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Separation of overlapping spectra from evolving systems using factor analysis. 1. Theory to obtain real spectra

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Abstract

A method based on factor analysis is developed that permits us to retrieve the real spectra and multiplication factors (or concentrations) of individual species in spectra of evolving complex systems with no *a priori* information. The application of constraints of non-negative intensities and non-negative concentrations to the matrices of abstract factors gives the unoptimized real spectra. Then searching for the most likelihood spectra by using the maximum entropy criterion gives the real spectra. Mixtures of experimental spectra are used to illustrate the method.

Keywords: Factor analysis; Eigenspectra; Real spectra; Constraints; Absorption spectroscopy; Fluorescence spectroscopy

1. Introduction

In the spectroscopic study of evolving chemical and specially biological systems one is often confronted with intractable intermediates species. These species are not easily isolated and are therefore difficult to characterize. One is left to speculate about their structures and their characteristics.

We encountered some of these difficulties when we wanted to identify the spectra of the ionic species in the fluorescence spectra of hematoporphyrin IX (Hp) [1]. Hp is a sensitizer used in photodynamic therapy for the treatment of cancer. The determination of the ionic species of Hp at physiological pH is essential to evaluate the biodistribution of the photosensitisers. Several fluorescence studies were unable to determine the exact ionic composition of Hp present in the pH range 3 to 8 [2–8]. The difficulty resides in the presence of several ionic species and aggregates in this pH range.

Another system difficult to study by spectroscopy is amphotericin B (AmB), an important antibiotic used to treat mycotic infections. The usefulness of this drug is limited by its toxicity due to the formation of aggregates. Much effort is now directed towards improving its solubility in aqueous media. One of the most useful analytical methods to study the aggregation properties of this antibiotic is absorption spectroscopy. The difficulty of this method to study AmB resides in the presence of several species in aqueous solu-

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tions. The concentrations of the species vary as the composition of the solvent varies. It is therefore difficult to identify the spectra of the monomers, dimers or aggregates and consequently determine which species is responsible for the toxicity of AmB [9–13].

The preceding studies are only two examples of the difficulties encountered in the study of many biological and chemical systems where it is tedious to obtain the spectra of intermediate species. To overcome the difficulties encountered in the study of the fluorescence spectra of Hp and the study of absorption spectra of AmB, we used the method of factor analysis [14,15].

When we first tried the method of factor analysis, it soon became apparent that the method presently used for the treatment of spectra requires some target spectra or requires that the spectra of the pure species are known in order to obtain real spectra [16,17]. By the nature of evolving biological or chemical systems it is impossible to isolate intermediate species to obtain their spectra. In this paper we present the modifications that we have made to the factor analysis procedure so that we could surmount these difficulties. This method that necessitates no *a priori* information on a system can be used to study a wide variety of situations and not only the two aforementioned examples.

Factor analysis is a computer method that permits the association of attributes in a large data bank containing information on the system under study. In spectroscopy, factor analysis can be used to determine: (1) the number of absorbing or emitting species in a series of spectra of an evolving system; (2) the concentration of each species in this system; and (3) the spectrum of each species [16]. The spectra and the concentration of the species retrieved by factor analysis are in abstract form which are not readily useful for analytical purposes. To obtain the real spectra of the species and their real multiplication factors (MF) from the abstract factors, some *a priori* information must be furnished like target spectra. For the systems aforementioned and similar systems no *a priori* information is available and consequently the real spectra and their concentrations cannot be obtained directly.

To surmount the difficulties of analyzing complex systems by factor analysis some constraints can be applied in the procedure that will permit the retrieval of the real spectra and the real MF of evolving chemical and biological systems. The MF are directly related to the concentration in

