CONTENTS

ABBREVIATIONS

ATB – antibiotics

FQs – fluoroquinolones

OFLO – ofloxacin

NOR – norfloxacin

CIP – ciprofloxacin

ENRO – enrofloxacin

MIC – Minimum Inhibitory Concentration

EDTA – ethylene diamine tetraacetic acid

CH_3OH – methanol

ERA – Environmental Risk Assessments

FDA – Food and Drug Administration

QT – interval in electrocardiogram

WWTPs – ~~water~~ waste-water treatment plants

1. Introduction

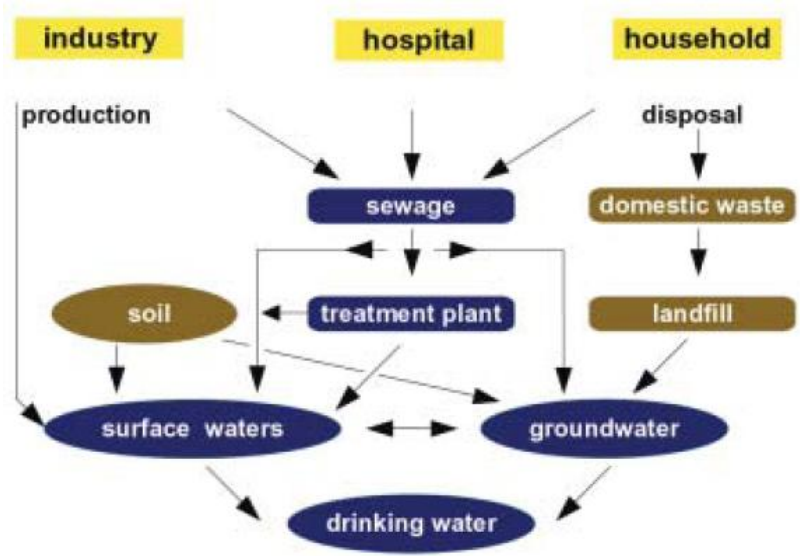
Pharmaceuticals are a class of emerging environmental contaminants that are extensively and increasingly being used in human and veterinary medicine.

The issue of pharmaceuticals and their metabolites in the aquatic environment has raised increasing concern in recent years. There is an increasing scientific interest of knowing the consequences for the ecosystem and public health that their presence in the environment may cause.

Among a wide variety of pharmaceutical compounds, antibiotics (ATB) assume special significance due to their extensive use in human and veterinary medicine.

Most of the antibiotics are poorly absorbed by humans and animals after intake, with about 25% to 75% of added compounds leaving the organisms unaltered via faeces and urine. [1] ~~(Chee-Sanford et., Apll. Environm. Microbiol. 67, 2001, 1494-1502).~~

The routes for entering ATB to the aquatic environment are different for human and agricultural antibiotics. For antibiotics used in human medicine, the excreted (the fraction of the dose excreted in its original form) antibiotics undergo the treatment processes in the ~~wastewater~~waste-water treatment facilities before entering surface water [1~~20~~]~~,~~ by animal's excrements, especially by stock cattle, by fertilizations, disposal of waste



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(unused medicine), and fodder in fish farms (where ATB in mess are addedads).

The amount of antibiotic excreted by the animal is estimated based upon the antibiotic dosage reported by the UCS and in the Feed Additive Compendium (2000) [20] assuming up to 80 percent of the administered antibiotic is excreted unmetabolized. [120] ATB goes to waste-water, then to river and lake waters, ground-water and soil.

Recently, several studies have indicated the presence of antibiotic residues at a pg/mL level in environmental water including municipal waste-water effluents and surface waters (REF. Pena et al , JSS 2008).. Numerous antibiotics have been detected in waste and natural water resources, sediments, soils and aquatic biota [120].

Some antibiotics are more retain due to their chemical structure and this, coupled with their continual input, may enable them to remain in the environment for a significant period of time (Jones et al., Water Res. 36, 2002, 5013). For example, fluoroquinolones (FQs) and tetracycline (TCs) antibiotics are fixed more than the others. These ATB makes bond with ions such Ca^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+}. ATB excreted by animals and humans can enter the environment already metabolized to an extent that depends on substance species (in animals), age and condition. For example, FQs elimination half-life may fluctuate from 1.5 to 16 hours, which means that FQs are excreted largely unchanged (lower than 25% metabolized).

Pharmaceuticals in the environment have been regulated in the USA by the US Food and Drug Administration (FDA) since 1977 under the auspices of the National Environmental Policy act of 1969. Regulation occurs through the environmental review process for New Drug Applications submitted to the FDA. In the late 1980s, additional information was required from pharmaceutical companies by the FDA and more extensive information was provided in environmental risk assessments that accompanied New Drug Applications. However, an evaluation of the data submitted from the late 1980s through the mid-1990s led the FDA to revise the regulations in 1997 to

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minimise environmental risk assessment data required in New Drug Applications[~~2~~2]

~~Authorisation Approval.~~ Revised draft guidelines for European ERAs were recently reviewed by various stakeholders and the final guidelines are expected to be available in mid-2004 [3~~2~~].

Recently, concern has been raised regarding public health issues over the presence of antibiotics in the environment and by indications of increased bacteria resistance in waste effluents from hospitals, pharmaceutical plants and animal husbandry.

~~(Goni-Urriza et al., Appl. Environm. Microbiol. 66, 2000, 125).~~

The concern with antibiotic residues in the environment is the inducement of resistance in bacterial strains. Concentration below therapeutic levels may be important in the development of resistance in some bacteria, and its genetic transfer. Exposure of bacteria to sub-therapeutic antimicrobial concentrations is thought to increase the speed at which resistant strains of bacteria develop.

The World Health Report 1998 of the World Health Organization (WHO) described the increasing occurrence of resistant bacteria and their quick spreading in the world population as one of the biggest health problem of the 21 st century ~~(WHO The World Health Report 1998, 1998; WHO, Fifty-First World Health Assembly, A51/44, 1998.)~~. [4~~33~~]

Regulations in~~s~~ the USA and in the European Union (EU) share the same objective of controlling pathogens and pollutants in sewage sludge, although differences exist in specific requirements, not only between the EU and the USA, but also among the EU member countries. Despite regulations to reduce the risk from sewage sludge, public opposition to sewage sludge land application is growing in the EU, just as it is in the USA. Government agencies in the EU have issued regulations to limit the risk from pathogens and pollutants.

EU has 27 members and though occupied smaller area than the USA population is larger. Therefore, sewage sludge management may be more urgent issue in the EU, since Europe produces more sewage sludge and has less

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agricultural area available for recycling of the material. Some individual countries have adopted lower heavy limits, or have included limits for pathogens or organic pollutants.- [5~~23~~]

Recently, ATB residues were detected in all parts of environment. To what extent antibiotics are present in waste water, their fate there, whether ground water is contaminated with them are largely unknown, as about their contribution to the level of bacterial resistance in the environment and its significance. ~~(Guardabassi et al., Appl.Environm Microbiol. 64, 1998, 3499).~~

Resistance can be transferred to other bacteria, living in other environments, for example ground water of drinking water.

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Ciprofloxacin, for example, was found in concentrations of between 0.7 and 124.5 µg/L in hospital effluent ~~(Hartmann et al., Archives Environmental Contamination and Toxicology, 36, 1999, 115-119)~~. Ampicillin was found in concentrations of between 20 and 80 µg/L in the effluent of a large German hospital ~~(Kummerer Chemosphere 45, 2001, 957-969)~~. CIP and NOR were detected in Switzerland at 40-570ng/L in surface waters that received ~~wastewater~~waste-water discharge. In the USA CIP and OFLO were repeatedly detected in various municipal ~~wastewater~~waste-waters at 80ng/L to 2µg/L, and for FQs (CIP, NOR, ENRO, SARA) were reported with mean concentrations from nondetectable to 0.12µg/L in surface waters. The highest concentrations of FQs were reported in hospitals waste waters, with 0.7-124.5 µg/L of CIP [2~~6~~1].

Hospital ~~wastewater~~waste-water is one of the main source of contamination. Antibiotic concentrations calculated and measured in hospital effluents are of the same order of magnitude as the minimum inhibitory concentrations for susceptible pathogenic bacteria[7] ~~(Kummerer, Clinical Microbiology and Infection, 2003)~~. The dilution of hospital effluents by municipal sewage will lower the concentration of antibiotics only moderately, because municipal waste water also contains antibiotic residues from households, veterinary sources and from livestock.

FQs adsorb strongly onto sewage sludge, soils and sediments and were not biodegraded in tests with sediments. FQs may be persist within

environmental compartments because of their strong sorption properties, and are rather resistant to microbial degradation. ~~(Arch.Environm.Contam.Toxicol. 37, 1999,158;, Environm Toxicol.Chem. 19,2000,2467; Chemosphere 40,2000,701).~~ Therefore, their determination in soils is important because their high persistence in this type of environmental matrix. On the other hand, there are only few related articles published in scientific literature.

It is important to perform more efficient and reliable environmental monitoring in order to know the stability of the FQs in the environment.

