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Four-way calibration applied to the processing of pH-modulated fluorescence excitation-emission matrices. Analysis of fluoroquinolones in the presence of significant spectral overlapping

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ABSTRACT

A new methodology involving four-way multivariate calibration with a balanced number of data points in all modes is presented. The method is based on fluorescence excitation-emission matrices modulated by a double pH gradient obtained in a flow injection system. This data array was employed for the quantitation of ciprofloxacin, ofloxacin and norfloxacin in unprocessed urine samples. Due to the presence of potential interfering compounds with overlapping profiles in the analyzed samples, it is required to achieve the second-order advantage. The four-way arrays obtained were processed by parallel factor analysis (PARAFAC), attaining satisfactory results with relative errors of prediction (REP%) between 3% and 7.5% in the analyzed samples for all analytes. The average limit of detection (mg L^{-1}) was 0.035 for norfloxacin and ofloxacin and 0.028 for ciprofloxacin.

1. Introduction

Chemical multi-way calibration has gained widespread acceptance by the analytical community, since it provides better quality of the results when developing analytical methods to quantify analytes of interest in complex matrices. Nowadays, the research dedicated to the development and testing of multivariate algorithms applied to progressively more complex chemical systems is very extensive, as can be seen from the literature in relevant analytical, chemometrics and applied journal [1,2]. The main reason for this continuing interest is that second-order and higher-order data exhibit the so-called "second order advantage". This property allows to accurately quantify the calibrated analytes, even in the presence of interfering compounds not included in the calibration set [1–3]. Higher-order data might also exhibit other advantages that could improve the predictive ability. They would provide richer analytical information, allowing the development of more stable methods towards concerning interference and matrix effects, and less prone to minor changes in experimental conditions [4].

A variety of second- and higher-order instrumental data can be produced using modern instrumentation. However, it is interesting to note that while the use of second-order data is widespread, only in a few cases third-order data have been recorded and used to construct quantitative calibration models and to develop analytical methodologies [1,2]. This may be attributed to the fact that the experimental acquisition of these data arrays is still difficult to implement. Examples of four-way/third-order data are bidimensional chromatographic systems equipped with detection based on time of flight mass spectrometry (TOFMS) or diode array detection (DAD), leading to GC-GC–TOFMS [5] or LC-LC–DAD, and LC–DAD as a function of reaction time [6], and more recently ultra-fast high performance liquid chromatographic data with fluorescence excitation-emission detection [7–10]. On the other hand, excitation-emission fluorescence or phosphoresce matrices as a function of reaction time [11–14] or decay time [15], and also UV spectra-time reaction-pH data [16], are also some of the examples of the use of multi-way analysis using third-order data.

In this work we present an innovative form of third-order data consisting of excitation-emission matrices (EEMs) modulated by a double pH gradient. A fast scanning spectrofluorimeter allowed recording a complete EEM in a short time, and flow injection analysis (FIA) was used to generate the pH mode. Thus excitation-emission-pH third-order data, with a reasonably balanced number of sensors in all modes, have been easily measured for each experimental sample and used to construct a four-way calibration model. This calibration was applied to quantify fluoroquinolones (FQs) in unprocessed urine samples, which contain other fluorescent compounds with significant overlapping profiles. The fluoroquinolones ciprofloxacin (CIP), ofloxacin (OFLO) and norfloxacin (NOR) were selected as a model to show the potentiality of the proposed strategy of third-order data generation and the corresponding modeling. FQs and other strongly pH-dependent

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compounds have already been determined in urine samples, using pH-modulated second-order fluorescence signal [17,18].

Several algorithms are available for the convenient processing of multidimensional data, achieving the second-order advantage. Four-data arrays have been usually processed by resorting to the well-known parallel factor analysis algorithm (PARAFAC) [19,20]. The algorithms based on latent variables, unfolded (U-PLS) and multi-way partial least-squares (N-PLS), can also be applied, combined with residual trilinealization (RTL) [7,21,22] in order to achieve the second-order advantage. Additionally, multivariate curve resolution coupled to alternating least-squares (MCR-ALS) [23] can be used to process these data by first unfolding them into matrices. The selection of the proper algorithm depends on whether the multidimensional array complies or not with the so-called multilinearity condition. This may be briefly defined as the possibility of expressing a multi-way data array for a set of samples as a linear function of component concentrations and profiles in the different data modes. Third-order data that meet this property are called quadrilinear.

When the pH mode is obtained in a flow system, the obtained four-data array might not be strictly quadrilinear due to: a) irreproducibility in the pH gradient generation, as a consequence of the lack of synchronization among samples in the flow system; b) pH evolution while measuring each EEM. In addition, in the presence of a pH gradient a closure relationship exists between pH-equilibrating species, implying that they might be mutually correlated. Correlations may complicate the resolution of the multi-way array [3].

In this report, the four-way arrays were processed with PARAFAC, which provided satisfactory predictions in all the analyzed systems. The results suggest that the pH mode, in the selected experimental conditions, does not produce a breaking of the quadrilinearity of the data. Furthermore, PARAFAC can handle the presence of different unmodeled compounds exhibiting significant spectral overlapping signal with those for the analytes.

In summary, the proposed experimental system enabled us to obtain, in a simple way and in a reasonable time, four-way data arrays useful to quantify FQs in urine samples without pretreatment and in the presence of interferent compounds.

