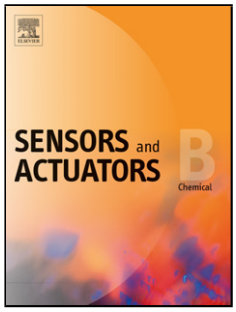

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Authors: S. Sanllorente, L. Rubio, M.C. Ortiz, L.A. Sarabia

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SIGNAL TRANSFER WITH EXCITATION-EMISSION MATRICES BETWEEN A PORTABLE FLUORIMETER BASED ON LIGHT-EMITTING DIODES AND A MASTER FLUORIMETER

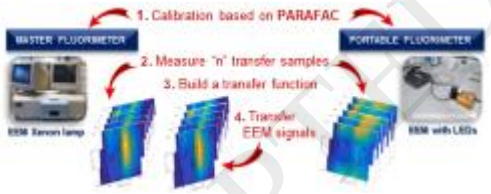
S. Sanllorente[a], L. Rubio[a], M.C. Ortiz[a, 1], L.A. Sarabia[b]

[a]*Department of Chemistry,* [b]*Department of Mathematics and Computation, Faculty of Sciences, Universidad de Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos (Spain)*

[1]Corresponding author. Telephone number: 34-947-259571. *E-mail address*: mcortiz@ubu.es (M.C. Ortiz).

[2]Absolute values of the relative errors (AVRE), capability of detection (CCβ), charge-coupled device (CCD), confidence interval (CI), confidence interval length (CIL), core consistency diagnostic (CORCONDIA), decision limit (CCα), direct standardization (DS), dissolved organic matter (DOM), excitation-emission matrices (EEM), light-emitting diodes (LEDs), master fluorimeter (MF), maximum residue level (MRL), parallel factor analysis (PARAFAC), piece-wise direct standardization (PDS), portable fluorimeter (PF).

Graphical abstract



HIGHLIGHTS

- EEM transfer between instruments with LEDs and a Xenon lamp as excitation sources
- The EEM signal transfer makes possible the unequivocal identification of analytes
- The transfer approximates the precision of the portable to the master fluorimeter
- There was no bias in the determination of enrofloxacin using the EEM signal transfer

ABSTRACT

In this work, the transfer of the excitation-emission matrices between a portable fluorimeter based on LEDs and a master fluorimeter based on a xenon source was carried out. Enrofloxacin was the analyte of interest and it was measured alone or in binary mixtures with flumequine (partially overlapped signals) or with ciprofloxacin (fully overlapped signals). The maintenance and transfer of the unequivocal identification of the fluorophores between both instruments are shown. The precision in the determination performed with the portable fluorimeter approximated to that made with the master fluorimeter using this transfer and it did not introduce bias. The correlation coefficients of the calibrations based on PARAFAC using EEM signals were higher than 0.999, whereas the values of the capability of detection ranged from 14.8 to 26.9 μg L^{-1} for probabilities of false positive and false negative fixed at 0.05. These results contribute to the effort to perform the fluorimetric detection outside the laboratory and to promote the use of databases of fluorescence spectra for the unequivocal identification in remote of fluorophores of interest and/or regulated.

Abbreviations[2]

Keywords: Signal transfer; portable fluorimeter; excitation-emission fluorescence; PARAFAC; capability of detection; enrofloxacin.

1. Introduction

The use of excitation-emission matrices (EEM) together with three-way decomposition methods such as parallel factor analysis (PARAFAC) have turned fluorescence spectroscopy into a powerful method of analysis due to the second-order property [1]. This property enables the unequivocal identification and quantification of a fluorophore even in the presence of interferents. As a consequence, the applications related to the determination of pesticides [2], migrants from food contact materials [3] and residues of veterinary medicinal products [4] have increased. In the environmental analyses, the advantages and disadvantages of using EEM are reviewed in [5] and their applications in [6]. EEM together with PARAFAC has also been used for the determination of polycyclic aromatic hydrocarbons in environmental samples [7], oil-field wastewaters [8], atmospheric aerosols [9] and food samples [10]. EEM-PARAFAC has been used for the determination of dissolved organic matter (DOM), including humic substances, proteins and other aromatic or aliphatic organic compounds, that plays an important role in both natural and engineered water systems. Recent advances can be seen in [11,12].