The goal of this work~~,~~ was the determination of four FQs, namely, OFLO, NOR, CIP and ENRO, since they are the most used, in soil samples.

Due to quinolones and FQs are only partially metabolised by patients, they are eliminated mainly parent compounds, being consequently discharged into hospital sewage or municipal waste water. Unfortunately, sewage water treatment plants are not able to completely remove these compounds and thus important quanti~~ta~~ties of the active ingredient are transported to the environmental aqua~~e~~tic systems. Beside this, it has been reported that irrigation of crops with treated waste water can introduce antibiotics in surface waters through agricultural runoff (Gau et al., Drug Res. 36, (II), 1986, 1545; Pedersen et al., J.Agric.Food Chem.53,2005,1625). On the other hand, the use of both sewage sludge and livestock manure as fertilizers in agricultural crops in several countries is favouring the accumulation of antimicrobials in soils [~~(Diáz-Cruz et al., Trends Anal.Chem. 22, 2003,340)~~8].

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2. Purpose

The aim of this work was the development and validation of an analytical methodology, sensitive, accurate and precise for determination of Fluoroquinolones ~~s~~ in soil samples. ~~Determination should be making by~~The chosen analytical methodology was Liquid Chromatography with fluorescence detector. Samples were from Waste Water Treatment Plant in Coimbra (Portugal) and from meadow in Tovim (Coimbra, Portugal).

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3. Theoretical part

3.1. Antibiotic Resistance

Development of resistance in bacterial populations is an extremely complex subject. The origin of drug resistance may be non-genetic because inherent resistance to some bacterial is always present in the population. However, most drug-resistant bacteria emerge as a result of genetic change and subsequent selection processes by antibiotics.

Further compounding the problem is that harmless bacteria with resistance genes can transfer these genes to pathogenic bacteria that enter the same environment. The genetic elements that are transferred often carry factors that impart resistance to more than one type of antibiotic. Over the past few decades, the use of antibiotics has enabled us to control many serious infectious diseases. However, as resistant strains become more widespread due to natural and inevitable evolutionary adjustments, antibiotics will cease to be the effective tool they have been for physicians and patients to control infectious diseases. In recent years, antimicrobial resistant pathogens have been emerging in human medicine and spreading more rapidly than in previous decades.

Adverse consequences include increase in the incidence of human infections caused by resistant pathogens, and potential therapeutic failure in humans. More often, bacterial resistance increases therapeutic costs because more diagnostics are required, more costly and sometimes more toxic drugs are needed, and hospitalization may be extended.

Concentrations below therapeutic levels may play a role in the selection of resistance and its genetic transfer in certain bacteria. Exposure of bacteria to sub-therapeutic antimicrobial concentrations is thought to increase the speed at which resistant strains of bacteria are selected. Resistance can be transferred to other bacteria living in other environments such as ground water or drinking water. In general, knowledge of sub-inhibitory concentrations and their effects against environmental bacteria is poor, especially with respect to resistance. There are a number of recent and older

publications about the mechanisms of very low antibiotic concentrations on the expression of bacterial virulence factors.

Antimicrobials may have qualitative and quantitative effects upon the resident microbial community of sediments, as summarized by Nygaard *et al.* [35] Resistant bacteria may be selected by antibiotic substances in hospital effluent, municipal sewage, aeration tanks, the anaerobic digestion process of STPs or in soil. Furthermore, resistant bacteria are excreted and discharged into sewage or soil and other environmental compartments. Resistant and even multi-resistant pathogenic bacteria have been detected in ~~wastewater~~waste-water and STPs, as well as in other environmental compartments ~~[35]~~.[7] Furthermore, in arid regions, ~~wastewater~~waste-water containing resistant bacteria and antibiotics is used for irrigation, and sewage sludge serves as a fertilizer. This allows resistant bacteria to enter the food chain directly.

Treatment of resistant infections is increasingly hampered due either to the prohibitive cost of existing new-generation drugs or to a total lack of effective antibiotics on the market. Antimicrobial resistance has become a global problem, affecting developed and developing countries, and it is rapidly spreading between continents through international travel. There is no question that bacteria develop resistance to antibiotics, and that they can transfer their resistance to other bacteria, even of other species.

Even though they are found in very low concentrations, there is still a lack of knowledge about long-term risks that that the presence of a large variety of drugs may pose for non-target organisms as well as for human health. [~~2~~9]

In the investigated liquid manure and soil samples some antibiotics (study was for tetracyclines, fluoroquinolones β-lactams antibiotics and sulphonamides) were found at mg/kg level and µg/kg level in manure and soil samples, respectively, which indicates a high stability of some antibiotics, especially by considering, that the soil was manure at least three months before

sampling, whereas other administered antibiotics are degraded within short time and may have hardly an effect on the amounts in the environment. [10~~1~~]

Results of toxicity studies have revealed important toxic activities of hospital ~~wastewater~~waste-water on aquatic organisms. Furthermore, studies have found increased prevalence of resistant bacteria in sewers receiving hospital ~~wastewater~~waste-water effluent. [11~~30~~]

3.2. Risk Assessment and management

Most compounds or at least most groups of compounds acting via the same mechanisms are found in hospital effluent and in some cases even in municipal sewage in concentrations that are high enough to warrant further risk assessment and risk management. It is imperative that we obtain a better database of the sources, fate and effects of both antibiotics and resistant bacteria in the environment. This information is necessary if appropriate and, in the long run, successful measures for sound risk assessment and proper risk management are to be taken.

The emission of antibiotics into the environment should be reduced as an important part of the risk management. For this reason, unused therapeutic drugs should not be flushed down the drain and physicians must be made aware that antibiotics are not completely metabolized by patients. On the contrary, antibiotics and other pharmaceuticals are often excreted largely unchanged, i.e. as active compounds. Doctors and patients as well as pharmacists play an important role in reducing the release of antibiotics, other pharmaceuticals, and disinfectants into the environment. The environmental significance of therapeutic drugs, disinfectants and diagnostics should be included in the undergraduate curricula of medical students and pharmacists. Patients should be made aware that antibiotics help against bacterial diseases but not against the common cold, which is caused by viruses. These issues should be addressed as part of a sustainable development in medicine and for the environment.

3.3. Occurrence and persistence of ATB in environment [~~20~~1]

The persistence of a drug in a sediment or soil mostly depends on its photo-stability, its binding and adsorption capability, its degradation rate, and leaching in water. Also the rate of sedimentation is highly related with the half-life of a chemical. Strongly sorbing pharmaceuticals tend to accumulate in soil or sediment. By contrast, highly mobile pharmaceuticals tend to leach into groundwater and be transported with groundwater, drainage water, and surface run-off to surface waters.[1~~20~~]

The sorptive exchange of chemicals between a water phase and a solid phase (sorbent, soil or sediment) is represented by the sorption coefficient K_d, solid, which is defined as the ratio between the concentration of the compound in the sorbent and in the water at equilibrium.

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As far as compound sorption to soil and sediments is concerned, tetracyclines adsorb most strongly followed by quinolones and macrolides. The literature review indicates that sulphonamides and fluoroquinolones followed by macrolides are more likely to persist and transport in the aquatic environments.

3.4. Fluoroquinolones

The FQ are synthetic family of broad spectrum ATB. FQ, derivates of quinine, were discovered in the early 1960s. In two decades the FQ moved from relatively small and unimportant group of drugs, used predominantly for treatment of urinary tract infections, to a worldwide class. These compounds have now been used in human therapy, veterinary treatment, and agriculture for over a decade, and during this time their input into the environment has been continuous. Ciprofloxacin (CIP) is the most widely prescribed FQ in the world; the second is ofloxacin (OFLO). Norfloxacin

(NOR) is very common in Europe. Most important veterinarian-used FQ is enrofloxacin (ENRO).

Base of FQ is nalidixic acid (piperazinyl derivates) (Fig 1-5). All FQs have fluoro group attached the central ring system, usually at the 6-position. Ofloxacin (OFLO), NOR and ENRO have two relevant ionisable functional groups, the 3-carbonyl group and N-4 of piperazine substituent. The antimicrobial activity of these compounds is also pH-dependent. ENRO is the most lipophilic compound. ~~CIPRO, ENRO and OFLO have high sorption coefficient, they move rapidly from the water compartment into solids (Table 1).~~

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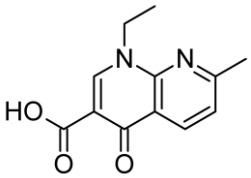

FQs inhibit bacterial DNA gyrase or the topoisomerase IV enzyme (a process that depends both on pH and acid concentration), there by inhibiting DNA replication and transcription. FQ are often using to treat intracellular pathogens. For many gram-negative bacteria DNA gyra~~z~~se is target, whereas topoisomerase IV is the target for many gram-positive bacteria. Eukaryotic cells do not contain DNA gyrase or topoisomerase IV. Resistance of FQs can develop rapidly, even during a course of treatment. FQs have 4 generations divided because of their antibacterial spectrum.

