BERNARDO LUÍS DE CASAIS MEIRA

On-line SPE-LC-FD to Quantify Fluoroquinolones in Aquatic Environments

DISSERTAÇÃO DE CANDIDATURA AO GRAU DE MESTRE EM CIÊNCIAS FORENSES SUBMETIDA AO INSTITUTO SUPERIOR DE CIÊNCIAS DA SAÚDE - NORTE

Orientadora
Profª. Doutora Maria Elizabeth Tiritan
Professora Auxiliar

INSTITUTO SUPERIOR DE CIÊNCIAS DA SAÚDE – NORTE

Abstract

In recent years the use of antibiotics has reached a very high consumption. With the desire to fight off infections, the increased variability of antibiotics and better economies, the pharmaceutical industries rise, and waste is produced. In light of the recurrent use in human and veterinary medicine the antibiotics can be located in diverse environmental sections through the excretion, uncorrected disposal, the constant drainage of surface runoff and release from wastewater treatment plants (WWTPs), which are not designed to complete elimination of small molecules during the treatment process. Given that these antibiotics potential threats to the environment, their incidence in freshwater systems is the focus of great concern.

The objective of this study is to provide a report on the intricacies of the on-line Solid Phase Extraction (SPE) methodology, and to create an efficient novel procedure to assess fluoroquinolones (FQs) occurrence. The FQs with higher environmental occurrence were selected, including Ciprofloxacin (CPF), Ofloxacin (CPF) and Norfloxacin (NOR), and their detection in WWTP's located around Porto, Portugal were also the focus of this work. For that an on-line SPE with Liquid Chromatography with Fluorescence Detection (on-line SPE-LC-FD) method was developed. The analytical method consisted in a two-dimensional liquid chromatography (2D-LC) with a Lichrospher® RP-18 ADS (Alkyl-Diol-Silica) column, which has a pore diameter of approximately 60 Å, particle diameter 25 µm, 25 x 4 mm (internal diameter) in the first dimension and a Luna PFP (2), pore size 100 Å, particle size 3 µm, 150 x 4.6 mm (internal diameter) in the second dimension. The mobile phase of the analytical column (second dimension) was aqueous 0.1% of triethylamine (TEA) adjusted to pH=2.2 with trifluoroacetic acid (TFAA) and Ethanol (EtOH) (76:24 *v/v*) at a 0.5 mL/min of flow rate. Water/Methanol (MeOH) 80:20 (*v/v*) mixture at a 1.5 mL/min flow rate was used in ADS column (first dimension).

The method was validated and demonstrated good selectivity, linearity (R^2>0.999), accuracy (always around 100%) and precision (intra-batch:

0.30<RSD<1.60; inter-batch: 0.40<RSD<1.80) in the range of 0.3 ng/mL – 30 ng/mL. The quantification limits were 0.3 ng/mL for all three target FQs.

The method was used to quantify wastewater sample effluents from four WWTPs. OFL was found at concentrations between 0.305 and 0.340 ng/mL and CPF was detected in all samples.

Resumo

Recentemente o consumo de antibióticos tem atingido níveis muito elevados. Com o desejo de melhorar a luta contra as infeções, o aumento da variabilidade de antibióticos e com as melhorias das condições económicas, estão impostas as condições necessárias para um grande crescimento da indústria farmacêutica, e com esta um aumento dos resíduos. Dado o uso recorrente de antibióticos na medicina humana e veterinária, estes podem ser encontrados em diversos sectores ambientais devido à excreção, à incorreta eliminação e à constante drenagem do escoamento superficial e libertação através de estações de tratamento de águas residuais (ETARs). No entanto as ETARs não estão devidamente preparadas para a eliminação de pequenas moléculas durante o processo de tratamento. Dado que os antibióticos são potenciais ameaças para o ambiente, a incidência dos mesmos nos recursos hídricos é uma área de grande preocupação.

O objetivo deste estudo é fornecer um estudo aprofundado sobre as complexidades que envolvem a metodologia da extração em fase sólida (SPE) *on-line* e criar um novo método para detetar e avaliar a ocorrência de fluoroquinolonas (FQs). As FQs com maior ocorrência ambiental foram escolhidas, incluindo a Ciprofloxacina (CPF), a Ofloxacina (OFL) e a Norfloxacina (NOR) e a deteção das mesmas em ETARs localizadas na área do Porto, Portugal foi também foco deste trabalho. Para tal, foi então desenvolvido um método de SPE *on-line* com Cromatografia Líquida com deteção por Fluorescência (on-line SPE-LC-FD).

O método analítico desenvolvido consistiu numa cromatografia líquida a duas dimensões (LC-2D) onde uma coluna Lichrospher® RP-18 ADS (Alkyl-DIOL-Silica), com um diâmetro de poro apróximado de 60 Å, diâmetro de partícula 25 µm e 25 x 4 mm (diâmetro interno), atuou na primeira dimensão e uma coluna Luna PFP (2), com um diâmetro de poro de 100 Å, diâmetro de partícula 3 µm e 150 x 4.6 mm (diâmetro interno), como segunda dimensão. A fase móvel da coluna analítica (a segunda dimensão) foi uma mistura aquosa com 0.1% de trietilamina (TEA) ajustada para um pH de 2.2 com ácido trifluoroacético (TFAA) e Etanol (76:24 *v/v*) a um fluxo de 0.5 mL/min. Uma mistura de

Água/Metanol (80:20 *v/v*) com um fluxo de 1.5 mL/min foi usada na coluna ADS (primeira dimensão).

O método foi posteriormente validado e demonstrou ter uma boa seletividade, linearidade (R^2>0.999), exactidão (sempre próxima de 100%) e precisão (*intra-batch*: 0.30<RSD<1.60; *inter-batch*: 0.40<RSD<1.80) na gama 0.3 ng/mL – 30 ng/mL. Os limites de quantificação foram 0.3 ng/mL para as três FQs selecionadas.

Por fim, o método foi usado para quantificar amostras de efluentes de quatro ETARs. A OFL foi encontrada na gama dos 0.305 aos 0.340 ng/mL, e a CPF foi detetada dentro dos limites estabelecidos.

List of abbreviations

2D-LC – 2 Dimensional-Liquid Chromatography
ACN – Acetonitrile
ADS – Alkyl-Diol-Silica
ARG – Antibiotic resistance gene
CPF – Ciprofloxacin
DAD – Diode array detection
DLLME - Dispersive liquid-liquid microextraction
DNA – Deoxyribonucleic acid
EC – European Community
ECG – Electrocardiogram
EtOH – Ethanol
FD – Fluorescence detection
FQ – Fluoroquinolone
GC – Gas chromatography
HF-SLM – Supported liquid membrane extraction with single hollow fiber
HLB – Oasis Hydrophilic-lipophilic-balanced reversed-phase sorbent
HPLC – High-performance liquid chromatography
ICH – International Conference in Harmonization
LLE – Liquid-Liquid Extraction
LOD – Limit of Detection
LOQ – Limit of Quantification
MeOH – Methanol
MM – Minimal salts medium
MS – Mass spectrometry
NOR – Norfloxacin
OFL – Ofloxacin
pH – Potential Hydrogen
PP – Protein precipitation
PPCP - Pharmaceutical and personal care product
R^2 – Coefficient of determination

RAM – Restricted-access media

RSD – Relative Standard Deviation

SPE – Solid-phase extraction

SPME-MD – Solid-phase microextraction with micellar desorption

TEA – Triethylamine

TFAA – Trifluoroacetic acid

TFC – Turbulent flow chromatography

TT – Transfer phase time

UFLC – Ultra Fast Liquid Chromatography Prominence System

USA – United States of America

USFDA - United States Food and Drug Administration

UV – Ultraviolet

WWTP – Wastewater Treatment Plant

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Acknowledgements

A thesis is not work it is dedication. The idea that it revolves around the agglomeration of pre-existing knowledge is naïve. A thesis is evolution, as it is growth of spirit and mind well beyond the expectations of a thoroughbred student sheltered from the responsibilities of life. As such, the pure notion that something like this is accomplishable by individual effort alone is not only egotistical but also self-defeating. Helen Keller once said: “Alone we can do so little; together we can do so much”. This project and the people that lead me in it, that befriended me, taught me to become a better scientist and person.

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Finally I would like to dedicate this thesis to my mother, and hope that she is in peace and that I do her proud.

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Introduction

I.1 General aspects

Antibiotics are one of the most noteworthy groups of pollutants inside the class of emergent contaminants (Schriks et al., 2010). In light of the recurrent use of these in human and veterinary medicine, the constant drainage of surface runoff and release from wastewater treatment plants (WWTPs), antibiotics can be located in diverse environmental sections. Given that they are potential threats to the environment, their incidence in freshwater systems is the focus of great concern (Meritxell et al., 2009, Kim and Carlson, 2007, Kümmerer, 2009) To great dismay, the occurrence of these chemicals has been reported not only on ground and surface waters but occasionally also in tap water (Fatta-Kassinos et al., 2011, Escher et al., 2011). Despite being in very low concentrations (ng to µ/L) its continuous inflowing in the environment through the excretion, uncorrected disposal, the constant drainage of surface runoff and release from wastewater treatment plants (WWTPs), poses a hazard to the ecosystems due to antibiotic resistance phenomenon. This is derived from their preordained biochemical effect on microorganisms, which in turn presents a significant impact in the processes managed by native biological communities (Maul et al., 2006, Muñoz et al., 2009, Rowan, 2010, Le-Minh et al., 2010).

The constant exposure of bacteria is the reason for one of the biggest concerns regarding the presence of antibiotics in the environment, leading to the occurrence and dissemination of antibiotic resistance genes (ARGs), since this occurs even at low antibiotic concentrations (Kümmerer, 2009, Martinez, 2009, Zuccato et al., 2008). Within these ARGs there are some that encode central bacterial enzymes, which play part in the unwinding of the deoxyribonucleic acid (DNA) double helix for the replication and transcriptions processes. Some antibiotics have the capability to inhibit these enzymes, a few of which are part of the Fluoroquinolones (FQs) group (Robinson et al., 2005a). As an example, in Northern Ireland, where FQs are considered the third most used antibiotics among 9 different drugs, resistance to them has already been observed in waterborne bacteria (Moore et al., 2010, Baquero et al., 2008, Martinez, 2009).

Despite FQs having been recurrently identified at low levels in surface waters (López-Serna et al., 2010, Pena et al., 2007) and at concentrations of μg/L in wastewaters (López-Serna et al., 2010, Le-Minh et al., 2010), the Water Frame Directive of European Union does not regulate the levels of FQs in aquatic environments (Hering et al., 2010), and this gives rise to the irrefutable necessity of a innovative method to determine the presence of these analytes in water systems.

The detection of FQs in groundwater, surface water, and wastewater has been defined in literature as feasible through the use of several chromatographic analytical methods, which are based on mass spectrometry (MS) detection (López-Serna et al., 2010, Ferdig et al., 2005, Kurie and Hiroyuki, 2006, Tamtam et al., 2009), diode array detection (DAD) (Poliwoda et al., 2010, Ferdig et al., 2005, Seifrtová et al., 2008), and fluorescence detection (Pena et al., 2007, Ferdig et al., 2005, Seifrtová et al., 2008, Herrera-Herrera et al., 2008). Although different methods are available for the identification of the analytes they are not present stand-alone in the environment, but most commonly in a complex matrix. FQs are present at trace levels in water systems, which is a complex matrix being this small concentration easily masked by a large amount of interfering compounds, and as a result a cleanup and augmentation step is always required (Smith, 2003).

In an effort to perform the pretreatment for the purification, pre-concentration, and isolation of FQs in environmental waters, the solid-phase extraction (SPE) technique is normally used (Babić et al., 2010). This is achieved through the usage to commercial sorbents constructed from alkyl-modified silica like Oasis HLB (Pena et al., 2007, Tamtam et al., 2009, Nageswara Rao et al., 2008, Shao et al., 2009) or Strata X (Babić et al., 2010, Lombardo-Agüí et al., 2010) and C18 (Ferdig et al., 2005, Ferdig et al., 2004) polymers. The extraction of FQs from environmental water samples has been, in recent years, attempted through SPE using magnetic molecularly imprinted polymers (Chen et al., 2010) and simple molecularly imprinted polymers (Benito-Peña et al., 2008). Due to the zwitterionic feature of FQs the mixed-mode sorbents (strong cation-exchange and reverse-phase) like the Oasis MCX cartridge (Lee et al., 2007) or in other instances an anion-exchange cartridge in tandem with an Oasis HLB

(Seifrtová et al., 2008) or a C18 (Turiel et al., 2005) cartridge are the most popular choices.

Even though off-line SPE is the most used method it bears some disadvantages such as time-consumption and the depletion of great quantities of organic solvents, which in turn led into the exploration of novel, more ecologically friendly and effective sample pretreatment procedures. The first of these being implemented in the extraction of multiple FQs in environmental water samples were: supported liquid membrane extraction with single hollow fiber (HF-SLM) (Poliwoda et al., 2010, Ramos Payán et al., 2011) and solid-phase microextraction with micellar desorption (SPME-MD) (Montesdeoca Esponda et al., 2009), leading to a lessening of organic solvent usage. Despite this, these approaches have drawbacks when it comes to time-consumption given that in the two applications of HF-SLM it takes 2 and 5.5 h for the extraction, or alternatively 60 min for extraction and 15 min for desorption in the SPME-MD process.

Ultimately a new approach was developed which can reach higher sensitivities with lesser sample volumes, with the advantage that the total extracted analytes get transferred into the chromatographic system. This approach was entitled as on-line SPE, and it can not only conquer the disadvantages connected to off-line SPE but also enable better precision and accuracy (Gil García et al., 2012).

I.2 Fluoroquinolones

I.2.1 Historical background

In 1962, George Lesher and coworkers generated from chloroquine, which at the time was used as antimalarial drug, nalidixic acid (Fig. 1). This was the origin of the first quinolone used in clinical approaches (Appelbaum and Hunter, 2000). When looking into this new compound it was observed that not only did it possess a small half-life (aprox. 1.5h) but also a near 90% protein binding action, as well as being active against several Gram- bacteria (Sharma et al., 2009). Although it was a progress, bacteria rapidly evolved a resistance to this reagent (Sharma et al., 2009, Sarkozy, 2001).

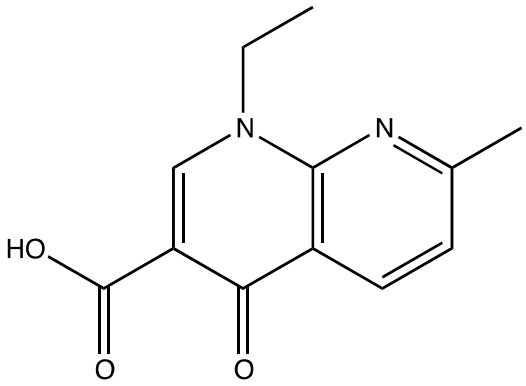


Figure 1 Nalidixic Acid

The United States Food and Drug Administration (USFDA) shortly after (1968) approved a new oxolinic acid (Fig. 2), synthesized by Kaminsky and Melfezer (Sharma et al., 2009). This paved the way for a new movement where there was no effort wasted in the development and creation of a vast group of active drugs of this class. Such was the dedication to this breakthrough that many analog design techniques (homologation of side chains or branching chains; stereochemistry; molecular modification for the lead optimization by bioisosteric replacements) were used in the advancement of new FQs which displayed broad spectrum activity and minimum toxicity (Table 1) (Sharma et al., 2009).

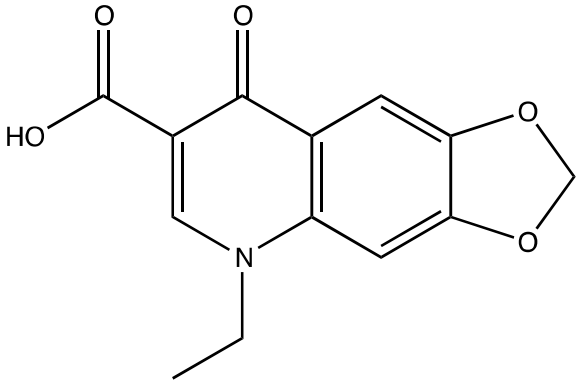


Figure 2 Oxolinic Acid

Many FQs have been patented (norfloxacin (NOR), 1978; ciprofloxacin (CPF), 1981; ofloxacin (OFL), 1982) of which flumequine (Fig. 3) was the first, and in light of the amplitude of compounds that have been originated, the FQ class achieved an advantage over their predecessors mainly because their broad spectrum of action. The great breakthrough came in the 1980's with the production of enoxacin (Fig. 3), an analog of nalidixic acid, that proved to have a notably larger spectrum of activity against Gram - or Gram + bacteria (Appelbaum and Hunter, 2000).

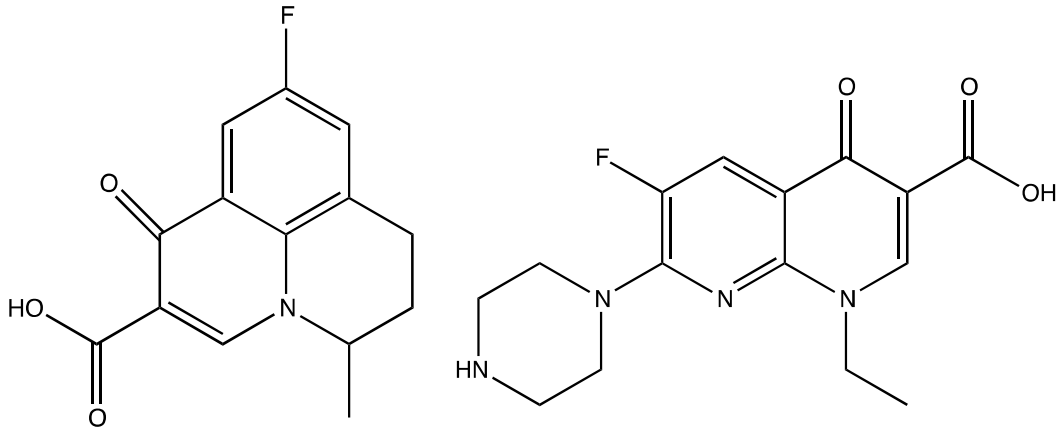


Figure 3 Flumequine and Enoxacin, respectively

Although enoxacin paved the way, the most successful and broadly used FQ is the CPF. Introduced into the market in 1986, it only aided in increasing the value of FQs in the treatment of innumerable infections, giving this class of antibiotics high recognition in society (Appelbaum and Hunter, 2000, de Almeida et al., 2007). As such, it is established that, in comparison to previously existing bactericidal drugs, the quinolone class has superior pharmacokinetic properties and numerous and potent activities that function counteracting many parasites, bacteria and mycobacteria, as well as their resistant strains (de Almeida et al., 2007, Anquetin et al., 2006).

