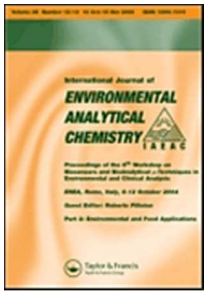

Screening fluorescent method for the fluoroquinolone family in groundwater samples from intensive livestock production systems

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Screening fluorescent method for the fluoroquinolone family in groundwater samples from intensive livestock production systems

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Screening fluorescent method for the fluoroquinolone family in groundwater samples from intensive livestock production systems

A fast and simple screening fluorescent method was developed and applied for detection of quinolones in groundwater samples. The experimental conditions for quinolone family detection ~~were performed~~ in 96-well plates were ~~in~~ acid media (pH = 4.85) from acetate buffer solution (0.1 mol L^{-1}) and 5.8 x 10^{-3} mol L^{-1} of anionic surfactant sodium dodecyl sulfate (SDS) as micellar media ~~presence of 5.8 x 10^{-3} mol L^{-1} sodium dodecyl sulfate (SDS) and acetate buffer solution (0.1 mol L^{-1}, pH = 4.85)~~ at excitation and emission wavelengths of 280 and 450 nm, respectively. The developed method was validated to guarantee the quality of the results reported. Thus, t~~T~~he decision limit (CCα) of ciprofloxacin of 6.8 µg L^{-1}, the most prescript quinolone in our country, was selected as cut-off level to classify the water samples as "suspect" or "negative" referred to quinolone content. ~~Due to the absence of certified materials, the method was validated using~~ The method showed good recoveries ranging between 80 to 114% for 6.8 µg L^{-1} ciprofloxacin with ~~recovery assay with spiked groundwater samples obtained good quinolone recoveries from 80 to 120% with~~relative standard deviation (RSD~~%~~) values lower than 1~~0~~3%. Moreover, other families of antibiotics such as aminoglycoside, penicillin, macrolide, sulfonamide and tetracycline did not present interference in the quinolone detection. Groundwater samples from Argentine regions with intensive livestock activities were analyzed by this method and the results had a good correlation with a reference method based on Ultra Performance Liquid Chromatography coupled to Mass Spectrometry (UPLC-MS).

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Keywords: Screening method, Fluoroquinolone, Fluorometry, Emerging contaminant, Livestock groundwater.

1. Introduction

Antibiotics are more and more a focus point of research due to their high detection frequency in the environment [1, 2]. Fluoroquinolones (FQs) are of interest since they are wide spectrum antibacterials with an increasing use in hospitals, households, and veterinary applications and they had included as emerging contaminants [13]. Particularly, veterinary drugs result in a direct input to soils and subsequently, via manure, to groundwater. Moreover, the use of veterinary drugs in farming activities, typically in the form of feed additives, leads to their direct entrance into the aquatic environment [24]. In addition, in wastewater treatment plant effluents one quinolone (ciprofloxacin) has been classified as one of the prevalent antibiotics [35]. They are active against both Gram-positive and Gram-negative bacteria through the inhibition of their DNA gyrase [6]. The presence of quinolone residues in food or water gives concerns to public health due to the development of drug resistance in intestinal bacteria populations [47, 8].

Moreover, the presence of these pseudo-persistent compounds in the environment can induce toxic effects on aquatic organisms [59]. The lack of biodegradation and high adsorption affinity results in long residence times in the environment, with reported half-life times of 10.6 days in surface water [610] and up to 580 days in soil matrices [711]. Generally, quinolones and other antibacterial agents have been detected at the ng to low μg L^{-1} levels in the aquatic environment in Europe and America [4, 812-14]. However, do not exist legislation for these emerging contaminants in water. On the otherrder hand, with the purpose of ensure the safety of food for consumers, several control agencies such as the United State (US) Food and Drug Administration (FDA) [915], European Union (EU) [106, 17], and SENASA in

Argentina [118] have set maximum residue limits (MRLs) in milk and target tissue of animal different for a wide range of antibiotics, including FQs.

Thus, scientists had developed methods to detection FQs using techniques like liquid chromatography with fluorescence detection [129, 20], capillary electrophoresis [1321], chemiluminescence [2142], high-performance thin-layer chromatography tandem mass spectrometry [1523] and electrochemical detection [1624, 25]. However, most of the above methods are time-consuming owing to the requirement of expensive and sophisticated instruments and have limited sensitivity. Also, these techniques are usually preceded by liquid–liquid extraction and different purification approaches, habitually solid phase extraction, new liquid extractions, or even microdialysis [2617] or automated online extraction procedures coupled chromatography which improve performance of analytical method but also increase the cost of analysis [18]. Alternatively, the fluorometric method has attracted greater attention because of the combination of convenience, simplicity and high sensitivity [27]. The sensitivity of these methods can be even further enhanced by use of organized media. The latter is a term used to describe different systems such as cyclodextrin and micelles, which can compartmentalize organic compounds, sequestering them from the bulk environment, and improving in this way their fluorescence properties [19]. Moreover, the most of FQs display strong fluorescence properties and thus more readily detected using a fluorescence detector without the need for lengthy derivatization procedures [28-3020]. Both the fluorescence properties and the use of micellar media in fluorometry for the FQs detection had been exploited by some articles [21,22] or automated online extraction procedures coupled chromatography which improve performance of analytical method but also increase the cost of analysis [20]. However, few direct fluorometric methods have been reported for determination of quinolones in water

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samples up to now. ~~To improve the sensitivity and selectivity of these analytical methods, surfactants can be used because of their ability to function as encapsulating systems to provide selective microenvironments [31, 32]~~. On the other hand, the full procedure and the methodologies for confirmatory analysis are costly in time, equipments and chemicals. Thus, it is very important to develop screening methods that allow the analysis of a large number of samples in short periods of time [23~~33~~]. Therefore, the present work proposed the development of a fast, sensitive and simple screening method based the fluorescent detection of quinolone family in 96-well plates. In order to do that, the additions of different organized media (surfactants) were studied and the experimental conditions were optimized by response surface methodology~~chemometric design~~. T~~hen, t~~hen, ~~developed method was first validated~~ the performance characteristics of the developed method (CCα, the detection capability – CCβ-, recovery assay, matrix effect and cross selectivity) were determined in water sample~~les~~. Since there is no specific directive guideline to determine residues of veterinary medicines in water samples, we thought that the guideline Directive 2002/657/EC for residue in food [24] would be the most appropiated and this guideline applied to our samples to determinate some analytical parameters (CCα and CCβ) was followed. Finally applied to analysis of groundwater samples from regions with intensive livestock activities.

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2. Materials and methods

2.1 Reagents and solutions

All antibiotics and surfactants used in this paper were purchased from Sigma ~~-~~Aldrich of Argentina SRL (Buenos Aires, Argentina). Stock standard solutions of quinolones were prepared in methanol. Working standard quinolone (100 and 10 mg mL^{-1}) and surfactant

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(0.1 mol L^{-1}) solutions were prepared by serial dilution of the stock standard solution with high-purity water.

Britton-Robinson (BR) buffer solution (pH from 3.0 to 7.0) were prepared in the usual way from 0.04 mol L^{-1} acetic acid (99.5%, Cicarelli), 0.04 mol L^{-1} sodium borate (Carlo Erba) and 0.04 mol L^{-1} phosphoric acid (85%, Cicarelli) solutions. The working buffer was 0.1 mol L^{-1} acetic acid/acetate at pH = 4.85. The pHs of buffer solutions were adjusted by addition of 0.20 mol L^{-1} sodium hydroxide o phosphoric acid solutions.

2.2 Water samples

Groundwater samples were collected in different localities in Argentina according the following Argentine protocol [2543~~4~~] and a "Non-probability sampling design for convenience" [26~~5~~35]. The samples come from places with intensive livestock production systems such as dairies, beef calves and poultry farms in Santa Fe, Córdoba and Santiago del Estero provinces. Samples were stored in dark glass bottles at 4 ◦C. Previous to the quinolone analysis, pH and turbidity expressed as the UV absorbance at 254 nm ($UV_{254\ nm}$) were measured in the samples. Then, the ionic force and pH were adjusted to 0.1 mol L^{-1} and 4.85, respectively (in analogy to the working buffer). In order to do that and not to dilute the water sample, 16 mg of acetate sodium and 5 µL of acetic acid were added to 2 mL of sample. Finally, ~~and~~ a concentration of 5.8 x 10^{-3} mol L^{-1} SDS was achieved~~dded~~ in each sample for their further analysis by a screening fluorescent method.

