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Departament Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal

Evaluación del riesgo en humedales costeros mediterráneos de la presencia de contaminantes químicos orgánicos

Evaluació del risc en aiguamolls costaners mediterranís per la presència de contaminants químics orgànics

Evaluation of the risk in Mediterranean coastal wetlands derived from the presence of organic contaminants

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Tesis Doctoral Internacional

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CERTIFIQUEN QUE:

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6**.- Spatial distribution of illicit drugs in surface waters of the natural park of Pego-Oliva Marsh (Valencia, Spain)**, P. Vazquez-Roig, V. Andreu, C. Blasco, F. Morillas, Y. Picó, Environmental Science and Pollution Research, 19 (2012) 971-982 (factor d'impacte: 2.651)

7**.- Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego-Oliva Marshlands (Valencia, eastern Spain),** P. Vazquez-Roig, V. Andreu, C. Blasco, Y. Picó, Science of the Total Environment, 440 (2012) 24-32 (factor d'impacte: 3.286)

8.- **Evaluation of the presence of pharmaceuticals and heavy metals in waters of a Mediterranean coastal wetland: Behavioral interrelations and the influence of the environment**, V. Andreu, E. Gimeno, P. Vazquez-Roig, Y. Picó, Journal of Hazardous Materials, (2013) Enviat (factor d'impacte: 4.173)

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Set dels deu treballs han estat presentats pel doctorand com a primer autor i en els altres tres signa en segon i tercer lloc, atés que es tracta de treballs realitzats en col·laboració amb investigadors del departament de degradació i conservació de sòls del centre d'investigacions sobre desertificació (CIDE) que són experts en algunes de les aplicacions desenvolupades com a processos d'adsorció en sòls, metalls pesats o maneig de GIS i han format al doctorand en aquestes tècniques. A més no hi ha cap article que haga estat o vaga a estar utilitzat implícita o explícitament per a la realització d'una altra tesi, per la qual cosa, autoritzem la seva presentació per a optar al grau de doctor.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS 1

AIMS AND SCOPE 5

CHAPTER 1: Introduction 15

Scientific publication 1: Advances in the analysis of legal and illegal drugs in the aquatic environment 19

1.1 Pharmaceuticals 33

1.2 Illegal drugs 34

1.3 Toxicity studies of pharmaceuticals and drugs of abuse 35

1.4 Risk assessment for aquatic organisms 36

1.5 Wetlands 38

1.5.1 L´Albufera of Valencia Natural Park 39

1.5.2 Pego-Oliva Marsh Natural Park 39

1.6 Legislation 40

CHAPTER 2: Analysis of tetracylines in soils 43

Scientific publication 2: Determination of tetracycline residues in soil by pressurized liquid extraction and liquid chromatography tandem mass spectrometry 47

CHAPTER 3: Analysis of pharmaceuticals in soils and sediments 61

Scientific publication 3: Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry 65

CHAPTER 4: Determination of illicit drugs in waters from L´Albufera of Valencia 83

Scientific publication 4: SPE and LC-MS/MS determination of 14 illicit drugs in surface waters from the Natural Park of L´Albufera (València, Spain) 87

CHAPTER 5: Determination of pharmaceuticals in L´Albufera of Valencia 105

Scientific publication 5: Assessment of the occurrence and distribution of pharmaceuticals in a Mediterranean Wetland (L´Albufera, Valencia, Spain) by LC-MS/MS 109

CHAPTER 6: Ocurrence of illicit drugs in Pego-Oliva Marsh 131

Scientific publication 6: Spatial distribution of illicit drugs in surface waters of the Natural Park of Pego-Oliva Marsh (Valencia, Spain) 135

CHAPTER 7: Pharmaceuticals in Pego-Oliva Marsh: distribution and risk assessment 149

Scientific publication 7: Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego-Oliva Marshland (Valencia, eastern Spain)..... 153

CHAPTER 8: Analysis of heavy metals in Pego-Oliva Marsh..... 175

Scientific publication 8: Evaluation of the presence of pharmaceuticals and heavy metals in waters of a Mediterranean coastal wetland: Behavioral interrelations and the influence of the environment..... 179

CHAPTER 9: Relationship between irrigation systems and levels of illicit drugs in L´Albufera of Valencia 209

Scientific publication 9: Presence of illicit drugs in surface waters of protected natural wetlands connected to traditional irrigation systems and urban areas. Management of water resources in protected areas 213

CHAPTER 10: Stereoisomeric profiling of drugs of abuse and pharmaceuticals in wastewaters..... 223

10.1 Introduction 224

10.2 Material and methods..... 225

10.2.1 Sampling 225

10.2.2 Chemicals 226

10.2.3 Analytical methodology 226

10.3 Results and discussion..... 228

10.3.1 Ocurrence of chiral drugs in wastewaters of Valencia 231

10.3.2 Enantiomeric profiling of wastewaters..... 231

10.3.3 Removal of chiral drugs during wastewater treatment..... 233

10.3.4 Drug usage at the community level in main urban areas of Valencia 234

10.4 Conclusions 236

SUMMARY OF RESULTS AND DISCUSSION 245

1 Development of analytical methodologies 247

1.1 Liquid chromatography tandem mass spectrometry determination..... 247

1.2 Solid sample extraction 249

1.2.1 PLE extraction for tetracyclines 249

1.2.2 PLE extraction for multi-class pharmaceuticals 250

1.3 SPE isolation of waters and PLE extracts from solid samples 252

1.3.1 SPE for pharmaceuticals 252
1.3.2 SPE for illicit drugs 254
2 Monitoring and risk assessment in L´Albufera of Valencia 255
2.1 Ocurrence of illegal drugs: levels and spatial distribution 255
2.1.1 Spatial analysis with Geographical Information Systems (GIS) 258
2.2 Ocurrence of pharmaceuticals: levels and implications for the aquatic fauna 259
2.2.1 Environmental implications 262
3 Monitoring and risk assessment in Pego-Oliva Marsh 263
3.1 Ocurrence of illegal drugs in waters of the Marsh 263
3.2 Ocurrence of pharmaceuticals and risk assessment 267
3.2.1 Water samples 268
3.2.2 Sediment samples 269
3.2.3 Soil samples 271
3.2.4 Spatial distribution 272
3.2.5 Statistical analysis 273
3.2.6 Risk assessment of the presence of pharmaceuticals in waters 274
3.3 Ocurrence of heavy metals in water samples 275
3.3.1 Statistical analysis and relationship with pharmaceuticals 276
CONCLUSIONS 279
RESUMEN EN CASTELLANO 285
RESUM EN VALENCIÀ 297
BIBLIOGRAPHY 309
INDEXES 325
INDEX I: List of tables 327
INDEX II: List of figures 333

LIST OF ABBREVIATIONS

APCI, Atmospheric-pressure chemical ionization

APPI, Atmospheric pressure photoionization

CTC, Chlortetracycline

DAD, Diode array detector

DC, Doxycycline

DME, Dispersive matrix extraction

EDTA, Ethylenediaminetetraacetic acid

EIC, Extracted ion chromatogram

ESI, Electrospray ionization

ETM, Erythromycin

EU, European Union

FD, Fluorescence detection

FQs, Fluoroquinolones

GC–MS, Gas chromatography with mass spectrometry

GIS, Geographic information system

HILIC, Hydrophilic interaction liquid chromatography

HLB, Hydrophilic-lipophilic-balanced

LC-MS^2, Liquid chromatography tandem mass spectrometry

LIDs, Legal and illegal drugs

LLE, Liquid-liquid extraction

LOD, Limit of detection

LSD, Lysergic acid diethylamide

MAE, Microwave-assisted extraction

MAME, Microwave assisted micellar extraction

MDA, 3,4-methylenedioxyamphetamine

MDMA, 3,4-methylenedioxymethamphetamine

MEC, Measured environmental concentration

PNEC, Predicted no-effect concentration

MSPD, Matrix solid-phase dispersion

N.D., Not detected

NI, Negative ionization

NSAIDs, Non-steroidal anti-inflammatory drugs

OTC, Oxytetracycline

PI, Positive ionization

PLE, Pressurized liquid extraction

PNEC, Predicted no effect concentrations

QqQ, Triple quadrupole

QTOF, Quadrupole-time of flight

RAM, Restricted access materials

SAs, Sulphonamides

SFE, Supercritical fluid extraction

SPE, Solid-phase extraction

SRM, Selected reaction monitoring

TBA, Tetrabutylammonium

TC, Tetracycline

TFC, Turbo-flow chromatography

TGD, Technical guidance document

THC, Δ^9-tetrahydrocannabinol

THC-COOH, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol

TP, Transformation products

UHPLC, Ultra high performance liquid chromatography

US, Ultrasonic extraction

WFD, Water framework directive

WWTP, Wastewater treatment plant

AIMS AND SCOPE

A swift development of population in the cities has created an intense impact in the environment. Nowadays, the coastal strip in most European countries is the area of most rapid social and economic development. Several punctual data coming from different sources can give a general idea of the importance of the demographic growing in these areas. Today, approximately 3 billion people — about half of the world's population — live within 200 kilometres of the coastline. Two-fifths of cities with populations of 1 million to 10 million people are located near coastlines. In Belgium, Portugal and Spain, the population density within 10 kilometres of the shoreline is twice that of the interior areas. Today, about 70 million of the 455 million citizens of EU (including new member states), i.e. 16 % of the population, live in coastal municipalities, although the coastal line is only 11 % of the EU's total area. The Mediterranean coast of Spain, along with Ireland, has the fastest growing population in Europe, with an increasing above 50 % over the past decade. Furthermore, in Spain, 1.7 million houses, mostly located along the coastal strip, are secondary residences.

During long time, decision-makers have focused their efforts to fulfil the needs of this growing population in detriment of the ecosystem. In recent years, new emerging contaminants as pharmaceuticals and illicit drugs have gained the attention of scientists, because they are good indicators of this anthropic development as their concentrations increase in parallel to the growing population. Pharmaceutical and drug residues in the environment, and their potential toxic effects, are recognized as an emerging research area in environmental chemistry [Richardson et al. 2011]. A better knowledge of the occurrence and fate of legal and illegal drugs (LIDs) release to the environment will attain a proper risk assessment for river basins, wetlands and others related ecosystems. Fig. A1 shows that pharmaceuticals enter the environment mainly as a result of their excretion in urine of humans and animals as well as of the aquaculture treatments. It is now well established that pharmaceuticals and drugs of abuse are widespread contaminants of wastewater effluents, surface and drinking waters [Ferrer, I. et al. 2010, Pailler et al. 2009]. Worldwide, sewage is recognized as the largest source of environmental contamination, and discharges have increased dramatically in the past three decades. In many cases, the sewage waters are not appropriately processed either by a lack of sewage treatment plants or by overloaded

the capacity of them. This last situation is typical in the Valencian Community (Spain), where there is a huge increase of tourism in summer. In addition, this season coincide with a scarcity of rain, which puts in danger water quality. An insufficient water volume makes the dilution and drainage of the contaminants difficult [Ginebreda et al. 2010]. As consequence of this, pollutants reach natural water systems and may be a hazard for aquatic environment, and consequently for human population.

This circumstance is particularly critical in the quiet films of water, lagoons and wetlands that cover the Valencian littoral because these protected areas play important roles as water reserve, flood control, charging of aquifers, etc [EEA 2012]. Furthermore, these protected wetlands provide habitat to many different species of wildlife, being important key points in the route of migratory birds. The increasing human pressure and the socio-economic development have caused the disappearance of the half of the world's wetlands in the 20th century, threatening seriously the future conservation of these ecosystems.

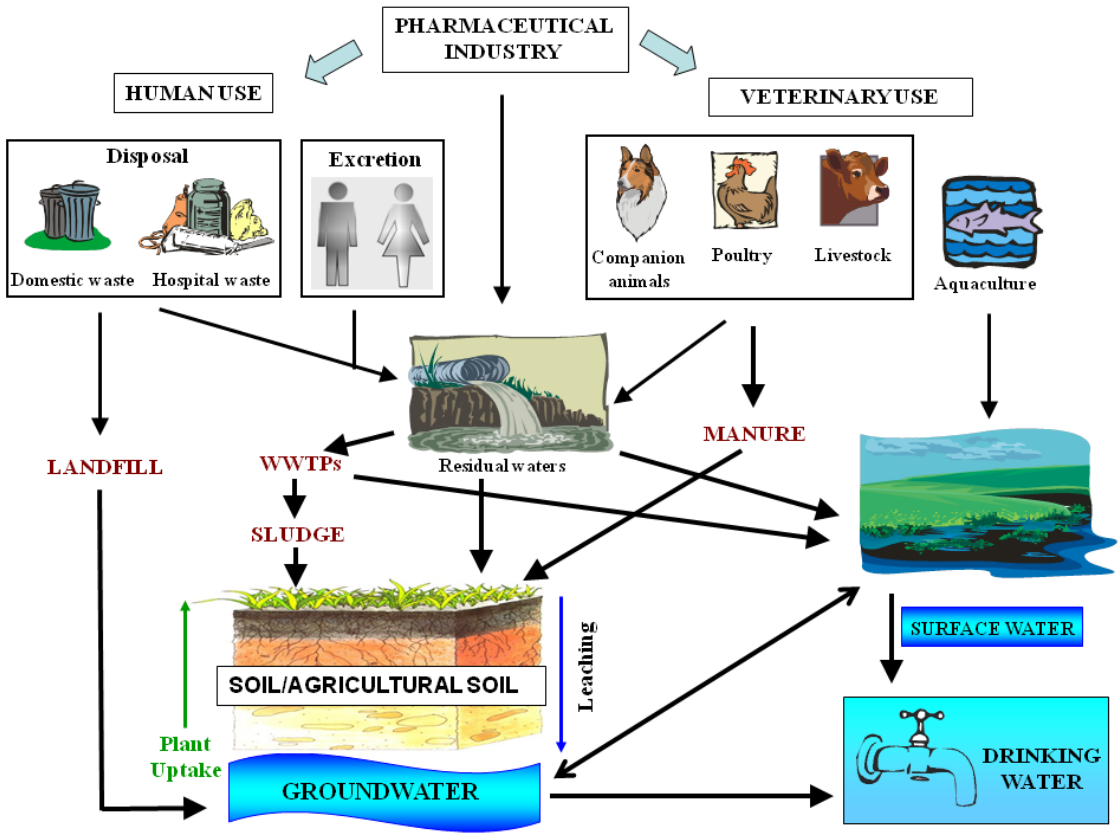

Figure A1. Sources and pathways of pharmaceuticals in the environment

At the beginning of this thesis and to our knowledge, the incidence of these contaminants in natural wetlands had never been studied. Furthermore, the simultaneous investigation of LIDs in water, sediment and soil samples was also nonexistent, and only a few studies dealt with the simultaneous analysis in water and sediments [Yang et al. 2011, Silva et al. 2011, Kim et al. 2007a] or in water and soils [Tso et al. 2011, Raich-Montiu et al. 2007]. There were already several methods to determine pharmaceuticals and drugs of abuse in the aquatic environment [Kasprzyk-Hordern et al. 2008a, González-Mariño et al. 2010, López-Serna et al. 2013]. They mainly consisted in solid-phase extraction (SPE) or solid-phase microextraction (SPME) for isolation and enrichment, and liquid chromatography tandem mass spectrometry (LC-MS/MS) or derivatization following gas chromatography–mass spectrometry (GC-MS) for quantification. However, there were much fewer methods available for the extraction and quantification of LIDs at trace levels in solid matrices. One possible reason for this lack of analytical methods for soils and sediments is the complexity of their interactions with pharmaceuticals or illegal drugs, which may have neutral, cationic, anionic, or zwitterionic charge under different pH conditions. Therefore, their physico-chemical properties such as Log K_{ow}, sorption behavior to solids or degradation may change with pH. Moreover, specific interactions (cation exchange, cation bridging, surface complexation, metal chelating, etc.) can bind LIDs chemically with organic matter and clays, making of sediments and/or soils a reservoir for these compounds (e.g. fluorquinolones and tetracyclines) and complicate their isolation. This remarks the need of developing new analytical methods to assess these matrices.

Under this situation, the overall objective of this Doctoral thesis was to give an overview of the quality of the waters of some Natural Parks of the Valencian Community, trying to understand whether the presence of pharmaceuticals and illicit drugs involves a risk for the aquatic fauna of these protected areas. For that, the specific objectives were:

1. Development and validation of analytical methodologies based on PLE and off-line solid phase extraction (SPE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) for the determination of pharmaceuticals belonging to

different therapeutic classes and drugs of abuse in water, soil and/or sediment samples.

2. To study the concentrations, distribution and fate of these anthropogenic contaminants in Mediterranean coastal wetlands of the Valencian Community (L´Albufera of Valencia and the Pego-Oliva Marsh), trying to determine the origin and patterns behind the spatial distribution of these compounds.

3. To assess the hazard derived from the presence of these pollutants for the aquatic fauna, based on available toxicological data (determined in laboratories or estimated by computer software) of pharmaceuticals and drugs of abuse. The target organisms were those belonging to the three different levels of the trophic chain, to obtain a whole picture of the potential impact of these substances to the aquatic environment.

For this, the thesis has been divided in ten chapters. Chapter 1 is a general introduction, in which several aspects are covered. Firstly, the state-of-art of analytical methods are considered, giving an overview of the most novel and advantageous techniques for the analysis of licit and illicit drugs in solids and liquid environmental matrices and discussing the factors that must be considered when creating and optimizing these methods. Examples of applications are provided, proposing when possible, future directions not already fully explored.

Furthermore a short and informative outline of the importance of pharmaceuticals and drugs of abuse as environmental contaminants highlighting toxicity studies, risk assessment and applicable legislation concludes this bibliographic chapter.

Chapter 2 to 10 presents the experimental work undertaken in this PhD, compiled in form of publications, which was planned and designed in order to achieve the proposal objectives.

In chapter 2, a method to simultaneously determine four TCs—TC, OTC, CTC, and DC—in soil, using PLE and SPE extraction followed by LC-MS/MS with a triple quadrupole (QqQ) analyzer was developed and optimized. This was the starting point of this thesis. Tetracyclines are strongly binding to soil and sediment since they have multiple ionizable functional groups at environmentally relevant pH values [Jacobsen et al. 2004]. They can exist as cations, zwitterions, or net negatively charged ions,

which complicates the prediction of their sorption, availability, and transport. The method was applied to soil samples of different locations and after different sludge treatments. To the best of our knowledge, this was the first finding of TC residues in typical Spanish agricultural soils.

In chapter 3, the method previously developed was adapted and optimized as multi-residue method to determine 17 pharmaceuticals (β-blockers, antidepressants, anti-epileptic drugs, analgesics, non-steroidal anti-inflammatory drugs, lipid regulators and antibacterials) in soils and sediments.

In chapter 4, an analytical method to analyze fourteen drugs of abuse and some of their metabolites in waters was developed. One aim of this study was to enlarge the range of tested SPE cartridges for fourteen illicit drugs. For this, seven SPE-sorbents were compared and evaluated. Sample pH for extraction on the SPE cartridge, the amount of solid sorbent and the water volume analyzed were studied. Another aim of this work was to evaluate, for the first time, the occurrence of illicit drugs in surface waters of L'Albufera Natural Park. This data complement and enlarge the scarce studies carried out on this topic in surface waters of spanish areas.

In this Natural Park, a monitoring program was conducted to know the incidence of pharmaceuticals in it. Results are presented in chapter 5. The aim of this work was to study the spatial distribution of pharmaceuticals among water, soil and sediment in water courses and channels of L'Albufera Natural Park. In the protocol, SPE was used to isolate and concentrate the chemicals from the water. For sediment and soil samples, the extraction results obtained by PLEs and ultrasonic shaking were compared. PLE conditions (dispersing agent, elution solvent, static time, number of cycles) were based on those used in the chapter 3.