Table 1 Classification of Fluoroquinolones (Sharma et al., 2009)

Generation	Drug	Characteristic Features
First	Nalidixic acid Oxolinic acid Pipemidic acid	-Active against some Gram - bacteria -Highly protein bound drugs -Short half life
Second	Norfloxacin Enoxacin Ciprofloxacin Ofloxacin Lomefloxacin	-Protein binding (50%) -Longer half life than previous agents -Improved activity against Gram - bacteria
Third	Temafloxacin Sparafloxacin Grepafloxacin	-Active against Gram - bacteria -Also active against + bacteria
Fourth	Clinafloxacin Trovafloxacin Moxifloxacin Gatifloxacin	-Show extended activity against both Gram + and - bacteria -Active against anaerobes and atypical bacteria

I.2.2 Essential structural features

All clinically relevant chemical structures of the FQ class are originated from fluorinated analogues of a 1,8-naphthyridine that possess a 4-quinolone nucleus called nalidixic acid (Moshirfar et al., 2008).

The 4-pyridone-3-carboxylic acid with a ring in the positions 5 or 10, as per Figure 4, is widely regarded as the pharmacophore required for relevant antibacterial activity.

Despite all the different changes that have been done to effectively improve antibacterial activity (Sharma et al., 2009), the original 1,8-naphthyridine nucleus was found to have two key pathways for the lead optimization. Both approaches are based in the variation of 6-fluoro and 7-piperazinyl quinolone (Blondeau, 1999). It is of relevance to mention a feature of many FQs with beta lactams possess. A mutual relationship is observed in both the classes where, increasing the Gram + activity displays a mutual decrease in Gram - activity (Appelbaum and Hunter, 2000).

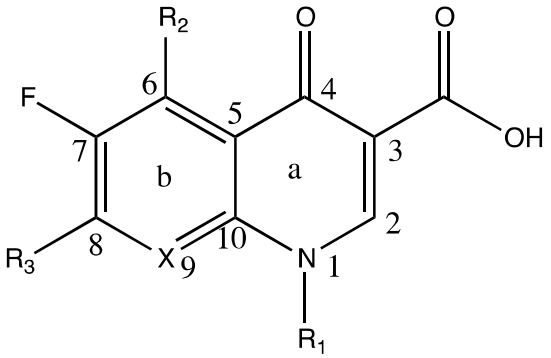


Figure 4 General structure of FQ

The initial path taken in the lead optimization approached the *"X"* position (Fig.4), where a carbon atom replaced a nitrogen atom. This was further complemented with other side chain modifications, which originated in FQs like our targets: 1-cyclopropanyl (CPF) and alternative variants of 1,8-cyclo compounds (OFL and levofloxacin) (Blondeau, 1999).

The alternate main path of possible chemical modifications was in the preservation of the naphthyridine core, of which enoxacin and tosufloxacin are prime examples of successful compounds (Ball et al., 1998).

I.2.2.1 Dynamic approach towards optimization

The FQ class is vast, as such there are several ways for the lead compound (Fig. 4) to be optimized. The most critical features in the development of a FQ are as follows:

- *Position 1*: The potency of the agent is considerably affected by the side chain (R_1) chosen to be connected to this ring nitrogen. An ethyl group was initially picked as the optimal substituent at this position given that many of the original compounds displayed this, such as nalidixic acid, pipemidic acid, norfloxacin (NOR) and many others (Sharma et al., 2009). Nonetheless when looking further into other options, the cyclopropyl and difluorophenyl chains caused augmented potency (Appelbaum and Hunter, 2000);
- *Positions 2 and 3*: It is of great significance to maintain the stereochemistry of this area as it is generally accepted that the carboxylic functional group at position 3 is the section of the pharmacophore that binds to the DNA gyrase of a bacterial cell (Appelbaum and Hunter, 2000). Normally the alterations of the 3-carboxylic acid group generate compounds with decreased activity, and as such this part of the molecule is rarely considered for modifications in these positions (Dax, 1997, Appelbaum and Hunter, 2000). In addition decreasing the 2,3-double bond terminates the activity (Sharma et al., 2009);
- *Position 4*: 4-Oxo group is fundamental for the activity (Sharma et al., 2009);
- *Position 6*: Alterations and substitutions at this site have demonstrated increased antibacterial activity against Gram + strains (Appelbaum and Hunter, 2000). In an effort to better antibacterial activity several substituents have been tested and it was observed that the bulky ones when placed in this position reduce said activity prominently. Despite this, other attempts have shown significant improvement, such as methyl and amino substituents, like in grepafloxacin and sparfloxacin, respectively (Appelbaum and Hunter, 2000, Domagala, 1994);

- *Position 7*: Here rarely changes are made since it seems that the fluorine atom is essential as it has function in the binding of the compound with the DNA topoisomerase enzyme of bacteria (Sharma et al., 2009);
- *Position 8*: Initial compounds, such as pipemidic acid, NOR and CPF, display examples of alterations in this position using unsubstituted piperazine, which has been observed to generate good activity against Gram - strain of viable bacteria. This is important since it is now accepted that modifications in this position of the molecule gave rise to great progress in improving the quinolone class, as changes here often give origin to major variations in potency. The bonding of a heterocyclic nitrogen containing rings affects the pharmacokinetics of the compound but also significantly shows better activity (Stein, 1988, Tillotson, 1996);
- *Position 9*: This position has been related to the *in vivo* properties and antibacterial activities as variations here affects these, especially against anaerobes (de Almeida et al., 2007). There are many options for modification at this site, like adding a fluorine or chlorine substituent which in turn originates likely active compounds (Appelbaum and Hunter, 2000), but one of the important changes to mention which is also significantly relevant to us can be observed in OFL. In its third ring the stereochemistry of the methyl group has proven to possess noteworthy effect on the antibacterial activity (Patrick, 2001). Not only is the (*S*)-enantiomer (levofloxacin) more soluble than the racemate, but also it is amazingly 10 times more potent than the (*R*)-enantiomer.

The amphoteric nature is associated with the presence of both acidic (carboxylic acid) and basic (tertiary nitrogen) groups in their pharmacophore, presenting low water solubility in the range of pH 6 to 8.

FQs bind to DNA causing the inhibition of its synthesis through the formation of a ternary complex involving the drug, the bound segment and the enzyme. FQs bind themselves in a stacking arrangement in such a way that the aromatic ring is coplanar while also permitting bonding interaction to occur between the substituents at the first position (Patrick, 2001). While the carbonyl and

carboxylate bind with DNA through the means of hydrogen bonds, the fluoro substituent, which is normally at carbon-7 (Fig. 4), and the carboxylate ion engage in the interactions that bind the enzyme (Fig. 5). When looking to increase activity and pharmacology of the FQs class the approach is primarily on the stacking domain and the enzyme and DNA binding sections, which is possible since the compound formed in the aforementioned binding has such stability that the activity of FQs rises (Foroumadi et al., 2005).

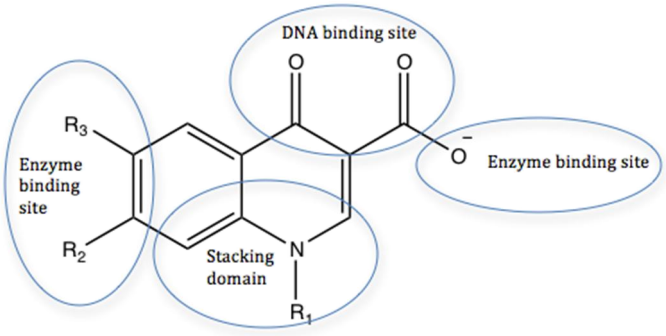


Figure 5 FQ binding sites

I.2.3 Metabolism and Elimination

Thanks to their longer serum half-life, once a day dosing in permitted, and as a result the maximum plasma concentration increases leading to wider coverage over the minimum inhibitory concentration. Not only this but the higher volume of distribution allows for a much more efficient tissue penetration. As such, in recognition of these traits, it is considered that the pharmacokinetic profile of novel analogs of the FQ class are far more advanced than that of the original compounds (Sharma et al., 2009).

FQs have displayed formidable penetration into different fluids and body tissue, with exception to the central nervous system. Consequently, novel FQs can reach a serum concentration well above that which is necessary for efficacy against infections (Cheng et al., 2007, Oliphant and Green, 2002). Generally this is also an effect of the FQs superior distribution to tissues, in comparison to the majority of other drugs, since the binding that could occur with plasma proteins is small (Sharma et al., 2009). An astonishing level of drug can be reached in the kidney, prostate gland, liver and lungs. The compounds

concentration present in the urine is above that which has been found to be the minimum concentration necessary of inhibition and as a result this makes FQs effective against urinary tract infections (Scholar, 2002). This shows that FQs can reach excretion organs in their unmetabolized state.

In fact, in terms of removing FQs from the system, there is a great variation in the level of elimination, whether it happens via being metabolized in the liver or by renal excretion. Their metabolism, which is mostly done by the conjugation of glucuronides at the 3-caboxylic group, is characterized as inactivating (Sharma et al., 2009). But the foremost pathway of elimination for most FQs occurs by tubular secretion and then via glomerular filtration via the kidney. Both peritoneal dialysis and hemodialysis present themselves as a less effective second pathway of elimination via the liver (Scholar, 2002). The result of having unmetabolized FQs in the urinary system, leads to the secretion in this state which in turn will result in the introduction of FQ compounds into the wastewater system (Sörgel and Kinzig, 1993).

I.3 Source and pathways of antibiotics in the environment

Fluoroquinolones (FQs) can reach the environment in many different ways from the point of usage (Fig. 6). FQs are most commonly excreted unchanged and the pathways through which they move in the environment diverge between human or veterinary usage, in which the main routes are via human excretion into wastewaters or dissolution of fertilizer onto agricultural soils, respectively (Sörgel and Kinzig, 1993, Golet et al., 2003). And although most are excreted unchanged it is also possible to observe mixtures of metabolites or even conjugates where an inactivating substituent is present in the molecule, depending on the targets pharmacological properties (Rang, 1991). The following present themselves as the most common routes (Halling-Sørensen et al., 1998):

- Human medicinal use;
- Growth promoters for livestock production;
- Therapeutics in livestock production;
- Coccidiostat used for poultry production;

- Therapeutics for treatment of livestock on fields;
- Feed additives in fish farms.

It is also common practice to apply sludge retrieved from wastewater treatment activities straight to land as fertilizer and the incidence of FQs has already been observed in topsoil samples where this practice had been performed (Golet et al., 2003).

The wastewater from medical establishments is believed to be a great contributor to the influx of large amounts of unchanged FQs into the environment (Hartmann et al., 1998). This is not the only contribution to environmental aquatic contamination. Other probable situations are the environmental adulteration with antibiotics by pharmaceutical production sites proceed with the disposal of their waste and when improper landfills are created, where waste from municipal and the aforementioned clinical settings is deposited (Sousa et al., 2012, Sukul and Spiteller, 2007). It is noteworthy to observe that in countries where regulation is more loosely implemented, like India, a FQ such as CPF has been found in the order of mg/L, which is an alarming amount (Larsson et al., 2007).

Although this is not a major problem in modern landfills, which are prepared to deal with this situation using protective layers and leachate collection systems, many others that have been built prior to this approach are the source of potential contaminations (Sukul and Spiteller, 2007). During the disposal in these problem areas (unlined landfill sites), shallow groundwater and surface waters can be adulterated by drug residues in the leachate (Holm et al., 1995).

This was in regard to human consumption but FQs are also prominent in agricultural and veterinary activities. Surface and ground waters are prime

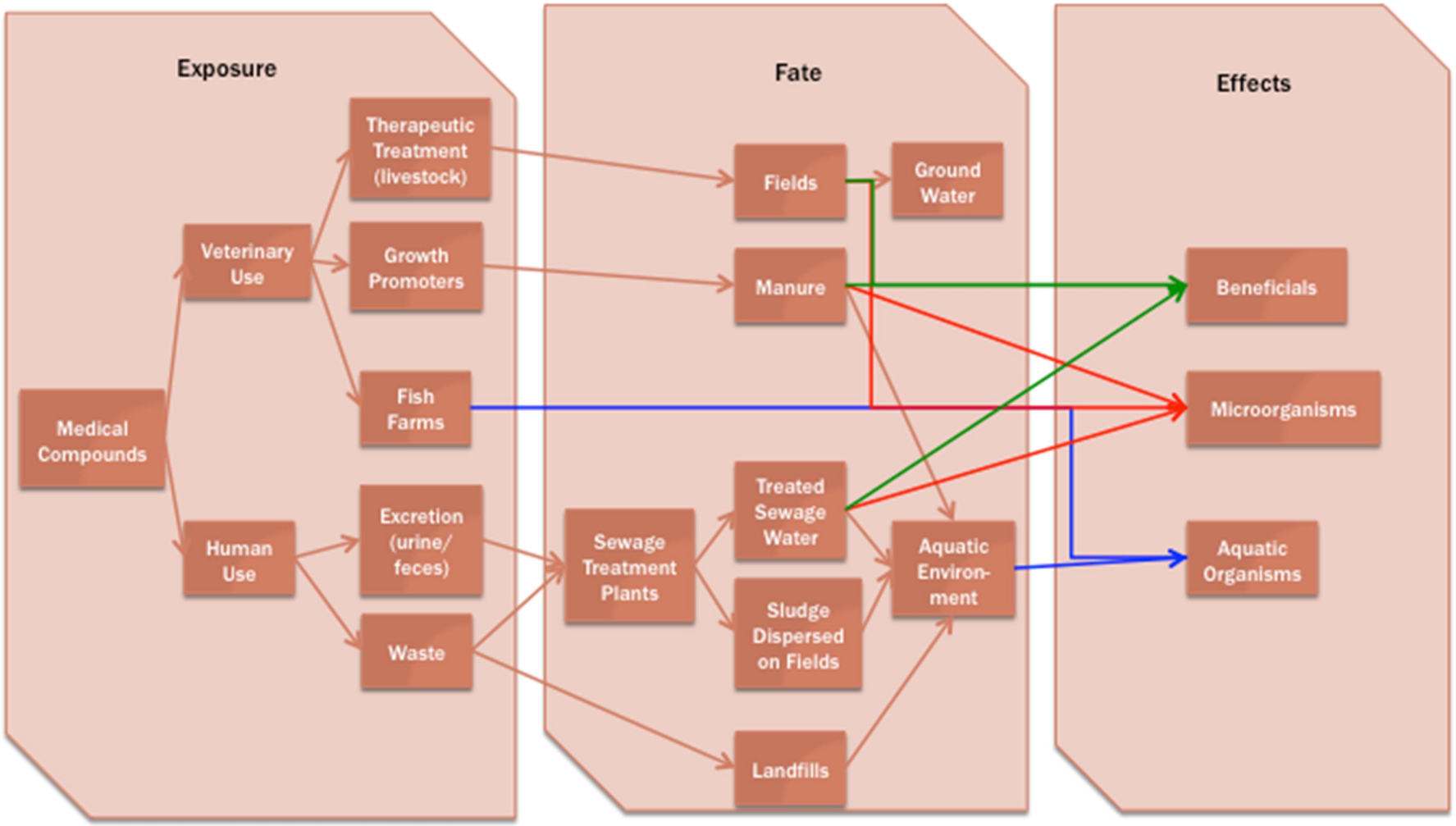


Figure 6 Pathways of Fluoroquinolones in the environment (adapted from (Halling-Sørensen et al., 1998)

targets for contamination via manure, which is generally how animal waste comes in contact with the soil, and as result, the animal drugs (metabolized or unmetabolized) that are present in solid or liquid manifestations of this waste after reaching the soil are transmitted to the aforementioned water systems (Hamscher et al., 2000, Hamscher et al., 2004). When performing intensive fish farming, antimicrobial agents that get released straight into the water generally take the shape of feed additives and are used to fight off infections. Consequently, given the direct influx in the aquatic environment, a buildup of antibiotic compounds occurs in the water and sediments (Sukul and Spiteller, 2007).

I.4 Fluoroquinolones occurrence in the environment

With the increased consumption of medical agents the occurrence of pharmaceutically active compounds in the environment has become the target of various studies throughout the world. More than 80 of these pharmaceutically active compounds have been positively identified within sewage, surface and groundwater in ranges up to the mg/L at countries like Austria, Brazil, India, China, Canada, Croatia, England, Germany, Greece, Italy, Spain, Switzerland, the Netherlands and the United States (Heberer, 2002).

The occurrence of antibacterial drugs in the inlet and effluents of WWTPs or surface waters, of several FQs have been identified in numerous studies (Table 2). Regarding well developed countries the concern is factual, among which, Germany (Alexy et al., 2006, Ohlsen et al., 2003), Switzerland (Golet et al., 2001), the United States (Kolpin et al., 2004) and Canada (Arlos et al., 2014). However emerging countries like China (Bai et al., 2014), India (Pravin and Atul, 2013) and Brazil (Beretta et al., 2014) are also worried about this subject (Na et al., 2014).