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2.3 Instrumentation

Electronic absorption measurements were carried out on a Perkin-Elmer Lambda 20 UV-VIS spectrophotometer. Fluorescence measurements were carried out on Perkin-Elmer (Llantrisant, United Kingdom) LS 55 luminescence spectrometer with surface readers. White F96 maxisorp microwell plates were acquired from Nunc (Roskilde, Denmark).

The pH measurements of buffer solutions were obtained using a combined glass electrode connected to a digital pH-meter (ORION, model 720A) by Merck (Darmstadt, Germany).

2.4 Optimization of individual quinolone detection

The optimization of multiple factors was ~~ere~~ performed by response surface methodologies [276]. In order to do that, a~~A~~ 3 x 3 chemometric design was used to analyses the effects of SDS concentration (5.0×10^{-3}, 1.0×10^{-2} and 1.5×10^{-2} mol L^{-1}) and pH (3, 5 and 7) in the response about six FQ systems. The quinolone concentrations were varied from 0.025 to 7 mg L^{-1}. Immediately, the responses in the 96-well plates were measured by luminescence spectrometer as fluorescence intensity (FI). The individual calibration curves of FI vs. the quinolone concentration (mg L^{-1}) were performed by ordinary least-square (OLS) calibration. The excitation and emission wavelengths for each quinolone are showed in Table 1. The limits of detections (LOD) and quantifications (LOQ) were calculated as 3.3 and 10 times the standard deviation of blank samples obtained from the calibration curves, respectively [28736].

The statistical analysis of the effects of each factor on the calibration curves were performed according to bibliography [37298] using a multiple regression model included in the StatGraphics Plus Centurión XVI software (StatGraphics, 2008) (Princeton, New Jersey, US), according the following equation:

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$$R_{ijkl} = FIR_{ijkl} = \beta_0 + \beta_1 A_i + \beta_2 B_j + \beta_{12} A_i B_j + \beta_{11} A_i^2 + \beta_{22} B_j^2 + e_{ijk} \quad (1)$$

where R_{ijkl}: regression model; FIR_{ijkl}: fluorescence intensity response; β_0, β_1, β_2, β_{12}, β_{11}, β_{22} : estimated parameters from model; A_i: SDS concentration; B_j: pH; A_iB_j: SDS and pH interaction; A_i^2: SDS squared; B_j^2: pH squared and e_{ijk} = residual error.

Then, optimal pH and SDS concentration in the quinolone detection were selected by the desirability function [26~~38~~] included in the StatGraphics Plus Centurión XVI software (StatGraphics, 2008).

2.5 Screening fluorescent method for quinolone family

The quinolone fluorescent detection was performed in working buffer at pH = 4.85 and SDS of 5.8 x 10^{-3} mol L^{-1}. The fluorescence intensities were registered at λ_{ex} = 280 nm and λ_{em} = 450 nm.

The validation study was performed according to Directive 2002/657/CE [24~~9~~39] and other related literature sources [30~~40~~, 31~~41~~].

Decision limit (CCα) was defined as three time the signal-noise ratio for twenty blank water samples, while detection capability (CCβ) was calculated as follows:

$$CC\beta = CC\alpha + 1.64\left(SD_{CC\alpha}\right) \quad (2)$$

where $SD_{CC\alpha}$ is the standard deviation of twenty blank samples spiked at CCα level. The spiked quinolone was ciprofloxacin.

Posteriori, groundwater samples were analyzed by developed screening method and classified as "negative" or "suspect" according the CCα level of ciprofloxacin.

2.6 Reference method (UPLC-MS)

Ultra Performance Liquid Chromatography was employed using an ACQUITY UPLC™ System (Waters, Milford, MA, USA) coupled to a triple quadrupole mass

spectrometer (Micromass TQ Detector from Waters, Manchester, UK) through an orthogonal-Z-spray ionization source. Separations were performed using an ACQUITY UPLC® BEH C18 RP Shield (1.7 µm 2.1 x 100 mm) column from Waters at a flow rate of 0.3 mL min^{-1} and 40 °C temperature. Mobile phase consisted of A (0.5 mM NH_4F + 0.1% formic acid in water) and B (0.5 mM NH_4F + 0.1% formic acid in methanol). Moreover, were evaluated aspects related to chromatographic methodology, ionization conditions, and operative detection variables in multiple-reaction monitoring (MRM) mode of the triple quadrupole mass spectrometer. In relation to mass detection, two transitions from each compound's pseudomolecular ion ($[M^+H]^+$) were used for identification in addition to the retention time, while for quantification the most abundant transition was used. MassLynx v4.1 software (Waters, Manchester, UK) was employed for instrumental operation, data acquisition and analysis. This method was applied for five quinolone detection: ciprofloxacin (CIPRO), enrofloxacin (ENRO), enoxacin (ENO), difloxacin (DIFLO) and ofloxacin (OFLO).

3. Results and Discussion

3.1 Fluorometry

The quinolones studied were danofloxacin (DANO), OFLO, CIPRO, norfloxacin (NOR), sarafloxacin (SARA) and ENRO which are widely used in veterinary practices. The spectrofluorimetric scans for six quinolones at pH from 3.0 to 7.0 allow selecting the wavelengths of excitation and emission in each system (see Fig. i in supplementary material).

These pH ranges were selected in based to the FQs are luminants in their neutral and cationic forms while there are no luminants in anionic form [3242]. In the Table 1, the wavelengths of excitation and emission selected for each quinolone are summarized.

While all FQs presented the same excitation and emission wavelengths at pH from 3.0 to 5.0, slightly blue shifts at pH 7.0 were obtained. Also, the fluorescence intensities were relatively stable and strong in the range of pH analyzed for the six FQs. These results are similar to the declared by Doorslaer et al. [3343] that found that the fluorescent intensity of CIPRO was stable in the range of pH from 2 to 7. This stable fluorescence can be attributed to the fact that the acid constants (K_{a1} of $H_3^{2+}A$, K_{a2} of $H_2^{+}A$ and K_{a3} of $H^{+}A^{-}$) are near to each other (see Table 1) [3343]. The positions of the different ionisable groups of six FQs are available in the Fig. 1.

Table 1

Figure 1

Additionally, the effect of surfactants on the fluorescent property of FQs was studied in order to increase the FQ responses and improve sensitivity of method. It is found that cationic surfactant with positive charge has no notable influence on the property of CIPRO in aqueous solution. This can be attributed to repulsion of the same charges between cationic forms of CIPRO and surfactant [3242]. Therefore, non-ionic [Triton X-100 (TX-100), Tween 20 (Tw20) and Tween 80 (Tw80)] and anionic [SDS and Sodium Lauryl Sulfate (SLS)] surfactants, which vary in size and the properties of the monomers, were evaluated on the fluorescence intensity of 0.8 mg L^{-1} NOR and 0.2 mg L^{-1} ENRO as model quinolones in BR buffer at pH = 5.0. According to bibliography, surfactants should be used above their critical micelle concentration (CMC) to have a good function as encapsulating systems [21,2231, 32]. Therefore, both CMC of each surfactant and specific bibliography [3444-3747] were considered in the selection of the concentration ranges. The concentrations evaluated for de non-ionic surfactants were lower than anionic ones due to the fact that the CMC values of ionic surfactants are higher than non-ionic ones [4838]. The results are showed in the Fig. ii

in supplementary material. On the one hand, the non-ionic surfactants (TX-100, Tw20 and Tw80), whose CMC are (2.1 – 2.4) x 10^{-4} mol L^{-1} [4939], 5 x 10^{-5} mol L^{-1} [5040] and (0.9 – 1) x 10^{-5} mol L^{-1} [5141], respectively, have a little quenching effect on the fluorescence of quinolones at concentrations above their CMC. This effect can be due to the fact that the non-ionic surfactants decrease of polarity of micelle micro-environment and, consequently, this factor can reduce the fluorescence response. On the other hand, the anionic surfactants (SLS and SDS) hadve different behaviors. SDS enhanced two-fold the fluorescent response ofshowed fluorescent enhancement for NOR and ENRO at concentrations higher than 3.0 x 10^{-3} mol L^{-1}, whose value is closer than its CMC of 7.0 x 10^{-3} mol L^{-1} [3949], while SLS had not significantive influence about the fluorescent responses. The different chemical structures between SDS and SLS and the average micellar weight of SLS lower than SDS could explicate that effect. SinceAs, micellization of anionic form of SDS with cationic form of quinolones would create a microenvironment of producing favorable polarity, improving solubility of quinolones in aqueous media and adopting certain conformations to protect the fluorescence of the analytes from external quenching effects. Therefore, SDS was selected as surfactant detergent and their concentration was optimized in posteriori assays for each FQ system.

