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Sledování norfloxacinu, ciprofloxacinu a enrofloxacinu pomocí HPLC-FD ve vzorcích odpadních vod a jejich vliv na životní prostředí.

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Occurrence of norfloxacin, ciprofloxacin and enrofloxacin by HPLC-FD in samples collected from piggeries in Portugal and evaluation of environmental impact.

Marie Spurná **2009**

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Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury.

Abstract

Fluoroquinolones (FQs) are used for therapy and prophylaxis of human and animal diseases and they are also used as growth promoters of animals. The usage of antibiotic is in connection with development of antibiotic resistance among bacterial populations. Since swine production industry is considered to be a relevant source of antibiotic resistant bacteria, measures to control the use of antimicrobial agents in animal husbandry have been adopted in recent years (1).

The presence of FQs in wastewater from swine farms can introduce antibiotics in surface waters through agricultural runoff. FQs are rather resistant to microbial degradation and these compounds may be persisting within environmental waters because of their sorption properties, favouring the accumulation in sewage sludge, manure and soil (2).

To trace the sources and ways of contamination, and evaluate the environmental impact of an outdoor swine production system, the occurrence norfloxacin (NOR), ciprofloxacin (CIP) and enrofloxacin (ENRO) residues were evaluated in wastewater and surface water samples. A LC-FD method based on the application of monolithic column (Chromolith Performance RP-18e (100 x 4.6 mm) successfully developed in previous studies (3,4,5) was applied. FQs were isocratically eluted using a mobile phase consisting of 0.025 M Phosphoric acid solution at pH 3.0 with tetrabutylamonium and methanol (90:10, v/v), at a flow rate of 2.2 mL/min, at an excitation and emission wavelengths of 278 and 450 nm respectively.

The limits of quantification (LQs) of these FQs, expressed as the lowest tested level with acceptable RSD was 25 ng/l. Recoveries yield of spiked samples ranged from 66.4% to 114%. Within-day accuracy and precision (expressed as RSD) data obtained were under 17%.

The method was successfully applied for determination of NOR, CIP and ENRO in water samples, collected from an intensive piggery, in Portugal.

1. INTRODUCTION

The knowledge of concentration of pharmaceuticals in water is very important. The concern of these compounds as environmental contaminants is based in the fact that they are developed with the intention of performing a biological effect and they often have the same type of psycho- chemical behaviour as other harmful xenobiotics (persistence in order to avoid the substance to be inactive before having a curing effect, and lipophilicity in order to be able to pass membranes) (6).

The primary source of antibiotics in the environment is the excretion of incompletely metabolized antibiotics by humans and animals.

With increasing usage of antibiotics increase also their occurrence in environment and their potential risk.

The impact may be on any level of the biological hierarchy: cells ± organs ± organisms ± population ± ecosystems ± the ecosphere (7).

Fluoroquinolone antibiotics (FQs) were shown to display high genotoxicity at concentrations detected in hospital effluent (3000 to 87,000 ng /L). Genotoxic substances are often mutagenic and carcinogenic and are, therefore, potentially suspect in the development of antibiotic resistant organisms (8).

FQs are probably among the most important class of synthetic antibiotics in human and veterinary medicines worldwide (9).They are widely used in the treatment of a wide variety of diseases. They are effective against Gram- negative bacteria and Gram- positive bacteria as well.

The aim of this work was to determine the concentration of FQs antibiotics, namely norfloxacin, ciprofloxacin and enrofloxacin in wastewater and surface water from an intensive piggery, in Portugal.

2. THEORETICAL PART

2.1 USAGE OF ANTIBIOTICS

Thousands of tons of pharmaceuticals are used yearly with different purposes such as the prevention, diagnosis, cure and mitigation of diseases or just to improve the state of health not only in humans but also in animals such as cattle, swine, poultry, and fish (10, 11). Antimicrobial agents are administered to livestock at therapeutic doses or to prevent illness (prophylaxis) at much lower doses (subtherapeutic). Antimicrobial agents are also used as feed additives to increase the rate of growth and to improve feed efficiency (12).

2.2 SOURCE OF ANTIBIOTICS IN ENVIRONMENT

Most of the compounds used in medicine are only partially metabolized by patients and are then discharged into the hospital sewage system or directly into municipal waste water if used at home (13).

About 25% to 75% of compounds leaving the organisms unaltered via feces or urine (14).
Metabolism may result in chemicals that are either more or less biologically active than the form in which they were consumed (15).

Along with excreta, they flow with municipal waste water to the sewage treatment plant (STP) (7).
Residential (private residences, dormitories, hotels, and residential care facilities) and commercial facilities (including hospitals) are also known contributors of antibiotics to municipal wastewater (8).

After the administration of veterinary medicinal products in livestock husbandry, parent compounds and metabolites are excreted by production animals via urine and feces. (16)

Veterinary medicinal products that are not degraded during manure storage and they are applied to farmland or grassland soils as organic fertilizers. Therefore, they can enter aquatic and soil environments via this entry route. This fact is namely considered relevant by the authorization procedure of veterinary medicinal products (16).

Numerous studies on fate and behaviour of veterinary medicinal products in pig manures clearly demonstrated possible effects of manure application to fields on the fate of veterinary medicinal products (16). These antibiotics may either end up in soil or sediment or in ground water (13).

Antimicrobial agents are also used to treat infections in intensive fish farming where they are added directly into the water, resulting in high local concentrations in the water compartment and abutting sediments. (13)

Some antibiotics such as streptomycins are used in fruit growing, others in bee-keeping (13).

Treatment of raw wastewater (which includes a mix of domestic sewage, industrial wastewater, and stormwater runoff, depending on the wastewater treatment plants (WWTPs) may remove a proportion of these compounds, but there is the potential for residues of antimicrobials to be released in treated effluent into the aquatic environment (11).

Nevertheless, there is a lack of the fate of veterinary medicinal products environmental compartment until today. (17)

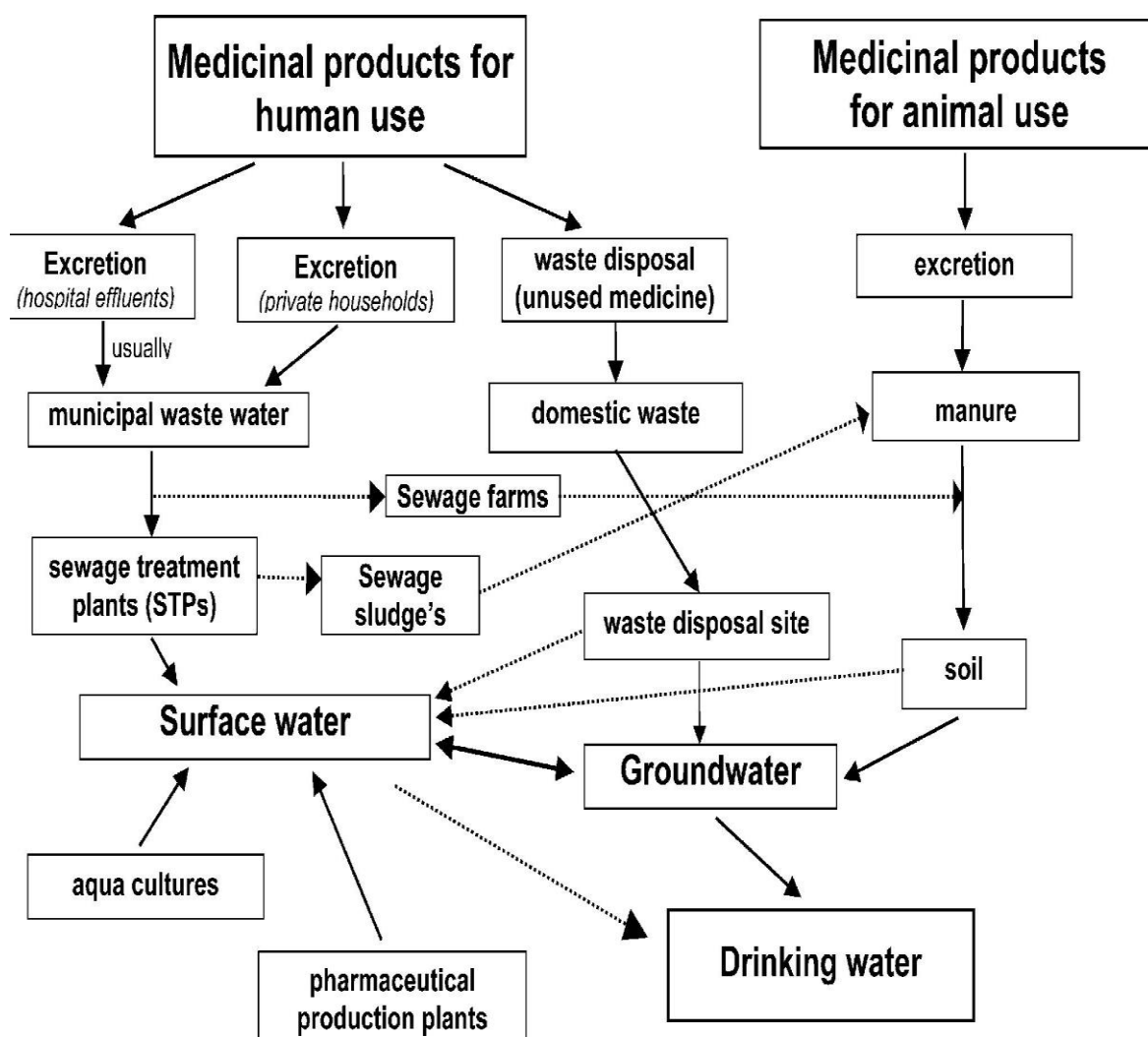*Fig. 1. Scheme showing possible sources and pathways for the occurrence of pharmaceutical residues in the aquatic environment.(19)*

