



## Calibration, standardization, and quantitative analysis of multidimensional fluorescence (MDF) measurements on complex mixtures (IUPAC Technical Report)

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## IUPAC Technical Report

Alan G. Ryder\*, Colin A. Stedmon, Niels Harrit and Rasmus Bro

# Calibration, standardization, and quantitative analysis of multidimensional fluorescence (MDF) measurements on complex mixtures (IUPAC Technical Report)

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**Abstract:** This IUPAC Technical Report describes and compares the currently applied methods for the calibration and standardization of multi-dimensional fluorescence (MDF) spectroscopy data as well as recommendations on the correct use of chemometric methods for MDF data analysis. The paper starts with a brief description of the measurement principles for the most important MDF techniques and a short introduction to the most important applications. Recommendations are provided for instrument calibration, sample preparation and handling, and data collection, as well as the proper use of chemometric data analysis methods.

**Keywords:** calibration; chemometrics; excitation emission matrix; fluorescence; IUPAC; multidimensional; Rayleigh scatter; standardization.

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## 1 Introduction

### 1.1 Background

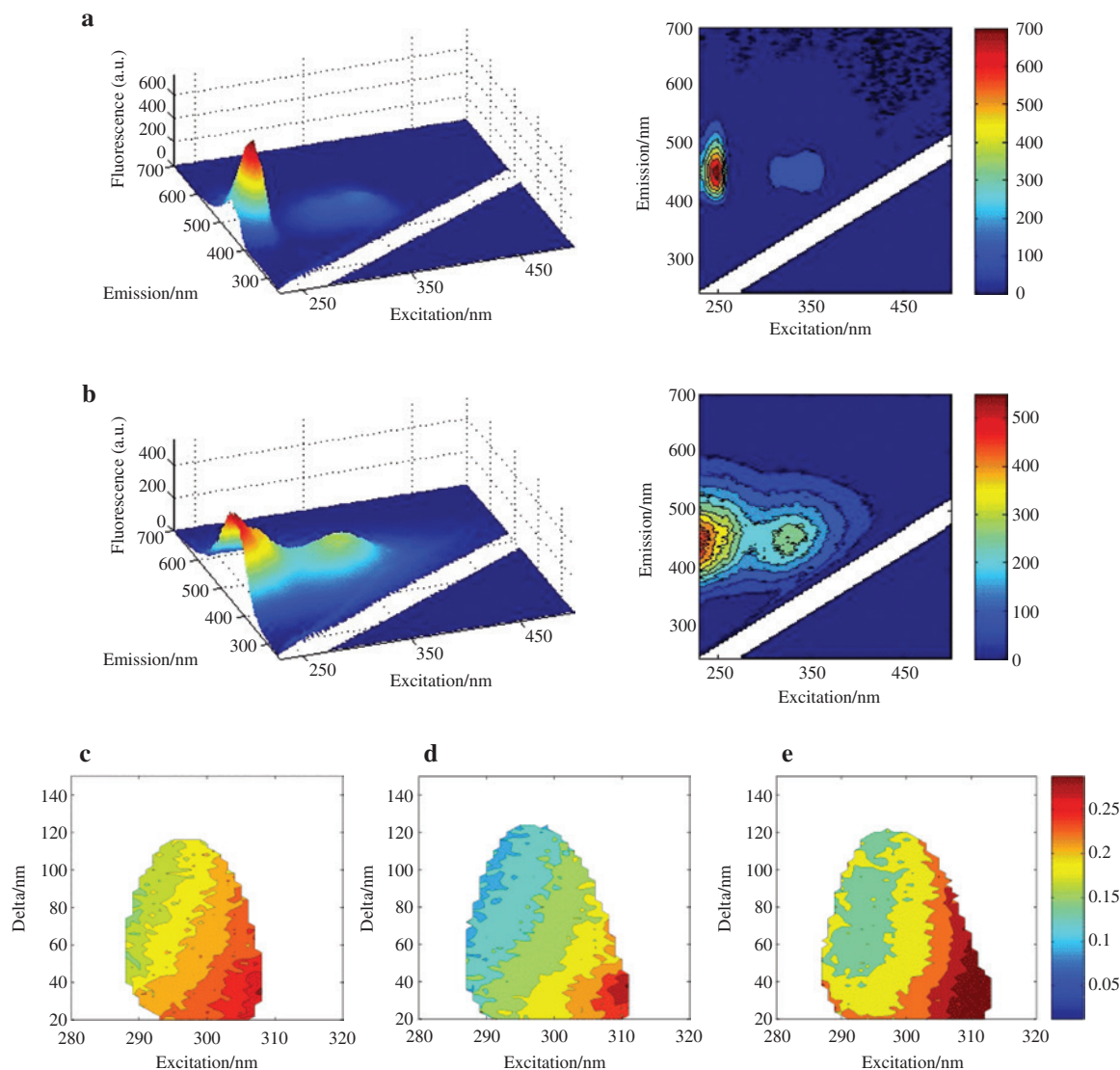
A paradigm shift is occurring in analytical chemistry and spectroscopic analysis. The focus is moving towards *in-situ* measurements that can be easily automated, particularly for the analysis of molecularly complex materials by fluorescence. New fluorescence techniques that involve the measurement of several parameters simultaneously are being developed and applied. These multi-dimensional approaches offer a greater potential for more accurate quantitative and qualitative characterization than conventional spectroscopy. Methods such as excitation emission matrices (EEM) and synchronous fluorescence scan (SFS) spectroscopy involve the scanning of both excitation and emission channels and often require the extensive use of computational data analysis. These data, and their correct interpretation, are therefore very susceptible to variations in instrumental procedures, sample preparation and presentation, instrumentation, and data analysis. The analyst who wishes to utilize EEM/SFS for accurate analytical purposes should ensure that an appropriate calibration methodology that corrects for all forms of bias arising from the instrument, the sample, and the data analysis is adopted.

These multi-dimensional techniques offer a greater potential for more accurate quantitative and qualitative characterization than traditional two-dimensional techniques due to the wealth of data obtained [1]. In contrast to many other spectroscopic techniques, fluorescence of a single sample is intrinsically two-way (a 'landscape'), as the signal intensity from a fluorophore in the absence of interferants<sup>1</sup> varies depending on its sample concentration as a function of excitation (absorption) and emission (fluorescence) properties. The emission spectrum from an optically dilute and interferant free solution of a single fluorophore is usually independent of excitation wavelength (Kasha/Vavilovs rule), whilst the intensity of fluorescence depends on the excitation parameters and absorption properties. When two or more fluorophores are present, the true shape of the emission spectrum changes with excitation wavelength in accordance with the properties and relative concentrations of all the chromophores and fluorophores present [2]. The measured emission shape can also be very sensitive to excitation/emission geometry. For samples with relatively low analyte concentrations, like water [3], the emission properties can be relatively easily modelled and understood. However, the situation becomes much more complicated in complex biogenic samples, such as cell culture media [4], food [5], or petroleum [6], where there are large numbers of fluorophores and quenchers present with many simultaneous competing photophysical processes.

### 1.2 MDF measurement techniques

When a series of emission spectra are measured for a range of excitation wavelengths, the data can be combined into an excitation emission matrix (EEM) representing a landscape of a subset of the fluorophore

<sup>1</sup> An interferant can be defined here as any additional constituent which can cause fluorescence quenching, energy transfer, or result in scattering/absorption of excitation or emission light.



**Fig. 1:** Landscape and contour plots of representative MDF data: (a) EEM of a solution of  $50 \times 10^{-9}$  quinine sulfate in 0.105 M  $\text{HClO}_4$ ; (b) EEM of an aqueous solution of Suwannee River Fulvic Acid; (c–e). Changes in the aniso-TSFS contour plots (from ARMES) for Human Serum Albumin (HSA)  $1 \text{ mg mL}^{-1}$  in buffer undergoing thermal unfolding: (c)  $10^\circ\text{C}$ , (d)  $50^\circ\text{C}$ , and (e) after cooling from  $70^\circ\text{C}$  to  $20^\circ\text{C}$  (colour scale denotes the anisotropy) [7].

properties: excitation wavelength ( $\lambda_{\text{ex}}$ ), emission wavelength ( $\lambda_{\text{em}}$ ), and Intensity ( $I_{\text{ex/em}}$ ). Alternatively, the EEM can be represented by a contour plot (Fig. 1). In synchronous fluorescence scan spectroscopy (SFS), excitation and emission monochromators are scanned simultaneously with a defined energy (or wavelength) separation. If a series of SFSs are run with different offsets between excitation and emission wavelengths, it is termed Total Synchronous Fluorescence Scan (TSFS) Spectroscopy [8]. The primary advantages of TSFS over EEM are that it eliminates Rayleigh scatter once an appropriate separation is used and that it can be slightly faster to implement using standard scanning spectrometers. Irrespective of the scanning mode, an EEM is obtained. More recently, TSFS/EEM has been combined with anisotropy measurements to generate a 4-dimensional data matrix [7, 9, 10]. If generated via TSFS, the 4-dimensional data matrix comprises excitation wavelength ( $\lambda_{\text{ex}}$ ), wavelength offset ( $\Delta\lambda$ ), Intensity ( $I_{\text{ex}/\Delta\lambda}$ ), and anisotropy ( $r_{\text{ex}/\Delta\lambda}$ ). Anisotropy Resolved Multidimensional Emission Spectroscopy (ARMES) can provide information about fluorophores size, environment, and mobility in mixtures and proteins [7, 9] (Fig. 1c–e).

