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PAPER

White light excitation fluorescence (WLEF)

Part II.† Analysis of complex multifluorophoric systems‡

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The scope of white light excitation fluorescence in analyzing multifluorophoric systems has been explored. White light excitation fluorescence (WLEF) spectra retain the resultant fluorescence signature of all the fluorophores and their interactions although, in contrast to excitation–emission–matrix fluorescence (EEMF) spectra, resolution along the excitation axis is lost. The demonstration of the advantages of WLEF with regard to fiber optic compatibility, sensitivity, selectivity and simultaneous detection of multiple fluorophores suggests that WLEF based analytical techniques can be methods of choice in certain cases, especially with regard to low cost *in situ* analysis of an analyte. The analysis of multifluorophoric systems by WLEF using a fiber optic fluorimeter clearly shows the effectiveness of WLEF (i) in two-fluorophoric systems at low fluorophore concentrations in which the spectral additivity is retained and (ii) in a complex multifluorophoric system like diesel–kerosene mixtures.

1. Introduction

Complex multifluorophoric systems contain a large number of unidentified fluorophores at unknown concentrations. Typical examples are petroleum products, like petrol, diesel, kerosene and transformer oil, biological systems, like blood plasma, and soil humus. The electronic absorption spectra of these systems are often broad and without any specific spectral features. The fluorescence spectral features, like spectral shape and intensity, vary strongly with excitation wavelength, and there is a strong overlap of absorption and emission spectra over the entire wavelength range. The proportionality of fluorescence intensity with concentration of the corresponding fluorophore is also absent in these systems due to the presence of inner-filter effects and excitation energy degrading interactions, like resonance energy transfer, quenching and other excited state bimolecular interactions.[1] Techniques like excitation–emission–matrix fluorescence (EEMF) and synchronous fluorescence scan (SFS) spectroscopy have often been successfully used for the analysis of complex multifluorophoric systems.[2–4] The EEMF spectrum is a fluorescence 'fingerprint' presented in the form of an excitation/emission iso-intensity contour diagram or as an excitation–emission–intensity three dimensional (3-D) plot. The lamp scatter profile is usually present as a ridge along the diagonal in these plots. Thus an EEMF spectrum, also known as a total fluorescence spectrum (TFS), contains in it the complete fluorescence spectral signature of the sample for all excitation–emission wavelength combinations.[5,6] EEMF measurement is done by holding the excitation wavelength constant and scanning the emission wavelength over the region of interest. Repeating this process at progressively varying excitation wavelengths gives the entire dataset. Obtaining an EEMF spectrum requires a scanning monochoromator for light excitation and either a scanning monochromator or diode array detector for collecting the spectrum. Typically the time required for recording an EEMF spectrum is longer than a single scan spectrum.[7] In SFS, both the monochromators are scanned simultaneously with constant scan rate and a constant wavelength interval, $\Delta\lambda$ is kept between λ_{emi} and λ_{exc}. The spectral data is usually plotted as the variation of fluorescence intensity as a function of λ_{exc}. For a complex multifluorophoric system, the SFS spectrum for a given choice of $\Delta\lambda$ represents emission from a group of fluorophores that have adequate fluorescence at λ_{emi} when excited

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‡ Electronic supplementary information (ESI): Fig S-1A: (a) WLEF, BPEF at the absorption maximum of (b) Rh-B and (c)Rh-6G of the bifluorophoric system Rh-B + Rh-6G in TDW (0.1% Methanol). Fig S-1B: (a) WLEF, BPEF at the absorption maximum of (b) Flu and (c)Rh-B of the bifluorophoric system Flu + Rh-B in TDW (0.1% Methanol). Fig S-2A: Normalized calibration plot for Rh-B in Rh-B + Rh-6G mixture under WLEF (black dots) and BPEF (red dots) with 5% error bar and the best linear fit of WLEF (blue line) and BPEF (green line) respectively. Fig S-2B: Normalized calibration plot for Fluin Rh-B + Flu mixture under WLEF (black dots) and BPEF (red dots) with 5% error bar and the best linear fit of WLEF (blue line) and BPEF (green line) respectively. Fig S-2C: Normalized calibration plot for Rh-6G in Flu + Rh-6G mixture under WLEF (black dots) and BPEF (red dots) with 5% error bar and the best linear fit of WLEF (blue line) and BPEF (green line) respectively. Table S-3: Reference value and predicted value of relative kerosene fraction present in the kerosene adulterated diesel using PLSR and PCR model. See DOI:10.1039/c0ay00704h