3.2 Optimization of individual quinolone detection

In order to determinate the quinolones individually, the excitation and emission wavelengths for each quinolone were selected according section 3.1 and then, Cthe calibration curves for each onesindividual quinolone were performed underin the experimental conditions plannedindicate by a 3 x 3 chemometric design. The LODs obtained from curves were used as response of design and are showed in Table 2. The sensitivities obtained in each calibration curve

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decreased in the sequence of DANO (~ 1500 mg^{-1} L) > ENRO > NOR > CIPRO > OFLO > SARA (~ 100 mg^{-1} L) (see Table i in supplementary material). Our goal was minimize the LODs in order to can detect quinolones in low concentration in water matrixes.

Table 2

After analysis of results, the equations obtained by applying order second multiple regression models are showed in the Table 3. For all assayed quinolones (excluding DANO) negative lineal ($-\beta_2$) and positive quadratic ($+\beta_{22}$) effects for pH were evidenced, demonstrating the presence of a relative minimum. On the other~~rder~~ hand, SDS concentration showed a similar behavior ($-\beta_1$ and $+\beta_{11}$) for DANO, CIPRO, NOR and ENRO.

Table 3

With the purpose of visualize the interaction effects of design variables about the studied quinolone LODs, Fig. 2 shows the response surface curves built for each studied quinolone. As can be seen, all studied quinolones have a minimum LOD area within the ranges selected in the design (pH from 3 to 7 and SDS concentration from 5.0 x 10^{-3} to 1.5 x 10^{-2} mol L^{-1}). On the other~~rder~~ hand, ENRO showed a deeper tendency to minimum LOD values to low SDS concentrations and high pH than NOR and CIPRO, that have similar structures and fluorescence behavior. These differences could be attributed to six times higher interaction effect (β_{12}) and four times lower quadratic effect of SDS concentration (β_{11}) for ENRO than for the other similar quinolones.

To determine the best condition to the quinolone detection at low levels, minimization criteria of LODs were applied for all quinolones because do not exist allowed maximum limits in environmental samples. Moreover, the weight criteria were fixed at 1.0 for all quinolones and the importance criteria were 5.0 for the quinolones

more frequently used (CIPRO and ENRO), 3.0 for this moderately used (NOR) and 1.0 for those less used (DANO, OFLO and SARA). The optimal conditions obtained by design were 5.8 x 10^{-3} mol L^{-1} SDS and pH = 4.85, with desirability function of 0.95. The convenience of low SDS concentration and the average pH value had already been observed in Fig. 2. In these conditions, the LODs predicted were 3.9, 25.6, 37.9, 11.9, 19.5, 27.5 and μg L^{-1} for DANO, OFLO, CIPRO, NOR, SARA and ENRO, respectively.

Figure 2

3.3 Optimization of screening fluorescent method for quinolone family

3.3.1 Study of quinolones in buffer solution

First, the selection of one only pair of excitation and emission wavelengths was performed with the finality of developing a screening fluorescent method in which can detect the quinolone family. The Fig. iii in the supplementary material shows the fluorescent responses of 100 μg L^{-1} quinolones at three wavelength pairs assayed (λ_{ex1} = 270 nm and λ_{em1} = 440 nm; λ_{ex2} = 280 nm and λ_{em2} = 450 nm; λ_{ex3} = 300 nm and λ_{em3} = 500 nm). The worst wavelength pair was the third because almost all quinolones had low fluorescence except OFLO. Therefore, 280 and 450 nm were selected as excitation and emission wavelenghts, respectively, due to the fact that in this condition the most quinolones showed higher response, specially quinolones most frequently reported in different environmental matrices [33~~43~~].

Second, individual calibration curves were built for each quinolone in the concentration range from 1 to 150 μg L^{-1} (nine levels for triplicate) in working buffer at pH = 4.85 and SDS of 5.8 x 10^{-3} mol L^{-1}. The fluorescence intensities were read to 280 and 450 nm as excitation and emission wavelenghts, respectively. The Fig. iv in the

supplementary material shows the curve obtained by CIPRO as example of quinolone. The critical values (LC), LOD and LOQ were calculated as 1.64, 3.3 and 10 times the standard deviation (DE) of blank samples obtained from the calibration curves for each quinolone, respectively [27~~36~~] and are showed in the Table 4. Our limits were similar to obtained by quantitative methodologies considered as confirmatory methods based on (1) ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) [15~~23~~], (2) dispersive liquid–liquid microextraction (DLLME) procedure combined with ultra-high performance liquid chromatography with diode-array detection [~~52~~42], (3) second-order capillary electrophoresis data modeling with multivariate curve resolution-alternating least squares [8~~13~~] (4) micellar liquid chromatography with fluorescent detection [21] and (~~5~~4) solid-phase extraction (SPE) procedure with fluorescent detection [43~~53~~]. Moreover, our limits were lower than detection limit of solid-phase UV spectrophotometric method [44~~54~~] and slightly higher than the reported limits for method based in solid-phase extraction and liquid chromatography with fluorimetric detection [35~~45~~] being the developed method simpler than those.

Table 4

~~*3.3.2 Study of quinolones in water sample*~~

~~Due to the absence of certified materials, a blank groundwater sample with $UV_{254\ nm}$ of (0.0023 ± 0.0004) was used as matrix in the recovery study at three levels (50, 100 and 150 µg L^{-1}) of six quinolone. The recoveries were calculated from individual calibration curve for each quinolone and are showed in the Table 5. The recoveries obtained were ranged from 80 to 120 % with precisions calculated as relative standard deviation (RSD %) lower than 10 %. These values are considered acceptable according to~~

~~recommendations for analytical method validation about precision requirements in the phase of method development [55].~~

~~Table 5~~

3.3.2~~3~~ Selection of congener quinolone

The figures of merits for each quinolone in Table 4 were taken into account to select the congener quinolone. Although DANO had higher sensitivity than other quinolones, CIPRO was selected as congener of quinolone family due to the fact that it is the most widely prescribed FQ in our country (Argentine) [45~~56~~, 46~~57~~] and in the world [47~~58~~]. Also, it is the most frequently detected in environmental matrices [33~~43~~].

Then, the effect of the fluorescent response when several quinolones are present in one matrix was studied. In order to do that, the response of CIPRO at their LC (2.5 μg L^{-1}) was evaluated opposite to the other quinolones in concentrations from LC (2.5 μg L^{-1}) to 10 LC (25 μg L^{-1}). Curves of CIPRO responses (μg L^{-1}) vs. the quinolone concentrations (μg L^{-1}) were performed by OLS calibrations whose sensitivities, estimated by curve slopes, increased from NOR ~ SARA (1.9 μg CIPRO L^{-1} μg quinolone^{-1} L) > ENRO ~ OFLO (3.9 μg CIPRO L^{-1} μg quinolone^{-1} L) > DANO (14 μg CIPRO L^{-1} μg quinolone^{-1} L). These results indicated synergistic responses for all quinolones in the concentration range evaluated, being the response overestimated from 1.9 to 14 times according to expectations. This fact would be beneficial for the implementation of the screening method in the quinolone family detection in water samples.

