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ORIGINAL ARTICLE

Optimizing Parallel Factor (PARAFAC) Assisted Excitation-Emission Matrix Fluorescence (EEMF) Spectroscopic Analysis of Multifluorophoric Mixtures

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Abstract
Parallel factor (PARAFAC) analysis is the most commonly used mathematical technique to analyse the excitation-emission matrix fluorescence (EEMF) data sets of mutifluorophoric mixtures. PARAFAC essentially performs the mathematical chromatography on the EEMF data sets and helps in extracting pure excitation, pure emission and contribution profiles of each of the fluorophores without requiring any pre-separation step. The application of PARAFAC requires the initialisation of the spectral variables that is usually achieved by performing the singular value decomposition (SVD) analysis on EEMF data sets. One of the problem with SVD based initialisation is that it orthogonalises the data sets and makes the PARAFAC modelling of the EEMF data sets computationally challenging task that needs to be taken care. To address this issue, the present introduces an alternate approach for initialising the spectral variables for performing the PARAFAC analysis. The proposed approach essentially involve initialisation of the spectral variables with random numbers in a constraint manner. The proposed approach is found to provide the desired computational economy, robustness and analytical effectiveness to the PARAFAC analysis of EEMF data sets.

Keywords Excitation-emission matrix fluorescence · PARAFAC · Random initialisation · Fluorophores · Spectral variables · Modelling · Biomolecules · Analytical utility

Introduction

Parallel factor (PARAFAC) analysis [1–8] is a chemometric technique used for analysing the multi-way arrays. PARAFAC decomposition of three-way array $\underline{X}$ of dimension $I \times J \times K$ can be described using the Eq. 1

$$X_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \quad (1)$$

In the above equation, F denotes the number of factors, X_{ijk} a_{if}, b_{jf}, c_{kf}, e_{ijk} are elements of $\underline{X}$, A, B, C and $\underline{E}$, respectively. The matrix A, B and C are the loading matrices of dimensions of $I \times F$, $J \times F$, $K \times F$, respectively. $\underline{E}$ is the three way array of dimension $I \times J \times K$. PARAFAC analysis is known to provide the unique solution i.e. it does not have problem of rotation ambiguity. The uniqueness arises because PARAFAC model not only determines the subspace but also defines the position of the axes defining that subspace [1–8]. In addition to this, PARAFAC model also provides the second order advantage i.e. it can be used to analyse the chemical components even in the presence of unknown interferences [1–8]. PARAFAC analysis has been mostly used for analysing the three-way data sets. One of the perquisites to apply PARAFAC algorithm is that three-way data set must have a trilinear structure [1–8]. A three-way array can be classified as trilinear if these three following criterion are satisfied. (a) A three-way array has equal number of factors along each mode (i.e. axis). (b) Each factor has unique profile along these modes. (c) Shape of the profiles for each of these factors along any specific mode is invariant to the changes in other two modes [1–8].

Excitation-emission matrix fluorescence (EEMF) is a multi-parametric fluorescence technique that captures the fluorescence response of all the fluorophore in a single three-dimensional spectrum [9–13]. EEMF has been successfully used for the analysis of petroleum products [14], vegetable oils [15], soil samples [16], water samples [17], ovarian tissue [18], and human skin tumours [19]. A number of

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successful application of EEMF in different fields could be seen in a recent review article reported by Kumar et al [13].

The fluorescence response of a fluorophore in EEMF mode essentially depends on the concentration, excitation wavelength and emission wavelength. Mathematically, it can be summarised using the mathematical expression summarised in Eq. 2

$$I = kClE_{ex}(\lambda_{ex})E_{em}(\lambda_{em}) \quad (2)$$

In the above equation, I is the intensity of a fluorophore in EEMF mode, E_{ex} is the excitation wave-function, E_{em} is the emission wave-function, C is the concentration, l is the path length, λ_{ex} is the excitation wavelength, λ_{em} is the emission wavelength and K is the constant. The trilinearity to EEMF data sets comes naturally and it is so because the E_{ex} and E_{em} wave-functions are independent of the λ_{em} and λ_{ex}, `respectively. The EEMF spectral data sets collected over wide concentration range are truly trilinear in nature. PARAFAC assisted EEMF analysis have been found to be highly efficient in analysing the mixtures of fluorophores having complex spectral overlap [9–13].