Fluorescence measurements of complex mixtures contain a large amount of information that until relatively recently was not being utilized to its full potential. Often, only the emission maxima or ratios of

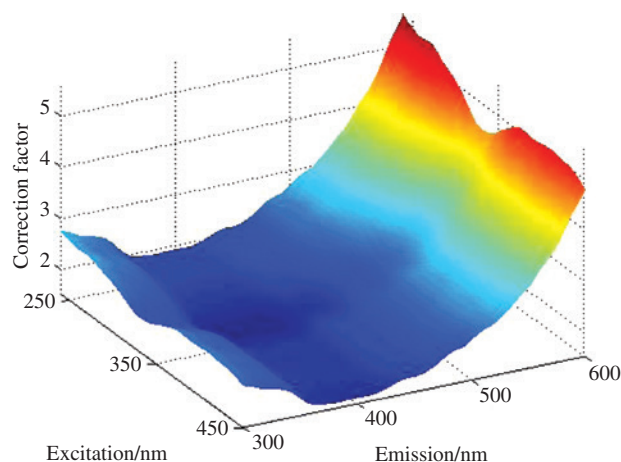
fluorescence at different wavelengths were used to describe changes in fluorescence properties (e.g. [11]). The multi-dimensional nature of EEM, SFS, and ARMES measurements requires chemometric data analysis to properly extract the wealth of important information contained in the data. This aspect of the analytical method has progressed with the increased computational power of inexpensive desktop computers, as well as more readily available, user-friendly software and an explosion in the published literature. These advances have made MDF techniques more easily accessible to a wide range of scientists, particularly those involved with the analysis of complex environmental- or biomedical-based materials.

The aim of this technical report is to, first, emphasize the importance of calibration and correction issues associated with EEM/SFS spectroscopy, and second, to stress the need for the correct use of chemometric data analysis in the calibration and standardization of EEM/SFS data. The six key issues presented here are: 1) Excitation, 2) Emission, and 3) Intensity corrections; 4) sample absorption (inner filter effects); 5) Sample stability and handling; and 6) Multivariate data analysis. All these must be carefully considered before undertaking measurements in order to achieve reproducible data for quantitative or qualitative applications. The first three issues are instrumentation-related. Many aspects of these have been detailed in various IUPAC technical reports [12–14]. The fourth and fifth factors relating to the samples are critical here, because many of the samples being tested by EEM/SFS/ARMES are biogenic in origin. Thus, a careful assessment of the chemical and physical stability of the sample should be undertaken before MDF measurements. The final issue deals with the fact that multivariate (chemometric) approaches are generally required to explore and analyse complex EEM/SFS data. Just as important is the fact that chemometrics also provides the best approach for accurately measuring method reproducibility, visualizing and understanding the sources of spectral variance, implementing calibration transfer, and validating the overall analytical method. Many of the necessary tools and procedures to deal with these issues are available to the community and are continually being developed further by academia, instrument and software vendors, and IUPAC committees. However, there is still scope to refine and simplify these processes further, develop practical EEM/SFS test standards, and eventually develop a simple one-step automated calibration procedure for EEM/SFS fluorescence in the not-too-distant future.

## 2 Instrumental calibration

Excitation calibration of a spectrofluorometer compensates for the fact that both the light source output (measured in photons/second) and the light transmission and detection by the emission side optics varies with wavelength. Likewise, the calibration of the emission side corrects for the wavelength-dependent sensitivity of the detector system and the emission side optics. Both procedures are relative—not quantitative—corrections of a non-ideal situation and ensure that any changes in excitation and emission wavelength patterns reflect properties of the sample, not the instrument. The key parameter being measured in fluorescence is the intensity of a single light beam. Since the electronic output from the detector is dependent on the experimental and instrumental circumstances, fluorescence intensities are routinely reported in arbitrary units (*a.u.*). The 2009 ASTM standard [15, 16] and a recent IUPAC technical report [13] cover many of these issues in detail.

Like a fingerprint, MDF spectra can represent data totally unique to a sample. However, since most fluorescence data are uncorrected, the measured spectra are not only unique for the sample, but also for the conditions under which the data were collected (e.g. instrumental parameters, sample placement, *etc.*). Traditionally, analysts try to minimize instrumental bias by running all the samples on the same instrument in the shortest possible time. In order for any technique to become more widely acceptable as a routine analytical method, it is mandatory that data from different instruments or data taken at different times are comparable. However, if the promise of a practical application is to be fulfilled, the calibration procedures should not be too lengthy or overly complicated. The calibration should involve the same instrumental routine as the measurement of the unknown samples. Ideally, this could be a range of stable standards containing a mixture of fluorophores either in a sealed cuvette or polymer matrix fabricated for front surface or 90-degree sampling geometries. To date, no such multi-fluorophore standards exist, and consequently both excitation



**Fig. 2:** Representative example of an EEM correction factor derived by multiplying the excitation and emission correction factors. A more detailed discussion of the process is included in Ref. [24].

and emission should be calibrated independently using conventional procedures. The reader is directed elsewhere for more detailed discussion of the aspects of standard spectral correction [12, 16–23].

Excitation and emission corrections rectify instrument-specific spectral biases which arise from a range of factors. These include variations in the spectral output of the light source and inevitable flaws in the instrument components' ability to transmit light across the wavelength range of interest. Once the excitation and emission correction factors have been derived, an overall correction factor EEM (Fig. 2) can be generated<sup>2</sup> and applied to all the subsequently measured EEMs (or, in a similar fashion, the SFSs). An example of the correction methodology for water analysis is given by [25]. An additional correction might be needed, due to the fact that almost every modern spectrograph scans with a spectral width being constant in wavelength units, rather than frequency units.

### 3 Quantification of fluorescence intensity

Before fluorescence measurements are quantitatively comparable between instruments – or indeed between measurements with different instrumental settings on the same instrument – an intensity quantification of the system must be performed. Although fluorescence is often used to characterize samples qualitatively, it can also be useful to quantitatively compare fluorescence intensity signals between samples. When working with known fluorophores, this is a simple issue, since standard intensity-concentration plots can be easily generated and the fluorescence signal subsequently converted to concentrations of fluorophore [26]. With the fluorescence of complex mixtures of unknown fluorophores, however, this is not possible and an alternative approach is required. The calibration procedure does not influence the shape of the EEM/SFS data and therefore serves only to provide units for the fluorescence intensity measurements on unknown analytes. Two such approaches are described here.

#### 3.1 Quinine sulfate method for quantification of fluorescence intensity

A common quantification method involves using the fluorescence of quinine sulfate [27] from excitation at 350 nm and emission at 450 nm [28, 29]. The feasibility of this approach relies on the fact that the quantum

<sup>2</sup> This is done by measurement of the EEM of a standard. The standard is measured on the instrument and compared to the reference spectrum. The ratio of the measured intensity at each point ( $\lambda_{ex}/\lambda_{em}$ ) then provides the correction (emis\_ex-em) for each  $\lambda_{ex}/\lambda_{em}$  pair for any new spectra. The process is similar to that implemented for simple 2D spectra.

yield of quinine sulfate is considered to be a well-determined molecular constant. The reference measurements are made with the same instrumental set up as used for the analysis of samples (sample placement, slit widths, detector gain, *etc.*) and a factor converting the raw fluorescence counts (signal) to “quinine sulfate equivalents” is derived and applied. A complete EEM is not necessary, but routine scans can be useful for additional monitoring of the performance of one’s excitation and emission correction procedure (although only within the wavelength range of quinine sulfate fluorescence). A published ASTM standard E578-01 [26] provides a sample preparation method that can be used for both this task and linearity testing.

