UNIVERSITY OF PÉCS

Doctoral School of Physics

Improvement of optical spectroscopic methods

PhD Thesis

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Thesis

Improvement of optical spectroscopic methods

Introduction and aims

Fluorescence spectroscopy methods and procedures are widely used in the medical, biochemical and biophysical research. These popular methods help not only the purely chemical or physical measures, their borders are increasingly merged. The combination of disciplines is suitable to show certain components in a mixture by means of more sensitive, more effective methods which can be useful in a drug test, or to show the effects of some processes in a biological sample. These spectroscopic methods' indisputable advantages are the extraordinary sensitivity and temporal resolution.

However theory needs to keep up with the evolution of techniques. The smaller the time interval measured, the greater the chance to occur a systematic error and by increasing the sensitivity of the measurement the noise also increases. With sufficiently high energy illumination other unexpected effects may occur, such as complexation, side reactions, or other energy transfer-associated internal conversion. By measuring with modern devices we get sufficiently high resolution data set, therefore throughout the data processing we have to mainly focus on the applied methods. The more accurate the applied smoothing, fitting and search algorithm, the better the sensitivity and reproducibility of the procedure.

The main objective of my research activity was the evaluation and comparison of known optical spectroscopic techniques and the development of such new

numerical methods which can further enhance the reliability and accuracy of evaluations. In the first part of my research I investigated and developed further the detection methods for mixtures consisting of components with similar quantum efficiency. In the second part I investigated the possibility of sorting by certain properties occurring as a result of unknown biological and chemical processes.

Results

1. *Fitting methods based on derivative matrix isopotential synchronous fluorimetry and least squares*

During my work I was looking for some techniques which have a sufficiently good resolution and sufficiently resistant to measurement errors. I compared two methods, which are powerful in resolving spectral components. The first increasingly popular method tested was the Derivative Matrix Isopotential Synchronous Fluorimetry (DMISF) [1-5]. This is based on derivative analysis of spectra, which are capable to eliminate the effects of background components during the measurements. Second, a well-established classical method, based on the Least Squares Fitting (LSF) was tested [6-7]. Both methods have their own advantages and drawbacks. The resolution sensitivity of the methods may be classified as an advantage while their noise dependency resulting from the used methods is a drawback. 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 of spectroscopy. It is well known, that measured spectra always have systematic and random errors which disturb the precise comparison of different

methods. That is why I chose using model spectra, which approximate very well the original ones, but their noise can be controlled.

T1 Obtaining the two methods' detection and noise tolerance limit using measured and simulated data

1.1, I developed programs to produce simulated data in good agreement with the measured ones and to set their noise levels.

To test these methods I generated simulated model spectra beside the measured ones. With the help of these data we can bypass systematic errors during the measurement and the signal-to-noise ratio can be precisely adjusted by the corresponding algorithms. Lognormal shape was chosen for both excitation and emission model spectra. It is a widespread and theoretically established approximation in cases of several organic molecules [8-10]. In steady-state measurements, the noise is best described by Poisson-type distribution. That is why I chose adding Poisson-noise to the pure lognormal shape model matrices. To generate this kind of noise I used Poisson-deviates with rejection method.

1.2, I developed a program to produce MISF spectra in case of two and three component mixtures.

I developed a program in Origin C to determine the desired component's intensity maximum and to search for the isopotential trajectory at this level in the interfering background component's matrix. Identical intensity points along the selected trajectory are generally not in the grid-points of excitation-emission matrix (EEM). At this step, Lagrange-interpolation was applied to find the excitation-emission wavelength pairs belonging to the given fluorescence intensity value. Then, an advanced 5 points-weighted Bezier-interpolation

algorithm was 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. I got approximate values within 1% error level with 5 points-weighted Bezier-polynomial. The found series of non-equidistant spectral coordinates belong to the points of an isopotential trajectory. Along on this path by measuring the mixture's fluorescence intensity, we got its MISF spectrum. I used inverse Bezier-interpolation to find these MISF spectra. Before producing their derivatives, smoothing was necessary with Savitzky-Golay algorithm.

1.3, I developed a program package to the use of LSF method in case of two and three component mixtures.