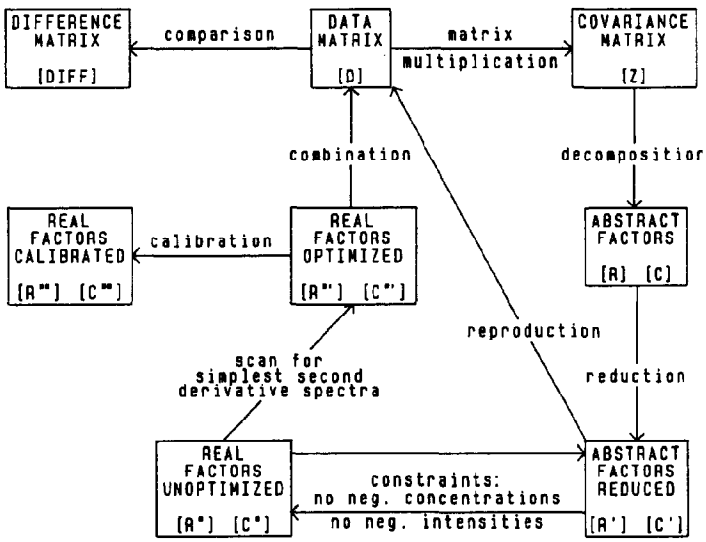

Fig. 1. Steps (indicated with numbers) in factor analysis to retrieve real spectra from a series of experimental spectra.

absorption spectroscopy. For fluorescence spectroscopy, the MF are also related to the concentrations as long as the quantum yield of fluorescence for each species is determined. In factor analysis, the term multiplication factors (MF) that we use in this paper is better suited than the term concentration which is often used.

To illustrate the method that we have developed and to avoid the difficulties of dealing with unknown compounds we employ in this first paper the absorption spectra obtained from three known standards. By mixing the spectra of these compounds in different proportions, we obtained the equivalent of experimental spectra with characteristics similar to evolving biological or chemical systems.

2. Theoretical considerations

2.1. Mathematical considerations

The mathematical formulation of factor analysis can be found in the book of Malinowski and Howery [16] and in other works [17–20]. Here we give only the key equations and the ones that we have incorporated in the procedure to introduce the constraints that permits us to obtain the real spectra with no *a priori* information on the systems. The different operational steps are given in Section 2.2. and in Fig. 1.

The n spectra of the system under study are expressed in digital form to give the elements d_{ik}. These $n \times k$ elements form the data matrix [**D**]. The covariance of [**D**] is obtained by premultiplying it by its transpose

$$[\mathbf{Z}] = [\mathbf{D}]^{\mathrm{T}} \cdot [\mathbf{D}]. \tag{1}$$

The covariance matrix [**Z**] is a $n \times n$ matrix. The diagonalization of this matrix is obtained by finding the eigenvalues Λ_j of the matrix by the operation

$$[\mathbf{Q}]^{-1} \cdot [\mathbf{Z}] \cdot [\mathbf{Q}] = [\Lambda]. \tag{2}$$

From this equation we obtain the set of equations:

$$[\mathbf{Z}] \cdot \mathbf{Q}_j = \Lambda_j \mathbf{Q}_j, \tag{3}$$

where the eigenvalues Λ_j are the elements of the diagonal and the eigenvectors $\mathbf{Q}_j$ is the jth column of [**Q**]. These orthogonal eigenvectors are normalized. From these properties we have:

$$[\mathbf{Q}]^{-1} = [\mathbf{Q}]^{T}, \tag{4}$$

where the matrix $[\mathbf{Q}]^{\mathrm{T}}$ is related to the data matrix [**D**] by:

$$[\mathbf{D}] = [\mathbf{U}] \cdot [\mathbf{Q}]^{\mathrm{T}}, \tag{5}$$

which are expressed by:

$$[\mathbf{D}] = [\mathbf{R}] \cdot [\mathbf{C}]. \tag{6}$$

[**U**] and $[\mathbf{Q}]^{\mathrm{T}}$ are directly related to the abstract factors [**R**] and [**C**] which contains the elements of the orthogonal spectra and MF, respectively. At this stage the dimensions of [**R**] is the same as that of [**D**]. The next operation is to reduce the abstract factors to obtain the minimum eigenvectors that will reproduce the data:

$$[\mathbf{R}] \Rightarrow [\mathbf{R}']$$
$$[\mathbf{C}] \Rightarrow [\mathbf{C}']. \tag{7}$$

This key operation is monitored by the following operations. First the data are reproduced by:

$$[\mathbf{R}'] \cdot [\mathbf{C}'] = [\mathbf{D}'] \tag{8}$$

and compared to the original data

$$[\mathbf{D}'] - [\mathbf{D}] = [\mathbf{DIFF}] = [\mathbf{0}]. \tag{9}$$

The minimum number of rows in [**R**] and the minimum columns in [**C**] are used to satisfy relation (9) within experimental error. The number of rows in [**R**'] and the number of columns in [**C**'] is m which gives the number of evolving species present in the system. The dimension of [**R**'] is $m \times k$.