### 3.2 The Raman integral method

Another approach [30–33] is to use the Raman scattering signal (O–H stretching vibration) from pure water, which is shifted by approximately  $3400\text{ cm}^{-1}$  from the Rayleigh peak at the excitation wavelength (Fig. 3). The integrated intensity of the Raman band ( $A_{\text{rp}}$ ) is directly proportional to the product of the excitation intensity ( $I_{\text{ex}}$ ) times the Raman cross-section ( $R_{\text{cs}}$ ) at any specific wavelength at a specified temperature:

$$I_{\text{ex}} \propto \frac{A_{\text{rp}}}{R_{\text{cs}}} \quad (1)$$

The fluorescence intensity ( $I_{\text{f}}$ ) is proportional to the excitation intensity and the quantum yield:

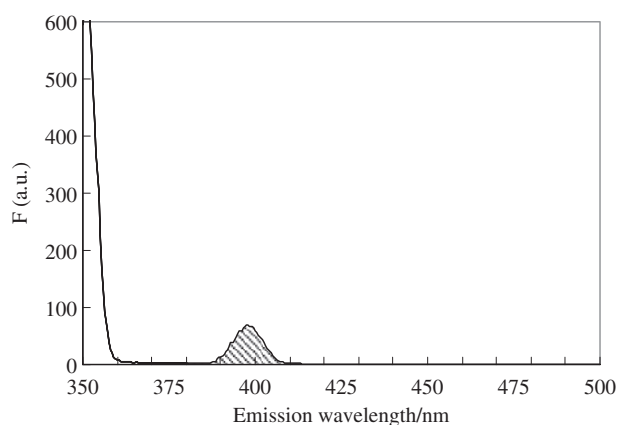
$$I_{\text{f}} \propto I_{\text{ex}} \times \Phi_{\text{f}} \quad (2)$$

Instrumentally,  $I_{\text{ex}}$ ,  $I_{\text{f}}$ , and  $A_{\text{rp}}$  are measured in the same “arbitrary units”, so these relationships open up the possibility to introduce a “Raman unit” for comparing fluorescence intensities on different instruments or with different settings. To obtain the fluorescence intensity in “Raman units” (R.u.), the signal in arbitrary units is divided by the area of the Raman peak:

$$I_{\text{f}}(\text{R.u.}) = \frac{I_{\text{f}}(\text{a.u.})}{A_{\text{rp}}(\text{a.u.})} \quad (3)$$

However, to do this accurately, several factors must be understood and appropriate experimental measures must be undertaken:

1. The excitation and the emission sides of the instrument must be spectrally calibrated according to standard methods [12, 16–18, 20–23].



**Fig. 3:** Emission scan of MilliQ water (excitation wavelength 350 nm). The first peak is due to Rayleigh scatter (elastic) and the second to non-elastic Raman scatter (shaded area =  $A_{\text{rp}}$ ).

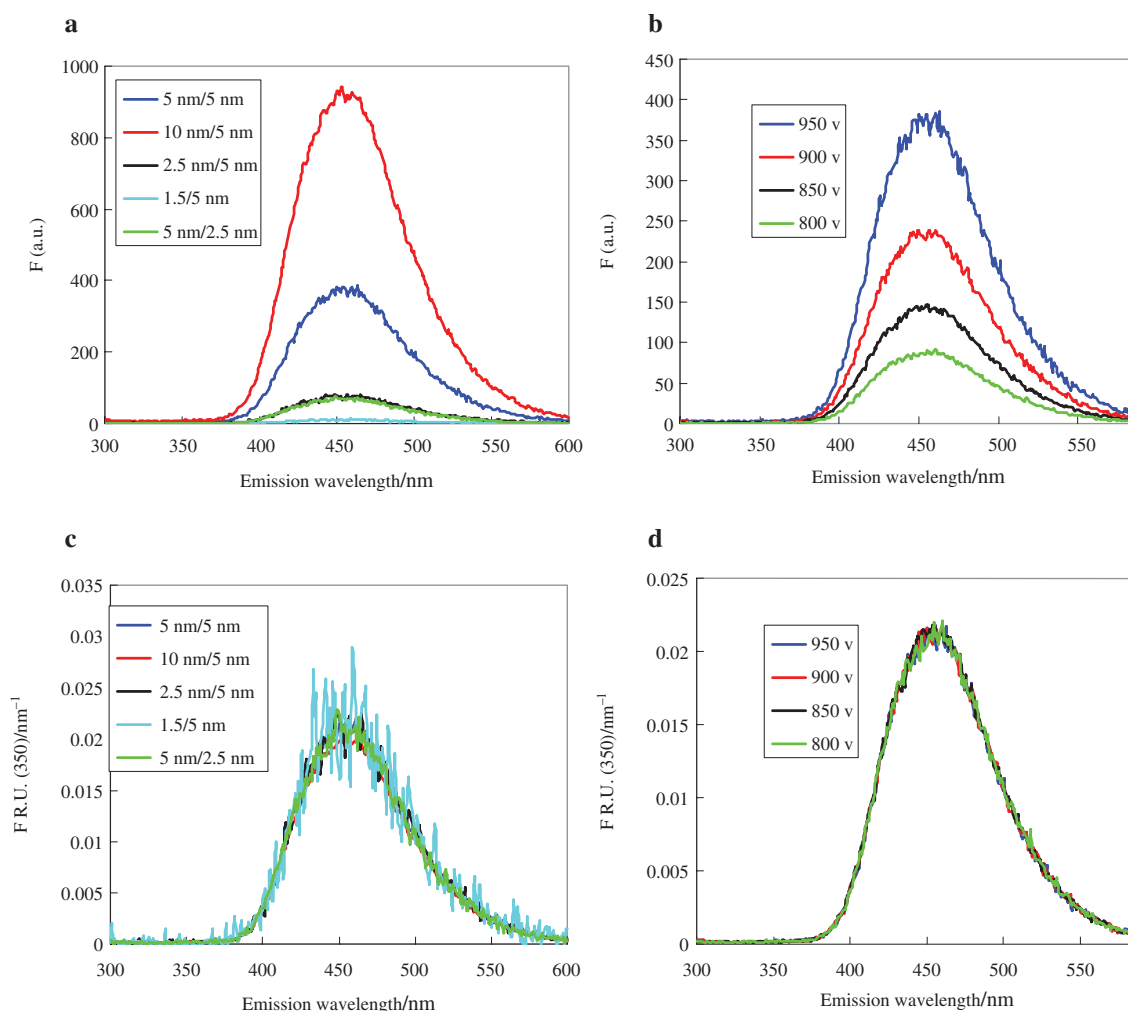
2. The excitation wavelength for obtaining  $A_{\text{rp}}$  must be specified, since  $R_{\text{cs}}$  depends on wavelength [34]. Unless somehow prohibited, 350 nm is suggested as a standard excitation wavelength, since the signal/noise ratio of an instrument is conventionally determined using the Raman scatter signal from this wavelength. The quantified signal could be reported in units abbreviated to R.u. (350) ( $\text{nm}^{-1}$ ).
3.  $A_{\text{rp}}$  depends strongly on instrumental parameters, most notably on the slit widths. Therefore, quantification and conversion of  $I_{\text{f}}$  into R.u. is only meaningful if  $A_{\text{rp}}$  is obtained using the *same instrumental settings* as the sample measurements.
4. The water must be of the highest quality, filtered to remove any suspended particles. *e.g.* reverse osmosis filtered water with a resistivity of 18 M $\Omega$  and preferably treated with a UVB lamp to destroy any remaining dissolved organic material. Alternately, a sealed water Raman standard can be purchased from several suppliers (*e.g.* Starna Raman Ultra-Pure Water, Certified Reference material, RM-H2O).
5. Quartz cuvettes should be handled only with gloves to prevent fingerprints. The quartz cuvettes used should be carefully cleaned internally and externally to ensure that no fluorogenic residue is present. Surfaces should also be clear of scratches, which can act as scattering surfaces that will lead to additional unwanted baseline signal.
6. The Raman signal intensity is temperature dependent (it governs the Stokes to Anti-Stokes band intensities, and thus one needs to explicitly state that samples and water standards are collected at the same temperatures and that this is fixed for a specific calibration).
7. In order to calibrate the whole EEM, the measured data is normalized to  $A_{\text{rp}}$  from one chosen excitation wavelength (*e.g.* 350 nm). It is important to note that this EEM quantification (normalization) can be done using the Raman integral value from one excitation wavelength only (provided spectral calibration has been carried out following conventional methods). If a set of data obtained on different instruments have been quantified in Raman units obtained at different excitation wavelengths, there are two optional methods for comparison. The reported values of  $R_{\text{cs}}$  of water at different wavelengths [34] can be used to convert between the different excitations wavelengths. It may be more convenient to obtain  $A_{\text{rp}}$  at the two wavelengths directly on the particular instrument, either from pure water or from the sample if it is an aqueous solution and the analyte fluorescence does not overlap with the Raman band. This option can be extended and integrated in the EEM measurement routine. A comparison with the correction factors thus obtained at each excitation wavelength with the spectral variance of  $R_{\text{cs}}$  with wavelength [34] will be an indirect control of the preceding conventional excitation/emission correction.