The interest in taking the chemical analyses from the laboratory to the field and/or the supply chains has led to a constant development of portable instruments. Molecular fluorescence instruments are following this trend towards portability due to their high sensitivity, although

most of the works register the emission spectra at a single excitation wavelength. Low consumption portable spectrometers have also been marketed using light-emitting diodes (LEDs) as excitation source that are playing increasingly important roles in analytical chemistry [13,14] such as the monitoring of DOM in aquatic environments [15,16].

The first use of LEDs for obtaining EEM was described in [17] where an imaging spectrometer (Oriel MS260i) with a cooled charge-coupled device (CCD) detector and seven LEDs at wavelengths between 370 and 636 nm were used. A fully portable system using LEDs and an Ocean Optics spectrometer was reported in [18]. This EEM-capable system was smaller, and the eight wavelengths used ranged from 405 to 640 nm. Another prototype that used the same compact spectrometer with 6 LEDs (between 265 and 340 nm) as excitation source to monitor the presence, migration and biodegradation of naphthenic acids can be seen in [~~15~~ 19], although EEM were not used in that work.

The IUPAC report [20] states that EEM together with PARAFAC represents a current consolidated advance of fluorescent methods and that the development of transfer procedures for EEM is needed together with the offline analyses to take advantage of the potential of the fluorescence spectral libraries [21,22].

In this work, a portable fluorimeter with 4 interchangeable LEDs as excitation source and a portable (StellarNet) spectrometer were used to: i) Evaluate the figures of merit of the quantitative results obtained in the determination of enrofloxacin alone and with binary mixtures (with flumequine or ciprofloxacin) which provide EEM signals with increasing overlap. The results were compared to the ones obtained for the same samples measured in a conventional fluorescence spectrometer; ii) Transfer the EEM signals from the portable fluorimeter based on LEDs to the master which is of interest to maintain a network of portable instruments referenced to a master instrument. This increases the use of fluorescence spectral libraries. As far as the authors are aware, this is the first time that the transfer has been carried out between instruments with different LEDs and a xenon lamp as excitation sources.

Enrofloxacin has been used as the analyte of interest and is a synthetic antibacterial agent which belongs to the fluoroquinolones group widely used in veterinary medicine and supplied through the water of poultry drinking troughs. The maximum residue limit (MRL) for enrofloxacin has been set between 100 and 300 μg kg^{-1} depending on the animal species and on the target tissue. Its use is banned in animals from which eggs are produced for human consumption. If applicable, these MRLs are applied to the sum of enrofloxacin and ciprofloxacin which is its most important active metabolite [23]. The labelling of the products that contain enrofloxacin has been recently regulated due to its antibiotic resistance. The waiting time after a treatment with this antibacterial for chickens and turkeys (meat) must be set at 7 and 13 days, respectively [24]. Therefore, a fast and portable method that enables the unequivocal identification of enrofloxacin in the presence of other analytes is needed to comply with the control of the drinking troughs.

2. Material and methods

2.1 Chemicals and standard solutions

The details are contained in the Supplementary Material (Section S1).

2.2 Instrumental

Fluorescence measurements were performed at room temperature with two different instruments. The master fluorimeter (MF) was a PerkinElmer LS50B Luminescence Spectrometer (Waltham, MA, USA) equipped with a xenon discharge lamp. The EEM were recorded in the following ranges: emission (300-600 nm, each 1 nm) and excitation (265-295 nm, each 5 nm). Excitation and emission monochromator slit widths were both set to 5 nm. The scan speed was 1500 nm min^{-1}.

The slave instrument was a portable spectrometer system (PF) (StellarNet Inc., Florida, USA) preconfigured for fluorescence which consisted of an SL1-LED excitation source, a LED kit including four LEDs (265 nm, 275 nm, 280 nm and 295 nm), and a high-performance fiber optic spectrometer compact SILVER-Nova Super Range TE Cooled. Liquid samples were measured using the CUV-F liquid fluorescence. The EEM were recorded in the following ranges: emission (300-600 nm, each 1 nm) and excitation (265 nm, 275 nm, 280 nm and 295 nm). The detector integration time was 10000 ms. A 10 mm quartz SUPRASIL® cell with cell volume of 3.5 mL by PerkinElmer (Waltham, MA, USA) was used in both instruments.

2.3 Software

The FL WinLab (PerkinElmer) and the SpectraWiz (StellarNet) software programs were used to register the fluorescent signals. The data were imported to MATLAB [25] using home-made functions to import data from both instruments, build the corresponding EEM at the same wavelengths and insert missing values into the matrix in the wavelengths that correspond to the Rayleigh effect. PARAFAC models were performed with the PLS_Toolbox 8.5.2 for use with MATLAB. The least squares regressions were built and validated with STATGRAPHICS Centurion XVII [26]. Decision limit (CCα) and capability of detection (CCβ) were determined using DETARCHI [27].