at the corresponding λ_{exc}. Thus a typical SFS for a particular $\Delta\lambda$ represents only a subset of all the fluorophores that could be present in the system. It has been shown that a judicious choice of $\Delta\lambda$, which is often a time consuming exercise, can enable the effective use of SFS for the analysis of complex multifluorophoric systems.[8,9] By its very nature SFS requires scanning monochromators on the excitation as well as the emission side. SFS is a single scan data acquisition process and hence it requires less time compared to EEMF. The problem of contamination of lamp scatter profile in SFS is easily avoided[4] by proper choice of $\Delta\lambda$. In recent times the combination of multivariate techniques with EEMF and SFS has been shown to offer significant advantages in the analysis of complex multifluorophoric systems.[10–13]

In the preceding paper, it has been shown that the white light excitation fluorescence (WLEF) spectrum is essentially equivalent to the spectrum in which the EEMF data is compressed along the excitation axis.[14] Thus the WLEF spectrum is expected to retain the resultant fluorescence signature of all the fluorophores and their interactions, although the resolution along the excitation axis is lost. As explored in the preceding paper, WLEF has significant advantages with respect to good fiber optic compatibility, low cost instrumentation, good spectral integrity, enhanced sensitivity and intensity, possibility of lamp scatter profile removal (difference spectrum) and ease of spectral data acquisition. The combination of the ability of WLEF to reflect the fluorescence signature of the entire multifluorophoric system and the advantages of the WLEF technique makes a strong case for the examination of WLEF as an analytical method for multifluorophoric systems. This is the general objective of this work.

The following are two specific objectives which have been studied, each based on a different condition of analysis.

(a) In order to examine the effectiveness of WLEF with respect to the analytical accuracy and selectivity, three two-fluorophoric systems were chosen such that the concentrations of the fluorophores in the system were low. Under this condition, the proportionality of the intensity of each individual fluorophore to its concentration is maintained, which is the basic operative condition for conventional analytical fluorimetry.

(b) In order to examine the applicability of WLEF for complex multifluorophoric systems, the analysis of diesel–kerosene mixtures was chosen as a test case. This system has been chosen as the analysis of this system using SFS and EEMF techniques has already been reported[18–21] and it would be easy to see whether WLEF based analysis matches with them favorably. Multivariate methods have been used for the present analysis.

2. Experimental

2.1. Instrumentation

A fabricated fiber optic fluorimeter[14] was primarily used for this study and a Fluoromax4 spectrofluorimeter (Horiba Jobin Yvon Inc.), with excitation and emission monochromator slit widths kept at 2 nm, used for supporting measurements.

2.2. Materials and methods

2.2.1. Chemicals. The flurophores: Rhodamine-6G (Rh-6G), Rhodamine-B (Rh-B) and fluorescein (Flu) (all from S.D. Fine Chem. Ltd.) were as used as received. The solvent methanol purchased from Sisco Research Laboratories was of spectroscopic grade. Triple distilled water (TDW) was also used as a solvent. Diesel and kerosene were collected from the authorized local vendors in Chennai.

2.2.2. Spectroscopy. Electronic absorption spectral measurements were carried out with a Lambda 25 UV/Visible spectrometer (PerkinElmer) at a scan rate of 240 nm s^{-1}. Although the fabricated fiber optic fluorimeter was primarily used for this study, a Fluoromax4 spectrofluorometer (Horiba Jobin Yvon Inc.), with excitation and emission monochromator slit widths kept at 2 nm, was used for supporting measurements.