PARAFAC model on trilinear data set is usually fitted using alternate least square (ALS) algorithm [4–8]. It requires the initialisation of either of the excitation or emission profiles for each of the F factors to initiate the ALS algorithm [4–8]. The initialisation is usually achieved by carrying out the singular value decomposition (SVD) analysis on suitably unfolded-EEMF data sets [4–8]. It is well known that SVD algorithm essentially orthogonalise the data sets and hence to start with destroy the shape of the spectral profiles associated with each of the F factors. As a result, SVD assisted PARAFAC analysis requires more number of iterations and significantly large amount of computational effort and time. Therefore, a constant effort is required towards optimising the PARAFAC algorithm so that a robust and computationally economical analytical procedure could be developed. Towards this, the present work proposes a novel approach for initialising the spectral variables so that PARAFAC analysis can be carried out (i) with the variables that are not generated under the orthogonal constraints and (ii) in a computationally economical manner. To carry out the present work, EEMF data set of complex mixtures of four biomolecules Catechol, Hydroquinone, Indole and Tryptophan having complex spectral overlap is taken as the test case.

Table 1 Concentrations of catechol, hydroquinone, indole and tryptophan in S1-S25 samples of the designed calibration set

Sample	Catechol ($\times10^{-5}$)	Hydroquinone ($\times10^{-5}$)	Indole ($\times10^{-5}$)	Tryptophan ($\times10^{-5}$)
S1	1.079	2.296	0	0
S2	8.704	2.238	0.128	0.186
S3	4.352	1.119	0.256	0.186
S4	8.704	2.238	0.256	0.744
S5	8.704	2.238	0	0
S6	6.528	1.678	0	0.372
S7	2.176	0.559	0.128	0.372
S8	8.704	2.238	0.384	0.558
S9	6.528	1.678	0.128	0.744
S10	4.352	1.119	0.384	0
S11	2.176	0.559	0.512	0.744
S12	4.352	1.119	0.128	0.558
S13	0	0	0.256	0
S14	0	0	0.384	0.558
S15	8.704	0	0.512	0.744
S16	4.352	1.119	0	0.372
S17	8.704	2.238	0.512	0.186
S18	0	2.296	0	0.384
S19	0	1.148	0.125	0.384
S20	0	0	0.499	0.384
S21	2.176	0.559	0.256	0.744
S22	2.176	0.559	0.384	0.744
S23	6.528	1.678	0.512	0.558
S24	2.176	0.559	0	0.186
S25	6.528	1.678	0.384	0

Table 2 Various instrumental parameter used for EEMF data acquisition on Varian fluorimeter

Parameter	Value
Excitation Wavelength	230–320 nm with a step size of 5 nm
Emission Wavelength	230–500 nm with a step size of 2 nm
Slit width (Excitation and Emission)	5 nm
PMT Voltage	600 V
Scan rate	1920 nm/min

Material and Methods

EEMF Data Sets

In order to carry out the present work a calibration set of 25 samples is designed from more than 500 samples whose composition is reported in the literature [20, 21] and can be downloaded from the website http://www.models.life.ku.dk/datasets. Each of these samples consist of varying concentrations of these four biomolecules namely Catechol, Hydroquinone, Indole and Tryptophan. For simplification, the selected 25 samples are labelled as S1-S25. The concentration of Catechol, Hydroquinone, Indole and Tryptophan in S1-S25 samples are reported in Table 1. The fluorescence measurements were carried out using Varian Eclipse spectrofluorimeter. Various instrumental parameters that were used to collect the EEMF data sets are summarised in Table 2.

Computational Platform

All the computational work is carried out on MATLAB platform. All the graphic plots are created using the suitable MATLAB inbuilt commands.

Results and Discussion

Data Arrangement, Data Pre-Processing and Mathematical Background of PARAFAC

Each of the acquired 25 EEMF spectra contain non-trilinear components Raman and Rayleigh scattering (both 1st and 2nd order) signals that must be mitigated before processing them with PARAFAC analysis. In the present case, Raman scattering signals are mitigated by subtracting the blank EEMF spectrum from each of the analysed 25 samples. The Rayleigh scattering signals are mitigated by setting the variables to zero in the wavelength range $\lambda_{em} \leq \lambda_{ex}$ and (ii) $\lambda_{em} \geq 2*\lambda_{ex}$. The processed EEMF spectrum of sample S4 is shown in Fig. 1. The scattering eliminated EEMF data sets of S1-S25 samples are arranged in a three-way array of dimension 25 (sample) × 136 (emission-wavelength) × 19 (excitation-wavelength) that can safely be processed using PARAFAC analysis.

