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A RATIO METHOD FOR FLUORESCENCE SPECTRAL DECONVOLUTION.(U)
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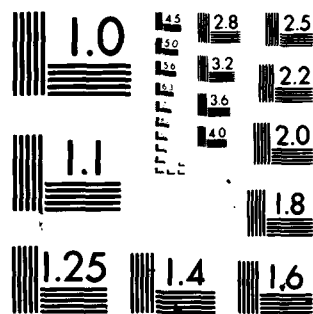
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Ratio Method for Fluorescence

Spectral Deconvolution

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Ratio Method for Fluorescence
Spectral Deconvolution

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Brief

A ratio method is used to deconvolute one and two-dimensional fluorescence data. Fluorescence quenching is shown to be an aid in this deconvolution scheme.

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ABSTRACT

A procedure is described for the spectral deconvolution of multicomponent fluorescence data using a ratio method. The only criteria for deconvolution is that each component must have a spectral region where it is the only emitter and that the acquired data sets have a reasonable signal to noise ratio. The ratio method described is an extension of an algorithm previously developed for deconvolution of one-dimensional infrared data. Examples of applications of this algorithm for one-dimensional and two-dimensional fluorescence data are provided. In addition, an example is given as to how fluorescence quenching can be used to aid in spectral deconvolution.

Ratio Method for Fluorescence Spectral Deconvolution

Spectroscopic data has often been used as a diagnostic or analytical tool to complement other analytical techniques. Thus, one can combine spectroscopic data with the measurement of widely differing techniques into a single data set, e.g. spectroelectrochemical data (1). Such techniques might be termed multiparametric in that the analytical signal is measured as a function of more than one parameter. In recent years, multiparametric methods have been found to be highly efficient in data collection and thus represent a large savings in the time necessary to gather data. However, a concomitant disadvantage is the increased time required to reduce these large data sets to pertinent information. Consequently, to make the trade-off of analysis time for data reduction time worthwhile, new strategies to reduce the data are required. These reduction strategies need to be efficient and simple in structure such that meaningful information can be rapidly extracted from a large data set.

In the past few years, many algorithms have been used in the reduction of multiparametric chemical data. In particular, the techniques of factor analysis and pattern recognition can be cited as examples. Although both techniques are in their infancy with regards to chemical data reduction, they have found extensive use in the social sciences where acquisition of multiparametric data is a common occurrence. Pattern recognition and factor analysis are used to reduce the bulk of data by elimination of those elements which are not associated with the properties of interest. In addition, pattern

recognition is used to classify via these reduced patterns using a pre-established criterion set up in a training set. A number of applications of factor analysis can be found including spin labelling and mass-spectral interpretation (2,3,4). Some recent applications of pattern recognition in biology and artificial intelligence modeling have appeared in the literature (2,5,6).

In the area of fluorescence spectroscopy, the video fluorometer can be cited as an example of multiparametric instrumentation. Two designs of this system can be cited, one by Johnson et al. (7) and the other by Warner et al. (8). In the latter design, the video fluorometer operating strictly as a fluorometer is capable of acquiring a mixtrix of data consisting of 50 emission spectral scans obtained at 50 different excitation wavelengths in 0.05 to 0.5 seconds. The acquired emission-excitation matrix (EEM) then consists of fluorescence intensity as a function of two parameters (excitation wavelength and emission wavelength). In addition, this instrument can be used as an HPLC detector (9,10,11) or as a multi-dimensional dectector for following reaction kinetics (12). Thus, a third parameter of time can be acquired.

The properties of the EEM have been described previously (13,14,15). However, a brief discussion is warranted here. The EEM of an ideal, noise-free, single component matrix is formed by the vector product of that component's emission and excitation spectra (both normalized to unity), multiplied by a scaler quantity containing concentration information. A system of "n" components can be represented by the summation of the "n" single component EEM's, i.e.

$$M_{\lambda_0} = \sum_{i=1}^n \alpha_{i0} \underline{x}_i \underline{y}_i^t \quad (1)$$

This equation is applicable provided that the absorbance is low and no synergistic effects such as energy transfer occur in the acquired data. Here, $\underline{x}_i$ and $\underline{y}_i$ represent column vectors of the emission and excitation spectra, respectively. The concentration term α_{i0} , represents the concentration of component i in matrix M_{λ_0} . In linear algebra, a matrix that can be composed from "n" linearly independent row or column vectors is said to be of rank "n". Eigenanalysis of such a matrix is known to give an arbitrary basis set of "n" vectors that are linearly independent. The addition of noise to the EEM complicates the form. The matrix can no longer be described by "n" vectors. The number of vectors necessary to describe a matrix with random noise as a component is usually equal to the number of rows and/or columns. The eigenvalues of the component vectors, however, are often significantly larger than those that are attributable to noise. Thus a noise threshold can be selected to discriminate true signal from noise. Since some components can be lost in the noise if their concentrations are small and since some components can be linear combinations of others, a matrix rank determined in this manner can only be considered an approximation to the number of components (a lower bound).

While eigenanalysis is relatively straight-forward, and many "canned" routines are available, it has some disadvantages that restrict its use in reduction of an EEM. Eigenanalysis has already, successfully, been employed for a qualitative (14) as well as quantitative (15) evaluation of the EEM. Recently it has been employed in a rank reduction technique (16,17). Two major problems are encountered in using linear

algebraic methods. First, the eigenvectors derived usually form an orthonormal set. Thus, for more than one component, these vectors contain both positive and negative elements. True spectra are non-negative. The eigenvectors can be rotated to a non-negative basis, but this procedure (14) is practically limited to a two component mixture. The resulting deconvolution does not have a unique solution in most cases. The second problem arises from performing eigenanalysis computations on minicomputers. A diagonalization of a 50 X 50 matrix can take as long as 30 minutes. A power method of analysis (18) can be used to extract only the largest eigenvalues and eigenvectors, but fails to extract them in order if large fluctuations occur in the matrix. For more than 5 eigenvalues, or when the magnitude of the eigenvalues are very close, the time of analysis approaches that of a standard diagonalization procedure.

A method requiring no prior knowledge of the sample has been used for the spectral deconvolution of multicomponent infrared data (19). This procedure involves generating a number of data sets of the same mixture while varying the relative ratios of the concentrations of each component in the data sets. The number of components in the matrix, as well as the relative concentration factors can be obtained directly from the data without any knowledge of the composition of the mixture. It has occurred to us that this ratio method should be applicable to spectral deconvolution of the EEM. In addition the use of fluorescence quenchers should provide some unique possibilities for changes in the apparent concentration (fluorescence intensity) of each fluorophore.

Theory

It should be apparent from our earlier discussion that the EEM of a multicomponent system can be represented as the summation of the vector product of the excitation and emission vectors multiplied by a scalar concentration factor for each component (Equation 1). Without loss of generality, one can define a standard component matrix

$$N_i = \alpha_{i0} (\underline{x}_i \underline{y}_i^t). \quad (2)$$

If we then rewrite Equation 1 based on this arbitrarily selected concentration basis, we obtain

$$M_j = \sum_{i=1}^n \alpha_{ij}^i N_i \quad (3)$$

where α_{ij}^i is now equivalent to α_{ij}/α_{i0} . This establishes the matrix M_0 as a basis for any set of matrices in this coordinate system since all the expansion coefficients in M_0 are unity.

If one can obtain a set of "n" matrices ($j=0, 1, 2 \dots n-2, n-1$) containing "n" components one can establish a set of "n" equations in "n" unknowns. This system can be represented in matrix form by

$$M^* = AN^* \quad (4)$$

The matrix A consists of an $n \times n$ matrix of coefficients α_{ij}^i . In Equation 4, M^* consists of the series of mixture matrices, M_j , and N^* represents the series of standard component matrices, N_i . This linear system has a solution if the matrix A is invertible, i.e.

$$A^{-1}M^* = N^*. \quad (5)$$

The invertibility of A can be verified by obtaining the determinant of

A. This system shows that a simple linear combination of a series of mixture matrices can yield each individual component matrix if one can find the set of α'_{ij} 's. Since the solutions are the set of N_i 's, the concentration information contained in M_0 is retained in this solution.

The problem reduces to determining the α'_{ij} factors that form A. If one exactly knows the composition of the sample, and can effectively alter concentrations in some standard manner (e.g. standard addition), the determination of each α'_{ij} is trivial. However in routine analyses, the composition is usually unknown. In that case, a ratio of two data sets containing the same components in differing amounts will give a matrix consisting of a series of plateaus wherever components of the sample are sole emitters, i.e.

$$\sum_{i=1}^n \alpha'_{ij} N_i / \sum_{i=1}^n N_i = R_j \quad j = 0, 1, 2 \dots n-2, n-1 \quad (6)$$

In the spectral region where a compound is a sole emitter, all elements of N_i except those due to the emitting species are zero. Therefore a plateau of height α'_{ij} occurs in this region. Regions in which no signal occurs leads to division by zero which is undefined, but in the limit these areas have a magnitude of one. In regions connecting plateaus, the function becomes dependent on the magnitudes of the N_i 's as well as the α'_{ij} 's. These intermediate areas connect the plateaus by sloping functions that are asymptotic to the ratio values at the plateau regions. In cases where two components with the same α'_{ij} overlap, the overlap region will also be flat and have value α'_{ij} . Thus, the appearance of a single plateau is given.

This technique is best illustrated using an example. In Figure 1, we display three data sets of three components each. The peaks are

formed using gaussian functions of a specified height and width. A concentration factor was arbitrarily selected for each component in each matrix. This data was generated without noise, and represents the idealized case. In addition, for illustration, we have elected to show components that are widely separated in emission and excitation wavelengths. In a real analysis one could obtain pure emission and excitation spectra of each component by selective excitation and monitoring at a selected emission wavelength. In this example, we do have overlap of all components since we are using gaussian functions. The ratioing of matrix 3 into 1 and 2 was performed with the result as displayed in Figure 2. Each ratio matrix shows 3 flat regions, one due to each component. These areas are perfectly flat and differ only in rounding considerations from element to element in the plateau. Three ratio values (α'_{ij} 's) were selected from each ratio EEM and set up as elements of the matrix $\hat{A}$. It is important to notice that any ratios close to unity would be lost in the background. However, as we shall see later the addition of noise to the EEM serves to outline the plateau regions. The α'_{ij} 's are deposited in $\hat{A}$ such that each row of $\hat{A}$ corresponds to ratio values extracted from a given ratio matrix, and each column corresponds to the different ratios among the same component through the series of matrices. Matrix $\hat{A}$ is then inverted, and the linear combinations of EEM's dictated by Equation 5 performed. The results show each component to be uniquely deconvoluted (Figure 3).