2.4 Case studies and samples

Three case studies with increasing complexity of the EEM signals were designed: i) CASE I (only enrofloxacin), ii) CASE II (binary mixtures with flumequine) and iii) CASE III (binary mixtures with ciprofloxacin). The signals of CASE II were partially overlapped, whereas the emission spectra of both fluoroquinolones in CASE III had the same shape but the signals of both analytes differed in sensitivity (see Figure 1).

Several calibration samples were prepared: 7 for CASE I and 19 for the rest. In the three cases, 8 transfer samples which were used to transfer the EEM signals from the PF to the MF and 5 test samples were also prepared (see Table 1).

Table 1 Concentration of each analyte in the calibration, transfer and test samples for each case

	CASE I	CASE II		CASE III	
	ENR ($\mu g\ L^{-1}$)	ENR ($\mu g\ L^{-1}$)	FLU ($\mu g\ L^{-1}$)	ENR ($\mu g\ L^{-1}$)	CIP ($\mu g\ L^{-1}$)
Calibration samples					
1	0	0	0	0	0
2	50	100	0	75	0
3	100	200	0	150	0
4	200	300	0	225	0
5	300	400	0	300	0
6	400	0	250	0	75
7	500	100	250	75	75
8		300	250	225	75
9		0	500	0	150
10		200	500	150	150
11		400	500	300	150
12		0	750	0	225
13		100	750	75	225
14		300	750	225	225
15		0	1000	0	300
16		200	1000	150	300
17		400	1000	300	300
18		200	0	200	0
19		0	0	0	0
Transfer samples					
1	75	200	250	150	75
2	150	400	250	300	75
3	250	100	500	75	150
4	450	300	500	225	150
5	200	200	750	150	225
6	300	400	750	300	225
7	400	100	1000	75	300
8	500	300	1000	225	300
Test samples					
1	350	150	375	112.5	112.5
2	100	150	875	112.5	262.5
3	175	200	500	150	150
4	275	350	375	262.5	112.5
5	425	350	875	262.5	262.5

ENR: Enrofloxacin. FLU: Flumequine. CIP: Ciprofloxacin

Three poultry drinking water samples were collected from different farms and prepared adding 5 mL of the buffer solution in a 50-mL volumetric flask and completing to the mark with each sample.

The change of the LED in the PF is manual, so changes in the recorded fluorescent intensity may appear. To minimize this effect, all the emission spectra were registered with the same LED, then another LED was placed, and the emission spectra were recorded again. This procedure avoids the overlapping problem [~~13~~ **Error! Bookmark not defined.**] of the regions in which each LED emits that are not strictly monochromatic.

3. Theory

3.1 PARAFAC

The EEM signals registered for different samples can be arranged in a data cube (array) which contains the fluorescence intensity recorded for each excitation and emission wavelengths.

The PARAFAC decomposition of a three-way array **X** with dimension (I×J×K) provides three loading matrices, **A** (I×F), **B** (J×F) and **C** (K×F) being their elements a_{ij}, b_{if}, and c_{kf}. The trilinear decomposition, with F factors, is found [28] by minimizing the sum of squares of the residuals, e_{ijk}, in the model:

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

where x_{ijk} is the element in the position i, j, k of the array **X**.

An array **X** is trilinear if the model of Eq. (1) is adequate for its decomposition. In this case, the least squares solution is unique, that is, there are no other loadings that achieve the same minimum. Therefore, the factors correspond to chemical factors (different fluorophores) present in the sample. The unequivocal identification of the factor associated to a fluorophore and its quantification are possible even in the presence of uncalibrated interferents. This is the second-order advantage in analytical chemistry [**Error! Bookmark not defined.**] which is relevant to perform analyses with a LED-based instrument outside the laboratory.

Two criteria (core consistency diagnostic, CORCONDIA, [29] and split-half analysis [30] together with the percentage of variance explained by the model) have been used to establish the number of factors of the PARAFAC model which is key to guarantee trilinearity. The closer to 100 these values are, the more suitable the model will be.

3.2 Signal transfer

The calibration or signal transfer for two-way data arrangements (I×J matrices) has been widely developed. The Piece-wise Direct Standardization (PDS) method establishes “local”

linear models that relate the response of the instrument to be standardized over a range of frequencies to the response of the standard instrument at a single frequency [31]. However, Direct Standardization (DS) uses the whole spectrum [32].