2.2.3. Two-fluorophore mixture solutions. Three pairs of two-fluorophore mixture solutions were prepared for the study; Flu + Rh-B, Flu + Rh-6G and Rh-B + Rh-6G (all were 10^{-6} M in 0.1% methanol solution in TDW) were chosen for WLEF emission and calibration studies of two-fluorophoric system and were made by varying the % concentration of the probe from 0 : 100, 10 : 90 *etc.* up to 100 : 0 (11 samples).

2.2.4. Diesel–kerosene mixtures. Diesel samples with different relative kerosene fractions (%, v/v) were prepared by adding appropriate volumes of neat kerosene to neat diesel. A calibration set of 32 samples and a validation set of 5 samples containing varying amounts of diesel and kerosene were prepared. The relative kerosene fraction (%, v/v) in the samples varied from 0 to 100%. For 0% (100% diesel v/v fraction) to 20% (80% diesel v/v fraction) kerosene v/v fraction in diesel, the interval maintained was 1% and for the range 20% to 100% the interval was 5%. The 1% interval was chosen in the composition range 0% to 20% kerosene v/v fraction in diesel because this is the range in which adulteration of diesel by kerosene is expected.

3. Results and discussion

3.1. WLEF analysis of two-fluorophore mixtures

In a multifluorophoric system, conventional fluorescence spectroscopy provides the unique advantage of the selective excitation of a particular fluorophore. Similar selectivity can also be exercised in choosing an emission wavelength corresponding to a particular fluorophore. This twin selectivity in terms of the choice of excitation and emission wavelength is a unique strength of conventional fluorimetry. This feature is absent in conventional electronic spectroscopy as well as in WLEF. The analysis of multiple fluorophoric samples by WLEF and multiple chromophoric samples by electronic absorption spectroscopy are, in essence, closely similar to each other. Selectivity in WLEF can only be exercised with regard to the choice of emission wavelengths. The possibility of simultaneous determination of two fluorophores by WLEF was examined for Rh-B, Rh-6G and Flu.

The fluorescence spectrum of the Flu + Rh-B pair is shown in Fig. 1, it shows the nature of WLEF and conventional band pass excitation fluorescence (BPEF) of the Flu + Rh-B pair. Spectral details of the other pairs were tested and are given as ESI (S-1 (A and B)).‡ It is seen that in every case, the WLEF gives the fluorescence of both the fluorophores present in the system. But in BPE (conventional fluorimetry) an increased fluorescence of

