Master in Bioengineering

Occurrence of Veterinary Antibiotics in Aqueous Environments

Master's Thesis

of

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Quanto faças, supremamente faze.
Mais vale, se a memória é quanto temos,
Lembrar muito que pouco.
E se o muito no pouco te é possível,
Mais ampla liberdade de lembrança
Te tornará teu dono.

In Odes, Ricardo Reis

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I dedicate this thesis to my parents.

Abstract

Antibiotics are used for over 60 years to improve both human and animal health, being their discovery considered one of the most significant scientific achievements of the 20th century. However, it is recently recognized that antibiotics are micro-pollutants, emerging the global concern about occurrence, fate, and effects of this pharmaceuticals in the environment.

The main purpose of this work was to develop, optimize, and validate an analytical methodology by high performance liquid chromatography (HPLC) with diode array detection (DAD) for analysis of amoxicillin and doxycycline, the two antibiotics most consumed in Portuguese veterinary medicine, in aqueous samples. The presented method can be applied to the direct and simultaneous analysis of the target antibiotics at concentrations levels of μg/L in tap water. The linearity interval of calibration curves ranged from 33 to 456 μg/L for amoxicillin and from 36 to 479 μg/L for doxycycline. The precision of the method was determined by repeatability (0.9 - 4.6%) and intermediate precision (1.0 – 3.8%). The recovery of analytes in tap water ranged from 97 to 103%.

Preliminary studies to clean-up and concentrated complex aqueous samples in the analytes by solid-phase extraction (SPE) were performed. The SPE parameters sorbent type, sample pH, washing step, and eluting solvent were tested. The acidification of samples at pH 2 prior extraction with the Oasis HLB sorbent, the omission the washing step, and the usage of methanol as eluting solvent were the experimental condition to achieve the highest recoveries of doxycycline (98%). Due to instability of amoxicillin in the extraction conditions, particularly low pH and elution with methanol, it was not recovered in any assay.

The stability of the target antibiotics in solutions were evaluated over the time under different storage conditions. It was verified that doxycycline degradation was mainly influenced by storage temperature, whereas amoxicillin was sensitive to both methanol solvent and storage temperature.

The development and optimization of a methodology for multiclass antibiotic analysis in environmental water samples is difficult and challenging, due principally to the different physicochemical properties of analytes and the complexity of the matrixes, being required a compromise in the selection of experimental conditions. Further work is necessary to assess the occurrence of antibiotics in aqueous environmental samples by SPE-HPLC-DAD and to confirm their identity as well as relevant antibiotics metabolites and degradation products by analytical methods based on liquid chromatography coupled to mass spectrometry detection.

Key words: veterinary antibiotics, stability, SPE, HPLC-DAD analysis, environment, Portugal

Resumo

Há mais de 60 anos que os antibióticos são utilizados para melhorar a saúde humana e animal, sendo a sua descoberta considerada uma das conquistas científicas mais importantes do século 20. No entanto, estes fármacos são atualmente considerados poluentes emergentes, existindo uma crescente preocupação global sobre a ocorrência, o destino e os efeitos destes fármacos no meio ambiente.

O principal objetivo deste trabalho foi desenvolver, otimizar e validar um método analítico para quantificar em amostras aquosas os dois antibióticos mais consumidos na medicina veterinária em Portugal, a amoxicilina e a doxiciclina. Baseada na cromatografia líquida de alta eficiência (HPLC) com deteção por arranjo de díodos (DAD), a metodologia apresentada pode ser aplicada na análise direta e simultânea dos analitos presentes em água da torneira em níveis de concentração na ordem dos µg/L. O intervalo de linearidade das curvas de calibração foi de 33 - 456 µg/L para a amoxicilina e 36 - 479 µg/L para a doxiciclina. A precisão foi avaliada pela da repetibilidade (0.9 – 4.6%) e da precisão intermédia (1.0 e 3.8%). A recuperação dos analitos em água de torneira variou de 97 a 103%.

Estudos preliminares de extração em fase sólida (SPE) foram realizados a fim de se estabelecer e otimizar as condições experimentais para posterior *clean-up* e concentração nos analitos de amostras aquosas complexas. O tipo de adsorvente, o pH da amostra, a etapa de lavagem e o solvente de eluição foram os parâmetros experimentais testados. A acidificação das amostras a pH 2 antes da extração com o sorvente Oasis HLB, a omissão do passo de lavagem e a utilização de metanol como solvente de eluição foram as condições experimentais que permitiram obter maiores recuperações de doxiciclina (98%). Devido à instabilidade da amoxicilina nas condições de extração, em particular baixos valores de pH e eluição com metanol, não foi possível recuperar o composto em nenhum ensaio realizado.

A estabilidade da amoxicilina e da doxiciclina em solução foi avaliada ao longo do tempo sob diferentes condições de armazenamento. Verificou-se que a degradação da doxiciclina foi principalmente influenciada pela temperatura de armazenamento, enquanto que a amoxicilina revelou-se sensível ao solvente metanol e à temperatura de armazenamento.

O desenvolvimento e otimização de uma metodologia para analisar múltiplas classes de antibióticos em amostras de água ambientais é difícil e desafiante, nomeadamente devido às diferentes propriedades físico-químicas dos analitos e à complexidade das matrizes, sendo necessário um compromisso na escolha das condições experimentais. São necessários mais estudos para avaliar a ocorrência de antibióticos em amostras ambientais aquosas por SPE-HPLC-DAD e para confirmar a sua identidade, bem como a dos seus metabolitos e produtos de degradação, através de métodos analíticos baseados na cromatografia líquida e deteção por espectrometria de massa.

Declaration

The author declares, under oath, that this work is original and that all non-original contributions were properly referenced.

Content

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Notation and glossary

Roman symbols

A	Pre-exponential factor	
a	Slop of calibration curve	mAU
b	Intercept of calibration curve	(mAU.L)/µg
E_a	Activation energy	J/mol
k	Rate constant of a chemical reaction	
K_d	Soil-water partition coefficient	
K_{ow}	Octanol-water partition coefficient	
pK_a	Acidic dissociation constant	
R	Universal gas constant	J/(K.mol)
S/N	Signal-to-noise ratio	
s_a	Standard deviation of slop	(mAU.L)/µg
s_b	Standard deviation of intercept	mAU
T	Temperature	K
ts_a	Confidence interval for slope	(mAU.L)/µg
ts_b	Confidence interval for intercept	mAU

Abbreviations and acronyms

ALE	Alentejo
AMOX	Amoxicillin
APA	*Agência Portuguesa do Ambiente*
ARB	Antibiotic resistance bacteria
ARG	Antibiotic resistance genes
ARH	*Administrações de Região Hidrográfica*
ATCvet	Anatomical Therapeutic Chemical Classification System for veterinary medicinal products
BOD_5	5-day biochemical oxygen demand
CADRM	Comissão de Ambiente e Defesa da Ribeira dos Milagres
CAS#	Chemical abstract services registry number
CCDR	*Comissão de Coordenação e Desenvolvimento Regional do Centro*
CE	Capillary electrophoresis
CL	Coastline
COD	Chemical oxygen demand
DAD	Diode array detector
DGAV	*Direção Geral de Alimentação e Veterinária*
DGS	*Direção Geral de Saúde*
DOX	Doxycycline
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ENEAPAI	*Estratégia Nacional para os Efluentes Agro-Pecuários e Agro-Industriais*
ESI	Electrospray ionization
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
ETAR	*Estação de Tratamento de Águas Residuais*
ETES	*Estação de Tratamento de Efluentes Suinícolas*
EU	European Union
FAO	Food and Agriculture Organization of the United Nation

FL Fluorescence
GNR *Guarda Nacional Republicana*
GPP *Gabinete de Planeamento e Políticas*
HF-LPME Hollow fiber liquid-phase microextraction
HPLC High performance liquid chromatography
HR Hydrographic region
HRMS High resolution mass spectrometry
i.e. That is
INE *Instituto Nacional de Estatística*
IT Ion trap
LC Liquid chromatography
LIF Laser induced fluorescence
LOD Limit of detection
LOQ Limit of quantification
MADRP *Ministério da Agricultura, do Desenvolvimento Rural e das Pescas*
MAMAOT *Ministério da Agricultura, do Mar, do Ambiente e do Ordenamento do Território*
MAOTDR *Ministérios do Ambiente, do Ordenamento do Território e do Desenvolvimento Regional*
mAU Mili arbitrary unit
MERCK Micellar electrokinetic chromatography
MIPs Molecularly imprinted polymers
MISPE Molecularly imprinted solid-phase extraction
MS Mass spectrometry
MW Molecular weight
n.a. Not applicable
n.d. Not detected
n.p. Information not provided
OIKOS *Associação de Defesa do Ambiente e Património da Região de Leiria*
PBS Phosphate buffered saline
ProDer *Programa de Desenvolvimento Rural*
QIT Quadrupole ion trap
QqQ Triple quadrupole
RRLC Rapid resolution liquid chromatography
RSD Relative standard deviation
RW Ribatejo and West
SDS Sodium dodecyl sulfate
SPE Solid-phase extraction
SPME Solid-phase microextraction
TBA Tetra-n-butylammonium hydrogen sulphate
TBAB Tetrabutylammonium bromide
TEA Trimethylamine
TFA Trifluoroacetic acid
THAM Tris(hydroxymethyl)aminomethane
UPLC Ultra high performance liquid chromatography
UV Ultraviolet
Vis Visible
WHO World Health Organization
WHOCC World Health Organization Collaborating Centres Database
WWTP Wastewater treatment plants

1 Background and aims

The discovery of antibiotics revolutionized both human and veterinary medicine. However, the existence of antibiotics in the environment and the consequent selective pressure on the natural ecosystems microbiota have recently received particular concern among the medical and scientific community (Cruickshank et al. 2014).

Around the world, antibiotics are extensively used in animal food production to control or prevent disease, enhance growth, or improve feed efficiency, accounting for up to 50% of total sales (M Teuber 2001; Philpott 2013). Moreover, it is estimated that the consumption of these drugs will consecutively increase in the coming decades (Van Boeckel et al. 2015). The major concern related to the widespread use of these drugs in veterinary medicine is their impact on organisms in the environment and on human health. Since animal husbandry excrete 75% to 90% of administered antibiotics (Marshall & Levy 2011), these pharmaceuticals and their metabolites reach the terrestrial and aquatic environment by the application of manure or slurry to agriculture soils, or by pasture-reared animals excreting directly on the land, followed by run-off to surface water or leaching to groundwater (Halling-Sørensen et al. 1998; Chee-Sanford et al. 2009; Kümmerer 2003). The complex vicious cycle of biotransformation and bioaccumulation of antibiotics in the environment contribute to the emergence and global spread of antibiotic resistance bacteria, some of which are pathogenic to humans and animals. In this context, studies about occurrence, fate, and effects of veterinary antibiotics in the environment has increased during the last decade (Kumar et al. 2005). Nevertheless, subtle and longer-term effects of these medicines continued to be unknown (Chee-Sanford et al. 2012). Therefore, further researches on antibiotics as emerging environmental micro-pollutants are required. Additionally, the development and validation of new analytical techniques is crucial to obtain accurate data on the concentrations of these compounds and their metabolites in the different environmental compartments.

The main goal of this work was developed, optimized, and validated an accurate and sensitive method based on high performance liquid chromatography coupled to diode array detection for simultaneous determination of amoxicillin and doxycycline, the two antibiotics most commercialized for veterinary practice in Portugal, in aqueous samples. The evaluation of the stability of the analytes in solution under different storage conditions as well as the development and optimization of a clean-up and pre-concentration methodology for target antibiotics using solid-phase extraction were additional objectives of this study.

2 Introduction

2.1 Veterinary antibiotics

2.1.1 History

The discovery of antibiotics is considered one of the most significant scientific achievements of the 20th century. Before the widespread use of this organic molecules, it was much more common people and animals die from bacterial infections and other diseases. Consequently, since its discovery and introduction in human and veterinary medicine, lives of millions of people and animals have been saved (Cruickshank et al. 2014).

The first antibiotic was accidentally discovered in 1928 when Alexander Fleming, a Scottish bacteriologist, noted that the growth of *Staphylococcus aureus* was inhibited around a colony of *Penicillium chrysogenum* (earlier known as *Penicillium notatum*) that had contaminated the Petri dish. In subsequent studies, Fleming concluded that the fungus *Penicillium* produces a metabolite with the observed antibacterial activity against *Staphylococcus* (Wright et al. 2014). Fleming and his assistants, Stuart Craddock and Frederick Ridley, purified the lytic agent, which he dubbed "penicillin", and performed assays in animal tissues, specifically wounds and human eyes, to evaluate the toxicity and irritability of the isolated compound. However, Fleming did not extend his work to clinical study since he was not able to purify enough penicillin for the experiments. At the outbreak of World War II, 12 years after breakthrough discovery of Fleming, it was emerged the need to research the chemical and biologic properties of potential antibacterial substances for the treatment of war victims. Consequently, in the following years, Howard Florey and Ernest Chain described the purification of penicillin quantities sufficient for clinical testing and reported successful use of penicillin to cure infections in mice, rats, and cats. In 1945, a year after the Nobel Prize for Medicine was awarded to Fleming, Florey, and Chain, penicillin started to be produced at industrial scale and commercialized in the open market, and therefore, a new era for medicine, called the "era of antibiotics", begun (Kong et al. 2010; Aminov 2010). The period between the 1950s and 1970s was certainly the golden era of antibiotic discovery, as more than 20 novel classes of antibiotics were discovered. Ever since, only two new classes of antibiotics have been marketed (Coates et al. 2011).

The therapeutic use of the discovered antibiotics in veterinary medicine has usually followed after their application in human medicine. Nevertheless, some antibiotics have been developed specifically for animal health and production (Giguère et al. 2013). During the last years of the World War II, penicillin started to be used in veterinary practice to treat bovine mastitis. Shortly afterwards, the growth promoter effect of other antibiotics was discovered and reported (Gustafson & Bowen 1997; Dibner & Richards 2005). However, European Union (EU) legislation has forbidden the use of antibiotics as growth promoters since 2006 as a EU's food safety strategy and due to wider considerations of public health (European Commission 2003a; European Commission 2003b).

This study is concerned with the use of antibiotics in veterinary medicine. Therefore, the topics presented in the following sections highlight the antibiotics administered to animals.

2.1.2 Definition and classification

Historically reformulated, the term "antibiotic" is currently defined as a chemotherapeutic agent which inhibits or destroys susceptible bacteria, fungi, or protozoa by specific interactions with the microorganism targets without having toxic effects on the host (Davies & Davies 2010). There are hundreds of antibiotics in veterinary use, most of which belong to a few major classes.

The antibiotics can be classified according to different criteria. A scheme presented in annex A1.1 summarizes the different classifications. Generally, the antibiotics can be grouped based on their **origin/source** (natural, semisynthetic or synthetic), **spectrum of activity** (broad-spectrum, intermediate-spectrum or narrow-spectrum, depending on the range of bacterial species susceptible to the antibiotic), **effect on bacteria** (bactericidal, bacteriostatic or both), **mode of action** (inhibition of cell wall, nucleic acids or protein synthesis, plasma membrane function or other metabolic processes) or **chemical structure** (Mendes et al. 2006; Kadam 2008) . The classification of the antibiotics according to its chemical structure is the most important due to substances belonging to the same group generally exhibit similar effect on bacteria and/or modes of action. Variations in the properties of the antibiotic within the same class results from the presence of different side chains, giving them different pharmacokinetic/pharmacodynamic index (Quinn et al. 2011; Reeves 2011).

On the other hand, the Anatomical Therapeutic Chemical Classification System for veterinary medicinal products (ATCvet) is widely used in Europe to classify veterinary drugs. Based on the same overall principles as the ATC system for substances used in human medicine, ATCvet system divides the active ingredients into different groups according to their therapeutic categories, attributing to them a specific code. In accordance with this system, veterinary medicines can be classified through 5 levels: 1st level, anatomical main group; 2nd level, therapeutic main group; 3rd level, therapeutic subgroup; 4th level, chemical/therapeutic subgroup; and 5th level, subgroup for chemical substance (WHOCC 2015). The therapeutic groups and respective ATCvet codes in which veterinary antibiotics are used in highest quantities are intestinal anti-infectives (QA07), gynecological anti-infectives and antiseptics (QG01), antibacterials for systemic use (QJ01), antibacterials for intramammary use (QJ51), and antiprotozoals (QP51). Table 2.1 lists the major antibiotic classes according to their chemical structure and ATCvet system for systemic use (Chu et al. 1989; Tillotson 1996; Khosla et al. 1999; Brooks 2001; Spízek et al. 2004; Mendes et al. 2006; Picó & Andreu 2007; Kadam 2008; Falagas & Vardakas 2010; Madigan et al. 2010; Romich 2010; Gómez-Caro et al. 2011; Quinn et al. 2011; Papp-Wallace et al. 2011; Rahim et al. 2011; Reeves 2011; Bbosa et al. 2014; WHOCC 2015). The classification presented in table 2.1 will be the used along this work.

Table 2.1. Antibiotics according to their chemical structure and ATCvet system for systemic use.

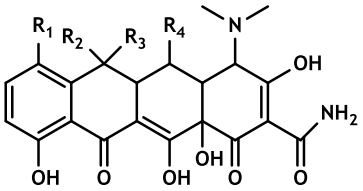
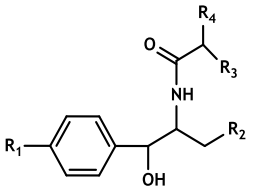
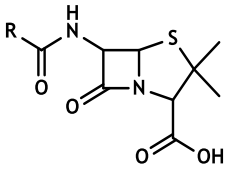
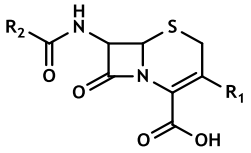
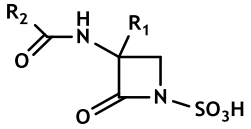
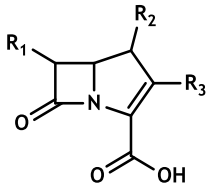
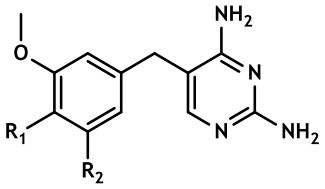
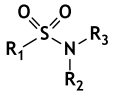
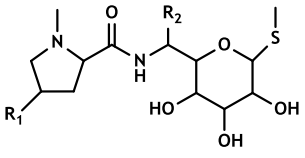
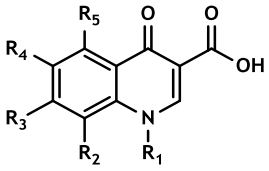
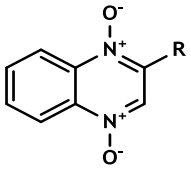
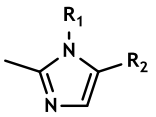
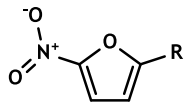
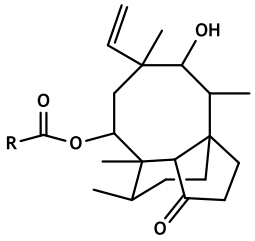
ATCvet code - Antibiotic classe	Main chemical structure	
QJ01AA – Tetracyclines	**Tetracyclines** are represented by the naphthacene carboxamide nucleus which consists of four linear fused, six membered rings.	
QJ01BA – Amphenicols	**Amphenicols** are characterized by a phenypropanoid structure.	
ß-lactams		
QJ01CA - Penicillins with extended spectrum **QJ01CE - ß-lactamase sensitive penicillins** **QJ01CF - ß-lactamase resistant penicillins** **QJ01CG - ß-lactamase inhibitors**	**Penicillins** are componsed by a thiozolidine ring fused with a four member cyclic amide, the ß-lactam ring, a free carboxyl, and one or more substituted amino group in the side chain.	
Other ß-lactams		
QJ01DB - 1st generation of cephalosporins **QJ01DC - 2nd generation of cephalosporins** **QJ01DD - 3rd generation of cephalosporins** **QJ01DE - 4th generation of cephalosporins** **QJ01DI - Other cephalosporins**	The core structure of the **cephalosporins** consists of ß-lactam ring condensed with dihydrothiazine ring.	
QJ01DF - Monobactams	**Monobactams** contain a sulfonic acid group bound to the N-atom of amide group at the ß-lactam ring and an acyl substituents at C3.	
QJ01DH - Carbapenems	**Carbapenems** structure are similar to the penicillins structure, but the sulfur atom has been replaced by a carbon atom and an unsaturation has been introduced.	
Trimethoprim and sulfonamides		
QJ01EA - Trimethoprim and derivatives	**Trimethoprim and derivatives** are diaminopyrimidine, i.e. their structure include two amino groups on a pyrimidine ring.	
QJ01EQ - Sulfonamides	**Sulfonamides** have a sulfonyl functional group connected to an amine group.	
Macrolides, lincosamides and streptogramins		
QJ01FA - Macrolides	**Macrolide**s are characterized by a large macrocyclic lactone, generally with 12, 14, or 16 members, attached to one or more sugars.	
QJ01FF - Lincosamides	Chemical structure of **lincosamides** consists in an amino acid linked to an amino sugar by an amide bond.	

Table 2.1. Antibiotics according to their chemical structure and ATCvet system for systemic use (*continuation*).

ATCvet code - Antibiotic classe	Main chemical structure
Macrolides, lincosamides and streptogramins	
QJ01FG - Streptogramins	**Streptogramins** are a group of cyclic peptides which consist of two types of molecules, group A and group B. Group A streptogramins are olyunsaturated macrolactones and group B streptogramins are cyclic hexadepsipeptides.
Aminoglycosides	
QJ01GA - Streptomycins **QJ01GB - Other aminoglycosides**	**Aminoglycosides** consist of two or more amino sugars bounded to a hexose (2-deoxystreptamine) centrally placed ring by a glyosidic linkage. Particularly, streptomycins have two amino sugars linked to a hexose (streptidine).
Quinolones and quinoxalines	
QJ01MA - Fluoroquinolones **QJ01MB - Other quinolones**	**Quinolones** are characterized by a core molecule quinoline ring substituted in the positions 1, 5, 6, 7, and 8. Particularly, **fluoroquinolones** have a C6 fluorine atom attached to ring (R_4 substituint). 
QJ01MQ - Quinoxalines	**Quinoxalines** contain a benzene ring fused to a pyrazine ring. 
Other antibiotics	
QJ01XA - Glycopeptides	Basic structure of **glycopeptide** antibacterials are glycosylated cyclic or polycyclic nonribosomal peptide.
QJ01XB - Polymyxins	The general structure of **polymyxins** consists of a cyclic heptapeptide, a linear tripeptide, and a fatty acid tail linked to the N-terminal of the tripeptide.
QJ01XD - Imidazole derivatives	**Imidazole derivatives** are characterized by an imidazole ring, a 5 membered ring with two fused N atoms. 
QJ01XE - Nitrofuran derivatives	Structural component of **nitrofuran derivatives** is a furan ring with a nitro group. 
QJ01Q - Pleuromutilins	**Pleuromutilins** consists of a common tricyclic mutilin core and a C21 keto group. 
QJ01XX - Other antibacterials	Active substances with different chemical structures are classified as **other antibiotics**: fosfomycin, xibornol, clofoctol, spectinomycin, methenamine, mandelic acid, nitroxoline, linezolid, daptomycin, bacitracin, methenamine, furaltadone, and novobiocin.

Considered the classification presented in table 2.1, it can be verified that antibiotics belonging to the same class have similar structural skeleton. Additionally, many antibiotic classes have acidic and/or basic functional groups. Consequently, both pH of the medium and acidic dissociation constants of antibiotics (pK_a values), which define their ionization state, influence antibiotics physicochemical characteristics. In fact, the different ionic species that are present in aqueous solutions at different pH values often have different properties, including water solubility, volatility, ultraviolet (UV) absorption,

and reactivity with chemical oxidants (Bérdy 1975; Qiang & Adams 2004). As a result, in studies related to antibiotics, such as occurrence, fate and effects, and control of antibiotics, it is crucial considering the pK_a values of antibiotics and control the pH of the solutions in order to obtain reproducible and comparable results.

In summary, it is concluded that chemical structure enables the antibiotics unambiguously distinction, determining all their chemical, physical, microbiological, pharmacological, and clinical properties. Therefore, the chemical characteristics and the consumption of this drugs are understandably interrelated. In the next sub-chapter, the main statistical data on consumption of veterinary antibiotics in Portugal are presented.

2.1.3 Consumption

Around the world, antibiotics are extensively used in livestock production for pro- and metaphylaxis medication, and animal growth promotion, accounting for up to 50% of antibiotic sales (M Teuber 2001; Philpott 2013). It is estimated that antibiotic consumption in livestock worldwide could rise by 67% between 2010 and 2030, from 63,151 to 105,596 tonnes (Van Boeckel et al. 2015). Countering this expected tendency, studies conducted by European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) revealed that antibiotics for use in animals decreased overall by 15% between 2010 and 2012 in Europe (EMA 2015). The European statistics on the subject have not been considered the usage of veterinary antibiotics to improve growth, once all growth promoters have been banned from European agriculture since 2006, as previously mentioned (European Commission 2003b).

Direção Geral de Alimentação e Veterinária (DGAV) is the Portuguese National Authority for Animal Health that provides to ESVAC data on consumption of antibiotics for animal production in Portugal and also published detailed information about the use of veterinary antibiotics in Portugal for the years 2010 and 2011. Considering that in Portugal the commercialization of veterinary antibiotics requires a medical prescription, DGAV assumed that their sale and consumption were properly communicated to regulatory authorities (DGAV et al. 2010; DGAV et al. 2011). The distribution of sales of veterinary antibiotics between 2010 and 2012 in Portugal are showed in figure 2.1 (EMA 2010; EMA 2011; EMA 2012).

Analyzing the graph of figure 2.1, it is noted that in Portugal the amount of antibiotic marketed between 2010 and 2012 decreased consecutively. It is also verified that the active compounds belonging to classes of veterinary antibiotics tetracyclines and penicillins were the two most commercialized, by decreasing order. It should be noted that sales of drugs belonging to the class of macrolides were significantly consumed in 2011, accounting for 13.4% of the total amount of consumed antibiotics in this year.

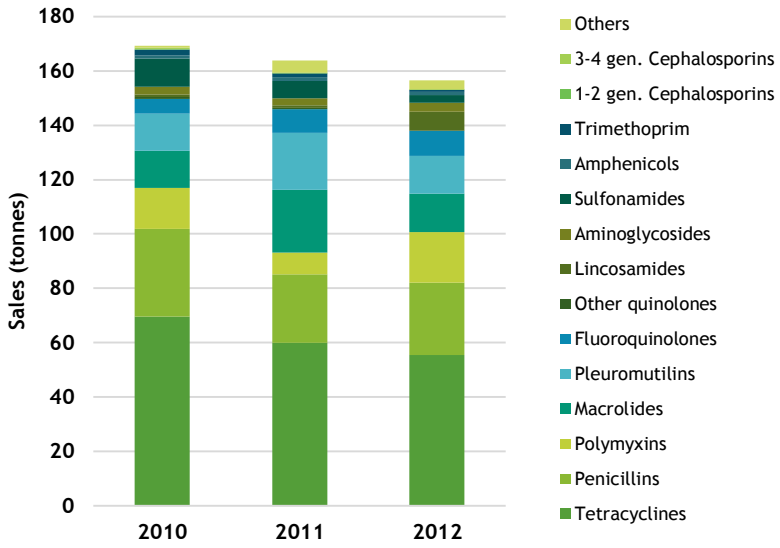


Figure 2.1. Proportion of the total sales of the different veterinary antibiotic classes in Portugal between 2010 and 2012 (tablets excluded; values expressed in tonnes; veterinary antibiotics classification based on ATCvet system).

Figure 2.2 shows the percentage of consumption in Portugal during 2011 of each active agent belonging to tetracyclines, penicillins, and macrolides classes (DGAV et al. 2010; DGAV et al. 2011). The detailed information presented was obtained by consulting the reports published by DGAV, which to date has not provided data for the year 2012. It can be seen that the consumption pattern of tetracyclines varied significantly from 2010 to 2011, contrary to the penicillins and macrolides. For tetracyclines class, oxitetracycline and doxycycline were the top selling active agents in 2010 and 2011, respectively. This noticeable difference is due to the fact that the quantity consumed oxitetracycline decreased from 46.5 to 13.3 tonnes from 2010 to 2011, while the marketed quantity of doxycycline and tetracycline remained approximately constant. In its turn, for the period under review the active agent of penicillins class that was the most consumed was the amoxicillin, verifying a slight increase in percentage terms. For macrolides, tylosin followed by the tilmicosin were the most consumed drugs.

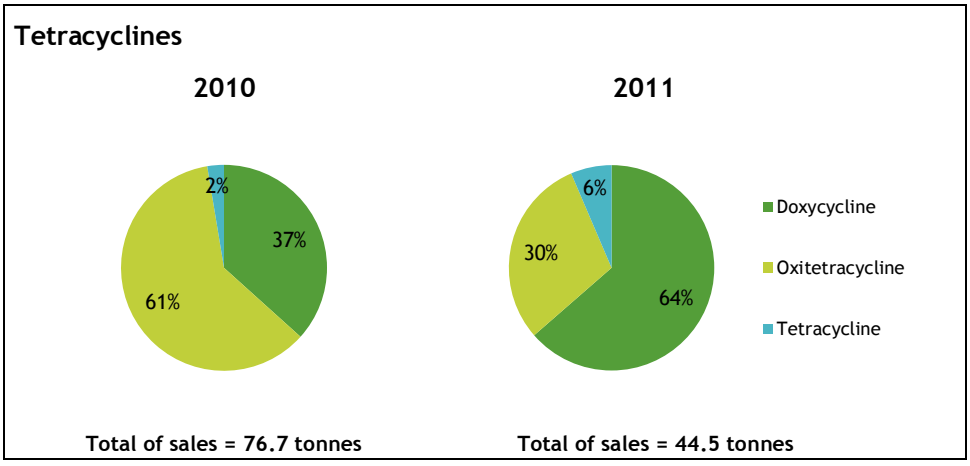


Figure 2.2. Distribution of sales of tetratcyclines, penicillins, and macrolides for food-producing animals and companion animals in Portugal in 2010 and 2011 (tablets included; veterinary antibiotics classification based on ATCvet system).