2. Experimental

2.1. Reagents

All experiments were performed with analytical grade chemicals. The following solutions were employed: acetic acid (HAc) 0.025 mol L^{-1}, prepared from commercial HAc (Merck, Darmstadt, Germany); sodium hydrogen carbonate ($NaHCO_3$) 5×10^{-3} mol L^{-1}, prepared from commercial $NaHCO_3$ (Analar, Poole, England). Ultrapure water provided by a MilliQ purification system was used.

Ofloxacin and norfloxacin were purchased from Sigma (Seelze, Germany) and ciprofloxacin was provided by Fluka (Seelze, Germany). Fluoroquinolone stock solutions (all 200 mg L^{-1}) were prepared by dissolving the exact amount of the corresponding compound in 50 mmol L^{-1} HAc solution. These solutions were stored at 4 °C and were stable for at least a month.

Stock solutions of sodium salicylate (Merck, Darmstadt, Germany) and naproxen (Sigma, Seelze, Germany), both 200 mg L^{-1}, were prepared weighing the required amount of the corresponding compounds and dissolving them in MilliQ water.

2.2. Apparatus

Two Gilson Minipuls Evolution peristaltic pumps (Gilson, Middleton, WI, USA) were consecutively used for the propulsion of the carrier solution. All sample solutions were manually injected into the carrier system using a dual proportional Upchurch injection valve (Upchurch scientific, Oak Harbor, WA, USA). The flow was injected into a quartz Hellma flow cell model 176.752-QS, 25 μL inner volume, 1.5 mm optical path length (Hellma, Müllheim, Germany).

Fluorescence measurements were done using a fast scanning Varian Cary Eclipse spectrofluorimeter (Varian Inc., Mulgrave, Victoria, Australia), equipped with two Czerny-Turner monochromators and a xenon flash lamp, and connected to a PC microcomputer via an IEEE 488 (GBIP) Serial interface. Excitation-emission fluorescence matrices were collected under the following conditions: for OFLO, excitation wavelength range, 275–375 nm, emission wavelength range 425–540 nm; for CIP, excitation wavelength range, 255–355 nm, emission wavelength range 370–485 nm and for NOR, excitation wavelength range, 250–350 nm, emission wavelength range 375–490 nm (in all cases, excitation ranges each 5.5 nm and emission ranges each 3.6 nm). The slit widths for the excitation and emission monochromators were fixed at 5 nm, and the detector voltage was set at 850 for OFLO and CIP, and 800 V for NOR. A wavelength scanning speed of 18,000 nm/min was employed, so that a complete excitation-emission fluorescence matrix was obtained in few seconds, collecting 45 successive matrices in 15 min.

The complete data were arranged into a third-order array of size $18 \times 32 \times 45$ data points, saved in ASCII format and transferred to a PC for subsequent manipulation with the multivariate program.

2.3. Calibration and validation samples

Three different calibration sets were prepared, one for each fluoroquinolone, having six duplicate concentration levels, equally spaced in the range 0.00 to 1.00 mg L^{-1}, which was established on the analysis of the linear fluorescence-concentration range for each analyte. In order to obtain the desired concentrations, appropriate aliquots of standard solutions were measured and placed in 20.00 mL volumetric flasks, completing to the mark with HAc 0.025 mol L^{-1} to be employed as carrier, or with $NaHCO_3$ 5×10^{-3} mol L^{-1} to be injected into the flow system. In order to test the method performance, a validation set was prepared for each analyte, employing different concentrations than those used for calibration and following a random design, i.e., choosing the validation concentrations by generating random numbers, equally distributed within the analyte calibration ranges.

Since test urine samples were diluted 1/200 (see below), the calibration concentration range, once converted to urine concentrations, covers the therapeutic ranges of the analytes in the urine samples of patients administered with the three studied drugs. All samples were measured in random order.

2.4. Urine samples

With the purpose of evaluating the feasibility of the method to quantify FQs in complex samples, sets of urine were prepared, one for each analyte, spiked at concentrations given by random numbers in the range of 0–200 mg L^{-1} (therapeutic range).

Besides, for OFLO a set of spiked urine samples containing the analyte was prepared with the addition of salicylate (SA) as interferent. For NOR, as well as for CIP, different sets of spiked urine samples were prepared containing the corresponding FQ, with the addition of SA or naproxen (NX) as interferent. Both analyte and interferent concentrations in all cases were in the range of 0–200 mg L^{-1}. These test samples are intended to mimic truly unknown samples composed of uncalibrated substances, where a responsive background may occur. The inclusion of known chemical components in these samples has the purpose of checking whether the multivariate algorithm is able to successfully retrieve their corresponding profiles, and accurately quantify the analytes. All spiked samples were diluted 1/200 with HAc 0.025 mol L^{-1} to be employed as carriers, or with $NaHCO_3$ 5×10^{-3} mol L^{-1} to be injected into the flow stream as calibration and validation sets, and measured in random order.

