Universidade de Aveiro Departamento de Química
2014

CARLA PATRÍCIA GONÇALVES SILVA

OCORRÊNCIA E DESTINO DE ESTROGÉNIOS E ANTIBIÓTICOS NO AMBIENTE, AVALIADOS POR METODOLOGIAS ANALÍTICAS DE BAIXO CUSTO

OCCURRENCE AND FATE OF ESTROGENS AND ANTIBIOTICS IN THE ENVIRONMENT EVALUATED BY LOW-COST ANALYTICAL METHODOLOGIES

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Valdemar Inocêncio Esteves, Professor Auxiliar do Departamento de Química da Universidade de Aveiro

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Aos meus pais.

o júri

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palavras-chave

17β-estradiol; 17α-etinilestradiol; sulfametoxazol; cafeína; ELISA; DLLME; fotodegradação.

resumo

Os disruptores endócrinos e os fármacos constituem grande preocupação, a nível ambiental. Nas últimas duas décadas, os estudos relacionados com a ocorrência e destino destes contaminantes emergentes no ambiente aquático, têm recebido grande atenção por parte da comunidade científica internacional. A sua presença no ambiente é particularmente importante, uma vez que se sabe que podem causar efeitos adversos nos ecossistemas, mesmo em concentrações extremamente baixas.

Os estrogénios e os antibióticos, em particular, são identificados como sendo capazes de induzir disrupção endócrina e contribuir para o aparecimento de bactérias multirresistentes, respetivamente. Uma melhor avaliação e compreensão do impacto real destes contaminantes no ambiente aquático implicam a análise da sua ocorrência e destino, o que constitui o principal objetivo desta Tese.

Os estrogénios 17β-estradiol e 17α-etinilestradiol e o antibiótico sulfametoxazol foram os contaminantes estudados neste trabalho, tendo sido avaliada a sua ocorrência em águas superficiais e residuais através da implementação de ensaios imunológicos (ELISA). Os ensaios foram otimizados por forma a conseguir-se: (i) a análise de amostras aquosas de matriz complexa, dando-se especial atenção aos efeitos de matriz, e (ii) aumentar a sensibilidade.

Uma vez que os níveis destes contaminantes no ambiente são extremamente baixos, foi desenvolvida, também, uma metodologia de pré-concentração. A microextração líquido-líquido dispersiva (DLLME) foi utilizada para a pré-concentração do E2 e EE2, subsequentemente quantificados por cromatografia líquida de alto desempenho (HPLC) e pelo imunoensaio ELISA previamente otimizado.

Além disso, o uso de marcadores antropogénicos, ou seja, indicadores da presença ou atividade humana, tem sido discutido como uma ferramenta válida para seguir a origem e tipo da contaminação. Desta forma, foi também desenvolvido um imunoensaio para a quantificação da cafeína, como marcador antropogénico, de modo a avaliar a ocorrência de poluição de origem humana em águas superficiais portuguesas.

Por último, a fotodegradação é considerada como um dos principais processos que afetam a persistência dos poluentes no ambiente aquático. Foram, assim, avaliadas as fotodegradações direta e indireta do E2 e do EE2. Uma vez que se sabe que as substâncias húmicas (HS) representam uma importante influência na fotodegradação dos poluentes, e, com o intuito de mimetizar o ambiente aquático real, foi dada especial atenção à influência da presença e concentração de diferentes frações de HS na fotodegradação das duas hormonas.

keywords

17β-estradiol; 17α-ethinylestradiol; sulfamethoxazole; caffeine; ELISA; DLLME; photodegradation.

abstract

Endocrine disruptors and pharmaceuticals are considered to be concerning environmental contaminants. During the last two decades, studies dealing with the occurrence and fate of these emerging contaminants in the aquatic environment have raised attention and its number is constantly increasing. The presence of these contaminants in the environment is particularly important since they are known to induce adverse effects in the ecosystems even at extremely low concentrations.

Estrogens and antibiotics, in particular, are identified as capable of induce endocrine disruption and contribute for the appearance of multi-resistant bacteria, respectively. A better assessment and understanding of the real impact of these contaminants in the aquatic environment implies the evaluation of their occurrence and fate, which is the main aim of this Thesis.

Two estrogens (17β-estradiol and 17α-ethinylestradiol) and an antibiotic (sulfamethoxazole) were the contaminants under study and their occurrence in surface and waste waters was assessed by the implementation of enzyme linked immunosorbent assays (ELISAs). The assays were optimized in order to accomplish two important aspects: to analyze complex water samples, giving special attention to matrix effects, and to increase the sensitivity.

Since the levels of these contaminants in the environment are extremely low, a pre-concentration methodology was also object of study in this Thesis. Dispersive liquid-liquid microextraction (DLLME) was developed for the pre-concentration of E2 and EE2, subsequently quantified by either high-performance liquid chromatography (HPLC) and the previously optimized ELISAs.

Moreover, the use of anthropogenic markers, i.e. indicators of human presence or activity, has been discussed as a tool to track the origin and type of contamination. An ELISA for the quantification of caffeine, as an anthropogenic marker, was also developed in order to assess the occurrence of human domestic pollution in Portuguese surface waters.

Finally, photodegradation is considered to be one of the most important pathways contributing for the mitigation of pollutants' presence in the aquatic environment. Both direct and indirect photodegradation of E2 and EE2 were evaluated. Since the presence of humic substances (HS) is known to have a noticeable influence on the photodegradation of pollutants and in order to mimic the real aquatic environment, special attention was given to the influence of the presence and concentration of different fractions of HS on the photodegradation of both hormones.

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GLOSSARY

Abbreviations and Symbols

ABBREVIATIONS

4PL – **Four**-**P**arametric **L**ogistic Equation

Ab – **A**nti**b**ody

Ag - **A**nti**g**en

AOP – **A**dvanced **O**xidation **P**rocesses

AST – **A**ctivated **S**ludge **T**reatment

BSA – **B**ovine **S**erum **A**lbumin

CDOM – **C**hromophoric **D**issolved **O**rganic **M**atter

CE – **C**apillary **E**lectrophoresis

CR – **C**ross-**R**eactivity

DLLME – **D**ispersive **L**iquid–**L**iquid **M**icro**e**xtraction

DLLME–SFO – **D**ispersive **L**iquid-**L**iquid **M**icro**e**xtraction with **S**olidification of a **F**loating **O**rganic Drop

DMA – **D**i**m**ethyl**a**cetamide

DMF – N,N-**D**i**m**ethyl**f**ormamide

DOC - **D**issolved **O**rganic **C**arbon

DOM – **D**issolved **O**rganic **M**atter

DRE – **D**ouro **R**iver **E**stuary

E1 – Estrone

E2 – 17β-Estradiol

E3 – Estriol

EC – **E**merging **C**ontaminant

ED – **E**ffluent **D**ischarge

EDC – **E**ndocrine **D**isrupting **C**ompound

EDTA – **E**thylene**d**iamine**t**etra**a**cetic acid

EE2 – 17α-Ethinylestradiol

EF – **E**nrichment **F**actor

ELISA – **E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssay

EPA – **E**nvironmental **P**rotection **A**gency

ER – **E**xtraction **R**ecovery

ESI – **E**lectro**s**pray **I**onization

FA – **F**ulvic **A**cids

FD – **F**luorescence **D**etector

GC-MS –**G**as **C**hromatography-**M**ass **S**pectrometry

GC-MS/MS – Gas Chromatography-tandem Mass Spectrometry

HA – **H**umic **A**cids

HF-LPME – **H**ollow-**F**iber **L**iquid-**P**hase **M**icro**e**xtraction

HPLC-FD – **H**igh **P**erformance **L**iquid **C**hromatography with **F**luorescence **D**etector

HRP – **H**orse**r**adish **P**eroxidase

HS – **H**umic **S**ubstances

IBCF – **I**so**b**utyl **C**hloro**f**ormate

Ig – **I**mmuno**g**lobulin

IHSS – **I**nternational **H**umic **S**ubstances **S**ociety

LC-DAD – **L**iquid **C**hromatography-**D**iode **A**rray **D**etector

LC-DAD-MS – **L**iquid **C**hromatography-**D**iode **A**rray **D**etector-**M**ass **S**pectrometry

LC-MS – **L**iquid **C**hromatography–**M**ass **S**pectrometry

LC-MS/MS – Liquid Chromatography-tandem mass spectrometry

LLE – **L**iquid-**L**iquid **E**xtraction

LOD – **L**imit **o**f **D**etection

LOQ – **L**imit **o**f **Q**uantification

LPME – **L**iquid-**P**hase **M**icro**e**xtraction

MALDI-TOF – **M**atrix **A**ssisted **L**aser **D**esorption **I**onization – **T**ime **o**f **F**light

MBR – **M**embrane **B**io**r**eactor

Mol. Wt. – **Mol**ecular **W**eigh**t**

NMM – 4-Methylmorpholin

NMR – **N**uclear **M**agnetic **R**esonance

NWWS – **N**orth STP **W**astewater **S**amples

OD – **O**ptical **D**ensity

PBS – **P**hosphate **B**uffer **S**olution

PPCP – **P**harmaceutical and **P**ersonal **C**are **P**roducts

QqQ – Triple-quadrupole analyzer

RIA – **R**adio**i**mmuno**a**ssay

ROS – **R**eactive **O**xygen **S**pecies

RSD – **R**elative **S**tandard **D**eviation

SA - **S**ulfon**a**mide

SMX – **S**ulfa**m**etho**x**azole

SOM – **S**uspended **O**rganic **M**atter

SPE – **S**olid **P**hase **E**xtraction

SPME – **S**olid **P**hase **M**icro**e**xtraction

SRT – **S**olids **R**etention **T**ime

STP – **S**ewage **T**reatment **P**lant

succ-SMX – **Succ**inamyl-**S**ulfa**m**etho**x**azole

SWS – **S**urface **W**ater **S**amples

SWWS – **S**outh STP **W**astewater **S**amples

T – **T**racer (enzyme conjugate)

TBABH – **T**etra**b**utyl**a**mmonium **B**oro**h**ydride

TF – **T**rickling **F**ilters

TMB – **T**etra**m**ethyl**b**enzidine

TOC – **T**otal **O**rganic **C**arbon

TRIS – Tris(hydroxymethyl)aminomethane

UPLC-Q-TOF-MS – **U**ltra **P**erformance **L**iquid **C**hromatography-**Q**uadrupole-**T**ime **o**f **F**light **M**ass **S**pectrometry

UV – **U**ltra**v**iolet

SYMBOLS

A – OD for an infinitely small analyte concentration ("blank")

$Abs_{est.}$ – Absorbance of estrogens

$Abs_{est.+HS}$ – Absorbance of the mixture (estrogens + HS)

b – Slope of the regression line

B – Slope at the inflection point

C – Concentration value at the inflection point *or* Concentration of estrogen at a given irradiation time

C_0 – Initial concentration of the analyte within the sample *or* Initial concentration of estrogen

C_{sed} – Analyte concentration in the sedimented phase

C_{std} – Parameter of the 4PL giving the antigen concentration at the inflection point

C_{test} – Concentration of the cross-reacting compound at its inflection point

D – OD for an infinite analyte concentration (standard excess)

^{3}HS*– Humic substances in the triplet state

k – Rate constant

k_{calc} – Calculated first-order rate constant for degradation of estrogens in presence of HS acting only as inner filter

K_{ow} – Octanol/water partition coefficient

n_0 – Total analyte amount (DLLME procedure)

n_{sed} – Analyte amount which was extracted to the sedimented phase (DLLME procedure)

$^1O_2$ – Singlet oxygen

OH$^\bullet$– Hydroxyl radicals

r – Correlation coefficient

ROO$^\bullet$ – Peroxyl radicals

S_w – Water solubility

$s_{y/x}$ – Statistical parameter that estimates the random errors in the y axis

t – Time

$t_{½}$ – Half-life time

t_T – Tracer incubation time

Y_N – Normalized OD

σ_y – Standard deviation of the response for replicate measurements

THESIS' MAIN OBJECTIVES AND LAYOUT

In the late 1990s, the so-called emerging contaminants (ECs) have raised great interest due to their potential to cause negative effects in the aquatic environment. Their continuous introduction in the environment may lead to high long-term concentrations, promoting unnoticed but continual adverse effects on a variety of organisms. It is therefore crucial to follow their occurrence and fate pathways in the environment, which should be done in a broader base. For this purpose it is necessary the development of simple, fast, efficient and cost-effective analytical methods for their pre-concentration and quantification and for the assessment of their fate in water matrices.

The main objective of the work herein presented was to contribute for a better understanding of some compounds belonging to the wide group of ECs. With that purpose, low-cost methodologies for their quantification in water samples were implemented and optimized and their fate was evaluated. Special attention was given to organic matter presence (and effects), in order to mimic a real aquatic environment. In Portugal, these type of studies are limited; consequently, it was also aimed to contribute to an assessment of the aquatic environment in our country.

This thesis has seven chapters, starting with an introductory section (Chapter 1) and finalizing with a "final remarks" section (conclusions and future work). The experimental part of the thesis is allocated into six chapters and divided into three main parts: Part I - Development of low-cost analytical methodologies for quantification of estrogens, antibiotics and anthropogenic markers in water samples; Part II - Development of a low-cost pre-concentration methodology for estrogens quantification by different techniques, and Part III - Evaluation of the fate and persistence of estrogens in the aquatic environment. Experimental part of the thesis highlight three essential aspects when dealing with the evaluation of ECs in the environment: a) the assessment of their occurrence by simple and rapid techniques without an additional pre-treatment step; b) the necessity to use, in some cases, this pre-treatment step, being itself simple, rapid, environmentally friendly and low-cost; and c) the requirement of better understand the fate of these pollutants in the environment by means of photolysis studies, since photolysis is known to be one of the major processes determining the fate of polluants in the aquatic environment. Due to financial restrictions that laboratories have to deal with, it was kept in mind the general objective of developing cost-effective methodologies for the assessment of pollutants in the environment, since, nowadays, this is an essential issue in order to regulate their presence.

Thesis is then organized as follows:

CHAPTER 1: INTRODUCTION

The introductory chapter aims to present an overview on endocrine disrupting compounds (EDCs) and pharmaceutical and personal care products (PPCPs), their effects, sources and possible pathways in the environment. Amongst the wide group comprised by EDCs and PPCPs, 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and sulfamethoxazole (SMX) are object of concern because of their capacity to cause endocrine disruption and bacterial resistance. Therefore, their occurrence and fate in the aquatic environment, analytical methodologies for their quantification in waters and removal strategies to apply before their final release into the environment are addressed. Moreover, the use of the so-called anthropogenic markers to follow the occurrence of human domestic pollution in surface waters, is also presented.

Some of the aspects detailed in the Introduction are published in the following review article:

Silva, C.P.; Otero, M.; Esteves, V.I.; Processes for the elimination of estrogenic steroid hormones from water: A review; *Environmental Pollution* 165 (2012) 38-58.

PART I - Development of low-cost analytical methodologies for quantification of estrogens, antibiotics and anthropogenic markers in water samples

CHAPTER 2: Development of enzyme linked immunosorbent assays for direct determination of E2 and EE2 in water samples

Determination of E2 and EE2 in surface and waste water samples collected in Aveiro, Portugal, by enzyme linked immunosorbent assay (ELISA). This research work involved the optimization of both assays (for E2 and EE2) in order to quantify the referred estrogens in environmental complex matrices, with high salinity and organic matter contents, without any sample pre-treatment. This study resulted in the following publication:

Silva, C.P.; Lima, D.L.D.; Schneider, R.J.; Otero, M.; Esteves, V.I.; Development of ELISA methodologies for the direct determination of 17β-estradiol and 17α-ethinylestradiol in complex aqueous matrices; *Journal of Environmental Management* 124 (2013) 121-127.

CHAPTER 3: Development of an enzyme linked immunosorbent assay for SMX determination in water samples

Determination of SMX in surface and waste water samples collected in Aveiro, Portugal, by ELISA. The optimization of the assay performance was accomplished in order to quantify the antibiotic in environmental complex matrices, without any sample pre-treatment. The work comprised also the quantification of SMX and its main metabolite, n-acetyl-SMX, in the same samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This study was carried out in Federal Institute for Materials Research and Testing - BAM (Berlin, Germany).

CHAPTER 4: Caffeine as a human pollution marker in waters of the north and center of Portugal

Determination of caffeine in surface and waste water samples collected in north and center of Portugal, by an ELISA optimized for the quantification of caffeine without any sample-treatment, even in more complex matrices. The assessment of the occurrence of human domestic pollution in Portuguese surface waters through the use of an anthropogenic marker – caffeine – was made. Also, results were validated by LC-MS/MS. Part of the work presented in this Chapter was performed in BAM (Berlin, Germany). This study resulted in the following publication:

Silva, C.P.; Lima, D.L.D.; Schneider, R.J.; Otero, M.; Esteves, V.I.; Evaluation of the anthropogenic input of caffeine in surface waters of the north and center of Portugal by ELISA; *Science of the Total Environment* 479-480 (2014) 227-232.

PART II - Development of a low-cost pre-concentration methodology for estrogens quantification by different techniques

CHAPTER 5: Development of dispersive liquid–liquid microextraction technique for estrogens' quantification by HPLC with detection by fluorescence

Implementation and optimization of dispersive liquid–liquid microextraction (DLLME), a pre-concentration methodology based on a ternary component solvent system, in order to accomplish the quantification of E2 and EE2 in water samples (from Minho and Aveiro regions, Portugal) in concentrations lower than the ones attained in Chapter 2.

Quantification technique in this work was high performance liquid chromatography with a fluorescence detector (HPLC-FD). This work is published in:

Lima, D.L.D.; Silva, C.P.; Otero, M.; Esteves, V.I.; Low cost methodology for estrogens monitoring in water samples using dispersive liquid–liquid microextraction and HPLC with fluorescence detection; *Talanta* 115 (2013) 980-985.

CHAPTER 6: Application of dispersive liquid-liquid microextraction for estrogens' quantification by enzyme linked immunosorbent assay

Evaluation of the DLLME process in order to be applied on ELISA. Conjugation of the previously optimized ELISA assays for E2 and EE2 (Chapter 2) and the previously optimized DLLME procedure (Chapter 5). This work resulted in a publication:

Lima, D.L.D.; Silva, C.P.; Schneider, R.J.; Otero, M.; Esteves, V.I.; Application of dispersive liquid–liquid microextraction for estrogens' quantification by enzyme-linked immunosorbent assay; *Talanta* 125 (2014) 102-106.

PART III - Evaluation of the fate and persistence of estrogens in the aquatic environment

CHAPTER 7: Photosensitized degradation of E2 and EE2 by humic substances

Study of the direct and indirect photodegradation of E2 and EE2, giving special attention to the effects of different fractions of humic substances (HS; humic acids (HA), fulvic acids (FA) and XAD-4 fraction) in the photodegradation rates. This work resulted in the following submitted publication:

Silva, C.P.; Lima, D.L.D.; Otero, M.; Esteves, V.I.; Photosensitized degradation of 17β-estradiol and 17α-ethinylestradiol by humic substances; *Chemosphere*, under review.

FINAL REMARKS

In this section, final conclusions and future work are addressed.

CHAPTER 1

INTRODUCTION

Silva, C.P.; Otero, M.; Esteves, V.I.; Processes for the elimination of estrogenic steroid hormones from water: A review; *Environmental Pollution* 165 (2012) 38-58.

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Summary

Nowadays, endocrine disrupting compounds (EDCs) and pharmaceutical and personal care products (PPCPs) are considered to be major groups of environmental contaminants. Since sewage treatment plants (STPs) have not been designed for the removal of emerging contaminants (ECs) from water, most of them are not effective barriers for these pollutants, which are consequently continuously discharged into the environment. Therefore, the main way of entrance of these compounds into the environment is through discharge of domestic sewage effluents.

Among ECs, E2, EE2 and SMX, have become a subject of attention in recent years and are now, with the improvement of the detection methods, recognized as nearly ubiquitous pollutants in waters. The occurrence of these substances in aquatic ecosystems may affect the endocrine system of humans and wildlife and may account for the appearance of resistant bacteria, which in turn may result in a major human health threat in the near future.

Extensive research on the occurrence and fate of these pollutants in the environment has been carried out during the last decades. Nonetheless, much is yet to be done in order to better understand the impact of their presence in the aquatic environment.

This *Introduction* chapter aims to give an overview of the effects, sources, fate, occurrence and processes of removal of these pollutants. Moreover, the use of anthropogenic markers for the assessment of human pollution-contaminated areas is also addressed.

Contents

1.1. EMERGING CONTAMINANTS IN THE ENVIRONMENT

In the late 1990s, a topic started to receive growing attention by environmental scientists. The so-called *emerging contaminants* (ECs) have raised great interest due to their potential to cause negative effects in the environment and later in living organisms (Jakimska et al., 2014; Petrović and Barceló, 2007).

ECs can be defined as natural or synthetically occurring substances that are not commonly monitored in the environment but that can induce known or suspected undesirable effects on humans and ecosystems (Meffe and Bustamante, 2014). These compounds are not necessarily newly developed ones; they may have been present in the environment for long time although only recently their presence has been acknowledged thanks to the advances in analytical techniques.

In the European context, surface water and groundwater quality standards are regulated under the Water Framework Directive (European Union, 2000). This directive required the monitoring of priority contaminants in the aquatic environment such as certain pesticides and their degradation products, chlorinate solvents, polycyclic aromatic hydrocarbons, disinfection by-products, volatile organic compounds and biocides (Meffe and Bustamante, 2014). Nevertheless, a large number of contaminants was not included in the list of chemicals to be supervised. Therefore, the number of compounds to be regulated by legislation was prone to be extended, which occurred in 2013.

ECs include a wide range of pollutants, such as disinfectants, industrial chemicals, detergents, pesticides, nanomaterials, flame retardants and the groups of contaminants so-called *endocrine disrupting compounds* (EDCs) and *pharmaceuticals and personal care products* (PPCPs).

1.1.1 Effects and sources

The endocrine-disrupting phenomena is a relatively new area of concern, first brought to light during the 1980s when deformities in fish were observed in certain stretches of United Kingdom rivers (Gomes et al., 2003). In more recent years, a growing body of scientific research indicates that some substances in the environment may interfere with the normal function of the endocrine system of humans and wildlife (Lintelmann et al., 2003).

The action of EDCs usually happens by mechanisms that, either temporarily or permanently, alter the feedback loops in various components of the endocrine system (Gore, 2001; Roy et al., 2009). These adverse effects can arise from either indirect or direct disturbances of endocrine function: when happens an indirect disturbance, the EDC affects a systemic target organ first, which in turn may influence the endocrine system; conversely, a direct-acting EDC affects the endocrine system first, which in turn results in toxicity in other organ systems (Kavlock et al., 1996).

EDCs play some mechanisms that have the ability to (i) mimic the effect of endogenous hormones; (ii) antagonize the effect of endogenous hormones; (iii) disrupt the synthesis and metabolism of endogenous hormones, modifying their levels and function; (iv) disturb the synthesis of the specific hormone receptors and (v) block, prevent and alter hormonal binding to hormone receptors or influence cell signalling pathways (Caliman and Gavrilescu, 2009; Caserta et al., 2008; Goldman et al., 2000; Matozzo et al., 2008; Mendes et al., 2002; Roy et al., 2009; Stoker et al., 2000). Some possible mechanisms played by EDCs are schematized in Fig. 1.1.

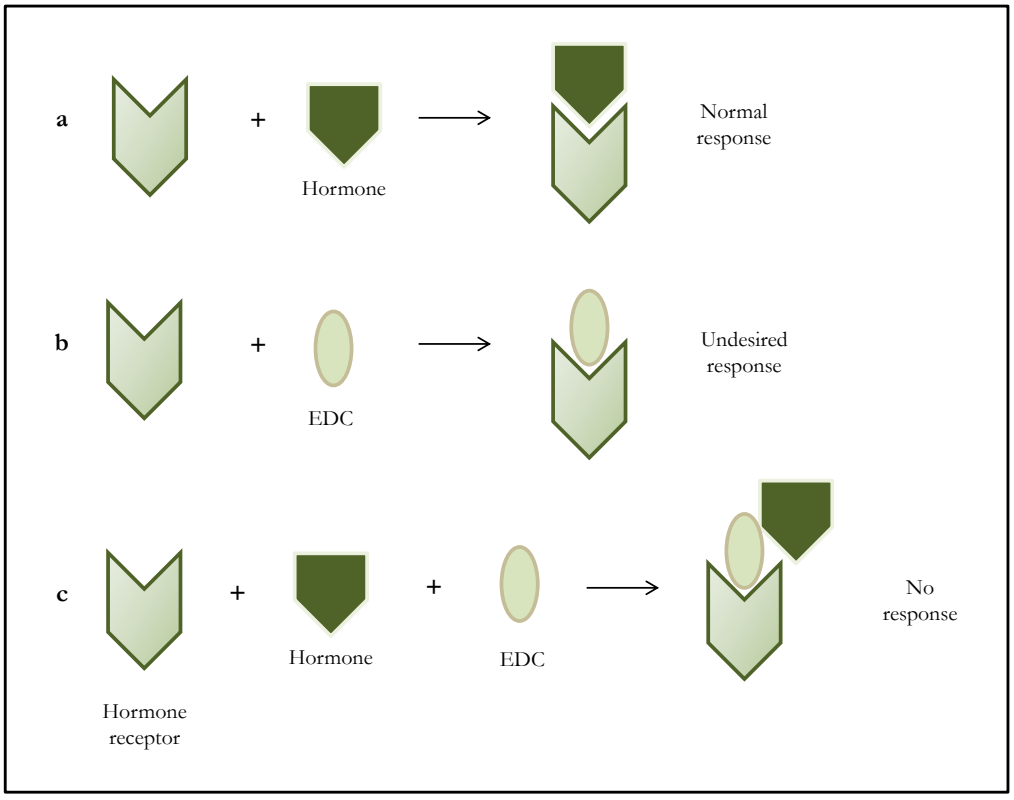


Fig. 1.1: Some possible endocrine-disrupting mechanisms: (a) normal functioning, (b) agonist, and (c) antagonist.

Also, some important features contribute to the risk associated to EDCs:

(i) EDCs are soluble in adipose tissues (Quan et al., 2005);

(ii) in general, chemicals are present in the form of mixtures and the compound interactions may result in additive, synergistic or antagonistic effects (Onesios et al., 2009; Quan et al., 2005): very strong endocrine disruption takes place when more than one compound simultaneously exists, although their individual action may be weak (Quan et al., 2005; Safe et al., 2002);

(iii) EDCs and their metabolites have a persistent nature in the different environmental compartments (Porte et al., 2006).

Therefore, it is essential to know if the levels of EDCs existing in the environment are high enough to exert adverse health effects on the general population. In any case caution is required since it is already acknowledged that the normal functions of all organ systems are regulated by endocrine factors. Thus, small disturbances in endocrine function can lead to profound and lasting effects, especially in some specific periods of life (Caserta et al., 2008; Kavlock et al., 1996), in which any affecting factor can result in significant damage to the host.

EDCs include a wide range of chemicals, among which are steroid hormones, a group of biologically active compounds that are synthesized from cholesterol and have in common a cyclopentan-o-perhydrophenanthrene ring (Ying et al., 2002).

PPCPs refer to any product used by individuals for personal health or cosmetic reasons or used by farming to enhance growth or health of livestock (USEPA, 2012). The scope comprises both anthropogenic and naturally occurring substances (Petrović and Barceló, 2007).

Consumption of pharmaceuticals continuously increases and their introduction rate into the environment may exceed the degradation rate. The exact risk associated with decades of persistent exposure to random combinations of low levels of pharmaceuticals is not yet well recognized (Jakmiska et al., 2014). However, there is a high possibility that these compounds may be the reason for the increase of bacterial resistance to antibiotics (Jakmiska et al., 2014; Richardson and Ternes, 2011). Furthermore, some pharmaceuticals (especially antidepressants and antibiotics) may be subjected to bioaccumulation processes in aquatic organisms, mostly fish (Jakmiska et al., 2014). Also, new concerns arise related to the antibiotics' ability to decrease biodegradation of leaf and other plant materials, which serve as the primary food source for aquatic life in rivers and streams (Richardson and Ternes, 2011).

Steroid estrogens and pharmaceuticals differ from other environmental organic pollutants (e.g. solvents, herbicides) because they are exposed to biochemical metabolism and consequently they will enter the aquatic environment in a modified form. Those that remain unaltered will share a resistance to biochemical transformation (Petrović and Barceló, 2007).

One type of metabolic modification is conjugate formation, in which the parent compound or its metabolite is covalently bound to a small organic fragment. Typical conjugates are glucuronide, sulphate, acyl, methyl and glutathione adducts. These modifications may complicate the already difficult environmental monitoring since they are potentially reversible. On the other hand, even when a compound is extensively metabolized, its metabolites may retain the biological activity of the parent compound (Halling-Sørensen et al., 1998; Petrović and Barceló, 2007; Racz and Goel, 2009; Richards and Cole, 2006). Therefore, significant amounts of the parent compound in the unmetabolized form, as well as in form of metabolites, are continuously excreted into the sewage systems.

The main source of these pollutants into the environment is, in fact, the human and animal excretion and consequent incomplete removal during the waste water treatment processes (Gomes et al., 2003; Leech et al., 2009; Liu et al., 2009), which shows the inadequacy of the treatment applied in sewage treatment plants (STPs) for this type of contaminants. However, there are other routes for the entrance of these pollutants into the environment. Fig. 1.2 schematizes the possible sources and fate of ECs in the environment. Although with minor expression, the effluents and sediments of pharmaceuticals' industrial STPs, as well as the improper disposing of unused or expired drugs in toilets or trash, also account for the load of ECs in the environment (Kotchen et al, 2009; Ternes et al., 2004).

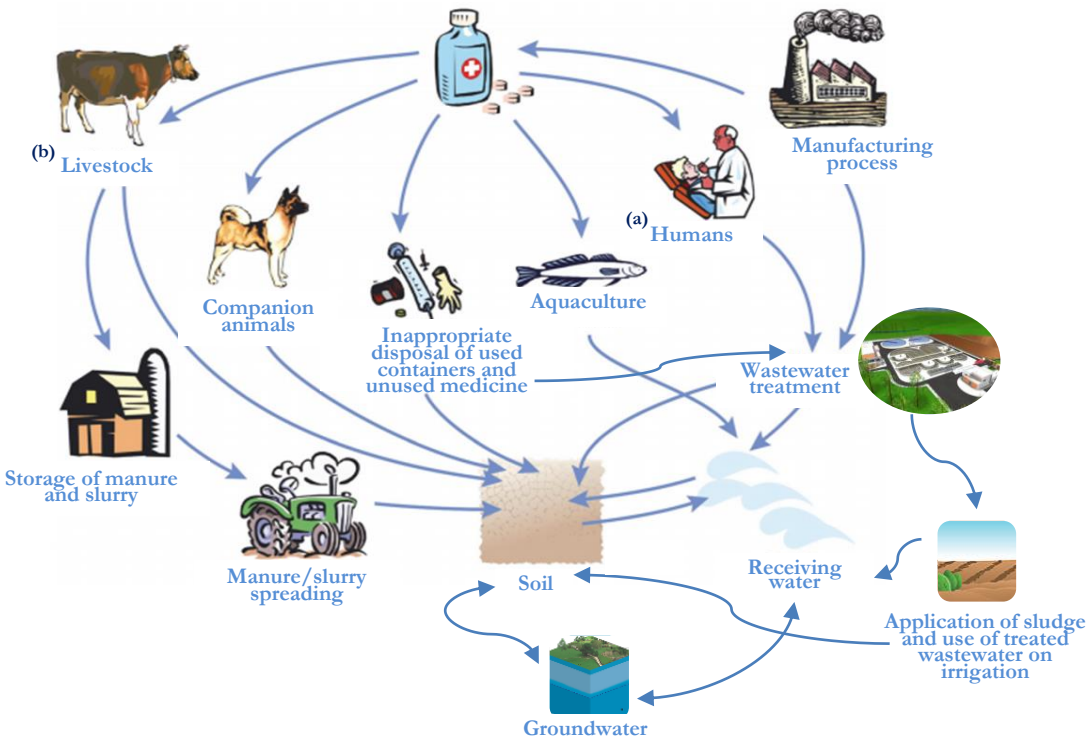


Fig. 1.2: Routes of pharmaceuticals entering the environment. Routes for endogenous EDCs, as estrogens, can also be represented following this diagram from (a) and (b) (adapted from Boxall (2004)).

The connection between the different environmental compartments makes it possible for contaminants to be present in soil, surface water and groundwater, and therefore, these compounds pose a risk to drinking water itself. Nowadays, the drinking water industry faces a challenge as regulatory bodies and the public become aware of the presence of these compounds, previously not detected, in water (Rahman et al., 2009).

1.1.2 Pathways and fate

The prediction of the fate of EDCs and PPCPs and their distribution in the environment is of great importance. Once these pollutants reach the environment they can undergo different pathways, as schematized on Fig. 1.3.

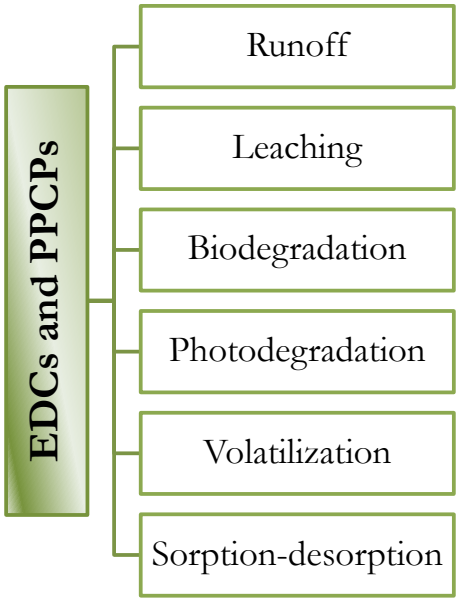


Fig.1.3: Pathways of pollutants' degradation and transport.

The transport and/or degradation of EDCs and PPCPs in the environment are determined by their physicochemical properties and site-specific environmental conditions (Ying et al., 2002), which can be useful tools to predict and understand their behaviour and fate (Lintelmann et al., 2003; Petrović and Barceló, 2007).

Important physicochemical parameters are the *water solubility* (S_w) that, in surface water, strongly depends on parameters as temperature, pH, ionic strength, or existence of dissolved and suspended organic matter (DOM and SOM, respectively); and the *octanol/water partition coefficient*, K_{ow}, a parameter that describes the partition of a non-polar organic substance between water and the organic solvent octanol (Lintelmann et al., 2003).

On the other hand, some environmental conditions may also influence the lifetimes of pollutants in aquatic systems, thus determining the magnitude of their effects (Caliman and Gavrilescu, 2009). Examples of these conditions are: the light action and its intensity (Caliman and Gavrilescu, 2009), salinity, total organic carbon (TOC) content (Lai et al., 2000), as well as dissolved organic carbon (DOC) (Leech et al., 2009; Paul et al., 2004).

Transport of pollutants comprises distinct processes, such as volatilization, leaching and surface runoff. A fundamental process is leaching, by which pollutants are transported from the soil profile by the action of percolating liquid water. This process has been identified as the major cause of groundwater contamination. Surface runoff has also impact on surface and groundwater quality (Haygarth and Jarvis, 2002).

Sorption occurs when pollutant molecules bind to particulate matter. This binding can vary from complete reversibility to total irreversibility and interaction may be physical (van der Waals forces) and/or chemical (electrostatic interactions) (Lima, 2011). The type of interaction depends not only on the pollutant's properties but also on the particle's properties. For example, the type of sediment has been shown to affect significantly the sorption of certain pollutants (Peuravuori and Pihlaja, 2009).

Degradation is one of the most important processes for the decrease of pollutants' load in the environment. Biodegradation is the process by which microbial organisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment. However, degradation may also be an abiotic process, including hydrolysis, oxidation/reduction and photolysis (Petrović and Barceló, 2007).

Most EDCs and PPCPs have been found to be photoactive because their structural compositions consist of aromatic rings and functional groups that can either absorb solar radiation or react with already photogenerated by-product species in natural waters (Peuravuori and Pihlaja, 2009). Therefore, sunlight-induced photochemical processes should be considered as an essential mechanism and one of the major pathways to mitigate the presence of this sort of pollutants in the environment. Photodegradation will be assessed in detail in Chapter 7.

1.2 EDCs AND PPCPs UNDER STUDY

In this work, two estrogens and one antibiotic were studied: 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and sulfamethoxazole (SMX). Their main properties are depicted in (Table 1.1).

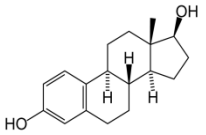
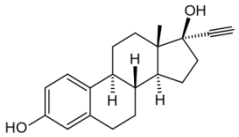
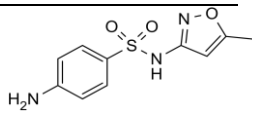
Natural estrogen, E2, and the synthetic estrogen, EE2, are among the most potent endocrine disruptors, even at levels as low as ng L^{-1}, and amongst the most commonly found estrogens in waste water (Racz and Goel, 2010). E2 is predominantly a female hormone, important for maintaining the health of the reproductive tissues, breasts, skin and brain, while EE2 is a synthetic steroid, used mainly as contraceptive.

SMX is an antibiotic, belonging to the class of sulfonamides (SAs), widely used in both human and veterinary medicine against bacterial infections. In human medicine, antibiotics

constitute the third biggest group amongst all pharmaceuticals in terms of prescriptions. In veterinary medicine, more than 70% of all consumed pharmaceuticals are antibacterial agents (Petrović and Barceló, 2007). In addition, both estrogens and antibacterials are also used in livestock and poultry production to increase the rate of growth.

Although present in the environment at low levels, antibiotics have a long life-time, can accumulate in organisms and may cause bacterial drug resistance (Černoch et al., 2012).

Table 1.1: Compounds under study: E2, EE2 and SMX (Petrović and Barceló, 2007; Ying et al., 2002; Zhong et al., 2013).

Compound	Group	Mol. wt. (g mol^{-1})	pK_a	S_w (mg L^{-1})	Log K_{ow}	Structure
E2	Natural estrogen	272.4	10.4	13.0	3.94	
EE2	Synthetic estrogen; acts mainly as oral contraceptive	296.4	10.4	4.8	4.15	
SMX	Antibacterial	253.3	pK_1 5.7 pK_2 1.8	384.7	0.89	

As it was already stated, the coefficient K_{ow} is a key parameter to predict the fate of chemicals in the environment. K_{ow} has been found to be related to S_W, soil/sediment adsorption coefficients and bioconcentration factors for aquatic life. Chemicals with low K_{ow} values (less than 10) may be considered relatively hydrophilic; they tend to have high S_W, small soil/sediment adsorption coefficients, and small bioconcentration factors for aquatic life. Conversely, chemicals with high K_{ow} values (greater than 10^4) are very hydrophobic and have high sorption potential (Jones-Lepp and Stevens, 2007).

Synthetic estrogens have lower solubility than natural estrogens, but both have very low vapour pressures (2.3×10^{-10}, for E2, and 4.5×10^{-11}, for EE2; values not shown in Table 1.1), indicating their low volatility (Ying et al., 2002). On the whole, it can be said that

estrogens are compounds of low volatility and hydrophobic nature and weakly acidic.

SAs mainly behave as weak acids due to the N-H bond of the sulfonamidic group and tend to form salts in strongly acid or basic media (Petrović and Barceló, 2007). SMX have a high S_W which explains its low adsorption to soils and sediments.

1.2.1 Occurrence of E2, EE2 and SMX in the aquatic environment

Numerous studies show evidence of E2 and EE2 in influents, but also in effluents of STPs and receiving waters (e.g. Atkinson et al., 2012; Baronti et al., 2000; Belfroid et al., 1999; Desbrow et al., 1998; Jacquet et al., 2012; Jeannot et al., 2002; Kuch and Ballschmiter, 2001; Li et al., 2013; Lin and Tsai, 2009; Rao et al., 2013; Schultz et al., 2013; Snyder et al., 1999; Ternes et al., 1999a; Ternes et al., 1999b; Zhang et al., 2014), as well as drinking water (Kuch and Ballschmiter, 2001).

Several countries have been conducting studies dealing with the quantification of estrogens in water. E2 was found in Brazil, in STPs influents' samples, with an average concentration of 21 ng L^{-1} (Ternes et al., 1999b), while in Italy, Baronti et al. (2000) found the estrogenic steroids E2 and EE2 in STPs' influents in concentrations of about 12 and 3 ng L^{-1}, respectively (Baronti et al., 2000). Likewise, Cargöuet et al. (2004), in France, quantified E2 and EE2 in influents and effluents of four STPs and in river waters. Mean values for E2 were 14.3, 6.7 and 2.3 ng L^{-1}, for influents, effluents and surface waters, respectively; while mean values for EE2 were lower, being: 6.1, 3.7 and 1.8 for influents, effluents and surface waters, respectively. More recently, E2 and EE2 were found in Canadian raw sewage at levels of 66.9 and 5.7 ng L^{-1}, respectively (Atkinson et al., 2012). In what concerns surface waters, E2 and EE2 were quantified in Chinese river waters, in concentrations up to 31.4 and 24.4 ng L^{-1}, respectively (Rao et al., 2013). Results for the quantification of these hormones in Portugal will be addressed in detail in Chapter 2.

The occurrence of antibacterials is also not restricted to waste waters. In fact, the presence of SAs has been reported in all kind of water samples. This wide detection may be related to their rather poor chelating ability and low sorption tendency (Petrović and Barceló, 2007). SMX has been detected even in drinking water – Stolker et al. (2004) detected concentrations below 25 ng L^{-1} of SMX in two drinking water samples out of 22, from The Netherlands – and in groundwater – Lindsey et al. (2001) quantified the antibiotic (220 ng L^{-1})

in a groundwater sample from USA. More SMX quantification values reported in literature will be addressed in Chapter 3.

1.2.2 Analytical methods for the quantification of E2, EE2 and SMX in water matrices

The environmental analysis of pollutants constitutes a difficult task due to both the complexity of environmental matrices and the usually very low concentrations of the target compounds. Both reasons make it necessary the use of highly sensitive and selective analytical techniques, and that, in turn, make the chromatographic methods the methods of choice to quantify both estrogens and antibiotics.

Steroid estrogens have been quantified by gas chromatography–mass spectrometry (GC–MS) (e.g. Kuch and Ballschmiter, 2001; Peck et al., 2004; Williams et al., 2003), GC–MS/MS (e.g. Huang and Sedlak, 2001), liquid chromatography–diode array detector (LC–DAD) (e.g. López de Alda and Barceló, 2001a; Perez and Escandar, 2014), LC–DAD–MS (e.g. López de Alda and Barceló, 2000; López de Alda and Barceló, 2001b), liquid chromatography–mass spectrometry (LC–MS) (e.g. López de Alda and Barceló, 2000), LC–MS/MS) (e.g. Cui et al., 2006; Díaz-Cruz et al., 2003; Rodríguez-Mozaz et al., 2004), LC–MS/MS with electrospray ionization (ESI) (e.g. Baronti et al., 2000; Di Carro et al., 2010) and ultra-high performance LC (UHPLC) coupled to MS/MS (e.g. Ripolles et al., 2014).

In the case of antibiotics, the use of GC is quite limited because of their properties – these compounds are rather polar, non-volatile and in some cases thermal labile. Therefore, derivatization is always required, which makes the analysis difficult and, in general, worsen results (Petrović and Barceló, 2007). Despite those disadvantages, a promising GC-atomic emission detection method for the quantitative analysis of several SAs, including SMX, was developed by Chiavarino et al. (1998), but no application in environmental analysis was reported. Therefore, LC is the most used technique in the quantification of antibiotics, specifically, SMX. HPLC-MS (e.g. Ferguson et al, 2013; Lindsey et al, 2001), HPLC-MS/MS (e.g. Christian et al., 2003; Vaicunas et al., 2013; Wang and Gardinali, 2012), HPLC-DAD (e.g. Teixeira et al., 2008) and UHPLC-MS/MS (e.g. Tamtam et al., 2008; Tamtam et al., 2009) have been used in the quantification of SMX.

