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Photo-induced fluorescence of magnesium derivatives of tetracycline antibiotics in wastewater samples

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ABSTRACT

An analytical strategy, for the determination of tetracyclines (TCs), based on a HPLC system coupled with a photo-reactor followed by post-column derivatization was developed. Higher fluorescence emission after coupling the resulting photo-fragments with magnesium ions was observed for the determination of minocycline (MC), epitetracycline (ETC), tetracycline (TC) and doxycycline (DC). The manifold included a HPLC system with a photo-reactor (PTFE tubing helically coiled around a low-pressure mercury lamp), a mixing T-piece and a fluorescence detector. The derivatization reagent was delivered at 0.5 mL min^{-1} by a pump.

After HPLC separation using a gradient system with a mobile phase containing oxalic acid 0.02 M and acetonitrile, TCs were irradiated for 60 s, and the resulting photo-fragments were mixed with the post-column derivatization reagent, and the magnesium derivatives of TCs were detected by fluorimetry (λ_{exc} 386 nm, λ_{em} 500 nm).

The results obtained showed a significant increase of sensitivity due to photodegration of TCs, 45.4%, 37.6% and 25.3% for MC, TC and ETC respectively. For DC an increase of only 1.5% was observed.

The developed method was successfully applied to TCs determination in hospital and municipal wastewater samples using solid phase extraction with Oasis HLB cartridges.

The LOQs were 0.25, 0.15, 01 and 0.25 μg L^{-1} for TC, ETC, MC and DC, respectively. The recovery values oscillated between 107.1% and 92.4% for fortification of 2.5 μg L^{-1} of each antibiotic.

1. Introduction

The issue of pharmaceuticals and their metabolites in the aquatic environment has raised increasing concern in recent years. There is an increasing scientific interest on knowing the consequences for the ecosystem and public health caused by their presence in the environment. Most drugs are designed to be persistent, so that they retain their chemical structure long enough to do their therapeutic work and this, coupled with their continual input, may enable them to remain in the environment for a significant period of time [1].

Antibiotics are among the emerging contaminants in water because of the concern of their potential adverse effects on human health, mainly due to the increased bacterial resistance on the ecosystem. They have been found in a wide range of environmental samples including surface water, groundwater, wastewater and drinking water [2–4].

There is still a lack of fundamental data on the occurrence, fate and effects of antimicrobials in the environment needed for proper risk assessment and risk management both for humans and the environment. This is probably the most relevant reason for the need of sensitive methods of analysis for antibiotics in environmental waters.

Tetracyclines (TCs) are a group of broad-spectrum antibacterial compounds which has afforded the medical profession a number of powerful antibiotics active against a wide of human and animal pathogens.

The analytical methodology usually applied to TCs analysis in surface water samples is based on multiresidue determination, together with other antibiotics or even other pharmaceuticals [5]. Liquid chromatography (LC) has become the technique of choice for multiclass analysis, especially when coupled to mass spectrometry (LC–MS) and tandem MS (LC–MS–MS). However, due to the complexity of the matrix, in most cases an extraction step for sample clean-up and preconcentration is required before analysis in order to achieve the required sensitivity [6]. Capillary electrophoresis has also been reported for the determination of antibiotics in environmental samples [7].

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Solid phase extraction coupled with LC–FD usually allows selectivity and sensitivity for monitoring TCs in the aquatic environment, representing a simple, robust and more economic alternative than MS based methods [5].

TCs have no native fluorescence in solvents such as methanol, acetonitrile or in their aqueous binary mixtures, but during UV irradiation they can be photolyzed into fluorescent photoproducts, whose fluorescence intensity increases quickly with the irradiation time [8].

