

# Sensitivity Test of Derivative Matrix Isopotential Synchronous Fluorimetry and Least Squares Fitting Methods

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**Abstract** Determination of concentrations of spectrally overlapping compounds has special difficulties. Several methods are available to calculate the constituents' concentrations in moderately complex mixtures. A method which can provide information about spectrally hidden components in mixtures is very useful. Two methods powerful in resolving spectral components are compared in this paper. The first method tested is the Derivative Matrix Isopotential Synchronous Fluorimetry (DMISF). It is based on derivative analysis of MISF spectra, which are constructed using isopotential trajectories in the Excitation-Emission Matrix (EEM) of background solution. For DMISF method, a mathematical routine fitting the 3D data of EEMs was developed. The other method tested uses classical Least Squares Fitting (LSF) algorithm, wherein Rayleigh- and Raman-scattering bands may lead to complications. Both methods give excellent sensitivity and have advantages against each other. Detection limits of DMISF and LSF have been determined at very different concentration and noise levels.

**Keywords** Excitation-Emission Matrix · Least squares fitting · Derivative matrix isopotential synchronous fluorescence · Mixture analysis

## Introduction

There are different methods for the determination of concentrations of spectrally closely overlapping compounds in solutions. Earlier, both direct and simultaneous determination of this kind of components have been proposed, such as zero-crossing first-derivative spectrophotometric method [1, 2], synchronous fluorescence based methods [3–6], second derivative spectrophotometric methods [7–9], parallel factor analysis and N-way partial least squares regression [10].

We searched for methods, which have high sensitivity and can tolerate noisy data. A relatively novel fluorometric method called Derivative Matrix Isopotential Synchronous Fluorescence Spectrometry (DMISF) [11–15] and a more widely known method called Least-Squares Fitting (LSF) [16–20] were compared. Although these methods give good results in detection of hidden components, the accuracy of their mathematical procedures has not yet been tested systematically and this comparison is still missing from the literature. We analyzed several measured and computed model spectra to find the sensitivity limits of both DMISF and LSF at different circumstances. It is well known, that measured spectra always have systematic and random errors which disturb the precise comparison of different methods. That is, why we chose using model spectra and this paper presents the results of computational analysis.

As a main goal, error levels and sensitivity limits of detection will be compared to find the main advantages and disadvantages between DMISF and LSF.

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## Experimental details

To establish the model calculations, we present EEMs of rhodamine dyes, which serve as bases for constructing model spectra and matrices. Compounds having similar absorption coefficient and quantum-yield were used for the sake of simplicity. When measuring real spectra, we used the following apparatus and parameters.

### Apparatus

The fluorometric measurements were performed on a Jobin-Yvon Fluorolog Tau3 spectrofluorometer, equipped with a 450 W xenon lamp. As a steady-state fluorometer, it works in single-photon counting mode. Slits' bandpasses of both the excitation and emission monochromators were set to 3 nm. All the measured spectra were obtained using the following parameters:

excitation wavelengths: 400 nm–600 nm, steps: 2 nm;  
emission wavelengths: 500 nm–650 nm, steps: 2 nm.

The temperature of the samples was set to 21 °C and was controlled within  $\pm 0.1$  °C using a Wavelength Electronics LFI-3751 Peltier device. Hellma quartz cuvettes with 1 cm optical path length were used in the measurements.

### Chemicals

The fluorophores were Rhodamine 6G and Rhodamine B, purchased from Sigma-Aldrich and diluted in ethanol of spectroscopic grade. Stock solutions of  $1 \cdot 10^{-5}$  M were prepared and kept in dark at room temperature.  $1 \cdot 10^{-7}$  M solution were prepared by mixing the appropriate amounts of stock solutions of fluorophores with ethanol.