Chromatographic methods, although highly sensitive and specific, have several potential drawbacks, such as the expensive instrumentation/maintenance, the requirement of a very high level of technical expertise for operation, the need to perform extensive clean-up procedures, and the unsuitability for screening purposes, thus inhibiting an application on a broader base (Farré et al., 2007; Hintemann et al., 2006; Pu et al., 2008). Therefore, one of the main challenges in the monitoring of EDCs and PPCPs, as E2, EE2 and SMX, in water samples, is the implementation of sensitive, but also simple and low-cost analytical methods.

Immunochemical techniques, as enzyme linked immunosorbent assays (ELISA), offer high sensitivity, cost-effectiveness, rapid analysis and the possibility of analysing a large number of samples simultaneously. Also, small sample volumes are used and instrumentation may be available in portable format (useful in field studies) (Caron, 2010; Farré et al., 2006; Mispagel et al., 2009; Roda et al., 2006; Swart and Pool, 2007). In addition, contrarily to traditional analysis, ELISA do not require any prior derivatization (Gray and Sedlak, 2005) and is therefore not subjected to laboratory errors and costs associated with this extra step, being especially useful in situations where analysis by conventional methods is either impossible or prohibitively expensive. Quantification of E2, EE2 and SMX by immunoassays will be addressed in Chapters 2 and 3.

Due to the very low levels of these compounds in the environment, in essentially all cases analyte enrichment is necessary and solid phase extraction (SPE) is usually applied prior to analysis. However, there is an increasing need of performing large screenings of potentially polluted areas, which requires simplicity, quickness and cost-effectiveness. This can be accomplished either by techniques as immunoassays, for the reasons explained above, and that can be used without sample pre-treatment (*cf.* Chapter 2 and 3), or by a pre-concentration procedure, prior to analysis, itself simple and low-cost, as well as environmentally friendly (contrarily to SPE, that needs a large solvent consumption), as it will be addressed in Chapters 5 and 6 of this thesis.

1.2.3 Fate of E2, EE2 and SMX in the environment: photolysis as a transformation pathway and effect of dissolved organic matter

As it was already stated, photodegradation is one of the key transformation pathways of EDCs and PPCPs in the environment. In what concerns pharmaceuticals, photolysis may

even be the only relevant abiotic loss process in sunlit aquatic systems (Petrović and Barceló, 2007). Therefore, understanding this phenomenon is essential for the study of the fate of these pollutants in the environment.

In a study by Fonseca et al. (2011), capillary electrophoresis (CE) was used to follow the degradation of estrogens by solar radiation. CE results showed that, under direct solar radiation, the degradation rate varied between 75 and 100%. Authors also concluded that estrogens were not degraded during 126 d in darkness, under the effect of temperature (4, 20 and 30 ºC). Natural sunlight has been shown to degrade estrogens to some degree in both seawater (Zuo et al., 2006) and river water (Lin and Reinhard, 2005). EE2 undergoes a rapid photodegradation in estuarine seawater under natural sunlight irradiation, with a half-life of less than 1.5 d in spring sunny days, as observed by Zuo et al. (2006). However, because of the natural seasonal and diurnal variations in solar irradiance, as well as spatially (with both depth and shading), one would predict high variability in photolytic degradation under natural conditions.

Under laboratory studies, main limitation is that, often, they do not simulate conditions found in the natural aquatic environment (Atkinson et al., 2011). That is why it is essential trying to mimic some environmentally important factors. Chowdhury et al. (2011) studied the photodegradation of E2 and the influencing water parameters: under the presence of natural water constituents as NO_3^-, Fe_3^+ and humic acids (HA), the photodegradation rate increased significantly, while the presence of HCO_3^- decreased the degradation rate. Lin and Reinhard (2005) found that the presence of dissolved and suspended substances may increase the photodegradation rate of estrogens. Leech et al. (2009) showed that E2 photodegradation increases from ~26% to ~40–50% in presence of 2.0–15.0 mg L^{-1} of DOC, highlighting that a significant proportion of the observed degradation was due to radicals formed from the photolysis of DOC. Also Canonica et al. (2008) highlighted the photolysis rate enhancement of EE2 in presence of DOC and hypothesized that it acts as a photosensitizer, producing excited triplet states and radicals that react with the estrogen. The composition of the matrix played a significant role also in the photodegradation of SMX. Under simulated solar radiation, SMX degraded relatively quickly with half-lives of 1.5 h (Batchu et al., 2014). Also Jasper and Sedlak (2013) have shown the importance of significant levels of DOC in wetland water in increasing the photodegradation, as well as Andreozzi et al. (2003), that reported a faster photolysis in presence of HA and nitrate. Lee et al. (2014) also showed that SMX was amenable to direct photodegradation and the photodegradation rate had a slight increase in

presence of organic matter. Lam et al. (2005) and Niu et al. (2013), however, concluded that the presence of organic matter inhibited the photodegradation.

Therefore, dual roles may be played by DOC, which is a key parameter in photodegradation pathway, whether enhancing it or inhibiting it. These effects will be addressed in detail later in this thesis (*cf.* Chapter 7).

1.2.4 Removal processes for E2, EE2 and SMX in STPs

In order to avoid the potential risks caused by organic pollutants in aquatic environments, their removal from sewage at STPs before final release into the environment is considered significantly important. Research on different ways to remove them from water has been carried out by scientists from all around the world, especially during the last decades.

Biological degradation and *sorption* are the most common mechanisms by which organic pollutants are removed from water at STPs; though, as it will be shown below, the removal efficiencies are normally incomplete and advanced treatments should be applied (Calisto, 2011).

Sewage treatment at conventional STPs basically involves two stages: a primary and a secondary (or biological) treatment. Zhou et al. (2010) highlighted that the synthetic estrogen (EE2) was mostly removed in the primary treatment; however, primary treatment has been shown to have a weak impact on estrogens removal from waste water (Jiang et al., 2005; Johnson et al., 2005; Muller et al., 2008; Servos et al., 2005; Zhou et al., 2010). Generally, estrogens are mainly removed from waste water during the secondary treatment, particularly by the activated sludge treatment (AST) (Johnson and Sumpter, 2001). AST is the most widely applied biological process for sewage treatment (Clouzot et al., 2010) and, as early as 1999, E2 and EE2 removal by this process was found to be 99.9% and 78%, respectively (Ternes et al., 1999b). When compared with trickling filters (TF) treatment, AS provided higher estrogenic removal (81%), while TF provided 28% (Svenson et al., 2003). Similar results were found by Johnson et al. (2005), Schlüsener and Bester (2008) and Servos et al. (2005). However, membrane bioreactor (MBR) (Clara et al., 2005a; Joss et al., 2004) and fixed bed reactor systems (Joss et al., 2004) provided similar, and even better, estrogens' removal efficiencies than conventional AST. MBR technology is considered to be a hopeful solution for the removal from water of the synthetic EE2, which biodegradation seems to be more difficult

than that of natural estrogens (Clouzot et al., 2010). Solids retention time (SRT) seems to be a key parameter for effluent concentrations, since degradation may be only expected to occur above a critical value that allows for the growth and higher accumulation of microorganisms (Andersen et al., 2003; Clara et al., 2005a; Clouzot et al., 2010; Hashimoto and Murakami, 2009; Holbrook et al., 2002; Joss et al., 2004).

In what concerns SMX, Perez et al (2005) achieved 80% removal in AST, while Gao et al. (2012) attributed 50% of the 90% SMX removal achieved overall during conventional STP treatment, to AST. MBR results for SMX have demonstrated consistent results, with removals ranging from 52 to 70% (Clara et al., 2005b; Kim et al., 2007; Reif et al., 2008).

Nonetheless, removal of pollutants in STPs is not complete, possibly due to their fluctuating levels in the influent, type of process applied and/or operational conditions. Moreover, it is known that microorganisms present in STPs can convert the excreted conjugates back to the active unconjugated forms (Racz and Goel, 2010; Ying et al., 2002). Additionally, it should be noted that the biodegradation products can be more harmful than the parent substance (International Union of Pure and Applied Chemistry, 1993).

Recently, research on alternative microorganisms that may help on the degradation of organic pollutants has been carried out. Enzymes have been studied as suitable microorganisms for the degradation of aromatic compounds, even those with low solubility (e.g. Auriol et al., 2008; Blánquez and Guieysse, 2008; Cajthaml et al., 2009; Sei et al., 2008; Suzuki et al., 2003; Tamagawa et al., 2006). Also, microalgae have been studied with the same purpose (e.g. Della Greca et al., 2008; Shi et al., 2010).

Even though biodegradation is suggested to be the main removal mechanism by AST in STPs (Andersen et al., 2003; Muller et al., 2008), sorption onto AS has been pointed out to be more important than biodegradation by some authors (Mastrup et. al., 2001; Urase and Kikuta, 2005). Although it is more likely that sorption is the first stage in biological degradation of estrogens (Clara et al., 2004b) and that biosorption and biodegradation interact during AS treatment, it is still unclear which plays the predominant role in the elimination of pollutants from sewage (Ren et al., 2007) and results obtained by different studies are quite inconsistent. Suzuki and Maruyama (2006), Johnson and co-workers (Johnson et al., 2000) and Urase and Kikuta (2005) pointed out that sorption and biodegradation of E2 and EE2 were both responsible for their removal in AS plants. Andersen et al. (2005) and Li et al. (2005) stated that the role of sorption by biomass was less significant in the removal of steroid estrogens in aerobic sludge process. Very recently, Alvarino et al. (2014) also found that

removal of both estrogens and SMX by sorption onto sludge was insignificant compared to biodegradation, as well as Gao et al. (2012), for SMX only.

Apart from the sorption occurred during AST at STPs, other sorption approaches have been explored and different adsorbents have been identified and investigated for this purpose, as next: activated carbon (e.g. Fukuhara et al., 2006; Jung et al., 2013; Kumar and Mohan, 2011; Zhang and Zhou, 2005), molecularly imprinted polymers (e.g. Fernández-Álvarez et al., 2009; Le Noir et al., 2007) and membranes (e.g. Bolong et al., 2009; Nghiem et al., 2004; Yoon et al., 2004; Yoon et al., 2006).

Advanced oxidation processes (AOP) have to be highlighted as a widely investigated area as alternative for both secondary waste water effluent treatment and disinfection step of drinking water pre-treatment. AOP refers specifically to processes in which the oxidation of organic contaminants occurs primarily through reactions with hydroxyl radicals (Glaze et al., 1987). *Ultraviolet* (UV) irradiation is widely used to disinfect water and waste water (Coleman et al., 2004; Liu and Liu, 2004; Mazellier et al., 2008). Under the use of radiation also lies the *heterogeneous photocatalysis* consisting in the capacity of semiconducting materials to act as sensitizers for light-reduced redox processes due to their electronic structure. Both E2 and EE2 and antibiotics have been shown to be amenable to degradation by this process (e.g. Adamek et al., 2012; Puma et al., 2010). *Strong oxidizers* have also been used in order to accomplish the removal of organic pollutants from waters (e.g. Jiang et al., 2009; Lee and von Gunten, 2009; Miralles-Cuevas et al., 2014). At last, *sonolysis* is a treatment consisting in the irradiation of ultrasound waves at low to medium frequency (20–1000 kHz) into a liquid medium (Adewuyi, 2001; Augugliaro et al., 2006; Suri et al., 2007) that has been shown to degrade organic chemicals (Suri et al., 2007; Suri et al., 2010).

Although the referred methods are effective in the removal of pollutants, it must be pointed out that under certain circumstances, an AOP can be a cause of concern itself, since it may render harmful by-products or transformation products whith similar or increased negative effects relative to the parent compounds (Bila et al., 2007; Lee and von Gunten, 2009). On this basis, the disappearance of the original compound does not necessarily imply the efficiency of the treatment.

1.3 ANTHROPOGENIC MARKERS FOR PHARMACEUTICALS INPUT IN ENVIRONMENTAL WATERS

One of the main objectives of drinking water suppliers is to be aware of the influences on their raw water sources used for drinking water production, to ensure potable water of high quality (Scheurer et al., 2011). It is therefore necessary to identify markers that are able to provide an early indication of contamination and that can be used for the quantification of the waste water burden. Moreover, for wells unaffected by waste water the capability of an early detection of traces of the marker can be used as an early warning system of a waste water breakthrough, e.g. an occurring leakage in the sewer system.

Traditionally, bacterial indicators, such as *Escherichia coli* have been used to test water quality. However, this type of indicators requires at least 24 h for obtaining data, being time-consuming in analysis, and do not discriminate between animal and human faecal sources, lacking selectivity (Carvalho, 2011; Ericksson, 2002; Scheurer et al., 2011). Recent literature shows that numerous other markers have been used to assess the contamination by domestic waste water (Buerge et al, 2003a; Buerge et al., 2006; Clara et al., 2004a; Managaki and Takada, 2005; Nakada et al., 2008).

An ideal marker should allow the unambiguous recognition of the pollution source. For domestic waste water, constant loads of the marker, as well as high concentration in effluent, to permit quantification after dilution in the receiving waters, are pre-requisites (Kahle et al., 2009; Scheurer et al., 2011). Therefore, organic trace pollutants with anthropogenic origin are suitable candidates and have become more and more popular as markers for waste water impact in the last decade.

Caffeine (1,3,7-trimethylxanthine; Fig. 1.4) is a xanthine alkaloid widely found in derived food products, as tea, cocoa, chocolate, energy drinks and, of course, coffee. It is also present in a large number of prescriptions because of its diuretic properties and benefits associated with improvements in alertness, learning capacity and exercise performance. About 80% of the caffeine dosage is metabolized in the liver to paraxanthine (1,7-dimethylxanthine), 10% to theobromine (3,7-dimethylxanthine) and 4% to theophylline (1,3-dimethylxanthine) (Bueno et al., 2011).

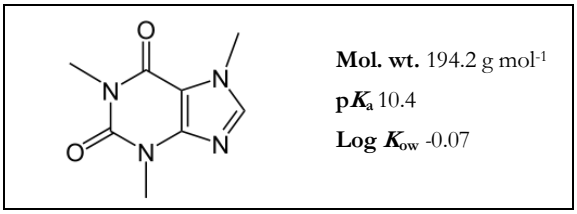


Fig. 1.4: Caffeine structure and some physicochemical properties (Petrović and Barceló, 2007; Ying et al., 2002).

Caffeine has been found in most of the studies where pharmaceuticals were monitored: in waste water effluents (Bartelt-Hunt et al., 2009; Buerge et al., 2006; Choi et al., 2008; Lin et al., 2008; Santos et al., 2007), surface waters (Metcalfe et al., 2003; Verenitch et al., 2006), groundwater (Barnes et al, 2008; Seiler et al., 1999); untreated (Focazio et al.; 2008) and treated drinking water (Hummel et al., 2006; Loos et al., 2007). Consequently, caffeine seems to be a serious candidate to become a chief marker for pharmaceuticals input in natural waters, becoming already an environmental marker of choice for human pollution contamination (Buerge et al., 2003b; Buerge et al., 2006; Ferreira, 2005; Ferreira et al., 2005; Glassmeyer et al., 2005; Nicolardi et al, 2012; Sauvé et al., 2012). Caffeine and its role as an anthropogenic marker will be addressed in Chapter 4 of this thesis.

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PART I

Development of low-cost analytical methodologies for quantification of estrogens, antibiotics and anthropogenic markers in water samples

CHAPTER 2

Development of enzyme linked immunosorbent assays for direct determination of E2 and EE2 in water samples

Silva, C.P.; Lima, D.L.D.; Schneider, R.J.; Otero, M.; Esteves, V.I.; Development of ELISA methodologies for the direct determination of 17β-estradiol and 17α-ethinylestradiol in complex aqueous matrices; *Journal of Environmental Management* 124 (2013) 121-127.

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Summary

This study comprises the development of E2 and EE2 ELISAs for use in complex aqueous matrices without any sample clean-up procedures. Salinity and dissolved organic matter were selected as potential interfering agents in the analysis of E2 and EE2. The optimization was performed in order to (i) overcome matrix effects, and to (ii) increase sensitivity. The addition of a sample buffer containing bovine serum albumin (BSA) prior to the sample was found to decrease the influence of matrix effects. Moreover, adjustments of this buffer's pH together with the optimization of tracer (T) dilution and incubation time were undertaken in order to lower the quantification range. The optimized methods allowed the quantification of E2 and EE2 in the ranges 0.03-200 μg L^{-1} and 0.02-10 μg L^{-1}, respectively. The assays were applied to real complex aqueous samples. It was possible to do a first approach to the levels of E2 in Portuguese surface and waste waters, by ELISA; however, it was not feasible to quantify EE2 in the samples tested.

Contents

2.1 CONTEXTUALIZATION

During the last years, many efforts have been devoted to the development of analytical methodologies sensitive enough to allow the determination of estrogens in environmental samples (Farré et al., 2007). A crucial point is, as it was already stated, the ability of the method to detect and quantify these compounds at very low concentrations (Roda et al., 2006). Also, it is important that the method presents simplicity and cost-effectiveness.

Therefore, the major objective of this chapter was to develop a low cost, simple and rapid methodology for the quantification of E2 and EE2 in water samples of different and complex matrices. Attention was paid to important aspects like influence of matrix effects and sensitivity. To the best of the author's knowledge the work conducted in this chapter is the first one dealing with the quantification of E2 and EE2, by ELISA, in Portugal.

2.1.1 Quantification of E2 and EE2 in water samples

2.1.1.1 Quantification of E2 and EE2 in Portugal

Despite the fact that the presence of estrogens in the environment is very concerning, studies on their quantification in Portuguese surface and waste waters are very recent (Table 2.1).

Table 2.1: Studies conducted in Portugal concerning E2 and EE2 quantification in water samples.

Water samples	Employed technique	Concentrations measured (ng L^{-1})	Observations	Reference
Douro River estuary (DRE)	SPE-HPLC-DAD[a]	EE2 up to 56.0	First time data about EDCs in the DRE	Ribeiro et al. (2007)
Mondego River estuary	SPE-HPLC-DAD	EE2 <LOD E2 <LOD	- Seasonal sampling between 2005 and 2006	Ribeiro et al. (2009a)
Sado River estuary	SPE-HPLC-DAD	EE2 <LOQ E2 <LOD	- Sampling in 2006 - Spatial and seasonal study	Ribeiro et al. (2009b)
DRE	SPE-HPLC-DAD	E2 <LOD EE2 up to 101.9	- Sampling in 2005/2006 - Spatial and seasonal study	Ribeiro et al. (2009c)
DRE and Atlantic Ocean	SPE-GC-MS[b]	E2 6.3–14.4 EE2 <LOD–2.8	- Sampling during March 2009	Rocha et al. (2011)
DRE and Oporto coastline	SPE-GC-MS	E2 5.2–5.7 EE2 1.0–1.3	- Sampling from late March to late May 2009	Rocha et al. (2012a)
Leça River and Oporto coast	SPE-GC-MS	E2 3.3–5.9 EE2 2.1–4.4	- Sampling on 2009 - Seasonal study	Rocha et al. (2012b)
Sado River estuary	SPE-GC-MS	E2 1.2–11.6 EE2 1.1–3.1	- Sampling throughout 2010 - Spatial and seasonal study	Rocha et al. (2013a)
Ria Formosa lagoon	SPE-GC-MS	E2 1.2–10.1 EE2 12.1–25.0	- Sampling on 2010 - Spatial and seasonal study	Rocha et al. (2013b)
DRE and Oporto coastline	SPE-GC-MS	E2 5.4–8.5 EE2 <LOD–4.5	- Sampling on 2010 - Spatial and seasonal study	Rocha et al. (2013c)
Ave River and Vila do Conde coastline	SPE-GC-MS	E2 1.6–9.4 EE2 0.3–20.4	- Sampling throughout 2010 - Spatial and seasonal study	Rocha et al. (2013d)
Ria de Aveiro lagoon and 13 Rivers[c]	SPE-GC-MS	E2 n.d. –11.5 EE2 n.d. –<LOD	- E2 >LOQ in Ave (8.9 ng L^{-1}), Lima (11.5 ng L^{-1}) and Tâmega (9.5 ng L^{-1}) Rivers	Rocha, S. et al. (2013)
Ria de Aveiro lagoon and Aveiro's STPs	SPE-LC-MS/MS[d]	E2 <LOD–9.2 EE2 <LOD	- Sampling in 2005/2006 - Seasonal study	Sousa et al. (2010)

n.d. - non detected; [a]SPE followed by high performance liquid chromatography with diode-array detector; [b]Solid phase extraction followed by gas chromatography–mass spectrometry; [c]Minho, Lima, Cávado, Ave, Vizela, Ferro, Douro, Sousa, Ferreira, Tâmega, Paiva, Vouga and Águeda; [d]Solid phase extraction followed by liquid chromatography–tandem mass spectrometry.

High levels of EE2 (up to 101.9 ng L^{-1}) and atypical comparing with literature, were detected in DRE by Ribeiro et al. (2009c). Authors justified this high value (obtained only in one sample site) with the low drainage rates of the River associated to the most severe drought in 60 years in the region that occurred in 2005. This was supported by later results in the same sampling sites, where EE2 levels became undetectable.

Rocha et al. (2013b) detected levels of E2 and EE2 up to 10.1 and 25.0 ng L^{-1}, respectively, in Ria Formosa lagoon. Authors stated that the studied area was impacted by the

effluents coming from 28 STPs, known to have functional problems. Apart from that, authors accounted also with the direct discharges coming from innumerous recreational boats and non-treated sewages contributing to the estrogenic load of the area. Since it was a seasonal study, in summer, when the number of inhabitants is significantly higher, an almost twofold increase of the E2 and EE2 levels was observed. The authors pointed out that E2 and EE2 were present in amounts able of inducing estrogenic effects in fish and other animals, including bivalves that are utterly important for the local economy.

The existence of STPs within the sampling area was also used as justification for the high estrogenic load observed in the Ave River (Rocha et al., 2013d), with E2 levels similar to to those measured in The Netherlands, Belgium, Italy and France (Baronti et al., 2000; Belfroid et al., 1999; Cargöuet et al., 2004; Noppe et al., 2007).

It is important to highlight that in the works carried out in the Aveiro region (Rocha, S., 2013; Sousa et al., 2010), neither E2 nor EE2 were detected in the estuarine samples. An E2 concentration of 9.2 ng L^{-1} was found in an Aveiro's STP effluent sample (Sousa et al., 2010), but it is important to state that, at present, this STP is no longer operative.

2.1.1.2 Quantification of E2 and EE2 by ELISA

Commercial ELISAs have been commonly used to detect estrogens in water matrices such as waste waters from STPs (e.g. Dorabawila and Gupta, 2005; Drewes et al., 2005; Hintemann et al., 2006; Lee et al., 2006; Suzuki and Maruyama, 2006; Swart and Pool, 2007).

Farré et al. (2006) compared different ELISA kits in the analysis of estrogens E2 and EE2. The linear ranges obtained for the different ELISA kits were in the range 500–5000 ng L^{-1} and it was necessary to perform a pre-concentration step prior to analysis. Authors compared the results with HPLC-MS/MS based on triple-quadrupole analyzer (QqQ) and found similar results by both techniques. These techniques and a third one - a method based on ultra performance liquid chromatography–quadrupole time of flight mass spectrometry (UPLC–Q-TOF-MS) - were also compared by Farré et al. (2007) in the determination of E2. Authors observed a moderate overestimation of the results by ELISA, especially in the analysis of complex waste water samples. However, results obtained by the three techniques were in good agreement. Huang and Sedlak (2001) developed an ELISA procedure for the determination of E2 and EE2 in a secondary waste water effluent and surface water,

determined concentrations ranging from 0.2 to 4.1 ng L^{-1}. Results were validated by GC-MS/MS. Authors performed sample extraction with C18 disks prior to analysis by ELISA. A similar approach, with slightly lower sensitivity, was described by Shishida et al. (2000) for determination of E2 in waste water. After SPE with SepPak C18 cartridges, the method LOD achieved was 10 ng L^{-1}. Valentini et al. (2002) validated an electrochemical ELISA procedure by LC-ESI-MS/MS showing that the electrochemical ELISA assay was suitable as a screening tool for the analysis of E2 in waste waters. Dorabawila and Gupta (2005) analysed the presence of E2 in surface water samples from ponds, rivers and coastal bays. Samples were filtered and E2 extracted by C18 cartridges. Concentrations in river waters varied between 1.9 and 6.0 ng L^{-1}. Highest E2 concentrations in river waters were observed immediately downstream of STPs. E2 concentrations in all the coastal bays tested were 2.3-3.2 ng L^{-1}.

Despite the good results on the quantification of estrogens in water, immunoassays are not immune to difficulties, which are especially troubling in the application of ELISA to environmental water samples. These drawbacks include narrow specificity: experiments conducted by Goda et al. (2000) suggested that ELISA may give overestimated values because of cross-reactivity. Besides, studies have revealed that the analysis of environmental water samples can yield conflicting results due to matrix effects (Hanselman et al., 2004; Mispagel et al., 2009). In fact, most efforts in environmental analysis have to be focused on the minimization of matrix effects. Suppression or enhancement of the analyte signal is a complex effect whose extent seems to be dependent on several experimental and instrumental conditions. Therefore, it is indispensable to account with matrix effects when developing and optimizing an analytical method for pollutants' quantification.

Also, as it can be seen by a quick search in literature, SPE is usually applied prior to analysis by ELISA in order to achieve lower detection levels. Therefore, there is much to be done in order to optimize some parameters that may permit to accomplish two main issues: decrease the matrix effects observed when working with real samples and decrease the limits of detection for direct determination.

2.1.2 Immunochemical methods

Immunochemical methods were first applied in clinical situations. Clinical chemists used the sensitivity and selectivity of these methods and developed highly successful

diagnostic techniques for medical research and health-care applications (Van Emon, 2001). Yalow and Berson (1959) presented a brand new methodology for the determination of protein hormones in blood, the fundamental principle of which utilized the ability of these hormones to stimulate antibodies formation. Yalow ended up winning the Nobel Prize in 1977 "for the development of radioimmunoassays of peptide hormones" (Nobel Prize Organization, 2014). Radioimmunoassay (RIA) is now widely used. However, radioisotopes are known to be hazardous, costly and monitoring and disposal procedures are difficult (Mikkelsen and Cortón, 2004). A suitable alternative to RIA would be substituting the radioactively-labeled antigens or antibodies for non-radioactive labeled ones. The first paper about ELISA, which was published in 1971, was the one by Engvall and Perlmann (1971), who quantified immunoglobulin G in rabbit serum using alkaline phosphatase as label. Meanwhile, pesticide chemists started to realize the potential benefits of immunochemical methods (Van Emon, 2001) and the first assay for the pesticides aldrin and dieldrin was developed by Langone and Vanvunakis (1975).

Later, in the 1980s, the utility of RIA and ELISA in environmental monitoring was recognized by the Environmental Protection Agency (EPA) and other specialized agencies, which became interested in these innovative methods for analyzing matrices of environmental significance (Van Emon, 2001).

Regarding the environmental monitoring of pharmaceuticals, efficient methodologies are required to detect trace levels of contamination. In this sense, immunoassays offer simplicity and can provide rapid screening information or quantitative data to fulfil rigorous data quality objectives (Petrović and Barceló, 2007; Van Emon, 2001). Because of their sensitivity and selectivity, immunoassays have proven to be reliable for measurement of various contaminants at trace concentrations. Immunoassays can also provide supplemental data by detecting complex environmental or biological conjugates not amenable to instrumental methods (Van Emon, 2001).

2.1.3 Antibodies

2.1.3.1 Structural properties and interaction with antigens

Immunochemical techniques are based on the affinity of an antibody (Ab) against an antigen (Ag), being this interaction very specific (Petrović and Barceló, 2007). Abs are

produced as an immune response to an immunogen and, on the other hand, Ags are species that are able to bind selectively to Abs, but not necessarily capable of generating an immune response (Mikkelsen and Cortón, 2004).

Abs are large Y-shaped macromolecules (Fig. 2.1) that belong to a glycoproteins' family, structurally related, and called immunoglobulins (Ig), present in the blood serum of all mammals. These proteins are naturally formed by reaction with Ags ("strange" substances to the organism). Five different classes of Ig are known: IgG, IgA, IgM, IgD and IgE, differing of each other in size, charge, aminoacids composition and carbohydrates content. The common structural features enable Igs to do two things: (i) recognize and bind specifically to a unique structural entity on an Ag (the epitope) and (ii) perform a common biological function after combining with the Ag (Benjamini and Leskowitz, 1991; Mikkelesen and Cortón, 2004).

These molecules consist of two identical light (L) chains and two identical heavy (H) chains linked by disulfide bridges (Fig. 2.1). In the resultant structure, the portion of the molecule that binds the Ag consists of an area composed of the amino-terminal regions of both H and L chains. Thus, each Ig molecule composed of 2H and 2L chains is symmetric and is capable of binding two identical epitopes, either on the same Ag molecule or on two different molecules (Benjamini and Leskowitz, 1991).

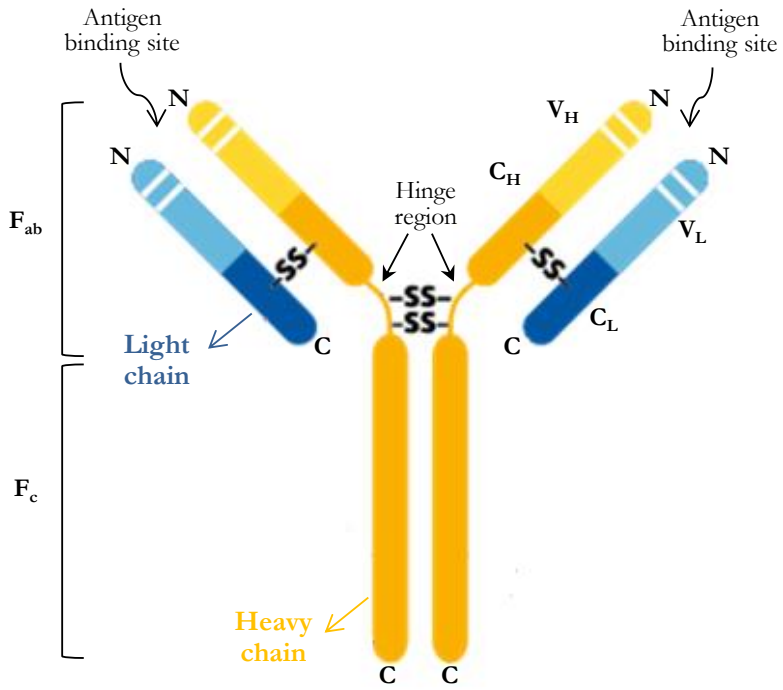

Fig. 2.1: Schematic representation of the basic structure of an Ab (IgG) (C_L – constant domain, light chain; C_H – constant domain, heavy chain; V_L – variable domain, light chain; V_H – variable domain, heavy chain; F_{ab} – fragments wich are monovalent antigen binding proteins; F_c - fragment which is part of the constant region of the two heavy chains linked through disulphide bridges) (adapted from eBioscience (2014)).

The selectivity of Ag-Ab interactions is analogous to the selectivity of substrate-enzyme interactions. The Ag binding site of an Ab has a structure that allows a complementary fit with structural elements and functional groups on the Ag (Mikkelsen and Cortón, 2004). The immune complex is stabilized by the combination of weak interactions that depend on the precise alignment of the Ag and Ab. Binding interactions between Ag and Ab involve hydrogen bonds, van der Waals forces, coulombic interactions and hydrophobic interactions. These interactions can occur between side chains or the polypeptide backbones (Hammock and Gee, 1995; Harlow and Lane, 1999; Mikkelsen and Cortón, 2004).

Ags may be classified according to the total number of binding sites and the number of different types of sites (Mikkelsen and Cortón, 2004).

2.1.3.2 Production of Abs

Abs for compounds of low molecular weight - as it is the case of some environmentally concerning compounds - can be difficult to develop because, although they may be antigenic, they cannot stimulate Ab production. The small molecule, or frequently a derivative of the compound (termed "hapten"), must be conjugated to a carrier molecule, such as a protein or a polymer, to form an immunogen. Frequently, when forming the hapten, a chemical functionality, such as OH, COOH, NH_2, or SH, is introduced onto the target analyte for conjugation with the carrier protein. The hapten portion of the immunogen should mimic as closely as possible the structure of the target molecule (size, shape, and electronic properties). The ideal approach is to develop a large library of haptens for Ab production (Van Emon, 2001).

To produce specific Abs, a selected Ag is injected into a laboratory animal and serum samples are collected. This serum becomes a source of Abs that can bind specifically to the Ag (Harlow and Lane, 1999). Abs can be monoclonal or polyclonal. Monoclonal Abs are produced by fusing Ab-producing spleen cells with mutant tumour cells derived from myelomas. Somatic cell hybridization enables the fusion between the myeloma cell and the Ab-producing spleen cell from an immunized animal. Once a hybridoma produces the desired Ab it is cloned for large-scale production. The result is the preparation of a single Ab population (Harlow and Lane, 1999; Mikkelsen and Cortón, 2004; Van Emon, 2001).

Polyclonal Abs are the most commonly used reagents for immunochemical techniques

(Harlow and Lane, 1999) and rabbits and goats are the animals more frequently used to produce them (Hammock and Gee, 1995). In this case, antiserum contains several different populations of Abs with varying degrees of selectivity towards the immunogen. Thus, the activity of a polyclonal antiserum is a combination of the responses from the different existing Abs (Van Emon, 2001). This type of Abs generally has higher affinity for a given analyte than monoclonal ones and is less expensive to produce (Van Emon, 2001).

Immunoassay performance is a function of the affinity, selective recognition and binding properties of Abs that result in a product (Ab–Ag) that can be measured (Mikkelsen and Cortón, 2004; Van Emon, 2001). This measurement is possible using the so-called tracer (T) – a labelled Ag or Ab. Its synthesis will be addressed later in this thesis (*cf.* Chapter 3).

2.1.4 Immunoassays' classification and ELISA

The classification of an immunoassay relies on (i) which species (Ab or Ag) is labelled; (ii) the type of label employed, and mainly (iii) whether they are heterogeneous – wherein a separation of bound and free Ab is required –, or homogeneous – requiring no such separation prior to measurement (Mikkelsen and Cortón, 2004; Petrović and Barceló, 2007; Van Emon, 2001).

Homogeneous immunoassays rely on labelled Ag species that show large signal changes upon Ab binding, so that separation of the bound and free fractions of the label is unnecessary (Mikkelsen and Cortón, 2004). These immunoassays are highly matrix dependent because the colour or turbidity of samples interferes with the signal from the coloured end-product of the assay (Van Emon, 2001). Usually, the labels used in this type of immunoassays are fluorophores and enzymes (Mikkelsen and Cortón, 2004).

ELISA are the most well-known and frequently used heterogeneous enzyme immunoassays formats (Mikkelsen and Cortón, 2004; Petrović and Barceló, 2007) and may be competitive or noncompetitive.

Noncompetitive ELISAs are based on sandwich assays (Fig. 2.2): an Ab is immobilized in excess, quantitatively binding the Ag; a second Ab, enzyme-labelled, is then allowed to react with the bound Ag, forming a sandwich that is detected by measuring enzyme activity bound to the surface of the support. Resulting calibration curves show an enzyme activity that increases with increasing free Ag concentration (Mikkelsen and Cortón, 2004).

This method can only be applied when the analyte of interest possesses at least two binding sites. Thus, it is not appropriate for very small molecules (Hennion and Barceló, 1998).

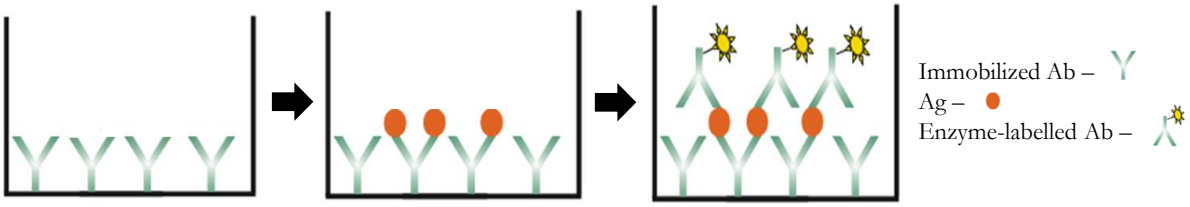

Fig. 2.2: Sandwich ELISA procedure (adapted from Schubert-Ullrich et al. (2009)).

A competitive ELISA may be based on direct binding or indirect competition (Petrović and Barceló, 2007; Van Emon, 2001). In the direct format, usually the immunoreagent immobilized onto the well is the Ab. The analyte in the sample competes with a known amount of labelled analyte for binding sites on the Ab. After a washing step, the unbound reagents are removed and the amount of label bound to the Ab is measured. Signal is inversely proportional to the amount of analyte in the sample (Petrović and Barceló, 2007; Van Emon, 2001). This was the format used in this work and its procedure will be addressed later (*cf.* Section 2.2.3). In the indirect format (Fig. 2.3), it is the Ag to be coated on the plate, but in this case, the amount of analyte present in the sample is indirectly measured by measuring the bound Ab with a second one that is conveniently labelled (Petrović and Barceló, 2007). Competitive ELISAs (indirect and direct) yield calibration curves in which enzyme activity decreases with increasing Ag concentration (Mikkelsen and Cortón, 2004).

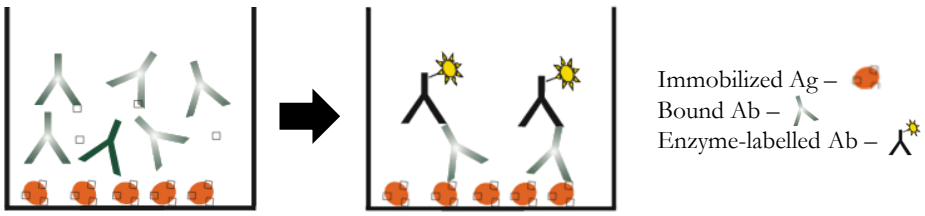

Fig. 2.3: Competitive indirect ELISA procedure (adapted from Schubert-Ullrich et al. (2009)).

2.1.5 ELISA calibration curve

In a competitive ELISA format the photometric determination of the enzyme activity by measuring absorbance is related to the analyte concentration via a dose-response curve (Fig. 2.4). This type of curve has a sigmoidal shape, with a linear central region.

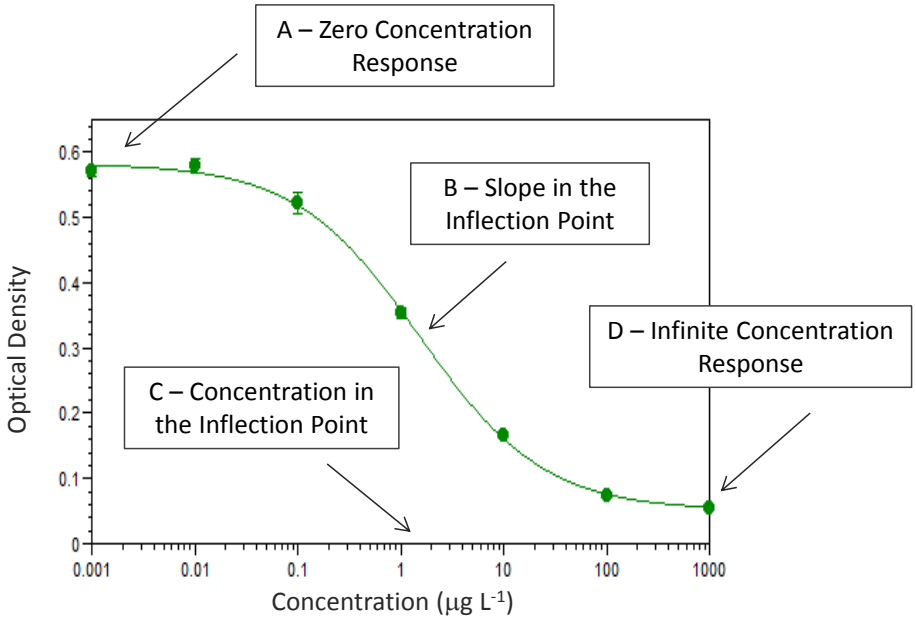

Fig. 2.4: Typical 4-parameter logistic function graph for an ELISA.

This shape is formed by fitting the data to a four parametric logistic equation (4PL) (Dudley et al., 1985):

$$y = \left[\frac{A-D}{\left[1+\left(\frac{x}{C}\right)^{B}\right]}\right] + D \qquad \text{(Eq. 2.1)}$$

where y is the optical density (OD); x, the antigen concentration; A, the OD for an infinitely small analyte concentration ("blank"); B, the slope at the inflection point; C, the concentration value at the inflection point; D, the OD for an infinite analyte concentration (standard excess).

At high concentrations of analyte, there is little binding to the solid phase (100% inhibition), while at low concentration of analyte there is maximal binding to the solid phase (0% inhibition) (Harrison et al., 1990).

In order to compare several standard curves, the OD data should be normalized between 100% (OD of a zero standard) and 0% (OD of a standard excess), according to the equation:

$$Y_N = \frac{Y - D}{A - D} \qquad \text{(Eq. 2.2)}$$

where Y_N is the normalized OD, Y, the OD, A and D, parameters of the 4PL (Schneider et al., 2005).

Moreover, from the dose-response curve one can conclude about the sensitivity of the immunoassay, which can be expressed by the limit of detection (LOD), considered as the lower concentration that produces a signal considerably different from the blank signal. There is a general consensus to define LOD by selecting the dose that inhibits 10% of the enzyme T binding to the Ab (Hennion and Barceló, 1998). However, other definitions can be found: LOD can be defined as the concentration that yields a signal that is equal to the mean of the blank signal plus two or three standard deviations (Mikkelsen and Cortón, 2004). LOD can be used to compare different immunoassay methods at the lower concentration limit, however it says nothing about the reliability of the assay, and so, detection limits should be used in conjugation with precision profiles (Mikkelsen and Cortón, 2004).

2.1.6 Precision profile and quantification range

From the OD standard deviations and the slope (1st derivative) at each individual standard concentration, a relative error of the analyte concentration readings is calculated in order to set up the *precision profile*. Precision profile undoubtedly represents the performance characteristics of the assay/operator combination with regard to random errors and is thus one of the fundamental indices of assay quality (Ekins, 1981).

Method by Ekins (1981) defined the precision profile as a graphical representation of the random error in the analyte measurement at each value of the analyte concentration. Both the error of the response and the slope of the calibration curve vary from point to point. The error of concentration is directly proportional to the error of the response and indirectly proportional to the slope of the calibration curve. Therefore, at a certain point the error of concentration ($error_x$) is given by:

$$error_x = \frac{\sigma_y}{slope} \quad \text{(Eq. 2.3)}$$

where σ_y is the standard deviation of the response for replicate measurements. The determination of this quotient for a number of points along the ELISA calibration curve allows building the precision profile. The slope of the calibration curve, at a given concentration value, is given by the first derivative of the 4PL function (Law, 2005). Precision profile in terms of relative error of concentration (%) is given, at each point, by:

$$\text{Relative error of concentration} = \frac{\sigma_y}{slope \times x} \times 100\% \quad \text{(Eq. 2.4)}$$

For instrumental methods, LOD is the lowest concentration that can be distinguished from a blank value within an established confidence limit, estimated from the mean of the blank and σ=3 times its standard deviation. In allusion to this "three-sigma-criterion" a relative error of 30% is usually stipulated (Ekins, 1981; Grandke et al., 2013) as the maximum allowable error for quantification.

The precision profile is also considered the best method to determine the quantification range that is defined as the maximum and minimum concentration quantifiable with an acceptable degree of precision (Ekins, 1981; Law, 2005).

2.1.7 Cross-reactivity

The assay specificity describes the ability of an Ab to produce a measurable response only for the analyte of interest. Cross-reactivity (CR) is a measurement of the Ab response to substances other than the analyte and has a critical importance for immunoassays in which a particular analyte is assayed in the presence of very similar species. CR is calculated as the ratio of molar concentrations at the inflection points (midpoints, C parameter in the 4PL) of the corresponding calibration curves and expressed in percentage relative to the midpoint for the Ag (Schneider et al., 2005):

$$CR = \frac{C_{std}}{C_{test}} \times 100 \quad \text{(Eq. 2.5)}$$

where *CR* is the cross-reactivity, C_{std} is the parameter of the 4PL giving the Ag concentration at the inflection point and C_{test} is the concentration of the cross-reacting compound at its inflection point (Schneider et al., 2005).

