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Two- and Three-Dimensional Strategies for Determining Consecutive Acidity Constants of Ciprofloxacin and Enrofloxacin Using Fluorescent Excitation-Emission Measurements

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Multidimensional analysis is getting more popular by the day thanks to technical improvements of analytical instruments and development of chemometric methods. Here, we describe the application of two different chemometric methods to the analysis of three-dimensional spectrofluorimetric dataset for green determination of dissociation constants of ciprofloxacin and enrofloxacin with minimal use of consumables. Three-dimensional spectrofluorimetric datasets of both drugs were obtained by recording excitation-emission matrices of only six sample solutions having different pH values. Parallel factor analysis (PARAFAC) and multivariate curve resolution (MCR) methods with the use of three components were used to decompose three-dimensional excitation-emission-pH arrays. The number of components support the presence of two pKa values. The individual profiles of H_2A, HA^- and A^{2-} species in excitation, emission and pH modes were obtained by the application of PARAFAC and MCR. The intersection points in the pH modes were used to predict pKa values. pKa_1 and pKa_2 of ciprofloxacin was reported as 6.35, 9.15 and 6.42, 9.40 by PARAFAC and MCR-ALS, respectively. For enrofloxacin, consecutive pKa values were predicted as 6.11 and 9.06 by PARAFAC and 6.10 and 9.10 by MCR. A detailed comparison to literature was done for the estimated pKa values.

Introduction

Fluoroquinolones are a class of broad-spectrum antibiotics with intrinsic fluorescence properties, containing a fluorine atom attached to a quinolone structure. Ciprofloxacin and enrofloxacin, both in fluoroquinolone class are effective against gram-negative and gram-positive bacteria.[1] Ciprofloxacin (CIP) is second-generation fluoroquinolone with the chemical formula of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid as illustrated in Figure 1a. It is indicated for a wide range of bacterial infections including urinary track, respiratory track, intra-abdominal, skin, bone and joint.[2] Enrofloxacin (ENR), 1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid, is a veterinary antibiotic, of which chemical formula was given in Figure 1b. It is commonly used to treat bacterial infections in various species including dogs, cats, cattle, pigs, sheep, goats, poultry, as well as exotic animals.[3]

In pharmaceutical and medicinal research, the pharmacokinetic and metabolic behaviors of active compounds depend on their physicochemical properties. The parameters such as lipophilicity, solubility, permeability and apparent acid dissociation constant (pKa) of a molecule determine its absorption, bioavailability and its interaction with enzymes. Such properties are also very important for understanding the mode of action of a drug. The acid-base dissociation constant (or pKa) is a significant chemical parameter that relates to the degree of dissociation of molecules in aqueous solution at different pH media. Chemical, physical and biological characteristics of natural and synthetic compounds depend on acidic and basic groups. An accurately determined acid dissociation constant is required to understand and predict the behavior of a drug under in vivo conditions.[4]

Various analytical methods have been used for the determination of acid-base ionization constants of analytes used in commercial drug and food products. Potentiometric pKa analysis is one of the most commonly used method in analytical chemistry and related pharmaceutical and food industries. However, it requires a large amount of samples and reagents. Nowadays, instrumental methods such as UV-VIS spectropho-

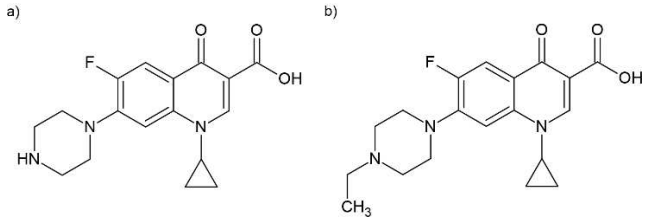


Figure 1. Chemical formula of **a)** CIP and **b)** ENR.

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tometry, high-performance liquid chromatography, voltammetry have been used for quantifying acid-base dissociation constants of biologically active compounds used in pharmaceutical and food products.[5] Each instrumental method has their advantages and disadvantages in terms of accuracy, repeatability, robustness, feasibility, cost and sustainability.