2.3 FATE OF ANTIBIOTICS IN ENVIRONMENT

On release via urine and faeces into the environment, antibiotics disperse through a variety of transport mechanisms. The introduction of drugs into the environment is a function of the combination of several factors. A number of physical and chemical

processes are responsible for the antibiotics moving through the feedlot or the open pasture into the environment. Sorption, leaching and degradation are the three important processes in the soil–water systems. These processes are driven by the physico- chemical properties of the antibiotics, such as their molecular structure, size, shape, solubility and hydrophobicity, dissociation constants and also by the quantity manufactured, the dosage (amount, frequency and duration), the excretion efficiency of parent compound and metabolites, the adsorption/desorption on soil capability and the metabolic decomposition in sewage treatment. Before discussing their fate and transport in the environment, the basic chemistry of these compounds should be understood because may help determine whether a compound is likely to concentrate in the aquatic, terrestrial, or atmospheric environment (20, 21) Concentration of pharmaceutical in the environment, their temporary evolution and their possible synergic and antagonist effects depend also on geographical area and climate conditions (22).

The drugs used by humans will be discharged to the sewer systems together with the urine and faeces and enter the STP. The possible fate of drugs in the STP may be divided as follows:

(i) The drug or metabolites of the parent drug is mineralized by micro-organisms to carbon dioxide and water, e.g., aspirin.

(ii) The drug or metabolites of the parent drug can persist in the STP. It depends mainly on the lipophilicity or other binding possibilities e.g. ionic bindings, a part of the substance will be retained in the sludge. If the sludge is used as soil conditioner drugs may be dispersed on agricultural fields. Again the fate of the drugs depends on the lipophilicity or other ability of binding to sludge or soil.
Drug molecules often have many functional groups e.g., carboxylic acids, aldehydes and amines, which makes the binding capacities of the molecules to solids dependent on pH or other constituents (e.g., complexation) in the solids matrix. Drugs that are mobile in the soil may be a threat to the ground water or leach to a nearby stream. Depending on the ability of the drug to bind solids either organisms in the terrestrial ecosystem or aquatic ecosystem may be exposed.

(iii) The drug or metabolites of the parent drug is persistent and at the same time very polar and nonbinding to solids. The substance will thus not be retained neither degraded in the STP and therefore easily reach the aquatic environment, and may affect the aquatic organisms (7).

An unknown portion of drugs marketed for human treatment ends in the sewer system as surplus medical substances (7).

Most of the drugs used for veterinary purposes will end up in manure which is conserved in tank systems before being dispersed on fields. As previously explained the mobility of the drugs or drug metabolites in the soil system predicts if the drug will threat the ground water, affect the terrestrial organisms, or the aquatic organisms, due to leaching from fields (7).

If the drugs are applied to foraging animals drug or drug metabolites will be urinated or defecated directly on the field and that way may give much higher local environmental loadings of drugs (7).

Drugs used as a feed additive in fish farming will be discharged directly to the receiving water as parent compound, because a large portion of the applied medicated feed is not eaten by the fish. A local water treatment plant is often treating the water from aquaculture before going to the aquatic environment. The sludge from these treatment plants are also used as soil conditioner so drugs used in fish farming may also end on agricultural soil and undergo the same fate as previously described for the growth promoters and therapeutically used drugs (7).
Probably, it was assumed that they occur in sufficiently low concentrations to be harmless for the environment independent on their biological activity. It may be the case if all drugs discharged to the environment would be diluted in the total environment. But several drugs are discharged locally, for instance antibiotics in manure which is spread on a few hectares of agricultural land, or hospital waste water discharged via a relatively small treatment plant to a local stream or lake (7).

The amount of pharmaceuticals and their bioactive metabolites being introduced into the environment is probably low. However, their continual input into the environment

may lead to a high, long-term concentration and promote unnoticed adverse effects on aquatic and terrestrial organisms. Effects can accumulate so slowly that changes remain undetected until they become irreversible (20).

Gathering baseline information about the existence and extent of these environmental pollutants is an essential first step toward understanding the environmental impact or human health (12).

Recent evidence suggests that the interaction between bacterial organisms and antimicrobial agents in the environment may contribute to the development of antimicrobial-resistant bacterial strains, with groundwater serving as a potential source of antimicrobial-resistant pathogens in the human food chain (12).

Recent analytical studies show that some pharmaceuticals are poorly removed in STP and are consequently detectable in surface water (rivers, lakes, seas) in the ng/L up to the µg/L range. STPs might therefore be the important point source of contamination, but for the majority of pharmaceuticals little information is available on their behaviour and ultimate fate in STPs and in the receiving surface water (23).

Thus, the antibiotics may accumulate and reach concentrations in the Minimal inhibitory concentration (MIC) range. If they are still effective after sorption, resistant bacteria may be selected by antibiotic substances due to their application in animals, their use as growth promoters and in soil. (13)

2.3.1 Occurrence in Sediments and Sludge

The persistence of a drug in a sediment or soil depends mostly on its photostability, binding and adsorption capability, degradation rate, and leaching into water. In addition, the rate of sedimentation conditions the halflife of the chemical. Strongly sorbing pharmaceuticals accumulate in the soil or sediment more than highly mobile pharmaceuticals, which tend to leach to the groundwater and be transported with the groundwater, drainage water, and surface run-off to surface waters. (24)

The ratio between the concentration of the compound in the sorbent and in the water at equilibrium is called sorption coefficient ($K_{d,solid}$). This coefficient, however, is hard to calculate under the multiple and variable conditions of each particular environment. In addition, the $K_{d,solid}$ of many neutral hydrophobic organic chemicals has been shown to vary depending on the organic carbon content of the sorbent. For such compounds, use of the so called organic carbon-normalized sorption coefficient (K_{oc}) is recommended. Another aspect that favors the use of K_{oc} as a measure of the sorption in environmental risk assessment is the strong correlation existing between this coefficient and the octanol-water partition coefficient (K_{ow}) an easily obtained physicochemical property from which the K_{oc} can be easily calculated. Quite a number of different mechanisms are involved in drug sorption, the most important being sorption to organic matter, surface adsorption to mineral constituents, ion exchange, complex formation with metal ions (such as Ca^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+}), and H-bonding (24).

The main processes of substance elimination in the environment, especially in waste water, sediments, and soil are due to bacteria. The concentration of antibiotics may be much higher if the active compounds are persistent and accumulate. In these cases, the role of antimicrobial concentration could be different from that in water. It is not known how strongly the antibiotics are sorbed and under what circumstances they are still available and active after sorption. (13)

Fluoroquinolones (FQs) are sorbed by organic matter. Therefore, they can accumulate. It is not yet known to what degree and under what circumstances the compounds are effective after sorption or whether they are released and may contribute to resistance. Antimicrobials may have qualitative and quantitative effects upon the resident microbial community of the sediments. (13)

3.3.2 Occurrence in drinking water

Although their concentration are extremely low (ranging from hundreds of μg/L to less than ng/L), we currently do not know what impacts long- term exposure to these

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compounds at low levels will have on people consuming contaminated drinking water. The main areas of interest focus on PPCPs role in endocrine disruption and antibiotic resistance. (15)

The drinking-water-treatment facility utilizes a conventional treatment process that consists of the following sequence of physical and chemical treatments:

I. Raw-water screening – the movement of raw water past a stationary bar rack and two travelling screens to remove coarse debris.

II. The addition of powdered activated carbon to remove taste- and odor causing compounds as well as organic chemicals.

III. The addition of sulphuric acid or caustic soda for pH control.

IV. Coagulation – the addition of coagulant salts and polymers to destabilize colloidal particles and facilitate their flocculation with other suspended particles.

V. Primary disinfection – the addition of Na hypochlorite to inactivate pathogenic microorganisms.

VI. Flocculation – the agitation of coagulated water to promote the aggregation of suspended materials.

VII. Sedimentation – the stilling of flocculated water to promote the settling of suspended solids and floccules.

VIII. Filtration – the movement of water through tanks that contain sand and either bituminous granular activated carbon (GAC), lignite GAC, or anthracite to retain remaining fine solids and bacteria.