3.3.3~~4~~ CCα and CCβ

CCα and CCβ for CIPRO as congener quinolone in groundwater were defined as three times the signal-noise ratio for twenty blank water samples and CCα plus 1.64 times standard deviation of CCα, respectively [29~~39~~]. The CCα and CCβ calculated were 6.8 and 7.6 μg L^{-1}, respectively. The CCα was used as cut-off level (discriminating value) to classify the water samples as "negative" or "suspect" [48~~57~~]. The advantage to fix the cut-off level starting from the results of the representative blank samples rather than from the results of the spiked ones [30~~40~~] was that it did not depend on the spiking level. As consequence, the screening test is used at its maximum performance in terms of detectability, since the estimated cut-off level substantially corresponds to the classical LOD. This is an important aspect~~,~~ since the scope of the monitoring plans is detect contaminant to low concentrations by simple and fast method.

3.3.4 Recovery assays

Six blank groundwater samples with $UV_{254\ nm}$ lower than 0.22 were used as matrix in the recovery study at CCα level of CIPRO (6.8 μg L^{-1}). The recoveries obtained were ranged from 80 to 114 % with precisions calculated as relative standard deviation (RSD %) lower than 13 % (see Table 5). These values are considered acceptable according to recommendations for analytical method validation about precision requirements in the phase of method development [49].

Table 5

3.3.5 Matrix effect

The matrix effect of a sample of turbid superficial water with undetected quinolones (CCα = 6.8 μg L^{-1}) was evaluated. The dissolved organic matter (DOM) expressed as the $UV_{254\ nm}$ value of (0.738 ± 0.002) was obtained in the sample initially. The recovery

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study was performed in this matrix by spiking of 100 μg L^{-1} CIPRO in the same conditions of the working buffer (0.1 mol L^{-1} and pH = 4.85) with 5.8 x 10^{-3} mol L^{-1} of SDS. A low mean recovery (62 ± 2) % was obtained which might indicate fluorescence response of CIPRO is reduced by the turbid sample matrix. As it is well known, the turbidity affects significantly the fluorescence responses. The presence of practically unavoidable scatterers and background absorbers in turbid media such as biological tissue, cell suspensions or suspended particles can significantly distort the shape and intensity of fluorescence spectra of fluorophores and, hence, greatly hinder the in situ quantitative determination of fluorophores in turbid media [~~59~~50]. Therefore, the relation between CIPRO recovery at 100 μg L^{-1} in water and $UV_{254\ nm}$ value at different superficial water sample dilutions was studied and the obtained results are showed in Fig. 3. Recoveries higher than 80% were imposed as acceptable minimal ones for this study due to the fact that it is the estimated average recovery in function analyte concentration (80-110 mean recovery %) by Association of Official Analytical Chemists (AOAC) [~~60~~49~~51~~]. This value can be achieved when $UV_{254\ nm}$ in the sample was lower than 0.30. As a consequence, previous to the quinolone detection by screening fluorescent method, the $UV_{254\ nm}$ value should be measured. If it is lower than 0.30, the quinolone detection in the water sample can be directly analyzed. On the other~~rder~~ hand, if $UV_{254\ nm}$ is higher than 0.30, the sample should be pretreated by cleanup process such as solid-liquid extraction or solid-phase extraction (SPE).

Figure 3

3.3.6 Cross selectivity

On the one hand, the cross selectivity intra-family, the responses of DANO, OFLO, CIPRO, NOR, SARA and ENRO at 2.5 μg L^{-1} (LC level of CIPRO) in blank

groundwater sample were evaluated and expressed as CIPRO equivalents in μg L^{-1}. The results were 36 (± 1), 5.2 (± 0.8), 2.5 (± 0.3), 6.2 (± 0.9), 2.6 (± 0.2) and 5.0 (± 0.5) μg L^{-1} for DANO, OFLO, CIPRO, NOR, SARA and ENRO, respectively. All quinolones could be detect using CIPRO as congener quinolone being, DANO, OFLO, NOR and ENRO overestimated from 2 to 15 times that is according to the higher sensitivities obtained for these quinolones individually by this screening method.

On the other hand, the cross selectivity by other antibiotic families (aminoglycoside, β-lactams, macrolides, sulfonamides and tetracyclines) was evaluated due to the fact that these families are frequently used in intensive livestock production systems [5016~~1~~]. Neomycin, penicillin, erythromycin, sulfamethoxazole and oxytetracyclin were evaluated in the concentration range from 15.5 to 1550 μg L^{-1} by optimized fluorescent method in blank groundwater sample. The fluorescent intensities obtained for potentially interfering species were related with the CIPRO concentrations. Fig. v in the supplementary material shows the responses as CIPRO concentrations versus interfering concentrations. As it expected because these antibiotic families have not native fluorescence in the wavelengths assayed, all responses were lower than CCα of CIPRO (6.8 μg L^{-1}) which suggest that the families of drugs evaluated not interfere in the detection of quinolones. Therefore, this fluorescent screening method is very selective for quinolones and there is no masking effect of the other antibiotics typically found in groundwater from intensive livestock production systems.

3.4 Quinolone detection in groundwater samples by screening fluorescent method

Seventeen groundwater samples from intensive livestock production systems were tested by screening fluorescent method for quinolone family. In most cases, the animals had been treated with quinolones in the last days according the farmers.

Table 6 shows the characteristics of samples (location in Argentine, source, pH and DOM expressed as $UV_{254\ nm}$ value). Also, the amounts of quinolones as CIPRO equivalents were calculated for each sample and classified as "suspect" or "negative" according the quinolone concentration were higher or lower than CCα (6.8 μg L^{-1}), respectively. Four samples (one sample of dairy and three samples of poultry farms) were "suspect" by screening fluorescent method with values from 8 to 13 μg L^{-1} of CIPRO equivalents. The pollution with FQs could be due to (1) percolation from sludge or manure fertilized acres into the deeper soil layers, (2) landfill leachate from dairies or animal husbandries if there are no collection barriers, or (3) via FQ polluted surface waters.

Table 6

Although it is widely known the use of quinolones in veterinary medicine in our country, we have not found publications that evaluate these antibiotics in groundwater samples. On the other~~rder~~ hand, values of quinolones in wastewaters from 0.2 to 2.5 μg L^{-1} were found in the province of Granada (Spain) [5123] and CIPRO groundwater pollution (0.7–14 μg L^{-1}) were observed in the Pantacheru region in India [6252]. Densely populated areas are also vulnerable for FQ groundwater pollution, as is observed by Teijon et al. [6353] and López-Serna et al. [5464], where FQ concentrations up to 0.5 – 1 μg L^{-1} are reported in the region of the metropolis of Barcelona (Spain). However, data about quinolone detection in groundwater are much scarcer than for surface water.

Also, these samples were evaluated by UPLC-MS, detecting quinolones in six samples (two samples had a concentration lower than CCα). This indicates a concordance of 88% in the evaluated samples, which suggest satisfactory analytical performance in the CIPRO detection by developed screening method. Therefore, the

developed method would be applicable to groundwater samples near areas with intensive use of quinolones such as animal farming. Moreover, the screening methods have advantages as simplicity, low cost and speed whereby they are very useful for the fast discrimination between "negative" and "suspect" samples, being the latter sent to confirm by reference method.

4. Conclusion

This work describes a simple screening fluorescent method for the detection of quinolone family with CCα of 6.8 $\mu g\ L^{-1}$ expressed as CIPRO equivalents in groundwater samples from intensive livestock production systems. The presence of FQs represents a threat to not only human health associated to antibiotic resistance but also ecological system where changes in the environment can affect diversity and function of species. In contrast with the traditional methods, this screening method will allow the simple, fast and cheap detection of quinolones without the demands of toxic analytical reagents and instrumentation sophisticated, reducing the environmental impact of the analysis. The total analysis time was of 30 min *per* 96-well plate with minimal pretreatment of samples (only conditioning of ionic force and pH). Besides, the method allows the analysis of a large number of samples per day from small volume of samples and reagent, so it tends to comply with the principles of "Green Chemistry".