Table 2 Occurrence of 4 Fluoroquinolones in the Environment

Fluoroquinolone	Matrix	Analytical method	Pre-Treatment	Environmental concentration detected	Sampling local (Country)	Ref.
Ofloxacin	Urban Wastewater	HPLC-ESI-MS-MS	SPE (-)	0.075-0.082 µg/L	Germany	(Alexy et al., 2006)
	Hospital Wastewater	LC-MS	SPE (C2/ENV+ Column)	0.2-7.6 µg/L	Sweden	(Lindberg et al., 2004)
	Hospital Wastewater	HPLC-ESI-MS-MS	Not mentioned	31 µg/L	Germany	(Ohlsen et al., 2003)
	River Water/Sediments	HPLC-MS-MS	SPE (Oasis HLB Column)	Max: 0.632 µg/L	China	(Bai et al., 2014)
	Wastewater Effluent	LC-ESI-MS-MS	SPE (Oasis HLB Column)	0.094 µg/L	Canada	(Miao et al., 2004)
Norfloxacin	Urban Wastewater: Primary Effluent Tertiary Effluent	Reversed-phase LC-FLD	SPE (MPC Column)	 0.270-0.367 µg/L 0.048-0.120 µg/L	Switzerland	(Golet et al., 2001)
	Hospital Wastewater	HPLC-FLD	SPE (MPC Column)	0.9-17 µg/L	Vietnam	(Duong et al., 2008)
	(Up/Down)town Streams	LC-MS	SPE (-)	0.03 µg/L	USA	(Kolpin et al., 2004)
	Wastewater Effluent: Raw Sewage Final Effluent	HPLC-FD	SPE (MPC Column)	 0.255-0.553 µg/L 0.036-0.073 µg/L	Switzerland	(Golet et al., 2002)
	Hospital Wastewaters	HPLC-ESI-MS-MS	Not mentioned	44 µg/L	Germany	(Ohlsen et al., 2003)
	River Water/Sediments	HPLC-MS-MS	SPE (Oasis HLB Column)	Max: 0.256 µg/L	China	(Bai et al., 2014)
	Wastewater Effluent	LC-ESI-MS-MS	SPE (Oasis HLB Column)	0.118 µg/L	Canada	(Miao et al., 2004)

Ciprofloxacin	Urban Wastewater: Primary Effluent Tertiary Effluent	Reversed-phase LC-FLD	SPE (MPC Column)	 0.249-0.405 µg/L 0.045-0.108 µg/L	Switzerland	(Golet et al., 2001)
	Hospital Wastewater	Reversed-phase HPLC-FLD	None or TITAN HPLC syringe filters	3-87 µg/L	Switzerland	(Hartmann et al., 1998)
	Hospital Wastewater	HPLC-FLD	SPE (MPC Column)	1.1-44 µg/L	Vietnam	(Duong et al., 2008)
	Hospital Wastewater	LC-MS	SPE (C2/ENV+ Column)	3.6–101.0 µg/L	Sweden	(Lindberg et al., 2004)
	(Up/Down)town Streams	LC-MS	SPE (-)	0.03 µg/L	USA	(Kolpin et al., 2004)
	Hospital Wastewater	HPLC	None or TITAN HPLC syringe filters	0.0007-0.125 µg/L	Germany	(Hartmann et al., 1999)
	Wastewater Effluent: Raw Sewage Final Effluent	HPLC-FD	SPE (MPC Column)	 0.313-0.568 µg/L 0.062-0.106 µg/L	Switzerland	(Golet et al., 2002)
	Hospital Wastewater	HPLC-ESI-MS-MS		51 µg/L	Germany	(Ohlsen et al., 2003)
	River Water/Sediments	HPLC-MS-MS	Not mentioned	Max: 0.185 µg/L	China	(Bai et al., 2014)
	Wastewater Effluent	LC-ESI-MS-MS	SPE (Oasis HLB Column)	0.4 µg/L	Canada	(Miao et al., 2004)
	Wastewater Effluent	HPLC/LCQ-ESI-MS-MS	SPE (Oasis HLB Column)	28000–31000 µg/L	India	(Larsson et al., 2007)
Enrofloxacin	(Up/Down)town Streams	LCMS	SPE (-)	0.01 µg/L	USA	(Kolpin et al., 2004)
	River Water/Sediments	HPLC-MS-MS	SPE (Oasis HLB Column)	Max: 0.070 µg/L	China	(Bai et al., 2014)

FQs, primarily CPF and NOR, have shown to be persistent contaminants in urban wastewaters, observed by (Golet et al., 2001) in primary and tertiary waste effluents in samples ranging in 48-367 ng/L and 45-405 ng/L for NOR and CPF respectively. In addition, hospital effluents have also shown to contribute to this environmental contamination, analyzed between 3-87 µg/L of CPF in Switzerland by (Hartmann et al., 1998). These unsuccessfully treated wastewaters are of great concern as FQs, like other compounds, come with toxic effects which in certain amounts can damage the environment.

I.5 Toxicity

FQs are proven to have toxicity for several organisms. Several reported cases with chronic and acute toxic effects have been noted (Table 3). OFL has been shown to produce chronic toxic effects in *Synechococcus leopolensis* cyanobacterium in quantities as low as 5 µg/L (Ferrari et al., 2004), as well as acute mutagenic effects in other organisms (Isidori et al., 2005). Further in one case, CPF has shown to cause an inhibition of algae growth (Wilson et al., 2003). The interfering with cellular reproduction was observed in response to concentrations of 0.015-1.5 µg/L, range often found in aquatic environment (Table 2).

FQs are also associated with several side effects such as (Sharma et al., 2009):

- Skin photosensitivity reactions, presented in varied severity and incidence depending on the FQ used (Blondeau, 1999, Scholar, 2002);
- Bone and joint damage in children associated with CPF (Sharma et al., 2009);
- Central nervous system disorders including: dizziness, insomnia, mood swings, seizures or hallucinations (Blondeau, 1999, Sarkozy, 2001, Scholar, 2002, Oliphant and Green, 2002);
- Modifications to the sense of palate (Lode et al., 1999);
- In unique occasions anaphylaxis and agranulocytosis were observed (Scholar, 2002);
- Dyglycemic reactions (Onyenwenyi et al., 2008, Malak et al., 2007);

- When used in multidrug resistant tuberculosis treatments, invasive pneumococcal diseases have been observed in children (von Gottberg et al., 2008);
- Increase in the QT interval in electrocardiogram (ECG) associated with sparfloxacin and grepafloxacin (Lode et al., 1999, Lipsky et al., 1999);

Some important drug interactions are further reported by Sharma et al. (2009) as significant interactions:

- Higher levels of cyclosporine in serum;
- CPF leads to a diminished concentration of antineoplastics in serum;
- Azlocillin, cimetidine and probenecid cause the concentrations of CPF and NOR to oscillate in the excretion (Sharma et al., 2009, Efthymiopoulos et al., 1997);
- Crystalluria can occur as a result of NOR having its solubility decreased when interacting with drugs similar to sodium bicarbonate, citrates or carbonic anhydrase inhibitors.

The presence of FQs in our daily lives has at this point been considered indispensable to modern medicine, as such it will be a continuous threat to our environment. For this reason it is vital that the influx of FQs is monitored and tightly controlled. In response to this the development of analytical methodologies that can efficiently detect and quantify FQ agents is a priority and has been approached by many in diverse ways.

Table 3 Toxic effects of Fluoroquinolones (FQ) in various organisms

FQ	Tested organism	Concentration	Effect	Obsevations	Ref.
Ofloxacin					
	S. leopolensis	0.005 mg/L (Chronic)			(Ferrari et al., 2004)
	C. meneghiniana	0.0312 mg/L (Chronic)	Inhibition of	–	(Ferrari et al., 2004)
	P. subcapitata	2.5 mg/L (Chronic)	DNA gyrase		(Ferrari et al., (2003,2004))
	B. calyciflorus	12.5 mg/L (Chronic)			(Ferrari et al., 2003)
	C. dúbia	10.0 mg/L (Chronic)			(Ferrari et al., (2003,2004))
	L. gibba	0.121 (EC10) mg/L (Chronic)			(Brain et al., 2004)
	P. Subcapitata	1.44 mg/L (Chronic)			
	B. Calyciflorus	0.53 mg/L (Chronic), 29.88 mg/L (Acute)			
	C. dúbia	3.13 mg/L (Chronic), 17.41 mg/L (Acute)	Genotoxic and	All values L(E)C50	(Isidori et al., 2005)
	V. Fischeri	100 mg/L (Acute)	Mutagenic		
	T. Platyurus	33.98 mg/L (Acute)			
	D. Magna	31.75 mg/L (Acute)			
	D. Rerio	1000 mg/L (Acute)			
	M. aeruginosa	21 µg/L			
	L. minor	126 µg/L	–	All values L(E)C50	(Robinson et al., 2005b)
	P. subcapitata	12100 µg/L;			
Norfloxacin					
	L. gibba	0.206 (EC10) mg/L	Inhibition of DNA gyrase	–	(Brain et al., 2004)
Ciprofloxacin					
	L. gibba	0.106 (EC10) mg/L	Inhibition of DNA gyrase	–	(Brain et al., 2004)
	Natural Algae Communities	0.015-1.5 µg/L	Variations in Algae growth	Increase in the diatom Synedra at 0.012 µg/L and 0.12 µg/L, as well as significant reduction of the Navicula at 0.12 µg/L. Reductions in the green alga Chlamydomonas at 0.12 µg/L and 1.2 µg/L, and for Sphaerocystis at 1.2 µg/L	(Brittan et al., 2003)

	Bacteria (activated sludge)	0.61 mg/L			
	S. Capricornutumb	2.97 mg/L	–	All values EC(50)	(Halling et al., 2000)
	M. Aeruginosa	0.005 mg/L			
	M. aeruginosa	17 µg/L			
	L. Minor	203 µg/L	–	All values L(E)C50	(Robinson et al., 2005)
	P. Subcapitata	18700 µg/L			
Enrofloxacin					
	M. Aeruginosa	49 µg/L			
	L. Minor	114 µg/L	–	All values L(E)C50	(Robinson et al., 2005)
	P. Subcapitata	3100 µg/L			
Levofloxacin					
	L. gibba	0.013 (EC10) mg/L	Inhibition of DNA gyrase	–	(Brain et al., 2004);
	M. Aeruginosa	7.9 µg/L			
	L. Minor	51 µg/L	–	All values L(E)C50	
	P. Subcapitata	7400 µg/L			(Robinson et al., 2005)
Lomefloxacin					
	Lemna gibba	0.008 (EC10) mg/L	Inhibition of DNA gyrase	–	(Brain et al., 2004);
	L. Minor	106 µg/L			
	M. Aeruginosa	186 µg/L	–	All values L(E)C50	(Robinson et al., 2005)
	P. Subcapitata	22700 µg/L			
Clinafloxacin					
	L. Minor	62 µg/L			
	M. Aeruginosa	103 µg/L	–	All values L(E)C50	(Robinson et al., 2005)
	P. Subcapitata	1100 µg/L			

I.6 Analytical methods for trace quantification of fluoroquinolones

When it comes to what analytic methods are best for the analysis of FQs, liquid chromatography (LC) has been far the most utilized in recent years. However not only for FQs, but it is also used for a variety of compounds that do not perfectly fit the profile required for other methods, such as gas chromatography (Yang et al., 2013). As a result the optimization of the reliable and well-established LC procedure has been the focus of great attention (Gupta et al., 2010).

Most pharmaceutical and personal care products (PPCPs), which enclose antibiotics, are polar, non-volatile and/or thermally labile agents, and given this they are considered largely incompatible with GC. Because of these properties a great disadvantage is created by GC in comparison to LC, which is the need for the derivatization of hydroxyl- and carboxyl- groups prior to performing the analysis (Chunyan et al., 2007).

Considering analyses of complex matrices as biological and environmental a great efforts have always to be done to eliminate the interferences of undesired compounds and enhance the concentration of the targets. The first step of the analytical process consists in sample preparation and is one of the most important in the method establishment.

I.6.1 Sample Preparation

The sample preparation is a vital part of any analytical method that handles biological or environmental samples, as it affects the output efficiency of each analysis. It is one of the most time consuming step of the method and often accounts for 80% of the analysis time (Sousa et al., 2012, Ribeiro et al., 2014). The purpose of sample preparation is to selectively single out a target compound from the sample matrix while discarding its endogenous compounds. At the same time it helps to protect and expand the lifespan of the analytical column while minimizing interferences that might appear during the chromatographic analysis (Nováková and Vlčková, 2009, Unceta et al., 2011, Kole et al., 2011).

The choice of a sample preparation method is derived from the research target, the tendencies that accompany the matrix of targets and the purpose of the investigators, consequently the extraction of FQs is already well established in literature and the most usual techniques found to extract them from biological and environmental matrices are as follows (Sousa et al., 2012):

- Protein Precipitation (PP)
- Liquid-Liquid Extraction (LLE)
- Solid-Phase Extraction (SPE)
- Ultrafiltration
- Dilution

PP is a non-selective preparation method for biological matrices. It is considered to have several advantages such as being the shortest method (in terms of time), one of the easiest to perform and to have a limit of quantification low enough for the desired objective of analytical methods engineered for FQ analysis. (Kole et al., 2011).

LLE was one of the first methods created for the purpose of sample preparation, and it involves the translocation of the target analyte from the aqueous sample to a water-immiscible organic solvent (Nováková and Vlčková, 2009, Kole et al., 2011). The creation of LLE methods for FQs is quite laborious and several limits accompany this method (Sousa et al., 2012):

- Large sample volumes are necessary;
- Consumes great quantities of organic solvents;
- Recovery is not accurate;
- Hydrophilic analytes cannot be processed;
- Complicated extraction in analytes with different lipophilicities.

In spite of this the use of LLE in the sample preparation of biological fluids is still observable in current events (Nováková and Vlčková, 2009, Kole et al., 2011). However for environmental analysis is quite impracticable. An innovative methodology based on LLE to overcome these drawbacks has emerged with the dispersive liquid-liquid microextraction (DLLME) (Ribeiro et al., 2014).

DLLME is a more environmentally friendly extraction technique with the added advantages of being faster, cheaper and simpler than LLE (Rezaee et al., 2006). This method makes use of two solvents (Ribeiro et al., 2014):

- A high-density, extraction-capable, and water-immiscible extracting solvent (like chlorobenzene, chloroform, carbon tetrachloride, or tetrachloroethylene);
- And a disperser solvent with high miscibility in both extracting and aqueous phases (MeOH, EtOH, ACN, or acetone)

It is critical to consider that the success of the extraction is highly dependent on the chosen solvents as well as their volumes (Vasil et al., 2012, Hyötyläinen and Riekkola, 2008, Rezaee et al., 2006).

As for the procedure itself, a swift injection of a mixture containing the aforementioned solvents is done into an aqueous sample that holds our target analytes. The extractant solvent then diffuses into the sample originating a cloudy solution (water/disperser solvent/extracting solvent), which has a large surface area separating the aqueous sample from the emulsified extracting solvent. This large area provides great interaction between the aqueous solution and the very small droplets of the extractant, as a result equilibrium amid the two is achieved quickly (Demeestere et al., 2007, Ribeiro et al., 2014).

To analyze the solution the mixture can be either centrifuged and the sediment phase analyzed, or instead the extractant solvent can be separated by solidification after cooling (Vasil et al., 2012, Rezaee et al., 2006, García Pinto et al., 2011). Lastly, DLLME has been found to deliver a high recovery and concentration analyses compatible with both GC and LC (Farré et al., 2010, Tankiewicz et al., 2011).

I.6.2 Solid-Phase Extraction (SPE)

SPE is the most observed method in literature and is considered the main preparation method given its better extraction of compounds, the greater removal of unwanted endogenous compounds and as a result it is the

methodology that records the best recovery values (Nováková and Vlčková, 2009, Kole et al., 2011, Hendriks, 2009). Two major attributes affect the retention of target compounds: polarity and ionic interactions. As a result no expense has been overlooked when developing the existing wide range of SPE sorbents that offer us many possibilities in terms of extraction capacity and selectivity (Nováková and Vlčková, 2009).

When applied to the extraction of FQs there are two major sorbents used: Silica-based reversed-phase cartridges and Polymeric reversed-phase sorbents.

Although the most used technique it does not come without its disadvantages, one of which is the expensive nature of this procedure. In response to this many methods are continuously being developed to eliminate the limitations brought on by SPE and increase its performance (Kole et al., 2011).

With the development of novel methods and technologies SPE took a step in the next direction and automated systems where the SPE sample preparation methods can be performed within the LC separation processes are now possible. The use of these with direct injection of plasma samples (Nguyen et al., 2004, Tasso and Dalla Costa, 2007) as well as FQs has already be referenced in literature (Tasso and Dalla Costa, 2007, Djabarouti et al., 2004, Lemoine et al., 2000) thanks to the fact that they carry numerous advantages:

- Less contact with dangerous samples;
- Better reproducibility given the decrease in human interactions;
- High-throughput analysis.

Currently there are several types of on-line SPE columns that are used in this preparation method (refer to Table 2 for other examples). However the three main types are the restricted access media (RAM), which was the one chosen in this project, the turbulent flow chromatography (TFC) and the polymeric adsorbents such as the Oasis Hydrophilic-lipophilic-balanced reversed-phase sorbent (HLB) columns (Kole et al., 2011, Laban-Djurdjević et al., 2006, Tagiri-Endo et al., 2009). These can be used as precolumns when joined with an analytical column or simply carry out their purpose as an analytical column by themselves (Sousa et al., 2012).

On-line SPE functions by having a pre-column before the analytical column to perform the SPE. But that is not all, the extraction performed is not a single event process, it means that is not a one directional process where sample is injected and goes straight to the analytical column. On-line SPE is a two dimensional procedure divided into two distinct phases controlled by a switch valve that shifts and allows for different connections (Fig.7).

PHASE 1- Load Position

In this first phase the sample is sent from the autosampler to the pre-column (first dimension), and the solvents chosen for the cleanup of the sample pass through the column continuously for a predetermined time and get discarded to the waste. Here the objective is to trap the target analytes and push the undesired contaminates out of our sample (cleanup).

PHASE 2- Inject Position

When satisfied with the cleanup an order is given out to the valve by the software and will cause it to shift allowing for a different pathway to be created that connects the pre-column to the analytical column. Now the analytes trapped in the pre-column will now be subjected to the solvents determined as the optimal mobile phase designated to perform the analytical separation in the analytical column (second dimension). The mobile phase will drag the analytes out of the pre-column into the analytical one and consequentially to the detector. The purpose here is to drag the analytes trapped in the SPE column to the analytical column.