Muller and Steele used the transformation matrix [**T**] ($m \times m$) to change the abstract factors into real factors [18,19]:

$$\begin{aligned}[\mathbf{D}'] &= [\mathbf{R}'] \cdot [\mathbf{C}'] \\ &= [\mathbf{R}'] \cdot [\mathbf{T}] \cdot [\mathbf{T}]^{-1} \cdot [\mathbf{C}'] \\ &= [\mathbf{R}''] \cdot [\mathbf{C}'']. \end{aligned} \tag{10}$$

These authors impose on the diagonal of [T] the values of ± 1 while the rest of the elements vary to produce real positive absorbance and concentrations. In our case the starting point in [T] is a unit matrix. All the elements in this matrix are modified at each iteration cycle to make the elements in the row and column matrices non negative. The row matrix contains the intensities of the spectra and the column matrix contains the MF. By definition these values cannot be negative for absorption or emission spectra. All the elements in [T] are varied until convergence is obtained.

While maintaining the constraint of non-negative terms, we add another constraint to the matrix [T] by scanning for the most likelihood spectra [20]. This maximum entropy criterion is done by minimizing one or all the following summations. For the spectra we have:

$$\sum_{i=1}^{m}\left(\sum_{j=1}^{k-1}|r_{i,j+1}-r_{i,j}|\right) \tag{11a}$$

where r_j is an intensity element of the spectrum i. The difference term in this summation is the difference between two successive intensity points in a spectrum. $r_{i,j}$ is an element of the intensity matrix [R″]. For the first derivative of the spectra we use the Savitsky and Golay differentiation method [21]. The summations of the first derivative spectra (11b) proceed as in (11a):

$$\sum_{i=1}^{m}\left(\sum_{j=1}^{k-1}\left|\frac{\mathrm{d}r_{i,j+1}}{\mathrm{d}\lambda}-\frac{\mathrm{d}r_{i,j}}{\mathrm{d}\lambda}\right|\right) \tag{11b}$$

where λ is the wavelength and $\mathrm{d}r_{i,j}/\mathrm{d}\lambda$ is the intensity element of the first derivative of the spectra at a given point. For the summations of the second derivative spectra we proceed as for the first derivative to obtain the summations in (11c):

$$\sum_{i=1}^{m}\left(\sum_{j=1}^{k-1}\left|\frac{d^2r_{i,j+1}}{\mathrm{d}\lambda^2}-\frac{\mathrm{d}^2r_{i,j}}{\mathrm{d}\lambda^2}\right|\right) \tag{11c}$$

where $\mathrm{d}^2r_{i,j}/\mathrm{d}\lambda^2$ is the intensity element of the second derivative of the spectra at a given point.

The real factors optimized thus obtained can be combined to give [D′]

$$[\mathbf{R}'''] \cdot [\mathbf{C}'''] = [\mathbf{D}'] \tag{12}$$

which can be compared with the original data matrix [D].

2.2. Operational steps in factor analysis

The different steps in factor analysis used to retrieve real spectra from a series of experimental spectra are illustrated in the diagram shown in Fig. 1. This diagram which is a modification of the one given by Malinowski [16] incorporates the modifications that we have introduced. The spectra is first transformed into the data matrix, [D], the starting point in the factor analysis process.

Step 1 transforms [D] by matrix multiplication into the covariance matrix, [Z].