2.5. Flow injection methodology

In order to generate the double pH gradient inside the flow stream, the alkaline sample was injected into the acid sample used as carrier. Each of the studied samples was diluted with HAc 0.025 mol L^{-1} and used as the carrier stream. The composition of the injected sample was identical to that of the carrier, except that the dilution was carried out with $NaHCO_3$ 5×10^{-3} mol L^{-1}. This mode of generating the pH gradient has already been employed [17,18], preventing changes in total analyte concentrations in the flow stream and achieving higher sensitivity. As shown in Fig. 1, two peristaltic pumps were used consecutively for the propulsion of the carrier solution, in order to change the flow rate during the experiment as follows: from the injection to the first seven minutes, the flow rate was 0.23 mL min^{-1} and then changed to 0.17 mL min^{-1}, using a switching valve. The acid sample used as carrier was sent through a tube (1.02 mm i.d.). After the alkaline sample (1.00 mL) was injected, the flow was sent to the spectrofluorimeter flow cell, passing first through a Teflon tube mixer (14 cm length, 1.5 mm i.d.) and then, through a Teflon reactor (total length 5 m, 0.5 mm i.d.). Spectral measurements were done 5 min after the sample injection, collecting excitation-emission fluorescence matrices under the conditions detailed in Section 2.2. The total experimental time for a given sample was 20 min.

3. Theory

3.1. PARAFAC

For third-order EEMs-pH data processing with PARAFAC algorithm, a set of I_{cal} calibration samples $\mathbf{X}i$,cal (each of them as a $J \times K \times L$ array, where J,K and L are the number of data points in each mode) are joined with the unknown sample data matrix $\mathbf{X}_u$ into a four-way data array $\boldsymbol{X}$, whose dimensions are $[(I_{cal} + 1) \times J \times K \times L]$. Provided $\boldsymbol{X}$ follows a quadrilinear PARAFAC model, it can be written in terms of four vectors for each responsive component, designated as $\mathbf{a}n$, $\mathbf{b}n$, $\mathbf{c}n$ and $\mathbf{d}n$ and collecting the relative concentrations $[(I_{cal} + 1) \times 1]$ for component n, and the profiles in the three data modes ($J \times 1$, $K \times 1$ and $L \times 1$, respectively). The specific expression for a given element of $\boldsymbol{X}$ is [24]:

$$X_{ijkl} = \sum_{i=1}^{N} a_{in}\, b_{jn}\, c_{kn}\, d_{ln} + e_{ijkl} \quad (1)$$

where N is the total number of responsive components, a_{in} is the score of relative concentration of component n in the ith sample, and b_{jn}, c_{kn} and d_{ln} are the fluorescence intensities at the emission wavelength j, excitation wavelength k and pH l, respectively. The values of e_{ijkl} are the elements of the array $\mathbf{E}$, which is a residual error term of the same dimensions as $\boldsymbol{X}$. The column vectors $\mathbf{a}n$, $\mathbf{b}n$, $\mathbf{c}n$ and $\mathbf{d}n$ are collected into the corresponding loading matrices $\mathbf{A}$, $\mathbf{B}$, $\mathbf{C}$ and $\mathbf{D}$ (the instrumental vectors $\mathbf{b}n$, $\mathbf{c}n$ and $\mathbf{d}n$ for each mode are usually normalized to unit length). A successful decomposition of $\boldsymbol{X}$, usually accomplished through an alternating least-squares minimization scheme [25,26], gives access to the emission spectral profiles ($\mathbf{B}$), excitation spectral profiles ($\mathbf{C}$) and pH profiles ($\mathbf{D}$), as well as relative concentrations ($\mathbf{A}$) of individual components in the $(I_{cal} + 1)$ mixtures, whether they are chemically known or not, providing the basis of the second-order advantage.

There are some significant topics for the application of the PARAFAC model to the calibration of four-way data array: (1) initializing the algorithm, (2) applying restrictions to the least-squares fit, (3) establishing the number of responsive components, (4) identifying specific components from the information provided by the model and (5) calibrating the model in order to obtain absolute concentrations for a particular component in an unknown sample.

Initialization can be done using several options implemented in the PARAFAC package [27]: (1) singular value decomposition (SVD) vectors, (2) random orthogonalized values and (3) the best-fitting model of several models fitted using a few iterations. The first of these alternatives was employed in the present work.

Constraints during the PARAFAC fit might be employed for retrieving physically recognizable profiles in the different modes. However, in the present case such restrictions were not necessary.

The number of responsive components (N) can be estimated by several techniques, such as CORCONDIA, a diagnostic tool considering the PARAFAC internal parameter known as core consistency [28,29]. Another useful technique is the consideration of the PARAFAC residual error (SSE), i.e., the sum of the squared elements of the array $\mathbf{E}$ in Eq. (1) [25]:

$$SSE = \sum_{i=1}^{Ical+1} \sum_{j=1}^{J} \sum_{k=1}^{K} \sum_{l=1}^{L} (e_{ijkl})^2 \quad (2)$$

Usually this parameter decreases with increasing N, until it stabilizes at a value corresponding to the optimum number of components. In the presently studied case, the progression of residual fits was more reliable than the CONCORDIA test.

Identification of the chemical constituents under investigation is done with the aid of the estimated profiles, mainly the emission and

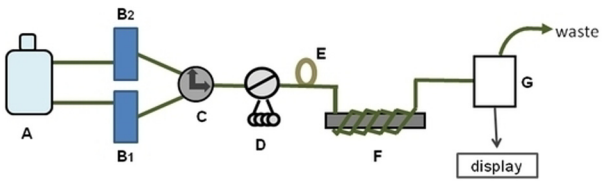

Fig. 1. Flow injection analysis assembly: (A) carrier, (B1) and (B2) peristaltic pumps, (C) switching valve, (D) injector, (E) mixer, (F) reactor, (G) spectrofluorimeter.