IX. Secondary disinfection – the addition of Na hypochlorite to maintain a chlorine residual in the distribution system.

X. The addition of caustic soda to adjust the pH at 7.8 to 8.2 for corrosion control (25).

2.4 POTENTIAL EFFECT OF ANTIBIOTICS IN ENVIRONMENT

Antibiotics can cause adverse effects on ecological systems and also on humans, e.g. allergic reactions. However, the main worry arises from the potential generation and/or spreading of new strains of resistant bacteria.

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 (26).

2.4.1. Bacterial Resistance

The resistance is a description of the relative insusceptibility of a microorganism to a particular treatment under a particular set of conditions. For bacterial resistance it is usually quantified as the minimum concentration required to assert a definable effect (e.g. growth inhibition) on a population of cells. Wherever there is a change in susceptibility that renders an agent ineffective against a certain organism, this organism is referred to as resistant. Many organisms have always been insensitive to and are thereby intrinsically resistant to a particular agent by nature of their physiology or biochemistry. Susceptible organisms can become insensitive by mutation or by incorporation of the genetic information which encodes the resistance (13).

The bacterial resistance is a natural part of the regulatory factors in any ecosystem. Bacteria have presented on the Earth for more than 3.8 milliards years and they have to adapt to environmental influences all the time. Therefore it is obvious that genes coding for resistance have existed as long as microbes. The increased use of antibiotics during the last five decades has caused a genetic selection of more harmful bacteria. The genetic pool of micro-organisms in nature has changed significantly, simply due to our increasing production and consumption of antibiotics (7, 18).

Antibiotic-resistant bacteria were detected in drinking water as early as the 1980s and later in the 1990s. Authors found that resistant bacteria identified using classical microbiological methods, i.e. standard plate counting, occurred within the distribution network of drinking water supply systems. They concluded that the treatment of raw

water and its subsequent distribution selects for antibiotic-resistant bacteria. In agreement with these data, increased phenotypic resistance rates were also detected at the drinking water sampling points in the study by Schwartz et al. (13).

For example, FQs usage in poultry husbandry have promoted the evolution of fluoroquinolone- resistant *Campylobacter jejuni* (Gaunt and Piddock, 1996), an important human pathogen. Exposure to FQs can result in a high fluoroquinolone MIC, for example of 4 mg/L for *C. jejuni*. Development of resistance to FQs typically occurs within 2 years of their widespread application in veterinary medicine (14).

2.4.2. Direct toxicity to microorganism
Apart from the issue of resistance, it has to be kept in mind that bacteria form one of the most important groups of organisms in soil and in other environmental compartments as well as in natural or technical sewage treatment. Residues of antimicrobials may also be directly toxic to microorganisms. Without bacteria, water would not be clarified. Bacteria are essential for the closing of nutrient and geochemical cycles such as the carbon, nitrogen, sulphur and phosphorous cycle. Without bacteria, soil would not be fertile and organic matter such as straw or leaves would accumulate within a short time. In soil, naturally occurring antibiotics from bacteria and fungi amongst others control the dynamics of bacterial populations. In contrast to these, most of the compounds used nowadays are semi-synthetic or synthetic. They are often much more stable and are not biodegradable by bacteria. They may therefore persist in the environment. Furthermore, they often have a different, e.g. broader, activity spectrum. (11, 13).

2.4.3. Detection of negative effects
Detection of these negative effects in the environment is difficult; although in vivo and in vitro laboratory tests generally show that the toxic effects of these compounds are not seen at the low levels currently detected in the environment, the possibility of variations in sensitivity, chronic exposure, and mixture effects such as concentration addition and synergism mean that other negative effects cannot be ruled out (27).

2.5 PROPERTIES OF FQs

Antimicrobially active compounds make up a large group of pharmaceuticals, which are widely used for the abatement of bacterial infections with humans and also with animals. Therefore, they are some of the most prescribed pharmaceuticals. In the European Union (EU) (including Switzerland) in 1997 the total amount of antibiotics used was 12 752 t. 7 659 t were used in human therapy and 5 093 t were applied in the veterinary sector, which is subdivided in:

1) antibiotics for therapeutic use with animals (in order to defeat or prevent an infection),
2) antibiotics, which are used as feed additives to accelerate growth (growth promoters).

For the first purpose in 1997 3 494 t were used, and for the second (i.e., as growth promoters), 1 599 t. In the last years a couple of substances has been prohibited by EU legislation for their use as growth promoters, due to the assumption that an increasing medication with antibiotics in subtherapeutic dosage can cause resistance in bacteria, which makes therapy more difficult, because less substances are able to cure an infection. Perhaps due to this regulation, the amount of antibiotics used as feed additives has lowered to its half, to 786 t in 1999,while the amount for human and also for animal therapy increased by 12%, so that in 1999 an overall mass of 13 216 t of antibiotics has been used (28).

2.5.1. Usage
Qs and FQs represent a highly potent group of modern antibiotics. They are nowadays broadly used in the treatment of a wide variety of diseases since they are not only more effective against Gram-negative bacteria but also moderately active against Gram-positive bacteria. They are used in the treatment of a great variety of respiratory diseases and enteric bacterial infections, treatment of Gram-negative urinary tract infections in humans and animals.
Quinolones are also used to treat livestock and fish in the aquaculture industry and used at sub-therapeutic levels to promote growth for animals.

FQs are antibacterial agents widely used for various infections because of characteristic of their broad activity spectrum and good oral absorption, good tolerance with less resistance. Compared with other drugs, Qs have a rapid bactericidal effect against most susceptible organisms, that is very important for patients who are seriously ill or for patients with defective or impaired immune system. In addition, they penetrate into tissues and mammalian cells extremely well which enables their widespread use in clinical medicine. Generally, FQs are prescribed 300–600 mg/day to the patient for therapeutic treatment.

The FQs administered to humans or animals are almost excreted as unchanged compounds in urine, and are mainly effluent from WWTP, but these compounds are not carried out monitoring on WWTP (11, 29, 30, 31, 32).

2.5.2. Mechanism of activity

Their activity is based on inhibition of DNA gyrase (topoisomerase II), a bacterial enzyme involved in DNA replication, recombination and repair, leading to bacterial cell damage (32).

2.5.3. Chemical structure

FQs are piperazinyl derivates of the quinolone nalidixic acid (NAL) and represent the second generation of this family of antibiotics.

Quinolones (Qs) are originated from antimalarial agent chlorquine, but NAL, the first generation quinolone antibiotics synthesised in 1962, had only modest antibacterial activity against gram-negative species and showed poor oral absorption. Introduction of a piperazinyl side chain at position 7 improved the activity against gram-negative bacteria and increased the penetration into bacterial cell wall. Fluorination at position 6, which was called fluoroquinolone antibiotics, increased their activity against gram-positive bacteria. Modification of molecular structure by substitution of both piperazinyl group and fluorine atom improved their efficiency. NOR, Enofloxacin (ENO), Fleroxacin (FLE), CIP and Ofloxacin (OFL) had broad antibacterial spectrum extending to most gram-positive species but their effect is still inadequate to treat respiratory infections. Recently developed newer generation Qs are trying to solve these problems by enhancing activity against gram-positive or anaerobic bacteria.

They are also active against atypical organisms like mycoplasma, chlamydiae, legionella or some mycobacteria (29, 32).

2.5.4. Physico- chemical properties

I. pH dependence

All compounds contain the keto oxygen at C-4 and carboxylic acid side chain at C-3, both of which have now been found to be essential to activity.

Due to carboxyl group and amine groups, the acid–base behaviour will be influenced by the physicochemical properties of solvent. (32)

Also, the antibacterial activity is pH-dependent, because these drugs act by inhibition of bacterial DNA gyrase, a process that depends upon both the pH and concentration of the acid. To this effect, the behaviour of Qs in vivo is significantly influenced by their degree of ionization. It has often been shown that the presence of charged groups is necessary for biological activity and solubility. However, the unionized form has a more favourable partition coefficient toward no aqueous solvents. Therefore, knowledge of the physico- chemical properties of these drugs such as dissociation constants may be essential for practical purposes and for the interpretation of structure–activity relationships. (32)

Therefore, Qs having different structures and substituents exhibit different antibacterial responses in various environments. Especially, the two groups of drugs, OFL, NOR, ENO etc., and NAL, Flumequine (FLU), Oxolinic acid (OXO) etc., show completely different antibacterial responses and chemical properties as the physicochemical properties of solvents are changed. These differences are governed by the presence of piperazinyl group at 7-carbon atom. With OFL and NOR, a pH increment from 5.6 to 8.3, which is the pH range that occurs in urine, progressively increases their activity in nutrient agar. On the other hand, NAL, FLU and OXO become more active in nutrient agar as the pH falls. (32)

Between Qs and some divalent cations 1:1 complexes are formed by ion–dipole interaction using the 4-keto oxygen and the ionised 3-carboxylic acid group. So when Mg^{2+} is added to an achievable urinary concentration (5.6 mM), the activity of these drugs is reduced at virtually every pH tested, but the antagonistic action of Mg^{2+} on these two groups of drugs, with or without piperazinyl group, does not exhibit similar

pH dependence. These observations suggest that there is a major difference between the two groups of drugs, with respect either to their mode of action, or to their mechanism of penetration into bacteria. (32).