In the case of three-way data (I×J×K arrays), several solutions for coupled chromatographic techniques have been proposed. An adaptation of PDS to standardize two-dimensional responses (e.g., GC/MS, LC/UV) has been developed in [33]. The array is trilinear with EEM data and samples diluted enough, so a signal transfer based in fitting a linear regression for each channel, i.e. for each (λ_{exc}, λ_{em}), can be used. The procedure has been developed in [34] and it is more efficient than unfolding the data cube to obtain a matrix and apply DS or PDS. Moreover, in that case, the second-order advantage is lost. The procedure enables to transfer the signals from several PF to a MF, so LEDs with different wavelengths can be used in the PF.

3.3 Procedure

Figure 2 shows a flowchart of the procedure:

i) The EEM signals are registered in each instrument and the four wavelengths of the LEDs used in the PF are selected in the EEM recorded with the MF. Finally, the arrays that contain the calibration samples of each of the CASES of Table 1 are built.

ii) Perform a calibration based on PARAFAC in both instruments (MF and PF) with the same calibration samples. Then, each calibration is applied to obtain the concentration of the test samples.

iii) Measure a set of "n" transfer samples in both instruments and the transfer function of the EEM signal of the PF to the MF is built.

iv) Transfer the EEM signals obtained for the test samples in the PF to the MF.

v) The new matrices are projected in the PARAFAC model and the calibration function is applied to the sample loadings.

Figure 3 shows the transferred signal of test sample 2 of CASE II of Table 1. The 8 transfer samples have been used and a univariate linear regression has been built for each channel. The transfer function is built considering all those regressions. This function is applied to the EEM recorded in the PF (Figure 3b) to obtain the transferred EEM (Figure 3c) which is similar to the one registered with the MF (Figure 3a).

4. Results and discussion

4.1 PARAFAC models

The dimension of the array **X** of CASE I is 7×270×4, whereas 19×270×4 is the dimension of the arrays of CASE II and III. Those dimensions correspond to the number of calibration samples (Table 1) and the emission and excitation wavelengths, respectively. As can be seen in Table 2,

the EEM data were trilinear with both instruments in the three cases since the CORCONDIA index was 100 and the similarity obtained in the split-half analysis was higher than 97% considering 2 factors for CASE I and II and 3 factors for CASE II. In CASE III, only a common factor was extracted for enrofloxacin and ciprofloxacin with MF and PF as expected due to the severe signal overlapping (see Figure 1). The explained variance was higher in the PARAFAC models with the EEM recorded in the MF than the ones registered in the PF, so the PF had a worse signal-to-noise ratio.

The percentage of explained variance of **X** was different depending on the instrument where the signals have been registered (MF or PF) and on their distribution according to the factor. If the identification of each factor was considered, the background showed the greatest change from 1.70 to 20.44%, from 3.52 to 51.00% and from 2.68 to 57.83% for CASE I, II and III, respectively. These changes indicate that the relative fluorescence intensity of the fluorophores was different in both instruments. PF seems to be more sensitive to the background signal particularly in the EEM of the binary mixtures (CASE II and III). However, this ~~which~~ did not prevent the identification of the analytes present in the samples.

Figure 4 shows the PARAFAC models obtained for CASE II. Figures 4a, 4c and 4e are the model obtained with the array recorded with MF, whereas 4b, 4d and 4f are the one obtained with the data registered with PF. The sample profiles (Figures 4a and 4b) are coherent with the composition of each sample. The shape of the emission spectra is different, and the contribution of the noise is higher in the profile obtained for PF at high wavelengths in the background and at low wavelengths for flumequine (Figures 4c and 4d). The excitation profiles (Figures 4e and 4f) are completely different for enrofloxacin and for the background.

Table 2 Characteristics of the PARAFAC models obtained in each case.

	CASE I		CASE II		CASE III	
Instrument	MF	PF	MF	PF	MF	PF
Number of factors	2	2	3	3	2	2
CORCONDIA	100	100	100	100	100	100
Split-half analysis*	99.5	99.9	99.8	96.9	99.8	99.6
Total variance Captured (%**X**)	92.99	86.23	87.28	75.17	90.87	76.50
Variance Captured (% Model)						
First factor	98.30	79.56	89.62	51.00	97.32	57.83
Second factor	1.70	20.44	6.86	40.75	2.68	42.17
Third factor	----	----	3.52	8.25	-----	----
Identification						
First factor	ENR	ENR	ENR	BACK	ENR+CIP	BACK
Second factor	BACK	BACK	FLU	ENR	BACK	ENR+CIP
Third factor	----	----	BACK	FLU	----	----

*Similarity measure of splits and overall model in percent

ENR: enrofloxacin. FLU: flumequine. CIP: ciprofloxacin. BACK: background.