In chapter 6, illicit drugs were monitored in waters of the Pego-Oliva Marsh in order to understand the pollution status and recommend future rationalization for controlling, reducing and eliminating releases of these compounds. The results of this study should be of value not only to control the contaminants in the Pego-Oliva Marsh but also to guarantee the safety of drinking water.

In chapter 7, pharmaceuticals were monitored in this Marsh, and a risk assessment derived from the presence of pharmaceuticals in waters was carried out in

this area. A total of 34 water, 17 sediment and 23 soil samples were collected in different points, covering the different environmental and land uses (agricultural, redbird, etc.) of this wetland. We sought to understand the incoming sources, distribution and fate of these contaminants in the Pego–Oliva marsh, to determine several patterns behind the spatial distribution of these compounds and to assess the risk for the aquatic fauna on the basis of available long-term data.

In Chapter 8, heavy metals were analyzed in waters of Pego-Oliva marsh, making a correlation between their presence and the previously reported concentrations of pharmaceuticals in the same sampling points. Synergies between contaminants, such as pharmaceuticals and heavy metals were, until now, scarcely studied and can be relevant because of their widespread distribution not only in waters but also in soils and sediments.

Chapter 9 has been focused on the development of a combined methodology based on environmental forensic principles to identify illicit drugs and its spatial sources and implications. Population distribution, traditional irrigation system and existent wastewater treatment plants were studied to determine their weight in the introduction the illicit substances in the waters of the L´Albufera Natural Park.

Finally Chapter 10, developed in the University of Bath, was focused on the understanding the behaviour of pharmaceuticals and drugs of abuse in the WWTPs through their chiral analysis. The measurement of the levels of LIDs, before and after treatment, proves to be invaluable in the verification of the contribution of wastewaters to pollution in natural parks, and the possible formation of some LIDs by degradation of their conjugated metabolites in the WWTPs. Besides, the enantiomeric profiling of LIDs lets us know if WWTPs are degradating them enantioselectively, since toxicity of these compounds is generally isomer-dependent.

CHAPTER 1

Introduction

Scientific publication 1:

Advances in the analysis of legal and illegal drugs in the aquatic environment

P. Vazquez-Roig, C. Blasco, Y. Picó

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abstract

We review the current methods developed for the analysis of legal and illegal drugs (LIDs) and their metabolites in environmental samples. We discuss the advantages and the pitfalls of multi-class methods with emphasis on new strategies for sample preparation and recent technical developments. Finally, we present the applicability of these methods to the analysis of LIDs in protected environmental areas.

Contents

1. Introduction . . . 66
2. Analytical protocols . . . 67
2.1. Analyte preservation through the process . . . 67
2.2. Extraction of water samples . . . 67
2.2.1. Off-line SPE . . . 69
2.2.2. On-line SPE . . . 71
2.2.3. Solvent-less methods . . . 71
2.3. Extraction of solid samples . . . 72
2.4. Determination . . . 73
3. Occurrence of LIDs in protected areas of the environment . . . 73
4. Conclusions and future trends . . . 76
Acknowledgments . . . 76
References . . . 76

Abbreviations: ACN, Acetonitrile; AMPs, Amphetaminics; APCI, Atmospheric-pressure chemical ionization; APPI, Atmospheric pressure photoionization; ASE, Accelerated solvent extraction; CANNAs, Cannabinoids; COCs, Cocainics; DAD, Diode-array detector; DI, Direct injection; DW, Drinking water; EDTA, Ethylenediaminetetraacetic acid; EPI, Enhanced product ion; ESI, Electrospray ionization; ETM, Erythromycin; EU, European Union; EW, Seawater; FD, Fluorescence detection; FQ, Fluoroquinolone; GC-MS, Gas chromatography with mass spectrometry; GW, Ground water; HILIC-MS, Hydrophilic interaction chromatography mass spectrometry; HLB, Hydrophilic-lipophilic-balanced; HRMS, High resolution mass spectrometry; IDA, Information dependent acquisition; LC-MS/MS, Liquid chromatography tandem mass spectrometry; LIDs, Legal and illegal drugs; LLE, Liquid-liquid extraction; LOD, Limit of detection; LOQ, Limit of quantification; LSD, Lysergic acid diethylamide; LYSs, Lysergic acid derivatives; MAE, Microwave-assisted extraction; MAME, Microwave-assisted micellar extraction; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxy-N-methamphetamine; MeOH, Methanol; MSPD, Matrix solid-phase dispersion; NI, Negative ionization; NSAID, Non-steroidal anti-inflammatory drug; OPIs, Opiates; PI, Positive ionization; PLE, Pressurized liquid extraction; QqLIT, Quadrupole linear ion-trap; QTOF, Quadrupole time of flight; RAM, Restricted access materials; SA, Sulfonamide; SD, Sediment; SFE, Supercritical fluid extraction; SI, Sludge; So, Soil; SPE, Solid-phase extraction; SPME, Solid-phase microextraction; SRM, Selected reaction monitoring; SW, Surface water; TBA, Tetrabutylammonium; TC, Tetracycline; TFC, Turbo-flow chromatography; THC, K^9-tetrahydrocannabinol; THC-COOH, 11-nor-carboxy-K^9-tetrahydrocannabinol; TMCS, Trimethylchlorosilane; TP, Transformation product; UHPLC-MS/MS, Ultra-high-performance liquid chromatography tandem mass spectrometry; US, Ultrasonic extraction; WW, Waste water; WWTP, Wastewater-treatment plant.

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1. Introduction

Contaminants of emerging concern, mainly human and veterinary pharmaceuticals and drugs of abuse, are considered pseudo-persistent as consequence of their continuous entry into the environment, mainly through human excretion. It has been reported that the elimination of some pharmaceutical compounds in the waste water treatments plants (WWTPs) is rather low and, as a result, they are found in surface waters, drinking water and groundwater [1,2]. Other compounds have long half-lives (e.g., erythromycin, naproxen, and clofibric acid) and will remain unchanged several years after their discharge. More hydrophobic pharmaceuticals tend to accumulate in river and sea sediments. The persistence of some of them has also been confirmed in soils fertilized with contaminated sewage sludge [3].

In 2010, the world pharmaceutical market was valued at US$875bn [4]. Due to the peculiar system suggested by the World Health Organization to register the consumption of pharmaceuticals (Anatomic Therapeutic Chemical Classification/Defined Daily Dose system), it is not possible to know exactly the tons consumed worldwide. However, in the EU alone, about 4000 pharmaceuticals are currently in use [5]. Their registration and marketing are exempt from the REACH Regulation and, currently, a procedure to establish their possible environmental impact is not required.

Several toxicity studies have been carried out to assess the potential risk of pharmaceuticals to the aquatic environment [6]. For human health, the appearance of resistance in bacteria due to the continuous presence of antimicrobials in the aquatic ecosystem is a clear threat. Also we are still far from knowing

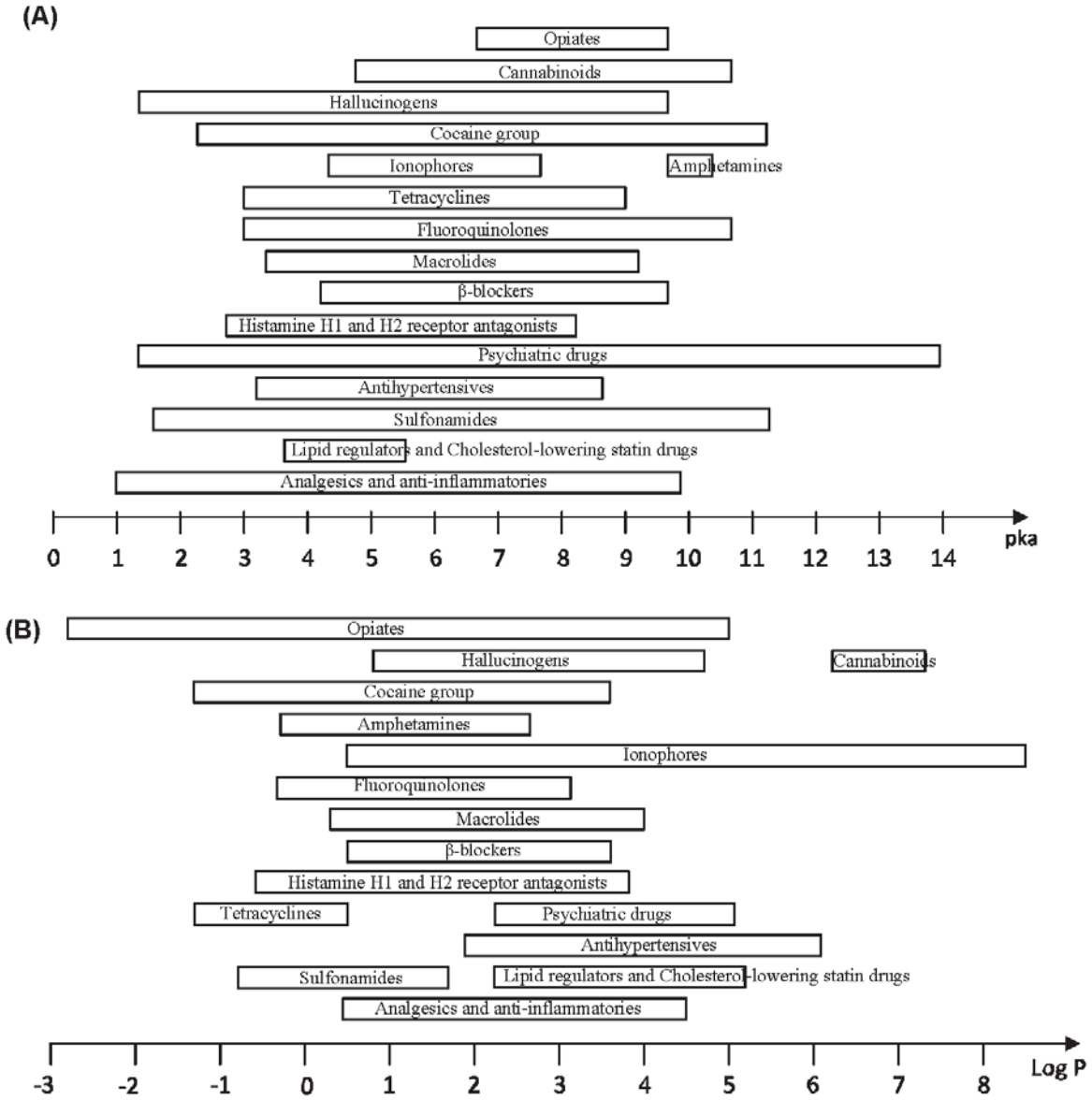

Fig. 1.1. Properties of the families of compounds studied in this review: (A) pka and (B) octanol/water partition coefficient (expressed as Log P).

the toxic effect of the complex mixture of pharmaceuticals and their metabolites.

Illegal drugs are widely consumed despite their prohibition. About 230 million people are estimated to have used an illicit drug at least once in 2010, and about 27 million are problem drug users [7]. Estimation of their use is traditionally obtained by means of statistics or, more recently, by wastewater analysis [8]. Because illicit drugs are release in huge quantities, toxic effects cannot be ruled out, but their effects on the aquatic environment are completely unknown.

A continuous survey of the levels of LIDs in the aquatic ecosystems is necessary to establish their sources, levels, fates, persistence and metabolites in the environment. This is a key step to make a realistic risk assessment of these compounds to protect the aquatic fauna and flora. For this purpose, it is necessary to use multi-residual methods covering a large variety of therapeutic compounds in various matrices. Moreover, new automated methods are appearing in the literature, increasing the sample throughput, diminishing costs and improving the productivity of personnel and instruments.

In Fig. 1.1, the pk_a and octanol/water partition coefficient (ex- pressed as Log P) of LIDs usually studied in the literature are repre- sented by horizontal bars. Each family of compounds includes diverse chemical groups that, except in a few cases, differ consid- erably in their polarities and acid-base properties. This leads to the need to apply a great amount of solvents and SPE cartridges for comprehensive sample extraction.

In contrast to wastewaters, environmental samples have analytes at very low concentrations (typically low ng/L in waters or ng/g in soils and sediment), so analytical methods have to provide enough low limits of detection (LODs).

Different reviews have already focused their attention in general [9] and specific aspects of sample preparation [10], or final determination [11]. Other recent reviews dealing with both aspects of the analytical methodology are too general due to the wide range of classes of contaminants studied [12]. There is no recent review dealing with the complete analysis of LIDs in environmental matrices.

Consequently, the objectives of this critical review are to give the state of the art of the most novel, advantageous techniques for the analysis of LIDs in solid and liquid matrices, discussing the factors that must be considered when creating and optimizing these methods. We provide examples of applications, proposing when possible, future directions that have not already been fully explored.

2. Analytical protocols

Relevant methods applied to the analysis of LIDs in surface waters together with their analytical performance are outlined in Tables 1.1 and 1.2 for off-line and on-line solid-phase extraction, respectively. Table 1.3 listed selected methods applied to sediments and soils.

2.1. Analyte preservation through the process

In general, LIDs have a polar character and most of them are present in the environment in ionized form. Consequently, samples must preferably be placed in plastic containers rather than glass, to minimize analyte losses by adsorption to glass walls {e.g., tetracyclines [38]}. If not, sylanization of the glass, or glassware treatment with a 10% nitric acid bath for 8 h has been reported as an alternative [38].

Some LIDs are rapidly degraded, so, once samples are taken from the environment, they are refrigerated in dark place to avoid analyte decomposition during transport [13,21]. Once at the laboratory, it is common practice to prevent analyte removal in water samples as consequence of microbiological activity through acidification (typically pH = 2) with hydrochloric acid [18,43,44]. However, this entails a risk for compounds suffering acid-catalyzed hydrolysis (e.g., methotrexate), and also enhances the adsorption of target compounds on natural organic matter. If the samples are not acidified, sodium azide [21,37] and formaldehyde [45] could be effective preservatives.

Water samples can be stored refrigerated without loss of any analytes for 1–2 days after collection. However, after 3 days of storage, losses of >40% of clofibric acid and ibuprofen from seawater were reported [13]. Erythromycin, mefenamic acid, diclofenac, propanolol, trimethoprim, sulfamethoxazole and dextropropoxyphene showed no degradation after 10 days of storage [13]. Only salicylic acid showed rapid degradation after only 1 day.

Some drugs (e.g., cocaine and 6-acetylmorphine) suffered rapid degradation at environmental temperatures and pH, while no degradation was observed at pH = 2 [14,18]. Ecgonine methyl ester shows a degradation rate similar to cocaine. By contrast, benzoylecgonine was stable at room temperature for a long time [44]. In tap-water samples, sodium thiosulfate was added in order to eliminate residual chlorine, avoiding oxidation of analytes, such as opiates and amphetamines [22].

To achieve higher recoveries, water is usually filtered through glass-fiber filters with pore sizes of 0.45–1.6 μm. However, filtration also removes the fraction of target compounds sorbed to suspended solids. It was therefore recommended to wash the filters with methanol after filtration [11]. Usually after filtration, surrogate standards are added to the sample for accurate quantification. Freezing is always recommended for sample storage. In final methanolic vials, no degradation has been reported after 8 days at −20°C [20]. If sample processing had to be interrupted prior to elution extraction, dried cartridge can be stored for at least 3 months at −20°C without significant degradation or interconversion reactions of illicit drugs [21].

There are few studies dealing with the stability of pharmaceuticals in solid samples under storage conditions. Antimicrobials present in the samples (common situation in real samples) diminish the degradation of other compounds [46]. Although some antibiotics are susceptible to photodegradation {e.g., fluoroquinolones (FQs) and tetracyclines (TCs) [37]} or biodegradation {e.g., macrolides and penicillins [47]}, it has no significant effect on their concentration in soils, since fixation and penetration into soil protect them. To avoid microbial degradation and other decomposition process (e.g., hydrolysis), drying the sample is recommended for solid samples. It could be done at room temperature with air, to avoid decomposition of analytes (e.g., macrolides), or freeze-drying, which is becoming popular for its effectiveness and performance.

The next step is to powder the dried sample in a mortar, or to sieve and to homogenize it directly in order to decrease the particle size and facilitate the extraction process.

2.2. Extraction of water samples

LIDs are frequently detected in concentrations slightly above their method LODs, so pre-concentration is mainly aimed at achieving these low LODs. Additional clean-up could be necessary to diminish matrix effects in MS, especially when an ESI source is utilized.

SPE has been established as the default technique to analyze LIDs in water samples. In FQs and TCs, a chelating agent (citric acid or Na_2EDTA) is added to the sample after filtration to improve the extraction recovery of these antibiotics [32,33,48]. However, it

Table 1.1
Overview of off-line SPE methods applied to LIDs in waters.

Compounds	Matrix	Pre-treatment	Sample vol. (mL)	SPE cartridge	Solvent	Recovery (%)	LOD (ng/L)	Detection	Ref.
12 (analgesics, antibiotics, lipid regulators, anti-hypertensive, anti-cancer, anti-depressant, anti-inflammatories)	DW,SW,EW,WW	pH 6, filter 0.7 μm (filter was analyzed too)	1000	Strata X (200 mg)	MeOH	5–114[b]	0.03–0.96	LC–MS/MS	[13]
6 (clofibric acid, ibuprofen, carbamazepine, naproxen, ketoprofen and diclofenac)	GW, WW, DW, SW	pH 2–3	500	Oasis HLB (60 mg)	MeOH	77–92[c]	1–8[a]	GC-MS	[14]
81(7 metabolites)	WW, EW, GW, SW, DW	filter 1 + 0.45 μm, 0.1% EDTA	100[b]	Oasis HLB (60 mg)	MeOH	30–158[b]	0.03–15.2[b]	UPLC-QqLIT	[15]
70 (based on US EPA Method 1694)	WW, SW, DW		200	Oasis HLB (500 mg)	MeOH	10–123[b]	20–20,000	LC-MS/MS	[16]
47 (2 metabolites)	WW, SW		100	Oasis HLB (60 mg)	MeOH	52–135[b]	0.5–76[a]	UHPLC–MS/MS	[17]
2 COCs, 5 OPIs and THC-COOH	WW,SW	Centrifuged, filter 0.45 μm, pH 3	500	Oasis MCX (200 mg)	MeOH (5% NH_4OH)	38–77	0.5–1	LC-MS/MS (fused core)	[18]
4 AMPs, 3 COCs, 5 OPIs and 2 CANNAs	SW	filter 1.6 μm	250	Oasis HLB (200 mg)	MeOH	57–120	0.01–1.5	LC-MS/MS	[19]
5 AMPs, 2 COCs, LSD, phencyclidine, fentanyl and ketamine	WW,SW	filter 1.6 μm	100	Oasis HLB (200 mg)	MeOH	75–99[b]	0.1–3.1[b]	UPLC-MS/MS	[20]
5 AMPs, 3 COCs, 4 OPIs and 2 CANNAs	WW,SW	filter 0.45 μm, pH 8.5	500[b]	Oasis HLB (200 mg)	ethyl acetate, acetone	74–125[b]	0.8–13[b]	GC-MS/MS	[21]
27 illicit drugs	DW	$Na_2S_2O_3$	200	Oasis HLB (200 mg)	MeOH	65–103	0.1–50[a]	UPLC-MS/MS	[22]
5 AMPs, 5 COCs and THC-COOH	WW,SW	centrifuged, pH 2	50	Oasis MCX (150 mg)	MeOH (2% NH_4OH)	73–106[b]	0.05–30	UPLC-MS/MS	[23]

MeOH, methanol; SW, surface water; WW, wastewater; DW, drinking water; GW, groundwater; EW, sea water; US EPA, United States Environmental Protection Agency.
[a] Limit of quantification.
[b] In surface water.
[c] In deionized water.

Table 1.2
Overview of the on-line methods applied to pharmaceuticals in waters.