FQs in human medicine [124]

Quinolones is using since 60s of last century. In last 25 years there are FQs used for their good tolerance and per os bioavailability. Because of unexplained metabolism is not allow to use~~d~~ any quinolones under 18 years old and during breast-feeding. Resistance of bacteria is increase very fast during treatment and there is cross-resistance. For example, resistance of *Escherichia coli* on CIP increased from 8% in 2001 to 20% in 2005 in Czech

Republic. Resistance is also increasing on *Kleibsitela pneumoniae* and *Pseudomonas aeruginosa.*

Drugs interaction of quinolones. Antacids, salts of ferrum and zinc decreased plasmatic concentration of all FQs. Therefore is says to use these compounds 2 hours after or before FQs administration. Several FQs make interval QT keep by other medicines longer (antiarytmics of I and III class – cisaprid, terfenadin etc.), which can caused death of the patients. For this interaction the most dangerous is moxifloxacin.

Structure of FQs:

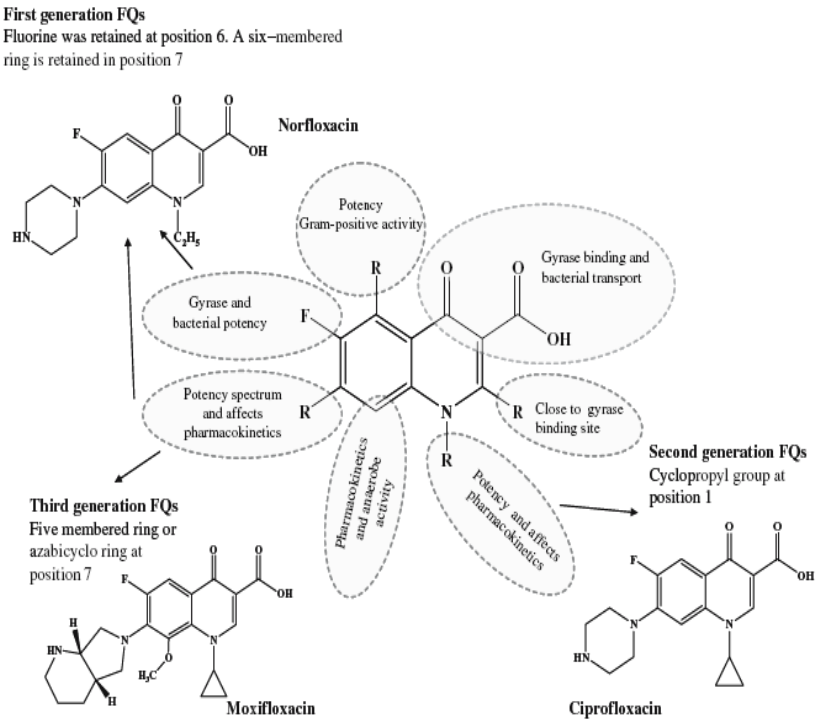

Figure 1- Structure of FQs

Ofloxacin:

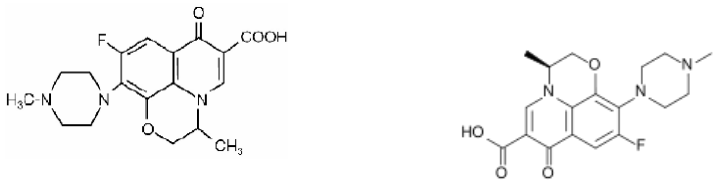

Figure 2 – A) OFLO (racemic mixture); B) Levofloxacin (biologically active)

IUPAC name: (+/-)-9-fluoro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)- 7-oxo-7*H*- pyrido [1,2,3-de]-1, 4-benzoxazine-6-carboxylic acid

Norfloxacin:

Structure sees above at figure 1

IUPAC name: 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1*H*-quinoline-3-carboxylic acid

Ciprofloxacin:

Structure sees above at figure 1

IUPAC name: 1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid

Enrofloxacin:

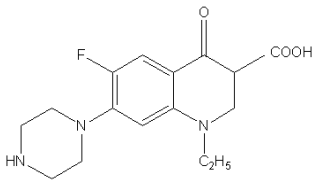

Figure 3 - ENRO

IUPAC name: 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid

FQ	pk_{a1}	pk_{a2}	Mol. mass g/mol	Melting point; °C	Time of elimination (hours)
Ofloxacin	5,97	8,28	361.368	250-257	9
Norfloxacin	6,23	8,55	319.331	227-228	3-4
Ciprofloxacin	5,90	8,89	331.346	255-257	4
Enrofloxacin	6,27	8,62	359.395	219-233	3-4

Table 1 – Properties of FQs

Freundlich equation isotherms

FQs are compound their properties can be described by Freundlich equation:

$\text{Log } q_e = n \log C_e + \log K_f$ and $q_e = K_d * C_e$

q_e – concentration of FQs in soil (mg/g) and calculate as

C_e – equilibrium concentration of FQs in aqueous phase (mg/L)

K_f – Freundlich coefficient

K_d – sorption distribution coefficient (L/g)

n – Freundlich linearity parameter ($K_f = K_d$ when n=1)

$V (C_t - C_e)/m$ where:

V – volume of aqueous phase (L)

C_t – concentration of FQs in soil free aqueous solutions after 24 h after agitation (mg/L)

m – mass of soil (g)

The study in of M. Ötker Uslu et al. shows that the K_d and K_f values for CIPRO and ENRO were found to be slightly different. The value of the slope in ranged between 0.63-0.74 for all soils, indicating a nonlinear relationship where proportionally more of both antibiotics were stored at lower concentrations.

3.5. Degradation of FQs

FQs are rather resistant to microbial degradation ~~(Arch.Environm.Contam.Toxicol. 37, 1999,158;, Environm Toxicol.Chem. 19,2000,2467; Chemosphere 40,2000,701)~~ and these compounds may be persisting within environment because of their strong sorption properties.

On the other hand, degradation of antibiotics, including photolysis ~~(Zou, Int.J.Pharm.110,1994,55; Burhenne et al.,Chemosphere 38, 1999, 1279; Fasani et al., J.Org.Chem. 64,1999,5388; Mella et al., Helv.Chim.Acta 84, 2001,2508) and chemical oxidation (Adams et al., J.Environm.Eng. 128, 2002, 253; Huber et al., Environm. Sci. Technol. 37, 2003, 1016; Dodd Environm. Sci. Technol. 37, 2003, 1016)~~ may be significant on their environmental ecosystem.

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Degradation of quinolone and tetracycline antibiotics is expected to be slow when exposure to sunlight is limited. Marengo et al. (1997) reported very slow aerobic biodegradation of sarafloxacin. Oxytetracycline, quinolone derivatives, and sulphonamide antibiotics were found to be persistent in model marine aquaculture sediment.

Furthermore studies have shown substantial degradation of CIPRO and ENRO by hydroxyl radical-mediated enzyme systems characteristics of brown-fungi (sp. Gloeophylum striatum). [13~~24~~]

Degradation by *Mucor Ramannianus*

Cultures of wood-decaying basidiomycetes, including strains found in manure, have been shown to convert ENRO to CO_2 and least 11 other

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metabolites. Several fungi already have been shown to metabolize fluoroquinolones [14~~25~~]

For instance, the zygomycete *Rhizopus arrhizus* demethylates the N-methylpiperazine ring of danofloxacin. *Gloeophyllum striatum* and other wood-decaying basidiomycetes metabolize ENRO and CIP by hydroxylation, decarboxylation, defluorination and removal of part or all of the piperazine ring. One of the ENRO metabolized of *G. striatum*, produced by removing two ethylene carbons from the piperazy~~r~~ine ring, was also a metabolite of *M. ramannianus*. CIP is metabolized by *M. ramannianus* to N-acetylciprofloxacin, which has now also been identified as a metabolite of ENRO. They propose that ENRO is first converted to CIP by N-dealkylation and that the resulting CIP is N-acetyled to give N-aceltylciprofloxacin. CIP was not detected in these experiments, presumably because the acetylating step occurred quickly. The transformation of ENRO by *M.ramannianus*, including N oxidation, N-dealkylation, N-acetylation and the breakdown of the piperayine ring, is similar to the mammalian metabolism of other FQ. For CIP, the major mammalian metabolites have significantly less antibacterial activity than the parent compound. The fungal ENRO metabolites previously reported also appear to have less antibacterial activity. [14~~25~~]

Degradation by *Gloeophyllum striatum*

In mammals, FQ can be metabolized by gluronidation, sulfation, N-dealkylation, and oxidation of the amine substituent. However, a major fraction is excreted unchanged and introduced into the environment via animal waste. Strong binding can be expec~~p~~ted to delay degradation and may partly explain the apparent recalcitrate of FQs. They have shown in vitro degradation of ENRO by white rot fungus *Phanetochaete chysosporium* and by *Gloeophyllum striatum*, representing the brown rot fungi. [~~2~~134~~4~~]

Brown rot fungi preferentially degrade the cellulose and hemicelluloses components of plant cell walls, while lignin has been shown to be modified primarily by hydroxylation and demethylation and to a minor extent by depolymerization.