Although fluorescence spectroscopy can be only used for fluorescent analytes, it is usually more sensitive and selective compared to traditional UV-VIS spectrometry. Another disadvantage of the spectrofluorimetry is the decrease of fluorescence intensity of the sample because the buffer components may lead to pH dependent fluorescence quenching.[4a,6] However, in our study, the change of fluorescence depending on pH in fluorescence measurements as a function of excitation and emission has been turned into an advantage in the development of fast, inexpensive, reliable and sustainable chemometric strategies for spectrofluorometric pKa determination. The proposed method is based on the differentiation of the excitation and emission bands of the acid and conjugated base forms of the analytes in dissociation equilibrium depending on the acidity of the medium. The change of the medium pH was obtained by using the universal Britton-Robinson buffer and methanol was used as a modifier to ensure the solubility of the drugs. A literature survey shows that various methods are available to determine the acidity constants of the studied drugs.[7] Table S1 and Table S2 in supporting information were given to summarize the pKa values of CIP and ENR in the literature.

The acid dissociation constant, usually depicted as K_a, is a quantitative measure of the tendency of a molecule to release H^+ and generate a hydronium ion (H_3O^+), which means the strength of an acid in solution. Generally, drug molecules have weak acid or weak base structures. These drug substances may have one or more equilibrium constants according to the ionization reactions. For example, a drug molecule with a diprotic acid nature exists in two successive dissociation equilibria among H_2A, HA^-, and A^{2-} species. These two acid-base equilibria in aqueous solution can be expressed by Equation 1 and 2:

$$H_2A + H_2O \rightleftharpoons HA^- + H_3O^+ \quad (1)$$

$$HA^- + H_2O \rightleftharpoons A^{2-} + H_3O^+ \quad (2)$$

where H_2A is the diprotic acid structure, HA- is the monoanion form of H_2A, and A^{2-} is the dianion form of H_2A. The acid-base equilibrium constants for the dissociation reactions depicted in Equations 1 and 2 can be given in Equations 3 and 4, respectively:

$$Ka_1 = \frac{[HA^-][H_3O^+]}{[H_2A]} \quad (3)$$

$$Ka_2 = \frac{[A^{2-}][H_3O^+]}{[HA^-]} \quad (4)$$

The first dissociation reaction illustrated in Equation 1 yields at low pH level, while the second one in Equation 2 occurs at high pH level. The strength of acids varies by orders of magnitude for the acid ionization constant (K_a). In practice, it is more intuitive to represent such numbers on a logarithmic scale. In the logarithmic scale of acidity constants, the term pKa represents the negative logarithm (base 10) of K_a.

In the Henderson-Hasselbalch equation (see Equation 5), pH is related to the pKa and the concentrations of chemical species in dissociation equilibrium.

$$pH = pK_a + log([A^-]/[HA]) \quad (5)$$

Therefore, it is possible to evaluate the pK_a value from the instrumentation response, which is measured as a function of pH, depending on the concentration of acid-base forms in equilibrium with various techniques. Based on this perspective, the focus of our study is that parallel factor analysis (PARAFAC) and multivariate curve resolution-alternating least squares (MCR-ALS) modelling of changes in the excitation and emission bands of a drug molecule at different pH allow for a quantitative estimation of the successive pK_a values of the drugs studied.

In this paper, highly sensitive and green pKa determination methods were presented based on PARAFAC and MCR-ALS modelling of spectroscopic data, obtained from fluorescence measurements It should be mentioned that these PARAFAC and MCR-ALS tools have already been used by the authors to determine pKa values of other active compounds.[8] However, until now (to our present knowledge), they have not been used to determine successive pKa values of CIP and ENR.

The most important aspect of this research paper is presenting alternative perspectives with two- and three-dimensional strategies to determine individual excitation-emission bands of chemical species (H_2A, HA^- and A^{2-}) in acid dissociation equilibria and to determine sequential pKa values. Identification of fluorescence bands and determination of pKa values were simultaneously estimated by direct application of MCR-ALS and PARAFAC to the same experimental datasets without using a classical titration procedure and an additional calculation treatment. In contrast, it is not possible to solve such analytical problems (identification and quantification) simultaneously with traditional analysis methods. This demonstrates the ability of the proposed two- and three-dimensional strategies with MCR-ALS and PARAFAC tools to solve complex problems with a second-order advantage.