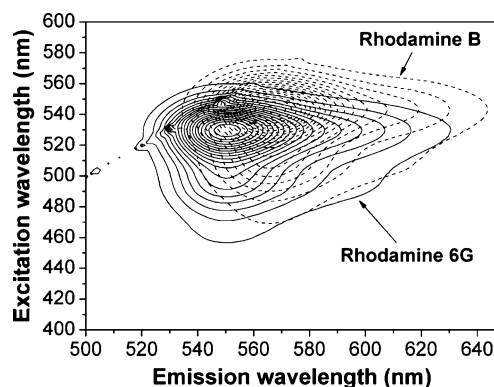
### Excitation-Emission Matrix of real compounds

Figure 1 shows EEMs of Rhodamine 6G and B. The two spectral maxima are very close to each other. The spectral distances of maxima are roughly 15 nm both in excitation and emission wavelength directions. It is obvious from Fig. 1 that determination of concentration can be problematic when the concentration ratio of the compounds is lower than 1:10.

## Details of simulations

### Lognormal spectral shape

Lognormal shape was chosen for both excitation and emission model spectra. It is a widespread and theoretically established approximation in cases of several organic



**Fig. 1** Contour plot of EEMs of closely overlapping compounds:  $10^{-7}$  M Rhodamine 6G ( $\lambda_{\text{exc}}=530$  nm;  $\lambda_{\text{emi}}=550$  nm) and  $10^{-7}$  M Rhodamine B ( $\lambda_{\text{exc}}=542$  nm;  $\lambda_{\text{emi}}=566$  nm). The wavelengths given are the spectral coordinates of maximal intensities

molecules [21–23]. Figure 2 displays an example. According to [22], the  $I(\lambda)$  intensity function is:

$$I(\lambda) = I\left(\frac{10^4}{\lambda_m}\right) \cdot \exp\left\{-\left\{\frac{\ln 2}{\ln^2 \rho}\right\} \cdot \ln^2\left[\frac{a - \frac{10^4}{\lambda}}{a - \frac{10^4}{\lambda_m}}\right]\right\} \text{ at } \lambda < a, \quad (1)$$

$$I(\lambda) = 0 \text{ at } \lambda \geq a. \quad (2)$$

$\rho$  is the band asymmetry parameter:

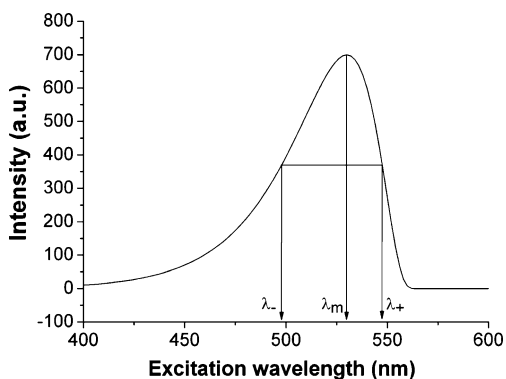
$$\rho = \frac{\left(\frac{10^4}{\lambda_m} - \frac{10^4}{\lambda_-}\right)}{\left(\frac{10^4}{\lambda_+} - \frac{10^4}{\lambda_m}\right)}, \text{ where} \quad (3)$$

$\lambda_+$  and  $\lambda_-$  are the wavelength positions of the left and right half-maximal amplitudes.  $\lambda_m$  is the wavelength position of the maximal amplitude. In Eq. 1 the function limiting point  $a$  is as follows:

$$a = \frac{\frac{10^4}{\lambda_m^2} - \frac{10^4}{\lambda_- \lambda_+}}{2 \cdot \frac{10^4}{\lambda_m} - \left(\frac{10^4}{\lambda_-} + \frac{10^4}{\lambda_+}\right)}. \quad (4)$$

Originally, all the functions in Eqs. 1–4 were given in wavenumber space [22]. The real measurements are made with spectrofluorometers which practically always measure spectral data in equidistant wavelength points. That is, why Eqs. 1–4 have to be written in wavelength space. The wavenumber ( $\bar{\nu}$ ) and wavelength ( $\lambda$ ) are generally measured in  $\text{cm}^{-1}$  and in nm, respectively, thus the conversation between them is:

$$\bar{\nu} = \frac{10^4}{\lambda}. \quad (5)$$



**Fig. 2** Lognormal shape and its parameters for model spectra

Rayleigh-scattering

In the next step, the band of Rayleigh-scattering is also added to the EEM. It was simulated using a simple Gauss shape and to be similar to Rayleigh-band of the measured matrices.