Step 2 diagonalises and decomposes [Z] into the abstract factors [R] and [C]. The elements with the highest weight factor are situated in the first row of [R] and the first column of [C], respectively. The next rows and columns have decreasing importance. Step 2 is made with the use of the subroutine EIGEN given by Shurvell and Bulmer [17]. This subroutine uses the Jacobian method to determine the characteristic roots of a matrix [22]. Step 1 and step 2 can be verified by reproducing the data matrix.

Step 3 reduces [R] and [C] into the abstract factors reduced [R′] and [C′] by using the minimum number of rows and columns, respectively. These matrices are transformed into spectra and MF curves to evaluate the result of the operation.

Step 4 reproduces the data matrix called now [D′] by combining [R′] and [C′].

Step 5 subtract [D′] from [D] to give [DIFF] which is converted into spectra to evaluate the effectiveness of step 3. If the elements of [DIFF] is not near zero, then another row in [R] and another column in [C] are added to make the new [R′] and [C′], respectively.

Steps 3 to 5 is used to determine the number of components in the system. When the difference spectra are at the zero intensity unit within experimental errors then the reduction process is

satisfactory and the number of components determined is suitable to describe the system. Alex and Savoie have developed a numerical method to determine the number of species in a series of spectra [23]. The later method can be used in conjunction with steps 3 to 5 to verify the reduction process in step 3.

Step 6 is the application of the constraints to the abstract factors [**R**′] and [**C**′] to obtain the real factors unoptimized [**R**″] and [**C**″]. The constraints are: non-negative MF and non-negative spectral intensities. The BOTM algorithm developed by Powell [24] is used to apply these constraints. The success of this operation can be verified by reproducing the data matrix.

Step 7 is used to further optimize the real spectra by scanning for the most likelihood spectra while maintaining the constraints imposed in step 6.

Step 8 combines the real factors optimized [**R**‴] and [**C**‴] to generate the data matrix [**D**′] which can be compared with the original data [**D**].

Step 9 is a calibration procedure since no *a priori* information is furnished to the factor analysis procedure. With the MF curves obtained from [**C**‴] one determines where only one species is present. The MF at that point and the spectrum obtained for that species generates a spectrum which is compared with the equivalent experimental spectrum to give the constant that will calibrate the other MF. Similarly [**R**‴] is calibrated to give [**R**⁗] which can be transformed into spectra.

3. Experimental

3.1. Spectroscopy

The spectra were taken from 400 to 1000 nm on a Shimadzu UV–Vis recording spectrophotometer model UV-160 with a resolution of 3 nm and a precision of the data points of ± 0.5 nm. Three spectra were taken for each sample. The samples were obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA): didynium glass filter (standard reference material 2009a); holmium oxide solution (standard reference material 2034); and glass filter number 50-041 (standard reference material 1930). The band positions agreed perfectly with the NIST standard positions.

3.2. Computer treatment of the data

The data points ($I(\lambda)$ vs. λ (in nm)] stored on floppy diskettes were transferred to a central computer (IBM RS6000) where the computations were carried out. First, the average of three spectra of all the samples was made. These average spectra are called the original spectra.

Secondly, a first series of ten spectra, called the experimental spectra, is made by numerically combining in different proportions the original spectra. These spectra are transformed into the data matrix, [**D**] (600×10), which are successively transformed into abstract factors and into real factors by the method described in Section 2.1. The abstract and real factors are transformed into spectra and MF which are traced on a plotter (IBM 6187-2) to give the figures presented here.

By the same procedure a second series of nine spectra is made with two spectra of the didynium glass filter separated by 2 nm. In this case the dimension of [**D**] is 600×9.

4. Results and discussion

4.1. Mixture of three spectra

Figure 2 shows the ten experimental spectra obtained from the combination of the original spectra of the didynium glass filter, the holmium solution, and the glass filter.

The ten orthogonal spectra obtained from the abstract factors [**R**] are given in Fig. 3. The first three spectra show some positive and negative bands, which is an indication of the number of species in the system and the positions of the bands in the spectra. The top of Fig. 3 shows the first three reduced orthogonal MF. The curves of the seven other MF contained only noise.

