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RAPID ULTRASENSITIVE CHEMOMETRICS-FLUORESCENCE METHODOLOGY TO QUANTIFY FLUOROQUINOLONES ANTIBIOTICS RESIDUES IN SURFACE WATER

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A sensitive method for the determination of fluoroquinolones in surface waters at trace concentration level is presented. The proposed two-step methodology consists in a solid-phase extraction using C-18 membranes followed measurement of the emission molecular fluorescence spectra over extracted membrane without elution of the analytes. Membrane background signal was removed by the used of chemometrics calculations, in addition chemometrics was as well used for the direct and simultaneous determination of the studied compounds. The method was optimized for the analysis of three fluoroquinolones: enoxacin (ENO), norfloxacin (NOR) and ofloxacin (OFLO). The fluorescence of these compounds increase drastically when they are into the membrane, thus with this method low concentrations are possible to be determined, as the concentration in which these compounds appear in surface water. Limits of detection at the ng · L^{-1} level were estimated for ENO, NOR and OFLO.

Keywords: fluoroquinolones, emerging contaminants, surface water, chemometrics, fluorescence.

Introduction

Fluoroquinolones are an important group of broad-spectrum synthetic antibacterial agents derived from nalidixic acid, present a potent effect against different gram (-) bacteria [1]. The great advantage of these drugs, in order to activity and spectral characteristics, is due to the presence of a fluorine atom in position six of the quinolonic ring (Fig. 1). The introduction of the fluorinated quinolones represents important therapeutic advantages, because this group of antibiotics shows higher antibacterial activity than parent compounds [2]. They are used to treat human and veterinary diseases and also to prevent diseases in food producing animals [3, 4]. Their main excretion pathway is urinary and low amounts are found in plasma [5].

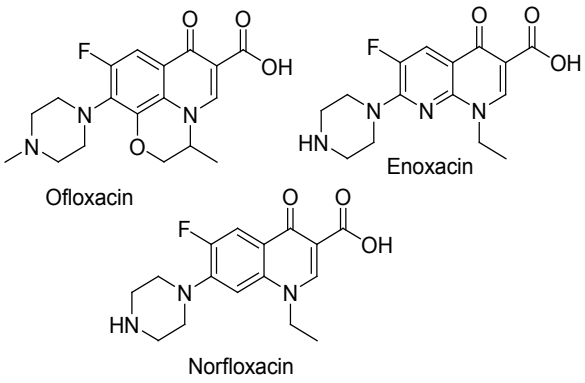

Fig. 1. Structures of the studied analytes.

Urinary or faecal excretions introduce human pharmaceuticals into wastewater. Veterinary drugs result in a direct input to soils and subsequently to groundwater. Moreover, the use of veterinary drugs in fish farming activities leads to their direct entrance into the aquatic environment. Several pharmaceuticals can be degraded in human and veterinary body, but others are excreted in their active forms. In that sense, antibiotics assume special significances due to their occurrence in the environment may contribute to the development of drug resistant bacterial strains. Fluoroquinolones are excreted mostly unchanged [6].

Many pharmaceuticals have been detected (at the low range of nanograms up to micrograms *per litre*) in the environment [7, 8]. That means the procedures for wastewater treatment are not able to completely remove these compounds. Ofloxacin (OFLO) and norfloxacin (NOR) are among the fluoro-

quinolones most frequently detected in wastewater effluents around the world [9 – 11]. Although such low concentrations are probably not active to humans, they are potentially hazardous to bacteria and other micro-organisms. Fluoroquinolones may be directly toxic or be the source of resistant human pathogens representing a possible risk to human health [12].

The importance of this antibiotic group is highlight by the huge numbers of reviews and articles published in the last decade. The studies are performed in different matrices such as biological fluids, pharmaceuticals, feeds and environmental samples [9, 13, 14].

To evaluate the fate of these drug residues and to control the quality of the aquatic medium, sensitive analytical methods are needed. The high fluorescence quantum yield exhibited by quinolones and fluoroquinolones allow us highly sensitivite detection. Among the analytical techniques, Liquid chromatography is the most widely applied for the determination of these compounds and both, UV and fluorescence detection, are usually employed [8, 15 – 18] and, also, mass spectrometry detection [19]. For multi-residue analysis, sample pre-treatments are necessary to extract drugs with different physical/chemical properties. In general, these methods employ solid phase extraction procedures [9].