The addition of noise to the matrix modifies the expected results of Equation 6. In low signal areas, the magnitude of the noise may approach that of the signal. In these low signal areas the possibility

of division of a real value by numbers close to zero will generate large fluctuations in the ratio EEM. However, in areas with a good signal-to-noise (S/N), the plateau region is still apparent. The randomly fluctuating noise regions serve to frame and set off the areas of interest. Thus, the possibility of losing a plateau region in the noise due to a ratio value of one is remote. Weak signals will tend to be noisy and result in noisy plateau areas. Maximizing the S/N will give a smoother plateau area, and less error in determining the coefficient corresponding to the height.

Thus, two conditions are necessary for this ratio technique to work: 1) each component should have a spectral region where it is the only emitter with a reasonable S/N ratio and 2) some simple method should be available to provide the required change in the relative ratio of each component in the multicomponent mixture. With regards to requirement #2, Hirschfeld (19) has previously identified several methods such as solvent extraction and preferential volatilization for this requirement. However, either of these two procedures can be time-consuming and tedious if several components are involved. For example, if we have a mixture containing ten components, then at least nine different extractions would be required. In addition, preferential volatilization assumes that all components are reasonably volatile which is not always the case. We have developed a method of changing the apparent concentration (fluorescence intensity) in the mixture using non-selective quencher molecules. This method can best be explained using the Stern-Volmer relationship

$$\frac{F_0}{F} = K_Q [Q] + 1 \quad (7)$$

where F and F_0 are the observed fluorescence intensity in the presence and absence of quencher molecules of concentration $[Q]$, respectively. The parameter K_Q is referred to as the Stern-Volmer constant and in the limit, as quenching encounters approach unity, can be approximated by

$$K_Q = k_q \tau_0 \quad (8)$$

where k_q approaches the diffusion controlled biomolecular rate constant and τ_0 is the mean fluorescence lifetime of the molecule. If we assume that k_q is approximately the same for most large fluorescent molecules (e.g. polynuclear aromatics), then the relative ratio of unquenched and quenched fluorescence is directly proportional to the lifetime of the molecule at a fixed quencher concentration. The implication here is obvious. One can change the apparent concentration ratio (fluorescence intensity) by the simple addition of a quencher molecule since the mean fluorescence lifetime (τ_0) tends to vary with the molecule.

Experimental

Apparatus. Video Fluorometer. The video fluorometer used for this study has been described previously (8). This system has been recently interfaced to a Hewlett Packard 9845T minicomputer through an RS232C serial interface coupled directly to the EG&G Princeton Applied Research Corporation OMA-2 console (Model 1215). The one-dimensional data presented in this study were spectrally deconvoluted on the OMA-2 console. Two-dimensional data were transferred via the serial interface to the HP9845T minicomputer and then deconvoluted using the algorithm

described here. All data were acquired without spectral correction. Maximum absorbance of all samples at all wavelengths were kept below 0.01. Reagents. The five polynuclear aromatic compounds, fluorene, carbazole, fluoranthene, anthracene and 9,10-dimethylanthracene were purchased from Aldrich Chemical Company, Sigma Chemical Company, Matheson, Coleman, and Bell, and used without further purification.

Results and Discussion

The algorithm described here is applicable to any data type where the measured signal intensity varies as a function of a single factor (e.g. concentration). All other parameters affecting the data set must only provide qualitative information. To illustrate, a three component system consisting of fluorene, carbazole and fluoranthene was deconvoluted in both a normal one-dimensional mode as well as a two-dimensional mode (EEM). The three mixtures shown in Figure 4 correspond to the set of ternary mixtures acquired as conventional emission spectrum excited at 322 nm. The ratio spectra were formed by dividing spectrum a into b and c (Figure 5). Three plateau areas are apparent in both ratio spectra. The extremes of these scans show the typical noise fluctuations found in areas of no signal. Some of the larger points in this area have been removed to aid viewing. From the heights of these plateaus, the values of α'_{ij} for each was extracted by averaging over the flat areas. The resulting inversion and linear combinations resulted in the deconvolutions shown in Figure 6. The spectra of fluorene and carbazole (Figure 6A) appear to be totally resolved. The spectrum of fluoranthene is totally

resolved in the area in which fluoranthene emits. However, another extraneous signal appears in the area that overlaps the emission region of fluorene and carbazole. It is not apparent whether this residual is in fact part of the carbazole and/or fluorene signal, or whether it is some impurity associated with the fluoranthene, or partially due to a light scattering peak. This deconvolution was done directly on the EG&G Princeton Applied Research OMA-2 1215 console. Matrix inversion was performed by a hand held calculator.

The identical solutions were used to test the algorithm in a two-dimensional mode of data acquisition. By providing a second dimension of spectral dispersion (excitation) the possibility of a compound being a line emitter in some part of the spectrum is enhanced. The three mixture matrices are shown in Figure 7. As was demonstrated in the previous example, this ternary mixture is already separable in one-dimension. It should be apparent from this data that it is possible to obtain a pure excitation spectrum of each component by monitoring at a selected emission wavelength. However, a pure emission spectrum of each component is not possible via selective excitation. In two-dimensions, one should be able to examine the ratio plateaus in the areas of maximum S/N for each component. The ratio EEM's are shown in Figure 8. The second ratio (Figure 8b) shows three distinct plateau areas. In the first set (Figure 8a) there appears to be two different plateau heights in the fluorene area. The large peaks to the center-rear are attributed to scattered light. The result of this dual tiering is seen in the deconvoluted spectra (Figure 9). The fluorene spectrum (Figure 9a) and the carbazole spectrum (Figure 9b) are totally resolved.

However, the fluoranthene (Figure 9c) is again separated but contains a structure very similar to that of the fluorene. This partition of the fluorene between two different matrices was predicted by the tiered structure that was apparent in Figure 8b. The cause for this effect lies in either some form of residual scattered light in the upper excitation band of the fluorene, energy transfer or an inner filter effect altering the fluorene profile. Nonetheless, the mixture matrix has been considerably simplified. This deconvolution may be improved by using a new ratio value (either tier) for a second deconvolution. The result shown here points to some unusual effects occurring in solution.

In the real world, it is impractical to modify one's sample to obtain the number of data sets necessary for a deconvolution. To our knowledge, there is no method other than the ratio technique described here that is capable of deconvoluting more than two components in an EEM without some prior knowledge of the components. For particular situations the ratio method becomes extremely practical. The speed at which an EEM can be acquired (typically .05-.5 sec) allows interesting possibilities as a chromatographic detector. In such a case, data is gathered along three dimensions, fluorescence intensity as a function of emission wavelengths, excitation wavelengths, and retention volume or time. Unresolved chromatographic peaks provide the necessary gradients along the chromatographic peak profile to generate combinations of components in which the relative ratios of their concentrations have changed. The data sets are generated without addition to the sample. An advantage of such an approach is that one can now sacrifice

chromatographic resolution in favor of speed. The video fluorometer can, with the aid of this algorithm, provide the necessary discrimination to make this possible. Such use of this algorithm to resolve chromatographic peaks has been previously suggested by Hirschfeld (19).

The ratio algorithm assumes no prior knowledge of the composition of concentrations involved. However, some interesting possibilities can be seen for systems in which some or all of the components are known. In such a situation, a standard addition with the known components can be performed either singly or in combination. One must remember that the coefficients derived from such a procedure must form an invertible matrix. This could also be done using standard library spectra. Matrices can be formed by summing library spectra, thus eliminating the need for excess data collection. In fact any standard matrix that will form a plateau when ratioed with the mixture matrix implicates that standard as a component in the mixture, provided there is an area in which it is the sole emitter. As a test for the identity of the components, this requirement can be relaxed if one can observe the sloped overlap regions between two component areas.

A theoretical possibility lies in predictably altering the fluorescence intensity of components using selective quenchers. The use of quenchers as aids in fluorescence analysis has been demonstrated (20-26). However, many quenchers alter the emission and/or excitation profile of the molecule being quenched. Consequently, one must be careful in his selection of quenchers since the ratio deconvolution cannot tolerate such distortions. The magnitude of the quenching is

predictable by prior experimentation. Our previous discussion of Equations 7 and 8 has shown that the Stern-Volmer relationship predicts the differences in quenching between various fluorescence molecules in interactions with the same quencher. Therefore, one needs only to add enough quencher to obtain the desired α'_{ij} . As an example of this use, a mixture of anthracene and 9,10-dimethylantracene was used. The EEM of this mixture was obtained, and then a small amount of methyl iodide was added and the spectrum acquired again (Figure 10). The α'_{ij} 's were obtained, again by averaging over the plateau area (Figure 11). This set is an example of two components that would be difficult to resolve in one-dimension. While the first emission band of anthracene is free, the remainder of the emission spectrum overlaps with that of the dimethyl derivative. The first excitation band of 9,10-dimethylantracene is relatively pure however. Thus, the necessary requirement for determining the α'_{ij} 's is obtained. The resulting deconvolution is shown in Figure 12. As shown, a virtually clean, spectral deconvolution has been accomplished.

For a comparison, the results of an eigen deconvolution are shown in Figure 12. As we discussed earlier, the eigenanalysis is practically limited to a two component mixture (14). Even for a two component mixture the ratio deconvolution is accomplished in nearly one-half the execution time. If a suitable set of coefficients is found, the ratio deconvolution is always unique (aside from noise). The uniqueness of the eigenanalysis result is dependent on the inter-relationship of the components in solution as well as the spectral range covered (14). The eigenanalysis does have the advantage that noise is apparently stripped

from the solution matrix. However, previous studies have shown that this apparent enhanced signal/noise ratio can be deceiving (14).

The technique of ratioing data sets in order to perform deconvolutions with no prior knowledge of the components involved has only been applied to infrared and fluorescence data. As we have seen, the procedure is simple and the underlying theory similarly so. This technique is applicable to any type of data where the single parameter that is being measured is the only variable. The other parameters may serve qualitative purposes, but must not contribute to variations in the measured parameter (i.e. input values used as measurement parameters). The number of independent variables is unlimited. As more multiparametric instrumentation become available the demand for such techniques as the ratio deconvolution will also increase.

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Credit

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Figure Captions

- Figure 1. Three component mixtures formed according to equation 1 using synthesized gaussian emission and excitation functions. Component one consists of four peaks in the upper right, component two, of three peaks in the upper left and component three a single peak in the central foreground. The concentration parameters (α 's) are A) mixture one; component one 0.270, component two 0.934, component three 0.999 B) mixture 2; component one 0.963, component two 0.807, component three 0.351 C) mixture 3; component one 0.854, component two 0.719, component three 0.343.
- Figure 2. Isometric plots of the ratios of the data presented in Figure 1. A) mixture 1/mixture 3 $\alpha'_{11}=0.316$, $\alpha'_{12}=1.299$, $\alpha'_{13}=2.913$ B) mixture 2/mixture 3 $\alpha'_{21}=1.127$, $\alpha'_{22}=1.122$, $\alpha'_{23}=1.023$.
- Figure 3. Deconvolutions derived from the data used for Figure 1C using the ratioing algorithm A) component one B) component two C) component three.
- Figure 4. Emission spectra of three component mixtures of fluorene, carbazole, and fluoranthene in pentane excited at 322 nm.
- a) mixture 1 $3.28 \times 10^{-5}M$ fluorene, $6.24 \times 10^{-6}M$ carbazole, $1.21 \times 10^{-6}M$ fluoranthene
 - b) mixture 2 $1.69 \times 10^{-5}M$ fluorene, $4.16 \times 10^{-6}M$ carbazole, $1.21 \times 10^{-6}M$ fluoranthene
 - c) mixture 3 $1.64 \times 10^{-5}M$ fluorene, $2.08 \times 10^{-6}M$ carbazole, $1.21 \times 10^{-6}M$ fluoranthene

Figure 5. Ratio spectra of the data presented in Figure 4.