4.2 Calibration

A univariate linear regression was built between the PARAFAC sample loading and the concentration of the calibration samples to evaluate the figures of merit of the procedure. The equations of these calibration lines and their evaluation are included in Table S1 (Supplementary Material, Section S2).

All these regressions were significant (p-value < 0.05). There were enough degrees of freedom in CASE II and III to perform the lack of fit test and it was concluded that there was no lack of fit (p-value > 0.05) except for enrofloxacin and flumequine in the MF (CASE II). However, there was no lack of fit at a 3% and at a 4% significance level in both cases, respectively (see Table S1 of the Supplementary Material). The percentage of explained variance, R^2, was higher than 99% except for CASE III in which that value decreased to 93%. This was due to samples with the same sum of concentrations but with different ratio when ENR+CIP was calibrated (see Figure 1).

The intercept and the slope of the accuracy line (predicted concentration vs true concentration) were equal to 0 and 1 (p-values > 0.05), respectively, in all cases. Therefore, there was neither constant nor proportional bias in any of the eight calibrations based on PARAFAC.

The capability of detection (CCβ) and the decision limit (CCα) are defined by the ISO 11843-2 [~~35~~]. The need to evaluate the probability of false positive, α, and of false negative, β, has also been recognized by the IUPAC [36], and in the EU it is mandatory for the determination of toxic residues or residues that come from veterinary treatments in products for human consumption [37].

The values of CCα and CCβ are included in Table S1 (Supplementary Material, Section S2). The CCα value for enrofloxacin varied between 7.30 and 13.47 μg L^{-1} in CASE I and II with both instruments. This value was higher (≈ 83 μg L^{-1}) in CASE III due to the high residual standard deviation, $s_{y/x}$. CASE I and II show that the procedure can be used as screening method with both instruments.

4.3 Quantification of the test samples and trueness

The procedure (see Section 3.3) has been applied to the 5 test samples of Table 1 for each analyte and CASE. The mean and standard deviation of the absolute values of the relative errors (AVRE) are included in Table 3. The means of AVREs corresponding to the sum of ENR+CIP were the only ones that exceed 5% due to the different ratio of the analytes in samples 2 and 4.

Table 3 Mean and standard deviation of AVRE in each case.

	Origin of EEM signals		
	MF	PF	TtMF
CASE I /ENR			
Mean of AVRE (%)	1.51	1.04	1.45
Standard deviation of AVRE (%)	0.76	1.35	1.31

CASE II/ ENR			
Mean of AVRE (%)	1.12	3.72	2.79
Standard deviation of AVRE (%)	0.86	1.56	1.84
CASE II/ FLU			
Mean of AVRE (%)	2.75	2.20	2.60
Standard deviation of AVRE (%)	2.30	2.26	1.20
CASE III/ ENR+CIP			
Mean of AVRE (%)	5.90	6.96	8.80
Standard deviation of AVRE (%)	4.98	5.43	8.12

A statistical study of the error in the determination of the test samples and the way the signals are registered (MF, PF or TtMF) has been carried out (see the Supplementary Material, section S3 and table S2). The conclusion reached after this study was that there were no differences in the trueness with the signals registered with MF, PF or TtMF. However, there were differences in the precision (see s_{yx} in Table 3). The mean of the confidence interval length (CIL) at a 95% confidence level for the 5 test samples is collected in Table 4. The precision of PF was equal or worse in all the cases except for CASE I. It must be taken into account that the signal transfer procedure involved an approximation of the precision of PF to the one of MF.

Table 4 Mean and standard deviation of the confidence interval length (CIL) at a 95% confidence level.