On the other hand, multivariate techniques have been incorporated to the analytical protocols in the last decade [20]. In particular, full-spectrum multivariate calibration methods offer the advantage of their speed, because the separations steps may be avoided. Analytical methods for determination of fluoroquinolones in several matrices using chemometrics have been reported. Enoxacin (ENO), OFLO and NOR was determined in urine samples by partial least squares (PLS) coupled to emission molecular fluorescence spectra, the limits of detection in urine were 10.0; 0.5 and 0.8 ng · mL^{-1}, respectively [21]; and it was proposed an excitation emission molecular fluorescence technique with PARAFAC calibration for enrofloxacin determination in feeding water from poultry farms; also proposed a spectrofluorimetric method coupled with PARAFAC for danofloxacin determination in milk samples [22], the limit of detection was under maximum residues level (MRL) fixed by European Union [23]. ENO, OFLO and pefloxacin were analyzed in urine samples [24] by synchronous fluorescence spectroscopy with PLS-1 and using excitation emission matrix fluorescence along with N-way partial least squares regression (N-PLS and U-PLS).

All above method has been applied to liquid samples. Solid-phase fluorescence spectroscopy permits the development of fast analytical methods, with minimum consumption of reagents, low costs for analysis and presents good

characteristics of sensitivity and selectivity [25]. Therefore we provide a method with high sensitivity via a simple experimental procedure consisting in a solid phase extraction and then measuring the emission molecular fluorescence spectra over extracted membrane without elution of the analytes. Emission molecular fluorescence spectra recorded were processed with a PLS method.

Experimental

Apparatus and software. Fluorescence measurements were made on a Fluorolog-3 spectrofluorimeter (ISA, JobinYvon-Spex, model FL3-11), equipped with a continuous source (450 W Xenon) for sample excitation.

All calculations were done using MATLAB 7.10 [19]. The routine for PLS-1 used was written following a previously known algorithm [26].

Reagents. All solvents used were of analytical reagent quality. Enoxacin, ofloxacin and norfloxacin were purchased from "Sigma Aldrich" (Madrid, Spain). Standard solutions of each compound (100 μg · mL^{-1}) were prepared by dilution in ethanol (avoiding exposure to direct light and maintained at 4°C). Buffer solution of pH 4 was purchased from "Fisher Scientific" (Madrid), and the C-18 membranes from "Agilent" (Madrid).

Solutions preparation. Standards solution of each compounds were prepared by dilution in ethanol. Working solutions were prepared in pure water, adjusting the pH to 4 adding 400 μl the buffer solution to 10 ml of sample. To keep a minimum ethanol volume ratio in the aqueous solution, highly concentrated stock solutions were used.

Solid phase extraction (SPE). First, the membranes were conditioned with 2 · 1 ml of methanol, 1 · 1 ml of pure water and 1 · 1 ml of the commercial buffer solution (pH 4). Water sample pH was adjusted to pH 4 and 10 ml of sample were percolated through the membrane using a 10 ml glass syringe (Fig. 2). Then 3 · 100 ml of air was percolated through the membrane to dry it. Finally, the fluorescence was measured directly in the solid surface.

Fluorescence measurements. The fluorescence spectra were directly registered from the C-18 membranes after the analyte retention. Membrane disks were appropriated placed in the lab-made membrane disk holder [27]. The direct measurement produces an important increase of the fluorescence signal allowing increasing the sensitivity of the methodology. Fig. 3, shows the significant gain of fluorescence produced when the analytes are retained into the membrane, being the signal in membrane 200 times higher than in solution for ENO, 118 for NOR and 88 for OFLO.