II. Complex formation

The complex formation between Qs and divalent cations plays an important biological role. The ability of these drugs to interact with some cellular components is mediated by this complexation. Several studies have shown that a DNA gyrase cannot bind quinolones in the absence of DNA and the amount of Qs bound to DNA is modulated by the Mg^{2+} concentration. The photocarcinogenic potential of some Qs could be related to their ability to interact with DNA. (32)

III. Resistance

Some studies have demonstrated that bacterial resistance against FQs in the hospital wastewater is higher than that in the sewage treatment plant wastewater. These antibiotics are rather resistant to microbial degradation and these compounds may be persisting within environmental waters because of their strong sorption properties. On the other hand, degradation of antibiotics, including photolysis and chemical oxidation may be significant on their environmental ecosystem. Effluence of FQs into the environmental waters occurs mainly as the parent compounds and as a consequence of inadequate treatment of human and animal excreta. It is apprehensive that bacteria exposed with antibiotics in environmental waters may acquire resistances against antibiotics (30).

IV. Photosensititivity

It is reported that these drugs are able to induce photosensitivity reaction in human skin by sunlight. This effect will occur in any subject with sufficient cutaneous photosensitiser exposed to enough irradiation of appropriate wavelength, but the mechanism remains still unknown. (32).

Drug-induced photosensitivity can be divided into phototoxicity and photoallergy.

(i) Phototoxicity is an adverse cutaneous response to the combined actions of a chemical agent such as a drug and a physical agent such as UV radiation. The Qs are one of typical photosensitiser. Phototoxicity owing to these antibiotics was caused by the reactive oxygen species (ROS). Oxygen-dependant release of ROS induces phototoxic damage on cell surface, DNA and lysosome. In an in vivo mouse experiment, phototoxicity increased UVA-induced edema, sunburn, cell formation and local suppression of immune response. Increment of prostaglandin E2 production and depletion of Langerhans cells owing to phototoxicity would be responsible for this local immune suppression. Clinically, phototoxicity shows features of exaggerated sunburn, and long-term intake of a photosensitiser may induce cataract of ocular lens or photo-aging of skin. Phototoxicity also may increase skin cancers like squamous cell carcinoma and malignant melanoma. Lomefloxacin (LOM) or FLE, which have another fluorine atom at C-8, exhibited increased photocarcinogenic potentials. Most quinolones show phototoxicity in various in vitro methods, which examine fluorescence or phototoxic killing of organisms or phototoxic destruction of cells, and in vivo methods, which measure swellings or erythema (32)

Substitution of position 8 with halogens increases phototoxicity, but substitution of this position with methoxy group reduces phototoxicity (32).

(ii) Photoallergy shows features of allergic contact dermatitis like poison ivy in exposed areas. Without UV radiation, they are usually inert. In the presence of UV light, Qs are activated to bind with skin protein, which becomes complete antigen and induces allergic dermatitis. (32)

V. Spectroscopic properties

To study the spectroscopic properties of Qs, OFL, NOR and FLU were selected as a model compounds. In aqueous solution, the absorption spectrum of OFL and NOR contain two major bands. In organic solvents, the strong band shifts toward the long wavelength side by ~15 nm. In aqueous solution, the emission spectra of OFL and NOR are strong, broad structureless band with large Stokes' shift. In organic solvents, the emission intensities of OFL and NOR are very weak, the lifetimes

become long and another small band appears in short wavelength region (350–400 nm). Moreover, the red shift of main emission band is observed in CH_3OH and CH_3CN compared with that in aqueous solution. The absorption and emission spectra of OFL and NOR in organic solvents have roughly mirror image relationships, especially for OFL. Such observations indicate that the geometry change upon excitation is small in organic solvents. For all three molecules, the changes of dipole moment upon excitation are small in gas phase. In aqueous phase, this dipole moment changes of OFL and NOR are very large. Because the internal conversion rate of $S_1 \rightarrow S_0$ for OFL and NOR will be very fast in organic solvents owing to the similar geometrical structures and dipole moments between these states, the fluorescence quantum yield becomes very low. Furthermore, the lifetimes of OFL and NOR in organic solvent are longer than those in water. (32).

The OFL and NOR have two electron releasing nitrogen atoms; the one is the nitrogen at position 1 and the other is the nitrogen of the piperazinyl group attached directly to the C-7 atom (N-16). The keto oxygen of OFL and NOR will serve as a good electron acceptor. Having both electron donors and acceptor, OFL and NOR may become one of the good donor– acceptor conjugated molecules (32).

VI. Biodegradability

The Closed bottle test (CBT) is recommended as a first, simple test for the assessment of the biodegradability of organic compounds (Nyholm, 1991; OECD, 1992). The CBT was performed according to test guidelines (OECD, 1992) in the dark at room temperature (20. 1°C), as described elsewhere in detail (Kummerer et al., 1996a). The standard test period of the CBT is 28 days, In the toxicity controls of the tests with the FQs CIP and OFLO, an inhibition of the biodegradation was observed within the first days of the test. The inhibition disappeared after 8 and 11 days, respectively. The CIP concentration in the test vessels was analysed by HPLC. Within the analytical error no elimination of CIP was found (data not shown). A prolongation of the test period did not influence the results (33).

  Fluoroquinolone carboxylic acids are photodegradable in aqueous solution. In the case of entering the aquatic environment via sewage water, the photo-degradation route is only of minor importance. Only if the substances are not eliminated in the

STP a possible photo-degradation in surface water must be taken into account, if they are not adsorbed to sediments (33).
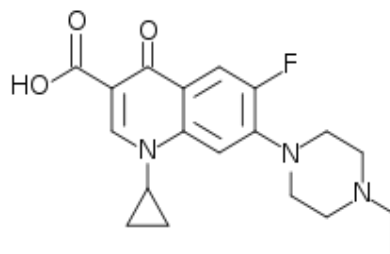
2.6. ENROFLOXACIN

Fig. 2. Enrofloxacin

1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-Fluoro-1,4-Dihydro-4-Oxo-3-Quinolonecarboxylic Acid

Enrofloxacin (ENRO) is used exclusively in veterinary medicine. It is available in several oral and parenteral formulations and is effective in controlling a wide range of bacteria. (34)

It is a lipophilic and amphoteric antibiotic which is extensively distributed from the bloodstream into different tissues including those colonised by pathogenic bacterial strains. ENRO undergoes deethylation to CIP (the active compound used in human medicine). ENRO and its metabolite CIP are effective against microorganisms which are resistant to other antimicrobial agents used for the treatment of endometritis, such as aminoglycosides, tetracyclines, macrolides and β-lactams (34). Endometritis is uterine infection due to bacteria ant it is one of the most common causes of infertility in mares. In one study, one third of all barren mares were found to be infected. (35)

Chronic infectious endometritis and venereal diseases are caused mainly by *Streptococcus zooepidemicus, Escherichia coli, Klebsiella pneumoniae, Pseudomona aeruginosa* and *Taylorella equigenitalis*.

Despite its obvious potential, ENRO is not currently recommended for use in horses. One reason for this is the concern raised by some authors over the possible

association between intravenous and/or oral ENRO administration and arthropathy in young horses.

The pharmacokinetic behaviour of ENRO in horses has not been fully characterized although several recent studies have been undertaken in adult animals and foals with the aim of optimizing the use of ENRO in the equine (10, 34).
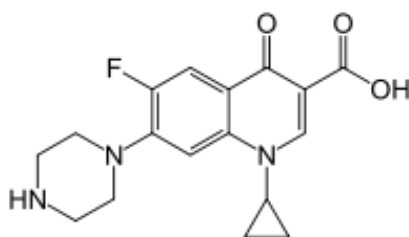
2.7 CIPROFLOXACIN

Fig.3. Ciprofloxacin

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

2.7.1 Distribution

The binding of CIP to serum proteins is 20 to 40% which is not likely to be high enough to cause significant protein binding interactions with other drugs.

After oral administration, CIP is widely distributed throughout the body. Tissue concentrations often exceed serum concentrations in both men and women, particularly in genital tissue including the prostate. CIP is present in active form in the saliva, nasal and bronchial secretions, mucosa of the sinuses, sputum, skin blister fluid, lymph, peritoneal fluid, bile, and prostatic secretions. CIP has also been detected in lung, skin, fat, muscle, cartilage, and bone. The drug diffuses into the cerebrospinal fluid (CSF), however, CSF concentrations are generally less than 10% of peak serum concentrations. Low levels of the drug have been detected in the aqueous and vitreous humors of the eye (36).

2.7.2 Metabolism

Four metabolites (desethylenciprofloxacin, sulphociprofloxacin, oxociprofloxacin and formylciprofloxacin) have been identified in human urine which together account for approximately 15% of an oral dose. The metabolites have antimicrobial activity, but are less active than unchanged CIP.

CIP is an inhibitor of human cytochrome P450 1A2 (CYP1A2) mediated metabolism. Coadministration of CIP with other drugs primarily metabolized by CYP1A2 results in increased plasma concentrations of these drugs and could lead to clinically significant adverse events of the coadministered drug (36).