Poisson-noise

In single-photon counting measurements, the noise is best described by Poisson-type distribution. In spectrofluorometry, it is a widespread detection technique and our JY Fluorolog device uses it, too. That is, why we chose adding Poisson-noise to the pure lognormal shape model matrices. To generate this kind of noise we used Poisson-deviates with rejection method [24]. We use the formalism given in [24]. Continuous distribution is given by:

$$q_x(m)dm = \frac{x^{[m]}e^{-x}}{[m]!} dm, \tag{6}$$

Using the given algorithm in each data-generation cycle we get  $\psi$  with an expected value  $m$ .

$$\psi = Poidev(m);$$

Using the results of several runs, it was thoroughly checked, that the expected value is equal to the variance, as it always should be in case of Poisson-noise.

Finally, the values of EEMs will be

$$I_{i,j}^* = I_{i,j} + \sqrt{I_{i,j}} \cdot (\psi - m) \cdot \zeta, \tag{7}$$

Where  $I_{i,j}$  are the intensity values of the noiseless EEM,  $I_{i,j}^*$  are the intensity values of the noisy EEM ( $i$  is the row,  $j$  is the column serial number),  $\zeta$  is the error level factor. In the calculations  $m=100$  was chosen. In this case, e.g.  $\zeta=0.2$  means 0.2% noise level.

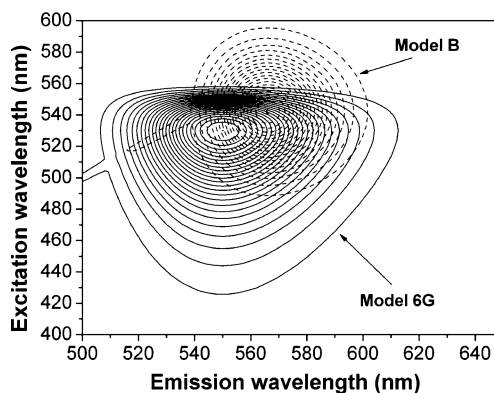
Figures 3 and 4 show contour and surface plots of EEMs of model matrices having lognormal shape in both spectral directions and Poisson-noise. The comparative test of DMISF and LSF method was done using these model functions. In our test, Model 6G serves as the background component and Model B as the component to be quantitatively determined from the mixture of Model 6G and Model B.

EEMs having lognormal spectral shape and Poisson-noise were created with OriginPro® 7 with its built-in C program. Several series of EEMs of model systems of two compounds were analyzed. Every series contained constant quantity of Model 6G and varying amount of Model B in the range of  $10^{-9}$ – $10^{-7}$  M. Series differed in the magnitude of noise, which was set from 0% to 10%.

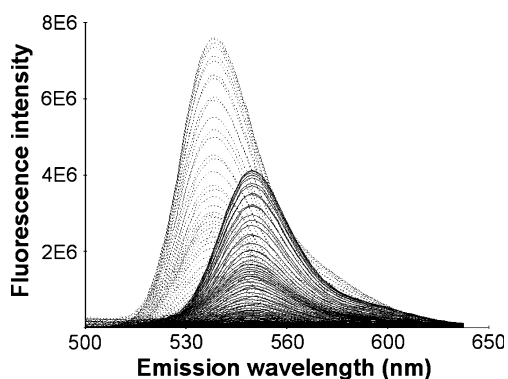
DMISF method

To create a DMISF spectrum, an isopotential trajectory of the background mixture’s 3D EEM at the intensity level of the wanted compound’s (Model B) EEM-maximum (Fig. 3) must be selected first. The concentration of the wanted compound can then be determined by taking the peak-to-peak intensity value of first derivative of the DMISF spectrum.