Before describing the alternate approach for initialising the spectral variables for subsequent ALS algorithm, it is important that briefly mathematical background of PARAFAC analysis be presented. The PARAFAC model described using Eq. 1 can also be described in a more simplified manner using the Khatri-Rao Product [4–8]. The simplified version of PARAFAC equation is summarised using Eq. 3

$$X_1 = A(C|\otimes|B)^T \tag{3}$$

Where X_1 is the matrix of dimension I × JK obtained by unfolding the three-way array $\underline{X}$ of dimension I × J × K. Traditionally, the PARAFAC model is fitted by making an initial estimate for the matrices B and C with SVD approach. In the second step, with the estimated B and C the matrix A is calculated. The above-described calculation is summarised using the Eq. 4

$$A = X_1 Z_1 \left(Z_1{}^T Z_1\right)^{\dagger} \text{ where } Z_1 = (C|\otimes|B) \tag{4}$$

With the calculated A and SVD estimated C, the matrix B is calculated using the Eq. 5

$$B = X_2 Z_2 \left(Z_2{}^T Z_2\right)^{\dagger} \text{ where } Z_2 = (A|\otimes|C) \tag{5}$$

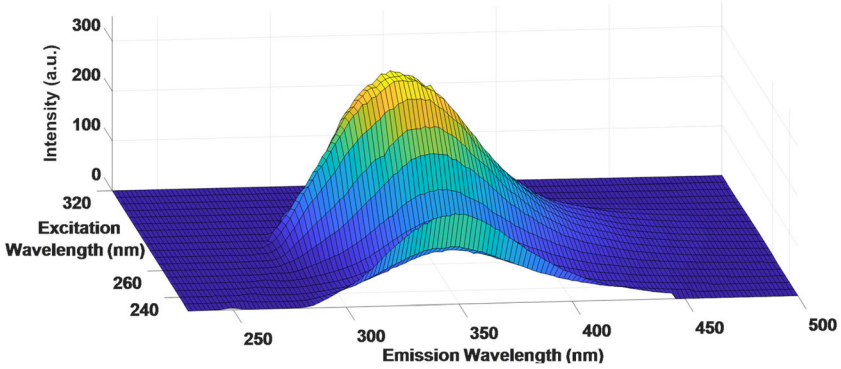

Fig. 1 Rayleigh-Raman scattering eliminated EEMF landscape of sample S4 containing Catechol, Hydroquinone, Indole and Tryptophan

As summarised in Eq. 6, with the calculated A and B, the matrix Cis calculated

$$C = X_3 Z_3 \left(Z_3{}^T Z_3\right)^{\dagger} \text{ where } Z_3 = (B|\otimes|A) \quad (6)$$

In the above equations, X_2 and X_3 are the matrices of dimensions J × KI and K × IJ, respectively obtained by suitably unfolding the three-way array $\underline{X}$. Using the ALS algorithm a series of iterations are carried out with the calculated A, B and C until the least square criteria is achieved.

Novel Spectral Initialization Approach for Initiating the Alternate Least Square Algorithm

As discussed earlier in the present work, the initialisation of the spectral data sets with SVD algorithm essentially destroy the shapes of the excitation and emission profiles for each fluorophores. Thus, SVD assisted PARAFAC analysis can require more number of iterations and significantly large amount of computational effort and time. To address this issue and make the analysis more robust and analytically useful the