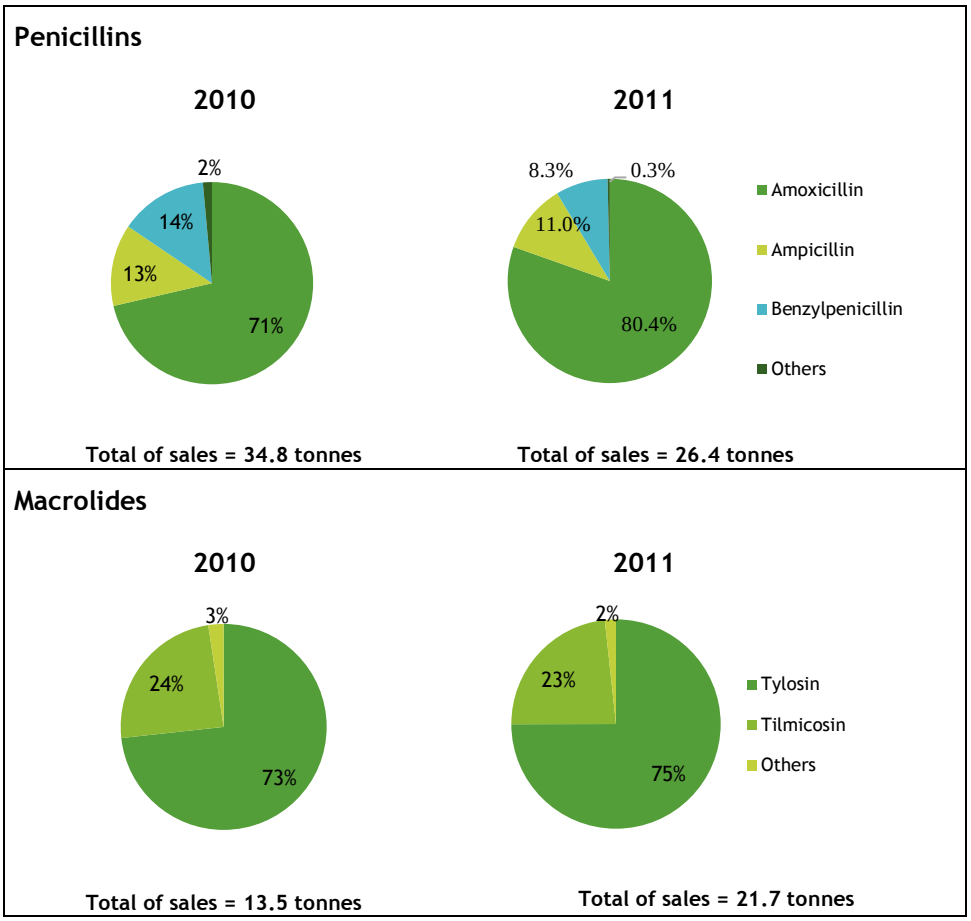


Figure 2.2. Distribution of sales of tetratcyclines, penicillins, and macrolides for food-producing animals and companion animals in Portugal in 2010 and 2011 (tablets included; veterinary antibiotics classification based on ATCvet system) (*continuation*)**.**

The environmental contamination by veterinary antibiotics or their metabolites is related with their massive consumption. The presence of these substances in the environment is currently subject of a major environmental concern due to their interference with the natural ecosystem dynamics. For a better understand of the magnitude of this global problem, the next section provides information about the possible pathways of the introduction of veterinary antibiotics in the environment.

2.1.4 Pathways into the environment

The existence of antibiotics in the environment has recently aroused an increasingly attention by the scientific community. These pharmaceuticals, massively used in livestock production, can take several routes into the environment. The major anticipated exposure pathways of veterinary antibiotics in the environment are displayed in figure 2.3 (Boxall et al. 2003; Jjemba 2002; Kemper 2008; Kumar et al. 2005; Kümmerer 2003; Kümmerer 2008; Li 2014; Rehman et al. 2013).

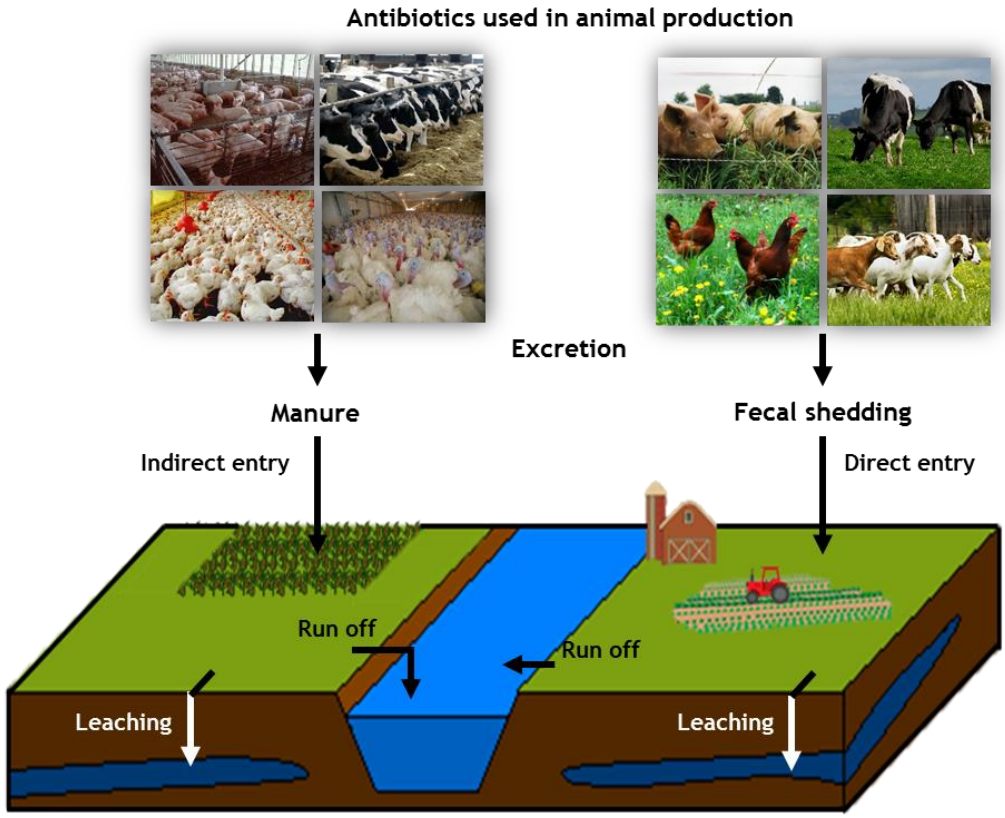


Figure 2.3. Principal anticipated exposure pathways of veterinary antibiotics in the environment.

The importance of individual pathways into the environment for different types of veterinary antibiotics depends on the type of treatment and livestock category. The main routes of entry to the terrestrial environment are from the use of veterinary antibiotics in intensively reared livestock and in pasture-reared animals (Kümmerer 2008).

After administration, antibiotics are partially metabolized by the organism. It is estimated that 75% to 90% of antibiotics used in food animals are excreted via urine and feces, largely unmetabolized (Marshall & Levy 2011). Excretion rates depends on the substance, mode of application, excreting species, and time of residence (Kemper 2008). As original compounds, polar antibiotics are eliminated more efficiently by the excretory organs than compounds which have high lipid solubility. For instance, excretion rates of amoxicillin (logarithm of octanol-water partition coefficient (log K_{ow}) = 0.87) and doxycycline (log K_{ow} = -0.22) are 80 - 90% and 90 - 95%, respectively, whereas chloramphenicol (log K_{ow} = 1.14) are excreted to about 5 - 10% (Kumar et al. 2005). On the other hand, antibiotic metabolites excreted can be revert back to the original active ingredient (e.g. sulphonamides) (Zhou et al. 2013). Consequently, via the application of slurry and manure to land, or direct deposition of manure by grazing animals, excreted antibiotics and their metabolites can reach the environment. Once in soils, veterinary antibiotics may also enter the aquatic environment indirectly via surface runoff or leaching to groundwater (Halling-Sørensen et al. 1998; Chee-Sanford et al. 2009; Kümmerer 2003). Additionally, livestock effluents are usually stored in lagoons and pit systems prior to application on agricultural fields. As a result, seepage and runoff into watershed systems mobilize the constituents, exposing humans and other animals to the contaminants (Chee-Sanford et al. 2001).

Furthermore, wastewater of livestock raising, pre-treated or not, can be routed through wastewater treatment plants (WWTP) (Kümmerer 2003; Rehman et al. 2013). Nevertheless, elimination rates of antibiotics residues during wastewater treatment are incomplete (Brown et al. 2006; Jjemba 2002). Hence, this pharmaceuticals may enter the aquatic environment through WWTP effluents, reaching surface water and subsequently groundwater and drinking waters (Kümmerer 2008; Li 2014).

An alternative pathway into the aquatic environment derives from the application of antibiotics for aquaculture. Incident release of products from spills or discharges are also possible pathways of antibiotic residue entry into the environment. Finally, emission during the manufacture, disposal of unused drugs and containers, atmospheric dispersal of feed and manure dust containing antibiotics, and inputs from companion animal treatments are expected to be minimal in comparison (Hirsch et al. 1999; Chee-Sanford et al. 2012).

The presence of veterinary antibiotics in animal wastes, surface and groundwaters, river sediments, and soils at concentrations that could have potential impacts on the ecosystems are reported by an increasing number of studies around the world. Once in the environment, antibiotics can be transported, degraded, absorbed through drinking water by fish and meat sources, and agricultural products, or bioaccumulated. Therefore, understanding the dynamic and behavior of antibiotics in the environment is of utmost importance to realize their impact on ecosystems (Kümmerer 2009a). In view of this, fate of veterinary antibiotics, along with their potential risks to the environment, is discussed in the next section.

2.1.5 Fate in the environment

Livestock production is an important source of antibiotic compounds that reach the environment, persisting through a cycle of partial biotransformation and bioaccumulation and gradual deposition in soil and groundwater. The life-cycle of this pharmaceutics in the environment is governed by a number of biological and physicochemical processes in the soil–water systems, particularly stability, sorption, leaching, and degradation, which depend on the physicochemical properties of antibiotics, soil properties, and weather conditions (Kumar et al. 2005). Chemical relevant information for common used antibiotic classes is provided in table 2.2 (Thiele-Bruhn 2003; Reeves 2011). The significantly differences presented in the table 2.2 among the wide range of veterinary antibiotics used is consistent with the information of the section 2.1.2. In fact, antibiotics are a diverse group of chemicals, often characterized by complex molecules with different functional groups (Kümmerer 2008).

Table 2.2. Typical ranges of physicochemical properties from classes of antibiotics mainly used in animal agriculture.

Antibiotic class	Molar mass (g/mol)	Water solubility at 25 ºC (mg/L)	Log K_{ow}	pK_{a1}/ pK_{a2}/ pK_{a3}/ pK_{a4}
Aminoglycosides	332.4 - 615.6	10,000 - 500,000	-8.1 - -0.8	6.9 - 8.5
B-lactams	334.4 – 470.3	22 – 10,100	0.9 – 2.9	2.8 - 2.7/ 7.2 - 7.3*/ 9.5*
Fluorquinolones	229.5 – 417.6	3.2 – 17,790	-1.0 – 1.6	3 – 4/ 6/ 7.5 – 9/ 10 – 11
Imidazoles	171.5 – 315.3	6.3 – 407	-0.02 – 3.9	2.4
Macrolides	687.9 – 916.1	0.45 – 15	1.6 – 3.1	7.5 - 9
Tetracyclines	444.5 – 527.6	230 – 52,000	-1.3 – 0.05	3 – 4/ 7 – 8/ 9 – 10
Sulfanamides	172.2 – 300.3	7.5 – 1,500	-0.1 – 1.7	2 – 2.5/ 5 – 7.5

* ß-lactams: amoxicillin and ampicillin.

The mobility of antibiotics in the environment are related to sorption–desorption processes and has traditionally estimated using the K_{ow} (Sukul et al. 2008). Nevertheless, soil sorption behavior of polar or ionizable compounds, such as many antibiotics, may be inaccurate by predictions based on K_{ow}, since this parameter only reflects hydrophobic interactions. In fact, the sorption behavior of antibiotics are related to hydrophobic partitioning and a number of hydrophobicity independent mechanisms. Electrostatic interactions, surface complexation, hydrogen bonding, cation exchange, bridging, and environmental factors can also affect sorption and are not accounted for by K_{ow} values (Boxall et al. 2003; Jechalke et al. 2014).

Soil-water partition coefficient (K_d) can be used to describe the sorption potential of pollutants and their mobility into the environment. Using K_d instead of K_{ow} will result in a more correctly assessment of sorption behavior in a specific soil. Generically, the mobility of the compounds in the soil decreases with the increase of K_d values. Drugs with lower K_d values are weakly bound to the soil, being more likely to be transported to either surface or groundwaters than compounds with high K_d values. On the other hand, strongly bound antibiotics are more likely to be transported with sediments via surface runoff (Boxall et al. 2002). Studies demonstrated that sorption of sulphonamides (e.g. sulfachloropyridazine) to biosolids are weak, while ciprofloxacin, tetracycline, doxycycline, and clindamycin have high sorption potential, being therefore more likely to remain on the solids in the top layer of the soil (Davis et al. 2006; Wu et al. 2009).

Adsorption commonly reduces the antimicrobial activity of the antibiotics, principally when the bioactive functionality are associated with the exchange sites. Nonetheless, these so-called sequestration process does not completely eliminate the antimicrobial activity of antibiotics. Since this is a reversible process, subsequent slow release of sequestered antibiotics can back into a bioavailable form (Thiele-Bruhn 2003; Jechalke et al. 2014). Sulfonamides, for instance, are detectable for prolonged periods of time at small sub-inhibitory concentrations (Boxall et al. 2002; Förster et al. 2009; Zarfl et al. 2009).

Additionally, biotic and abiotic degradation processes play an important role in the environmental fate of antibiotics. Multiple of these compounds are susceptible to photolysis, hydrolysis, or thermolysis (Kümmerer 2008). Photodegradation may be a major elimination process for light sensitive active agents in aqueous matrixes, particularly in surface water and during effluent treatment. The extent of the

process depends on location, season, and latitude, and the physicochemical properties of matrix (Werner et al. 2006). Laboratory tests shew that UV radiation plays a significant role in catalyzing the removal of tetracyclines (Wammer et al. 2011; Verma et al. 2007; Garcia-Rodríguez et al. 2013), sulfonamides (Batchu et al. 2014; Abellán et al. 2009), fluoroquinolones (Lorenzo et al. 2008), and nitrofuran antibiotics (Edhlund et al. 2006) from aqueous matrixes. However, the results of these assays must be evaluated carefully since they do not simulate all conditions of photochemical decomposition in the environment.

Hydrolysis and thermolysis are other important degradation processes for some antibiotics in the environment. For instance, penicillins and tetracyclines are instable in water, being not commonly expected to be found in the aquatic environment. Studies demonstrated that hydrolysis rates for penicillins and oxytetracycline increase as pH diverges from pH 7 and as temperature increases (Längin et al. 2009; Garcia-Rodríguez et al. 2013). The ß-lactam ring of penicillins can be opened by ß-lactamase, an enzyme present in bacteria (figure 2.4 (Gentry 2012)), or by chemical hydrolysis. On the other hand, sulfonamides and quinolones are stable against hydrolysis (Kümmerer 2009a).

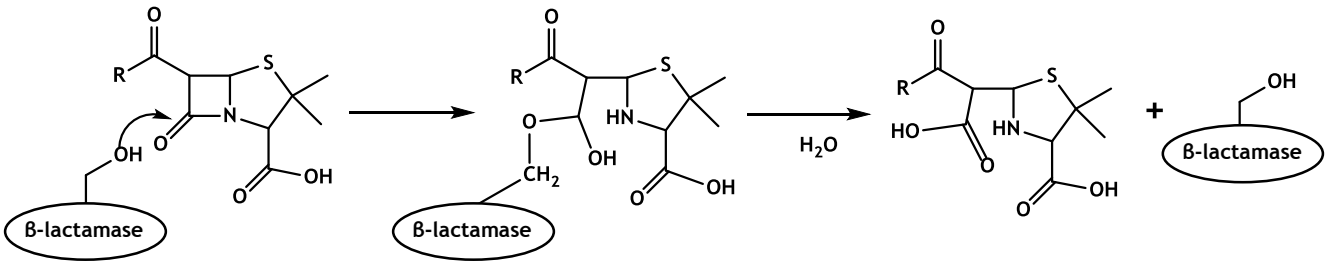


Figure 2.4. ß-lactamase-catalized hydrolysis of penicillins.

Only a few number of studies to evaluate the biodegradation of antibiotics have been conducted. This process depends on temperature, moisture, chemical composition of the environment, and the microbiota (Martinez 2009; Manzetti & Ghisi 2014). Since the microorganisms presence is essential to the biodegradation reactions, these bioactivity are higher in soil than in natural waters (Li 2014). However, due to the fact of antibiotics be biologically active compounds, most of them have limited biodegradability in both soil and water, being persistent in the environment (Kümmerer 2003; Abellán et al. 2009; Kümmerer 2009c). It should be noted that degradation processes can be incomplete, resulting intermediates that can be even more stable than the parent compounds (Kümmerer 2008). Consequently, the toxic effects of this process on the resident bacteria should be considered. However, subtle and longer-term effects of these medicines continued to be unknown (Chee-Sanford et al. 2012).

On the other hand, antibiotics and their metabolites that end up on arable land can be uptake and/or accumulate by plants or vegetable crops. Additionally, changes in the activities of soil microbial communities due to antibiotics contamination can affect the symbiotic plant–microbe relationship, inducing an additional stress to the plants (Rehman et al. 2013). These biological mechanisms of antibiotics fate has recently received considerable attention due to possible health hazard to consumers of tainted plants (Jjemba 2002; Chee-Sanford et al. 2012; Jechalke et al. 2014).

Once in the aquatic environment, antibiotics can be absorbed by animals (food producing animals and fishes) or reach the drinking water, inducing also possible long-term effects on humans as a continuous part of their diet through water or food at low concentrations (Thiele-Bruhn 2003; Manzetti & Ghisi 2014).

The presented diffusion of antibiotics in the environment may result in the development of microbial resistance with the potential of adversely affecting bacterial cycles/processes critical to aquatic ecology (nitrification/denitrification) or agriculture (soil fertility) and animal production. The emergence of antibiotic resistance has become a global concern, since many common bacterial infections are becoming increasingly difficult to treat as this phenomena spread across the world (Hernando et al. 2006; Sarmah et al. 2006; Watkinson et al. 2009). In this context, the next section provides a brief discussion about the risk of antibiotic resistance to human health.

2.1.6 Resistance risk

Resistance to antibiotics has been described as one of the greatest threats to public health throughout the world. The widespread use of antibiotics may cause the selection pressure on environmental microorganisms, contributing to the occurrence and dissemination of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARG) in various environmental media (Aminov 2009).

Antibiotic resistance is an adaptive genetic trait possessed or acquired by bacteria subpopulations, enabling them survive and grow in the presence of the antibiotic agent at therapeutic concentration that would normally inhibit or destroy these microorganism. Despite being a natural phenomenon, antibiotic resistance is worsened by the overuse of these medicines (Cruickshank et al. 2014; Kümmerer 2009b). Antibiotics used in animals contribute for the development and propagation of ARB. In this context, animals treat continuously with sub-lethal dosages of antibiotics can be considered as intermediaries, reservoirs and disseminators of ARB and/or ARG (Aryal 2001; Michael Teuber 2001; Davies 2006). Accordingly, misuse of antibiotics in animals may eventually result in increased human morbidity and mortality, reduced efficacy of related antibiotics used for human medicine, increased healthcare costs, and potentiate the emergence and spread of resistant human pathogens (WHO 2001; Marshall & Levy 2011).

Resistance to antibiotics is a reality worldwide and constitutes a serious problem in the treatment of infectious diseases. Some publications suggests that the return to pre-antibiotic era is inevitable as a result of the decline in the number of new antibacterials launched in the last 40 years and the rise of the resistance rates around the world (Coates et al. 2011; Wright et al. 2014). The lack of knowledge about the long-term risks of the presence of antibiotics, ARB, and ARG in the environment has recently motivated scientific studies on the subject, as well as national and international actions to prevent and mitigate this global problem (Tollefson 2004; Larsson 2014; WHO 2015). In Portugal, competent authorities monitors the national consumption of veterinary antibiotics, participating in the international project ESVAC, as mentioned in section 2.1.3. Additionally, the *Plano de Ação Nacional para a Redução do Uso de Antibióticos nos Animais* was implemented in 2014 by DGVA for promotion and protection of both animal and human health (DGAV 2013). However, there is a lack of literature and research on

occurrence, fate, and effects of veterinary antibiotics in the environment in Portugal. To the best of the author knowledge, there are only one publication about environmental occurrence of antibiotics in Portugal (Pena et al. 2010). Therefore, studies on the subject in the Portuguese territory are required, in order to develop plans of action and monitoring to limit the spread of resistance and maintain the effectiveness of antibiotics according to the national reality.

2.2 Study cases

2.2.1 Amoxicillin and doxycycline

Amoxicillin (a ß-lactam antibiotic) and doxycycline (a tetracycline antibiotic) are the antibiotics most commonly used in veterinary practice in Portugal (section 2.1.3). After administration to animals, these active agents are only poorly metabolized, being excreted in urine and faeces (Hirsch et al. 1999; Jjemba 2002). Consequently, these compounds and their metabolites can enter the environment via several pathways (section 2.1.4), persisting as parent compound, metabolites, or transformation products through a life-cycle (section 2.1.5) according their biological and physicochemical properties. Studies shew that the presence of these antibiotics in the environment may be related to the change of the natural ecosystems microbiota and the emergence of resistant bacteria, represented a global public health problem (Foti et al. 2009; de Toro et al. 2011; Harnisz 2013; Ayandiran et al. 2014). For these reasons, the study of the occurrence in the aquatic environment of amoxicillin and doxycycline used in livestock was considered important for the identification of these problem in Portugal.

2.2.1.1 Physicochemical characterization

Amoxicillin (AMOX, $C_{16}H_{19}N_3O_5S$) is an α-amino-substituted ß-lactam antibiotic (Reyns et al. 2008) and doxycycline (DOX, $C_{22}H_{24}N_2O_8$) is a tetracycline antibiotic obtained by modification of the oxytetracycline molecule. These antibiotics show an amphoteric behavior characterized by three acid dissociation constant as figures 2.5 (Homem et al. 2014) and 2.6 illustrate, respectively. Schematic representation of the dissociation equilibrium of amoxicillin and doxycycline are presented in annex A1.2 (Babić et al. 2007; Prankerd 2007). At pH range between 3 and 8 they are ionized. Amoxicillin and doxycycline exist in the cationic form at a pH below 3, as a zwitterion between pH of 3.1 and 7.5, and above that as an anion (Hamscher 2006).

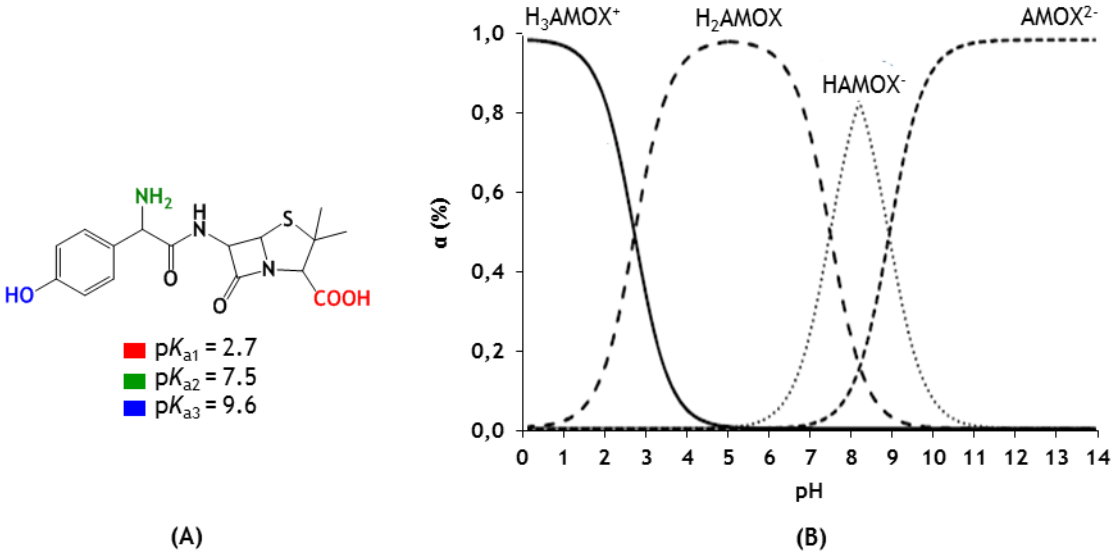


Figure 2.5. (A) Chemical structure of amoxicillin (AMOX); (B) Speciation diagram for AMOX.

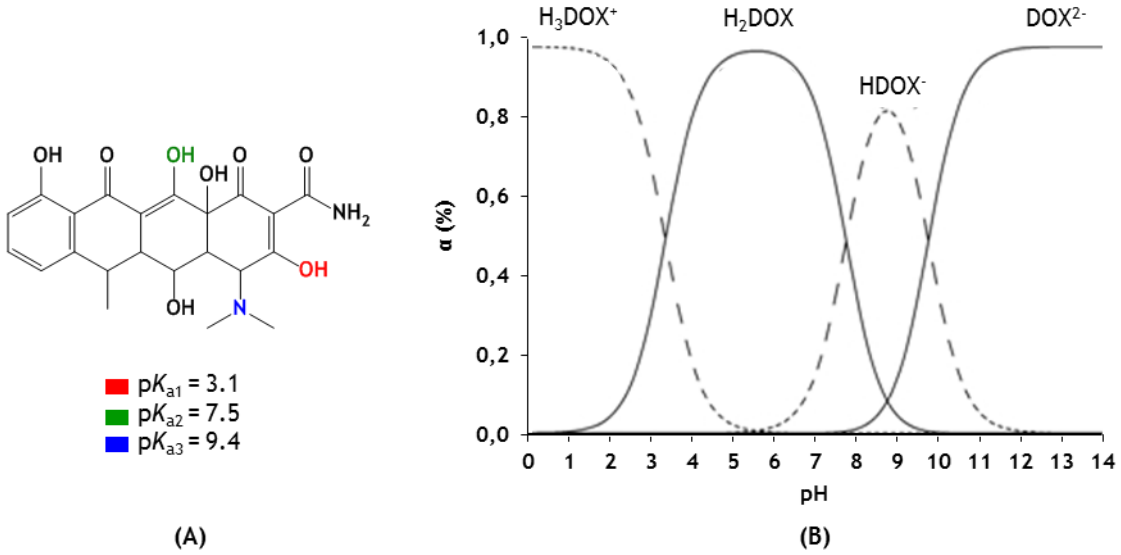


Figure 2.6. (A) Chemical structure of doxycycline (DOX); (B) Speciation diagram for DOX.

Other physicochemical properties of these compounds are summarized in table 2.3 (Williams et al. 2003; Hamscher 2006; Florence & Attwood 2011; Kogawa & Salgado 2012; Council of Europe 2015; PubChem 2015; DrugBank 2015).

Table 2.3. Physicochemical properties of amoxicillin and doxycycline.

Compound	Amoxicillin	Doxycycline
CAS#	26787-90-3	564-25-0
MW (g/mol)	365.41	444.45
Log K_{OW}	0.87	-0.02
Solubility	4000 mg/L (in water, 25 ºC)	630 mg/L (in water, 25 ºC)
	Slightly soluble in water and in methanol; insoluble in chloroform	Very slightly soluble in water; freely soluble in dilute acid and in alkali hydroxide solutions; sparingly soluble in alcohol; practically insoluble in chloroform and in ether.

CAS# - chemical abstract services registry number; MW – molecular weight; Log K_{OW} – logarithm of octanol-water partition coefficient.

As a penicillin antibiotic, amoxicillin is unstable, being sensitive to heat, light, extremes in pH, heavy metals, and oxidizing and reducing agents. This antibiotic contains a ß-lactam nucleus that is susceptible to cleavage by both abiotic and biotic processes (see annex A1.3) (Wood 1986; Crea et al. 2012; Gozlan et al. 2013; Merck 2015a). In turn, doxycycline, like other derivatives of tetracycline, is unstable in aqueous solution, particularly at alkaline pH, and sensitive to heat and light (Skúlason et al. 2003; Injac et al. 2007). This compound forms poorly soluble chelates with bivalent and trivalent cations, particularly calcium, magnesium, aluminum, and iron (figure 2.7 (Romich 2010; Gentry 2012)). Compared to other members of the tetracycline class, it is more lipophilic, resulting in higher intracellular penetration, longer half-life, and enhanced antimicrobial activity (Castro et al. 2009; Merck 2015b).

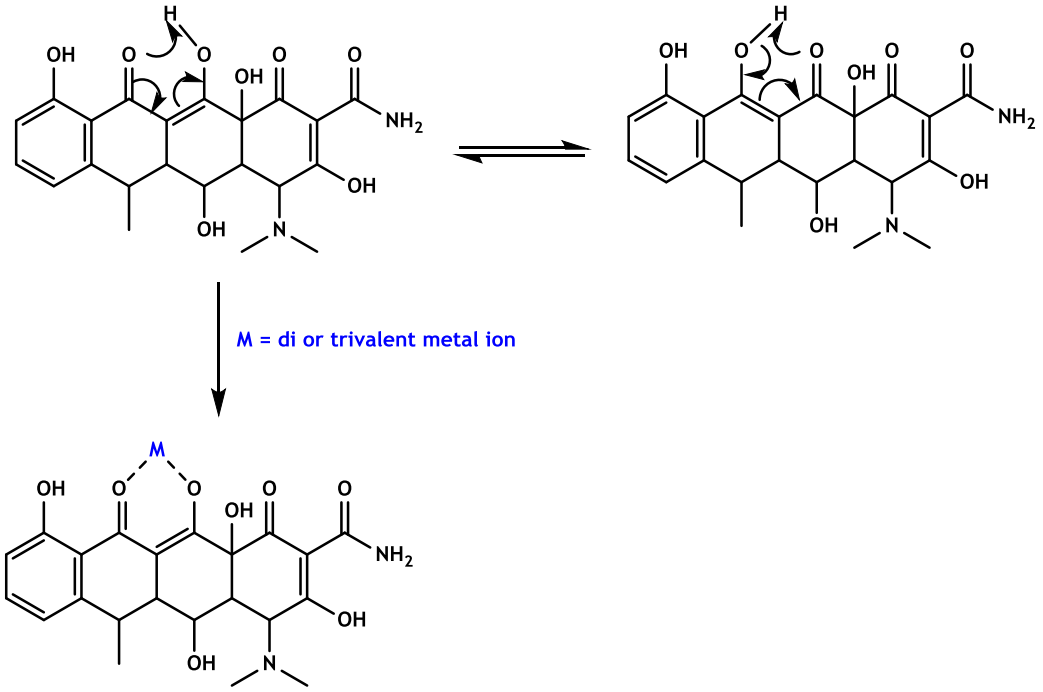


Figure 2.7. Metal chelation with doxycycline.

The physicochemical characteristics of the antibiotics are related with antimicrobial activity and applications. In the next sections, relevant information about biological properties and therapeutic use in animals of the amoxicillin and doxycycline are presented.

2.2.1.2 Biological properties

Antibiotics are special compounds with pharmacological and antimicrobial activity depending on their physicochemical characteristics. Biological information about amoxicillin and doxycycline are summarized in the table 2.4 (Schwarz et al. 2008; Castro et al. 2009; Riviere & Papich 2013; Eghianruwa 2014; Pomorska-Mól et al. 2014; Merck 2015a; Merck 2015b).

Table 2.4. Biological properties of amoxicillin and doxycycline.

Compound	Amoxicillin	Doxycycline
Spectrum of activity	Broad spectrum of activity against many Gram-positive and Gram-negative bacteria.	Broad spectrum of activity against a wide variety of microorganisms, including aerobic and anaerobic Gram-positive and Gram-negative bacteria, chlamydiae, rickettsiae, and mycoplasmas.
Effect on bacteria	Bactericidal.	Bacteriostatic.
Mode of action on the bacteria	Inhibition of the cell wall synthesis by binding to the enzymes which produce the protein cell wall peptidoglycan.	Inhibition of the synthesis of bacterial proteins by binding predominantly to 30S subunit of bacterial ribosome.
Absorption by the gastrointestinal tract	Very well absorbed; Effective blood concentrations are reached in 2 – 4 hours after administration.	About 95% of administered dose is absorbed; Effective blood concentrations are reached in 2 – 4 hours after administration.
Plasm half-life in animals	17 hours.	4.5 – 16.7 hours.
Excretion	About 80 – 90% is excreted in urine after intravenous or oral administration; Low metabolism.	About 90 – 95% (mostly unchanged) is excreted in urine and faeces after intravenous or oral administration; Low metabolism.