### 3.3 Demonstration of quantification in Raman units

An example of the effectiveness of the Raman-quantification approach with the emission spectra of a sample measured using different instrumental set-ups is shown in Fig. 4. In this example, only one emission scan is presented, in order to facilitate comparison and data presentation. However, the calculations can easily be performed on all the emission scans that make up an EEM. The graphs clearly show the substantial effects that different instrumental setups can have on the spectra measured. In Fig. 4a the excitation and emission slit widths were varied and in Fig. 4b the PMT voltage was changed. For each instrumental setup, a measurement of pure water (reverse osmosis filtered, 18 M $\Omega$  resistivity, UVB treated) was also run (excitation 350 nm), which allowed us to re-scale the spectra according to the method described above. The results are shown in Fig. 4c and d. The spectra now overlap, as they should, because it is the same sample being measured and the instrumental setup differences between measurements have been removed (apart from shot noise, which can be a significant issue). This approach is favourable, because it requires little sample preparation and because sealed water samples are often used for routine instrument signal-to-noise and sensitivity calibration.

Finally, we note that it is not yet common practice to report fluorescence spectra in relative Raman intensity units and so far only a few workers have investigated this option [30–33].





**Fig. 4:** Demonstration of the ability of the Raman quantification approach to remove instrumental setup effects on fluorescence intensity. The emission spectrum of a  $5 \times 10^{-9}$  solution of quinine sulphate is shown measured with (a) different excitation and emission slit widths and (b) different PMT voltage (detector gain). Plots (c) and (d) are the same data after Raman quantification relative to the Raman scatter from 350 nm [32].

### 3.4 Additional considerations

**Temperature:** It is best practice to always use a specified temperature whilst undertaking MDF measurements, preferably with a cell/sample holder that actively controls the sample temperature. The specifying of measurements being made at room temperature is insufficiently precise for accurate measurements, as room temperature can vary from (15 to 30+) °C depending on location, time of year, and the configuration of the laboratory. For the collection of large MDF datasets over multiple months [4, 35], the use of Peltier controlled sample holders and fixed temperature was found to be adequate for reproducible data collection. The supplemental information in reference [4] details the chemometric assessment of EEM data reproducibility under these conditions. Sample temperature will also affect rates of quenching, chemical reactions, protein structure, and other processes, all of which can affect the fluorescence emission. The degree of temperature dependence will be directly related to the sample type and the process under investigation. For example, the kinetics of most protein interactions are very sensitive to temperature in this range, and thus will influence fluorescence emission.

**Polarization:** Polarization (or anisotropy) phenomena in some samples must also be factored into any calibration procedure [36], as there can be a significant effect on measured intensity. This is particularly

important when the sample includes slow moving macromolecules like proteins [37], polymers, viscous petroleum fluids, or asphaltenes [38]. The specific design of fluorimeter being used for MDF measurements may also be important, as the spectral ranges covered in MDF measurements are much greater than in simple single excitation wavelength spectroscopic measurements.

**Data integrity:** Another factor that is increasingly important, and one which is critical to accurate implementation of MDF methods, is data integrity [39]. As the primary output of MDF measurements is easily manipulated digital data, and the primary analysis method normally involves chemometric analysis using software, it is thus easy for data to be unknowingly, or knowingly, corrupted, or otherwise modified. It is critical, therefore, that both the instrument operator and the data analyst be very mindful of the data. It is recommended that the original MDF measurement data (including the header file component) be saved with a readily identifiable filename and in a secure location. This data should then be copied into new folders for chemometric data analysis, ensuring that the original raw data is *always* available for consultation. These original raw data files should not be modified in any way and should always bear the date stamp of the original measurement time. This is critical for three reasons: 1) it maintains the original source data for archival and audit purposes; 2) it provides the source data with which one can validate chemometric data analysis processes and procedures; and 3) it provides the source data to allow a third party to verify and validate results based on MDF measurements. While this is becoming standard practice in industry, academia should, and will have to, follow suit.

## 4 Inner filter effects (IFE)

If samples are not optically dilute, then the fluorescence signal will be subjected to inner filter effects (IFE). This is due to dissolved analytes attenuating either the excitation, the emission light, or both as it passes through the sample. The excitation and the emission light are referred to as primary and secondary inner filter effects, respectively. IFE has a strong and dramatic effect on the shape and intensity of EEM/SFS data and is thus a critical factor in measurement and calibration processes. There are four methods for dealing with IFE for EEM/SFS measurements: 1) Mathematical correction, 2) Sample dilution, 3) Pathlength change, or 4) Explicit inclusion of the IFE. Sample dilution is relatively easy to implement, but it adds a sample handling penalty (which is a source of error) that may not always be appropriate for the application. The mathematical correction approach (detailed below) is appropriate for some samples where the optical density is not too high,  $A < 1.5$  [24]. The final option of essentially doing nothing (detailed below) and including IFE in the EEM/SFS analysis is also a valid option in applications where sample handling should be minimized.

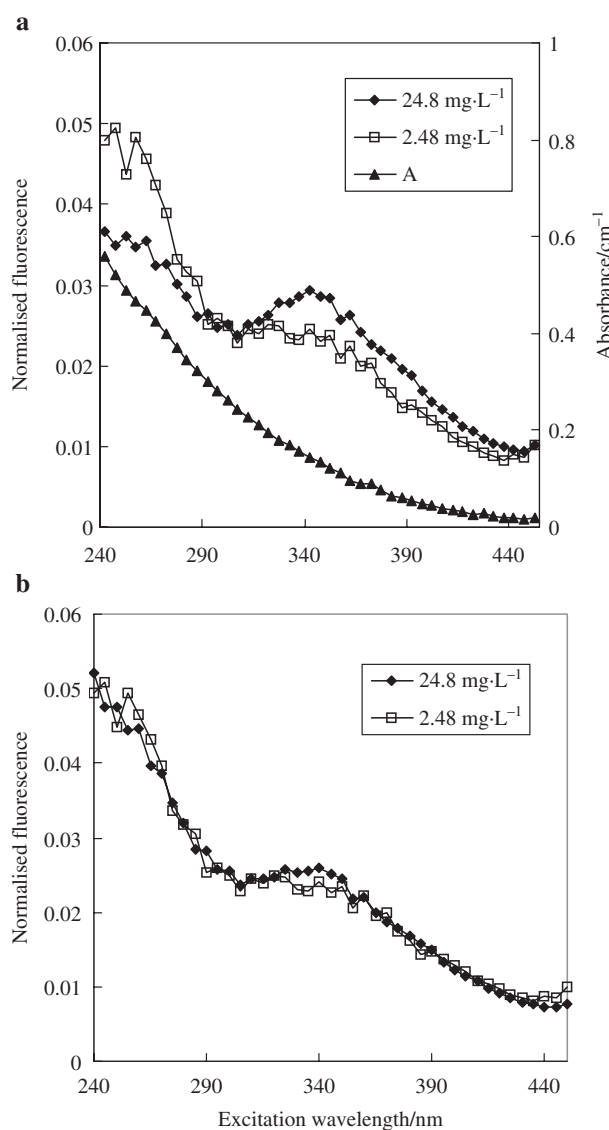
Changing the pathlength to reduce sample absorption is a quick and easy solution, as there are a wide variety of bespoke quartz cuvettes with variable pathlengths available from various vendors. However, in some cases, where the optical density is very high and the samples are very viscous *e.g.* food or petroleum oils, front surface excitation (FSE) is often the best option [6, 40]. For FSE, reproducible alignment of the sample cuvette is critical, otherwise, artefacts will be generated in the EEM/SFS spectra. Another consideration with FSE is variation in the optical density of the sample, for example with natural products *e.g.* milk, petroleum, etc. The intrinsic sample variation may generate large fluctuations in optical density and in those cases the effective pathlength sampled by the excitation beam will vary significantly. This can also generate unwanted artefacts. In these cases, it may be advisable to avoid the use of transparent cuvettes with short pathlengths (1–2 mm) as there may be reflection artefacts from the rear wall of the cuvette.

IFE can sometimes be approximated and corrected for, if the absorption properties of the sample are also known (measured) and fluorescence is measured with 90° geometry (angle between excitation and emission light paths). While a variety of equations with ranging complexity are available for IFE correction [40, 41] the simple equation 4, presented in Lakowicz [42, 43], will often suffice:

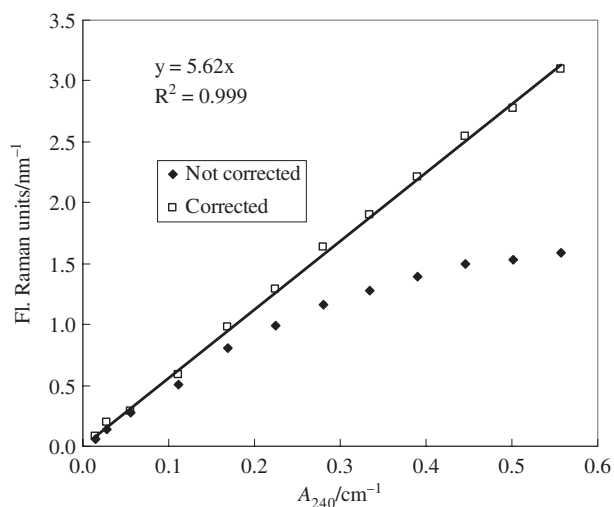
$$f_{\lambda_{\text{ex}}, \lambda_{\text{em}}}^{\text{IFE}} = 10^{(0.5(A_{\lambda_{\text{ex}}} + A_{\lambda_{\text{em}}}))} \quad (4)$$

Here  $f$  is the IFE correction factor, which varies depending on the excitation and emission wavelengths ( $\lambda_{\text{ex}}$  and  $\lambda_{\text{em}}$  respectively).  $A$  is absorbance measured in a 1 cm cuvette at both the excitation or emission wavelengths. Each measured intensity is simply multiplied by the corresponding correction factor. The equation assumes that the fluorescence takes place at mid-point in a 1 cm fluorescence cuvette, where the pathlength is 0.5 cm for both the excitation and emission filter effects.