	Origin of EEM signals		
	MF	PF	TtMF
CASE I /ENR			
Mean of CIL (μg L^{-1})	28.60	18.50	28.59
Standard deviation of CIL (μg L^{-1})	0.91	0.55	0.88
CASE II/ ENR			
Mean of CIL (μg L^{-1})	13.58	32.56	21.70
Standard deviation of CIL (μg L^{-1})	0.33	0.55	0.36
CASE II/ FLU			
Mean of CIL (μg L^{-1})	56.49	124.09	56.48
Standard deviation of CIL (μg L^{-1})	1.26	2.59	1.27
CASE III/ ENR+CIP			
Mean of CIL (μg L^{-1})	200.66	197.99	202.66
Standard deviation of CIL (μg L^{-1})	5.48	6.99	8.01

4.4 Determination of enrofloxacin in the poultry drinking trough samples

The same procedure of Section 3.3 has been applied to the three poultry drinking water samples. The results included in Table S3 (Supplementary Material, Section S4) have been obtained using PARAFAC and the calibration of CASE II. Enrofloxacin was not detected at a 95% confidence level. The uncertainty in the determination expressed as CIL was 13.78 μg L^{-1} for the signals registered with MF, whereas that value was triple (33.25 μg L^{-1}) with PF, being intermediate (22.21 μg L^{-1}) for TtMF which was coherent with the precision (see Table 4).

4.5 Inter-day stability

To evaluate the stability of the EEM signals over time, an "ad hoc" experiment has been carried out. So, a blank and a sample containing enrofloxacin at 200 μg L^{-1} was measured 8 different days. An array of dimension 16×270×4 was obtained for each instrument. The two-factor PARAFAC models explained 87.56 and 85.01% of the variance of the array of MF and PF, respectively. The CORCONDIA index was equal to 100% in both cases and the global similarity index obtained using the split-half procedure was 99.9 and 96.5% for the model with MF and PF, respectively. The sample loadings are represented in Figure 5. The sample loadings of enrofloxacin obtained with the MF were coherent with the amount present in each sample (Figure 5a), whereas there were irregularities in the loadings obtained with the PF due to the change from day to day and the manual operation of the instrument (Figure 5b). A device that avoids the manual change of LED is being designed.

5. Conclusions

The EEM signal transfer between a portable instrument that uses LEDs as excitation source and a master fluorimeter based on a xenon source is feasible and approximates the precision in the determination performed with the PF to the one of the MF. In addition, the unequivocal identification of the fluorophores is possible and there is no bias in the determination.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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BIOGRAPHIES

S. Sanllorente is a lecturer at Universidad de Burgos, Spain. She received her Ph.D. in Chemistry in 2010. She has published ~~21~~ 22 articles.

L. Rubio is a postdoctoral researcher at Universidad de Burgos, Spain. She received her Ph.D. in Chemistry in 2016. She has published ~~8~~ 11 articles.

M.C. Ortiz is Full Professor of Analytical Chemistry at University of Burgos, Spain. She received her Ph.D. in Chemistry at University of Valladolid, Spain, in 1988. She has published ~~130~~ 134 publications.

L.A. Sarabia is Full Professor of Statistics at University of Burgos, Spain. He received his Ph.D. in Mathematics at University of Valladolid, Spain, in 1979. He has published ~~130~~ 132 publications.

FIGURE CAPTIONS

Figure 1 Emission spectra recorded with MF at $\lambda_{excitation}$= 295 nm. A) enrofloxacin, B) ciprofloxacin.

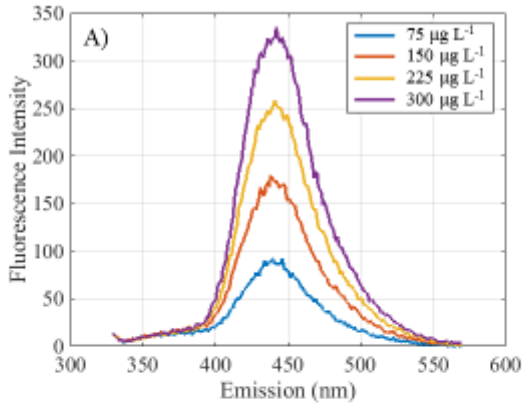

Figure 1

Figure 2 Flowchart of the procedure (Section 3.3).