Apart from physicochemical properties, the antimicrobial activity and pharmacokinetic features of amoxicillin and doxycycline differ significantly. However, it should be noted that both are effectively absorbed by the intestinal tract and poorly metabolized. Thus, these compounds or their degradation products (considering their stability) may be present in the environment (Kim et al. 2012). The risk of contamination of ecosystems by antibiotics depends, among other factors, on the therapeutic uses. The application of the studied antibiotics in veterinary medicine in Portugal is presented in the following section.

2.2.1.3 Application in veterinary medicine

In Portugal, amoxicillin and doxycycline are used in both human and veterinary medicine for treatment infections caused by the susceptible microorganisms. In regard to therapeutic use in animals, amoxicillin is administered to treat gastrointestinal, urinary, and respiratory tract infections (WHOCC 2015). In turn, doxycycline is used for respiratory and intestinal tract diseases (Fernández et al. 2004).

These veterinary antibiotics are used in different proportions for the different categories of animals (pets and farm animals). The sales of active ingredients amoxicillin and doxycycline by animal species for the years 2010 and 2011 is shown in figure 2.8 (DGAV et al. 2010; DGAV et al. 2011).

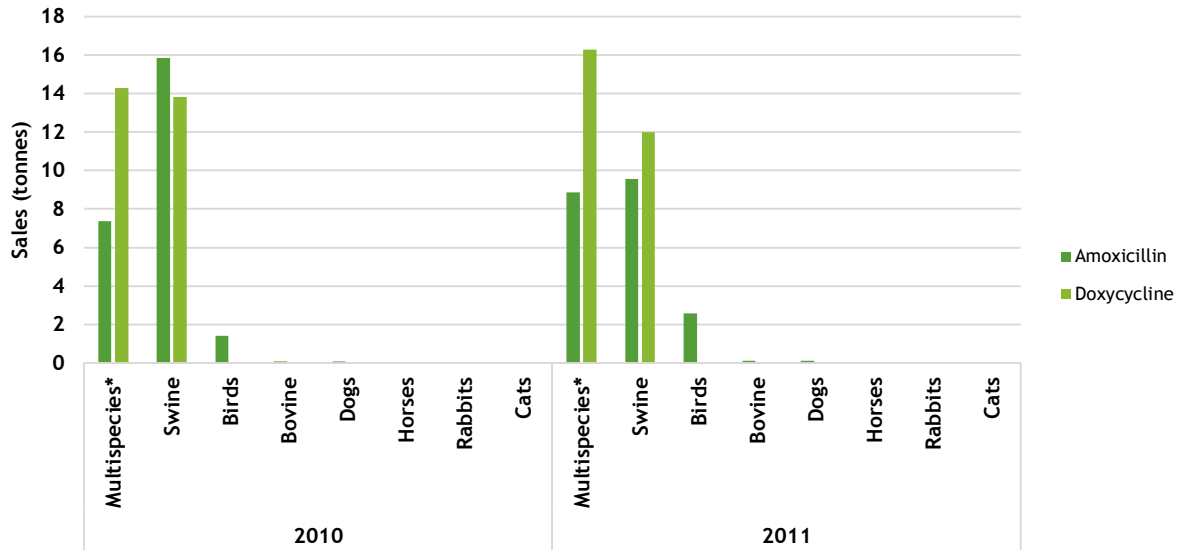


Figure 2.8. Sales of active substances amoxicillin and doxycycline for the different species of animals in Portugal in 2010 and 2011 (tablets included; values expressed in tonnes)**. *** The term "multispecies" refers to more than one specie for the same active substance.

Analyzing the presented graph and excluding the category multispecies, it can be verified that both antibiotics amoxicillin and doxycycline were mainly used in pigs. Information about swine production in Portugal is provided in the annex A1.4.

2.2.2 Analysis of amoxicillin and doxycycline in water

In last years, the growing concern about the presence of micro-pollutants, such as antibiotics, in different environmental matrixes resulted in the enormous progress in the development in analytical techniques for trace analysis of these compounds. The new analytical methods are crucial for the assessment of their environmental impact and effect on human health through monitoring plans, which requires accurate and reliable data (Buchberger 2011).

The analysis of amoxicillin and doxycycline in aqueous environmental matrixes is usually carried out by liquid chromatography (LC) after the sample clean-up and pre-concentration on the analytes by solid-phase extraction (SPE). In the next sections, the SPE method for sample extraction and the analytical method high performance liquid chromatography coupled to diode array detector (HPLC-DAD) are introduced.

2.2.2.1 Sample extraction by solid-phase extraction

Emerging in the mid-80s (Armenta et al. 2008), solid-phase extraction (SPE) is currently the most widely used methodology to perform extraction of many analytes in multiresidue environmental analysis (Kostopoulou & Nikolaou 2008; Bondi et al. 2009). The removal of the interferences from the matrix, the sample clean-up and pre-concentration, and the solvent exchange are aims of SPE. This procedure allows the subsequent detection of the analytes present in the initial aqueous sample at very low concentrations. The range of the concentration factors can extend to four orders of magnitude (Seifrtová et al. 2009). In SPE procedure, analytes from the aqueous phase are extracted to a solid phase.

Considering the sorbent functionality and the interaction that compounds establish between the liquid and the adsorbent, there are different types of SPE: reverse phase SPE (extraction of polar or hydrophobic organic analytes from aqueous matrixes), normal phase SPE (extraction of polar analytes from non-polar organic solvents) and ion exchange SPE (extraction of charged or ionizable analytes from aqueous samples or non-polar organic solvents) (Waters Corporation 2015b).

Isolating and cleaning up sample components of interest can be achieved by adsorption of matrix interferences while components of interest are unretained or adsorb components of interest while matrix interferences pass through the cartridge. The second mentioned strategy is typically used to extract amoxicillin and doxycycline from aqueous samples. In general, as figure 2.9 (Lucci et al. 2012) shows, SPE procedures comprises four steps: (i) conditioning of the cartridge, (ii) loading the sample, (iii) washing the cartridge, and (iv) eluting the analytes (and some interferences). In some applications, one or more steps are omitted (Bruno et al. 2001; Bailón-Pérez et al. 2009).

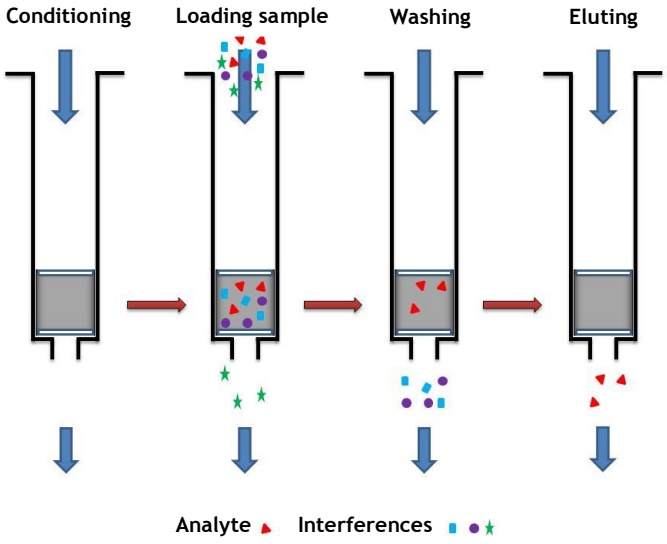


Figure 2.9. Typical four steps SPE method.

After the sorbent activation with a suitable solvent, samples passed through the bed onto which the analytes and some interferences are retained. In fact, during the retention step, multiple compounds which are present in the sample may be retained on the solid surface. In the sorbent washing step, the undesirable compounds less retained are removed. Therefore, washing step prevent the co-elution of analytes and many interferences during the last step. In elution, compounds of interest (and some interferences) are desorbed from solid phase matrix and can be collect in the liquid phase. Several types of solid phase matrixes are nowadays available. The choice of the sorbent, as well as the washing and elution solvents, should be based on the physicochemical properties of the analytes (Loos et al. 2010; Simpson & Martha 2000).

2.2.2.2 Analyzes by high performance liquid chromatography-diode array detector

Nowadays, high performance liquid chromatography (HPLC) is one of the most powerful tools in analytical chemistry, being able to separate, identify, and quantitate the analytes present in samples at trace concentrations. In HPLC, the compounds to be analyzed must be soluble in the mobile phase, interact with the stationary phase, and properly detected (Waters Corporation 2015a).

The components of a basic HPLC system are shown in figure 2.10 (Linde 2008).

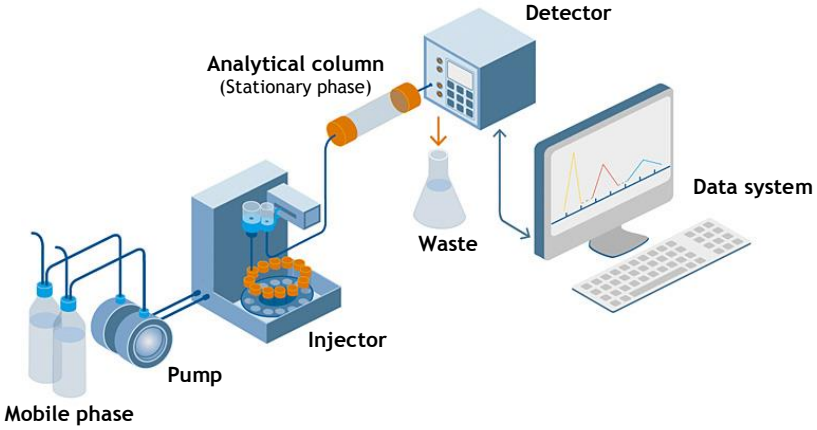


Figure 2.10. Schematic representation of the HPLC system components.

The sample is injected into the continuously flowing mobile phase (solvent) stream which is pumped at a specified flow rate, moving the components of the sample through the analytical column. The chromatography column contains the stationary phase (packing material). The chromatographic separation is obtained from differentiated interactions between the compounds of the sample and both stationary and mobile phases. The components of the mixture are distributed between the two phases according to their affinities. While compounds with higher affinity for the stationary phase move more slowly, those with low affinity for the stationary phase move more rapidly through the mobile phase. The polarity, electrical charge, and molecular size of the chemical compounds can be used to create HPLC separations. Separations based on polarity are generally used in the HPLC analysis of amoxicillin and doxycycline. After elution from HPLC column, the compounds pass by the detector which transmits an electrical signal to the data system. Through the signal received from the detector, the computer data station generates and displays a chromatogram, by which it is possible to identify and quantify the concentration of the sample constituents (Hanai 1999).

Several detectors are currently available for HPLC analysis. According to analytes characteristics, refractive index, ultraviolet/visible (UV/Vis), fluorescence (FL), electrochemical, and mass spectrometry (MS) detectors can be used. The detection of amoxicillin and doxycycline in HPLC analysis is typically carried out by UV/Vis and/or MS detectors. The combination of UV/Vis and MS detectors provides, from a single injection, more comprehensive information about the analytes (Waters Corporation 2015a). MS detectors are a powerful tool to perform simultaneously detection of multicomponent in HPLC analysis, allowing the identification and quantification of analytes present in the sample at very low concentrations (in the order of ng/L). In fact, the limits of detection obtained by LC-MS analysis are lower than analysis by HPLC couple to UV/Vis detector. However, the MS operation requires an expertise

user and have high cost (Honikel et al. 2014). Consequently, in this work the simultaneous detection of amoxicillin and doxycycline were performed by a UV/Vis detector, the diode array detector (DAD).

The molecular absorption properties of compounds are measured by UV/Vis detectors. DAD, a specific type of UV/Vis detector, are able to perform spectroscopic scanning and absorbance readings at wavelengths from 190 to 800 nm while the compounds pass though the detector flow cell. Figure 2.11 (Aboul-Enein & Serignese 2005) shows a diagram of a diode array detector.

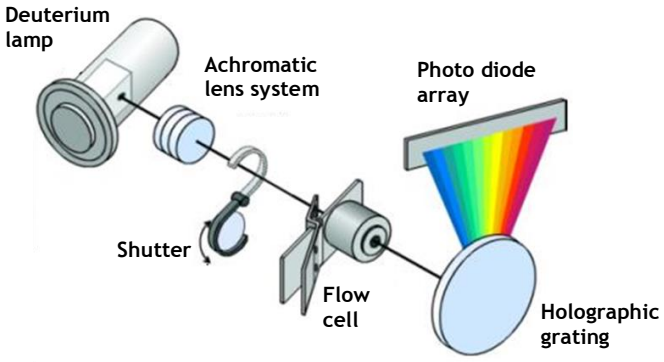


Figure 2.11. Schematic representation a diode array detector.

In this type of detector, the achromatic lens system collimate the light emitted from the broad emission source, such as deuterium lamp. The rays of light accurately parallel pass through the detector cell, being then dispersed in a holographic grid. The separated light reach the array that contain hundreds of diodes. The electrical signal produced is proportional to the amount of light received. Therefore, the absorbance of the sample compounds can be simultaneously determined for all diffracted wavelengths (Beesley & Scott 1998; Scott 2011).

3 State of the art

In this section, published works on the determination of antibiotics amoxicillin and doxycycline in aqueous environment samples and wastewaters will be presented and discussed. Table 3.1 summarizes the studies in which the simultaneous analysis of these antibiotics, among other drugs, were performed, including the sample preparation and extraction procedures and the analytical methods, as well as the results obtained. Additional information about the methodologies developed to determine only one of these antibiotics, among other pharmaceuticals, in aqueous environment matrixes is presented in the table of the annex A2. The publications reported in the tables were organized according to the country where the real sample was collected.

Antibiotics, widely used in veterinary medicine, represent a group of emerging chemicals of environmental concern, being considered pseudo-persistent contaminants, since they are continuously introduced into the environment (Seifrtová et al. 2009). For monitoring these compounds in the aquatic environment, sensitive and selective methods are required (Hernández et al. 2007). Strategies successfully used for routine analysis of amoxicillin and doxycycline in aqueous samples currently available include a clean-up and pre-concentration step by solid-phase extraction (SPE), followed by liquid chromatography (LC) in combination with diode array detector (DAD) or mass spectrometry (MS) as detector (Buchberger 2007). Since environmental sample matrixes are complex and often contain interfering elements which can mask or interfere with the compounds of interest, direct analysis may not be possible (Pavlović et al. 2007). Therefore, clean-up and extraction techniques allow to obtain accurately clean extracts for sensitive analysis, being an important procedure previously analysis (Kostopoulou & Nikolaou 2008).

In the existing literature for trace analysis of the studied pharmaceuticals in water samples is normally suggested the sample centrifugation and filtration through < 1 μm glass-fiber filters prior to the pre-concentration technique, to remove the suspended matter and increase the extraction efficiencies (Buchberger 2011). Na_2EDTA, a strong chelating agent, was usually added in protocols for analysis of doxycycline, since it has a high tendency to complex with ions, resulting in lower extraction recoveries. Moreover, amoxicillin and doxycycline are amphoteric molecules with several ionizable functional groups (different pK_a values), being their structure and stability, and subsequently their extraction efficiency, dependent on the pH of the samples. Therefore, acidification of water samples is necessary (Fatta et al. 2007). Generally, multiresidue sample pH is adjusted within the range from 2 to 3 to obtain their acidic forms, which may significantly improve the extraction recoveries for the majority of compounds (Gros et al. 2013).

The pre-concentration and purification of the analytes prior to their analysis are also crucial, since their concentration in the environment are generally low (ng/L). Nowadays, SPE is the most popular sample clean-up and pre-concentration method (He et al. 2007), being the most used procedure for extraction the amoxicillin and doxycycline in aqueous samples. The effectiveness of SPE for each compound studied mainly depends on the sorbent type and the elution solvent. Both these parameters need to be tested in order to optimize the simultaneous extraction of different classes of antibiotics.

Nowadays, the copolymer of divinylbenzene and vinylpyrrolidone, commercialized under the trade name Oasis HLB by Waters, is one of the stationary phases most widely used to pre-concentrate the aqueous samples on different antibiotics simultaneously. In fact, Oasis HLB and mixed mode sorbents based on Oasis HLB and containing ion-exchange groups (such as Oasis MCX and Oasis WCX containing strong and weak cation exchange groups, and Oasis MAX and WAX containing strong and weak anion exchange groups) represent the state of the art for SPE of antibiotics in aqueous samples.

Grujić et al. (2009) tested the efficiency of the following SPE cartridges to extract diverse pharmaceuticals, including amoxicillin and doxycycline, from surface and groundwaters: Speedisk Octadecyl C18 and H_2O-Philic DVB, Bakerbond SDB-1, Supelclean ENVI-18, ENVI-Chrom P, ENVI-Carb, and LC-SCX, and Oasis MCX and HLB. The highest recoveries (81 - 115%) were obtained using Oasis HLB cartridges. Particularly, amoxicillin and doxycycline were extracted with recoveries of 47 and 85%, respectively. The pH value and the volume of the water sample, as well as the elution solvent (methanol) volume were optimized. The tested pH values were 3, 4.5, 6 and 7.5. For Oasis HLB sorbent, the results have shown that amoxicillin and doxycycline recoveries were the highest at pH 7.5 and 3, respectively. For elution of Oasis HLB SPE cartridge (200 mg/ 6 mL), methanol volumes of 7 – 15 mL were tested. The results have shown that for doxycycline and amoxicillin 7 mL of methanol was sufficient for their complete elution from the column. Finally, sample volume of 100 mL provided high recoveries and significantly shortens the time of the SPE procedure. It was also observed a significant loss of doxycycline (up to 60%) for sample volume of 500 mL.

Gros et al. (2013) compared the extraction recoveries of a wide range of antibiotic classes under different conditions and polymeric phases, in order to develop a procedure that enables the simultaneous analysis of the analytes in one single extraction step from urban and hospital wastewaters. The sample acidification before extraction with Oasis MCX or HLB sorbents was tested. Higher recoveries for amoxicillin (20 - 126%) and doxycycline (50 – 125%) were obtained when Oasis HLB with sample acidification at pH 2.5 was used.

Rossmann et al. (2014) tested SPE extraction efficiency of the multiclass antibiotic from wastewater with two different cartridges at pH 2.5, 3.5, and 6.5: Strata-X, a hydrophilic–hydrophobic balance polymeric cartridge, and Oasis HLB. For simultaneous extraction of amoxicillin and doxycycline, Oasis HLB sorbent at pH 3.5 provided higher recoveries (about 35 and 110%, respectively).

Kasprzyk-Hordern et al. (2007) also optimized SPE procedure for the determination of pharmaceuticals in surface water, testing the following variables: type of adsorbent, pH value of the sample, and elution conditions. Extraction efficiency of Oasis HLB, MCX, MAX, WCX, and WAX, Chromabond C18, and Isolute ENV$^+$ and HCX cartridges were studied. Oasis MCX was found to be the most efficient for the majority of studied pharmaceuticals at pH 2.5. The elution of the analytes with 1mL of methanol and 2 mL of 5% ammonium hydroxide in methanol at a rate of 1 mL/min provided higher extractions efficiencies. However, the recovery of doxycycline (26.8%) in the optimized conditions for this study was significantly lower than the obtained in studies with the sorbent Oasis HLB. In turn, this difference did not influence the extraction of amoxicillin, since the recovery of this analyte was 40.6%.

In some cases, two cartridges with different properties have been used in tandem. Locatelli et al. (2011) employed in series the anion exchange Strata-SAX and polymeric Oasis HLB SPE cartridges for extraction of antibiotics belonging to different classes from water at pH 2.4. The SAX cartridge was placed on top of the HLB cartridge for removing negatively charged compounds, while the target antibiotics which are neutral or positively charged at tested pH were retained on the HLB cartridge. This can improve further retention of the target antibiotics on Oasis HLB cartridges.

On the other hand, the elution of cartridges is usually done by organic solvents such methanol, or acetonitrile. Retained analytes in cation exchange cartridges are eluted by the mixture of methanol and ammonia according to the specific sorbent.

In general, for the optimal SPE conditions in multicomponent analysis, low recovery percentages of amoxicillin are obtained, which mainly results from hydrolysis of the ß-lactam ring during the extraction process. In recent years, molecularly imprinted polymers (MIPs) have attracted much attention due to their highly specific recognition ability for target molecules. This characteristic can be used to overcome the unsatisfactory selectivity of traditional sorbents to extract a specific analyte or a class of compounds in complex environmental samples. Therefore, MIPs for SPE have been developed and commercialized (He et al. 2007). Unfortunately, their high specificity can also be a disadvantage since these materials are not appropriate for multiclass analysis. As a result, their applications for routine monitoring programs limited and unattractive (Buchberger 2011). Yin et al. (2010) develop a selective molecularly imprinted solid-phase extraction (MISPE) protocol to be applied to the environmental monitoring of ß-lactam antibiotics in river and tap waters. In this study, the application of the pseudo-template nafcillin to make the MIP sorbent overcomes the template bleeding that occurred using the target compound as the template for the MIPs synthesis. The results suggest that the sorbent synthesized can selectively recognize the analyzed ß-lactam antibiotics (penicillin, amoxicillin, ampicillin, and mezlocillin), since the average recoveries of the analytes was in the range of 60-90%.

Other methods reported in the literature to extract doxycycline from water include hollow fiber liquid-phase microextraction (HF-LPME) and dispersive solid-phase microextraction (dispersive-SPME). Shariati et al. (2009) developed a pre-concentration method using carrier mediated HF-LPME prior to chromatography analysis for simultaneous determination of trace amounts of highly hydrophilic tetracycline antibiotics (tetracycline, oxytetracycline, and doxycycline) in different real samples. The extractions were carried out using an Accurel Q3/2 and three caprylil methyl ammonium chloride was chosen as cationic carrier. In this study, the doxycycline extraction efficiency was reduced (recovery percent of 24.8%). Tsai et al. (2009) tested different silica-based and polymeric sorbents for simultaneously pre-concentration of tetracyclines from aqueous or organic solutions by dispersive-SPME. For all types of tested sorbents, extraction efficiencies of tetracyclines were higher from the organic solvent extract than those from water. The recoveries of tetracyclines in water for all sorbents were lower than 40%. Additionally, results shew that the silica-based sorbents are the most effective than polymeric materials under both aqueous and organic environment. Under the optimal conditions, using the silica-based material PSA as a sorbent material and acetonitrile as extraction solvent, recoveries of doxycycline in surface waters range from 100.6 to 104.1%. Therefore, dispersive-SPME method can be efficiently used for the determination of doxycycline in environmental water samples.

Analytical methods based on LC have been used for sensitive and selective identification and quantification of antibiotics as environmental contaminations, being the most used. The application of ultraviolet (UV) and DAD detection were use in analysis of analytes of few antibiotic classes. Benito-Peña et al. (2006) developed a method based on SPE and high performance liquid chromatography (HPLC) with DAD for the simultaneous determination of β-lactam antibiotics in wastewater. Recovery of 78% was achieved for amoxicillin and its limit of detection (LOD) range was 4.2 – 5.9 μg/L.

Fluorescence (FL) detectors are also applied in the analysis of doxycycline. However, derivatization using toxic reagents are usually required, limiting its application. Pena el al. (2010) investigated the occurrence of tetracyclines in wastewaters by SPE followed by HPLC with FL detection. Post column derivatization with magnesium reagent was carried out. The limit of quantification (LOQ) of doxycycline was 5 μg/L.

In turn, in majority studies mass spectrometry (MS) detection is used to identify and quantify the analyte or confirm its molecular structure. MS detection involving atmospheric pressure ionization such as electrospray ionization (ESI) is nowadays state of the art. Quantitative analysis of analytes present in simple matrixes, like drinking water, can be performed using LC–MS. Nevertheless, in complex matrixes, such as wastewaters, LC–MS/MS is required for quantitation and unequivocal confirmation the identified compounds. Additional LC-MS/MS allows LODs in the range of ng/L which makes its application in the detection of antibiotics present in environmental matrixes very common. Zhou et al. (2012) extracted and determined different classes of antibiotics simultaneously in various environment waters by rapid resolution liquid chromatography - tandem mass spectrometry (RRLC–MS/MS) equipped with ESI source. The SPE of samples at pH 3 using Oasis HLB was performed prior to their analysis. The recovery of doxycycline was 76 - 146% and its LODs range was 1.28 – 18.5 ng/L. Although amoxicillin has not been analyzed, other antibiotics belonging to the class of β-lactam antibiotics as cloxacillin typically had recoveries < 50%. LODs were in the range of 1.71 – 18.4 ng/L.

Nowadays, more sophisticated mass analyzers than single-quadrupole instruments (e.g. triple quadrupole (QqQ)) are frequently employed in multiresidue analysis, since they offer higher sensitivity and selectivity (Buchberger 2007; Hernández et al. 2007). Grujić et al. (2009) determined amoxicillin and doxycycline, among other antibiotics, in environmental waters by LC coupled to a QqQ-MS/MS with ESI source. Amoxicillin and doxycycline LODs were 0.78 and 8.06 ng/L, respectively.

Capillary electrophoresis (CE) and micellar electrokinetic chromatography (MEKC) has also been reported for the determination of amoxicillin in environmental samples. Bailón-Pérezet al. (2008) developed and validated a CE-DAD method for trace determination of β-lactam antibiotics in environmental water with previous SPE sample clean-up and pre-concentration using Oasis HLB cartridge without pH adjustment. The amoxicillin recovery ranged between 94 and 97% and the analyte LOD was 0.80 μg/L. Serrano et al. (2007) quantified the β-lactam antibiotics in environmental waters by MEKC coupled to laser induced fluorescence (LIF) detection and a SPE pre-concentration step using a laboratory made weakly basic exchange column at pH 5.5. The amoxicillin recovery and its LOD were 96.4% and 45 ng/L, respectively.

Mostly, C18 and C8 analytical columns were used for the separation of analytes. In multiresidue studies reported gradient elution of mixtures of water/acetonitrile or water/methanol were regularly used. Volatile additives such as formic acid, acetic acid, and ammonium acetate at different concentration were used to modify mobile phase in order to improve the ionization of analytes and sensitivity of MS detection in the analysis of antibiotics, as well as to control pH. Lindberg et al. (2004) developed and validated method for determination of different antibiotic classes in wastewater based on LC using C18 reversed phase analytical column coupled to a MS detector with ESI source. A gradient elution program was performed to elute the antibiotics using acetonitrile and water, both with 0.01% of formic acid, as mobile phase. Prior to analysis, sample at pH 3 was clean-up and pre-concentrated by SPE using C2/ENV^{+} cartridge. 54% of amoxicillin and 55% of doxycycline were recovered. LOQs of these analytes were 0.37 and 0.64 ng/L, respectively.

To avoid time and labor intensive pre-concentration procedures, direct injections methods for multiresidue analysis have been published. Teixeira et al. (2008) used direct injection of wastewater samples by HPLC-DAD for a multiresidue analysis of different antibiotic classes. The LOD of amoxicillin was 14 µg/L. Vosough et al. (2015) direct analyzed amoxicillin, among other antibiotics, in wastewater by a HPLC-DAD method. The amoxicillin LOD was 1.3 µg/L.

Aforementioned approaches in antibiotics analysis enable a realistic evaluation of their occurrence and fate in the environment. An increasing number of studies on the presence of antibiotics in several aqueous environmental matrixes from different countries has recently been published. The concentration range at which amoxicillin has been detected in wastewater (30 – 6,940 ng/L) was higher than in surface water (4 – 1,284 ng/L), being also found in sea water at 0.74 – 76 ng/L. According to the matrix, the concentration at which doxycycline has been detected also varies. The occurrence of this antibiotic in wastewater at 10 – 685,600 ng/L was reported, whereas in surface water it was found at 5.61 – 400 ng/L. It is verified that amoxicillin and doxycycline can be found in wastewater at high concentrations. However, this contamination results primarily from the use of antibiotics in human medicine.

In conclusion, antibiotics have mainly been detected in wastewater and surface water. Nevertheless, their diverse physicochemical properties and low concentrations (ng/L) in the environment, and the complexity of the matrixes make their simultaneous determination difficult and challenging. The optimization of multiclass antibiotic analysis of environmental water samples generally requires a compromise in the selection of experimental conditions. Consequently, the best performance for each compound may not be obtained. However, the modern clean-up and extraction procedures couple to the recent advances in the analytical have provided accurately clean extracts for sensitive and specific analysis. The pH control of the sample and the mobile phase are important considerations for maximizing the SPE retention of the antibiotics and the resolution of LC analysis, respectively.

Table 3.1. Methods for simultaneous extraction and analysis of amoxicillin and doxycycline, among other drugs, in aqueous environmental matrixes.