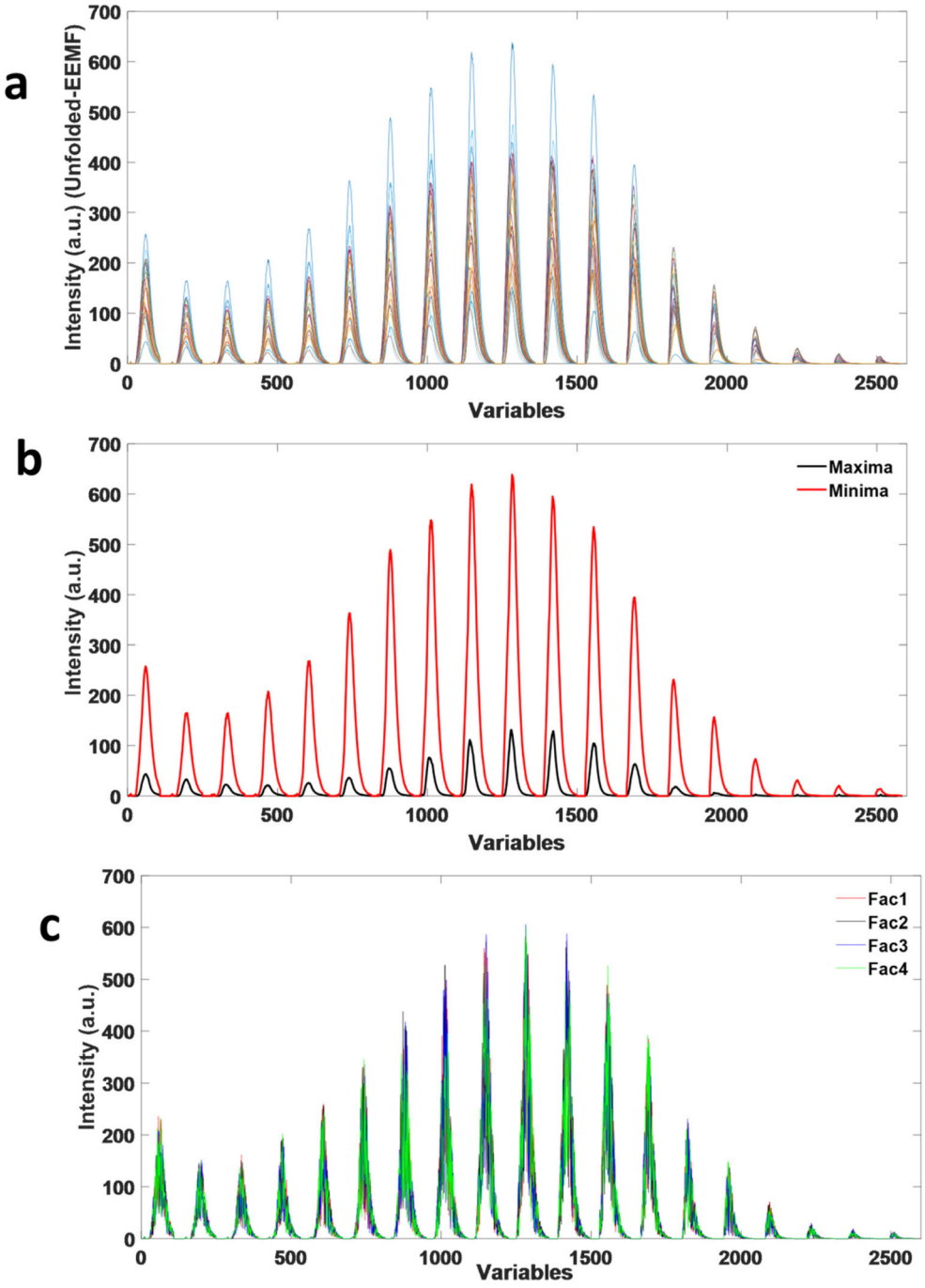

Fig. 2 **a** Unfolded-EEMF data sets of samples S1-S25, **b** Unfolded-EEMF spectra corresponding to minima and maxima, **c** Randomly initialised unfolded-EEMF spectra of Fac1-Fac4 in the space spanned by minima and maxima limits of each variables