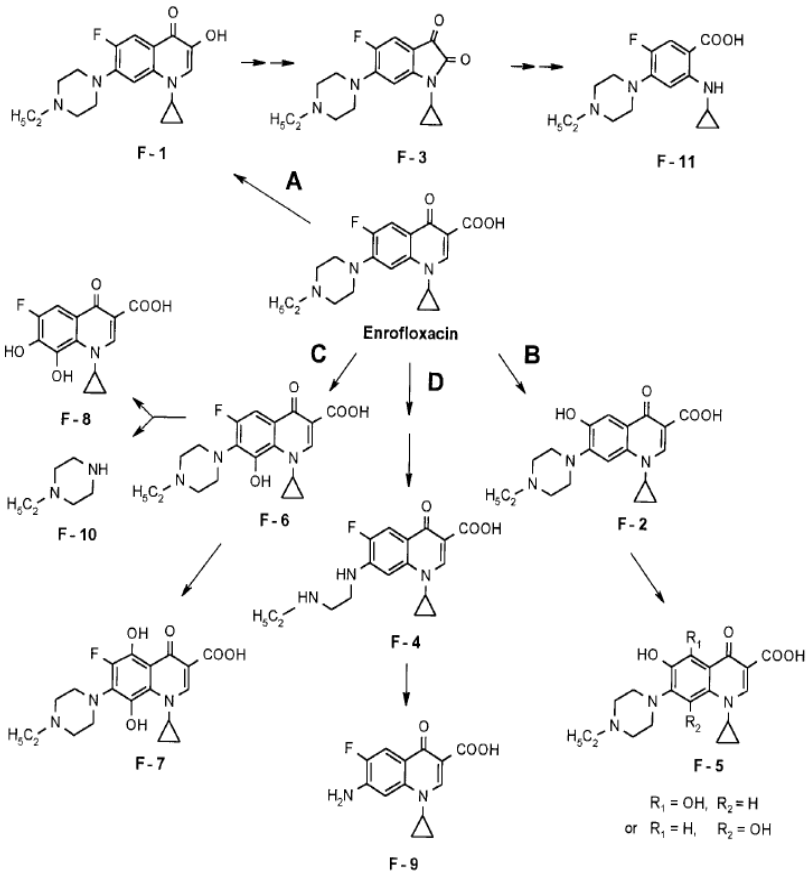


Figure 3-Degradation of ENRO[24]

F-1... 1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-3-hydroxy-4-*1H*-

quinolinone
F-2... 1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-1,4-dihydro-6-hydroxy-4-oxo-3-quinolinecarboxylic acid
F-3...1-Cyclopropyl-6-(4-ethyl-1-piperazinyl)-5-fluoro-*1H*-indole-2,3-dione
F-4...1-Cyclopropyl-7-{[2-(ethylamino)ethyl]amino}-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid
F-5...1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-1,4-dihydro-6,8(5,6)-dihydroxy-4-oxo-3-quinolinecarboxylic acid
F-6...1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-8-hydroxy-4-oxo-3-quinolinecarboxylic acid
F-7...1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-5,8-dihydroxy-4-oxo-3-quinolinecarboxylic acid
F-8...1-Cyclopropyl-6-fluoro-1,4-dihydro-7,8-dihydroxy-4-oxo-3-quinolinecarboxylic acid
F-9...7-Amino-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid
F-10...1-Ethylpiperazine
F-11...2-Cyclopropylamino-4-(4-ethyl-1-piperazinyl)-5-fluorobenzoic acid

Route A would be initiated by an oxidative descarboxylation. This irreversibly inactivates the drug, because the carboxyl group is essential for antibacterial activity of FQs. Route B would be initiated by defluorination of ENRO. This eliminates the xenobiotic structural element and reduces the antibacterial potential of metabolite F-2 to ≤ 3%. Route C could be initiated by hydroxylation of ENRO at position C-8. This modification reduces the antibacterial potential of F-6 to ≤ 5% and most likely enhances its further degradation. Dihydroxylated congeners are included in routes B and C. Such autoxidizable structures are prone to undergo further oxidative degradation, which might even include the cleavage of the homoaromatic part of ENRO. Route D shows an oxidative degradation of the piperazinyl moiety. This sequence of reaction is apparently initiated by the formation of a carbonyl group, as was shown for CIP.

Degradation of ENRO was very sensitive to hydroxyl- radical-scavenging agents like ethanol and dimethyl sulfoxide. The proposed degradation routes may reflect different sites of initial attack of ENRO by hydroxyl radicals. Each of the primary metabolites offers various sites for secondary attack, which would cause branching of the routes, resulting in the

formation of a network of metabolites. ENRO was also chemically degraded by Fenton´s reaction, in which hydroxyl radicals are generated from hydrogen peroxide and Fe^{2+}:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^-$$

Key steps in the degradation of compounds containing structural elements also found in ENRO, 3-carboxy-pyridine, 3,4-dihydroxypyridine, quinoline, 1H-4-oxoquimoline, and anthranilic acid, are catalyzed mostly by molybdenum – containing dehydrogenases, dioxygenases, or monooxygenases. FQs not are readily accessible for such enzymes. Due to the fluorine substituent and to the carbonyl as well as the carboxyl group, the heterocyclic core of ENRO tends to be electron deficient. In addition, the high degree of substitution might prevent an enzymatic attack due to steric hindrance. Thus, the radical–based mechanism is potentially employed by wood–rotting. Basidiomycetes may provide the most suitable, if not the only, means to initiate degradation of such complex compounds. [14~~25~~]

Complexation by MnO_2

Sorption the soil, sediments, or dissolved organic matter was indicated to be an important FQs pathway into environment. For example, Nowara et al. [16]~~(REFERENCE)~~ reported strong interactions of ENRO with clay minerals and also found that greater than 90% of FQs adsorbed to different soil samples with only small variations in the sorption coefficient K_d. The strong adsorption of FQs to soils and sediments may result in a lower concentration of freely dissolved species and thus reduced the photo-degradation and biodegradation potential of FQs.

δ-MnO_2, with a reduction potential of 1.23V has been shown to be an effective oxidant for pollutants including substituted phenols and anilines.

Naformátováno: není zvýrazněné

FQs	Adsorption %
OFLO	76 ±6[a]
NOR	62
CIP	69
ENRO	68

Table 2 – adsorption of FQs, [a] only at this case there was more than one Measuring

Typically, the differences in measured parent concentrations from the initial several time increments (reaction time<2 h) were averaged to represent the adsorption extent of FQs and model amines [2~~27~~].

Tests showed that acidification improved the stability and analytical results of the parent FQ compound; thus 100µL of 1 M H_3PO_4 was immediately added to sample aliquots (from experiments quenching by ascorbic acid) or centrifugation supernatants (1mL) after quenching [~~27~~2]**.** The samples were then stored in amber vials at < 5°C and analyzed within a couple of days.

In addition, experiments with FQs and Mn^{2+} in contact with air but without MnO_2 yielded negligible degradation of FQs after 5 days, indicating that the potential catalytic ability of Mn^{2+}/O_2 observed in degradation of some compounds.

In the absence of MnO_2, FQs were stable under the experimental conditions and no measurable loss of the compounds could be detected after a week. The reaction kinetics was complicated and apparently derivate from pseudo-first-order decay.

Adsorption of CIP to MnO_2 surfaces-determined by comparing the measured CIP concentrations after quenching by ascorbate addition *versus* by centrifugation – also decreased with increasing pH.

The experiments showed rapid degradation of six out of the seven FQs by MnO_2 within 3 h at pH 6. The adsorption to MnO_2 was strongest for CIP, ENRO, NOR and OFLO at 62-76% [227]

In summary, the redox reaction is initiated by generating a surface complex between the FQs and MnO_2. The results that the rate of CIP oxidation by MnO_2 increases as the adsorption of CIP to MnO_2 increased when the system pH varied agrees with above expectation.

In contrast, the present study shows that FQs do not necessarily adsorb to MnO_2 more strongly than model amine 1-phenylpiperazine (PP). The study shows that adsorption of CIP to MnO_2 increases when the pH is decreased from 8 to 4. On the basic of the reported pH of 2.4 for δ-MnO_2, the MnO_2 surfaces have an overall negative charge under the experimental conditions [227].

Experimental data of FQs also support the idea that the N_1 atom is more reactive to oxidation. For example, CIP, NOR, and ENRO do not differ from each other at their N_1 atom but differ at position of quinolone ring N atom or the N_4 atom. The fact that CIP, NOR, and ENRO have comparable reaction rates with MnO_2 suggest that the main reaction centre is not associated with the quinolone ring N or the piperazyl N_4 atom[227].

PP is about 3 times more reactive than CIP is likely due to the electron-withdrawing substituent effect in CIP that is absent in PP. The fact that the reaction rate of FQ oxidation by MnO_2 shows as pH increases can also be explained by this oxidation power of MnO_2.

Although it is clear now that the initial oxidation site of FQs is the piperazyl N_1 atom, the N_4 atom also participates in the overall reaction on the basis of the results of product identification and a close examination of the observed compound reactivity.

The study demonstrates high reactivity and fast reaction of FQs with manganese oxides. Reaction of FQs with manganese oxides yielded various N-dealkylated, hydroxylated, and possibly coupling oxidation products.