Photochemically induced fluorimetry (PIF), based on the conversion upon UV irradiation of non-fluorescent analytes into strongly fluorescent photoproducts, is a recent approach applied to pharmaceutical quantitative analysis. Generally, PIF methods are considered as efficient fluorophore-generating systems for stationary media [9–10] as well as for flowing devices such as HPLC post-column photoreaction [11–14] or FIA [15–18]. Among the various parameters controlling the analyte conversion photoreaction and method sensitivity two are particularly important namely, the optimum UV irradiation time (time of irradiation optimum, corresponding to the maximum PIF signal) and the type of solvent [19]. Time of irradiation optimum and PIF intensity values vary significantly with the solvent polarity and its protic or aprotic character. For analytical purposes, the solvent selected should be the one giving the shortest time of irradiation optimum and the largest PIF signal.

This study deals with an analytical strategy based on coupling a HPLC system with a photo-reactor and post-column derivatization of TCs with magnesium ions. In this work, we observed that fluorescence emission was higher for minocycline (MC), epitetracyclie (ETC) and tetracycline (TC). For DC only an insignificant increase was observed.

A simple and low cost analytical methodology, representing an important tool to achieve sensitive, specificity and accuracy, is proposed for routine analysis of TCs residues in hospital and municipal wastewaters.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of Gilson Model 321, 305 and 307 pumps (Gilson, Medical Electronics, Villiers-le-Bel, France) and a Model 7725 loop injector (Rheodyne, Cotati, CA) with a 100 μL loop. The fluorescence detector was a Model LC 305 instrument (LabAlliance, LabAlliance, State College, PA) operating at an excitation wavelength of 386 nm and an emission wavelength of 500 nm. The spectral bandwidth was 5 nm for both excitation and emission. The results were recorded on a Model 3390 integrator (Hewlett-Packard, Philadelphia, PA) and a Unipoint Gilson data system (Gilson, Medical Electronics, Villiers-le-Bel, France). The HPLC column used was a Hichrom Lichrosorb RP8-10 (250 mm × 4.0 mm) (Hichrom Limited, Reading, United Kingdom). The mixing T for derivatization reaction was directly coupled through a 30 cm (0.25 mm i.d.) tube to the detector. The reaction temperature was about 22 °C.

2.2. Reagents and preparation of standard solutions

All the solvents were HPLC grade and were purchased from Carlo Erba (Italy). Oxalic acid, magnesium acetate, boric acid, potassium hydroxide, sodium hydroxide and Na_2–EDTA were analytical reagent grade chemical obtained from Merck (Germany). MC, TC and doxycycline (DC) were obtained from Sigma Chemical (Spain). ETC was purchased from European Pharmacopoeia.

Table 1
HPLC gradient programme used for determination of TCs.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	90	10
7.0	60	40
7.5	50	50
12.95	90	10
13	End run	

Double-distilled water was produced by distillation and passage through Milli-Q system (Millipore, Ireland). The water was filtered through a 0.45 μm filter (Millipore, Ireland) under vacuum and degassed by ultrasonication.

Solid phase extraction (SPE) cartridges, Oasis HLB 6 cm^3/200 mg, were purchased from Waters Corporation (United States).

Individual stock standard solutions were prepared at 1 mg mL^{-1} for MC, ETC and DC and at 2 mg mL^{-1} for TC, and were stored at 4 °C in brown glass vials for a maximum period of one month. The working solutions were a mixture of the four compounds prepared by serial dilutions of the stocks and were stored in brown glass vials at 4 °C. These solutions were prepared immediately before use.

The post-column reagent was prepared daily by dissolving 6.0 g of magnesium acetate, 6.1 g of boric acid and 2.5 g of potassium hydroxide in 950 mL of distilled water. Then the pH value was adjusted to 9.0 with 1 M sodium hydroxide solution and the final volume make up to 1 L. It is important to follow the order of addition of these reagents as precipitations may occur. This solution was filtered through a 0.45 μm filter under vacuum, degassed by ultrasonication and held in amber glass bottle [20].

The mobile phase and post-column reagent were filtered through a 0.45 μm filter under vacuum, degassed by ultrasonication.

2.3. Stability studies

To investigate the presence of possible degradation products, stability studies of standard solutions were performed in solvent (standard solutions) and in matrix (sample purified extracts).