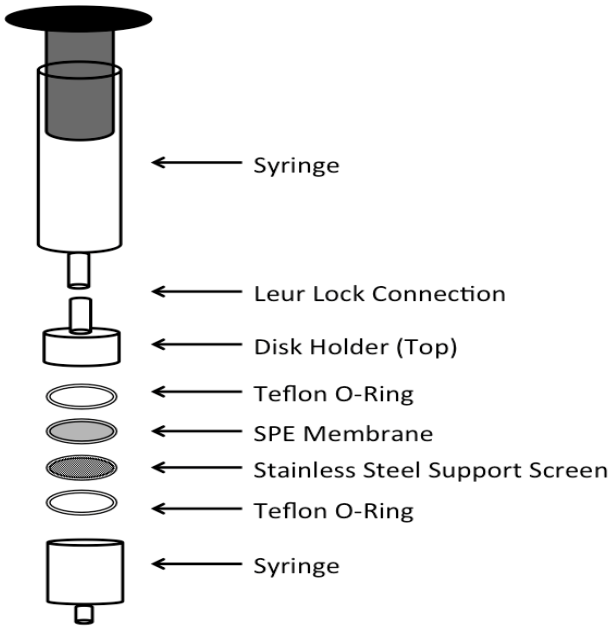

Fig. 2. Membrane solid-phase extraction device.

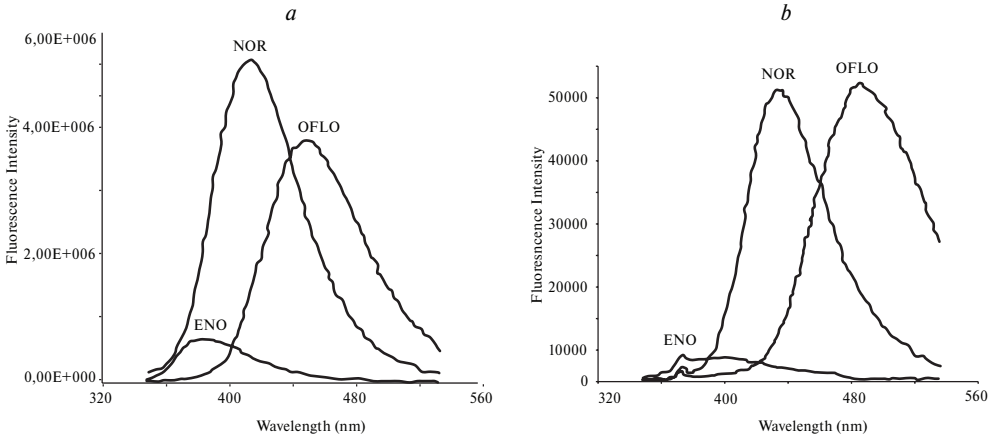

Fig. 3. Emission spectra, for ENO, NOR and OFLO, into the membrane (a), and in water solution (b), in the same concentration and instrumentals conditions.

Results and discussion

SPE and membrane retentions. Firstly, the retention capacity of the selected membranes was studied based on the amount of analyte effectively retained in the membrane. 10 different fluoroquinolones solutions in the range of norfloxacin, 0.0 – 80.0 ng · L^{-1}, enoxacin, 0.0 – 200.0 ng · L^{-1}, and ofloxacin,

0.0 – 80.0 ng · L^{-1} were prepared as described above and extracted according to the SPE methodology. Fluorescence signal was registered before and after of sample percolation through the extraction system, and the membrane retention efficiency was calculated by comparing the signals obtained before and after. Table 1 summarized the results obtained in this experiment. The recovery values were from 82 to 94% for the different drugs.

*Table 1. Retentions of enoxacin, norfloxacin and ofloxacin into the C-18 membrane**

Analyte	$\lambda_{exc}/\lambda_{em}$ **, nm	Retentions, %***
Enoxacin	347/386	88.5 ± 1.4
Norfloxacin	332/419	94.3 ± 0.6
Ofloxacin	340/460	82.8 ± 0.2

* Ten millilitres of water sample was used; ** fluorescence excitation (λ_{exc}) and emission (λ_{em}) maxima; *** retention was calculated based on fluorescence intensity before and after extraction.

PLS calibration. The PLS-1 method involves a calibration step in which the relation between bi-dimensional emission spectra and analyte concentrations is estimated from a set of reference samples, and a prediction step in which the results of the calibration are used to estimate the component concentrations in unknown samples.