Now, let us go into details of creating DMISF spectra. In DMISF method, first, we have to search for the spectral position (excitation and emission wavelength coordinates) of fluorescence intensity maximum of the wanted compound’s EEM. Then we can use these spectral coordinates in finding isointensity values of fluorescence of the background component’s EEM. These isointensity values will result in an isopotential trajectory. Now, the interpolated isopotential trajectory’s points from the 3D matrix are to be collected. Identical intensity points along the selected trajectory are generally not in the grid-points of EEM. At this step, Lagrange-interpolation [12] was applied to find the excitation-emission wavelength pairs belonging to the given fluorescence intensity value. Then, an advanced 5



**Fig. 3** Contour plot of EEMs of lognormal model matrices



**Fig. 4** Surface plot of model EEMs of closely overlapping compounds.  $10^{-7}$  M Model 6G (dot) and  $10^{-7}$  M Model B (straight)

points-weighted Bezier-interpolation algorithm is used to obtain the EEM's third coordinates: the intensity values. To get these values at the calculated  $x, y$  coordinates, different precision of Bezier-splines (3, 5 and 7 points-weighted) were compared. We got approximate values within 1% error level with 5 points-weighted Bezier-polynomial using the OriginPro<sup>®</sup> 7 built-in function:

$$B(t) = (1-t)^5 P_0 + 5t(1-t)^4 P_1 + 10t^2(1-t)^3 P_2 + 10t^3(1-t)^2 P_3 + 5t^4(1-t) P_4 + t^5 P_5; \quad (8)$$

$$t \in [0, 1],$$

where  $B(t)$  defines the interpolating curve between the first and last point,  $P_n (n=1...5)$  are the weighting points (two for the left side and two for the right side) of the region where the calculations were done. To reach precise approximation, 1,000 points are evaluated for  $t$  between 0 and 1.

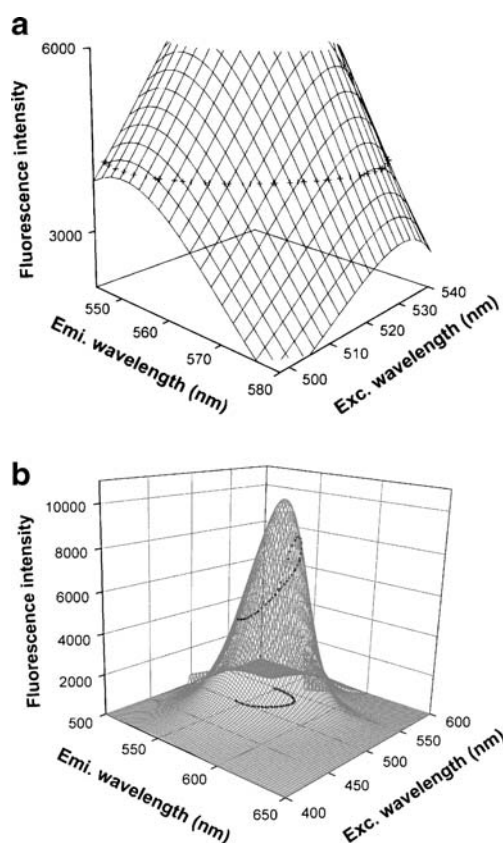
Once the isointensity route had been defined (discrete crosses in Fig. 5a), the belonging intensity values were extracted from the mixture's EEM using the inverse Bezier-interpolation. Figure 5b shows both the isopotential trajectory's spectral coordinates (lower bold dots) and the found intensity values from the mixture's matrix (upper bold dots). The above mentioned calculations were done using another program written by our group in OriginPro<sup>®</sup> 7 C.

The isointensity route means a "determination series", where every point has excitation and emission coordinates. The intensity values of the mixture were obtained along this trajectory. Now we have the Matrix Isopotential Synchronous Fluorimetry (MISF) spectrum. When we find the intensity values of a mixture's matrix, we see the sum of the constant intensity values of the background component and the wanted compound's contribution to the total fluorescence intensity. These spectra has series numbers as the independent coordinate, where behind all of the series

numbers there are excitation and emission wavelength pairs.