2.4. Chromatographic conditions and detection settings

An analytical method based on a HPLC system coupled with a photo-reactor followed by post-column derivatization was developed. The manifold included a HPLC system with a photo-reactor (PTFE tubing helically coiled around a low-pressure mercury lamp), a mixing T-piece and a fluorescence detector. The derivatization reagent was delivered at 0.5 mL min^{-1} by a pump (Fig. 1).

The mobile phases used for analysis consisted of aqueous oxalic acid solution 0.02 M (mobile phase A) and acetonitrile (mobile phase B). The mobile phase flow was 1 mL min^{-1} and the gradient conditions are shown in Table 1. Fig. 2 shows the HPLC–FD chromatogram of the standard solution of MC, ETC, TC and DC at 0.1, 0.05, 0.25 and 0.5 μg L^{-1}, respectively. Mean retention times were for MC, ETC, TC and DC 5.89, 7.63, 8.25 and 10.51 min, respectively.

The post-column reagent was prepared daily. It is important to follow the order of addition of these reagents, because precipitations may occur. This reagent was mixed with the photo-fragments in a T-piece positioned between the low-pressure mercury lamp and the detector.

The wavelengths of excitation and emission were optimized to provide comparable fluorescence intensity for every tetracycline. The spectra for each compound was obtained and the absorption observed for values above 300 nm was associated with the fluorophore formed by the ionized phenolic group b-diketone. Based on these values, the fluorimetric detection of MIN, ETC, TC and DC

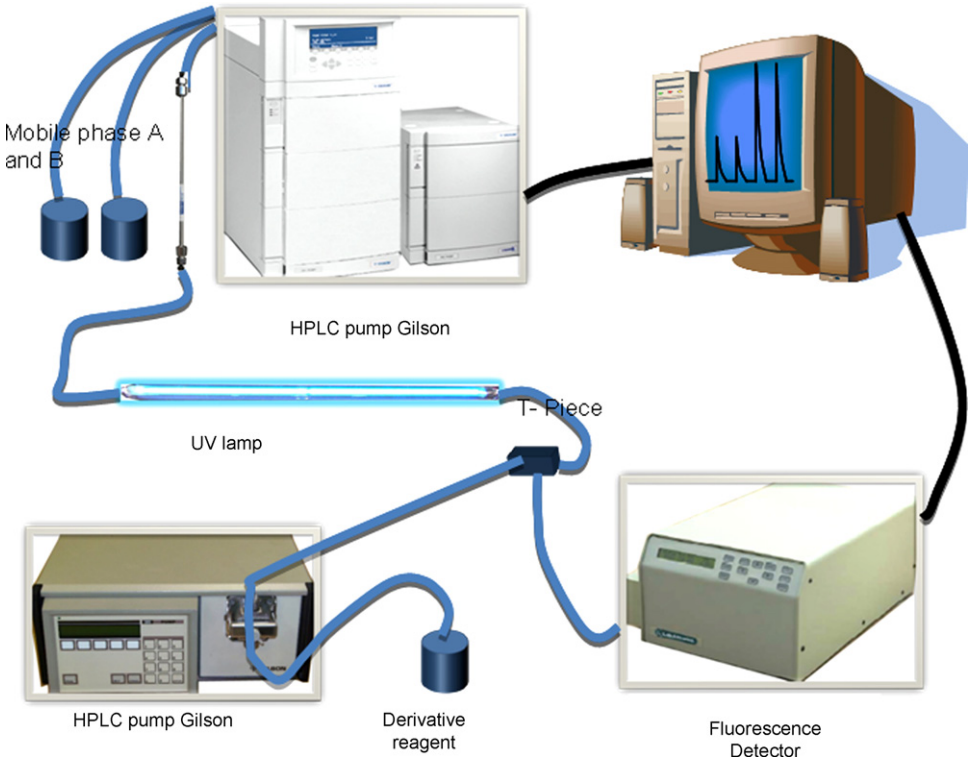

Fig. 1. Scheme of the HPLC-PIF method.

was established for the wavelengths of excitation and emission of 386 and 500 nm, respectively.