There are two methods to perform the transfer of analytes to analytical column: *forward flush* or a *back flush*. In the first a sample gets pushed to the analytical column in the same direction by which the sample was clean (example left to right). On the other hand in the *back flush* the transfer occurs in the reverse direction, as such the analytes go through the same path from which they came

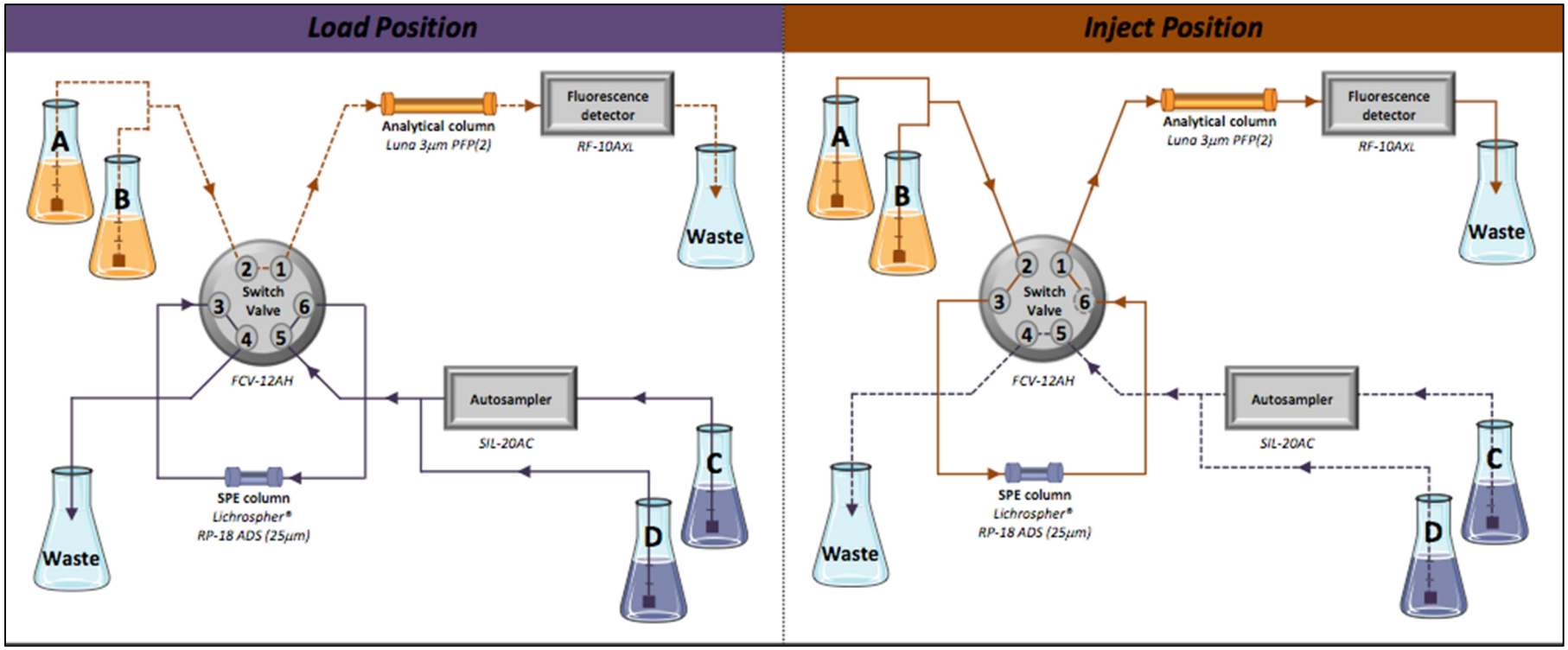


Figure 7 On-line SPE Liquid Chromatography system

(input: left, output: left). For this project the *back flush* was chosen because it was generally accepted that FQs were trapped in the start of the column and as such it would be simpler and more efficient to transfer them in the reverse direction, also preventing compounds that are further down the column to be transferred to the analytical column.

On-line SPE is less time-consuming and more economic than off-line SPE, and leaves less room for human mistakes as interaction with the samples is reduced. It is a tool which can prove to be vital in producing consistent and precise high quality results in the FQ and other PPCP analysis (Chunyan et al., 2007).

I.7 Significance of environmental analyses for Forensic Fields

A forensic science is any scientific area or method that can be used to assist in the judicial (criminal or civil) process by helping to establish proof (SAGE Publications). As such there is a myriad of applications and fields of study in the area of forensic studies.

In our media driven age, forensics are commonly associated with aggressive and violent crimes, but the reach of this area of study is much wider. The key is to understand how forensics assist in the investigation of a litigious situation. The idea that these methods are used to singlehandedly establish guilt is naïve and innocent, as the objective is not to simply instigate a individual but to ascertain the method by which an illegality was committed, the motive behind the behavior and the time and place at which it was perpetrated.

Given this, the methods created in the assistance of an investigation can be applied to a wide range of situations. Someone who performs an illegal act might be and individual or/and an organization, a weapon can be physical or chemical and reason can be emotional, financial or even sociologically irrational.

The idea intended to be transmitted is that inter-personal crimes are not the only focus of forensic, there are environmental illegal activities, and even within said inter-personal crimes, the environment is an important aspect of the crime, as it can influence the many factors involved in the situation.

As Edmund Locard once stated, in his principle of exchange (summarized), every contact leaves a trace (Chisum and Turvey, 2011). With this affirmation the ways the environment is connected to the forensic sciences can be summarize as:

- The environment as a victim of a litigious act
- The environment as a witness of a litigious act

By this we mean that not only can there be crimes against the environment but it is also a tool in the acquisition of proof needed in the forensic area.

Analytical methods are indispensible in the forensic sciences but even more when dealing with environmental issues. When in the role of witness the environment can lead to geological identification of areas where criminal action took place. They can place an individual within said area by matching trace elements (like pollen, soils, particular water samples) found in the possession of said person (or belongings) to a specific region or in cases involving chemical weapons, such as poisons, chemical analysis can prove useful beyond the usual identification use as a means to trace a disposed substance in waters or soils.

On the other hand many times the environment itself is target of abuse and illicit actions, like the destruction of habitats, the insufficient treatment of waste or even the deliberate poisoning/pollution of the ecosystem. This project is more entwined in these cases where many times chemicals are disposed of in surface waters or soils to avoid payment for the proper treatment methods. By having to analyze a contaminated scene, in the judicial process, it is highly important to identify the origin of the contamination, the time of occurrence of it and, a major factor, the distribution and affected surface or subsurface areas as well as the implication of the contamination in these (Morrison, 2000).

Forensics are an important part of our society as an efficient method of establish order and proof.

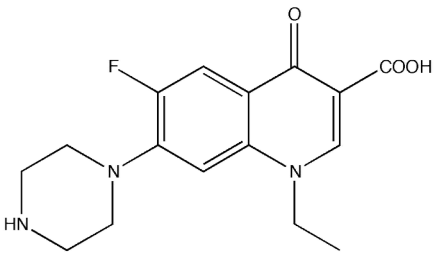
I.8 Aims

The general objective of this study is to provide and in depth report on the intricacies of the on-line SPE methodology, and to create an efficient novel procedure to assess FQs occurrence in aquatic environmental compartments. The specific objectives were:

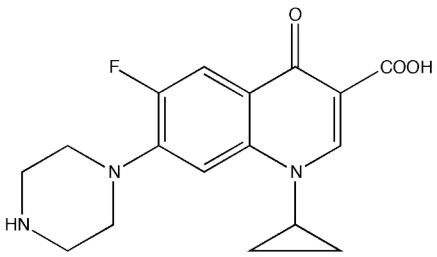
-To develop an on-line SPE method to quantify the FQs namely ofloxacin (OFL), norfloxacin (NOR) and ciprofloxacin (CPF) – (Fig. 8);

-To validate several parameters of the method developed according to International Criteria;

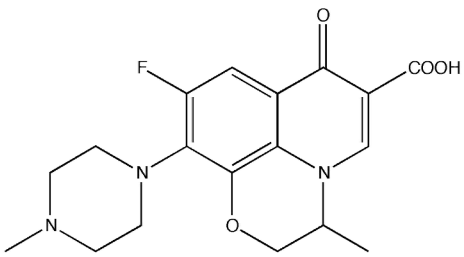
-To quantify the target FQs in WWTP's effluents located in around Porto, Portugal.



Norfloxacin



Ciprofloxacin



Ofloxacin

Figure 8 Target Fluoroquinolones

II. Materials and methods

II.1 Chemicals

The fluoroquinolone (FQs) antibiotics; ofloxacin (OFL), norfloxacin (NOR) and ciprofloxacin (CPF) standards were purchased from Sigma Aldrich (Product from China). All the used standards presented a purity degree above 98%. OFL, NOR and CPF stock standard solutions were prepared at 1000 µg/mL in water/acetic acid 10% (50:50 *v/v*) after which these stock standard solutions were stored at -20 ºC in amber bottles.

All the work standard solutions were prepared weekly and obtained via dilutions of the stock solutions performed in ultra-pure water/acetic acid 10% or in minimal salts medium (MM) composed of: $Na_2HPO_4.2H_20$, 2.67 g; KH_2PO_4, 1.40 g; $MgSO_4.7H_20$, 0.20 g and $(NH_4)2SO_4$, 0.5 g per liter and added 10 mL of a trace elements solution with the following composition per liter: NaOH, 2.0 g; $Na_2EDTA_2.2H_2O$ 12.0 g; $FeSO_4.7H_20$ 2.0 g; $CaCl_2$, 1.0 g; Na_2SO_4, 10.0 g; $ZnSO_4$, 0.4 g; $MnSO_4.4H_2O$, 0.4 g; $CuSO_4.5H_2O$, 0.1 g; $Na_2MoO_4.2H_2O$, 0.1 g and H_2SO_4 98%, 0.5 mL.
The Ethanol (EtOH) HPLC grade was purchased from Fisher Scientific (Product from the UK). Triethylamine (TEA) with ≥ 99% purity was obtained from Sigma Aldrich (Product from the USA). The acetic acid, trifluoroacetic acid (TFAA) and sulfuric acid were purchased from VWR (Product from the EC), Merck (Product from Germany) and Sigma-Aldrich (Product from Germany) respectively. Ultra-pure water was supplied by a Milli-Q water system. Finally while HPLC grade methanol (MeOH) was acquired from Sigma-Aldrich (Product from Israel).

II.2 Chromatographic instruments and conditions

The column chosen to perform this work was a Luna PFP (2), pore size 100 Å, particle size 3 µm, 150 x 4.6 mm, a modified reverse phase silica-based column, from Phenomenex (Product from the USA). And the pre-column used to performed the on-line SPE method was a special reverse phase Lichrospher® RP-18 ADS (Alkyl-Diol-Silica) with a pore diameter of approximately 60 Å, particle diameter 25 µm, 25 x 4 mm (length by diameter), (RAM) from Merck (Product from Germany).

The chromatographic equipment used in the analytical analysis was a Shimadzu UFLC Prominence System equipped with four LC-20AD Pumps, an SIL-20AC Autosampler, a CTO-20AC Column Oven, the Shimadzu FCV-12AH flow control valve, a DGU-20A5 Degasser, a CBM-20A System Controller and a LC Solution, Version 1.24 SP1 (Shimadzu). The Fluorescence Detector (FD) coupled to the LC System was a Shimadzu RF-10AXL.

The mobile phase consisted in an isocratic mixture of ultra-pure water with 0.1% TEA and pH=2.2 adjusted with TFAA (Eluent A) and EtOH (Eluent B) 76:24 (v/v) and 0.5 mL/min of flow rate in the pump A/B system which conditioned the analytical column and transfered analytes from the pre-column. In the C/D pump system, which conditioned the pre-column and performed the sample cleanup, an ultra-pure water (Eluent C)/methanol (Eluent D) 80:20 (v/v) mixture was used at a 1.5 mL/min flow rate. The analysis was executed with an injection volume of 500 µL and a column oven temperature of 45 ºC. The FD was set to an excitation wavelength of 290 nm and an emission wavelength of 460 nm, this factor was inherited from previous projects. The ideal combination of emission and excitation wavelengths had been observed and studied for all three of our target FQ compounds for an optimal signal that would reduce time in the analysis process and maintain the acquisition of a good quality signal (Maia et al., 2014).

II.3 Parameters validation

II.3.1 Linearity

Calibration curves were performed with eight different concentrations (each one done in triplicate) of the standard solutions in the minimal salts medium for every compound: 0.3 ng/mL, 0.5 ng/mL, 0.7 ng/mL, 5.0 ng/mL, 10.0 ng/mL, 15.0 ng/mL, 20.0 ng/mL and 30.0 ng/mL. The aforementioned curves were obtained by linear regression corresponding to the correlation between the peak area and the nominal concentration.

II.3.2 Accuracy

Method accuracy was determined to each analyte using three different concentrations, 0.9 ng/mL, 13.0 ng/mL and 25.0 ng/mL, which represent 3

times the limit of quantification, approximately 50% of the upper limit of the curve and approximately 75% of the upper limit of the linear calibration curve, respectively, as per the FDA Guidelines for method validations. All concentrations were done in the MM in triplicate, and after analysis they were plotted in the calibration curve from where their concentration was extrapolated and expressed in percentage when compared to the nominal concentration.

II.3.3 Precision

Method precision was considered by the analysis, in triplicate, of three different concentrations of each compound in the MM and expressed as relative standard deviation (RSD) of intra-day (three determinations executed in the same day) and inter-day (three determinations executed in three different days) assays.

II.3.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LODs and LOQs were determined based on the signal-to-noise ratio. Determination of signal-to-noise ratio was performed by comparing measured signals from samples with low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected or quantified. A signal-to-noise ratio of 3:1 is generally acceptable to estimate the LOD as a signal-to-noise ratio of 10:1 is typically considered to estimate the LOQ.

III. Results and Discussion

III.1 LC-FD method development

Liquid chromatography (LC) is the most common method for the analysis of FQs, but even within LC there are different variations, mostly in terms of detection, that give rise to various opinions and preferences in methodologies. The reported methods for detection of FQs in biological and environmental matrices are normally the following:

- Ultra-violet (UV) (Marazuela and Moreno-Bondi, 2004);
- FD (FDA, 2012) (Shen et al., 2004);
- Mass Spectrometry (MS) (Nakata et al., 2005);

When faced with the need to ascertain the presence of a target compound the preferred method is normally LC hyphenated to mass analyzer given that its highly sensitive, selective and allows for the determination of multiple compounds (Schneider and Donoghue, 2003) and, depending on type of mass analyzer, the possibility to predict the metabolites (Maia et al., 2014).
This feature of the detector is not the only possible amendment to the methods, the manner to the pre-treatment of samples is also an important feature, which brings us to the on-line SPE method. Aforementioned above is the necessity for biological matrices to go though some pretreatments prior to analysis. On-line SPE permits to perform the sample pre-treatment (SPE) within the LC system.

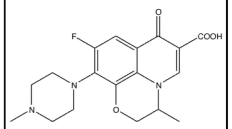
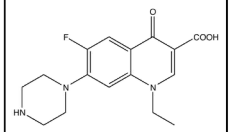
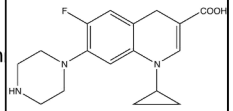
The LC analytical method has been chosen to analyse the target FQs (Table 4) since it carries the benefits of being simple, quick and has the possibility to be associated with sample preparation in the two dimensional system (2D-LC).
Although an UV detector has the capability to detect FQs, the chosen and preferred is the FD, which was mainly selected given the ability of FQs to fluoresce, which in turn allows us to take advantage of the sensitive and selectivity provided by this type of detector, which minimizes the matrix interferences (Maia et al., 2014).
The final objective when trying to develop a novel LC method is normally to be able to achieve separation between all analytes in the least possible time of analysis interval. With this in mind, multiple variables and chromatographic

paramaters require adjustment. Variables such as: column oven; temperature; mobile phase; flow rate; or even injection volume.

All samples were prepared by dilution of the mixtures previously stated in section II.1. It is important to mention thought that given the properties of wastewater effluents and their bacterial components, plus FQs physical chemical properties, all samples were acidified using sulfuric acid to pH 2.8.

Before it is possible to validate the method and display its use in the application step, it is required that as much as possible the chromatographic parameters of the method are adjusted and fine tuned until the best results overall are achieved. As such it is required that multiple attempts at a given step be performed and, even then after one parameter is optimized another might suffer as a consequence and needs further optimization. It has to be acknowledged that the method was based on previously work developed in the group (Maia et al., 2014).

Table 4 Structure, Pharmacokinetics and Properties of 3 target Fluoroquinolones (adapted from (Sharma et al., 2009))

Name	Structure	Pharmokinetics			Properties				Clinical information	Ref
		AUC (mg/h/L)	1/2 life (h)	Cmax (mg/L)	Mol. Weight	pKa	Hidrosulability (g/L)	LogP		
Ofloxacin		7,67	5,32	0,87	361,37	5.97; 8.00	1,44	-0,39	Complicatedurinary tract infections; Gastroenteritis with severe diarrhea prostatitis and nosocomial infections; STD's; Anthrax	(Cheng et al., 2007, Yuk et al., 1991, Immanuel et al., 2002, Hameed et al., 2002, Israel et al., 1993, Lode et al., 1987)
Norfloxacin		1,77	3,7	0,33	319,33	6.36; 8.70	1,01	-1,03	Uncomplicated urinary tract infections	(Cheng et al., 2007, Lee and Ronald, 1987, Corrado et al., 1987, Heseltine and Corrado, 1987, Kaplowitz et al., 1987, Hooper and Wolfson, 1985, Adhami et al., 1984)
Ciprofloxacin		2,56	4,16	0,56	331,34	6.09; 8.62	1,35	0,28	Complicatedurinary tract infections; Gastroenteritis with severe diarrhea prostatitis and nosocomial infections; STD's; Anthrax	(Oliphant and Green, 2002, Lubasch et al., 2000, Dieter et al., 2004)

III.1.1 Analytical Column Mobile Phase

The mobile phase chosen for the primary pump system (A/B), which transfers the FQs (OFL, NOR, CPF) into the analytical column as well as conditioning it, was optimized to allow the best performance for the two dimensional system.

The mobile phase used was a mixture of two eluents: aqueous solution of 0.1% TEA acidified to pH = 2.20 with TFAA (eluent A) and EtOH (eluent B). Eluent A was chosen for the presence of the basic component TEA, which aided in the improvement of the resolution and peak symmetry, by lessening the "tailing", which is a commonly enough problem that occurs in reverse mode chromatography of basic analytes, by blocking the available silanol groups in the stationary phase. The use of TFAA was mostly to assist in the suppression of ionization of acid analytes, which in turn result in slimmer chromatographic peaks (Maia et al., 2014). Eluent B (EtOH) on the other hand had much more direct reasons for its choice. In spite of its efficiency in accomplishing its role in the analytical method, its choice went in hand with its environmental characteristics, which are more ecologically responsible than other organic solvents vastly used in LC, such as MeOH and ACN. Not only this but EtOH carries a slightly smaller financial cost and is more readily available industrially than its counterparts. After many attempts the optimized mobile phase for the separation of targets FQs consisted on 0.1% of TEA adjusted to pH=2.2 with TFAA and EtOH (76:24 *v/v*), at a flow of 0.5 mL/min (Fig. 9).