Fluorescence intensities can also be corrected to account for IFE by using more complex models, such as those described by [44, 45]. Discussions on the efficacy of the different methods are given by [46, 47]. The analyst should consider carefully the choice of correction methodology and whether it is appropriate for the sample type being examined. For example, in samples with low analyte concentrations and relatively low absorbance, IFE correction is a good option. For samples with low to medium analyte concentrations, and relatively high absorbance's, sample dilution can be considered as a more suitable method for dealing with IFE.



**Fig. 5:** Correcting for inner filter effects. (a) Graph of the normalized excitation spectrum of Suwannee River Fulvic Acid at two concentrations. Additionally, the measured UV-Vis absorbance properties are shown (24.8 mg·L<sup>-1</sup>). (b) The same normalized excitation spectra after correction for inner filter effects using equation 3.



**Fig. 6:** Example of the ability of the inner filter correction procedure to correct for non-linearities between the fluorescence and absorbance of an analyte (in this case Suwannee River Fulvic Acid).

The influence of IFE and the effectiveness of equation 4 for correcting them can be demonstrated using a dilution series of Suwannee River Fulvic Acid (International Humic Substances Society). A stock solution of  $24 \text{ mg} \cdot \text{L}^{-1}$  was made and diluted to create a series of standards. The fluorescence and absorption properties of the solutions were then measured. Due to the absorption characteristics of the material (increasing with decreasing wavelength, see Fig. 5a), the primary IFE dominates and are therefore shown here as an example. This figure also shows the excitation spectra ( $\lambda_{\text{em}} = 550 \text{ nm}$ ) of two standards with an order of magnitude difference in concentration. The spectra have been normalized to their integral area in order to remove quantitative differences and reveal the effect that inner filter processes have on the shape of the excitation spectra. The higher concentration standard has a flatter spectrum. Figure 5b shows the same spectra corrected for IFE using equation 4. It is clear that the deviations due to IFE can be corrected using this simple procedure. The effects can also be seen in Fig. 6, where the fluorescence ( $\lambda_{\text{ex}} = 240 \text{ nm}$ ,  $\lambda_{\text{em}} = 450 \text{ nm}$ ) is plotted against the absorbance at 240 nm. It is again clear that this correction method is suitable for minimizing the effect of IFE. This equates to an A of approximately  $0.09 \text{ cm}^{-1}$ , which agrees well with threshold values reported in the literature [43, 48, 49].

An alternative approach to the issue of IFE with complex, relatively concentrated samples is to accept that IFE is a characteristic property of the sample and is thus of use in certain cases. For analytical applications, where one needs to minimize sample handling by using dip probes and/or data processing, then one can more accurately and simply implement a defined sampling geometry rather than undertaking an IFE correction as outlined above. This approach has been implemented successfully for the quantitative analysis of various complex biogenic samples from, *e.g.* wine [50], milk [40, 51], food [5, 52, 53], and cell culture media [4, 35, 54]. In many of these applications, there are generally multiple fluorophores present, often at high concentration. It is the spectral variation due to compositional changes (both absolute and relative) that is of interest. In essence, the contours of the EEM/SFS plot provides the information, and IFE is integral to the shape of the plot. It is of minor concern if these variances are due to IFE, since this is inextricably linked to concentration. In highly regulated industries, there is a requirement that the media and raw materials be consistent, so we do not need to correct explicitly for IFE. However, this approach still requires instrument and spectral calibration to ensure that instrument performance does not impair measurements. It also requires the verification of the quality of the cuvettes prior to use and, ideally, the use of a fluorescence standard for intensity calibration. We note these sample types are much less optically dense than many petroleum fluids. As such, this approach is favoured over front face excitation due to better measurement reproducibility.

## 5 Sample handling

While primary sample errors are the largest cause of problems in any analytical method, the use of MDF-based measurements has some specific issues in relation to sample handling that can also contribute significantly to the total analytical error.

### 5.1 Sample handling issues

Once the hardware for the EEM/SFS measurements has been properly calibrated, the issues surrounding the collection of data from samples need to be carefully addressed. One needs to be cognizant of the fact that many of the samples types routinely analysed by EEM/SFS methods are complex chemical mixtures (*e.g.* water, media, food, *etc.*). This intrinsic chemical complexity produces sample instability and sensitivity to change induced by temperature, light, *etc.* Thus, all the benefits of instrument calibration may be lost if the control, calibration, and test samples are not properly assessed and handled during storage and data collection. It is particularly important if one uses a complex mixture as a day-to-day control, calibration, or validation sample for instrument or chemometric model performance qualification. The issue of standard samples for EEM/SFS based methods is not trivial – often the need is for control samples that undergo the same sample handling, preparation, and data collection steps as the test samples. Since EEM/SFS data is in itself complex, it may not always be possible to identify uncontrolled sample changes. It is, therefore, important to implement careful sample handling procedures to minimize physiochemically induced measurement variance between samples, and in particular between control samples. This is very important in the context of both calibration transfer activities and EEM/SFS measurements made over extended periods of time.

The primary sample issues to be managed, via an appropriate experimental design, are:

1. **Chemical instability:** Most biogenic materials, such as cell culture media [4], river waters, biological fluids, and others, are complex mixtures of molecules, many of which can react with each other when in solution. For example, if cell culture media are stored at (2–8) °C in liquid form, one can observe subtle chemical changes [55], which may or may not have an impact on the EEM/SFS data. For samples containing proteins and glucose [56], glycation of proteins can cause observable changes in fluorescence. There are many other chemical reactions that can and will occur in solution so the analyst should always undertake a chemical risk assessment to establish the short, medium, and long-term stability of the samples and adjust the handling and data collection procedures accordingly.
2. **Photochemical stability:** Some complex biogenic liquids, such as cell culture media, often contain photosensitive compounds like riboflavin, which will photo-degrade rapidly under ambient light and also cause the degradation of other components. This has a very large impact on fluorescence emission [57].
3. **Physical instability:** Liquid samples that contain proteins are also sensitive to physical stresses. These can cause changes in the sample, which adversely affect the MDF data. One common problem is the formation of bubbles inside cuvettes during transfer of high concentration protein samples. This can act as sites of intense scatter. Some proteins can also aggregate [58] if shaken, again changing sample properties and the MDF data.
4. **Freeze-thaw/defrosting:** Samples which are stored frozen at low temperature must be defrosted before EEM/SFS measurement and this can cause variation in sample emission. For example, in the case of some cell culture media, inadequate defrosting times led to the formation of micro-crystalline particles, which are clearly observable using Raman spectroscopy [59]. In EEM data, these particles will increase the amount of Rayleigh scatter and can cause problems. The physicochemical properties, and thus the optical properties of protein containing samples, can also be affected by freezing, and this can impact on EEM measurements [60].

All these factors can cause changes in EEM/SFS plots that may obscure the compositional changes the analyst is seeking to monitor. This can result in erroneous conclusions. Thus, for proper and accurate EEM/SFS meas-

urements, one should develop a careful sample handling procedure informed by the chemical and physical properties of the control, standard, and tests samples under investigation. One should never assume that complex (particularly biogenic) samples are stable without proper care.

## 5.2 Sample handling recommendations

The diversity of sample types being tested by EEM/SFS methods is too large to for a specific set of recommendations. However, the key point is that the analyst should develop a detailed experimental plan before embarking on MDF measurements that includes a risk assessment of the sample and its physicochemical behavior under the analysis conditions (*e.g.* concentration, temperature, ionic strength, *etc.*). As a general point of principle, the more compositionally complex the sample, and the more biogenic in nature, the more critical the handling requirements. For example, consider some typical liquid samples with low, medium, and high compositional complexities:

1. **Low dissolved analyte load *e.g.* seawater:** Samples should be filtered to remove microbes (*e.g.* 0.2  $\mu\text{m}$ ) and stored refrigerated in dark coloured bottles. An inter-laboratory study [61] indicated that short term storage (<2 months) presented no significant storage issues, although this is dependent on the origin of the samples and its susceptibility to bacterial regrowth.
2. **Medium dissolved analyte load *e.g.* cell culture media:** For complex biogenic liquids extracted from bioprocesses (biotechnology processes), such as media (particularly those containing photosensitive compounds such as riboflavin), it is recommended that the samples be stored in the dark at  $-70\text{ }^{\circ}\text{C}$  (to avoid any enzymic based processes) [57] and only removed for measurement. Further, the thawing procedures should be carefully controlled to ensure that all components have redissolved prior to measurement.
3. **High dissolved analyte load *e.g.* blood:** For each material, a careful assessment should be undertaken, and in general these materials should be stored in the dark and frozen (at  $-20\text{ }^{\circ}\text{C}$  or  $-70\text{ }^{\circ}\text{C}$ ) using appropriate containers for the sample material. As an example, detailed storage protocols have been published for human blood and urine for bio-banking purposes [62, 63].