- a) mixture 1/mixture 3 $\alpha'_{11}=1.96$, $\alpha'_{12}=3.69$, $\alpha'_{13}=1.10$
- b) mixture 2/mixture 3 $\alpha'_{21}=1.00$, $\alpha'_{22}=2.30$, $\alpha'_{23}=1.10$
(component 1 is fluorene, component 2 is carbazole, and component 3 is fluoranthene).

Figure 6. a) Deconvolution of mixture 3 in Figure 4.

b) Standard spectra offered for comparison.

a) fluorene, b) carbazole, and c) fluoranthene.

Figure 7. Isometric projections of three component mixtures of fluorene, carbazole and fluoranthene (see Figure 4) acquired in a two-dimensional acquisition mode A) mixture 3 B) mixture 1 C) mixture 2.

Figure 8. Ratio spectrum of the data presented in Figure 7. A) mixture 1/mixture 3 $\alpha'_{11}=1.96$, $\alpha'_{12}=3.69$, $\alpha'_{13}=1.09$ B) mixture 2/mixture 3 $\alpha'_{21}=1.00$, $\alpha'_{22}=2.29$, $\alpha'_{23}=1.06$ (component 1 is fluorene, component 2 is carbazole and component 3 is fluoranthene).

Figure 9. Deconvoluted spectra of mixture 3 in Figure 7 using the ratioing deconvolution algorithm A) fluorene B) carbazole C) fluoranthene D) standard spectrum of fluorene E) standard spectrum of carbazole F) standard spectrum of fluoranthene.

Figure 10. A two component mixture of anthracene (1.12×10^{-7} M) and 9,10-dimethylantracene (1.27×10^{-8}) in ethanol/water (50:50). A) no quencher added B) 0.052 M in ethyliodide as quencher.

Figure 11. Ratio spectrum of the data displayed in figure 10
(unquenched/quenched). $\alpha'_{11}=1.52$, $\alpha'_{12}=2.27$ (component 1
is anthracene, component 2 is 9,10-dimethylantracene).

Figure 12. A) ratio deconvoluted spectrum of anthracene B) ratio
deconvoluted spectrum of 9,10-dimethylantracene C)
eigenanalysis (14) deconvolution of anthracene D) eigenanalysis
(14) deconvolution of 9,10-dimethylantracene.

FIGURE 1

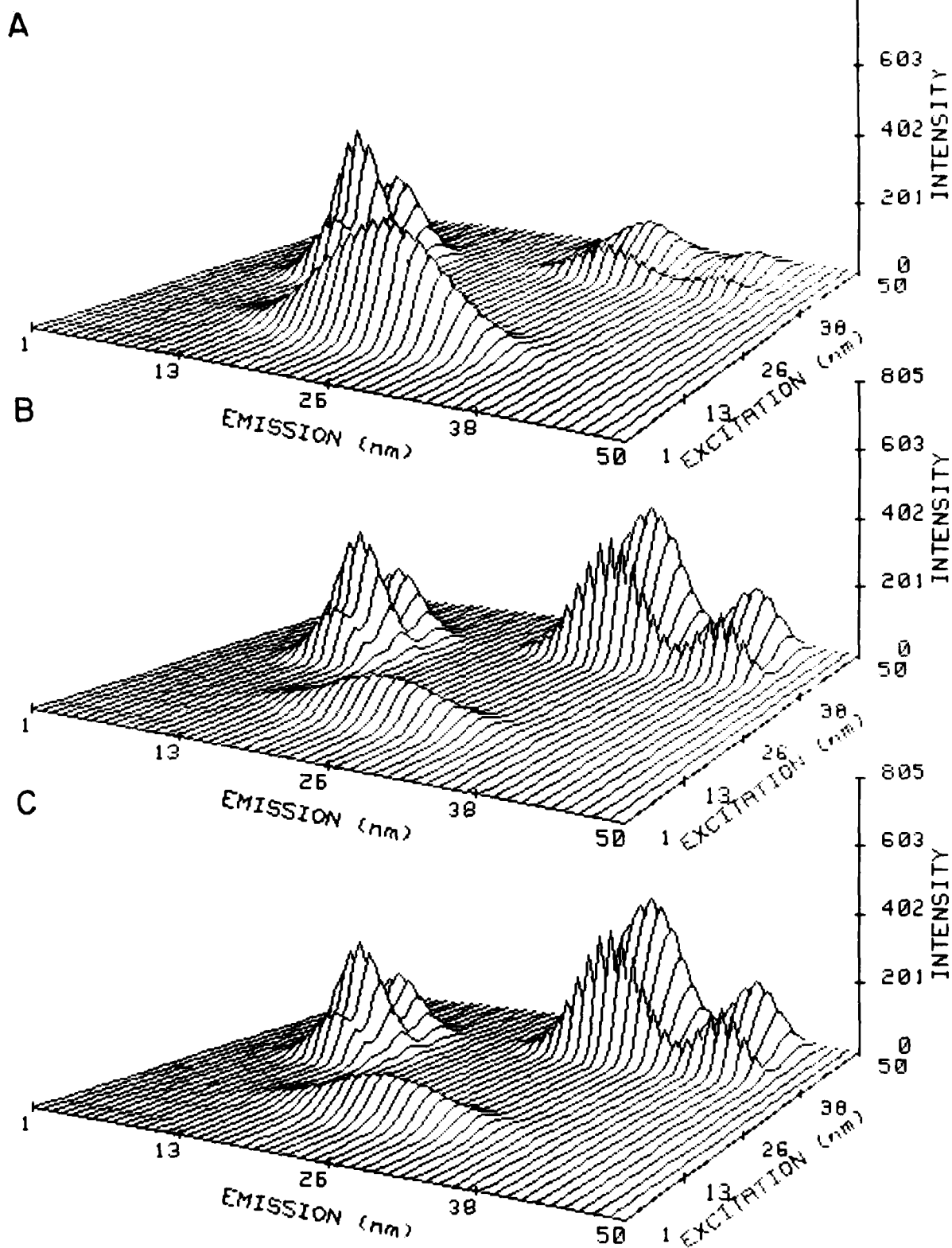


FIGURE 2

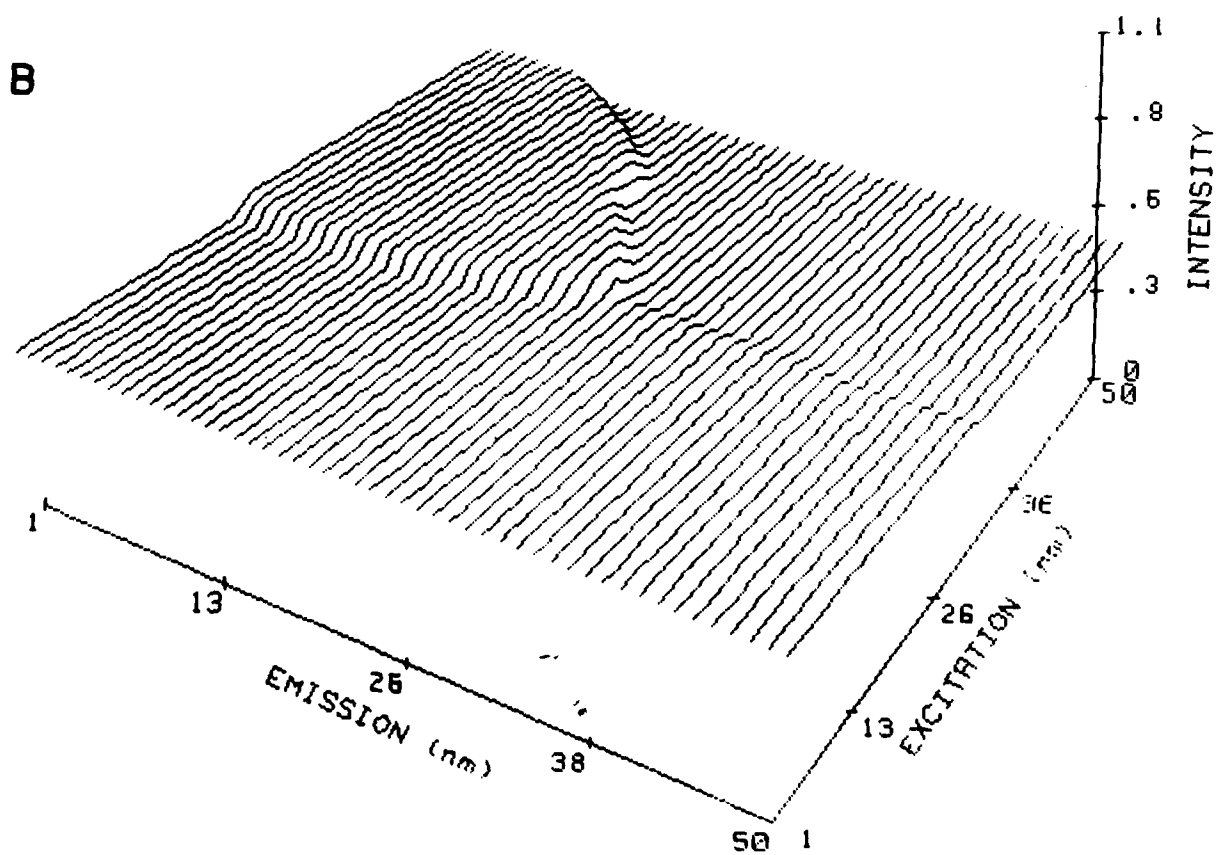
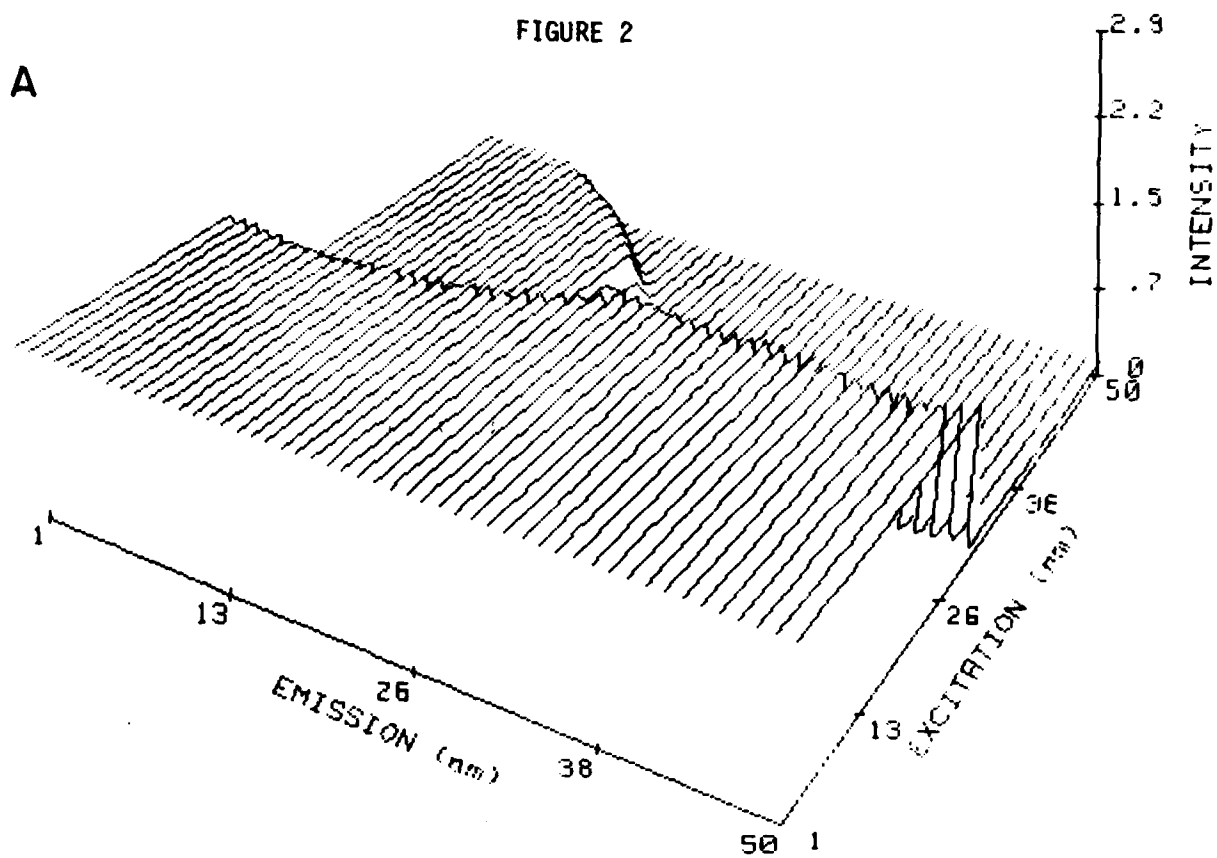