2.5. Sample collection and preparation

A total of twelve 24-h composite wastewater samples obtained from the four hospitals located in Coimbra city, Portugal, and from the influent and effluent of the local wastewater treatment plant (WWTP) were collected during the autumn season of 2007.

During transportation wastewater samples were kept in amber glass bottles in a cooler with ice. After delivery to the laboratory, samples were filtered with 0.45 μm glass fibre filter to remove the suspended matter and stored in the dark at 4 °C until extraction, which occurred within two days.

2.6. Sample extraction and clean-up

Wastewater samples were percolated through Oasis HLB 200 mg SPE cartridges. The cartridge was previously conditioned with 5 mL of methanol, 5 mL of deionized water and 5 mL of formic acid buffer at pH 3.4. Wastewater samples were added of 3 mL of Na_2–EDTA and pH adjusted to pH 3.0. Then 250 mL of sample were passed through the cartridge, following a wash step with 10 mL of a mixture of water:methanol (95:5). The elution of the antibiotics was performed with a mixture of methanol acidified with trifluoroacetic acid 1%.

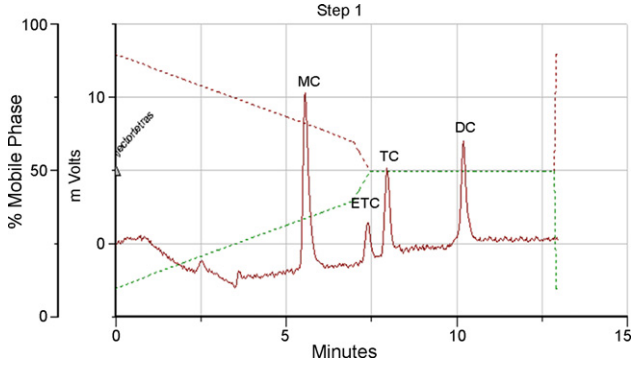

Fig. 2. HPLC–FD chromatogram of the standard solution of MC, ETC, TC and DC at 0.1, 0.05, 0.25 and 0.5 $\mu g\,L^{-1}$, respectively. Mean retention times were for MC, ETC, TC and DC 5.89, 7.63, 8.25 and 10.51 min, respectively.

3. Results and discussion

3.1. Study of photodegradation of the TCs

PIF main advantages are as follows: (i) the use of photons for analyte conversion instead of a chemical reagent does not require a mixing system, and therefore, the analyte must not be diluted; (ii) since most photochemical reactions take place *via* free radicals, the reactions rates are generally fast, resulting in short conversion times; (iii) the reaction takes place at room temperature in opposite to the higher temperature required in thermally initiated derivatization; (iv) the technique requires low cost equipment and is suitable to various experimental conditions.

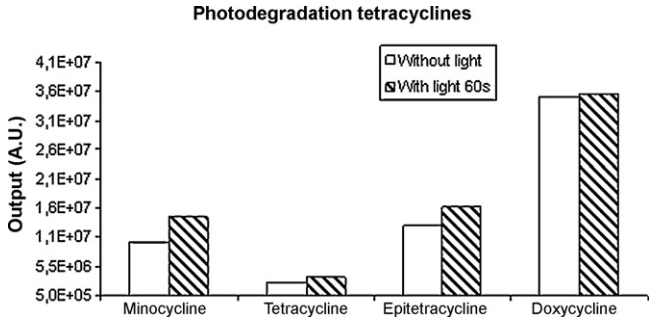

Fig. 3. .Results of the sensitivity increase obtained by TCs photodegradation.