First derivative of the MISF spectrum was generated after it had been smoothed using Savitzky-Golay algorithm [25], which performs a local polynomial regression and generates smoothed values for each points, resulting in less fluctuation in the first derivative. To retrieve the wanted fluorophore's concentration in a more noisy mixture, advanced smoothing is necessary. OriginPro<sup>®</sup> 7's Diff/Smooth built-in function was used with different smoothing parameters. Now, we are to get the first derivative of MISF spectrum, which will be called DMISF spectrum. Let us note, that—although in our modeling test, the background component's concentration and thus its fluorescence intensity is constant—here we meet the main advantage of using MISF spectra. Determination of the isointensity trajectory in the background component's EEM means, that using this trajectory, the derivative (DMISF) spectra are independent of the concentration of the background component.

Finally, peak-to-peak [11, 15] evaluations were made to obtain proper intensity vs. concentration graphs in case of the tested series.



**Fig. 5** **a** Selected MISF trajectory (crosses) on the background's ( $10^{-7}$  M Model 6G) EEM. **b** Selected MISF trajectory (lower bold dots) and found values (upper bold dots) on the mixture's ( $10^{-7}$  M Model 6G and  $10^{-7}$  M Model B) EEM

**LSF method**

Using classical LSF, the constituents’ ratio can be determined by solving a linear equation system. The least-squares method minimizes  $\chi^2$ , the sum of the squared residuals of all the spectral points. The applied mathematical routines are fast and they can be used even in case of complex mixtures.

In LSF method, a MathCAD® 14 program was used to solve linear matrix equations to get components’ ratios. We used a domain-limitation to avoid the calculations at unwanted regions of EEM’s (Fig. 6).

This method assumes that  $\chi^2$  is minimal in case of the best fit. Let  $F_1(i,j)$  and  $F_2(i,j)$  stand for the first (let us say the background) and the second (wanted) component’s EEM, respectively. The so-called measured spectrum  $Y(i,j)$  was created by linear combination of the two components (Model 6G and Model B) using Poisson-noise. Now, let us see, how to get the quantities of the two components. Let A and B stand for the factors of the two components. The squared sum of the residuals  $\chi^2$  can be calculated as

$$\chi^2 = \sum_{i,j} (Y(i,j) - (A \cdot F_1(i,j) + B \cdot F_2(i,j)))^2. \tag{9}$$

$$\begin{pmatrix} \sum F_1^2(i,j) & \sum F_2(i,j) \cdot F_1(i,j) \\ \sum F_1(i,j) \cdot F_2(i,j) & \sum F_2^2(i,j) \end{pmatrix} \cdot \begin{pmatrix} A \\ B \end{pmatrix} = \begin{pmatrix} \sum F_1(i,j) \cdot Y(i,j) \\ \sum F_2(i,j) \cdot Y(i,j) \end{pmatrix}. \tag{10}$$

Solving the inverse matrix equation

$$\overline{M} \cdot \overline{X} = \overline{N} \Rightarrow \overline{X} = \overline{M}^{-1} \cdot \overline{N}, \tag{11}$$

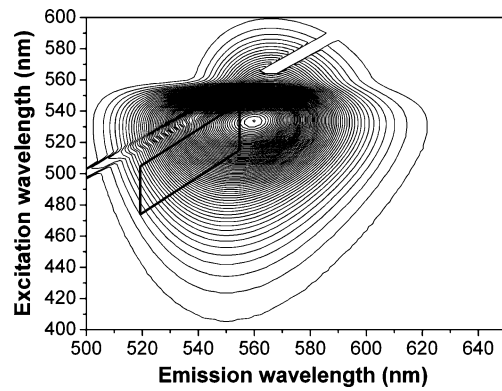
we get:

$$A = \frac{DETA}{DET} = \frac{((\sum F_1(i,j) \cdot Y(i,j)) \cdot (\sum F_1^2(i,j))) - (\sum F_1(i,j) \cdot F_2(i,j)) \cdot (\sum F_2(i,j) \cdot Y(i,j)))}{(\sum F_1^2(i,j)) \cdot (\sum F_2^2(i,j)) - (\sum F_1(i,j) \cdot F_2(i,j))^2}, \tag{12}$$

$$B = \frac{DETB}{DET} = \frac{((\sum F_2(i,j) \cdot Y(i,j)) \cdot (\sum F_1^2(i,j))) - (\sum F_1(i,j) \cdot F_2(i,j)) \cdot (\sum F_1(i,j) \cdot Y(i,j)))}{(\sum F_1^2(i,j)) \cdot (\sum F_2^2(i,j)) - (\sum F_1(i,j) \cdot F_2(i,j))^2}. \tag{13}$$