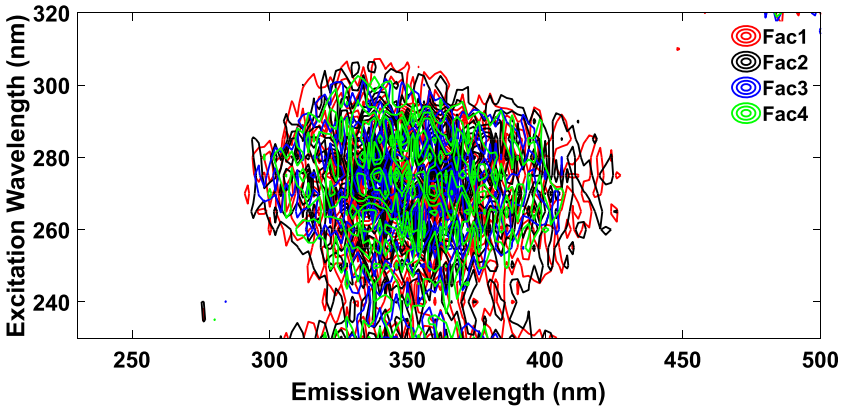

Fig. 3 Initialised EEMF spectra of Fac1-Fac4. The EEMF spectra are obtained by reshaping the initialised unfolded-EEMF spectra of Fac1-Fac4

present work introduces an alternate way of initialising the spectral variables for the subsequent ALS algorithm.

The proposed approach of initialising the spectral variables for fitting the PARAFAC model to a trilinear data set essentially involves the following steps

(i) In the first step, three-way array $\underline{X}$ of dimension I × J × K is unfolded to generate an unfolded-EEMF data sets of dimension I × L (=JK)

(ii) In the next step, the space [$L_{l,min}$, $L_{l,max}$] spanned by minima ($L_{l,min}$) and maxima ($L_{l,max}$) of each of the l^{th} variables is estimated. In other words, an unfolded-EEMF spectra corresponding to minima and an unfolded-EEMF spectra corresponding to maxima of each of the l^{th} variables are generated

(iii) In the next step, using the Eq. 7, the unfolded-EEMF spectral profiles are initialised for each of the underlying F factors

$$\text{Unfolded-EEMF}_{l,F} = \left(L_{l,max} - L_{l,min}\right) * \text{random number} + L_{l,min} \quad (7)$$

(iv) The initialised unfolded-EEMF spectral variables for each of the F factors are reshaped to create their EEMF spectra

(v) The emission and excitation spectral variables are extracted from the initialised EEMF spectra of each of F factors and are stored in the loading matrices B and C for the subsequent ALS algorithm.

In the following section, the proposed approach is validated on the EEMF data sets of the dilute aqueous mixtures of Catechol, Hydroquinone, Indole and Tryptophan.

Initialising the Statistical Parameters to Carry out the PARAFAC Analysis on EEMF Data Sets

The optimum number of factors required to carry out the analysis are estimated by carrying out the principal component analysis (PCA) on the unfolded-EEMF data sets of dimension 25 × 2584. The analysis suggest that there are four significant component one for each fluorophore. The obtained results clearly show that mathematical and chemical rank of the data sets correlates well with each other. Thus, PARAFAC analysis can be safely carried out with four factors. The PARAFAC analysis is carried out with non-negative constraints and it is so because the concentration and fluorescence intensities are always positive. The negative values in each iterations are replaced with a value of 10^{-6}. The maximum number of iterations is set to 10,000. The convergence criteria are achieved when the relative or absolute change in fit are equal to 10^{-6}.

Table 3 Various mathematical parameters obtained from four-factor PARAFAC analyses on EEMF data sets with proposed and traditional initialisation approaches

Parameter	Proposed Random Initialisation assisted PARAFAC analysis	Traditional SVD assisted PARAFAC analysis
Constraints	Non-Negative on each mode	Non-Negative on each mode
Relative change in fit	3.25×10^{-8}	9.25×10^{-7}
Absolute change in fit	3255	7895
Number of iterations	45	221
Iteration or calculation time	15 s	65 s
Cause of termination	Relative Change in fit	Relative Change in fit
Core consistency analysis (CORCONDIA) value	100	93.62
Variance	99.56%	94.35%

Implementing the Novel Initialisation Approach for Carrying out the PARAFAC Analysis