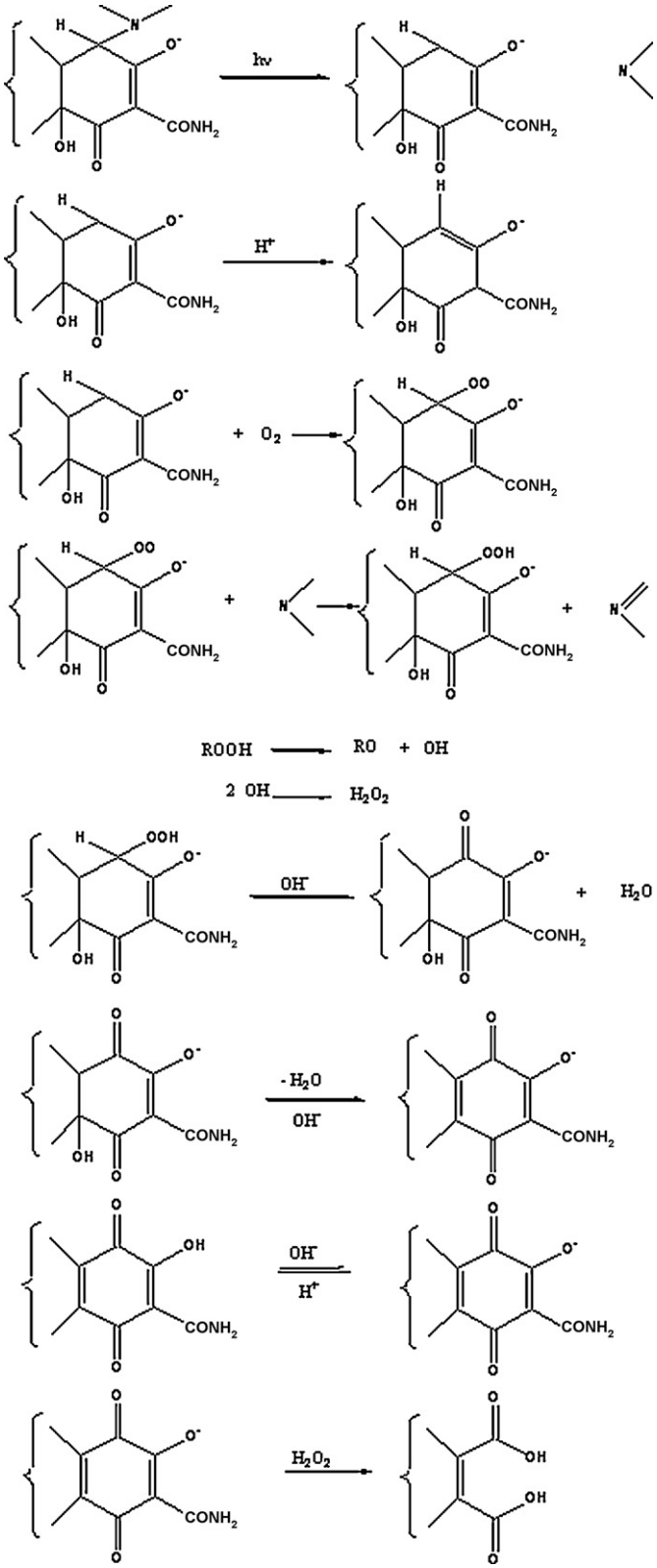

Fig. 4. Photodegradation pathway for the tetracyclines [21].

The study of photodegradation of the different TCs was carried out with a photo-reactor, which consisted of a 150 cm length and 0.3 mm internal diameter PTFE tubing helically coiled around a 30 W low-pressure mercury lamp (Philips TUV 30W G30T8 UV-C long life) for germicidal use. The photo-reactor length allowed the