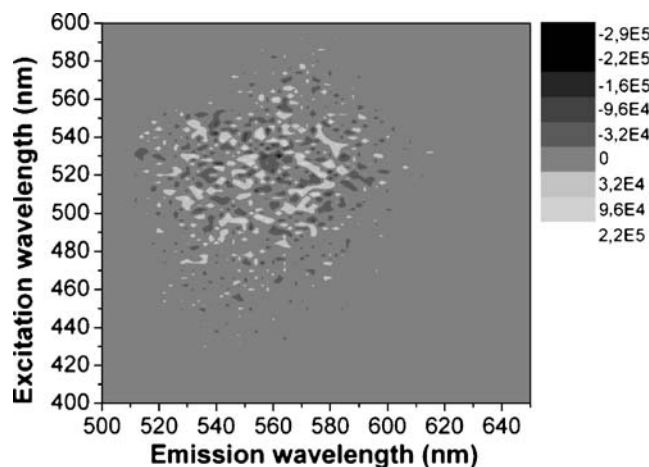
The next step is the determination of a selectable range of EEM. Different spectral regions were analyzed to avoid properly the Rayleigh- and Raman-scattering bands (Fig. 6) and to improve hereby the selectivity. When measuring an EEM, Rayleigh- and Raman bands generally form sharp bands across the EEM. A very small, unavoidable uncer-

tainty in the monochromator positions of the fluorometer can yield several percent change in these band’s intensities. This effect would produce unnecessary error in determining the components’ factors. That is, why LSF procedure should be restricted into a part of the EEM. Rayleigh-scattering has much higher cross section then Raman-



**Fig. 6** Contour plot of mixture of  $10^{-7}$  M Model 6G and  $10^{-7}$  M Model B and the processed area (bold line). Rayleigh- and Raman-scattering regions are excluded from the calculations

The minimum of  $\chi^2$  is reached when  $\partial\chi^2/\partial A = 0$  and  $\partial\chi^2/\partial B = 0$ . The resulted two equations form a linear equation system, which can be expressed in matrix form as follows:

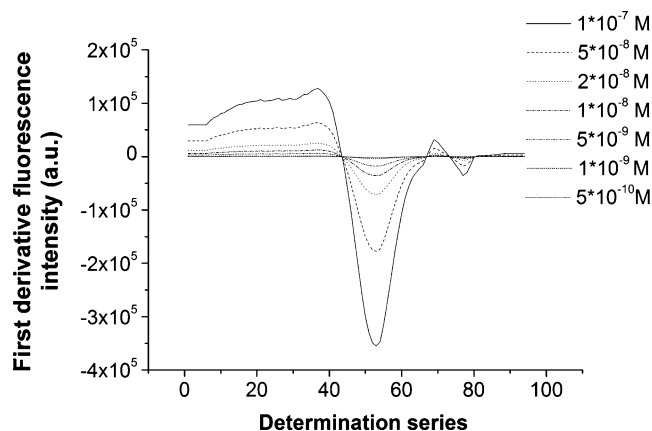


**Fig. 7** Contour plot of matrix of residuals using LSF method. Here, the absolute maximum value is less than 1% of maximum of the mixture's EEM