To apply this method I enhanced a program in MathCad® 14 environment. The program solves a linear matrix equation which generates the components' multiplication factors relative to their input matrices. The more data points the algorithm uses, the more accurate is the alignment it allows. However, the outlier data points can easily degrade its efficiency. Therefore calculations within a limited area of evaluation is used, avoiding the matrix points of Rayleigh and Raman scattering provinces in order to improve the accuracy of determination. The program makes it possible to quantify the individual components of mixtures. During the evaluation a residual was calculated from the difference of original and calculated matrices. These positive and negative differences showed small intensity values and random distribution. Otherwise the fitting model is not adequate. Either a spectral shift appeared during the measurement

(incorrect monochromator settings), or we have to apply some additional spectral components throughout the fitting procedure.

1.4, I determined the detection limits and noise-tolerance of DMISF and LSF in case of two and three component mixtures.

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. 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. In case of MISF the detection limits of the desired compound were:

- 1% of the background when the noise level was 0%
- 10% of the background when the noise level was 0.5%
- 50% of the background when the noise level was 2%

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. Detection limits are:

- 0.1% of the background when the noise level was 0%
- 1% of the background when the noise level was 5%
- 10% of the background when the noise level was 10%

T2. Improving the Derivative Matrix Isopotential Synchronous Fluorimetry

2.1, I have improved the DMISF method. I have proved the efficiency of the development with simulations in case of two- and three-component mixtures.

I found that two data points from a three-component mixture's EEM are enough to establish a reliable concentration determination of a desired compound in the presence of two known background compounds of unknown concentration. By selecting proper isopotential trajectories, the wanted component's concentration can be determined both in case of one-component or even two-component background of unknown concentration. It is enough to measure fluorescence intensity just in two spectral points, and the intensity difference [$I(\lambda_{exc1}, \lambda_{emi1})$-$I(\lambda_{exc2}, \lambda_{emi2})$] is proportional to the concentration of the wanted compound. Measurements were done with this new Quick Matrix Isopotential Synchronous Fluorescence (QMISF) method in case of two- and three-component real and simulated mixtures.

2.2, I determined the new method's noise sensitivity

The QMISF method is capable to quantitatively determine fluorophores being in less than 1:200 fluorescence intensity ratio compared to each other even at medium noise level (1%-2% of intensity of the background component). This new method is a significant (approximately two orders of magnitude) improvement compared to the classical DMISF method. At the final form, when the investigation of a certain complex sample has been done, only two spectral points are to be selected from the interpolated EEM. It means that a very sensitive, extremely fast, almost real time measurement can be established for

determining the concentration of a wanted component very precisely. The most important features of the improved MISF are:

- the concentrations of background components are practically indifferent
- the sensitivity is much higher compared to classical DMISF
- measurements only at two spectral positions are enough

A main force of QMISF is that the wanted component can be found at a reasonable measuring time at even - relative concentration compared to the background (assuming similar quantum yields both for wanted and background components). Based on these results, fast routine measurements could be implemented.

2. Analysis of Normalized relative reflectance- and ISSI spectra

T3. Identification of Fusarium Graminearum infection severity of wheat grains by digitally aided spectroscopy

3.1, I investigated the possibilities of the identification severity of wheat grains by fluorescence spectroscopic methods.

I measured EEMs of differently infected wheat grains. Fluorescence emission attributed to the consequence of infection was found with a maximum of λ_{exc}=365 nm and λ_{em}=445 nm. The spectral shape and its position change slightly with the degree of infection. Nevertheless, fluorescence is found to be inadequate for establishing a reliable discrimination method. Reflectance spectra show more significant relation between the degree of infection and the spectral shape. To use these spectra one needs a reference spectral series measured on differently infected seeds of the same wheat type. The comparison of measured and

reference spectra can yield a very good estimation of degree of infection. A 2-5 percentage-point error level can be achieved.

