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Role of non-fluorescent chromophores in inner filter effect correction and PARAFAC decomposition

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Abstract: Numerous studies have shown the impact of inner filter effect (IFE) on the fluorescence signal. IFE reduces the fluorescence intensity and distorts the fluorescence peak shape and position, through the absorption of the emitted radiation by the sample components. In this study, we aimed to understand the role of a non-fluorescing chromophore in IFE correction and PARAFAC decomposition. Solutions of three fluorophores, tryptophan, fluorescein and quinine sulfate, and an absorbing compound, green ink, have been prepared using the controlled dilution approach (CDA). PARAFAC identified three components associated with quinine sulfate, fluorescein and an IFE artifact, which was caused by a shift in peak position. Results showed that the absorption of the chromophore plays an important role in component determination. We observed that the CDA-PARAFAC was able to correct the quinine sulfate and fluorescein signals, and to suppress the IFE artifact component. However, the method was not effective in removing the IFE impact at high concentrations. The results have significant implications on the analysis of samples that contain complex mixtures of fluorophores and chromophores, such as colored natural organic matter or nutrients, like NO_3^{2-} .

Keywords: Inner filter effect, fluorescence spectroscopy, controlled dilution approach, PARAFAC

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1. Introduction

Fluorescence spectroscopy has found several applications in environmental science for water quality monitoring of surface water, ground water, untreated and treated drinking and

wastewater [1]. The technique has been intensively used due to its advantages such as fast measurement, no reagents needed and high sensitivity [2,3]. However, issues have been encountered with its application as several factors can change the fluorescence response. These factors include: the inner filter effect (IFE), temperature, pH or the presence of metal ions [2]. Among these factors, the IFE is the most severe as it can impact the shape of the spectra, reduce the fluorescence intensity and change the position of the peak, making the spectra more difficult to correct [4–7]. The approaches used to correct the IFE, for example absorption- and dilution-based methods, are relatively impractical. The absorption-based method often requires another instrument, while the dilution approach reduces the signal to noise ratio and may contaminate the sample [4,8,9]. Another approach includes the use of specific cuvette configurations to increase the path length, during measurements [7]. However, this method depends on the type of instrument or analyst [7]. The controlled dilution approach (CDA), involving a series of low dilution factors, was proposed by Luciani et al. [8] as a fast and safe solution to IFE correction. Combined with Parallel Factor Analysis (PARAFAC), it can turn into a powerful tool to avoid error and misinterpretation of fluorescence data [8].

In the past decade, PARAFAC has been commonly applied to analyze fluorescence spectra of water organic matter [10]. PARAFAC decomposes excitation-emission matrices (EEMs) into underlying chemical components [11]. Since IFE can distort EEMs, issues arise with PARAFAC decomposition of uncorrected EEMs [11]. For instance, Luciani et al. [8] found the worst results at PARAFAC decomposition on IFE uncorrected EEMs, when measuring overlapping sample components. Several studies have been made so far to show the role of various fluorophores in producing IFE and the optimum method of correcting IFE [4,5,8,12–15]. However, no study was made so far to assess how well the IFE can be corrected, with the CDA-PARAFAC approach, when a non-fluorescent chromophore is present in a solution.

In this study, we aim to understand the role of a non-fluorescent chromophore in IFE correction and PARAFAC decomposition. We applied the CDA-PARAFAC on EEMs recorded for solutions of three fluorophores and one chromophore. We also tested if the approach can be used at different concentration levels.

2. Methods and materials

2.1 Sample preparation

Solutions of fluorescein, quinine sulfate and tryptophan were prepared in 0.1 M H₂SO₄ (Aldrich). All chemicals were analytically graded. The fluorophores were chosen due to their high quantum efficiency and peak overlapping characteristics [16]. Fluorophore solutions were prepared in mixtures of 0 %, 33 %, 66 % and 100 % of an initial concentration of fluorescein (F₀, 75 mg/L) and quinine sulfate (QS₀, 250 mg/L), while tryptophan (170 mg/L) represented 33 % of the total analyzed volume (Table A1). To each mixture, commercially available green ink was added as an absorbing component, in concentrations of 0 %, 10 % and 100 %, corresponding to maximum absorption peak values of 0, 0.1 and 1. A total of 45 solutions were obtained.