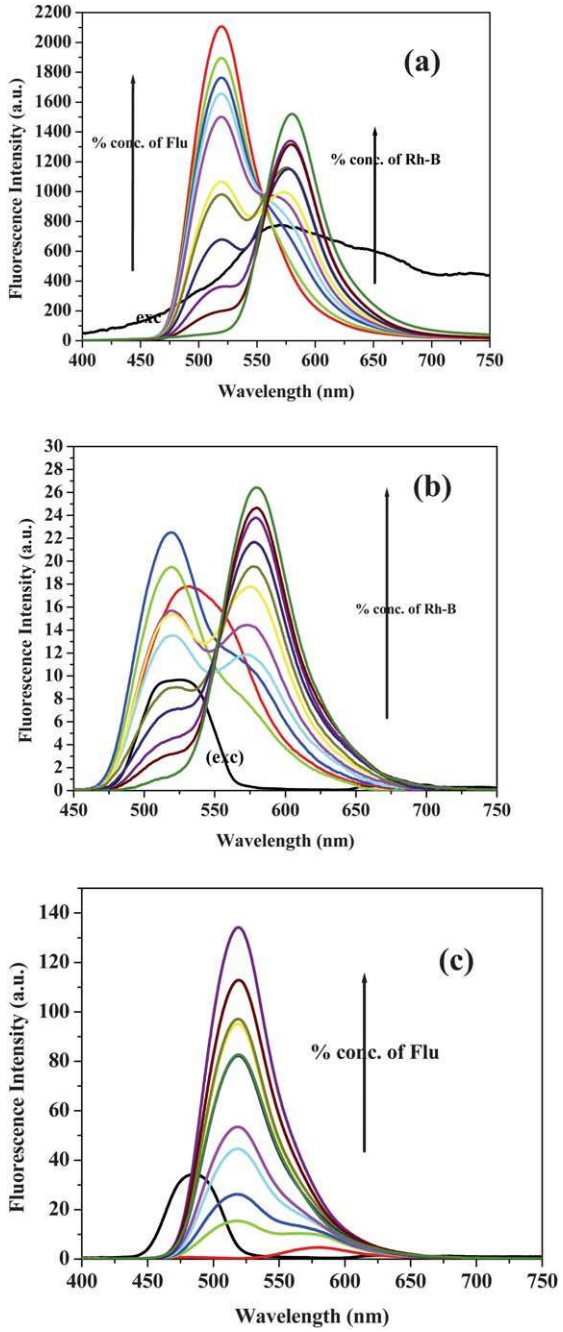

Fig. 1 (a) WLEF, BPEF at the absorption maximum of (b) Rh-B and (c) Flu of the two-fluorophoric system Rh-B + Flu in 0.1% methanol in TDW.

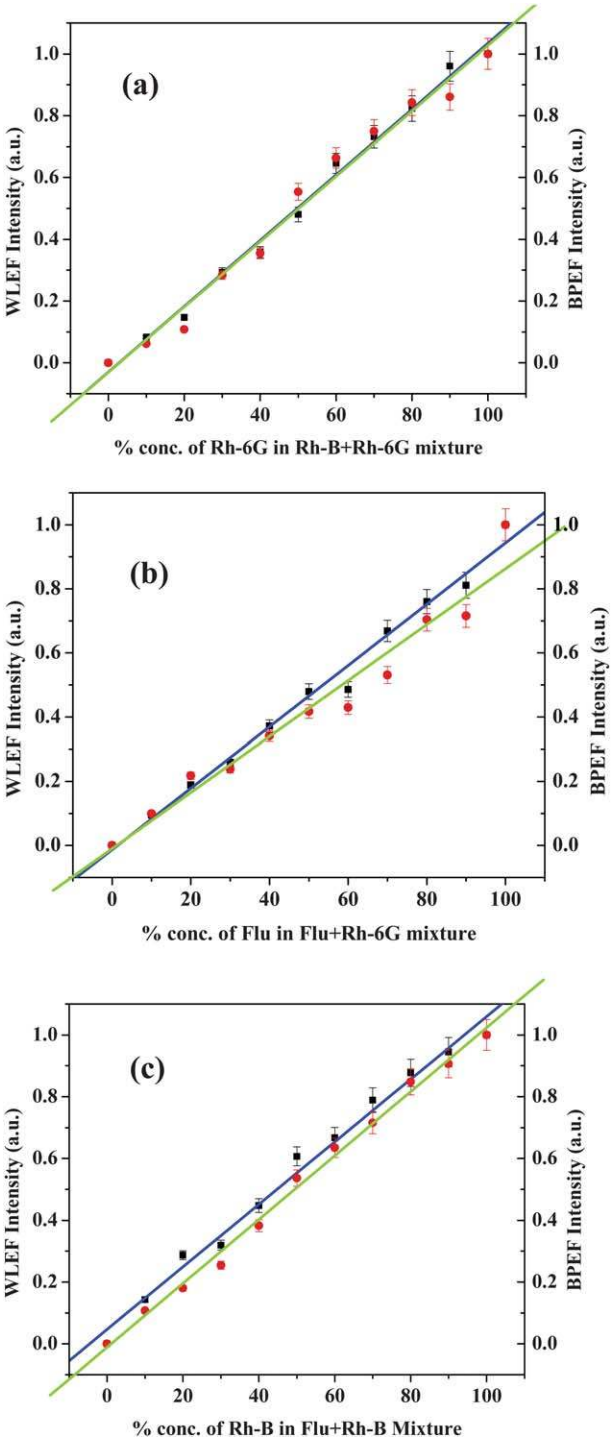

Fig. 2 Normalized calibration plots for Rh-6G, Rh-B and Flu in the mixtures Rh-6G + Rh-B, Rh-B + Flu and Flu + Rh-6G, under WLEF (black dots) and BPEF (red dots) with 5% error bar and the best linear fit of WLEF (blue line) and BPEF (green line) respectively.