Chemometric Methods

Two-way data analysis

MCR-ALS is a bilinear model to decompose the experimental row data (or augmented data) into matrices, which correspond to individual contributions of analyzed species. The bilinear decomposition of the augmented data (or two-way data) array of analyzed system is given in Equation 6:

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$$X = C.S^T + E \quad (6)$$

where X is the two-way data array with overlapping experimental information or the fluorescence measurements for different samples at various pH values of the temporal dimensions, the columns in C and the rows in S^T represents pure contributions of analyzed species in the row and column directions of X, respectively, and E is an error matrix in the model. The details of the MCR-ALS method and its applications in many areas of analytical chemistry were described by Roma Tauler and co-workers in references.[9]

Three-way data analysis

PARAFAC is one of multiway data analysis methods, and is a decomposition method for three-way array (or N-way array) which can be considered as a generalization of bilinear PCA to higher order arrays.[10] PARAFAC model and its theoretical basis in applications are explained in the references.[10–11] For the qualitative and quantitative analysis in analytical chemistry, PARAFAC is one of the most commonly used method for modelling fluorescence excitation and emission data matrix (EEM). PARAFAC model is based on the decomposition of the fluorescence signals X into trilinear components. Trilinear component number is the number of fluorophores in the analyzed samples. For example, when trilinear component number is F, the PARAFAC model of an EEM array is expressed as Equation 7:

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \quad (7)$$

where $x_{ijk}$is the fluorescence intensity for sample *i*, at excitation wavelength *j*, and emission wavelength *k*. e_{ijk} denotes to the error term for the model. In Equation 7, a_{if}, b_{jf}, and c_{kf} are the i^{th}, j^{th}, and k^{th} elements of the f^{th} sample, f^{th} excitation and f^{th} emission of components, respectively.

PARAFAC is a three-way (or N-way) analysis method that has second order advantage to get individual contributions of fluorophores in the analysis of complex mixtures. Moreover, the fluorophores can be analyzed even in the presence of modelled interferents.

Results and Discussion

The main purpose of this article was to establish green alternatives for the determination of the successive pKa values of CIP and ENR with low-cost and high-speed. To estimate the acid dissociation constants, new, selective, rapid and robust chemometric strategies were developed based on the fluorescence excitation and emission measurements of CIP and ENR, which are natural fluorescent species.

In the first stage of the method development, the fluorescence intensity of CIP and ENR at different pH media was measured as a function of excitation and emission wavelengths. In this stage, the excitation and emission matrix (EEM) of each analyte at pH 5 was obtained from the measurement of the fluorescence intensities. Similar procedure at six different pH levels (from pH 5 to pH 10) was repeated as displayed in Figure 2a. The excitation and emission matrices, which correspond to the six different pH levels were stacked into a so-called three-way array as illustrated in Figure 2b. As can be seen in Figure 2c, after building the three-way data array, the PARAFAC decomposition directly yielded the excitation profiles, emission profiles, and relative concentrations (or pH profiles) for each of the acid-base species of the analytes. The excitation and emission profiles were used for qualitative analysis of acid-base species of the analyte in acid ionization equilibrium, while pH windows were used for the pKa determination. As it was displayed in Figure 2d, the augmented data was obtained by unfolding three-way array into a two-way data matrix. Then, the fluorescence excitation-emission profiles and pH profiles of the species were obtained by applying the MCR-ALS to the augmented data matrix. The individual curves of acid-base forms were identified and pKa values of analytes was determined from the pH windows.

For the spectrofluorometric pKa determination of CIP and ENR with chemometric strategies, the practical application of PARAFAC and MCR-ALS models were described below. In order to estimate pKa_1 and pKa_2 of CIP and ENR with bilinear component and trilinear component models, a set of six samples containing 20 μg/mL of each analyte in the methanolic aqueous solution at six pH levels from pH 5 to pH 10 was prepared by using the above mentioned stock solutions of analytes. Three-dimensional fluorescence spectra or fluorescence EEMs of the sample set for both CIP and ENR at six different pH levels were recorded at emission wavelengths between 360–600 nm ($\Delta\lambda = 2$ nm), at different excitation wavelengths between 208–352 nm ($\Delta\lambda = 4$ nm) as displayed in Figure 3a and b, respectively. As described above, the recorded fluorescence EEMs of the samples for both analytes at six different pHs were separately collected and organized as a