The unfolded-EEMF spectral data sets for each of the 25 samples are shown in Fig. 2a. Unfolded-EEMF spectral profiles corresponding to minima and maxima for each of the 2584 variables are generated, and these profiles are shown in Fig. 2b. Using the Eq. 6, the unfolded-EEMF spectral profiles are initialised for each of the four factors (Fac1-Fac4). The initialised spectral profiles for Fac1-Fac4 are shown in Fig. 2c. The initialised unfolded-EEMF spectral profiles for Fac1-Fac4 are further reshaped to generate their EEMF spectral profiles, shown in Fig. 3. From these EEMF spectral profiles of Fac1-Fac4, the loading matrix B of dimension 176×4 that contains the estimate for emission spectrum and the loading matrix C of dimension 19×4 that contains the estimate for the excitation spectrum are initialised. With these initialised B and C matrices and other parameters that are specified above, the ALS algorithm is used to carry out the PARAFAC analysis of the EEMF data sets. Various parameters such as relative change fit, absolute change in fit, number of iterations, cause of termination, computational time elapsed before the termination of the ALS algorithm are summarised in Table 3. In order to assess the trilinearity of the developed PARAFAC model, a parameter called Core consistency diagnostic (CORCONDIA) [4–8] value is estimated and reported

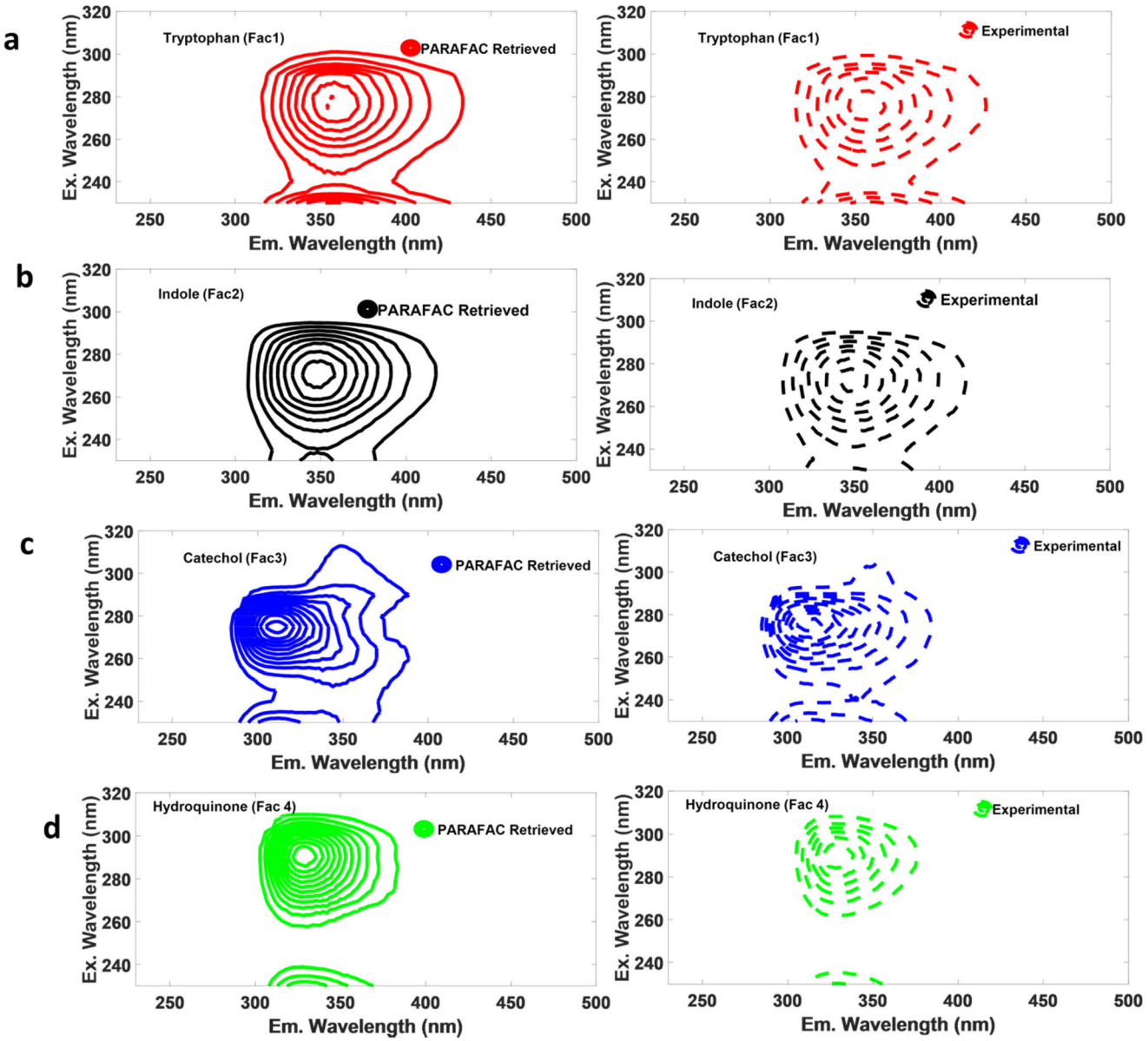

Fig. 4 PARAFAC retrieved and experimentally acquired EEMF spectra of (**a**) Tryptophan (Fac1) (**b**) Indole (Fac2), **c** Catechol (Fac3) and **d** Hydroquinone (Fac4). The obtained results clearly suggest close correspondence between experimentally acquired EEMF and PARAFAC retrieved EEMF spectra of each of the four fluorophores

Table 4 Assessing the correspondence between the experimentally and PARAFAC retrieved EEMF spectral profiles of the Tryptophan, Indole, Catechol, Hydroquinne

PARAFAC model	Fluorophore	Similarity index (SI)
Proposed random initialisation assisted PARAFAC analysis	Tryptophan	0.998
	Indole	0.999
	Catechol	0.985
	Hydroquinone	0.995
Traditional SVD assisted PARAFAC analysis	Tryptophan	0.956
	Indole	0.964
	Catechol	0.961
	Hydroquinone	0.962

in Table 3. Amount of variance captured by the developed PARAFAC model is also reported in Table 3. The PARAFAC retrieved EEMF spectral profiles of Fac1-Fac4 are shown in Fig. 4. It can be seen that, retrieved EEMF spectral profile of Fac1, Fac2, Fac3 and Fac4 correlates well with the experimentally acquired EEMF spectral profiles of Tryptophan, Indole, Catechol and Hydroquinone, respectively. In order to quantify the similarity or resemblance between the experimentally acquired and mathematically retrieved EEMF profiles of Catechol, Hydroquinone, Indole and Tryptophan, a parameter called similarity index (SI) is calculated for each of these fluorophores using the Eq. 8

