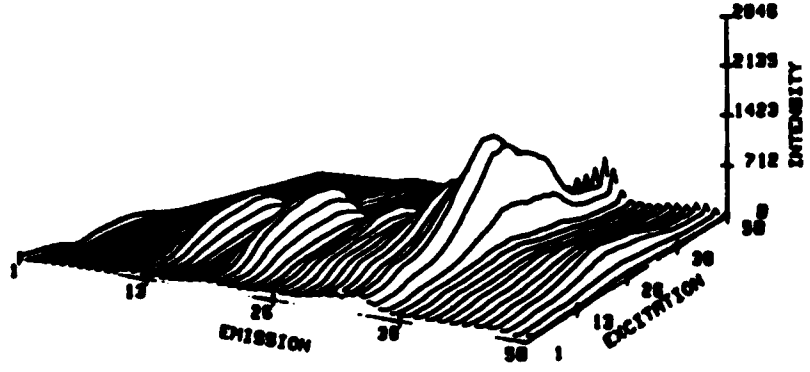

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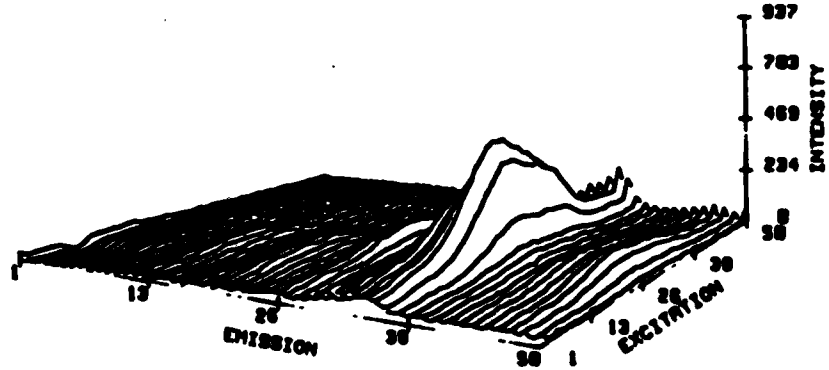
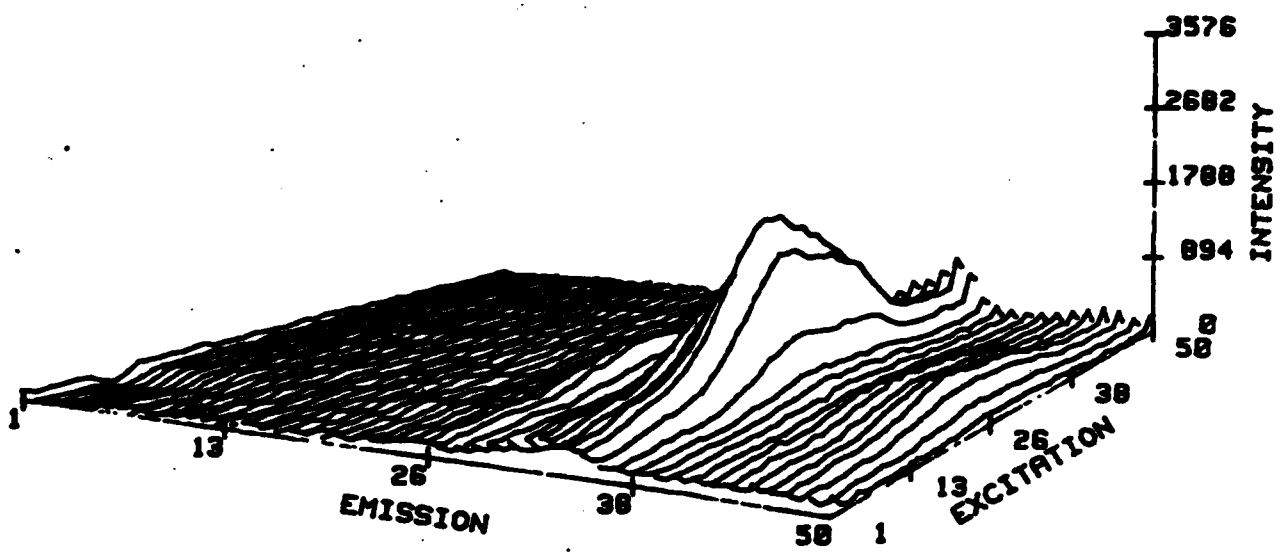
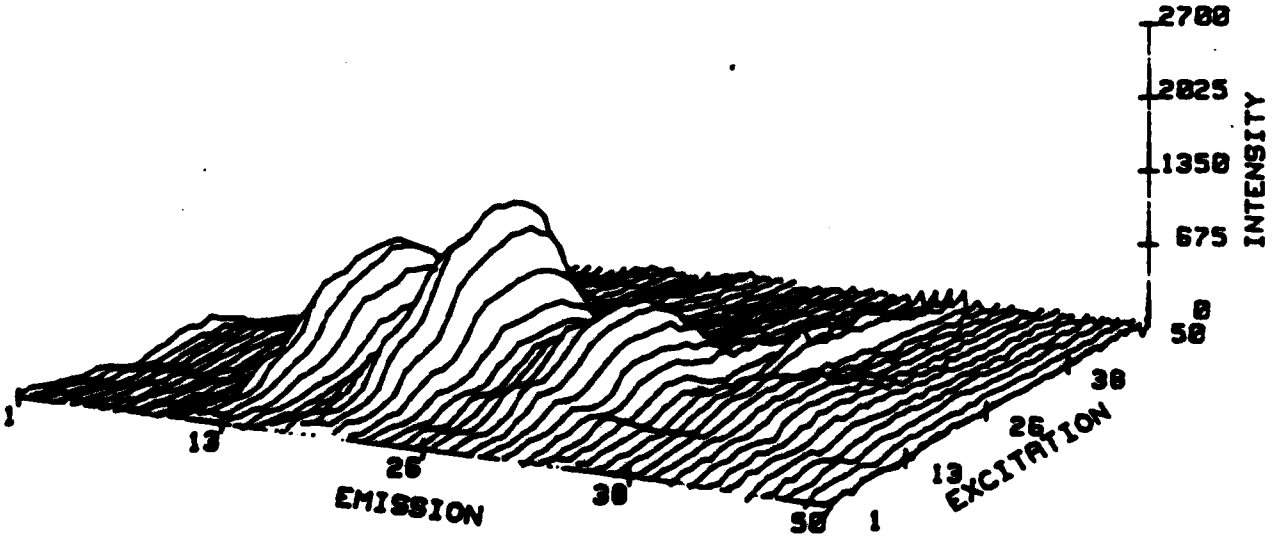