one probe having absorption maximum very closed to BPEF maximum is observed and its partner gives feeble fluorescence due to the poor overlap of the absorption and excitation lamp profile.

The selective BPE of the probes was carried out at their absorption maxima, Flu at 485 nm, Rh-6G at 526 nm and Rh-B at 554 nm. BP selective excitation at 550 nm not only gives the fluorescence of Rh-B but also the fluorescence of Flu since there is enough overlap of the excitation source profile and absorption spectrum of Flu. The gradual variation in fluorescence of the fluorophores at WLE and BP selective excitation is visible from the emission plots presented in Fig. 1 and a linear calibration fit is also made for ensuring the strength of WLEF as an analytical tool. Normalized calibration plots for Rh-6G in the mixtures Rh-B + Rh-6G, Rh-B in Flu + Rh-B and Flu in Rh-6G + Flu are shown in Fig. 2 and the remaining three calibration plots are given as ESI (S-2 (A–C)).‡

Even though white light was used as an excitation source linear calibration plots were obtained at the emission maximum of all the fluorophores present in the system. To obtain the calibration plots signal intensities were measured at the emission maximum, Rh-B (580 nm), Rh-6G (554 nm) and Flu (535 nm), and the experiment was repeated 5 times for all the above flurophore combinations. The consistency and reproducibility of the results obtained from WLEF makes this technique good and comparable to conventional BPEF methods.

The emission spectra show a gradual variation in fluorescence intensity even in the presence of second fluorophores. Calibration plots were drawn for each of the fluorophores at their emission maximum under WLEF and conventional BPEF. The fluorescence intensity of each of the fluorophores at their emission maximum was obtained by subtracting the percentage contribution of its partner fluorophores at that emission maximum. The calibration parameters are shown in Table 1. In general the coefficient of linearity values (R-value) of $\geq$0.991 imply good linearity of the calibration. Similarly the standard deviation (S.D.) values of $\leq$ 0.046 imply that the measurement errors are within the acceptable limits. Both R and S.D. values for WLEF and the conventional BPEF methods compare fairly well with each other.

3.2. WLEF and EEMF of a multifluorophore-diesel

In the preceding paper it was shown that the EEMF spectrum and the WLEF spectrum are related to each other. This was done by using a Fluoromax-4 spectrofluorometer instrument because it has the provision (0 nm selection of the excitation monochromator) of low intensity white light for calibration purposes, as well as the provision of obtaining EEMF spectrum. Using the EEMF data matrix, an area plot was constructed by integrating the excitation profiles at different emission wavelengths and plotting the integrated area against the corresponding emission wavelength. This area plot was shown to match closely with the WLEF spectrum. Since WLEF is expected to contain in it the spectral signatures of all the fluorophores present in the system, the area plot obtained from the diesel EEMF spectrum should also show a close match with the diesel WLEF spectrum. The 3-D and contour plots of diesel fluorescence, obtained by scanning the emission monochromator from 380 nm to 650 nm (increment 2 nm) and the excitation monochromator from 300 nm to 552 nm (increment 4 nm) at slit widths 2/2 nm is shown in Fig. 3.

The area plot which is a projection of EEMF along the emission axis was constructed from the EEMF data. The comparison of the spectral profile of the area plot and that of WLEF is given in Fig. 4.

As is observed in Fig. 4, even for a complex multifluorophoric system like diesel there is a close match of the WLEF with the excitation-compressed EEMF area plot.