Subsequently, these solutions were diluted to apply the CDA correction. Details regarding the CDA methodology can be found in Luciani et al. [8]. The CDA correction takes into account the fluorescence intensity of the undiluted sample ($I_0(\lambda_{ex}, \lambda_{em})$), the intensity of the diluted sample ($I_1(\lambda_{ex}, \lambda_{em})$) and the dilution factor (p), as shown in Eq. (1):

$$\text{Corrected EEM} = \left(\frac{p \cdot I_1(\lambda_{ex}, \lambda_{em})}{I_0(\lambda_{ex}, \lambda_{em})} \right)^{\frac{1}{p-1}}$$

Dilution factors from 2 to 16 were used on the 45 solutions, by mixing them with 0.1 M H₂SO₄. (Fig. A1), based on Eq. (2):

$$D_1 = 2 \cdot D_2 = 8 \cdot D_3 = 16 \cdot D_4 \quad (2)$$

The concentrations D1 and D2 corresponded to a high IFE, while D3 and D4 corresponded to the quasilinear part of the IFE curve. Reference dilutions were prepared to represent the solutions with no IFE and physico-chemical changes [8]. The reference solutions were obtained by mixing 100 μ L of initial solution to 1,900 μ L of 0.1 M H₂SO₄.

2.2 Measurements

The absorption spectra were registered, within the range of 200 to 800 nm, with a Shimadzu-1800 spectrometer using 1 cm quartz cell. The EEMs were acquired with a HITACHI F4500 fluorimeter. The EEMs were measured in the emission wavelength range of 200-600 nm (slit 2.5 nm) and in the excitation wavelength range of 200 to 550 nm (slit 2.5 nm), every 5 nm. The PM voltage was fixed at 700 V and the scan speed at 2,400 nm/min (response 0.1 s). The absorption and fluorescence spectra of each component are presented in Figure A3. The CDA-PARAFAC analysis was detailed by Luciani et al. [8]. Four groups of EEMs were considered for CDA-PARAFAC correction: the EEMs group measured on reference

solutions, the uncorrected EEM group, the absorption corrected EEM and the CDA corrected EEMs. These groups represented the four 3-way tensors with the dimensions 7 x 46 x 51, as detailed by Luciani et al. [8]. Before the CDA-PARAFAC procedure Raman and Rayleigh scattering were removed from the EEMs, according to the method of Zepp et al. [17], CP/PARAFAC algorithm, with “progmeef” Graphical User Interface developed by R. Redon (<http://woms18.univ-tln.fr/progmeef/>) was used for EEM decomposition. The number of components was selected when we obtained the higher component number with a core consistency score above 60%, according to Mounier et al. [18].

3. Results and discussions

3.1 Fluorescence PARAFAC components

Three PARAFAC components were obtained (Fig. 1). Component 1 (C_1) belonged to quinine sulfate, with excitation and emission wavelengths at 250 & 350 / 430 nm. The second component (C_2) was attributed to fluorescein, at $\lambda_{ex/em} = 440 / 510$ nm. The third component (C_3), $\lambda_{ex/em} = 350 / 460$ nm, was believed to be an artifact, as it could not be attributed to any of the substances that were used in the mixtures. C_3 reflected the impact of the IFE on PARAFAC decomposition. The IFE causes a peak shift to shorter or longer wavelengths, depending on component aggregation and solutions composition [5,19]. In the present analysis, the IFE peak position change determined PARAFAC to extract a false third component. Yu et al. [20] also found that component peak shift undermined the global PARAFAC model.

PARAFAC was not able to extract tryptophan. Tryptophan fluorescence intensity may have been quenched by the fluorophores or chromophore.. Galinha et al. [12] and Wang et al. [21] proved that tryptophan-like fluorescence intensity may be quenched by other components competing for similar excitation wavelengths or by complex formation with other components present in the solution. Another explanation may be that the concentration of tryptophan was constant in the solutions and could not be identified as an individual component.

Fig. 1 PARAFAC decomposition of EEMs of fluorescing and absorbing compounds mixtures. C₁ – quinine sulfate; C₂ – fluorescein; C₃ – IFE artifact.

3.2 Chromophore impact on PARAFAC decomposition

The fluorescence response of C₁ (quinine sulfate) and C₂ (fluorescein), at each dilution and chromophore concentration, is presented in Figures 2 and 3. Results showed that only the low dilutions, D₃ and D₄, preserved the linearity of C₁ fluorescence response; however, the chromophore substantially reduced the fluorescence response of C₁. In addition, the fluorescence intensity of quinine sulfate decreased with the chromophore increasing concentration. The fluorescence intensity of D₃ was higher compared to D₄, at the solutions with no chromophore added. However, when the maximum quantity of chromophore was added to the solution, both D₃ and D₄ dilutions displayed the same fluorescence intensity. This finding proved that chromophores can have a substantial impact on the fluorescence signal, even at low fluorophore concentrations, but that the impact is greater at higher concentrations. The fluorescence intensity decreased with ~ 44 %, at D₄ dilution, from the the solution with no chromophore, to the solution with maximum concentration of chromophore. While a ~ 68 % decrease was observed at D₃, at maximum chromophore concentration. Also, the addition of the maximum concentration of chromophore, reduced the fluorescence intensity of D₂ with ~ 67 %. The highest reduction of fluorescence intensity was seen at D₁, of ~ 80 %.