2.7.3 Excretion

The serum elimination half-life in subjects with normal renal function is approximately 4 hours. Approximately 40 to 50% of an orally administered dose is excreted in the urine as unchanged drug. After a 250 mg oral dose, urine concentrations of CIP usually exceed 200 µg/mL during the first two hours and are approximately 30 µg/mL at 8 to 12 hours after dosing. The urinary excretion of CIP is virtually complete within 24 hours after dosing. The renal clearance of CIP, which is approximately 300 mL/minute, exceeds the normal glomerular filtration rate of 120 mL/minute. Thus, active tubular secretion would seem to play a significant role in its elimination. Co-administration of probenecid with CIP results in about a 50% reduction in the CIP renal clearance and a 50% increase in its concentration in the systemic circulation. Although bile concentrations of CIP are several fold higher than serum concentrations after oral dosing, only a small amount of the dose administered is recovered from the bile as unchanged drug. An additional 1 to 2% of the dose is recovered from the bile in the form of metabolites. Approximately 20 to 35% of an oral dose is recovered from the faeces within 5 days after dosing. This may arise from either biliary clearance or transintestinal elimination (36).

2.7.4 Microbiology

CIP has in vitro activity against a wide range of gram-negative and gram-positive microorganisms. The mechanism of action of FQs, including CIP, is different from that of penicillins, cephalosporins, aminoglycosides, macrolides, and tetracyclines;

therefore, microorganisms resistant to these classes of drugs may be susceptible to CIP and other Qs. There is no known cross-resistance between CIP and other classes of antimicrobials. In vitro resistance to CIP develops slowly by multiple step mutations.

CIP is slightly less active when tested at acidic pH. The inoculum size has little effect when tested in vitro. The minimal bactericidal concentration (MBC) generally does not exceed the minimal inhibitory concentration (MIC) by more than a factor of 2 (36).

2.7.5 Indications and Usage

I. Adult Patients:

Urinary Tract Infections caused by *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Serratia marcescens, Proteus mirabilis, Providencia rettgeri, Morganella morganii, Citrobacter diversus, Citrobacter freundii, Pseudomonas aeruginosa, methicillin-susceptible Staphylococcus epidermidis, Staphylococcus saprophyticus, or Enterococcus faecalis.*

Acute Uncomplicated Cystitis in females caused by *Escherichia coli* or *Staphylococcus* saprophyticus.

Chronic bacterial prostatitis caused by *Escherichia coli* or *Proteus mirabilis.*

Lower Respiratory Tract Infections caused by *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis, Pseudomonas aeruginosa, Haemophilus influenzae, Haemophilus parainfluenzae*, or penicillin-susceptible S*treptococcus pneumoniae*. Also, *Moraxella catarrhalis* for the treatment of acute exacerbations of chronic bronchitis.

Acute sinusitis caused by *Haemophilus influenzae*, penicillin-susceptible *Streptococcus pneumoniae*, or *Moraxella catarrhalis.*

Skin and Skin Structure Infections caused by *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis, Proteus vulgaris, Providencia stuartii, Morganella morganii, Citrobacter freundii, Pseudomonas aeruginosa,* methicillin-susceptible *Staphylococcus aureus* methicillin-susceptible *Staphylococcus epidermidis*, or *Streptococcus pyogenes.*

Bone and joint infections caused by *Enterobacter cloacae, Serratia marcescens*, or

Pseudomonas aeruginosa.

Complicated intra-abdominal infections (used in combination with metronidazole) caused by *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae,* or *Bacteroides fragilis.*

Infectious Diarrhea caused by *Escherichia coli* (enterotoxigenic strains), *Campylobacter jejuni, Shigella boydii†,Shigella dysenteriae, Shigella flexneri* or *Shigella sonnei†* when antibacterial therapy is indicated.

Typhoid fever (Enteric fever) caused by *Salmonella typhi*.

Uncomplicated cervical and urethral gonorrhea due to *Neisseria gonorrhoeae.*

II. Pediatric patients (1 to 17 years of age):

Complicated urinary tract Infections and pyelonephritis due to *Escherichia coli*.

Adult and Pediatric Patients:

Inhalational anthrax (post-exposure): To reduce the incidence or progression of disease following exposure to aerosolized *Bacillus anthracis*.

Ciprofloxacin serum concentrations achieved in humans served as a surrogate endpoint reasonably likely to predict clinical benefit and provided the initial basis for approval of this indication.4 Supportive clinical information for Cip for anthrax post-exposure prophylaxis was obtained during the anthrax bioterror attacks of October 2001 (36).

2.7.6 Biodegradability

CIP was not biodegradable in the CBT (37). CIP was found in concentrations between 0.7 and 124.5 lg/l in efluents from hospitals and was assumed to be the main source of genotoxic effects measured with the umuC test in hospital efluents (38). Additionally, resistant bacteria may be selected by antibiotic substances in the aeration tanks or anaerobic digestion process of STPs (33).
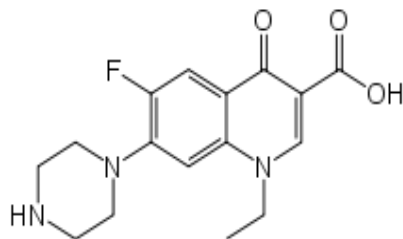
2.8 NORFLOXACIN

Fig.4. Norfloxacin

1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1H-quinoline- 3-carboxylic acid

2.8.1 Absorption

NOR is rapidly absorbed; 30% to 40% absorbed in fasting patients. Food and dairy products decrease absorption. Steady state is two days, C_{max} is 0.8 to 2.4 mcg/mL, and T_{max} is approximately 1 h after dosing.

2.8.2 Distribution

Protein binding is 10% to 15% and crosses the placenta.

2.8.3 Metabolism

Suggested as first-pass metabolism; however, further study is needed.

2.8.4 Elimination

NOR is eliminated in urine (26% to 32% as NOR, 5% to 8% as active metabolites) and feces (30%).

2.8.5 Indications and Usage

Oral treatment of urinary tract infections caused by susceptible organisms, treatment of STDs caused by *Neisseria gonorrhoeae* ; ocular solution for treatment of superficial ocular infections due to strains of susceptible organisms; prostatitis caused by *E. coli* (39).

CIP and NOR are the two most consumed FQs in human medicine. Therefore, their presence in sewage sludge may be notorious due to their strong adsorption properties. This hypothesis is in concordance with the data reported by Golet et al., according to which the extent of elimination for FQs during municipal wastewater treatment would be in the range of 79–87%. An study conducted in several municipal wastewater treatment plants in Switzerland reveals that the concentrations of the two FQs in raw sewage sludge were in the range 1.4–2.03 mg/kg, being in the same range as those found in digested sludge, 2.13–2.42 mg/kg. This high concentration level indicates their high affinity towards solid phases, as sludge can be in a wastewater treatment process (40).

2.9. MONITORING OF FQS

Analysis of FQs for the drug monitoring in human serum or urine and the determination in aqueous samples have been carried out mainly by high-performance liquid chromatography (HPLC) with UV and fluorescence detector (FD), liquid chromatography/mass spectrometry (LC/MS), or LC–tandem mass spectrometry (LC/MS/MS). 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. Most of these techniques, however, require large sample volumes. The in-tube solid-phase microextracation (SPME) technique, using an open tubular fused-silica capillary with an inner surface coating as extraction device, is simple and can be easily coupled on-line with HPLC, LC/MS and LC/MS/MS (30).

2.9.1 High-performance liquid chromatography (HPLC)

Samples were analyzed by HPLC with fluorescence detector. HPLC instruments consist of reservoir of mobile phase, a pump, an injector, a separation column and detector. Compounds are separated by injecting a plug of the sample mixture onto into the column. The different compounds in the mixture pass through the column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and stationary phase (41).

2.9.2 Fluorescence detector

Fluorescence detector is used almost exclusively in liquid chromatography and is a specific detector that senses only those substances that fluoresce. A flow cell is used as the sensor through which the excitation light passes axially. A photocell is situated at the side of the cell to receive radialy emitted light. The cell wall is often made of Pyrex glass to prevent the excitation light (usually UV light) from reaching the photo cell. When a solute that fluoresces in the excitation light is situated in the cell, the fluorescent light passes through the walls of the cell onto the photo cell, the output of which is electronically processed and the output passed to a computer. The excitation light may be UV at 254 nm produced by the mercury lamp or it may be light of any wavelength selected from the light produced by a deuterium lamp using a monochromator. A monochromator may also be used to analyze the fluorescent light and, thus a fluorescent spectrum can be produced for excitation light of any specific wavelength and an excitation spectrum produced for fluorescent light of any specific wavelength. To improve the specificity of an LC analysis, a fluorescent derivative of the substance of interest may be prepared (employing an appropriate fluorescent reagent). The substance may then be selectively detected from other solutes which, (if they do not fluoresce) need not be resolved from each other by the chromatographic column (42).