2.2 EXPERIMENTAL SECTION

2.2.1 Reagents and materials

All reagents were of analytical grade and were used as received. Polyclonal Abs and Ts were kindly provided by Federal Institute for Materials Research and Testing - BAM, Berlin, Germany and their production and synthesis are described in Hintemann et al. (2006).

E2 (≥97%, HPLC) and EE2 (≥98%, HPLC) were supplied by Sigma. Tetramethylbenzidine (TMB, puriss), tetrabutylammonium borohydride (TBABH, >97%), dimethylacetamide (DMA), tris(hydroxymethyl)aminomethane (TRIS, p.a.), bovine serum albumin (BSA, for electrophoresis, 98%) and commercial HA (technical) were purchased from Sigma. Sodium phosphate dibasic dihydrate (>99%), sodium phosphate monobasic dihydrate (>99%), potassium sorbate (>99%), potassium dihydrogen citrate (>99%), hydrogen peroxide (30%), Tween™ 20 and sulfuric acid (95-97%) were from Fluka. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, p.a.) and sodium chloride (99.5%) were from Panreac. Sodium azide was from Riedel-de Haën.

Ultrapure water, used in the preparation of solutions, was obtained using a Millipore water purification system (Milli-Q plus 185).

Transparent 96 flat-bottom well microtiter plates with high binding capacity (MaxiSorp™) were purchased from Nunc (Thermo Scientific). Washing steps were carried out using an automatic 8-channel plate washer (Atlantis, ASYS Hitech). Plates were shaken using a plate shaker (Titramax 100, Heidolph). OD was read at 450 nm and referenced to 620 nm using a microplate spectrophotometer (UVM340, ASYS Hitech).

Filters (pore size 0.45 μm), used to filtrate real samples, were from Millipore.

2.2.2 Water samples

Water samples were collected (250 mL) in cleaned dark glass bottles (previously washed 3 times with a few millilitres of the sample to be collected), in and around Aveiro. Samples were collected in May 2010 and February 2011 in the Aveiro district, on the Northwest coast of Portugal (Fig. 2.5). Immediately after collection, all the samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at 4ºC until

analysis. Samples were not subjected to any other cleaning procedures or extraction or enrichment processes.

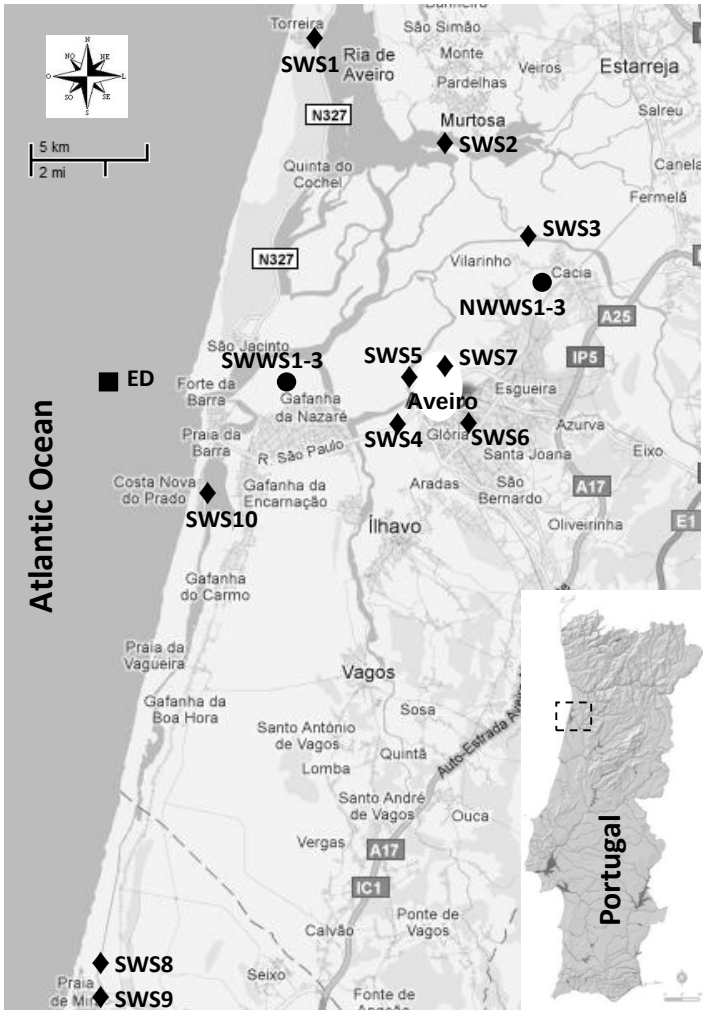

Fig. 2.5: Schematic representation of the sampling region in and around Aveiro: surface water samples from Ria de Aveiro, SWS1-10 (♦); waste water samples from the North STP, NWWS1-3, and waste water samples from South STP, SWWS1-3 (●); location of the effluent discharge of the two STPs, ED (■).

2.2.2.1 Surface water samples

Surface water samples were collected from different locations of Ria de Aveiro in: (i) rural areas (SWS2 and SWS3), (ii) urban areas (SWS4-7) and (iii) coastal areas (SWS1; SWS8-10) (Fig. 2.5 and 2.6).

Ria de Aveiro is a shallow lagoon (average depth of 1 m) situated in the Northwest Atlantic coast of Portugal (40º38'N, 8º45'W) with 45 km long and 10 km large. The lagoon

receives freshwater from two main rivers, Antuã River and Vouga River. Also, Ria de Aveiro has a number of channels, the more important being S. Jacinto and Espinheiro channels. Building and land occupation and agricultural and industrial activities have been growing near Ria de Aveiro margins, resulting in a constant input of anthropogenic nutrients and contaminants (Dias and Lopes, 2006; Lopes et al., 2006).

Fig. 2.6: Images from (a) SWS3; (b) SWS4; (c) SWS1; (d) NWWS1; (e) NWWS2.

2.2.2.2 Waste water samples

Waste water samples were collected in the two STPs serving Aveiro city ("North" and "South" STPs) and three collection points were selected in each one: after primary decantation (WW1), after secondary biological treatment (WW2) and after secondary decantation (which corresponds to the final treated effluent, WW3) (Fig. 2.5 and 2.6).

North STP was dimensioned to serve a population of 272 000 inhabitants and an average daily flow of 48 705 m^3. In the liquid phase, this STP performs the treatment of domestic and industrial effluents, following the steps: pre-treatment, primary decantation (both constituting primary treatment), biological treatment and secondary decantation (both constituting secondary treatment). From the sewage treatment results a solid phase (sludge) that is anaerobically treated at the STP to produce biogas (to obtain energy) and digested and dehydrated sludge (to apply in agricultural land). South STP was projected to serve a population of 159 700 inhabitants and an average daily flow of 39 278 m^3. Aqueous and solid

phases' treatment is similar to the treatment applied at North STP, as detailed above. However, South STP only performs the treatment of domestic effluents (SIMRIA, 2014).

The treated aqueous effluents are discharged into the Atlantic Ocean by a submarine outfall located at 3.3 km from the coast (ED, Fig. 2.5). Before rejection, effluents are controlled by analytical analysis in order to safeguard the quality of the receiving environment, including water quality at beaches for bathing purposes.

2.2.3 ELISA procedures

Direct competitive ELISA was used in the analysis of E2 and EE2, as schematized in Fig. 2.7.

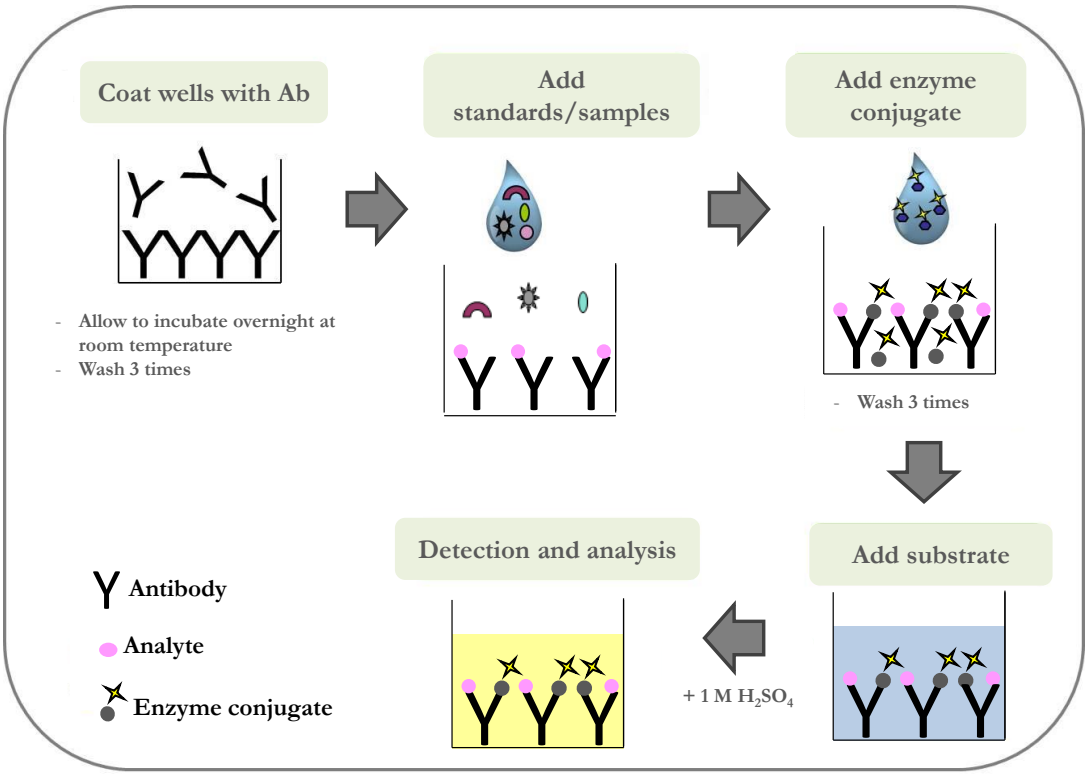

Fig. 2.7: Scheme of the ELISA experimental procedure for detection of E2 or EE2.

Microtiter plates were coated with polyclonal Ab serum diluted 1:10 000 for E2 and 1:50 000 for EE2 in coating buffer (15 mmol L^{-1} Na_2CO_3, 35 mmol L^{-1} $NaHCO_3$, 3 mmol L^{-1}

NaN_3, pH 9.6), using 200 µL per well. Plates were covered with Parafilm™ to prevent evaporation. After overnight incubation at 20 ºC in the plate shaker, at 750 rpm, the plates were washed three times with washing buffer concentrate (43 mmol L^{-1} KH_2PO_4, 375 mmol L^{-1} K_2HPO_4 1.33 mmol L^{-1} sorbic acid potassium salt and 3% Tween™ 20, pH 7.6), diluted 60 times. When applied, sample buffer (1 mol L^{-1} $C_4H_{11}NO_3$, 1.5 mol L^{-1} NaCl, 107 mmol L^{-1} $Na_2EDTA.2H2O$, 1% (w/v) BSA, pH 8.6, 7.6 or 6.4) was added after these washing steps (25 µL per well). Then, standards/samples were added to the plate (100 µL per well) and the plate shaken at room temperature for 30 min. This was followed by addition of the respective enzyme conjugate (T; 100 µL per well) in phosphate buffer (PBS; 10 mmol L^{-1} $NaH_2PO_4.2H_2O$, 70 mmol L^{-1} $Na_2HPO_4.2H_2O$, 145 mmol L^{-1} NaCl, pH 7.6). Enzyme conjugate was used at dilutions 1:25 000 and 1:50 000 for E2 and 1:75 000 and 1:100 000 for EE2. In order to improve sensitivity, the influence of enzyme conjugate incubation time was tested (10 and 30 min). This incubation step was performed at room temperature and followed by a second three-cycle washing step. At last, the final substrate solution was added (200 µL) and incubated for 30 min. Final substrate solution was freshly prepared for each run and consisted in 540 µL stabilized TMB solution (prepared according to Frey et al. (2000), using 41 mmol L^{-1} $C_{16}H_{20}N_2$ (TMB) and 8 mmol L^{-1} $C_{16}H_{40}BN$ (TBABH), in 10mL DMA), 22 mL citrate buffer (220 mmol L^{-1} $C_6H_7KO_7$, 0.5 mmol L^{-1} $C_6H_7KO_2$, pH 4.0) and 8.1 µL H_2O_2. The enzyme reaction was stopped by addition of H_2SO_4 1 mol L^{-1} (100 µL per well). SoftMax® Pro Software (version 5.3, Molecular Devices) was used for the data analysis.

2.2.4 ELISA calibration curve and precision profile

To obtain the ELISA calibration curves, analyte stock solutions (1000 mg L^{-1} E2 or EE2) were prepared in methanol and then further diluted with ultra-pure water to obtain standard solutions with concentrations ranging between 0.0001 and 1000 µg L^{-1}.

The OD mean values were fitted to a 4PL previously described (Dudley et al., 1985). All determinations were at least made in triplicate. To determine the quantification range (defined as the highest and lowest concentration which can be determined with a given degree of precision), 16 standard solutions were assayed (with 6 replicates each). Standard solutions were randomly distributed over the 96 wells of the microtiter plate to level out the influence

of possible systematic errors that might arise from signal drifts across the plate. Subsequently, the relative error of the E2 and EE2 concentration was calculated to obtain the precision profile of the assay as described by Ekins (1981). A maximum relative error of 30% for the quantification of E2 or EE2 in a sample was established as the criterion to define the quantification range of the assays.

2.2.5 Determination of cross-reactivity

The immunoassay selectivity for E2 or EE2 was determined by assaying a dilution series of structurally related estrogens in water (estrone, E1, and estriol, E3). CR was calculated as the ratio of molar concentrations at the inflection points (midpoints) of the corresponding calibration curves and expressed in percentage relative to E2 or EE2 (Eq. 2.5).

2.2.6 Evaluation of matrix effects

Matrix effects are a major issue when analyzing environmental samples due to their complexity and because they can probably affect Ab or enzyme performance (Schneider et al., 2005). Organic matter and salinity were selected as potential interfering agents to study matrix effects, due to the need of analyzing water samples with high DOC and high salinity levels.

The mean DOC concentration of lakes, streams and rivers is between 2.0 and 10 mg L^{-1} (Leech et al., 2009) and in final STP effluents, DOC values are reported to vary between 3.7 and 22 mg L^{-1} (Escalas et al., 2003; Hintemann et al., 2006; Ueda and Hata, 1999). The influence of the presence of organic matter was evaluated using HA with concentrations in the range 0.5-20 mg L^{-1}. Calibration curves between 0.0001 and 1000 μg L^{-1} containing 1, 10 and 20 mg L^{-1} HA were obtained for both E2 and EE2 and compared to those in the absence of HA. Furthermore, recovery tests were performed using 1 μg L^{-1} E2 or EE2 standard solutions. To these standards adequate volumes of 1 g L^{-1} HA stock solution were added so as to obtain concentrations of 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 mg L^{-1} HA. Trying to overcome the strong interference caused by dissolved organic matter, the effect of the addition of a 1% (w/v) BSA sample buffer (pH 7.6) was tested. BSA is an agent that potentially binds organic matter (Calisto et al., 2011) and was added to the wells prior to the addition of the analyte.

Afterwards, calibration curves were constructed in order to evaluate its effect.

Typical salinity values for surface water samples from Ria de Aveiro range between those of freshwater and those of marine water, which can reach 36 PSU (Dias et al., 1999; Vaz et al., 2005). Salinity was simulated using NaCl with concentrations in the range 10-30 g L^{-1}. Recovery tests were performed spiking 1 μg L^{-1} E2 or EE2 standard with different volumes of NaCl, in order to obtain concentrations of 10, 20 and 30 g L^{-1}. Results were compared to those in absence of NaCl.

Recovery rates for both E2 and EE2 were also established in surface and waste water samples, by spiking them with 0.5, 1.0 and 1.5 μg L^{-1} E2 or EE2.

2.3 RESULTS AND DISCUSSION

2.3.1 Assay performance: Ab and T dilutions

For the E2 assay, Ab and T were tested for different dilutions (Ab 1:10 000, T 1:10 000; Ab 1:10 000, T 1:25 000; Ab 1:25 000, T 1:10 000 and Ab 1:25 000, T 1:25 000) and curves and respective 4PL parameters were obtained and are presented in Fig. 2.8 and Table 2.2, respectively.

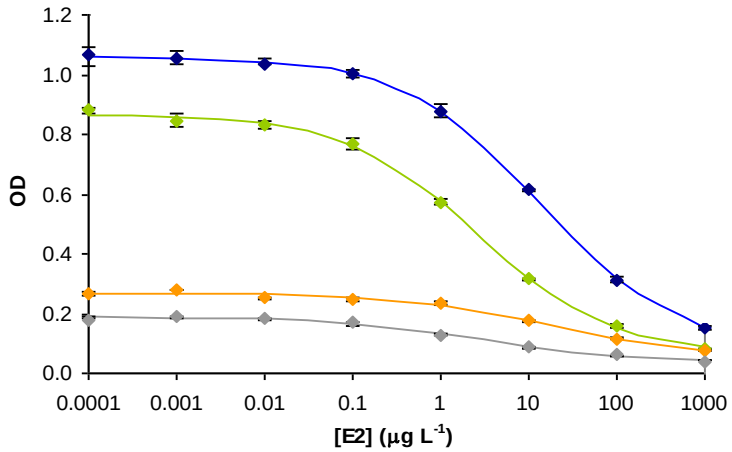

Fig. 2.8: Calibration curves obtained using different Ab/T dilutions for direct ELISA, for E2 measurements. Ab 1:10 000, T 1:10 000 – blue; Ab 1:10 000, T 1:25 000 – green; Ab 1:25 000, T 1:10 000 – orange; Ab 1:25 000, T 1:25 000 – gray.

Table 2.2: Parameter values obtained for the 4PL using different dilutions of polyclonal Ab and T for E2.

Ab and T dilutions	A	B	C	D	r^2
Ab 1:10 000; T 1:10 000	1.06	0.569	13.8	0.0730	1.000
Ab 1:10 000; T 1:25 000	**0.869**	**0.570**	**2.67**	**0.0610**	**0.999**
Ab 1:25 000; T 1:10 000	0.269	0.496	23.1	0.0438	0.990
Ab 1:25 000; T 1:25 000	0.189	0.531	2.47	0.0386	0.993

The combination chosen was Ab 1:10 000, T 1:25 000. This combination and the combination Ab 1:25 000, T 1:25 000 presented the lowest C values. However, among these two combinations, Ab 1:10 000, T 1:25 000 presented a higher difference between the OD value of the lower and the higher standards (higher difference between A and D parameters), and thus higher sensitivity.

In the case of EE2, the chosen combination was Ab 1:50 000, T 1:75 000 (Table 2.3). This combination did not present the lowest C parameter value; however, it was close to the lowest value and also was the one that presented the highest difference between A and D parameters.

Table 2.3: Parameter values obtained for the 4PL using different dilutions of polyclonal Ab and T for EE2.

Ab and T dilutions	A	B	C	D
Ab 1:50 000; T 1:50 000	1.25	0.767	1.32	0.0580
Ab 1:50 000; T 1:75 000	**1.32**	**0.767**	**0.963**	**0.0467**
Ab 1:75 000; T 1:50 000	0.281	0.937	1.20	0.0583
Ab 1:75 000; T 1:75 000	0.315	0.686	0.599	0.0680

2.3.2 Accuracy

Accuracy can be defined as the fundamental ability that the assay has to measure the true concentration of an analyte (Mikkelsen and Cortón, 2004). In order to evaluate the accuracy of the method, a 1.0 μg L^{-1} E2 or EE2 standard was replicated 50 times. Mean for the 50 measurements was 0.93 ± 0.077 μg L^{-1} (RSD 8.3%), for E2, and 1.0 μg L^{-1} ± 0.064 μg L^{-1} (RSD 6.3%), for EE2.

The concentration obtained for each well was plotted and a 25% upper and lower deviation from the real standard concentration was considered acceptable (Fig. 2.9).

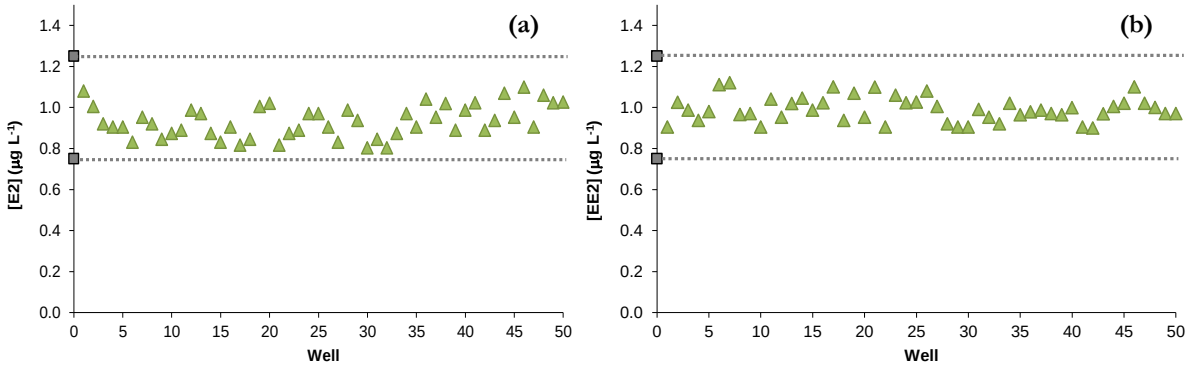

Fig. 2.9: Measured concentration in each of 50 wells for the 1.0 µg L^{-1} E2 (a) and EE2 (b) standard. Upper and lower deviation limits are also shown.

All the concentrations measured were within the previously mentioned limit, therefore both assays were considered accurate.

2.3.3 Cross-reactivity

Immunoassays that are developed for specific compounds often recognize structurally similar compounds as well. As it was already said, it is possible to use the 4PL to identify the response to cross-reactive compounds and to account for non-specific binding (Fare et al. 1996; Van Emon, 2001).

The relative sensitivity of E2 and EE2 ELISAs towards other steroid hormones (EE2, E1 and E3, for E2, and E2, E1 and E3, for EE2) was determined. Molar CRs obtained are presented in Table 2.4.

Table 2.4: CR (%) of selected hormones at the center points of their calibration curves.

Hormone	E2 CR (%)	EE2 CR (%)
E2	100	0.62
EE2	0.52	100
E1	4.78	0.21
E3	3.91	0.19

CR were very low (<5%, for E2, and <1%, for EE2), indicating that both assays present high specificity. Therefore, it is not expected any interference on the determination of E2 or EE2 from the tested compounds when present in the same sample.

2.3.4 Quantification range

The quantification range of both assays was obtained as described in Section 2.1.6, based on the maximum relative error accepted of 30% (Ekins, 1981; Grandke et al., 2013). Quantification ranges of 0.06-10 μg L^{-1} for E2 (Fig. 2.10) and 0.03-40 μg L^{-1} for EE2, were obtained.

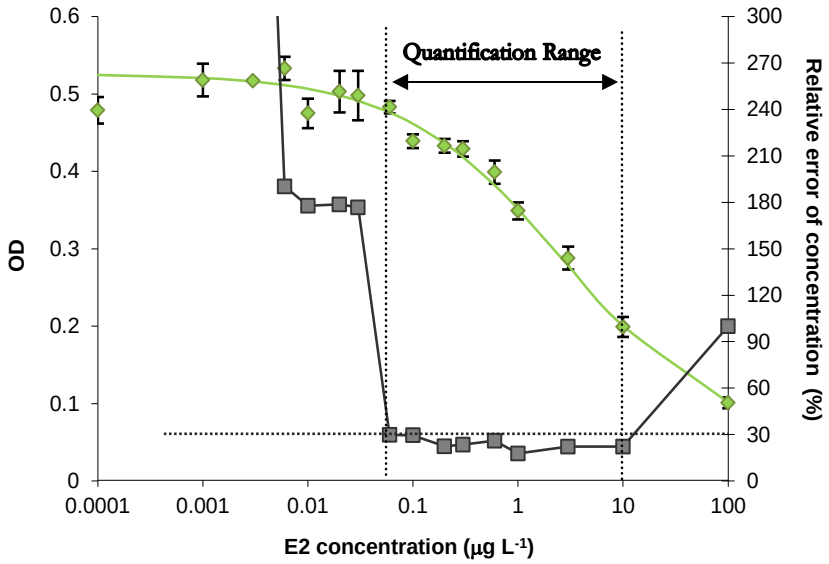

Fig. 2.10: Calibration curve (green marks) of E2 ELISA (A = 0.526; B = 0.568; C = 2.64; D = 0.0478; r^2 = 0.993) and precision profile (gray marks). Ab 1:10 000; T 1:25 000. The precision profile and determination of the relative error of concentration were calculated in accordance with Ekins (1981).

2.3.5 Matrix Effects

Dissolved organic matter and salinity were selected as the most relevant matrix interferents to study. The presence of organic matter was simulated by the addition of known amounts of HA stock solution to standards.

For the E2 assay (Fig. 2.11a), a decrease of the OD_{max} (A parameter) was observed with an increase in the HA concentration, being 36% lower for 20 mg L^{-1} HA, in comparison with ultrapure water (0 mg L^{-1} HA). Therefore, the presence of HA interferes with the performance of the assay. The mechanism of this interference is not well understood. However, the hypothesis is that matrix components have a denaturing impact on proteins and enzymes. Signal generation involves the formation of a stable linkage between the T and the

Ab, which is probably affected by unspecific binding of HA to the Ab or to the enzyme protein, or both. In either case, the decrease in OD with the increase of HA concentration may generate an overestimation of the E2 concentration.

To overcome this interference, a BSA-based sample buffer (pH 7.6) was added to the wells prior to the addition of the analyte. The use of sample buffer was found to solve the organic matter interferences. Fig. 2.11b shows that the four E2 calibration curves, obtained in presence of sample buffer, maintained the sigmoidal shape.

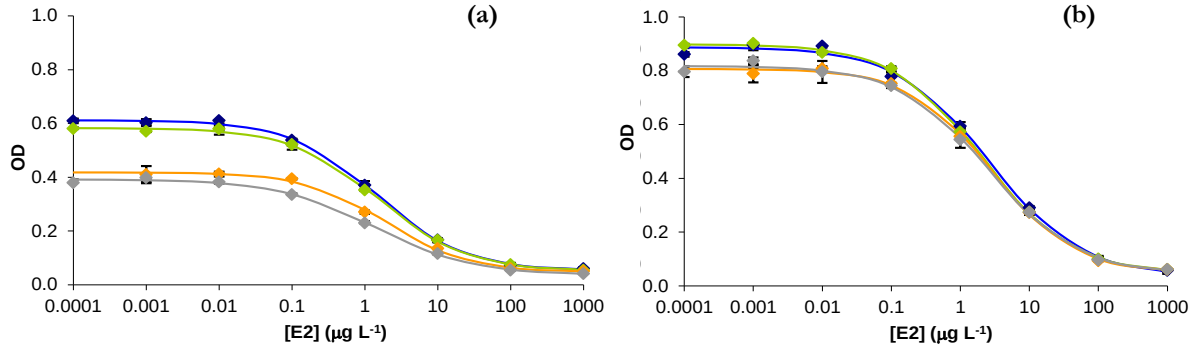

Fig. 2.11: Evaluation of the organic matter effect on the E2 ELISA calibration curve in (a) absence and (b) presence of BSA buffer. Standards prepared in ultrapure water (0 mg L^{-1} HA - blue); 1 mg L^{-1} HA (green); 10 mg L^{-1} HA (orange); 20 mg L^{-1} HA (gray). Ab 1:10 000; T 1:25 000. [Curve parameters: **(a)** 0 mg L^{-1}: A - 0.612, B - 0.725, C - 1.46, D - 0.0522; 1 mg L^{-1}: A - 0.583, B - 0.722, C - 1.54, D - 0.0509; 10 mg L^{-1}: A - 0.418, B -0.771, C - 1.93, D - 0.0467; 20 mg L^{-1}: A - 0.392, B - 0.650, C - 1.35, D - 0.0360; **(b)** 0 mg L^{-1}: A - 0.888, B - 0.648, C - 2.63, D - 0.0337; 1 mg L^{-1}: A - 0.899, B - 0.667, C - 2.14, D - 0.0442; 10 mg L^{-1}: A - 0.807, B - 0.727, C - 2.84, D - 0.0472; 20 mg L^{-1}: A - 0.818, B - 0.670, C - 2.60, D - 0.0425].

The slight OD_{max} (A parameter) decrease observed for 10 and 20 mg L^{-1} HA corresponds only to 9.1 and 7.9%, respectively, in comparison with ultrapure water (0 mg L^{-1} HA). However, it may be a problem for the region of the curve where the decrease happens, i.e. at concentrations $\leq$0.1 µg L^{-1}.

In the case of EE2, although small concentrations of HA (such as 1 mg L^{-1}) did not interfere with quantification, a flattening of the calibration curve, as well as the increase of the C parameter, occurred with the increase of HA concentration (calibration curves not shown).

The interference and its resolution by using BSA sample buffer was confirmed by recovery tests (mean recoveries). For E2, in absence of BSA, recoveries were acceptable only in the range [0-0.5] mg L^{-1} HA rising up to 1063 ± 6%, for 20 mg L^{-1}, clearly demonstrating that E2 quantification is strongly affected by the presence of HA. Recovery rates in presence of BSA were in the range 83 ± 1-107 ± 1%. Therefore, results showed that in presence of the

BSA sample buffer, E2 quantification was not affected by the presence of HA even at concentrations as high as 20 mg L^{-1}.

For the EE2 assay (calibration curves not shown), the effect was much less pronounced compared to the one observed in the E2 assay; however, some effect of the presence of HA was noticed, the recovery rates reaching 156.1 ± 0.4%, for 20 mg L^{-1} HA. The EE2 assay also exhibited a robust behaviour when buffer was added, even for HA concentrations as high as 20 mg L^{-1} (mean recoveries in the range 95 ± 3-120.1 ± 0.2%).

Earlier studies have already shown the beneficial effects of BSA addition to buffer solutions in suppressing/reducing matrix effects on immunoassays (Winklmair et al., 1997; Zhang et al., 2007). Calisto et al. (2011) tested the addition of BSA for its capacity to suppress matrix effects of 10 mg L^{-1} synthetic HA in a carbamazepine ELISA, which had a negative effect on the sensitivity of the assay. The hypothesis is that BSA, as a net negatively charged protein, has a combined effect of attracting positively charged interferences (such as cations), as well as inactivating any contaminants that might cause a denaturation of proteins (Bahlmann et al., 2009), thus protecting the immunochemicals (Ab and enzyme molecules). However, in this work, an increase in the C parameter values of the calibration curves obtained in presence of sample buffer was observed, in comparison to those without it (Fig. 2.11). To elucidate if the buffer affected the precision profile, working range was obtained in these conditions. The lower limit of the quantifiation range was changed from 0.06 μg L^{-1} (not using buffer) to 0.3 μg L^{-1} (using buffer), for E2, and from 0.03 μg L^{-1} (not using buffer) to 0.1 μg L^{-1} (using buffer), for EE2. Therefore, it seems that interferences due to organic matter can be overcome using a buffer with BSA; however, the associated increase in the lower limit of the quantification range should not be overlooked.

To evaluate the salinity effect on the performance of the assay no calibration curves were obtained; however, this effect was studied by means of the recovery rates. Presence of salinity was simulated by the addition of NaCl to the 1.0 μg L^{-1} E2 or EE2 standard in order to obtain concentrations ranging from 10.0 to 30.0 g L^{-1} NaCl. Recovery rates ranged from 83 to 121% (absence of sample buffer) and 81 to 98% (presence of sample buffer), for E2, and from 80 to 98% (absence of sample buffer) and 80 to 96% (presence of sample buffer), for EE2. Results demonstrated that salinity does not interfere with the quantification of both estrogens.

2.3.6 Evaluation of T incubation time, T dilution and BSA solution pH

As it was previously shown, the use of BSA sample buffer is beneficial to lower the interference of matrix effects. However, the quantification range under these conditions has a very high lower limit considering the expected concentrations of E2 and especially EE2 in real samples. Therefore, it was necessary to optimize the assays when using the BSA sample buffer in order to shift the quantification range to lower concentrations.

With this purpose new conditions were tested: pH of BSA solution (pH 8.7 and 6.4), T incubation time (t_T; 10 min) and T dilution (1:50 000). Fig. 2.12 shows E2 calibration curves obtained for a t_T of 10 min. Two T dilutions were used (T 1:25 000 – Fig 2.12a, and T 1:50 000 – Fig. 2.12b) and in both cases the variation of the BSA sample buffer pH was tested.

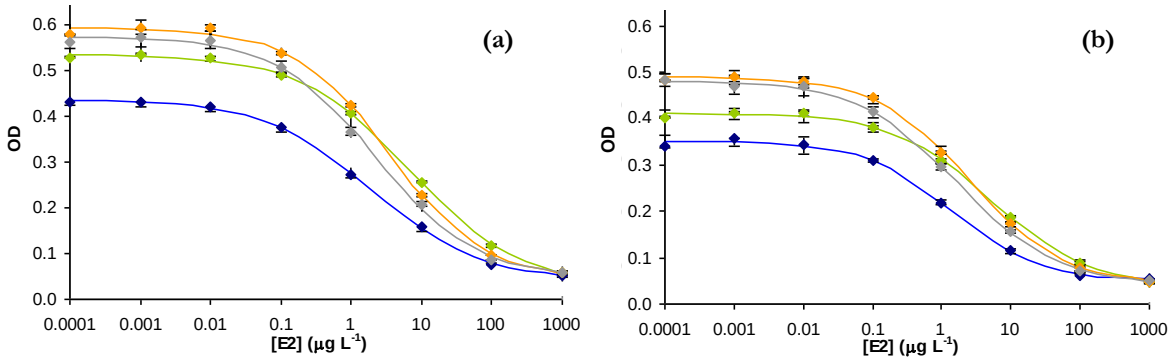

Fig. 2.12: Evaluation of the BSA sample buffer pH effect on the ELISA calibration curve: (a) T 1:25 000; t_T = 10 min; (b) T 1:50 000; t_T = 10 min. In both (a) and (b): without sample buffer (blue); pH of BSA sample buffer 8.7 (green); pH of BSA sample buffer 7.6 (gray); pH of BSA sample buffer 6.4 (orange). [Curve parameters: **(a)** without sample buffer: A - 0.437, B - 0.566, C - 2.02, D - 0.0409; pH 8.7: A - 0.536, B - 0.566, C - 6.88, D - 0.0280; pH 7.6: A - 0.594, B -0.652, C - 3.47, D - 0.0434; pH 6.4: A - 0.575, B - 0.612, C - 2.30, D - 0.0455; **(b)** without sample buffer: A - 0.351, B - 0.681, C - 1.45, D - 0.0500; pH 8.7: A - 0.412, B - 0.611, C - 5.43, D - 0.0331; pH 7.6: A - 0.489, B - 0.650, C - 2.59, D - 0.0404; pH 6.4: A - 0.481, B - 0.607, C - 1.75, D - 0.0395].

As it can be seen, either from the calibration curves and/or the parameters' values (Fig. 2.12), the new T dilution of 1:50 000 with an incubation time of 10 min seems to be a good approach for the E2 determination, comparatively with the 1:25 000 T dilution (also with an incubation time of 10 min). As it can be seen, C parameter is, in all the cases tested (without sample buffer, $pH_{BSA\ sample\ buffer}$ = 8.7, $pH_{BSA\ sample\ buffer}$ = 7.6; $pH_{BSA\ sample\ buffer}$ = 6.4), lower for the case of T dilution of 1:50 000. Fig. 2.13, shows its variation with sample buffer pH, for different values of T dilution and t_T, for E2.

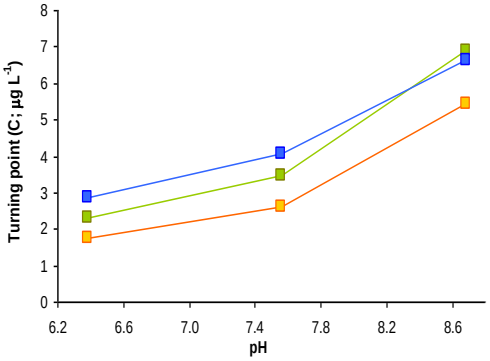

Fig. 2.13: Variation of the 4PL curve turning point (C parameter) with BSA solution pH, using different T dilutions and T incubation time: T 1:50 000, t_T = 10 min (orange); T 1:25 000, t_T = 10 min (green); T 1:50 000, t_T = 30 min (calibration curves not shown in Fig. 2.12) (blue). Lines are shown only for clarity purposes.

The tendency was similar for the three cases represented in Fig. 2.13, the C parameter (turning point) being higher for higher values of BSA solution pH. In the case of 1:50 000 T dilution, the C parameter value obtained for the lower pH is very similar to that obtained for the case of no addition of sample buffer (1.46).

These results brought the perspective of improving the lower limit of the quantification range in presence of BSA solution. Considering these results, T dilution of 1:50 000, T incubation time of 10 min and BSA solution pH of 6.4, were the conditions applied in the subsequent E2 experiments.

In the case of EE2, combinations T 1:50 000, t_T = 10 min; T 1:100 000, t_T = 30 min and T 1:100 000, t_T = 10 min were studied. The behaviour of the EE2 assay was analogous to the E2 assay, i.e. the decrease of sample buffer pH lowers the C value for each combination. Similarly to E2, the optimized conditions corresponded to the higher T dilution (1:100 000) in combination with shorter incubation time (t_T = 10 min) and lower sample buffer pH (6.4).

In order to confirm the above results, the precision profiles were calculated using the same methodology as before. Quantification ranges of 0.03-200 µg L^{-1} and 0.02-10 µg L^{-1} were obtained for E2 and EE2, respectively (Fig. 2.14). Therefore, it was possible to establish a considerable decrease of the lower limits of the quantification ranges (up to 10-fold lower in the case of E2, than under the previous conditions). These limits were even lower than those obtained in the absence of sample buffer.

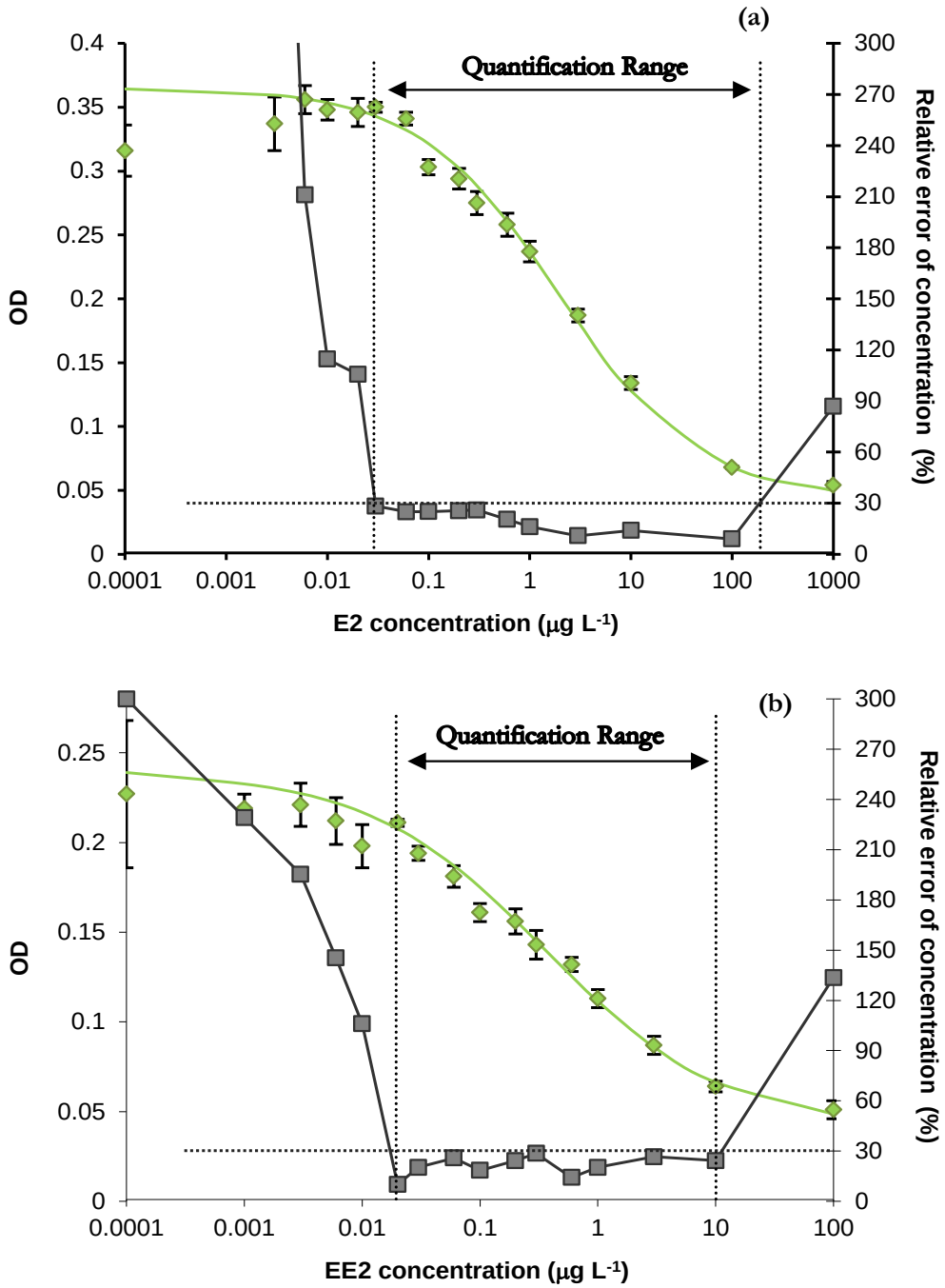

Fig. 2.14: Calibration curve (green marks) and precision profile (gray marks) in presence of BSA sample buffer pH 6.4 of (a) E2 ELISA (A = 0.365; B = 0.626; C = 1.93; D = 0.0434; r^2 = 0.997); Ab 1:10 000; T 1:50 000; t_T 10 min and (b) EE2 ELISA (A = 0.239; B = 0.579; C = 0.357 D = 0.041; r^2 = 0.995); Ab 1:50 000; T 1:1000 000; t_T 10 min. Precision profiles and determination of the relative error of concentrations were calculated in accordance with Ekins (1981).

Matrix effects were evaluated again in order to confirm the good performance of the new conditions. First of all, four calibration curves were obtained for standards in absence or

presence of HA (up to 20 mg L^{-1}) (Fig. 2.15).

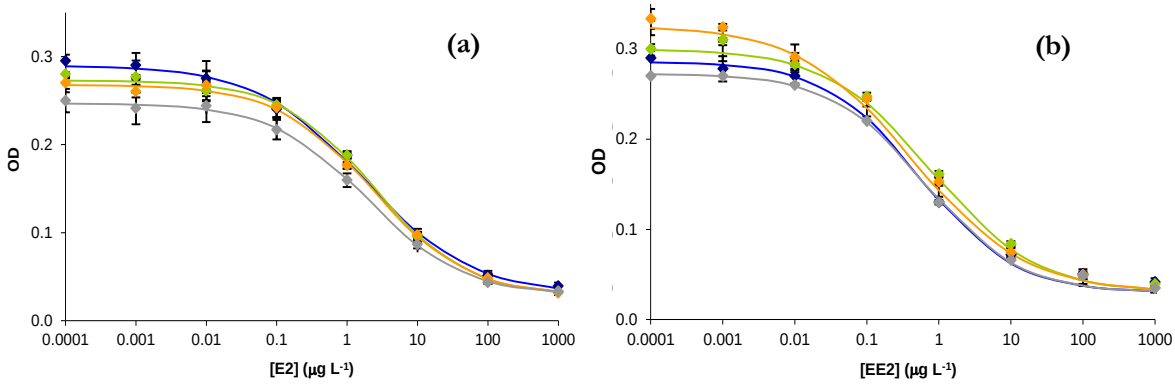

Fig. 2.15: Evaluation of the organic matter effect on the ELISA calibration curves in presence of BSA sample buffer, pH 6.4: (a) E2 ELISA - Ab 1:10 000; T 1:50 000; t_T 10 min and (b) EE2 ELISA - Ab 1:50 000; T 1:100 000; t_T 10 min. Standards prepared in: water (0 mg L^{-1} HA) – blue; 1 mg L^{-1} HA – green; 10 mg L^{-1} HA – orange; 20 mg L^{-1} HA – gray. [Curve parameters: **(a)** 0 mg L^{-1}: A - 0.290, B - 0.568, C - 1.76, D - 0.0296; 1 mg L^{-1}: A - 0.273, B - 0.659, C – 2.42, D - 0.0282; 10 mg L^{-1}: A - 0.268, B - 0.642, C – 2.31, D - 0.0281; 20 mg L^{-1}: A - 0.274, B - 0.638, C - 1.95, D - 0.0285; **(b)** 0 mg L^{-1}: A - 0.297, B - 0.663, C - 0.550, D - 0.0406; 1 mg L^{-1}: A - 0.306, B - 0.603, C - 0.795, D - 0.0355; 10 mg L^{-1}: A - 0.333, B - 0.555, C - 0.425, D - 0.0365; 20 mg L^{-1}: A - 0.289, B - 0.669, C - 0.650, D - 0.0459].