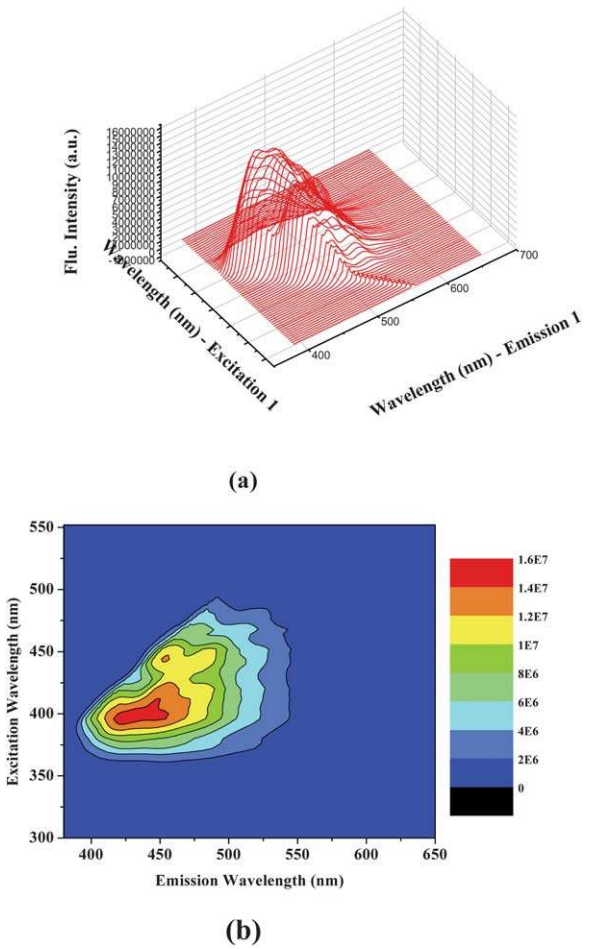

Fig. 3 (a) 3-D and (b) contour plot of pure diesel.

Table 1 Calibration plot data for entire fluorophores in the mixtures RhB + Flu, Rh-B + Rh-6G and Flu + Rh-6G in 0.1% methanol in TDW under WLEF and conventional BPEF

Fluorophores in mixtures	Mode	Intercept (A)	Slope (B)	R-value	S.D.
Rh-B in Rh-B + Flu	WLEF	0.0469	0.0101	0.99_4	0.03_8
	BPEF	−0.0110	0.0103	0.99_7	0.02_7
Rh-B in Rh-B + Rh-G	WLEF	0.0593	0.0096	0.99_4	0.03_5
	BPEF	0.0788	0.0086	0.98_1	0.05_9
Flu in Rh-B + Flu	WLEF	0.0023	0.0099	0.99_5	0.03_6
	BPEF	0.0291	0.0095	0.99_0	0.04_8
Flu in Flu + Rh-6G	WLEF	−0.0130	0.0096	0.99_4	0.03_5
	BPEF	−0.0099	0.0087	0.97_8	0.06_5
Rh-6G in Rh-B + Rh6G	WLEF	−0.0297	0.0106	0.99_7	0.03_0
	BPEF	−0.0309	0.0106	0.99_2	0.04_9
Rh-6G in Flu + Rh-6G	WLEF	0.0283	0.0099	0.99_1	0.04_6
	BPEF	−0.0219	0.0102	0.99_2	0.04_4

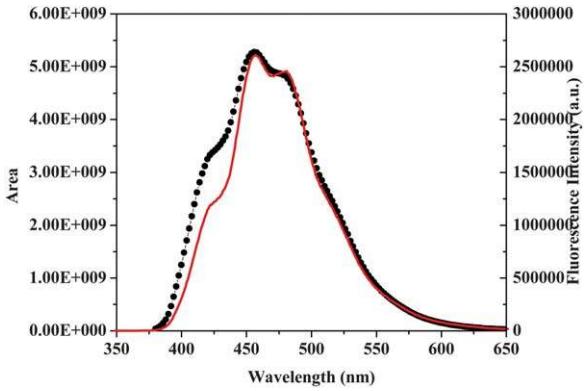

Fig. 4 A projection of EEMF along the emission axis (area plot) and WLEF spectrum of diesel.