Compounds	Matrix	Pre-treatment	Sample Vol. (mL)	SPE cartridge	Solvent	Recovery (%)	LOD (ng/L)	Detection	Ref.
8 quinolones	SW	filter 0.45 μm, acidic pH	15	C18	ACN/Water (20:80)	82–126	1–80	LC-LC-FD	[24]
74 pharmaceuticals	GW,WW,SW	filter 1 + 0.45 μ n, 0.1% EDTA	2.5	HySphere Resin GP	PI mode: ACN/0.1% ormic acid NI mode: ACN/MeOH (50:50)/H_2O	42–287[a]	0.01–23.5	LC–MS/MS	[25]
58 pharmaceuticals and 19 metabolites and transformation products	GW,WW,SW	filter	2.5[b]	TFC (Cyclone P, C18-P XL and Cyclone MAX)	MeOH	51–345[a]	0.03–49.3	TFC-LC-MS/MS	[1]
19 sulfonamides	GW,WW,SW	1 + 0.45 μ n filter 0.45 μm	5[c] 15[a]	Oasis HLB	ACN/water 0.1% formic acid	7–130	0.02–4.52[a]	LC-QqLIT	[26]
4 macrolides	SW	filter 0.45 μm	1	Capcell Pak MF Ph-1 column packed with restricted access material	Water/ACN both with 0.1% formic acid and 10 mmol/L ammonium acetate	87–98	2–6	LC–MS/MS	[27]
4 ionophore antibiotics and two avermectin antiparasitics	SW	0.02% EDTA, pH 7	3	SPE on chromatographic column (C18)	MeOH/water both with 0.1% formic acid	91–120	1–7	LC–MS/MS	[28]
8 pharmaceuticals and illicit drugs	WW,SW	filter 0.45 μ n, pH 4.5	10	in-house synthesized hypercrosslinked HXLPP	15 mM CH_3COONH_4/CH_3COOH at pH 4.5 and ACN	78–102[a]	1–5[a]	HILIC–MS/MS (fused core)	[29]
5 AMPs, 3 CANNAs, 3 COCs, 5 OPIs and 3 LYSs	WW	filter 1 μ m and 0.45 μ n	5	PLRP[b] Oasis HLB[c]	ACN/water	8–121[d]	0.01–1.15[d]	LC-MS/MS	[30]

ACN, acetonitrile.
[a] In river water.
[b] In positive ionization mode.
[c] In negative ionization mode.
[d] In deionized water.

seems that an excess of EDTA chelates not only metals but also TCs and ionophore polyethers, resulting in lower extraction efficiencies [48].

Depending on the target analytes, pH adjustment could be necessary to enhance extraction retention. However, it is necessary take into account that the use of polar solvents and acidic conditions also helps to extract the humic substances, leading to an increase in the matrix effects [38].

2.2.1. Off-line SPE

In off-line SPE, Oasis HLB (polymeric sorbent) is the cartridge most frequently utilized in LID analysis (see Table 1.1). Elution from these cartridges is usually performed with polar solvents (e.g., methanol). This cartridge has a high versatility working at differ- ent pHs and with analytes of different polarities and acid-base properties. The number of compounds simultaneously extracted is rising constantly.

Using this cartridge, Gracia-Lor et al. extracted 47 multi-class pharmaceuticals (including 26 antibiotics) from 100 mL of environmental and wastewater samples, obtaining satisfactory recoveries (≥70%) for 43 of the compounds studied [17]. Only sulfadiazine, sarafloxacin, tylosin and erythromycin were not satisfactorily recovered. The selected analytes were almost totally restricted to parent pharmaceuticals; the exceptions were salicylic acid (metabolite of acetylsalicylic acid), which is frequently found in surface waters [15], and 4-aminoantipyrine (metabolite of dipyrone). In particular, dipyrone (metamizole) is rarely detected in WWTPs, while metabolites 4-aminoantipyrine, 4-acetylaminoantipyrine and 4-formyl-aminoantipyrine have been detected in WWTP effluents and surface waters [49].

Gros et al. [15] increased the number of compounds simultaneously detected to 81 (including metabolites 2-hydroxycarbamazepine, 10,11-epoxycarbamazpine, acridone, norfluoxetine, desloratadine, azaperol, hydroxy-metronidazole and norverapamil) in both environmental waters (drinking, ground, sea and surface) and wastewaters. Most of the compounds (rv70%) yielded relative recoveries higher than 70% in surface waters. Some substances (e.g., hydrochlorothiazide, salbutamol, losartan, thiabendazole, metronidazole and hydroxy-metronidazole) were practically not recovered. This highlights the difficulty of obtaining good extraction efficiencies for all compounds when there is great chemical variability among them. Carbamazepine metabolites were detected at higher concentrations than the parent compound in surface waters, seawater and wastewaters. Metabolites norfluoxetine, desloratadine, azaperol, hydroxy-metronidazole and norverapamil were not detected in any type of sample.

Of a total of 90 pharmaceuticals, 52 not previously determined in water samples were included in an analytical protocol developed by Grabic et al. [50]. Pharmaceuticals were chosen based on their potency (effect/concentration ratio) and potential to bioaccumulate in fish. The recoveries of the method for real matrices were in the range 40–130% for surface waters (except for glibenclamid, glimepirid and meclozine).

In a study by De Jongh et al. [49], 17 common pharmaceuticals and nine TPs were determined in surface and drinking waters (from treated surface or ground waters). All the TPs (two derivatives from metamizole and one from phenazone, O-desmethyltramadol, Carbamazepine-10,11-epoxide, O-desmethylvenlafaxine) were found in surface waters. In drinking water (aerated, filtrated over active carbon and disinfected with UV light in the laboratory), no pharmaceuticals could be quantified.

A method developed for the analysis of 15 top prescribed pharmaceuticals in Belgium and four of their metabolites in influent wastewater [51] reported, for the first time, the presence of telmisartan, enalaprilat and perindoprilate (metabolites of enala-

Table 1.3
Main methods utilized for the analysis of LIDs in solid samples.

Compounds	Matrices	Pre-treatment	Extraction	Solvent	Clean-up	Recovery (%)	LOD (ng/g)	Detection	Ref.
43 pharmaceuticals	Sd	lyophilized	PLE	MeOH-water (1 + 2)	SPE (HLB)	33–206	0.01–3.2	LC-QqLIT-MS/MS	[31]
32 pharmaceuticals	Sd, So	Air-dried (30 °C)	PLE	MeOH/water/NH_4OH (pH = 9)	SPE pH = 7(MAX-HLB)	PLE: 60–114 SPE: 33–87	0.1–2.1	LC-MS/MS	[32]
17 pharmaceuticals	Sd, So	lyophilized	PLE	water	SPE (HLB)	62–119	0.1–6.8	LC-MS/MS	[33]
21 pharmaceuticals	Sd, So, Sl	lyophilized	MAE	MeOH/water (3 + 2)	automated SPE(HLB)	91–101	0.0008–0.0051	GC-MS	[34]
12 pharmaceuticals	Sd	Air-dried	MSPD (C18)	Acetonitrile/5% oxalic acid (60:40)	–	47–119	0.1–500	LC-MS/MS	[35]
5 FQs	Marine sediment, Sl	Air-dried	MAME	Water 5% HTAB	–	73–96[a]	0.15–0.55[a]	LC-MS/MS	[36]
50 antibiotics (11 classes)	Sd, manure, Sl, water	Freeze-dried	US	ACN and citric buffer (pH 3)	SPE (SAX + HLB)	20–284[a]	0.19–1.75[a]	LC-MS/MS	[37]
18 antibiotics (FQs, TCs, SAs)	So	–	US	Potassium phosphate/ACN (50/50, pH 3.2)	SPE (SAX + HLB)	56–99	0.1–8.9	LC-MS/MS	[3]
9 sulfonamides, 7 TCs	So, water	–	PLE	Water/MeOH/acetone (50/25/25) and 25 mM EDTA, NaCl and 2%NH_4OH	SPE pH4(HLB)	22–115	0.01–1	LC-MS-MS	[38]
4 SAs	So	Air-dried	DMAE	ACN for DMAE and 0.3% acetic acid for SPE extraction	Online SPE (neutral alumina)	83–94	1.4–4.8	LC-MS-MS	[39]
22 SAs (5 acetylated metabolites)	So, Sl	Freeze-dried	PLE	Sl: ACN-water (25:75) 50°C So: MeOH-water (90:10) 100°C	SPE (HLB)	60–130	0.01–4.19[b]	LC-QqLIT-MS/MS	[40]
4 illegal drugs and 8 pharmaceuticals	Sd, Sl	Freeze-dried	PLE	MeOH with 0.1% formic acid	Centrifugation	56–128[a]	1–50[a]	UHPLC-MS-MS	[41]
Amphetamine	Sl	Centrifugation	US	50 mM formic acid/MeOH (80/20)	SPE pH10 (HLB)	–	–	LC-MS-MS	[42]
60 Pharmaceuticals and illicit drugs	So, WW particulate matter	Centrifugation, filtration and dried (40°C)	PLE	MeOH/water (1/1)	SPE (MCX)	13–145[b]	0.01–1.3[b]	LC-MS-MS	[43]

DMAE, Dynamic microwave-assisted extraction, Sd, sediment; Sl, sludge; So, soil.
[a] Data in sediments.
[b] Data in soils.

pril and perindopril respectively), and the major carboxylic metabolites of clopidogrel and losartan in water systems.

Cation exchange (e.g., Oasis-MCX) and hydrophilic-lipophilic sorbents (e.g., Oasis HLB) have commonly been chosen for the analysis of illegal drugs with similar results [18,19,23]. Pedrouzo et al. [18] tested four SPE adsorbents (Oasis HLB, Oasis MCX, Strata-X, Strata-XC, all with 200 mg) at two different sample pH (3 and 7) for the extraction of five drugs of abuse and four metabolites from 500 mL of surface waters. In Strata-X and Oasis HLB, elution was performed with 10 mL of methanol, whereas, in Strata-XC and Oasis MCX, it was with 8 mL methanol (5% NH_4OH). The best performance was obtained with Oasis MCX, without practically any influence of the pH of the sample on the recoveries of compounds. Only 11-nor-carboxy-K^9-tetrahydrocannabinol (THC-COOH) was not satisfactorily recovered (38%). This low recovery was not observed by Zuccato et al. [52], who, utilizing the same cartridge, obtained a 69% extraction efficiency for this compound. The reason for the low recovery [18] could be the quantity of analyte in the spiked sample (5000 ng against 25 ng), but also the amount of cartridge sorbent (200 mg against 60 mg). An excessive bed weight has already been related to lower recoveries for benzoylecgonine [44].

Other comparative studies of a broad range of solid sorbents corroborated the previous results. Oasis HLB and Oasis MCX gave the highest recoveries for the majority of compounds [19]. However, MCX needs acidic pH for extraction (which promotes the storage stability of the studied compounds) and the possibility of using organic solvent acidified for washing step to remove acidic and neutral compounds (providing cleaner extracts than HLB) [53]. The worst recoveries are always obtained for THC and THC-COOH. These compounds are non-polar, so the polar solvents used commonly as eluent do not provide good recoveries [19,20,30,52]. This problem has been settled by Gonzalez-Mariño et al. [21] using ethyl acetate followed by acetone to extract 14 compounds successfully (including the following metabolites: cocaethylene, THC-COOH and benzoylecgonine) from an Oasis HLB, obtaining recoveries of 97% for THC and 93% for THC-COOH.

Other preconcentration techniques (e.g., molecularly-imprinted polymers) have shown high selectivity in retaining specific target analytes and their effectiveness in reducing co-extracted matrix compounds, but they have not often been utilized because multi-residue analysis is not achievable using them.

2.2.2. On-line SPE

On-line preconcentration of LIDs in an SPE column means minimal sample handling in one of the most time-consuming tasks in water analysis. Although its tune up is more laborious than in the off-line approach, it allows a great saving of time if it is going to be used as a routine method. If the SPE column has a highly retentive sorbent, the mobile phase might not have enough strength to elute the analytes trapped in the SPE, since reversed-phase usually starts with low levels of organic solvents. Another problem is the widening of the peaks, which would lead to integration problems and increase the LOD of the analytes.

López-Serna et al. [25] developed a multi-residue method for 74 pharmaceuticals, passing 2.5 mL of the water sample with 0.1% EDTA through a HySphere Resin GP cartridge (after comparing extraction efficiency with PRLP-s and Oasis HLB). Absolute recoveries achieved were in the range 50–150% for the 61% of target compounds in surface water. For polar compounds (e.g., atenolol), low absolute SPE recoveries were obtained. However, the use of 51 surrogates significantly increased the percentages of compounds with relative SPE recoveries around 100%.

Columns packed with restricted access material (RAM) remove macromolecules by the size-exclusion mechanism. Only small molecules are able to penetrate into the pores of the RAM and interact with a stationary phase bonded on the inner surface, while large molecules are discarded with the washing solvent. Using this on-line technology, an SPE-LC-MS/MS method was developed for the determination of four macrolides (erythromycin, roxithromycin, tylosin and tilmicosin) [27]. Matrix effects were not observed up to 100 mg/L of humic acid. Recoveries were higher than 87–98% and LODs of 2–6 ng/L.

Turbulent flow chromatography (TFC) involves a new advance using RAM, combining size-exclusion and chemical-selectivity mechanisms. This new technology utilizes a column (TFC column) filled with big particles of 30 μm, which are supplied with different chemical properties, depending on the type of column. When sample arrives at this column, only small molecules will diffuse into the pores. Choosing the appropriate chemistry of the cartridge particles, only the small molecules of interest will bond to the cartridge, while the rest of the small molecules will be flushed to waste with the mobile phase. This system was applied for the first time to determine 58 pharmaceuticals and 19 metabolites and TPs in environmental aqueous samples [1]. Due to the large number of analytes determined in this method, three cartridges (with different chemical properties) were used. Relative recoveries in surface water were higher than 70% for 67 compounds.

A variant of the typical on-line method involves doing sample preconcentration and chromatographic separation in the same column. This ingenious method was applied to the analysis of four ionophore antibiotics and two avermectin antiparasitics [28], all highly hydrophobic. Recoveries were 91–120% with LODs of 1–7 ng/L. This method is limited to analytes of similar polarity to prevent premature elution of the most polar while part of the sample is still arriving to the column.

For illicit drugs, Postigo et al. [30] developed an on-line method for wastewaters what was further extended to the analysis of non-wastewater samples with good results [54]. In this method, 5 mL of the water sample is passed through a PLRP-s cartridge for analytes measured in PI mode (all but cannabinoids) and through an Oasis HLB cartridge for analytes measured in NI mode. Only THC was almost unrecovered.

To date, there is only other published on-line SPE method to analyze drugs of abuse in surface waters [29]. In this method, on-line SPE is coupled to hydrophilic interaction chromatography tandem MS (HILIC–MS/MS) to determine cocaine, morphine, codeine and metabolites (benzoylecgonine, 6-acetylmorphine and dihydrocodeine) and two pharmaceuticals (trimethoprim and atenolol). The HILIC technology is based on a hydrophilic column eluted with high percentages of organic solvent. This resolved some of the drawbacks of on-line SPE, achieving low column back pressure and increasing ionization efficiency in the electrospray chamber and retention of the high polar analytes, thus providing narrower peaks. The method yields recoveries of 78–102% in river waters.

2.2.3. Solvent-less methods

The consumption of solvents makes analysis more expensive. This is one of the reasons why liquid liquid extraction (LLE) was falling into disuse. LLE has been replaced by miniaturized liquid-extraction procedures. Using only 2 μL of organic solvent and 1 μL of the derivatization reagent, Zhang et al. determined four acidic compounds (ibuprofen, ketoprofen, naproxen and clofibric acid) in water samples using dynamic hollow-fiber liquid-phase microextraction followed by GC injection-port derivatization and MS determination [55]. Relative recoveries were close to 100%, LODs were 10–50 ng/L.

In the same way, 4-isobutylacetophenone, a toxic metabolite of ibuprofen, was extracted and analyzed in river and sewage waters using hollow-fiber microporous membrane LLE and GC-MS. LODs were 7 ng/L and 14 ng/L, respectively, with high repeatability and reproducibility [56].

Two cannabinoids, THC and THC-COOH, were extracted from surface water and wastewater samples using SPME and determined by GC-MS, after derivatization with N-methyl-N (trimethylsilyl)trifluoroacetamide (MSTFA) [57]. Satisfactory precision (RSD <3%) and trueness (94–96% relative recovery) in surface waters, and LODs at low ng/L were achieved (1.0 ng/L and 2.5 ng/L for THC and THCCOOH, respectively).

Direct injection (DI) of samples in a spectrometer offers several advantages (i.e. increase in sensitivity and minimal cost and sample handling) since no SPE cartridges are utilized. This fast, accurate alternative to both off-line and on-line SPE has been applied to the simultaneous analysis of 12 drugs of abuse and their metabolites in wastewater, river and lake water samples [58]. Using HPLC-MS/MS as the characterization technique, most drugs could be detected with limits of quantification (LOQs) of 0.2 ng/L in surface water and recoveries of 92–138%.

2.3. Extraction of solid samples

Sediment and soils act as reservoirs for LIDs in the aquatic environment. In general, soils have less organic matter content, which might result in slightly better efficiency of extraction and a lower matrix effect [32,33]. Even for those compounds having a polar character, some specific interactions (e.g., cation exchange, cation bridging, and surface complexation) bind them chemically with organic matter and clays, complicating their extraction (e.g., FQs) [3]. Depending on the pH of the extraction solvent, distribution-ratio K_d of the compounds can vary considerably. The log K_{ow} of ionizing compounds changes considerably in a pH range around the pK_a (e.g., amphoteric sulfonamides increase their adsorption to the soil when pH decreases, relating to their ionization) [35].

Analytes from solid samples are generally extracted using one of these techniques:

- pressurized liquid extraction (PLE) [31–33];
- microwave-assisted extraction (MAE) [34];
- ultrasonic extraction (US) [3,37];
- matrix solid-phase dispersion (MSPD) [35]; or,
- microwave-assisted micellar extraction (MAME) [36].

Off-line SPE is usually performed for purification and preconcentration. Before SPE, the organic-solvent content of the extract has to be reduced to less than 5% to prevent early breakthrough of analytes from the cartridges.

MAE generally provides higher recoveries, but special care with temperature and irradiation time is required to avoid degradation of analytes. High microwave powers, in combination with long times, accelerate decomposition of pharmaceuticals, as also reported for clofibric acid, metoprolol and propranolol [34].

The main methods utilized for the analysis of LIDs in solid samples and their performance are outlined in Table 1.3.

Due to the novelty of the analysis of illicit drugs in the environment, there is only one method that analyzes these compounds in river sediments and sludge. Amphetamine, benzoylecgonine, cocaine, and methamphetamine were extracted from sediments using methanol with 0.1% formic acid using an accelerated solvent extraction (ASE) system and subsequent centrifugation [41]. Recoveries were in the range 85–131%, with LODs of 2–5 ng/g. There are few optimized methods yet for the analysis in soils, but analysts could pay attention to methods already published for:

- sewage sludge using sonication with formic acid and methanol followed by adjustment of the pH to 10 and clean-up with Oasis HLB [42]; or,
- suspended particulate matter using PLE before acidic adjustment and SPE preconcentration with Oasis MCX cartridges (also covers method validation in soils) [43].

In pharmaceutical analysis, very high recoveries (>92%) were obtained using microwave and continuous SPE clean-up with Oasis HLB to extract 18 compounds (analgesics, antibacterials, anti-epileptics, b-blockers, lipid regulators and non-steroidal anti-inflammatories) from soils, sediments and sludge [34]. Elution for cartridges was automated using two valves with ethyl acetate. After silylated derivatives were added, extract was injected by GC-MS to obtain really good LODs (0.8–5.1 pg/g) with very low RSDs.