3.2, I developed a new method to rapidly determine the infection level of wheat grains

It can be concluded that the degree of infection causes visible changes mainly in the shorter wavelength regions. The intensity data of the reflectance spectra may vary quite a lot according to the unique shape and reflectance of every single seeds. A normalization method is needed to make these spectra comparable. For this purpose we utilized the fact that there is a long wavelength spectral region where no influence of infection can be observed. From the Normalized relative reflectance spectra it seems obvious that healthy seeds have at least 25-30% less reflection at shorter wavelengths than the infected ones. Based on these facts I used a three-color characterization of the seeds. R, G and B components of seeds' images have been used for spectral analysis. To characterize these spectra I introduced the following function:

$$ISSI(\lambda_1,\lambda_2)=\frac{I_{\lambda_1}-I_{\lambda_2}}{I_{\lambda_1}+I_{\lambda_2}},$$

where λ_1 and λ_2 get their values from the 8 bit R, G or B intensities. Infection sensitive spectral index (ISSI) functions can be used very well for ordering new spectral parameters to each pixel. The use of ISSI (RG), ISSI (RB) and ISSI (GB) functions opens the way to use special, new characterizing parameters. X_{max} and FWHM values of histograms of ISSI-images are simpler but still very useful parameters. I developed a program to get the RGB values from the images and for the preparation of ISSI images.

3.3, I obtained the necessary parameters of the ISSI method for further analysis.

ISSI-analysis of digital images of seeds seems a very reliable and fast method in determining the degree of infection. The most significant change with the level of infection is found in ISSI (RG) images, thus its parameters are the most proper for characterizing the degree of infection. Higher infection means lower X_{max} and FWHM values of ISSI (RG). The largest change can be seen between the healthy and the slightly infected cases. When the level of infection is increasing, the change in X_{max} is less expressed. It means, that a very fast and simple method can be established for deciding whether the sample is infected at all or not. Using digital cameras, a fast single image shot is the first step. The second step is to prepare the histogram of ISSI (RG) image, and finally the X_{max} of this histogram has to be calculated. I used these parameters to estimate the infection level of diseased wheat grains. I found that quantitative determination of the level of infection is not possible. However this method is capable for its primary purpose, to separate infected wheat grains from the healthy ones.

Publications

The publication the thesis is based on:

1. **G. Makkai**, A. Buzády, J. Erostyák:
   Sensitivity Test of Derivative Matrix Isopotential Synchronous Fluorimetry and Least Squares Fitting Methods J. Fluor. 20 (1) January (2010) 87-94.
2. **G. Makkai**, J. Erostyák: *Quick Multicomponent Analysis Based on Matrix Isopotential Synchronous Fluorimetry* ISRN Spectroscopy (2012) http://www.hindawi.com/isrn/spectroscopy/aip/917879/

In preparation:

1. **G. Makkai**, Á. Mesterházy, J. Erostyák:
   Spectroscopy Study of Fusarium graminearium infection of wheat seeds by image histograms
   Optical Engineering (Under Review)

Posters:

1. **G. Makkai**, Á. Mesterházy, J. Erostyák: *Spectroscopy Study of Fusarium graminearium infection of wheat seeds by image histograms*; XV International Symposium on Luminescence Spectrometry (ISLS 2012), June 19-22, (2012), Barcelona; Spain PP43.

2. **G. Makkai**, A. Buzády, J. Erostyák*: Improved sensitivity of matrix isopotential synchronous fluorimetry;* XIV International Symposium on Luminescence Spectrometry (ISLS 2010), July 13-16, (2010), Prague; Czech Republic P082.

3. **G. Makkai**, A. Buzády, J. Erostyák: *Fluorescence Spectroscopy Study of Fusarium graminearium infection of wheat seeds*; 11th International Conference on Methods and Applications of Fluorescence (MAF11), September 6-9, (2009), Budapest; Hungary PO 172.
4. **G. Makkai**, A. Buzády, J. Erostyák: *Matrix Isopotential Synchronous Fluorometry vs. Least Square Fitting. Comparative study of mixture analyzing methods;* XIII International Symposium on Luminescence Spectrometry (ISLS 2008), September 7-11, (2008), Bologna; Italy PO123.
5. **G. Makkai**, A. Buzády, J. Erostyák: *Sensitivity Limits of Matrix Isopotential Synchronous Fluorimetry;* 9th Symposium on Instrumental Analysis (9th SIA), June 29-July 2, (2008), Pécs; Hungary P-41.

Other publications:

1. J. Erostyák**, G. Makkai**, A. Buzády, P. Molnár, S. V. Kukhlevsky:
   Relation between fluorescence decays and temporal evolution of excited states
   J. Fluor. 16 (3) May (2006) 301-307.

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