$$SI = \frac{x^T y}{||x||\,||y||} \tag{8}$$

In the above equation x represents the experimentally acquired data set and y represents the mathematically retrieved data set. An SI value close to unity would suggest true correspondence between the mathematically retrieved and experimentally acquired spectral profiles. The SI values obtained for Catechol, Hydroquinone, Indole and Tryptophan are reported in Table 4. It can be seen that values are close to unit for each of the fluorophores clearly suggests that retrieved profiles are in close corresponds with the experimentally acquired spectra for each of the four fluorophores. The loading matrix A contains the PARAFAC retrieved contribution values of each of the four fluorophores and it is regressed against their actual concentration to develop the calibration model. The regression equation correlating the actual and predicted concentrations are summarised in Table 5. To assess the robustness of the developed calibration model two statistical parameters namely square of the correlation coefficients (R^2) [22] and root mean square of calibration (RMSEC) [23, 24] values are also calculated for each of the four fluorophores and the obtained values are summarised in Table 5. The obtained statistical parameters clearly suggest that there is good agreement between the actual and predicted concentrations of each of the four fluorophores in the analysed 25 dilute aqueous mixtures.

All the statistical parameters clearly suggest that proposed approach of randomly generating the variables in a prescribed manner for subsequently carrying out the PARAFAC modelling is workable and must be considered. In order to further demonstrate the analytical utility of the present work, the proposed approach is further compared with traditional way of carrying out the PARAFAC analysis where the spectral variables are initialised using the SVD approach.

Comparing the Proposed Novel Initialisation Approach with Traditional SVD Initialisation Approach

PARAFAC analysis with traditional SVD initialisation approach is carried out with the four factors with the same constraints and parameters that were used in the PARAFAC analysis assisted with proposed approach involving the random initialisation of the spectral variables. For example, non-negative constraints are imposed on all the three modes (i.e. concentration (A), emission (B) and excitation (C) profiles) for each of the four factors. A non-negative tolerance value of

Table 5 The regression equation Y = m*x + c, relating the actual and predicted concentrations of Tryptophan (Fac 1), Indole (Fac 2), Catechol (Fac 3), Hydroquinone (Fac 4). Y is the predicted concentration and X is the actual concentration, m is slope and c is intercept. RMSEC is measure of calibration error R^2 is the square of correlation coefficient between the actual and predicted concentrations