FIGURE 3

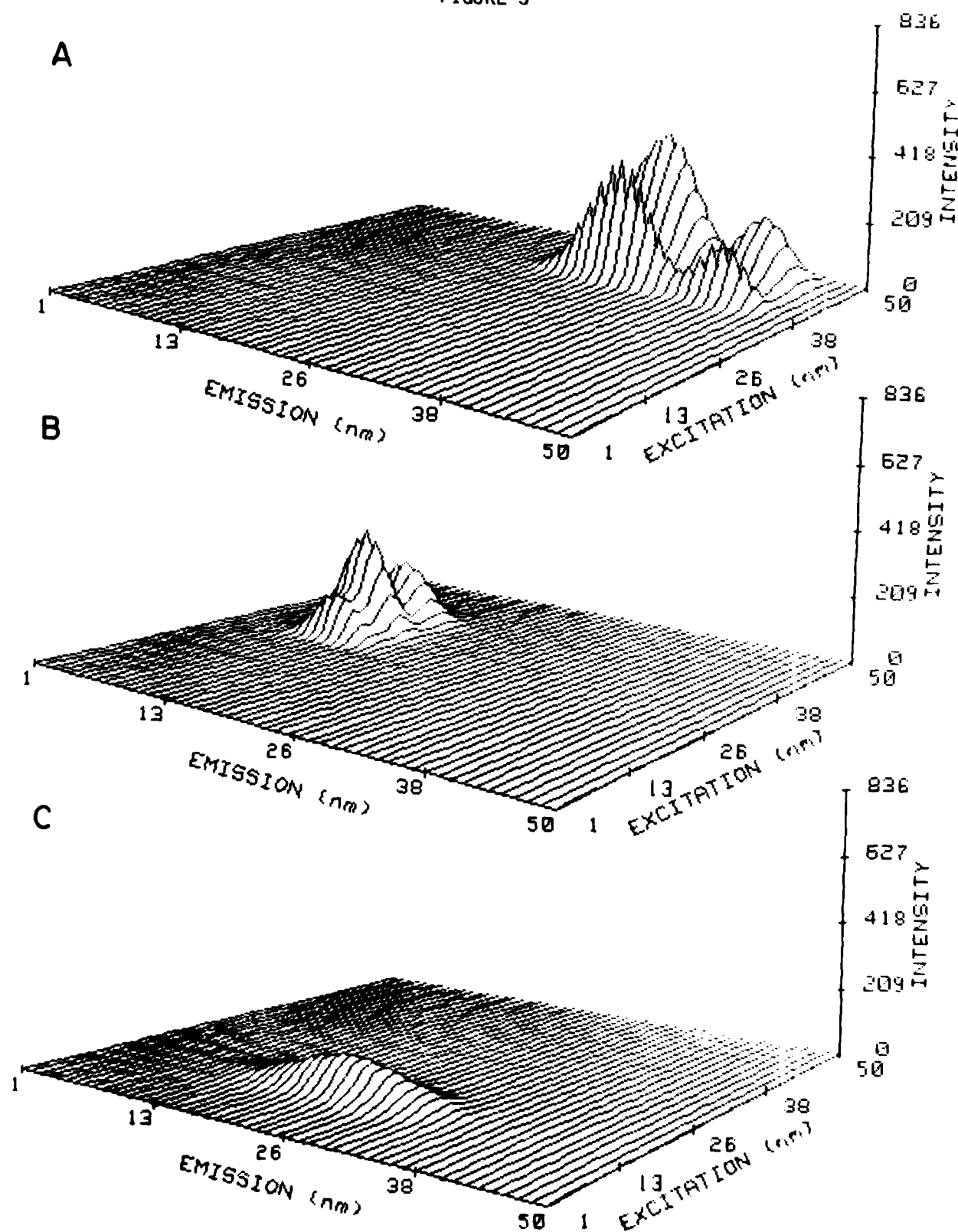


FIGURE 4

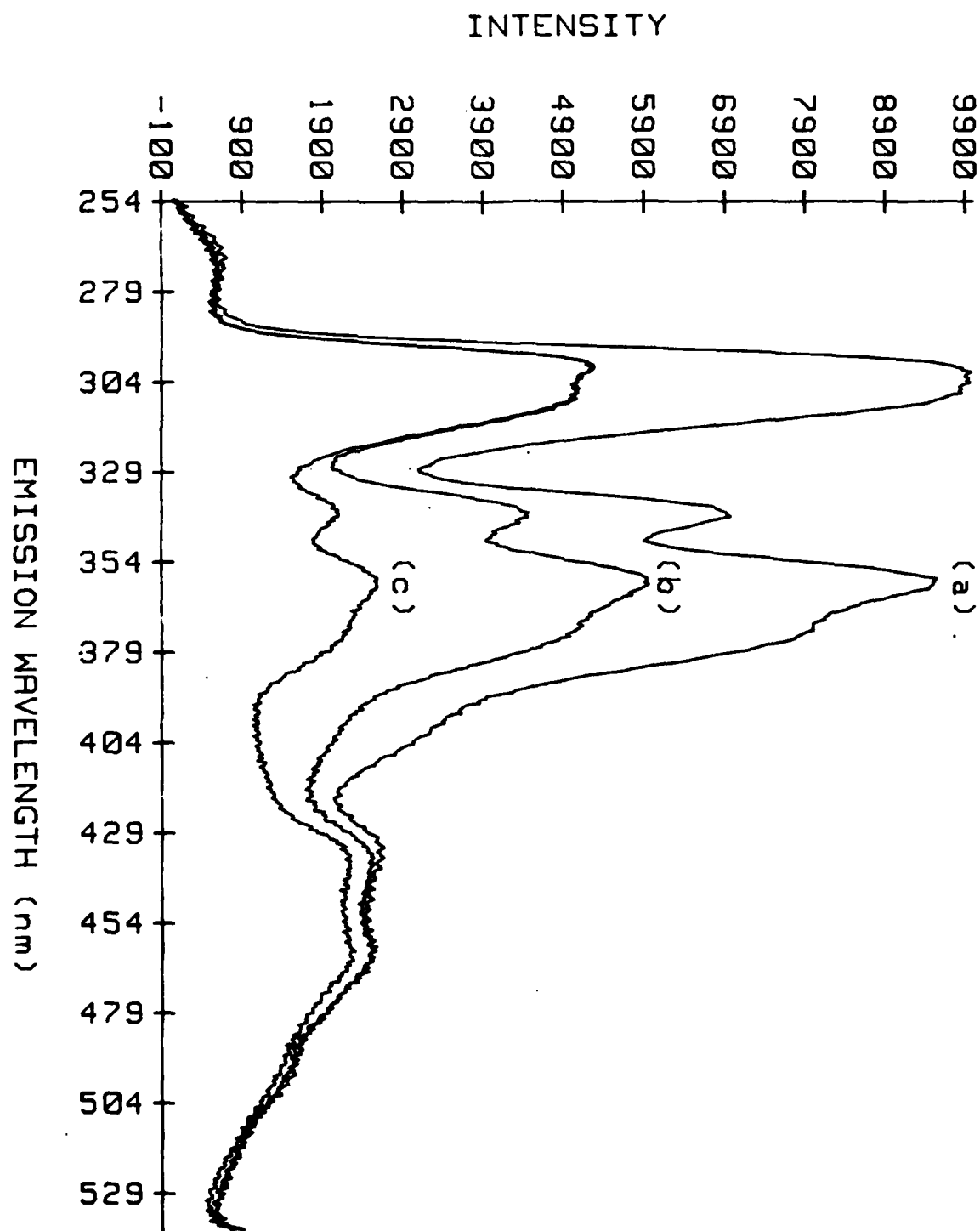


FIGURE 5

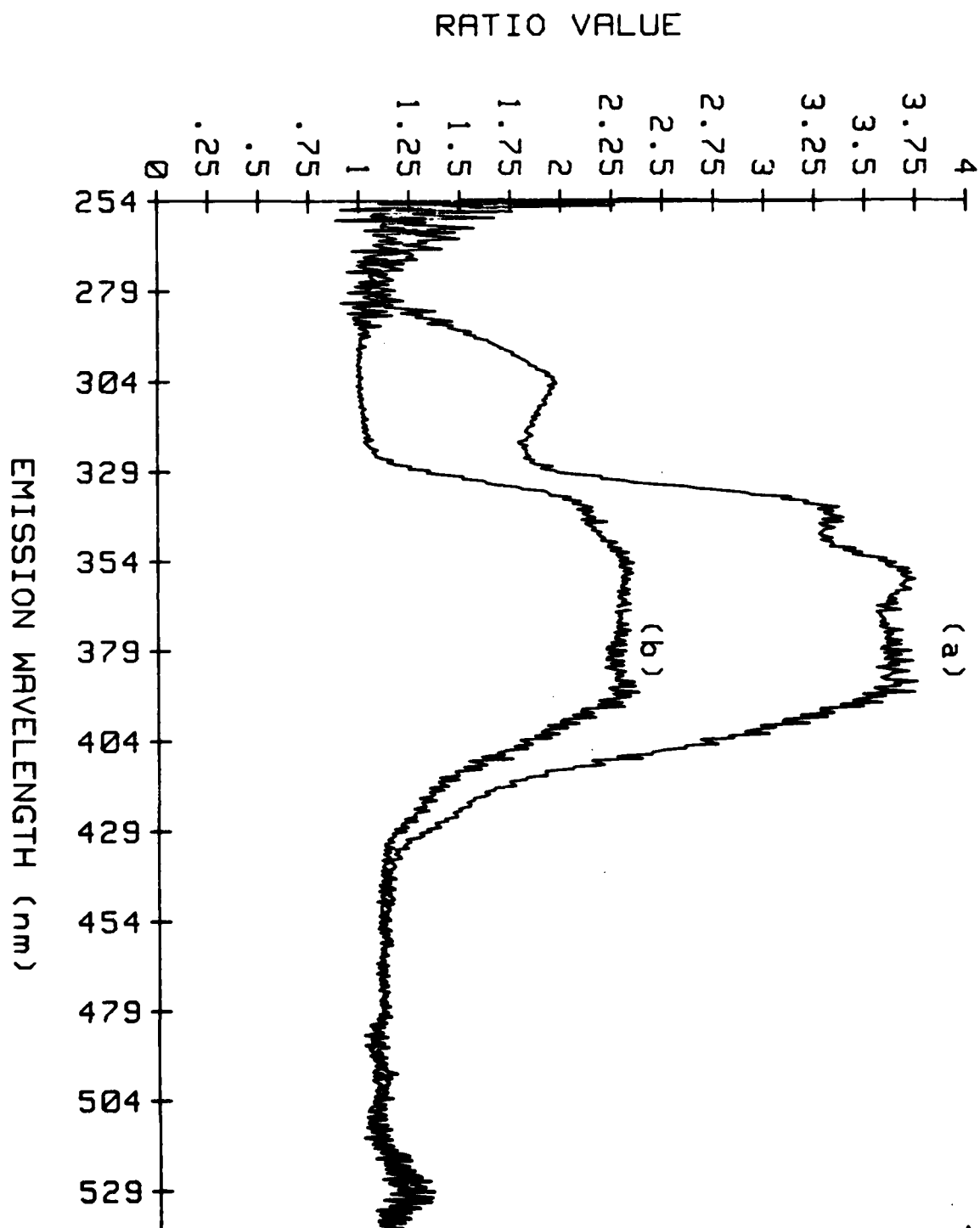


FIGURE 6A