Finally, the application of this method in the detection of groundwater samples in Argentina is a contribution very important due to the fact that we did not found reports in our region about this, in spite of the intensive livestock activity carried out there.

Acknowledgments

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Screening fluorescent method for fluoroquinolone family in groundwater samples from intensive livestock production systems

Supplementary Material

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3. Results and Discussion

3.1 Fluorometry

The excitation and emission spectra of DANO, OFLO, CIPRO, NOR, SARA and ENRO solutions at concentrations of 0.1, 1.0, 0.8, 1.2, 1.0 and 0.2 mg L^{-1}, respectively, were evaluated at pH from 3.0 to 7.0. Initially, spectrophotometric studies were performed for each system, selecting the absorption peak. From these results, the excitation and emission maximum were obtained by spectroflurimetric scans. The Fig. i shows the spectra of CIPRO obtained by fluorescence detection at pH of 3.0, 5.0 and 7.0.

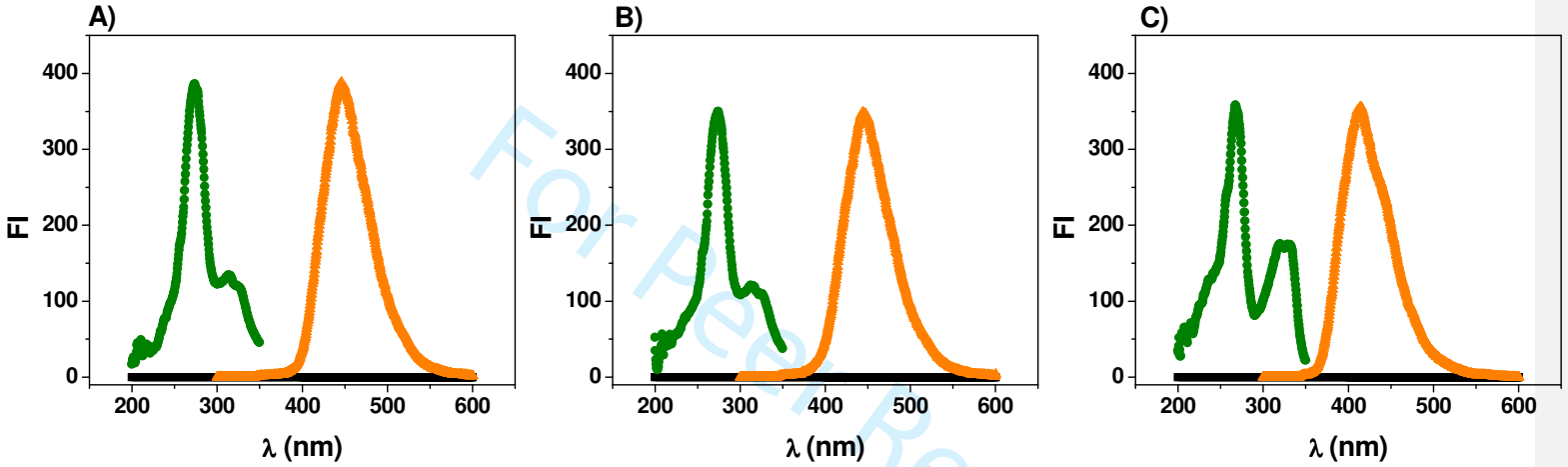

Fig. i. The spectra of blank (black line) and 0.8 mg L^{-1} CIPRO by LS 55 luminescence spectrometer (green and orange lines for excitation and emission spectra, respectively) in BR buffer solutions at pH of 3.0 A), 5.0 B) and 7.0 C).

The effect of the TX-100, TW20, TW80, SDS and SLS surfactants were evaluated on the fluorescence intensity of 0.8 mg L^{-1} NOR and 0.2 mg L^{-1} ENRO as model quinolones in BR buffer at pH of 5.0. The concentrations of surfactants were selected in based to their critical micelle concentrations. The analyzed ranges were from 1 x 10^{-5} to 5 x 10^{-2}, from 5 x 10^{-4} to 1 x 10^{-1}, from 3 x 10^{-5} to 1 x 10^{-2}, from 1 x 10^{-5} to 2 x 10^{-4} and from 3 x 10^{-5} to 5 x 10^{-4} mol L^{-1} for SLS, SDS, Tw20, Tw80 and TX-100 respectively. The Fig. ii shows the effects of different surfactants about the response of NOR and ENRO.

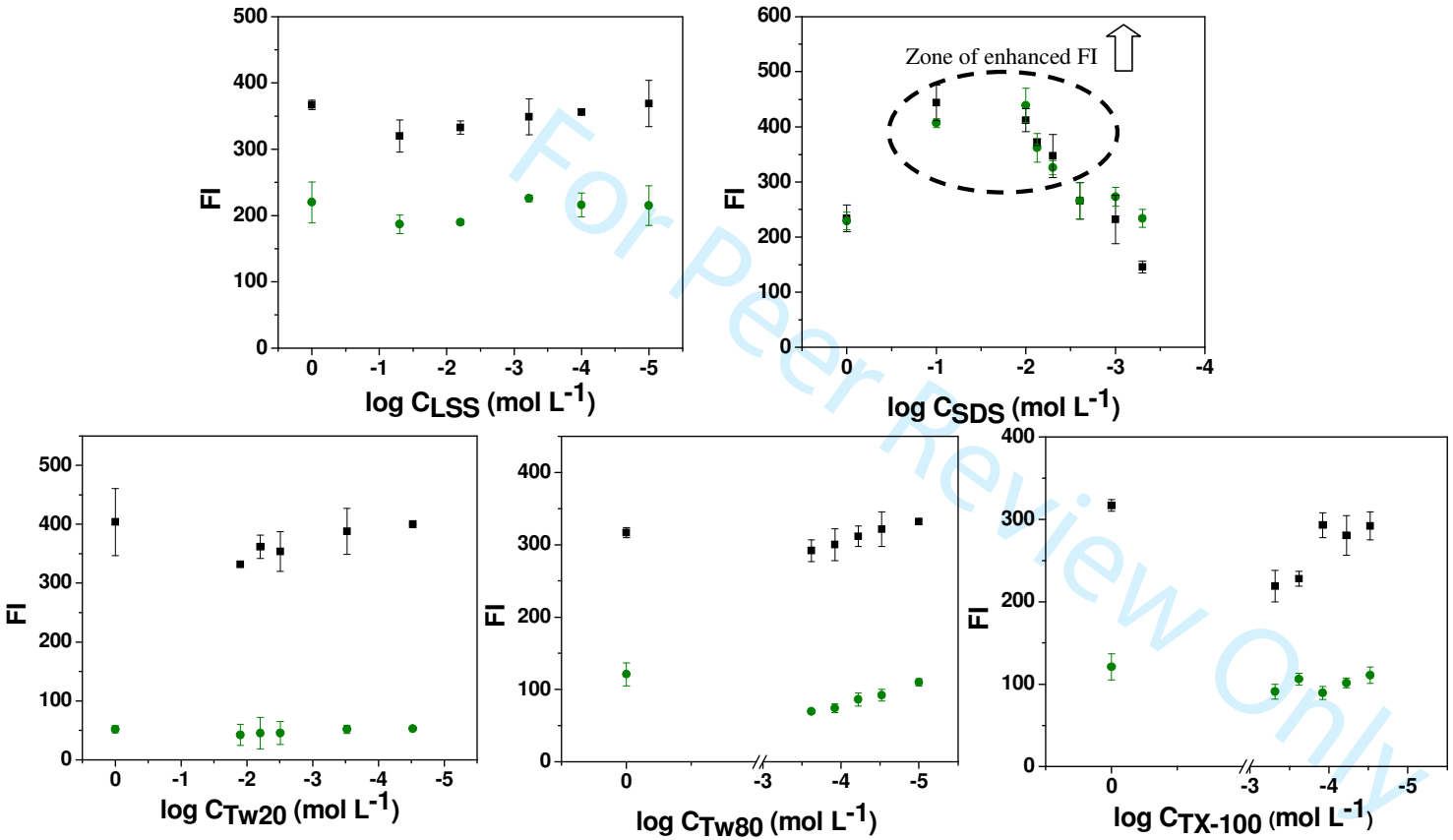

Fig. ii. The fluorescence intensity of 0.8 mg L^{-1} NOR (black square) and 0.2 mg L^{-1} of ENRO (olive circle) in BR buffer solutions at pH = 5.0 in presence of different surfactants (1 x 10^{-5} - 1 x 10^{-1} mol L^{-1}). The absence of surfactants is represented with 0 in X. Data: λex = 274 nm and λem = 444 nm. The error bars show the standard deviation for N = 3.