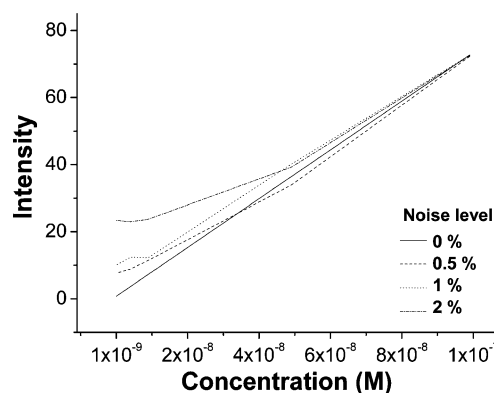
scattering, thus the Rayleigh-band across the EEM is much stronger than the Raman-band. After selecting the proper spectral region, by evaluating the given functions (Eqs. 9–13), we get the concentrations of the components, which is a product of the factors  $A$  or  $B$  and the base concentrations of the components. Also, we get a 3D graph of residuals (Fig. 7), reflecting the accuracy of fitting. The residuals are defined as

$$Res(i, j) = \sum_{i, j} (Y(i, j) - (A \cdot F_1(i, j) + B \cdot F_2(i, j))). \quad (14)$$

The randomly distributing positive and negative value areas of residuals reflect that the optimal factors have been found.



**Fig. 8** DMISF spectra of model mixtures. Concentration of Model 6G is  $10^{-7}$  M in every case. The varying component is Model B, concentrations of it are inserted on the Figure. The noise level is equal to zero



**Fig. 9** Linearity at different concentration / noise ratios using DMISF. Analyzed component is Model B

## Results and discussion

0.0, 0.5, 1.0, 2.0, 5.0 and 10.0 percent of the maximum fluorescence intensity were added to the mixtures as random noise for mapping detection limits at various circumstances. Every single matrix has a unique data set similarly to that of real spectra, which always differ measurement by measurement. To draw reliable conclusions, all the matrix generation and fitting procedures were repeated several times. Statements below are based on the averaging of these independent calculations. We found, that averaging of five independent calculations' results can give stable and reliable data.

For practical reasons, we chose to add Poisson-noise to the model spectra. Other types of noise could be tested, of course, but we believe, that the conclusion of this comparative test would not change significantly.

## DMISF

The peak-to-peak intensity in a DMISF spectrum is proportional to the wanted compound's concentration (Fig. 8) [11–15]. After averaging the results of more calculations, the linearity of fluorescence intensity of Model B in the presence of Model 6G is already adequate. Figure 9 shows the intensity vs. concentration graph using mixture of Model 6G in constant concentration and Model B in varying concentration. The method is sensitive to the noise. It means that even it gives very good finding of concentration at low noise levels, the error increases significantly at higher noise level. The higher the noise level, the bigger the deviation from the correct concentration value. The calculated concentration is always higher than the exact one. This effect is due to the fact that the noise appears as a virtual component. In other words, when the noise level is comparable to the wanted component's intensity, the noise at least partly raises its intensity. For detailed data see Table 1.

**Table 1** Sensitivity limits at different concentration / noise ratios using DMISF. Accepted region (concentration determination within 5% error level) is marked with light grey background. Searched component is Model B

Noise/Conc.	0%	0,5%	1%	2%	5%	10%
<b>1.00E-7</b>	1.00E-7	0.99E-7	0.99E-7	0.99E-7	1.26E-7	2.12E-7
<b>5.00E-8</b>	5.00E-8	4.95E-8	5.21E-8	5.38E-8	9.99E-8	19.9E-8
<b>1.00E-8</b>	1.00E-8	1.21E-8	1.68E-8	3.26E-8	7.16E-8	15.6E-8
<b>5.00E-9</b>	5.00E-9	12.1E-9	27.1E-9	31.5E-9	69.4E-9	168E-9
<b>1.00E-9</b>	1.00E-9	10.5E-9	13.7E-9	38.4E-9	52.2E-9	134E-9

## LSF

After fitting in the selected region, a matrix of residuals was always calculated to display the accuracy of LSF method (Fig. 7). This difference matrix has to show a random distribution of positive and negative value areas. If not, the fitting model is inadequate. In other words, (1) further spectral component should be supposed and used in the fitting procedure, or (2) a spectral shift error in the measurements of matrices occurred (it is not so rare at all as one would think).

Figure 9 shows the excellent linearity of determined concentration of the wanted component. Data in Table 2 show, that even at very high (>2%) error level, the finding of the original concentration is excellent. The wanted component can be qualitatively identified at more than two orders of magnitude lower concentration, than that of the background component.