As it can be seen in Fig. 2.15, the addition of HA did not constitute a major influence in the curve formats nor in the calibration curve parameters. This was considered a good indication for the recovery tests.

Based on the established quantification range, 0.05, 0.1 and 1.0 µg L^{-1} E2 or EE2 standards were used to perform recovery tests, spiking them with different volumes of HA stock solution (to obtain HA concentrations in the range 0.5-20 mg L^{-1}). The graphical variation of the obtained concentration for the 0.05 and 0.1 µg L^{-1} standards in both assays is presented in Fig. 2.16.

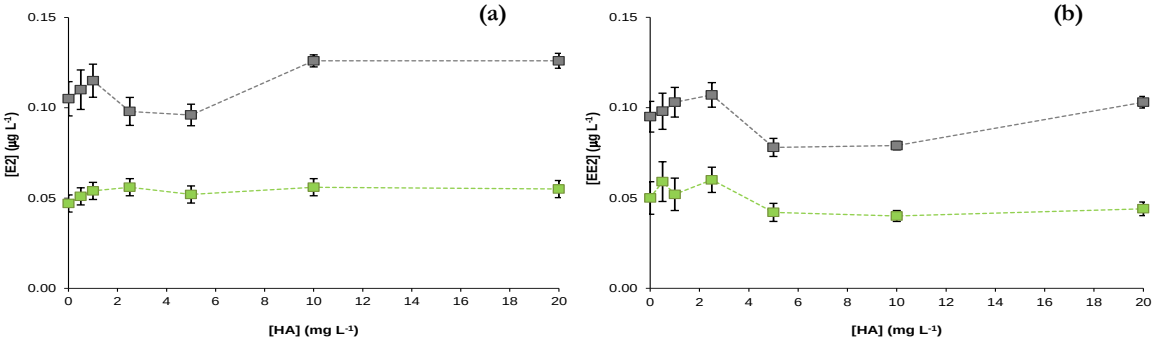

Fig. 2.16: Mean concentration obtained for 0.05 µg L^{-1} standard (green) and for 0.1 µg L^{-1} standard (gray), in presence of increasing concentrations of HA and BSA sample buffer, pH 6.4 (n=9): (a) E2 ELISA - Ab 1:10 000, T 1:50 000; t_T 10 min and (b) EE2 ELISA - Ab 1:50 000, T 1:100 000; t_T 10 min.

Mean recoveries for the three standards tested ranged between 82 and 120% and from 80 to 120%, for E2 and EE2, respectively. These results showed that E2 and EE2 quantification was not affected by the presence of HA under these conditions.

The effect of the presence of salinity was also evaluated under the new conditions, again with good results (mean recoveries 85-115%, for E2, and 92-117%, for EE2).

2.3.7 Recovery tests in water samples

One waste water sample (from after primary decantation, NWWS1) and a surface water sample (SWS4) were spiked with 0.5, 1.0 and 1.5 μg L^{-1} E2 or EE2. The concentration results were plotted against spiked levels and linear regression parameters were obtained (Fig. 2.17).

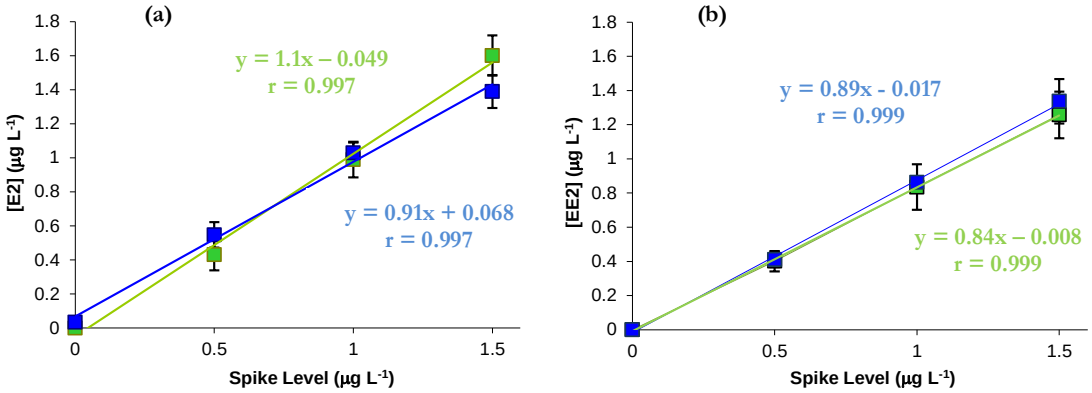

Fig. 2.17: E2 (a) and EE2 (b) concentrations for three spiking levels in waste water sample, NWWS1 (blue marks) and surface water sample, SWS4 (green marks), in presence of sample buffer pH 6.4 (n = 6). E2 ELISA - Ab 1:10 000, T 1:50 000; t_T 10 min; EE2 ELISA - Ab 1:50 000, T 1:1000 000; t_T 10 min.

Recovery rates were calculated multiplying the slope by 100%. For the case of E2, in the STP sample, the recovery rate obtained was 91 ± 5% (r = 0.997) while for the surface water sample, the recovery rate was 107 ± 6% (r = 0.997). For EE2, the recovery rate was 89 ± 2% (r = 0.999) in the waste water sample, while for the surface water sample was 84 ± 1% (r = 0.999). Good assay performances were observed and no major interferences due to matrix effects occurred in real samples when optimized conditions were applied.

2.3.8 Quantification of E2 and EE2 in water samples

E2 was quantified in two waste water samples and one surface water, as it can be seen in the table presented below (Table 2.5).

Table 2.5: Quantification of E2 and EE2 in the surface and waste water samples collected.

Samples	Concentration (μg L^{-1})		Samples	Concentration (μg L^{-1})	
	E2	**EE2**		**E2**	**EE2**
SWS1	<LOD	<LOD	**NWWS1**	0.035 ± 0.002	<LOD
SWS2	< LOD	<LOD	**NWWS2**	0.068 ± 0.002	<LOD
SWS3	<LOD	<LOD	**NWWS3**	<LOD	<LOD
SWS4	<LOD	<LOD	**SWWS1**	<LOD	<LOD
SWS5	< LOD	<LOD	**SWWS2**	<LOD	<LOD
SWS6	<LOD	<LOD	**SWWS3**	<LOD	<LOD
SWS7	<LOD	<LOD			
SWS8	0.085 ± 0.010	<LOD			
SWS9	<LOD	<LOD			
SWS10	<LOD	<LOD			

In what concerns waste water samples, it can be observed that the concentration of E2 in the sample from the effluent of primary treatment (NWWS1) was lower than that quantified in the sample from the effluent of biological treatment (NWWS2): concentrations of 0.035 ± 0.002 μg L^{-1} and 0.068 ± 0.002 μg L^{-1}, respectively. Although it would be expected that the levels of contaminants would decrease with treatments (i.e. that would be lower after biological treatment than after primary treatment), the same tendency has been widely reported in literature. A possible explanation is that a fraction of the hormones is excreted in its original form, while another fraction is metabolized and excreted in the form of inactive conjugates (sulphates and glucuronates). However, by the action of the microorganisms present in the STPs' biological treatment, these inactive conjugates are transformed back to the original forms, incrementing concentrations in effluents compared to those in influents (Baronti et al., 2000; Lai et al., 2000; Petrović and Barceló, 2007; Ternes et al., 1999; Ying et al., 2002).

Also, it was possible to quantify E2 in a surface water sample, with a concentration of 0.085 ± 0.010 μg L^{-1}. This concentration was higher than expected, but, considering the region in question, it was believed that run-off from manure or disposal of animal untreated wastes were the possible explanations for this E2 concentration.

EE2 was not present in a quantifiable concentration in any of the samples tested, possibly because the lower limit of the quantification range of the assay was not low enough. In fact, concentrations of this estrogen in water samples are known to be very low. In surface waters, concentrations of 1-10 ng L^{-1} are often reported in literature (Hintemann et al., 2006; Martínez et al., 2010; Ying et al., 2002). Even in STP effluents, while other estrogens are present at levels ranging from a few ng L^{-1} to several dozens ng L^{-1}, this estrogen varies from non-detected to a few ng L^{-1} (Janex-Habibi et al., 2009).

2.4 CONCLUSIONS

In this work, ELISA methodologies were optimized in order to quantify the hydrophobic organic pollutants E2 and EE2, in water samples.

The ELISA assay proved to be adequate for the detection of E2 in complex matrix samples, namely with high concentrations of dissolved organic matter and sodium chloride. The optimization of the conditions led to a methodology able to have a good performance even in samples as complex as waste water. These samples were analyzed without any sample cleanup procedure, except for the filtration step after sampling. E2 was quantified in two waste water samples and one surface water sample in concentrations ranging between 0.035 ± 0.002 μg L^{-1} and 0.085 ± 0.010 μg L^{-1}. Good recoveries were attained for both waste and surface waters (between 91 ± 5% and 107 ± 6%).

A methodology for EE2 was also developed. The assay performance for the optimized conditions was not influenced by matrix effects. Even though the sensitivity was improved and the optimized lower limit of the quantification range was established to be 20 ng L^{-1}, it was not possible to determine EE2 at a quantifiable level in any of the samples tested. However, good recoveries were obtained (between 84 ± 1% and 89 ± 2%), demonstrating that the optimized method is suitable for application in samples of complex matrix.

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CHAPTER 3

Development of an enzyme linked immunosorbent assay for SMX determination in water samples

Summary

This study encompasses the development of an ELISA for the quantification of SMX in complex aqueous matrices without any sample clean-up procedures. Salinity and dissolved organic matter were selected as potential interfering agents in the analysis of SMX. The addition of containing BSA-sample buffers of different pH prior to the standards/samples was tested. Sample buffer with pH 7.6 was found to decrease the influence of both organic matter and salinity effects. The optimized method allowed the quantification of SMX in the range 0.1-30 μg L^{-1}. The assay was applied to real aqueous samples and it was possible to do a first assessment of the levels of SMX in surface and waste waters from the Aveiro region. Nonetheless, when trying to validate results by LC-MS/MS, correlation between the two techniques was found to be unacceptable.

Contents

3.1 CONTEXTUALIZATION

SAs are a class of broad-spectrum and low-cost synthetic antibiotics used for bacterial and protozoan diseases such as gastrointestinal, urinary and respiratory infections in humans. In addition, are widely used for therapeutic, prophylactic and growth-promoting purposes in livestock farming (Shen et al., 2005; Zhang et al., 2010). As it was already stated, even though these compounds are normally present in the environment at low levels, they have a long life-time and the chronic exposure to them can cause accumulation in organisms from freshwater systems implying side effects such as the spread of bacterial drug resistance and direct toxicity to biota through bioaccumulations and transfer by the food chain (Černoch et al., 2012; Shelver et al., 2008; Zhang et al., 2010).

It is, therefore, important to understand the occurrence of SAs in the aquatic environment which entails the development of analytical methodologies presenting high sensitivity and throughput. For this effect various methods have been developed, among which LC-MS/MS is the most widely adopted (e.g. Batt and Aga, 2005; Conley et al., 2008; Díaz-Cruz et al. 2003; Hartig et al., 1999; Kolpin et al. 2002; Tamtam et al., 2008; Wang and Gardinali, 2012; Ye et al., 2007). Once again, it has to be highlighted that, despite the high sensitivity of this method, the expensive instrumentation, high-cost operation, relatively low throughput and need of extensive sample cleanup, limit its application in routine monitoring analysis. Once more, a valid alternative are the immunoassays.

In this chapter, an ELISA was applied to the quantification of SMX in surface and waste waters, collected in Aveiro (Northwest Portugal). This study aimed the evaluation and optimization of the assay performance in presence of high concentrations of organic matter and salinity, in order to develop an ELISA method adequate to perform large environmental screenings, without any sample pre-treatment. This work also intended to present, for the first time, results concerning SMX contamination levels in the region of Aveiro. Moreover, to the best of the author's knowledge this is the first study in our country dealing with the detection of SMX by ELISA. The experimentation described in this Chapter was carried out in BAM (Berlin, Germany), within a 2-months grant for a guest scientist stay in this Institute.

3.1.1 Quantification of SMX: worldwide scenario

From the wide spectrum of SA drugs, SMX, followed by sulfapyridine and their acetyl metabolites, are the most frequently detected compounds in the aquatic environment (Černoch et al., 2012). Recent studies have reported that the elimination of antibiotics during waste water treatment processes is incomplete, with efficiencies ranging between 60 and 90% (Pastor-Navarro et al., 2009). In fact, Peng et al. (2008) stated that approximately 30% of SMX might not be degraded during the primary clarification and biological treatment processes at STPs due to its hydrophilic character.

SMX has been detected worldwide in waste and surface waters, and even in ground and drinking water (Table 3.1). Back to 1999, in Germany, Hirsch et al. (1999) detected SMX in surface water and STP effluents up to 0.48 and 2.0 μg L^{-1}, respectively, while in another study, Hartig et al. (1999) reported values for the same type of samples - between 0.030 and 2.5 μg L^{-1}. Some years later, Christian et al. (2003) quantified a 0.052 μg L^{-1} maximum SMX concentration in various surface water samples. In France, SMX concentrations in Seine River samples were stated to be between 0.013 and 0.54 μg L^{-1} (Tamtam et al., 2008; Tamtam et al., 2009), while in Switzerland, values of 0.34, 0.34 and 0.35 μg L^{-1} for the primary, secondary and tertiary effluent, respectively, were reported by Göbel et al. (2004), showing that treatment is not efficient for the SMX removal. In Canada and in Australia, SMX values in STPs' effluents were reported to reach a maximum of 0.87 and 0.21 μg L^{-1} (UV disinfected effluent), respectively (Le-Minh et al., 2012; Miao et al., 2004). In the USA, several studies were performed, for different types of water matrices. SMX concentrations in effluents of STPs were reported to be between 0.32 and 1.3 μg L^{-1} (Batt and Aga, 2005; Renew and Huang, 2004; Wang and Gardinali, 2012; Yang and Carlson, 2003). Regarding a pond directly affected by irrigation with reclaimed water from a STP, quantified values reached 0.014 μg L^{-1} (Wang and Gardinali, 2012). In surface waters, values ranged from 0.0054 to 5.2 μg L^{-1} (Cahill et al., 2004; Ferguson et al., 2013; Lindsey et al., 2001; Vanderford et al., 2003). Lindsey et al. (2001) reported a surprisingly high SMX concentration (0.22 μg L^{-1}) given in account that it was obtained in a groundwater sample. Finally, in drinking water, Ye et al. (2007) reported a mean value of 0.0032 μg L^{-1}.

So far, determination of SMX in water matrices by ELISA has been performed in a very limited number of studies (Pastor-Navarro et al., 2009; Shelver et al., 2008; Zhang et al.,

2010), concentrations of SMX ranging from <LOD to 0.09 μg L^{-1}, in surface waters and from n.d. to 111 μg L^{-1}, in waste water samples. Vaicunas et al. (2013) used an ELISA also, but only for screening purposes before detection by LC-MS/MS.

As it can be seen in Table 3.1, in Portugal, studies on the analysis of SMX in water are very scarce and none of them based on the use of ELISA. Maximum concentration values were determined in surface waters, not exceeding 0.0080 μg L^{-1} (Gaffney et al., 2014). However, in another study on waste waters (Teixeira et al., 2008), SMX was not detected.

Table 3.1: Quantification of SMX worldwide.

	Sample	Country	Detection method	SMX levels (μg L^{-1})	Reference
SMX detection by techniques other than immunoassays	**Drinking water**	USA	HPLC-ESI-MS/MS	0.0032	Ye et al. (2007)
	Groundwater	USA	HPLC-MS	0.22	Lindsey et al. (2001)
	Surface water	Germany	HPLC-ESI-MS/MS	0.030-0.085	Hartig et al. (1999)
		Germany	HPLC-ESI-MS/MS	0.48	Hirsch et al. (1999)
		USA	HPLC-MS	1.0	Lindsey et al. (2001)
		Germany	HPLC-MS/MS	0.052	Christian et al. (2003)
		USA	HPLC-ESI-MS/MS	0.0054-0.035	Vanderford et al. (2003)
		USA	HPLC–ESI-MS	5.2	Cahill et al. (2004)
		France	UPLC-MS/MS	0.072-0.54	Tamtam et al. (2008)
		France	UPLC-MS/MS	0.013-0.026	Tamtam et al. (2009)
		USA	HPLC-MS/MS	<LOD-0.014	Wang and Gardinali (2012)
		USA	HPLC-MS	0.0015-0.22	Ferguson et al. (2013)
		USA	HPLC-MS/MS	0.011	Vaicunas et al. (2013)
	Wastewater	Germany	HPLC-ESI-MS/MS	2.0	Hirsch et al. (1999)
		Germany	HPLC-ESI-MS/MS	Primary effluent - 2.5 Secondary effluent - 1.5	Hartig et al. (1999)
		Switzerland	HPLC-MS/MS	Primary effluent - 0.34 Secondary effluent - 0.34 Tertiary effluent - 0.35	Gobel et al. (2004)
		Canada	HPLC-ESI-MS/MS	0.87	Miao et al. (2004)
		USA	HPLC-MS/MS	1.3	Batt and Aga (2005)
		USA	HPLC-MS	0.40-0.58	Renew and Huang (2004)
		USA	HPLC-MS/MS	0.41	Wang and Gardinali (2012)
		USA	HPLC-MS/MS	0.0030-0.41	Wang and Gardinali (2012)
		USA	HPLC-MS	0.32-0.50	Yang and Carlson (2003)
		Australia	HPLC-MS/MS	Influent - 1.7 UV disinfected effluent - 0.21	Le-Minh et al. (2012)
SMX detection by immunoassays	**Surface water**	USA	ELISA	<LOD-0.09	Shelver et al. (2008)
	Wastewater	China	TRFIA	Hospitals' effluents - nd-0.44 STP influent - 0.85 STP effluent - 0.35	Zhang et al. (2010)
		China	ELISA	Hospitals' effluents - nd-0.49 STP influent - 0.67 STP effluent - 0.29	Zhang et al. (2010)
		USA	ELISA	0.6-3.1	Shelver et al. (2008)
		Spain	ELISA	<LOD-111	Pastor-Navarro et al. (2009)
SMX detection in Portugal	**Drinking water**	Portugal	UPLC-ESI-MS/MS	<LOD-0.00027	Gaffney et al. (2014)
	Groundwater	Portugal		<LOD-0.0013	
	Surface water	Portugal		0.00039-0.0080	
	Wastewater	Portugal	HPLC-DAD	n.d.	Teixeira et al. (2008)

n.d. – non-detected

3.1.2 Tracer synthesis for ELISA analysis

As mentioned before, immunoassays rely on the specific interaction between Ab and Ag and such interaction is only quantifiable due to the use of a labelled Ag or Ab, denominated as tracer. The ideal label for an immunoassay would be inexpensive, safe and stable. Labelling has a minimal effect on the binding behaviour, which means that labelled and unlabelled reagents behave similarly with respect to Ab-Ag binding (Mikkelsen and Cortón, 2004). Different label types can be used, depending on the immunoassay; for instance, radioisotopes, fluorophores, chemiluminophores and enzymes (the more common of which are horseradish peroxidase (HRP) and alkaline phosphatase) (Hennion and Barceló, 1998; Mikkelsen and Cortón, 2004). Alternative labels have also been investigated, including red blood cells, viruses and free radicals (Mikkelsen and Cortón, 2004; Van Emon, 2001). Nowadays, radioisotopes have clearly been surpassed by current applications of fluorescent labelling methods and enzyme labels and in environmental appplications, enzymes with colorimetric substrates constitute the most common (Van Emon, 2001).

Haptens, a modified analyte derivative, can be used for the synthesis of the enzyme tracers, using HRP (Maqbool et al., 2002). The production of the derivative starts with the introduction of a carboxylic group that will act as a linker and should be attached as far as possible from the recognition sites and to be capable of binding covalently to a carrier protein. Small molecules need a spacer arm in the linker to favour the recognition by the immune system. Spacer lengths between three to six carbon atoms have proven to be most favourable (Hennion and Barceló, 1998). Then, the coupling of the hapten to the HRP is performed. One method to do so is the mixed anhydride method described by Munro and Stabenfeldt (1984) and used in this work. One version of this method uses isobutylchloroformate (IBCF) to generate a mixed anhydride (Fig. 3.1). This mixed anhydride reaction product is reactive towards primary amine groups of the HRP protein (Mikkelsen and Cortón, 2004).

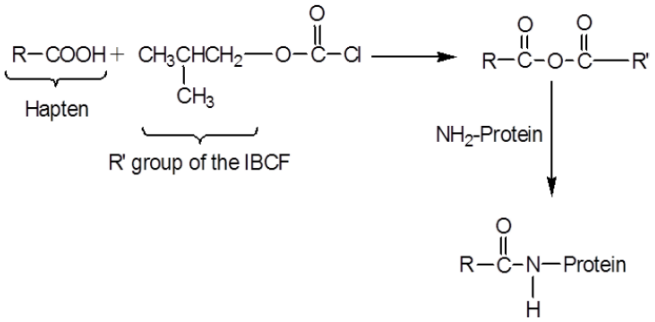


Fig. 3.1: Coupling of the hapten to HRP using the mixed anhydride method.

3.2 EXPERIMENTAL SECTION

3.2.1 Reagents and materials

All reagents were of analytical grade and were used as received. A secondary polyclonal Ab against rabbit IgG (goat, purified R1364P) was from ACRIS Antibodies, while the immunization of the primary Ab, anti-SMX-pAb (second bleeding) was performed by SeqLab Sequence Laboratories. Synthesis of HRP conjugate for SMX was performed for this work, following the procedure of Munro and Stabenfeldt (1984), as described in the next section (*cf.* Section 3.2.2). HRP (EIA grade) was obtained from Roche. Guardian™ (peroxidase conjugate stabilizer/diluent) was purchased from Thermo Scientific. Succinamyl-sulfamethoxazole (succ-SMX) was synthesized in-house in 2010, N,N-dimethylformamide (DMF, puriss.), 4-methylmorpholin (NMM) and isobutyl chloroformate (IBCF) were from Fluka. SMX (VETRANAL™, analytical standard) used for the preparation of standards was from Fluka. TMB (research grade), and Tween™ 20 (pure) were from Serva. TBABH (≥97%), DMA (puriss. ≥99.5%), sodium phosphate dibasic dihydrate (>99%), sodium phosphate monobasic dihydrate (>99%), potassium sorbate (>99%), potassium dihydrogen citrate (>99%), sodium chloride (99.5%) and hydrogen peroxide (30%) were from Fluka. TRIS (p.a.) was from Merck. Sulfuric acid (95-97%) was from J.T. Baker. BSA (for electrophoresis, 98%), EDTA (>99%) and sodium azide (>99%) were from Sigma.

Different types of humic substances (HS) were used: commercial HA (technical) were purchased from Sigma and leonardite HA, Suwannee River HA and Suwannee River fulvic acid (FA) standards were from International Humic Substances Society (IHSS).

Ultrapure water, used in the preparation of solutions, was obtained using a Millipore water purification system (Millipore Synthesis A10).

Sephadex™ columns were obtained from GE Healthcare. Transparent 96 flat-bottom well microtiter plates with high binding capacity were purchased from Greiner Bio-One. Washing steps were carried out using an automatic 96-channel plate washer (BioTek Instruments, ELx405 Select™). Plates were shaken using a plate shaker (Titramax 101, Heidolph). OD was read at 450 nm and referenced to 620 nm using a microplate spectrophotometer (SpectraMax Plus384, Molecular Devices). Data were analyzed using Softmax® Pro 5.3 software.

3.2.2 Tracer synthesis

Enzyme conjugate synthesis was performed analogously to Munro and Stabenfeldt (1984), using the NMM and IBCF -21ºC/0ºC method. Succ-SMX, $C_{14}H_{15}N_3O_6S$ (M = 353.36 g mol^{-1}), was used as hapten to synthesize the SMX conjugate.

Synthesis was performed in two steps (activation and conjugation), as follows:

• *Activation* (in Thermobox at -21ºC, under Ar atmosphere)

a) 2.5 mg hapten were dissolved in 50 μL DMF;

b) 0.4 μL NMM were added and mixture was stirred;

c) 0.4 μL IBCF were added;

d) mixture was incubated for 30 min, at -21 ºC, while stirring.

• *Conjugation*

a) water/DMF mixture (5:3) was prepared at room temperature;

b) 2.2 mg HRP were dissolved in a solution of 25 μL water and 15 μL DMF, at room temperature; mixture was stirred and placed at -21 ºC;

c) activated hapten solution was added, drop by drop, to HRP solution, at -21 ºC;

d) mixture was incubated 60 min, at -21 ºC, while stirring;

e) mixture was slowly warmed to 0 ºC;

f) mixture was incubated 2 h, at 0 ºC, while stirring;

g) to purify the conjugate, PBS was used for the conditioning and elution of the enzyme conjugate on a Sephadex™ column;

h) after conditioning the column, the conjugate was added to the top of the column and after entering the column, more PBS buffer was added;

i) fractions of the eluted solution were collected in a microtiter plate, 3 drops in each well;

j) after elution, the OD of each well was measured at 405 nm.

3.2.3 Matrix assisted laser desorption ionization – time of flight-mass spectrometry (MALDI-TOF) analysis

Matrix assisted laser desorption ionization – time of flight-mass spectrometry (MALDI-TOF) spectra were acquired on a Bruker Reflex III MALDI mass spectrometer

(Bruker-Daltonik) operated with a nitrogen laser and at 20 kV acceleration voltage. 10 μL HRP, were loaded onto a Zeba™ Micro Desalt Spin Column, centrifuged for 90 seconds at 10 000 rpm, eluted with 10 μL water and mixed with 50 μL of matrix. The sinapic acid matrix was freshly prepared as a 10 g L^{-1} aqueous solution that contained 50% acetonitrile and 0.1% trifluoroacetic acid. The sample target was precoated with a droplet of 0.5 μL of matrix solution and dried for 5 min. Then, 0.5 μL of protein sample was added onto the same spot and air-dried for one hour. Data was processed using Origin™ 8.0 (OriginLab). The mass peaks were fitted with a Lorentzian function and the centers of the fitting curves were assigned to HRP and the conjugate masses. MALDI-TOF analysis was kindly performed by Sabine Flemig from BAM.

3.2.4 Water samples

Water samples were collected (250 mL) in cleaned dark glass bottles (previously washed 3 times with a few millilitres of the sample to be collected), in the same locations as detailed in Chapter 2 (*cf.* Section 2.3.2). Samples were collected in late October 2012. Immediately after collection, all the samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at 4 ºC until analysis. Samples were not subjected to any other cleaning procedures or extraction or enrichment processes.

3.2.5 ELISA procedure

A direct competitive ELISA was used in the analysis of SMX. When not detailed, composition of buffers/solutions is as referred in Chapter 2. Microtiter plates were coated with secondary Ab serum diluted 1:1000 in PBS, using 200 μL per well. Plates were covered with Parafilm™ to prevent evaporation. After overnight incubation at 20ºC in the plate shaker at 750 rpm, the plates were washed three times with washing buffer concentrate, diluted 60 times. After that, anti-SMX Ab was added to the microtiter plate, diluted 1:50 000 in PBS (200 μL per well), and allowed to incubate for 30 min, after which another washing step was applied. When applied, sample buffer (1 mol L^{-1} $C_4H_{11}NO_3$, 1.5 mol L^{-1} NaCl, 107 mmol L^{-1}

$Na_2EDTA.2H2O$, 1% (w/v) BSA, pH 7.6; 1 mol L^{-1} $C_2H_5NO_2$, 3 mol L^{-1} NaCl, 2% (w/v) $Na_2EDTA.2H2O$, 1% (w/v) BSA, pH 9.5; or 1 mol L^{-1} $C_6H_7KO_7$, 3 mol L^{-1} NaCl, 1% (w/v) BSA, pH 4.5) was added (25 mL per well). Then, standards/samples were added to the plate (100 μL per well) and the plate shaken at room temperature for 15 min. This was followed by addition of the respective enzyme conjugate diluted 1:50 000 (100 μL per well) in TRIS (10 mmol L^{-1} $C_4H_{11}NO_3$, 150 mmol L^{-1} NaCl, pH 8.5), incubated for 30 min. This incubation step was performed at room temperature and followed by a second three-cycle washing step. At last, the final substrate solution was added (200 μL per well) and incubated for 15 min. Final substrate solution was freshly prepared for each run and prepared according to Frey et al. (2000). The enzyme reaction was stopped by addition of H_2SO_4 1 mol L^{-1} (100 μL per well). SoftMax® Pro Software (version 5.3, Molecular Devices) was used for the data analysis.

3.2.6 ELISA calibration curve and precision profile

To obtain the ELISA calibration curves, an analyte stock solution (1000 mg L^{-1} SMX) was prepared in methanol and then further diluted with ultra-pure water to obtain standard solutions with concentrations ranging between 0.0001 and 1000 μg L^{-1}.

The mean values of OD were fitted to a 4PL, as explained in Chapter 2. All determinations were, at least, made in triplicate. Quantification range was also determined following the method explained in Chapter 2.

3.2.7 LC-MS/MS procedure

LC–MS/MS experiments were carried out using an Agilent 1100 liquid chromatograph (Agilent Technologies) and an API 4000 triple-stage quadrupole mass spectrometer from Applied Biosystems. A C18 reversed-phase column, 250 mm × 2.1 mm, 5 μm (Phen, UltraSep ES, SepServ) was used and the ionization performed in electrospray positive ion mode. The temperature of the column oven was kept at 40 ºC. A binary gradient consisting of 10 mM ammonium acetate and 0.1% acetic acid in water (A) and methanol (B) was used: starting with 80% A, isocratic for 3 min, linear decrease to 5% A within 20 min, kept at 5% A for 10 min.

The flow rate was maintained at 0.5 mL min^{-1} and the sample injection volume was 20 μL. Samples were previously enriched by SPE, in an automatic SPE workstation, AutoTrace (Thermo Scientific Dionex). Phenomenex® cartridges (500 mg/6 mL Strata™-X 33 μm polymeric reversed phase) were preconditioned twice with methanol, then once with Milli-Q water and loaded with 100 mL of sample. Elution of compounds was performed with 10 mL methanol and evaporation was carried out with a nitrogen stream. Samples were reconstituted in ultrapure water (concentration factor 1000-fold). SPE and LC-MS/MS were kindly performed by Marvin Engel and Andreas Lehmann, respectively, from BAM.

3.2.8 Evaluation of matrix effects

Dissolved organic matter and salinity were once more selected as potential interfering agents to study matrix effects, as explained in Chapter 2.

The influence of the presence of organic matter was evaluated using different types of HS: commercial HA and standards of IHSS - leonardite HA, Suwannee River HA and Suwannee River FA. For the commercial HA, calibration curves between 0.0001 and 1000 μg L^{-1} containing 1, 10 and 20 mg L^{-1} HA were obtained and compared to the one in the absence of HA. Furthermore, recovery tests were performed using 0.6 and 3.0 μg L^{-1} SMX standard solutions. Adequate volumes of 1 g L^{-1} comercial HA or IHSS HS stock solutions were added to these standards as to obtain concentrations of 1.0, 10.0 and 20.0 mg L^{-1} HA.

Trying to overcome the strong interference caused by dissolved organic matter, a 1% (w/v) BSA sample buffer (pH 7.6) was added to the wells prior to the addition of the analyte. Afterwards, recovery rates for the same standards as above were obtained in order to evaluate the effect of the sample buffer. Also, the effect of the BSA sample buffer pH was evaluated and calibration curves were obtained in presence of BSA sample buffer of pH 4.5, 7.6 and 9.5 and compared to the one obtained in absence of BSA sample buffer.

Salinity was simulated using NaCl with concentrations in the range 10-30 g L^{-1}. Calibration curves were performed spiking standards with different volumes of NaCl, in order to obtain concentrations of 10, 20 and 30 g L^{-1} NaCl. Results were compared to those in absence of NaCl (calibration curve in ultrapure water). Furthermore, recovery tests were performed using 0.6 and 3.0 μg L^{-1} SMX standard solutions. Adequate volumes of 50 g L^{-1}

NaCl stock solution were added to these standards as to obtain concentrations of 10.0, 20.0 and 30.0 g L^{-1} NaCl.

Recovery rates in a surface water sample (SWS3) were also obtained, spiking it with 1.5, 3.0 and 5.0 μg L^{-1} SMX.

3.3 RESULTS AND DISCUSSION

3.3.1 Enzyme conjugate preparation

After activation and conjugation steps, fractions of the eluted solution of HRP-succ-SMX were collected in a microtiter plate and the OD of each well was measured at 405 nm (Fig. 3.2).

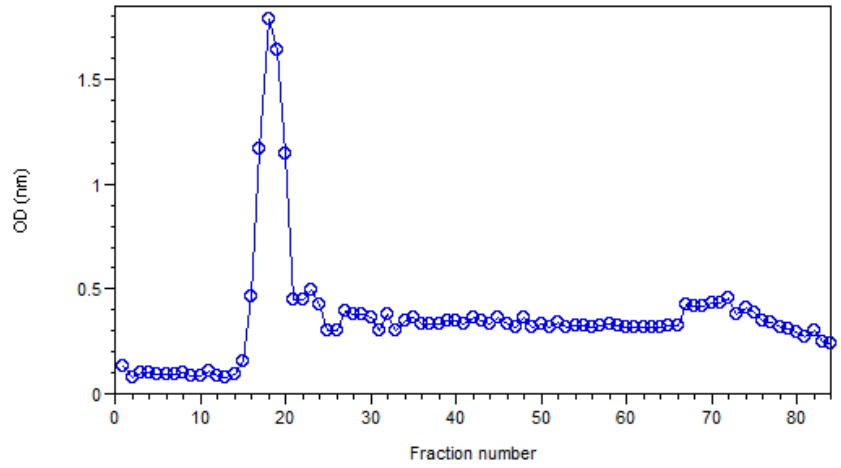


Fig. 3.2: OD results for the collection of conjugate fractions of SMX, after separation by means of a Sephadex column.

After that, three different fractions were collected and stored: HRP-succ-SMX pre-fraction, HRP-succ-SMX main-fraction and HRP-succ-SMX post-fraction, and later analysed by MALDI-TOF. Fig. 3.3 represents HRP-succ-SMX coupling ratios determined by MALDI-TOF for the three different fractions.

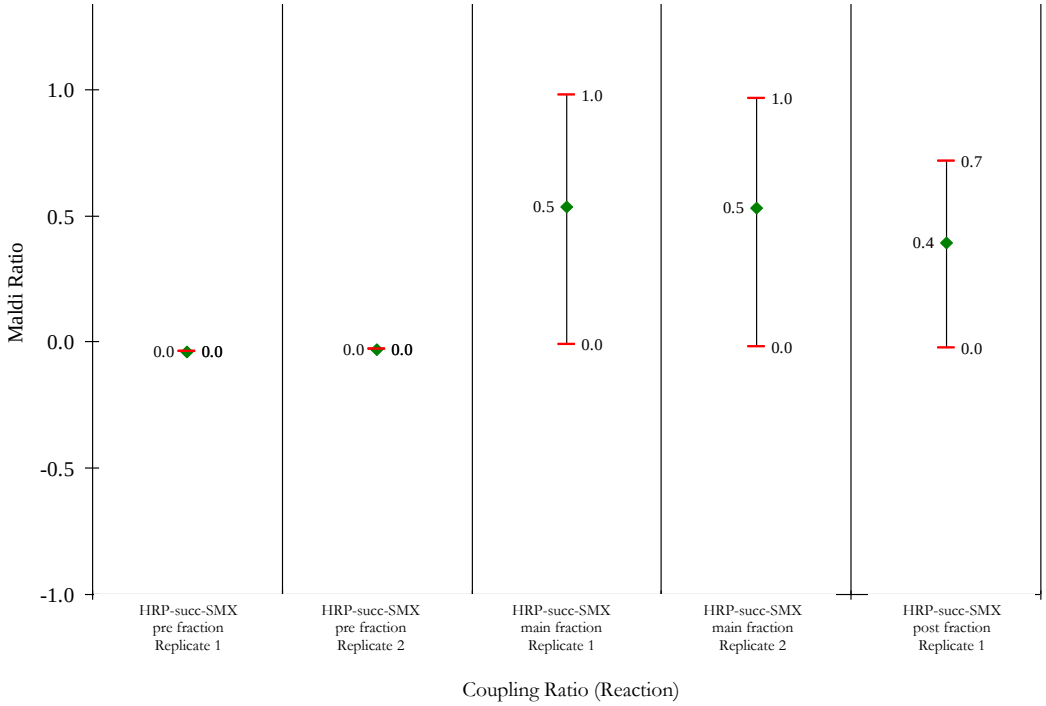


Fig. 3.3: MALDI-TOF coupling ratios for HRP-succ-SMX.

Results showed that the conjugation effectively took place and the hapten was linked to HRP. There was also some hapten not linked, which has no effect on the immunoassay performance as it has no anchor and is washed away.

3.3.2 Matrix effects

3.3.2.1 Organic matter

In order to evaluate organic matter effects on SMX assay performance, four calibration curves were obtained with standard solutions containing 0.0, 1.0, 10.0 and 20.0 mg L^{-1} HA (Fig. 3.4).

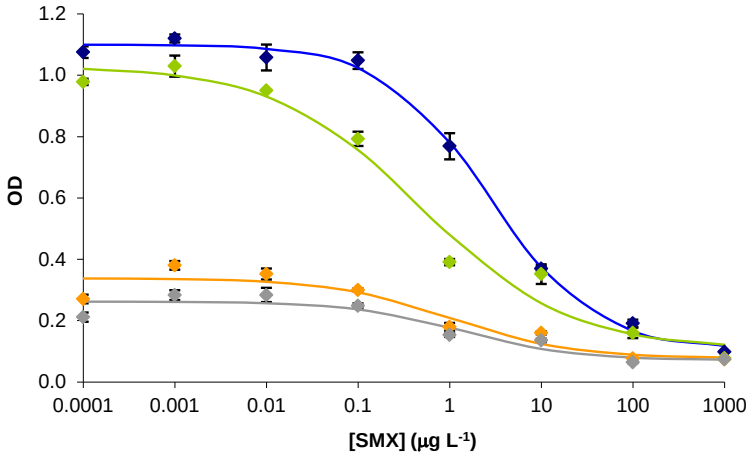


Fig. 3.4: Evaluation of the organic matter effect on the SMX ELISA calibration curve: standards prepared in ultrapure water (0 mg L^{-1} HA - blue); 1 mg L^{-1} HA (green); 10 mg L^{-1} HA (orange); 20 mg L^{-1} HA (gray). Ab 1:50 000; T 1:50 000. [Curve parameters: 0 mg L^{-1}: A - 1.10, B - 0.757, C - 2.68, D - 0.106; 1 mg L^{-1}: A – 1.03, B - 0.544, C – 0.489, D - 0.107; 10 mg L^{-1}: A - 0.338, B - 0.664, C - 1.03, D - 0.0769; 20 mg L^{-1}: A - 0.262, B - 0.719, C - 1.35, D - 0.0711]

A decrease of the OD_{max} (A parameter) was observed with an increase of the HA concentration, being 76% lower for 20 mg L^{-1} HA compared to 0 mg L^{-1} HA. Therefore, the presence of HA interferes with the assay performance, resulting in an OD decrease.

Effect of organic matter was further evaluated calculating recovery rates for SMX standards 0.6 and 3.0 µg L^{-1}. The interference of HA was confirmed by the extremely high mean recoveries obtained (reaching 5599% and 2267%, for 0.6 µg L^{-1} and 3.0 µg L^{-1} SMX standards, respectively). Only for ultrapure water (0.0 mg L^{-1} HA) it was possible to work without interference, with recoveries of 100.0 ± 0.1 and 111.1 ± 0.5%, for 0.6 and 3.0 µg L^{-1} SMX standards, respectively.

Trying to overcome this strong interfering role of HA, BSA sample buffer was added to the plate prior to the standards. Once again, recovery rates were calculated. Acceptable recovery rates (103-145%, for 0.6 µg L^{-1} SMX standard, and 93-121%, for 3.0 µg L^{-1} SMX standard) were obtained using the BSA sample buffer, showing that BSA, as a potential agent that binds organic matter, has a positive effect in the assay performance, when in presence of organic matter.

Moreover, the pH of sample buffer was also evaluated. BSA sample buffers with pH 4.5 and 9.5 were also applied. Under these conditions, both calibration curves and recovery tests were performed. Fig. 3.5 shows the comparison between the four conditions tested: assay

without sample buffer, with BSA sample buffer 7.6, with BSA sample buffer 4.5 and with BSA sample buffer 9.5.

In what concerns recovery rates, were calculated to be in the ranges: 99 to 218%, for 0.6 μg L^{-1} SMX standard and 89 to 124%, for 3.0 μg L^{-1} SMX standard, for BSA sample buffer pH 4.5; and 122 to 185% and 85 to 120%, for 0.6 and 3.0 μg L^{-1} SMX standard, respectively, for BSA sample buffer pH 9.5 (Table 3.2).

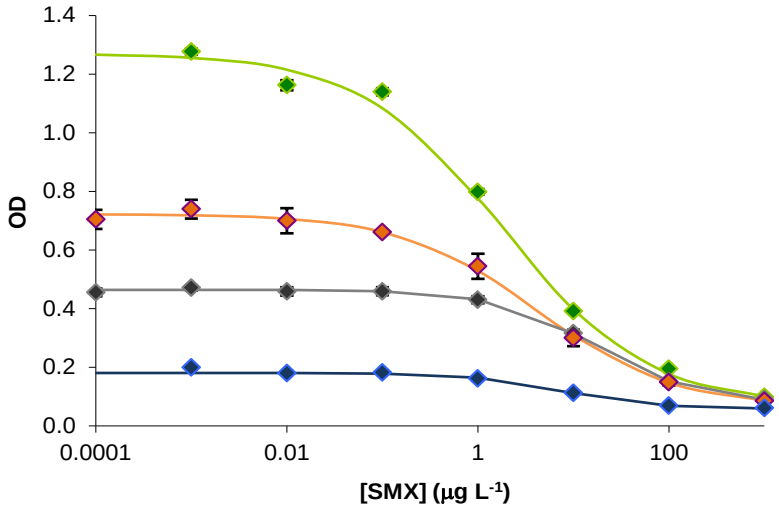


Fig. 3.5: Effect of the pH of BSA sample buffer and comparison with calibration curve in absence of buffer (green): sample buffer pH 7.6 (orange), sample buffer pH 4.5 (gray), sample buffer pH 9.5 (blue). [Curve parameters: absence of sample buffer: A - 1.27, B - 0.5800, C - 1.85, D - 0.0688; pH 7.6: A - 0.731, B - 0.588, C - 3.73, D - 0.061; pH 4.5: A - 0.464, B - 0.823, C - 17.9, D - 0.0479; pH 9.5: A - 0.18, B - 0.921, C - 7.64, D - 0.058].

Since BSA sample buffer pH 7.6 presented the best compromise between OD signal, turning point and recovery rates for both standards tested, this was the sample buffer used in the subsequent work.