3.2 Optimization of individual quinolone detection

The effects of SDS concentration (5.0 x 10^{-3}, 1.0 x 10^{-2} and 1.5 x 10^{-2} mol L^{-1}) and pH (3, 5 and 7) in the fluorescence response about six fluoroquinolone systems were evaluated by 3 x 3 design. The quinolone concentrations were varied from 0.025 to 7 mg L^{-1}. Nine calibration curves of fluorescence intensity vs. quinolone concentration were built for each quinolone. Table i shows the analytical sensitivities obtained in each calibration curve.

Table i: Sensitivities from calibration curves for each quinolones

Factors		Analytical sensitivities (mg^{-1} L)					
SDS (mol L^{-1})	pH	DANO	OFLO	CIPRO	NOR	SARA	ENRO
1.5 x 10^{-2}	3	1642	141	167	242	107	241
1.0 x 10^{-2}	3	1721	220	183	365	117	278
5.0 x 10^{-3}	3	1321	129	128	185	106	228
1.5 x 10^{-2}	5	1535	214	309	607	135	921
1.0 x 10^{-2}	5	1488	230	208	355	138	450
5.0 x 10^{-3}	5	1353	276	206	355	107	417
1.5 x 10^{-2}	7	1333	85	87	150	35	182
1.0 x 10^{-2}	7	1330	77	86	103	37	142
5.0 x 10^{-3}	7	1089	83	85	112	34	151

3.3 Optimization of screening fluorescent method for quinolone family

3.3.1 Study of quinolones in buffer solution

First, one pair of wavelengths for six fluoroquinolones was selected in the development of this screening method. In order to do that, the response of quinolones in working buffer at pH = 4.85 and 5.8 x 10^{-3} mol L^{-1} SDS were evaluated at three excitation and emission wavelengths (λ_{ex1} = 270 nm and λ_{em1} = 440 nm; λ_{ex2} = 280 nm and λ_{em2} = 450 nm; λ_{ex3} = 300 nm and λ_{em3} = 500 nm). The three subgroups were selected based on the pairs for individual detection of the quinolones (Table 1). The subgroup 1 would allow detecting to CIPRO, ENRO and NOR, the subgroup 2 to DANO and SARA and the subgroup 3 to OFLO.

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In preliminary assays performed in cuvettes (data not showed), the emission wavelength was close to 500 (489 ± 20) nm for OFLO. As our instrument does not allow performing scans in microplates, a media emission wavelength was fixed in 500 nm. The media fluorescent responses with their confidence intervals in three conditions were plotted for each quinolone (see Fig. iii).

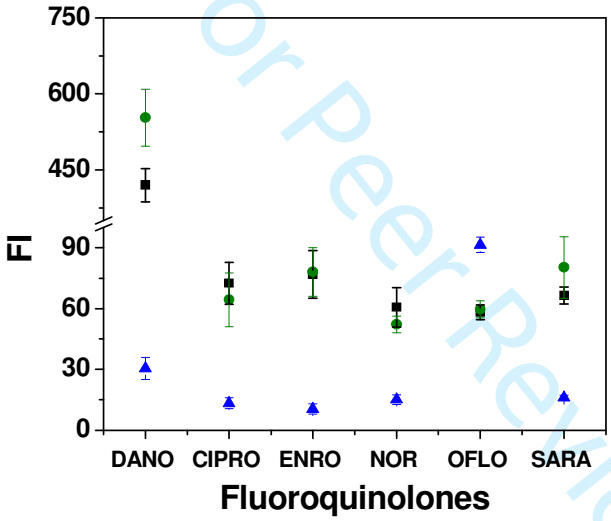

Fig. iii. The fluorescence intensity of 100 µg L^{-1} quinolones in working buffer at pH = 4.85 and 5.8 x 10^{-3} mol L^{-1} SDS at three wavelength pairs: (1) λex = 270 nm and λem = 440 nm (black square), (2) λex = 280 nm and λem = 450 nm (olive circle) and (3) λex = 300 nm and λem = 500 nm (blue triangle). The error bars show the standard deviation for N = 3.

Second, the calibration curves for six fluoroquinolones were built in the concentration range from 1 to 150 µg L^{-1} in working buffer at pH = 4.85 and SDS of 5.8 x 10^{-3} mol L^{-1}. The fluorescence intensities were registered at λ_{ex} = 280 nm and λ_{em} = 450 nm. In the Fig. iv an example of calibration curve is reported. The final curve was prepared using nine standard solutions of ciprofloxacin in dilution buffer at the following concentrations: 3.4, 6.8, 10, 25, 50, 75, 100, 125 and 150 µg L^{-1}. The LC, LOD and LOQ were 2.5, 5.1 and 15.6 µg L^{-1}, respectively.

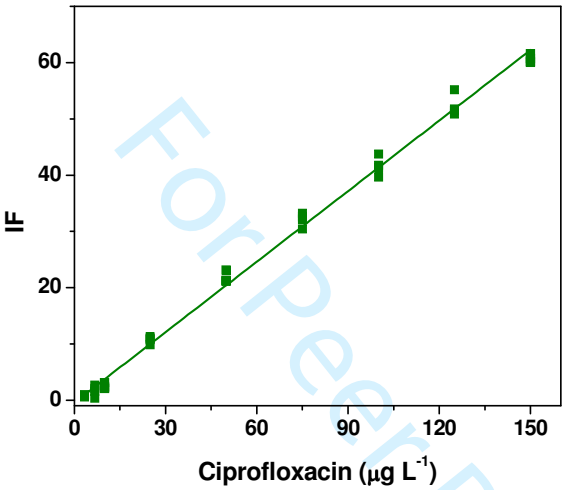

Fig. iv. Calibration curve of ciprofloxacin. Each point represents the results of four replicates.

3.3.6 Cross selectivity

The cross selectivity of the fluorescent method was evaluated by determination of the responses of several families of veterinary drugs. In order to do that, oxytetracycline (a tetracyclin), penicillin (a β-lactam), neomycin (an aminoglycoside), erythromycin (a macrolide) and sulfamethoxazole (a sulfonamide) were analyzed at five levels (15.5; 77.5; 150; 775 and 1550 μg L^{-1}) in working buffer at pH = 4.85 and SDS of 5.8 x 10^{-3} mol L^{-1}. The fluorescence intensities were registered at λ_{ex} = 280 nm and λ_{em} = 450 nm. Fig. v shows the responses obtained for potential interfering species as ciprofloxacin concentration versus interfering concentration.

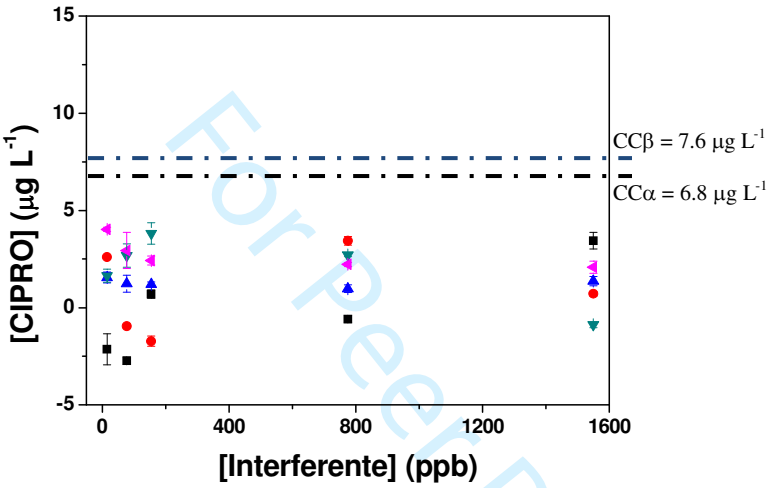

Fig. v. Selectivity assay for penicillin (black square), neomycin (red rhombus), erythromycin (blue triangle), oxytetracycline (olive triangle) and sulfamethoxazol (magenta triangle) in the range of 15.5 to 1550 µg L^{-1}. The error bars show the standard deviation for N = 3.