A 15 samples set was built for calibration with the PLS-1 model. The analyte concentrations corresponded to a central composite design, formed by a three-component full-factorial design at two levels (i.e. (2^3) 8 samples, a central point (one sample), and a star design (2x3) 6 samples), making a total of 15 samples. The extreme concentrations for the design were as follows: norfloxacin 0.0 – 80.0, enoxacin – 0.0 – 200.0, and ofloxacin – 0.0 – 80.0 ng · L^{-1}. The membrane background was important, so the model was modified in order to obtain better results, introducing 10 blank samples. The total number the samples for the calibrations was 25; 15 of them corresponds to the central composite design and the other 10 to the blank solutions.

In order to determine the correct number of loading vectors to be used for the modeling of the data, a cross-validation calculation for all the samples in the training set, was performed to calculate the prediction residual error sum of squares (PRESS). The selection of the number of factors, to be use in the calibration with the PLS algorithm, is very important in order to achieve the best predictions for to model the system without overfitting the concentration data. To select the optimum number of factors, the criterion proposed by Haaland and Thomas was used [26].

The optimum wavelength ranges were 350 – 550 nm for ENO, 395 – 445 nm for NOR and 350 – 550 nm for OFLO, using 4 factors in the three cases. On the other hand, determination coefficients R^2 of 0.9568, 0.9167 and 0.92491 were calculated for ENO, NOR and OFLO. The poorest results were obtained for NOR (Table 2).

Table 2. Optimum number of factors and calibration statistical parameters by applying PLS algorithm to resolve the mixture enoxacin, norfloxacin and ofloxacin in C-18 membrane using the emission spectra (λ_{exc} = 334 nm) in natural water samples

Statistical Parameter	Enoxacin	Norfloxacin	Ofloxacin
Range (nm)	350 – 550	395 – 455	350 – 550
FACTORS	4	4	4
PRESS	$3.604 \cdot 10^3$	$1.366 \cdot 10^3$	$8.997 \cdot 10^2$
R^2	0.9568	0.9167	0.92941

The emission spectra were registered between 350 – 550 nm each 0.5 nm, and the excitation wavelength was 334 nm. The emission and excitation slits were fixed at 1.2 nm (Fig. 4).

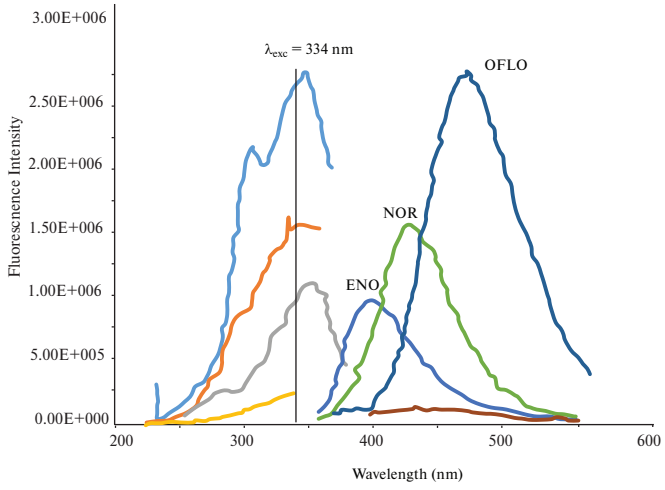

Fig. 4. Excitation and emission spectra in membrane of ENO (1.0 μg/mL), NOR (1.0 μg/mL), OFLO (1.0 μg/mL) and the blank signal.

Analytical figures of merit. The selectivity (SEL), sensitivity (SEN) and limit of determination of an analytical method (named figures of merit) to determine the quality of an analytical technique can be calculated. Selectivity can be expressed as:

$$\mathrm{SEL}_K = \frac{1}{\| b_k \| \| s_K \|} = \frac{\| \mathrm{NAS}_K \|}{\| s_K \|}.$$

Ranging from 0 to 1 is a measure of how unique the spectrum of the analyte of interest is compared with other species. A value of 0, means that analysis is impossible because the analyte spectrum is equal to a linear combination of the interference spectra. A value of 1 indicates that the interferences do not interfere. The part of the signal that relates uniquely to the analyte of interest is more important than the total signal (s_k). This unique signal, is named the net analyte signal (NAS) and is a vector related to the regression vector *b*. NAS_k designates the square root of the sum of each element in the *b*.