Dealkylated products similar to those found in this study have also been identified in the FQ photodegradation mixtures [227].

Fasani et al. reported that OFLO photo-degrades much more slowly than CIP, ENRO and NOR due to its 5-oxygen electron-withdrawing substituent on the aromatic ring, and it undergoes primarily defluorination while the other three FQs undergo primarily dealkylation. Thus may be postulated that the dealkylated products have much lower antimicrobial activity than the defluorinated products. If the above reasoning were indeed correct, the dealkylated oxidation products of FQs with manganese oxide would mean reduction in antimicrobial activity.

Degradation by Wood-Rotting Fungi

Enrofloxacin, the first FQ developed for veterinary medicine, is used to treat various infections in pets and livestock. During its passage through the animal, enrofloxacin is degraded only to a small extent, i.e., most of the rug is excreted in urine and feces. Additionally, manure from livestock is often disposed of by spreading onto agricultural soils and pastures. However, FQs are tightly bound to feces and soils and are hardly bioavailable which also may contribute to their apparent recalcitrance.

The non-specificity of these radical-mediated reactions also enables white rot fungi to degrade recalcitrant xenobiotics like polyaromatic hydrocarbons, polychlorinated biphenyls, chlorinated pesticides (dichloro-diphenyl-trichloroethane [DDT] and lindane), and explosives (trinitrotoluene [TNT]).[126]

The results of this investigation [216]demonstrate that woodrotting fungi in pure culture are able to mineralize the carbonyl group of enrofloxacin. It is remarkable that all three strains of the brown rot fungus *G. striatum* showed degradation rates which were much higher than those of the white rot fungi tested.

[14C]enrofloxacin preadsorbed to native soil could be mineralized at a small but constant rate. This can be explained by the reported low bioavailability of quinolones ~~(11)~~, a lack of indigenous microorganisms with sufficient degradation potential in this specific soil, or other factors.

As straw was degraded by up to 80%, enrofloxacin probably became directly available to the fungi.

3.6. Adsorption and composition of Clay

The adsorption of antibiotics at soil components were primarily occures on clay minerals and humic substances. The content of clay in the different soils varies between 2.5% and 41.7% and consists mainly of montmorillonite and kaoline. Five soils of different geographic origin with varied quantities of clay and organic carbon represents a broad spectrum of different soil types from important cultivation areas. The large clay fractions and the huge amounts of pure clay minerals provide a high adsorption surface. Consequently, the desorption is very low. Kaolinite soils show K_d values based on the clay mineral content that are considerably lower on the those of montmorillonite-contining soils. The isolated clay fractions of the different soils sorb a very high amount of the added substrate. Different desorption results from isolated and pure clay minerals lead to the assumption that a high amount of phyllosilicate in the clay fraction of the soils increases the adsorbability and decreases the desorption. [~~2~~178~~8~~]

The fundamental element leading to a high adsorption of fluoroquinolones seems to be the β-keto acid structure. This element is not present in the case of decarboxylated ENRO, and therefore this compound is adsorbed to a cantly lower extent.

Naformátováno: Odsazení: První řádek: 1,27 cm

Naformátováno: Odsazení: První řádek: 0 cm

Clay mineral	Adsorption %	pH	Desorption %
kaolinite	98.9	6.0	49.9
illite	99.3	6.1	47.1
vermiculite	99.7	5.8	42.0
montmorillonite	99.8	5.9	32.4

Table 3 – Adsorption and desorption of various type of soil [17~~28~~]

Due to the pH values measured in the soil solutions and clay suspensions equilibrated with the weak acid ENRO, the degree of dissociation can be calculated to about 10%. This amount of ionized ingredient is able to interact with cations on the base surfaces as well as with cations in the interlamellar space of expandable clay minerals. This sorption leads to a further dissociation of ENRO to maintain the equilibrium. The high capacity of the soil and clay minerals is able to adsorb the applied compound ionically. Along this process the abstracted protons can be buffered by the soil and do not influence the pH of the soil solutions significantly.

Naformátováno: Odsazení: První řádek: 1,27 cm

Soils with a variety of different pedological parameters readily adsorb the FQs derivates nearly 100%, and the desorption is low. This behaviour is attributed to the clay mineral's ability to preferably adsorb plane anionic substrates between the mineral layers and at the outer surfaces via Columbic interactions.

3.7. Coulomb's law

Coulomb's law was developed by French physicist Charles Augustin de Coulomb in 1780s. The form of the law is follows: The magnitude of the electrostatic force between two point electric charges is directly proportional to the product of the magnitudes of each charge and inversely proportional to the square of the distance between the charges.

Power has longer range in compare with other non-covalent interaction and depended on pH, magnitude of charges, on permittivity of environment.

Naformátováno: Odsazení: První řádek: 1,27 cm

3.8. Analytical methods for determination of FQ in soil

Unfortunately there are only a few articles about determination of FQs in soil. There is two possibility of extraction in acid pH, where the FQs are in cationic form or in basic pH, where the FQs are in anionic form. There is no concordate opinion which way is better. Some papers published in scientific literature achieved better results at low pH, using a diluted acid as extraction solvent. Analytical methods available in the scientific literature for determination of FQ in soil by organic solvents are presented in table 4.

It is known that quinolones and FQs are able to form stable complexes with several divalent and trivalent metal ions. Among them Ca(II), MG(II) and AL(II) lead to complexes with the highest stability constants [18] ~~(Durán et al. Analyst 125, 2000, 1471; El Kommos et al., Talanta 60, 2003, 1033)~~ and thus, the ability of these cations to desorbs and extract quinolones and FQs from soil was evaluated [~~(Tuiel et al., Anal.Chimica acta 562, 2006,30-35)~~2]. Due to this fact, the use of exhaustive extraction methods, such as accelerated solvent extraction (ASE) or microwave-assisted extraction, were required to the determination of FQs in soil samples [2]~~(Golet et al., Anal.Chem. 74, 2002, 5455;Morales Muñoz et al., J.Chromat A1059, 2004,25).~~

Analysis~~–~~ of FQs has~~ve~~ been carried out mainly by HPLC with fluorescence detector ~~(Nakata et al., Chemosphere 58, 2005, 759; Golet et al., Anal.Chem. 73, 2001, 3632)~~ LC-MS ~~(Nakata et al., Chemosphere 58, 2005, 759; Reverte et al., J.Chromat. A1010, 2003, 225; Renew and Huang J.Chromat. A 1042, 2004,113)~~ or LC-MS-MS ~~(Lindberg et al., Environm. Sci.Technol. 39, 2005, 3421)~~. These methods are coupled with off-line or on-line solid-phase-extraction (SPE) techniques for extraction and concentration of FQs in environmental water samples ~~(Ferdig et al., J.Chromat. A 1047, 2004, 305; Reverte et al., J.Chromat. A1010, 2003, 225; Renew and Huang~~

Naformátováno: Odsazení: První řádek: 1,27 cm

Naformátováno: Odsazení: První řádek: 1,27 cm, Řádkování: Přesně 22 b.

Naformátováno: Odsazení: První řádek: 1,27 cm

Naformátováno: Odsazení: První řádek: 1,27 cm, Řádkování: Přesně 22 b.

~~J.Chromat. A 1042, 2004,113; Nakata et al., Chemosphere 58, 2005, 759; Golet et al., Anal.Chem. 73, 2001, 3632)~~.

Liquid chromatography-mass spectrometry (LC-MS) ~~(Kolpin et al., Environm. Sci.Technol. 36, 2002, 1202 Renew and Huang, J.Chromat. A 1024, 2004, 113 ; Lindsey et al., Anal. Chem. 73, 2001 4640)~~ or LC-MS-MS ~~(Hirsch et al., Sci.Total Environm. 225, 1999, 109; Miao et al., Environm. Sci. Tecnhol. 38, 2004, 3533; (Kolpin et al., Environm. Sci.Technol. 36, 2002, 1202; Yang et al. Rapid Commun. Mass Spectrometry 18, 2004, 2131,Gobel et al., Anal.Chem. 76, 2004, 4756,Hamscher et al., Anal. Chem., 74, 2002 1509)~~ has been used in the analysis of antibiotics because of its high sensitivity and ability to provide compound information. [18]

Naformátováno: Odsazení: První řádek: 1,27 cm

Naformátováno: není zvýrazněné

Specific FQ	***Extraction conditions***	***Determination conditions***	***Recovery***
Ciprofloxacin Enrofloxacin	Oasis-HLB elution with MeOH:H_2O, 75:25	FLD:λ 278-320nm; λ emission: 365-500	-
Norfloxacin, Ciprofloxacin	Acetic acid and acetone, Oasis-HLB cartridge, elution with NH_4OH (2%) in methanol	Gradient: 20 $mmolL^{-1}$ ammonium acetate with 0.1% acetic acid and acetonitrile with 0.1% acetic acid	95-98%
Enrofloxacin	SE; Adjusted to pH 6.8., dichlormethane	Gradient :20 $mmolL^{-1}$ H_3PO_4(pH2.9):CH_3CN, flow-rate 1mL min^{-1}	100% ($0.6\mu L^{-1}$)
Ofloxacin	Elution with 5% NH_3 in 15% MeOH; 0,01 $molL^{-1}$ $CaCl_2$ SPE cleanup; water HBL cartridges 50mg	LC-UV Gradient: 0.02 mmolL NaH_2PO_4 (pH7): CH_3OH λ: 280nm	-
Norfloxacin	Ultrasound-assisted extraction (30 min) in small columns using 8mL 50% (w/v) aqueous $MgNO_3$ with 4% NH_3 in acetone	LC-UV Gradient: H_2O, (acidified with formic acid):CH_3CN λ: 260 nm	82% - 104% (0.15 – 0.25 mg kg^{-1})