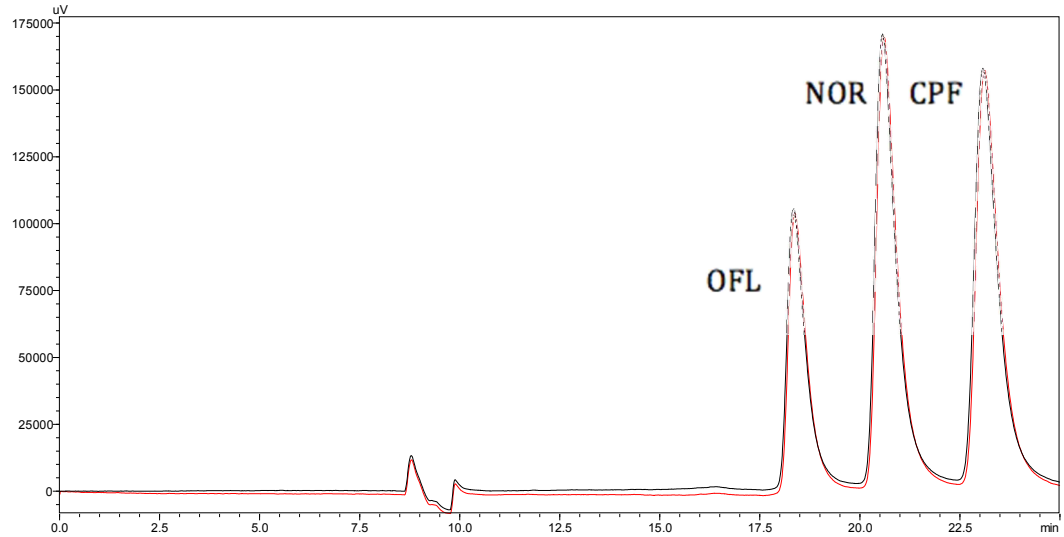


Figure 9 FQ Standards 12 ng/mL - Final Conditions

III.1.2 RAM column mobile Phase – First Dimensional Optimization

Firstly it is important to establish the objective of the RAM column, which is not to separate our target compounds. The RAM column bears the same task as SPE used in off-line sample preparation, as such, its intended purpose is to clean the matrix of as many impurities as possible.
The SPE-LC-FD system works in two dimensions, the first dimension is the extraction procedure and the second dimension is the analyses of the target. The mobile phase in the first dimension acts in two separate steps: the cleanup and the washing. The cleanup step is the initial procedure in our method, where the raw sample is injected, the analytes are trapped, and where it is necessary to eliminate matrix interferences. On the other hand the wash step is where the RAM column should be cleared of any remains that did not get transferred to the analytical column, being it analytes or impurities. From these operational objectives it was established that we required a strong organic solvent to remove matrix interferences, and at the same time a weaker one that would not be strong enough to drag our analytes out of the RAM column.
As such the optimized mobile phase was a mixture of ultra-pure water and MeOH in 80:20 (*v/v*), with the water acting as the cleaning agent and the MeOH as the washing agent, and thus we were able to achieve the desired effects through manipulation of this balance during each run. It is important to mention that MeOH was not our first choice, as the method first was applied with 100% of ultra pure water and then with 5% of ACN in water.

The developing method was attempted with two different matrixes: a sample of a complex matrix that had been submitted to pre-concentration by SPE using HLB Oasis cartridges and standard samples made in acidified water. These were chosen to accurately observe the methods ability to clean a sample and its recovery of the target FQs, respectively.

The pre-concentrated samples consisted in very complex matrix, it was clear that the cleanup step was not being effective, as such, water alone was no longer a viable choice for the mobile phase for the cleanup.

Furthermore, it is important to note that the optimized conditions were demonstrated to be feasible for quantifying the FQs in effluent of WWTPs and bioreactors, after filtration. This is because it is similar to the original complex matrix used for the optimization conditions. The limit of quantification (LOQ) and limit of detection (LOD) was determined in both matrices.
Finally, the validation was carried out in a MM, which completely resembled the effluent of WWTP and bioreactors. This was to avoid performing the validation in a matrix which contains endogenous FQs. The MM consisted in a complete blank matrix.

To improve the aforementioned cleanup, many steps were taken to fully optimize the method. The first of which was changing the pH of the mobile phase.

Water pH variation

Lowering the pH of the water (Eluent C) produced two relevant results:
Firstly, the recovery rate of the target analytes increased considerably (Fig.10). After testing water at pH 2.3 and 4 it was noticed that at both interferences were becoming more visible and as a result resolution was worst. At pH 3 though, with the exception of an artifact before the OFL peak, the chromatograph seemed cleaner but the peaks had become slightly broader. The most efficient result was at pH 3.

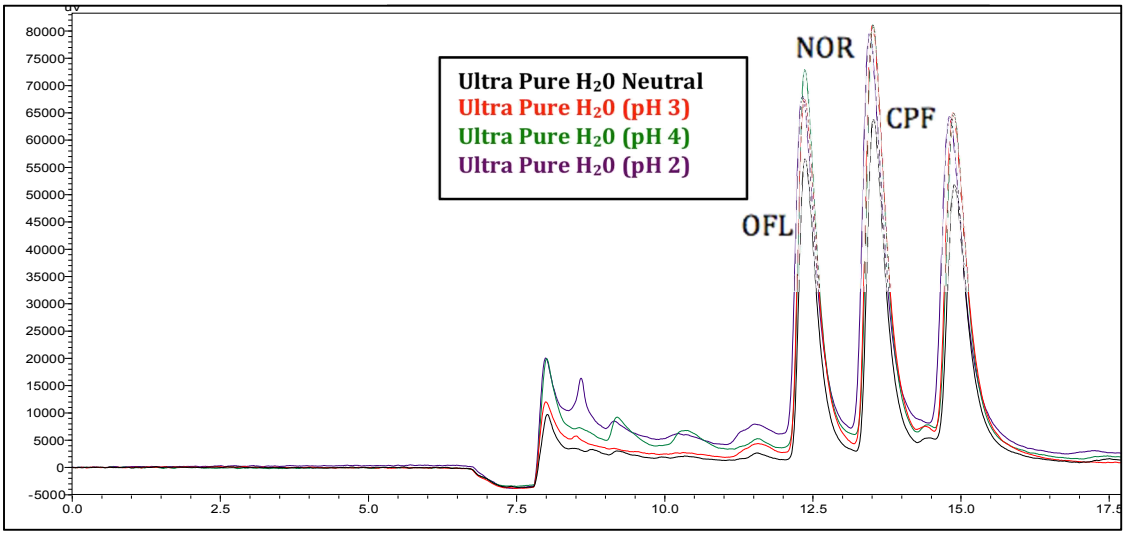


Figure 10 Eluent C: water pH variation in fluoroquinolone standards

And lastly, when performing tests on a more complex mixture, it was observed that this increase in recovery is a result of a less efficient cleanup of the sample and in spite of having better peaks in lower pH values, it was still clear that the least compromising value that provided better recovery was around 3 (Fig.11).

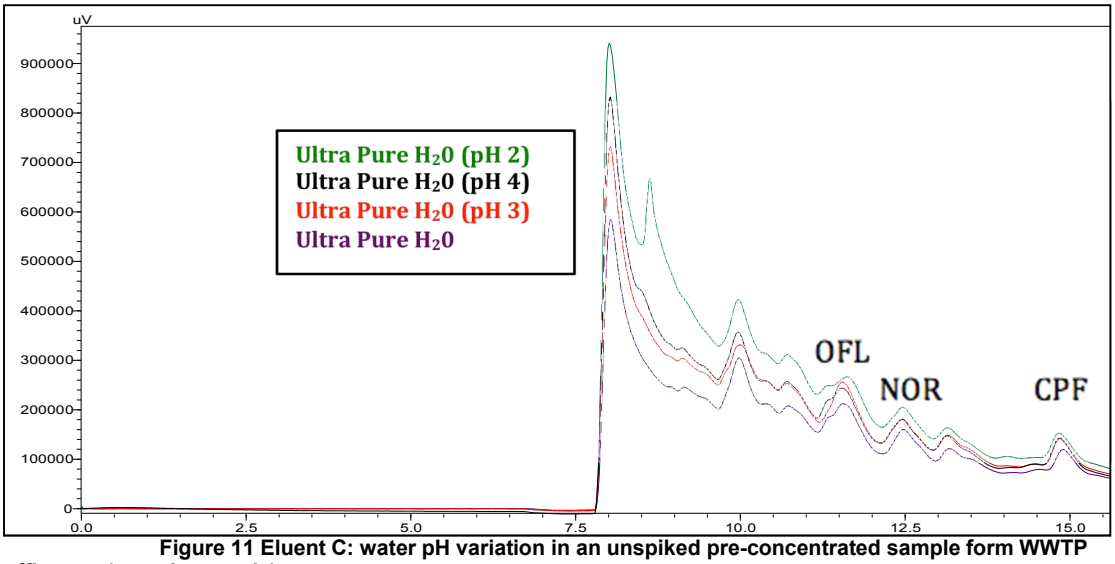


Figure 11 Eluent C: water pH variation in an unspiked pre-concentrated sample form WWTP effluents (complex matrix)

Due the zwitterionic form of the FQs alkalinization was also attempted but no positive result was achieved so it was immediately discarded (data not shown). With these results it was clear that water alone was not adequate to produce good results in recovery and cleanup. As such the next step would be to introduce an organic eluent in contrast to the water, and acetonitrile was chosen. The organic eluent has greater affinity towards the FQs and as such interacts more strongly with them.

Acetonitrile (ACN) proportion

The 100% water was replaced with a mixture of 5% ACN (in water) and 10% ACN (in water). The resulting chromatographs of FQs standards had an approximate 2% peak area increase in 5% ACN given that the baseline lowered in comparison to our original method, which was already a sign that the cleaning process was occurring in a more efficient fashion (Fig.12). Although there was a slight improvement, as we increase the ACN % the baseline did not display any further enhancement, worsening in the 3rd peak, and the peak height decreased as well, leading to an overall recovery rate drop in the range of 7 to 14%. At this point the stage where the ACN strength was too high had

been reached and not only removed impurities but cleared our samples as well (Fig.12).

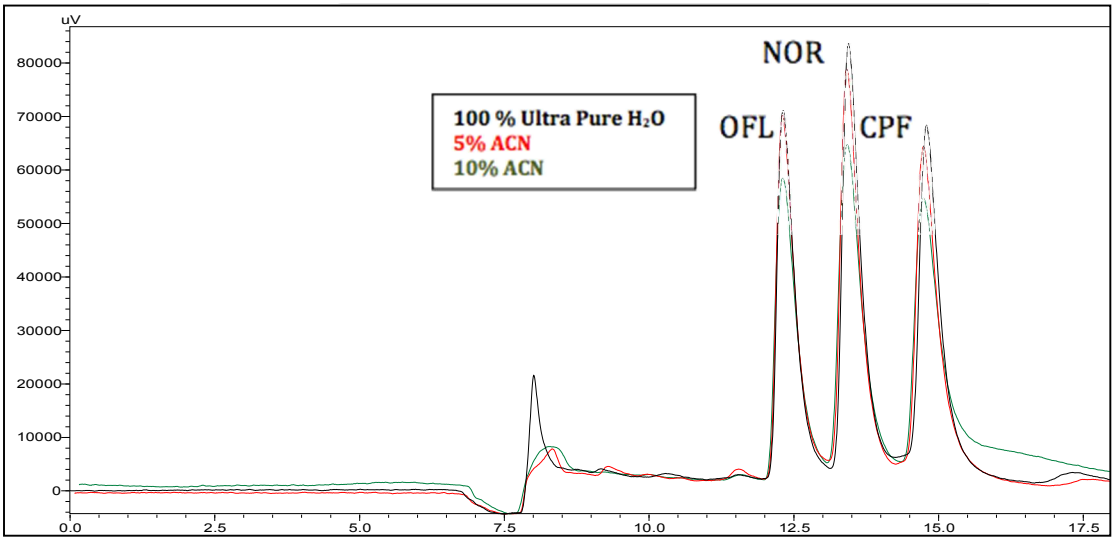


Figure 12 Eluent C: eluent variation in standards; 100% H_2O vs 5% ACN vs 10% ACN

On the other hand, if we observe the chromatograms for the complex matrix (Fig.13) samples, substantial progress had been made in the cleanup of the samples. However peak height greatly decreased, some even being eliminated, which in part was due to another factor that also contributed to this outcome, which was a considerable broadening of peaks.

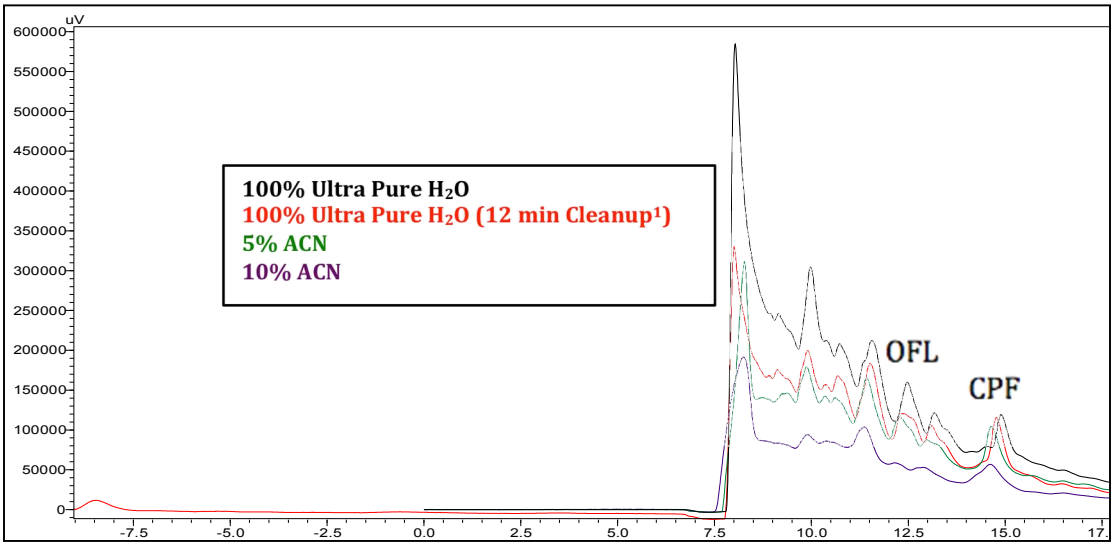


Figure 13 Eluent C: eluent variation in a complex matrix; 100% H_2O vs 5% ACN vs 10% ACN;

1- Initial cleanup was 3 min, 12 displayed better results, and will be mentioned in other section;

At this point it was opted to use the 5% ACN method as the standard procedure, but there was one more option to be tested, MeOH, and as such better results were still a possibility. This was due to the fact that MeOH has

chemical properties which make it a weaker organic solvent than ACN, but presented stronger interactions with our samples than water. This opened the prospect of achieving a method that, with enough MeOH, could clean like ACN and not drag our components.

20% methanol *vs* 100% ultra-pure H_2O *vs* 5% acetonitrile

Further ahead will be displayed the test results which allowed us to reach the conclusion that 20% was the ideal percentage of MeOH, but to sum it up, it was the value which produced the most similar result to a 10% ACN cleanup (the best cleanup we had) without significantly compromising the recovery.

From Fig. 14 it is observable that both the 5% ACN and 20% MeOH increased our recovery rates given their greater affinity to our samples. Although visually the others seem superior, the MeOH produces lower baselines and a better cleanup of the sample, effectively improving our peak areas and, as a result, the recovery rates.

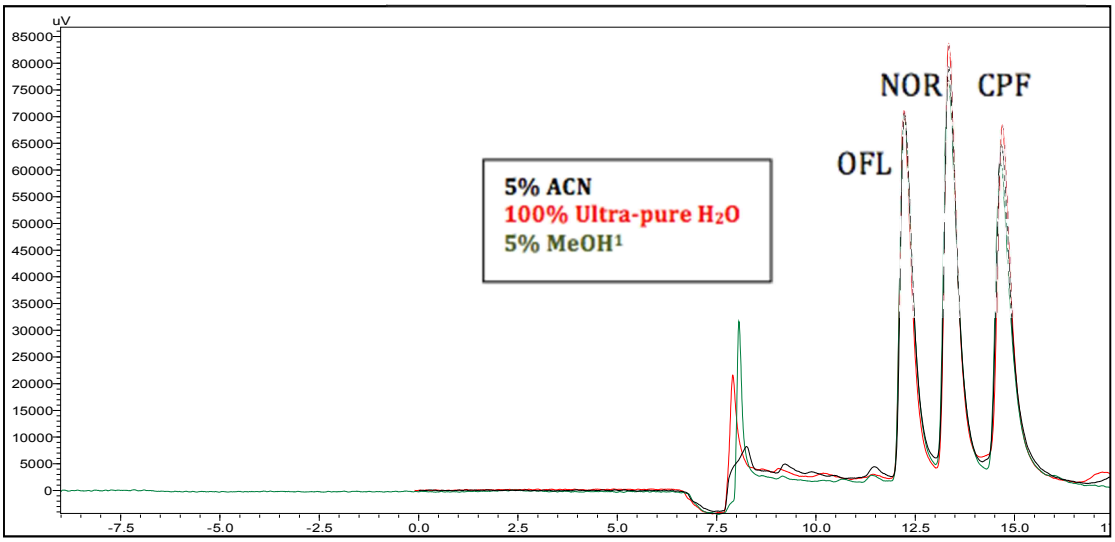


Figure 14 5% methanol VS 100% ultra-pure H_2O VS 5% acetonitrile (fluoroquinolone standards)

1- 20% methanol decreased peak height as it will be shown, but not the results from the cleanup outweigh the loss of analyte

Although for standards 5% ACN might appear superior if 5% MeOH was already so similar the great difference came when verifying results in the complex matrix observable in Fig. 15. There are by far more gains in taking advantage of MeOH's reduced organic strength to use a higher percentage of it in the cleanup while maintaining good compound recovery.