Table 3.2: Mean recoveries for 0.6 and 3.0 μg L^{-1} SMX standards in absence and presence of BSA sample buffer of different values of pH (4.5, 7.6, 9.5).

	SMX standard (μg L^{-1})	[HA] (mg L^{-1})	Recovery (%)
Absence of BSA sample buffer	0.6	0	100 ± 0.1
		1	363.3 ± 0.4
		10	2794.0 ± 4.1
		20	5599.1 ± 5.9
	3.0	0	111.1 ± 0.5
		1	284.3 ± 1.3
		10	1304.6 ± 5.4
		20	2267.0 ± 1.3
BSA sample buffer pH 4.5	0.6	0	99.1 ± 0.2
		1	127.5 ± 0.1
		10	161.1 ± 0.2
		20	218.3 ± 0.4
	3.0	0	88.7 ± 0.1
		1	94.0 ± 0.02
		10	122.1 ± 0.1
		20	124.0 ± 0.2
BSA sample buffer pH 7.6	0.6	0	103.5 ± 0.03
		1	105.2 ± 0.1
		10	108.1 ± 0.2
		20	145.4 ± 0.2
	3.0	0	107.0 ± 0.1
		1	93.2 ± 0.2
		10	118.0 ± 0.3
		20	121.2 ± 0.5
BSA sample buffer pH 9.5	0.6	0	122.0 ± 0.3
		1	127.9 ± 0.5
		10	135.3 ± 0.9
		20	185.1 ± 0.2
	3.0	0	85.1 ± 0.5
		1	85.4 ± 0.4
		10	120.1 ± 0.3
		20	115.4 ± 0.6

3.3.2.1.1 Effect of different humic substances

The addition of different HS was tested in order to understand if the assay responds differently depending on the type of HS. Leonardite HA, Suwannee River HA and Suwannee River FA standards were tested and mean recoveries' results for the 3.0 μg L^{-1} SMX standard compared to the previously presented results obtained using commercial HA (Table 3.3).

Table 3.3: Comparison of the mean recoveries' results for the 3.0 μg L^{-1} SMX standard obtained for different HS, in presence and absence of BSA sample buffer pH 7.6.

Type of humic substance	Concentration (mg L^{-1})	Presence of sample buffer			
		NO		**YES**	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Commercial Hunic Acid (Sigma)	0	111	5.4	107	5.3
	1	287	5.4	93	1.2
	10	1304	1.9	118	8.3
	20	2267	4.5	120	4.5
Leonardite Humic Acid Standard INTERNATIONAL HUMIC SUBSTANCES SOCIETY	0	86	7.0	89	15.0
	1	311	3.2	88	8.6
	10	2821	4.6	91	7.3
	20	7469	1.1	115	7.3
Suwannee River Humic Acid Standard INTERNATIONAL HUMIC SUBSTANCES SOCIETY	0	86	10.8	93	14.6
	1	108	4.9	83	12.1
	10	213	4.1	83	9.4
	20	315	9.9	93	8.3
Suwannee River Fulvic Acid Standard INTERNATIONAL HUMIC SUBSTANCES SOCIETY	0	86	3.2	87	8.7
	1	85	9.4	86	10.0
	10	83	1.3	81	1.9
	20	81	8.4	83	9.1

As it can be seen, immunoassay has the same behaviour for all the HA tested, i.e. in absence of sample buffer recovery rates are not acceptable, reaching acceptable values in presence of sample buffer (83-120%). In the case of FA, it was interesting to see that performance of the assay is not negatively affected by their presence when no sample buffer was added. Given that the molecular weight of FA is lower than that of HA, it may be for this reason that FA did not create an unspecific linkage to the Ab or T.

3.3.2.2 Salinity

In order to evaluate salinity effects on SMX assay performance, four calibration curves were obtained with standard solutions containing 0.0, 10.0, 20.0 and 30.0 g L^{-1} NaCl (Fig. 3.6).

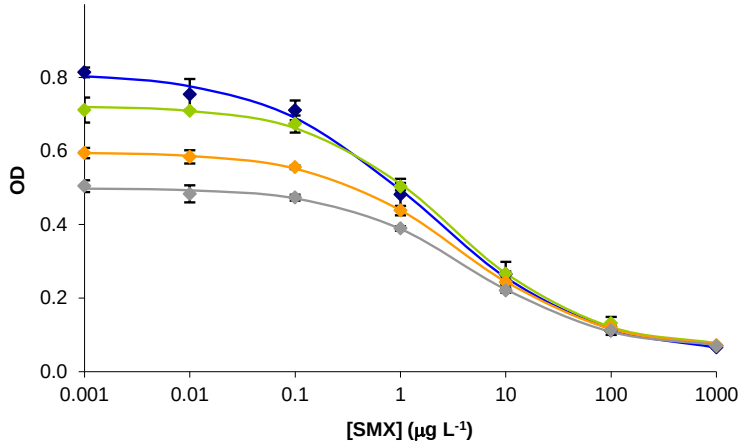


Fig. 3.6: Evaluation of the salinity effect on the ELISA calibration curve. Standards prepared in ultrapure water - 0 g L^{-1} NaCl (blue); 10 g L^{-1} NaCl (green); 20 g L^{-1} NaCl (orange); 30 g L^{-1} NaCl (gray). [Curve parameters: 0 g L^{-1} NaCl: A – 0.814, B - 0.5690, C - 1.82, D - 0.0445; 10 g L^{-1} NaCl: A - 0.723, B - 0.672, C - 3.01, D - 0.0643; 20 g L^{-1} NaCl: A - 0.597, B - 0.662, C – 3.72, D - 0.0617; 30 g L^{-1} NaCl: A - 0.499, B - 0.701, C - 4.64, D - 0.0628].

A decrease of the OD_{max} (A parameter) was observed with an increase of the NaCl concentration, being 39% lower for 30 g L^{-1} NaCl in comparison with 0 g L^{-1} NaCl. Therefore, as well as the presence of organic matter, the presence of salinity also interferes with the assay performance, resulting in an OD decrease.

Effect of salinity was further evaluated calculating recovery rates for SMX standards 0.6 and 3.0 µg L^{-1}. Recovery tests were performed in absence and presence of BSA sample buffer pH 7.6 (Fig. 3.7).

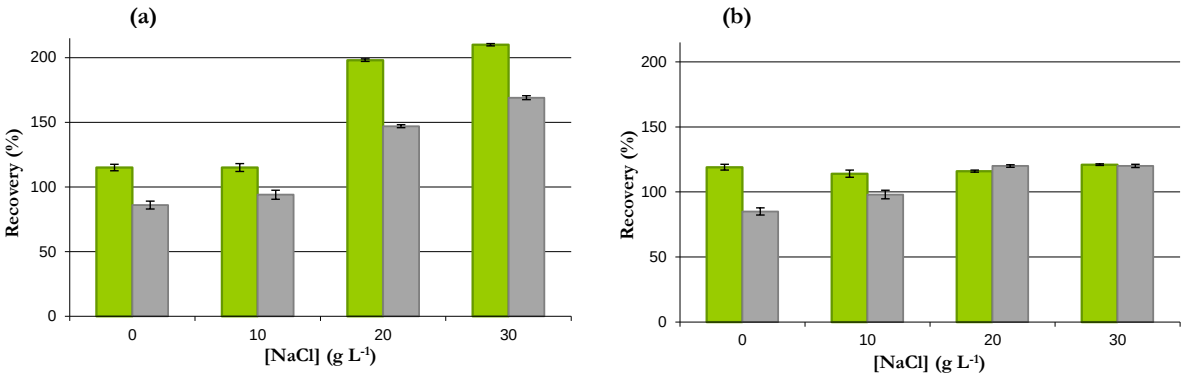


Fig. 3.7: Mean concentration obtained for 0.6 (green) and 3.0 µg L^{-1} (gray) SMX standards, in presence of increasing concentrations of NaCl (n=9) in (a) absence of BSA and (b) presence of BSA pH 7.6. Ab 1:50 000; T 1:50 000.

The interference was noticeable when not using sample buffer, mean recoveries being between 115 and 210%, for 0.6 μg L^{-1} SMX standard, and between 86 and 169%, for 3.0 μg L^{-1} SMX standard. When BSA sample buffer was added to the plate prior to the standards, mean recoveries were calculated to be in the range 119-121%, for 0.6 μg L^{-1} SMX standard, and in the range 85-120%, for 3.0 μg L^{-1} SMX standard.

Therefore, the use of the BSA sample buffer was shown to be advantageous not only to overcome the interference of organic matter, but also the interference of salinity.

3.3.3 Precision profile and quantification range

To determine the quantification range, 16 standards were used (6 replicates) and the precision profile was set up as described by Ekins (1981) and explained before (*cf.* Chapter 2). Quantification range was determined to be between 0.1 and 30 μg L^{-1} (Fig. 3.8).

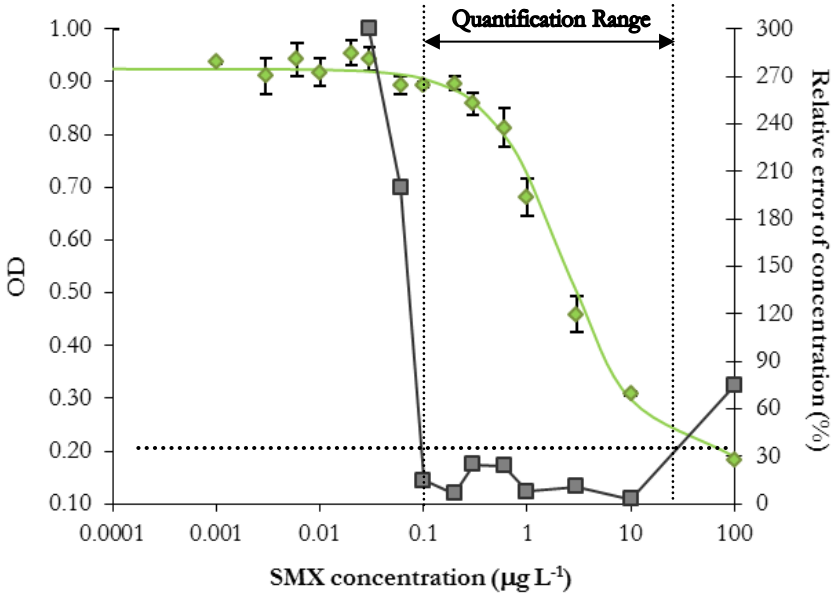


Fig. 3.8: Calibration curve (green marks) of SMX ELISA (A = 0.924; B = 1.16; C = 2.37; D = 0.180; r^2 = 0.997) and precision profile (gray marks), in presence of BSA sample buffer pH 7.6; Ab 1:50 000; T 1:50 000.

3.3.4 Recoveries and quantification of SMX in water samples

Recovery rates were obtained by spiking one surface sample (SWS3) with SMX. The concentration results were plotted against spiking levels and linear regression parameters were obtained (Fig. 3.9).

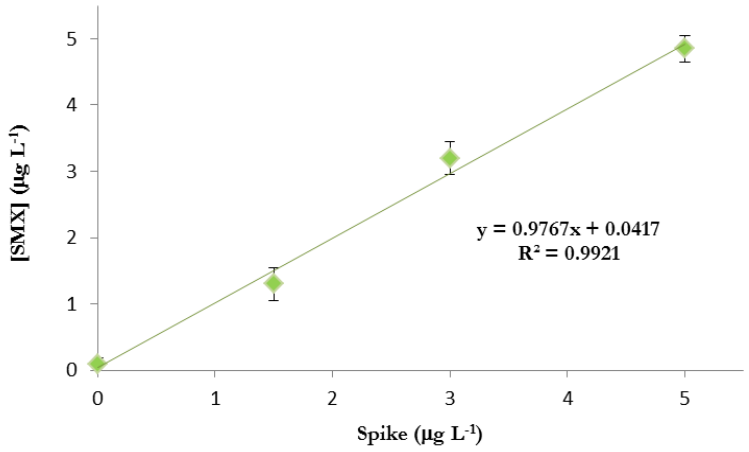


Fig. 3.9: SMX concentrations for three spiking levels in a surface water sample, SWS3, in presence of BSA sample buffer pH 7.6.

Recovery rates were calculated, being 98 ± 6% (r = 0.996). Therefore, a good assay performance was observed and no major interferences due to matrix effects seemed to occur in an environmental water sample.

The optimized assay was applied in the quantification of SMX in surface and waste water samples. Results obtained by ELISA were compared with those obtained by LC-MS/MS (Table 3.4). Additionally, n-acetyl-SMX, the main human metabolite of SMX (Göbel et al., 2004), was also quantified by LC-MS/MS.

Table 3.4: Quantification of SMX by both ELISA and LC-MS/MS. Quantification of n-acteyl-SMX was obtained by LC-MS/MS.

Sample	$[SMX]_{ELISA}$ ($\mu g\ L^{-1}$)	$[SMX]_{LC\text{-}MS/MS}$ ($\mu g\ L^{-1}$)*	$[n\text{-}Acetyl\text{-}SMX]_{LC\text{-}MS/MS}$ ($\mu g\ L^{-1}$)*
NWWS1 (after primary treatment)	11 ± 0.53	0.18	0.22
NWWS2 (after biological treatment)	8.2 ± 0.36	0.084	0.10
NWWS3 (final effluent)	8.3 ± 0.83	0.077	0.11
SWWS1 (after primary treatment)	9.5 ± 0.78	0.18	0.50
SWWS2 (after biological treatment)	4.3 ± 0.14	0.11	0.08
SWWS3 (final effluent)	8.3 ± 0.36	0.16	0.13
SWS1	0.73 ± 0.082	0.0040	0.0061
SWS2	0.60 ± 0.052	0.0020	0.0023
SWS3	0.095 ± 0.011	0.0010	0.0029
SWS4	0.29 ± 0.017	0.0050	0.0042
SWS5	0.76 ± 0.069	0.0040	0.0030
SWS6	0.45 ± 0.050	0.0010	<lod
SWS7	0.13 ± 0.022	0.0170	0.000056
SWS8**	---	---	---
SWS9	0.15 ± 0.011	0.0020	0.00040
SWS10	0.90 ± 0.078	0.0010	0.00153

*LC-MS/MS results were supplied with no s.d. values

**Missed sample

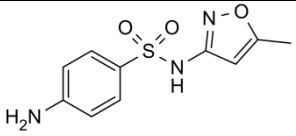
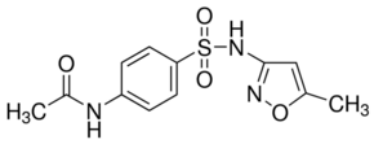
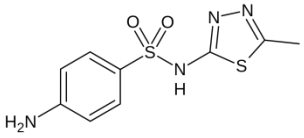
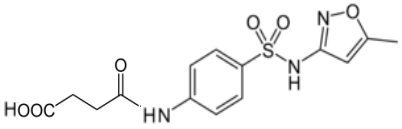
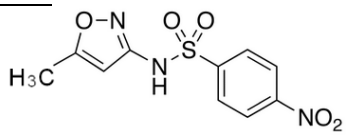
It can be observed that in the case of South STP, final effluent concentration almost doubles biological treatment concentration. Some other studies have already shown higher SAs' concentrations in the final effluents (Chang et al., 2008; Senta et al., 2008) pointing out for the possible retransformation of the main metabolites to the active parent SA during the waste water treatment (Černoch et al., 2012).

Overall, comparison between ELISA and LC-MS/MS showed that results did not correlate well, with ELISA overestimating the results. In a previous study by Zhang et al. (2010), it was also observed the tendency of obtaining higher results by ELISA than by LC-MS/MS. Authors attributed this fact to matrix effects and cross-reactivity of SAs' metabolites in the ELISA method and/or a low signal-to-noise ratio resulting from low concentrations and high organic content in LC-MS/MS method (Zhang et al., 2010).

In the present study, it seems that, although matrix effects play a role on the immunoassay performance and these problems had been solved by the addition of a sample buffer, other problems are affecting the SMX assay. The hypothesis is that some cross-reactivity problems are occurring, and so the immunoassay is overestimating the results. In fact, it has been found that SMX presents high cross-reactivity with some structure-related compounds (Table 3.5; data from BAM). This is in accordance with Shelver et al. (2008), who

reported that the SMX ELISA cross-reacts with several compounds.

Table 3.5: CR of SMX with structure-related compounds (data supplied by BAM).

Compound	Structure	Molar mass (g mol^{-1})	CR (%)
Sulfamethoxazole		253.3	100
N-Acetyl-Sulfamethoxazole		295.3	540
Sulfamethizole		270.3	232
Succinamyl-Sulfamethoxazole		353.4	1125
4-Nitro Sulfamethoxazole		283.3	469

Moreover, there is also the possibility that the cross-reactivity phenomena is happening with compounds other than the ones structurally related, but highly present in surface and waste water samples, as, for instance, other pharmaceutical compound. Bahlmann et al. (2009) reported that an Ab initially raised against the anticonvulsant carbamazepine also recognized the antihistamine cetirizine.

3.4 CONCLUSIONS

In this work, an ELISA methodology was optimized in order to quantify the antibiotic SMX in water samples. The ELISA assay was expressively affected by organic matter and salinity and therefore it was tried to overcome these interferences. The optimization of the

conditions included the testing of the presence of a sample buffer and also its pH. It was observed that matrix effects were minimized by using a BSA sample buffer with a pH of 7.6 prior to standards/samples. Recovery rates obtained in these conditions were acceptable: 93-121% and 85-120% (3.0 μg L^{-1} SMX standard) for the presence of organic matter and salinity, respectively. The assay performance was good even in an environmental water sample, with a recovery rate of 98 ± 6% (r = 0.996).

SMX was quantified in all samples tested with concentrations ranging from 4.3 to 11.0 μg L^{-1}, in waste water samples, and from 0.095 to 0.90 μg L^{-1} in surface water samples. Significantly lower results by LC-MS/MS analysis, which were obtained for comparison and validation, were achieved, highlighting the overestimation of the ELISA method. Since organic matter and salinity interferences were overcome in the optimized assay, the discrepancy between results may be related to CR between SMX and other compounds in solution.

Even though ELISA results did not correlate well with the reference method (LC-MS/MS), the SMX ELISA optimized in this work may be used as screening analytical tool.

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CHAPTER 4

Caffeine as a human pollution marker in waters of the north and center of Portugal

Silva, C.P.; Lima, D.L.D.; Schneider, R.J.; Otero, M.; Esteves, V.I.; Evaluation of the anthropogenic input of caffeine in surface waters of the north and center of Portugal by ELISA; *Science of the Total Environment* 479-480 (2014) 227-232.

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Summary

Caffeine is used to assess anthropogenic inputs in the aquatic environment. For this purpose, the present study comprises the development of an ELISA for the quantification of caffeine in complex aqueous matrices without any sample clean-up procedure. The quantification range of the developed method was 0.1–100 μg L^{-1}. Quantification of caffeine was possible in 43 out of 51 real aqueous samples, at values between 0.10 and 15 μg L^{-1}. Results correlated well with those obtained by LC–MS/MS. To the best of author's knowledge this is the first study dealing with the quantification of caffeine in Portuguese surface waters.

Contents

4.1 CONTEXTUALIZATION

The huge number (which is increasing constantly) and the variety of pharmacologically active pollutants, as well as their metabolites, present in natural waters make it difficult and costly to monitor all of them. However, this monitoring is crucial to assess the quality of water resources in order to determine for which purpose they may be used (drinking water, recreation, industrial and agricultural activities, such as irrigation and livestock watering, etc.). Moreover, a minimum quality is required to maintain aquatic and associated terrestrial ecosystem function. An approach that has been discussed to track the origin and type of contamination is the use of anthropogenic markers, i.e. indicators of human presence or activity (Bahlmann et al., 2012, Buerge et al., 2003).

Caffeine has already been proposed as an anthropogenic marker for waste water contamination of surface waters (Bahlmann et al., 2012; Buerge et al., 2003; Kurissery et al., 2012; Peeler et al., 2006; Seiler et al., 1999; Standley et al., 2000). Caffeine is the most widely consumed substance at a world scale; furthermore, it is relatively stable under variable environmental conditions, has high water solubility and mobility and a negligible volatility (Buerge et al., 2003; Kurissery et al., 2012).

Very few studies (Bahlmann et al., 2012; Carvalho et al., 2010; Nicolardi et al., 2012) have been carried out on the applicability of ELISA for quantitative analysis of caffeine in water monitoring.

In this work, an ELISA based on a monoclonal Ab was developed to measure caffeine, for the very first time, in organic-rich saline waters. From a practical point of view, this is especially important if we consider that estuaries are normally heavily populated areas and that about 60% of the world's population live along estuaries and the coast. A secondary objective was to assess the occurrence of human domestic pollution in Portuguese surface waters using caffeine as a marker. To the best of author's knowledge, such an assessment had never been carried out in Portugal.

Part of the experimental work presented in this Chapter (ELISA results and validation by LC-MS/MS of the samples from the October 2012 sampling campaign) were performed in Federal Institute for Materials Research and Testing - BAM, Berlin (Germany).

4.2 EXPERIMENTAL SECTION

4.2.1 Reagents and materials

Anti-caffeine monoclonal Ab and T were provided by Federal Institute for Materials Research and Testing - BAM, Berlin, Germany. Origin of both Ab and T is described elsewhere (Carvalho et al., 2010). The secondary (capture) Ab, a polyclonal anti-mouse IgG whole molecule, from sheep (R1256P, 2.17 mg mL^{-1}), was from Acris Antibodies (Aachen, Germany). Caffeine (puriss.) was from Sigma-Aldrich (St. Louis, USA).

Other reagents and instrumentation for ELISA were as described in Chapter 2.

TOC was measured using a TOC-V_{CPH} Analyzer, from Shimadzu.

4.2.2 ELISA procedure

Direct competitive ELISA was used for the analysis of caffeine, performed at room temperature (20 ± 1ºC). If not detailed, composition of buffers/solutions is as referred in Chapters 2 and 3. Microtiter plates were coated (200 μL per well) with the polyclonal anti-mouse Ab (1:2200) in PBS, covered with Parafilm and incubated overnight in the plate shaker at 750 rpm. The plates were then washed three times with diluted washing buffer, using the plate washer. The anti-caffeine monoclonal Ab (1:75 000) was diluted in TRIS buffer, added to the pre-incubated plate (200 μL per well) and shaken for 90 min. After washing the plate three times, the BSA sample buffer (pH 7.6; 25 μL per well, when applied), the caffeine standards or samples (100 μL per well) and the T (1:300 000) diluted in TRIS-NaCl buffer (100 μL per well) were incubated together, for 40 minutes, with permanent shaking. This incubation step was performed at room temperature and followed by a second three-cycle washing step. At last, the final substrate solution was added (200 μL per well) and incubated for 30 min. Final substrate solution was freshly prepared for each run and prepared according to Frey et al. (2000). The enzyme reaction was stopped by addition of 1 mol L^{-1} H_2SO_4 (100 μL per well). SoftMax Pro Software (Version 5.3, Molecular Devices) was used for the data analysis.

4.2.3 Immunoassay performance

To obtain the ELISA calibration curves, a 1000 mg L^{-1} caffeine stock solution was prepared in methanol. Standards were subsequently obtained by diluting the stock solution with ultrapure water. Eight standard solutions logarithmically distributed, with concentrations ranging from 0.0001 to 500 μg L^{-1}, were used for all calibration curves. A 4PL was fitted to the mean (3 replicates) OD values, as explained in Chapter 2.

In order to optimize the calibration curve, four combinations of anti-caffeine Ab and T dilutions were tested - anti-caffeine Ab 1:100 000, T 1:100 000; anti-caffeine Ab 1:100 000, T 1:300 000; anti-caffeine Ab 1:100 000, T 1:500 000; anti-caffeine Ab 1:75 000, T 1:300 000.

To determine the quantification range, 16 standards were used (6 replicates). As previously described in Chapter 2 and in order to set up the "precision profile", the relative error of the caffeine concentration readings was calculated from the respective OD standard deviations and the slope (1^{st} derivative) at each individual standard concentration (Ekins, 1981). In order to evaluate the accuracy and precision of the method, a 0.3 μg L^{-1} caffeine standard was determined (58 replicates), allowing a 25% deviation from the real standard concentration.

4.2.4 Evaluation of matrix effects

In order to evaluate organic matter effects, ELISA calibration curves were obtained for caffeine using standard solutions containing 1.0, 10.0 and 20.0 mg L^{-1} of commercial HA. Calibration curves were compared with the ones obtained with caffeine standard solutions in absence of HA. Also, recovery tests were performed with the 0.3 μg L^{-1} caffeine standard. This standard was spiked with 1 g L^{-1} HA stock solution to obtain HA concentrations between 1.0 and 20.0 mg L^{-1}. To evaluate salinity effects, ELISA calibration curves were obtained for caffeine using standard solutions containing 10.0, 20.0 and 30.0 g L^{-1} of NaCl. Calibration curves were compared with those obtained with caffeine standard solutions in absence of NaCl. Recovery tests using the 0.3 μg L^{-1} caffeine standard were performed, spiking it with 50 g L^{-1} NaCl stock solution to obtain NaCl concentrations between 10.0 and 30.0 g L^{-1}. Also, recovery tests were performed using water samples by spiking them with

proper amounts of caffeine (1.0, 1.5, 3.0 μg L^{-1} caffeine, for surface waters, and 15.0, 30.0, 45.0 μg L^{-1} caffeine, for a waste water sample).

A BSA-containing buffer (pH 7.6) was added to the plate prior to the standards/samples because of its probable beneficial effects in suppressing/reducing matrix interferences on immunoassays, possibly due to a combined effect of attracting positively charged interferents, as well as attenuating the denaturing impact that matrix components might have on proteins (Bahlmann, 2009; Silva et al., 2013).

4.2.5 Water sampling for caffeine quantification

In order to prove its applicability for water monitoring, the developed ELISA was employed in the quantification of caffeine in water samples. A total of 51 samples were collected in July and November 2012 and in April 2013 at different locations in the north and center of Portugal.

Samples numbered 1 to 20 were surface water samples collected from 10 different sites (described in more detail in Chapter 2) of the estuarine shallow lagoon Ria de Aveiro (northwest coast of Portugal). Samples numbered 21 to 33 were surface samples, collected in the north and center of Portugal. Samples from 34 to 39 were taken from public fountains providing potable water. The last numbered samples (40 to 51) were waste water samples from different stages of the treatment of two STPs (STPs described in Chapter 2). Samples 40, 41 and 42 are from North STP, and were sampled after primary decantation, after biological treatment and from the final effluent, respectively; samples 43, 44 and 45 correspond to the same stages of the treatment, but from South STP. The same applies to samples 46-48 (North STP) and 49-51 (South STP), but sampled in a different sampling campaign (April 2013).

In all cases, 250 mL of water were collected in cleaned dark glass bottles (previously washed 3 times with a few millilitres of the sample to be collected). Immediately after collection, all the samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at 4 ºC until analysis. Samples were not subjected to any other cleaning procedure, extraction or enrichment processes.

Samples 1 to 21 were analysed using a TOC-V_{CPH} Analyzer and TOC values ranged from 2.9 to 12.6 mg L^{-1}.

4.2.6 Assay validation

To validate the ELISA method, random control samples were measured also by LC–MS/MS. The LC–MS/MS experiments were carried out using the same equipment as described in Chapter 3. The chromatographic method for caffeine was previously described in detail by Carvalho et al. (2010). Samples were previously enriched by SPE, in an automatic SPE workstation, AutoTrace (Thermo Scientific Dionex). Phenomenex® cartridges (500 mg/6 mL Strata™-X 33 μm polymeric reversed phase) were preconditioned twice with methanol, then once with Milli-Q water and loaded with 100 mL of sample. Elution of caffeine was performed with 10 mL methanol and evaporation was carried out with a nitrogen stream. Samples were reconstituted in ultrapure water (concentration factor 1000-fold). SPE and LC-MS/MS were kindly performed by Marvin Engel and Andreas Lehmann, respectively, at BAM.

4.3 RESULTS AND DISCUSSION

4.3.1 Immunoassay calibration curve

Table 4.1 summarizes the results for experiments using different combinations of Ab and T dilutions. Combination anti-caffeine Ab 1:75 000, T 1:300 000 was the one selected. Although it did not present the lowest C parameter value, it presented both a good C value and a high difference between the OD values of the lower and the higher standards (A and D parameters, respectively), which is an indicator of higher sensitivity (Table 4.1).

Table 4.1: Parameter values obtained for the 4PL using different anti-caffeine Ab and T dilutions.

Anti-caffeine Ab/T dilutions	A	B	C	D	r^2
Ab 1:100 000; T 1:100 000	0.790	0.983	0.452	0.0309	0.993
Ab 1:100 000; T 1:300 000	0.296	0.652	0.277	0.0248	0.987
Ab 1:100 000; T 1:500 000	0.182	0.873	0.451	0.0228	0.988
Ab 1:75 000; T 1:300 000	**0.454**	**0.923**	**0.379**	**0.0317**	**0.996**

4.3.2 Evaluation of matrix effects

A decrease of the OD_{max} (A parameter), in absence of BSA, was observed for high HA concentrations, being 61% lower for 20 mg L^{-1} HA, in comparison with 0 mg L^{-1} HA (Fig. 4.1a). The presence of HA interferes with the performance of the assay, possibly due to unspecific binding of HA to the anti-caffeine Ab or to the enzyme protein, or even both. In either case, the decrease in OD with the increase of HA concentration may generate an overestimation of the caffeine concentration.

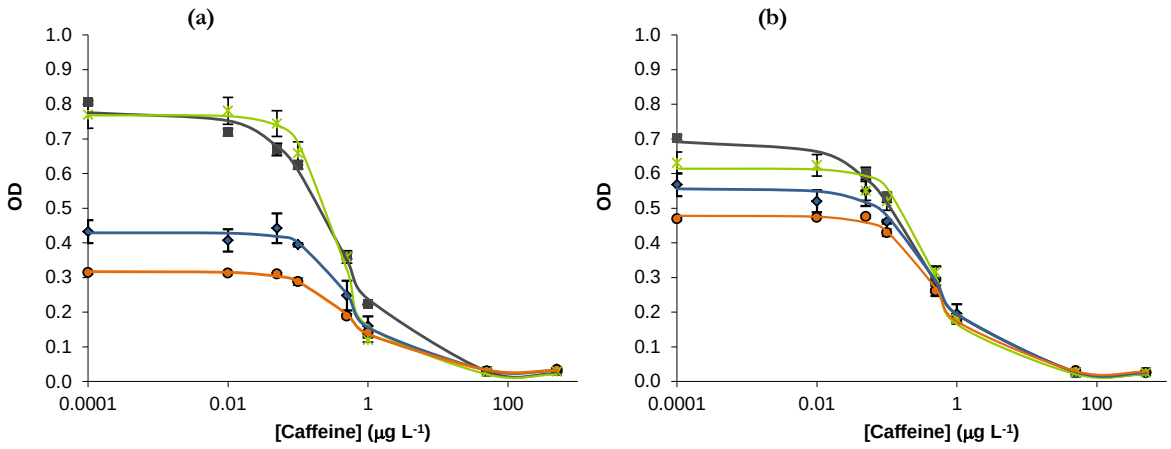

Fig. 4.1: Evaluation of the organic matter effect on the caffeine ELISA calibration curve in (a) absence and (b) presence of BSA buffer. Standards prepared in ultrapure water: 0 mg L^{-1} HA - (gray); 1 mg L^{-1} HA (green); 10 mg L^{-1} HA (blue); 20 mg L^{-1} HA (orange). Anti-mouse Ab 1:2200; anti-caffeine Ab 1:75 000; T 1:300 000. [Curve parameters: **(a)** 0 mg L^{-1} HA – A = 0.776, B = 0.943, C = 0.380, D = 0.0218; 1 mg L^{-1} HA – A = 0.768, B = 1.57, C = 0.391, D = 0.0202; 10 mg L^{-1} HA – A = 0.429, B = 1.46, C = 0.583, D = 0.0316; 20 mg L^{-1} HA – A = 0.317, B = 1.18, C = 0.632, D = 0.0315; **(b)** 0 mg L^{-1} HA – A = 0.692, B = 0.905, C = 0.313, D = 0.0200; 1 mg L^{-1} HA – A = 0.614, B = 1.48, C = 0.465, D = 0.0205; 10 mg L^{-1} HA – A = 0.556, B = 1.10, C = 0.499, D = 0.0244; 20 mg L^{-1} HA – A = 0.478, B = 1.28, C = 0.558, D = 0.0280].

Fig. 4.1b shows the four caffeine calibration curves obtained in presence of BSA buffer. From these curves it is clear that the loss of signal (decrease of the OD) for high concentrations of HA is much less pronounced when using this buffer.

Although solving the influence of organic matter, it was previously observed (*cf.* Chapter 2) that BSA sample buffer (pH 7.6) decreased the sensitivity. However, in the present case, and using the C parameter as a measure of the assay sensitivity, it can be seen that the addition of the sample buffer did not cause an increase in the C parameter ($C_{\text{in absence of sample buffer}}$ = 0.380; $C_{\text{in presence of sample buffer}}$ = 0.313), i.e. it did not imply a decrease in sensitivity (Fig. 4.1).

Besides the observation of the curves and the respective parameters, the organic matter effect was also evaluated by recovery tests, using the 0.3 μg L^{-1} caffeine standard. In absence of sample buffer, recoveries were only acceptable in the range 0-10 mg L^{-1} HA, rising up to 145% for 20 mg L^{-1}. These revoveries demonstrate the overestimation effect of high organic matter contents on caffeine quantification. On the other hand, when using the BSA-based sample buffer (pH 7.6) prior to the addition of the analyte, recovery rates were improved (between 93.1 ± 1.2% and 103.0 ± 1.1%), showing that this buffer minimizes the organic matter interferences, as it was observed by the calibration curves.

When studying the effect of salinity, four calibration curves were also obtained for different levels of added NaCl, both in absence and presence of BSA (Fig. 4.2). As it can be seen, in absence of BSA sample buffer, the effect of salinity is much less pronounced than that of organic matter. Still, BSA sample buffer has an advantageous effect on the performance of the assay. Also in this case, BSA buffer did not affect the sensitivity, as in the case of HA.

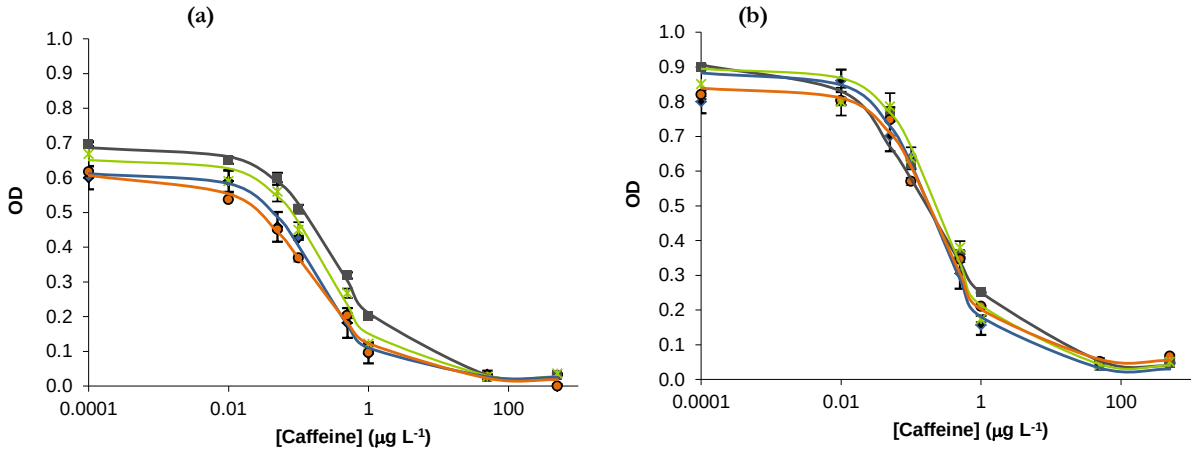

Fig. 4.2: Evaluation of the salinity effect on the caffeine ELISA calibration curve in (**a**) absence and (b) presence of BSA buffer. Standards prepared in ultrapure water: 0 g L^{-1} NaCl - (gray); 10 g L^{-1} NaCl (green); 20 g L^{-1} NaCl (blue); 30 g L^{-1} NaCl (orange). Anti-mouse Ab 1:2200; anti-caffeine Ab 1:75 000; T 1:300 000. Curve parameters: **(a)** 0 g L^{-1} NaCl – A = 0.687, B = 0.895, C = 0.351, D = 0.0234; 10 g L^{-1} NaCl – A = 0.651, B = 1.00, C = 0.248, D = 0.0269; 20 g L^{-1} NaCl – A = 0.612, B = 1.03, C = 0.178, D = 0.0252; 30 g L^{-1} NaCl – A = 0.608, B = 0.829, C = 0.158, D = 0.0177; **(b)** 0 g L^{-1} NaCl – A = 0.910, B = 0.698, C = 0.201, D = 0.0350; 10 g L^{-1} NaCl – A = 0.895, B = 1.04, C = 0.268, D = 0.0398; 20 g L^{-1} NaCl – A = 0.883, B = 1.02, C = 0.221, D = 0.0296; 30 g L^{-1} NaCl – A = 0.839, B = 1.03, C = 0.241, D = 0.0547.

In the case of salinity, recovery rates were also calculated for the 0.3 μg L^{-1} caffeine standard, confirming the small influence of NaCl in the performance of the assay, especially in the presence of BSA buffer. Recovery rates ranged from 86.1 ± 3.2% to 107.4 ± 7.2%, in absence of BSA, and between 95.2 ± 1.9% and 102.3 ± 1.4%, in presence of BSA.

4.3.2.1 Recovery tests in water samples

Several samples were subjected to caffeine spiking in order to calculate the recovery rates (Table 4.2). The concentration results were plotted against spiking levels and linear regression parameters were obtained.

Samples selected were considered representative of high levels of organic matter. TOC values of the surface water samples (all the samples presented in Table 4.2, with exception to sample 46 – wastewater sample) ranged between 2.9 and 12.6 mg L^{-1}. As it can be seen in Table 4.2, good assay performances were observed. All mean recoveries were in the range 81-118%, showing that no major interferences occurred in the quantification of caffeine in real samples, when optimized conditions were applied.

Table 4.2: Recoveries for samples subjected to the addition of caffeine spikes.

Sample	Spike (μg L^{-1})	Equation	Recovery (%)	Sample	Spike (μg L^{-1})	Equation	Recovery (%)
1	1.0	y = 0.9124x + 0.553	91.24 ± 0.06	13	1.0	y = 1.0429x + 0.1768	104.29 ± 0.04
	1.5	R^2 = 0.9904			1.5	R^2 = 0.9969	
	3.0				3.0		
3	1.0	y = 0.8275x + 0.4679	82.75 ± 0.09	15	1.0	y = 0.8136x + 0.1887	81.36 ± 0.04
	1.5	R^2 = 0.977			1.5	R^2 = 0.9999	
	3.0				3.0		
5	1.0	y = 0.8377x + 0.2639	83.77 ± 0.09	17	1.0	y = 0.8063x + 0.0806	80.63 ± 0.01
	1.5	R^2 = 0.9789			1.5	R^2 = 0.9856	
	3.0				3.0		
7	1.0	y = 0.8476x + 0.359	84.76 ± 0.04	19	1.0	y = 1.1824x + 0.1728	118.24 ± 0.02
	1.5	R^2 = 0.9962			1.5	R^2 = 0.9973	
	3.0				3.0		
9	1.0	y = 1.1502x + 0.2005	115.02 ± 0.02	21	1.0	y = 1.0001x + 0.0255	100.01 ± 0.07
	1.5	R^2 = 1			1.5	R^2 = 0.9995	
	3.0				3.0		
11	1.0	y = 0.9829x + 0.408	98.29 ± 0.03	46	15.0	y = 1.0527x + 12.14	105.3 ± 0.3
	1.5	R^2 = 0.9976			30.0	R^2 = 0.9956	
	3.0				45.0		

4.3.3 Immunoassay performance

The quantification range (Fig. 4.3) was obtained via the precision profile, calculated using the model proposed by Ekins (1981) and considering a maximum acceptable relative error of 30% (Grandke et al., 2013; Silva et al., 2013), as explained in Chapter 2.

The quantification range obtained was 0.1-100 μg L^{-1}. Therefore, this assay can be used over three orders of magnitude in analyte concentration.

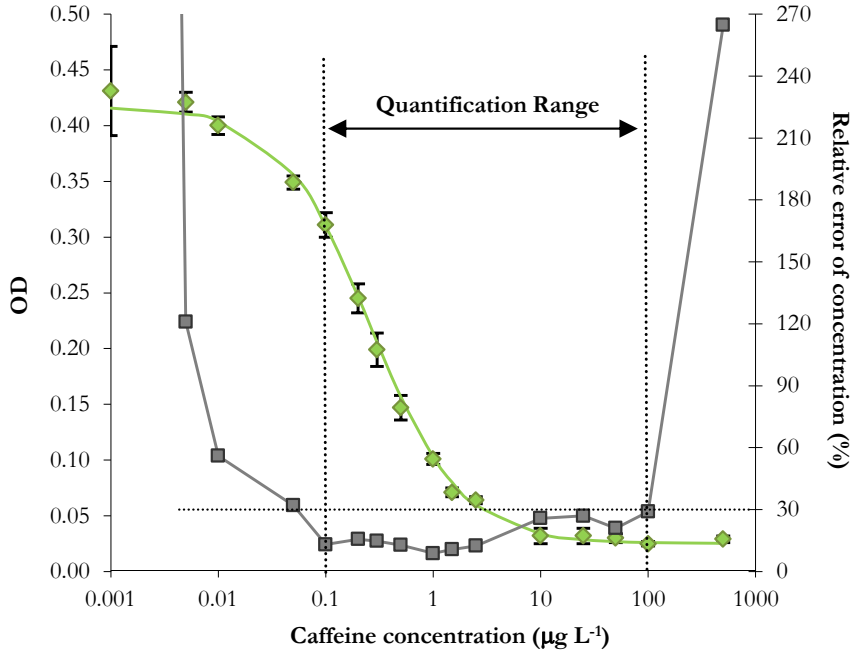

Fig. 4.3: Caffeine ELISA calibration curve (green) (A = 0.417; B = 1.03; C = 0.258; D = 0.0252) and precision profile (gray). Anti-mouse Ab 1:2200; anti-caffeine Ab 1:75 000; T 1:300 000. The precision profile and determination of the relative error of concentration were calculated in accordance with Ekins (1981).

Results for the accuracy at the 0.3 μg L^{-1} caffeine level demonstrated that all the concentrations measured were within the 25% pre-established limit, i.e., between 0.225 and 0.375 μg L^{-1}, indicating that the method can be considered accurate (Fig. 4.4).

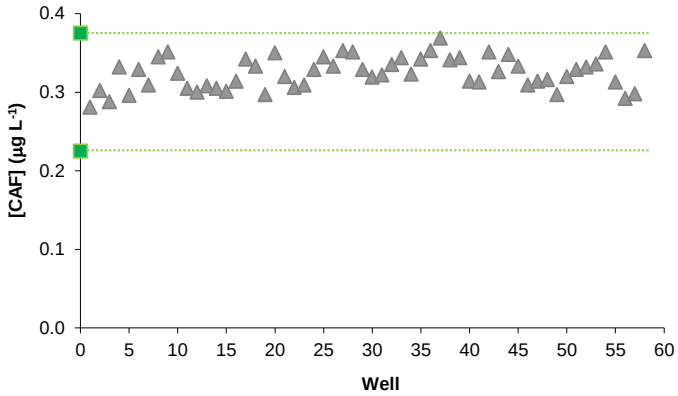

Fig. 4.4: Measured concentration in each of 58 wells for the 0.3 μg L^{-1} caffeine standard. Upper and lower deviation limits are also shown.

The mean obtained for the 58 replicates was 0.32 ± 0.02 μg L^{-1}, with a relative standard deviation of 6.3%. Thus, besides accurate, the method can also be considered precise.

4.3.4 Quantification of caffeine in water samples

Caffeine was quantified in 43 out of 51 samples (Fig. 4.5 and Table 4.3) in values ranging between 0.10 and 15 μg L^{-1}.

