Determination of relative concentrations in two-component mixtures with unknown composition by laser-induced fluorescence spectra

E O Tsibulskaya and N A Maslov
Khristianovich Institute of Theoretical and Applied Mechanics SB RAS, 4/1 Institutskaya st., Novosibirsk, 630090, Russia
euglenaria@gmail.com

Abstract. The paper is devoted to the development of a new methodology of statistical analysis of laser-induced fluorescence excitation-emission matrices. The method allows one to calculate the number of fluorescent components, their excitation and fluorescence spectra in samples under study without using any prior information about their nature. The algorithm is based on the principal component method and is modified for analysis of excitation-emission matrices. Its features were tested on two-component optical phantoms, namely, optically thin mixtures of fluorescent dyes (pyridoxine and fluorescein). The calculated excitation and fluorescence spectra of components coincided with the initial ones with good accuracy (deviation less than 5% in intensity). The weight coefficients proportional to concentrations were calculated by the new algorithm. The comparison of its ratios with those calculated from known concentrations also showed good agreement (deviation of 5%).

1. Introduction
The method of laser-induced fluorescence (LIF) has found wide application in aerophysics and combustion physics for registration of gas flows and flames [1]. In medicine, this method is used in the optical diagnosis of various diseases and the assessment of the biological tissues’ state [2]. Its advantages are fluorescence’s high sensitivity to changes in a substance and the possibility of non-invasive studies.

Contribution to the fluorescence spectra is determined by all fluorophores, which compose the samples. However, in condensed matter, the molecules are located close to each other. As a result, the energy levels overlap, and the lines in the fluorescence spectra expand, turning into continuous bands. Therefore, in multi-component samples, composition analysis of the samples by spectra is significantly complicated due to overlapping spectra.

The most existing studies use a single laser wavelength to excite fluorescence. However, in multi-component samples, changing the excitation wavelength leads to radical changes in the fluorescence spectra shape due to the different excitation efficiency of the components. That is, the measurement of excitation-emission matrices (sets of fluorescence spectra at different excitation wavelengths) provides additional information that can also be used in data analysis.

At the present moment, the topical problem is a development of sample diagnosis method which includes obtaining information about a number of fluorophores in the samples and its spectral characteristics using only their statistical differences. First of all, the presented problem is important for the study of samples with a previously unknown composition, when there is no any information on
fluorophores available. Also, many substances show spectral variability depending on changes in their environment (temperature, polarity, pH, etc.), which is difficult to evaluate in advance. Therefore, the new algorithm had been proposed to solve this problem and analyze the excitation-emission matrices [3]. It is based on the tensor version of the principal component analysis (PCA). The standard PCA algorithm allows one to best describe the statistical differences in the initial data. However, the calculated principal components are orthogonal, and, consequently, they have a sign variable shape.

This paper investigates the ability of the proposed method to calculate the positive excitation and fluorescence spectra, and to estimate the relative concentrations of fluorophores in the sample. This algorithm was applied to the analysis of optically thin two-component samples’ spectra (optical phantoms) to refine the technique. Concentrations of fluorescent substances were monitored during their creating in presented experiment.

2. Materials and methods

2.1. Experimental setup

Figure 1 shows the experimental setup for measuring of the laser-induced fluorescence spectra. Pulsed laser system with an optical parametric generator (Vibrant (HE) 355 II + UV, Opotek Inc, USA) was used to excite fluorescence. It allows of tuning the wavelength in the range 210 - 350 nm with step of 10 nm.

![Figure 1. Scheme of experimental setup for laser-induced fluorescence spectroscopy.

Figure 2. The excitation-emission matrix of mixture No. 2.](image)

The system of spherical mirrors and the aperture were used to remove the scattered light from the laser system. Laser beam was divided into two beams which were sent to the examined sample and the photodiode to measure the pulse energy. Acton SpectraPro SP2300 spectrograph with a Pixis 256 CCD matrix (Princeton Instruments, United States) was used to record the fluorescence spectra. The 300 nm high pass filter was used to protect the spectrometer matrix from laser radiation.

The excitation-emission matrices were measured in 220-290 nm excitation wavelength range. The pulse energy was limited to 200 J per pulse, because the fluorescence intensity linearly depends on the excitation energy in this range. At higher energy densities, the saturation of the fluorescence signal may occur [4]. So the linear relationship of fluorescence intensity on the absorbed dose was controlled during the measurements of the spectra. Fluorescence spectra were recorded in the wavelength range 340 - 700 nm.