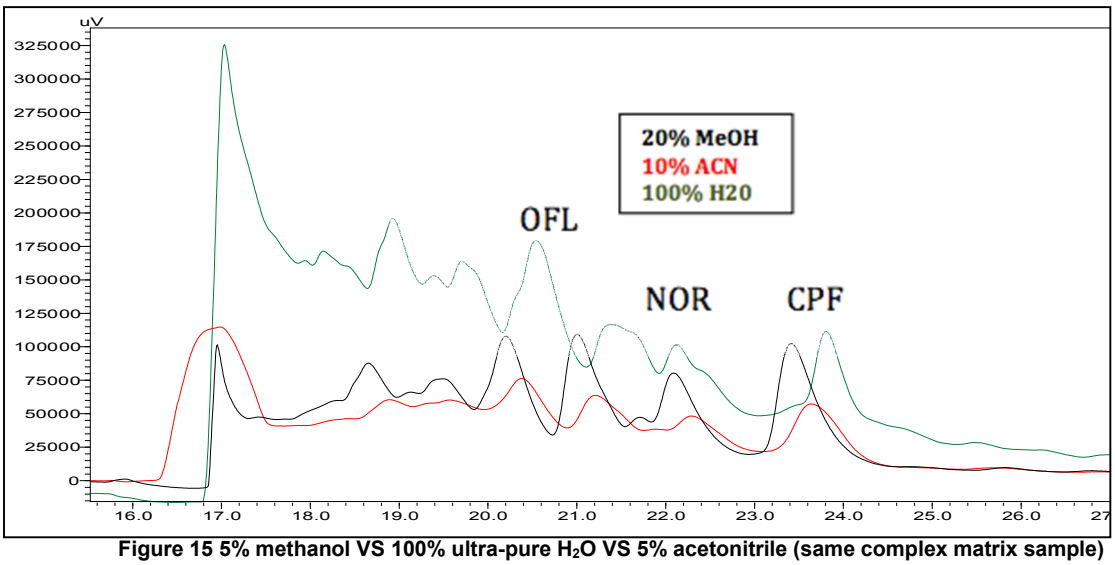


Figure 15 5% methanol VS 100% ultra-pure H_2O VS 5% acetonitrile (same complex matrix sample)

With 20% MeOH acting as strong as 10% ACN and still maintain such peak resolution in a sample that has so many impurities, it was chosen as the optimal eluent for our RAM column pump system. The final mixture proportions were set at 80:20 (v/v), Ultra-pure water:methanol.

Once these major factors were established, what were left were the fine adjustments required for the optimal chromatographic results with the two dimensional system.

- Column oven temperature
- Fine adjust of the mobile phase
- Mobile phase flow in the analytical column
- Mobile phase flow in the RAM column
- Transfer phase time
- Cleanup phase time
- Wash phase time
- Injection volume

III.1.3 Final Optimization

Column oven temperature

The column backpressure and viscosity of mobile phase are limiting factors, temperature plays an important role in the system performance as increasing it can lead to a reduction back pressure column and increase resolution in a shorter analysis time. The effects of temperature modification as a means of obtaining better peak shape and faster separations has been reported (Gotta et al., 2010). The selected temperature to take full advantage of this inherent effect was 45 ºC, as our column had a limit of 60 ºC and, as a preventive measure, if not necessary to further increase temperature we would keep it at approximately 70% of the maximum.

This variable remained unchanged throughout the other attempt variations. Next step was adjusting the composition of the mobile phases.

Analytical mobile phase adjustment in the second dimension (Analytical column)

Increasing the EtOH percentage in the second dimensional (analytical column) effectively provided a better recovery rate, but it came with consequence as the peaks also eluted sooner, decreasing resolution and causing co-elution (Fig. 16).

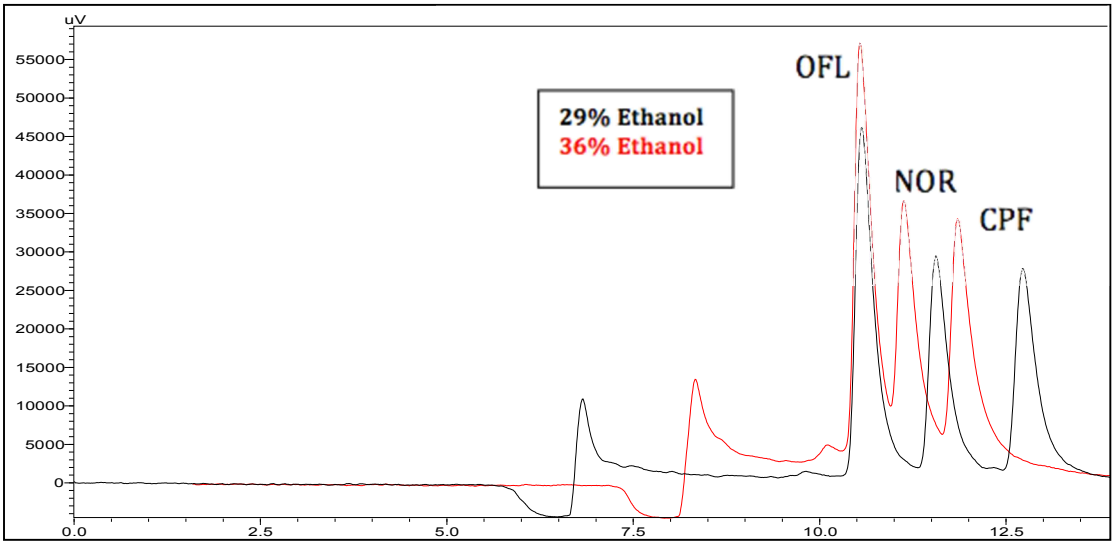


Figure 16 Effects of ethanol percentage in the analytical mobile phase

The optimal percentage was found to be between 24-30%, this range is important because as we adjust other variables, peaks may co-elute or peak

recovery might worsen. Higher EtOH % would provide better peak signal but worse the resolution and lower EtOH % worse the recovery and increase resolution. As a result of all the fine adjustments the final ratio was established as 0.1% of triethylamine (TEA) adjusted to pH=2.2 with TFAA and EtOH (76:24 *v/v*).

RAM mobile phase adjustment (First dimension)

A conclusion reached from the results of previous experiments was that the MeOH required the use of higher percentages to achieve a similar or superior result to that of the ACN while at the same time help increase peak recovery.

In Fig. 17, as expected of a solvent stronger than the ultra-pure water, as the percentage of mobile phase increases more amounts of the analytes were dragged deeper into the RAM column, these result in lower peak height. But this difference not only is small but when precisely analyzing the results we can objectively say that the peak areas as MeOH increases are not significantly affected. As this happens the baseline also lowers providing better peak resolution that in turn adds to the individual peak areas. Furthermore, the real positive aspect of this methodology is observable in the complex matrix (Fig.18).

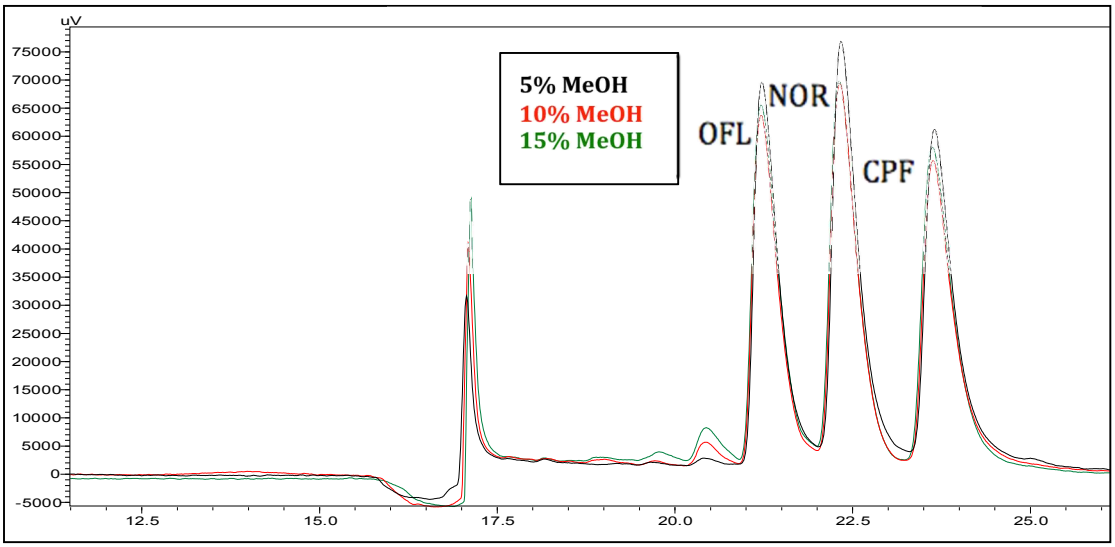


Figure 17 Effects of methanol percentage in the precolumn mobile phase to standard samples

As presented previously, 20% of MeOH in water is far superior than 100% of water and seems to be functionally equivalent to that of 10% acetonitrile without major losses. Given these results, the factor that we did not wish to sacrifice

any more recovery and that cleanup in our target did not display significant improvement, the optimal value kept in 20% MeOH (Fig. 18).

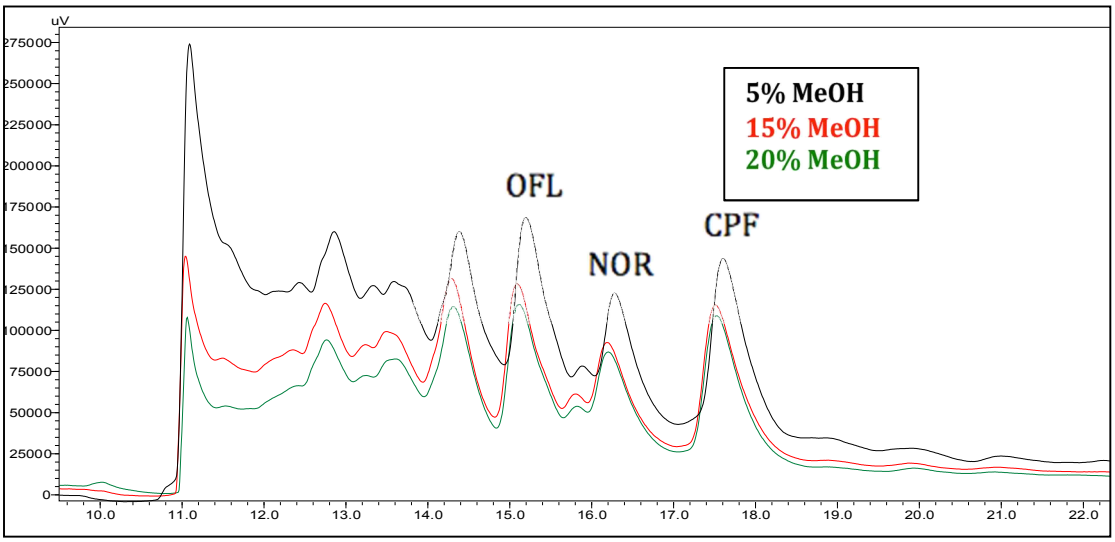


Figure 18 Effects of methanol percentage in the precolumn mobile phase to a complex matrix

With the mobile phases defined we moved to the adjustment on to the flow rate of each dimension.

Analytical column mobile phase flow

As per Fig. 19, reducing the flow leading to the analytical column we managed to, within the same run time, delay the elution of the analytes, granting us better separation without compromising the results in any way.

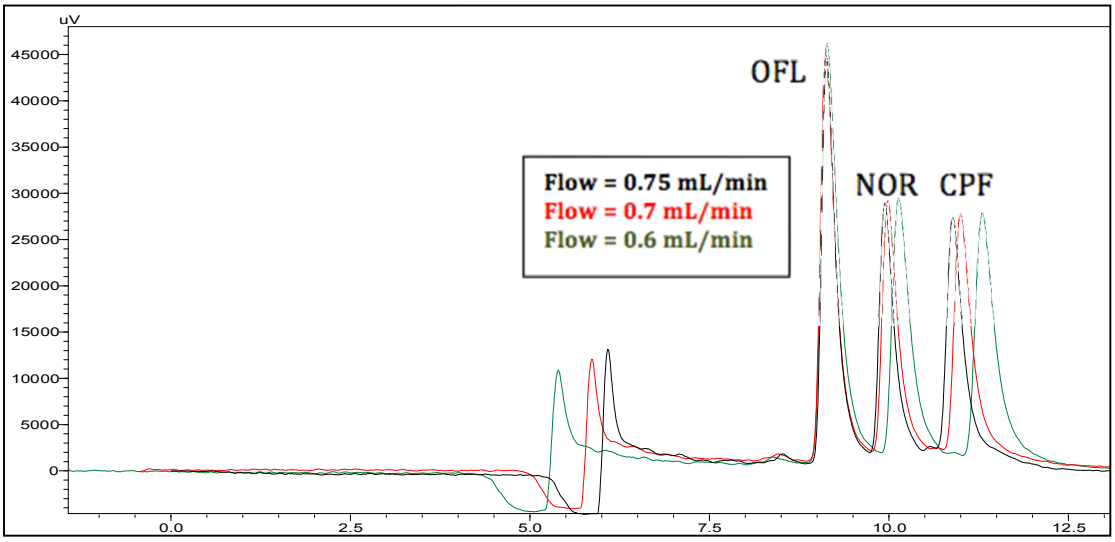


Figure 19 Effect of column mobile phase flow on standard samples

The minimum achieved flow without compromising resolution was 0.5 mL/min, which was selected as the default flow. In spite of no major disadvantages present in this factor, with exception to run time, lowering the flow too much

prevents analytes from being efficiently acquired and compromises their transference through the analytical column

RAM column mobile phase flow

Varying the RAM flow allowed to obtain slimmer peaks thus improving overall resolution. In addition several impurities observable in the chromatograms were removed or decreased (Fig. 20).

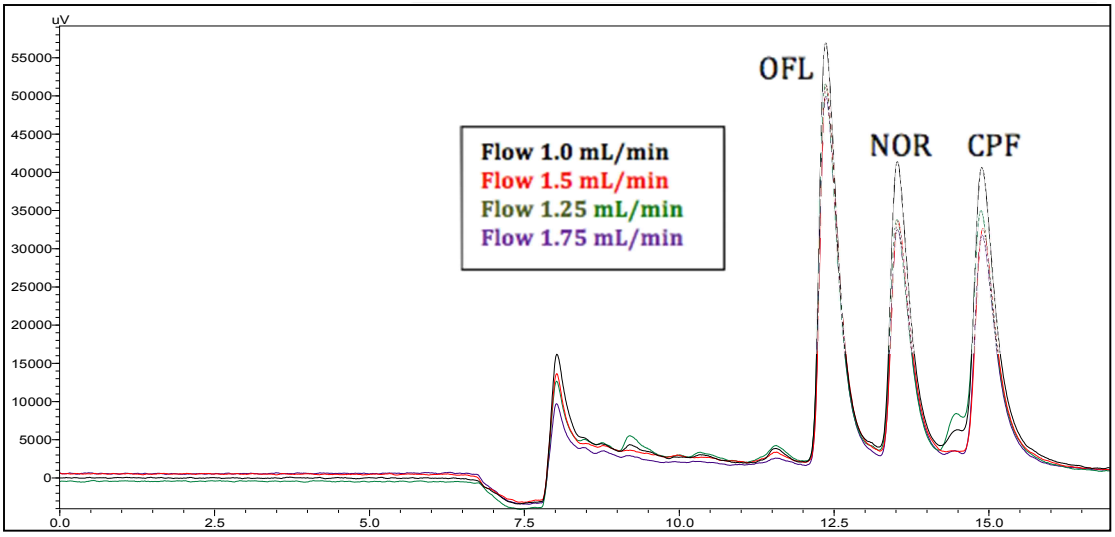


Figure 20 Effect of precolumn mobile phase flow on standard samples

In spite of having achieved better resolution the peak areas suffered, this is a result of analytes getting dragged further along the RAM column and being eliminated in the cleanup or not transferred. The final setting chosen here was 1.5 mL/min as any value above this presented no advantage, and values bellow worsen significantly the cleanup efficiency.

Transfer phase time

Visually observable right from the start is the advantage of increasing the transfer time (TT). In spite of there being slight increases in the levels of impurities detected, this increase appears to be of a smaller order than that of the cleanup achieved following the increase of the RAM column flow. But the major advantage noticeable when implementing the increase in transfer time was the increased recovery rate (Fig. 21).

This recovery is due to analyte transfer becoming considerably more efficient and as a result analytes which previously remained in the RAM column, causing *carryover* (noticeble with an EtOH injection made to check for this negative effect), are completely transferred to the analytical column.

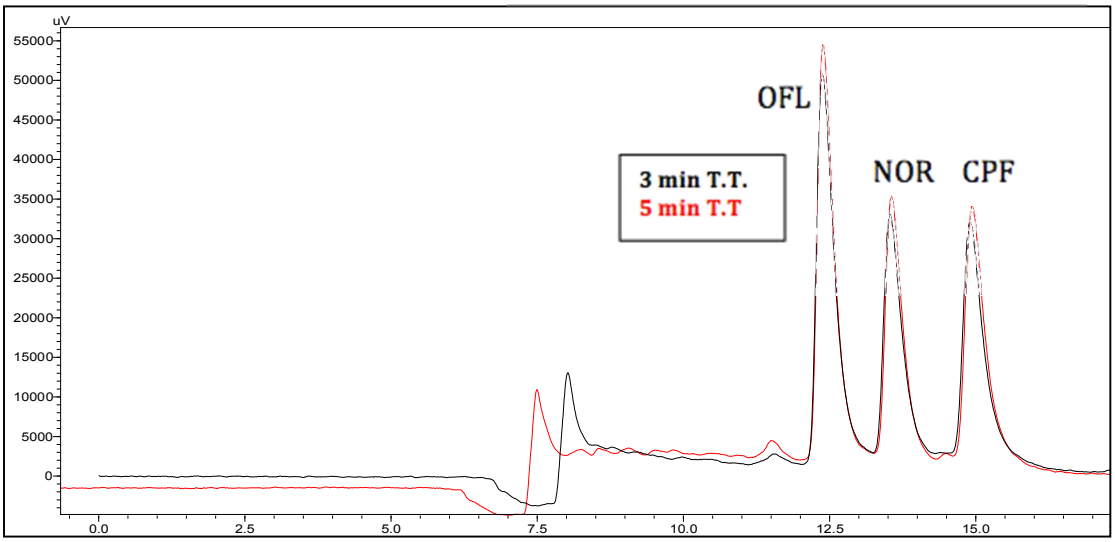


Figure 21 Effect of transfer time on standard samples

The method chosen to reach the optimal value was to perform a standard injection followed by an EtOH injection until there was no visible carryover or it was negligible. Through this system the ideal transfer time was set at 6 min as more presented no advantage and increased the run time far too much.