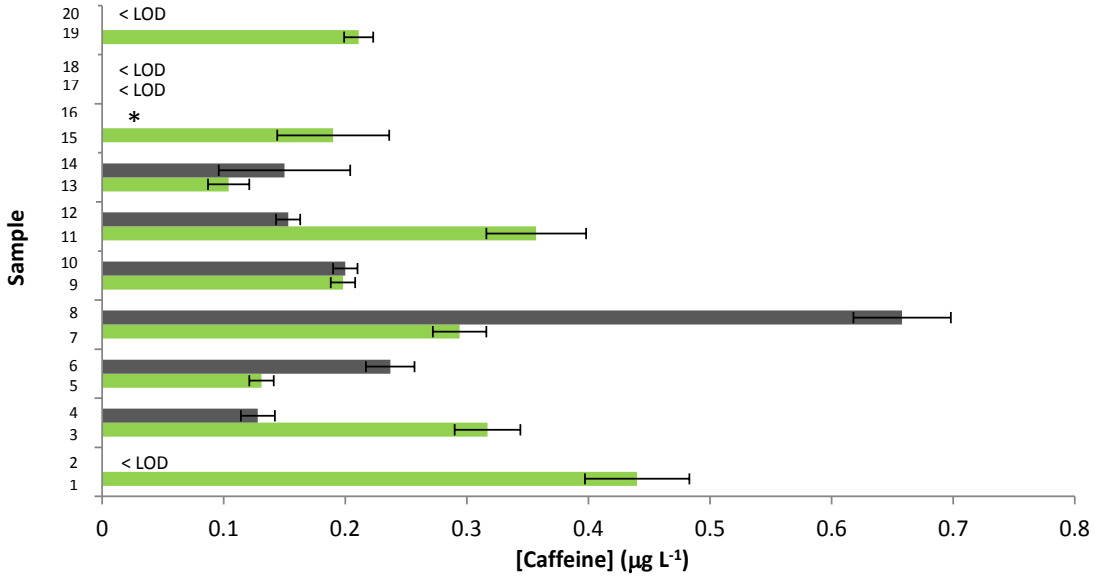

Fig. 4.5: Quantification of caffeine in surface waters from Ria de Aveiro (Portugal) sampled in July (green) and November 2012 (gray) (*missed sample).

Results showed that samples 1 and 19 presented caffeine detectable concentrations in the sampling campaign carried out in July but not in the November campaign. A possible explanation is that these samples were from a coastal bathing area where probably there was a higher amount of people consuming caffeine in the summer, i.e. in vacation months. The population increase generally observed on coastal areas during summer is known to enhance the anthropogenic load to surface waters due to the larger discharge of domestic waste water (Buerge et al., 2003). Results <LOD observed in samples 2 and 20 (samples corresponding to 1 and 19 sites, but collected in November (winter time)) corroborate the assumption that caffeine concentration increases in summer time and decreases in winter in coastal bathing

areas. It is important to highlight that for the November sampling campaign, the higher value found (sample 8 - 0.66 μg L^{-1}) was from a city center area; also, high values found in samples 10, 12 and 14 (between 0.15 and 0.20 μg L^{-1}) correspond to urban areas as well. Leakages from septic systems cannot be excluded overall.

Table 4.3: Quantification of caffeine in surface, potable (from public fountains) and waste waters.

Sample[a]	[Caffeine] (μg L^{-1})	Sample[a]	[Caffeine] (μg L^{-1})
21	< LOD	37	0.14 ± 0.01
22	0.31 ± 0.01	38	0.58 ± 0.05
23	0.54 ± 0.01	39	0.23 ± 0.01
24	0.16 ± 0.01	40	15 ± 1
25	0.57 ± 0.01	41	1.0 ± 0.1
26	0.53 ± 0.03	42	0.92 ± 0.03
27	0.13 ± 0.01	43	6.4 ± 0.4
28	0.14 ± 0.01	44	0.60 ± 0.02
29	0.18 ± 0.03	45	0.58 ± 0.03
30	0.109 ± 0.001	46	11.1 ± 0.1
31	0.32 ± 0.03	47	0.4 ± 0.1
32	9 ± 1	48	0.17 ± 0.05
33	0.25 ± 0.01	49	14.2 ± 0.3
34	< LOD	50	0.26 ± 0.05
35	0.16 ± 0.01	51	0.20 ± 0.09
36	< LOD		

[a]Samples 21-33 – Surface water samples. Samples 34-39 – Potable water (from public fountains) samples. Samples 40-51 – Waste water samples (40 and 46 – after primary decantation in North STP; 41 and 47 – after biological treatment in North STP; 42 and 48 - final effluent in North STP; 43 and 49 – after primary decantation in South STP; 44 and 50 – after biological treatment in South STP; 45 and 51 - final effluent in South STP).

In what concerns surface waters (samples 21-33), it was observed, once again, that some of the highest values were found in urban areas (samples 22, 23, 25, 26 and 31), which can be attributed to leakages from old septic tanks. On the other hand, considering that sample 21 is from an uninhabited area, it makes sense that caffeine concentration had been below LOD, which enforces the use of caffeine as a marker of human presence. In the remaining samples, caffeine concentrations were in accordance with values found in literature (e.g. Bahlmann et al., 2012; Kurissery et al., 2012; Nicolardi et al., 2012). An exception is the relatively high caffeine concentration (9 μg L^{-1}) of the surface water sample 32. This sample was collected right after the discharge of a small STP into the river. In any case, even when considering an incomplete mixing of the effluent with the receiving surface water, this caffeine

concentration points to a poor waste water treatment at the STP. In fact, Buerge et al. (2006) suggested that the presence of caffeine in receiving waters should be indicative of untreated waste water discharge, as elimination efficiencies in most STPs are over 99%.

Results in waters from public fountains (samples 34-39) were surprisingly high, especially taking into account that some of these fountains (sample 37, for instance) are supplied with spring water. From 6 samples, 4 were contaminated with caffeine, in concentrations ranging from 0.14 to 0.58 $\mu g\ L^{-1}$. It was already stated that caffeine, because of its high mobility, may also be an adequate marker for confirming groundwater contamination (Buerge et al., 2003). This contamination (and the possibly related bacterial contamination) poses a threat to drinking water quality.

Finally, and as it can be seen in the STP samples (samples 40-51), the treatment causes a decrease in the caffeine concentration (removal from after primary decantation and final effluent ranged between 91 and 99%). Even so, concentrations in final effluents ranged between 0.17 and 0.92 $\mu g\ L^{-1}$.

Results, as a whole, are representative of the anthropogenic input in surface waters.

4.3.5 Assay validation

To validate the ELISA results, some samples (6 estuarine samples and 5 waste water samples) were also measured by LC–MS/MS. Although some overestimation by ELISA was observed in the case of waste water samples (due, possibly, to a more complex matrix), caffeine was satisfyingly measured by ELISA in both surface and waste waters. The methods were correlated according to the following linear regression equation: $[\text{Caffeine}]_{\text{ELISA}} = (1.50 \pm 0.01) \times [\text{Caffeine}]_{\text{LC–MS/MS}} - (0.040 \pm 0.021)$ ($r^2 = 0.9996$).

A *t test* usually used for mean comparison is not appropriate in this case because of the differences between the samples' concentrations, which will disguise any variation due to the method. Therefore, a *paired t-test* was applied in order to establish if the results obtained by both methods were not, in fact, significantly different. Since the calculated *t* value (1.16) was lower than the critical *t* value (2.23), for 10 degrees of freedom, at a 95% confidence level, it can be stated that there were no significant differences between the results obtained by ELISA and LC-MS/MS.

4.4 CONCLUSIONS

The developed ELISA proved to be adequate for the quantification of caffeine in samples with a complex matrix, like samples from an estuarine area and from STPs. The use of a BSA sample buffer reduced interferences from the sample matrix, namely high concentrations of organic matter. Therefore, caffeine was satisfyingly measured by ELISA.

LC-MS/MS validation of results proved that ELISA is a very useful tool for large sampling campaigns allowing high-throughput analysis at very low cost. For monitoring programs, the developed ELISA assay may be used for large screenings in order to identify contaminated areas, thus reducing time and analytical costs compared to LC-MS/MS.

Since it is crucial to maintain the quality of natural waters, quantification of caffeine by the developed ELISA can be a suitable tool to assess contamination owed to human pollution.

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PART II

Development of a low-cost pre-concentration methodology for estrogens quantification by different techniques

CHAPTER 5

Development of dispersive liquid–liquid microextraction technique for estrogens' quantification by HPLC with detection by fluorescence

Lima, D.L.D.; Silva, C.P.; Otero, M.; Esteves, V.I; Low cost methodology for estrogens monitoring in water samples using dispersive liquid–liquid microextraction and HPLC with fluorescence detection; *Talanta* 115 (2013) 980-985.

http://dx.doi.org/10.1016/j.talanta.2013.07.007

Summary

A new low cost methodology for estrogens' analysis in water samples was developed in this work. Based on dispersive liquid–liquid microextraction (DLLME) followed by high-performance liquid chromatography with fluorescence detection (HPLC-FD), the developed method is fast, cheap, easy-to-use, uses low volumes of organic solvents and has the possibility of a large number of samples to be extracted in parallel. Under optimum conditions (sample volume: 8mL; extraction solvent: 200 μL of chlorobenzene; dispersive solvent: 2000 μL of acetone), the enrichment factor (EF) and extraction recoveries (ER) were 145 and 72%, for E2, and 178 and 89%, for EE2, respectively. LODs of 2.0 ng L^{-1}, for E2 and 6.5 ng L^{-1}, for EE2 were achieved, allowing the detection and quantification of these compounds in surface and waste water samples in concentrations ranging from 12 to 32 ng L^{-1}, for E2, and from 11 to 18 ng L^{-1}, for EE2. Also, recovery tests were performed to evaluate possible matrix effects. Recoveries between 98 and 106% were obtained using HA to simulate the effect of organic matter, and between 86 and 120%, in environmental water samples.

Contents

5.1 CONTEXTUALIZATION

As it was already stated, due to their extremely low environmental concentrations, E2 and EE2 direct quantification in water samples may require instrumentation with high sensitivity as GC-MS and GC-MS/MS, as well as LC-MS and LC-MS/MS. Compared with LC–MS and LC–MS/MS, HPLC coupled either to UV or fluorescence detector (FD) is a simpler, faster, easy-to-use and widely available technique. Still, detection limits are not as good as with MS detectors so a pre-concentration step is required. Among pre-concentration methodologies that can be applied are SPE and liquid-liquid extraction (LLE). An SPE coupled online with LC-ESI-MS/MS has been used for determination of hormones in water samples with limits of quantification between 0.02 and 1.02 ng L^{-1} (Rodríguez-Mozaz, et al., 2004). However, SPE implies a high consumption of organic solvents. In this sense, SPME has an advantage over SPE, which is the minor solvent consumption. Nevertheless, for SPME, the fragile, expensive, limited life time and sample carryover of the fiber is also an issue (Du et al., 2010). Regarding classical LLE, main weaknesses are that it is time-consuming and requires large volumes of toxic organic solvents. Nonetheless, liquid-phase microextraction (LPME) overcomes many disadvantages of LLE as well as some of those of SPME (Rezaee et al., 2010). Among LPME techniques, several have been applied successfully to steroid hormones pre-concentration. Hollow-fiber LPME (HF-LPME) combined with GC–MS was optimized for the determination of steroid hormones with LODs of 1.6-10 ng L^{-1} (Zorita et al., 2008). Chang and Huang (2010) applied dispersive liquid-liquid microextraction with solidification of a floating organic drop (DLLME–SFO) followed by HPLC, obtaining LODs ranging from 0.8 to 3.1 μg L^{-1}.

DLLME, which was first introduced in 2006, by Rezaee and co-workers (Rezaee et al., 2006), is a simple and fast microextraction technique based on a ternary component solvent system that may be an interesting pre-concentration option for the HPLC analysis of hormones. This is particularly important at a moment when it is crucial to develop new and low cost methodologies able to concentrate and determine, at environmentally relevant levels, these ECs that have raised great concern in the last years.

Therefore, the main purpose of this work was the development of a low cost methodology for the analysis of steroid hormones in environmental samples, focusing in the sample preparation and on lowering the LOD. DLLME-HPLC-FD was optimized for

simultaneous determination of E2 and EE2 in tap, surface and waste water samples. Also, matrix effects, such as presence of organic matter, which can decrease the extraction efficiency, were evaluated.

5.1.1 Dispersive liquid-liquid microextraction (DLLME)

Dispersive liquid-liquid microextraction (DLLME) has become a very popular environmentally benign sample-preparation technique, because it is fast, inexpensive and easy to operate, presents high enrichment and recovery factors, offers the possibility of a large number of samples to be extracted in parallel and consumes low organic solvent volumes (Rezaee et al., 2010). It has been applied to the extraction and pre-concentration of several compounds (e.g. Berijani et al., 2006; Farina et al., 2007; Herrera-Herrera et al., 2013; Kozani et al., 2007; Panagiotou et al., 2009).

In this pre-concentration technique (Fig. 5.1), few microliters of an appropriated organic solvent – extracting solvent – together with a dispersive solvent (with high miscibility in both extractant and aqueous phase) are rapidly injected into the sample, producing high turbulence. Such turbulence originates the formation of small droplets, which are dispersed throughout the aqueous sample. After the formation of the cloudy solution and after the equilibrium state is achieved, the mixture is centrifuged and the sedimented phase containing the analyte is collected.

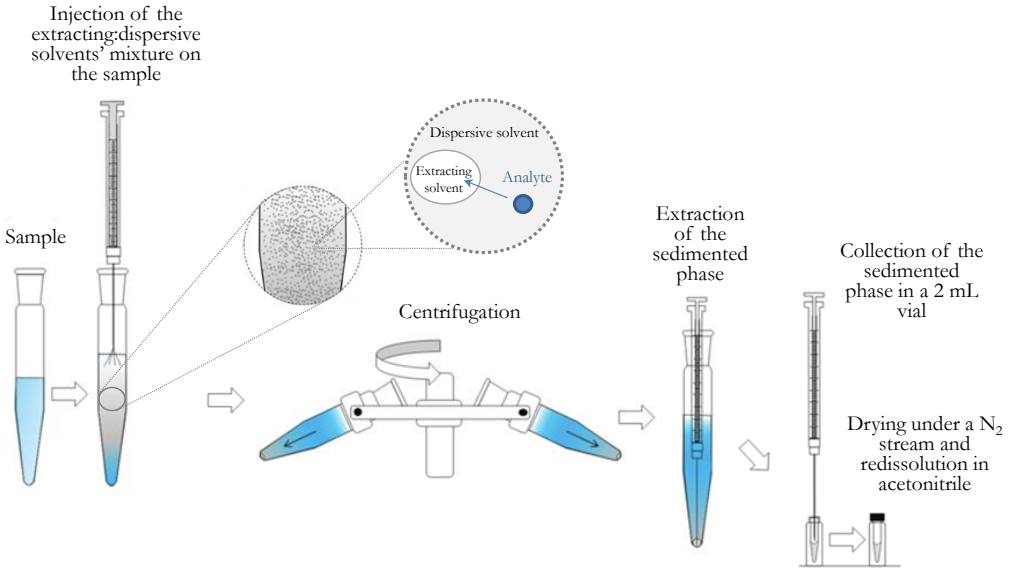

Fig. 5.1: DLLME procedure (adapted from Caldas et al. (2011)).

5.1.1.1 Extracting and dispersive solvents

The selection of appropriate extracting and dispersive solvents is very important for the DLLME process.

The properties of a good extracting solvent for DLLME are: (i) higher density than water, so it can sediment easily; (ii) low solubility in water, making the separation between the extracting solvent and the aqueous solution easier; (iii) great extraction capability of the analyte, in order to obtain high recoveries; and (iv) good chromatographic behavior, allowing to evaluate the extraction efficiency (Hadjmohammadi and Ghoreishi, 2011). All these requirements reduce the number of good extracting solvents, which difficults the application of this technique to the simultaneous pre-concentration of different pollutants.

On the other hand, dispersive solvent should be miscible with both water and the extracting solvent (Hadjmohammadi and Ghoreishi, 2011), acting as an emulsion stabilizing agent. That means that it should support the high superficial area of contact between the aqueous solution and the extracting solvent.

5.1.1.2 Extraction time

Extraction time in DLLME is defined as the interval time between the solvents' mixture injection and the starting time of the centrifugation (Du et al., 2010). The extraction time should be enough for the analyte to reach the chemical equilibrium between the two phases. However, after the formation of the cloudy solution, the surface area between the extracting solvent and the aqueous sample becomes very large, so the equilibrium state is achieved very quickly (Rezaee et al., 2010). Therefore, the extraction time for this technique is very short, in the seconds order. In fact, this is considered to be the main advantage of DLLME (Rezaee et al., 2010).

5.1.1.3 Enrichment factor and extraction recovery

The enrichment factor (EF) is defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration of the analyte (C_0) within the

sample (Hadjmohammadi and Ghoreishi, 2011):

$$EF = \frac{C_{sed}}{C_0} \quad \text{(Eq. 5.1)}$$

The extraction recovery (ER) is defined as the percentage of the total analyte amount (n_0) that was extracted to the sedimented phase (n_{sed}) (Hadjmohammadi and Ghoreishi, 2011):

$$ER = \frac{n_{sed}}{n_0} \times 100\% = \frac{C_{sed} \times V_{sed}}{C_0 \times V_{aq}} \times 100\% \quad \text{(Eq. 5.2)}$$

5.2 EXPERIMENTAL SECTION

5.2.1 Reagents and standards

Steroid hormones, E2 (≥ 97%) and EE2 (≥ 98%), and chlorobenzene (99.9%) were supplied by Sigma. Trichloroethylene (99%) and carbon tetrachloride (99.9%) were obtained from Panreac. Methanol, acetone and acetonitrile, all for HPLC, 99.9%, were from Fischer Chemical, Carlo Erba and HiPerSolv CHROMANORM, respectively. Chloroform (99%) was from Scharlau and dichloromethane (99.8%) was purchased from Riedel-de Haën. Commercial HA (technical) were obtained from Sigma.

Ultrapure water was obtained from a Milli-Q water purification system from Millipore.

Individual standard stock solutions of E2 and EE2 were prepared in methanol at a concentration of 100 mg L^{-1}. Each solution was further diluted to the appropriate concentration using ultrapure water. A stock HA solution of 1 g L^{-1} at pH 9 (in 1 mol L^{-1} ammonium hydroxide) was also prepared.

5.2.2. Instrumentation

E2 and EE2 analysis was performed on a Shimadzu High-Performance Liquid Chromatograph Prominence system equipped with a FD. This device consists of a degasser DGU-20A5, a bomb LC-20AD, a column oven CTO-10ASVP. A new ACE® C18 column-PFP (5 μm, 150 mm x 4.6 mm) connected to an ACE® 5 C18 4.6 mm i.d. guard column was used for the separation. The mobile phase consisted in a water:acetonitrile mixture (50:50, v/v), at a flow rate of 0.8 mL min^{-1} with an injection volume of 20 μL. Detection was

performed using a Shimadzu Prominence RF-20A XS fluorescence detector at an excitation wavelength of 280 nm and an emission wavelength of 310 nm (Yoon et al., 2003). Column temperature was maintained at 25°C.

Water and acetonitrile used in the mobile phase were pretreated by filtering through a 0.2 μm polyamide membrane filters from Whatman. A Lab Dancer Mini Vortex from VWR was used to perform the agitation during the extraction procedure.

5.2.3 DLLME procedure

Aliquots of 8 mL of samples or ultrapure water spiked with E2 or EE2 were placed in 12 mL glass centrifuge tubes with conical bottom. Afterwards, a mixture containing 2000 μL of acetone and 200 μL of chlorobenzene was added to each tube, which was immediately shaken during 30 s using a vortex. After the formation of the cloudy solution, as a result of the dispersion of fine droplets of chlorobenzene in aqueous sample, the tubes were centrifuged at 4000 rpm, for 5 min. Chlorobenzene organic phase, sedimented at the bottom of the conical centrifuge tube, was transferred to a 2 mL vial, dried under a nitrogen stream and redissolved using 40 μL of acetonitrile. The redissolved fraction was then analysed by HPLC-FD.

5.2.4 Optimization of extracting conditions

5.2.4.1 Selection of extracting solvent

Among the organic solvents with good characteristics for being an extracting solvent of choice, halogenated hydrocarbons, such as carbon tetrachloride, chloroform and chlorobenzene, are usually selected due to their high density.

From all the organic solvents available, carbon tetrachloride, chloroform, chlorobenzene, trichloroethylene and dichloromethane were tested to extract E2 and EE2 from water samples. The procedure consisted in injecting into an 8 mL sample, spiked with 1 μg L^{-1} E2 or EE2, a mixture containing 500 μL of methanol and 50 μL of one of the following solvents: tetrachloride, chlorobenzene, trichloroethylene; or 75 μL of chloroform (a higher volume was used in this case so to obtain a similar volume of sedimented phase). For

dichloromethane, 8 mL sample spiked with 1 μg L^{-1} E2 or EE2 was injected with only 200 μL of dichloromethane (no dispersive solvent was used), since using methanol as dispersive solvent no two-phase system was formed.

5.2.4.2 Selection of dispersive solvent

As already stated, miscibility of dispersive solvent in both aqueous phase and extracting solvent is an essential factor to its selection. Acetone, methanol and acetonitrile are usually the solvents of choice. In this work, those three dispersive solvents were tested along with the three extracting solvents with the best results in the previous section. Also, an extraction without the use of a dispersive solvent was performed for comparison.

5.2.4.3 Selection of volume of extracting and dispersive solvents

The optimal ratio between extracting and dispersive solvent volumes should ensure high extraction efficiency. This ratio affects directly the formation of the cloudy solution, the dispersion degree of the extracting solvent in aqueous phase and also the extraction efficiency (Rezaee et al., 2010). The procedure consisted in injecting 500 μL of acetone containing different volumes of chlorobenzene to an 8 mL sample spiked with 1 μg L^{-1} E2 or EE2. The different volumes of chlorobenzene resulted in a different extracting solvent:dispersive solvent ratio. The volumes tested were 50, 70, 85 and 100 μL, resulting in the ratios 1:10, 1:7.1, 1:5.9 and 1:5, respectively.

5.2.4.4 Salt and agitation time effect

It is well known that, generally, the increase in the ionic strength of sample solution results in a decrease of analyte solubility and enhances ER, due to the salting-out effect (Chang et al., 2010; Hadjmohammadi and Ghoreishi, 2011). The effect of the addition of salt on the extraction efficiency was tested by adding NaCl 5% (w/w). Moreover, the effect of agitation time was evaluated between 30 s and 5 min.

5.2.5 Matrix effects

Since the DLLME method is intended to be applied directly to environmental samples, it is necessary to evaluate possible interferences by water matrix. The influence of organic matter was first simulated using HA with concentrations ranging from 0 to 30 mg L^{-1}. Also, and in order to evaluate real matrix effects, the extraction recovery rates for E2 and EE2 were determined by spiking 15, 30 and 60 ng L^{-1} of E2 and EE2 into three different types of water samples - tap, surface and waste water.

5.2.6 Determination of E2 and EE2 in environmental water samples

Finally, and in order to evaluate the applicability of the proposed DLLME, tap, surface and waste water samples were collected in cleaned glass containers and subjected to the optimized method. Collection of surface and waste water samples took place on December 2012. Surface water samples were collected in Rivers of the Minho region, while waste water samples were collected in North STP (described in Chapter 2), at three different points of the treatment: after primary decantation, after secondary biological treatment and after secondary decantation (which corresponds to the final treated effluent). Immediately after collection, all samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at 4°C prior to extraction.

5.3 RESULTS AND DISCUSSION

5.3.1 Optimization of extraction conditions

5.3.1.1 Selection of extracting solvent

From all the extracting solvents tested, chloroform and chlorobenzene presented similar results for EE2. However, for E2, the extraction capacity of chlorobenzene was higher than that of chloroform. Results obtained on the selection of the extracting solvent are presented in Fig. 5.2.

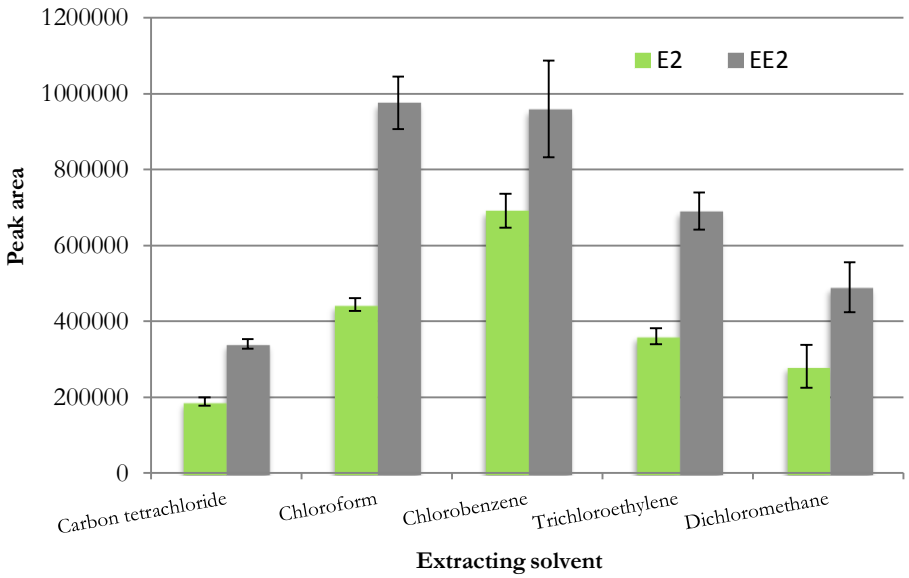

Fig. 5.2: Effect of extracting solvent (n=3) on the chromatographic peak area obtained for E2 and EE2 by HPLC-FD analysis. Extraction conditions: 8 mL of sample spiked with 1 μg L^{-1} E2 or EE2; extracting solvent: 50 μL carbon tetrachloride, chlorobenzene, trichloroethylene, 75 μL chloroform, 200 μL dichloromethane; dispersive solvent: 500 μL of methanol, except with dichloromethane, where no dispersive solvent was used; extraction time: 30 s.

For both estrogens, carbon tetrachloride and dichloromethane as extracting solvents presented lower peak areas, thus suggesting a lower extraction capacity. In fact, it was observed that the formation of the cloudy solution was not evident.

5.3.1.2 Selection of dispersive solvent

Since the extraction capacity of the organic solvent is also influenced by the dispersive solvent, the three extracting solvents with the best results (chloroform, chlorobenzene and trichloroethylene) were chosen to be tested with different dispersive solvents (acetone, methanol and acetonitrile). For all extracting solvents tested, acetone seems to be the more suitable dispersive solvent for the extraction of both compounds (Fig. 5.3). The only exception was chloroform, for which methanol seems to be a more efficient dispersive solvent for EE2 extraction. The combination chlorobenzene-acetone was the one chosen between all the possibilities tested due to the higher peak areas obtained. It was also possible to observe that extraction without dispersive solvent is generally poorer than using a dispersive solvent.

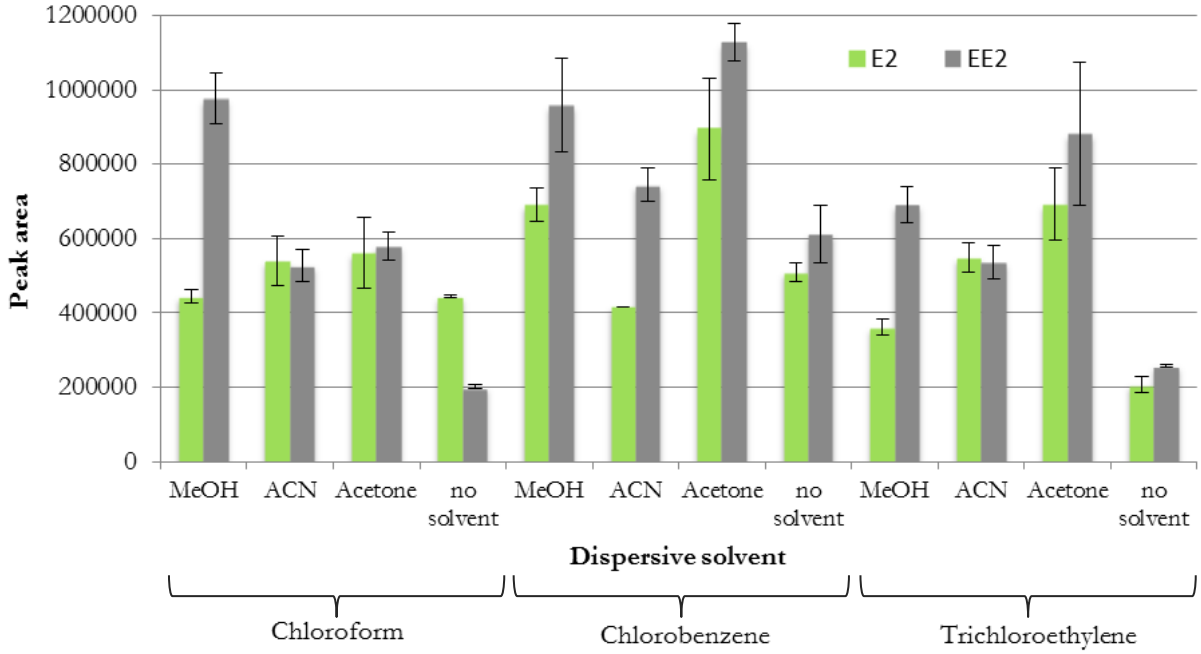

Fig. 5.3: Effect of dispersive solvent (n=3) on the chromatographic peak area obtained for E2 and EE2 by HPLC-FD analysis. Extraction conditions: 8 mL of sample spiked with 1 μg L^{-1} E2 or EE2; extracting solvent: 75 μL chloroform, 50 μL chlorobenzene, 50 μL trichloroethylene; volume of dispersive solvent: 500 μL; extraction time: 30 s.

5.3.1.3 Selection of volume for extracting and dispersive solvents

The volumetric ratio between extracting and dispersive solvents was also tested and results are shown in Fig. 5.4. It was possible to observe a different behaviour for E2 and EE2. While for EE2 the peak area tends to decrease when increasing the ratio between extracting and dispersive solvents, for E2 there was not such an obvious trend. The ratio of extracting solvent:dispersive solvent giving the best results was not the same for both compounds. For E2, 85 μL of extracting solvent (1:5.9 ratio) was the best choice; however, for EE2, 50 μL of extracting solvent (1:10 ratio) was a better solution. These results required a compromise in order to choose a common suitable ratio. In any case, it must be considered that, generally, the EE2 concentration in water samples is lower than that of E2. For this reason, the ratio extracting solvent:dispersive solvent chosen was 1:10 (50 μL of extracting solvent and 500 μL of dispersive solvent), which provides a higher EF for EE2.

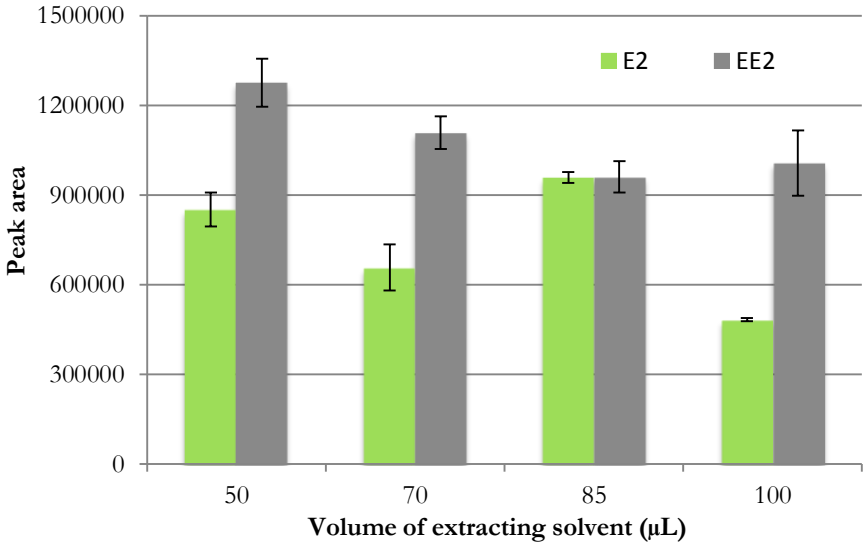

Fig. 5.4: Effect of extracting solvent volume (n=3) on the chromatographic peak area obtained for E2 and EE2 by HPLC-FD analysis. Extraction conditions: 8 mL of sample spiked with 1 µg L^{-1} E2 or EE2; extracting solvent: chlorobenzene; dispersive solvent: 500 µL of acetone; extraction time: 30 s.

The extracting solvent:dispersive solvent total volume has a direct influence on the EF. Increasing the volume will directly increase the volume of sedimented phase, thus decreasing the EF. However, the halogenated hydrocarbons are not compatible with the reverse-phase-HPLC mobile phase and thus, an extra step was needed to evaporate them before final analysis. Previously to HPLC analysis, the fraction was redissolved in acetonitrile. Considering the acetonitrile volume as the sedimented volume to obtain the EF, the volume of the extracting mixture can be increased to improve the extraction efficiency without a direct influence on the EF.

A mixture with a 1:10 ratio between extracting and dispersive solvents was prepared and different volumes of this mixture were used to perform the DLLME (Fig. 5.5).

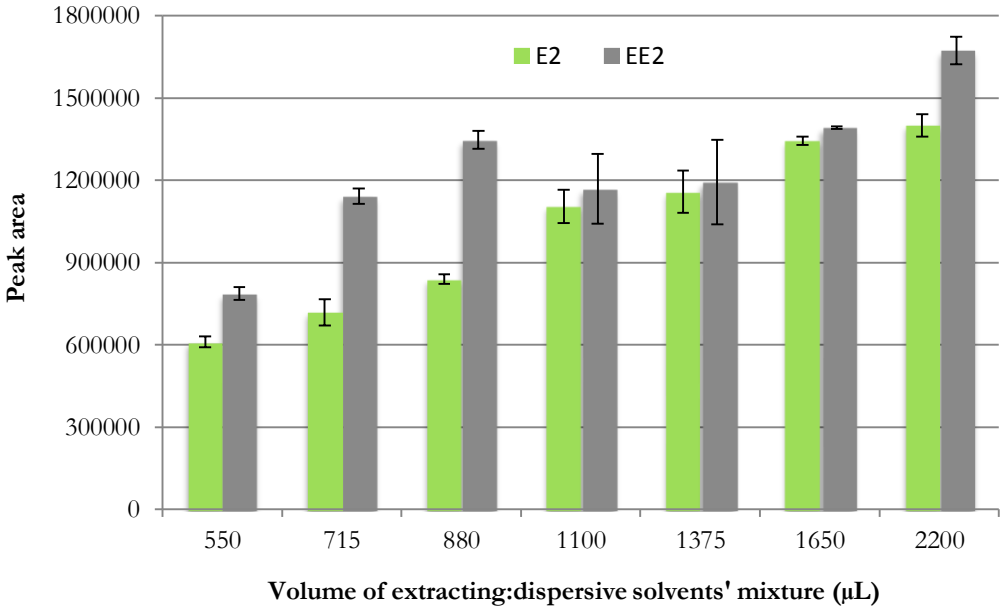

Fig. 5.5: Effect of extracting:dispersive solvents' mixture volume (n=3) on the chromatographic peak area obtained for E2 and EE2 by HPLC-FD analysis. Extraction conditions: 8 mL of sample spiked with 1 µg L^{-1} E2 or EE2; extracting solvent:dispersive solvent ratio 1:10; extraction time: 30 s.

The results showed a predictable increase in the peak areas with the increase of the mixture volume. However, this increase would result in a higher organic solvent volume for evaporation prior to analysis. For EE2, using 2200 µL, the recovery rates reached around 90%, a value considered very good for this pre-concentration procedures. For E2, the recovery was around 70%, a value that should be improved. However, when the volume of the mixture increased from 1650 to 2200 µL, the E2 peak area (and thus the associated recovery), did not change significantly, suggesting that a volume higher than 2200 µL would not improve the extraction. For this reason, the extracting:dispersive solvents' mixture volume of 2200 µL was the one selected.

5.3.1.4 Salt and agitation time effect

Results showed that the addition of NaCl 5% (w/w) had no influence in the recovery of E2 and EE2. Also, no difference in the recovery was observed for agitation times between 30 s and 5 min, being possible to conclude that 30 s are enough to reach the maximum extraction yield.

5.3.2 Analytical performance

Under optimized conditions, linear range, correlation coefficient (*r*), linearity, LOD, ER and EF of the DLLME method for both estrogens were calculated and are presented in Table 5.1. The performance of the method was evaluated by the *r* value, LOD and linearity (Lin (%) = 100 – RSD_b, where RSD_b is the relative standard deviation of the slope). LOD was calculated from each calibration curve as $a + 3\ s_{y/x}$, where *a* is the intercept of the regression line and $s_{y/x}$ is the statistical parameter which estimates the random errors in the *y*-axis (signal). Linear range was 10-300 ng L^{-1} for E2 and 10-500 ng L^{-1}, for EE2, while LOD was 2.0 ng L^{-1}, for E2 and 6.5 ng L^{-1}, for EE2. Both estrogens presented good *r* and Lin values in the linear range used in this study. ER was also calculated, being 72%, for E2, and 89%, for EE2, leading to an EF of 145 and 178, respectively.

Table 5.1: Quantitative parameters for typical analytical curves obtained by DLLME-HPLC-FD.

Analyte	Linear Range (ng L^{-1})	r	Linearity (%)	LOD (ng L^{-1})	ER (%)[a]	EF[a]
E2	10-300	0.99967	98.182	2.0	72 ± 4[b]	145 ± 8[b]
EE2	10-500	0.99996	99.466	6.5	89 ± 3[b]	178 ± 7[b]

[a] Values obtained for 0.1 μg L^{-1} E2 and EE2 extracted simultaneously (n=3). Extraction conditions: 8 mL of E2 or EE2 standard; extracting solvent: 200 μL of chlorobenzene; dispersive solvent: 2000 μL of acetone; extraction time: 30 s.
[b] Mean value ± standard deviation (n=3).

5.3.3 Matrix effects

Results obtained for the influence of organic matter (simulated by the presence of HA) in the peak areas, are shown in Fig. 5.6, which represents the peak area for E2 and EE2 standards subjected to the previously optimized DLLME procedure in absence and presence of different HA concentrations.

Applying a one way ANOVA to compare the peak area means obtained with different HA concentrations it was established the null hypothesis as being: peak area means do not

differ significantly. By the obtained results for P=0.05, it was possible to observe that the obtained peak areas did not differ significantly, indicating that HA present in sample solution upon the extraction procedure did not interfere in any way the extraction efficiency.

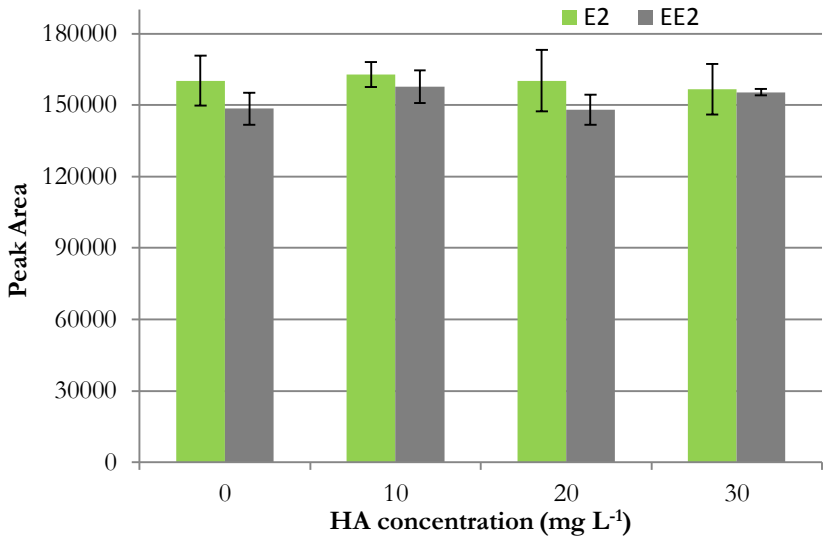

Fig. 5.6: Effect of HA concentration on the extraction efficiency (n=3). Extraction conditions: 8 mL of 0.1 μg L^{-1} E2 or EE2 standard with HA concentrations ranging from 0 to 30 mg L^{-1}; extracting solvent: 200 μL of chlorobenzene; dispersive solvent: 2000 μL of acetone; extraction time: 30 s.

Also, E2 and EE2 recoveries were determined and are presented in Table 5.2. Results obtained, between 98 and 107%, for E2, and from 100 to 106%, for EE2, confirmed that HA did not influence the extraction efficiency.

Table 5.2: Effect of HA concentration on the recovery of E2 and EE2 (n=3).

	Recovery (%)	
[HA] (mg L^{-1})	E2	EE2
0	119 ± 6	102 ± 5
10	102 ± 7	106 ± 7
20	100 ± 10	100 ± 6
30	98 ± 9	105 ± 5

Extraction conditions: 8 mL of 0.1 μg L^{-1} E2 or EE2 standard with HA concentrations ranging from 0 to 30 mg L^{-1}; extracting solvent: 200 μL of chlorobenzene; dispersive solvent: 2000 μL of acetone; extraction time: 30 s.

Although results obtained using HA to simulate organic matter revealed the absence of matrix effects in the proposed procedure, this was applied in collected water samples in order to evaluate a real matrix influence.

The chromatogram of a surface sample spiked with 0.1 μg L^{-1} E2 and EE2 subjected to DLLME-HPLC-FD is presented in Fig. 5.7(a). Also, the chromatogram of the surface sample (without spiking) subjected to the same procedure is presented in Fig. 5.7(b).

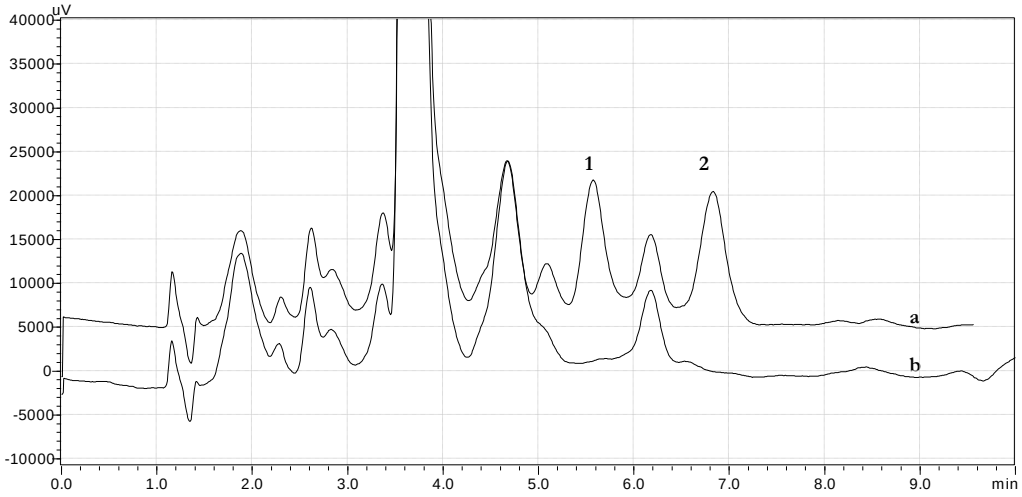

Fig. 5.7: Chromatogram of a surface water sample (a) with and (b) without a 0.1 μg L^{-1} spike of E2 and EE2 subjected to DLLME-HPLC-FD. Peaks: 1 - E2; 2 - EE2. Extraction conditions: 8 mL of sample; extracting solvent: 200 μL of chlorobenzene; dispersive solvent: 2000 μL of acetone; extraction time: 30 s.

Several peaks, other than the ones attributed to E2 and EE2 can be observed in both chromatograms. These peaks were also present when ultra-pure water (control sample) was subjected to DLLME-HPLC-FD, ensuring that its provenance is not from the water sample, but due to the extraction procedure itself. It is important to notice that, as it can be seen in Fig. 5.7, these peaks did not interfere with the E2 and EE2 quantification.