From a purely chemical point of view, greater compositional complexity leads to a higher probability of uncontrolled/unknown sample change with improper storage or handling. The degree of sample change is also very concentration dependent, so water samples from a river with low analyte concentrations should be significantly more stable than a food or bioreactor sample.<sup>3</sup> The photophysical complexity of the sample also increases with compositional complexity. This too has an impact on the measurement process (see below). In summary, for each new sample type and application, the analyst should carefully consider all of the above, produce a detailed experimental design and chemistry-led Standard Operating Procedures (SOP) for the sample handling and data collection tasks.

## 6 Chemometric data analysis for calibration and validation

Correct calibration of EEM/SFS data by the methods described above allows the analyst to generate complex and information-rich data sets. However, interpreting these data can be difficult because of the broad, usually featureless fluorescence spectra and the sheer size of the data matrix (samples  $\times$  EEM/SFS spectra). Furthermore, the fluorescence properties of natural materials like proteins are often very complex due to the nature of the sample (frequently, these are mixtures of large numbers of compounds) and also the range of photo-physical phenomena occurring (*e.g.* energy and electron transfer) and operational artefacts (IFE, polarization effects) present. Thus, interpretation of the multi-way data often requires the use of multivariate analysis

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<sup>3</sup> In this context, we are not considering changes due to microbial action here, as that is a separate issue.

(i.e. chemometrics). Proper use of chemometric data analysis on MDF data requires careful consideration of sample photo-physics and an in-depth understanding of the methods [64, 65] and their use [66–70].

The initial and final steps in the implementation of EEM/SFS-based analytical methods generally requires the use of chemometrics, first to assess the reproducibility and robustness of the measurement itself, and second to produce an output in terms of a qualitative result or quantitative value. The second aspect is outside the scope of this paper, but there are many reviews available describing the various applications in biotechnology, food science [71], and environmental analysis [72–77], for example. The application of chemometrics to the fluorescence data analysis used for calibration/standardization can generally be split into four distinct elements or steps:

1. **Data pre-processing.** Depending on the application and the specific chemometric methods being employed, one may have to pre-treat the raw spectral data prior to analysis.
2. **Exploratory analysis.** This is where the EEM/SFS data is assessed for reproducibility, sources of variance, and robustness. Typically, one can utilize multi-way versions of Principal Component Analysis.
3. **Chemical profiling.** Here the unique properties of certain fluorescence models (e.g. PARAFAC or MCR based) are used to provide an analytical chemical fingerprint directly from complex mixtures.
4. **Multivariate regression.** Here one seeks to correlate spectral variance in the EEM/SFS data with a specific external factor. The most widely used methods are based on Partial Least Squares (PLS) regression [78, 79], although there are a variety of alternative multi-way methods.

## 6.1 Data pre-processing

Here we summarize the most important steps for data pre-processing prior to chemometric analysis and outline the most common pitfalls and problems. This is an important consideration in all of the standardization and calibration processes that are required prior to effective analytical method development.

- **Rayleigh scatter:** Since Rayleigh and Raman scatter are generally unrelated to the compositional properties of the normal samples and the scatter peaks do not behave linearly (or trilinearly), they may complicate and bias fluorescence data modelling unless dealt with appropriately. The use of SFS measurements provides a simple way of eliminating the Rayleigh scatter during measurement. However, this may not always be a valid solution. Various computational/mathematical methods are available for dealing with the Rayleigh scatter issue in EEM. For example, one could insert zero values outside of the fluorescence data area [80]. For PARAFAC modelling, one can insert NaN values in the scatter regions combined with a non-negativity constraint [81]. Jiji and Booksh [82] used data point weighting to make the scattering band insignificant during trilinear decomposition, whereas Wentzell *et al.* [83] chose to eliminate scatter by using weighted PCA on the unfolded EEM matrix before refolding and subsequent decomposition of the EEM. There are various other strategies available, including interpolation [84] and modelling [85, 86].
- **Normalization:** In many cases the instrument and intensity calibration processes will generate EEM/SFS data that is well corrected for the small variations in instrument response function [33]. However, in some cases one can also implement a post-measurement normalization strategy to reduce small instrument induced variances.

The recommendation for the data-pre-processing step is that the exact same methods be implemented throughout the analytical method development cycle. In other words, the same methods are to be used, in the same sequence, and using the same code/software.

## 6.2 Exploratory analysis: principal component analysis

A critical issue in the use of EEM/SFS measurements is the identification of sources of variance in the measurement process and sample handling prior to developing and implementing an analytical method. Because

of the multi-way nature of the data, this is best done using multivariate analysis combined with chemical knowledge of the sample, *i.e.* via the application of chemometrics. Many applications of chemometrics to EEM data make use of traditional multivariate analysis tools, such as PCA and PLS regression [87]. PCA can extract *latent variables* from a set of measurements, regardless of their origin [64]. While methods based on PCA are very powerful for assessing sample variance and fingerprinting of samples, there is another family of methods based on component resolution that are able to extract even more information from EEM data. This is especially true when the fluorescence data are obtained in something approximating an ideal situation, where IFE and Förster Resonance Energy Transfer (FRET) is minimized. These methods can be used for more accurate chemical profiling of sample variance in terms of changes to specific fluorophores.

### 6.3 Component resolution and analysis

The benefit of using methods like parallel factor analysis (PARAFAC) [81, 88, 89] or Multivariate Curve Resolution (MCR) [90–93] for the analysis of MDF data is that, under favourable conditions (*i.e.* minimal IFE and FRET), they can extract the excitation and emission profiles of some or all of the most significant fluorophores contributing to the measured emission. This can be particularly important in the initial stages in developing an EEM/SFS-based analytical method, where one needs to acquire information about the photophysics of the samples being used for standardization, calibration, and testing.

In 1961 it was shown that the properties of fluorescence make it possible to mathematically separate the signal from a simple mixture of fluorophores into its underlying components [94]. Seemingly unrelated work in psychology [95] led to the development of a general model for so-called three-way data called PARAFAC [96]. When applying PARAFAC models to the fluorescence of relatively simple mixtures, the underlying components can be mathematically resolved. These components can be fluorophores if the mixture contains non-interacting fluorophores and there are minimal effects from interferants. This is similar to what is done in traditional physical chromatographic separations, as the approach carries no assumptions on the number of fluorescent components or their spectral characteristics. This can be termed “*mathematical chromatography*”.

PARAFAC analysis [81, 97] separates the EEM fluorescence signal from a series of samples (mixtures) into a set of tri-linear terms and a residual array. In order to provide a chemical interpretation, it assumes that the measured fluorescence is approximately the sum of the fluorescence of the individual fluorophores present and the instrumental noise:

$$x_{ijk} = \sum_{f=1}^F a_{if} b_{jf} c_{kf} + e_{ijk}, \quad i = 1, \dots, I; j = 1, \dots, J; k = 1, \dots, K \quad (5)$$

Here  $x_{ijk}$  represents an element in a three dimensional “box” of fluorescence intensity data for  $I$  samples, at  $J$  emission wavelengths and  $K$  excitation wavelengths. It has been shown that the PARAFAC model is intrinsically unique [98], hence estimating the parameters  $a_{if}$ ,  $b_{jf}$ , and  $c_{kf}$  from the fluorescence intensities  $x_{ijk}$ , will provide unique estimates of these parameters. This is crucial, as it allows one to ‘un-mix’ mixture measurements. Assuming that the fluorescence data behave approximately as described (linearity and additively), the uniqueness of the PARAFAC model implies that, if the correct number of components ( $F$ ) is used, then the parameters will be estimates of chemically meaningful parameters. The score  $a_{if}$  is proportional to the concentration of analyte (fluorescent component)  $f$  in sample  $I$ , while  $b_{jf}$  is proportional to the quantum yield and emission distribution of analyte  $f$  at emission wavelength  $j$ . Likewise,  $c_{kf}$  is related to the molar absorption coefficient at excitation wavelength  $k$  and  $e_{ijk}$  is the residual representing the unexplained signal. The PARAFAC model derives scores and loadings for each component, resulting in an emission spectrum loading and an excitation spectrum loading vector. As in PCA, the components can be considered latent variables reflecting the underlying variation, but because of the uniqueness of the model, the loading vectors are potential estimations of the real chemical spectra. Therefore, the score will also be more than merely the amount of an abstract phenomenon – it will be the relative concentration of whatever the spectral loadings represent. In summary, PARAFAC under ideal conditions can resolve an EEM spectrum into its component fluorophores (the loadings) and the relative contribution (the scores).