Cleanup phase time

The cleanup phase is important as it functions effectively as the SPE, and obtaining the least impurities is important when dealing with wastewater samples. For our standard samples a 3-minute increase (from 3 min to 6 min) in the cleanup time proved to be of no relevant consequence (Fig. 22). The only change was the expected elution of our target analytes 3 minutes later in the 6 min cleanup changing the elution times.

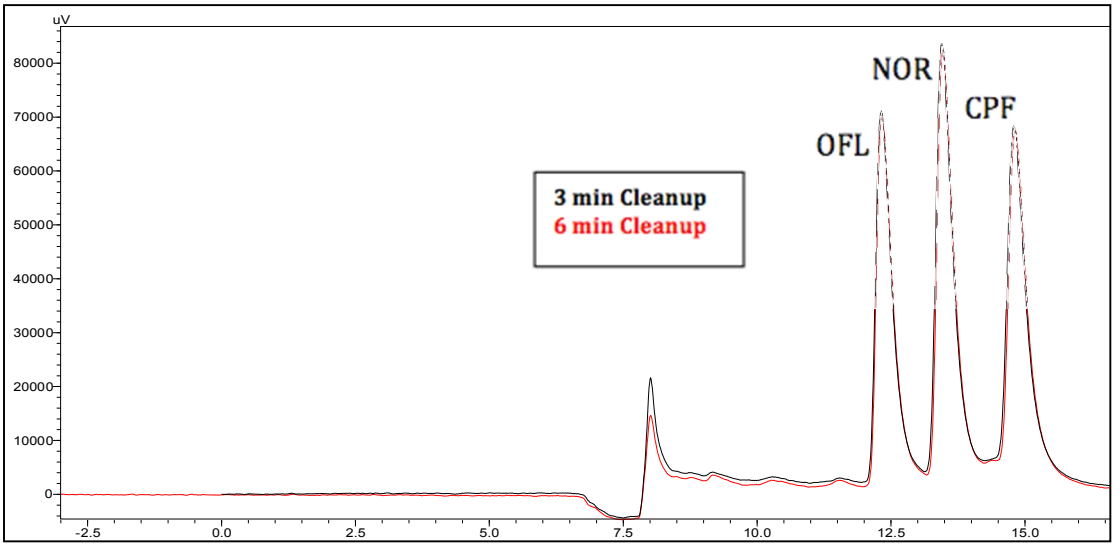


Figure 22 Effects of increasing cleanup time in standard samples

On the other hand, when applying that same change to an HLB extract sample (complex matrix) there was somewhat of an improvement to the sample cleanup. In response an attempt to further improve said cleanup was performed by further increasing the time from 6 to 12 min (Fig. 23).

It was noted that a substantial improvement had occurred and that despite some peaks becoming smaller, overall the cleanup was better. The final test was with a cleanup time of 15 minutes, at which point no further improvement in relation to the 12-minute cleanup was detected.

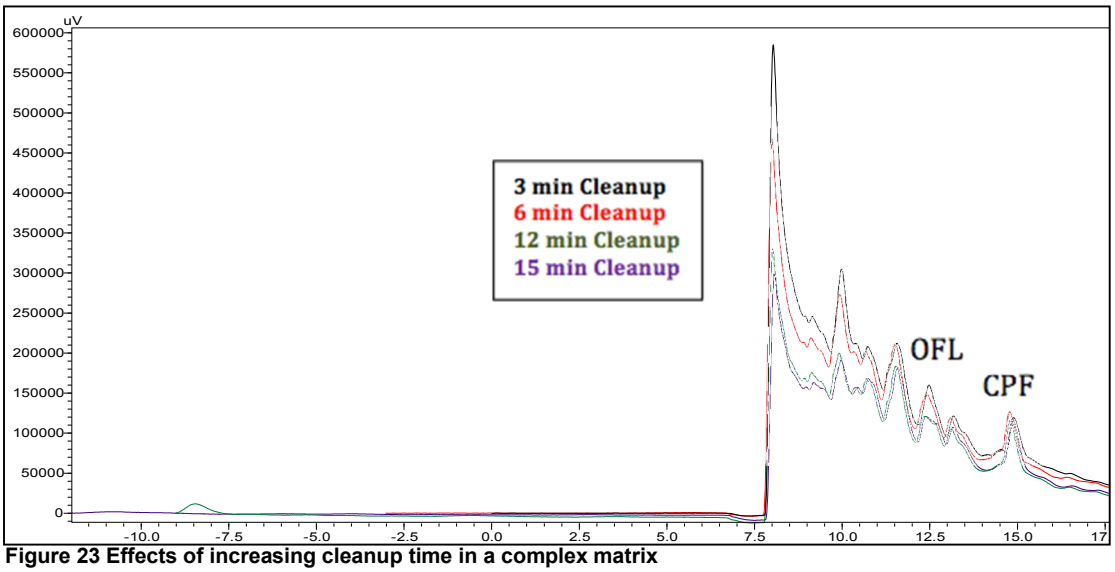


Figure 23 Effects of increasing cleanup time in a complex matrix

Even with the improvement in the removal of impurities it was deemed that a 12-15 min Cleanup Phase far too much time to consider optimal. Not only is there a lot of waste time when dealing with an extensive number of samples but there is chemical waste and it became impractical. In addition after further tests

were performed further in it proved to be acceptable to select a 5 min Cleanup Phase.

Wash phase time

Here it's a simple objective: to keep the total run time as short as possible while guaranteeing that the RAM column completely washed off analytes and interferences, being ready for a fresh injection. First step was simply to increase the amount of MeOH running through the RAM column after the transfer is done. As seen in the function of the on-line system (Fig. 7), in this position (the starting position) mobile phase goes straight through the RAM column to the waste. By increasing the MeOH to a 10:90 (v/v) water:methanol ratio we assure that the solvent with highest organic strength is carrying as efficiently as possible everything in the RAM column to the waste. All that was left was to make sure that when a new run started in an automated fashion the pressure of the pump system was stable at the original values, as such, five minute before the end of the run the proportion was return to the original values. It was concluded that a total of 14 min wash phase was enough to acquire the data of the target analytes, clean and re-stabilize the RAM column pressure in the most efficient matter.

Injection volume

Lastly, the idea of increasing the injection volume (initial 100 µL) as to also intensify the signal obtained from each analytes was attempted. In Fig. 24 the advantages of this are clear but the consequence in increasing the volume is that the base line between the targets is higher and as such the resolution decreases. The increase to 500 µL was considered quite beneficial and the drawback not relevant, not only this but, any further rise to the injection would become inconvenient towards the automation of the process given the vial volume restrictions and saturation of detector signal.

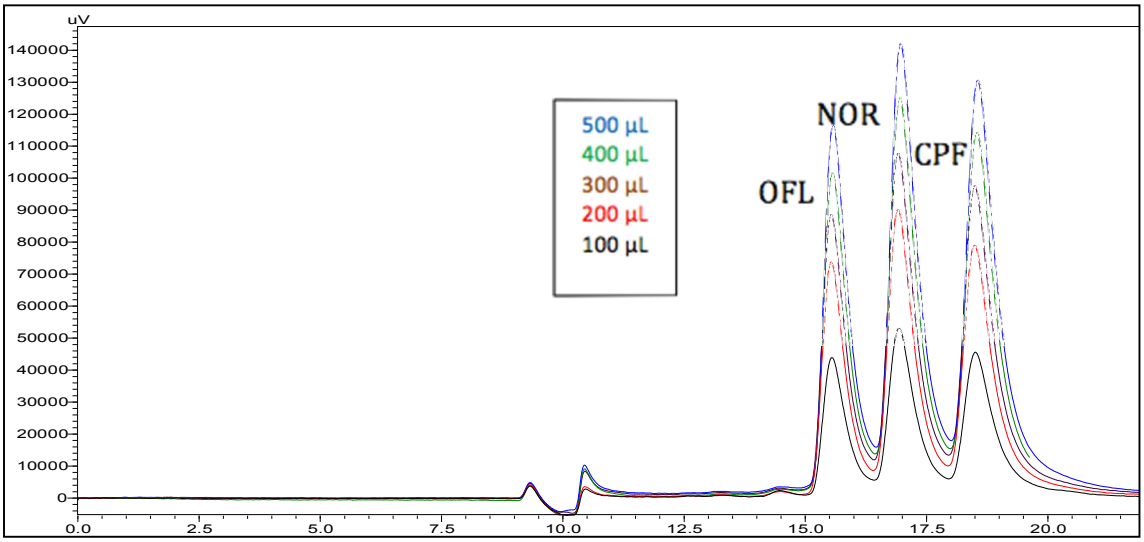
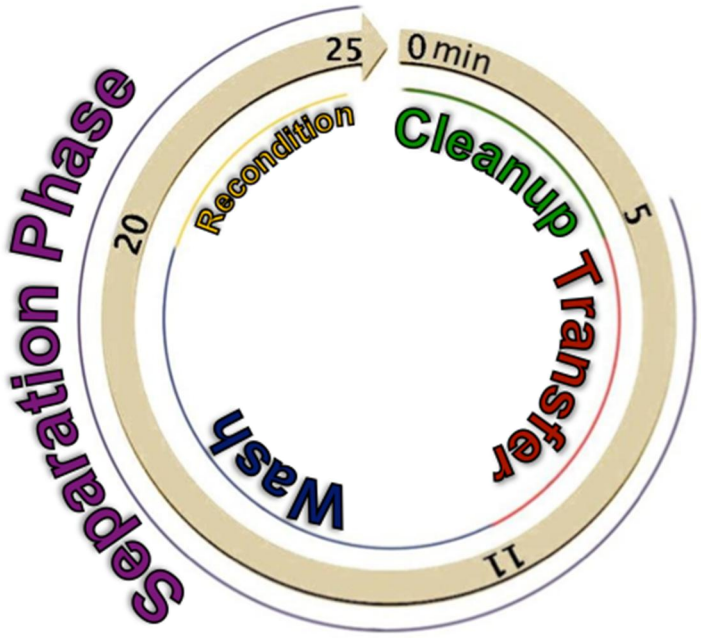


Figure 24 Injection volume comparison in FQ standards

III.1.4 Final Method

- RAM column flow: 1.5 mL/min
- Analytic column flow: 0.5 mL/min
- Temperature: 45ºC
- Injection vol.: 500 µL
- A/B analytic column pump system eluent ratio: 76:24 (v/v), 0.1% TEA:Ethanol
- C/D precolumn pump system eluent ratio: 80:20 (v/v), Water:Methanol
- Cleanup phase: 5 min
- Transfer phase: 6 min
- Wash phase: 14 min
- Total run time: 25 min



Mobile Phase Ram Column (Cleanup/Transfer/Recondition): 80:20 (v/v), Water:Methanol; Flow 1.5 mL/min

Mobile Phase Ram Column (Wash): 10:90 (v/v), Water:Methanol; Flow 1.5 mL/min

Mobile Phase Analytical Column (Cleanup/Separation): 76:24 (v/v), 0.1% TEA:Ethanol; Flow 0.5 mL/min

Figure 25 Final method chart

III.2 Method validation

The validation of an analytical method is necessary to show that the chosen methodology is appropriate to achieve the desired analytical purpose, as per the International Conference in Harmonization Q2B (ICH, 2005). The following paramaters were considered in the validation of this on-line SPE-HPLC-FD method:

- Linearity
- Accuracy
- Precision
- LOD
- LOQ

When performing the method validation, this should be carried out in samples with the target compounds in a matrix similar to that which will be encountered when applying the method. The method was tested in wastewater treatment plant (WWTP) effluents, bioreactor effluents, and in minimal salts medium (MM). Considering that both matrices (WWTP and bioreactor effluents) presented the target FQs and were similar enough to MM, the validation was carried out in the MM instead of the WWTP or bioreactor effluents.

III.2.1 Linearity

The capability of within a range achieving test results, which are proportional to the concentration of a given analyte in the sample, is the definition of an analytical procedure's linearity. For an LC method to have this characteristic there must be a linear relation between the absorbance and the concentration of the target compound's chromatographic peaks, within a defined range. The range of the analytical method is the interval where the lowest and highest concentration limits of an analyte in the target compound have shown to have an appropriate degree of precision, accuracy and linearity. As such, in the development of the linearity test for this work the concentrations chosen represent a range between the limit of quantification and the highest concentration that could be reached before saturation of the detector. According (ICH, 2005) a minimum of five different concentration levels is required to demonstrate linearity within the linear range, we chose to perform 8 to be able to follow the the US Food and Drug Administration's (FDA, 2012) guidelines as well. The results were statistically manipulated and treated using the software that accompanies the LC system, LC Solution, Version 1.24 SP1 (Shimadzu), which applied the necessary calculations and linear regressions. The calibration curves were performed with eight different concentrations, where each one was done in triplicate and each injected once, of the standard solutions in the MM for every compound: 0.3 ng/mL, 0.5 ng/mL, 0.7 ng/mL, 5.0 ng/mL, 10.0 ng/mL, 15.0 ng/mL, 20.0 ng/mL and 30.0 ng/mL. The calibration curves are here displayed in Fig. 26 - 28, and each was guaranteed to have correlation coefficients above 0.999.

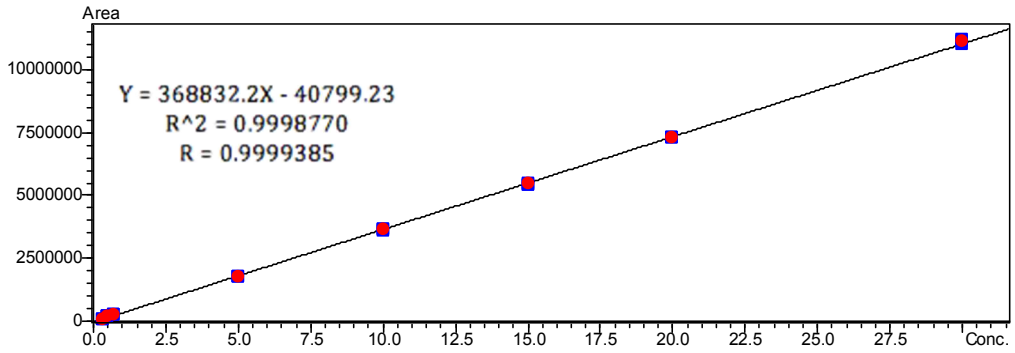


Figure 26 Ofloxacin calibration curve

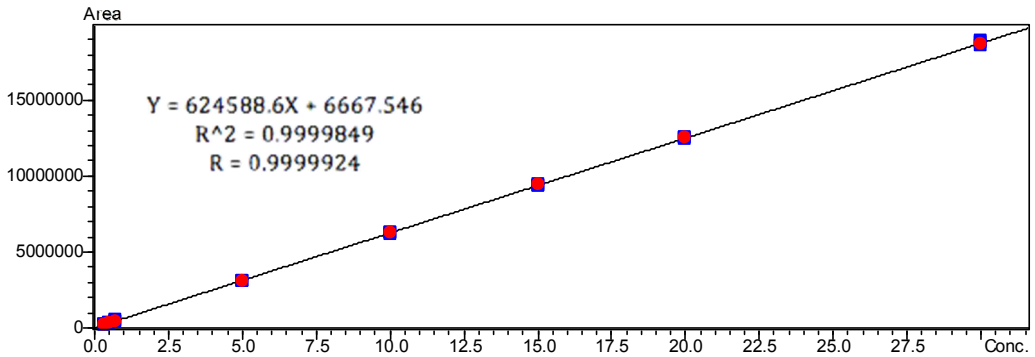


Figure 27 Norfloxacin calibration curve

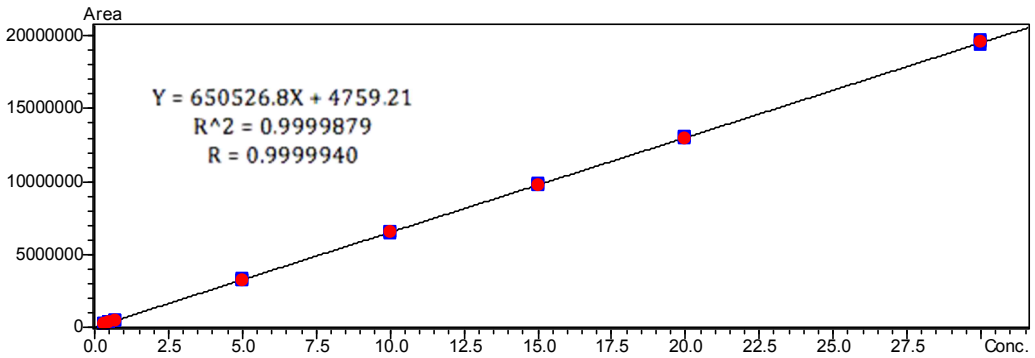


Figure 28 Ciprofloxacin calibration curve

III.2.2 Accuracy

The accuracy is the representation of how close the values found using the analytical method resemble the values that are accepted as the true value or a reference true value. As per the Q2B (ICH, 2005) guidelines, accuracy must be achieved through the use of three different concentrations in triplicate, and calculated by finding the correlation between the concentrations obtained via the peak area of the standards and the nominal concentrations. ICH recommends accuracy values for pharmaceutical compounds between 70% and 130% and the FDA between 80%-120%. The concentrations used to

determine accuracy were 0.9 ng/mL (three times the limit of quantification), 13 ng/mL (approximately 50% of the maximum calibration value), and 25 ng/mL (approximately 75% of the maximum calibration value). This was repeated during 3 consecutive days.