2.2. Modified PCA algorithm

In [3], we had proposed a modified PCA algorithm for calculating the excitation and fluorescence spectra of fluorophores from excitation-emission matrices for samples with initially unknown composition. To calculate the principal components, the tensor form of NIPALS (non-linear iterative...
partial least squares) algorithm [5] was used with several modifications. The total number of fluorophores was chosen based on the assumption of the samples’ nature. The first approximation of principal components was calculated according with the standard algorithm. After that, components were sequentially refined over several hundred iterations using new algorithm of the minimization of initial data’s functional. Unlike the standard NIPALS algorithm, iterative refinement of the principal components allows us to switch from sign variable spectra of the principal components to positive ones. Then, they can be identified using a database of known fluorophores.

3. Results and discussion
The modified PCA algorithm was tested using an optical phantoms, mixtures of two fluorescent dyes, namely, pyridoxine and fluorescein with fluorescence maxima of 395 and 514 nm, respectively. Total amount of examined samples was 3. All mixture samples had been prepared using water solutions with initial concentrations of pyridoxine 5 µg/ml and fluorescein 0.12 µg/ml. At these concentration levels, resulting mixtures were optically thin, i.e., the absorption depth of laser radiation was greater than the thickness of the sample. Series of subsequent dilutions showed that fluorescence intensity linearly depended on the concentration of the substances.

Figure 2 presents the example of excitation-emission matrix for mixture No. 2. All obtained excitation-emission matrices were analyzed using the modified PCA algorithm. After 200 iterations, the RMS deviation of the normalized calculated spectra from the initial ones was within 5% of the maximal intensity.

Figure 3 shows the principal components (PC1, PC2) been obtained as a result of PCA analysis and their comparison with the spectra of the initial fluorophores. The 1st component corresponds to pyridoxine and the 2nd component corresponds to fluorescein, and the calculated spectra repeat the initial ones rather well.

![Figure 3](image-url)  
Figure 3. The principal components: (a) fluorescence spectra, (b) excitation spectra. PC1, PC2 are calculated principal components; P, F are initial substances (pyridoxine and fluorescein, respectively).

The first and the second columns of table 1 present the weight coefficients obtained as a result of PCA analysis. They determine the contributions of the corresponding components to mixtures’ matrices. The ratios of the 2nd principal component’s weight coefficients to 1st ones were calculated for each sample and compared with the concentrations of the initial substances in the mixtures (table 1). It can be seen that the contribution ratios of the principal components coincide with the concentration ratio with deviation of 5%.
Table 1. Calculated weight coefficients, comparison with fluorophores’ concentration ratios

| Sample      | Weight coefficients PC1 | Weight coefficients PC2 | Weight components’ ratios normalized to unity for the first mixture PC2/PC1 | F/P |
|-------------|--------------------------|--------------------------|-----------------------------------------------------------------------------|-----|
| Mixture 1   | 3252.64                  | 666.83                   | 1                                                                           | 1   |
| Mixture 2   | 3043.38                  | 1280.63                  | 2.06                                                                        | 2   |
| Mixture 3   | 6071.1                   | 592.85                   | 0.48                                                                        | 0.5 |

The accuracies of the reconstructed excitation and fluorescence spectra are shown in table 2. They had been calculated as the standard deviation normalized to the spectral maximum intensity and expressed as a percentage. It can be noted that the modified PCA algorithm allows us to calculate the fluorescence and excitation spectra of the initial components from matrices of two-component mixtures and to estimate the relative contributions of these components with sufficient accuracy.

| Fluorophore | Fluorescence spectra (%) | Excitation spectra (%) |
|-------------|--------------------------|------------------------|
| Pyridoxine  | 1                        | 2                      |
| Fluorescein | 5                        | 5                      |

4. Conclusion
The present study showed that the modified PCA algorithm allows one to carry out spectral analysis of sets of optically thin two-component samples of unknown nature. It can calculate with high accuracy the fluorescence and excitation spectra of individual fluorescent components from the excitation-emission matrices. For optical phantoms the principal components’ weight coefficients calculation error was within 5%. The calculated excitation and fluorescence spectra also had coincided in intensity with the initial ones with discrepancies of 5% and lower. The presented algorithm does not use any prior information about the spectra of fluorophores and can be used to study samples with initially unknown composition, like biological tissues.

Acknowledgments
The research was carried out within the framework of the Program of Fundamental Scientific Research of the state academies of sciences in 2013-2020 (project No. AAAA-A18-118021590029-2).

References
[1] Lozano A, Yip B and Hanson R K 1992 Exp. Fluids. 13 369–76
[2] Razhev A M, Iskakov I A, Churkin D S, Orishich A M, Maslov N A, Tsibul'skaya E O, Lomzov A A, Ermukova O V, Trunov A N and Chernykh V V 2018 Quantum Electron. 48 481-86
[3] Maslov N A and Papaeva E O 2016 Tech. Phys. Lett. 42 718-21
[4] Maslov N A 2018 J. Fluoresc. 28 689-93
[5] Geladi P and Kowalski B R 1986 Anal. Chim. Acta. 185 1-17