There are, however, certain assumptions that have to be met in order for PARAFAC to provide meaningful results. Each fluorophore must first have a characteristic emission and excitation spectrum and second vary independently in concentration from the remaining fluorophores. This is rarely the case for many sample types measured by EEM/SFS methods (particularly for proteins). Thus, one must carefully evaluate the suitability of using PARAFAC (or any other method with these criteria) in terms of the sample photo-physics. For example, in a dilute sample of individual small molecule fluorophores, when the combined absorbance is less than  $0.05 \text{ cm}^{-1}$  ( $A_{\text{ex}} + A_{\text{ex}} < 0.05 \text{ cm}^{-1}$ ), it can be assumed that IFE are minimal. In this situation PARAFAC analysis is very well suited for characterizing and identifying even severely overlapping fluorophores in complex mixtures. However, when the absorbance increases (values between  $0.05$  and  $0.5 \text{ cm}^{-1}$ ), corrections for IFE must be applied according to Eq. 4 before such PARAFAC analysis can be optimally pursued. For dilute solutions of protein, where there are multiple intrinsic fluorophores located in close proximity ( $<100 \text{ \AA}$ ), FRET is a very important factor and one must be very careful in the interpretation of the results from PARAFAC or MCR modelling, as there is a loss in tri-linearity of the data.

In optically dense samples ( $A > 0.5 \text{ cm}^{-1}$ ), it is normally no longer appropriate to assume that a direct, real chemical interpretation can be obtained. Still, there might be characteristic features in the spectra, and these can be characterized by chemometric methods like PCA with no requirement for a “molecular” origin of the collective signal. This approach often includes other independently obtained qualitative properties of the sample. This is typically the assignment of the sample to a group. For example, if the sample is a body fluid or tissue, a sample group could be *e.g.* people with a specific disease.

Another elegant application of PARAFAC is to extract the analyte signal from the signal of other components [99], which then allows analyte quantification. This offers an attractive, mathematical alternative to the more laborious standard addition or modified standard addition methods [100]. Finally, PARAFAC has also been used to model Rayleigh scatter in EEM data, which can then be used to remove the scatter signal [10, 101].

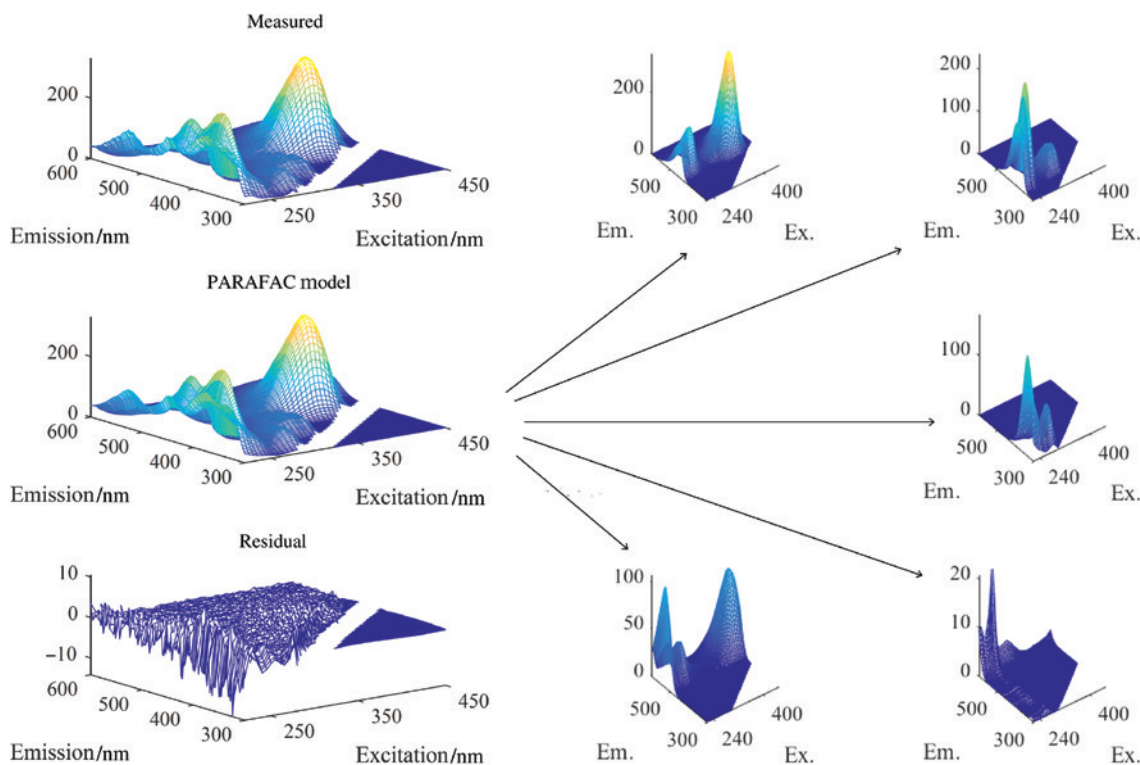
## 6.4 Combining chemometric characterization with spectral calibration

To date, calibration routines such as those discussed in this paper have focused on calibrating the excitation and emission elements independently and involve time consuming measurements. Scans are performed slowly and replicated to minimize the influence of instrumental noise on the correction factors. Additionally, in order to cover a broad wavelength range, a series of standards have to be measured individually. The ideal solution for MDF applications would be to have a stable mixture of standards which could be analysed simultaneously using the same (comparatively) fast scan speeds used for samples. The major problem with this approach would be the poorer signal-to-noise ratio of the measurements influencing the quality of the subsequently-derived correction factor. However, in theory this could be circumvented by integrating chemometric data analysis into the instrument spectral calibration procedure. This approach was explored (internally in the Stedmon lab, using a standard Varian Exclipse spectrometer, unpublished results) with the five commercially-available BAM fluorescence standards [18, 20]. A more rigorous inter-laboratory study with multiple instrument and software platforms will be required to validate this method for operational use. However, this exercise is included to demonstrate the feasibility of such a chemometrics based approach [18, 20].

Five mixtures containing all five standards at different concentrations were made (Table 1) and EEMs of the mixtures were recorded with a fast integration time ( $0.1 \text{ s}$  – corresponding to  $1200 \text{ nm/min}$ ). The time

**Table 1:** Volumes of fluorescence standards used to create 5 mixtures (samples 1–5) of 4 mL each.

Standard	BAM-F001 ( $\mu\text{L}$ )	BAM-F002 ( $\mu\text{L}$ )	BAM-F003 ( $\mu\text{L}$ )	BAM-F004 ( $\mu\text{L}$ )	BAM-F005 ( $\mu\text{L}$ )
Sample 1	600	700	800	900	1000
Sample 2	700	800	900	1000	600
Sample 3	800	900	1000	600	700
Sample 4	900	1000	600	700	800
Sample 5	1000	600	700	800	900



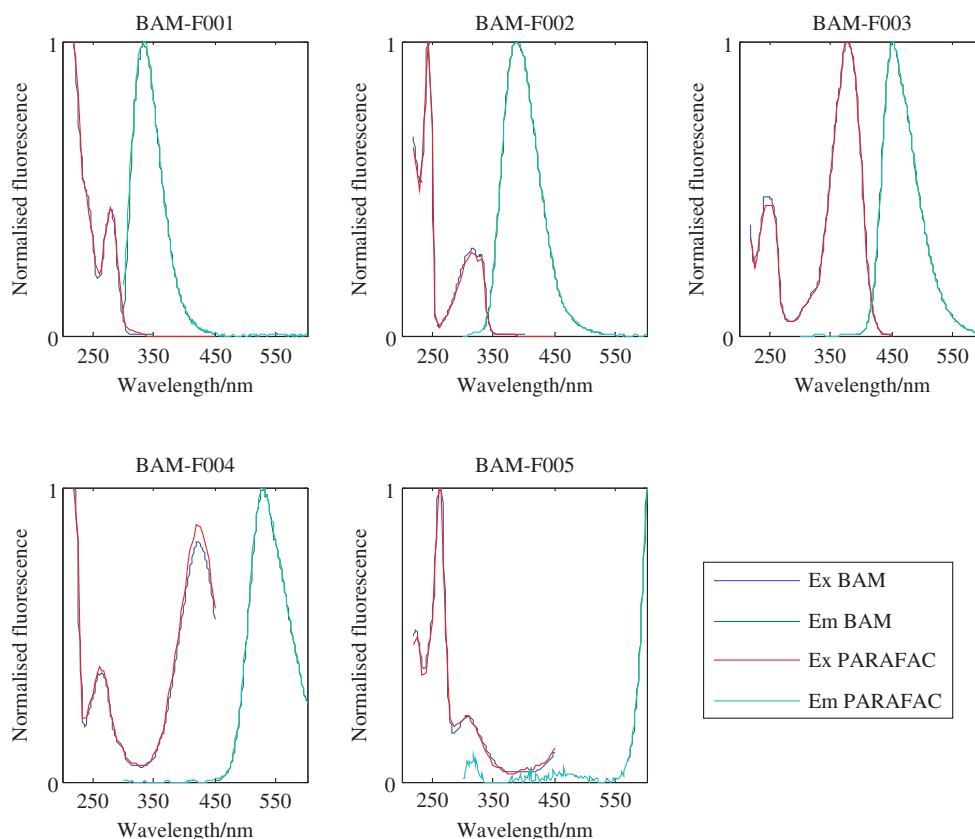
**Fig. 7:** The results of a five component PARAFAC model of a mixed solution of the fluorescence standards. Left: The measured EEM is a mixture of five fluorophores (BAM-F001, BAM-F002, BAM-F003, BAM-F004, BAM-F005). The model is the five-component model derived by PARAFAC. The residual EEM is the unexplained fluorescence signal and represents largely instrumental noise. Right: The 3D representations of the five PARAFAC components which correspond to each of the BAM fluorophores.