The obtained values are entirely included in both intervals mentioned and are as follows:

- Day 1 t_0

Table 5 Accuracy results t_o

	OFL		NOR		CPF	
	Avg. Acuracy	RSD%	Avg. Acuracy	RSD%	Avg. Acuracy	RSD%
0.9 ng/mL	105%	0.590%	98%	1.262%	100%	0.901%
13 ng/mL	100%	0.355%	99%	0.488%	100%	0.415%
25 ng/mL	101%	0.330%	101%	1.260%	102%	1.241%

- Day 2 t_{24}

Table 6 Accuracy results t_{24}

	OFL		NOR		CPF	
	Avg. Acuracy	RSD%	Avg. Acuracy	RSD%	Avg. Acuracy	RSD%
0.9 ng/mL	107%	1.381%	100%	0.840%	101%	1.247%
13 ng/mL	100%	0.527%	98%	0.664%	98%	0.657%
25 ng/mL	102%	0.553%	101%	0.603%	101%	0.606%

- Day 3 t_{48}

Table 7 Accuracy results t_{48}

	OFL		NOR		CPF	
	Avg. Acuracy	RSD%	Avg. Acuracy	RSD%	Avg. Acuracy	RSD%
0.9 ng/mL	108%	1.162%	101%	1.297%	102%	1.247%
13 ng/mL	100%	0.306%	98%	0.741%	97%	0.670%
25 ng/mL	102%	0.510%	100%	0.570%	100%	0.576%

III.2.3 Precision

Precision, in contrast to accuracy, is not having the ability to achieve the true result, but instead it's being able to obtain the same result when all conditions are the same. It is the expression of how close a series of measurements of a sample under the same analytical conditions are from each other. There are several ways to express this characteristic of the method, the common ones being: the variance, standard deviation or the coefficient of variation of a group of measurements. This work considered the attribute through the parameters of intra-batch precision and inter-batch precision in which the results were express in the form of % of relative standard deviation (RSD). The ICH recommends that both should be calculated from nine determinations, and from three different concentrations. The FDA recommends using a minimum of five determinations per concentration and having a minimum of three concentrations. Given these guidelines three concentrations were chosen: 0.9 ng/mL (three times the limit of quantification), 13 ng/mL (approximately 50% of the maximum calibration value), and 25 ng/mL (approximately 75% of the maximum calibration value). All of these were made in triplicates and each one of the 9 solutions (three times three concentrations) was injected twice.

Intraday

Intra-batch precision was studied throughout three consecutive days, by the analyses of the 9 solutions, all performed in the same day each day with controlled work conditions.

- Day 1 t_0

Table 8 Intra-batch precision Day 1 %RSD

	OFL		NOR		CPF	
	x	RSD%	x	RSD%	x	RSD%
0.9	308005.33	0.668%	552499.33	1.260%	591109.5	0.894%
13	4762520.17	0.358%	8022812.17	0.488%	8446905.17	0.415%
25	9295041	0.332%	15751069.2	1.260%	16552771.3	1.240%

- Day 2 t_{24}

Table 9 Intra-batch precision Day 2 %RSD

	OFL		NOR		CPF	
	x	RSD%	x	RSD%	x	RSD%
0.9	314085.333	1.561%	564001.5	0.839%	596698.67	1.237%
13	4739230.17	0.531%	7948357.67	0.664%	8315831.67	0.657%
25	9346782	0.555%	15756188.2	0.603%	16472184.2	0.606%

- Day 3 t_{48}

Table 10 Intra-batch precision Day 3 %RSD

	OFL		NOR		CPF	
	x	RSD%	x	RSD%	x	RSD%
0.9	317525.833	1.311%	567104.5	1.296%	741.972	1.237%
13	4757646.33	0.308%	7917994.5	0.741%	55245.826	0.670%
25	9327676.5	0.512%	15599140.5	0.570%	93624.006	0.576%

Every %RSD is below 1.6% as such no result is considered to be significantly different from one another and intra-batch precision has been established.

Interday

Inter-batch precision was observed by the analyses of the nine determinations during three consecutive days, with controlled work conditions. After which an average peak area value and standard deviation was calculated using all the results obtained throughout the three days for each concentration. Then the %RSD was extrapolated from those to values.

Table 11 Inter-batch precisions in 3 concentration ranges

	0.9 ng/mL	13 ng/mL	25 ng/mL
	RSD%		
OFL	1.743%	0.442%	0.499%
NOR	1.579%	0.825%	0.962%
CPF	1.229%	1.164%	1.132%

III.2.4 Limit of Detection

The LOD of the analytical method is the lowest concentration at which an analyte can be identified but not accurately quantified. Here it was chosen to calculate the detection limit through the signal-to-noise ratio. As defined in Q2B by the ICH (ICH, 2005) to determine the signal-to-noise ratio a comparison between blank samples and the signals from samples with known concentrations. This was done in a wastewater matrix to most accurately resemble working conditions, as such, the blanks might present signals at the locations desired. To prevent misinterpretations of the signal-to-noise ratios two methodologies were adapted to calculate these.

Given that some signal was already present in the blank in this method we calculated the noise differently. Like before the time interval was chosen for the low and high points avoiding the target signals in the blank. Then it was measured the peak height of the blank signals and they were subtracted from the sample peak height.

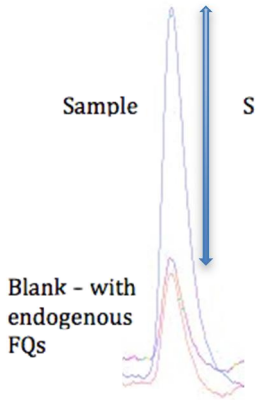


Figure 29 Example of the calculation method of the signal (S) used in the S/N ratio

A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the LOD.

Table 12 Limits of Detection

	Conc. ng/mL
OFL	0.2-0.3
NOR	0.07-0.08
CPF	0.07-0.08

III.2.5 Limit of Quantification

The LOQ follows the same rules and principles of the LOD, but the accepted signal-to-noise ratio is of 10:1. The LOQ is the lowest concentration at which an analyte in a sample can be accurately and precisely quantified.

Table 13 Limits of Detection

	Conc. ng/mL (Min)
OFL	0.3
NOR	0.3
CPF	0.3

III.3 Quantification of targets FQs in Portuguese WWTP effluents

Lastly, after the method was optimized and validated it was carried out as to observe its performance in actual, fresh, samples obtained from WWTPs.

Although the FQs in the samples recovered are bellow the established LOQ, it was possible for OFL to be quantified (Table 16) in the analyzed samples (Fig. 30-33) having and average concentration of 0.3 ng/mL, with exception of sample 2 (Fig. 31) where it is below the LOQ. NOR was not detected in any sample. CPF was clearly detected in every sample but proved to be below the LOQ, ranging from 0.15 to 0.21 ng/ml (Table 14). It is relevant to reinforce that

the LOQ could be lower if the same matrix (MM) used in the standards was used to determinate the LOQ.

Table 14 Detection of 3 fluoroquinolones in 4 different WWTP samples

OFL	Retention Time	Concentration (ng/mL)	Below LOQ
Sample 1	18.329	0.323	
Sample 2	18.282	0.205	x
Sample 3	18.348	0.297	x
Sample 4	18.373	0.322	
NOR	Retention Time	Concentration (ng/mL)	
Sample 1	-	-	
Sample 2	-	-	
Sample 3	-	-	
Sample 4	20.563	0.0289	x
CPF	Retention Time	Concentration (ng/mL)	
Sample 1	23.058	0.164	x
Sample 2	23.025	0.0316	x
Sample 3	23.105	0.213	x
Sample 4	23.141	0.157	x

If we observe the last peak in figures 30 through 33, correspondent to CPF, the previous statement regarding the LOQ becomes apparent as this peak is clearly separated in with high intensities but in spite of their good resolution they are below the LOQ.

Sample 1

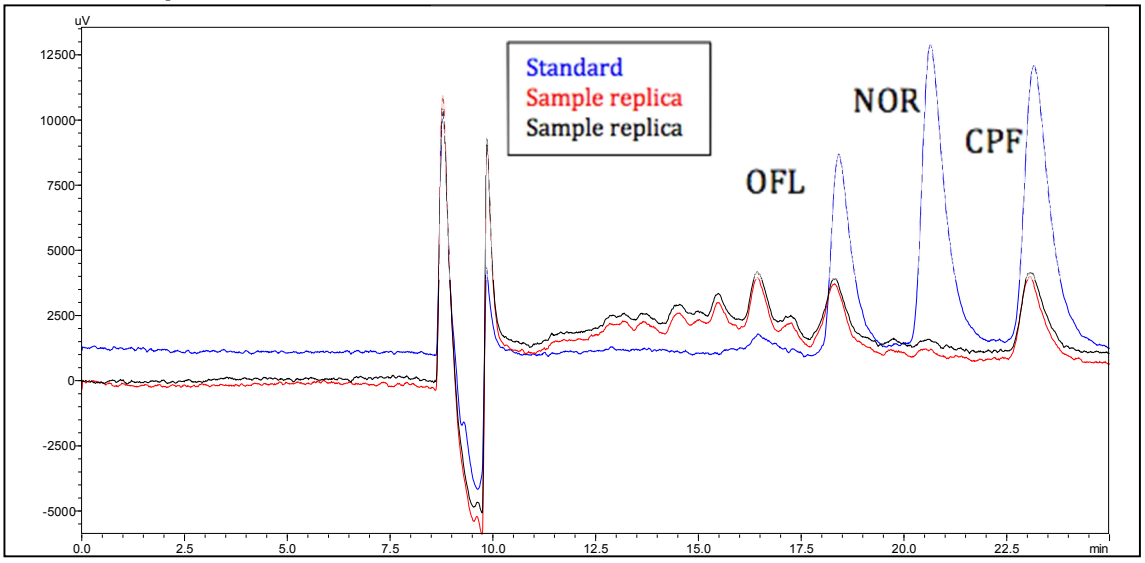


Figure 30 WWTP sample 1 analysis against 0.9 ng/mL standard sample

Sample 2

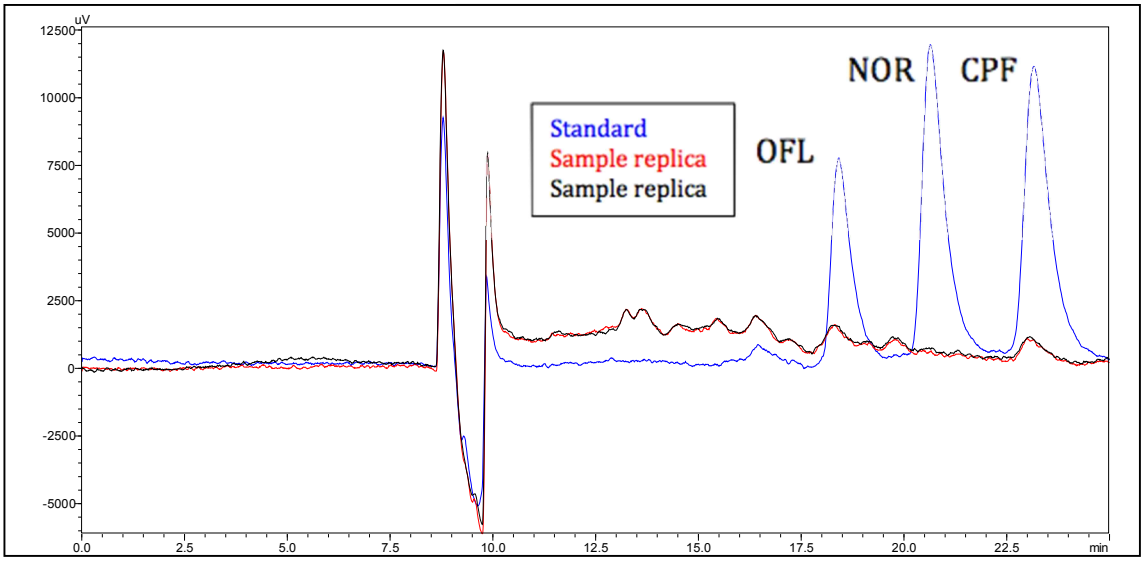


Figure 31 WWTP sample 2 analysis against 0.9 ng/mL standard sample

Sample 3

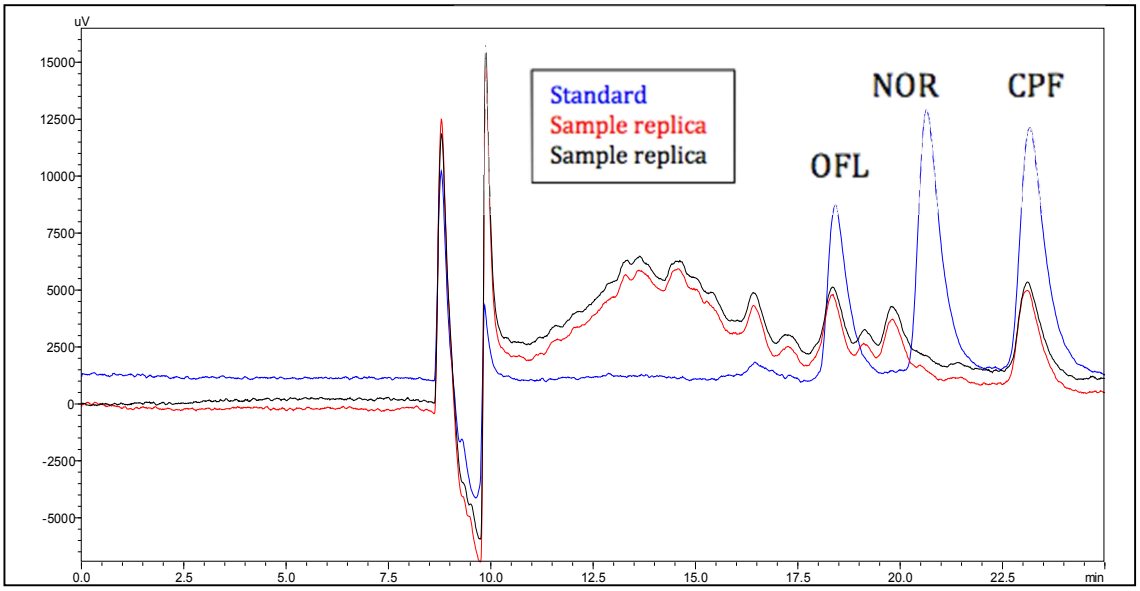


Figure 32 WWTP sample 3 analysis against 0.9 ng/mL standard sample

Sample 4

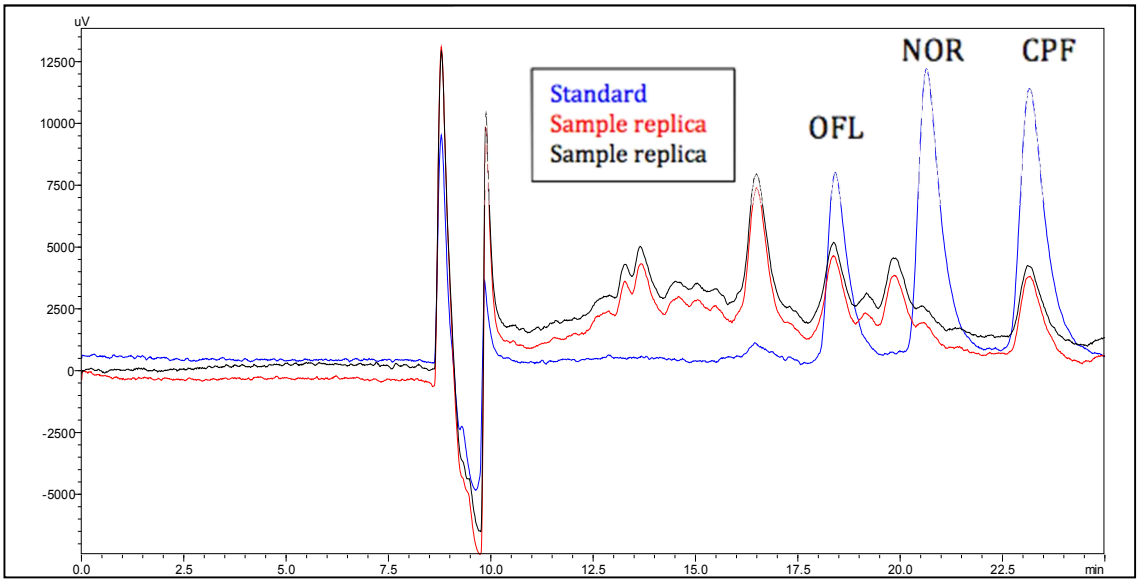


Figure 33 WWTP sample 4 analysis against 0.9 ng/mL standard sample

IV. Conclusions

This work represented the systematic development of an innovative analytical method to quantify three important fluoroquinolones (FQs) in WWTP effluents.

The analytical method consisted in a two dimensional liquid chromatography (2D-LC) with on-line SPE method for sample pre-treatment. The method was developed by changing diverse variables in order to optimize the chromatographic parameters and validate according to international criteria. The method proved to be successfully to quantify the target FQs (OLF, NOR and CPF) in an accurate and less solvent and time-consuming, beside the production of a minimal waste.

Through this novel on-line SPE-HPLC-FD method the three FQs antibiotics can be accurately quantified permitting not only the evaluation of wastewaters after being treated but also providing a helpful approach to determine contamination of water from illegal industrial waste disposal. The developed and validated method displays advantages such as a simple and more cost efficient way to analyze samples without the need of pretreatment columns such as off-line SPE, which are not reusable and are less selective. It was also demonstrated being less time consuming as samples are pretreated during the sample analysis process and thus it is not require to dispend several hours or days to treat individual samples.

The validation proved to be successful as the method displayed:

- Linearity with an R^2 above 0.999;
- Intra-batch precision with 0.30<%RSD<1.60 (range: 0.3 - 30 ng/mL);
- Inter-batch precision with 0.40<%RSD<1.80 (range: 0.3 - 30 ng/mL);
- Limit of Quantification of 0.30 ng/mL for all three analytes.

In the future the decrease of the LOQ and LOD should be attempted as these were calculate in an environmental sample which already contained an estimated 0.3 ng/mL of FQs OFL and CPF, thus making it impossible to achieve lower limits than those.

Finally, after focusing so much time in the method development it would be interesting to see if with small modifications this method could be applied to other compounds of this family or even take it one step further into other substances that might present forensic value in an investigation be it for legal reasons or for environmental profiling.

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