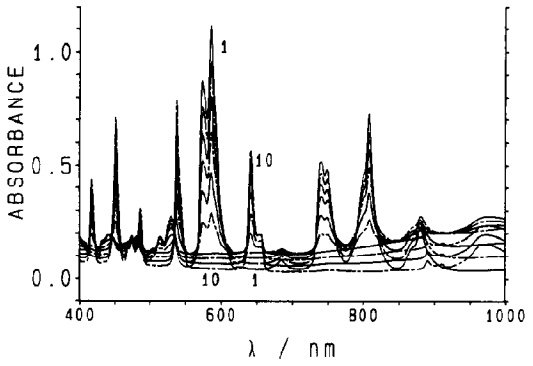

Fig. 2. Ten spectra obtained from the mixtures of the three absorption spectra: didynium glass filter, holmium oxide solution and neutral glass filter.

By recombining the abstract factors [**R**] and [**C**] to reproduce the data matrix [**D**] we verified that the matrix multiplication and decompositions proceeded properly. In this case the ten differ-

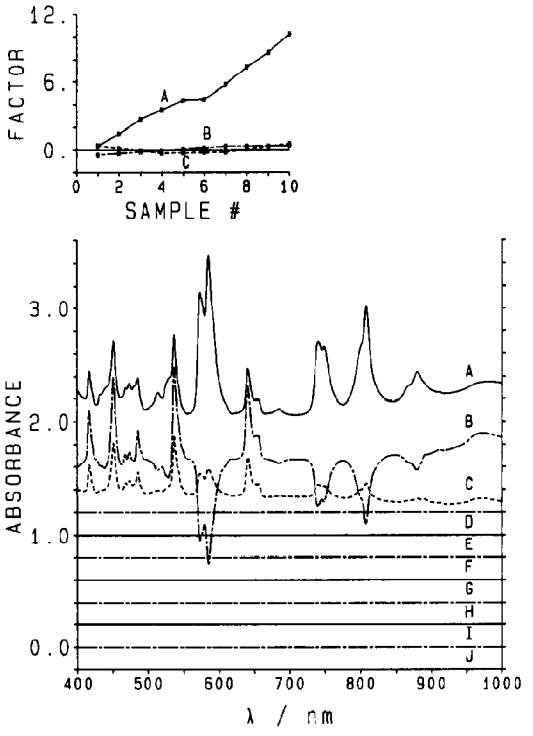

Fig. 3. Abstract factors. Bottom: the ten orthogonal spectra retrieved from the spectra of Fig. 2 (each displaced 0.2 A). Top: the first three MF.

ence spectra obtained by subtracting the original spectra from the reproduced spectra were at zero absorbance unit.

For analytical purposes the orthogonal factors are not very useful other than to indicate the number of species and to verify that the data matrix can be reproduced. From the results of Fig. 3 we see that three species are present because spectra A and B are intense and spectrum C although weak is present. The MF curve A is strong and the MF curves B and C are weak. We will study two possibilities: one with two orthogonal factors and one with three orthogonal factors. When the noise level is higher than the cases that we present here, one has to make the choice by trial and error and with the help of numerical recipes like the one given in Ref. [23].

4.1.1. Choice of two orthogonal factors

From Fig. 3 we take the two strongest orthogonal spectra (traces A and B) with their distribution factors to give the abstract reduced factors [**R'**] and [**C'**], respectively. The combination of these factors gave [**D'**]. The difference between these data and the original spectra of Fig. 2 gave the spectra at the top of Fig. 4. The intensities of these spectra differ considerably from the zero positions where they should be situated had the number of orthogonal spectra been correct. This situation indicates that at least another species is present.

After imposing on the orthogonal matrices the constraints of non-negative band intensities and non-negative concentrations we obtained the two real spectra shown in the bottom of Fig. 4. In the middle of the figure is the distribution factors obtained for this situation. These real spectra contain unrealistic features located on the base lines of the spectra. Here again, these features indicate that at least another species is present in the system under study.

4.1.2. Choice of three orthogonal factors

Next from Fig. 3, we choose spectra A, B, and C with their corresponding MF to give the abstract reduced factors.