Table 1. Wavelengths of emission and excitation selected for six quinolone systems at pH differents and B) acid constants according Doorslaer et al. [33].

Quinolone	A) Wavelengths of emission and excitation						B) Acid constants			
	pH = 3.0[a]		pH = 5.0[a]		pH = 7.0[a]					
	λex (nm)	λem (nm)	λex (nm)	λem (nm)	λex (nm)	λem (nm)	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
DANO (0.1 mg L^{-1})	279	440	279	440	275	433	-	-	6.18	8.78
OFLO (1.0 mg L^{-1})	292	491	292	489	286	451	-	5.20	5.98	8.00
CIPRO (0.8 mg L^{-1})	274	444	274	444	268	416	3.32	5.59	6.14	8.85
NOR (1.2 mg L^{-1})	274	444	274	444	268	416	3.10	5.55	6.27	8.71
SARA (1.0 mg L^{-1})	277	455	277	455	271	418	-	-	5.91	9.07
ENRO (0.2 mg L^{-1})	274	444	274	444	268	416	3.88	6.19	6.20	8.13

[a]BR buffer solutions (0.04 mol L^{-1} acetic acid, 0.04 mol L^{-1} sodium borate and 0.04 mol L^{-1} phosphoric acid).

Table 2. LODs obtained for each quinolone in the factor combinations

Factors		LODs ($\mu g\ L^{-1}$)					
SDS (mol L^{-1})	pH[a]	DANO	OFLO	CIPRO	NOR	SARA	ENRO
1.5×10^{-2}	3	5.7	31.0	67.3	23.9	38.0	25.3
1.0×10^{-2}	3	1.7	50.6	63.2	28.3	35.6	34.0
5.0×10^{-3}	3	7.6	50.0	131.6	50.7	54.0	113.7
1.5×10^{-2}	5	7.0	16.7	57.6	11.7	34.6	19.1
1.0×10^{-2}	5	2.0	52.0	37.9	13.5	29.6	45.4
5.0×10^{-3}	5	14.0	37.5	61.2	58.4	43.6	36.8
1.5×10^{-2}	7	2.2	41.3	147.8	72.4	114.0	10.3
1.0×10^{-2}	7	3.3	107.4	114.8	30.8	202.8	17.9
5.0×10^{-3}	7	12.3	63.0	179.7	113.4	133.5	35.0

[a]BR buffer solutions (0.04 mol L^{-1} acetic acid, 0.04 mol L^{-1} sodium borate and 0.04 mol L^{-1} phosphoric acid).

Table 3. Equations obtained from multiple regression models to six quinolone systems

Quinolones	$R_{ijkl} = FIR_{ijkl} = \beta_0 + \beta_1 A_i + \beta_2 B_j + \beta_{12} A_i B_j + \beta_{11} A_i^2 + \beta_{22} B_j^2 + e_{ijk}$	C[a]
DANO	$FIR = 0.008 - 4\,[SDS] + 0.008\,pH - 0.20\,[SDS]\,pH + 232\,[SDS]^2 - 0.0006\,pH^2$	95.1
OFLO	$FIR = 0.06 + 22\,[SDS] - 0.05\,pH - 0.07\,[SDS]\,pH - 1203\,[SDS]^2 + 0.005\,pH^2$	86.3
CIPRO	$FIR = 0.58 - 36\,[SDS] - 0.16\,pH + 0.8\,[SDS]\,pH + 1423\,[SDS]^2 + 0.016\,pH^2$	95.3
NOR	$FIR = 0.26 - 27\,[SDS] - 0.05\,pH - 0.3\,[SDS]\,pH + 1235\,[SDS]^2 + 0.006\,pH^2$	87.5
SARA	$FIR = 0.2 + 15\,[SDS] - 0.12\,pH - 0.09\,[SDS]\,pH - 789\,[SDS]^2 + 0.015\,pH^2$	87.9
ENRO	$FIR = 0.26 - 18\,[SDS] - 0.04\,pH + 2\,[SDS]\,pH + 304\,[SDS]^2 + 0.001\,pH^2$	80.7

[a]C: concordance efficient (%).

Table 4. Figures of merits obtained by each quinolones from individual calibration curve at λ_{ex} = 280 nm and λ_{em} = 450 nm.

Quinolone	Figures of merits			
	LC (μg L^{-1})[a]	LOD (μg L^{-1})[b]	LOQ (μg L^{-1})[c]	Analytical sensitivity (μg^{-1} L)
DANO	0.6	1.1	3.4	1898
OFLO	2.2	4.3	13.2	484
CIPRO	2.5	5.1	15.5	424
NOR	1.6	3.3	9.9	644
SARA	2.4	4.9	14.9	427
ENRO	2.3	4.7	14.2	450

[a] $LC = 1.64 \times \frac{DE\ blank\ samples*}{slope}$

[b] $LOD = 3.3 \times \frac{DE\ blank\ samples*}{slope}$

[c] $LOQ = 10 \times \frac{DE\ blank\ samples*}{slope}$

*DE blank samples was 0.637.

Table 5. Recoveries (%) for spiked blank water samples at CCα of CIPRO (6.8 μg L^{-1}).

Samples	DOM ($UV_{254\ nm}$)	Recoveries (%) (RSD %[a])
1	0.1230	114 (10 %)
2	0.0987	102 (6 %)
3	0.0845	89 (4 %)
4	0.0941	91 (13 %)
5	0.2145	80 (6 %)
6	0.0457	91 (11 %)

[a]RSD % = relative standard deviation % (N = 4).

Table 6. Analysis of groundwater samples by screening fluorescent (CCα = 6.8 μg L^{-1}) and reference methods

Characterization of the samples					Screening fluorescent method		Reference method (UPLC-MS)[d, e]					
Sample	Location in Argentina[a]	Source	pH	$UV_{254\ nm}$	Result (μg L^{-1})[b]	Interpretation	[ENO] (μg L^{-1})	[CIPRO] (μg L^{-1})	[ENRO] (μg L^{-1})	[OFLO] (μg L^{-1})	[DIFLO] (μg L^{-1})	Interpretation
1	Esperanza[a1]	Dairy	7	0.0114	< CCα	Negative	-	-	-	-	-	Negative
2	Esperanza[a1]		7	0.2280	< CCα	Negative	-	-	-	-	-	Negative
3	Esperanza[a1]		7	0.1706	< CCα	Negative	-	-	-	1.04	-	Detected
4	Morteros[a2]		7	0.0343	< CCα	Negative	-	-	-	-	-	Negative
5	Esperanza[a1]		6	0.0839	13 (± 1)[c]	Suspected	13.52	-	-	1.29	-	Detected
6	San Jorge[a1]	Beef calve	7	0.2365	< CCα	Negative	-	-	-	-	-	Negative
7	San Jorge[a1]		7	0.0805	< CCα	Negative	-	-	-	-	-	Negative
8	Esperanza[a1]		7	0.0809	< CCα	Negative	-	-	-	-	-	Detected
9	Selva[a3]		7	0.2976	< CCα	Negative	-	-	-	-	-	Negative
10	Avellaneda[a1]	Poultry farm	5	0.0749	< CCα	Negative	-	-	-	-	-	Negative
11	Avellaneda[a1]		6	0.0124	< CCα	Negative	-	-	-	-	-	Negative
12	Avellaneda[a1]		5	0.0706	8 (± 1)	Suspected	23.53	6.45	1.50	1.97	14.70	Detected
13	Santa Fe[a1]		7	0.0288	< CCα	Negative	-	-	-	-	0.51	Detected
14	Santa Fe[a1]		6	0.0691	< CCα	Negative	-	-	-	-	-	Negative
15	Santa Fe[a1]		7	0.0432	12 (± 3)	Suspected	-	1.33	11.12	-	-	Detected
16	Santa Fe[a1]		6	0.0605	< CCα	Negative	-	-	-	-	-	Negative
17	Valle María[a3]		5	0.0080	13 (± 1)	Suspected	16.19	-	1.34	-	0.92	Negative

[a]Santa Fe (a1), Córdoba (a2) and Santiago del Estero (a3) provinces.