In a multiclass method, Jelic et al. extracted 43 pharmaceuticals in sediments and sewage sludge, using PLE and methanol/water 1:2 (v/v) as extraction solvent [31]. Clean-up was by SPE with Oasis HLB (200 mg), and elution with methanol. Recoveries in sediments were 67–206% for all compounds except acetaminophen, atorvastatin, sulfamethazine, metronidazole and butalbital. Due to the great complexity of the specific sediment-pharmaceutical interactions, it is difficult to explain the low recoveries obtained for these compounds. For example, despite the hydrophilic nature of sulfamethazine (Log K_{ow} = 0.27), the distribution ratio K_d at pH 5 was up to 10^4 times greater than K_{oc} reported in the literature [59], which shows sulfamethazine has a great tendency to remain bound to the sediment.

PLE with methanol and aqueous ammonia solution (pH = 9) was utilized by Perez-Carrera et al. to extract 32 pharmaceuticals from agricultural soils and sediments [32]. Extracts obtained from PLE were passed through two SPE cartridges (MAX-HLB) at pH = 7. Subsequently, the HLB and MAX cartridges were dis-assembled, air-dried and washed with heptane. Then, the MAX cartridge was eluted with ethyl acetate, methanol and methanol containing 2% acetic acid; the HLB cartridge was eluted with ethyl acetate and methanol. Recoveries below 50% were obtained for citalopram, omeprazole, oxazepam and prednisolone. LODs were in the range 0.1–2.1 ng/g.

A green PLE method using EDTA-washed sand to disperse the solid sample and water as extracting solvent was developed for 17 pharmaceuticals in soils and sediments [33]. Two cartridges in tandem (SAX and HLB) were used for clean-up. Recoveries were higher than 70% except for those compounds with Log K_{ow} higher than 4.5 (fenofibrate and diclofenac), which were not satisfactorily extracted with this highly-polar solvent.

MSPD was applied to the extraction of 12 pharmaceuticals from sediments [35]. Samples were blended with C18, packed and eluted with acetonitrile acidified with oxalic acid. Recoveries were over 80% with the exception of roxithromycin (47%). This method is a good choice to analyze pharmaceuticals because of its simple usage, low cost per sample and good recoveries.

Several methods dealing with the analysis of antibiotics have been developed due to their wide use in farm and human medicine. An on-line method coupling dynamic microwave-assisted extraction to an SPE column packed with neutral alumina for the determination of four SAs in soil was successfully developed [39]. The extraction and clean-up carried out simultaneously improved precision and accuracy. The recoveries of SAs were 82.6–93.7%. The disadvantage of this method was that it can be prepared only one sample each time.

Recently, a comprehensive method to determine 50 target antibiotics (belonging to 11 different classes) in sediment, manure and sludge was developed by Zhou et al [37]. Extraction of solid samples was carried out by a combination of ultrasonic and vortex mixing using a mixture of acetonitrile and citric buffer at pH 3, then cleaned by tandem SPE (SAX + HLB). The anionic cartridge (SAX) removed negatively-charged fulvic and humic materials,

avoiding saturation of the polymeric cartridge with organic matter, so that it was more available to retain analytes of interest. After sample loading, the anionic cartridge was removed, and analytes eluted from the polymeric cartridge with methanol. Recoveries higher than 50% were obtained for 38 compounds and method LOQs for the target compounds were in the range 0.6–6.7 ng/g.

García-Galán et al. detected five acetylated metabolites of sulfonamides for the first time in sludge with a PLE method developed for 22 sulfonamides in sewage sludge and soil samples [40]. Purification was carried out by an Oasis HLB cartridge. The recovery efficiencies in soils were found to be over 62% for the majority of the sulfonamides (except sulfacetamide, sulfamethizole, sulfamethoxypyridazine, sulfapyridine, sulfathiazole and sulfisoxazole). The LOD achieved was in the range 0.01–4.19 ng/g for soil samples.

2.4. Determination

LIDs have a polar character, which makes them more suitable for analysis by LC than GC, as they do not require a derivatization step prior to analysis. To achieve sensitivity required to detect low concentrations of these substances in the environment, LC is usually coupled with tandem MS, although other detectors are still in use {e.g., diode array (DAD) [60] and fluorescence (FD) [5,24]}.

A relatively new development of LC is the use of apparatus and analytical columns that tolerate high pressures. This technique, known as UHPLC, uses columns with sub-2-μm-diameter particles, improving chromatographic performance [e.g., speed, sensitivity and resolution, reducing co-elution of interferences (and therefore diminishing matrix effects)] when compared to methods established with conventional HPLC. This technology has been applied to the analysis of drugs of abuse, reducing the time for chromatography to 6–8 min [23]. In pharmaceutical analysis, 81 compounds were determined in two injections of 5 min (137 transitions acquired in PI mode) and 2 min (52 transitions in NI mode) [15]. Gracia-Lor et al. determined 47 compounds in a single injection of 10 min [17]. These fast methods result in the capacity to process more samples per day and to save solvent. Fig. 1.2 highlights the superiority of UHPLC against HPLC, achieving the same resolution in a fifth of the time. This results in higher throughput and greater availability of the apparatus. New alternative to the use of sub-2 μm columns are fused-core-particle columns, which can be used in conventional LC equipment. These columns are designed to provide low back-pressures at high flow rates, which result in high efficiencies and shorter analysis times. They have been successfully applied to the analysis of drugs of abuse [18,29].

In MS/MS apparatus, two transitions are usually monitored for each compound, except for ketoprofen, ibuprofen and salicylic acid, which only give one due to their poor fragmentation [17]. To solve this confirmation problem, some authors proposed the use of tandem quadrupole-linear ion trap (QqLIT) instruments, performing an information-dependent acquisition (IDA) experiment [15,31]. It involves monitoring one selected reaction monitoring (SRM) transition per compound as a survey scan in combination with an enhanced product-ion scan (EPI) as a dependent scan. Compound identification was carried out by mass spectral library search, matching the EPI spectra against commercial or user-created libraries.

Measuring erythromycin-H_2O (ETM-H_2O) (m/z 716) instead of erythromycin (m/z 734) is a common approach when macrolides are extracted at low pH, as ETM-H_2O is a degradation product in acidic conditions as well as existing in the aquatic environment [48].

High-resolution MS (HRMS) is a powerful tool in pharmaceutical analysis due to the possibility of executing full-scan analysis and accurate-mass measurements, allowing the identification and the elucidation of unexpected compounds, metabolites and degradation products. LC-quadrupole time-of-flight (QTOF)-MS is also a powerful technique for large-scale screening of compounds, including parents and metabolites, as revealed for antibiotics [61], and illicit drugs [62]. Due to its superior resolving power, LC-QTOF-MS distinguishes isobaric co-eluting interferences from the signals of interest in complex matrices, achieving a reliable identification of the parent compounds. Furthermore, it allows retrospective analysis to identify non-target compounds (not included in the first screening), without the need for additional injection of sample extracts.

Non-target analysis of pharmaceuticals by UHPLC-QTOF-MS detected, for the first time in environment, 4-(3-methylphenyl) amino-3-pyridinesulfonamide (a key intermediate used in torsemide synthesis) and desmethylazithromycin (a by-product in azithromycin production) [63]. Fig. 1.3 shows the product-ion spectrum of desmethylazithromycin, which coelutes with azithromycin. Both compounds only differ in one -CH_2 group.

Recently, the use of HRMS also helped to discover that other metabolites of dypirone (4-formylaminoantipyrine and 4-acetylaminoantipyrine) produced 4-amino antipyrine via in-source fragmentation, and therefore shared the same transitions [64]. The examples shown illustrate the information attained using full-spectrum acquisition HRMS and that suitable chromatographic separation to reduce the possibility of bi-isobaric co-eluting 'interferences' when investigating metabolites cannot be underestimated.

The quantitative capabilities of the Orbitrap mass analyzer were recently utilized in the analysis of 24 drugs of abuse and relevant metabolites with satisfactory results [65].

Dealing with matrix effects is possibly the most difficult challenge in the analytical determination of polar compounds by LC-MS/MS. Matrix effects are particularly marked when ESI sources are utilized, being less pronounced with other sources [e.g., atmospheric-pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI)]. However, these other ionization sources are rarely utilized. Common strategies to remove matrix components are extensive clean-up steps after extraction, or to compensate for quantification errors using labeled surrogate standards, preparation of a matrix-matched standard curve [26,33,35,50] or single-point standard addition [38].

GC-MS analysis provides low LODs as good as LC-MS/MS, together with a very good resolution for the analysis of LIDs. However, the drawback of requiring previous derivatization of compounds has not helped to extend its use. It has been successfully utilized to determine drugs of abuse [21], multi-class pharmaceuticals [34] and NSAIDs [14]. Commonly-used derivatization reactions are silylation [21,34] and alkylation [14]. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was utilized as derivatizing reagent to analyze 18 acidic, neutral and basic pharmaceuticals [34]. Gonzalez-Mariño compared the performance of three derivatizing agents to analyze drugs of abuse {i.e. N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), BSTFA and N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) [21]}. Among them, the last two reagents failed to derivatize some of the aliphatic hydroxyl and the amine groups. Derivatization has also been carried out on-line in the injection-port using a large-volume (10 μL) sample injection with tetrabutylammonium (TBA) salts, achieving very reproducible results, with RSD in the range 1–10% [14].

3. Occurrence of LIDs in protected areas of the environment

As result of the high quantity of drugs of LIDs consumed, and the poor elimination of some of them in WWTPs, these substances reach rivers, lagoons and even protected areas of the environment

(e.g., estuaries, natural wetlands, and deltas). They are of particular interest due to their ecological and economic importance, as they support a great variety of endemic flora and fauna. In some wetlands, for example, these protected areas assure directly the needs of millions of people through rice farming [2]. Table 1.4 shows the existing literature dealing with the presence of pharmaceuticals in these protected areas. These studies are examples of the field application of the methods reviewed above. Researchers have usually analyzed illicit drugs in sewage-treatment plants to estimate the consumption of these substances in the population [8,54].

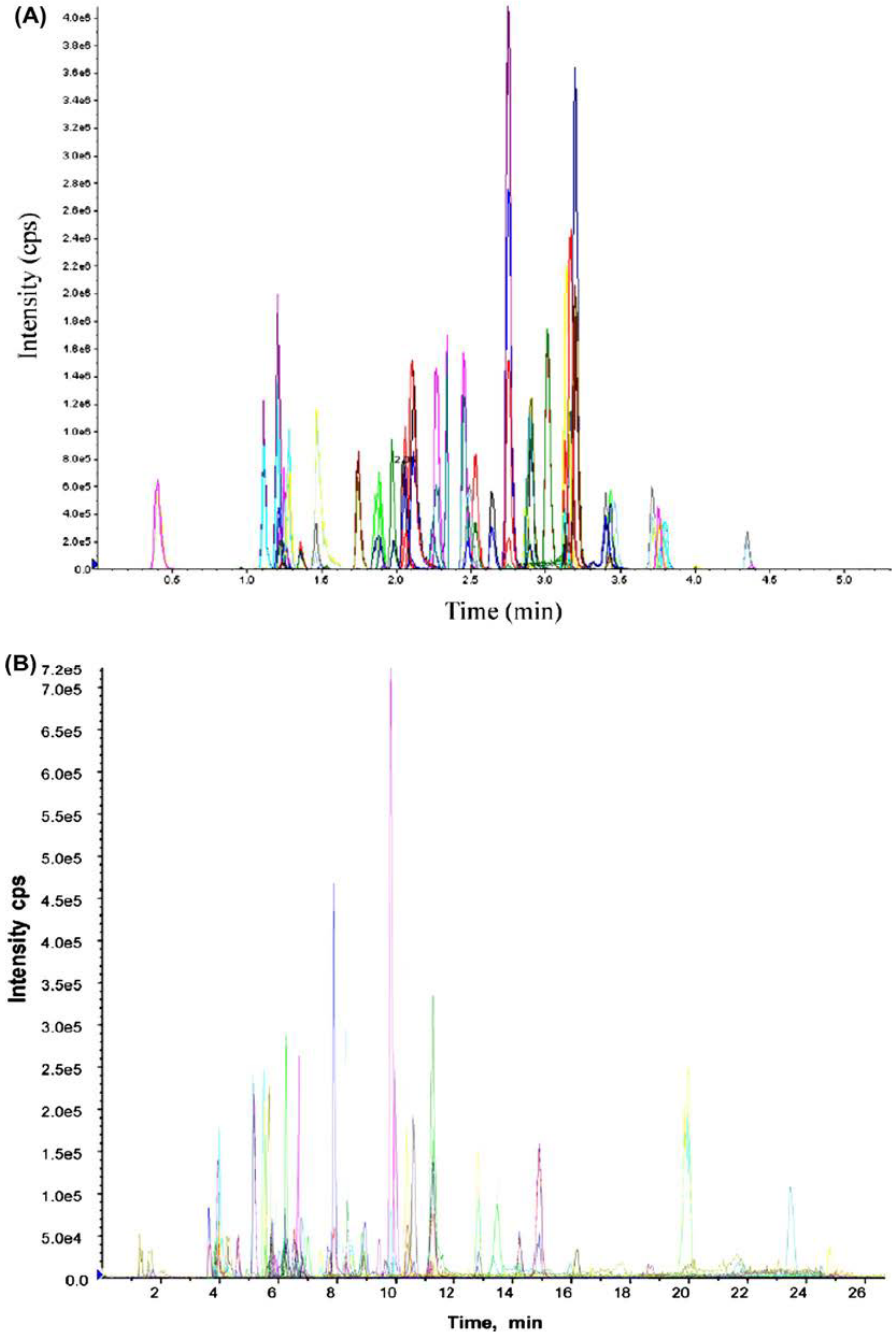

Fig. 1.2. Chromatograms in positive ESI mode of a standard mixture of pharmaceuticals: (A) 137 transition analyzed in less than 5 min with UPLC {[15], with permission}; and, (B) 146 transitions in 26 min with HPLC {[25], with permission}.

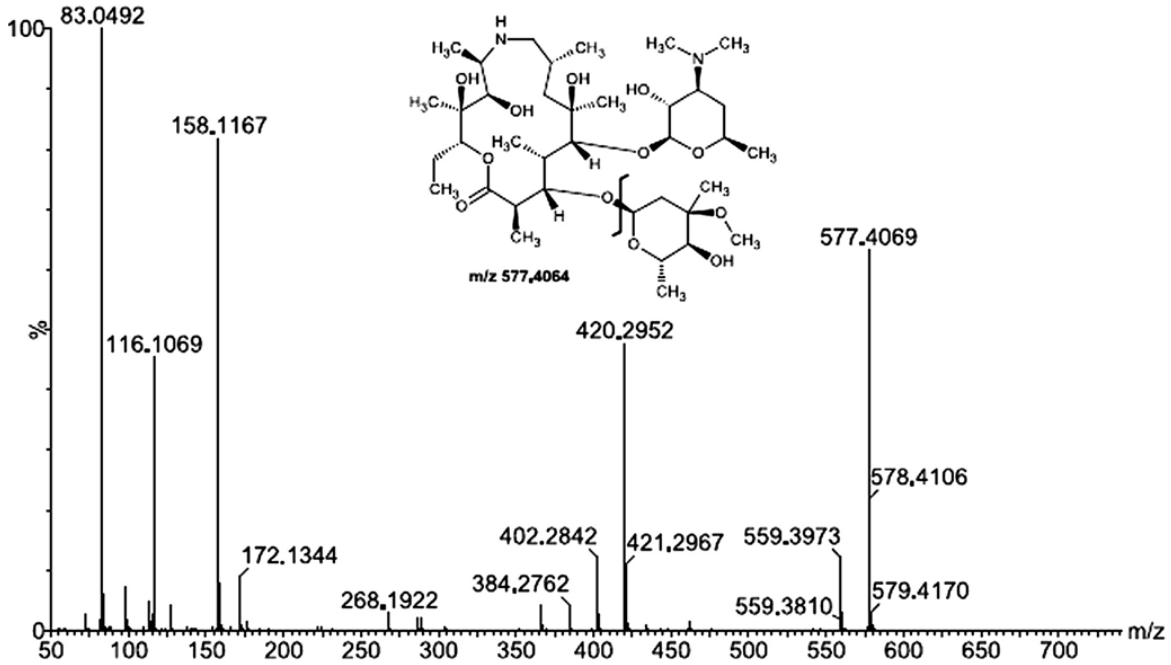

Fig. 1.3. Product-ion mass spectrum of desmethylazithromycin, reported for the first time in the environment, as reported by Terzic et al. [63].

Table 1.4
Mean concentration (ng/L) of pharmaceuticals monitored in wetlands and other protected areas of the environment

Wetland/ Substance	Pego-Oliva wetland (Spain)	L'Albufera wetland (Spain)	Doñana Park watershed (Spain)	Douro Estuary (Portugal)	Yangtze Estuary (China)	Pearl River Delta (China)	Ebro Estuary (Spain)
Carbamazepine	6	10	260	178	27		0.3–0.8
Clofibric acid	0.5	9	N.D.			8.6	0.1–0.3
Diazepam	2	2		4			N.D.
Diclofenac	1	39	90		N.D.	9.4	1–7
Fenofibrate	4	N.D.					N.D.
Furosemide							N.D.
Gemfibrozil			1490				8.2
Ibuprofen	16	290	3540			4.9	30
Indomethacine					226		0.2–1
Ketoprofen			N.D.				1060
Naproxen			1290			N.D.	29
Propranolol	2	2	310	3	13		
Sulfamethoxazole	2	27	N.D.	53	176		
Trimethoprim	0.1	8	N.D.	16			N.D.
Reference	[2]	[67]	[60]	[68]	[69]	[70]	[71]

N.D., not detected.

The presence of these substance in protected areas is poorly reported [19,66], so they are not included in Table 1.4. These studies revealed that heroin, 3,4-methylenedioxyamphetamine (MDA), methamphetamine and THC were not detected in these areas. Cocaine and its metabolite benzoylecgonine were frequently de- tected, together with methadone and 3,4-methylenedioxy-N- methamphetamine (MDMA). THC-COOH, the major metabolite of THC, was scarcely detected, as reported by other authors [52,54]. THC-COOH usually has bad recoveries due to its high hydrophobic- ity, which could explain the small number of positive samples.

Unlike illicit drugs, pharmaceuticals are widely studied in protected areas. In order to compare their concentrations in different areas studied, only compounds common to several studies are shown in Table 1.4. Carbamazepine, propranolol and ibuprofen were found in all sampling sites, ibuprofen having the highest concentrations. Some studies showed elevated concentrations of pharmaceuticals [e.g., Doñana Park (Spain) and Yangtze Estuary (China)]. In these areas, sample points are specially affected by various WWTPs, which discharged water upstream from where the samples were taken. At the same time, the effectiveness of a WWTP is greatly influenced by the types of treatment, the temperature and the quantity of water (strongly dependent on the season) and the input load of contaminants. So the moment when the sampling campaign is done and the WWTP characteristics, the manner which samples are taken (24-h composite or grab samples) and the analytical characteristics of the method utilized have critical roles in the results, so it is difficult to make comparisons among different studies.

4. Conclusions and future trends

Pharmaceuticals and illicit drugs are usually analyzed by methods not including illegal drugs, although they could be determined together [29,13]. Different types of technique have been described for their extraction (i.e. SPE both on-line and off-line, and solventless methods).

Multi-residue methods are able to analyze dozens of compounds in a single injection selectively and reliably. However, because of the disparity of their polarities and acidic properties, it is not always possible to achieve high efficiency in their extraction from the matrix. However, due to the appearance in the market of new, more sensitive spectrometers, low recoveries are not an obstacle to detecting LIDs in samples. Besides, the development of sub-2-μm and fused-core particle columns has helped to obtain high sensitivity and to decrease enormously the chromatographic time. As a consequence, we have a lower cost per sample and a higher throughput. Moreover, new on-line methods have been developed for water samples, saving the time of the analyst once the method is ready for its application.

Generally, analytical methods rarely included metabolites and TPs of pharmaceutical compounds, with clofibric acid, salicylic acid, fenofibric acid, oxazepam and norfluoxetine being typically included in the methods [32,67]. A list of the degradation pathways of some drugs of abuse can be found in the review of Castiglioni et al. [72]. Usually ecgonine methyl ester and cocaethylene (metabolite of cocaine when is taken with alcohol) are not found in any sample, while benzoylecgonine and THC-COOH gave higher concentrations than their parent compounds.