Fig. 2 The fluorescence intensity of quinine sulfate, at each dilution, in relation to the chromophore.

Fig. 3 The fluorescence intensity of fluorescein, at each dilution, in relation to the chromophore.

Similar results were detected for the fluorescein component. We recorded a linear response for D₃ and D₄ (Fig. 3), and a non-linear response for D₁ and D₂. Also, the fluorescence intensity decreased substantially when the highest concentration of chromophore was added to the solution. Solution D₄ showed a ~ 42 % decrease, D₃ a ~ 64 % decrease, D₂ a ~ 50 % decrease and D₁ showed a ~ 78 % decrease in fluorescence intensity, from the solution with no chromophore added. Unlike quinine sulfate, fluorescein dilutions D₁ and D₂ displayed the same fluorescence intensity at the solution with 10 % chromophore and with no chromophore. According to Murphy et al. [11], IFE is most severe at short wavelengths. Quinine sulfate has a higher absorption at shorter wavelengths compared to fluorescein (Fig. A1), indicating that quinine sulfate could be more sensitive to IFE at low chromophore concentrations. In addition, the absorption spectra of quinine sulfate and fluorescein overlap, resulting in further IFE.

The third component, C₃, attributed to the IFE, presented no signal at dilutions D₃ and D₄ because there was no IFE impact at these low concentrations. For dilutions D₁ and D₂, the component presented an ascending trend with each concentration (Fig. 4). The fluorescence intensity of D₂ solutions was similar at all chromophore concentrations. However, the fluorescence intensity was substantially lower at D₁ solution, with maximum chromophore concentration, compared to D₁ solutions with the other chromophore concentrations (10 % and 0 %). The fluorescence signal of C₃ contrasted the response of quinine sulfate and fluorescein, suggesting that C₃ values represented the reduction in fluorescence intensity due to IFE, which resulted in linearity loss, at dilutions D₁ and D₂ of quinine sulfate and

fluorescein. This suggested that PARAFAC may identify the IFE induced by fluorophores but not the effect of chromophores, because that trilinear part of PARAFAC composition is null (no fluorescence response).

Fig. 4. The response of the PARAFAC component associated with IFE in relation to chromophore concentration.

3.3 CDA correction of components

The correction was applied to the combined EEMs dataset of D_1 and D_2 , representing high IFE, and to the dataset of D_3 and D_4 , for low IFE. Based on the 60 % rule of core consistency provided by Mounier et al. [18], we obtained the best decomposition at 4 components, for the D_1 and D_2 dataset, and at only 3 components, for the D_3 and D_4 dataset (Fig. A4). However, the decomposition was forced to three components for better comparison between datasets, and because the fourth component, in the D_1 and D_2 dataset, was most likely another artifact produced by the IFE.

Figure 5 displays the CDA correction of quinine sulfate and fluorescein solutions, for the D_1 and D_2 dataset. CDA successfully established linearity for fluorescein and provided the same fluorescence intensity for each solution, irrespective of chromophore concentration. However, the CDA did not entirely remove the IFE impact on the solutions with the highest concentrations of quinine sulfate and chromophore. At the maximum concentration of quinine sulfate and chromophore, a difference of 38 % of fluorescence intensity from the solution with no chromophore was observed. This was potentially caused by the high susceptibility to

IFE impact of quinine sulfate, compared to fluorescein. C_3 was completely suppressed at all chromophore concentrations.

For low concentrations, D_3 and D_4 , the linearity of the signal was completely restored at both components, C_1 and C_2 (Fig. 5b). The CDA correction for C_3 displayed a flat signal noise.

Fig. 5 CDA correction of quinine sulfate, fluorescein and IFE artifact (C_3) for a) D_1 and D_2 , and b) D_3 and D_4 datasets.

4. Conclusions

In this study, we presented the application of CDA correction on a complex solution of three fluorophores and a chromophore, and the impact of IFE on PARAFAC decomposition. The IFE reduced the fluorescence intensity and produced a fluorescence peak shift, which was identified by PARAFAC as a separate component. In the presence of the chromophore, CDA correction was able to restore the quinine sulfate and fluorescein signals to linearity and to completely suppress the artifact component associated with IFE. Tryptophan signal was not identified by PARAFAC, even after CDA correction. Moreover, the correction method was not sufficiently effective at high concentrations of fluorophore and chromophore. These results have significant implications on the analysis of samples that contain a complex mixture of relatively unknown fluorophores and chromophores, such as the natural organic matter or nutrients, like NO_3^{2-} . Particularly, we recommend to use CDA or absorption correction, systematically, before EEM decomposition. Future studies are needed to find a method of extracting the non-fluorescent compounds, at the same time with fluorescing ones, and to enhance the IFE correction and detection at short wavelengths.

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