AIM

The aim of this work was the development and validation of an analytical methodology, sensitive, accurate and precise for determination of three fluoroquinolones (norfloxacin, ciprofloxacin and enrofloxacin) in wastewater and surface samples in an intensive piggery.

3. EXPERIMENTAL PART

3.1 REAGENTS AND MATERIALS

Standards of norfloxacin, ciprofloxacin and enrofloxacin were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile HPLC grade, and fosforic acid RPE-ACS were purchased from Carlo Erba (Milan, Italy). Sulphuric acid 95-97% "Baker Analyzed" (Deventer, Netherlands), tetrabuthylammonium and disodium ethylenediaminetetraacetate (Sigma-Aldrich, Steinheim, Germany). Water was purified by distillation and passage through Milli Q system (Millipore, Bedford, MA), EDTA (Merck, Germany).
Extraction cartridges Oasis HLB 6cc/ 200 mg (Waters Corp. Milford, MA).

3.2 EQUIPMENT

The HPLC method described here was developed using a HPLC system, consisting of two pumps (model 307, Gilson Medical Electronics, France), a injector Model 7125 (Rheodyne, Cotati, California, USA), a column oven (Technology, LDA), a Fluorimeter 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 a SP 4270 integrator (Hewlett Packard, Philadelphia, USA). NOR, CIP and ENRO were eluted isocratically using a mobile phase consisting of 0.025 M Phosphoric acid and Methanol (90:10) through a monolithic (Chromolith Performance RP-18e (100 x 4.6 mm) from Merck, Darmstand, Germany). The HPLC system was operated at room temperature and the flow rate was 2.2 mL/min.

3.3 ANALYTICAL METHODOLOGY

3.3.1 Sampling and storage of samples

Samples were collect from wastewaters and surface waters in an intensive piggery and transported at low-temperature. Samples were storage at 4°C in dark.

Sampling:
1. Waste lagoon primary
2. Waste lagoon secondary
3. Waste lagoon tertiary
4. Wastewater
5. Receiving piggery effluent 1
6. Receiving piggery effluent 2
7. Water river receiving piggery effluent 1
8. Water river receiving piggery effluent 2

3.3.2 Preparation of mobile phase

The mobile phase used for the analysis was consisting of 0.025 M phosphoric acid solution (adjusted to pH 3 with tetrabutylamonium) and methanol (90:10). It was filtered through a 0.45µm filter under vacuum and degassed by ultrasonication.

3.3.3 Preparation of standard fluoroquinolones solutions

Individual stock standard solutions (1 mg/mL) of the three fluoroquinolones were prepared by dissolving of standards in 0.005 M Sulphuric acid (0,025g of standard was dissolved in 25mL of sulphuric acid).

The working standard solutions (at concentration level 0.1µg/mL, 0.25µg/mL, 0.5µg/mL, 1µg/mL) were prepared by a serial dilution of the stock standard solutions in 0.005 M sulphuric acid. The volume of prepared working standard solutions was 10mL. Used volume of stock standard solution is shown in table 1.

Concentration of working solution (µg/mL)	Volume of stock solution (µL)
0.1	1
0.25	2.5
0.5	5
1	10

Table 1. Preparation of standard FQs solutions

3.3.4 Preparation of samples

The extractions were performed using Solid Phase Extraction (SPE) cartridges (Waters Oasis HLB, 200mg, 6 cc cartridge). The cartridge was conditioned with 5 mL of methanol and 5 mL of Citric acid 0.04M. The water samples were filtered through membrane filter (polyamid 0.2µm, NL 16 Schleicher and Schuell) and adjust to pH 4 with Sulphuric acid (1 M). Then 200mg of EDTA di-potassium salt was added. 50mL of the sample was percolated through the cartridge. Washing was performed by 5mL of Citric acid and 5ml of Milli-Q water at pH 4.2. Then the cartridge was eluted with 4mL of methanol. This eluate was evaporated to dryness in a water bath (40°C) under a gentle stream of nitrogen and the residue was redissolved in 0.5mL of mobile phase. Then final filtration through membrane filter 0.45µm was done. A volume of 50µl (respectively 20µl of samples 6-8) was injected for analysis.

4. RESULTS AND DISCUSSION

4.1 VALIDATION OF ANALYTICAL METHODOLOGY

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.

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

4.2 RETENTION TIME OF FQs

A volume of injection of 50µL of the standard solutions of NOR, CIP, ENRO at concentration 0.25µL were injected three times each day during consecutive three days, to evaluate intraday and interday precision.
The mean retention times and the precision value of the retention times are shown in table 2.

	RT (min)	intraday RSD (%)	interday RSD (%)
NOR	4.91	7	13
CIP	5.62	6	11
ENRO	8.00	9	17

Table 2. Retention times of Nor, Cip, Enro

Using the monolithic column in HPLC, the time of analysis can be decreased. In our case, all three fluoroquinolones were separated in about 8 minutes. The relatively high RSDs are due to the instability of the HPLC system used.
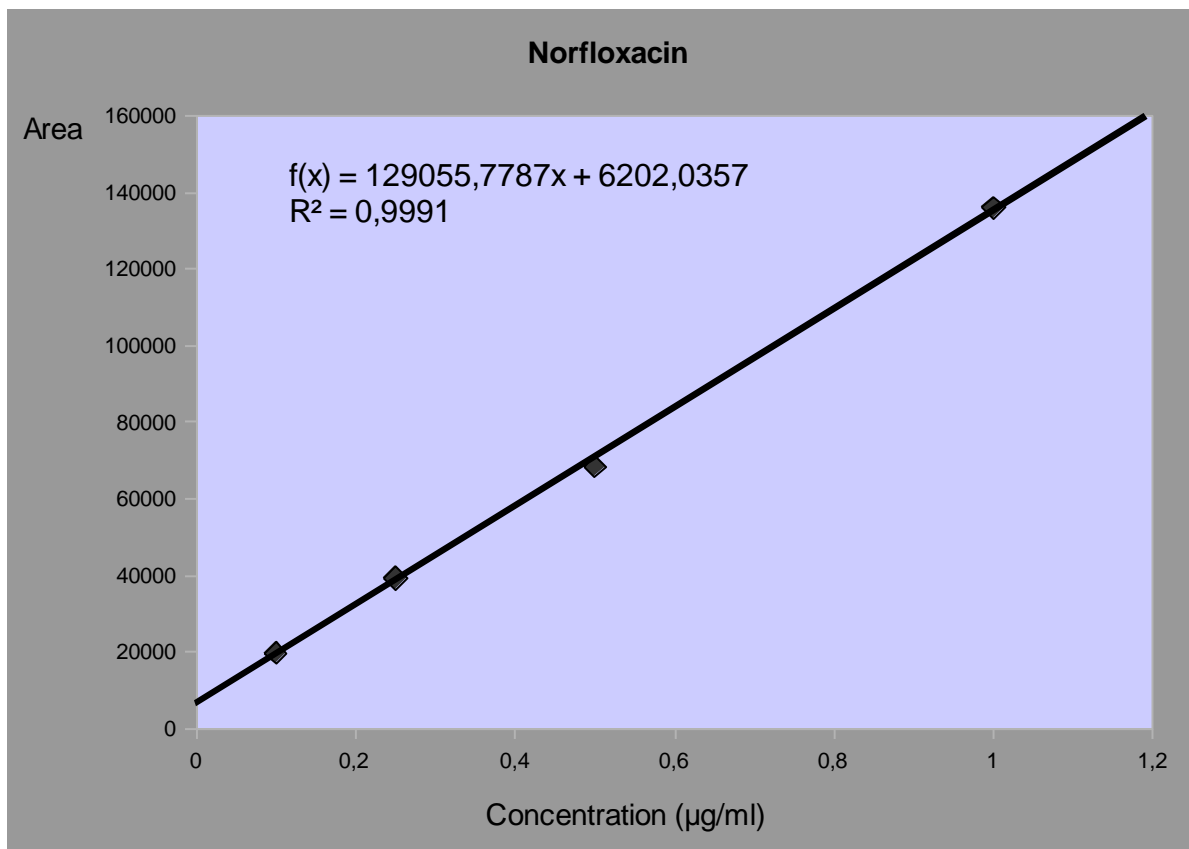
4.3 LINEARITY AND RANGE

The calibration curve was prepared by measurement of areas of FQs standard solutions in the range from 0.1 to 1μg/mL. Each level of concentration was injected three times.
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) were higher than 0.998 for all of them (Fig. 2-4).

4.4 SPECIFITY

In order to verify the absence of potential interfering substances around the retention time of OTA, water blanks were analyzed in order to assess the specificity of the method. No interferences were observed in the region of interest.