New TPs of pharmaceuticals have been detected for the first time in the environment thanks to the acquisition of non-target mass spectrometers (QTOF and Orbitrap). For a better understanding of the fate and the effects of LIDs in nature, it is necessary to include metabolites and TPs in the typical target analysis, which is still rare. The monitoring of the emerging compounds is extremely important to assure the quality of our drinking water.

The partition of LIDs between water and sediment gives an approach to the preferred routes that they take to their degradation in the environment and their bioaccumulation potential. However, there are intrinsic mechanisms in biota that could enhance their accumulation or promote their degradation. It is therefore necessary to develop analytical methods in biota from different trophic chain levels in order to obtain a more realistic risk assessment of LIDs, these ubiquitous substances.

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Unlike persistent contaminants, most of pharmaceuticals and illicit drugs are usually rapid transformed in the environment, but because the huge quantities poured into the water systems they can be considered as pseudo-persistent. They are commonly detected at low concentrations in environment, making it difficult to establish ecotoxicological effects. However, absence of effects cannot be discarded, since pharmaceuticals are developed to produce biological effects. Additionally the combined presence of compounds sharing a common mechanism of action could induce synergistic effects. To determine the consequences of the presence of these emerging pollutants in the water environment is a key issue to carry out a risk assessment for the aquatic fauna.

1.1 Pharmaceuticals

Pharmaceutical industry is one of the biggest businesses over the world. According to IMS Health (an international consulting and data services company), in 2010, world pharmaceutical market was valued at US$ 875 billion [SESRIC Report 2010]. According to data supplied by the European Federation of Animal Health [FEDESA Report 2001], in 1999 there were a total of 13,216 ton of antibiotics used in the European Union and Switzerland, 65% of which was applied in human medicine, and the rest in animals, agriculture and aquaculture [Kümmerer 2009]. In livestock facilities, antibiotics are commonly employed at sub-therapeutic doses like growth-promoters. This overuse of antibiotics has brought as consequence the development of resistant bacteria. Manure prepared from these farms contains high levels of antibiotics [Hamscher et al. 2002], spreading them when it is applied to agricultural soils. At the same time, sludge coming from hospital and urban waste are contaminated by a cocktail of pharmaceuticals, which could be also applied to soils. They can be washed off from the top soil after rain, reaching ground water and surface waters of rivers and lakes.

Pharmaceuticals are excreted unmetabolized (excretion rate is sometimes over 50% as the parent compound), or as metabolites in urine and feces. Thus, it is expected that metabolites or transformation products would be present in the

environment before they would be fully mineralized (being converted into inorganic salts). A complete elimination of pharmaceuticals is an endpoint difficult to determine it, since in most of the cases, metabolites formed can go unnoticed for a lack of specific methods to determine these compounds. Besides, when they reach WWTPs might be transformed or remain unchanged. Except a few of metabolites, which are well known and usually included in the analytical methods, there is scarce information about their occurrence and behavior in the environment, and which are predominant or have more stability [López-Serna et al. 2012a, de Jongh et al 2012].

Pharmaceuticals can be grouped by either their chemical structure or mechanism of action, but even inside of a group there is a high variability in their solubility, stability and acid-base properties. This makes their full extraction from matrix a great deal, having to reach a compromise to obtain acceptable recoveries for the most of them.

1.2 Illegal drugs

The use of drugs of abuse is increasing worldwide and causes not only a well-known serious social problem but also concern as environmental emerging contaminants. The most consumed drugs of abuse and their metabolites have been determined in the sewage system [Bijlsma et al. 2013], surface waters of natural ecosystems [Zuccato et al. 2008] and even in tap water [Rosa Boleda et al. 2011].
Recently, the measure of levels of illicit drugs or their main metabolites in WWTPs, has been utilized as a quantitative approach to estimate drug abuse in the population [Thomas et al. 2012].

Given the novelty of this issue and the limited research undertaken in this field, there is slender data and minimal understanding of the environmental occurrence, transport and fate of these compounds.

1.3 Toxicity studies of pharmaceuticals and drugs of abuse

Organisms dwell in aquatic media contaminated with pharmaceuticals can suffer changes in their ordinary development. Reproductive alterations, damages in organs and other undesirable effects (even in fishes) have been widely reported [Flippin et al. 2007, Huggett et al. 2002, Oetken et al. 2005]. However, there is not currently any regulation controlling the presence or the maximum levels of these substances in the environment. Different indexes and indicators have been used to assess and detect the loss of water quality and degradation processes of aquatic ecosystems. One of the most recent one is the determination of emerging contaminants and their subsequent assess of the potential risk for the aquatic environment.

Several toxicity studies have been addressed to study the potential hazard of pharmaceuticals for the species of different levels of the chain trophic. Different reviews addressing this issue have been published [Santos et al. 2010, Brausch et al. 2012].

Six weeks exposure assay to low concentrations of ibuprofen result in changes in the pattern of reproduction of Japanese medaka fishes [Flippin et al. 2007]. Less frequently reproduction but with a higher rate of fertilized eggs was observed. In fact, ibuprofen and other non-steroidal anti-inflammatory drug are known to inhibit ovulation in mammals, including humans [Hernando et al. 2006]. In the study conducted by Hoeger et al. (2005) estructural damages in organs and a reduction in the number of red blood cells was observed in trouts after 21 days of exposure to diclofenac, as previously related in other experiment [Schwaiger et al. 2004]. In a 4-week propanolol exposure, the total number of eggs produced by Medaka fishes and the number of viable eggs that hatched were decreased at concentrations as low as 0.5 µg/L [Huggett et al. 2002].

Carbamazepine can act blocking the metamorphosis of some insects at lower concentrations [Oetken et al. 2005]. In fishes, changes in their reproductive patterns [Flippin et al. 2007, Huggett et al. 2002], decreasing of hematocrit levels and damages in kidney, gills and liver have been also reported [Hoeger et al. 2005]. Oxazepam (an

anti-anxiety drug) alters fish behavior, make them more antisocial and voracious [Brodin et al. 2013]. Other pharmaceuticals have showed adverse effects in the growth, longevity and fertility of crustaceans [Dzialowski et al. 2006, Stewart et al. 1998] or endocrine-disrupt effects, being also a possible threat to biota [Ternes et al. 2002]. Implications for human health of the apparition of resistance in bacteria due to the continuous presence of antimicrobials in the aquatic ecosystem are a clear threat [Lee et al. 2008]. Also we are still far to know the toxic effect of the complex mixture of them and of their metabolites.

The biological effects of illicit drugs at low environmental concentrations have not been addressed yet, but because they produce several disorders in humans, their toxicity for aquatic animals cannot be a priori ruled out.

1.4 Risk assessment for aquatic organisms

In order to protect the aquatic ecosystems, the European Medicines Agency (EMEA) following European Directive 2001/83/EC, has created a guideline for the risk assessment of new and existing medicinal products [EMEA 2012a]. Supporting this, a technical Guidance Document (TGD) must be followed to conduct an adequate risk assessment [EMEA 2012b]. For the aquatic compartment, risk evaluation is performed calculating the risk quotient (RQ) as the ratio between predicted environmental concentration (PEC) and predicted no-effect concentration (PNEC). A common practice is substitute PEC values by measured environmental concentrations (MEC), since real concentration data are more accurate. Besides, highest concentrations of pharmaceuticals in the water samples used to be considered (to set in the worst-case scenario).

$$RQ = \frac{MEC}{PNEC}$$

PNEC is calculated by dividing the lowest short-term L(E)C50 or long-term NOEC (no-observed-effect-concentration) value available in the literature by an assessment factor (AF). The AF is an arbitrary factor to consider the uncertainty in the obtained

laboratory toxicity data when it is extrapolated to the field. If the ratio MEC/PNEC is higher to one, an environmental risk could be suspected. In addition to this, a certain risk could be expected for those substances with a RQ between 0.1 and 1. To cover all food chain in the water, RQ is calculated at three different trophic levels of the ecosystem, usually algae, daphnids and fishes. Other species belonging to each trophic level (primary producers, primary consumers and secondary consumers) can be utilized to calculate PNECs according to appendix IV of the TGD. Besides, as recommended in the guideline, blue-green algae (cyanobacterias) have to be utilized to evaluate the effects of antimicrobials as they are more sensitive indicator than green algae.

Due to the complexity and ambiguity of the TGD to clarify when a study can be considered as long-term or not, a widespread approach is the use of data from short-term studies (EC50 or LC50) to calculate PNECs [Vazquez-Roig et al. 2011, Kim et al. 2007b, López-Serna et al. 2012b]. But in order to do a more realistic evaluation of the hazard assessment, ecotoxicity long-term data must be utilized always as possible, since pharmaceuticals are usually in very low concentrations in waters and effects observed in chronic studies could go unnoticed in acute one. In this sense, regarding the large quantity of studies existing for some pharmaceuticals, an ecotoxicity pharmaceutical database is highly advisable to assist in the search for lowest L(E)C50 or NOEC. An almost complete free access database (called WikiPharma) was developed by Molander et al. (2009) and is available through http://www.wikipharma.org/. According to the TGD when using long-term data,it is necessary to know all the long-term studies available for one determined compound to choose the appropriate AF for each trophic level, and this involves a titanic task without the help of this database.

Because the disparity about when a study must be considered as long-term, only a few standardised long-term assays are available. In the risk assessment we <<should preferably follow the test protocols issued by the European Commission, Organization for Economic Co-operation and Development (OECD) or the International Organization for Standardization (ISO)>>, but <<it is recognized that there are other acceptable tests, which are capable of providing an equivalent environmental risk assessment>>, as those standard methods from international recognized institutions

(EPA, AFNOR, etc.) and non-standardised test methods. To consider a study as long-term the next criterion in the duration of the experiments isfollowed: 21 days for fishes, 7 for the daphnid group and 72h for algae (always than other long-term studies were availables for at least one of the other trophic levels, following the instructions of the TGD).

Conclusions obtained from a risk evaluation have to be considered together with their limitations. In one hand the lack of long-term toxicological studies and the unfeasibility to carry out chronic studies during lifespan of the organisms (especially in fishes), but on the other hand since mixture of compounds with the same pharmacological mechanism are present in waters, synergist effects could be expected, could being the real hazard greater than calculated.

Other consideration is that some effects could be acceptable. For example, De Lange et al. reported mobility variations in crustaceans in the presence of ibuprofen [De Lange et al. 2006], and Triebskorn et al. (2007) observed that metoprolol exposition produced structural changes in liver and gills of fishes. Therefore, a balance between human health-benefits and effects in the environment must be carefully evaluated for medical substances, although all dramatic adverse effects are certainly unacceptable [EPA 1991].

1.5 Wetlands

Wetlands have been always related with the development of civilizations, being necessary for their survival. They are areas of immense value in biological and economic aspects. They are unique in biological diversity and they offer multiple benefits constituting a great water reserve for the planet and to produce biomass and nutrients for the trophic chain. They control floods, since they act as sponges, capturing and releasing slowly the rain water. They are fundamental in the recharge of aquifers (groundwaters), controlling the erosion, retaining sediments and nutrients [Mitsch and Gosselin 1993, Papastergiadou et al. 2007]. Besides, wetlands have a filter function, preventing the rise of nitrates, avoiding eutrophication.

Nowadays, the coastal strip in most European countries is the area of most rapid evolution with regards to the social and economical development [Cundy et al. 2006] which is translated in an increasing pressure on these ecosystems. This is specially marked in the Mediterranean wetlands, which have suffered a decline of their extension, being drained and transformed in agricultural or urbanistic areas. In addition to this, the Mediterranean wetlands are even more fragile, because the lack of rains and the increase of temperatures [IPCC, 2008]. The impact of these anthropic and natural phenomena is progressively reflected by the loss of soils and water quality, mainly because of aquifers overexploitation and contamination processes.

1.5.1 L´Albufera of Valencia Natural Park

This park is one of the most important wetlands in Europe, included in the RAMSAR agreement of 2 of february of 1971, being a key point for migratory birds. It has a surface of 21,200 Ha. and is located 12 km south of the city of Valencia. It includes a coastal shallow lagoon, marshlands around it, dunes and pinewoods, surrounded by rice fields in its not urbanized part. The hydrological flow of the lagoon is maintained through almost sixty channels, five mouths of its hydrographical basin (Xuquer hydrological basin) and the treated water of the WWTPs of Pinedo, El Perelló, Palmar, Quart-Benager, Sueca and Albufera Sur. These WWTPs collect sewage water from Valencia City and twelve towns surrounded the Natural Park, discharging approximately 172,000 m^3/day into the lagoon. Besides, it suffers a high impact derived from the numerous industries of the region. Two artificial wetlands situated in the north and south of the Park ("Tancat de la pipa" and "Tancat de Milia" respectively) help to remove some of the contaminants of the waters.

1.5.2 Pego-Oliva Marsh Natural Park

The Pego-Oliva Marsh Natural Park is a humid area with an approx. surface of 1,290 ha. It is found in the extreme south of the Gulf of Valencia, and it is included in

the list of the RAMSAR Convention on wetlands. It has been designated a special protection zone on birds pursuant to Directive 79/409/EEC [Council Directive 1979] and has been funded by the Life Nature programme since 1992. Torrential rains are common in this area, with an annual average of 905 mm. Summer is hot and extremely dry. Several rivers flow into the Marsh, Vedat/Bullens River at the northern and Racons/Molinell River at the southern part of the marsh, connected by a network of irrigation channels. Citrus trees and rice plants are cultivated in the Park, and in the surrounded mountains new houses have been built in the last years. This settlement together with the sewage waters (coming mainly from the Pego WWTP) have increased considerably the impact over the Marsh.

1.6 Legislation

Drugs and pharmaceuticals have gained the attention the attention of scientist and policy-makers, since they are not necessarily persistent but hydrosoluble, being found in all water systems.

The Water Framework Directive (WFD) takes an integrated approach to water policy that focuses on water management at river basin level [WFD 2000]. It demands that must be available a River Basin Management Plan in all River Basin across the EU, in order to reach a good ecological and chemical quality in water bodies by the end of 2015. The WFD has settled a short list of priority substances that pose a significant risk to the EU aquatic environment, however there are none pharmaceuticals in this list. In order to achieve good chemical status, water bodies must meet the Environmental Quality Standards (EQS) set for these substances, the most dangerous of which are identified as priority hazardous substances (PHS). In 2011 a widening of this list was proposed, including 17alpha-ethinyl estradiol, 17beta-estradiol and diclofenac as priority substances [COM 2011]. Other substances (as carbamazepine, clofibric acid, etc.) are candidates to belong to future lists.

Pharmaceutical registration and marketing are exempted from the REACH Regulation and, currently, a procedure to establish their possible environmental

impact is not required. The dumping of these substances cause health and environmental problems not enough studied yet.

Illegal drugs have not a legal trade yet, being difficult with this situation take some control on their consumption and presence on waters.

CHAPTER 2

Analysis of tetracylines in soils

Scientific publication 2:

Determination of tetracycline residues in soil by pressurized liquid extraction and liquid chromatography tandem mass spectrometry.

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ORIGINAL PAPER

Determination of tetracycline residues in soil by pressurized liquid extraction and liquid chromatography tandem mass spectrometry

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Abstract An optimized extraction and cleanup method for the analysis of chlortetracycline (CTC), doxycycline (DC), oxytetracycline (OTC) and tetracycline (TC) in soil is presented. Soil extraction in a pressurized liquid extraction system, followed by extract clean up using solid-phase extraction (SPE) and tetracycline determination by liquid chromatography tandem mass spectrometry (LC-MS/MS) provided appropriate efficiency and reproducibility. Different dispersing agents and solvents for soil extraction and several SPE cartridges for cleanup were compared. The best extraction results were obtained using ethylenediamine tetraacetic acid-treated sand as dispersing agent, and water at 70 °C. The most effective cleanup was obtained using Strata-X™ sorbent in combination with a strong anion exchange cartridge. Recoveries ranged from 71% to 96% and precision, as indicated by the relative standard deviations, was within the range of 8–15%. The limits of quantification (LOQs) by using LC-MS/MS, based on signal-to-noise ratio (*S/N*) of 10, ranged from 1 μg kg^{-1} for TC to 5 μg kg^{-1} for CTC. These results pointed out that this technique is appropriate to determine tetracyclines in soils. Analysis of 100 samples taken in the Valencian Community revealed that, in soil, up to 5 μg kg^{-1} CTC, 15 μg kg^{-1} OTC, 18 μg kg^{-1} TC, and 12 μg kg^{-1} DC could be detected. Detection of the analytes in several samples, which typify great part of the Spanish agricultural soils, should be outlined as most important result of this study.

Keywords Antibacterials · Tetracyclines · LC-MS/MS · Pressurized liquid extraction · Environmental analysis · Soil

Introduction

Pharmaceutical residues in the environment are of increasing concern worldwide because of the large number of drugs used in human and veterinary medicine [1–4]. After excretion, these drugs and their metabolites reach the environment by passing sewage treatment plants or by soil amended with sewage sludges or manures [5–7]. New investigations show that more than 45 different drugs can be found in surface waters from the low to the very low micrograms per liter concentration range [8–10].

Among the first groups of antibiotics to come into use in human beings was the tetracyclines (TCs); these drugs have stood the test of time and continue being useful in treating a broad range of infections [11]. TCs have been found widely disseminated in water and sewage. However, little is known about their occurrence and impact in soils. Some data extracted from the literature show that residues of the commonly used veterinary drugs, tetracycline, and chlortetracycline (CTC) can be detected in soil surface (0–40 cm) fertilized with animal slurry [12–15]. Nevertheless, no leaching of TCs into deeper soil segments or groundwater has been observed [14, 15]. The half-life of oxytetracycline

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(OTC) in manure was 30 days, and the compound was still detectable in this matrix after 5 months. In the manured soils, OTC was detected at concentrations at least ten times lower than the European Agency for the Evaluation of Medicinal Products threshold (100 μg kg^{-1}) [16].

Detection of TCs in soil, sediments, and water and the growing concern of their potentially adverse effect on natural ecosystems have resulted in a need to understand their behavior in the soil system. TCs have multiple ionizable functional groups at environmentally relevant pH values. They may exist as cations, zwitterions, or net negatively charged ions, which complicates predicting their sorption, availability, and transport. The sorption of OTC, TC, and CTC by several soils varying in pH, clay content and type, cation exchange capacity, anion exchange capacity, and soil organic carbon was investigated. Strongest sorption was observed for clays, followed by humic substances, and then clay–humic complexes [17, 18]. The greater sorption in the Ca systems than in the K ones and the decreased sorption with increasing pH suggested that cation bridging and charge neutralization contribute to sorption. [19–21].

Summarizing, TCs enter in the environment in significant concentrations via repeated amendments with manure or sludges, build up persistent residues, and accumulate in soil. Therefore, TCs may have a potential risk, and investigations on their environmental effects are necessary [22].

In a recent review, O'Connor and Aga [7] discuss strategies for sample preparation, extraction, cleanup using solid-phase extraction (SPE) and molecularly imprinted polymers, as well as analysis of TCs and their transformation products in soils. This review also points out that available information about the environmentally relevant concentrations of TCs is limited, mostly due to analytical difficulties encountered when trying to analyze trace levels of these compounds in complex matrices. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become widely used in detecting antibiotics, including TCs. In applying LC-MS/MS for environmental investigations, the analyst is faced with two major challenges: poor detectability of TCs and highly variable matrix interferences, which compromise quantification. Efforts have been directed to attain high-throughput methods able to extract a large number of samples in a short time [15, 16, 23]. Pressurized liquid extraction (PLE) is a rather new technique that uses solvent at a relatively high pressure and temperature without reaching their critical point. This improves efficiency compared to extractions at room temperature and atmospheric pressure [7]. Recently, O'Connor et al. [24] optimized the extraction of TCs by using rapid and simple PLE procedures with a mixture of acetate buffer (pH 8) and methanol as the extracting solvent for soils. In the same way, Jacobsen et al. [25] reported the use of PLE with mixtures of methanol and citric acid (pH 4.7) to extract TC, macrolide, and sulfonamide antibiotics from agricultural soils. Other studies have applied PLE to extract veterinary drugs from food matrices [26–28] or different contaminants from soils [29, 30]. All these studies remarked at the technology's benefits in providing rapid and reliable analysis.