Location	Matrix / Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
Europe								
Germany	Wastewater AMOX, DOX, and other antibiotics (different classes)	AMOX = 1,270 DOX = 2,393	V = 500 mL Na_2EDTA addition Centrifugation Filtration pH 3.5 (CH_2O_2)	**SPE** ***Sorbent:*** Oasis HLB (30 mg) ***Condition:*** 1 mL $CH_4O/H_2O/CH_2O_2$, 90:9:1 (v/v/v) 1 mL deionized H_2O 1 mL 1 M Na_2EDTA ***Washing:*** 1 mL deionized H_2O ***Elution:*** 0.5 mL $CH_4O/H_2O/CH_2O_2$, 90:9:1 (v/v/v)	AMOX = 35 DOX = 110	**LC-ESI-MS/MS** ***Stationary phase:*** Nucleoshell HILIC (100 x 3.0 mm, 2.7 µm) ***Mobile phase:*** **Gradient elution** (1) CH_3CN/2 mM $C_2H_7NO_2/CH_2O_2$, 3:97:0.05 (v/v/v) (2) CH_3CN/2 mM $C_2H_7NO_2/CH_2O_2$, 95:5:0.05 (v/v/v)	LOD_{AMOX} = 2.8 LOQ_{AMOX} = 9.3 LOD_{DOX} = 8.8 LOQ_{DOX} = 29.2	(Rossmann et al. 2014)
Poland	Surface water AMOX, DOX, and other pharmaceuticals	AMOX < LOQ DOX = n.p.	V = 1,000 mL pH 2.5 (HCl) Filtration Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis MCX (60 mg) ***Condition:*** 2 mL CH_4O 2 mL 2% CH_2O_2 (pH 2.1) ***Washing:*** 2 mL 2% CH_2O_2 (pH 2.1) ***Elution:*** 1 mL CH_4O 2 mL 5% NH_4OH in CH_4O	AMOX = 40.6 DOX = 26.8	**UPLC-ESI- QqQ-MS/MS** ***Stationary phase:*** Acquity UPLC BEH C18 (100 x 1 mm, 1.7 µm) ***Mobile phase:*** **Gradient elution** (1) $H_2O/CH_4O/C_2H_4O_2$, 94.5:5:0.5% (v/v/v) (pH 2.8) (2) $CH_4O/C_2H_4O_2$, 99.5:0.5% (v/v/v) (pH 3.2)	LOD_{AMOX} = 2.5 LOQ_{AMOX} = 10 LOD_{DOX} = n.p. LOQ_{DOX} = n.p.	(Kasprzyk-Hordern et al. 2007)
Serbia	Wastewater Surface water Ground water AMOX, DOX, and other pharmaceuticals	AMOX = n.d. DOX = n.d. AMOX = n.d. DOX = n.d. AMOX = n.d. DOX = n.d.	V = 100 mL pH 3 ($C_2H_4O_2$) Filtration	**SPE** ***Sorbent:*** Oasis HLB (30 mg) ***Condition:*** 5 mL CH_4O 5 mL deionized H_2O (pH 3) ***Washing:*** n.a. ***Elution:*** 15 mL CH_4O	AMOX = 17 – 22 DOX = 98 - 118	**LC-ESI-QIT- MS/MS** ***Stationary phase:*** Zorbax XDB C18 (75 x 4.6 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O (2) CH_4O (3) 10% $C_2H_4O_2$	LOD_{AMOX} = 0.79 LOD_{AMOX} = 2.63 LOD_{DOX} = 8.06 LOD_{DOX} = 16.88	(Grujić et al. 2009)
Spain	Wastewater Surface water AMOX, DOX, and other antibiotics (different classes)	AMOX = 216 - 258 DOX = n.d. AMOX = 100 - 175 DOX = n.p.	V = 25 - 50 mL Filtration Na_2EDTA addition pH 2.5 (HCl)	**SPE** ***Sorbent:*** Oasis HLB (60 mg) ***Condition:*** 5 mL ultrapure H_2O (pH 2.5, HCl) ***Washing:*** 6 mL ultrapure H_2O ***Elution:*** 6 mL CH_4O	AMOX = 20 – 126 DOX = 50 - 125	**UPLC-ESI-QqQ-MS/MS** ***Stationary phase:*** Acquity HSS T3 (50 x 2.1 mm, 1.8 µm) ***Mobile phase:*** **Gradient elution** (1) ultrapure H_2O with 0.1% CH_2O_2 (2) CH_3CN	LOD_{AMOX} = 1.23 – 9.49 LOQ_{AMOX} = 4.39 – 31.63 LOD_{DOX} = 11.23 – 77.49 LOQ_{DOX} = 37.43 – 258.29	(Gros et al. 2013)

Table 3.1. Methods for simultaneous extraction and analysis of amoxicillin and doxycycline, among other drugs, in aqueous environmental matrixes (*continuation*)**.**

Location	Matrix / Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
Europe								
Sweden	Wastewater AMOX, DOX, and other antibiotics (different classes)	AMOX = n.p. DOX = 1,000 – 6,500	V = 200 - 500 mL Filtration pH 3 (H_2SO_4)	**SPE** ***Sorbent:*** C2/ENV+ (1 g) ***Condition:*** 5 mL CH_4O 5 mL 50% CH_4O 5 mL H_2O (pH 3) ***Washing:*** 6 mL H_2O (pH 3) ***Elution:*** 5 mL 5% TEA in CH_4O	AMOX = 54 DOX = 55	**LC-ESI-IT-MS/MS** ***Stationary phase:*** YMC Hydrosphere RP C18 (150 x 4.6 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) ultrapure H_2O with 0.1% CH_2O_2 (2) CH_3CN with 0.1% CH_2O_2	LOQ_{AMOX} = 0.37 LOQ_{DOX} = 0.54	(Lindberg et al. 2004)
UK	Surface water AMOX, DOX, and other pharmaceuticals	AMOX = 39 – 245 DOX = n.p.	V = 1,000 mL pH 2.5 (HCl) Filtration Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis MCX (60 mg) ***Condition:*** 2 mL CH_4O 2 mL 2% CH_2O_2 (pH 2.1) ***Washing:*** 2 mL 2% CH_2O_2 (pH 2.1) ***Elution:*** 1 mL CH_4O 2 mL 5% NH_4OH in CH_4O	AMOX = 40.6 DOX = 26.8	**UPLC-ESI- QqQ-MS/MS** ***Stationary phase:*** Acquity UPLC BEH C18 (100 x 1 mm, 1.7 µm) ***Mobile phase:*** **Gradient elution** (1) $H_2O/CH_4O/C_2H_4O_2$, 94.5:5:0.5% (v/v/v) (pH 2.8) (2) $CH_4O/C_2H_4O_2$, 99.5:0.5% (v/v/v) (pH 3.2)	LOD_{AMOX} = 2.5 LOQ_{AMOX} = 10 LOD_{DOX} = n.p. LOQ_{DOX} = n.p.	(Kasprzyk-Hordern et al. 2007)
Oceania								
Australia	Wastewater AMOX, DOX, and other antibiotics (different classes)	AMOX = 30 - 280 * DOX = 20 - 65 *	V = 200 mL Filtration pH 3 (H_2SO_4)	**SPE** ***Sorbent:*** Oasis HLB (60 mg) ***Condition:*** n.a. ***Washing:*** 3 mL CH_4O ***Elution:*** 2x 2 mL CH_4O	AMOX = n.p. DOX = n.p.	**LC-ESI- QqQ-MS/MS** ***Stationary phase:*** Synergi Hydro RP (50 x 2 mm, 4 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_4O	LOD_{AMOX} = 14,000 LOD_{DOX} = 4,000	(Watkinson et al. 2007)
	Wastewater Surface water Drinking water AMOX, DOX, and other antibiotics (different classes)	AMOX = 50 – 6,940 * DOX = 150 - 650 * AMOX = 200 DOX = 400 AMOX = n.d. DOX = n.d.	V = 200 – 1,000 mL Filtration pH 3 (H_2SO_4) Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (200 or 500 mg) ***Condition:*** n.a. ***Washing:*** 3 mL CH_4O ***Elution:*** 2x 2 mL CH_4O	AMOX = 29 DOX = 76	**LC-ESI- QqQ-MS/MS** ***Stationary phase:*** Synergi Hydro RP (50 x 2 mm, 4 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_4O	LOD_{AMOX} = 20,000 LOD_{DOX} = 5,000	(Watkinson et al. 2009)

* Range of maximum concentrations detected.

n.a. - not applicable; n.d. - not detected; n.p. – information not provided.

Abbreviations: AMOX – amoxicillin; DOX – doxycycline; ESI - electrospray ionization; IT – ion trap; LC- liquid chromatography; LOD – limit of detection; LOQ – limit of quantification; MS – mass spectrometry; QIT - quadrupole ion trap; QqQ – triple quadruple; TEA – trimethylamine; UPLC – ultra high performance liquid chromatography.

Molecular formulas: $C_2H_4O_2$ - acetic acid; CH_3CN – acetonitrile; $C_2H_7NO_2$ - ammonium acetate; NH_4OH - ammonium hydroxide; HCl - chloridric acid; Na_2EDTA - ethylenediaminetetraacetic acid disodium salt; CH_2O_2 – formic acid; CH_4O – methanol; H_2SO_4 - sulfuric acid; H_2O – water

4 Materials and methods

4.1 Reagents and materials

Analytical grade reference standards of amoxicillin (purity, > 900 µg/mg) and doxycycline (purity, 98%) were supplied from Sigma–Aldrich (Steinheim, Germany). Acetonitrile and methanol were of HPLC grade. These solvents were obtained from VWR (Fontenay-sous-Bois, France). The water used as mobile phase was double-distilled, and filtered through 0.45 µm nylon membrane filters obtained from Supelco, Sigma–Aldrich (Pennsylvania, USA). Ultrapure water was prepared with an Elix Essential Water Purification System (3 L/h) from Merck Millipore (Beeston, United Kingdom). *Ortho*-phosphoric acid (purity, 85%) was obtained from VWR (Fontenay-sous-Bois, France). Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA) was purchased from Merck (Darmstadt, Germany).

For solid-phase extraction (SPE), Oasis HLB (200 mg, 6 cm^3) and Oasis WCX (200 mg, 6 cm^3) cartridges were sourced from Waters Corporation (Dublin, Ireland). Chromafix Dry (Na_2SO_4) sorbents for drying organic samples were supplied by Macherey-Nagel (Düren, Germany). Glass fiber filter (1 µm) equipped with a GxF prefilter were obtained from Pall Life Sciences (Portsmouth, United Kingdom).

4.2 Stock and standard solutions stability

The evaluation of the influence of the solvent, storage temperature, and storage time on the stability of the antibiotics in the stock solution was carried out as described hereinafter. Different individual stock solutions of the amoxicillin and doxycycline were prepared on weight basis to a final concentration of about 100 mg/L. After stabilizing overnight at 4 ºC, each solution was divided in 10 mL aliquots, which were transferred into amber glass vials and storage at different temperatures (details in table 4.1). The stability were evaluated weekly and during a mouth by preparing fresh individual standard solutions at one level of concentration (200 µg/L) in ultrapure water from storage stock solutions, considering 0% of degradation.

Table 4.1. Information about individual stock solutions of the amoxicillin and doxycycline prepared to evaluate the influence of the solvent, storage temperature, and storage time on the stability of the antibiotics.

Antibiotic	Stock solution		
	Solvent	Concentration (mg/L)	Storage temperatures (ºC)
Amoxicillin	Ultrapure water	109	-20 and 4
	3% methanol	109	-20 and 4
	Methanol	108	-20 and 4
Doxycycline	3% methanol	120	-20 and 4
	Methanol	105	-20 and 4

The stability of mixture standard solutions prepared from stock solutions of amoxicillin in ultrapure water (109 mg/L) and doxycycline in 3% of methanol (120 mg/L) was also analyzed. Individual stock solutions were stabilized overnight at 4 ºC prior to use. An appropriate volume of these solutions was diluted in ultrapure water to yield mixture standard solutions with three levels of concentration (40, 200 and 400 µg/L). After stabilizing 2 hours at 4 ºC, each solution was divided in 2 mL aliquots, which were

transferred into twelve amber glass vials, six of them were storage at -20 ºC and the other ones at 4 ºC. During a week, the stability of the antibiotics in the storage solutions were daily evaluated.

4.3 Analytical method

4.3.1 Instrumentation

Chromatographic analyses were performed using a Merck Hitachi system (Tokyo, Japan) consisting of a L-7100 pump (Merck Hitachi), Rheodyne injector (Rohnert Park, USA) Model 7725 (100 µL loop), and a photo diode-array detector L-7450 A (Merck Hitachi). Chromatographic separation was carried out on an end-capped RP-18 column (250 x 4 mm, 5 µm of particle size) and a guard column Purospher RP-18e (4 x 4 mm) supplied by Merck (Darmstadt, Germany). The mobile phase constituents, (A) water adjusted with phosphoric acid (pH 2) and (B) acetonitrile, were used in a gradient elution program as follows; initial gradient conditions were set at 10% B and held for 10 minutes, then increased from 10 to 50% B during 5 minutes, and kept for 5 minutes. The mobile phase finally returned to the initial composition in 1 minute and the analytical column was allowed to get to the equilibrium before the next run. Total run time was 25 minutes. Injection volume was 100 µL and all separations were performed at room temperature. Mobile phase flow rate was 0.8 mL/min. The identification of antibiotics was performed by comparison of standards, concerning LC retention time and UV spectra of each analyte. The UV absorption of analytes were monitored over a range of wavelengths comprised between 220 and 400 nm. However, detection was carried out at the following wavelengths: 230 nm for amoxicillin and 360 nm for doxycycline. The data were collected and processed using HSM D-7000 software package (version 3.1) for HPLC. Quantification was carried out using external calibration.

4.3.2 Analytical method validation

Analytical method validation was evaluated in terms of linearity, repeatability (intraday precision), intermediate precision (interday precision), and accuracy.

Mixed working standard solutions at different concentrations were daily prepared by appropriate dilution with ultrapure water of the stock solution of amoxicillin in ultrapure water (109 mg/L) and doxycycline in 3% of methanol (120 mg/L) and doxycycline in 3% methanol, both storage at -20 ºC.

The linearity was investigated by an eleven points calibration curve (33 – 436 µg/L for amoxicillin and 36 – 479 µg/L for doxycycline). For each analyte, the limits of detection (LODs) and the limits of quantification (LOQs) were determined from an average (n = 11) signal-to-noise ratio (S/N) of 3 and 10, respectively. Repeatability was defined as the relative standard deviation (RSD, %) of a six-fold analysis of standard solutions at three different concentrations (n = 6). Intermediate precision was determined as the RSD from duplicate injections of analytical standards at three levels of concentration on three consecutive days (n = 6). Furthermore, the accuracy was determined by the standard addition method of six tests (n = 6) at three levels of concentration. Tap water from the laboratory was spiked with a known concentration and the recovery was determined by comparing the instrumental response with the expected response, considering the blank, a non-spiked sample.

4.4 Solid-phase extraction methodology

In this work, preliminary solid-phase extraction assays were performed to develop a method for simultaneous extraction of amoxicillin and doxycycline.

Preliminary experiments were carried out for distilled water spiked with pharmaceuticals to verify the recoveries of pharmaceuticals. Before extraction, 100 mL of distilled water were fortified with 1 mL of mixture standard solutions (prepared as described in section 4.3.2) to produce analytes concentration of 4.8, 1.0, and 0.5 µg/L. Then, 1 mL of an 800 mg/L Na_2EDTA solution was added per 100 mL of sample to prevent doxycycline complexing with Ca^{2+} and Mg^{+} ion and residual metals on the SPE cartridges.

Different operating conditions were tested to evaluate which of them yielded higher recoveries of target antibiotics. The preliminary experiments variables compared were type of adsorbent, pH value of the sample, washing step omission, and elution conditions. The cartridges used were lipophilic/hydrophilic balanced Oasis HLB and the reversed phase weak cationic exchange sorbent Oasis WCX. Three different sample pH were evaluated: one with no sample pH adjustment and the other ones by adjusting the sample pH at 2 or 3 using *ortho*-phosphoric acid, prior to extraction. Moreover, the analytes elution with methanol or acetonitrile were compared. Consequently, three different SPE procedures using Oais HLB or WCX as sorbents were tested. This methodologies are summarized in annex A3. In method 1, the cartridges were conditioned with 5 mL of methanol and equilibrated with 5 mL of ultrapure water at sample pH. After passage of the samples at flow rate of 1 drops per second, the cartridges were washed with 5 mL of ultrapure water and dried with air for 30 minutes. Elution were performed with 6 mL of methanol at 1 drop per second. After elution, cartridges were dried with air for 30 minutes, to remove excess of water. In method 2, extraction was carried out as in method 2, with the exception of the omission of the washing step after sample loading. Method 3 differs from the method 2 in the elution with 6 mL of acetonitrile instead of methanol. In all procedures, the water from extracts were removed using a Na_2SO_4 sorbents. Then, extracts were filtered through 1 µm glass fiber filters and then evaporated to dryness under a gentle nitrogen stream. Immediately prior to analysis by to HPLC-DAD as described in section 4.3, dryness extracts were reconstituted with 1 mL of 3% methanol.

The percentage of recoveries of amoxicillin and doxycycline in each experiment were calculated considering a blank (nonspiked) sample and the known added amount of analytes.

4.5 Storage, fate and treatment of wastes

Liquid wastes generated consisted on organic solutions containing acetonitrile and traces amounts of antibiotics, methanol, *ortho*-phosphoric acid and Na_2EDTA. Solid wastes comprised Oais HLB and WCX cartridges, Na_2SO_4 sorbents, and glass fiber filters. The sorbents housing of Oais HLB and WCX cartridges were reused for MIP studies in the research group, while the other wastes were collected in closed containers properly labeled and stored in protected locations of light and ignition sources for further treatment by *Sistema de Gestão Ambiental da FEUP* (EcoFEUP).

5 Results and discussion

5.1 Standard solutions storage and stability

Amoxicillin and doxycycline exhibit general poor stability, being subjected to extensive degradation under several conditions. Therefore, assess to the stability of analytes in the stock and standard solutions during their storage is important to conduct analytical studies. In this work, the stability of target compounds in different solvents was investigated under different storage conditions. Individual stock solutions of analytes were prepared in 3% of methanol or methanol and stored, protected from light, at 4 ºC or -20 ºC, being their stability evaluated weekly over a one month period. The stability of amoxicillin in a stock solution prepared in ultrapure and stored under the same mentioned conditions was also evaluated. The degradation extent of antibiotics was analyzed by comparing the peak areas of the analytes obtained during the analysis period with the initial peak area. The analyzed samples were prepared previous chromatography analysis by dilution of stock solutions in ultrapure water (details in section 4.2). The results are displayed in figures 5.1 and 5.2. In this study, amoxicillin was not detected in the stock solutions prepared in methanol.

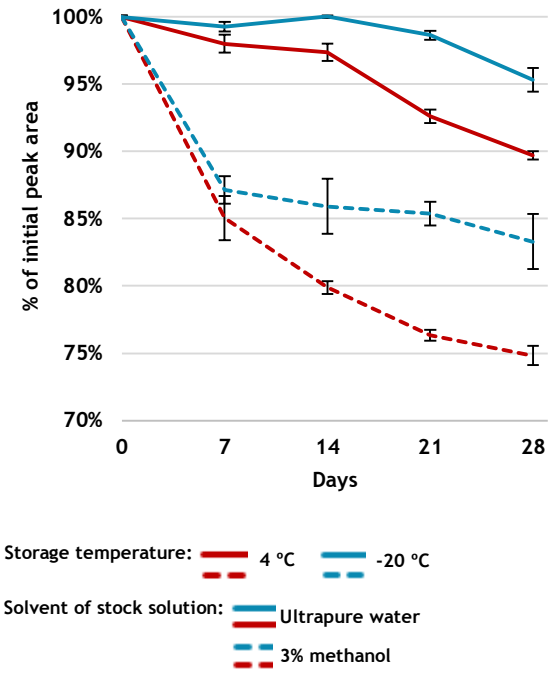


Figure 5.1. Influence of storage conditions on amoxicillin stability in stock solutions prepared in ultrapure water or 3% of methanol (the analyzed solutions (200 µg/L) were prepared from the stock solution considering 0% degradation; values are the mean of two chromatography analysis, n = 2).

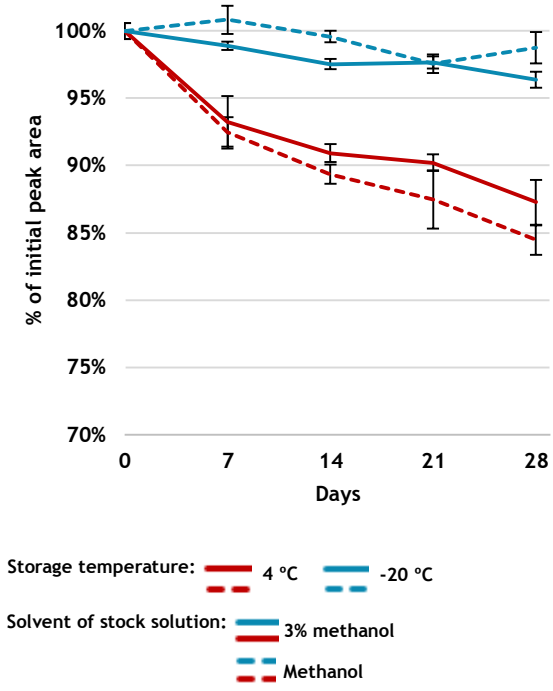


Figure 5.2. Influence of storage conditions on doxycycline stability in stock solutions prepared in 3% of methanol or methanol (the analyzed solutions (200 µg/L) were prepared from the stock solution considering 0% degradation; values are the mean of two chromatography analysis, n = 2).

Considering the tested conditions, it can be verified by analysis of figures 5.1 and 5.2 that the stability of amoxicillin stock solutions strongly depended on storage temperature and solvent, whereas the degradation of doxycycline in stock solutions was mainly influenced by storage temperature.

Different degradation kinetics were observed at 4 ºC and at -20 ºC, being the rates of degradation reactions of analytes higher at the highest temperature, regardless of the stock solution solvent. Amoxicillin and doxycycline were not stable at 4 ºC over the 7-day period (degradation > 10%), except amoxicillin in ultrapure water. Stability was greatly increased when stock solutions were stored at −20 ºC. The variation of the reaction rate with temperature and activation energy can be described by the Arrhenius' equation (equation 1):

$$k = Ae^{\frac{-E_a}{RT}} \qquad (1)$$

where, k is the rate constant of a chemical reaction, A is the pre-exponential factor, E_a is the activation energy, R is the universal gas constant, and T is the temperature (Atkins & Jones 2006).

Analyzing the Arrhenius' equation, it is concluded that reactions with higher activation energies are more sensitive to temperature variations. Through the slope of the graphs for each solvent, it appears that the stability of doxycycline is more temperature dependent than that of the amoxicillin. Therefore, it can be inferred that the activation energies of degradation reactions of doxycycline are higher than that of the amoxicillin. Nevertheless, further studies, particularly kinetic studies, which are beyond the scope of this work, are required to confirm this conclusion.

The degradation products of doxycycline identified in studies about thermostability of this antibiotic include its epimer 6-epidoxycycline (figure 5.3) and methacycline (figure 5.4) (Skúlason et al. 2003; Injac et al. 2007).

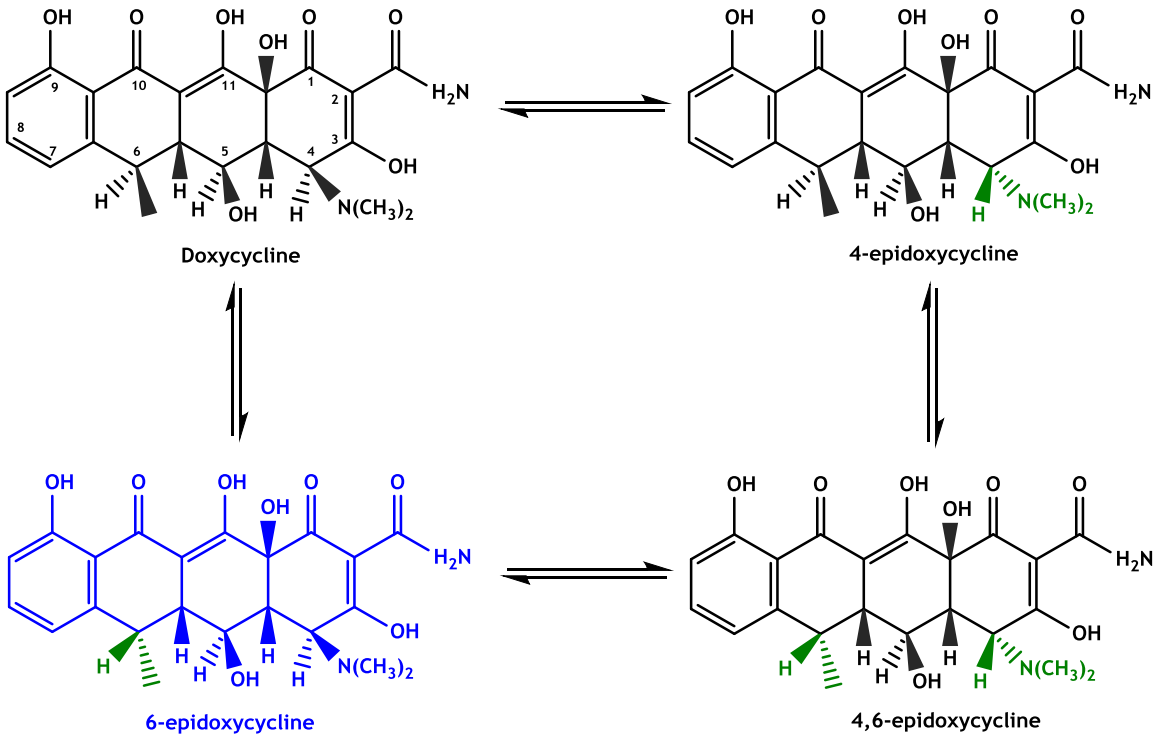


Figure 5.3. Doxycycline and its epimers.

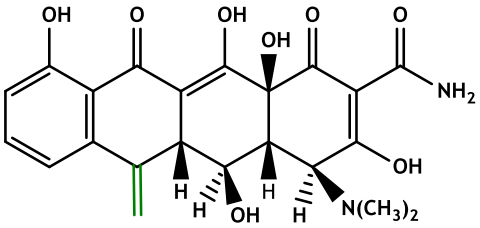


Figure 5.4. Chemical structure of methacycline, a common degradation product of doxycycline.

On the other hand, contrary to doxycycline, it was proved that amoxicillin react with alcohols to form more stable degradation products (Bruno et al. 2001; Deshpande et al. 2004). Regardless temperature, for stock solutions of amoxicillin prepared in 3% of methanol, after 7 days about 15% of the compound had been degraded, 6.5 times more than the degradation of the analyte in the solution prepared in ultrapure water. This might be due to methanol can act as a nucleophile in the nucleophilic substitution reaction, degrading the compound. The carbons susceptible to nucleophilic attack are indicated in blue in the figure 5.5. However, taking in account that as a penicillin, amoxicillin is characterized by the unstable, highly strained, and reactive ß-lactam amide bond, the carbon of the amide group in the ß-lactam ring are more likely to be attacked by the oxygen (electron donator) of methanol. Therefore, the four-membered ß-lactam ring can opens by methanol nucleophilic attack, yielding the product amoxicillin methyl ester. This reaction mechanism is shown in figure 5.6.

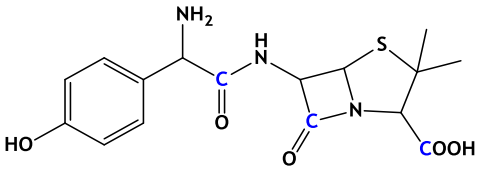


Figure 5.5. Indication of carbons susceptible to nucleophilic attack by methanol in the chemical structure of amoxicillin.

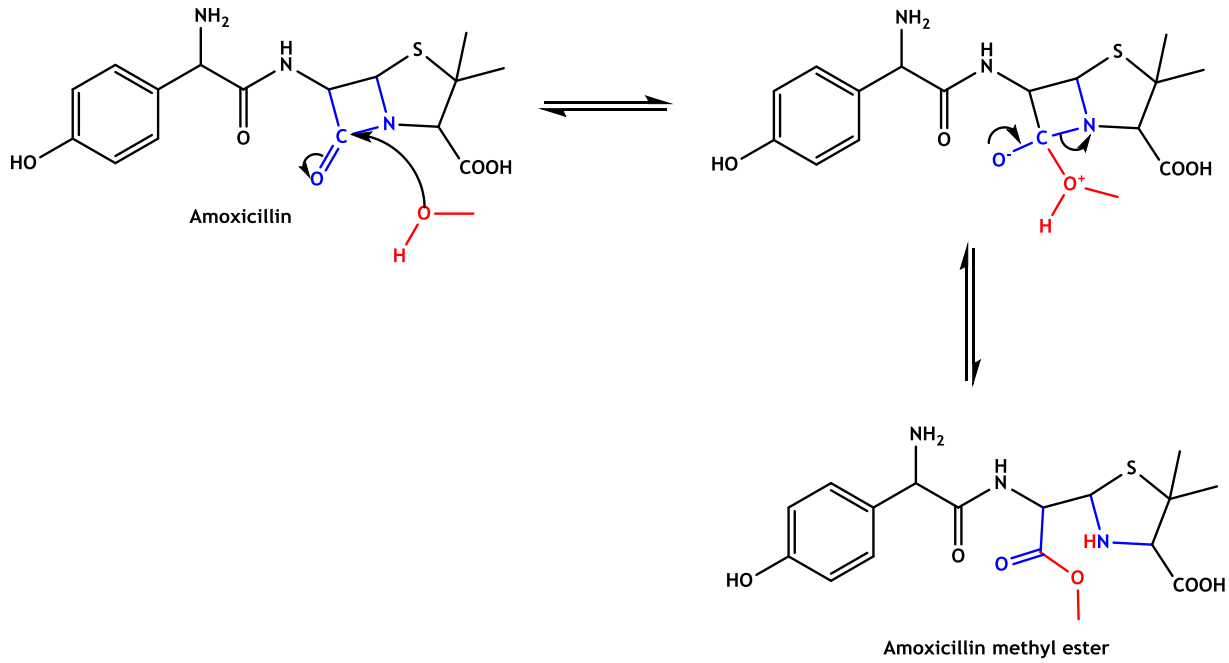


Figure 5.6. Amoxicillin ring opening by nucleophilic substitution reaction.

Amoxicillin methyl ester contains a free ester group and an amine group, which give a lower polarity to this molecule comparatively the amide group of the parental compound. This leads to a shift towards a later retention time in the reverse phase liquid chromatography separation, as presented in figure 5.7. However, it should be notice that to verify the identity of these compounds, further chromatographic analysis by LC-MS are required.

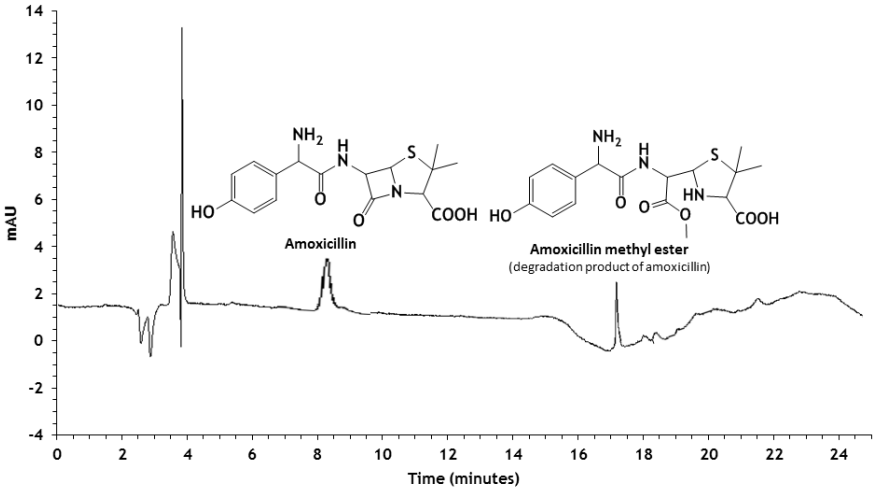


Figure 5.7. Chromatogram of the amoxicillin solution (200 µg/L) prepared from the analyte stock solution in 3% methanol stored at -20 °C during 7 days.

The nucleophilic substitution reaction schematized in figure 5.6 is an equilibrium reaction, so that applying the Le Chatlier's Principle, when methanol is in excess in the reaction medium, the equilibrium shifts toward the formation of products. Accordingly, when the amoxicillin stock solution was prepared in methanol, it was only detected the degradation product during the monitoring time at 4 °C or -20 °C (figure 5.8). Amoxicillin methyl ester for amoxicillin solution prepared in 3% of methanol was only detected after 7 days as a result of the reaction quotient at time 0 be higher than for the solution prepared in 100% of methanol.

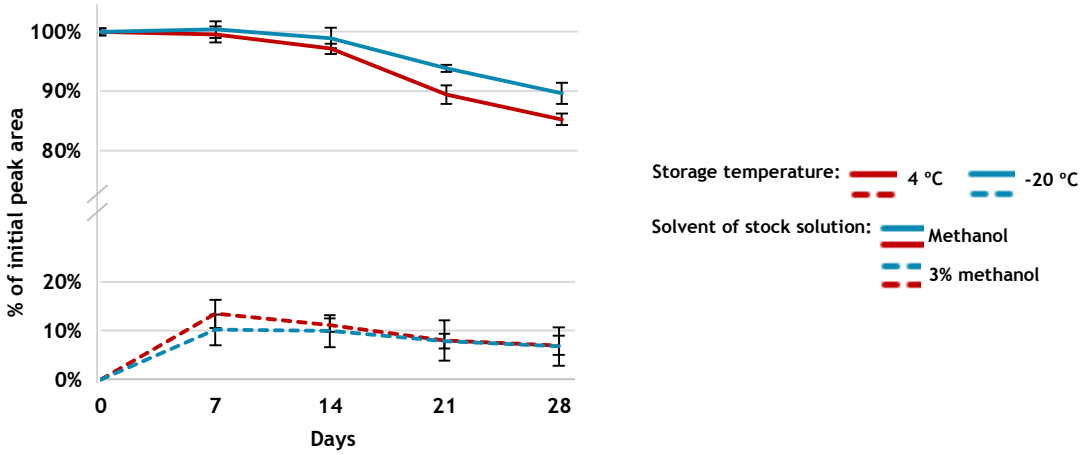


Figure 5.8. Monitoring amoxicillin degradation products in stock solutions prepared in 3% of methanol or methanol and stored at 4 °C or -20 °C (the analyzed solutions (200 µg/L) were prepared from the stock solution considering 0% degradation; values are the mean of two chromatography analysis, n = 2).