The top of Fig. 5 shows the difference between the combination of the orthogonal spectra with

the MF and the original spectra. The difference spectra are at zero absorbance which indicate that the choice of three species as the number of species for this system is adequate.

After imposing the constraints of non-negative concentrations and non negative intensities to the abstract factors reduced the spectra of Fig. 5 were obtained. The MF are shown in the middle of Fig. 5. By looking carefully at spectrum B (Fig. 5) we notice that the slope of the intensities near 600, near 750 and near 800 nm are negative. These positions correspond to some strong absorption in spectrum A and is due to a surcompensation phenomenon. The MF (insert) show some small anomalies: curve B do not start and do not finish at 0.0. These small anomalies indicate that the real spectra retrieved are not absolutely correct.

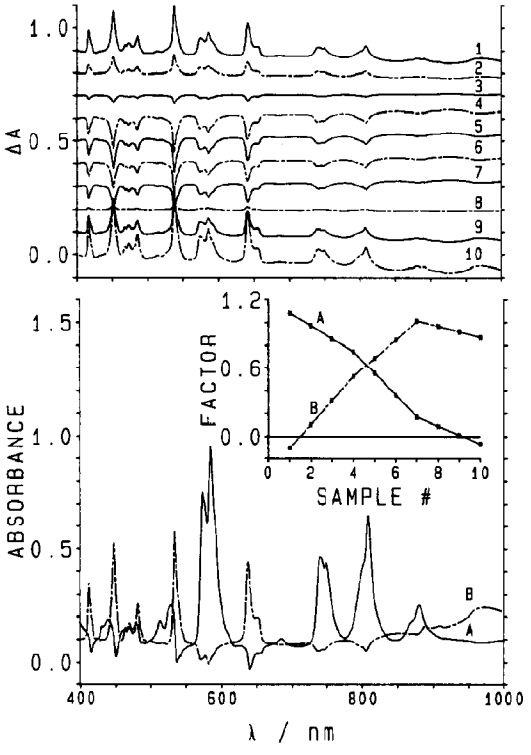

Fig. 4. Use of the first two set of orthogonal factors to obtain real spectra. Bottom, the unoptimized real spectra; insert, the unoptimized MF. Top, difference between the combination of the orthogonal factors (Figs. 3A and B) and the spectra of Fig. 2 (each displaced 0.1 A).

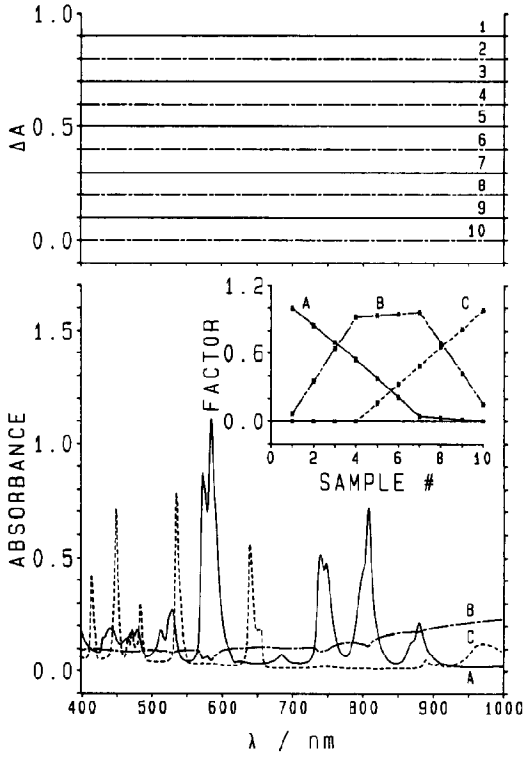

Fig. 5. Use of the first three set of orthogonal factors to obtain real spectra. Bottom, the unoptimized real spectra; insert, the unoptimized MF. Top, difference between the combination of the three spectra generated with the MF and the spectra of Fig. 2.

The search for the most likelihood spectra is added to the constraints. The result is given in the bottom of Fig. 6. The three spectra obtained show no anomalous features. The difference between these spectra normalized and the original spectra are given on the top of Fig. 6. As we see that these difference spectra are at the zero ΔA units which indicates that the spectra retrieved by this procedure are accurate within a normalizing factor.