The present study focuses on developing a method for the simultaneous determination of four TCs—TC, OTC, CTC, and doxycycline (DC)—in soil, using PLE and SPE extraction followed by LC-MS/MS with a triple quadrupole (QqQ) analyzer. Different dispersing agents and solvents for soil extraction, and several SPE cartridges for cleanup were compared. The method was applied to soil samples of different locations and after different sludge treatments. To the best of our knowledge, this is the first finding of TC residues in typical Spanish agricultural soils.

Experimental

Chemicals and standards

TC, OTC, CTC, DC and demeclocycline (DMC) were purchased from Sigma (St. Louis, MO, USA). DMC was used as internal standard (IS) because it is an obsolete antibiotic. The three epimers, 4-epitetracycline (e-TC), 4-epioxytetracycline (e-OTC), and 4-epichlortetracycline (e-CTC), were from Acros (Fisher Scientific, Schwerte, Germany). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Formic acid, citric acid monohydrate, sodium acetate anhydrous, sodium hydroxide pellets and ethylenediamine tetraacetic disodium salt (EDTA-Na_2) were of analytical grade (Aldrich, Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water system (Millipore, Bedford, MA, USA). All the solvents and solutions were filtered through a 0.45-μm cellulose filter from Scharlau (Barcelona, Spain) before use. Acidic, neutral, and basic alumina (Al_2O_3) were obtained from Merck, silica gel from Scharlau, Florisil® from Aldrich, sea sand from Panreac (Barcelona, Spain), and anhydrous sodium sulfate (analytical grade) from Scharlau. To block metal impurities, 60 g of solid sorbent was placed in a Buchner funnel, and 120 mL of 0.1 M EDTA-Na_2 was passed through the sorbent using vacuum. Strong anion exchange cartridges (SAX, 500 mg sorbent, 6 ml cartridge) were purchased from Isolute, IST. Oasis™ hydrophilic-lipophilic balance (HLB) extraction cartridges [poly(divinylbenzene-co-N-pyrrolidone), 6 cc, 200 mg] were from Waters Corporation (Milford, MA, USA) and Strata-X™ SPE cartridges [surface modified styrene divinylbenzene, 6 cc, 200 mg) from Phenomenex (Torrance, CA, USA).

Sample collection

Samples of soil were collected from the Ap horizon (0–20 cm) from 50 fields located in the south surrounding area of Valencia city (Spain) in early February 2007. The soils were of loamy texture, highly carbonated, representing typical Spanish agricultural soils. Some soil characteristics are listed in Table S2.5 (in the Electronic Supplementary Material). The last fertilization with sewage sludges was in September 2006 according to the data provided by the Conselleria de Medi Ambient. There were no data on the amount of sludge added to the field or the contamination levels of the sludges by these pharmaceuticals. Two control samples from fields without slurry fertilization since, at least, 5 years were also taken from this region. Samples were taken in plastic bags and immediately transported under cooling to the laboratory. To achieve homogeneous samples, the soils were air dried and sieved through a 2 mm sieve before further handling.

Sample preparation

Pressurized liquid extraction

The extraction of antibacterial agents from soil was performed by PLE, using an ASE 200 system from Dionex (Sunnyvale, CA, USA). The system was operated with pressure-resistant steel extraction cells with a volume of 22 ml and lined with glass-fiber filters from Dionex.

Approximately 5 g of soil was added to 10 μl DC (IS) solution of 10 μg ml^{-1} and blended with 5 g of EDTA-Na_2 washed sea sand for 5 min in a mortar using a pestle. This mixture was introduced into a stainless steel extraction cell, which was positioned in the PLE system connected to a four-bottle solvent controller. Nitrogen, at a pressure of 10 bar, was supplied to assist the pneumatic system and to purge the extraction cells. The extraction cells were preheated for 2 min, the analytes were extracted with deionized water (pH 7.0–6.8) at 70 °C and 1,500 psi for 10 min of static time, in one cycle, at 100% of flush; then, extraction cells were purged for 60 s with nitrogen to eliminate any trace of the extraction solvent. The total final volume of extract was approximately 40 ml and the pH 6.45.

Solid-phase extraction

Clean up and preconcentration was performed using a combination of SAX and Strata-X cartridges. Cartridges were placed in tandem to simultaneously remove negatively charged humic material (SAX) and retain the antibacterial agents (Strata-X). The SAX cartridge was placed on top of the Strata-X cartridge, and both columns were conditioned first with 2 ml methanol and then 2 ml water. PLE extracts (40 ml) were passed through both SPE columns at approximately 5 ml min^{-1} and, after extraction, the columns were washed with 2 ml water and dried under vacuum for 15 min. Then, the SAX cartridge was removed, and the antibacterial agents were eluted from the Strata-X sorbent with 2 ml methanol. The eluate was evaporated to dryness using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, MA, USA), and the residue was redissolved in 1 ml methanol–water (10:90).

Liquid chromatography-mass spectrometry

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (Waters Corporation) consisted of an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface, and a Mass Lynx NT software ver. 4.1 were used. Analysis was performed in positive ion mode. The electrospray ionization (ESI) source values were: capillary voltage, 3.00 kV; extractor, 2 V; radio frequency (RF) lens, 0.5 V; source temperature, 120 °C; desolvation temperature, 300 °C, and desolvation and cone gas (nitrogen 99.99% purity) flows, 600 l h^{-1} and 60 l h^{-1}, respectively. The analyzer settings were resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 2.0; entrance and exit energies, −1 and 1; multiplier, 650; collision gas (argon, 99.995%) pressure 2.79×10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s. The mass spectrometer was operated in scan and product ion scan modes to optimize the conditions and select the transitions, and in selected reaction monitoring (SRM) mode to confirm the identity of analytes in the samples by selecting two transitions for each one and to quantify. Table 2.1 shows the particular conditions and transitions for each analyte. The analytical column was a Xterra C_{18} (100×2.1 mm I.D., 3.5 μm) from Waters. The mobile phase consisted of methanol and water, both with 10 mM formic acid at 0.2 ml min^{-1} in gradient that begins with 10% methanol, increasing linearly in 15 min to 90% of methanol, maintaining this proportion for 5 min and returning to the initial conditions in 10 min. The injected volume was 20 μl.

Method validation

Recoveries for the entire procedure were determined using the two control samples taken from fields without fertilization. Soil samples were fortified with CTC, OTC, TC, and DC at three concentration levels (approximately 10, 50, and 100 μg kg^{-1} soil) and the IS at fixed concentration of 100 μg kg^{-1}. Six different extractions were performed at each level. The fortified samples were extracted and analyzed using the entire procedure. Recoveries were calculated as the percentage of extracted antibacterial agent compared to the spiked level.

Table 2.1 LC-MS/MS conditions for confirming and quantifying the selected tetracyclines in soil samples

Compound	t_r (min)	Quantifier SRM (m/z)	CV (V)	CE (V)	Qualifier SMR (m/z)	CV (V)	CE (V)	Ion ratios[a]
TC	8.23	445 → 410	18	12	445 → 427	18	18	0.536±0.003
e-TC	6.23	445 → 410	18	12	445 → 427	18	18	0.502±0.008
CTC	10.72	479 → 444	20	15	479 → 444	20	18	1.425±0.009
e-CTC	9.11	479 → 444	20	15	479 → 444	20	18	0.729±0.007
OTC	8.66	461 → 443	18	10	461 → 426	18	18	0.358±0.003
e-OTC	7.53	461 → 443	18	10	461 → 426	18	18	0.504±0.004
DMC (IS)	9.38	465 → 448	22	15	465 → 430	22	18	0.407±0.010
DC	12.60	445 → 428	20	18	445 → 321	20	30	0.060±0.001

The criteria for residue identification were (1) four identification points through the measurement of two product ions plus the precursor ion; (2) retention time of suspected analyte and reference standard within the tolerance interval of ±2.5%; and (3) the ion ratio for each analyte in samples matches that of the standards within the maximum permitted tolerances (±20% for TC, e-TC, CTC. e-CTC and e-OTC; ±25 for OTC, and ±50 for DC)

CV cone voltage, CE collision energy

[a] Ion ratios = the ratio of the intensities of the two most abundant transitions of each tetracycline determined from the analysis of standards prepared in methanol-water 10:90 (v/v) (n=15)

Experiments were performed to determine recoveries of the antibacterial agents CTC, OTC, TC, and DC for the tandem SPE (SAX + Strata-X) cleanup step only. Sample matrix was obtained by extracting control samples, using the described PLE method. These PLE extracts were fortified with antibacterial agents at two concentration levels (1.25 and 12.5 μg l^{-1}), corresponding to extracts obtained from extraction of soil samples with antibacterial agent contents of approximately 10 and 100 μg kg^{-1} soil, respectively. The fortified samples were passed through the SAX+Strata-X SPE cartridges as described above.

Day-to-day variations for the extraction procedure were determined by repeating the recovery experiment for concentration levels of 10, 50, and 100 μg kg^{-1} soil after 3 days.

The linearity of the analytical methods was demonstrated building the calibration curves for each compound. Calibration samples of TCs (10, 25, 50, 75, 100, and 500 μg kg^{-1} of each TC) and IS (100 μg kg^{-1}) were prepared by adding different volumes of stock solution of TCs and constant volume of IS in "blank extracts" of the soils without slurry treatment (matrix-matched standards) or directly in the soils without slurry treatment (for entire extraction procedure). Each level was prepared in triplicate. The calibration curve was obtained from the least-squares linear regression of the peak area ratio with spiked concentrations.

The limit of detection (LOD) was estimated at a signal-to-noise ratio (S/N) of 3, while the limit of quantification (LOQ) value was estimated by using a S/N of 10. LODs and LOQs were obtained by the transition with higher signal/noise in SRM mode. For the LOQ, the confirmatory transition should be, at least, visible in the chromatogram. Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure to ensure their feasibility.

Results and discussion

Soil extraction method optimization

Extraction solvent for extracting the TCs from soil was selected in accordance with their physico-chemical properties. Many different soil-adsorption mechanisms, such as hydrophobic interactions, hydrogen bonding, complexation and cation exchange, may affect the extraction of the compounds from soil [18, 22, 31]. TCs form strong complexes with di- and trivalent cations in the clay mineral interlayers or with hydroxyl groups at the surface of the soil particles [19–21]. As a starting point, a sample pretreatment by homogenization of soil with EDTA-Na_2 washed sand as dispersing agent, followed by PLE using water as extractant, was applied as previously employed for food analysis [27, 32].

The influence of the sorbent used for the homogenization and dispersion of soil was investigated by performing the homogenization with alumina (neutral and basic), Florisil®, silica and sea sand, all of them washed with EDTA-Na_2. The washing of the material was carried out because previous studies performed in food demonstrated that EDTA-Na_2-washed materials always provide better recoveries [27]. EDTA-Na_2 deactivates metal impurities present in the surface and probably also chelates metals present in soil facilitating the TCs decomplexation. Silica and sea sand provided the better recoveries for all TCs (up to 40%). Alumina, neutral or acid, does not recover these compounds, and Florisil® provides good recoveries for OTC, CTC, and their epimers but fails in recovering TC, e-TC, and DC.

In order to obtain the highest possible concentrations of the TCs in soil extracts, the performance of the PLE system was investigated for soil samples weighing up to 25 g.

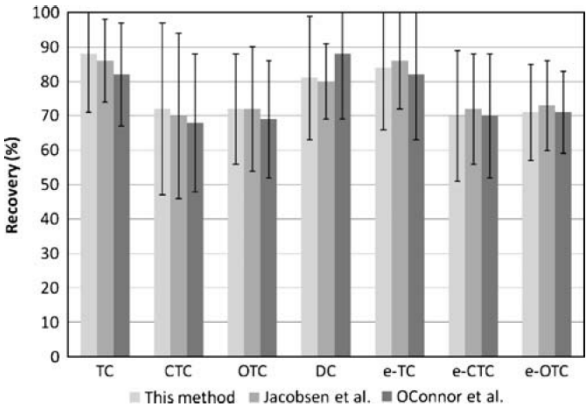

Fig. 2.1 Efficiency of the PLE system using different extractants. Soil samples were spiked at 50 μg kg^{-1}, and the extracts were analyzed by LC-ESI-MS/MS without further cleanup step

However, samples greater than 5 g were difficult to analyze owing to the larger number of matrix-producing peaks. To increase contact surface between soil particles and water and prevent metal complexation, as well as clogging of the extraction cell, 5 g EDTA-Na_2 washed sea sand was mixed into 5 g soil sample before extraction. In the development and optimization of the PLE method, several settings need to be considered such as pressure, temperature, and number of solvent cycles. The final operating parameters for the validation of the method are listed in "Experimental" (see "Pressurized liquid extraction").

Chemical transformation processes of TCs, such as isomerization and epimerization, have been reported giving rise to structurally related compounds [7]. The possible interconversion between TCs and their 4-epimeric (4-eTCs) forms during the extraction procedure was checked by spiking different soil samples only with the TCs or with the 4-eTCs. No interconversion between the TCs and its 4-epimers was observed, even though the extraction was performed at 70 °C.

The method was compared with two methods based on PLE, recently reported by Jacobsen et al. [25] and O'Connor et al. [24] (Fig 2.1). The former procedure is based on mix 10 g soil sample with 10 g Ottawa sand before adding to the extraction cell of 33 ml. The extraction buffer consisted of a 50:50 (v/v) mixture of methanol and 0.2 M citric acid in water with pH adjusted to 4.7 with NaOH. Extractions were performed at room temperature to avoid that the TCs were converted to their epi- or anhydro forms. Recoveries, achieved using this method, were above 40%, which were comparable to the recoveries for the proposed combination. The latter protocol involves extraction of 5 g of soil with 50:50 (v/v) methanol-acetate buffer (pH 8). O'Connor et al. [24] established that the percent recoveries of the optimized PLE method varied between the soils and ranged from 22% to 99%, depending on soil type, and more specifically, on clay content. In the present study, the method was applied only to one type of soil because it is representative of more than 50% of the Spanish agricultural soils with 20% of clay. Comparing the three extraction methods, i.e., the results shown in Fig. 2.1, all of them provided acceptable recovery levels of the studied TCs with overall recoveries of 77% for the method proposed by Jacobsen et al. [25], 74% for that proposed by O'Connor et al. [24] and 78% for the method developed in this study. The main advantages of the proposed procedure for the TC extraction from soils are that it only

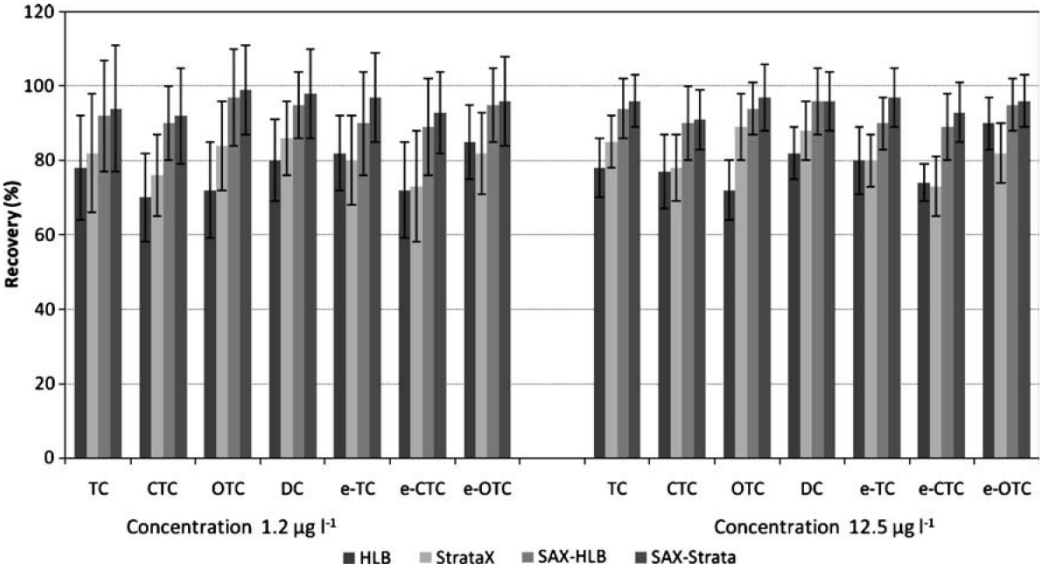

Fig. 2.2 Efficiency on the SPE cleanup using different SPE platforms at two different concentrations, 1.25 and 12.5 μg l^{-1}

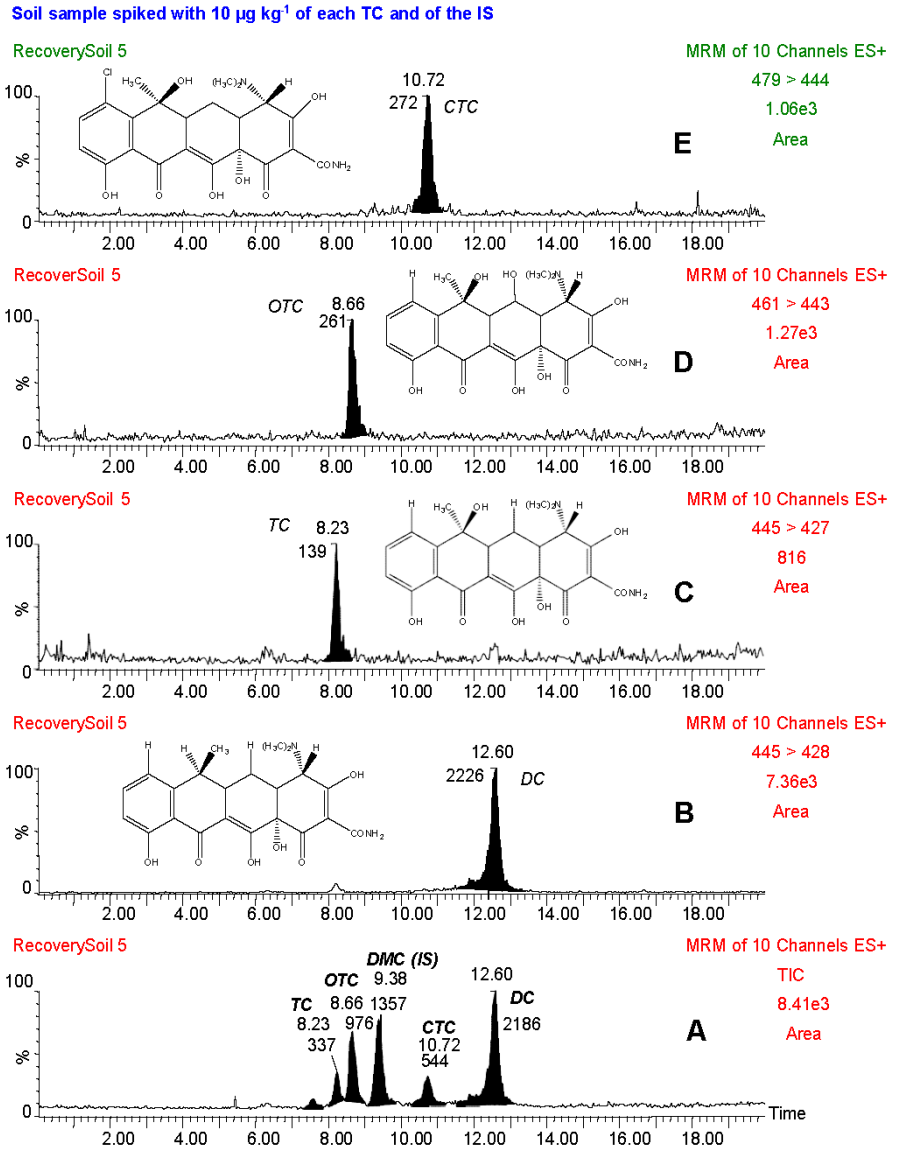

Fig. 2.3 LC-MS/MS chromatograms of soil spiked at 10 µg kg^{-1} of each tetracycline including the internal standard. a TIC of the ten precursor → product ion transitions reported in Table 2.1, b mass chromatogram for DC (445 → 428 transition), c mass chromatogram for TC (445 → 427 transition), d mass chromatogram for OTC (461 → 443 transition), and e mass chromatogram for CTC (479 → 444 transition)

uses water as extractant and the stability of the compounds through the extraction procedure.