DECONVOLUTED EEM = CORONENE



DECONVOLUTED EEM = PHENANTHRENE



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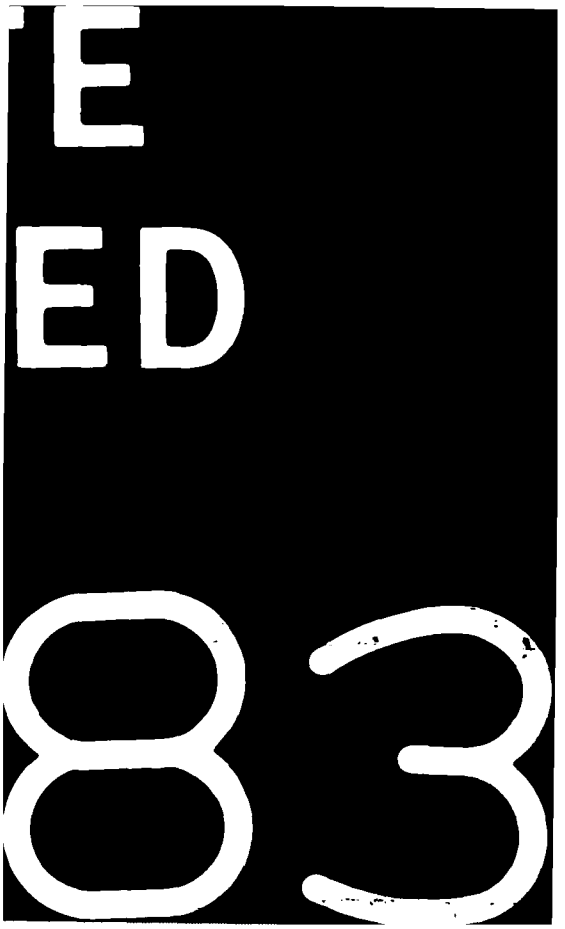
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