Table 4 – Examples of determination of FQs [197]

4. Experimental part

4.1. Reagents

Standards of OFLO, NOR, CIPRO and ENRO were purchased from Sigma-Aldrich (Steinheim, Germany). These FQs were in > 98% purity. Citric acid, hydroxide sodium, ammonnium 25%, magnesium sulphate, chloric acid 25%, were obtained from Merck (Darmstadt, Germany). LC grade methanol was supplied from Carlo Erba (Milan, Italy). Phosphoric acid RPE-ACS was acquired from (Carlo Erba, Milan, Italy), sulphuric acid 95-97%from Reagente "Baker Analized" (Deventer, Netherlands), tetrabuthylammonium (TBA) from (Sigma-Aldrich, Steinheim, Germany) and EDTA from (MercK, Germany). Water was HPLC grade. SPE cartridges, Oasis HLB 6cc/200 mg and AccuBOND II SAX Cartridges 6ml/ were purchased from Waters (Waters Corp., Millford, MA) and from Agilent (Agilent Technologies, Santa Clara, United States), respectively.

4.2. Apparatus and chromatographic conditions

The LC method described here consists of one pump (model 307, Gilson Medical Electronics, France), an injector Model 7125 (Rheodyne, Cotati, California, USA), fluorimeteric detector (LabAlliance, France) operated at an excitation wavelength of 278 nm and an emission wavelength of 450 nm. The spectral bandwidth was 10 nm for both excitation and emission. The results were recorded on an SP 4270 integrator (Hewlett Packard, Philadelphia, USA).

The four FQs were eluted isocratically using a mobile phase containing 0.025 M phosphoric acid solution (pH adjusted to 3.0 with TBA, methanol and acetonitrile (920:70:10, v/v/v). Analysis was performed through a monolithic column (Chromolith Performance RP-18e -100 x 4.6 mm) at a flow rate of 1.4 mL/min and room temperature.

Naformátováno: Odsazení: První řádek: 1,25 cm

4.3. Sampling and storage of samples

Samples were collect in first 10 cm from ground around WWTPs and transported at low-temperature. Other samples were collected at meadow, transported without cooling. Samples were stor~~ag~~ed at 4°C in dark, before analysis.

4.4. Standards and stability

Individual stock standard solutions (1 mg/mL) were prepared in 0.005 M sulphuric acid. The working standard solutions were a mixture of the four compounds prepared by appropriate dilution of the stock solutions in 0.005 M sulphuric acid at concentrations: 10 µg/mL for OFLO, 0.25 µg/mL for NOR, 0.3 µg/mL for CIP and 0.5 µg/mL for ENRO. Working standards were kept in dark and at 4ºC.

Naformátováno: Řádkování: Přesně 22 b.

4.5. Preparation, extraction of samples and clean-up

Based on method of extraction from T. Christian [10~~1~~] and Y. Picó [19~~7~~] we made pre-treatment of soil sample. Soil samples were heated at 100°C over night in dryer. Then the samples, which has room temperature ~~at room temperature~~ were extracted. Temperature of pre-treatment was adjusted therefore Czech Pharmacopoeia.

To one gram of the soil in tube 5mL of MeOH and 5mL of acetone 1g of EDTA were added, and the mixture was left in contact during 15 min at room temperature, in the dark. After 15 min in ultrasonic bath, the sample was centrifuged during 10 min at 2000g and supernatant was moved to other tube. Supernatant was evaporated under gentle nitrogen flow in a water bath (40°C) until dryness. The dried extract was dissolved in 20mL of mQ-H_2O, pH =4.5 and added with 0.2g of EDTA.

After conditioning of OASIS HLB cartridge with 5mL of methanol and 5mL of citric acid (pH 4.0), the sample was percolated through the column.

Then a wash step with 20mL of mQ H_2O was carried out., and the column was dried for 5 – 10 min. Eluted from OASIS HLB was performed with 7 ml of methanol (pH 7.75; pH was adjusted by NH_4OH). Methanol was evaporated under gentle nitrogen flow in a water bath (35°C), and the residue was dissolved in mobile phase and filtered through 0.45μm filter.

5. Results

5.1. Optimization of SPE method

In order to evaluate the losses and increase the accuracy of the analytical methodology, the studies were firstly performed with a standard solution (assay 1), and subsequently with a mixture of standards in Milli Q water (assay 2), according to the analytical methodology. In both cases pH was adjusted on 4.5. The results are presented in Table 5.

In this study we proceed to the evaluation of the clean-up methods. A ~~more~~ simpler one using only one single clean-up through a polymeric sorbent HLB 6 cc Oasis, ~~Dr. Angelina, please, can you fill up this information~~ and two amounts of the polymeric sorbent were evaluated: 200 and 500 mg, and a second method using a tandem double clean-up with a SAX (on the top) and a OASIS columns.

Naformátováno: Odsazení: První řádek: 1,25 cm

Naformátováno: Barva písma: Světle modrá

FQ	Standard Areas	Assay 1 Areas	recovery of assay 1; %	Assay 2 Areas	recovery of assay 2; %
OFLO	11962.0	9002.5	75.26	8276.5	69.19
NOR	37927.5	29325.0	77.32	28450.5	75.01
CIP	28280.0	21732.5	76.85	19573.5	68.78
ENRO	76445	56484.0	73.89	53933.5	70.55

Table 5 – Results of measuring of assay 1 and assay 2

For the tandem clean-up the cartridges were conditioned according M. Seifertová ~~Marcela~~ et al method [20]~~(ABC 2008)~~ ~~Jana please confirm if it was the same procedure.~~ . Recovery ranged between 68.8 to 75.0 %.

Next, we tried only one clean-up step with OASIS column and in order to optimize this analytical procedure, two amounts of the polymeric sorbent were evaluated: 200 and 500 mg. The results obtained are presented in Table 6, and according the better results wereas achieved with the 200mg column, with

recovery values between 86 and 101 %. The process was exactly same like with the tandem of SAX and OASIS.

FQ	Std Areas	500mg, Areas	200mg Areas	Recovery(%), 500mg	Recovery(%), 200mg
Ofloxacin	5906	4302	5263.3	72.84	89.11
Norfloxacin	7971.6	11414.5	8045.3	143.19	100.92
Ciprofloxacin	8237.3	6090	7080	73.93	85.95
Enrofloxacin	21743.6	12862.5	18843.3	59.15	86.66

Table 6 – Recovery of compare of OASIS columns

Since we obtain good blanks and good accuracy results after OASIS clean-up, this method was optimized with a soil sample.

5.2. Application to soil samples

When soil samples were fortified with standards and analysed according the proposed analytical methodology, very low recoveries were achieved. Therefore, different attempts were undertaken in order to explain and eliminate the losses origin.

At first, losses due to complexation of FQs were not a strong possibility during the analytical method since H. Zhang and C-H Huang [227], stated that it is necessary several hours, (3-4) hours to make a complex with ions in soils. Otherwise, in our particular case, since soil samples were submitted to an acidic extraction, FQs are in cationic form. Therefore, they not make complex with cations like Ca^{2+} or Mg^{2+} etc. But, there is possibility to make reaction with anions.

Naformátováno: Odsazení: První řádek: 1,25 cm

Since the addition of EDTA is referred by most authors (Barceló, Turiel), we proceed to the optimization of the amount of EDTA, in order to

improve the recovery of the proposed method. The results are presented in table **7.**

EDTA was added before standard and shacking for 45 minutes, than the blank was left 12 hours in dark at 4°C.Afterwards, 7 mL of HCl wasere added to the soil and 30 min shacked, 15 min centrifuged. Supernatant was moved to another tube and to the soil wasas added next 7mL of HCl and took to the ultrasonic bath for 30 min, than 15 min centrifuged and supernatant was removed to the tube. This was made one more time. All supernatants were centrifuged together for 20 min, pH were adjusted on 4.5 and than clean-up was done. For low amount of EDTA none of the FQs were detected, demonstrating losses during the process.

The results are presented in Table 7. The better results were obtained after the addition of 1.63 g of EDTA, but the recovery values were still low. Therefore, other interference in real soil samples occur which can make almost impossible detection of FQs added to the soil sample.