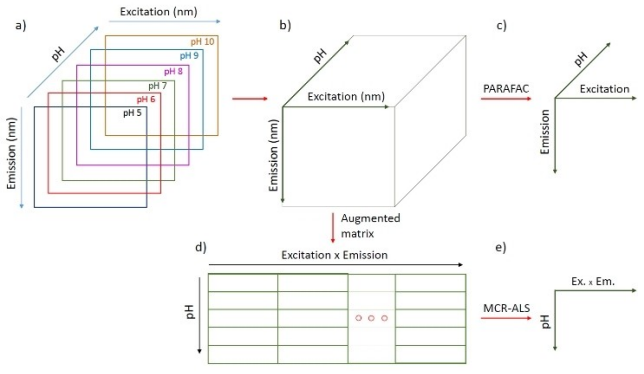


Figure 2. a) Recording excitation-emission data matrix in the six different pHs, **b)** obtaining cubic data (or three-way array) from data matrices, **c)** the graphical illustration of the application of PARAFAC to fluorescence cubic data, **d)** unfolding cubic data into two-way data matrix (or augmented data) and **e)** the graphical representation of the application of MCR-ALS to augmented data matrix.

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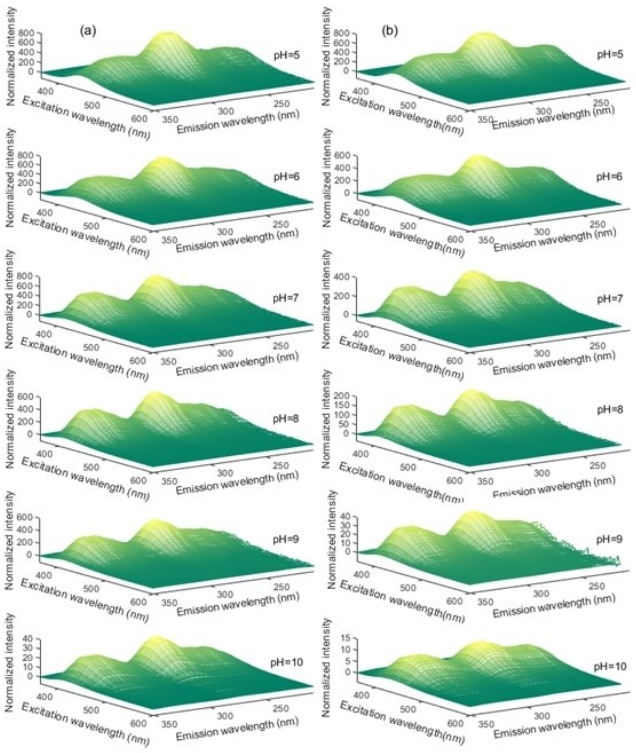


Figure 3. Three-dimensional spectrofluorometric plots of **a)** CIP and **b)** ENR obtained as a function of excitation and emission wavelengths at six different pH levels.

three-way array with dimensions emission, excitation and pH (121×37×6).

Fluoroquinolones show significant fluorescence only in neutral and moderately acidic solutions, their emission is much weaker and the overall quantum yield of fluorescence decreases in basic media. CIP and ENR exists in their anionic forms at higher pH conditions than their pKa_2 value, having a carboxylate group and deprotonated amine group in piperazine ring.[13] The significant decrease of fluorescence intensity in higher pH media can be seen in Figure 3 for both drugs. The quenching of the fluorescence may be due to the poor emission ability from anions and deactivation of the singlet excited state caused by intramolecular electron transfer from deprotonated piperazine ring to the quinolone ring.[14]

For the quantification of the acidity constants of CIP and ENR based on the decomposition of EEM fluorescence three-way array into trilinear matrices using PARAFAC model, the pure contributions of acid and its conjugate base species of analyte in the ionization equilibrium were obtained. In practice, the number of components for the trilinear component model was identified for the application of the PARAFAC algorithm considering H_2A, HA^-, A^{2-} species of both CIP and ENR in their dissociation equilibria. In order to check the number of components, the core consistency diagnostic method was used.[12] Three-component PARAFAC model with constraints and without constraints in three modes were tested. Then, a model with non-negative constraints in was found to be suitable for modelling the fluorescence three-way array. It was observed from this implementation that core consistency diagnostics was 97.2% and 98.4% for CIP and ENR, respectively. The variance explained in the model was reported as 99.05% and 99.60% in 1210 and 1324 iterations for CIP and ENR, respectively.