The recovery rates were determined by spiking 15, 30 and 60 ng L^{-1} of E2 and EE2 into three different types of water samples - tap, surface and waste water. The obtained recoveries are presented in Table 5.3.

Table 5.3: Effect of water sample matrix on the extraction recovery of E2 and EE2 (n=3).

	Recovery (%) Spiking level: 15 ng L^{-1}		**Recovery (%) Spiking level: 30 ng L^{-1}**		**Recovery (%) Spiking level: 60 ng L^{-1}**	
Water samples	E2	EE2	E2	EE2	E2	EE2
Tap water	95 ± 13	119.7 ± 0.1	90 ± 15	110 ± 7	96 ± 10	104 ± 6
Surface water	104 ± 3	115 ± 3	96 ± 6	93 ± 12	99 ± 5	117 ± 6
Waste water	94 ± 5	114 ± 23	86 ± 15	119 ± 20	106 ± 8	117 ± 2

Extraction conditions: 8 mL of spiked water sample; extracting solvent: 200 μL of chlorobenzene; dispersive solvent: 2000 μL of acetone; extraction time: 30 s.

As it may be seen, recovery rates obtained in tap water ranged between 89.9 and 96.3%, for E2 and between 104 and 119.7%, for EE2, while in surface water results ranged between 96.4 and 104.1%, for E2 and between 93.2 and 117.3%, for EE2. When using waste water samples, which comprise a much more complex matrix, results ranged between 86 and 106%, for E2 and between 114 and 119%, for EE2. Observing results in Table 5.3, it is possible to see an increase in standard deviation for recoveries obtained in the waste water sample, especially for lower concentrations. This can be explained by the matrix complexity of such type of water, which can interfere with the extraction process. However, results obtained can be considered acceptable considering the very low concentrations used.

5.3.4 Analysis of water samples

From all river and tap water samples analysed, only one river water presented E2 and EE2 in a quantifiable level. The collected sample was obtained near to a STP discharge point, which can explain the 12.05 ± 0.08 ng L^{-1} concentration obtained for E2 and 11 ± 2 ng L^{-1} concentration obtained for EE2. Considering the three waste water samples analysed, both E2 and EE2 were detected only after primary decantation with concentrations of 32 ± 3 ng L^{-1}, for E2, and 18 ± 3 ng L^{-1}, for EE2. Concentrations obtained are within the values generally obtained by other authors (e.g. Janex-Habibi et al., 2009; Johnson et al., 2000; Ternes et al., 1999), confirming the applicability of the developed method.

5.3.5 Comparison with other methods

In order to compare results obtained on the analysis of E2 and EE2 in this work with other methods reported in literature, Table 5.4 is presented. As it may be seen, the LOD values that Patrolecco et al. (2013) obtained for E2 and EE2 using SPE-HPLC-FD are comparable to those obtained in this work. The same applies to the LOD values that Aufartová et al. (2012) obtained for EE2 by SPME-HPLC-FD. However, comparing with SPE, DLLME presents advantages: is easier to implement, less time consuming and needs lower sample volume. When comparing with SPME, similar sample volumes are used, but extraction time is significantly reduced. Overall, the proposed DLLME procedure presents several advantages over other extraction techniques, showing that it is a suitable pre-treatment method for determination of E2 and EE2 in water samples.

Table 5.4: Comparison of DLLME-HPLC-FD with other methods used for the quantification of E2 and EE2 in water samples.

Method	Compounds	Recovery (%)	LOD (ng L^{-1})	Extraction time	Sample volume (mL)	Reference
SPE-HPLC-UV	E2	85-105	78.1	n.a.	50	Wang et al. (2008)
SPE-HPLC-FD	E2 EE2	76-86 72-100	3 15	n.a.	500-1000	Patrolecco et al. (2013)
SPME-HPLC-UV	E2 EE2	n.a	300 400	45 min	3.5	Peñalver et al. (2002)
SPME-HPLC-FD	EE2	80.9-81.6	5	n.a.	1	Aufartová et al. (2012)
DLLME-HPLC-UV	E2	89.9-94.5	10	In seconds	5	Hadjmohammadi and Ghoreishi (2011)
DLLME-HPLC-FD	E2 EE2	86-106 93-119.7	2 6.5	In seconds	8	Present study

n.a. – non available

Hadjmohammadi and Ghoreishi (2011) used DLLME-HPLC-UV for the determination of E2, E1 and diethylstilbestrol and concluded that the extraction procedure was the most advantageous. This work (DLLME-HPLC-FD) is an improvement, since a

lower LOD (2 ng L^{-1} compared to 10 ng L^{-1}) was achieved for E2, using a FD. It must also be highlighted that this work presents the development of DLLME for EE2 and results for this hormone, which was not developed in the referred work (Hadjmohammadi and Ghoreishi, 2011).

5.4 CONCLUSIONS

A methodology based on DLLME-HPLC-FD was developed and optimized for the analysis of estrogens in water samples. The optimized DLLME-HPLC-FD procedure provided low LODs (2.0 ng L^{-1}, for E2, and 6.5 ng L^{-1}, for EE2), high EFs (145 ± 8, for E2, and 178 ± 7, for EE2) and also high ERs (72 ± 4, for E2, and 89 ± 3, for EE2) for the detection and quantification of E2 and EE2 in tap, surface and waste water samples. Also, recovery tests proved that water samples matrix does not interfere in the extraction efficiency.

DLLME is fast, inexpensive and easy-to-use, allowing the extraction and pre-concentration of a large number of environmental samples in parallel. Besides, it is an environmentally friendly technique, due to the low volume of toxic organic solvents used.

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CHAPTER 6

Application of dispersive liquid-liquid microextraction for estrogens' quantification by enzyme-linked immunosorbent assay

Lima, D.L.D.; Silva, C.P.; Schneider, R.J.; Otero, M.; Esteves, V.I; Application of dispersive liquid–liquid microextraction for estrogens' quantification by enzyme-linked immunosorbent assay; *Talanta* 125 (2014) 102–106

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Summary

Estrogens, such as E2 and EE2, are the major responsible for endocrine-disrupting effects observed in aquatic environments due to their high estrogenic potency, even at concentrations ranging from pg L^{-1} to ng L^{-1}. Thus, it is essential to develop analytical low-cost methodologies suitable for monitoring their presence in water samples. DLLME was used as a pre-concentration step prior to the quantification of E2 and EE2 by ELISA. Firstly, an evaluation of the effect of DDLME on the E2 and EE2 ELISA calibration curves was performed. Since the extraction procedure itself had an influence on the ELISA OD, it became necessary to subject, not only the samples, but also all the standards, to the DLLME process. The influence of organic matter, both in the extraction and quantification, was evaluated and it was observed that its presence in the solution did not considerably affect the calibration curve. Recovery rates were also determined, ranging from 77 to 106%, for ultrapure water and from 104 to 115%, for waste water samples, the most complex ones in what concerns matrix effects. Results obtained when applying the proposed method to water samples can be considered quite satisfying. Moreover, the obtained quantification ranges (1.2-8000 ng L^{-1}, for E2, and 0.22-1500 ng L^{-1}, for EE2) encompass values generally reported in literature, confirming the practical use of the method for environmental samples.

Contents

6.1 CONTEXTUALIZATION

As it was already stated, ELISA provides a valid alternative approach for estrogens' quantification in water samples. Moreover, it was proven in Chapter 2 of the present thesis that ELISA allows the analysis of E2 and EE2, without any sample pre-treatment, in water samples of complex matrices. Even though, accounting that levels of E2 and EE2 in environmental waters are extremely low, pre-concentration methodologies may be required in some samples.

Regarding works coupling ELISA to pre-concentration techniques, Huang and Sedlak (2001) developed an SPE-ELISA procedure for the determination of E2 and EE2 in secondary waste water effluent and surface water samples where concentrations ranged from 0.2 to 4.1 ng L^{-1}. A similar approach, with slightly lower sensitivity, was described by Shishida et al. (2000) for the determination of E2 in waste water, achieving a LOD of 10 ng L^{-1}. Also using an SPE-ELISA, Dorabawila and Gupta (2005) analysed the presence of E2 in water samples from ponds, rivers, STPs and coastal bays. LOD was 0.5 ng L^{-1} and concentrations ranged between 1.9 and 6.0 ng L^{-1}.

Several other authors refer to SPE as the pre-concentration step of choice prior to ELISA (e.g. Lee et al., 2006; Suzuki and Maruyama, 2006; Swart and Pool, 2007). However, SPE is time-consuming, implies not only a high consumption of organic solvents, but also the consumption of expensive cartridges, and requires high sample volume. As an interesting and valid alternative, DLLME is a simple and fast microextraction technique.

In Chapter 5, DLLME was successfully used to pre-concentrate E2 and EE2 present in water samples prior to quantification by HPLC-FD and the advantages of this extraction procedure over SPE (simplicity, low quantity of organic solvents, low cost, high recovery and enrichment factors, possibility of a large number of samples to be extracted in parallel) were there highlighted.

The main purpose of this work was therefore to combine the key advantages of the DLLME procedure already developed with the advantages provided by ELISA. Interference in ELISA due to the extraction had to be evaluated and overcome in order to apply the method to E2 and EE2 quantification in various water samples.

6.2 EXPERIMENTAL SECTION

6.2.1 Reagents and standards

Polyclonal Ab and T were provided by Federal Institute for Materials Research and Testing - BAM, Berlin, Germany, and their production/synthesis were described elsewhere (Schneider et al., 2004; Schneider et al., 2005; Hintemann et al., 2006). Reagents and buffers, as well as instrumentation, used for ELISA were as described in Chapter 2.

Individual standard stock solutions of E2 and EE2 were prepared in methanol at a concentration of 1000 mg L^{-1}. Each solution was further diluted to the appropriate concentration using ultrapure water (from a Millipore Milli-Q water purification system). A stock HA solution of 1000 mg L^{-1} at pH 9 (in 1 mol L^{-1} ammonium hydroxide) was also prepared.

6.2.2 DLLME procedure

The E2 and EE2 DLLME procedure used at this stage was previously optimized and is described in detail in Chapter 5. Briefly, a mixture containing 2000 μL of acetone and 200 μL of chlorobenzene was added to 8 mL aliquots of E2 or EE2 standards or samples and immediately shaken using a vortex for 30 s. The tubes were centrifuged at 4000 rpm, for 5 min, and the chlorobenzene organic phase, sedimented at the bottom of the tube, was transferred to a 2 mL vial, dried under a nitrogen stream and redissolved using 160 μL of ultrapure water. The redissolved fraction was then analysed by ELISA.

6.2.3 ELISA procedure

The E2 and EE2 ELISA procedure used at this stage was previously optimized and is described in detail in Chapter 2, as well as the method used to fit ELISA calibration curves. Concisely, microtiter plates were coated with polyclonal Ab serum diluted 1:10 000, for E2, and 1:50 000, for EE2, in coating buffer (200 μL per well). After overnight incubation and a washing step, sample buffer was added (25 μL per well) and followed by standards/samples

(100 μL per well), all incubated for 30 min. T was added to the plate (100 μL per well), diluted 1:50 000, for E2, and 1:100 000, for EE2, in PBS and incubated 10 and 15 min for E2 and EE2, respectively. After a second two-cycle washing step, the final substrate solution was added (200 μL per well) and incubated for 30 min. The enzyme reaction was stopped by the addition of 1 mol L^{-1} H_2SO_4 (100 μL per well).

6.2.4 Evaluation of the DLLME effect on ELISA performance

In order to evaluate the influence of the extraction procedure on the ELISA performance, several standards and one blank sample (ultrapure water) were subjected to the previously optimized DLLME procedure (*cf.* Chapter 5) and quantified by the previously developed ELISA procedure (*cf.* Chapter 2).

Also, to evaluate the extraction procedure, two ELISA calibration curves were obtained, with and without the DLLME procedure. Standards between 0.1 and 1×10^6 ng L^{-1} E2 or EE2 were analysed directly by ELISA. Standards between 2×10^{-3} and 2×10^4 ng L^{-1} E2 or EE2 were subjected to DLLME, resulting in concentrations 50 times higher after the extraction (between 0.1 and 1×10^6 ng L^{-1}). Therefore, it should be expected to obtain similar calibration curves.

6.2.5 Analytical performance

To determine the quantification range (defined as the highest and the lowest concentration that can be determined with a given degree of precision), 16 standards were used (n = 6). The relative error of the E2 and EE2 concentrations was calculated in order to obtain the precision profile as described by Ekins (1981). A relative error of 30% was established as the maximum allowable error for the quantification of both estrogens, as explained in Chapter 2.

6.2.6 Matrix effects

The application of the developed method for E2 and EE2 quantification to environmental water samples could be problematic due to matrix effects that may affect both the extraction and the ELISA procedures. As reported previously, 1% BSA buffer can be used to overcome matrix effects observed in ELISA, due to organic matter. Moreover, it was considered relevant to evaluate also the organic matter influence in the extraction procedure itself. Therefore, standards prepared in ultrapure water and in 30 mg L^{-1} HA were subjected to DLLME and subsequently analysed by ELISA.

Furthermore, to evaluate the effect of organic matter present in water samples, ultrapure, surface and waste waters were spiked with 25 and 50 ng L^{-1} E2 or EE2, subjected to DLLME procedure and analysed by ELISA.

6.2.7 Application to environmental water samples

In order to evaluate the applicability of the proposed method, several water samples (250 mL) from the North and Center regions of Portugal were collected in April 2013, using dark glass containers (previously washed 3 times with the sample to be collected). Water from public fountains, delivering potable water (samples 1-7) and surface water samples (samples 8-18) were collected. Locations of some of the surface waters are shown in Fig. 6.1. Moreover, waste water samples from two different STPs (STPs described in Chapter 2; samples 19 and 20) were also collected, both in the final effluent stage. Immediately after collection, all samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at 4°C prior to extraction.

Fig. 6.1: Locations of some of the collected surface water samples.

6.3 RESULTS AND DISCUSSION

6.3.1 Evaluation of the DLLME effect on ELISA performance

Extremely high recoveries (up to 332 ± 1% and 375.2 ± 0.5%, for E2 and EE2, respectively) obtained for standards subjected to DLLME and quantified by ELISA, demonstrated an overestimation of the E2 and EE2 concentrations. Moreover, the high E2 and EE2 concentrations obtained for the blank (ultrapure water) corroborated the high recovery results.

Two ELISA calibration curves, with and without the DLLME procedure, were obtained. A decrease in the signal (associated to an increase of concentration) was observed for both compounds, which can explain the previously observed overestimation.

If the extraction efficiency was not satisfactory, a calibration curve shifted to higher OD values would be obtained. However, it was observed exactly the opposite case, i.e. a decrease of the OD values. This fact can be explained by the use of an organic solvent in the

DLLME procedures that, although evaporated, can still influence the ELISA (known to be influenced by organic solvents).

However, the DLLME can be used as a pre-concentration step prior to ELISA quantification if both samples and standards are subjected to the same procedure (proved by good recovery results obtained for standards in ultrapure water, presented in Table 6.1).

6.3.2 Analytical performance

In order to determine the quantification range for both estrogens, the precision profiles (Fig. 6.2 and Fig. 6.3) were obtained.

In the case of E2 ELISA, standards with concentrations between 2×10^{-2} and 2×10^{5} ng L^{-1} E2 were used (Fig. 6.2). Considering the maximum relative standard deviation allowed of 30%, a 1.2–8000 ng L^{-1} quantification range was found for E2.

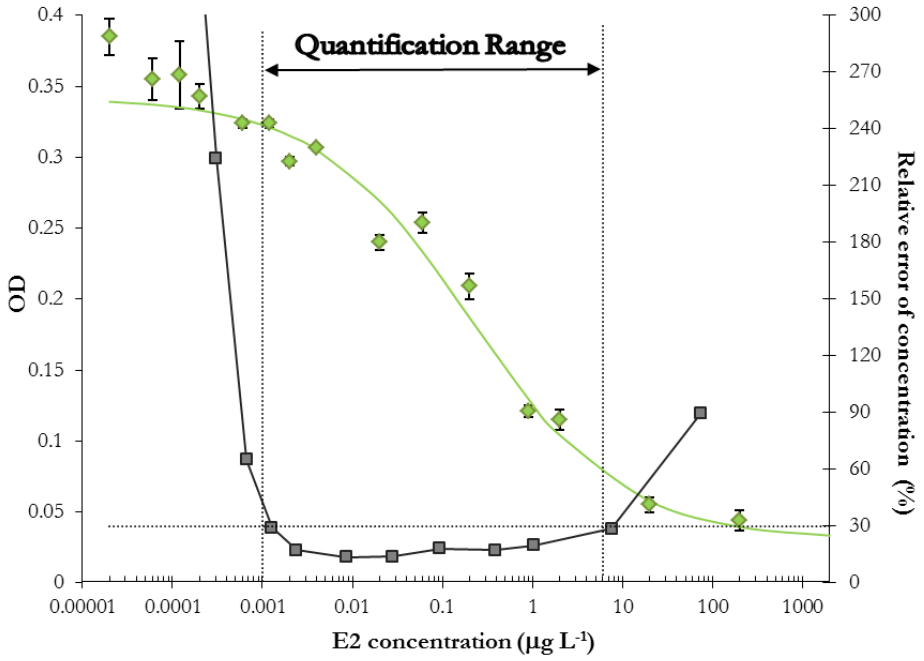

Fig. 6.2: Calibration curve (green) of E2 (A = 0.342; B = 0.512; C = 0.203; D = 0.0306; r^2 = 0.981) and precision profile (gray), in presence of BSA buffer pH 6.4. E2 ELISA conditions: Ab 1:10 000; T 1:50 000 incubated 10 min. The precision profile and determination of the relative error of concentration were calculated in accordance with Ekins (1981).

For EE2 ELISA, standards with concentrations between 2×10^{-3} and 2×10^{4} ng L^{-1} EE2 (Fig. 6.3) were used. Considering 30% as the maximum relative standard deviation allowed, a 0.22–1500 ng L^{-1} quantification range for EE2 was obtained.

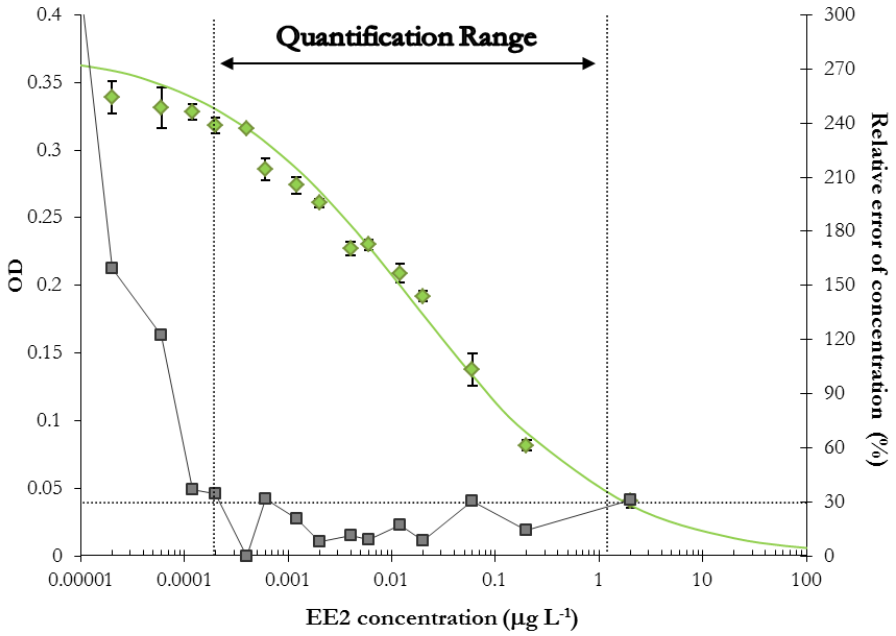

Fig. 6.3: Calibration curve (green) of EE2 ELISA (A = 0.377; B = 0.446; C = 0.0161; D = 0; r^2 = 0.992) and precision profile (gray), in presence of BSA buffer pH 6.4. EE2 ELISA conditions: Ab 1:50 000; T 1:100 000 incubated 15 min. The precision profile and determination of the relative error of concentration were calculated in accordance with Ekins (1981).

Comparing the quantification ranges obtained with DLLME-ELISA with the ones obtained for ELISA without the prior extraction (30–2×10^{5} ng L^{-1}, for E2, and 20–1×10^{4} ng L^{-1}, for EE2 (*cf.* Chapter 2), it was possible to conclude that this simple extraction procedure decreased the lower limit of both quantification ranges, approximately 30 times for E2 and 100 times for EE2. Therefore, the obtained quantification ranges, in the present conditions, easily allow the quantification of these estrogenic disruptors in surface waters, where the expected concentrations are extremely low.

6.3.3 Matrix effects

Calibration curves for both estrogens in the presence and absence of HA are presented in Figure 6.4.

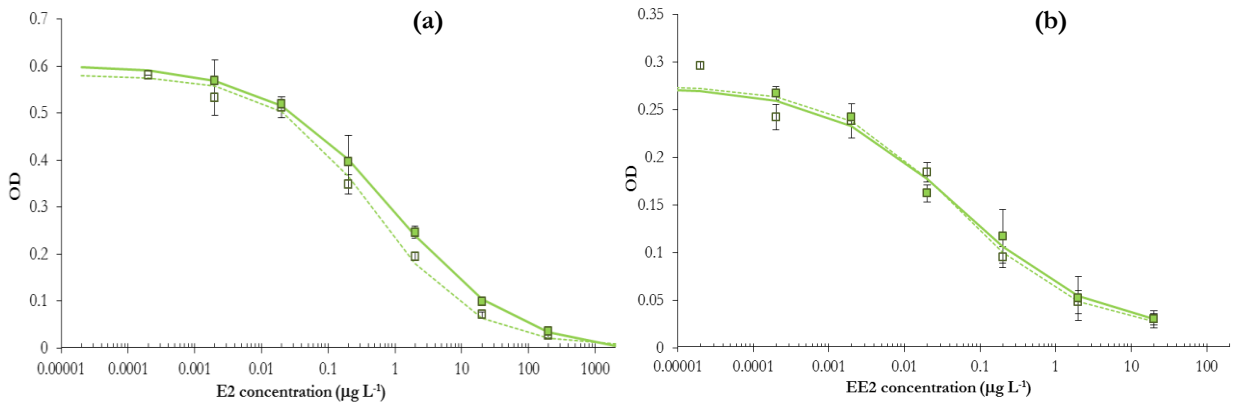

Fig. 6.4: Evaluation of the organic matter effect on the DLLME procedure and ELISA calibration curve of (a) E2 and (b) EE2. Standards prepared in ultrapure water - 0 mg L^{-1} HA (full line) and in 30 mg L^{-1} HA (dashed line). E2 ELISA conditions: Ab 1:10 000; T 1:50 000; t_T 10 min; EE2 ELISA conditions: Ab 1:50 000; T 1:100 000; t_T 15 min.

As it can be seen, the presence of HA did not affect the extraction considerably and, consequently, the calibration curve. It is important to highlight that this good behavior in presence of HA was achieved extracting the organic-aqueous interphase (which contains a small amount of analyte) together with the organic phase.

However, to confirm that organic matter present in the water samples did not affect the quantification, ultrapure, surface and waste waters were spiked with different concentrations of E2 or EE2, subjected to DLLME procedure and analysed by ELISA. Recovery results are presented in Table 6.1.

Table 6.1: Effect of water sample matrix on the extraction recovery of E2 and EE2 (n=3).

	Recovery (%) Spiking level: 25 ng L^{-1}		Recovery (%) Spiking level: 50 ng L^{-1}	
Water samples	**E2**	**EE2**	**E2**	**EE2**
Ultrapure water	88 ± 5	79 ± 18	77 ± 17	106 ± 29
Surface water	86 ± 2	120 ± 7	78 ± 17	112 ± 45
Waste water	104 ± 22	107 ± 19	115 ± 17	111 ± 12

Extraction conditions: 8 mL of spiked water sample; extracting solvent: 200 μL of chlorobenzene; dispersive solvent: 2000 μL of acetone; extraction time: 30 s.

For ultrapure water, recovery rates ranged from 77 to 106%, while for waste water samples (the most complex samples in what concerns matrix effects) recoveries ranged from 104 to 115%. Recovery results can be considered acceptable and the developed method suitable for application in water samples.

6.3.4 Application to environmental water samples

Several water samples were collected in public fountains providing potable water (samples 1–7). Only one sample (sample 4) contained E2 in a quantifiable amount (Table 6.2); however, EE2 was quantified in three (samples 4–6) of the seven samples tested.

Results obtained for surface water samples (8–18) collected in rivers, small streams and ponds were slightly different. In this case, E2 concentrations were in general higher than those of EE2, just as expected. Concentrations ranged from 4 to 34 ng L^{-1}, for E2, and from 0.3 to 24 ng L^{-1}, for EE2. In what concerns waste water samples, it was observed that, in both analyzed samples, E2 concentration was higher than EE2 concentration.

It is important to highlight that the quantified concentrations are in accordance with values generally obtained and reported in literature (e.g. Janex-Habibi et al., 2009; Johnson et al., 2000; Ternes et al., 1999), confirming the applicability of the developed method.

Table 6.2: Determination of E2 and EE2 in water from public fountains and in surface and waste water samples, subjected to DLLME and analysed by ELISA (n=3).

	Concentration (ng L^{-1})			Concentration (ng L^{-1})	
Samples[a]	E2	EE2	Samples[a]	E2	EE2
1	<LOD	<LOD	**11**	34 ± 11	2.4 ± 1.1
2	< LOD	<LOD	**12**	<LOD	<LOD
3	<LOD	<LOD	**13**	17.8 ± 0.9	<LOD
4	2.0 ± 0.1	1.32 ± 0.8	**14**	8 ± 2	24 ± 6
5	<LOD	0.37 ± 0.05	**15**	<LOD	5 ± 2
6	<LOD	0.5 ± 0.4	**16**	30 ± 1	16 ± 3
7	<LOD	<LOD	**17**	<LOD	0.8 ± 0.2
8	<LOD	0.4 ± 0.2	**18**	33.1 ± 0.1	0.3 ± 0 .1
9	4 ± 1	<LOD	**19**	77 ± 33	6 ± 1
10	<LOD	<LOD	**20**	21 ± 19	8.5 ± 0.6

[a]Samples 1-7 – samples from public fountains supplying potable water; Samples 8-18 – surface water samples; Samples 19-20 – waste water samples.

6.3.5 Comparison of DLLME-ELISA with DLLME-chromatographic analysis

In order to compare the developed method with DLLME-chromatographic analysis already reported in literature, Table 6.3 is presented.

Table 6.3: Comparison of DLLME-ELISA with other methods used for the quantification of E2 and EE2 in water samples after DLLME.

Method	Compounds	Recovery (%)	LOD (ng L^{-1})	Reference
DLLME-HPLC-UV	E2	89.9-94.5	10	Hadjmohammadi and Ghoreishi (2011)
DLLME-HPLC-FD	E2	86-106	2	Lima et al. (2013)
	EE2	93-120	6.5	
DLLME-ELISA	E2	77-115	1.2	Present study
	EE2	79-120	0.22	

When compared with HPLC, ELISA provides several advantages, such as sensitivity, specificity, simplicity and high throughput of samples. In what concerns LOD, generally ELISA presents lower detection limits, when compared to chromatographic techniques.

As it can be seen in Table 6.3, LOD for E2, using DLLME-HPLC-UV, is 10 ng L^{-1}, while using DLLME-HPLC-FD, is 2 ng L^{-1}. However, if DLLME-ELISA is used the reliable quantification range extends to 1.2 ng L^{-1}. The improvement observed for EE2 is even higher, from 6.5 ng L^{-1}, with DLLME-HPLC-FD, to a lower quantification range limit of 0.22 ng L^{-1}, using DLLME-ELISA.

Therefore, besides the advantages itemized previously, ELISA quantification after DLLME allows the quantification of E2 and, particularly important, of EE2, in water samples.

6.4 CONCLUSIONS

The main objective of this work was to combine DLLME and ELISA procedures in order to quantify E2 and EE2 in concentrations as low as few ng L^{-1}. However, some problems due to the extraction procedure interference on the ELISA had to be solved, since the extraction procedure itself yielded an influence on the ELISA OD, leading to an overestimation of the concentration. As a solution, the standards for ELISA calibration curves were also subjected to the DLLME procedure prior to ELISA in order to eliminate ELISA signal differences due to extraction. No influence of organic matter was observed in the extraction and quantification; recovery rates obtained were in the ranges 77-106%, using ultrapure water and 104-115%, using waste water samples. The simple extraction procedure adopted decreased the quantification range approximately 30 times for E2 and 100 times for EE2, comparing with the quantification ranges obtained without the DLLME pre-concentration step (*cf.* Chapter 2). Lower quantification range limits were 1.2 ng L^{-1}, for E2, and 0.22 ng L^{-1}, for EE2. This allowed for the quantification of estrogens in water samples in concentrations between 2 and 77 ng L^{-1}, for E2, and between 0.3 and 24 ng L^{-1}, for EE2.

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PART III

Evaluation of the fate and persistence of estrogens in the aquatic environment

CHAPTER 7

Photosensitized degradation of E2 and EE2 by humic substances

Silva, C.P.; Lima, D.L.D.; Otero, M.; Esteves, V.I.; Photosensitized degradation of 17β-estradiol and 17α-ethinylestradiol by humic substances; *Chemosphere,* under review.

Summary

Aquatic photodegradation of E2 and EE2 was investigated using simulated solar radiation. After 5 h, photodegradation in ultrapure water was very low – 16%, for EE2, and 6%, for E2. However, in spiked freshwater, estuarine water and waste water, irradiation led to a decrease of the hormones' concentration, photodegradation being in the ranges 44-94%, for EE2, and 27-95%, for E2. Since these matrices had a high chromophoric dissolved organic matter (CDOM) content, it was hypothesized that humic substances (HS) may pose a relevant photosensitizing effect on E2 and EE2. Photodegradation kinetics of these hormones in ultrapure water both in the absence and in the presence of the different fractions of HS (HA, FA and XAD-4) were compared. The three fractions of HS were responsible for a noticeable increase in the photodegradation rates. Half-life time ($t_{1/2}$) of EE2 decreased from 46 h, for direct photodegradation, to 6.4, 2.1 and 2.7 h, in presence of HA, FA and XAD-4, respectively. For E2, $t_{1/2}$ decreased from 94 h, for direct photodegradation, to 5.7, 2.9 and 3.1 h, in presence of HA, FA and XAD-4, respectively. Therefore, HS were shown to be of critical importance on the photodegradation of both estrogens, the photosensitizing effect of XAD-4 being similar to that of FA and higher than that of HA. The addition of scavengers showed that presumably singlet oxygen and hydroxyl radicals have a minor participation on the indirect photodegradation of EE2 and E2 for the type of matrix used in the experiments.

Contents

7.1 CONTEXTUALIZATION

To correctly evaluate the real ecological impact of pollutants, it is important to take into consideration their fate and persistence in aquatic environment. Once present in surface waters, steroid hormones are subjected to various transformation and removal processes, namely, biodegradation, sorption to colloids and sediments and photodegradation.

In fact, photodegradation is known to be one of the most important factors affecting the environmental persistence of pollutants, especially in surface waters (Calisto et al., 2011; Chowdhury et al., 2011). Indeed, natural or simulated sunlight has been shown to degrade estrogens to some degree (Chowdhury et al., 2011; Jürgens et al., 2002; Lin and Reinhard, 2005; Zhang et al., 2008; Zuo et al., 2006). However, very different degrees of photodegradation have been determined for these hormones. For example, EE2 and E2 were shown to be photodegraded in river waters with half-life times ($t_{½}$) of at least 10 d under 12 h of bright sunshine per day (Jürgens et al., 2002), while in sea water, a $t_{½}$ lower than 1.5 d was ascribed to the EE2 degradation (Zuo et al., 2006).

Differences in the rate and degree of estrogens photodegradation may be related, at least to some extent, to the occurrence of indirect photolysis, which is reliant on the medium composition. Actually, the photochemical fate of contaminants in natural aquatic environments may differ significantly from the fate of those present in pure water, owing to the presence of naturally occurring radiation absorbers, quenchers or sensitizers. Therefore, differences in chemical composition of natural waters invariably affect the photochemical function resulting in a variation in the lifetime of a pollutant (Lam et al., 2003).

Therefore, in this work and aiming to improve the understanding on the fate of E2 and EE2 in different aquatic environments, the degradation of these estrogens in aqueous solutions containing HS has been investigated. With this purpose, the direct photolysis of E2 and EE2 was compared to the photodegradation of these estrogens under the presence of the three different fractions of HS, namely HA, FA and XAD-4. XAD-4 is, to the best of author's knowledge, here assessed for the very first time.

7.1.1 Direct and indirect photodegradation in the environment

In the aquatic environment, both direct and indirect photolysis may occur (Fig. 7.1). Direct photolysis is possible when chromophoric groups can absorb light at wavelengths present in sunlight (λ>290 nm) (Peuravuori and Pihlaja, 2009). It involves absorption of photons, by the chemical itself, able to induce a chemical transformation (Calisto et al., 2011; Lin and Reinhard, 2005). Thus, the rate of direct photodegradation is a function of the intensity of available light, the hormone's capacity to absorb that light and the efficiency of the conversion of the absorbed light into photochemical reactions (Young et al., 2013).

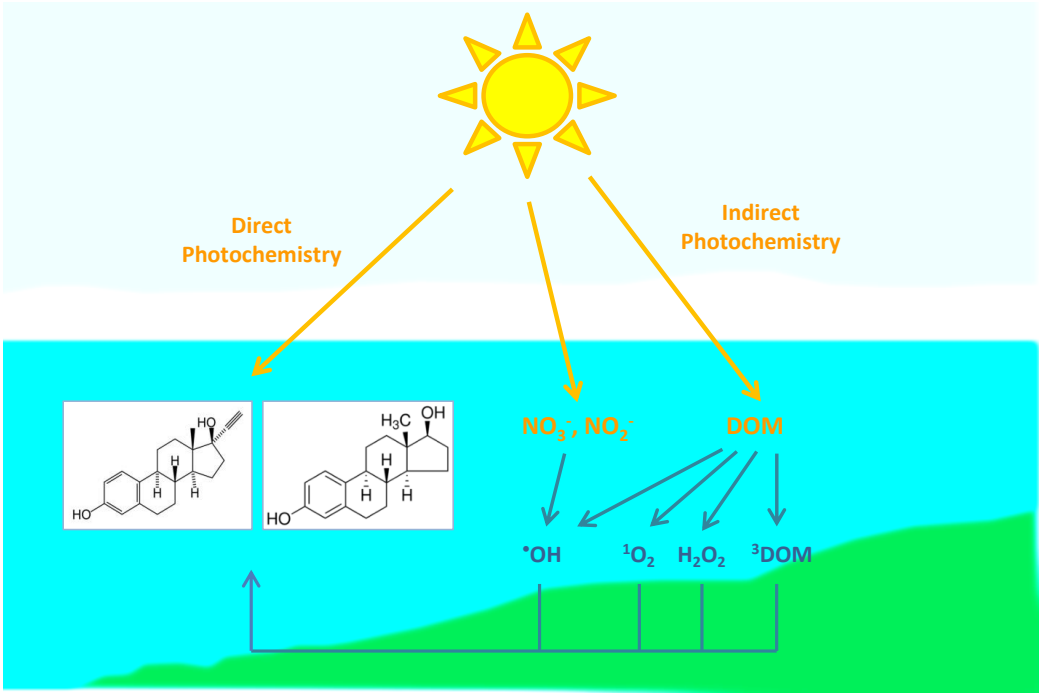


Fig. 7.1: Direct and indirect photoprocesses occurring in the aquatic environment.

Indirect photolysis happens when phototransformation is induced indirectly by other substance also present in water that absorbs solar radiation to reach an excited state, subsequently generating free radicals comprised of reactive oxygen species (ROS) (e.g., hydroxyl radicals ($OH^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and singlet oxygen ($^1O_2$)) and other non-ROS transients (Carlos et al., 2012; Lin and Reinhard, 2005), which then cause the degradation of the hormone. These substances are the so called photosensitizers, among which DOM is one of the most important (Lin and Reinhard, 2005).

7.1.2 Humic substances' effect on photodegradation

DOM in aquatic environments absorbs light, and such material is termed chromophoric DOM (CDOM). Although chemical forms of CDOM are poorly understood, HS are considered to be representative of CDOM. When absorbing photons in the UV and the visible region of the solar spectrum up to 500 nm, HS absorb energy within the range 58-98 kJ mol^{-1}, making a number of photochemical processes possible (Aguer et al., 1999).

On the basis of different studies, the photochemical properties of HS were proposed to result in part from intramolecular charge-transfer interactions between hydroxyl-aromatic donors and quinoid (or other) acceptors, which are formed through the partial oxidation of lignin and possibly other partially oxidized hydroxy-aromatics, i.e., polyphenols, tannins and melanins (Porras et al., 2014).

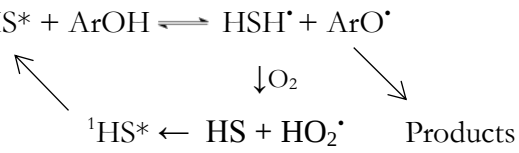
HS can both promote the transformation of organic contaminants under sunlight irradiation and also retard their phototransformation by screening sunlight (Carlos et al., 2012). It was found that the enhancement or inhibition role of HS in the photodegradation depends on the type and quality of DOM (Atkinson et al., 2011), as well as on the incident light intensity (Chen et al., 2013). Either way, HS are expected to have an important role on the photochemical fate of aquatic pollutants and their persistence in natural waters, as it has been already stated in literature (e.g. Carlos et al., 2012; Chowdhury et al., 2011; Leech et al., 2009).

HSs can hamper the degradation of pollutants, acting as absorbers of light, which can cause a reduced quantity of photons available for photoreactions and were also proven to act themselves as quenchers (Brezonik and Fulkerson-Brekken, 1998; Chen et al., 2013).

On the other hand, when acting as photosensitizers, their photochemical excitation can generate many reactive species, as it was already mentioned. These species may in turn induce the photodegradation of organic species. Sunlight irradiation of natural waters causes a transition of HS to an excited state, by absorption of the solar radiation (Aguer et al., 1999):

$$HS \xrightarrow{h\upsilon} {}^{1}HS^{*} \rightarrow {}^{3}HS^{*}$$

HS, as a photosensitizer in the triplet state ($^{3}HS^{*}$) can react in two ways. One mechanism involves hydrogen-atom abstraction or electron-transfer reactions between the excited state of the photosensitizer and a substrate to produce free radicals and free radicals' ions (Bancirova, 2011). The oxidation of the substrate by the reactive triplet states occur as follows (Aguer et al., 1999):



Another mechanism involves energy transfer between the excited triplet state of the photosensitizer and the ground state of molecular oxygen, thus generating the first excited state of oxygen, singlet oxygen (Bancirova, 2011; Chen et al., 2013):

$^3HS^* + O_2 \rightarrow HS + {}^1O_2$

Singlet oxygen, in turn, will react with the organic pollutant to form a peroxide (Zepp et al., 1977). The formation of hydroxyl radicals can be explained by the intermediate formation of hydrogen peroxide through the dismutation of $O_2^{\bullet-}/HO_2^{\bullet}$ (Aguer et al., 1999; Du et al., 2014).

Active species of molecular oxygen can be deactivated by either physical or chemical quenching agents. The quenching process can occur by electron transfer or energy transfer (Valencia et al., 2013). In this work, 2-propanol and sodium azide, as $OH^{\bullet}$ and $^1O_2$ scavengers, respectively, were tested. Sodium azide is mainly reported as a highly selective $^1O_2$ scavenger but is also known to react with $OH^{\bullet}$ (Prevot et al., 2011) and carbocations (Cosa, 2004).

7.2 EXPERIMENTAL SECTION

7.2.1 Reagents and materials

Steroid hormones E2 (≥97%) and EE2 (≥98%) were supplied by Sigma. Acetonitrile (for HPLC, 99.9%) was from HiPerSolv CHROMANORM. Ultrapure water was obtained using a Milli-Q Millipore system (Milli-Q plus 185). Sodium azide, NaN_3 (≥99%), was purchased from Riedel-de Haën, while 2-propanol (99.5%) was from Hoechst.

The irradiation experiments were performed with a Solarbox 1500 (Co.fo.me.gra, Italy) equipped with a 1500 W arc xenon lamp and special outdoor UV filters that restricted the transmission of light for wavelengths below 290 nm. A parabolic reflection chamber guaranteed the uniformity of the irradiation, whereas the temperature inside the irradiation chamber was maintained by an air cooled system. The irradiance was kept constant at 55 W m^{-2} (290-400 nm) and controlled, as well as the temperature, with a multimeter (Co.fo.me.gra, Italy) equipped with a black standard temperature sensor and a UV 290-400 nm large band sensor.

E2 and EE2 analysis was performed on a Shimadzu High-Performance Liquid Chromatograph Prominence system equipped with a fluorescence detector, as detailed in Chapter 5. The mobile phase consisted of a water:acetonitrile mixture (40:60, *v*/*v*), at a flow rate of 0.7 mL min^{-1} with an injection volume of 20 μL. Water and acetonitrile used in the mobile phase were filtered through a 0.2 μm polyamide membrane filters from Whatman. A linear calibration curve was obtained for each estrogen using eight standard solutions with concentrations ranging from 0.5 to 500 μg L^{-1}, analyzed in triplicate. Correlation coefficients were of 0.9997 and 0.9999, for both EE2 and E2, respectively, showing the excellent linear response in the studied range of concentrations. LODs, defined as a + 3 $s_{y/x}$ (where *a* is the intercept of the regression line and $s_{y/x}$ is the statistical parameter which estimates the random errors in the *y*-axis (signal)), were 3.1 and 1.5 μg L^{-1}, for EE2 and E2, respectively.

TOC was measured using a TOC-VCPH Analyzer, from Shimadzu.

UV-visible spectra were obtained with a T90 + UV/visible Spectrophotometer (PG Instruments Ltd.) using quartz cuvettes of 1 cm path length, between 200 and 550 nm.

7.2.2 Photodegradation experiments

Individual standard stock solutions of E2 and EE2 were prepared in methanol at a concentration of 1000 mg L^{-1}. Then, for photodegradation experiments, each solution was further diluted to an initial concentration of 50 μg L^{-1} using ultrapure water or environmental water samples. E2 and EE2 solutions (5 mL) were irradiated in quartz tubes (internal diameter × height = 1.8 × 20 cm) always in triplicate. Each set of experiments was accompanied by dark controls wrapped in aluminum paper. The dark controls were maintained inside the solar simulator during the same time as the irradiated solutions. After that time, aliquots (500 μL) of experiments and dark controls were stored in dark at 4 ºC and analyzed within 24 h. The photodegradation percentage at each experiment was always corrected in relation to the respective dark control. The experiments using collected water samples (spiked with 50 μg L^{-1} EE2 or E2) were conducted by irradiating during 5 h.

Kinetic photodegradation studies were carried out by irradiating individual EE2 and E2 solutions (50 μg L^{-1}) in ultrapure water during 168 h and 360 h, respectively, aliquots being collected every 24 h. In order to investigate the mutual influence of these hormones on their

degradation they were added together in ultrapure water solutions (50 μg L^{-1} of each hormone) and kinetic studies were performed using aliquots collected at 24, 48, 96, 120, 144, 168 and 182 h. For the assessment of the role of HS on E2 or EE2 photodegradation, kinetic studies were also performed. E2 and EE2 solutions (50 μg L^{-1}) in ultrapure water together with HS fractions (20 mg L^{-1} HA, FA or XAD-4 fraction) were irradiated for a maximum of 15 h, and aliquots were collected at 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 15 h. For the evaluation of the HS concentration effect on photodegradation, solutions of E2 or EE2 (50 μg L^{-1}) in ultrapure water and in presence of different HS concentrations (20, 30 and 40 mg L^{-1} HA, FA or XAD-4 fraction) were irradiated during 5 h.

Finally, in the final effluent wastewater sample, spiked with 50 μg L^{-1} E2 or EE2, the scavengers' addition experiments were performed by adding 2 mM sodium azide (as a singlet oxygen scavenger) or 0.26 M 2-propanol (as a hydroxyl radicals' scavenger) and irradiating the samples during 5 h.