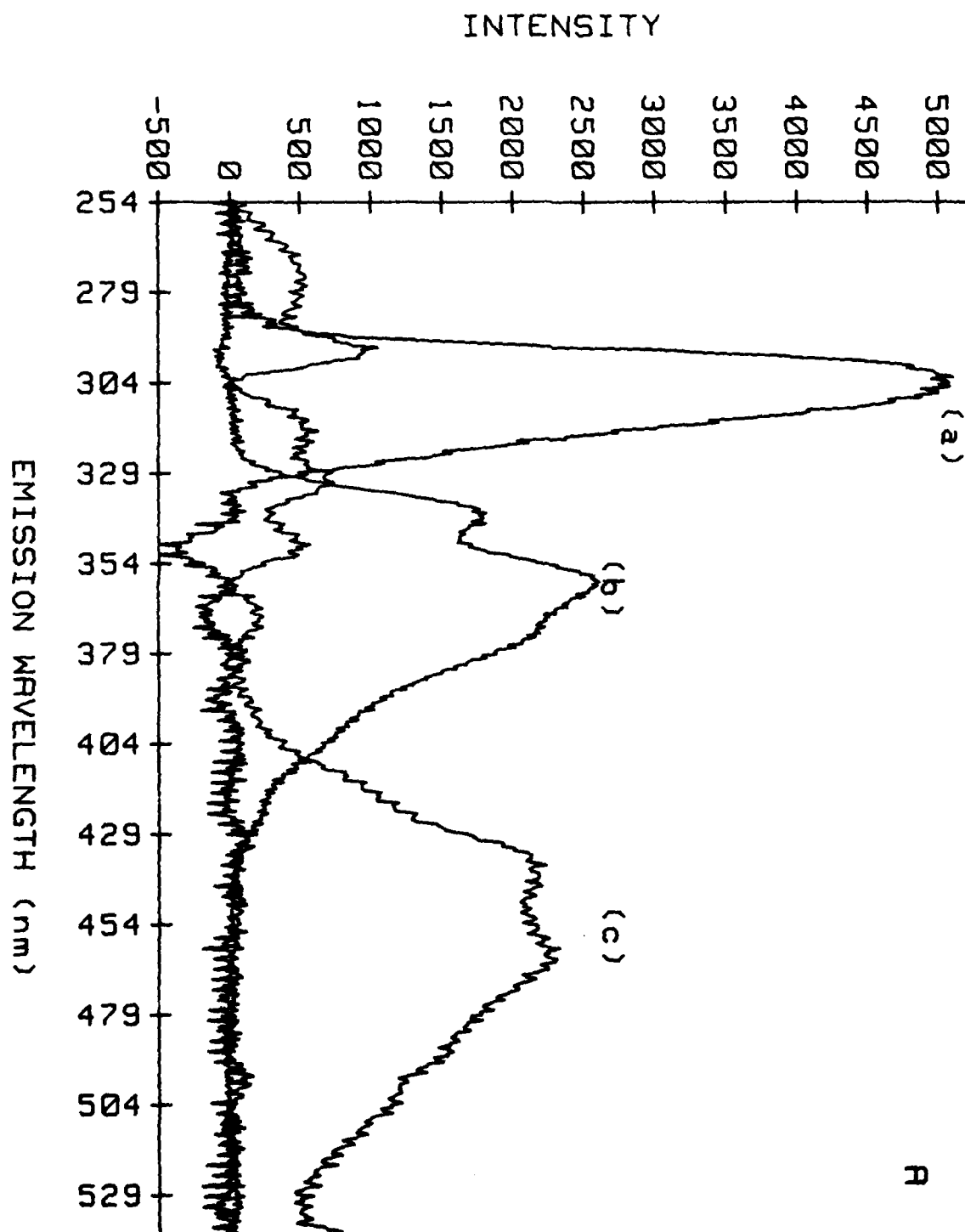


FIGURE 6B

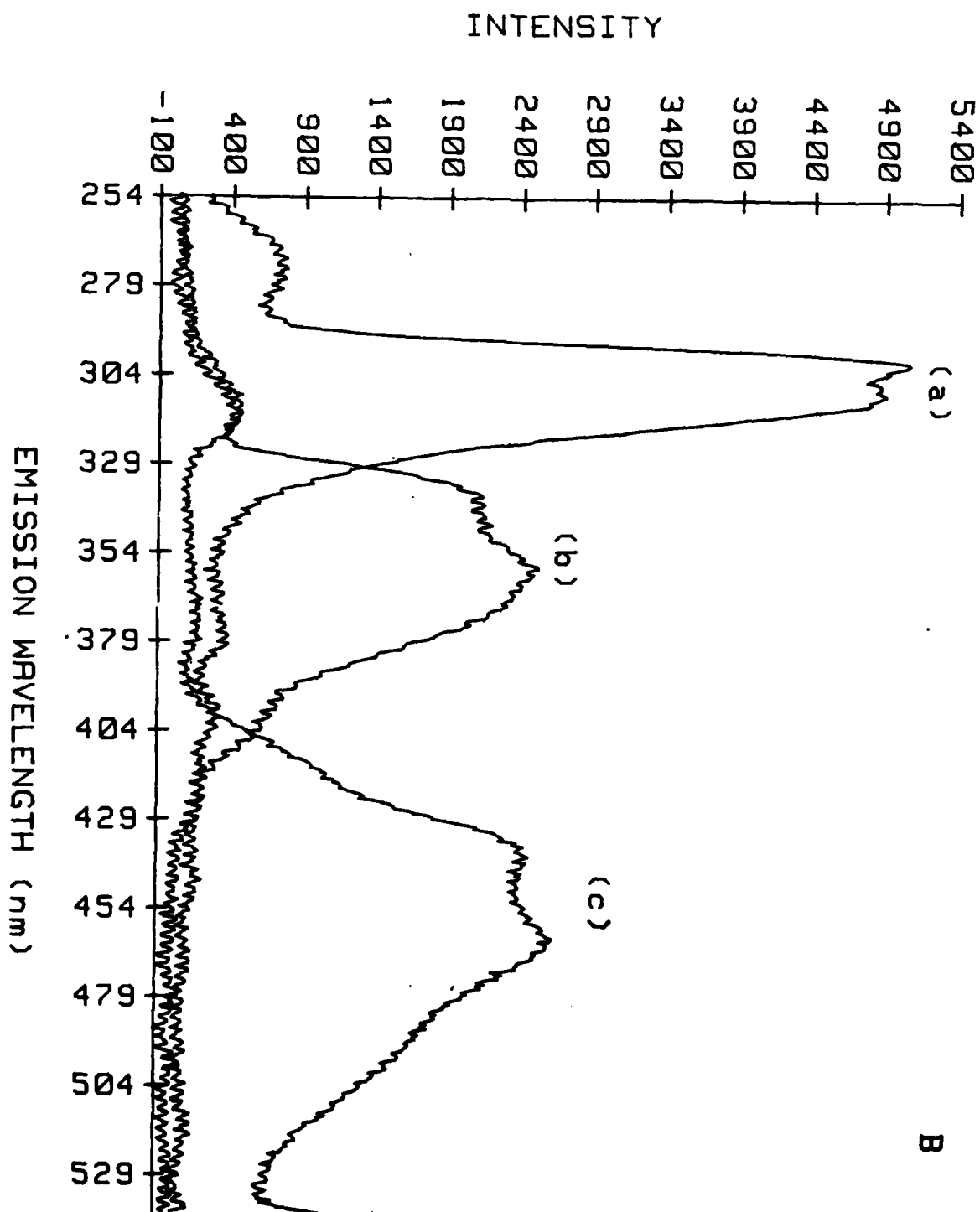


FIGURE 7

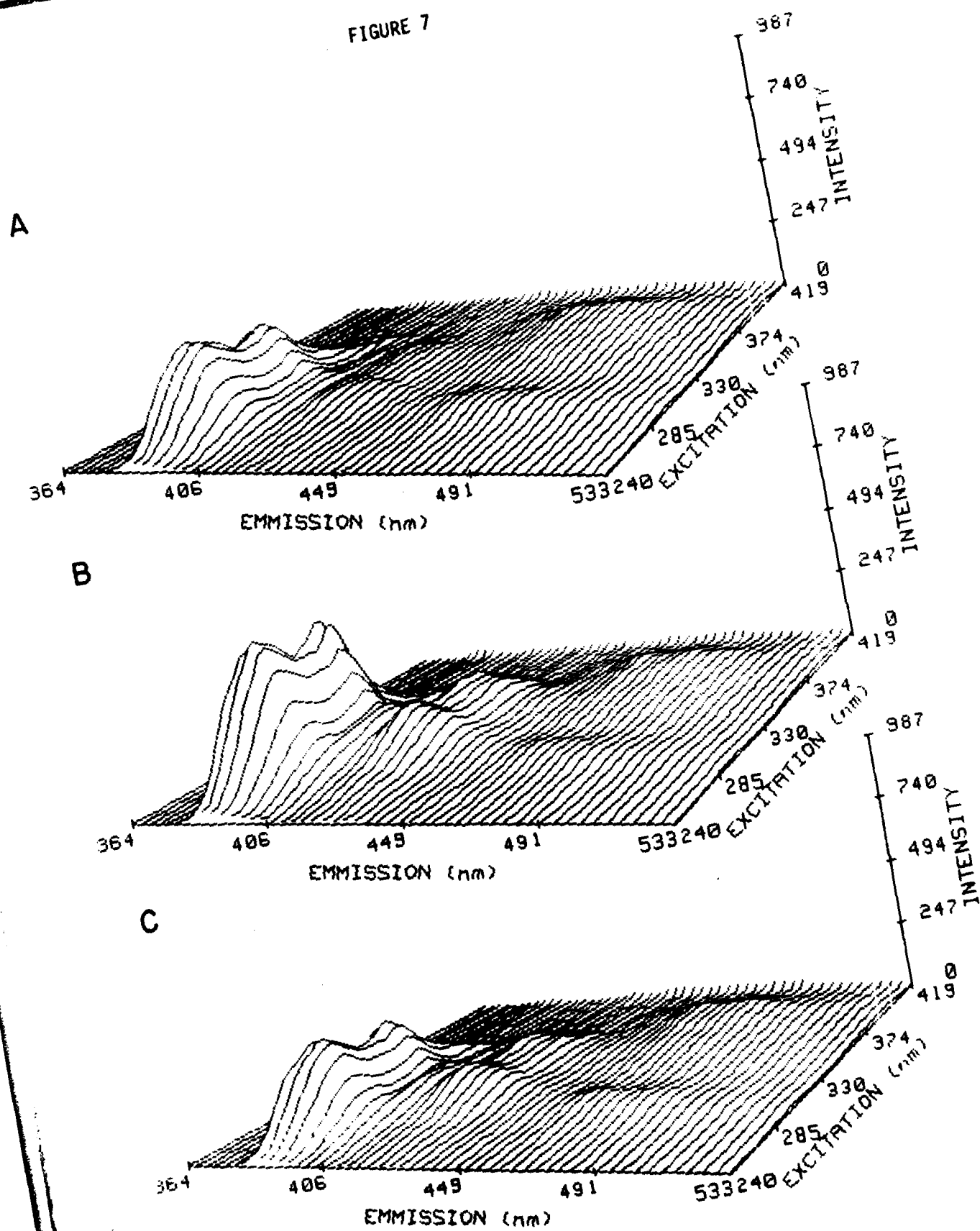


FIGURE 8