Sensitivity is expressed as:

$$\mathrm{SEN} = \frac{1}{\| b_k \|} = \| \mathrm{NAS}_k \|.$$

And it is proportional to the regression vector, because the inverse calibration model is used. The units of sensitivity are signal/concentration.

Other possibilities are to calculate the limit of detection (LOD) expressed as:

$$\mathrm{LOD} = 3 \cdot \| \varepsilon \| \cdot \| b_K \|,$$

being $||\varepsilon||$ the instrumental noise, and it can be calculated from blank signals.

Table 3 summarized the quality parameters calculated as described above. The limits of detection were between 11.0 and 78.1 ng/L. The highest values were obtained by the ENO. However, these LODs are adequate for the determination of these three antibiotics compounds in surface water. For the instrumental noise determination, 10 blank samples were used.

Table 3. Figures of merit of the analytical method (limit of detection, selectivity and sensibility)

Figure of merit	Enoxacin	Norfloxacin	Ofloxacin
LOD*	78.1	15.5	11.0
SEL	0.1915	0.3211	1.359
SEN**	1.955 · 104	3.279 · 104	1.387 · 105

*ng/L; ** FU · L · ng^{-1}.

Analysis of ENO, NOR and OFLO in surface water. The optimized method was used to simultaneously determine the concentration of ENO, NOR and OFLO from lake water (Orlando, Florida, USA). Water samples were collecting in different point of the lake using appropriate Teflon flasks. Samples were filtered and submitted to the analytical procedure. In all cases, no positive results were obtained, and therefore samples were spiked with different quantities of ENO, NOR and OLFO in the range of 20 – 150 ng/L. After the drug extraction from spiked water, fluorescence was measured from membranes and the PLS method was applied to quantify the fluoroquinolones contents. Table 4 summarized the results obtained from the different drug and concentration level.

Table 4. Recovery of ENO, NOR and OFLO added to natural water by PLS-1 calibration

Analyte	Added (ng · L^{-1})	Found (ng · L^{-1}) ± R.S.D*	% Recovery ± R.S.D*
ENO	150	148.9 ± 4.7	99.7 ± 3.1
	80	70.6 ± 2.9	88.8 ± 4.1
	70	64.9 ± 1.4	92.7 ±1.9
NOR	40	36.3 ± 1.5	90.7 ± 4.0
	20	15.0 ± 2.1	75.2 ± 1.9
	30	24.7 ± 2.3	82.3 ± 4.2
OFLO	40	45.3 ± 0.8	113 ± 2.0
	20	21.0 ± 2.0	105 ± 4.1
	40	42.5 ± 0.9	106 ± 2.3

* Mean of three determinations and R.S.D.: relative standard deviation.

Conclusions

A fast and accurate procedure was proposed to determine fluoroquinolones drugs in superficial waters. The high sensitivity obtained by combining

a solid-phase extraction using C-18 membranes followed measurement of the emission molecular fluorescence spectra over extracted membrane without elution of the analytes. PLS chemometric analysis was a practical alternative for the simultaneous determination of the studied drugs without further separation. In addition, with the aid of chemometrics the background signal was adequately removed from the signal. The method was optimized for the analysis of three fluoroquinolones: enoxacin, norfloxacin and ofloxacin. The direct fluorescence measurement over the C-18 extraction membrane increased drastically, increasing notoriously the method sensitivity. The calculated LOD for the three drugs were in the range of ng/L and therefore this method could be feasible to determine these drugs as emerging pollutant in superficial waters.

Acknowledgments

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References

[1] *Hooper D.C., Wolfson J.S.* // Quinolone Antimicrobial Agents. – [2nd ed]. – Washington: American Society for Microbiology, 1993.

[2] *Jackson L.C., Machado L.A., Hamilton M.L.* // Acta Medica,1998, **8**, P. 58 – 65.

[3] *Currie D., Lynas L., Kennedy D.G., McCaughey W.J.* // Food Additives and Contaminants, 1998, **15**, P. 651 – 660.