FQ	0.2g EDTA; %	0.8g EDTA; %	1.16g EDTA; %	1.63g EDTA; %	1.65g EDTA; %	1.71g EDTA; %	1.81g EDTA; %	2.0g EDTA; %	3.0g EDTA; %	5.1g EDTA; %
OFLO	12.9	19.43	37.29	43.77	37.28	33.14	-	-	5.44	-
NOR	15.3	17.80	16.01	40.04	16.01	20.63	2.00	9.63	7.52	-
CIP	-	18.96	10.55	20.46	10.55	20.66	12.00	-	1.41	-
ENRO	-	14.97	11.11	15.76	11.12	12.03	2.69	5.95	-	-

Table 7 – Recovery of samples with various amount of FQs

So, in order to confirm if the low recovery values reported were due to FQs complexation, an assay with standard solutions added with EDTA, without matrix, was carried out. Results obtained are presented in Table 8 and reveal that this issue has no effect in the recovery. So, we can conclude that the complex EDTA-FQs is not formed. It can be explained by the constant of dissociation of the complex FQs-EDTA when compared with constant of dissociation 15,36 EDTA-Ca [1~~34~~].

FQ	Standard Areas	Sample Areas	RECOVERY %
OFLO	18002	15028	83.48
NOR	50930	35764	70.22
CIP	31173	19274.5	61.83
ENRO	81340.5	71829.5	88.30

Table 8 – Standard with EDTA only

Since the problem is related with the matrix. Fungi and microbial degradation of FQs could be also the reason of these results. In order to eliminate the organic matter, that can interfere with FQs and make bounding on the soil easier, and the fungi and microorganisms which can broke piperazyl circle and therefore camouflaged the results. Therefore, an assay with a sand sample after submitted to a previous treatment was undertaken.

The sand sample after a pre-treatment with 10% of HCl overnight at 550ºC and then washed by water at pH 7. In this assay, we decreased the time of interaction between FQs and sand, to 15 min during the shaking step and 15 nim during extraction in ultrasonic bath. After 15 min of centrifugation, the extracts were cleanup according the method described above. The results are shown in the Table 9.

Naformátováno: Odsazení: První řádek: 1,25 cm

FQ	Standard Areas	Sample Areas	Recovery %
OFLO	17012	13784.5	81.02
NOR	47449	27665	66.74
CIP	29622.5	17388	58.69
ENRO	76781	51551	67.14

Table 9 – Recovery of sand sample

No interferences were observed in the region of interest where the FQs were eluted, and the recovery values were higher, according with those reported by the literature.

Therefore, this treatment that includes a acid washing step of the soil sample with 0,03M, followed by washing with water at pH 7 and heating at 550º C overnight, was applied to WWTP soil sample and to a loam sample from meadow. The recovery results obtained were remains very low, so they could not be explained either by FQs degradation.

Table10. resumes the values obtained for the different soil samples analysed in this study.

Matrix	Recovery of OFLO	Recovery of NOR	Recovery of CIP	Recovery of ENRO
Clay (1.63g of EDTA)	43.77	40.04	20.46	15.76
Clay + sand (1: 1)	7.57	5.63	–	–
sand	81.02	66.74	58.69	67.14

Table 10 – Recovery of various kind of soil

In order to improve the recovery of the analytical methodology, we proceed to the extraction with an organic solvent. Based on Pico et. al 2007 [19] ~~(REFERENCE),~~ extraction in ultrasonic bath for 30 min with a mixture of CH_3OH and water (80: 20) was assayed. After evaporation of the solvent the extract was dissolved in water. Since 2mL of water remains FQs standards

added were not detected. So, in next assay the sample was extract only with MeOH (without water). FQs recoveries were very low (for OFLO 7,56%; for NOR and CIP value was under limit of detection and for ENRO 12%).

Based on Barceló et al. [22](2003) a mixture of organic solvents CH_3OH and acetone (1:1) was assayed in order to improve the recovery. The results obtained are improved and according with those reported by other authors, namely Barcelo et al [2231]. Results from this method can be seen in the table 11.

FQs	Standard area	Sample area	Recovery; %
OFLO	11089.0	8670.0	78.18
NOR	40311.5	18983.0	47.09
CIP	33213.0	18311.5	55.13
ENRO	83588.5	52805.0	63.17

Table 11 – Results with organic solvents

5.3. Validation

Method validation is one of the measures universally recognized as a necessary part of a comprehensive system of quality assurance in analytical chemistry. Reliable analytical methods are required for compliance with national and international regulations in all areas of analysis providing data of the required quality.

Naformátováno: Odsazení: První řádek: 1,25 cm

Analytical method validation is completed to ensure that an analytical methodology is accurate, specific, precise and robust over the specified range that an analytes will be analysed.

The main aim of validation of an analytical method is to perform that the method is suitable for its intended purpose, such as implementation of legislation and for monitoring and risk assessment studies. It must be ensured that the method generates meaningful data and is accurate, specific,

reproducible and robust over the specified range that an analyte will be analysed.

5.4. Linearity and range

—The calibration curve was prepared by measurement of areas of FQs standard solutions in the range from 0.05 to 1 µg/mL (0.05; 0.1; 0.25; 0.5; 1 µg/mL)

—The linearity of the method was obtained by using the linear least squares regression procedure of the peak area versus concentration. The linearity for FQs, in the working standard solutions of four concentrations levels, was good as shown the fact that the determination of mean correlation coefficients (R) are 0.9945 for norfloxacin, 0.9974 for ciprofloxacin and 0.9982 for enrofloxacin.

Quantification limit

—Limit of quantification (LOQ) is For OFLO 5mg/L, for NOR is 0,083mg/L, for CIP is 0,116mg/L and for ENRO is 0,125mg/L.

5.5. Stability studies of standard solutions

The stability of standard solutions and of sample extracts was evaluated. The working standard solutions were stored at 4ºC and analysed during a one-week period. The scan of degradation started 3 day after the solution was prepared.

Concerning stock standard solution no degradation was observed after one month of storage

The sample extracts are stable till 48 hours at 4°C.

Naformátováno: Odsazení: První řádek: 1,25 cm

Naformátováno: Odsazení: První řádek: 1,25 cm

FQ	1.day Areas	2.day Areas	2.day; %	3.day Areas	3.day; %
OFLO	11992.0	12406.0	103.45	11962.0	99.74
NOR	35477.5	36321.0	102.37	37927.5	106.90
CIP	29833.0	28673.0	96.11	28280.0	94.79
ENRO	76428.5	73376.0	96.00	76445.0	100.02

Table 12 – Results of stability study from first 3 days

FQ	4.day Areas	4.day; %	5.day Areas	5.day; %
OFLO	12683.0	105.76	12560.0	104.73
NOR	43268.0	121.95	36480.5	102.82
CIP	33760.0	113.16	28382.5	95.13
ENRO	80011.0	104.68	77535.0	101.44

Table 13 - Results of stability study from last 2 days

6. Discussion

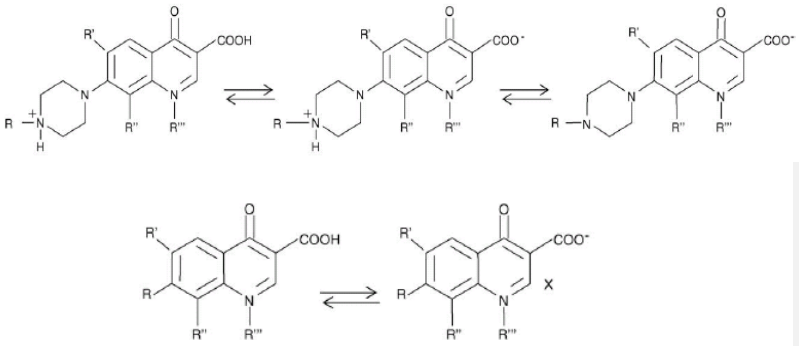
As was already said soil is very complex matrix, also with microbial and enzymatic activity against all organic contaminants including FQs. Because of that samples were stored in 4ºC in dark in plastic bottles. Dark conditions stopped photo-degradation, which is important at FQ. For example ENRO had an average half-life of 2 hours under summer sunlight conditions (the kinetics of photolysis decreased in the presence of humid acids).

Against ~~S~~stability study has FQs ~~has~~ good stability ~~stability~~ in mobile phase as well as in sulphuric acid (0.005M) minimal in 48 hours. It is common dissolve samples in mobile phase if it is possible, cause minimal interaction during detection.

FQ form stable complexes with several divalent and trivalent metal ions. These complexes are formed by ion-dipole interaction with the 4-keto oxygen and the ionized 3-carboxylic acid group. The observed strong adsorption of quinolones by soil, with their different acid-base properties, makes exhaustive optimization of the extraction step essential

According to Golet et al. ~~(2002)~~[23] use of high or low pH improves recoveries, which are maximum at acidic pH. These results suggest that complexes formed chelates with deprotonated carboxyl group of FQs. Because of these compounds are strongly adsorbed by soils use of exhaustive extraction methods, such as pressurized liquid extraction(PLE) or microwave-assisted extraction (MWAE) are required. [19~~7~~] From obtained results (Turiel et. al, 2006), it can be concluded that, in general, FQs are better extracted from soil using basic solutions that is in their anionic form. However, on the contrary, extraction of quinolones in their anionic form was almost impossible, whereas it was clearly favoured using acidic conditions that are in their neutral form. On the other hand, it can be seen that, among the three organic solvents tested (ACN, MeOH or acetone) acetone was in general most successful extractant. The highest recovery was observed using the mixture acetone/water/ammonia (50/25/25) for FQ. It is know that quinolones and FQ are able to form stable

complexes with several divalent and trivalent metal ions. Among them Ca (II), Mg (II) and Al(III) lead to complexes with the highest stability constants and thus, the ability of these cations to desorbs and extract quinolones and fluoroquinolones from soil was evaluated. Firstly, the influence of pH on the formation of the corresponding complexes and thus on the efficiency of the extraction was evaluated.