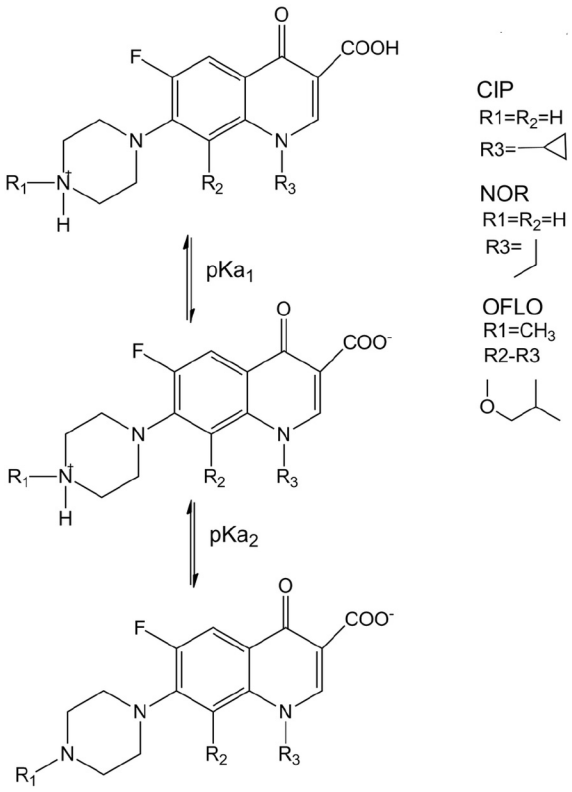

Fig. 2. Fluoroquinolone structures: cationic, zwitterionic and anionic species.

excitation spectra, and comparing them with those for a standard solution of the analyte of interest. This is required since the components obtained by decomposition of $\boldsymbol{X}$ are sorted according to their contribution to the overall spectral variance, and this order is not necessarily maintained when the unknown sample is changed.

Absolute analyte concentrations are obtained after calibration, because the four-way array decomposition only provides relative values ($\mathbf{A}$). Calibration is done by means of the set of standards with known analyte concentrations (contained in an $I_{cal} \times 1$ vector $\mathbf{y}_{cal}$), and regression of the first I_{cal} elements of column $\mathbf{a}n$ (provided by the calibration samples) against $\mathbf{y}_{cal}$:

$$k = \mathbf{y}_{cal}{}^{+} \times \boldsymbol{a}_n(1 \ldots I_{cal}) \quad (3)$$

where '+' implies taking the pseudo-inverse. Conversion of relative to absolute concentration of component n in the unknown proceeds by division of the last element of column $\mathbf{a}_n$ by the slope of the calibration graph k:

$$y_u = \boldsymbol{a}_n(I_{cal} + 1)/k \quad (4)$$

the above procedure is repeated for each new test sample analyzed.

It is noteworthy that even when there are several species in equilibrium for a given analyte (in the present work, acid-base species), the values contained in the vector $\mathbf{y}_{cal}$ are total analyte concentrations. On the contrary, the scores $\mathbf{a}_n$ are specific for a given analyte species. Therefore, several pseudo-univariate graphs can in principle be obtained, by regressing the scores for each analyte species against the nominal analyte concentrations. The most sensitive of these graphs is chosen to predict the analyte concentration, i.e., the one with largest value of the slope k in Eq. (3).

3.2. Software

All calculations were done using MATLAB 7.0, using different routines and graphical interfaces: MVC3 (Multivariate Calibration for third-order) [30], an integrated MATLAB toolbox for third-order calibration, freely available on the Internet [31].

4. Results and discussion

4.1. General considerations

As previously mentioned, FQs are compounds which exhibit pH-dependent fluorescence. The studied FQs present a carboxylic group at position 3 and a basic piperazinyl group at position 7. Therefore, in aqueous solution 7-piperazinylquinolones show three different species, which are cationic, zwitterionic and anionic (Fig. 2). The reported pKa values for these FQs fall in the ranges of 5.5–6.6 and 7.2–8.9 for pKa_1 and pKa_2, respectively [32]. In order to optimize the composition of the carrier and injection solutions, the pH behavior of the different FQs was previously evaluated in batch. According to the results, we have set the pH values of the carrier (HAc 0.025 mol L^{-1}, pH about 3) and the injection solution ($NaHCO_3$ 5×10^{-3} mol L^{-1}, pH about 7) so that the dominating species in our experiments are: the protonated species occurring below pH ≅ 6, and the neutral form (mainly zwitterionic) above this pH value (from here on, we will refer as acid and alkaline forms, respectively). These selected species exhibit a higher fluorescent