Analyzing the figure 5.8, it was found that the product of the methanol nucleophilic attack reaction in methanol solvent was also unstable, since the initial concentration of the compound decreased about 10% after one month of storage, regardless the temperature. Thus, it is concluded that amoxicillin methyl ester may be an intermediate for formation of other degradation products more stable in the tested conditions. Other pathways of amoxicillin degradation described in the literature include dimerization and hydrolysis depending on the pH (Wood 1986; Crea et al. 2012) (see annex A1.3). It should be noted that the solutions were prepared at pH 6 - 6.5, being expected that degradation of amoxicillin due to extreme pH were minimal.

Given the above results, along this work, the stock solution of amoxicillin and doxycycline were monthly prepared in ultrapure water and in 3% methanol, and stored at -20 ºC. Although it was found that methanol does not affect the stability of doxycycline, it was decided to prepare the stock solution of the compound in 3% methanol, in order to the amount of methanol in the mixture standard solutions be residual and, consequently, the degradation of amoxicillin be reduced.

Additionally, during a week, it was daily examined the stability of mixture standard solutions at three levels of concentration stored at 4 ºC or -20 ºC. The results displayed in figures 5.9 and 5.10 show that there was not a correlation between the stability of the analytes and their concentration in solution. Due to the test period be relatively short, the influence of temperature on the stability of amoxicillin and doxycycline was not as evidenced as described above. In turn, it was again demonstrated that amoxicillin was very reactive with methanol. Although methanol was present in solutions at trace amounts, amoxicillin was significantly degraded (> 10%) after 5 days. For more concentrated solutions of analytes (200 and 400 µg/L), the degradation product amoxicillin methyl ester was detected (figure 5.11)

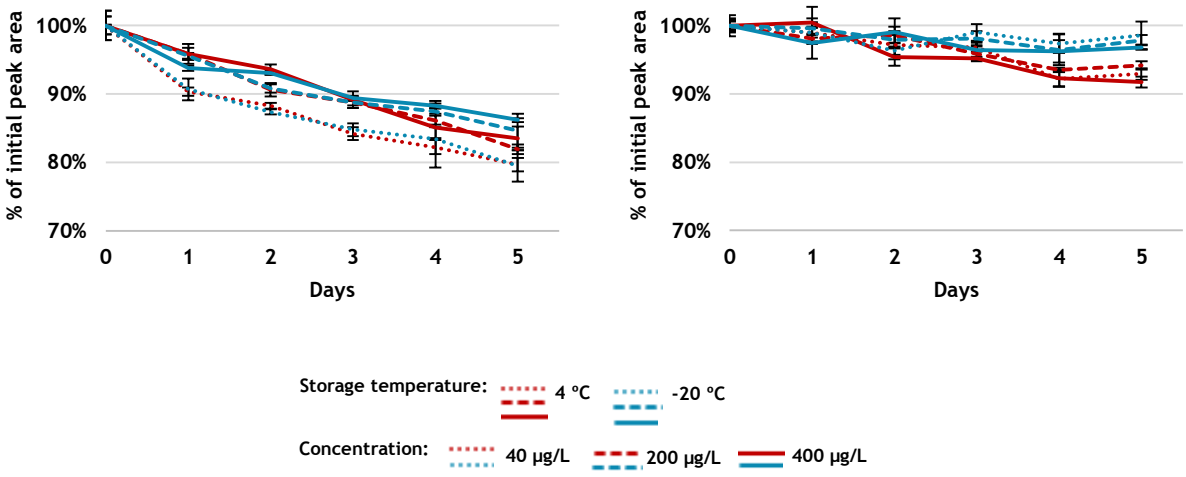


Figure 5.9. Influence of storage temperature (4 ºC and -20 ºC) on amoxicillin stability in mixed standard solutions (tested concentrations were 40, 200, and 400 µg/L; values are the mean of two chromatography analysis, n = 2).

Figure 5.10. Influence of storage temperature (4 ºC and -20 ºC) on doxycycline stability in mixed standard solutions (tested concentrations were 40, 200, and 400 µg/L; values are the mean of two chromatography analysis, n = 2).

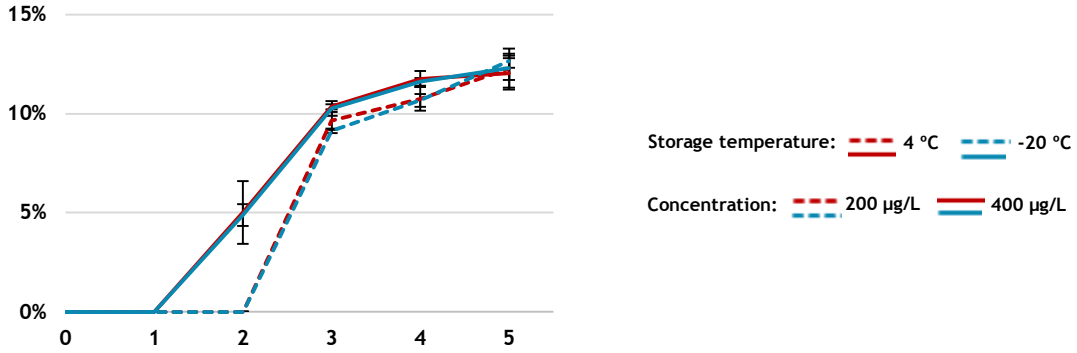


Figure 5.11. Monitoring amoxicillin degradation products in mixed standard solutions stock solutions stored at 4 °C or -20 °C (tested concentrations were 200 and 400 µg/L; values are the mean of two chromatography analysis, n = 2).

Considering the results presented, it was decided to prepare standard solutions daily and to discharge them after a day of use.

5.2 Validation of analytical method

The first main goal of this work was to develop, optimize, and validate an analytical methodology using high performance liquid chromatography coupled to diode array detector (HPLC-DAD) for a simultaneous, specific, and sensitive determination of amoxicillin and doxycycline in aqueous samples.

In a previous study conducted in the research group, an optimized analytical method to detect and quantify the antibiotic amoxicillin, among other pharmaceuticals, through the equipment referred in section 4.3 was developed and validated (Teixeira et al. 2008). Under optimal operational condition, the retention time of the amoxicillin was 5.4 minutes. On the other hand, doxycycline was separated from other tetracyclines with a retention time of 10.5 minutes under analytical procedure developed and optimized by Pena et al. (Pena et al. 2010). These studies were considered in the presented work for analytical method development and optimization.

Amoxicillin and doxycycline were identified by direct injection of individual analytes standard solutions, evaluating their retention time. Figures 5.12 and 5.13 show the chromatograms of a mixed standard solution of amoxicillin (436 µg/L) and doxycycline (479 µg/L) monitoring at 230 and 360 nm, respectively.

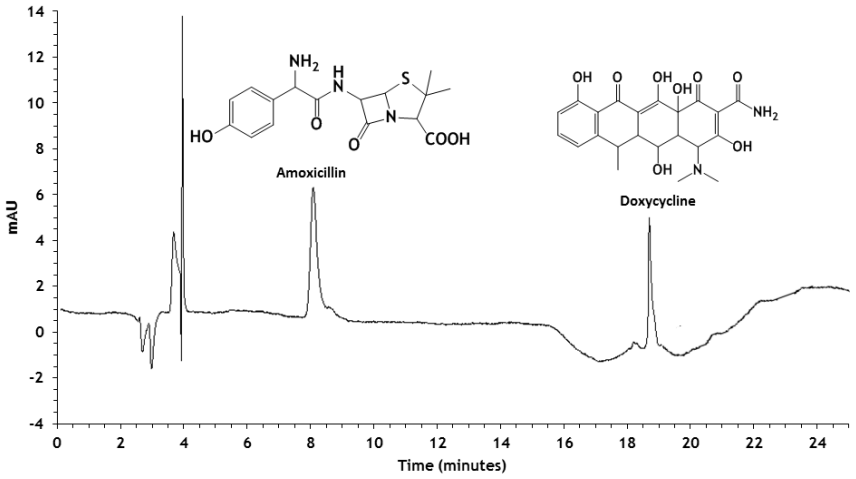


Figure 5.12. HPLC-DAD chromatogram of a mixed standard solution with a concentration of 436 µg/L for amoxicillin and 479 µg/L for doxycycline. Monitoring was performed at 230 nm.

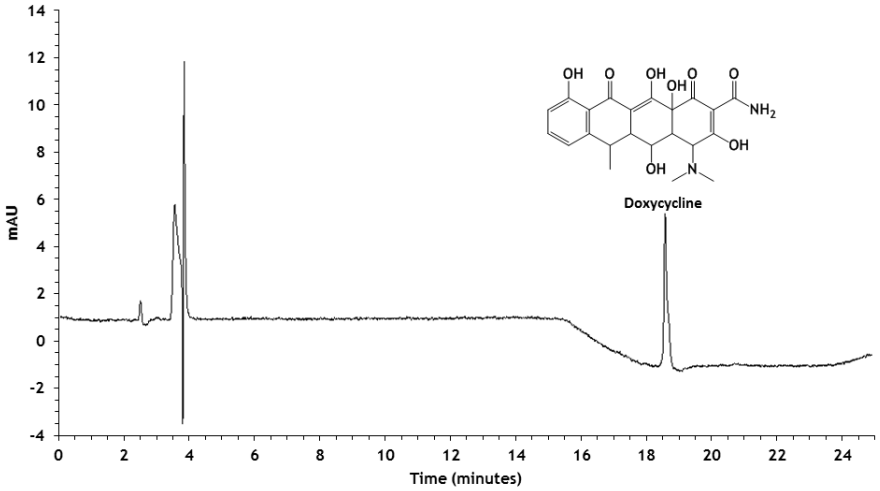


Figure 5.13. HPLC-DAD chromatogram of a mixed standard solution with a concentration of 436 µg/L for amoxicillin and 479 µg/L for doxycycline. Monitoring was performed at 360 nm.

Under the stated chromatographic conditions (section 4.3), whereas the doxycycline retention time kept constant during the experiments (18.5 minutes), the retention time of amoxicillin varied from 6.0 to 7.5 minutes, depending on the room temperature. The adsorption of amoxicillin to the stationary phase is favored by temperature reduction. As a result, the lower ambient temperature, the higher the retention time of the compound. To unambiguously identify the peak of the compounds in each experiment, a mixture standard solution was injected before the samples analysis.

Analyzing figures 5.12 and 5.13, it was concluded that doxycycline is less polar than amoxicillin. Therefore, the elution strength of the mobile phase required to elute the doxycycline in reverse phase chromatography was higher.

In turn, the purity of the obtained peaks can be assessed by the absorption spectrum of the studied compounds provided by the diode array detector (figures 5.14 and 5.15).

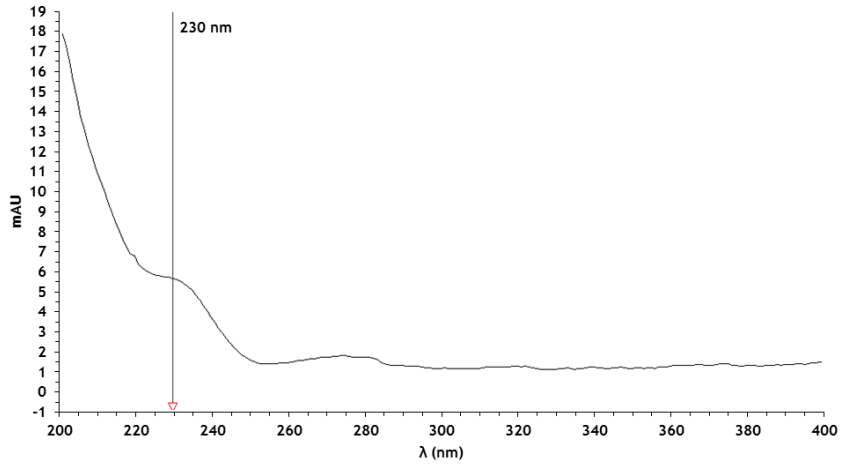


Figure 5.14. Absorption spectrum of amoxicillin in a mixed standard solution with a concentration of 436 µg/L for amoxicillin and 479 µg/L for doxycycline.

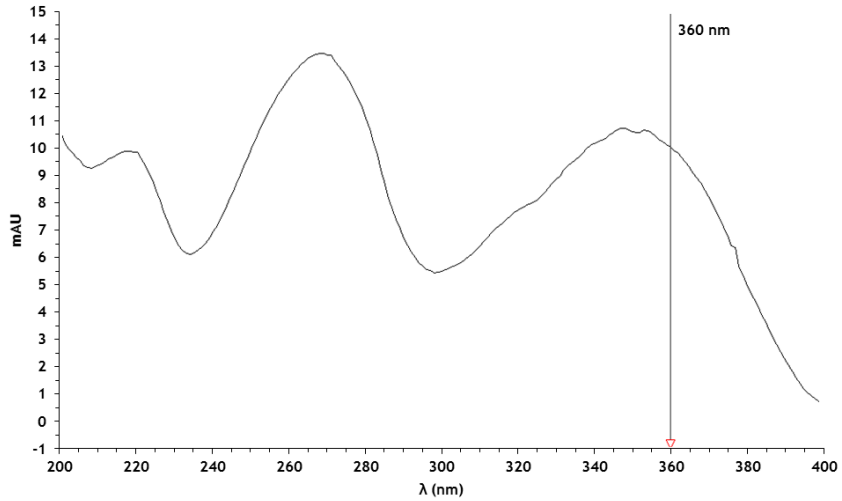


Figure 5.15. Absorption spectrum of doxycycline in a mixed standard solution with a concentration of 436 µg/L for amoxicillin and 479 µg/L for doxycycline.

The retention time and the UV spectrum of the compounds were used in the identification of the analytes.

The instrumental validation was performed evaluating the linearity, repeatability, intermediate precision, and accuracy.

5.2.1 Linearity and limits of detection and quantification

The calibration was carried out at eleven concentration levels, in the range of 33 – 436 µg/L for amoxicillin and in the range of 36 – 479 µg/L for doxycycline, being each standard solution analyzed in duplicate. Figure 5.14 shows the calibration curve of each analyte and its confidence limits (95%).

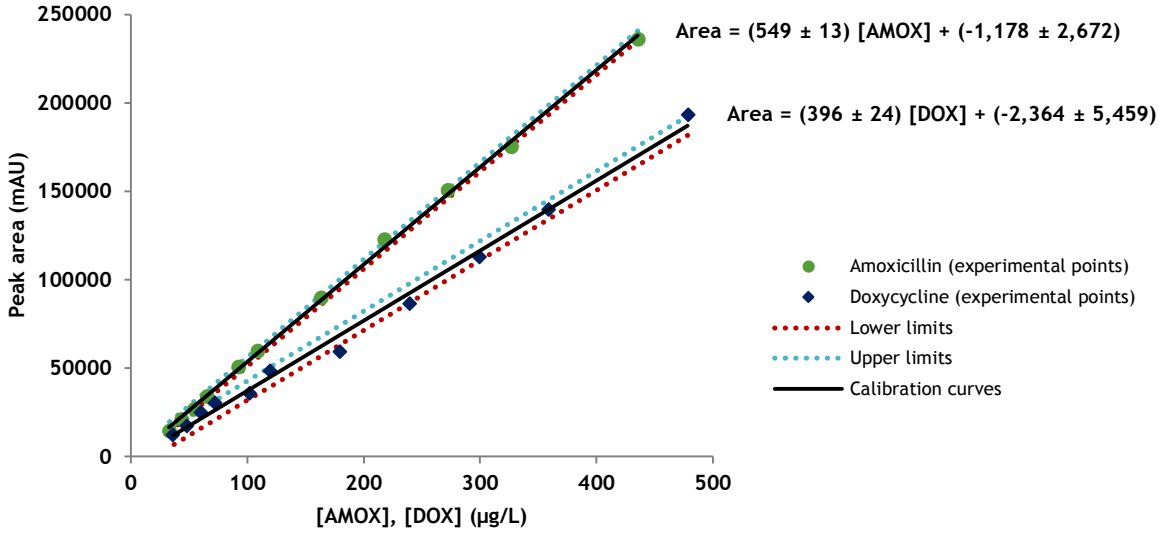


Figure 5.16. Calibration curve for amoxicillin (AMOX) and doxycycline (DOX) and their confidence interval (95%).

Table 5.1 summarizes the analytical parameters. The least squares method was used to calculate the slope, intercept and correlation coefficient of the regression line. The detection and quantification limits were calculated from an average (n = 11) signal-to-noise ratio (S/N) of 3 and 10, respectively.

Table 5.1. Analytical method parameters.

Analyte	Linearity range	Slope ± ts_a ((mAU.L)/µg)	Intercept ± ts_b (mAU)	Correlation coefficient	LOD (µg/L)	LOQ (µg/L)
Amoxicillin	33 - 456	549 ± 13	-1,178 ± 2,672	0.9995	28	92
Doxycycline	36 - 479	396 ± 24	-2,364 ± 5,459	0.9968	29	95

s_a - confidence interval for slope (8 degrees of freedom; 95% of confidence level); s_b - confidence interval for intercept (8 degrees of freedom; 95% of confidence level).

The analytical method is more sensitive for determination of amoxicillin than doxycycline, since slope of calibration curve is higher for the first mention antibiotic.

Quality control laboratories typically consider three criteria to validate a calibration curve: (i) relative standard deviation of the slope $\left(\frac{s_a}{a} \times 100\right)$ less than 5%; (ii) intercept contains the origin $(b - s_b < 0 < b + s_b)$; and (iii) correlation coefficient greater than 0.995 (Miller & Miller 2005).

The aforementioned criteria have been checked for calibration curves of both analytes. The relative standard deviations of the slopes for calibration curves of amoxicillin and doxycycline were 1.0% and 2.7%, respectively. Intercepts contained the origin: $b - s_b = -2{,}359 < 0 < b + s_b = 3$ and $b - s_b = -4{,}778 < 0 < b + s_b = 49$ for amoxicillin and doxycycline calibration curves, respectively. As presented in the table 5.1, correlation coefficients were higher than 0.995, indicating a good linearity in the concentration range studied. Consequently, can be stated that the obtained calibration curves were suitable for use in analysis.

5.2.2 Precision

The precision can be defined as the degree of scatter between a series of measurement of the same sample. In this study, intermediate precision and repeatability was determined to evaluate of the method. Repeatability expresses the precision under the same operating conditions over a short interval of time, being in this study estimated by the relative standard deviation (RSD, %) of six measurements of the same standard, under the same conditions and in the shortest period of time. The intermediate precision expresses variations within laboratories, being in this work determined by RSD of duplicate injections of analytical standards at three levels of concentration, by varying the day of analysis. The results are summarized in the table 5.2.

Table 5.2. Study of the precision of the analytical method with standard solutions.

	Amoxicillin			Doxycycline		
	44 µg/L	**218 µg/L**	**436 µg/L**	**48 µg/L**	**239 µg/L**	**479 µg/L**
Repeatability (RSD, %) (n = 6)	2.8	1.6	1.0	4.6	1.3	0.9
Intermediate precision (RSD, %) (n = 6)	3.8	1.8	1.0	2.5	1.3	1.1

Analyzing the table 5.2, it can be seen that as expected RSD decreased with increasing concentration, i.e. the precision increased with increasing concentration. The precision ranged from 1.0 to 4.6% and 1.0 to 3.8%, for intraday and interday conditions, respectively. The results indicate that the method were precise.

5.2.3 Accuracy

The accuracy of an analytical procedure can be defined as the closeness of agreement between the conventional true value or an accepted reference value and the value found. In this work, the accuracy was estimated through analytical recovery tests in order to evaluate the suitability of the developed method for the determination of the target antibiotics in real samples as clean as possible. The analytes present in complex aqueous environmental samples cannot be analyzed by this analytical procedure without a prior clean-up and extraction methodology. Therefore, the accuracy of the method were determined using tap water spiked at three levels of concentration. The results are summarized in table 5.3. The target antibiotics were not found in the blank sample (nonspiked tap water).

Table 5.3. Study of the accuracy of the analytical method in tap water.

	Amoxicillin			Doxycycline		
	44 µg/L	**218 µg/L**	**436 µg/L**	**48 µg/L**	**239 µg/L**	**479 µg/L**
Recovery ± RSD (%) (n = 6)	97 ± 3	103 ± 2	102 ± 2	103 ± 3	101 ± 2	98 ± 3

The average recovery obtained was 101 ± 3% and 101 ± 2% for amoxicillin and doxycycline, respectively. Since good recovery results and low RSDs were obtained, it was considered that the analytical method enables an accurate evaluation of the studied antibiotics in the aqueous samples which may be represented by tap water.

Figures 5.17 and 5.18 show the chromatograms of tap water fortified with a mixed standard solution of amoxicillin (436 µg/L) and doxycycline (479 µg/L) monitoring at 230 and 360 nm, respectively.

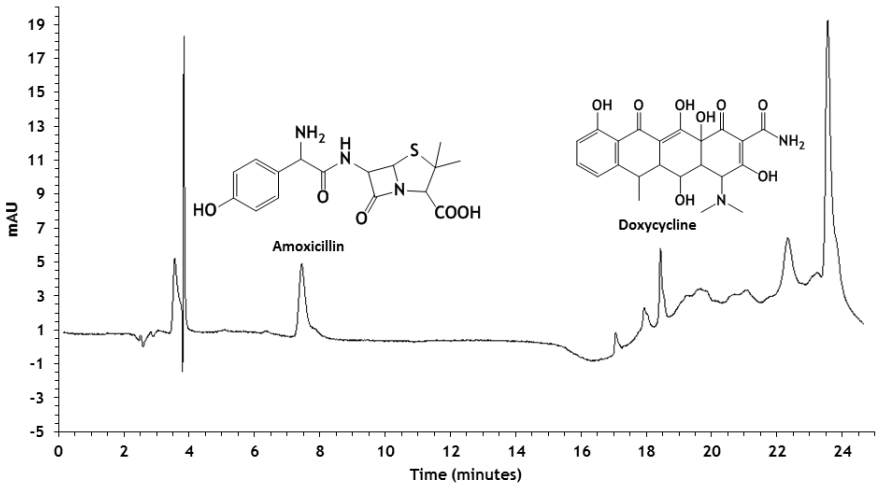


Figure 5.17. HPLC-DAD chromatogram of tap water spiked with amoxicillin (436 µg/L) and doxycycline (479 µg/L). Monitoring was performed at 230 nm.

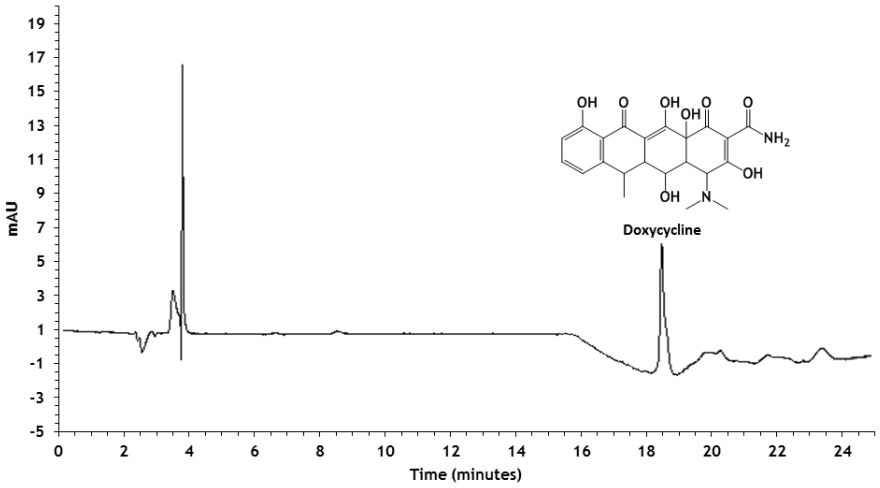


Figure 5.18. HPLC-DAD chromatogram of tap water spiked with amoxicillin (436 µg/L) and doxycycline (479 µg/L). Monitoring was performed at 360 nm.

Analyzing the figures 5.17 and 5.18, it can be seen that the proposed method allows to identify and quantify the analytes in tap water despite the interferences that are mainly eluted at higher elution strength.

5.3 Solid-phase extraction

In multiresidue analysis of antibiotics in aqueous environmental samples, pre-concentration is a required step for sample preparation since this compounds are usually present in these matrixes at trace levels. In this work, preliminary SPE studies were presented, in order to develop and optimize a methodology for the simultaneous extraction of amoxicillin and doxycycline from aqueous samples.

Since amoxicillin and doxycycline are characterized by different physicochemical properties, the choice of the SPE sorbent which allows to obtain a satisfactory recovery for both compounds is one of the main challenges. Two cartridges were evaluated to pre-concentrate the target analytes in distilled water: Oasis HLB and Oasis WCX. Oasis HLB is a reversed-phase hydrophilic/lipophilic balanced sorbent made from a specific ratio of two monomers, hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene (figure 5.19). Therefore, this sorbent exhibits retention capacity for a wider polarity spectrum of analytes, being stable in a wide range of pH (from pH 1 to 14). In turn, Oasis WCX, a mixed-mode sorbent consisting of a weak cation exchanger (carboxylic acid, pK_a about 5) and an N-vinylpyrrolidone/ divinylbenzene polymer (figure 5.20). Based on ionic interaction, this sorbent creates a strong attraction with opposite functional groups of the sample compounds, being used to extract strong basic compounds which have one or more positive charges.

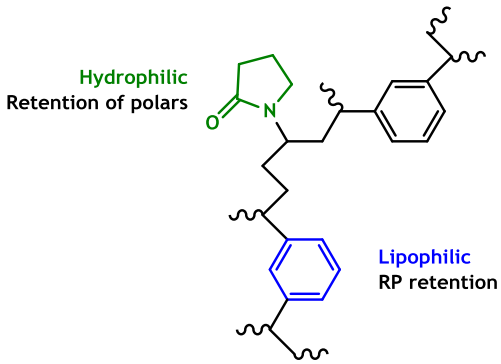


Figure 5.19. Chemical structure of sorbent Oasis HLB, a copolymer of N-vinylpyrrolidone/ divinylbenzene.

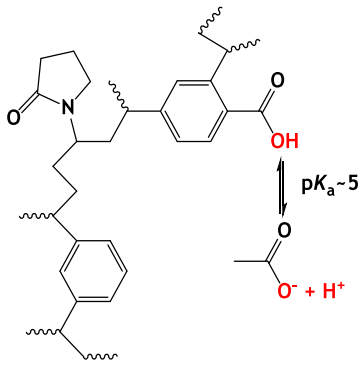


Figure 5.20. Chemical structure of sorbent Oasis WCX, a weak cation exchange sorbent.

Sample pH is a very important parameter to consider, once the published studies demonstrate that acidification of the sample generally improves the extraction recoveries for the majority of compounds for further multiresidue analysis.

On the other hand, sample interferences can be co-retained with compounds of interest during sample load. Therefore, a wash step is usually required to elute interferences without eluting compounds of interest, obtaining a cleaner extract after step elution. Additionally, the choice of the appropriate elution solvent to disrupt the analytes interactions with the sorbent is crucial. Typical elution solvents include water miscible organic solvents such as acetonitrile and methanol.

By the abovementioned reasons, in this study, the factors evaluated for extraction with both sorbents (Oasis HLB and WCX) include the study of the pH of the sample, washing step, and composition of the eluting solution. It should be notice that Na_2EDTA was added in all protocols, since chelation is an important feature of the chemical properties of the doxycycline. The acidic functions of the doxycycline

are capable of forming salts through chelation with metal ions (Gentry 2012), resulting in lower extraction recoveries. Therefore, the Na_2EDTA addiction prevent the formation of these salts of polyvalent metal ions, by strong complexation with metals or multivalent cations soluble in water.

The extraction recoveries of doxycycline for the distilled water spiked at 1.0 µg/L are graphically presented in figure 5.21. Table 5.4 shows the results for samples fortified at 4.8 and 0.5 µg/L. Amoxicillin was not recovered in the all experiments performed.

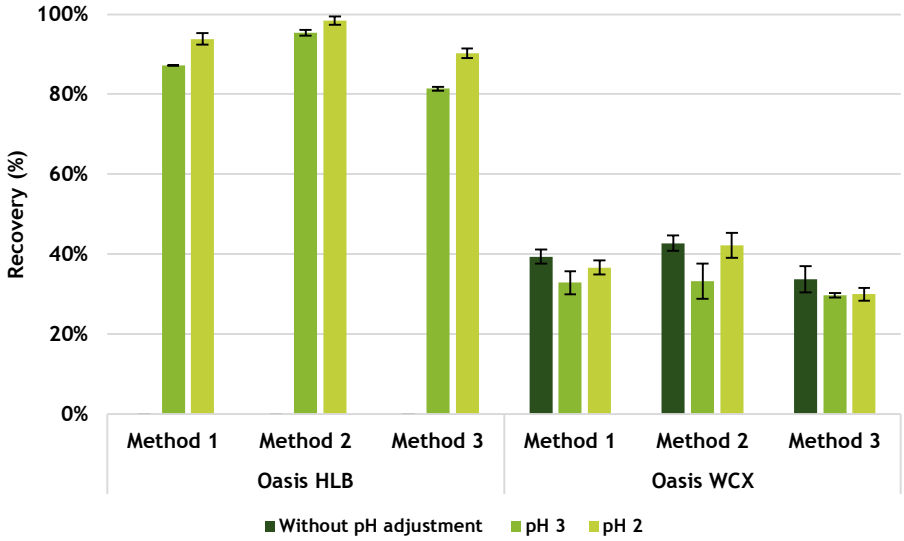


Figure 5.21. Doxycycline recoveries using Oasis HLB and WCX cartridges, with and without sample pH adjustment, for the distilled water spiked at 1.0 µg/L (values are the mean of two chromatography analysis, n = 2)**.** Method 1 - washing with 5 mL ultrapure water and elution with 6 mL methanol; method 2 - washing step not performed and elution with 6 mL methanol; and method 3 - washing step not performed and elution with 6 mL acetonitrile.

The results presented in figure 5.21 show that doxycycline exhibited pH dependent recoveries for extractions using Oasis HLB and WCX cartridges. However, this fact is more pronounced in the extraction with Oasis HLB sorbent. Although HLB (hydrophilic/lipophilic balance) sorbent is designed to extract acidic, neutral and basic analytes at wide range of pH values, a strong acidic environment (pH 2 or 3) is necessary for high extraction of this antibiotic. At pH of not acidified samples (pH 6 – 6.5), doxycycline exists in the zwitterionic form, being the tertiary amine positively charged and the hydroxyl negatively charged. At a pH below 2, doxycycline are fully protonated, being only the tertiary amine positively charged. At pH 3, both fully protonated and zwitterionic forms of doxycycline are in solution. Therefore, regardless of the tested method, doxycycline was not recovered from samples not acidified using HLB sorbents. This cartridge appears to retain more effectively the fully protonated form of doxycycline, since the recovery of the antibiotic from in samples at pH 2 was slightly higher than in samples at pH 3.

In turn, despite the poor performance of the Oasis WCX cartridge in retain the doxycycline under the tested conditions, the recovery of the compound from samples not acidified was possible using this sorbent. Since the retention mechanism of Oasis WCX is a mixed mode, basic, acidic and neutral substances can be retained at pH above pK_a of the cation exchanger (carboxylic acid, pK_a about 5): basic drugs are positively charged, being strongly bound to the polymer by positive cation exchanger, while neutral and acidic compounds are retained by reversed phase. When sample pH was not adjusted, the

carboxyl functional groups of the sorbent were predominantly negatively charged, establishing ionic interactions with the amine functional groups, which were positively charged. At pH 3 or 2, the carboxyl group of the sorbent was protonated, not establishing ionic interactions with charged groups of doxycycline.

Additionally, it was verified that when sorbents were washed with ultrapure water, small amounts of doxycycline are eluted, since slightly higher recovery were obtained when the washing step is omitted (method 2 compared to the method 1). In these assays, it was also found that the elution strength of methanol in elute the doxycycline is slightly higher than acetonitrile (method 3 compared to the method 2).