Cleanup and preconcentration using SPE

Solid phase extraction is traditionally used as a cleanup technique for extracts of environmental samples. The two reported procedures for tetracyclines used combination of SAX and HLB (polymeric) or SAX and Strata-X SPE sorbents, respectively. These sorbents attained cleanup and preconcentration of the TCs. The SAX column reduces matrix interferences by adsorbing anionic humic particles from the soil extracts, avoiding contamination, block, and overload of the HLB or Strata-X sorbent. At the pH of the extract (6.45), the TCs are overall neutral or cationic and, therefore, not retained on the SAX cartridge, while the polymer-based HLB or Strata-X cartridge simultaneously retain neutral, polar, and nonpolar, compounds, including the studied TCs. A study of the results obtained using HLB and Strata-X cartridges individually, and the combination SAX and HLB and SAX and Strata-X, was carried out. HLB and Strata-X cartridges, showed, as expected, an appropriate analyte isolation and strong adsorption of matrix compounds, since TCs were quantitatively eluted with 2 ml of methanol. The extract obtained, using either HLB or Strata-X cartridges, showed that extract still has an intense yellow-brown color indicative of co-extraction of soil components. Additional purification of the sample by

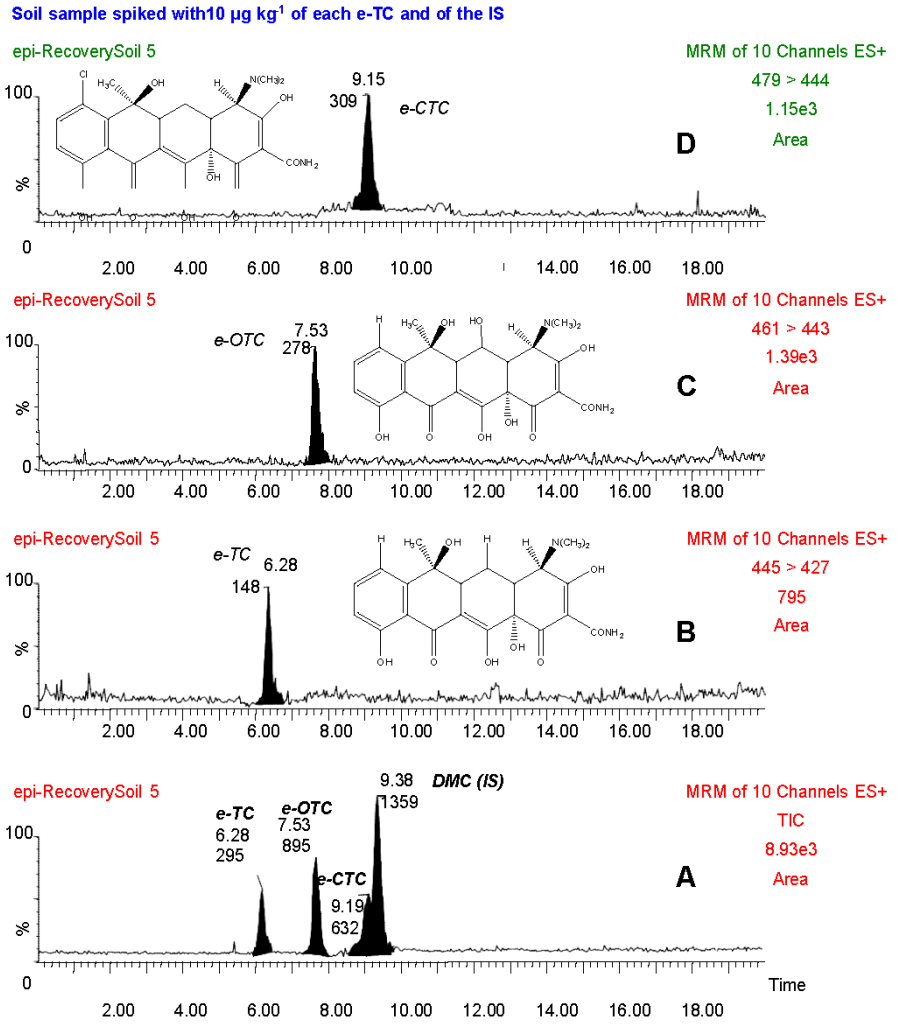

Fig. 2.4 LC-MS/MS chromatograms of soil spiked at 10 µg kg^{-1} of each tetracycline epimer and the internal standard. a TIC of the ten precursor→product ion transitions reported in Table 2.1, b mass chromatogram for e-TC (445→427 transition), c mass chromatogram for e-OTC (461 → 443 transition), and d mass chromatogram for e-CTC (479 → 444 transition)

SAX resulted in a considerable decrease in the humic acid content, providing almost transparent extracts. The interpretation of the chromatograms with low concentrations of the analytes improved at the same time as the lifetime of the LC column was prolonged.

Recoveries and corresponding 95% confidence intervals for the different SPE platforms, at two concentration levels (approximately 1.2 and 12.5 µg l^{-1} in the final extract) are shown in Fig 2.2. The recoveries obtained using the SAX+ Strata-X system were slightly better than those obtained with the SAX+HLB because of this, the former was selected for the definitive method.

Table 2.2 Linear regression parameters of TCs from soil calibration curves ranging from 10 to 500 µg kg^{-1} (six points, triplicate analyses), LODs, and LOQs

Compound	Spiked soil extracts			Entire soil extraction procedure			LODs (µg kg^{-1})	LOQs (µg kg^{-1})
	Slope	y-intercept	R^2	Slope	y-intercept	R^2		
TC	29.85	12.75	0.9985	27.25	28.25	0.9989	1	3
OTC	20.12	−4.74	0.9983	18.62	18.01	0.9984	2	6
CTC	10.45	14.28	0.9998	8.94	14.95	0.9996	3	10
DC	24.20	13.94	0.9996	21.09	19.54	0.9998	1	3
e-TC	28.30	−19.07	0.9996	25.82	12.01	0.9990	1	3
e-OTC	26.42	−12.73	0.9984	24.42	9.95	0.9972	2	6
e-CTC	15.62	25.73	0.9971	13.09	−24.83	0.9988	3	10

The linear regression analysis was carried out by plotting the peak area ratio of analyte and IS versus the analyte concentration

Table 2.3 Recoveries and the relative standard deviations of the studied TCs in soil spiked at different concentrations

TCs	Concentration					
	10 μg kg^{-1}		50 μg kg^{-1}		500 μg kg^{-1}	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
TC	86.7	14.8	93.8	8.6	96.0	10.8
OTC	74.2	12.3	73.7	10.6	77.3	10.2
CTC	75.9	13.1	76.5	11.1	76.4	9.0
DC	84.2	14.5	83.2	8.5	92.1	8.5
e-TC	83.2	14.5	83.2	8.0	92.1	8.5
e-OTC	73.0	11.6	71.2	8.6	75.7	7.7
e-CTC	71.2	12.5	73.2	8.5	72.1	8.5

Values are the mean of six independent determinations at each concentration

Table 2.4 Results of the investigation of 100 samples collected of the Ap horizon (0-20 cm) from 50 fields located in the South surrounding of Valencia city in February 2007

Sample[a]	TCs[b] (mean±SD, n=4)			
	OTC (μg kg^{-1})	CTC (μg kg^{-1})	TC (μg kg^{-1})	DC (μg kg^{-1})
1a	25.0±5.3	10.4±0.2		
1b	32.1±3.2	8.1±2.1		
4a	22.4±2.1	12.9±4.8	62.7±6.1	
4b	34.2±6.4	15.2±4.4	64.3±7.2	
7a	29.6±4.3			
10a	52.2±10.1	7.1±0.3		
10b	45.7±9.3	12.8±0.6		
15b	32.2±4.4	10.3±1.2	54.5±7.8	12.1±1.2
17a				45.2±4.3
17b				43.4±5.8
20a	15.7±1.3			
20b	32.3±5.0			
23a				
30a	105.4±12.8	14.3±0.4		
30b	95.8±11.6	5.8±0.2		
42b			34.0±2.7	
49b	34.5±3.3			45.7±8.2
57a	45.2±6.2		29.4±5.2	
57b	52.2±7.7		18.8±4.1	
72a	22.7±8.1			
72b	25.4±6.4			
83a	67.6±5.2	10.3±0.6	42.1±3.8	
94a	34.5±3.2		22.3±4.3	
94b	28.5±2.3		32.6±3.8	
97b	92.9±12.0	34.4±0.9		

[a] The two samples taken of each field were identified by the same number and the letter "a" or "b"

[b] The concentration of each TCs is the sum of the TC and the 4-epimer if it exists

Validation of the soil extraction procedure

Fig. 2.3 and 2.4 display a typical total ion current (TIC) and mass chromatograms obtained for a soil sample spiked with the four TCs plus the IS and with the three 4-epimers and the IS, respectively. The TIC chromatogram is a summation of the ion signal generated by all the precursor → product ions transition shown in Table 2.1. Chemical transformation processes of TCs, such as isomerization and epimerization, have been reported giving rise to structurally related compounds. For instance, CTC is converted to isochlortetracycline (iCTC) under alkaline conditions, while the epimerization has been found to be catalyzed in acidic solutions in a pH range from 2 to 6 [7]. The mass chromatograms (Fig. 2.3B–E and Fig. 2.4B–D) show that there is no interconversion between TCs and their 4-epimeric forms during the extraction procedure. In the present study, epimerization was observed in both standard and extract of the spiked samples, after preserving them more than 1 month as indicated in "Experimental." The formation of other degradation analogues was observed for CTC, which also displayed another peak that forms epi-analogues. This peak was previously reported and tentatively identified as iCTC based on the absence of the transition 679 → 444, which indicates the lack of hydroxyl group of the CTC [7].

Calibration curves were obtained by least-squares linear regression analysis of the peak area ratio of analyte/IS versus analyte concentration, in standards prepared in control soil extracts and in the same soil samples directly spiked with the TCs prior to apply the entire extraction procedure. These curves were linear with convenient regression coefficients (R^2)>0.99 in the range of 10–500 μg kg^{-1}. The corresponding equations are shown in Table 2.2. Slope values were similar, indicating only small differences attributed to the recovery results appearing in Table 2.3. Values obtained for

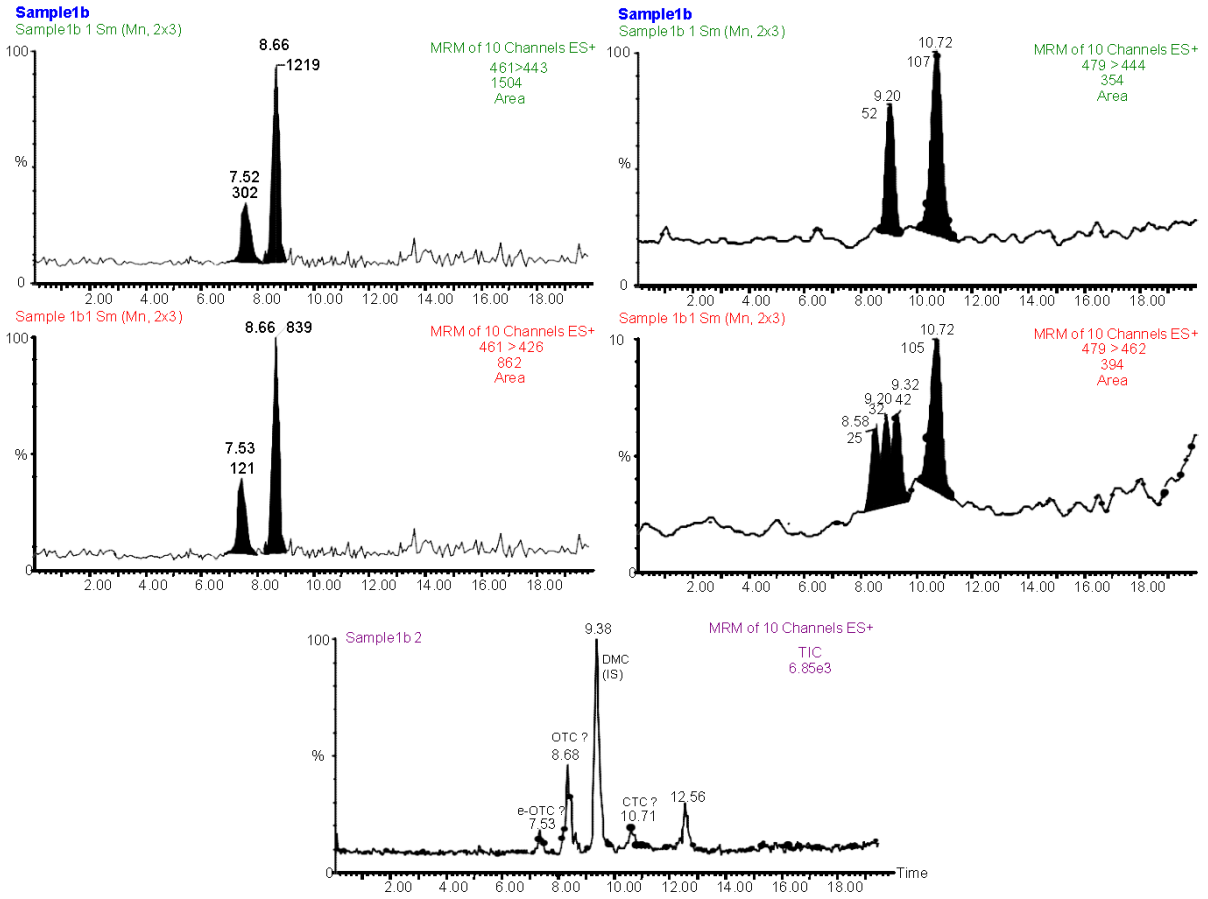

Fig. 2.5 LC-MS/MS chromatogram corresponding to sample 1b

LODs and LOQs were in the range <1–3 and 3–10 μg kg^{-1}, respectively, which covers the range expected for environmental soil samples. The low LODs and LOQs of the method make it suitable for analyzing the studied TCs.

Mean recoveries and corresponding confidence intervals for the PLE extraction of six replicate soil samples are listed in Table 2.3. Recoveries for TCs were between 71% and 96%, and the relative standard deviations (RSDs) on six replicate samples were between 8% and 15%. For the TCs (OTC and CTC), recoveries of approximately 71–78% were achieved, which is lower than recoveries obtained for the SPE method (Fig. 2.2), indicating that the compounds are not fully extracted from the soil. This is probably due to the many sorption mechanisms involved in the binding of TC to soil, resulting in very strong sorption and corresponds well with previous studies showing that TCs are sorbed to the clay fraction of the soil by complexation and hydrogen bonding, and bound to acid sites in the organic fraction (Table S2.5 in the Electronic Supplementary Material) [17–21].

Field study

Frequency, concentration, and identity of TCs found in the analyzed soil samples are outlined in Table 2.4. Of the 100 samples analyzed, TCs were detected in 25. The most commonly detected TC was OTC, followed by CTC, TC, and DC. OTC was detected in 21 samples at levels ranging from 15.7 to 105.4 μg kg^{-1}, CTC in 11 samples in the concentration range of 5.8–34.4 μg kg^{-1}, TC in nine samples at concentrations from 18.8 to 64.3 μg kg^{-1}, and DC in four at levels ranging from 12.1 to 45.7 μg kg^{-1}.

On the co-occurrence of TC residues, 12 samples contained two TCs that were in seven cases OTC and CTC, in four OTC and TC, and in one OTC and DC. Three samples contained three TCs that were OTC, CTC, and TC. Only one sample contained the four TCs studied.

The isomerization of the different TCs to their epimers in soil samples was also checked. Data reported in Table 2.4 were calculated for the sum of both the TCs and the

4-epimers. However, Fig. 2.5 illustrates a chromatogram of one sample containing OTC and CTC (sample 1b of Table 2.4). The results show that OTC epimerizes in low percentage, less than 25%, compared with CTC that shows its isomeric conversion product, iso-CTC and their 4- respective-epimers. As can be observed in the chromato- gram, the conversion of CTC to iso CTC also takes place in an important percentage. TC is also epimerized in a mean value between OTC and CTC. The quantification of iso- CTC was not possible because we did not have available the iso-CTC standard. These results indicated the need for a further study on TCs metabolites and the difficulties to perform it, starting with the lack of analytical standards.

These data confirm the recent findings reported in the literature [12–15] that TCs occur in relatively high concentrations and persist in the environment after repeated fertilizations of farmland with liquid manures or sludges. However, these data also include several novelties, such as: (1) the finding of DC; (2) the observation of co-occurrence of TCs in soil samples that have received an unknown treatment with sludge—this co-occurrence can be by the coexistence of different TCs in the manure or sludge or by the soil treatment with different sludges; and (3) the different isomerization patterns of TCs in real soil samples.

Conclusions

Simultaneous extraction of TC, CTC, OTC, and DC from soil was carried out using hot-water PLE, cleanup and concentration by SPE, and analysis by LC-ESI-MS/MS. Recoveries, LOD, and LOQ were satisfactory, demonstrating its applicability for simultaneous determination of TCs from soil. It can be concluded that the proposed PLE-SPE method is an interesting alternative extraction technique for the determination of TC residues in soil because it provides similar results to other techniques, reduces the use of organic solvents and complex buffers, and does not need pH adjustment. A preconcentration and cleanup step is necessary because of the large amount of co-extracted humic and fulvic acids. SAX+Strata-X was found to be an efficient cleanup step that selectively removed humic and fulvic acids. The proposed method compares well with the results obtained by the other recently reported procedures and presents the advantage of eliminating the use of organic solvent as extractants. Considering the savings in time and solvent consumption, which are both diminished by 90%, PLE is an attractive alternative for extracting TC residues from soil.

The application of the method made the detection of significant amounts of persistent TCs in the soil possible. This study pointed out that TCs, which are frequently used worldwide, are persistent in soil in significant amounts, and that these substances represent an actual environmental problem. Ecotoxicological studies, especially on soil micro-organisms, should be performed to estimate the risk for the soil flora and the spread of antibiotic resistance.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Determination of tetracycline residues in soil by pressurized liquid extraction and liquid chromatography tandem mass spectrometry

Vicente Andreu · Pablo V Roig · Cristina Blasco · Yolanda Picó

Table S2.5. Physico-chemical characteristics of the soil samples

Soil type	Haplic Calcisol
Sampling Depth (cm)	30
Morphology	Hill
Slope (%)	4-5
Altitude (m)	60
Parent material	Tertiary Marls
Soil use	Citrus crops and orchard
Textural Class	Loamy
Textural distribution (%)	
Very coarse sand (2-1 mm Ø)	0.8
Coarse sand (1-0.5 mm Ø)	3.3
Medium sand (0.5-0.25 mm Ø)	5.1
Fine sand (0.25-0.1 mm Ø)	15.8
Very fine sand (0.1-0.05 mm)	14.6
Coarse loam (0.05-0.02 mm Ø)	21.3
Fine loam (0.02-0.002 mm Ø)	19.1
Clay (Ø<0.002 mm)	20.0
pH (H_2O)	7.8
pH (KCl)	7.0
Electric Conductivity (dS m^{-1})	0.7
Organic matter (%)	3.4
Total N (%)	0.14
C/N Ratio	13.9
Mineral N (mg N 100 g^{-1})	1.14
Calcium Carbonate (%)	35.4
Cation Exchange Capacity ($cmol_{(+)}$ kg^{-1})	36.8
Ca ($cmol_{(+)}$ kg^{-1})	36.3
Mg ($cmol_{(+)}$ kg^{-1})	0.2
K ($cmol_{(+)}$ kg^{-1})	0.2
Na ($cmol_{(+)}$ kg^{-1})	0.1
Bases saturation (%)	100

CHAPTER 3

Analysis of pharmaceuticals in soils and sediments

Scientific publication 3:

Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry.