## Conclusion

It was found that the simultaneous detection of spectrally closely overlapping compounds is possible using both MISF method combined with derivative techniques and LSF method. The DMISF method has high sensitivity and selectivity, but at higher noise levels the sensitivity of detection decreases more rapidly (Fig. 9). DMISF is capable to discriminate fluorophores of similar quantum-yield being in not more than 1% concentration compared to interfering high concentration components at low noise level. Detection limits of the wanted compound were 10% of the background when the noise level was 0.5% and 50%

**Table 2** Sensitivity limits at different noise/concentration ratios using LSF. Accepted region (concentration determination within 1% error level) is marked with light grey background. Searched component is Model B

Noise/Conc.	0%	0,5%	1%	2%	5%	10%
<b>1.00E-7</b>	1.0E-7	1.0E-7	1.0E-7	1.00E-7	1.00E-7	0.998E-7
<b>5.00E-8</b>	5.0E-8	5.0E-8	5.0E-8	5.0E-8	5.09E-8	4.98E-8
<b>1.00E-8</b>	1.0E-8	1.0E-8	1.0E-8	1.0E-8	1.00E-8	1.00E-8
<b>5.00E-9</b>	5.0E-9	5.0E-9	5.0E-9	5.0E-9	5.00E-9	4.90E-9
<b>1.00E-9</b>	1.0E-9	1.0E-9	1.0E-9	1.0E-9	1.0E-9	1.10E-9

of the background component at 2% noise level (Table 1). In practically noiseless circumstances, DMISF is capable to discriminate compounds differing in their concentrations in more than two orders of magnitude.

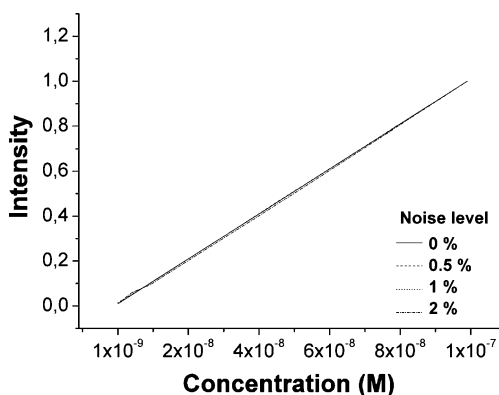
LSF is capable to discriminate 0.1% concentration even at higher noise level (Fig. 10). Detection limits are: 1% at 5% noise; 10% at 10% noise level (Table 2).

At low noise level, DMISF can detect more than one component at the same time and its results can be very reproducible and stable using proper smoothing algorithms. In routine measurements, DMISF needs much less data, which means less measuring time. It is enough to measure fluorescence intensity data along the isopotential trajectory calculated in the preliminary measurements.

At higher noise level and even in the presence of more unknown components in mixtures, LSF is the preferable method. It is able to detect one component even at 0.1% concentration compared to the background. We attribute this higher sensitivity to the higher number of data points used in the fitting algorithm.

Originally, both methods based on the same data sets, namely, on the EEMs. After the preliminary measurements, different subsets can be selected from EEMs to optimize the numerical procedures. But these subsets can only be well selected after measuring the whole matrices.

DMISF can be extended to not more than three component mixtures, where parallel determination of two components is possible. This kind of limitation is not present with LSF, where the number of components to be determined is not limited. Although LSF is unbeatable in finding components being in very low concentrations, DMISF has a significant advantage: it is not sensitive to the amount of the background component. Because of the derivative calculation technique,



**Fig. 10** Linearity at different concentration/noise ratios using LSF. Analyzed component is Model B

the constant background disappears from the DMISF spectra even if it was at higher or lower concentration. This property gives a very valuable advantage to DMISF. Unfortunately, in the practice of spectroscopists, not all of the spectrofluorometers can be programmed to scan along an arbitrary route in the EEM. When a spectrofluorometer can scan not only using equidistant spectral points, the measurement's time is reduced drastically and routine scans can be run in several seconds.

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