In the decomposition of the fluorescence three-way array with the PARAFAC algorithm, the individual contributions of H_2A, HA^- and A^{2-} forms of the analytes in three modes were indicated in Figure 4a and b for CIP and ENR, respectively. Figure 4a and b in I and II show the estimated excitation and emission spectra of the species for both CIP and ENR in the acid-ionization equilibria. The pH profiles of CIP and ENR, obtained by applying PARAFAC model to three-way fluorescence data arrays was presented in Figure 4a and b in III, respectively. As can be seen in these figures, the pH profiles which were obtained by plotting the loadings of the third mode vs the pH values for each species indicate the changes in the relative concentration of H_2A, HA^- and A^{2-} species of CIP and ENR as a function of the pH. In the Henderson-Hasselbalch (Equation 7), when the relative ratios of H_2A/HA^- and HA^-/A^{2-} are equal, the pKa_1 and pKa_2 values for CIP and ENR equal the pH_1 and pH_2 as shown in the pH profiles given in Figure 4a and b in III, respectively. The values of pKa_1 and pKa_2 for CIP and ENR were determined by using the pH profiles in Figure 4a and b in III, and listed in Table 1 and 2.

For both drugs, the dissociation of the carboxylic group corresponds to their first pKa value a while the deprotonation of the N_4 of the piperazynil ring gives the second.[13]

As it was described before, a two-way data matrix, (or augmented data matrix, 6x4235) with dimensions, pH x (excitation x emission) was obtained by unfolding the fluorescence three-way tensor into a matrix (see Figure 2d). The MCR-ALS was applied to the decomposition of two-way data matrix (augmented data) into a set of two matrices considering three number of components (the number of H_2A, HA^- and A^{-2} species for CIP and ENR in two consecutive equilibria) with non-

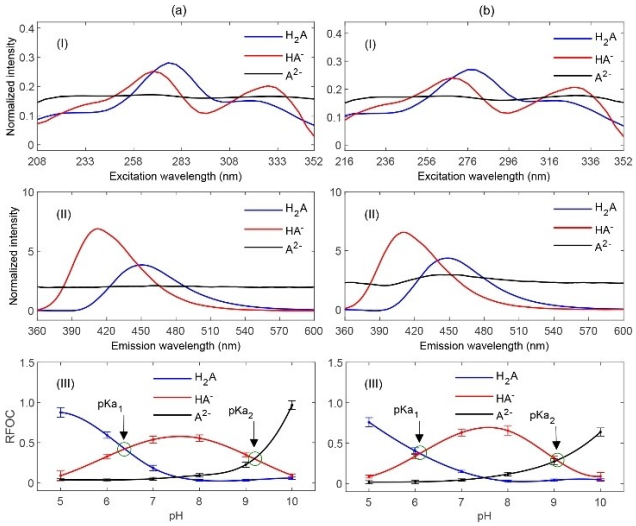


Figure 4. Excitation (I), emission (II), and pH (III) profiles of **a)** CIP and **b)** ENR obtained by applying PARAFAC to three-way EEM arrays. RFOC: relative fraction of concentration.

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Table 1. pKa values of ciprofloxacin obtained by PARAFAC and MCR-ALS models.

	PARAFAC		MCR	
	pKa_1	pKa_2	pKa_1	pKa_2
CIP1	6.23	9.13	6.32	9.32
CIP2	6.46	9.05	6.38	9.31
CIP3	6.38	9.28	6.57	9.58
Average	6.35	9.15	6.42	9.40
SD	0.11	0.12	0.13	0.15
RSD	1.79	1.29	2.05	1.62

SD: Standard deviation; RSD: Relative standard deviation

Table 2. pKa values of enofloxacin obtained by PARAFAC and MCR-ALS models.