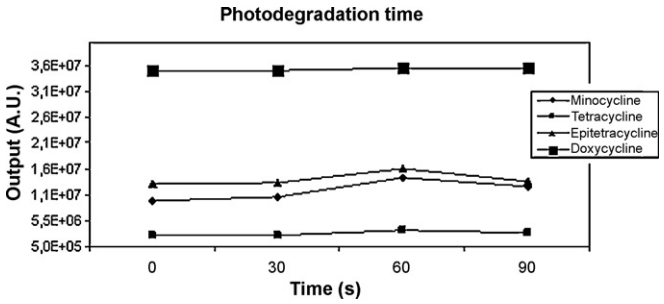

Fig. 5. Results obtained by photodegradation of TCs along the tested time range (0–90 s).

photodegradation of TCs during 60 s. The obtained results show an increase in fluorescence emission after irradiation, 45.4%, 37.6% and 25.3% for MC, TC and ETC respectively. For DC an insignificant increase of 1.5% was observed and thus no significant advantage over the method without irradiation was achieved for this compound (Fig. 3).

One possible photodegradation pathway for TC [21] is represented in Fig. 4. TC shows a photodegradation product more fluorescent than the TC itself, [20], this fact was confirmed by this work. Moreover, as shown by the obtained results the same occurred for MC and ETC. For DC the increase was not statistically significant.

3.2. Study of photodegradation time

This parameter can be controlled by modifying the length of the helically coiled PTFE tubing around the lamp and the chromatographic flow (1 mL min^{-1}). However, this last parameter could not be changed, since this flow rate was found to be optimum for chromatographic resolution. The tested interval, from 0 to 90 s, was controlled by changing the PTFE tube length. Results are shown in Fig. 5.

Sixty seconds allowed, for every antibiotic, the highest fluorescence increase, for which it was the selected interval for further experiments. The obtained results show a higher increase in fluorescence emission after irradiation for MC, TC and ETC comparatively to DC.

3.3. Method validation

In the validation procedure of the analytical method the following criteria were considered: sensitivity; linearity; recovery; precision; and evaluation of the matrix effect.

3.3.1. Linearity

The calibration curves were prepared using linear regression analysis and gave good fits over the concentration range studied for each antibiotic as shown in the Table 2.

The mean regression coefficients (R^2) were 0.9953 for MC, 0.9913 for ETC, 0.9944 for TC and 0.9987 for DC.

3.3.2. Stability studies

The stability of standard solutions and of sample extracts was evaluated. The stock standard solutions were stored at −20 °C and analysed during a one-month period, and the working standard solutions were stored at 4 °C and analysed during a one-week period. For the period of study we did not observe any degradation of TCs. The stability of TCs during sample storage at −20 °C was tested during one week, and no degradation was observed.

Table 2
Analytical figures of merit for the determination of TCs.

Antibiotic	LOQ ($\mu g\,L^{-1}$)	Linear range ($\mu g\,L^{-1}$–$mg\,L^{-1}$) (R^2)	Precision intra-day ($n=5$)[a] (%)	Precision inter-day ($n=5$)[a] (%)
Minocycline	0.25	0.25–1 (0.9953)	4.5	6.1
Epitetracycline	0.15	0.15–1 (0.9913)	4.3	5.8
Tetracycline	0.10	0.10–2 (0.9944)	0.7	1.5
Doxycycline	0.25	0.25–0.5 (0.9987)	2.1	1.9

[a] $5\,\mu g\,L^{-1}$.

Table 3
Accuracy and precision validation results obtained through fortification of wastewater samples.

Antibiotic	Levels of fortification ($\mu g\,L^{-1}$)	Recovery (%)	Precision intra-day (%RSD)[a]	Precision inter-day (%RSD)[b]
Minocycline	2.5	97.0	2.7	4.0
	5.0	95.3	2.1	3.0
Epitetracycline	2.5	92.4	1.3	2.7
	5.0	82.1	1.8	1.7
Tetracycline	2.5	102.0	5.5	7.1
	5.0	102.6	1.9	1.2
Doxycycline	2.5	107.1	2.7	3.3
	5.0	94.7	9.4	5.5

[a] ($n=3$).
[b] ($n=9$).

Table 4
Concentrations of antibiotic residues detected in the wastewater samples analysed.