Table 5.4. Recovery of doxycycline under the different extraction conditions for the distilled water spiked at 4.0 and 0.5 µg/L (values are the mean of two chromatography analysis, n = 2).

SPE conditions		Sample pH	Doxycycline recovery ± RSD (%) (n = 2)	
			Spiking level (µg/L)	
			4.8	0.5
Oasis HLB	Method 1 [a]	Without pH adjustment	n.d.	n.d.
		pH 3	8 ± 3	89 ± 3
		pH 2	8 ± 2	92.2 ± 0.3
	Method 2 [b]	Without pH adjustment	n.d.	n.d.
		pH 3	25.1 ± 0.4	94 ± 2
		pH 2	25 ± 2	99.8 ± 0.9
	Method 3 [c]	Without pH adjustment	n.d.	n.d.
		pH 3	25 ± 1	80 ± 6
		pH 2	25 ± 1	91.3 ± 0.6
Oasis WCX	Method 1 [a]	Without pH adjustment	11 ± 1	n.d
		pH 3	10 ± 1	n.d.
		pH 2	11 ± 2	n.d.
	Method 2 [b]	Without pH adjustment	11 ± 2	n.d.
		pH 3	10 ± 1	n.d.
		pH 2	11 ± 3	n.d.
	Method 3 [c]	Without pH adjustment	11 ± 2	n.d.
		pH 3	10 ± 2	n.d.
		pH 2	11 ± 2	n.d.

[a] Washing with 5 mL ultrapure water; elution with 6 mL methanol.

[b] Washing step not performed; elution with 6 mL methanol.

[c] Washing step not performed; elution with 6 mL acetonitrile.

n.d. – not detected.

Analysing the results presented in table 5.4, it can be noticed that the recovery of doxycycline under the experimental conditions was lower for samples spiked at 4.8 µg/L than for samples fortified at 1.0 or 0.5 µg/L of the target analyte. This is due to the retention capacity of the sorbents reaches the saturation point. When all adsorption sites in the sorbent for the analyte are occupied, the compound is not retained, resulting the reduction of the percentage of recovery. On the other hand, doxycycline was not detected after its extraction from samples spiked at the lowest level. The concentration factor for all extraction procedures was 100. This means that if 100% of doxycycline were recovered from the

samples spiked with 0.5 µg/L, the extract to analyze would have 50 µg/L, being this concentration value close to its limit of detection (considering the analytical method described in the section 4.3). Therefore, low recoveries of the analyte, such as those obtained for assays performed with Oasis WCX (see figure 5.21), result in the non-detection of the compound.

Concerning amoxicillin, its non-detection in any of tested extraction conditions may be due to its instability under acidic conditions and in the presence of methanol. In fact, it has been reported that the ß-lactam ring and its amide bond break open in the presence of acid, resulting in the formation of various complex products (see annex A1.3). In turn, methanol can act as a nucleophile, being the amoxicillin degraded by the nucleophilic ß-lactam ring cleavage (see section 5.1) (Bruno et al. 2001; Deshpande et al. 2004; Nägele & Moritz 2005; Ferrer et al. 2010). Mainly due to these characteristics, the extraction assays without pH adjustment and using acetonitrile as the eluting solvent were performed. However, it was not possible to recover the compound under these conditions using Oasis HLB or WCX sorbents. These results are consistent with some of the information published by other authors. In these studies, the percentage of amoxicillin recovery was less than 30% (Cha et al. 2006; Kasprzyk-Hordern et al. 2008; Watkinson et al. 2009; Locatelli et al. 2011) or the analyte was not detected (Cahill et al. 2004; Benito-Peña et al. 2006; K'oreje et al. 2012; Vergeynst et al. 2015). However, there are also publications which report the extraction of amoxicillin under slightly different conditions with recoveries higher than 80% (Bailón-Pérez et al. 2009; Bailón-Pérez et al. 2008; Serrano & Silva 2007). Thus, it is suggested that the operating condition described by these authors shall be reproduced in future studies of amoxicillin extraction by SPE, in order to obtain similar results.

6 Conclusions

Worldwide, antibiotics have been used in veterinary medicine to treat, control, and prevent diseases in animals for more than 60 years. However, it is recognized that livestock production practices contributes for the introduction of these drugs and their metabolites into the environment, being a key risk factor for the development and spread of antimicrobial resistance. In Portugal, according to the latest statistics, doxycycline and amoxicillin are the most consumed antibiotics in livestock production, being mainly administered to swine. Therefore, researches on occurrence of these antibiotics in the environmental are required to identify and quantify subtle and longer-term effects of these drugs as pseudo-persistent pollutants.

In this work, an analytical methodology by direct HPLC-DAD based on gradient elution was developed, optimized, and validated to analyze simultaneously the antibiotics amoxicillin and doxycycline in the aqueous samples which may be represented by tap water. The correlation coefficients of the calibration curves, that were higher than 0.9968 in ultrapure water, showing good linearity of the method in the range of 33 - 456 µg/L for amoxicillin and 36 - 479 µg/L for doxycycline. The limits of detection varied from 28 µg/L for amoxicillin to 29 µg/L for doxycycline, which made the method useful for the determination of these pharmaceuticals at concentration levels of µg/L in water samples. The repeatability and intermediate precision were average less than 5%, indicating that the method were precise. Good recovery results were obtained at three levels of concentration in tap water, being the presented method suitability for the determination of the target antibiotics in real matrixes with similar physicochemical characteristics to tap water.

The developed analytical method cannot be applied in the analysis of analytes without a prior methodology of clean-up and extraction. Subsequently, preliminary SPE tests were performed. The efficiency of Oasis HLB and WCX SPE cartridges to extract the studied analytes from distilled water under different conditions was evaluated. Higher recoveries of doxycycline (98%) were achieved in acidified sample at pH 2 before extraction with the Oasis HLB sorbent, omitting the washing step and using methanol as eluting solvent. Amoxicillin was not recovered in any assay, which may be due to its instability in the experimental conditions, particularly low pH and elution with methanol. In fact stability tests performed in this study showed that amoxicillin is very sensitive to methanol, being the ß-lactam ring hydrolyzed by the nucleophilic attack of this solvent. Through the stability tests, it was also found that the temperature increases the degradation reactions rate of both amoxicillin and doxycycline.

The distinct physicochemical properties of the amoxicillin and doxycycline make their simultaneous analysis difficult and challenging. Further researches are required to develop, optimize, and validate a methodology based on SPE-HPLC-DAD for simultaneous determination of amoxicillin and doxycycline in environmental aqueous samples, particularly swine wastewater. Moreover, confirmation the identity of antibiotics and their degradation products by a complementary analytical method such as LC-MS is also necessary.

7 Limitations and future work

Although the main objectives proposed for this work have been achieved, there were some limitations such as time, equipment and methods of extraction and analysis, as well the different physicochemical properties of the analytes and their degradation behavior.

Future studies should include analysis of antibiotics belonging to the class of macrolides, particularly tylosin and tilmicosin, in addition to doxycycline and amoxicillin. A methodology of extraction and analysis by SPE-HPLC-DAD should be developed, optimized, and validated for the multiclass antibiotics (β-lactams, tetracyclines, and macrolides) determination in aqueous environmental samples. More work is required for amoxicillin regarding SPE and optimization of recovery. Accordingly, SPE assays to extract β-lactams could be improved by using MIPs. However, due to their specific and selective properties, other alternatives for the multicomponent analysis in complex matrices should be considered. Therefore, SPME as alternative to SPE should be tested since this technique had been emerged as a viable approach to liquid sample preparation, due to simplicity of operation, rapidity, low cost, high recovery, and high pre-concentration factor, and being environment friendly (Pavlović et al. 2007). On the other hand, complementary techniques based in LC-MS for the confirmation and identification of antibiotics and their metabolites and degradation products should also be pondered. The nuclear molecular resonance (NMR) and infrared (IR) spectroscopy could also be used to determine more exactly the structure of unknown metabolites and degradation products of amoxicillin, doxycycline, tylosin, and tilmicosin.

In Portugal, further research is need to assess the impact of the antibiotics commonly used in veterinary medicine and their metabolites in the aquatic environment. Subsequently, the presence of these compounds in aqueous environmental samples from the most concentrated Portuguese regions for livestock such as Leiria should be evaluated and monitory. The determination of occurrence, fate, and effects of antibiotics and their metabolites and degradation products in other environmental matrixes (e.g. soils), plants, and animals is also essential to be performed in future works, since understanding how the presence of these compounds affects human health and environmental sustainability is crucial to mitigate the emergence of ARB.

Studies on the removal of the target antibiotics should be performed, besides the evaluation of the effectiveness of conventional wastewater treatment processes. Investigations of the occurrence these pharmaceuticals in various wastewater treatment steps can be exploited in order to evaluate wastewater treatment technologies with the respect to elimination of specific contaminants. In turn, knowing that microparticles can improve the efficacy of various medical treatments, mainly because of the ability to protect the encapsulated compound and to allow their controlled release, the effectiveness of microencapsulation in reduce the amount of the administered antibiotics required to cure the bacterial infections may be considered in future studies as preventive approach to decrease the environmental contamination by these drugs.

In conclusion, there are a lake of knowledge about the short- and long-term effects on massive consumption of antibiotics. Therefore studies about the occurrence, fate, risks, and ecotoxicity are essential, particularly in Portugal, where the number of the publications on this field is reduced.

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A1 Supplementary content to the introduction

A1.1 Veterinary antibiotics classification

The table A1.1 schematically summarizes the classification of the antibiotics according to various criteria.

Table A1.1. Classification of antibiotics used in animals according to their origin/source, spectrum of activity, effect on bacteria, chemical structure, and ATCvet system.

Criteria	Class
Origen/source	• **Natural:** antibiotic produced by microorganisms • **Semisynthetic:** antibiotic naturally produced and chemically modified • **Synthetic:** agent produced by chemical synthesis
Spectrum of activity	• **Broad-spectrum:** effective against a wider range of both Gram-positive and Gram-negative bacteria • **Intermediate-spectrum:** effective against some of both Gram-positive and Gram-negative bacteria • **Narrow-spectrum:** active against a limited group of bacteria
Effect on bacteria	• **Bactericidal:** antibiotic that kills bacteria • **Bacteriostatic:** agent that inibits the growth or reproduction of bacteria • **Bactericidal and bacteriostatic**
Mode of action	• **Inhibition of bacterial cell wall synthesis** • **Inhibition of protein synthesis** • **Inhibition of nucleic acid synthesis** • **Inhibition metabolic pathways** • **Disruption of bacterial plasma membrane**

Chemical structure	• **Aminoglycosides**
	  • Streptomycins
	  • Other aminoglycosides
	• **Amphenicols**
	• **ß-lactams**
	  • Carbapenems
	  • Cephalosporins
	  • Monobactams
	  • Penicillin
	• **Glycopeptides**
	• **Imidazole derivatives**
	• **Lincosamides**
	• **Macrolides**
	• **Nitrofuran derivatives**
	• **Pleuromutilins**
	• **Polymyxins**
	• **Quinolones**
	• **Quinoxalines**
	• **Streptogramins**
	• **Streptomycins**
	• **Sulfonamides**
	• **Tetracyclines**
	• **Trimethoprim and derivatives**
	• **Other antibiotics**

ATCvet system	• **1st level:** anatomical main group • **2nd level:** therapeutic main group • **3rd level:** therapeutic subgroup • **4th level:** chemical group or therapeutic sub-subgroup • **5th level:** chemical group or subgroup

A1.2 Dissociation equilibrium of amoxicillin and doxycycline

This annex presents the dissociation equilibrium of the antibiotics amoxicillin and doxycycline (figures A1.2 and A1.3, respectively).

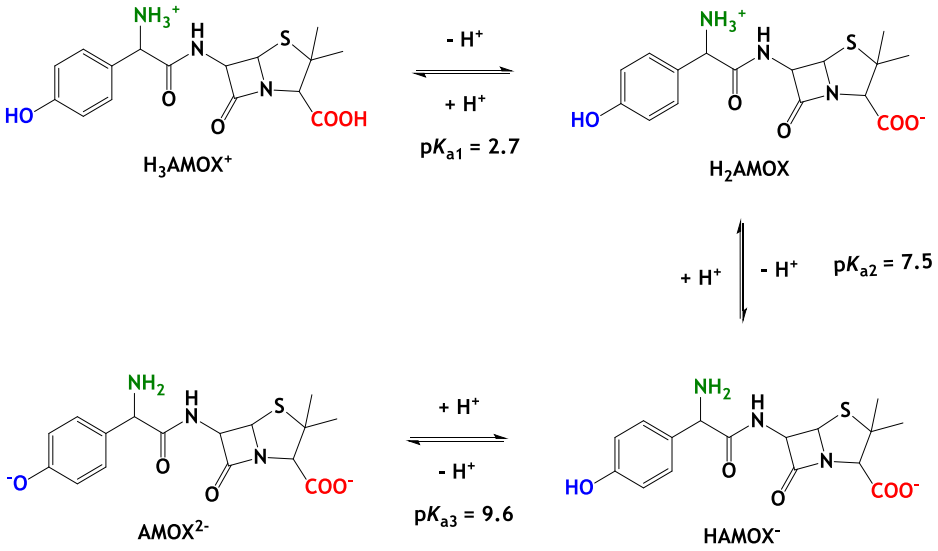

Figure A1.1. Schematic representation of the dissociation equilibrium of amoxicillin. Ionizable functional groups of this antibiotic are highlighted.

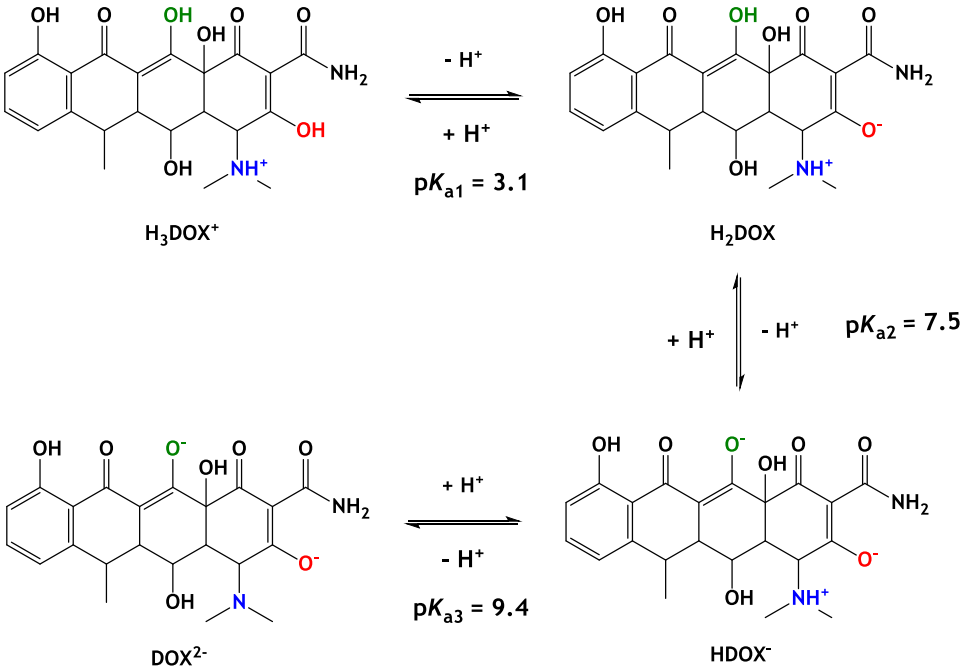

Figure A1.2. Schematic representation of the dissociation equilibrium of doxycycline. Ionizable functional groups of this antibiotic are highlighted.

A1.3 Pathways of ß-lactams degradation

This annex presents the degradation pathways of penicillins according to pH of the medium (figure A1.4 (Deshpande et al. 2004)), particularly the hydrolysis of the ß-lactam ring of amoxicillin and its dimerization in acid conditions (figure A1.5 (Nägele & Moritz 2005)).

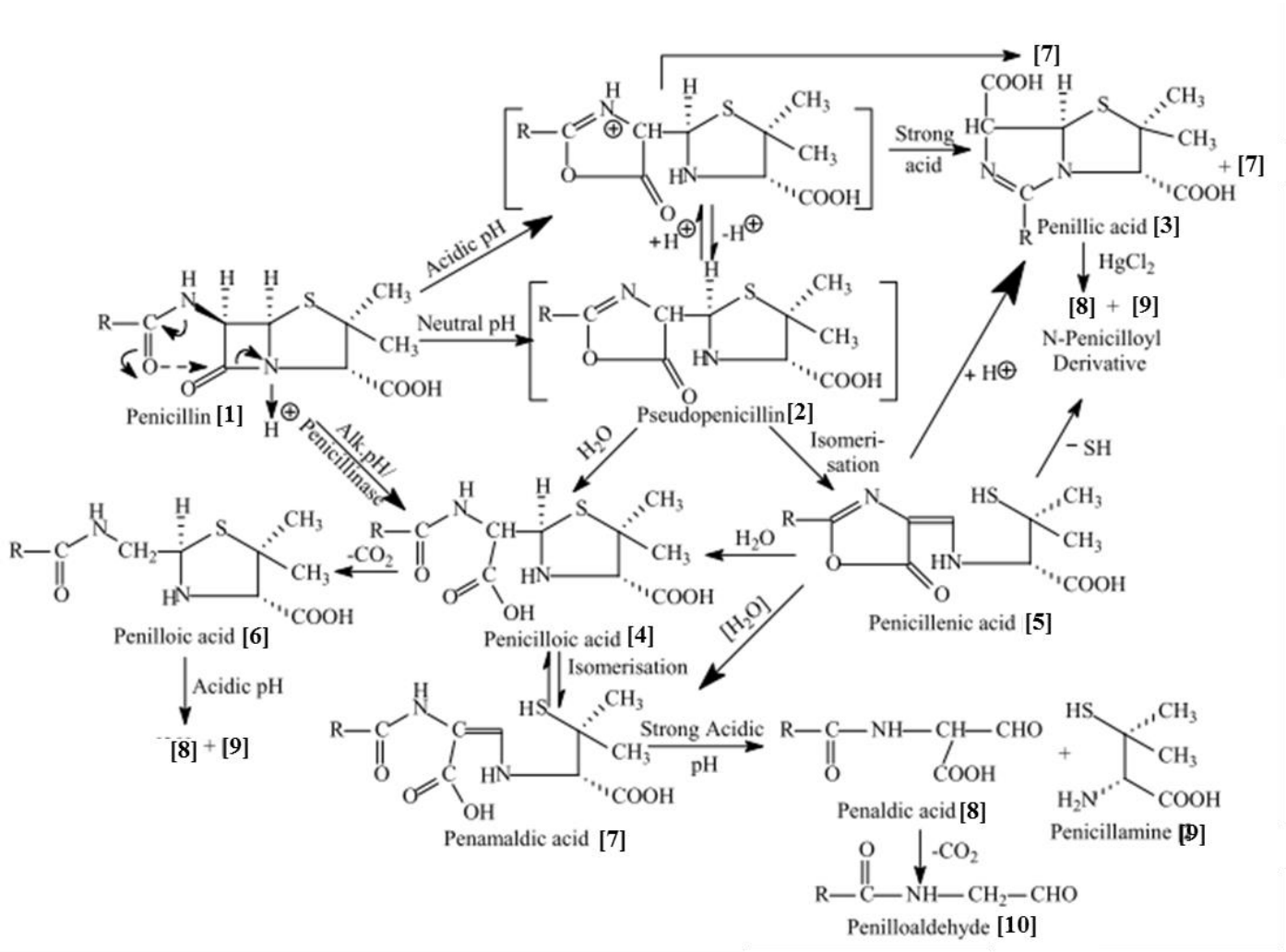

Figure A1.3. Pathways of degradation of penicillins in acidic and alkaline conditions.

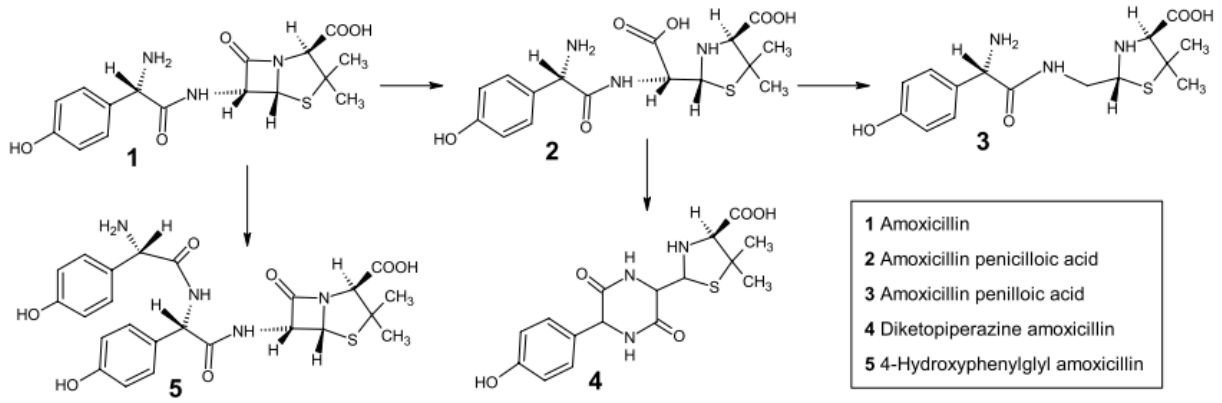

Figure A1.4. Pathways of degradation of amoxicillin in acidic conditions.

A1.4 Swine production

To assess the presence and distribution of veterinary antibiotics in surface and groundwaters, and wastewaters in Portugal, it would be interesting to study a region where livestock raising and the pollution by its wastes were significantly high. It should be notice that antibiotics detected in the environment mainly results from its use in both animal and human medicine.

Although the analysis of antibiotics in the real environmental samples is beyond the scope of this study, it should be included in future research on occurrence studies. Knowing that amoxicillin and doxycycline are mostly administered to pigs (see figure 2.8), it was performed the research of a region of Portugal with high both swine density and high aquatic environmental pollution by swine wastewater discharge into the local rivers. Therefore, in the sections of this annex, statistical information on the geographical location of pig farms in Portugal as well as the aquatic environmental impact of this agricultural sector are presented.

A1.4.1. Portugal statistics

Meat is a major source of proteins and essential amino acids which are fundamentals for human growth and development (DGS 2014). According to Food and Agriculture Organization of the United Nation (FAO), the global meat consumption in 2014 was 42.9 kg *per capita*, being 331.8 and 31.3 million tons of meat produced and traded, respectively. Pork is the most widely eaten meat in the world accounting for over 36% of the world meat intake (FAO 2014). In particular, Portuguese follow this global trend, having consumed about 43.9 kg *per capita* in 2014 (INE 2015a).

Across the EU, the size of the pig herd decreased for 7 consecutive years, having recovered in 2014, with the total pig population up by 0.9% on 2013 levels. Additionally, forecasts indicate a marginal increase in the EU pig net production, reaching around 22.6 million tons by the end of 2024 (close to 2012 levels) (European Commission 2014). In fact, over the past few years, the EU expansion of pig meat production have mainly been limited by environmental concerns, animal welfare rules and changes in consumption patterns (Westhoek et al. 2014). In Portugal, programs to support rural development have been implemented, aiming to improve viable food production, create employment across the country, sustainably manage of the natural resources, and prevent the climate changes (MAMAOT & GPP 2012).

The *Instituto Nacional de Estatística* (INE) is a Portuguese statistical authority, whose goals are to produce and publish official statistical information to the whole society, cooperating with other statistical offices, such as Eurostat (INE 2015b). In 2009, INE conducted the last agricultural census, whose report characterizes the Portuguese agriculture, the production structures, the rural population, and the methods of agricultural production in 2009 (INE 2009). Annually, INE publishes and actualizes the Portuguese agricultural statistics.

In Portugal, swine production has a significant importance in both economy and society, being a major sector of activity in some regions of the country (European Commission & DGA 2003; Tavares 2014). As shown in figure A1.6, the swine industry led the animal production between 2009 and 2013. However,

comparing the tonnes of pigs produced in 2013 with 2009, it turned out a decrease of about 7.5% (INE 2010; INE 2011; INE 2012; INE 2013; INE 2014).

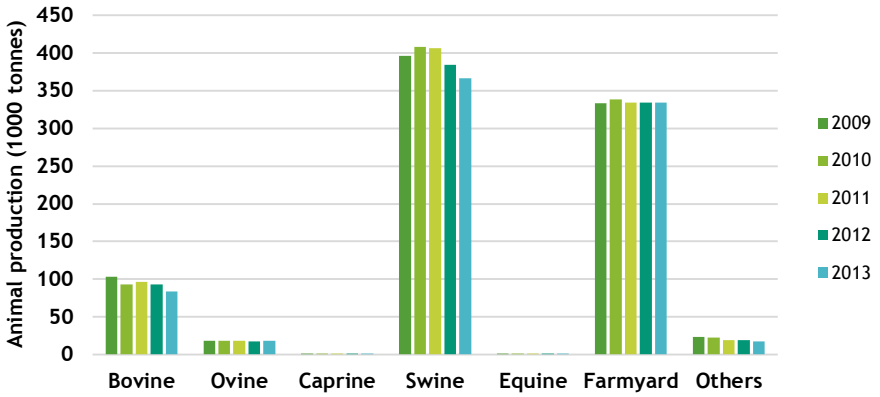

Figure A1.5. Production of different species of meat in Portugal between 2009 and 2013 (values expressed in 1000 tonnes).

Regional data on livestock numbers provide information as to where the most concentrated regions for pig farming are located. The geographic distribution of pig production across Portugal is presented in the map of figure A1.7 (INE 2010). It is verified that center of continental Portugal and Alentejo are the most important Portuguese regions for swine production. Although the swine population displayed on the map dates back to 2009, current publications show that there are no significant variations (figure A1.8 (INE 2010; INE 2011; INE 2012; INE 2013; INE 2014)).

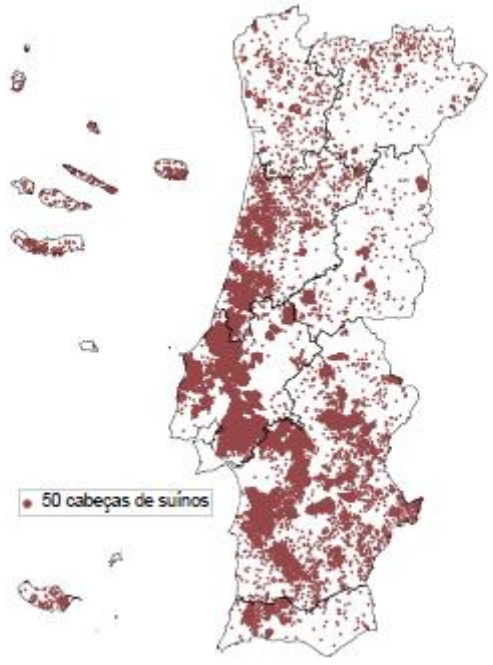

Figure A1.6. Regional concentration of swine produced in Portugal in 2009.

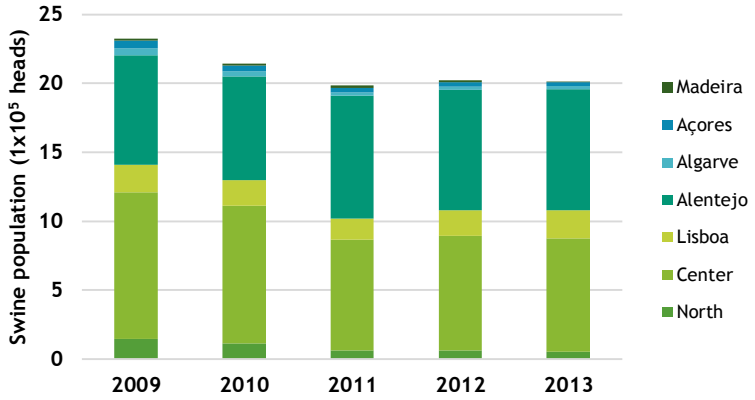

Figure A1.7. Regional distribution of swine population in Portugal between 2009 and 2013 (values expressed in $1x10^5$ heads).

The regional distribution of livestock population is important in assessing the environmental impact of this economic sector. Unless measures to protect the environment from discharges of pollutants generated are implemented, the environmental pressure exerted by pig farms in the regions with high swine density will continue to be significantly high (APA 2008).

A1.4.2. Environmental impact

In Portugal, pig production is a significant source of environmental pollution, since it mainly takes place in specialized landless farms, often without proper wastewater management systems. This problem is regularly aggravated regions where the density of pigs is high (APA 2008). The pollution of Portuguese pig farms is equivalent to the 7.6 million people (TSF 2003). Swine wastewater is usually characterized with high concentrations of organic matter and inorganic nitrogen and phosphate, which are the most significant causes of eutrophication in waterways. In addition, it contains micro-pollutants such as antibiotics and heavy metals, as well as pathogenic microorganisms. As mentioned in the section 2.1.4 conventional methods for wastewater treatment are not effective in removing most of the antibiotics. Moreover, the presence of antibiotics in the environment is nowadays one of the major threats to human health and the dynamic equilibrium of ecosystems, concerning the medical and scientific community (see sections 2.1.5 and 2.1.6). However, the presence of antibiotics in the water is not an evaluated parameter to assess the quality of the water.

The problems associated with the agro-livestock and agro-industrial effluents in Portugal was analyzed and identified in the context of the *Estratégia Nacional para os Efluentes Agro-Pecuários e Agro-Industriais* (ENEAPAI) published in Dispatch No. 8277/2007 of 9 May (MAOTDR & MADRP 2007). Table A1.2 indicates the pressure exerted by the swine production in each hydrographic region (HR) of continental Portugal established in the *Lei da Água*, mainly considering the loads pollutants produced.

Table A1.2. Pressure of swine production in the Portuguese hydrographic regions.

Hydrographic region	HR1	HR2	HR3	HR4	HR5	HR6	HR7	HR8
Swine production pressure				CL	RW	RW/ALE		

■ - very high pressure; ■ - high pressure; ■ - low or no pressure.

HR1 – Minho-Lima; HR2 – Cávado/Ave/Leça; HR3 – Douro; HR4 – Vouga/Mondego/Lis; HR5 – Tejo/Ribeiras do Oeste; HR6 – Sado/Mira; HR7 – Guadiana; HR8 – Ribeiras do Algarve.

CL – Coastline; ALE – Alentejo; RW – Ribatejo and West.

The effluents of the swine production have different environmental pressures in the various HR of continental Portugal. In fact, this activity is principally concentrated in the regions of West, Wetland of Tejo, Pinhal Litoral, and Alentejo, being responsible for a significant production of wastes which exert very high pressure in HRs of this zones. Particularly, the production of pigs in the farms of the water basin of Lis generates about 2,000 m^3 of effluent per day (Público 2009), which are punctually discharged into the Ribeira dos Milagres of Rio Lis without any appropriated pre-treatment (Câmara Municipal de Leiria 2010). In this region, there are about 200,000 inhabitants and 350,000 pigs, which produce four times more waste than humans (TSF 2003). In 1971, the news titled "A poluição das águas do Rio Lis (em Leiria) causa a extinção do peixe" published in the Diário de Lisboa warned for the first time the Portuguese population about the environmental problems caused by the direct discharge of pig wastes in this river (PAeM 2015). Over the years, pollution in Lis has worsened, being frequently reported in the media. Therefore, environmentalists and local society have appealed to the competent authorities and the government for viable and sustainable solutions. Some news of the Portuguese press that report this environmental problem over the years are cited below.