The close compatibility of WLEF with fiber optic fluorimetry (FOF) has been established in the preceding paper.[14] The FOF–WLEF spectrum of a complex multifluorophoric system like diesel will retain advantages like analytical precision, accuracy, selectivity and sensitivity. However, the difference spectrum technique for removal of the lamp scatter profile is not usable because of the absence of a proper reference for a neat diesel sample. Fortunately, diesel is a fairly transparent medium and the contribution from the lamp scatter profile is not expected to be significant.

3.3. WLEF of diesel–kerosene mixture

WLEF spectra of samples containing various compositions of diesel and kerosene are shown in Fig. 5. The peak at 484 nm shows a gradual decrease in intensity and a slight blue shift on increasing the relative kerosene % v/v fraction.

A calibration model for the kerosene contamination in diesel was made using 32 samples of known concentration with the help of the multivariate methods PLSR and PCR by employing the following sequence of steps:

3.3.1. Data pre-processing. To proceed with the multivariate regression process, data were arranged into a matrix characterized by samples as rows and WLEF intensities as columns. Data pre-processing was done using auto-scaling[15] which involves mean-centering followed by dividing each column by the standard deviation of the column.

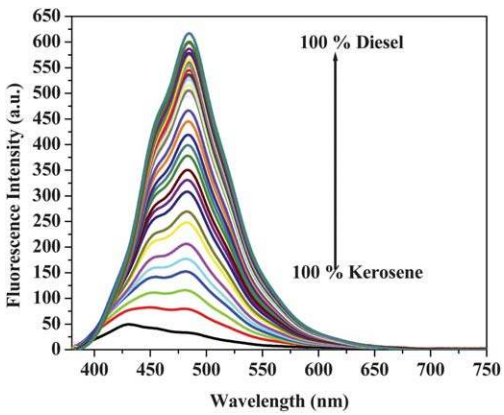

Fig. 5 WLEF spectra of diesel–kerosene mixtures measured using the fiber optic fluorimeter (FOF) instrument.

3.3.2. Optimum number of components to build the model. After pre-processing the data the next step in the multivariate regression method was the determination of the optimum number of factors that allow the system to be modelled without over fitting the concentration data. For this purpose a cross-validation method, contiguous block[15] with 6 splits of the data was used. The optimum numbers of components or latent variables with minimum root-mean-square error of cross-validation (RMSECV) and root-mean-square error of calibration (RMSEC) values was found to be six for both partial least squares regression (PLSR) and principal component regression (PCR)[15–17] models. Hence, a six component PLSR and PCR model was made, these factors explains the maximum variation in the respective models.

3.3.3. Accuracy and prediction ability of the model. To evaluate the accuracy and reliability of the model, the measured values of diesel concentration was plotted against the concentration values predicted by the model (Fig. 6).