The MF used to prepare the experimental spectra are given in the left of Fig. 7. The ones given in the right of the same figure are the ones that were retrieved by the factor analysis procedure. The only differences between the two sets

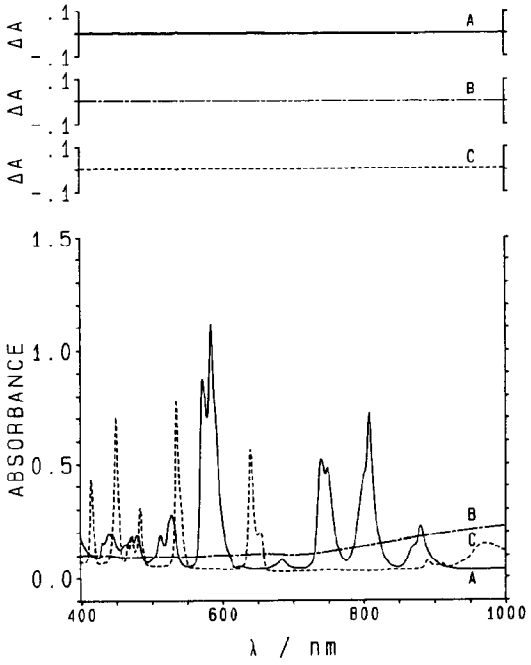

Fig. 6. The three real spectra refined: A, didynium glass filter; B, neutral glass filter; and C, holmium oxide solution. Top, difference between the retrieved and normalized spectra and the original spectra.

are in the slope of the different curves. Since we have furnished no indication on the absorption coefficient of the original species, the difference in the MF is compensated by the intensities of the retrieved spectra.

None the less, it is still possible to calibrate the MF and the spectra. Once we have identified in

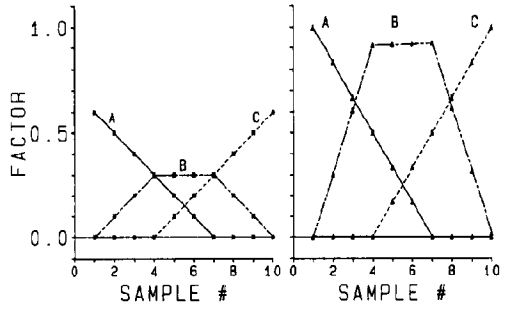

Fig. 7. MF curves for the spectra of Fig. 2. Left, original MF; right, refined real MF retrieved. A, didynium glass filter; B, neutral glass filter; and C, holmium oxide solution.

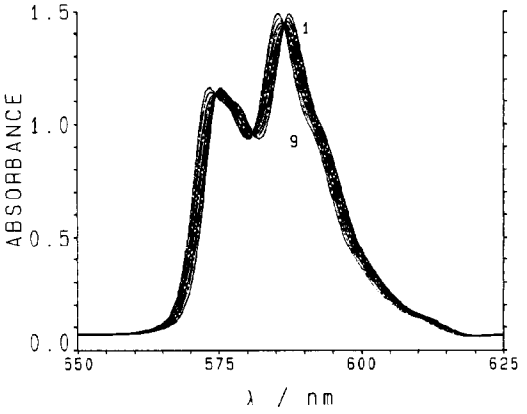

Fig. 8. Spectra of a mixture of two spectra of didynium glass filter separated by 2 nm. The section from 550 to 625 nm is shown.

the original experimental set a spectrum that contains only one species it can be used to determine the normalisation factor. In this case, samples 1 and 10 contain only species A and C, respectively. Either sample can be used to calibrate the other points on the MF curves and subsequently the spectra.

4.2. *Mixture of two identical spectra separated by a few nanometres*

To illustrate the effectiveness of the procedure to separate two close spectra, we used two spectra of the didynium glass filter that we separated by 2 nm. Recall that the resolution of the instrument is 3 nm. Different proportions of these two original spectra are used to make the series of nine experimental spectra. These spectra cover the region from 200 to 1100 nm but we give on Fig. 8 only the region from 550 to 625 nm so that we can distinguish the individual spectra. In biological systems such a situation can be encountered when the chromophores are situated in two media: one in an aqueous environment and the other in a lipidic environment. Such situations are observed when chromophores are dispersed in liposomes.