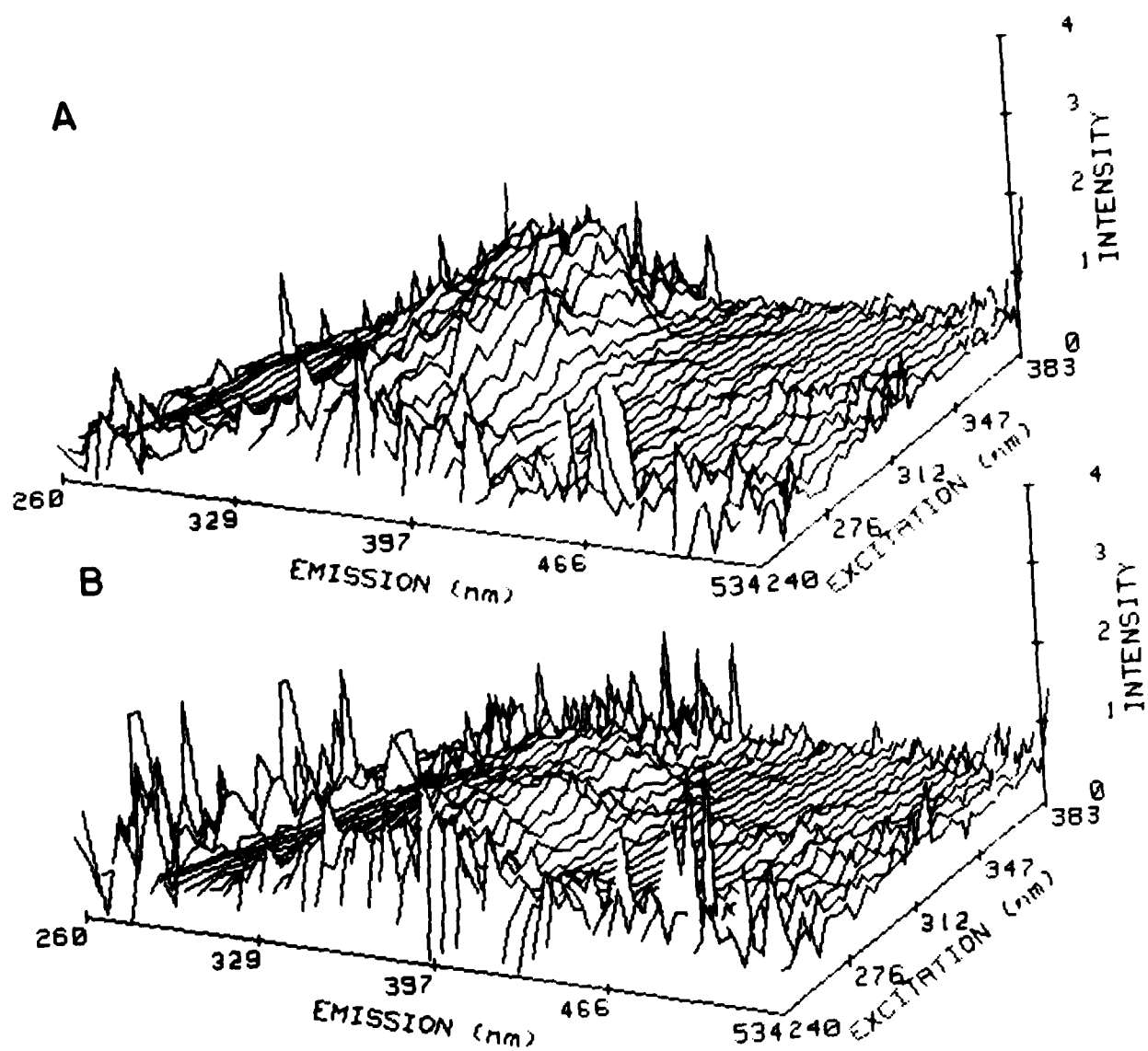


FIGURE 9

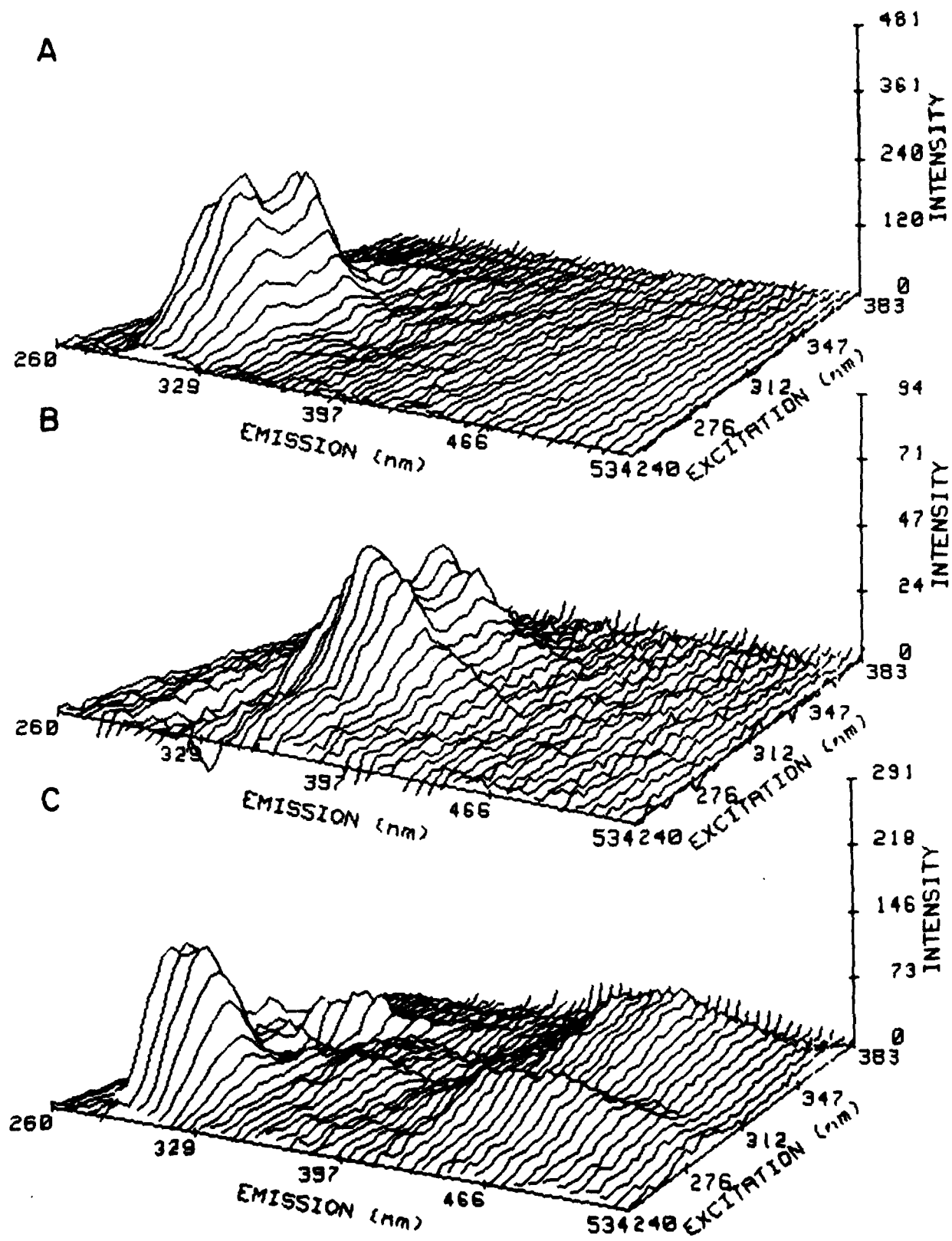
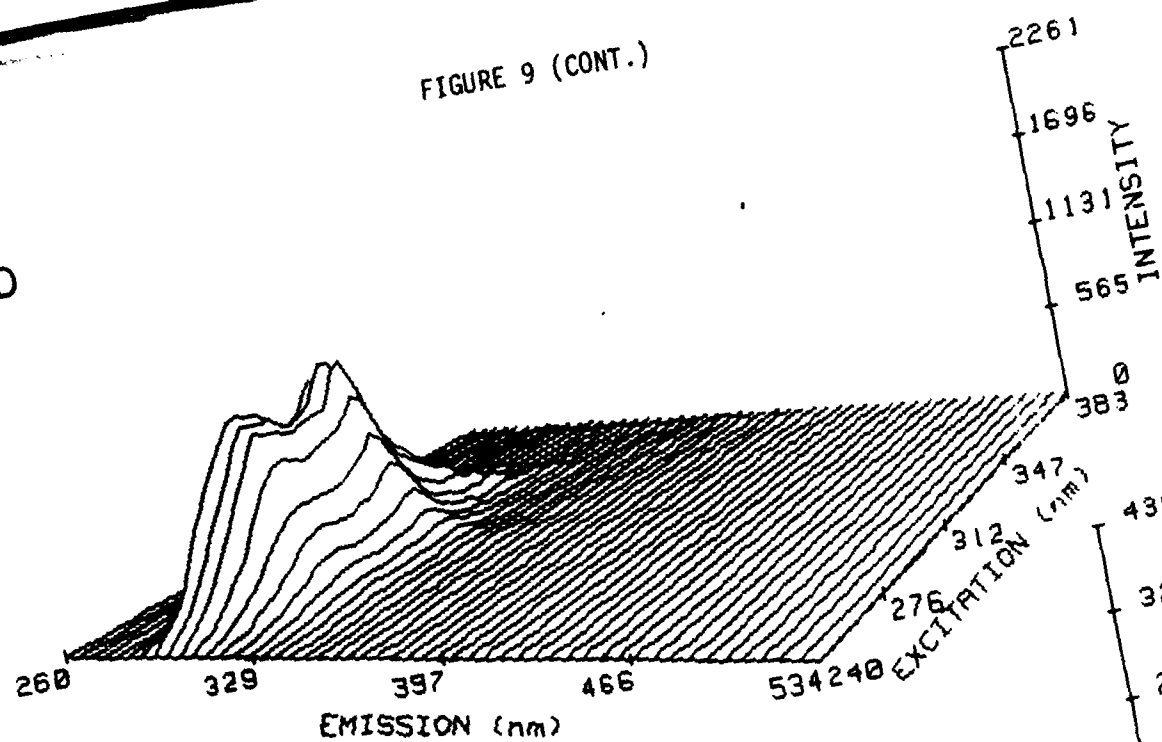
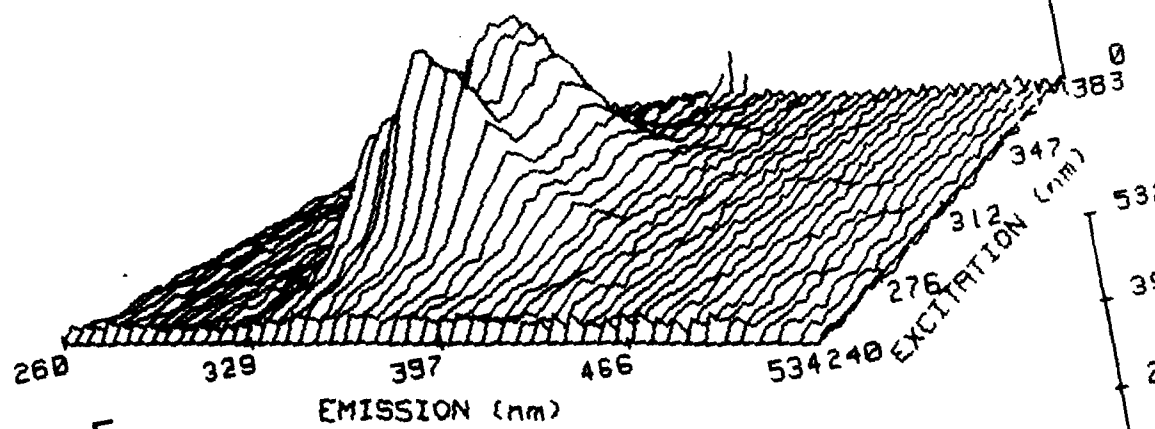


FIGURE 9 (CONT.)