	PARAFAC		MCR	
	pKa_1	pKa_2	pKa_1	pKa_2
ENR1	5.99	8.90	5.93	9.25
ENR2	6.24	9.07	6.13	8.92
ENR3	6.09	9.22	6.23	9.14
Average	6.11	9.06	6.10	9.10
SD	0.12	0.16	0.15	0.17
RSD	2.02	1.77	2.47	1.85

SD: Standard deviation; RSD: Relative standard deviation

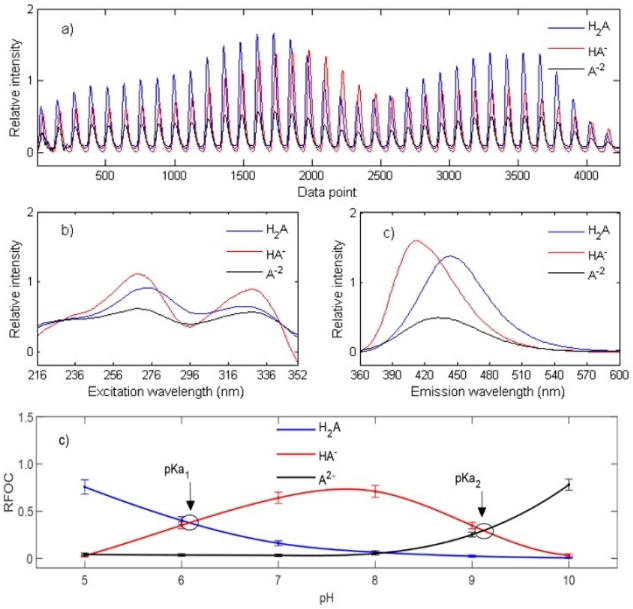


Figure 6. a) Excitation-emission data profile and its corresponding example **b)** excitation profile and **c)** emission profile, and **d)** pH profiles of ENR obtained by applying MCR-ALS to the augmented data matrix. RFOC: relative fraction of concentration.

negative constraints in two-mode. Both excitation-emission data and pH (or relative concentration) profiles of the studied drugs were illustrated in Figure 5b,c and d, and Figure 6b,c and d respectively. Figure 5b and c, and Figure 6b and c show the extracted excitation and emission spectra of CIP and ENR from Figure 5a and 6a, respectively.

The pH profiles were obtained by plotting the relative concentration of chemical species versus pH values as shown in Figure 5d and 6d. The individual curves of H_2A, HA^- and A^{2-} forms of CIP and ENR in acid-dissociation equilibria were identified from the pH profiles. In the pH profile given in Figure 5d and 6d, two intersections for the curves of H_2A/HA^- and HA^-/A^{2-}, which correspond to pKa_1 and pKa_2 of CIP and ENR were reported when the relative fraction of the relative concentration were equal to one (pH = pKa). The numerical values of pKa_1 and pKa_2 for CIP and ENR was determined from the profiles in Figures 5d and 6, which were listed in Table 1 and 2, respectively.

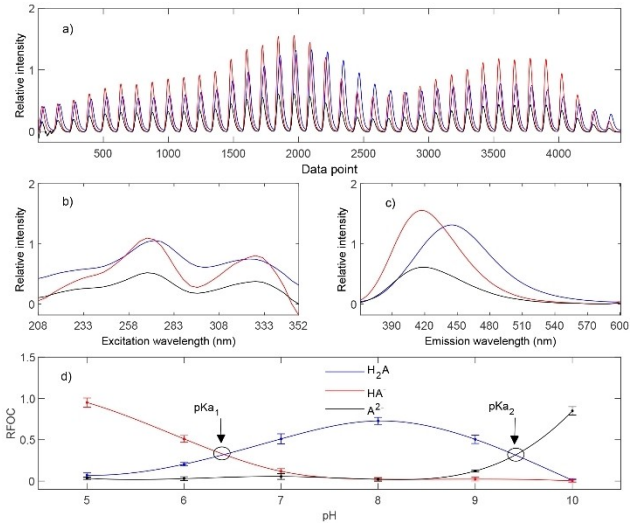


Figure 5. a) Excitation-emission data profile and its corresponding example **b)** excitation profile and **c)** emission profile, and **d)** pH profiles of CIP obtained by applying MCR-ALS to the augmented data matrix. RFOC: relative fraction of concentration.