4.5 CALIBRATION CURVES

Norfloxacin

$f(x) = 129055,7787x + 6202,0357$
$R^2 = 0,9991$

Area — axis label; Concentration (µg/ml) — x-axis label

4.5.1 Norfloxacin

Fig. 2. : Calibration curve of NOR
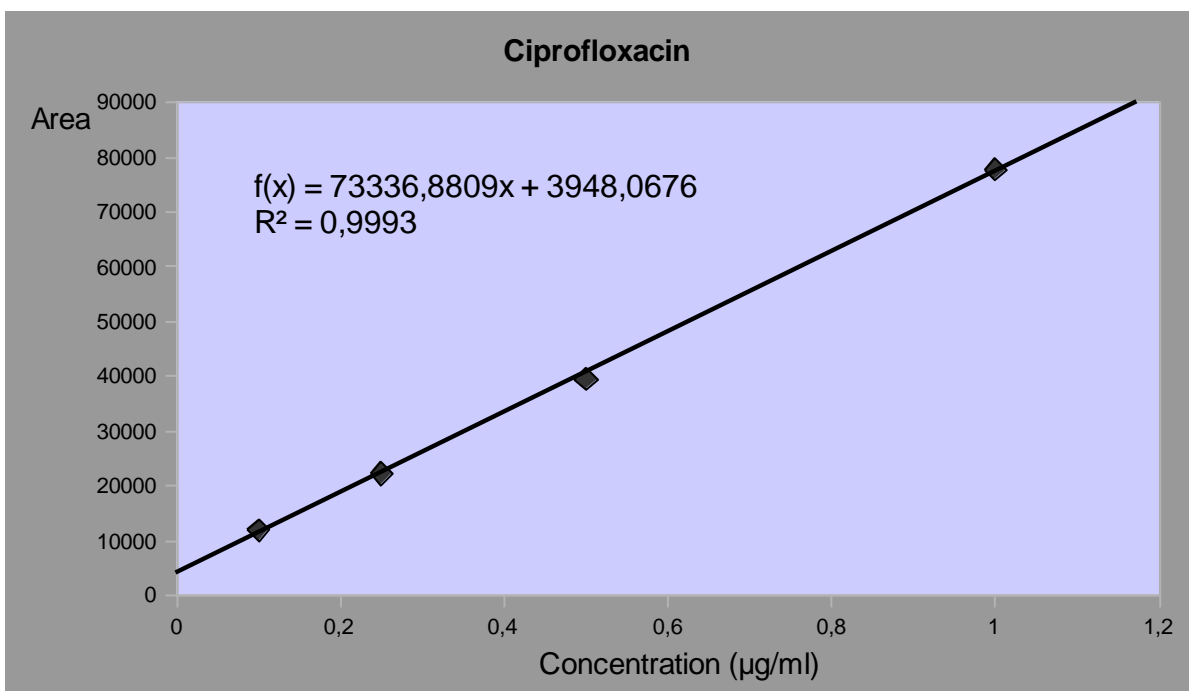
4.5.2 Ciprofloxacin

Fig.3. : Calibration curve of CIP
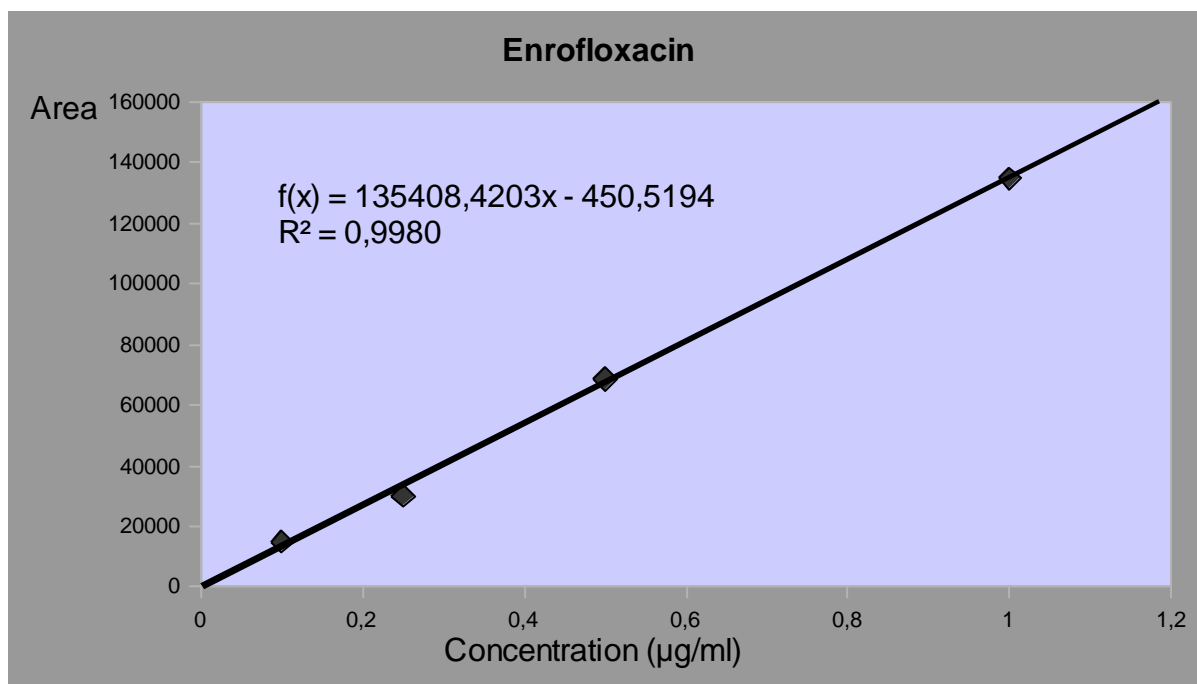
4.5.3 Enrofloxacin

Fig. 4. : Calibration curve of Enrofloxacin

4.6 RECOVERY ASSAYS

Fortified samples were prepared by same method like common sample but before SPE was an accurate amount of standard solutions (NOR, CIP, ENRO) added into 50ml of blank water sample. The accuracy of the method was studied by spiking water samples at three fortification levels: 0.2, 0.02, 0.01µg/mL. Extraction efficiency was determined by comparison of the peak areas obtained from fortified samples with those given by standard solutions.

Within-day accuracy and precision data were determined by analysing, on the same day, three replicates of a spiked sample at three fortification levels, and one blank (to check for interferences). The between-day accuracy and precision were also determined by extracting batches of three fortification levels and analysing them on three consecutive days.
 The recovery values ranged from 66.9% to 98.7% for NOR, from 99 %to 114% for CIP and from 66.4% to110% to ENRO. The average recovery was 89.5%. Value of inter and intra-day RSD were less than 19%.

4.7 CONCENTRATIONS OF FQS IN SAMPLES

A total eight samples of wastewaters and surface waters were analyzed under the conditions described and concentration of FQs in samples were calculated by comparing the peak areas of the samples and of the standards. Fig. 5 shows chromatogram of standard solution containing 0.25µg/mL of NOR, CIP and ENRO. Fig. 6 gives example of chromatogram of waste lagoon tertiary sample.
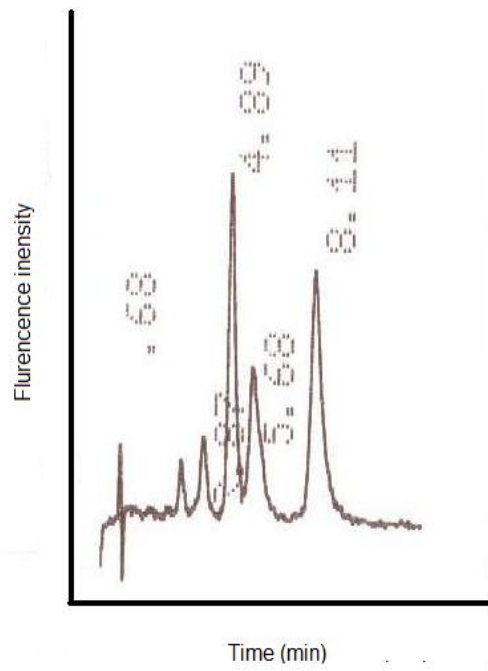
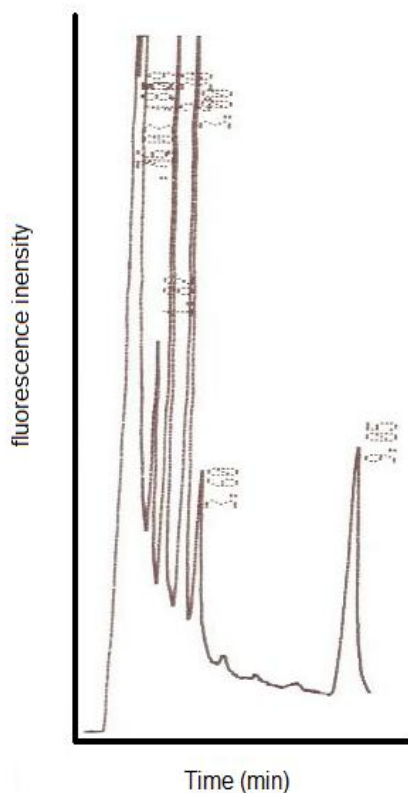Fig. 5.: *Chromatogram of standard mixture of 0.25*µg/mL of NOR, CIP, ENRO

Fig. 6.: Cromatogram of waste lagoon tertiary sapmle

Each sample was injected three times and the results obtained are presented:

1. Waste lagoon primary- NOR was detected at concentration of 0.6ng/ml, CIP 0.45ng/ml and ENRO 0.71ng/ml.

2. Waste lagoon secondary - NOR 0.28ng/ml.

3. Waste lagoon tertiary

4. Wastewater – NOR 4.4ng/ml, CIP 1.8ng/ml and ENRO 0.92ng/ml.

5. Receiving piggery effluent- NOR113.6ng/ml, CIP 15.2ng/ml, ENRO 2.28ng/ml.

6. Receiving piggery effluent 2- NOR 12.35ng/ml, ENRO 2.33ng/ml.

7.Water river receiving piggery effluent 1

8. Water river receiving piggery effluent 2

Samples were collected from one intensive piggery, to assess the frequency of occurrence in detectable concentrations and to some extent, fate of the monitored antibiotics. Leaching experiments are a part of environmental risk assessment (ERA)

to estimate the distribution and fate of these antibiotics in the environment. Therefore, a study on migration of antibiotic residues in the surround environment was undertaken. Wastewaters, superficial water immediately proximal to the piggery, collected during sampling period were analyzed.