D



E



F

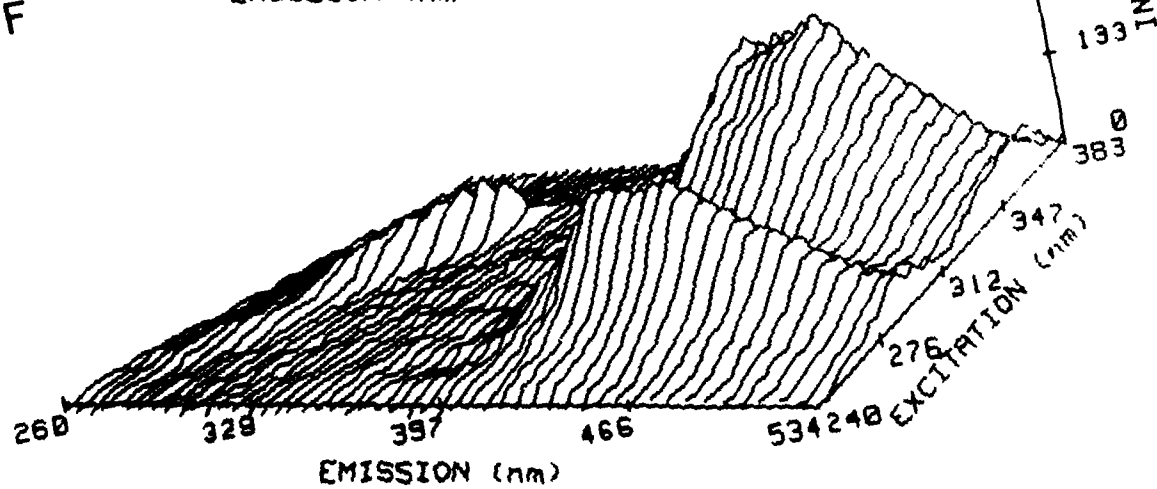


FIGURE 10

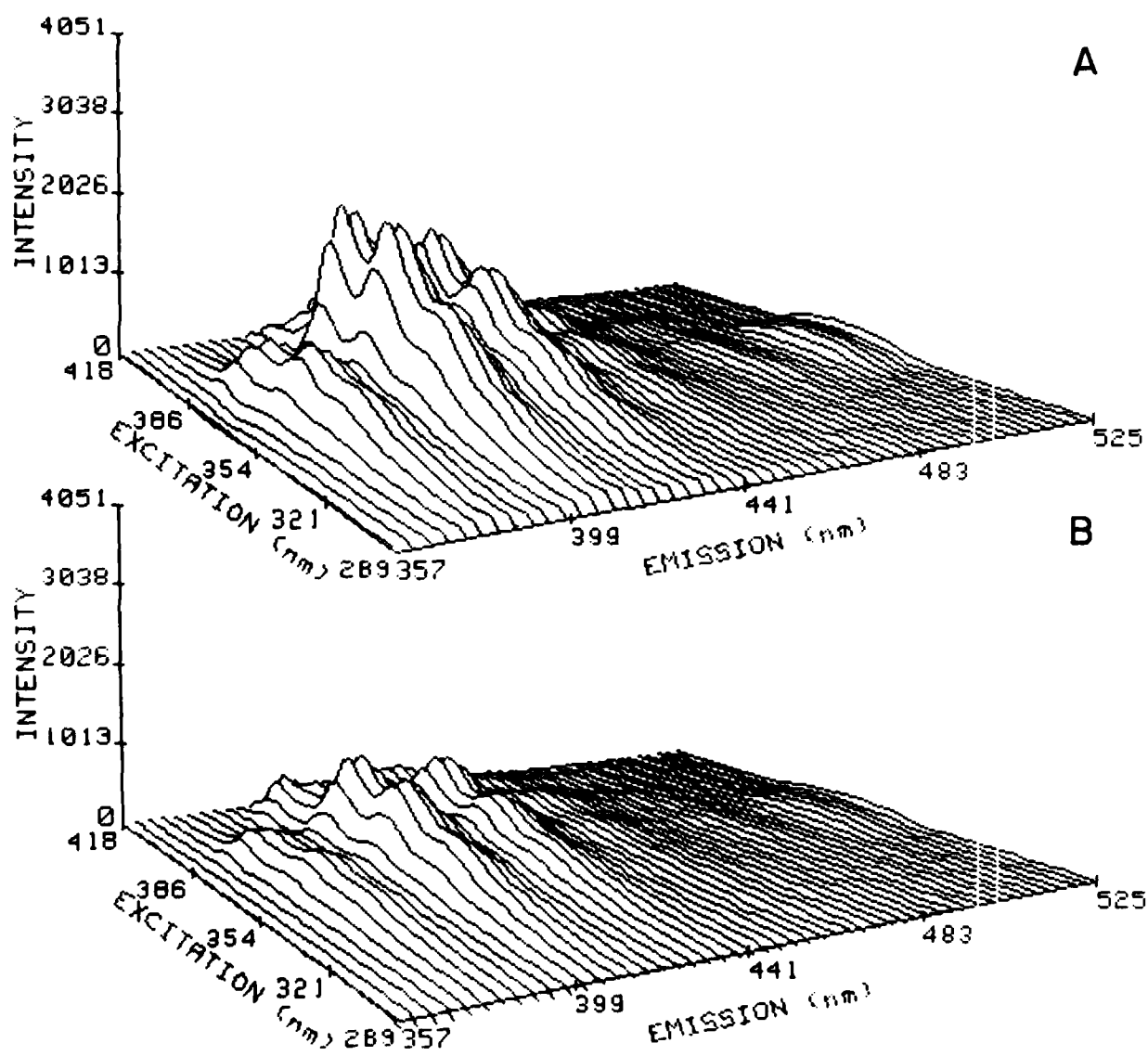


FIGURE 11

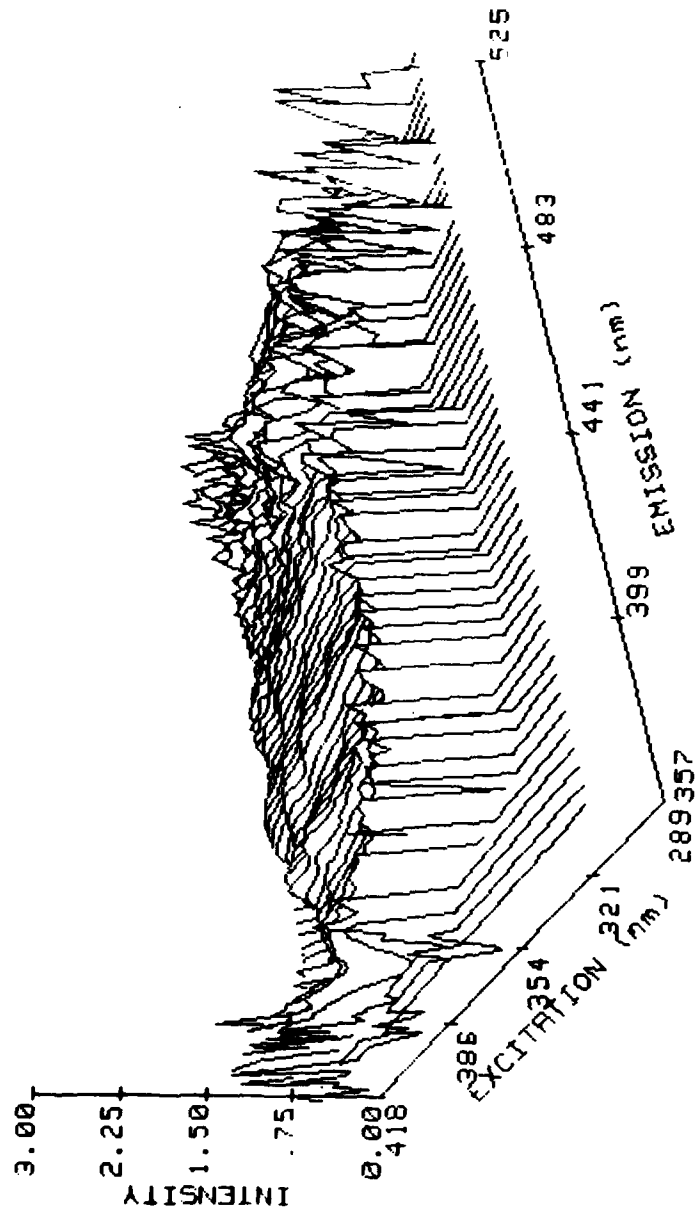