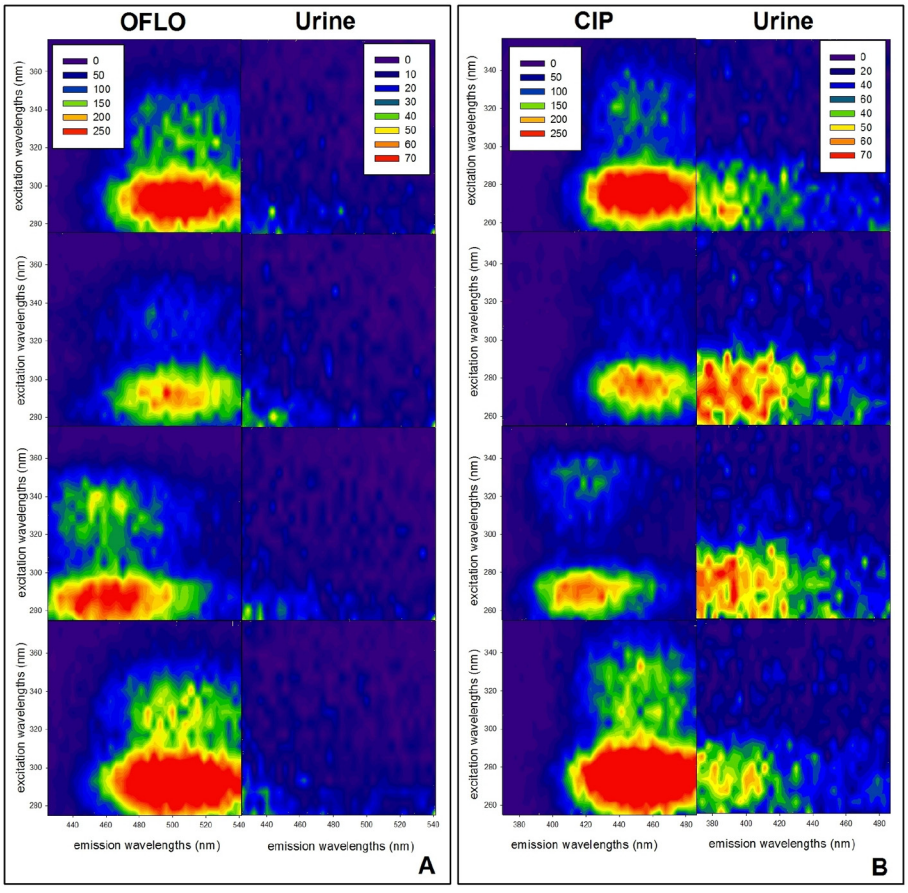

Fig. 3. Contour plots of the EEMs third-order data for: (A) OFLO 0.50 mg L^{-1} and a typical urine sample diluted 1/200, (B) CIP 0.50 mg L^{-1} and a typical urine sample diluted 1/200. Excitation and emission wavelengths are indicated in each case. Each contour plots correspond to a FIA point in the pH dimension, representing the evolution along the sample injection.

signal and a better spectral discrimination in comparison with the anionic form. In addition, isofluorescent points are found when plotting the emission spectra at different pH values, in accordance with the presence of two species in each case: 470 nm for OFLO, and 440 for CIP and NOR, as discussed in previous works [17].

Different FIA experimental conditions were tested (i.e. reactor lengths, injection loops and flow rate) in order to obtain a gradual pH variation, leading to suitable pH profiles. A high scanning rate and a very slow flow (0.17 mL min^{-1}) during the data collection were employed with the purpose of minimizing changes in the concentrations of the constituents as recording a complete EEM (about 20 s), while the pH evolves in the flow stream. The initial flow value (0.23 mL min^{-1}) described in Section 2.5 aims to reduce the analyses time.

When typical experimental samples are injected into the flow stream and EEMs are collected, third-order data are obtained whose contour plots are shown in Fig. 3. According to a previous report [17], FQs exhibit a shift to shorter wavelengths and a decrease in the intensity when pH changes from acid to alkaline values, related to the conversion from the cationic into the zwitterionic form (see plots on the left of Fig. 3A and B). In addition, the emission wavelengths of OFLO (both in protonated and neutral form) are longer than those corresponding to CIP. The pH-spectral behavior of NOR is similar to CIP, hence it is not shown. Finally, urine contour plots display no shifts in fluorescent emission as the pH changes (see plots on the right of Fig. 3A and B). However, the urine background is significantly overlapped with the excitation mode of both forms of CIP and NOR, and to a lesser extent with OFLO.

As has already been stated in the experimental section, the analyzed urine samples also present other compounds, SA and NX, unmodeled during the calibration step. Fig. 4A shows that for OFLO, the excitation mode presents the most significant overlapping, mainly for both alkaline and acid forms with SA at 300 nm. For CIP and NOR, which have similar spectral behavior, the emission mode of the alkaline form is affected by the presence of SA (Fig. 4B) whereas the excitation mode of both FQs forms is largely overlapped with NX (Fig. 4C). In conclusion, all the unmodeled compounds exhibit different degrees of overlapping with the analytes in the spectral modes, posing different challenges for the algorithms.

Finally, the EEM-pH data arrays obtained for the different sets of the studied FQs were analyzed using PARAFAC, considering that the experimental design was conducted to avoid the loss of multilinearity in the data array, a requirement for a successful PARAFAC decomposition. In addition, this algorithm allows attaining the second-order advantage, which is required taking into account the composition of the analyzed samples.

4.2. PARAFAC analysis

When PARAFAC analysis of the different experimental data sets was performed, the first step was the estimation of the number of responsive components. This can in principle be assessed using either the diagnostic tool known as the core consistency test or the consideration of the residual fit of the PARAFAC model, as the number of components is increased. For validation samples containing only FQ, the progression of residual fits, as well as the core consistency values indicates the stabilization at two components, in agreement with the expected responsive components: the fluorescent acidic and alkaline forms. When analyzing urine samples, either in the presence or absence of other interfering compounds (SA and NX), the consideration of the residual fit is more reliable than the CONCORDIA test: whereas the progression of the core consistency values (100, 48.5, 5.04 and negative value for 1–4 components) suggests two components, the changes in fitting residuals (24, 13, 11 and 11 from 1 to 4 components, respectively) indicate stabilization at three components, as expected: both FQ forms and at least one interferent. The fitting residual (11–16 units) is comparable to the estimated level of instrumental noise. In conclusion, for all FQs in urine