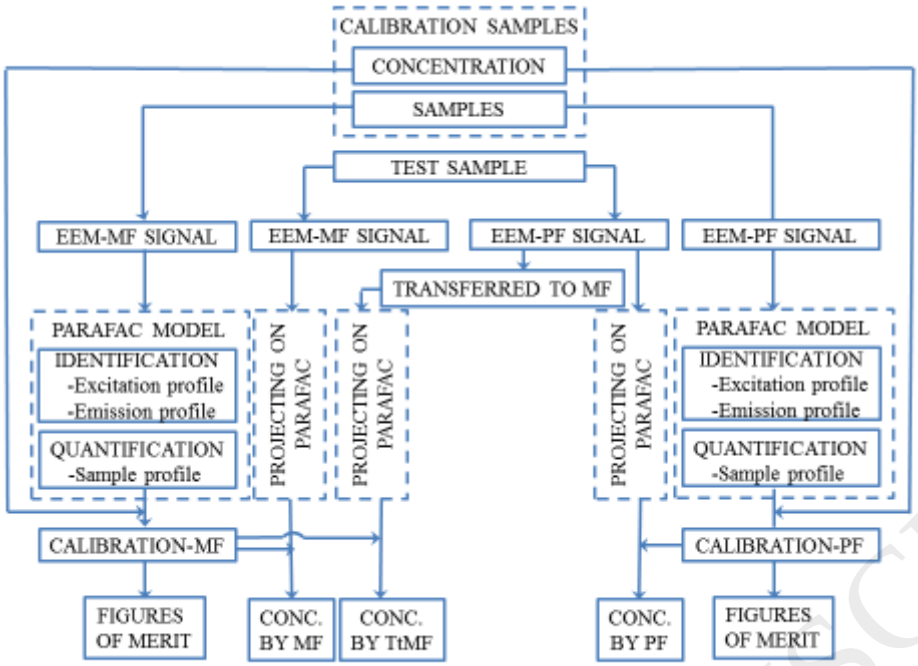

Figure 2

Figure 3 Contour plots of test sample 2 (CASE II) of Table 1. EEM recorded: A) in the MF, B) in the PF, C) transferred from PF to MF.

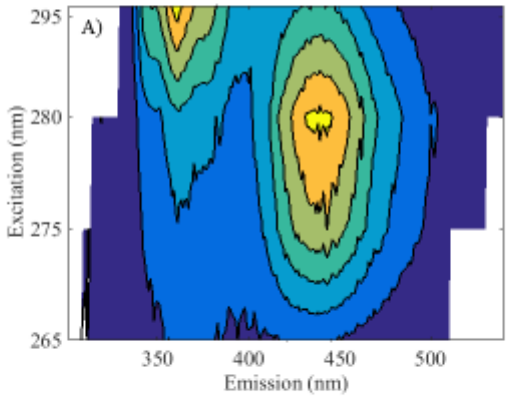

Figure 3 A

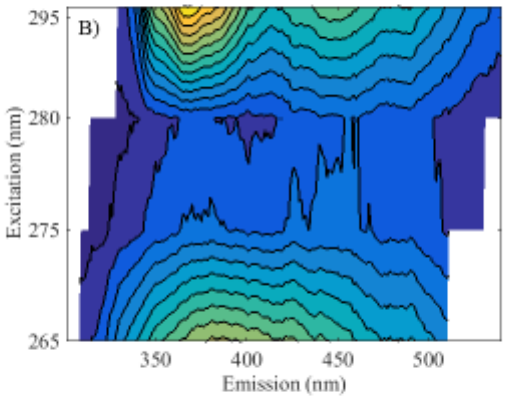

Figure 3 B

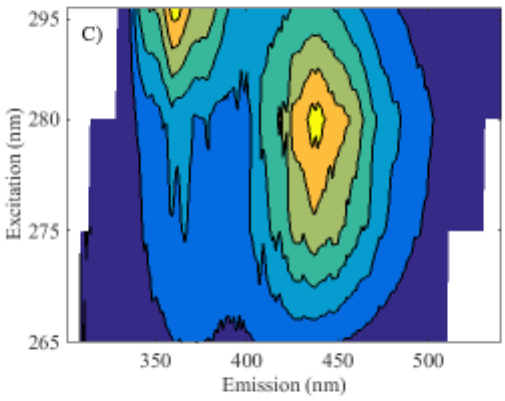

Figure 3C

Figure 4 PARAFAC models obtained for CASE II. A), C) and E) correspond to the loadings obtained with the data recorded with MF, whereas B), D) and F) are the ones obtained with PF.