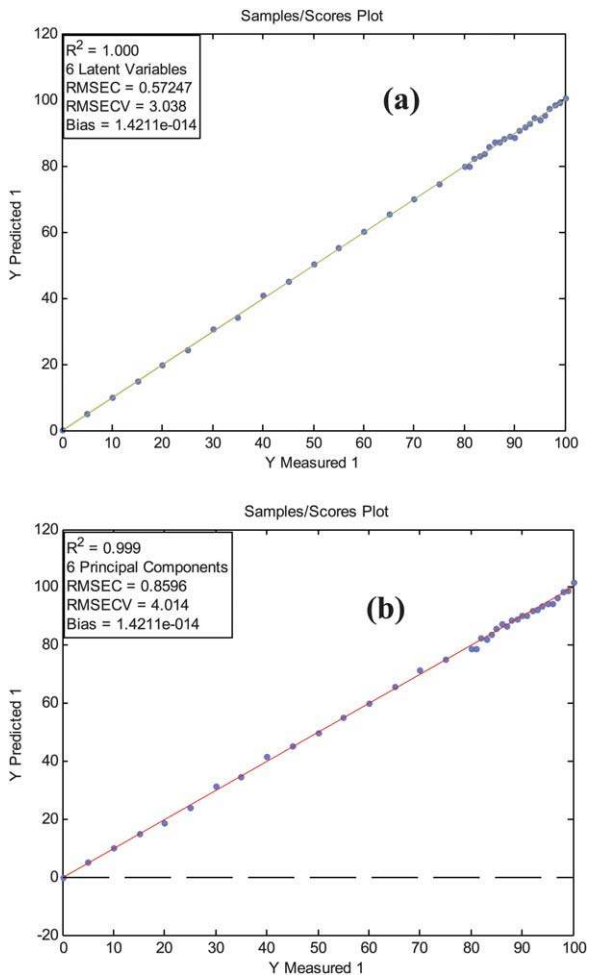

Fig. 6 Reference concentration *vs.* predicted concentrations of diesel–kerosene mixture in the calibration dataset developed by (a) PLSR and (b) PCR.

Table 2 Measured and predicted values of relative kerosene fraction present in the kerosene adulterated diesel for 5 sets and their average RMSEP using PLSR and PCR model[a]

Measured value (% v/v of kerosene)	Predicted value (% v/v of kerosene)		Prediction error for each sample	
	PLSR	PCR	PLSR	PCR
55	55.15_4	54.91_5	0.1539_7	0.0845_7
70	70.04_4	71.28_9	0.0443_1	1.2894_1
83	82.89_9	82.29_5	0.1006_1	0.7046_3
91	90.86_5	90.23_1	0.1350_6	0.7685_4
99	99.03_2	99.10_8	0.0318_0	0.1076_8
Average RMSEP			**0.1049**	**0.7441**

[a] RMSEP for PLSR and PCR methods are 0.1049 and 0.7441 respectively.

Both PCR and PLSR methods show very good correlation between the measured and predicted concentrations with correlation coefficients of 1 and 0.999 which shows a good linear fit (Fig. 6). The RMSEC values for PLSR and PCR were 0.57427 and 0.8596 respectively. The calibration dataset (reference value) and the values obtained on validation (predicted value) by cross validation (contiguous block) method are given as ESI (S-3).‡ A test set of five samples of known concentrations was used to test the prediction ability of the model. Quantitative determination of the amount of kerosene present in the mixture was achieved, which shows very close agreement with the measured value (Table 2).

This study shows that WLEF is a viable and acceptable method for the analysis of complex multifluorophoric systems like diesel–kerosene mixtures, with sensitivity up to 1% of kerosene contamination and an error prediction value of 0.10 for PLSR and 0.74 for PCR. This range of sensitivity and accuracy compares well with other analytical methods like EEMF[18,19] and SFS.[20,21]

4. Conclusion

This work on the analysis of multifluorophoric systems by WLEF using fiber optic fluorimeter (FOF) clearly shows the effectiveness of WLEF (i) in biflluorophoric systems at low fluorophore concentrations in which the spectral additivity is retained and (ii) in a complex multifluorophoric system like diesel–kerosene mixtures. As an analytical technique WLEF compares well with conventional BPEF as well as with techniques like SFS and EEMF.

The demonstration of the advantages of WLEF with regard to fiber optic compatibility, sensitivity, selectivity, simultaneous detection of more fluorophores *etc.* strongly suggest that this method of obtaining fluorescence spectral data can be a method of choice in certain cases, especially with regard to low cost *in situ* analysis of an analyte. One of the major strengths of conventional fluorimetry lies in its selectivity enabled by monochromatic excitation, which can be a source of weakness in simultaneously detecting multiple fluorophores in a solution. WLEF can thus be a nice complement to conventional fluorimetry (BPEF). This paper and the preceding paper examine the hitherto unexplored scope of WLEF in analytical fluorimetry.

5. Acknowledgement

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