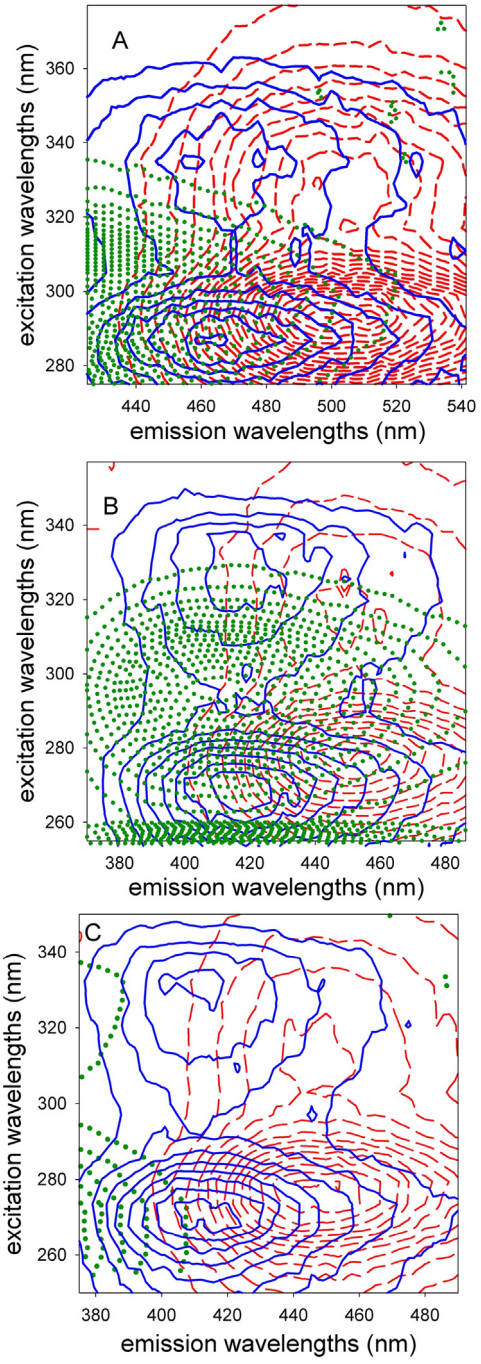

Fig. 4. Contour plots of FQs and interferents: (A) OFLO and SA (B); CIP and SA; (C) CIP and NX. FQs alkaline form, (solid line, blue); FQs acid form, (dashed line, red); SA and NX (dotted line, green). FQs and interferents at 1.00 mg L^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples, three components were enough to explain the variability of the four-data array.

PARAFAC was initialized using SVD, without constrains during the least-squares fit. It is noteworthy that uniqueness is preserved for a four-way data array, even in the presence of correlation between components in one mode, i.e. when a conjugate acid-base pair is present [33]. Furthermore, the best performance was obtained using a restricted pH range, from sensor 1 to 25 for OFLO and NOR, and from sensor 1 to 30 for CIP. As shown in Fig. 5, PARAFAC was able to satisfactorily retrieve the spectral profiles of the calibrated components. The retrieved pH profiles of FQs are consistent with their acid-basic behaviour, as previously

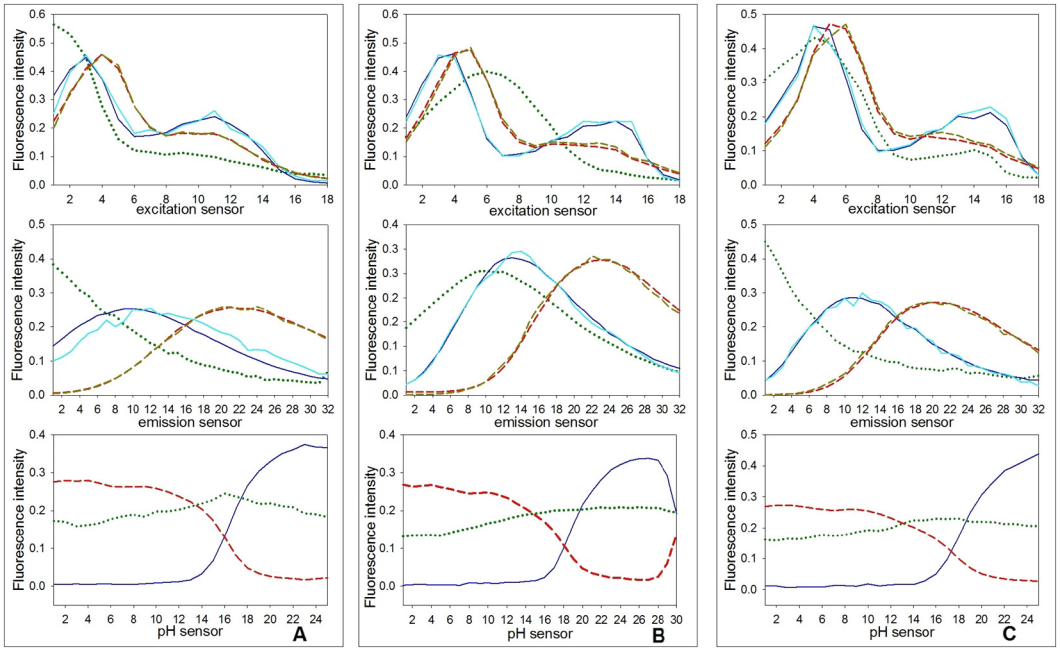

Fig. 5. Experimental and retrieved profiles when PARAFAC was applied for FQs determination. Profiles can be identified as: retrieved (dashed line, red) and experimental (dashed line, dark yellow) acid analyte form; retrieved (solid line, blue) and experimental (solid line, cyan) alkaline analyte form; interferents (dotted line, green), for urine samples spiked with (A) OFLO 0.10 mg L^{-1}, (B) CIP 0.25 mg L^{-1} and SA 0.80 mg L^{-1} (C) NOR 0.30 mg L^{-1} and NX 0.70 mg L^{-1}. All profiles were normalized to unit length. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