Sample[a] Antibiotic	Hospital 1 ($\mu g\,L^{-1}$) (RDS, %)	Hospital 2 ($\mu g\,L^{-1}$) (RDS, %)	Hospital 3 ($\mu g\,L^{-1}$) (RDS, %)	Hospital 4 ($\mu g\,L^{-1}$) (RDS, %)	ETAR influent ($\mu g\,L^{-1}$) (RDS, %)	ETAR effluent ($\mu g\,L^{-1}$) (RDS, %)
Minocycline	n.d.	n.d.	n.d.	n.d.	350 (1.2%)	n.d.
Epitetracycline	n.d.	18.9 (5.8%)	n.d.	n.d.	n.d.	n.d.
Tetracycline	42.2 (0.8%)	54.7 (2.2%)	n.d.	23.2 (3.2%)	n.d.	n.d.
Doxycycline	8.1 (3.1%)	n.d.	0.25 (9.8%)	n.d.	n.d.	n.d.

[a] ($n=3$).

3.3.3. Specificity

In order to assess the specificity of the method, the presence of potential interfering substances around the retention time of TCs in wastewater blank samples ($n=6$), was evaluated. No interferences were observed in the region of interest where the TCs were eluted.

In the present study, matrix effect was evaluated by comparison of the detector responses from TCs standard solutions in solvent with those from different Oasis HLB purified matrix extracts added of TCs at different concentration levels. From the calculated matrix effect results, it could be concluded, that the matrix effect for TCs is not significant. Therefore the proposed analytical methodology is suitable for analysis of trace levels of TCs in wastewaters.

3.3.4. Limit of quantification, accuracy and precision

LOQ and chromatographic method precision results are shown in the Table 2. Repeatability was evaluated by analysis of 5 consecutive injections (intra-day precision) of the same solution containing $5\,\mu g\,L^{-1}$ of each antibiotic. The same experiment was repeated on 5 different days with freshly prepared solutions (inter-day precision).

Table 3 summarises accuracy and precision results of the analytical method, obtained for the two fortification levels, 2.5 and $5\,\mu g\,L^{-1}$, carried out by adding each antibiotic to the wastewater samples.

As can be seen, recovery values ranged from 92.4% to 107.1% and from 82.1% to 102.6% for fortification of 2.5 and $5.0\,\mu g\,L^{-1}$, respectively showing good accuracy. For the two fortification levels, the precision was calculated through intra-day precision ($n=9$) and inter-day precision (3 non-consecutives days), with RSDs lower than 9.4% for intra-day repeatability and lower than 5.5% for inter-day precision, demonstrating good method precision.

3.4. Application to real samples

The optimized analytical method was applied to wastewaters samples collected from 4 hospitals and from influent and effluent WWTP and the results obtained are presented in the next table (Table 4).

Differences were observed in wastewaters from the different hospitals. MC was not present in any hospital, TC was detected in three hospitals, two hospitals contained DC and in one only TC and its epimer were detected. The concentrations ranged from 23.2 and $54.7\,\mu g\,L^{-1}$ for TC and from 0.25 and $8.1\,\mu g\,L^{-1}$ for DC. ETC, was also present in one sample at a concentration of $9.4\,\mu g\,L^{-1}$, indicating TC degradation.

In municipal wastewaters, only MC was detected in high concentrations, $350.0\,\mu g\,L^{-1}$ and the wastewater treatment resulted in a reduction of 100%, showing the effectiveness of the WWTP treatment.

4. Conclusions

A novel, simple, low cost and sensitive analytical strategy, based on coupling a HPLC system and a photo-reactor following post-column derivatization of the photo-fragments with magnesium ions, was developed for the routine analysis of TCs in environmental wastewaters. From the obtained results an increase of fluorescence emission is clear for MC, TC and ETC, providing higher sensitivity. For DC only an increase of 1.5% was observed that was considered not statistically significant. The accuracy and precision results testify the efficiency of the proposed method for the intended purpose.

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