taken to collect the entire EEM was approximately 10 min. In addition, the individual emission spectra of the five standards were measured in accordance with the fluorescence standard kit instructions. Four replicate emission scans were made for each standard and a scan speed of  $60 \text{ nm} \cdot \text{min}^{-1}$  was used. An excitation scan of each standard was also recorded, so that the excitation properties could be compared later.

The five EEMs were modelled using a five component PARAFAC model. An illustration of the PARAFAC model is shown in Fig. 7 (left), where the EEM measured on sample 2 is shown together with the PARAFAC model and the residual EEM. The 3D spectra of the five PARAFAC model components derived from the mixtures are also shown (Fig. 7, right). Figure 8 offers a more conventional representation and compares the excitation and emission spectra of the five pure standards with the spectral components extracted from the EEMs by PARAFAC. The emission spectra are identical for the first four standards (Fig. 8). BAMF005 is the poorest resolved component. However, this can be expected, as its fluorescence is weak within the region of the EEM because it has a fluorescence maximum above  $600 \text{ nm}$ .<sup>4</sup> The excitation properties of the PARAFAC components deviate to a slightly greater degree. This is to be expected, as the excitation wavelengths were varied in  $5 \text{ nm}$  steps in the EEMs, resulting in poorly resolved excitation peaks.

From this exercise, we can conclude that it is possible to mix these fluorescence standards and incorporate the calibration routine into a chemometric approach. Using the algorithms provided by [102], the spectral properties of the derived PARAFAC components can be used to derive an emission correction curve. This example is a proof of concept and future research should focus on simplifying and refining the approach. This would bring the fluorescence community one step closer to a simple, rapid, and robust calibration

<sup>4</sup> One should be careful when studying luminescence properties in the longer wavelength regions (above  $600 \text{ nm}$ ) as many standard instruments are not optimized for this spectral range. This is generally manifested in a detector with poor near-IR performance (PMT) and the common use of gratings blazed in the  $(400\text{--}500) \text{ nm}$  region.



**Fig. 8:** The excitation and emission spectra of the five BAM fluorescence standards measured individually (Ex BAM and Em BAM), plotted together with the five spectral components identified in the PARAFAC model of the mixture (Ex PARAFAC and Em PARAFAC). The emission scans of the pure standards are averages of four replicate scans as required by BAM.

procedure for EEM measurements, which in turn will greatly increase the number of applications in industry and research.

## 6.5 Chemometric methods for calibration transfer

Even with individual instrument calibration, there still remains the issue of calibration transfer for EEM/SFS methods between instruments. One study [103] has shown that variance between similar instruments can be very significant (~6–10% in intensity), even with careful calibration. The recent trend towards hosting open access online libraries of spectral data, which for example includes EEM data [104], makes it imperative that calibration transfer for EEM/SFS data become more widely adopted. The feasibility of using PARAFAC for calibration transfer has also been demonstrated [105].

## 6.6 Chemometric method integrity

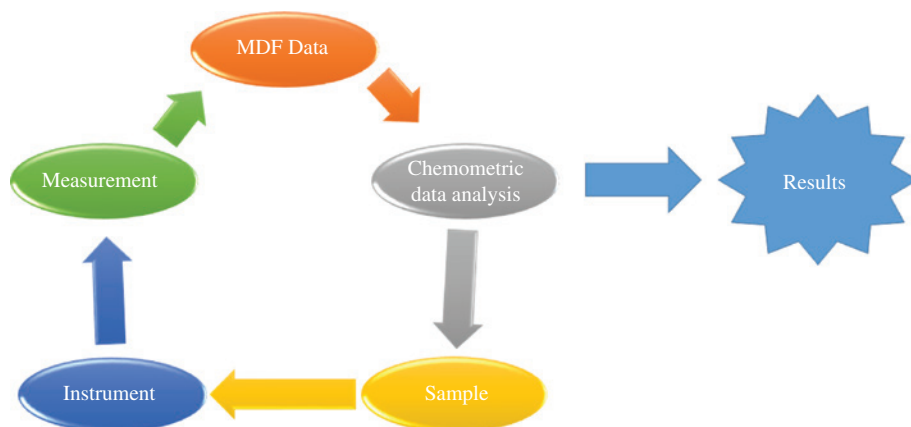
As outlined above in the context of the MDF data [39], it is important for the implementation of standardized analytical methods based on MDF that the chemometric data analysis procedure be accurately recorded and documented, to ensure that calibration and validation test methods as outlined above can be reproducibly performed in different laboratories. This should involve the development of robust procedures for describing each individual step in the process, saving and archiving the code/algorithms implemented, and noting the

specific software platforms used. It should be based on the use of standardized descriptions of methods, as per IUPAC recommendations [106].

## 7 Recommendations and perspectives

The use of multi-dimensional fluorescence measurements methods like EEM, SFS, and ARMES is expanding rapidly as new needs in the area of complex materials analysis emerge. With the physical measurement comes the increased use of chemometric data analysis, in essence a “mathematical chromatography” [1, 107]. These include, amongst others, the characterization of natural organic matter in soil and aquatic ecosystems [72, 108, 109], oil-spill forensics [110] and oil characterization [6, 111], monitoring of food quality and production [67, 71, 112], and diagnostics on body fluids *e.g.* [113, 114]. In the near future, we anticipate that there will be a significant increase in the use of EEM/SFS and chemometrics for routine and high-value analytical applications. This will be driven largely by biomedical diagnostics, environmental monitoring, and process analytical technology (PAT) needs [4, 35, 57, 115]. The fact that these measurements involve physically moving components, wide spectral ranges, and complex samples introduces many potential sources of error. Therefore, a prerequisite for the adoption of these methods in regulated environments is the development of simple, robust, reliable, and common calibration routines for both EEM/SFS measurement and data analysis procedures.

The analyst who wishes to utilize EEM/SFS for accurate analytical purposes should ensure that for each step in the analytical process (Fig. 9) appropriate calibration methodologies are adopted. These should minimize unwanted sample variation, correct for instrumental and sample bias, ensure correct data collection and the integrity of same, and finally ensure that the correct chemometric methods are adopted and validated. It is a three-phase operation with sample, instrument, and digital environments being *equally important* to the generation of accurate output results. Failure to properly standardize, calibrate, and validate *any* individual element in the process will undermine the validity of the data obtained and the results inferred from those data. Therefore, it is strongly recommended that the experimental planning for MDF measurements and applications be carefully undertaken and fully integrated. A very important consideration here is the need to minimize the disconnect that often occurs between the person who produces the samples, the analyst who collects the data, and the analyst who undertakes the chemometric analysis. There should, therefore, be a clear chain of custody for the samples, from collection to measurement to data, for the data, from the instrument to the computer, and for all steps in the chemometric analysis.



**Fig. 9:** The interlinking nature of the MDF operational cycle as applied to analytical methods. For each element in the cycle (data-instrument-measurement-data-analysis), appropriate controls and procedures need to be implemented to generate accurate and useful output results.

In conclusion, the combination of EEM/SFS fluorescence and chemometric analysis has proved to be a powerful analytical tool in scientific research, as well as in process and environmental monitoring applications. Most of the tools necessary for robust method calibration are available to the community and are continually being developed and simplified for non-specialist use. The calibration and correction procedures still need to be simplified further (*i.e.* integrated into the spectrometer software), and a set of standards will have to be developed for the chemometric methods. However, the results presented here suggest that a simple one step automated procedure for the calibration and standardization of EEM/SFS fluorescence measurements will be available in the not too distant future.

## Reference methods, standards and applications of photoluminescence

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