Based on the published literature and our knowledge we used HCl (0.15M) and because this methods used in the laboratory at other matrix had very good results. Thought this information we also tried extraction with methanol and water (8mL of methanol and 2mL of water per 1g of soil) and other extraction with 10mL of $Mg(NO_3)_2$+ 0.5mL of NH_3 per 1g of soil (1mg/L of $Mg(NO_3)_2$; 25% NH_3). In both case extraction was not successful. Because of that we change method of extraction several times. The first we used the original method (1x shacking and 2x 15 minutes in ultrasonic bath – the method was not published yet), than we used 1 time ultrasonic bath and twice shacking in various order. We also tried 3x 15 minutes ultrasonic bath, but ~~best~~ better results had~~was~~ 3x15 minutes of shacking. Between every shacking we used centrifugation. We tried 10 or 15 minutes of centrifugation but there was no different observed. So we used 10 minutes because of heftiness time. We tried various acids to make an extraction: H_2SO_4 (1M), H_2SO_4 (0.1M), H_3PO_4 (1.8M); the best results were with using HCl (0.15M). When the results with

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acidic extraction were not good we tried use organic solvents; methanol, methanol with water (8:2) and methanol with acetone (1:1). The last one was successful.

During optimizing of SPE extraction we followed "Strategy for optimizing the Generic SPE Method". The pH is decreased for acidic analytes (below the pk_a of compound) to increase retention. In our case was used pH lower than pk_{a1} of FQ. It says that is suitable used higher concentration of organic solvent to remove interferences. Amount of sample used in general method is same as well as at condition process, thought we do not keep it results were good.

For SPE extraction we originally used method developed by Seifrtová [2036]. For elution is recommended volume 4 ml (in study of Seifrtová; [200720]) and in Strategy for optimizing the Generic SPE Method as well), but we find out that elution by this amount is not enough in our case. We eluted 4mL of MeOH and than second 2mL of other MeOH and in the second elution still was rest of sample. Because of that 7 ml of methanol (pH 7.75) was used.

During optimization of this method was the concentration changed. 3 various concentrations of standards were used**.** The best shape of peak was at terminal concentration.

standard	**Concentration first**	**Concentration second**	**concentration terminal**
OFLO	7.5 µm /mL	6.5 µm /mL	10 µm /mL
NOR	0.25 µm /mL	0.25 µm /mL	0.25 µm /mL
CIP	0.5 µm /mL	0.3 µm /mL	0.3µm /mL
ENRO	0.5 µm /mL	0.5 µm /mL	0.5 µm /mL

Table 14 – Concentration which was used

We changed flow during detection. It was because of shape of peak again. Because of change flow retention time and pressure were changed also, of course. For all detection was used 1.4 ml/min. LC is very sensitive on outside conditions, retention times are changing because of temperature as well. In several days there was 1 min difference between measuring (6 hours between injections). From this reason is better injecting standard during day between measuring of samples.

FQ	RT (1.2 ml/min)	RT (1.4ml/min)
OFLO	5.73	4.88
NOR	8.77	7.46
CIP	10.37	8.77
ENRO	14.89	12.58

Table 15 – Retention time with various flows

We changed method ~~after~~ because we observed~~got~~ low recovery with long-time extraction. We tried decrease contact between FQs in fortification and soil, because of possibility of adsorption, complexation and degradation by the microorganism. Therefore extraction was only 15 min of shacking 15 min, of ultrasonic bath and 15 min of centrifugation. The extraction with sand and later results is only with the sort-time extraction. Anyway the results were not better.

FQs can be extracted of the soil with polar organic solvents, including mixtures of solvents, combined with blending, stirring, or use of ultrasound, or with aqueous solutions, then clean-up by SPE. The observed strong adsorption of quinolones by soil, with their different acid–base properties, makes exhaustive optimization of the extraction step essential. [19~~7~~]

7. Final conclusion

We find out that extraction with HCl (0,15M) is enough for water, and soil without organic matter and microorganisms (for example sand or sandy soil). At the beginning we tried also acidic type of extraction. Extraction was made with HCL, H_2SO_4, H_3PO_4 and HNO_3. In all cases we add EDTA because of interferences with other substances in samples. Best recoveries in acidic conditions were with HCl.

-Method with organic solutions was better for our conditions. Limit of quantification (LOQ) is fFor OFLO 5mg/L, for NOR is 0,083mg/L, for CIP is 0,116mg/L and for ENRO is 0,125mg/L. Mean recoveries ranged between 75% to 121%, for OFLO, NOR, CIPRO and ENRO. Because of time there are no real samples.

7. Abstract

Several pharmaceutics used in human and veterinary medicine could be detected in environment. To these compounds belongs Fluoroqinolones. Our work observed 4 fluoroqinolons (ofloxacin, norfloxacin, ciprofloxacin, enrofloxacin) in soils sample. Fluoroquinolones were determinate by LC-FD. For extraction was used mixture of organic compounds (methanol: acetone; 1:1) and 1.0g of EDTA. Sample was 15 minutes shacked, 15 minutes in ultrasonic bath and 15 minutes of centrifugation. After that organic solution was evaporated by gentle stream of nitrogen in water bath. Rest from evaporation was dissolved in mQ-H_2O and clean-up was done. After evaporation after clean-up the rest was dissolved in mobil phase. Mobil phase was H_3PO_4: MeOH: ACN (920: 70: 10) and flow was 1.4mL/min.

Limit of quantification (LOQ) is For OFLO 5mg/L, for NOR is 0,083mg/L, for CIP is 0,116mg/L and for ENRO is 0,125mg/L. Mean recoveries ranged between 75% to 121%, for OFLO, NOR, CIPRO and ENRO.

At the beginning we tried also acidic type of extraction. Extraction was made with HCL, H_2SO_4, H_3PO_4 and HNO_3. In all cases we add EDTA because of interferences with other substances in samples.

We find out, that no complex is build between flouroquinolones and EDTA. For clean-up from soil samples is better OASIS column HLB 6cc/200mg. Extraction in acidic conditions is suitable for extraction from water and sandy soil (without organic matter and microorganism). Stability study was done and Fluoroquinolones were stabile minimal for one week at 4°C. Samples are stabile minimal for 2 days at same temperature. Fluoroquinolones are decomposed at normal temperature and on the light (under UV).

8. Abstrakt v češtině

Některé látky využívané v humánní a veterinární medicíně jsou detekovatelné v prostředí. Mezi tyto látky patří i fluorochinolony. Naše práce se zabývá 4 flurochinolony (ofloxacin, norfloxacin, ciprofloxacin a enrofloxacin) ve vzorcích půdy.

Fluorochinolony byly detekovány metodou LC-FD. Pro extrakci byla použita směs organických rozpouštědel (methanol:aceton; 1:1) a 1.0g EDTA. Vzorky byly 15 minut třepány, 15 minut v ultrazvukové lázni a poté 15 minut centrifugovány. Po odpaření organických rozpouštědel mírným proudem dusíku ve vodní lázni byl odparek rozpuštěn v mQ-H_2O byl proveden clean-up (přes kolonu OASIS). Po odpaření methanolu z clean-up byl odparek rozpuštěn v mobilní fázi. Mobilní fáze byla H_3PO_4: MeOH: ACN (920: 70: 10) a průtok 1.4mL/min. Limit kvantifikace je pro OFLO 5mg/L, pro NOR je 0,083mg/L, pro CIP je 0,116mg/L a pro ENRO je 0,125mg/L. Rozsah hodnot výtěžnosti jsou 75% - 121%, pro OFLO, NOR, CIPRO a ENRO.

Z počátku byla prováděna extrakce v kyselém prostředí, ale výtěžnost byla nízká. Extrakce byla prováděna pomocí HCl, H_2SO_4, H_3PO_4 a HNO_3. Ve všech případech bylo přidáváno EDTA, aby se zabránilo interferencím s jinými látkami obsaženými ve vzorku.

Bylo zjištěno, že nevzniká komplex mezi EDTA a fluorochinolony. Pro clean-up je vhodnější OASIS HLB 6cc/200mg. Extrakce v kyselém prostředí je vhodná pro extrakci z vody nebo písečné půdy (bez organické složky a mikroorganismů). Byla provedena stabilitní studie, ve kteeréⁿ bylo dokázáno, že fluorochinolony jsou stabilní minimálně 5 dní při teplotě 4°C v temnu. Vzorky byly stabilní minimálně 2 dny při stejných podmínkách. Fluorochinolony ~~jsou~~ běžně podléhají rozkladu při pokojové teplotě a na světle.

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