PARAFAC Model	Fluorophore (Factor)	Regression equation	RMSEC	R^2
Proposed random initialisation assisted PARAFAC analysis	Tryptophan (Fac1)	$Y = 0.99*X + 3.10*10^{-7}$	$1.15*10^{-7}$	0.99
	Indole (Fac2)	$Y = 0.99*X + 2.00*10^{-8}$	$2.67*10^{-7}$	0.99
	Catechol (Fac3)	$Y = 0.98*X + 8.90*10^{-6}$	$9.31*10^{-6}$	0.98
	Hydroquinone (Fac4)	$Y = 0.97*X + 2.00*10^{-6}$	$3.27*10^{-6}$	0.97
Traditional SVD assisted PARAFAC analysis	Tryptophan (Fac1)	$Y = 0.91*X + 1.10*10^{-7}$	$7.15*10^{-7}$	0.91
	Indole (Fac2)	$Y = 0.94*X + 2.21*10^{-8}$	$6.67*10^{-7}$	0.94
	Catechol (Fac3)	$Y = 0.88*X + 6.92*10^{-6}$	$8.31*10^{-6}$	0.88
	Hydroquinone (Fac4)	$Y = 0.87*X + 2.21*10^{-6}$	$7.28*10^{-6}$	0.87

10^{-6} is used to replace the negative values in each iteration. The maximum number of iterations is set to 10,000. The convergence criteria are set to relative or absolute change in fit equivalent to a value of 10^{-6}. Amount of variance captured by the developed SVD assisted PARAFAC model is summarized in Table 3. The CORCONDIA value, relative change fit, absolute change in fit, number of iterations before the termination, cause of termination, computational time elapsed before the termination of the ALS algorithm are also summarised in Table 3. The SI values quantifying the similarities between the experimental and mathematically retrieved profiles are also calculated for Tryptophan, Indole, Catechol and Hydroquinone. The calculated SI values for each of the fluorophores are reported in Table 4. In order to develop the calibration model, the contribution values of Tryptophan, Indole, Catechol and Hydroquinone obtained from the SVD assisted PARAFAC models are regressed against the their actual concentrations. The regression equation correlating the actual and predicted concentrations, R^2 and RMSEC values for each of the four fluorophores are summarised in Table 5. The comparison of the results summarized in Table 3-5 clearly shows that proposed approach has clear advantages over the SVD assisted PARAFAC analysis. The advantages of the proposed approach of randomly initialising the spectral variables for carrying out the PARAFAC analysis are summarised below

(i) The proposed approach allows capturing the greater amount of variance of the EEMF spectral data sets
(ii) The proposed approach provides the PARAFAC model that has greater CORCONDIA values. In other words, the PARAFAC model developed with the proposed approach has more trilinear character
(iii) The proposed approach requires less number of iterations before the convergence
(iv) The proposed approach is computationally fast and requires less computational time
(v) The SI values for Catechol, Hydroquinone, Indole and Tryptophan clearly suggest that EEMF spectral retrieved with proposed approach has greater resemblance with experimentally acquired profiles than those retrieved obtained with traditional SVD analysis
(vi) The regression equation, RMSEC and R^2 values clearly suggests that proposed approach provides better calibration model for quantifying each of the four fluorophores.

In summary, the proposed approach clearly provides computationally economical and more robust procedure to fit the PARAFAC model to an EEMF data sets acquired for the multifluorophoric mixtures. It is believed that proposed approach will be useful in various research fields utilising PARAFAC and EEMF as a useful analytical tool.

Conclusion

The present work proposes an alternate way of initialising the spectral variables for fitting PARAFAC model to trilinear EEMF data sets. The approach essentially involves generating the spectral intensity values in a constraint fashion. The proposed approach is successfully tested by analysing the complex EEMF data sets acquired for the dilute aqueous mixtures of Catechol, Hydroquinone, Indole and Tryptophan. The proposed approach is found to be computationally fast and more robust compared to SVD assisted PARAFAC approach of analysing the EEMF data sets. To conclude, the proposed analytical approach can be useful in all the research fields that involve application of PARAFAC assisted EEMF spectroscopy as an analytical tool.

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