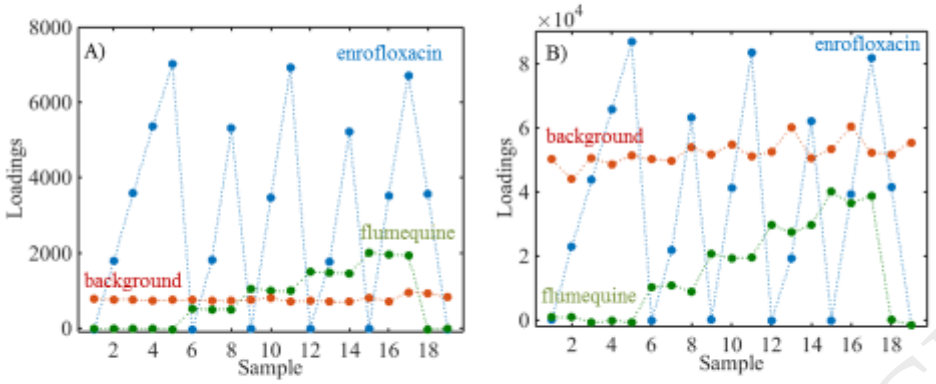

Figure 4A 4B

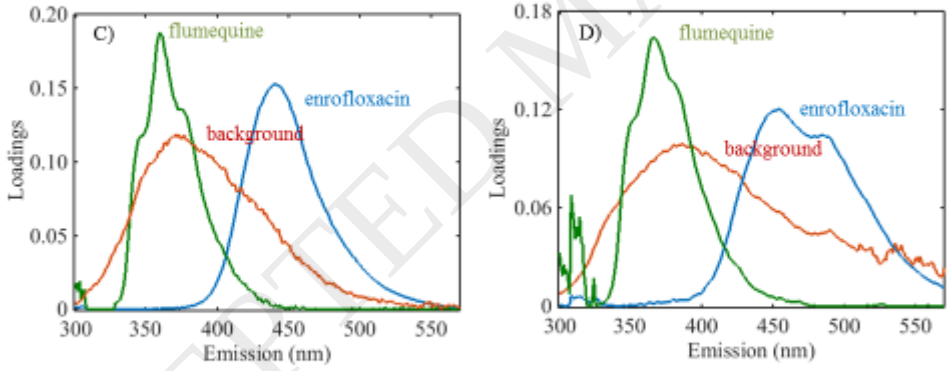

Figure 4C 4D

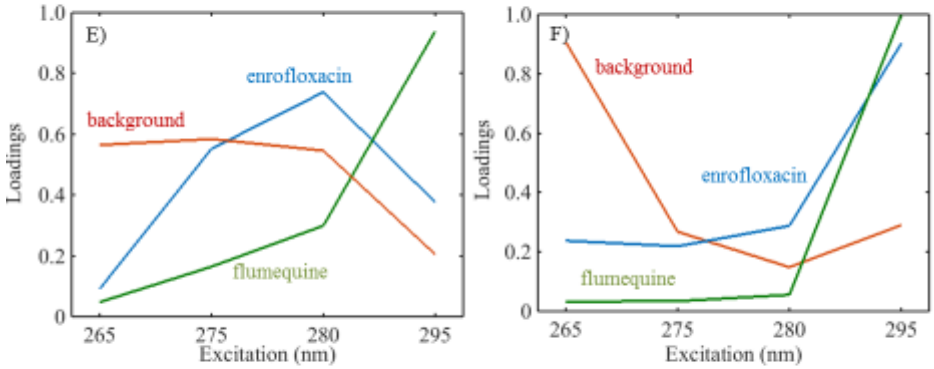

Figure 4E 4F

Figure 5 PARAFAC sample loadings obtained in inter-day stability study. EEM recorded with A) MF and B) PF.

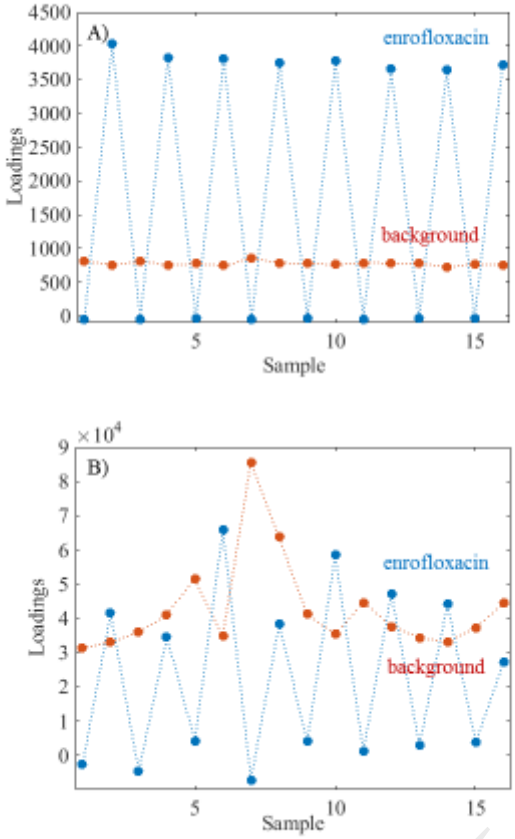

Figure 5