described. In general, the unmodeled compounds in the calibration step were recovered as a single component (urine, urine and SA or NX). PARAFAC was not able to discern between the profiles of each foreign constituent, retrieving the interference as a single unexpected constituent. However, this fact did not preclude the obtainment of good analytical results in these samples.

Once the analyte profiles were identified, prediction proceeded by the usual interpolation into the pseudo-univariate calibration graph, built with the scores for the species of the analyte which provided better sensitivity, in this case the acid form, as has already been stated in the PARAFAC theory Section 4.1.

In a first stage, validation samples for the three analytes were analyzed, providing the following results in terms of relative error of prediction (REP), expressed in % with respect to the mean calibration concentration: 4.6, 3.6 and 4.9 for OFLO, CIP and NOR, respectively (see Table 1). Afterward, the test urine samples were evaluated and the recovered root mean square errors (RMSE) and REP values are summarized in Tables 2, 3 and 4, showing accurate predictions for the different systems, with REP % values ranged from 3.3 up to 7.4.

PARAFAC satisfactory results allow to assert that there is no loss of multilinearity caused neither by irreproducibility in the pH gradient, nor by the finite time employed by the spectrofluorimeter in recording the matrix, in comparison with the time elapsed between successive pH points.

In order to qualify the method, figures of merit were calculated for the studied FQs. Very recently, expressions for multi-way calibration based on the concept of input and output noise in a given system were derived, and these expressions were used to compute the figures of merit in the present work. Sensitivity (SEN) measures the ratio of

Table 1
Predicted concentrations for FQs in validation samples.

OFLO		CIP		NOR	
Nominal[a]	Predicted[a]	Nominal[a]	Predicted[a]	Nominal[a]	Predicted[a]
0.20	0.23	0.15	0.16	0.15	0.13
0.30	0.31	0.30	0.33	0.30	0.29
0.50	0.51	0.50	0.52	0.50	0.51
0.66	0.62	0.60	0.61	0.70	0.75
0.75	0.77	0.75	0.73	0.85	0.87
0.90	0.94	0.85	0.86	0.95	0.94
RMSE[b]	0.024		0.018		0.024
REP[c]	4.7		3.6		4.9

[a] Concentrations are all given in the measuring cell, expressed in mg L^{-1}.
[b] RMSE, root mean square error, expressed in mg L^{-1}.
[c] REP%, relative error of prediction, expressed (in %) with respect to the mean of calibration concentrations (0.5 mg L^{-1}).

Table 2
Predicted concentrations for OFLO in urine samples.

Urine samples		Urine samples with SA		
Nominal[a]	Predicted[a]	Nominal[a]		Predicted[a]
OFLO		OFLO	SA	
0	0.02	0	0.40	0.002
0.10	0.11	0	0.80	0.02
0.20	0.22	0.15	0.20	0.15
0.30	0.30	0.25	0.80	0.25
0.38	0.41	0.30	0.70	0.32
0.50	0.50	0.40	0.40	0.43
0.66	0.69	0.52	0.60	0.52
0.75	0.74	0.60	0.52	0.63
0.90	0.90	0.72	0.20	0.72
1.00	1.02	0.90	0.30	0.85
		1.00	1.00	0.94
RMSE[b]	0.018			0.028
REP[c]	3.6			5.6

[a] Concentrations are all given in the measuring cell, expressed in mg L^{-1}.
[b] RMSE, root mean square error, expressed in mg L^{-1}.
[c] REP%, relative error of prediction, expressed (in %) with respect to the mean of calibration concentrations (0.5 mg L^{-1}).

Table 3
Predicted concentrations for CIP in urine samples.

Urine samples		Urine samples with SA			Urine samples with NX		
Nominal[a]	Predicted[a]	Nominal[a]		Predicted[a]	Nominal[a]		Predicted[a]
CIP		CIP	SA		CIP	NX	
0	0.001	0	0.40	0.02	0.15	0.20	0.18
0.15	0.16	0	0.80	0.01	0.30	0.70	0.31
0.30	0.33	0.15	0.20	0.16	0.50	0.50	0.46
0.42	0.42	0.25	0.80	0.24	0.60	0.87	0.64
0.50	0.51	0.30	0.70	0.33	0.70	0.30	0.66
0.65	0.62	0.42	0.90	0.39	0.85	0.30	0.90
075	0.74	0.50	0.40	0.54			
0.80	0.80	0.60	0.15	0.58			
0.90	0.88	0.70	0.30	0.73			
		0.85	0.80	0.83			
		0.90	0.30	0.87			
RMSE[b]	0.017			0.024			0.037
REP[c]	3.3			4.8			7.4

[a] Concentrations are all given in the measuring cell, expressed in mg L^{-1}.
[b] RMSE, root mean square error, expressed in mg L^{-1}.
[c] REP%, relative error of prediction, expressed (in %) with respect to the mean of calibration concentrations (0.5 mg L^{-1}).

output noise to input noise, and can be defined as the variation in net response for a given change in analyte concentration [34]. The obtained figures of merit (Table 5) when applying PARAFAC in urine samples were satisfactory, with detection limits on the order of 0.028–0.035 mg L^{-1} for all FQs, calculated according to Ref [35].