For consecutive pKa determinations of CIP and ENR two-way and three-way strategies based on the use of fluorescence excitation-emission measurements are more selective than the pKa determination methods presented in References.[7a–c,f–m] Therefore, the proposed chemometric models would not be affected by the impurities of the medium for the determination of pKa of analytes. In this respect, it was an advantage that the method was based on fluorescence measurement. For example, when comparing the MCR-ALS and PARAFAC results for CIP with those of the methods in References,[7d–f] it was observed the results were comparable. In this context, similar approaches for ENR in Reference[7k] may be expressed. The proposed methods with chemometric strategies requires less effort, time and consumables than the literature methods.

Conclusions

In this research article, novel chemometric strategies based on PARAFAC and MCR-ALS modellings of fluorescence measurements were improved to monitor curves of acid-base species of the drugs in consecutive ionization equilibria and to determine pKa values of CIP and ENR with satisfactory sensitivity. In this regard, modeling two-way and three-way data arrays, which

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were obtained from the fluorescence excitation and emission measurements at six different pH levels, enabled to determine the pKa constants of CIP and ENR with high sensitivity, low cost, green and practical manner. Also, thanks to the second-order advantage of the proposed models, they enabled the monitoring of the excitation and emission spectral profiles of acid-base species in the ionization equilibria. This is an advantage of PARAFAC and MCR-ALS over conventional pKa determination methods. Moreover, sample preparation and measurement steps were straightforward and only required preparation of 6 samples with 1 mL methanol and 30 mL aqueous buffer unlike conventional potentiometric titration method. The pKa_1 and pKa_2 results of both CIP and ENR obtained by PARAFAC and MCR-ALS models showed that the proposed two-way and three-way strategies would be green alternative approaches to traditional methods for the estimation of the acid dissociation constants.

Experimental Section

Data generation and analysis

A Cary Eclipse Fluorescence Spectrometer (Agilent, USA) was used for recording excitation and emission measurements of the analyzed samples. Excitation range for ciprofloxacin was 208–352 nm with an interval of 4 nm and emission spectra were recorded between 360–600 nm with an interval of 2 nm, resulting an EEM of (121×37) for each sample. For enrofloxacin, excitation and emission ranges were set as 316–352 nm ($\Delta\lambda = 4$ nm) and 360–600 nm ($\Delta\lambda = 2$ nm), respectively. Each EEM of enrofloxacin sample had a dimension of 121×35. Photomultiplier voltage was set at 580 for CIP, 540 V for ENR and the scanning speed was 1200 nm/min for all samples. Both excitation and emission bandwidths were set at 5 nm. The fluorescence data obtained were transferred from the instrument's Cary Eclipse software (Agilent, USA) to the Matlab (MathWorks, USA) platform for the two-way and three-way data treatments. Chemometric algorithms, quantitative calculations and illustrations were performed in Matlab.

Preparation of sample solutions

Ciprofloxacin (purity 99.6%) and enrofloxacin (purity 99.3%) were kindly donated by Vilsan Veterinary Pharmaceuticals (Ankara, Turkey). ACS reagent grade methanol, glacial acetic acid (CH_3COOH), phosphoric acid (H_3PO_4) and reagent grade sodium hydroxide (NaOH) was procured from Sigma Aldrich (USA). ACS reagent grade boric acid (H_3BO_3) was supplied by Sharlau (Spain). Ultra-pure water was obtained from a Millipore Milli-Q water filtration system (Germany).

Stock solutions of ciprofloxacin and enrofloxacin were prepared individually by dissolving 5 mg standard material in 1 mL methanol. Sample solutions were prepared in 5 mL calibrated flasks by diluting 20 μL of stock solution with the buffer solutions of required pH values (dilution factor = 250). The pH ranges for both drugs were designated as pH 5–10 with an interval of $\Delta pH = 1$. Overall, only six sample solutions were prepared to determine the pKa value of each drug with minimal use of methanol as organic solvent.

Universal Britton-Robinson buffer was the buffer of choice to obtain the required pH levels of the sample solutions. The pH of the Britton-Robinson buffers were adjusted by adding 6 M NaOH into the aqueous solution containing 40 mM CH_3COOH, 40 mM H_3BO_3 and 40 mM H_3PO_4.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: acid-dissociation constant · chemometrics · ciprofloxacin · EEM · enrofloxacin

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