NOR was detected in 4 samples, at concentrations between 0.28 µg/L and 13.6 µg/L, ENRO was found in three samples in a range between 0.92 µg/L and 2.33 µg/L and CIP, its degradation product, was presented in two samples between 1.8 µg/L and 15.2 µg/L. CIP detection is explained by N-dealkylation of ENRO.

In a study on migration of antibiotic residues in the surround environment, we noted the occurrence of FQs residues in waste lagoon (primary and secondary), in wastewater and in receiving piggeries effluents.

5. CONCLUSION

An HPLC with fluorometric detection was tested for determination of NOR, CIP, ENRO in wastewater and surface waters from piggery.

The best result was achieved by using mobile phase consist of Phosphoric acid 0.025M and methanol (90:10 v/v), flow rate 2.2mL/min, excitation wavelengths of 278 and emission wavelengths of 450 nm.

We tested the linearity of the calibration curves for concentration ranges that are normally measured in waste water. The correlation coefficient were higher than 0.998 for each of he compound of interest.

The instrumental repeatability and precision were assessed by repeated injection of standard mixtures. The values of inter and intra-day RSD were less than 17%.

LOQs expressed as the lowest tested level with acceptable RSD was 25 ng/L.

The prevalence of antimicrobials compounds in water samples proximal to swine farms, coupled with the results from the animal waste samples, provide evidence that animal waste stored in lagoons or applied to agricultural fields as fertilizer appears to act as a non-point source of antimicrobial residues in water sources.

Concluding from the experimental data, the concentration of FQs was highest for samples collected in waste lagoons (primary and secondary), wastewater and receiving piggery effluents.

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7. ABBREVIATIONS

FQs	Fluoroquinolone antibiotics
NOR	Norfloxacin
CIP	Ciprofloxacin
ENRO	Enrofloxacin
LC	Liquid chromatography
FD	Fluorescence detector
LQs	Limits of quantification
RSD	Relative standard deviation
STP	Sewage treatment plant
WWTPs	Wastewater treatment plants
PPCPs	Pharmaceuticals and personal care products
$K_{d, solid}$	Sorption coefficient

K_{oc}	Organic carbon-normalized sorption coefficient
Qs	Quinolone antibiotics
FLE	Fleroxacin
NAL	Nalidixic Acid
FLU	Flumequine
OXO	Oxolinic acid
ROS	Reactive oxygen species
LOM	Lomefloxacin
CBT	Closed bottle test
OFLO	Ofloxacin
ENRO	Enrofloxacin
CSF	Cerebrospinal fluid
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration

SOUHRN

Znalost koncentrace léčiv v životním prostredí je velice důležitá. Hlavním důvodem je to, že jsou to látky s účinkem na živý organismus a jejich fyzikálně- chemické vlastnosti jsou často podobné škodlivým xenobiotikům.

Antibiotika patří mezi nejčastěji předepisovaná léčiva. Fluorochinolonová antibiotika jsou používána k terapii i k profylaxi lidských i zvířecích onemocnění. Především k léčbě širokého spektra respiračních a enterických infekcí a infekcí močových cest. Kromě toho jsou také v subterapeutický dávkých užívány ke stimulaci růstu hospodářských zvířat.

Podstatou účinku fluorochinolonů je inhibice DNA-gyrazy. Jsou účinné proti gram-negativním i gram-pozitivním bakteriím.

Účinek fluorochinolonů je závislý na jejich chemické struktuře, nezbytná je přítomnost volné karboxylové skupiny v poloze 3 a oxoskupiny v poloze 4. Prostřednictvím těchto skupin dochází k vazbě na DNA-gyrázu. Karboxylová skupina a amino skupina určují jejich acidobazické vlastnosti. Jejich aktivita je tedy také významně závislá na pH prostředí.

25-75% podaných antibiotik je z organismu vyloučeno v nezměněné formě. Metabolismem vznikají sloučeniny, které mohou být méně nebo více učinné než původní látka.

Část těchto sloučenin je odstraněno v čistírnách odpadních vod. Určitý podíl ale ve vodě zůstává. Tak se fluorochinolony přítomné v odpadních vodách z nemocnic, domácností a vznikajících vlivem zemědělství, včetně prasečích farem, mohou odtokem dostat do povrchových vod.

Flurochinolony jsou poměrně odolné mikrobiální degradaci a díky sorpci pak mohou setrvávat v životním prostředí, především ve splaškovém kalu, v hnojivu a v půdě.

Rozsah sorpce a degradace je určen především fyzikálně chemickými vlastnostmi sloučenin jako jsou tvar a velikost molekuly, rozpustnost a lipofilita. Záleží také na kvalitě výroby, geografické poloze a klimatických podmínkách.

Tyto rezidua pak mohou negativně ovlivnit živé organismy a to na každé úrovni tzn. buňky, orgánu, organismu, populace, ekosystemu i celé ekosféry.

Nebezpečí vyplývá z jejich přímé toxicity na mikroorganismy, které jsou nezbytné pro proces čistění vody, ale také pro uzavření potravního řetězce a vzniku organických látek.

Antibiotika také mohou vyvolat u lidí alergické reakce.

Užívání těchto antibiotik je ale také spojeno s rozvojem rezistence u bakterií. Chov vepřů je považován za významný zdroj rozvoje antibakteriální rezistence a proto byly v posledních letech přijata různá kontrolní opatření pro používání antibiotik v chovu dobytka.

Cílem této práce bylo dopracovat a validovat přesnou a citlivou metodu pro stanovení fluorochinolonů v odpadních a povrchových vodách v oblasti intenzivního chovu vepřů v Portugalsku.

Ke stanovení norflofacinu (NOR), ciprofloxacinu (CIP) a enrofloxacinu (ENRO) byla použita úspěšně vyvinutá LC-FD metoda (4) založená na použití monolitické kolony (Chromolith Performance RP-18e (100 x 4.6 mm). Flurochinolony byly izokraticky eluovány mobilní fází tvořenou 0,025M roztokem kyseliny fosforečné o pH 3 (dosaženo tertabutyamoniem) a methanolem (90:10 v/v). Objemový průtok byl 2,2 ml/min, hodnota excitační vlnové délky byla 278nm, emisní vlnová délka byla 450nm.

Vzorky byly odebrány z odpadních a povrchových vod v oblasti intenzivního chovu vepřů. Do použití byly uchovávány v temnu v teplotě do 4°C.

Nejprve byly zjištěny retenční časy pro standardy. Pro NOR 4,91min, CIP 5,62 min, ENRO 8,00 min.

Úprava vzorků byla provedena metodou SPE (extrakce pevným sorbentem). SPE kolonky byly nejprve promyty 5ml methanolu a 5ml kyseliny citronové. Poté se nechalo perkolovat 50ml vzorku o pH 4, do kterého bylo přidáno 200mg didraselné soli EDTA. Poté se kolonka promyla 5ml kyseliny citronové a 5ml Milli-Q vody o pH 4.2. Eluce byla provedena 4ml methanolu. Tento eluát se nechal odpařit ve vodní lázni pod slabým proudem dusíku a znovu rozpustil v 0,5 ml mobilní fáze. Následovala závěrečná filtrace.

Pomocí roztoků standardů o koncentraci 0,1- 1µg/ml byly sestaveny kalibrační křivky závislosti plochy píku na koncentraci. Hodnota lineárního korelačního koeficientu (R) byla ve všech třech případech vyšší než 0,998.

Mez stanovitelnosti byla 25ng/L. Výtěžek (recovery) metody je v rozmezí 66,4- 114%. Hodnota správnosti a přesnosti měření (vyjádřena jako relativní směrodatná odchylka) byla nižší než 17%.

Nor byl zjištěn ve 4 vzorcích v koncentraci 0,28μg/ml- 13,6μg/ml, Enro ve 3 vzorcích v koncentraci 0,92μg/ml- 2,33μg/ml, Cip ve 2 vzorcích v koncentraci 1,8μg/ml- 15,2μg/ml. Přítomnost Cip je vysvětlována N-dealkylací Enro.