[4] *Ihrke P.J., Papich M.G., Demanuelle T.C.* // Veterinary Dermatol., 1999, **10**, P. 193 – 204.

[5] *Knapp J.S., Fox K.K., Tress D.L., Whittington W.L.* // Emerging Infectious Diseases, 1997, **3**, P. 33 – 39.

[6] *Espinosa Mansilla A., Muñoz de la Peña A., González Gómez D., Cañada Cañada F.* // J. Separ. Sci., 2006, **29**, P. 1969 – 1976.

[7] *Reemtsma T.* Organic Pollutant in the Water Cycle. – Weinheim: Wiley-VCH, 2006.

[8] *Sacher F., Lange F.T., Heinza-Jürgen B., Blankenhorn I.* // J. Chromatogr, A, 2001, **938**, P. 199 – 210.

[9] *Speltini A., Sturini M., Maraschi F., Profumo A.* // J. Separ. Sci., 2010, **33**, P. 1115 – 1131.

[10] *Xu W.H., Zhang G., Li X.D., Zou S.C., Li P., Hu Z.H., Li J.* // Water Res., 2007, **41**, P. 4526 – 4534.

[11] *Gao L., Shi Y., Li W., Niu H., Liu J., Cai Y.* // Chemosphere, 2012, **86**, P. 665 – 671.

[12] *Sturini M., Speltini A., Maraschi F., Pretali L., Profumo A., Fasani E., Albini A., Migliavacca R., Nucleo E.* // Water Res., 2012, **46**, P. 5575 – 5582.

[13] *Samanidou V., Evaggelia F., Evaggelopoulou N.* // J. Separ. Sci., 2007, **30**, P. 2549 – 2569.

[14] *Sousa J., Alves G., Fortuna A., Falcão A.* // Anal. and Bioanal. Chem., 2012, **403**, P. 93 – 129.

[15] *Cañada-Cañada F., Arancibia J.A., Escandar G.M., Ibañez G.A., Espinosa-Mansilla A., Muñoz De La Peña A., Olivieri A.C.* // J. Chromatogr., A, 2009, **1216**, P. 4898 – 4876.

[16] *Parrilla Vázquez M.M., Parrilla Vázquez P., Martínez Galera M., Gil García M.D.* // Anal. Chim. Acta, 2012, **748**, P. 20 – 27.

[17] *Poliwoda A., Krzyzak M., Wieczorek P.P.* // J. Chromatogr., A, 2010, **1217**, P. 3590 – 3597.

[18] *Wammer K., Korte A.R., Lundeen R.A., Sundberg J.E., McNeill K., William A.A.* // Water Res., 2013, **47**, P. 439 – 448.

[19] *Castiglioni S., Bagnati R., Calamari D., Fanelli R., Zuccato E.* // J. Chromatogr., A, 2005, **1092**, P. 206 – 215.

[20] *Olivieri A.C.* // Anal. Methods, 2012, **7**, P. 1876 – 1886.

[21] *Espinosa-Mansilla A., Muñoz De La Peña A., Salinas F., González-Gómez D.* // Talanta, 2004, **62**, P. 853 – 860.

[22] *Cañada-Cañada F., Espinosa-Mansilla A., Muñoz De La Peña A., Jiménez Girón A., González-Gómez D.* // Food Chem., 2006, **113**, P. 1260 – 1265.

[23] *Commission* Regulation (EC) No. 508/1999 of 4 March, amending Annexes I to IV to Council Regulation (EEC) No. 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. // Official J. – L 060, 09/03/1999, 16

[24] *Murillo Pulgarín J.A., Alañón A., Boras N.* // Spectrochim. Acta, A, 2012, **98**, P. 190 – 198.

[25] *Alves J.C.L., Poppi R.J.* // Anal. Chim. Acta, 2009, **642**, P. 612 – 616.

[26] *Haaland D.M., Thomas E.V.* // Anal. Chem., 1998, **60**, P. 1193 – 1202.

[27] *Escandar G.M., González-Gómez D., Espinosa-Mansilla A., Muñoz de la Peña A., Goicohechea H.C.* // Anal. Chim. Acta, 2004, **506**, P. 161 – 170.

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