7.2.3 Water samples

Surface water and waste water samples were collected in 250 mL dark glass containers. Sampling was carried out between February and April 2014. Immediately after collection, all samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at 4 °C prior to use.

One surface sample was from an estuarine shallow lagoon (Ria de Aveiro) (SWS4 in Fig. 2.5; *cf.* Chapter 2). The other surface water sample tested was a freshwater sample (SWS3 in Fig. 2.5; *cf.* Chapter 2). Two waste water samples were also collected at two different stages of the treatment – after primary treatment and from the final effluent – of the North STP serving the town of Aveiro (NWWS1 and NWWS3 in Fig. 2.5; *cf.* Chapter 2).

7.2.4 Humic substances

The HS used in this study had already been extracted and isolated from a riverine water sample, which was collected in a freshwater stream that flows into the Aveiro lagoon

(Esteves et al., 2009). In this riverine aquatic system, HS are mainly derived from the decomposition of herbaceous plants. The extraction and isolation of the different fractions of HS, which are described in detail by Santos et al. (1994) and Esteves et al. (1995), were performed by using two columns, one of Amberlite XAD-8 resin and other of Amberlite XAD-4, connected in series. The characterization of the purified fractions (HA, FA and XAD-4) by elemental analysis and solid-state ^{13}C-CPMAS NMR may be found elsewhere (Esteves et al., 2009).

7.3 RESULTS AND DISCUSSION

7.3.1 Characterization of water samples and humic substances

UV-visible spectra of the water samples and humic substances' solutions (Fig. 7.2) were performed in the wavelength range 200-550 nm.

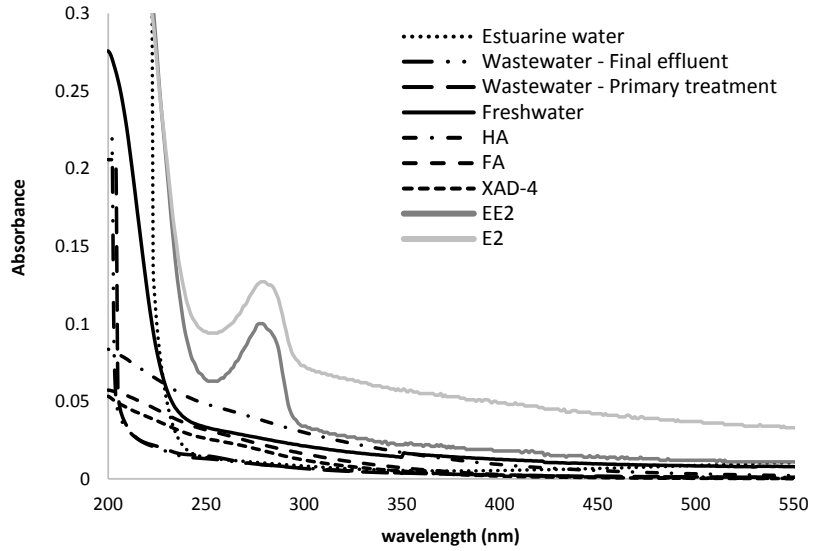


Fig. 7.2: UV–visible spectra of the solutions/samples used. Since solutions have different organic matter contents, spectra were normalized by dividing each one by the respective TOC value, for comparison.

UV–visible spectra for solutions of FA and XAD-4 fractions show a similar trend; for the HA fraction the higher incidence of aromatic moieties, comparing with FA and XAD-4 fractions, results in a higher absorption of UV light. Because of the structural heterogeneity of HS, they do not produce any well resolved peak and the absorbance increases monotonously as the wavelength decreases.

TOC content (Table 7.1) for the HS solutions ranged from 10.8 to 12.1 mg L^{-1}. The highest TOC content was, as expected, measured in waste water samples.

Table 7.1: TOC values for water samples and HS solutions.

Sample	TOC (mg L^{-1})
Freshwater	4.8
Estuarine water	16.7
Wastewater – Final effluent	45.2
Wastewater – Primary treatment	48.6
HA solution, 20 mg L^{-1}	12.1
FA solution, 20 mg L^{-1}	11.9
XAD-4 solution, 20 mg L^{-1}	10.8

7.3.2 Photodegradation of EE2 and E2 in water samples

Experiments carried out in collected water samples (Fig. 7.3) showed that photodegradation of E2 and EE2 was higher in all the water samples considered than in ultrapure water. In any case, the initial concentration of E2 and EE2 in dark controls remain the same throughout the experiments.

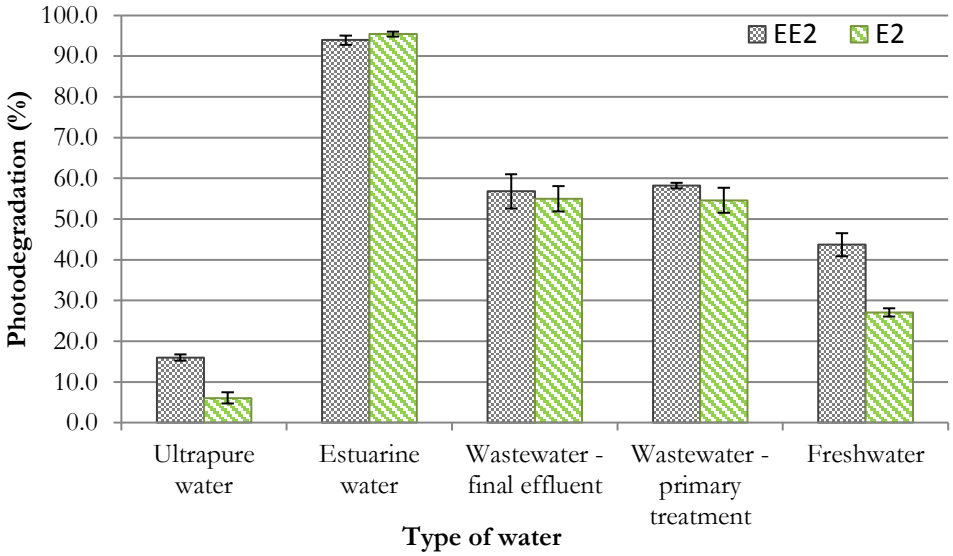


Fig. 7.3: Photodegradation (%) in ultrapure water and surface and waste water samples for 5 h of irradiation, for both EE2 and E2.

The water sample responsible for the major increase in photodegradation was the estuarine one. In comparison with photodegradation in ultrapure water, photodegradation of EE2 and E2 in estuarine water was 5.9 and 15.6 times higher, respectively. Also, photodegradation was determined in freshwater, being the results 2.7 and 4.4 times higher, for EE2 and E2, respectively, compared to photodegradation in ultrapure water. For waste water, photodegradation was performed in a sample from the final effluent and in a sample after the primary treatment. Photodegradation was 3.6 times higher for EE2, in both final effluent and primary treatment effluent, compared with direct photodegradation. In the case of E2, average photodegradation increased 9.0 times, for final effluent, and 7.3 times, for the primary treatment in comparison with photodegradation in ultrapure water. However, differences in EE2 and E2 photodegradation between the two types of waste water samples were not significant ($t_{EE2} = 0.5$; $t_{E2} = 0.06$; critical t value = 2.78, at a 95% confidence level).

The lower photodegradation of E2 and EE2 in ultrapure water compared to collected water samples may be related to the presence of photosensitizers in the latter. Therefore, while only direct photodegradation occurs in ultrapure water, both direct and indirect photodegradation of E2 and EE2 may occur in the collected water samples. As it has been shown in the previous section all these samples were characterized by a relatively high TOC content. Thus, the higher photolysis degree observed in TOC-rich water samples (compared

to the photodegradation in ultrapure water) could be attributed, at least partially, to photosensitization by DOM and/or other reactive chromophores or ions, which may have acted as precursor for photoreactive species, enhancing the overall rate of photolysis for the target compounds. In fact, Chowdhury et al. (2011) and Leech et al. (2009) already stated that the presence of organic matter increases de photodegradation of E2. In any case, it must be highlighted that these water samples constitute very complex and different matrices so other photosensitizing species, apart from DOM, may be affecting the photodegradation of both estrogens. Also, it must be taken into account that DOM may also slow down photodegradation of organic compounds by screening radiation. Moreover, the characterization of these samples in the previous section pointed to different types of functional groups and aromaticity in DOM, which may influence the photoprocesses differently.

7.3.3 Photodegradation Kinetics

In order to better understand the role of DOM, and, specifically, HS in the photodegradation of EE2 and E2, photodegradation kinetics were studied, both in ultrapure water (direct photodegradation) and in presence of the three different fractions of HS – HA, FA and XAD-4 (indirect photodegradation). In all experiments no concentration decrease of EE2 or E2 was observed in the dark controls, indicating that degradation was not by microbiological or thermal means, but only photo-induced.

7.3.3.1 Direct photodegradation kinetics

Kinetic results for both EE2 and E2 direct photodegradation were fitted to a pseudo first order kinetics. GraphPad Prism 5 (demo version) was used for the determination of nonlinear regression fittings. Experimental results are presented in Figure 7.4, together with the fittings to the pseudo-first order equation $C/C_0 = e^{-kt}$. In the referred equation, k is the rate constant, t is time, and C and C_0 are the concentration of estrogen at a given irradiation time and the initial concentration of estrogen, respectively.

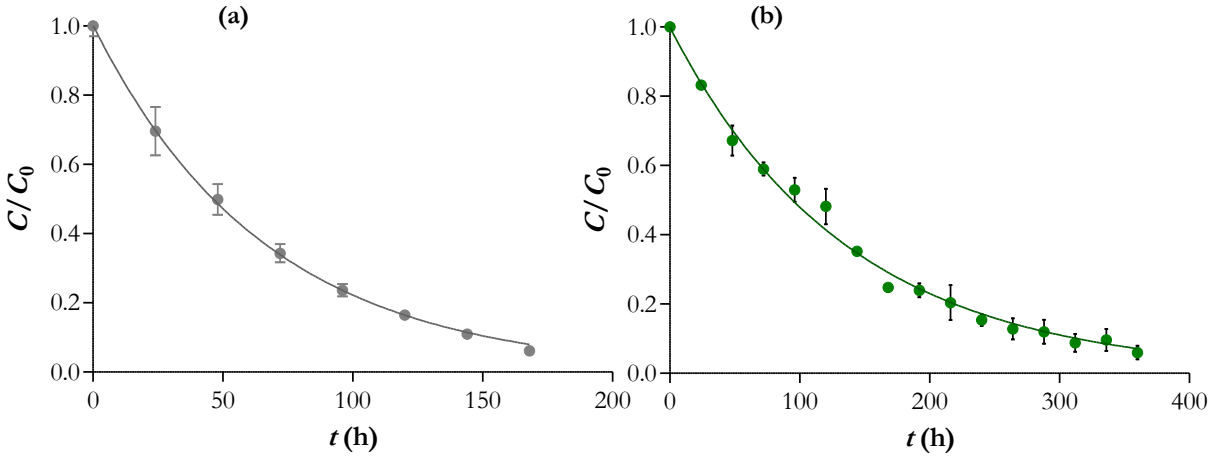


Fig. 7.4: Kinetics of (a) EE2 and (b) E2 photodegradation in aqueous solution and curves of pseudo-first-order decay fitted to the data by nonlinear regression. Shown error bars are standard deviations; n = 3.

Data were well fitted by the above mentioned equation (r_{EE2} = 0.9997 and r_{E2} = 0.9941) showing that the photodegradation of both EE2 and E2 in aqueous solution follows a pseudo-first order kinetics, which is in agreement with literature (Chowdhury et al., 2011; Jürgens et al., 2002; Leech et al., 2009).

Rate constants were 0.0151 ± 0.0002 h^{-1} and 0.0073 ± 0.0003 h^{-1}, for EE2 and E2, respectively. The $t_{½}$, calculated as ln2/k was 46 h, for EE2, and 94 h, for E2 (Table 7.2). These $t_{½}$ are lower than those obtained by Jürgens et al. (2002), who determined values of 124 h and 126 h for E2 and EE2, respectively. These authors (Jürgens et al., 2002) had already highlighted that direct photodegradation of both steroid hormones proceeded slowly and differences with the herein obtained rates may be due to the higher estrogens' initial concentration (100 μg L^{-1}) used by Jürgens et al. (2002). Concentration is known to influence the degradation since photolysis rate can be decreased due to photon limitations occurring at higher initial pollutants' concentrations (Chowdhury et al., 2011).

Regarding the kinetic photodegradation experiments on solutions containing a mixture of E2 and EE2 in ultrapure water, fittings of the obtained results to the pseudo-first order equation were also determined (using GraphPad Prism 5, demo version). Experimental results together with the corresponding fittings are represented in Fig. 7.5 and rate constants for each estrogen were calculated for their photolysis in the mixture (Table 7.2).

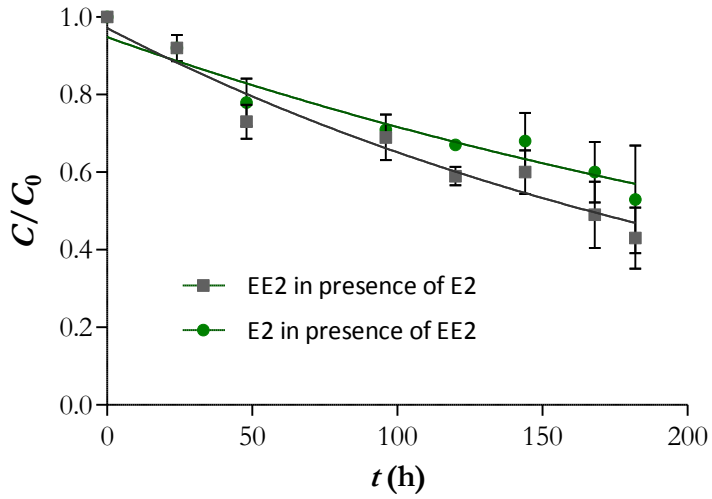


Fig. 7.5: Kinetics of EE2 and E2 photodegradation in the mixture aqueous solution (50 μg L^{-1} EE2 + 50 μg L^{-1} E2) and curves of pseudo-first-order decay fitted to the data by nonlinear regression. Shown error bars are standard deviations; n = 3.

Results showed lower degradation rates for each of the hormones in the mixture, compared to the degradation rates obtained for the single-compound experiments. The photodegradation of both EE2 and E2 in the mixture followed a pseudo-first order kinetics (r_{EE2} = 0.9599 and r_{E2} = 0.9619). Rate constants were of 0.0040 ± 0.0005 h^{-1} and 0.0028 ± 0.0004 h^{-1}, for EE2 and E2, respectively. The $t_{½}$ was 173 h, for EE2, and 247 h, for E2 (Table 7.2). A possible explanation for the decrease of the photodegradation rate may be the concentration effect explained above. In these experiments, when both hormones are present in solution, total concentration is higher (100 μg L^{-1}), which brings the related limitation of photons. Also, differences with respect to results obtained for E2 and EE2 in separate can be ascribed to the light screening effect due the presence of the other molecule, decreasing the light available for each target compound to undergo direct photolysis. Furthermore, indirect photoprocesses, attributable to interaction between compounds, can decrease the photodegradation rate by quenching processes, as stated by Carlos et al. (2012) for one pollutant in a mixture. To the extent of author's knowledge, this type of study had never been performed in publications dealing with photodegradation of EE2 and E2 under simulated solar radiation.

7.3.3.2 Indirect photodegradation kinetics

7.3.3.2.1 Effect of different types of humic substances

Each set of results was fitted to a pseudo-first order kinetics by using GraphPad Prism 5 (demo version). Experimental results and the corresponding fittings are shown in Fig. 7.6 and the inferred kinetic parameters can be compared with those for direct photodegradation (Table 7.2).

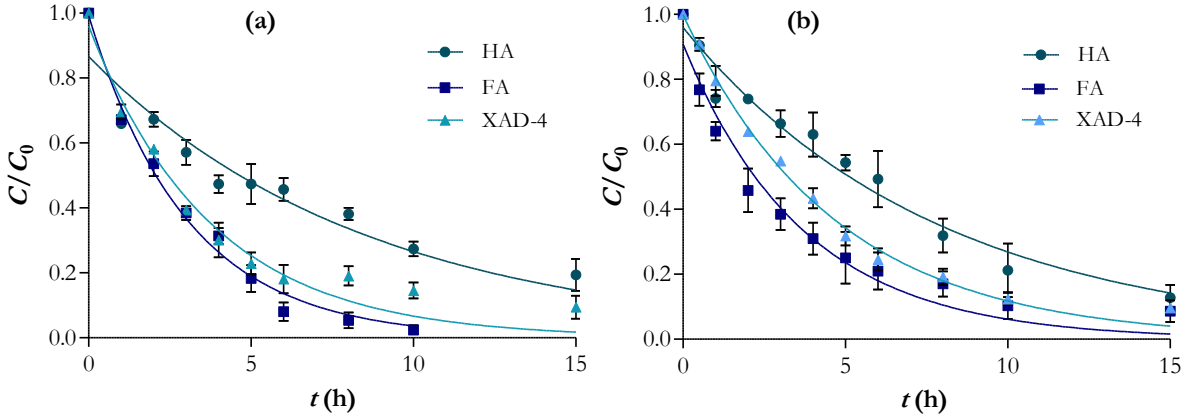


Fig. 7.6: Kinetics of (a) EE2 and (b) E2 photodegradation in aqueous solution, in presence of HS fractions – 20 mg L^{-1} HA, FA, XAD-4 – and curves of pseudo-first-order decay fitted to the data by nonlinear regression. Shown error bars are standard deviations; n = 3.

Table 7.2: Comparison of the results for r, k and $t_{1/2}$, for EE2 and E2 in absence and presence of different fractions of 20 mg L^{-1} HS.

	r	k (h^{-1})	$t_{1/2}$ (h)
EE2			
Ultrapure water	0.999	0.0151 ± 0.0002	46
Ultrapure water in presence of E2	0.960	0.0040 ± 0.0005	173
HA	0.964	0.11 ± 0.01	6.4
FA	0.994	0.32 ± 0.02	2.1
XAD-4	0.981	0.26 ± 0.03	2.7
E2			
Ultrapure water	0.994	0.0073 ± 0.0003	94
Ultrapure water in presence of EE2	0.962	0.0028 ± 0.0004	247
HA	0.982	0.12 ± 0.01	5.7
FA	0.993	0.24 ± 0.01	2.9
XAD-4	0.998	0.22 ± 0.01	3.1

HS are known to yield two opposite effects on the rate of photodegradation of organic molecules in water. Since they are able to absorb UV radiation in a broad range of wavelengths they can reduce the available energy for the organic molecules present in the solution and compete for photons and radicals, thus acting as an inner filter. Also, HS molecules submitted to irradiation are promoted to a transient excited state (triplet state), in which they may react with oxygen present in the solution forming reactive species as singlet oxygen, or to react directly with other organic species. Therefore, the overall effect of HS on the photodegradation of an organic substance will depend on the balance between these two opposite contributions (Andreozzi et al., 2003; Li et al., 2014). When HS act mainly as inner filter, their addition will result in a decrease of the photodegradation rate. On the opposite hand, if the promoting effect of HS prevails, an enhancement in the rate of photodegradation will happen.

The latter was the observed situation in the present study. In the presence of HS and whatever the fraction, a pronounced enhancement of E2 and EE2 photodegradation was observed when compared to direct photodegradation. Hydrophobic organic contaminants, like estrogen steroids, are likely to bind with DOM (Chen et al., 2012). Therefore, indirect photodegradation in presence of DOM, especially the intra-DOM reaction, may be a very important mechanism for their transformation in the environment. In fact, electron rich aromatic compounds are known to react with DOM generated photo-oxidants of various lifetimes: at high concentrations, degradation is dominated by short-lived species like ^{3}DOM, while at low concentrations reaction kinetics are a combination of both short-lived and long-lived species, which include peroxyl or phenoxyl radicals and excited states of DOM chromophoric constituents (Jacobs et al., 2011).

The photodegradation enhacement of both E2 and EE2 followed the same pattern in presence of HS, i.e., FA > XAD-4 >> HA. Surprisingly, results obtained with XAD-4 fraction were similar to the ones obtained with FA, which are considered the most photo-chemically active fraction of DOM in aquatic environments (Jacobs et al., 2011; Li et al., 2014). Photodegradation accomplished with FA and XAD-4 was similar, being the difference more accentuated between both these fractions and HA. HA increased the degradation rates of both E2 and EE2 but the corresponding $t_{1/2}$ were 2-3 times higher in comparison with FA and XAD-4 fractions (Table 7.2). These results may suggest the existence of a correlation between the hydrophobicity of the HS and the effect on photodegradation rates of both E2 and EE2, since HA are the most hydrophobic fraction (more enriched in aromatic and/or

chromophoric groups), while FA and XAD-4 are the most hydrophilic fractions. Considering the ^{13}C nuclear magnetic resonance (NMR) spectra of the here used HS (Esteves et al., 2009), they presented four well resolved bands characteristics of HS: 0-60 ppm (alkyl and methoxyl carbons), 60-90 ppm (O-alkyl carbons), 108-145 ppm (aromatic carbons) and 160-190 ppm (carboxylic and ester carbons). These HS were of freshwater origin, so having a high degree of aromatic moieties, which has been related to terrestrial influence, namely higher plants and soil-derived sources. Anyhow, it is to highlight that ^{13}C NMR spectra suggested a higher prevalence of aromatic moieties in HA than in FA or XAD-4 (108-145 and 145-160 ppm).

On the other hand, even though the observed photodegradation intensification of E2 and EE2 in presence of HS was quite clear, the apparent first order kinetic constant, k, corresponds to two degradation phenomena: the direct photodegradation and the degradation induced by the presence of HS. As it may be seen in Table 7.2, the direct photodegradation occurred with an apparent first order kinetic constant of 0.015 h^{-1}, for EE2 and 0.0073 h^{-1}, for E2. Under the presence of HS, neglecting their photosensitizing effect and considering only their inner filter effect, the apparent first-order rate photodegradation constant of both EE2 and E2 would be proportional to the fraction of light absorbed by each hormone in the mixture (hormone + HS fraction). When HS are present, it is expected that a considerable part of light will be absorbed by them. Therefore, the calculated first order rate constant of E2 or EE2 degradation in the presence of HS acting only as an inner filter, k_{calc}, can be determined as follows (Caupos et al, 2011):

$$k_{calc} = k \times \left[\frac{\left(\frac{I_a}{I_0} \right)_{est./HS}}{\left(\frac{I_a}{I_0} \right)_{est.}} \right] \qquad \text{(Eq. 7.1)}$$

where, $\left(\frac{I_a}{I_0} \right)_{est./HS}$ is the fraction of light absorbed by E2 or EE2 in presence of HS; $\left(\frac{I_a}{I_0} \right)_{est.}$ is the fraction of light absorbed by E2 or EE2 in absence of HS (in ultrapure water); k is the apparent first order rate constant of E2 or EE2 measured in absence of HS (in ultrapure water).

Knowing that:

$$\left(\frac{I_a}{I_0} \right)_{est./HS} = \left(\frac{I_a}{I_0} \right)_{est.+HS} \times \left(\frac{Abs_{est.}}{Abs_{est.+HS}} \right) \qquad \text{(Eq. 7.2)}$$

where $\left(\frac{I_a}{I_0} \right)_{est.+HS}$ is the fraction of light absorbed by the mixture (E2 or EE2 + HS); $Abs_{est.}$

represents the absorbance of E2 or EE2; $Abs_{est.+HS}$ is the absorbance of the mixture (E2 or EE2 + HS). Both Abs were measured at a wavelength of 295 nm. Considering also that $I_a = I_0(1-10^{-Abs})$, k_{calc} is obtained by:

$$k_{calc.} = k_{est.} \times \frac{\left(1-10^{-Abs}\right)_{est.+HS}}{\left(1-10^{-Abs}\right)_{est.}} \times \frac{Abs_{est.}}{Abs_{est.+HS}} \quad \text{(Eq. 7.3)}$$

Table 7.3 summarizes the k and k_{calc} values for photodegradation of both hormones in solutions without and with 20 mg L^{-1} HS. The comparison of both values for each hormone can give an idea about the real contribution of HS for the photodegradation rate of E2 and EE2.

Table 7.3: First-order rate constants (measured and calculated) and HS contribution on indirect photodegradation of E2 and EE2.

	k (h^{-1})	k_{calc} (h^{-1})	HS contribution (%)
EE2			
HA	0.11	0.0097	91.2
FA	0.32	0.011	96.4
XAD-4	0.26	0.012	95.3
E2			
HA	0.12	0.0049	96.0
FA	0.24	0.0058	97.6
XAD-4	0.22	0.0062	97.2

Values for $k/k_{calc.}$ much greater than 1 indicate the occurrence of indirect photoprocesses of high relevance. Thus, as it can be inferred from values showed in Table 7.3, the presence of HS had a key photosensitizing effect in the photodegradation of E2 and EE2. This was especially relevant for E2 in the presence of FA, which photosensitizing effect contributed approximately ≈98% for the photodegradation of this hormone.

7.3.3.2.2 Effect of the humic substances' concentration

The effect of the concentration of HS on photodegradation was studied for three levels of concentration – 20, 30 and 40 mg L^{-1}.

Results displayed in Fig. 7.7 were obtained for the two hormones and the three HS

fractions used (HA, FA and XAD-4), after 5 h under irradiation.

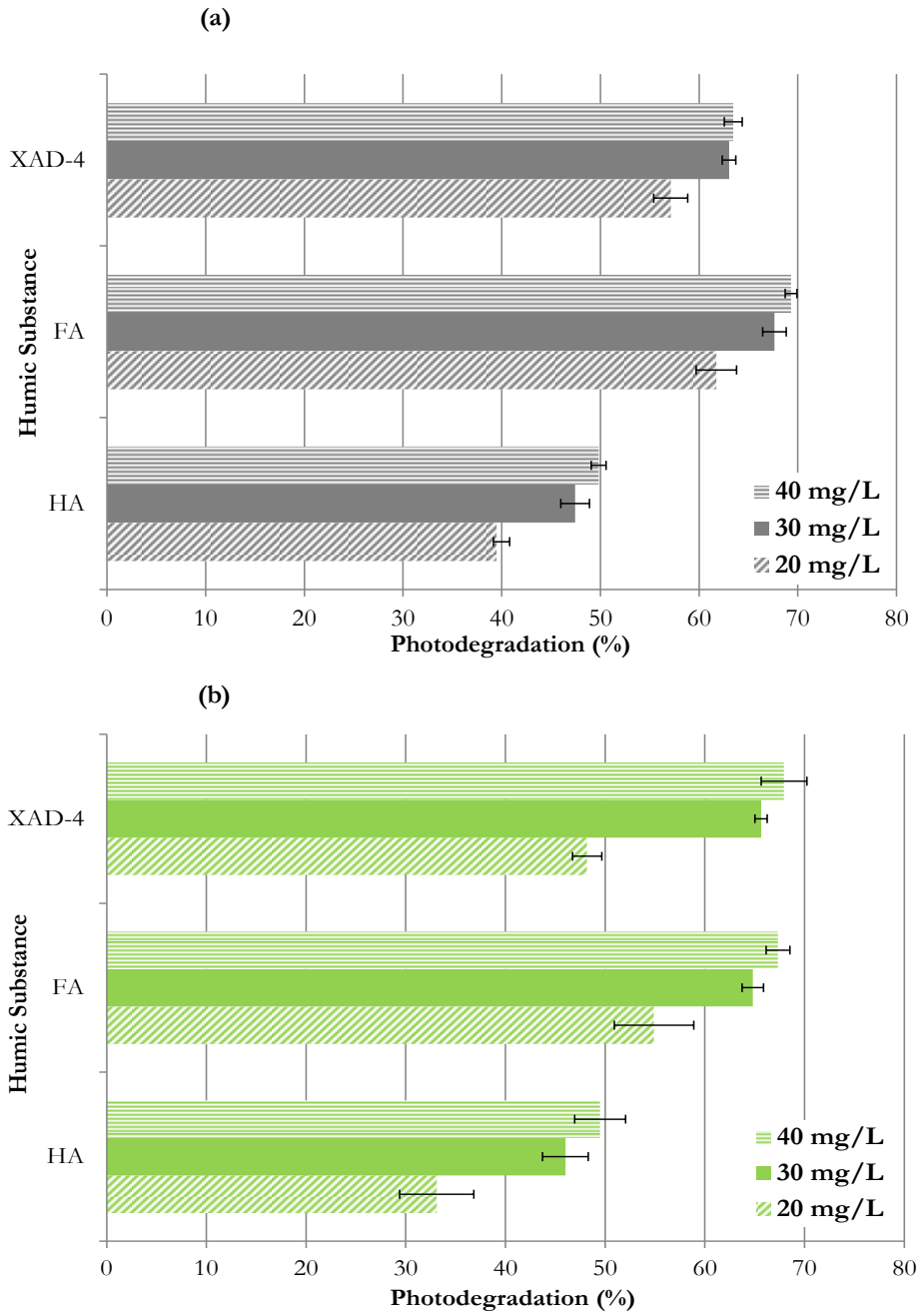


Fig. 7.7: Effect of the concentration of HS on the photodegradation of (a) EE2 and (b) E2, for 5 h of irradiation.

As it can be seen, hormones' photodegradation increased when increasing HS concentration from 20 to 30 mg L^{-1}. Considering the three types of HS, for EE2, photodegradation increases between 9 and 17%, and for E2, between 15 and 28%. Therefore,

the increase in HS concentration from 20 to 30 mg L^{-1} has a major expression in the case of E2. However, for both hormones, from 30 to 40 mg L^{-1} HS, even though a slight increase was observed, differences in photodegradation were found to be not significant, to a 95% confidence level ($t_{EE2,HA}$ = 2.7; $t_{EE2,FA}$ = 2.2; $t_{EE2,XAD-4}$ = 0.76; $t_{E2,HA}$ = 1.8; $t_{E2,FA}$ = 2.6; $t_{E2,XAD-4}$ = 1.7; critical *t* value = 2.78). These results are in agreement with those by Chowdhury et al. (2011), who determined an increment on the photodegradation rate of E2 with increasing HA concentration until reaching a plateau due to scavenging of reactive species, as well as possible light attenuation.

Results in this work point to the important role of HS chemical composition and concentration on their photochemical activity, which had already been stated by Calisto et al. (2011).

7.3.3.3 Addition of scavengers

Several species may be involved in the phototransformation of EE2 and E2 in the presence of HS. Experiments have been performed with the addition of 2 mM of sodium azide (as a singlet oxygen scavenger) or 0.26 M of 2-propanol (as a hydroxyl radicals scavenger), to a selected sample (waste water – final effluent; TOC = 45.2 mg L^{-1}). Samples were irradiated during 5 h and results were compared with those for waste water sample with no addition of scavengers (Fig. 7.8).

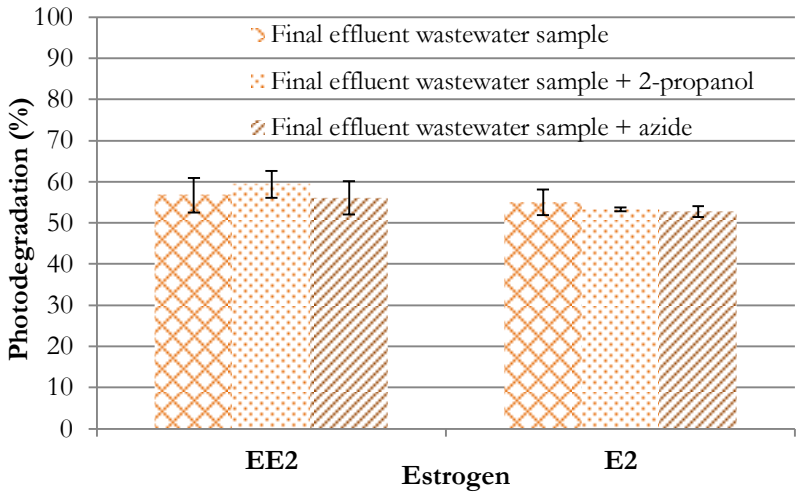


Fig. 7.8: Photodegradation (%) for both EE2 and E2 in the final effluent waste water sample in absence and presence of scavengers, for 5 h of irradiation (n = 3).

The addition of either 2-propanol or sodium azide did not have a significant influence on the photodegradation in comparison to the waste water photolysis in absence of the scavengers. A *t-test* was performed to establish if the mean photodegradation for each hormone in absence and presence of each one of the scavengers were significantly different. Since the calculated *t* values ($t_{EE2,prop}$ = 2.6; $t_{EE2,az}$ = 0.7; $t_{E2,prop}$ = 1.7; $t_{E2,az}$ = 2.2) were lower than that of the critical *t* value (2.78), for 4 degrees of freedom, at a 95% confidence level, it can be stated that there are no significant differences in the photodegradation of EE2 or E2 in presence of scavengers compared to the sample photodegradation without scavengers.

Therefore, results indicate that hydroxyl radicals and singlet oxygen play a minor role on the indirect photodegradation of EE2 and E2 in this type of matrix. The participation of other reactive species, other than hydroxyl radicals and singlet oxygen, in the photodegradation process is to be expected. Excited triplet states of chromophoric DOM constituents, radical cations of aromatic structures, solvated electrons or peroxyl radicals (Canonica et al., 2001) are possible photo-oxidants that can play a significant role on the phototransformation of EE2 and E2.

7.4 CONCLUSIONS

This study demonstrated that EE2 and E2 are hardly photodegraded in ultrapure water under simulated solar radiation. However, in collected water samples, under the same irradiation conditions and duration, photodegradation had a marked increase for both hormones. The major increase was observed in the estuarine water sample, where photodegradation rate was 5.9 and 15.6 times higher, for EE2 and E2, respectively, than in ultrapure water. This increase in these water samples was attributed to the photosensitizing effect of DOM.

In fact, the capacity of HA, FA and XAD-4 fractions to induce the photodegradation of both hormones was remarkable, with $t_{½}$ ranging between 2.1 and 6.4 h, for EE2 and from 2.9 to 5.7 h, for E2, in comparison with 46 and 94 h, for EE2 and E2, respectively, in absence of HS.

The experiments with scavengers showed that singlet oxygen and hydroxyl radicals have a minor participation on the indirect photodegradation of EE2 and E2 in the type of

matrix used (treated waste water). Thus, DOM excited triplet states directly reacting with the hormones might be a mechanism playing an important role on their photodegradation.

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FINAL REMARKS

Conclusions and Future work

CONCLUSIONS

The occurrence of EDCs and PPCPs in the aquatic environment has become a concerning subject in the international scientific community. Much research is being done in the last two decades in order to better understand the occurrence, fate and behavior of these pollutants in the environment. Despite these efforts, much is yet to be done in order to improve the understanding of these important issues. Therefore, the work presented in this thesis aimed to be part of this improvement, contributing especially for the knowledge of the Portuguese reality.

In the first part of this work, ELISA methodologies were developed and optimized in order to obtain a simple, fast and low cost method to follow the occurrence of the estrogens E2 and EE2, the antibiotic SMX and the anthropogenic marker caffeine, in water samples.

ELISA has proven to be a valid alternative to chromatographic methods hyphenated to MS detectors that are expensive in both instrumentation and maintenance, entail difficult sample pre-treatment methods and a high level of expertise for operation and are not applicable for screening purposes. Several advantages are attributed to ELISA: it is rapid, several samples can be analysed within the same experiment, requires low-cost equipments, it is characterized by operational simplicity and it is suitable to perform high-throughput environmental screenings. Moreover, one of the most remarkable advantages of immunoassays is that can be used without any sample pre-treatment. Despite all these advantages, ELISA is known to be prone to matrix effects' interferences, particularly in samples of highly complex matrices, such as waste water samples. Therefore, matrix effects were an imperative issue when developing the assays presented in this work.

In what concerns Part I of this thesis, it has to be highlighted that ELISA was used without any sample pre-treatment apart from filtration right after collection. ELISA was used with success in the quantification of the hormones E2 and EE2 after a proper optimization of the assay for being used in water samples of complex matrices. Related with the quantification of pollutants in the aquatic environment and the assessment of polluted areas is the quantification of anthropogenic markers. Caffeine was shown to be a valid anthropogenic marker in the identification of polluted sites with domestic and urban waste waters. Moreover, in the case of caffeine, results were validated by a reference method, LC-MS/MS. The SMX assay was shown to overestimate results when comparing with those obtained by LC-MS/MS. However, the assay may be used for screening purposes, in order to identify possibly

contaminated areas. Overall, these results show that ELISA is an inexpensive and time-efficient alternative, or at least a complement, to expensive and time-consuming chromatographic techniques.

It is known that levels of hormones in the environment, especially EE2, are very low, therefore demanding in some cases pre-concentration strategies. Consequently, in Part II of this thesis, a pre-concentration methodology was implemented and optimized for the subsequent quantification of E2 and EE2, either by HPLC-FD and ELISA. DLLME proved to be a simple, fast, inexpensive technique, allowing the extraction and pre-concentration of a large number of environmental samples in parallel. It is also environment-friendly since the volume of organic solvents used is low. After the optimization of the technique for quantification by HPLC-FD, the advantages of both DLLME and ELISA, already enumerated, were conjugated in what, to the best of author's knowledge, is the first approach dealing with DLLME-ELISA.

The use of immunoassays in environmental analysis is not entirely accepted yet and, in some cases, their application is still limited. Therefore, this thesis aimed to prove the applicability of this method in the quantification of the studied compounds.

Another goal of this work was to assess the fate of estrogens in the aquatic environment in what concerns photolysis, which is described in Part III. Photolysis is considered one of the most relevant processes for transformation/elimination of pollutants in the aquatic environment. Yet, many details related, for instance, with the environmentally relevant parameters that influence this process are still under study in the international scientific community. E2 and EE2 were shown to be resistant to direct photodegradation, but this work highlighted the enormous influence of HS in the enhancement of the photodegradation of both E2 and EE2. This is indicative that photodegradation of environmentally concerning compounds should be studied in conditions that mimic the real aquatic environment.

Detailed accomplishments and conclusions were as next:

- ELISA assays were implemented and optimized for the quantification of E2, EE2, SMX and caffeine without any sample pre-treatment except for the filtration step after sampling. These assays aimed to be simple, rapid, low-cost and applicable in large sampling campaigns allowing high throughput analysis.

In the case of both E2 and EE2 assays:

- The addition of a BSA-based sample buffer added to the plate before the standards/samples was proved to solve matrix effects due to dissolved organic matter and sodium chloride presence;

- The decrease of both the sample buffer pH (6.4) and the T incubation time was found to decrease the lower limit of the quantification range, allowing higher sensitivity;

- Quantification ranges of the optimized assays were of 0.03-200 μg L^{-1} and 0.02-10 μg L^{-1}, for E2 and EE2, respectively;

- The developed E2 ELISA assay proved to be adequate for the quantification of E2 in matrices as complex as waste water, permitting its quantification in two waste water samples and one surface water sample in concentrations ranging between 0.035 ± 0.002 μg L^{-1} and 0.085 ± 0.010 μg L^{-1};

- The optimized methodology for EE2 was also not influenced by matrix effects; however, it was not possible to determine EE2 at a quantifiable level in any of the samples tested.

In what concerns SMX assay:

- The SMX ELISA, meaningfully affected by organic matter and salinity presence, was optimized in order to overcome these interferences, which was accomplished by using a BSA sample buffer with a pH of 7.6 prior to standards/samples;

- All the recovery rates obtained using the sample buffer were good: 93-121%, 85-120% and 98 ± 6%, for the presence of organic matter (humic acids), presence of salinity and in an environmental water sample, respectively;

- The quantification range of the optimized assay was between 0.1 and 30 μg L^{-1};

- SMX was quantified in all samples with concentrations ranging from 4.3 to 11.0 μg L^{-1}, in waste water samples, and from 0.095 to 0.90 μg L^{-1}, in surface water samples;

- Nonetheless, ELISA analysis overestimated the results in comparison to a reference method – LC-MS/MS, which may be associated to CR between SMX and compounds in solution;

- SMX ELISA may be, even though, used as screening analytical tool.

In relation with caffeine assay and its analysis:

- The developed ELISA proved to be adequate for the quantification of caffeine in samples with a complex matrix, like samples from an estuarine area and STPs, by using a BSA

sample buffer (pH 7.6). In these conditions, a quantification range of 0.1-1000 μg L^{-1} was obtained;

- Caffeine was quantified in 43 out of 51 samples, in values between 0.1 and 15 μg L^{-1};

- Results by ELISA were successfully validated by LC-MS/MS;

- Caffeine ELISA was shown to be a suitable tool to assess contamination owed to human pollution, being possible to be used in campaigns of natural waters quality maintenance.

• A preconcentration methodology (DLLME) was developed in order to facilitate the quantification of E2 and EE2, usually present in very low levels in water samples. This methodology aimed to be simple, low-cost and environmentally friendly.

In what concerns the coupling of DLLME with HPLC-FD:

- Low LODs were obtained: 2.0 ng L^{-1}, for E2, and 6.5 ng L^{-1}, for EE2;

- Recovery tests proved that water samples matrix does not interfere in the extraction efficiency;

- Quantification of E2 and EE2 was possible in both surface and waste water samples with concentrations from 12 to 32 ng L^{-1}, for E2, and from 11 to 18 ng L^{-1}, for EE2.

Regarding the coupling of DLLME with ELISA:

- The extraction procedure was found to interfere on the assay performance, which was solved by subjecting the standards themselves to the DLLME procedure prior to ELISA;

- The simple extraction procedure developed decreased the lower limit of the quantification range approximately 30 times for E2 and 100 times for EE2 (in comparison with the quantification ranges obtained without the DLLME pre-concentration step);

- Lower quantification range limits were 1.2 ng L^{-1}, for E2, and 0.22 ng L^{-1}, for EE2;

- Quantification of both E2 and EE2 was possible in water samples of complex matrix in concentrations of 2-77 ng L^{-1}, for E2, and 0.3-24 ng L^{-1}, for EE2.

• In order to assess, not only the occurrence of the contaminants under study, but also their fate in the aquatic environment, photodegradation of E2 and EE2 was investigated using simulated solar radiation:

- E2 and EE2 were hardly photodegraded in ultrapure water under simulated solar radiation: for 5 h of irradtiation, E2 degraded 6% and EE2 16%;

- In collected water samples, under the same irradiation conditions and duration, photodegradation had a marked increase for both hormones: in the estuarine water sample,

photodegradation rate was 15.6 and 5.9 times higher, for E2 and EE2, respectively, in comparison with ultrapure water. This increase was attributed to the photosensitizing effect of DOM;

- HA, FA and XAD-4 fractions induced the photodegradation of both hormones: $t_{½}$ ranged from 2.9 to 5.7 h, for E2, and between 2.1 and 6.4 h, for EE2, in comparison with 94 and 46 h, for E2 and EE2, respectively, in absence of HS;

- For the type of matrix used (treated waste water), experiments with scavengers showed that singlet oxygen and hydroxyl radicals have a minor or no participation on the indirect photodegradation of both estrogens, therefore attributed to DOM excited triplet states.

FUTURE WORK

Antibiotics and estrogens are concerning contaminants due to their potential to increase resistance in pathogens and induce endocrine disruption, respectively. Photodegradation is assumed to mitigate their impacts in aquatic environment, but this may not occur if photoproducts retain parent compounds' activity. When undergoing photodegradation, pollutants result in various products and thus, apart from the fate of parent compounds, knowledge on phototransformation products is also critical.

Also it is known that the photoproducts' type and concentration is strongly influenced by the photodegradation mechanism, which in turn is largely affected by the chemical composition of aqueous matrices (e.g., presence of sensitizers, scavengers, changes in oxygen and organic matter concentrations, pH, salinity). Therefore, the study of organic pollutants' photodegradation under different environmental circumstances, the knowledge of photolysis' kinetics, mechanisms, main photoproducts and their activity is essential to predict the behavior and real implications of pollutants in natural waters.

With these aspects in mind, in the future it is aimed to focus efforts on the assessment of antibiotics' and estrogens' photodegradation, under environmentally important factors, elucidating photodegradation pathways, identifying the produced photoproducts and assessing their anti-bacterial and estrogenic activities, in order to better understand their real environmental impact.