TSF: September 21st, 2002

Poluição do Rio Lis teve origem em suinicultura

Isaltino Morais admitiu hoje que a poluição no Rio Lis, que provocou a suspensão do abastecimento de água à cidade de Leiria, possa ter sido causada pela descarga de explorações de suinicultura.

Público: June 15th, 2003

Promortec responsável pela descarga suinícola em afluente do Rio Lis

A descarga suinícola que sucedeu hoje na Ribeira dos Milagres, Leiria, foi causada pela empresa Promortec, do grupo PROMOR, que é um dos maiores produtores de suínos e de rações do país, com sede na freguesia de Barracão.

Público: July 6th, 2003

Nova descarga de efluentes suinícolas no Rio Lis

A descarga de há duas semanas obrigou à interdição dos banhos na Praia da Vieira.

TSF: July 25th, 2003

Quercus oferece dejetos de porco a Sevinate Pinto

A Quercus vai oferecer dois baús com 60 litros de dejetos de porco ao ministro da Agricultura, Sevinate Pinto. O objetivo da ação passa por denunciar os «problemas escondidos» do acordo entre o Governo e os suinicultores da região de Leiria.

RTP notícias: December 7th, 2004

Populares contestam novas descargas na Ribeira dos Milagres

A Comissão de Ambiente e Defesa da Ribeira dos Milagres denunciou hoje a realização de duas novas descargas de efluentes suinícolas naquele curso de água e criticou a "impunidade" dos empresários do sector.

(…) "recorremos ao Parlamento Europeu para ver se temos alguma ajuda" (…) Procurando "evitar que estes graves atentados continuem a acontecer sem qualquer punição" e que "a saúde pública, fauna e flora saiam a perder", os populares reclamam a "rápida intervenção" das instituições comunitárias (…)

Diário de Notícias: February 25th, 2005

Leiria: descarga suinícola na bacia do Rio Lis

RTP notícias: April 2nd, 2005

Populares alertam para descarga de efluentes na Ribeira dos Milagres

Uma nova descarga de efluentes suinícolas foi detetada na Ribeira dos Milagres, em Leiria, minutos depois de fortes chuvas terem assolado a zona, anunciou a Comissão de Ambiente e Defesa daquele curso de água.

(…) os dados recolhidos "indiciam" despejos de efluentes suinícolas na Ribeira dos Milagres que, misturados com outros focos de contaminação, aumentaram os níveis de poluição do Rio Lis para "valores incomportáveis para os peixes" (…)

RTP notícias: May 11th, 2005

Centenas de peixes mortos detetados no Rio Lis

Centenas de peixes mortos foram hoje detetados a boiar num açude do Rio Lis próximo de Monte Real, onde a água apresentava uma cor escura, indiciando o despejo de esgotos.

(…) No local está uma equipa de Ambiente da GNR a tentar investigar as causas da poluição, que terá tido origem numa descarga de efluentes (…)

RTP notícias: June 14th, 2005

Populares despejam baldes com efluentes suinícolas em frente à Câmara de Leiria

Dirigentes da Comissão de Ambiente e Defesa da Ribeira dos Milagres (Leiria) despejaram hoje, frente à Câmara de Leiria, parte de dois baldes com efluentes suinícolas detetados durante a manhã naquele curso de água.

RTP notícias: June 29th, 2005

Morte de peixes no Lis, em Maio, devido a poluição orgânica

A morte de centenas de peixes num açude do Rio Lis próximo de Monte Real, Leiria, em Maio, foi causada por poluição orgânica, revelou hoje fonte da Comissão de Coordenação e Desenvolvimento Regional do Centro (CCDR).

(…) os dados recolhidos "indiciam" despejos de efluentes suinícolas na Ribeira dos Milagres que, misturados com outros focos de contaminação, aumentaram os níveis de poluição do Rio Lis para "valores incomportáveis para os peixes" (…)

RTP notícias: July 3rd, 2005

Populares dos Milagres protestam contra aumento da poluição suinícola

Centena e meia de populares da freguesia dos Milagres manifestaram-se hoje numa entrada da vila em protesto contra a poluição suinícola nos cursos de água que tem aumentado desde o início da época balnear.

(…) Segundo José Carlos Faria, porta-voz da Comissão de Defesa do Ambiente da Ribeira dos Milagres, "desde que abriu a época balnear" verificam-se "descargas diárias" (…) esta manifestação foi mais uma forma de "mostrar a indignação aos nossos governantes", reclamando a aplicação da Lei de Bases do Ambiente à região (…) Isabel Gonçalves espera que a proposta de saneamento do sector suinícola, desenvolvida pela empresa Recilis, venha solucionar um problema com muitos anos e que já teve uma "primeira tentativa de solução" na década de 90 com a construção de duas Estações de Tratamento de Águas Residuais (ETAR) que nunca funcionaram bem (…)

RTP notícias: July 11th, 2005

Populares de Milagres convidam Jorge Sampaio a visitar ribeira poluída

A Comissão de Ambiente e Defesa da Ribeira dos Milagres (CADRM), Leiria, convidou hoje o Presidente da República a visitar o curso de água para constatar no local a poluição causada por efluentes suinícolas.

(…) O primeiro-ministro "nada fez mesmo estando totalmente informado dos graves atentados ambientais e à saúde pública que aqui perduram há mais de 30 anos, parecendo mais um canal de esgoto a céu aberto que uma ribeira propriamente dita" (…) Atualmente, está em curso um projeto de saneamento das explorações suinícolas que deverá estar concluído em 2007 (…)

RTP notícias: April 15th, 2006

Defensores da Ribeira Milagres protestam contra poluição com cartazes

Populares que contestam as sucessivas descargas de dejetos de suiniculturas na Ribeira dos Milagres, concelho de Leiria, exigiram hoje a despoluição daquele efluente do Lis, afixando cartazes com mensagens alusivas à situação do curso de água.

(…) elementos da Comissão de Ambiente e Defesa da Ribeira dos Milagres (CADRM) afixaram alguns cartazes nas margens da ribeira, onde se leem frases como "Oceano Atlântico continuará a substituir as estações de tratamento" e "Justiça. Há mais de 30 anos, onde estás?" (…) "Verificam-se descargas consecutivas, que os suinicultores dizem estar autorizadas" (…) A Ribeira dos Milagres tem sido palco, ao longo dos últimos anos, de grandes descargas de efluentes suinícolas, situação que se espera fique resolvida no âmbito do projeto de despoluição da bacia hidrográfica do Rio Lis (…)

RTP notícias: May 4th, 2006

Ribeira dos Milagres alvo de nova descarga poluente

A Ribeira dos Milagres, no concelho de Leiria, foi alvo ao início da manhã de hoje de uma descarga de dejetos de suinicultura, alertou a Comissão de Ambiente e Defesa daquele afluente do Rio Lis.

(…) Aproveitando o Dia Mundial da Água (22 de Março), a CADRM - comissão de populares que nos últimos anos tem contestado as sucessivas descargas de efluentes de suiniculturas para a Ribeira dos Milagres - tornou pública a petição, na qual acusava as autoridades portuguesas de não tomarem providências para a resolução do problema (…)

RTP notícias: December 6th, 2006

Nova descarga de efluentes poluentes na última madrugada

Ambiente e Defesa da Ribeira dos Milagres, concelho de Leiria, apresentou hoje queixa na GNR, depois de mais uma descarga de efluentes poluentes para aquele curso de água, registada durante a madrugada.

(…) a descarga "foi detetada durante a manhã de hoje", com a Ribeira dos Milagres a apresentar "espuma branca, água preta e um cheiro nauseabundo". "Pelo cheiro, não há dúvidas quanto à origem: é de suinicultura" (…)

RTP notícias: July 10th, 2007

Autoridades lançam campanha de fiscalização que pode encerrar suiniculturas

As autoridades iniciaram hoje em todo um país uma campanha de fiscalização das suiniculturas e caso sejam detectadas anomalias graves do ponto de vista ambiental algumas explorações serão encerradas.

(…) A região de Leiria é considerada uma das zonas mais críticas do ponto de vista ambiental devido à poluição suinícola pelo que o Governo deu recentemente prioridade a um projeto de despoluição da bacia do Rio Lis, através da construção de uma estação de tratamento e de uma rede de condutas, que serão geridas pelos próprios empresários. No total, a região tem cerca de 300 mil porcos espalhados por 900 explorações legais e cerca de 300 que não estão em situação regular (…)

Jornal de Notícias: July 18th, 2007

Recolha de assinaturas pelo Rio Lis

(…) um ambientalista (…) decidiu iniciar uma recolha de assinaturas. A intenção é mostrar o seu "descontentamento", e de quem se quiser associar, pela "poluição que atinge o Rio Lis, provocada pelas descargas suinícolas" (…)

RTP notícias: May 10th, 2008

GNR chamada para nova descarga poluente na Ribeira dos Milagres

Os serviços de Proteção da Natureza da GNR de Leiria foram hoje chamados para uma nova descarga de efluentes suinícolas na Ribeira dos Milagres, cujo alerta foi dado por uma associação de ambiente local.

(…) as descargas "têm sido frequentes" porque os empresários do sector utilizam a chuva para diluir os seus efluentes (…) A descarga de hoje foi mais um capítulo da história da ribeira, marcada por vários atentados ambientais atribuídos ao sector suinícola, que se debate com falta de locais onde fazer os despejos dos efluentes. Os problemas ambientais da ribeira levaram mesmo o Estado a atribuir apoios suplementares para uma empresa que irá criar e gerir um sistema de saneamento das explorações (…) Esta semana, foi aprovada a localização em Amor da nova Estação de Tratamento de Efluentes Suinícolas (ETES), que a empresa gestora quer construir no âmbito do projeto global de recuperação da bacia hidrográfica do Rio Lis. O projeto deverá estar concluído dentro de dois anos (…)

RTP notícias: September 6th, 2008

Comissão denuncia descarga poluente "impressionante" na Ribeira dos Milagres

A Comissão de Ambiente e Defesa da Ribeira dos Milagres denunciou hoje a existência de uma descarga "impressionante" de efluentes suinícolas naquele curso de água, afluente do Rio Lis.

RTP notícias: January 21st, 2009

Ministério do Ambiente pressiona Leiria com reforço da fiscalização

As entidades fiscalizadoras e inspetivas do Ministério do Ambiente abriram no ano passado 37 processos de contraordenação contra as suiniculturas da região do Lis, um número que representa 58 por cento dos processos abertos desde 2005, segundo dados do ministério a que a Lusa teve acesso (…) "Em 2003 a Ribeira dos Milagres era um esgoto. No ano passado houve nove queixas [por causa de descargas ilegais] e a GNR presumiu que apenas cinco delas foram das suiniculturas" (…)

RTP notícias: July 23rd, 2009

Descarga poluente afeta de novo Ribeira dos Milagres

A Comissão Ambiente e Defesa da Ribeira dos Milagres alertou hoje para a ocorrência de uma nova descarga poluente para a Ribeira dos Milagres, no concelho de Leiria. José Carlos Faria, membro da comissão, disse que "a descarga foi feita durante a noite, aproveitando a chuva", sendo que pela manhã ainda era visível "água turva, preta e com espuma". A jornalista Ana Isabel Costa traz-nos mais pormenores desta notícia.

RTP notícias: April 21st, 2010

Nova descarga poluente para a Ribeira dos Milagres

A Comissão Ambiente e Defesa da Ribeira dos Milagres alertou hoje para uma nova descarga poluente, durante a madrugada, na Ribeira dos Milagres, concelho de Leiria.

(…) esta descarga assume semelhanças com anteriores, dado ocorrer "à noite ou durante a madrugada" e sempre que "as condições meteorológicas apontam chuva", como foi o caso de terça feira.

Tinta Fresca: April 25th, 2010

Ambientalistas mascaram-se de porcos em protesto contra poluição na bacia do Rio Lis

Concentração junto à ponte da Ribeira dos Milagres.

RTP notícias: October 28th, 2011

Comissão de defesa da Ribeira dos Milagres denuncia atentado ambiental

A Comissão Ambiente e Defesa da Ribeira dos Milagres (CADRM) denunciou hoje "um atentado ambiental" em resultado de uma nova descarga poluente naquela ribeira, em Leiria

(…) o Ministério, na ocasião, não respondeu à questão colocada pela Lusa sobre a ETES de Amor, não se sabendo qual o ponto de situação atual de um projeto prometido há mais de uma década e que resolveria o problema dos efluentes suinícolas na região de Leiria (…)

RTP notícias: November 12th, 2011

Associação quer reunir com ministra do Ambiente após nova descarga poluente na Ribeira dos Milagres

A Comissão Ambiente e Defesa da Ribeira dos Milagres (CADRM) pediu hoje uma audiência à ministra do Ambiente após nova descarga poluente denunciada pela associação esta madrugada, a quarta em menos de três semanas.

A audiência solicitada (…) é motivada pelo "aumento de descargas tanto na frequência como na intensidade", bem como "uma inércia crescente por parte das entidades com responsabilidade nesta atividade e das autoridades e poder político local" (…)

RTP notícias: February 18th, 2013

Associação de Leiria denuncia nova descarga poluente na Ribeira dos Milagres

A Comissão Ambiente e Defesa da Ribeira dos Milagres (CADRM) denunciou hoje uma nova descarga poluente naquele afluente do Rio Lis, em Leiria.

(…) Em novembro, (…) estava prevista uma verba no Programa de Desenvolvimento Rural (Proder) de cerca de 10 milhões de euros, "para poder ajudar a suportar uma parte da construção da infraestrutura das suiniculturas da região de Leiria, que é um problema crítico ambiental que tem muitos anos" (…) o processo se arrasta há 13 anos.

Público: January 21th, 2014

Nova descarga de efluentes de suiniculturas na Ribeira dos Milagres

O ano de 2014 não trouxe nenhuma alteração ao panorama de descargas sistemáticas neste ribeiro. GNR vai investigar.

(…) As descargas para a Ribeira dos Milagres ocorrem há várias décadas, prevendo-se que o problema seja resolvido com a construção de uma ETES. A 28 de junho de 2013, a ministra da Agricultura, Assunção Cristas, presidiu à assinatura de um protocolo que previa construir a ETES em dois anos (…)

SIC notícias: August 14th, 2014

Porto Canal: February 5th, 2015

GNR identifica suinícola que fez descarga para a Ribeira dos Milagres, Leiria

(…) As descargas para a Ribeira dos Milagres ocorrem há várias décadas, prevendo-se que o problema seja resolvido com a construção da ETES (…) No final do ano passado, foi anunciado um financiamento de 45% por parte do ProDer (…) cujo concurso público deverá ser lançado em breve.

Since the 90's, associations to protect the environmental water of the Leiria region were created (PAeM 2015). In this context, meetings with political rulers and manifestations have organized and held by these associations with the support of the citizens and environmentalist. The *Associação de Defesa do Ambiente e Património da Região de Leiria* (OIKOS) and the *Comissão Ambiente e Defesa da Ribeira dos Milagres* (CADRM) have protested against pollution of water in the Ribeira dos Milagres caused by pig farms located in the region, in order to alert the authorities and the government to this problem and appeal to its effective resolution, as well as raise the awareness of the pig farmers for the environmental impacts caused by the discharge of swine effluents in the local rivers and subsequent effects on human health. Despite the efforts, episodes of water pollution of the river by pig waste Lis continued.

In 2006, the Minister of Environment announced the creation of a working group to define a national strategic plan for agricultural wastewater (*Estratégia Nacional para os Efluentes Agro-Pecuários e Agro-Industriais*, ENEAPAI), as well as a consortium to build a swine wastewater treatment plant (*Estação de Tratamento de Efluentes Suinícolas,* ETES) in Amores civil parish, Leiria. The ETES was projected to pre-treat the swine effluents, being subsequently forwarded to a WWTP, ETAR Norte, where they will be treated and then discharged into the Rio Lis. As a result, the discharge of untreated effluents in waterways and soils can be avoided. At the same time, the energy could be recovered and organic matter could be valorized in the projected ETES (Câmara Municipal de Leiria 2010). Although the initial operation of this infrastructure have been predicted for 2008, its conclusion has constantly been delayed due to economic issues, as can be seen in information reported by Portuguese media. Some of these news are cited below. Currently, the government considers that the conclusion of building of ETES in Leiria is a priority, since the contamination of the river Lis by pig waste continues to occur.

RTP notícias: December 28th, 2006

Primeira ETAR para suiniculturas será adjudicada em Janeiro

O processo de saneamento das suiniculturas na região Oeste vai dar o primeiro passo concreto em Janeiro, com a adjudicação da primeira Estação de Tratamento de Águas Residuais (ETAR) do Oeste, anunciou o governador civil de Leiria.

(…) A ETAR de São Martinho do Porto (…) será a primeira a ser construída de acordo com o projeto global de saneamento do sector que deverá estar concluído até Julho de 2009 (…) No Oeste, existem cerca de 500 suinicultores com cerca de 290 mil animais, a maior parte deles concentrados nos concelhos de Alcobaça e Caldas da Rainha (…)

RTP notícias: August 7th, 2008

Estação de tratamento de efluentes suinícolas deverá arrancar no fim deste ano

O presidente da Recilis, entidade que vai construir a ETES de Leiria, rejeitou hoje responsabilidades no atraso da sua construção, que deverá começar no final do ano ou início de 2009.

(…) A estação de tratamento vai ficar instalada na Freguesia de Amor, Concelho de Leiria, e a sua conclusão está prevista para "final de 2009 ou início de 2010". O projeto envolve um custo total superior a 30 milhões de euros e pretende resolver o problema de mais de 70 por cento da carga poluente do Rio Lis. No total, a Recilis estima em 270 mil o número de porcos na região, que produzem efluentes equivalentes a mais de um milhão de pessoas, recordando que o sector movimenta localmente por ano 600 milhões de euros e suporta dois mil postos de trabalho.

Público: January 30th, 2009

Leiria: "trauma" ambiental das suiniculturas será resolvido dentro de dois anos

O presidente da Recilis, entidade criada para resolver o problema da poluição dos efluentes suinícolas na bacia hidrográfica do Rio Lis, assegurou que o "trauma" das suiniculturas "vai ficar resolvido dentro de dois anos".

(…) A primeira solução passa pelo tratamento do efluente em estações próprias, a segunda contempla o espalhamento em terrenos agrícolas ou florestais e a terceira são descargas controladas na Ribeira dos Milagres (…) A Autoridade de Saúde de Leiria acrescentou que "a problemática das suiniculturas é um dos mais graves problemas de saúde pública do concelho de Leiria" (…)

Público: September 14th, 2009

Estação de efluentes suinícolas em Leiria aguarda aprovação do ProDer

A construção da estação de tratamento de efluentes suinícolas (ETES) de Leiria, que deverá pôr fim às descargas ilegais para a Ribeira dos Milagres, aguarda aprovação da candidatura por parte do Proder.

RTP notícias: November 6th, 2012

Recilis não tem dinheiro restante para estação de tratamento na Ribeira dos Milagres mas está a trabalhar

A Recilis, que é a empresa responsável pela gestão dos efluentes suinícolas da região do Lis e que integra a associação dos suinicultores da região, afirma que não tem o dinheiro restante para a construção de uma ETES para resolver as descargas na Ribeira dos Milagres.

RTP notícias: November 11td, 2014

Financiamento da estação de efluentes suinícolas de Leiria aprovado pelo ProDer

O financiamento para a construção da ETES de Leiria, que deverá pôr cobro às descargas para a Ribeira dos Milagres, foi aprovado pelo ProDer - Programa de Desenvolvimento Rural, disse hoje um dos parceiros.

(…) A ETES do Lis, projetada para a freguesia de Amor, vai tratar cerca de 900 metros cúbicos de efluente diário que, "a acrescer aos 280 m^3 já instalados na ETAR Norte (…) irá perfazer uma capacidade total de 1.180 m3 por dia". "Agrega mais de 400 explorações pecuárias em toda a bacia hidrográfica do Rio Lis" (…)

Confragi: December 12th, 2014

Ministra da Agricultura diz que regadio do Vale do Lis é «prioritário»

A ministra da Agricultura, Assunção Cristas, afirmou que o regadio do Vale do Lis, em Leiria, está inscrito como «prioritário». À margem da entrega formal do contrato de financiamento aprovado pelo ProDer, a todos os parceiros envolvidos na construção da ETES de Leiria, Assunção Cristas afirmou que o governo já tem aprovado «o plano estratégico dos regadios» (…)

In fact political rulers, environmental authorities, and population are mainly concerned with pollution caused by organic matter (determined by the chemical oxygen demand (COD) and 5-day biochemical oxygen demand (BOD_5)) and nitrogen and phosphorus compounds present in wastewater discharged into the rivers (MAMAOT & ARH Centro 2012), not being aware of the serious threat that the presence of antibiotics, ARB, and ARG in the environment may represent for humans and ecosystems. Thus, it should be noted that the projects in the implementation stage, particularly ETES, should consider the effectiveness of the elimination of antibiotics during the treatment process.

Taking into account the information presented in this annex, it is believed that the analysis and monitoring of amoxicillin and doxycycline in the aquatic environment the water basin of Lis, in particular the Ribeira dos Milagres, could contribute to better understand the impact that the common swine production practices related to the use of veterinary antibiotics have on the environment. Therefore, locations in the water basin of Lis basin to collect water samples in future works are proposed in the annex A1.5.

A1.5 Sampling in water basin of Lis

Located in the central region of continental Portugal, the water basin of Lis belongs to the HR 4 (*Região Hidrogrográfica 4, RH 4 - Vouga, Mondego, Lis e Ribeiras do Oeste*) under the jurisdiction of the *Administração da Região Hidrográfica do Centro, I.P.* (ARH Centro), according to the provisions of Decree Law No. 347/2007 (MAOTDR 2007). The geographical area of this basin is approximately 945 km^2, being delimited between by the parallels 39º 30' and 40º 00' latitude north, and meridians 8º 35' and 8º 58' longitude west. Rio Lis is located in water basin of Lis and has approximately 40 km. Its headwater is in village of Fontes (Leiria) and its stream is into the Atlantic Ocean at the northern of beach of Vieira. The main tributaries of the river Lis are Ribeiras do Sirol, Ribeira dos Milagres, and Rio Fora on the right bank and Rio Lena and Coletor de Amor on the left bank (Vieira 2007; APA & ARH Centro 2009).

As mentioned in annex A1.4, pig production in water basin of Lis is the high, constituting a very high pressure source in the aquatic environment of the region. Therefore, this Portuguese region are an interesting local of study to analyze and monitor antibiotics routinely used in swine production. Figure A1.9 shows five proposal locations (S1 – S5) for the collection of water samples to analyze the presence of analytes amoxicillin and doxycycline in the water basin of Lis. The choice presented considered the location of WWTP and de sites of direct discharged from swine production (MAMAOT & ARH Centro 2012). It should be noted that the identification and analysis of amoxicillin and doxycycline in real water samples are beyond the scope of this study but could be the objective of future research.

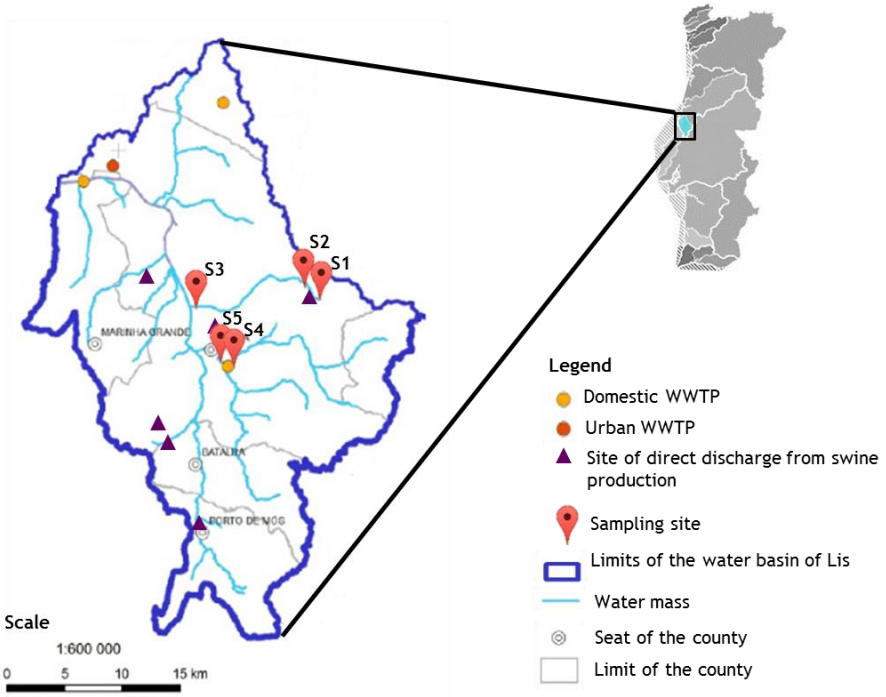

Figure A1.8. Map of the water basin of Lis identifying the proposal sampling location to analyze and monitor amoxicillin and doxycycline in environmental water, the domestic and urban WWTP, and the sites of direct discharged from swine production.

S1 and S2 are located on the Ribeira dos Milagres, being upstream and downstream of the discharge of swine production effluent, respectively. S3 is where the Ribeira dos Milagres flows into the Rio Lis, being a sample of surface water as S1 and S2. S4 and S5 are effluent and tributary of Olhalvas WWTP that treats swine production effluents, respectively (MAMAOT & ARH Centro 2012).

A2 Supplementary content to the state of art

Table A2.1.Methods for detection and analysis of amoxicillin or doxycycline, among other drugs, in aqueous environmental matrixes.

Location	Matrix / Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
Africa								
Kenya	Wastewater Surface water AMOX and other pharmaceuticals	AMOX = n.p. AMOX = n.p.	V = 500 mL Filtration pH 7 (NaOH) Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (200 mg) ***Condition:*** 5 mL CH_4O 5 mL ultrapure H_2O ***Washing:*** 5mL ultrapure H_2O ***Elution:*** 2x 5mL CH_4O	AMOX = n.p. AMOX = n.p.	**LC-ESI-MS** ***Stationary phase:*** Luna C18 (150 x 2.0 mm, 3 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_4O	LOD_{AMOX} = n.p.	(K'oreje et al. 2012)
America (USA)								
	Surface water Ground water AMOX and other pharmaceuticals	AMOX = n.d. AMOX = n.d.	V = 1,000 mL Filtration	**SPE** ***Sorbent:*** Oasis HLB (500 mg) ***Condition:*** 6 mL CH_4O 6 mL ultrapure H_2O ***Washing:*** 1 mL 5% CH_4O ***Elution:*** 2x 3 mL CH_4O 2x 2 mL CH_4O (pH 3.7, TFA)	AMOX = n.d.	**LC-ESI-MS** ***Stationary phase:*** MetaSil C18 (150 × 2.0 mm, 3 µm) ***Mobile phase:*** **Gradient elution** (1) 10 mM CH_5NO_2 in CH_2O_2 (pH 3.7) (2) CH_3CN	LOD_{AMOX} = n.p.	(Cahill et al. 2004)
	Wastewater Surface water AMOX and other ß-lactam antibiotics	AMOX = n.d. AMOX = n.d.	V = 200 mL Centrifugation Filtration Na_2EDTA addition pH 7.5 (NH_4OH)	**SPE** ***Sorbent:*** Oasis HLB (60 mg) ***Condition:*** 3 mL CH_4O 3 mL 0.5 N HCl 3 mL deionized H_2O ***Washing:*** 3 mL deionized H_2O ***Elution:*** 5 mL CH_4O	AMOX = 10 - 33	**LC-ESI-MS** ***Stationary phase:*** Xterra MS C18 (50 x 2.1 mm, 2.5 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_4O (3) CH_3CN	LOD_{AMOX} = n.p.	(Cha et al. 2006)
	Drinking water DOX and other antibiotics (different classes)	DOX = n.p.	V = 500 mL $C_6H_8O_6$ addition Filtration Na_2EDTA addition pH 3	**SPE** ***Sorbent:*** Oasis HLB (200 mg) ***Condition:*** 6 mL CH_4O 6 mL 0.1% CH_2O_2 in CH_4O 2x 6 mL H_2O ***Washing:*** 2x 6 mL H_2O ***Elution:*** 4x 2 mL 0.1% CH_2O_2 in CH_4O	DOX = 54	**LC-ESI-QqQ-MS/MS** ***Stationary phase:*** Pursuit C18 (150 x 2 mm, 3 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_3CN	LOD_{DOX} = 1.0 LOQ_{DOX} = 3.5	(Ye et al. 2007)
	Wastewater Surface water Drinking water DOX and other pharmaceuticals	DOX = n.p.	V = 200 mL	**SPE** ***Sorbent:*** Oasis HLB (500 mg) ***Condition:*** 4 mL CH_4O 6 mL ultrapure H_2O ***Washing:*** n.a. ***Elution:*** 5 mL CH_4O	DOX = n.d.	**LC-ESI-QqQ-MS/MS** ***Stationary phase:*** Zorbax C18 (100 x 2.1 mm, 3.5 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_3CN	LOD_{DOX} = 100	(Ferrer et al. 2010)

Table A2.1. Methods for detection and analysis of amoxicillin or doxycycline, among other drugs, in aqueous environmental matrixes (*continuation*)**.**

Location	Matrix Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
America (USA)								
	Surface water Ground water DOX and other antibiotics (different classes)	DOX = n.p. DOX = n.p.	V = 123 mL Filtration H_2SO_4 addition Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (60 mg) ***Condition:*** 3 mL CH_4O 3 mL 0.5 N HCl 3 mL distilled H_2O ***Washing:*** 20 mL destilled H_2O ***Elution:*** 5 mL CH_4O	DOX = 101	**LC-ESI-MS** ***Stationary phase:*** Luna C8(2) (100 x 4.6 mm, 3 µm) ***Mobile phase:*** **Gradient elution** (1) 10 mM CH_5NO_2 in H_2O/ CH_4O, 90:10 (v/v) with 0.3% CH_2O_2 (2) 10 mM CH_5NO_2 in CH_4O with 0.5% CH_2O_2	LOD_{DOX} = 100	(Lindsey et al. 2001)
America (Center)								
Costa Rica	Surface water DOX and other pharmaceuticals	DOX = 74 [1] DOX = 73,722 [2]	V = 350 mL Filtration Na_2EDTA addition pH 5 (H_2SO_4 or NH_4OH)	**SPE** ***Sorbent:*** Strata X (200 mg) ***Condition:*** 2 mL CH_4O 3x 2 mL 1% Na_2EDTA ***Washing:*** 2 mL 5% CH_4O ***Elution:*** 2x 3 mL CH_4O	DOX = 75 - 79	**LC-ESI-QqQ-MS/MS** ***Stationary phase:*** Supelco Discovery HS C18 (150 x 4.6 mm, 3 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_3CN	LOQ = 18 – 1,693	(Spongberg et al. 2011)
America (South)								
Brazil	Surface water AMOX and other antibiotics (different classes)	AMOX = 4.0 – 1,284	V = 1,000 mL Filtration pH 2.4 (HCl) Na_2EDTA addition	**SPE** ***Sorbents in series:*** (A) Strata SAX (500 mg) (B) Oasis HLB (500 mg) ***Condition:*** 6 mL CH_4O 6 mL ultrapure H_2O 6 mL 37% HCl (pH 2.4) ***Washing:*** (B) 6 mL deionized H_2O ***Elution:*** (B) 6 mL CH_4O	AMOX = 16.8 - 17.6	**LC-ESI-QqQ-MS/MS** ***Stationary phase:*** Zorbax SB C18 (30 x 2.1 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) deionized H_2O with 0.1% CH_2O_2 (2) CH_3CN with 0.1% CH_2O_2	LOD_{AMOX} = 0.14 LOQ_{AMOX} = 0.46	(Locatelli et al. 2011)

Table A2.1. Methods for detection and analysis of amoxicillin or doxycycline, among other drugs, in aqueous environmental matrixes (*continuation*)**.**