With the purpose of getting further insight into the accuracy and precision of the proposed method, nominal versus found concentration values were compared by application of the elliptical joint confidence region (EJCR) test [36], taking into account all urine samples for each FQ. The conclusion is that all the ellipses (at 95% confidence level) contain the ideal point and have a small size (see Fig. 6), indicating great analytical accuracy and precision, as expected. Specific results for the regressions are: OFLO, slope = 0.97 ± 0.03, intercept = 0.02 ± 0.02; CIP, slope = 0.98 ± 0.03, intercept = 0.01 ± 0.02; NOR, slope = 0.98 ± 0.03, intercept = 0.01 ± 0.01 (confidence intervals at the 95% confidence level). All slopes and intercepts are statistically comparable to 1 and 0, respectively.

Table 4
Predicted concentrations for NOR in urine samples.

Urine samples		Urine samples with SA			Urine samples with NX		
Nominal[a]	Predicted[a]	Nominal[a]		Predicted[a]	Nominal[a]		Predicted[a]
NOR		NOR	SA		NOR	NX	
0	0.00	0	0.40	0.02	0.15	0.20	0.18
0.15	0.14	0	0.80	0.01	0.30	0.70	0.33
0.20	0.19	0.15	0.20	0.14	0.50	0.50	0.55
0.30	0.31	0.23	0.80	0.24	0.60	0.87	0.62
0.45	0.42	0.30	0.70	0.31	0.70	0.30	0.68
0.50	0.47	0.40	0.70	0.40	0.85	0.30	0.86
0.60	0.59	0.50	0.50	0.54			
0.65	0.62	0.63	0.30	0.62			
0.75	0.74	0.70	0.30	0.68			
0.80	0.81	0.85	0.90	0.84			
		0.90	0.40	0.90			
RMSE[b]	0.033			0.017			0.029
REP[c]	6.7			3.4			5.8

[a] Concentrations are all given in the measuring cell, expressed in mg L^{-1}.
[b] RMSE, root mean square error, expressed in mg L^{-1}.
[c] REP%, relative error of prediction, expressed (in %) with respect to the mean of calibration concentrations (0.5 mg L^{-1}).

Table 5
Analytical figures of merit for PARAFAC method in urine samples.

Figures of merit	OFLO	CIP	NOR
SEN (mg^{-1} L)[a]	12,000	9600	7400
γ_n (mg^{-1} L)[b]	700	600	680
LOD (mg L^{-1})[c]	0.035	0.028	0.035
LOQ (mg L^{-1})[d]	0.11	0.08	0.11

[a] SEN: sensitivity.
[b] Analytical sensitivity, calculated as sensitivity/sd_{test}; $sd_{test:}$ residual fit of the test sample signal.
[c] LOD: limit of detection.
[d] LOQ: limit of quantitation.

5. Conclusions

Innovative third-order data based on excitation-emission fluorescence matrices modulated by a double pH gradient were obtained in a fast and simple way, and allowed the quantitation of fluoroquinolones in unprocessed urine samples. PARAFAC was able to give satisfactory results in all the studied systems, highlighting that in the experimental conditions the data array complies with the multilinearity conditions. Furthermore, the algorithm can handle the presence of unmodeled compounds with dissimilar degrees of overlap in the different spectral modes. PARAFAC, which uses all the data structure and has the property of uniqueness, satisfactorily retrieved the analytes and interferent profiles, which ensures reaching physically interpretable results.

As previously stated, the increase of data order might produce more stable methods towards background effects and changes in the experimental conditions, improving the prediction ability. Therefore, the use of higher-order data, as the presently reported third-order data, enables new analytical strategies for resolving analytical situations in complex samples.

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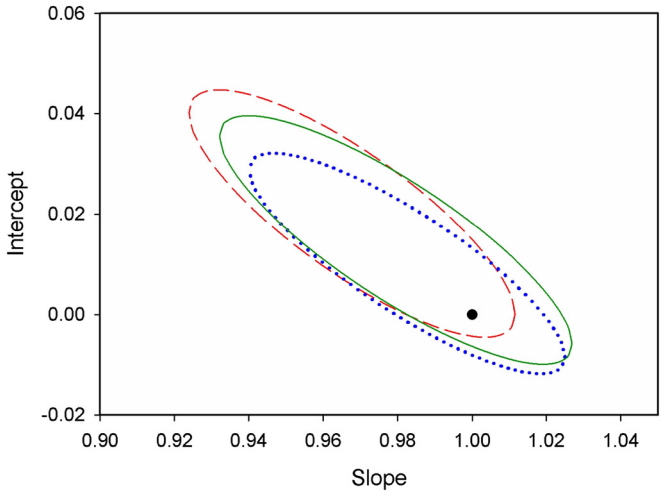

Fig. 6. Elliptical joint regions (at 95% confidence level) for the slopes and intercepts of the regressions for the corresponding predictions for OFLO (dashed line, red), CIP (solid line, green) and NOR (dotted line, blue). The black circle in the elliptical plots marks the theoretical (intercept = 0, slope = 1) point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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