A portion of the retrieved real spectra is illustrated on the bottom of Fig. 9. On the top of the figure is the difference between the original spec-

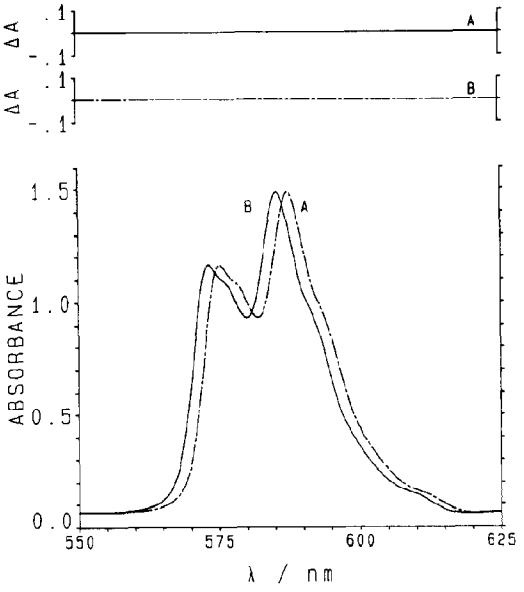

Fig. 9. The refined real spectra retrieved from the spectra of Fig. 8. Top, difference between the retrieved and normalized spectra and the original spectra.

tra and the normalized separated spectra. The difference between the two sets of spectra is at the zero absorbance unit which indicates that the separation procedure did work properly.

The MF used and retrieved for this system are given in Fig. 10. Here again the only difference between the two sets of curves is in the slope. These curves are readily normalized once a sample number of one species is identified. In this case the samples 1 and 9 contains only one species and therefore these points can be used for normalization and subsequently calibrate the other MF points and the spectra.

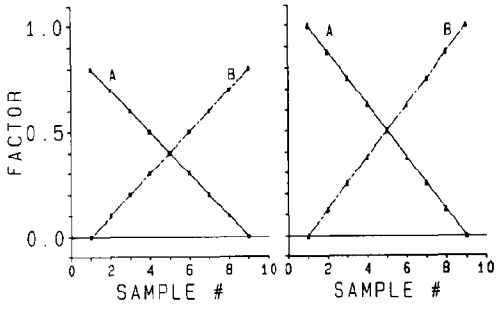

Fig. 10. MF curves used to obtain the spectra of Fig. 8. Left, original MF; right, refined MF retrieved.

5. Conclusion

The procedure that we have developed using the modified factor analysis method permit us to obtain the real absorption or fluorescence spectra of a system without *a priori* information on the system. This method can adequately transform the orthogonal spectra into real spectra using constraints imposed on the matrices of the abstract factors. A first series of constraints based on the definition of a spectrum is used. A second series of constraints based on the maximum likelihood method is added to further optimize the spectra. For these constraints, the use of derivative spectra is efficient to correct small unrealistic features in the base lines that were obtained after the first series of constraints.

The method is efficient in separating the spectra in complex mixtures even when some spectra have no prominent bands and when aggregation complicates the spectra. The separation between two spectra that we have achieved is better than the resolving power of the spectrometer.

Autocalibration of the spectra and MF is possible as long as one point in the multiplication factors is related to one species. If the autocalibration procedure can be achieved, then the precision on the intensities of the separated spectra and the MF would be of the same order of magnitude as that of the original spectra.

Five overlapping spectra identified with as many species were separated from the fluorescence spectra of aqueous solutions of porphyrins obtained in the pH range 0.1 to 13 [14]. In the absorption spectra of amphotericin B dissolved in mixtures of water and propanol, five overlapping spectra were separated [15]. The last two references are only examples of the utility of the method that we have developed. This method is quite general and can be applied to the study of a wide variety of systems using different spectroscopic or chromatographic techniques.

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