[b]Ciprofloxacin equivalents.

[c]RSD % in parenthesis for N = 3 (Screening fluorescent method).

[d]LOD for each quinolones: 0.35 ng L^{-1} (ENO), 0.34 ng L^{-1} (CIPRO), 0.37 ng L^{-1} (ENRO), 0.34 ng L^{-1} (OFLO) and 0.33 ng L^{-1} (DIFLO).

[e]RSD % in all case < 2 % for N = 3 (UPLC-MS).

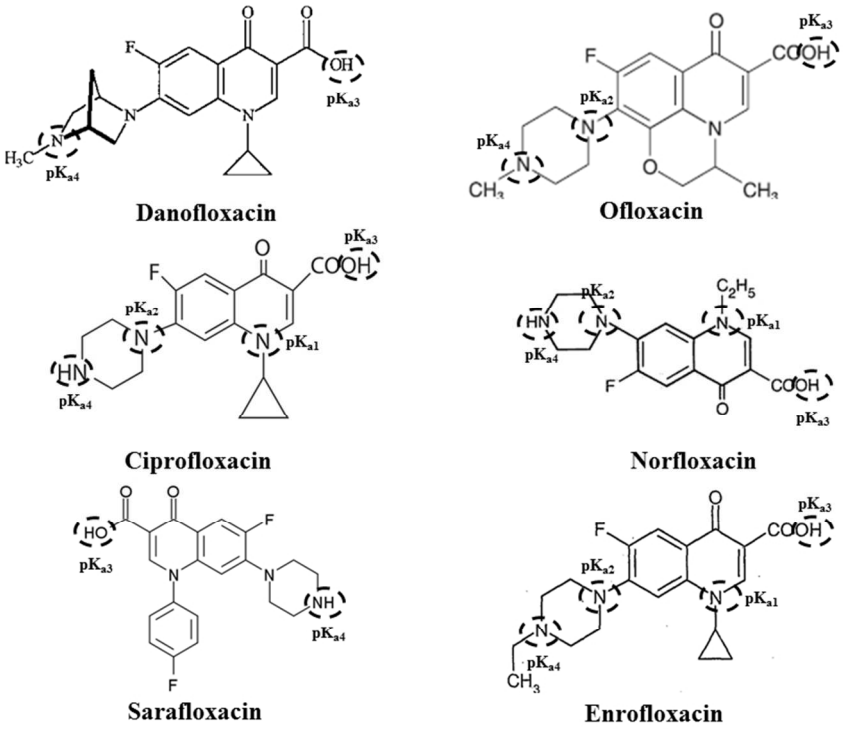

Fig. 1. Structural formulae of the quinolones under study (danofloxacin, ofloxacin, ciprofloxacin, norfloxacin, sarafloxacin and enrofloxacin).

254x190mm (96 x 96 DPI)

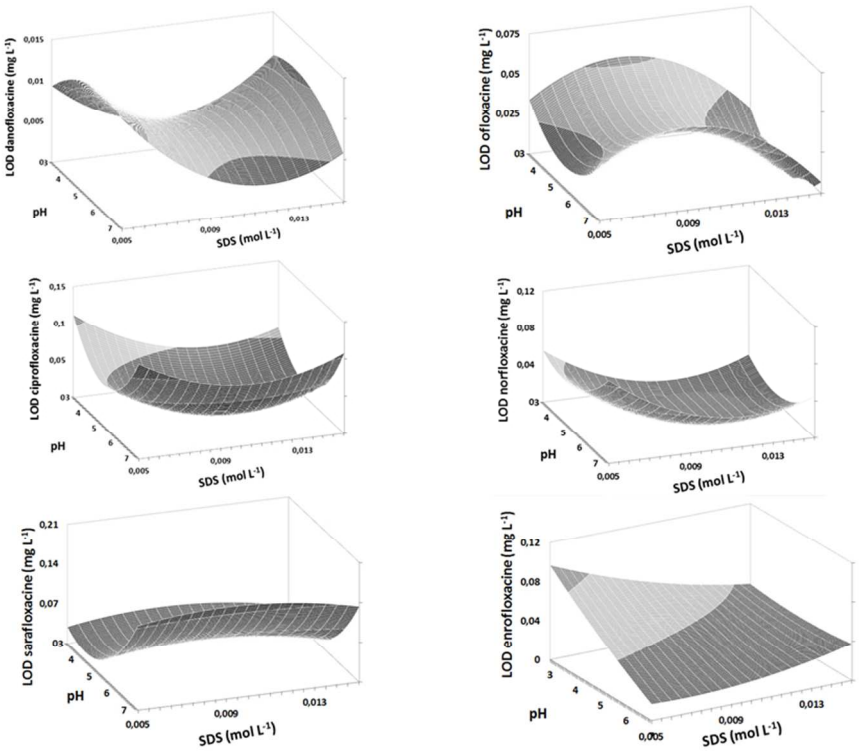

Fig. 2. Response surface showing the effects of SDS concentration and pH on the fluorescence intensity of six FQs.

254x190mm (96 x 96 DPI)

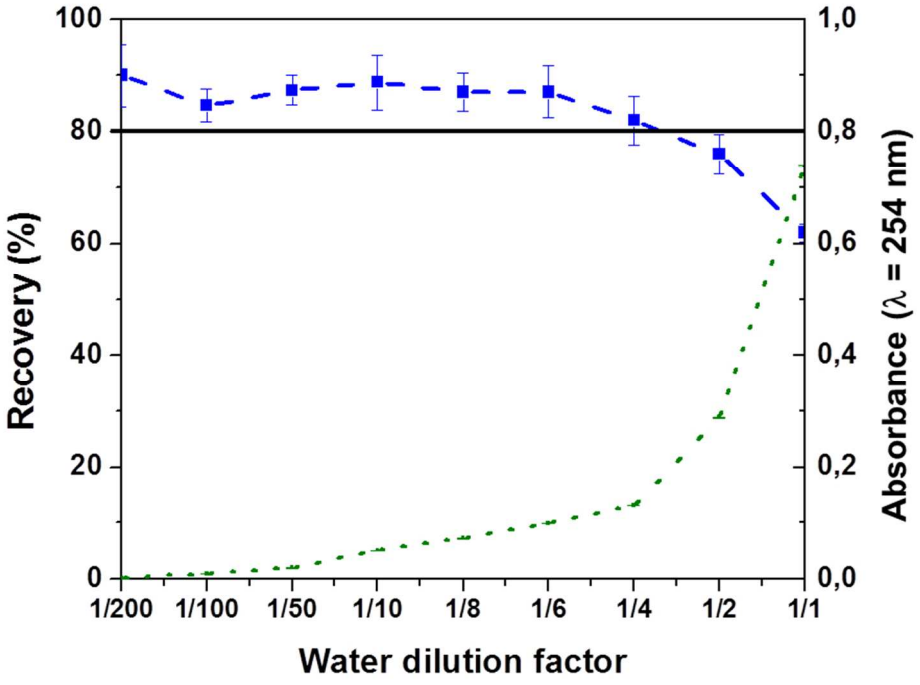

Fig. 3. Recovery of 100 µg L-1 CIPRO and DOM expressed as UV254 nm in water dilution factors. The solid black line represents the acceptable minimal limit of recovery (80%). The dash blue and the dot olive lines are the recoveries and absorbance values (at λ = 254 nm) obtained for each dilution factor. The error bars show the standard deviation for N = 3.

254x190mm (96 x 96 DPI)