FIGURE 12

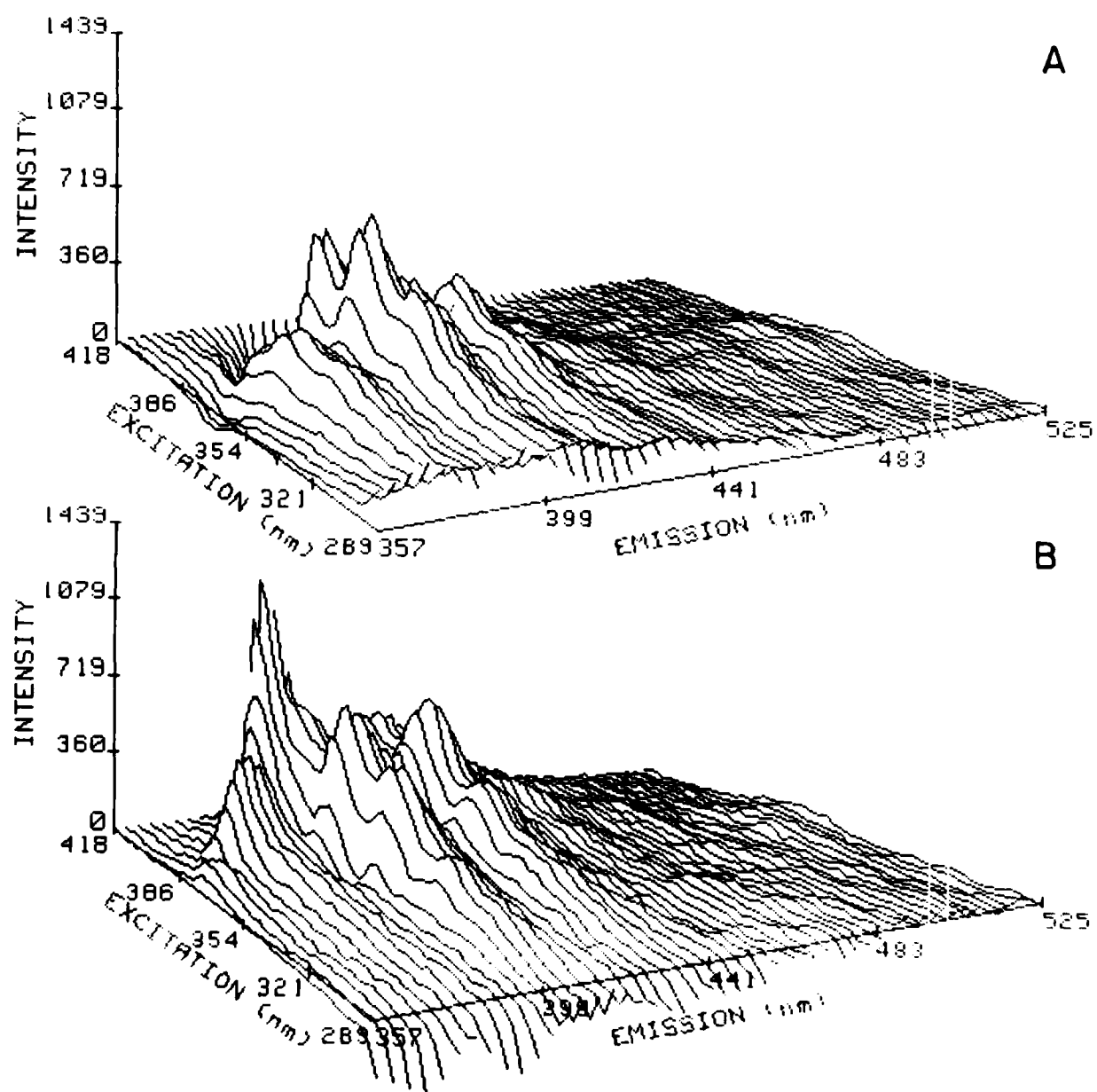
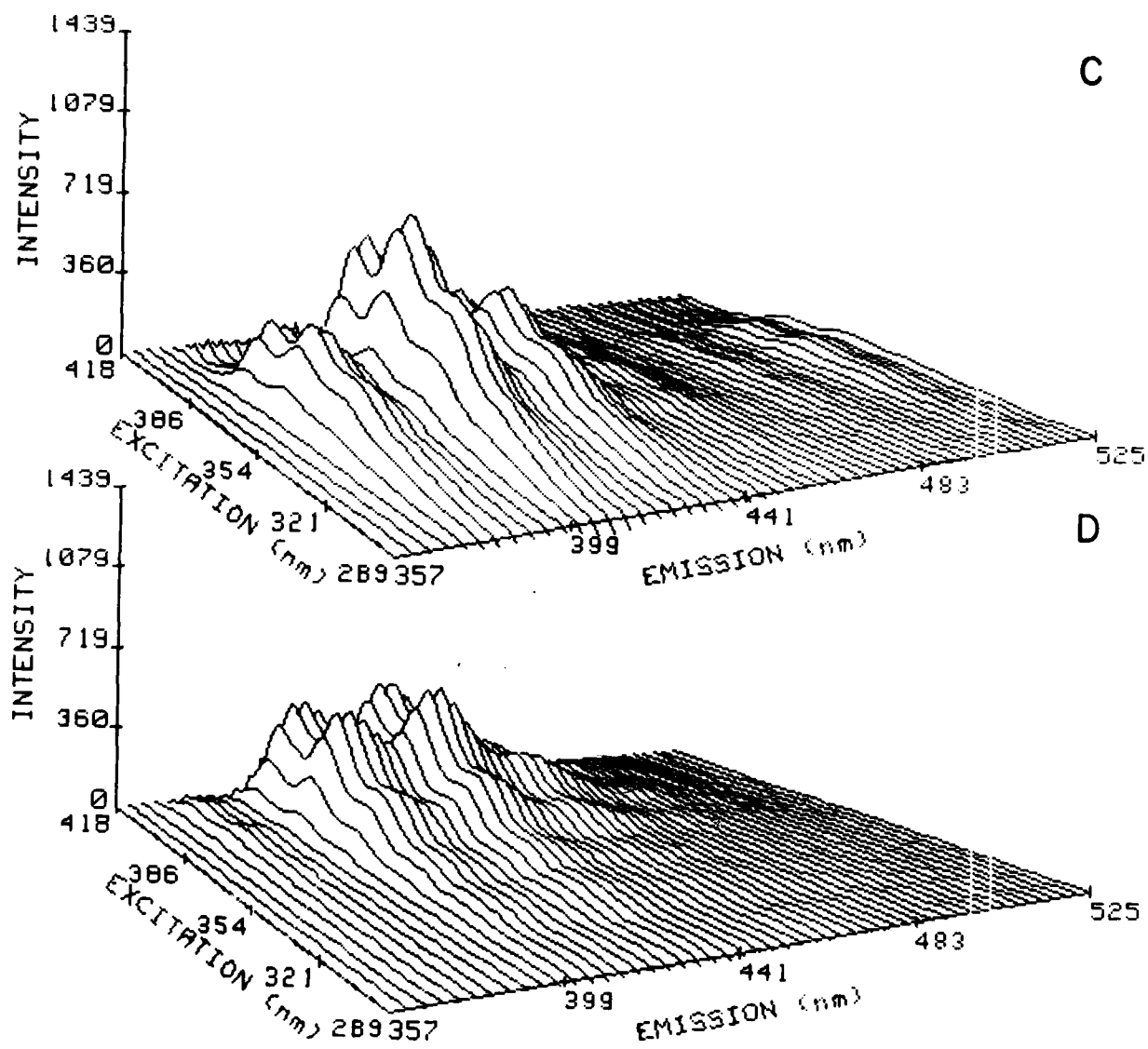


FIGURE 12 (CONT.)



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