Location	Matrix Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
Asia								
China	Wastewater Surface water DOX and other tetracycline antibiotics	DOX = n.p. DOX = n.p.	V = 150 – 900 mL Filtration Na_2EDTA addition pH 3 (HCl)	**2 sequential SPE** **SPE (A)** ***Sorbent:*** Oasis HLB (500 mg) ***Condition:*** 6 mL CH_2Cl_2 6 mL CH_4O 6 mL ultrapure H_2O with 0.5 g/L Na_2EDTA ***Washing:*** 6 mL ultrapure H_2O ***Elution:*** 6 mL CH_4O **SPE (B)** ***Sorbent:*** Oasis MAX (60 mg) ***Condition:*** 1 mL CH_4O 1 mL 5 N NaOH 1 mL ultrapure H_2O ***Washing:*** 1 mL 5% $NH_3 \cdot H_2O$ 1 mL CH_4O ***Elution:*** 3 mL 50% CH_4O with 1% CH_2O_2	DOX = 80 – 113	**LC-ESI-QqQ-MS/MS** ***Stationary phase:*** Acquity UPLC BEH C18 (100 x 2.1 mm, 1.7 µm) ***Mobile phase:*** **Gradient elution** (1) ultrapure H_2O with 0.1% CH_2O_2 (2) CH_3CN	LOD_{DOX} = 2.3 – 5.4 LOQ_{DOX} = 6.9 – 16.4	(Jia et al. 2009)
	Surface water DOX and other tetracycline antibiotics	DOX = n.p.	V = 4 mL	**Dispersive-SPME** ***Sorbent*:** Supelclean PSA (30 mg) ***Desorption:*** 300 µL CH_3CN/70-72% $HClO_4/H_2O$, 10:2:88 (v/v/v)	DOX = 100.6 – 104.1	**HPLC-DAD** ***Stationary phase:*** Intersil ODS (150 × 4.6 mm, 2.5 µm) ***Mobile phase:*** **Gradient elution** (1) 10 mM $C_2H_2O_4$ (2) CH_3CN	LOD_{DOX} = 3,500	(Tsai et al. 2009)
	Surface water DOX and other antibiotics (different classes)	DOX = 5.61 – 46.93	V = 1,000 mL Filtration pH 3.0 (H_2SO_4) Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (500 mg) ***Condition:*** 6 mL CH_4O 6 mL ultrapure H_2O (pH 3) ***Washing:*** 6 mL ultrapure H_2O (pH 3) ***Elution:*** 3x 3 mL CH_4O	DOX = 92	**LC-ESI-MS** ***Stationary phase:*** X Bridge C18 (150 x 2.1 mm, 3.5 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_3CN	LOD_{DOX} = 1.51 LOQ_{DOX} = 5.03	(Jiang et al. 2011)
	Wastewater Surface water DOX and other antibiotics (different classes)	DOX = 110 – 39,500 DOX = n.d.	V = 100 – 200 mL Filtration pH 7 (H_2SO_4 or NaOH) 5% Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (500 mg) ***Condition:*** 6 mL 10 mM Na_2EDTA (pH 7) ***Washing:*** 10 mL ultrapure H_2O ***Elution:*** 8 mL CH_4O	DOX = 84.8 – 87.2	**LC-ESI-QqQ-MS/MS** ***Stationary phase:*** Zorbax RX C8 (150 x 2.1 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_3CN with 0.1% CH_2O_2	LOQ_{DOX} = 50	(Wei et al. 2011)

Table A2.1. Methods for detection and analysis of amoxicillin or doxycycline, among other drugs, in aqueous environmental matrixes (*continuation*)**.**

Location	Matrix Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
Asia								
China	Wastewater DOX and other antibiotics (different classes)	DOX = 10 – 685,600	V = 500 mL Filtration pH < 3 (H_2SO_4) Na_2EDTA addition	**SPE** ***Sorbents in series:*** (A) SAX (500 mg) (B) Oasis HLB (500 mg) ***Condition:*** 10 mL CH_4O 10 mL ultrapure H_2O ***Washing:*** 10 mL ultrapure H_2O ***Elution:*** 0.1% CH_2O_2	DOX = 62.5 – 69.7	**UPLC-MS/MS** ***Stationary phase:*** Acquity UPLC BEH C18 (100 x 2.1 mm, 1.7 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_4O with 0.1% CH_2O_2	LOD_{DOX} = 0.1	(Chen et al. 2012)
	Wastewater DOX and other antibiotics (different classes)	DOX = 46 – 262	V = 200 - 1,000 mL Filtration pH 3 (H_2SO_4) Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (500 mg) ***Condition:*** 10 mL CH_4O 10 mL ultrapure H_2O ***Washing:*** 10 mL ultrapure H_2O ***Elution:*** 12 mL CH_4O	DOX = 74 - 141	**RRLC-ESI-QqQ-MS/MS** ***Stationary phase:*** Eclipse Plus C18 (100 x 2.1 mm, 1.8 µm) ***Mobile phase:*** **Gradient elution** (1) ultrapure H_2O with 0.2% CH_2O_2 and 2 mM $C_2H_7NO_2$ (2) CH_3CN Or (1) H_2O (2) CH_3CN	LOD_{DOX} = 1.18 – 18.5 LOQ_{DOX} = 4.27 – 61.7	(Zhou et al. 2012; Zhou et al. 2013)
Iran	Wastewater AMOX and other antibiotics (different classes)	AMOX = n.p.	Centrifugation Filtration	**Direct injection**	n.a.	**HPLC-DAD** ***Stationary phase:*** RP18 (70 × 4.6 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) 50 mM PBS (pH 3) (2) CH_4O	LOD_{AMOX} = 1,300 – 5,000	(Vosough et al. 2015)
	Drinking water DOX and other tetracycline antibiotics	DOX = n.p.	V = 11 mL Na_2HPO_4 (pH 9.1-9.5) addition	**HF-LPME** ***Fiber membrane:*** Accurel Q3/2 polypropylene (8.8 cm x 600 µm x 200 µm, 0.2 µm) ***Membrane phase:*** 10% (w/v) Aliquat-336 in $C_8H_{18}O$ ***Receiving phase:*** 25 µL 0.1 M H_3PO_4 and 1.0 M NaCl (pH 1.6)	DOX = 24.8	**HPLC-UV/Vis** ***Stationary phase:*** Supelcosil C18 (150 × 4.6 mm, 3 µm) ***Mobile phase:*** **Gradient elution** (1) 0.05 M $C_2H_2O_4$ (pH 2.4) (2) CH_3CN (3) CH_4O	LOD_{DOX} = 1,000	(Shariati et al. 2009)

Table A2.1. Methods for detection and analysis of amoxicillin or doxycycline, among other drugs, in aqueous environmental matrixes (*continuation*)**.**

Location	Matrix / Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
Asia								
Hong Kong	Surface water Ground water Sea water AMOX and other antibiotics (different classes)	AMOX = n.d. AMOX = n.d. AMOX = n.d.	V = 250 mL Filtration pH 3 (H_2SO_4) Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (500 mg) ***Condition:*** 6 mL CH_4O 6 mL ultrapure H_2O 6 mL 10 mM Na_2EDTA (pH 3) ***Washing:*** 10 mL ultrapure H_2O (pH 3) ***Elution:*** 3x 2 mL CH_4O	AMOX = 61 - 67	**LC-ESI-MS/MS** ***Stationary phase:*** ODS-P (250 × 4.6 mm, 3.5 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.2% CH_2O_2 (2) CH_3CN	LOQ_{AMOX} = 3.2 - 20	(Xu et al. 2007)
	Wastewater Sea water AMOX and other antibiotics (different classes)	AMOX = 66 – 1,330 AMOX = 0.74 - 76	V = 1,000 mL Filtration Na_2EDTA addition pH 3 – 3.5 (CH_2O_2)	**SPE** ***Sorbent:*** Oasis HLB (200 mg) ***Condition:*** 4 mL CH_4O 4 mL ultrapure H_2O ***Washing:*** 2x 2 mL ultrapure H_2O ***Elution:*** 2x 2 mL CH_4O	AMOX = 66 - 75	**LC-ESI-QqQ- MS/MS** ***Stationary phase:*** XBridge C18 (50 x 2.1 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) ultrapure H_2O with 10 mM CH_2O_2 (2) CH_4O with 10 mM CH_2O_2	LOD_{AMOX} = 0.35 - 13	(Minh et al. 2009)
Europe								
Belgium	Wastewater AMOX and other pharmaceuticals	AMOX = n.d.	V = 50 - 100 mL Filtration pH 7 (NaOH) Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (200 mg) ***Condition:*** 6 mL CH_4O 6 mL deionized H_2O ***Washing:*** 4x 6 mL deionized H_2O ***Elution:*** 5 mL CH_4O	AMOX = n.d.	**LC-ESI-HRMS** ***Stationary phase:*** Luna C18(2) (150 x 2.0 mm, 3 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_4O with 0.1% CH_2O_2 Or (1) H_2O (2) CH_3CN	LOD_{AMOX} > 25,000 LOQ_{AMOX} > 25,000	(Vergeynst et al. 2015)
France	Surface water AMOX and other antibiotics (different classes)	AMOX = 68	V = 500 mL Filtration Na_2EDTA addition pH 4 (H_3PO_4) or pH 7 (NaOH)	**SPE** ***Sorbent:*** C18 HD (2 mm x 10 mm) ***Condition:*** 3 mL CH_4O 3 mL ultrapure H_2O (pH 4 or 7) ***Washing:*** n.a. ***Elution:*** 1.8 mL ultrapure H_2O (pH 4 or 7)	AMOX = 64 - 70	**LC-ESI-QqQ-MS/MS** ***Stationary phase:*** Zorbax C18 (150 x 2.1 mm, 3.5 µm) ***Mobile phase:*** **Gradient elution** (1) ultrapure H_2O with 0.1% CH_2O_2 (2) CH_3CN with 0.1% CH_2O_2	LOD_{AMOX} = 12.0 LOQ_{AMOX} = 39.2	(Tuc Dinh et al. 2011)

Table A2.1. Methods for detection and analysis of amoxicillin or doxycycline, among other drugs, in aqueous environmental matrixes (*continuation*)**.**

Location	Matrix Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
Europe								
Germany	Wastewater Surface water Ground water DOX and other antibiotics (different classes)	DOX = n.d. DOX = n.d. DOX = n.d.	V = 100 mL Filtration Na_2EDTA addition	Lyophilization	n.a.	**LC-ESI-MS/MS** ***Stationary phase:*** n.p. ***Mobile phase:*** n.p.	LOQ_{DOX} = 50	(Hirsch et al. 1999)
Portugal	Wastewater DOX and other tetracycline antibiotics AMOX, DOX, and other pharmaceuticals	DOX = 8,100 [2]	V = 250 mL Filtration pH 3.4 (CH_2O_2) Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (200 mg) ***Condition:*** 5 mL CH_4O 5 mL deionized H_2O 5 mL CH_2O_2 buffer (pH 3.4) ***Washing:*** 10 mL 5% CH_4O ***Elution:*** 1% TFA in CH_4O	DOX = 89.8 -117.1	**HPLC-FL** ***Stationary phase:*** Lichrosorb RP8 (250 × 4.0 mm, 10 µm) ***Mobile phase:*** **Gradient elution** (1) 0.02 M $C_2H_2O_4$ (2) CH_3CN	LOQ_{DOX} = 5,000	(Pena et al. 2010)
Spain	Wastewater AMOX and other β-lactam antibiotics	AMOX = n.p.	V = 250 mL Filtration pH 7.5	**SPE** ***Sorbent:*** Oasis MAX (500 mg) ***Condition:*** 6 mL CH_4O 6 mL ultrapure H_2O 6 mL 0.05 M PBS (pH 7.5) ***Washing:*** 6 mL 0.05 M PBS (pH 7.5, 5% CH_4O) ***Elution:*** 2× 1 mL 0.05 M 98% TBA in CH_4O	AMOX = n.d. - 54	**HPLC-DAD** ***Stationary phase:*** Luna C18 (150 × 4.6 mm, 3.5 µm) ***Mobile phase:*** **Gradient elution** (1) ultrapure H_2O with 0.01% TFA (2) CH_3CN with 0.01% TFA	LOD_{AMOX} = n.d. – 5,900	(Benito-Peña et al. 2006)
	Surface water Drinking water AMOX and other β-lactam antibiotics	AMOX = n.p. AMOX = n.p.	V = 500 mL pH 5.5 Filtration	**SPE** ***Sorbent:*** Amberlite IRA-93 (200 mg) ***Condition:*** 20 mL ultrapure H_2O (pH 5.5) ***Washing:*** n.a. ***Elution:*** 500 mL derivatization buffer [3] with 15 mM TBAB	AMOX = 97.1	**MEKC-LIF** ***Stationary phase:*** Silica capillary (50 cm × 50 µm) ***Mobile phase:*** **Electrolyte** 35 mM sodium borate (pH 9.3; 0.5M HCl) 15 mM SDS	LOD_{AMOX} = 45	(Serrano & Silva 2007)
	Wastewater Surface water Ground water AMOX and other β-lactam antibiotics	AMOX = n.p. AMOX = n.p. AMOX = n.p.	V = 100 mL Filtration	**SPE** ***Sorbents in parallel:*** (A) Oasis HLB (60 mg) (B) Alumina N (500 mg) ***Condition:*** (A) 2 mL CH_4O 3 mL CH_3CN 5 mL deionized H_2O (B) 5 mL deionized H_2O 5 mL CH_3CN ***Washing:*** n.a. ***Elution:*** (A) 6 mL CH_3CN (B) 3 mL deionized H_2O	AMOX = 94 - 97	**CE-DAD** ***Stationary phase:*** Silica capillary (64.5 cm × 75 µm) ***Mobile phase:*** **Electrolyte** 175 mM THAM (pH 8, 1 M HCl with 20% C_2H_6O)	LOD_{AMOX} = 800 LOQ_{AMOX} = 2,000	(Bailón-Pérez et al. 2008)

Table A2.1. Methods for detection and analysis of amoxicillin or doxycycline, among other drugs, in aqueous environmental matrixes (*continuation*)**.**

Location	Matrix / Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
Europe								
Spain	Surface water Ground water AMOX and other ß-lactam antibiotics	AMOX = n.p. AMOX = n.p.	V = 250 mL Filtration	**SPE** ***Sorbent:*** Oasis HLB (60 mg) ***Condition:*** 2 mL CH_4O 3 mL CH_3CN 5 mL deionized H_2O ***Washing:*** n.a. ***Elution:*** 6 mL CH_3CN	AMOX = 92.5 - 97.1	**HPLC-DAD** ***Stationary phase:*** Luna C18(2) (150 × 0.5 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) ultrapure H_2O with 0.01% TFA (2) CH_3CN with 0.01% TFA	LOD_{AMOX} = 60 LOQ_{AMOX} = 200	(Bailón-Pérez et al. 2009)
UK	Wastewater Surface water AMOX and other pharmaceuticals	AMOX < LOQ AMOX < LOQ	V = 200- 1,000 mL pH 2 (HCl) Filtration	**SPE** ***Sorbent:*** Oasis MCX (60 mg) ***Condition:*** 6 mL CH_4O 2 mL 2% CH_2O_2 ***Washing:*** 2 mL CH_4O ***Elution:*** 2 mL 5% NH_4OH in CH_4O	AMOX = 16 - 41	**UPLC-ESI- QqQ-MS/MS** ***Stationary phase:*** Acquity UPLC BEH C18 (100 x 1 mm, 1.7 µm) ***Mobile phase:*** **Gradient elution** (1) $H_2O/CH_4O/C_2H_4O_2$, 94.5:5:0.5 (v/v/v) (pH 2.8) (2) $H_2O/ CH_4O/C_2H_4O_2$, 0:95.5:0.5 (v/v/v) (pH 3.2)	LOD_{AMOX} = 2.5 LOQ_{AMOX} = 12 - 87	(Kasprzyk-Hordern et al. 2008)
	Wastewater Surface water Drinking water AMOX, DOX, and other antibiotics (different classes)	AMOX = 50 – 6,940 [2] DOX = 150 - 650 [2] AMOX = 200 DOX = 400 AMOX = n.d. DOX = n.d.	V = 200 – 1,000 mL Filtration pH 3 (H_2SO_4) Na_2EDTA addition	**SPE** ***Sorbent:*** Oasis HLB (200 or 500 mg) ***Condition***: n.a. ***Washing:*** 3 mL CH_4O ***Elution:*** 2x 2 mL CH_4O	AMOX = 29 DOX = 76	**LC-ESI- QqQ-MS/MS** ***Stationary phase:*** Synergi Hydro RP (50 x 2 mm, 4 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 0.1% CH_2O_2 (2) CH_4O	LOD_{AMOX} = 20,000 LOD_{DOX} = 5,000	(Watkinson et al. 2009)

Table A2.1. Methods for detection and analysis of amoxicillin or doxycycline, among other drugs, in aqueous environmental matrixes (*continuation*)**.**

Location	Matrix / Analytes	Concentration (ng/L)	Pre-treatment	Extraction procedure	Recovery (%)	Analytical procedure	Sensitivity (ng/L)	References
n.p.								
	Wastewater Surface water Ground water Drinking water AMOX and other ß-lactam antibiotics	AMOX = n.p. AMOX = n.p. AMOX = n.p. AMOX = n.p.	V = 100 – 4,000 mL $Na_2SO_3.5H_2O$ addition	**SPE** ***Sorbent:*** Carbograph 4 (0.5 g) ***Condition*** 20 mL H_2O (pH 2, HCl) 5 mL distilled H_2O ***Washing:*** 20 mL distilled H_2O 2 mL CH_4O 10 mL CH_2Cl_2/CH_4O, 80:20 (v/v) ***Elution:*** **Normal elution** 6 mL CH_2Cl_2/CH_4O, 80:20 (v/v) with $C_2H_3NaO_2$ **Reverse elution** 10 mL CH_2Cl_2/CH_4O, 80:20 (v/v) with $C_2H_3NaO_2$	AMOX = 76 – 98	**LC-ESI-MS** ***Stationary phase:*** Alltima C18 (25 × 4.6 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O with 5 mM CH_2O_2 (2) CH_4O with 5 mM CH_2O_2	LOD_{AMOX} = 1.8 LOQ_{AMOX} = 24	(Bruno et al. 2001)
	Wastewater AMOX and other pharmaceuticals	AMOX = n.p.	V = 10 mL Centrifugation Filtration	**Direct injection**	n.a.	**HPLC-DAD** ***Stationary phase:*** Purospher RP18 (250 x 64 mm, 5 µm) ***Mobile phase:*** **Gradient elution** (1) H_2O (pH 2.5, H_3PO_4) (2) CH_3CN	LOD_{AMOX} = 14,000	(Teixeira et al. 2008)

[1] Median of the detected concentrations; [2] Maximum concentration detected; [3] Derivatization buffer - 30 mM sodium borate (pH 8.35) and 5 mM sodium dodecyl sulfate.

n.a. - not applicable; n.d. - not detected; n.p. – information not provided.

Abbreviations: AMOX – amoxicillin; CE – capillary electrophoresis; DAD – diode array detector; DOX – doxycycline; ESI - electrospray ionization ; FL – fluorescence; HPLC – high performance liquid chromatography; HRMS – high resolution mass spectrometry; IT – ion trap; LC- liquid chromatography; LIF - laser induced fluorescence; LOD – limit of detection; LOQ – limit of quantification; MS – mass spectrometry; QIT - quadrupole ion trap; QqQ – triple quadruple; RRLC- rapid resolution liquid chromatography; TBA - tetra-n-butylammonium hydrogen sulphate; TBAB - tetrabutylammonium bromide; TEA – trimethylamine; TFA - trifluoroacetic acid; THAM - tris(hydroxymethyl)aminomethane; PBS - phosphate buffered saline; SDS - sodium dodecyl sulfate; UPLC – ultra high performance liquid chromatography; UV/Vis – ultraviolet visible spectroscopy.

Molecular formulas: $C_2H_4O_2$ - acetic acid; CH_3CN – acetonitrile; $C_2H_7NO_2$ - ammonium acetate; CH_5NO_2 – ammonium formate; NH_4OH - ammonium hydroxide; $NH_3.H_2O$ - ammonia monohydrate; $C_6H_8O_6$ - ascorbic acid; HCl - chloridric acid; CH_2Cl_2 – dichloromethane; C_2H_6O – ethanol; Na_2EDTA - ethylenediaminetetraacetic acid disodium salt; CH_2O_2 – formic acid; CH_4O – methanol; $C_8H_{18}O$ – 1-octanol; $C_2H_2O_4$ - oxalic acid; $HClO_4$ - perchloric acid; H_3PO_4 - phosphoric acid; $C_2H_3NaO_2$ - sodium acetate; NaOH – sodium hydroxide; H_2SO_4 - sulfuric acid; H_2O – water.

A3 Supplementary content to the materials and methods

The figure A3.1 schematically presents the SPE methodology.

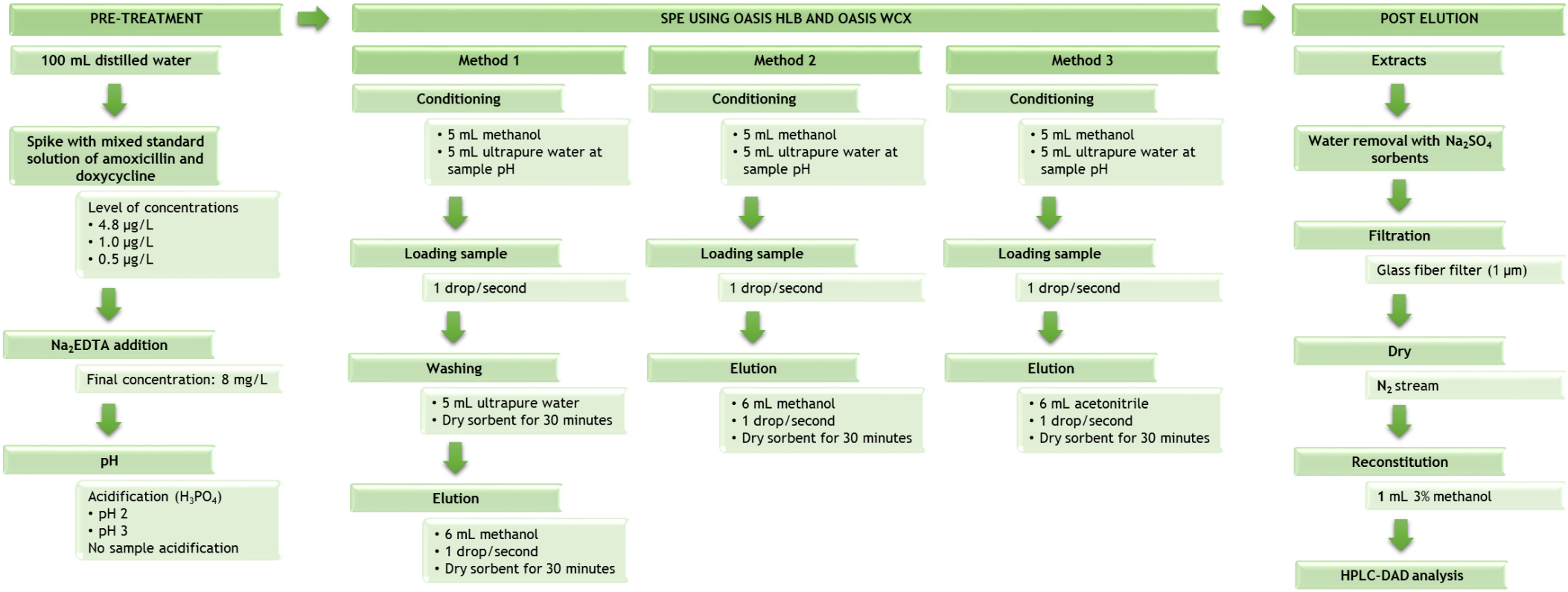

Figure A3.1. Schematic representation of the clean-up and extraction procedure.

A4 Scientific poster presented on IJUP 2015

In the context of this work, a scientific poster was presented in the 8th meeting of young research of University of Porto (IJUP 2015), held on 13 to 15 May 2015. In this section, the abstract (A) submitted for participation and the scientific poster (B) presented are displayed.

(A) **Veterinary antibiotics amoxicillin and doxycycline in aqueous environmental samples**

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The discovery of antibiotics is considered one of the most significant scientific achievements of the 20th century. Although they are used for over 60 years to improve both human and animal health, only recently the existence of antibiotics in the environment has received particular notice [1].

Around the world, antibiotics are massively used in animal food production, accounting for up to 50% of total sales. Moreover, it is estimated that antibiotic consumption in livestock worldwide could rise 67% by 2030. Since animal husbandry excrete 75% to 90% of administered antibiotics, these pharmaceuticals reach the environment by the application of manure to agriculture soils, or by pasture-reared animals excreting directly on the land, followed by run-off or leaching to water. The complex vicious cycle of biotransformation and bioaccumulation of antibiotics in the environment contribute to the emergence and global spread of antibiotic resistance bacteria [2].

In this context, studies about occurrence, fate and effects of veterinary antibiotics in the environment has increased during the last decade. However, further researches on veterinary antibiotics as emerging environmental micropollutants is required. Additionally, the development and validation of new analytical techniques is crucial to obtain accurate data on the concentrations of these compounds in the environmental. In this study, solid-phase extraction (SPE) and high-performance liquid chromatography-diode array detection (HPLC-DAD) analytical procedure for concentration and quantification amoxicillin and doxycycline in aqueous samples was developed, optimized and validated. The proposed methodology can be applied to the simultaneous determination of the target antibiotics, which are the two antibiotics most commercialized in Portugal for veterinary practice, in different water samples. Enabling the fast screening and effective determination of both compounds in the aqueous environment, the presented procedure might be important to understand their dynamic in the ecosystems.

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References:

[1] Cruickshank, M., Duguid, M., Gotterson, F. and Carter, D. (2014), *Taking action to preserve the miracle of antibiotics*, Australian Veterinary Journal, 92 (1-2), 3–7.

[2] Kümmerer, K. (2008), *Antibiotics in the Environment*, in Kümmerer, K., "Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks", Springer Science and Business Media, Heidelberg, pp. 75–94.

(B)

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Veterinary antibiotics amoxicillin and doxycycline in aqueous environmental samples

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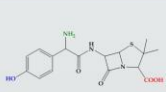
Introduction

The discovery of antibiotics is considered one of the most significant scientific achievements of the 20th century. Around the world, antibiotics are massively used in animal food production, accounting for up to 50% of total sales. According to the last report published by the Portuguese National Authority for Animal Health, Direção Geral de Alimentação e Veterinária (DGAV), amoxicillin and doxycycline were the two antibiotics most commercialized for veterinary practice in Portugal.



	Amoxicillin	Doxycycline
Water solubility	4000 mg/L at 25°C	630 mg/L at 25°C
pK_a values	pK_{a1} = 2.7 pK_{a1} = 7.5 pK_{a1} = 9.6	pK_{a1} = 3.1 pK_{a1} = 7.5 pK_{a1} = 9.4
Log $K_{O/W}$	0.87	-0.22

Since animal husbandry excrete 75% to 90% of administered antibiotics, these pharmaceuticals reach the environment by the application of manure to agriculture soils, or by pasture-reared animals excreting directly on the land, followed by run-off or leaching to water.

The complex vicious cycle of biotransformation and bioaccumulation of antibiotics in the environment contribute to the emergence and global spread of antibiotic resistance bacteria.

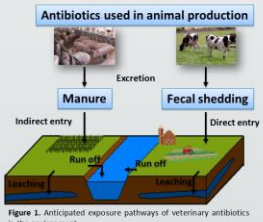


Figure 1. Anticipated exposure pathways of veterinary antibiotics in the environment.

In this context, the development and validation of analytical techniques is crucial to obtain accurate data on the concentrations of these compounds and their metabolites in the different environmental compartments. The aim of this study was develop, optimize and validate an accurate and sensitive high-performance liquid chromatography-diode array detection (HPLC-DAD) method for separation, and qualitative and quantitative determination of amoxicillin and doxycycline in aqueous samples.

Experimental

Preparation of standard solutions of amoxicillin and doxycycline

Stock solutions
[Amoxicillin] = 109 mg/L
[Doxycycline] = 120 mg/L

Mixed standard solution
[Amoxicillin] = 33 – 436 µg/L
[Doxycycline] = 36 – 479 µg/L

Chromatography analysis

Instrument	Merck Hitachi Elite LaChrom HPLC System with diode array detector
Stationary phase	Purospher STAR RP-18e (250 x 4 mm; 5 µm)
Mobile phase	Organic: acetonitrile (ACN) Aqueous: water (pH = 2, H_3PO_4)
Flow	0.8 mL/min
Temperature	Room temperature
Volume injection	100 µL
Wavelength	Amoxicillin: 230 nm Doxycycline: 360 nm

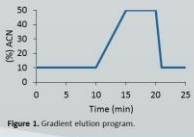
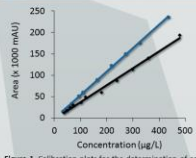


Figure 1. Gradient elution program.

Method validation study

Analytic parameters	Precision parameters
Linear range	Repeatability 3 levels of concentration 6 injections in the same day Defined as RSD
Pearson correlation coefficient (r)	
Limit of detection (LOD) Defined as signal-to-noise ratio of 3	Intermediate precision 3 levels of concentration 6 injections in 3 consecutives days Defined as RSD
Limit of quantification (LOQ) Defined as signal-to-noise ratio of 10	

Results



Area = (549 ± 13) [Amoxicillin] – (1178 ± 2672)
Area = (396 ± 20) [Doxycycline] – (2364 ± 5471)

Figure 1. Calibration plots for the determination of amoxicillin and doxycycline in aqueous samples.

❖ The analytical method is more sensitive for determination of amoxicillin than doxycycline.

Table 1. Analytic parameters of the method.

	Linear range (µg/L)	r	(%) RSD of the slope	LOD (µg/L)	LOQ (µg/L)
Amoxicillin	33 – 436	0.9995	1.0	28	92
Doxycycline	36 – 479	0.9968	2.2	29	92

Table 2. Analytical method precision.

	Amoxicillin			Doxycycline		
	44 µg/L	218 µg/L	436 µg/L	48 µg/L	239 µg/L	479 µg/L
Repeatability (%) RSD (n = 6)	3.1	1.7	1.1	5.0	1.4	0.5
Intermediate precision (%) RSD (n = 6)	3.8	1.8	1.0	2.5	1.3	1.1

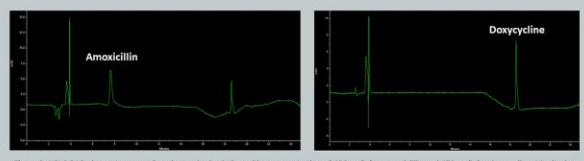


Figure 2. HPLC-DAD chromatograms of a mix standard solution with a concentration of 436 µg/L for amoxicillin and 479 µg/L for doxycycline. Monitoring was performed at 230 nm for amoxicillin (left) and 360 nm for doxycycline (right).

Conclusions

❖ The developed analytical methodology by HPLC-DAD is a fast, and simple chromatographic procedure based on gradient elution which enables the simultaneous analysis of the veterinary antibiotics amoxicillin and doxycycline.

❖ The proposed method is sensitive and precise enough for analysis of the for the monitoring of these antibiotics at low µg/L in environmental water samples after clean-up.

Future work

❖ Develop, optimize, and validate a clean-up and pre-concentration methodology for the posterior determination of amoxicillin and doxycycline in Portuguese environmental water samples by the presented HPLC-DAD analytical method.

❖ Investigate the occurrence of these antibiotics in wastewater from large animal feeding operations in Portugal.

References

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