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Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry

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abstract

The present work describes the development of a sensitive analytical method based on pressurized liquid extraction (PLE) and pre-concentration by solid-phase extraction (SPE), followed by liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS) for the determination of seventeen pharmaceuticals in soils and sediments. The method is based on sample homogenisation using Na_2–EDTA washed sand and extraction with water at 90 °C. Special emphasis was placed on the optimization of the extraction procedure to develop a green method that reduces, at a maximum, the use of organic solvents in order to eliminate matrix components during the clean-up. The proposed method was linear in a concentration range from 0.3 to 333 ng g^{-1}, with correlation coefficients higher than 0.993. Method detection (MDLs) and quantification (MQLs) limits ranged from 0.1 to 6.8 ng g^{-1} and from 0.25 to 23 ng g^{-1}, respectively. Absolute recoveries were analyte dependent, varying between 50% and 105% at the MQL level, except for fenofibrate (40%) and diclofenac (34%). The intra-day and inter-day precision was given by RSD values from 0.7% to 7.9% and from 1.6% to 14.5%, respectively. Acetaminophen, carbamazepine, ciprofloxacin, clofibric acid, codeine, diazepam, fenofibrate, metropolol, ofloxacin and propanolol were detected at concentrations from MDL to 35.62 ng g^{-1} in soils and sediments from marsh areas. Due to the low recoveries, results for fenofibrate and diclofenac can only be considered as semi-quantitative. The method was fully suitable for the other 15 pharmaceuticals.

1. Introduction

Thousands of tonnes of pharmacologically active substances are annually used in human medicine for treatment and/or prevention of illness. As an example, in Spain during 2006, a total of 30 tonnes of quinolones were consumed, which is equivalent to 3.2 defined daily doses per 1.000 inhabitants and day (DHD) [1–3]. In veterinary, there is also a great dependence on drugs—e.g. hormones, non-steroidal anti-inflammatories and antibiotics used as feed additives or as preventives [4].

Pharmaceutical residues in the environment, and their potential toxic effects, already have been recognized as an emerging research area in environmental chemistry [5]. A better knowledge of the occurrence and fate of pharmaceuticals release to the environment will attain a proper risk assessment for river basins, wetlands and others related ecosystems [6]. It is now well established that pharmaceuticals are widespread contaminants of wastewater effluents [7–10], surface and drinking waters [11,12], but limited publications treat their occurrence in terrestrial ecosystems [5]. To the best of our knowledge, up to now, the great majority of authors have focused their attention on antibiotics such as tetracyclines [13–16], macrolides [14,17], quinolones [15,18,19], sulfonamides [14,15] and β-lactames [15]. Only few studies deal with the analysis of pharmaceuticals other than antibiotics. Carbamazepine, clofibric acid, ibuprofen, salicylic acid, gemfibrozil, naproxen, ketoprofen, diphenhydramine and diclofenac have been analyzed by gas chromatography–mass spectrometry (GC–MS) [20–22] together with endocrine disrupting compounds or personal care products. Furthermore, Cuevas-Mestanza et al. [23] determined phenazone, carbamazepine, clofibric acid, ibuprofen, naproxen, ketoprofen, bezafibrate and propanolol in sediments by liquid chromatography with ultraviolet detection (LC–UV). Löffler and Ternes [24] simultaneously detected bezafibrate, clofibric acid, diclofenac, fenoprofen, gemfibrozil, ibuprofen, indomethacin, naproxen, ketoprofen, antibiotics and the antiparasitic ivermectin, in river sediment by LC with electrospray ionization (ESI) and tandem mass spectrometry (MS/MS). Radenović et al. [25] established a method for analyzing 31 pharmaceuticals (i.e. eight analgesics and anti-inflammatory drugs, five antibiotics, two psychiatric drugs, one antiulcer agent, one antiepileptic drug, four β-blockers, one diuretic, one hypoglycemic agent, five lipid regulator and cholesterol lowering statin drugs, and three antihistamines) from sewage

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sludge by LC–ESI-MS/MS. This method was the starting point adopted by the same research group to obtain optimum extraction conditions for analysis of 42 pharmaceuticals in sewage sludges and sediments [26].

Extraction procedures for pharmaceuticals from soils and sediments commonly involve ultrasonication [22,24,27], ultracentrifugation [22,24,28,29], microwave assisted micellar extraction (MAME) [21,23] and/or pressurized liquid extraction (PLE) [13–17,20,25,26]. The extraction is followed by a clean-up step with solid-phase extraction (SPE), mainly using reversed phases such as Oasis Hydrophilic Lipophilic Balance (HLB) [20,25,26], LiChrolute EN (Merck) [13], Isolute ENV+ (Separtis) [29], polymeric phase (SDB-2) [15] or C_{18} [22,28]. Some procedures combine two cartridges in tandem, one containing a strong anion exchange phase (SAX) [14] to remove organic matter, and the other one of the previously mentioned reversed phases. Determination of these pollutants not only in soils but also in other environmental matrices has been carried out by GC–MS [20,21,30], but preferably by LC, since no time consuming derivatization is needed, either with photodiode array detection (DAD) [23,28,29] or MS [13–15,17,24–26]. As DAD is a non-specific detector, interferences of other matrix components may cause false positives detection at ultra trace levels, because of this LC–MS and, particularly LC–MS/MS, is considered the best choice.

In this paper, a sensitive multi-residue method is proposed for the simultaneous extraction of seventeen commonly used pharmaceuticals from soils and sediments, with many different polarities and pK_a's (acids, basics and neutrals). We opted for these compounds on the basis of levels of use in Spain and reported aquatic toxicity effects [1–3,24,25]. Table 3.1 shows their chemical structures and lists some relevant physico- chemical properties. The selected pharmaceuticals belong to a great variety of different therapeutical classes: analgesics, β-blockers, antibiotics, anti-inflammatories, anticonvulsants, antidepressants and lipid regulators. The developed analytical method combines PLE using water as extractant, clean-up with SPE and determina- tion by LC–ESI-MS/MS. The advantage of this method over the few reported applications is its suitability for a wide range of compounds and the reduction of the use of organic solvents, which results in a decrease of the analysis cost and safeguards the integrity of the analyst and the environment.

2. Materials and methods

2.1. Chemicals and materials

Acetaminophen, codeine, carbamazepine, ciprofloxacin, clofibric acid, diazepam, diclofenac, fenofibrate, ibuprofen, metoprolol, norfloxacin, ofloxacin, oxytetracycline, sulfamethoxazole, tetracycline, propanolol, trimethoprim and 4-epitetracycline hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). 4-Epioxytetracycline was from Acros Organics (Morris Plains, NJ, USA). Ibuprofen-d_3, acetaminophen-d_3 and carbamazepine-d_2 (internal standards, ISs) were from CDN Isotopes (Quebec, Canada). All standards were of analytical grade (purity >95%). Stock solutions (1000 mg L^{-1}) of each pharmaceutical were prepared in methanol with the exception of ciprofloxacin, which was prepared in water. Stocks solutions were stored at −20 °C. Working solutions, at different concentrations, were prepared monthly by dilution of the standard stock solutions in methanol–water (25:75, v/v). A mixture of the ISs at concentrations of 10 ng μL^{-1} each was prepared in methanol and 10 μL were added in soil and sediment samples to obtain concentrations of 33 ng g^{-1}. Formic acid (reagent grade), acetonitrile and methanol (gradient grade for liquid chromatography), were purchased from Merck (Darmstadt, Germany). High purity water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). Citric acid, ethylenediaminetetraacetic disodium salt dihydrate (Na_2–EDTA), di-potassium hydrogen phosphate (K_2HPO_4) and hydrochloric acid (37%), all reagent grades were purchased from Scharlau (Ferosa, Barcelona, Spain). The 0.1 M Na_2–EDTA–McIlvaine buffer solution was obtained mixing 61.45 mL of 0.1 M citric acid and 38.55 mL 0.2 M K_2HPO_4 and adding 3.36 g of Na_2–EDTA, to prevent the pharmaceuticals from complexing with Ca^{2+} and Mg^{2+} ions. The pH of the solution was fixed at pH 4 with hydrochloric acid (37%).

Aluminium oxide 90 (neutral, acidic and basic) was from Merck, silica gel 60 (0.04–0.06 mm) from Scharlau, Florisil® 60–100 mesh from Sigma–Aldrich and sea sand for Panreac (Barcelona, Spain). EDTA and EDTA–McIlvaine washed sea sand were prepared by placing 60 g of sand into a Buchner funnel and passing 120 mL of the selected solution through it using vacuum. Partial drying of the sand was carried out by vacuum. Thereafter, sand was completely dried in an oven at 100 °C.

Oasis HLB 60 mg sorbent/6 mL cartridge (Waters Corp., Milford, MA, USA), Strata-X 33 μm Polymeric Reversed Phase 200 mg (Phenomenex, Torrance, CA, USA) and Isolute SAX 500 mg (Symta, Madrid, Spain) were used for SPE.

2.2. Sampling and sample preparation

Sediments and soils were collected in sixteen points of five marsh areas of the Valencian Community (Spain), L'Albufera Natural Park, Prat Torreblanca-Cabanes and the marshes of Oliva-Pego, Silla and Moros. The main physical and chemical properties of typical soil and sediment of these areas are given in Table S3.7, Supplementary material. These soils are characterized by pH > 7, loamy texture, high calcium carbonate content (>30%) and low levels of organic matter (≈2%).

Soil samples of the upper 20 cm horizon layer were collected. From each sampling point, of 1 m^2, two sub-samples were taken. Once in the laboratory, samples were dried and passed through a 2 mm Ø sieve, and then, the sub-samples of each sampling point were homogenised to create a composite one. The composite soil samples were extended in a layer of approximately 1 cm thickness on polypropylene trays and air-dried in darkness at 20 °C to moisture content of approximately 3% water. Then, samples were stored in sealed plastic bag at 4 °C.

Sediment samples, taken from irrigation channels and marshes, were of pH > 7.4, sandy loam texture, and with high content in calcium carbonate (>30%) and organic matter (>15%). These samples were weighed and approximately 800 g of each sample were placed in a polypropylene pot, frozen at −80 °C, lyophilised (Hetosicc CD4, Birkerod, Denmark), passed through a 2 mm Ø sieve, and homogenised. The process of lyophilisation was carried out at −90 °C and with 0.440 bar vacuum over 7 days for each sediment sample to water content <1%. Finally the lyophilised samples were stored in sealed plastic bags at −20 °C until the extraction.

Soil and sediment samples that do not show pharmaceuticals after a preliminary analysis were used as control blank and for the optimization and validation of the method.

2.3. Pressurized liquid extraction (PLE)

The soil and sediment samples were extracted by PLE using an ASE 200 system (Dionex, Sunnyvale, CA, USA). The selected sorbent was sea sand washed with Na_2–EDTA. Soil or sediment were weighed (3 g) into a mortar and added 10 μL of a 10 ng μL^{-1} mixture of the ISs. The sample was then mixed with approximately 25 g of Na_2–EDTA washed sea sand in the mortar. This mixture was put into a 22 mL extraction cell, then the cell was filled up with Na_2-EDTA washed sea sand. Whatman glass fiber filters were placed at

Table 3.1
Therapeutical classes, chemical structures and relevant physicochemical properties of the studied compounds.

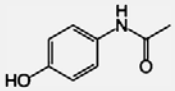
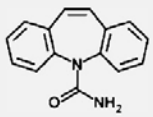
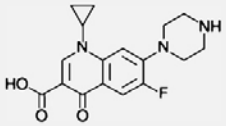
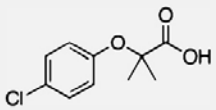
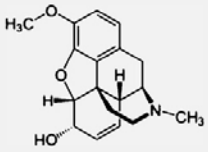
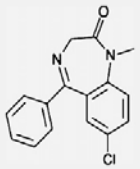
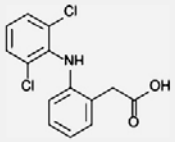
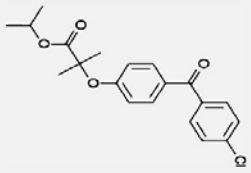
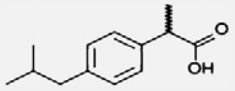
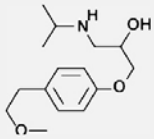
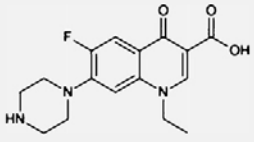
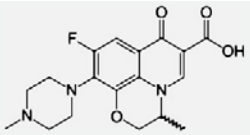
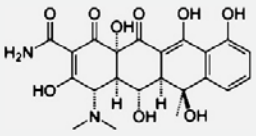
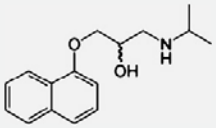
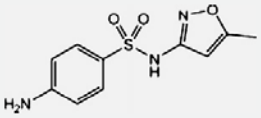
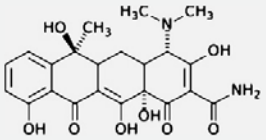
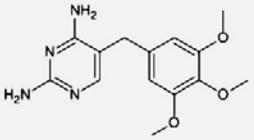
Compound	Therapeutical class	CAS no.	Molecular weight	Structure	pK_a	$\log K_{ow}$
Acetaminophen	Analgesic	103-90-2	151.17		9.38	0.46
Carbamazepine	Anticonvulsant	298-46-4	236.27		13.9	2.45
Ciprofloxacin	Antibiotic	85721-33-1	331.35		5.9/8.9	0.28
Clofibric acid	Lipid regulator	882-09-7	214.65		3.46	2.58
Codeine	Analgesic	76-57-3	299.36		8.2	1.52
Diazepam	Antidepressant	439-14-5	284.75		3.3	2.8
Diclofenac	Analgesic	15307-86-5	296.15		4.15	4.51
Fenofibrate	Lipid regulator	49562-28-9	360.83		–	5.19
Ibuprofen	Anti-inflammatory	15687-27-1	206.29		4.5	3.97
Metoprolol	β-Blocker	37350-58-6	267.36		9.7	1.88
Norfloxacin	Antibiotic	70458-96-7	319.33		6.22/8.51	−1.0

Table 3.1 (Continued)

Compound	Therapeutical class	CAS no.	Molecular weight	Structure	pK_a	$logK_{ow}$
Ofloxacin	Antibiotic	82419-36-1	361.37		6.05/8.22	−0.4
Oxytetracycline	Antibiotic	79-57-2	460.43		3.2/7.5/8.9	−1.3
Propanolol	β-Blocker	525-66-6	259.34		9.5	3.48
Sulfamethoxazole	Antibiotic	723-46-6	253.28		5.7	0.89
Tetracycline	Antibiotic	60-54-8	444.43		3.3/7.8/9.6	−1.2
Trimethoprim	Antibiotic	738-70-5	290.32		6.6	0.91

the bottom and top of the extraction cell to avoid the obstruction of the end caps by the soil or sediment particles. In the final method, the sample was heated to 90 °C with a static period of 7 min and extracted by a flush volume of 100% in three cycles using water. Pressure was set to 500 psi and a purge time to 1 min. The water volume ending up in the glass vial was approximately 30 mL, using a cell size of 22 mL.

2.4. SPE/clean-up

The process SPE/clean-up used in this work was based on that reported by Petrovic et al. [31] for the analysis of pharmaceuticals in water samples with slight modifications. SPE extraction was performed using a combination of SAX cartridge (strong anion exchange) and Oasis HLB cartridges [poly(divinylbenzene-co-N-pyrrolidone)]. The SAX cartridge was placed on top of the HLB cartridge. The conditioning of the SPE cartridges was performed with 5 mL of methanol followed by 5 mL of Milli-Q water at a flow rate of 1 mL min^{-1} through the cartridges using a vacuum system. The 40 mL of aqueous PLE extracts were loaded into the cartridges, the glass vials were rinse with 10 mL of distilled water that were also load into the cartridges. Samples were passed through the cartridges at a flow rate of 10 mL min^{-1}. The cartridges were rinsed with 5 mL of Milli-Q water and dried under vacuum for 15 min, to remove excess of water. Then, the SAX cartridge was removed and the analytes retained were eluted from the HLB sorbent with 6 mL of methanol at 1 mL min^{-1}. The extract was evaporated under a gentle stream of nitrogen and reconstituted with 1 mL methanol–water (25:75, v/v). Prior to injection, soil and sediment extracts were filtered using syringe PTFE filters (0.22 μm, Analisis Vinicos, Tomelloso, Spain).

2.5. LC–ESI-MS/MS

The LC separation was performed using an Alliance 2695 HPLC separation module (Waters). In positive ion (PI) mode, a column Sunfire C_{18} (4.6 mm × 150 mm, 3.5 μm, from Waters) and a Gemini C_{18} (4.0 mm × 2.0 mm) guard cartridge (Phenomenex) were used. The mobile phase combines eluent A (formic acid 0.1% in methanol) and eluent B (formic acid 0.1% in water) in a gradient programme that started at 20% A for 0.1 min, increased linearly to 90% A in 15 min, then increase to 98% A in 15 min, hold for 8 min, and returned to initial conditions after 1 min followed by 11 min of equilibration time. The flow rate was 0.2 mL min^{-1}. In NI mode, a column Luna C_{18} (2) 100 Å (2.0 mm × 150 mm particle size 3 μm) and Gemini C_{18} (4.0 × 2.0 mm) guard cartridge both from Phenomenex were used. The mobile phase was composite of acetonitrile/methanol (60:40, v/v) as eluent A and ammonium acetate 10 mM in water as eluent B, at a flow rate of 0.2 mL min^{-1}. The analytical column was preconditioned using 15% of acetonitrile and 85% of eluent B at the same flow rate for 11 min. A gradient programme was used as follows: 15% of eluent A for 0.1 min, followed by a linear increase to 98% in 5 min, held for 7 min. Then, a 3 min gradient returned to the preconditioning conditions 15%

of acetonitrile and 85% of eluent B. The injection volume was 20 μL.

The tandem MS analyses were performed on a Micromass Quattro triple quadrupole mass spectrometer (Manchester, UK). Instrument control, data acquisition and evaluation were done with the MassLynx NT software (v. 3.4).

In both PI and NI mode, the applied parameters were: radio frequency lens, 0.5 V; electrospray source block, 125 °C; low mass (LM) 1 resolution, 12.0; high mass (HM) 1 resolution, 12.0; LM 2 resolution, 12.0; HM 2 resolution, 12.0; multiplier 650 V; desolvation temperature: 350 °C; argon collision gas 2.5×10^{-3} mbar; cone nitrogen gas flow, 50 L h^{-1}; desolvation gas: 600 L h^{-1}. In PI mode, the extractor voltage was 2.0 V and capillary voltage 4.0 kV. In NI mode the parameters for the analysis were: extractor voltage, 1.0 V and capillary voltage 3.2 kV. The optimal quantification and confirmation transitions and their respective cone voltages and collision energies are listed in Table 3. 2.

2.6. Validation of the analytical procedure

The criteria applied to confirm the identity of a suspected pharmaceutical were: (i) the ratio of the relative (to the I.S.) retention time of the analyte to that of the same analyte in standard solution should be within ±2.5% tolerance; (ii) the presence of a signal at each of the two SRM transitions for the analyte; (iii) the peak area ratio of the confirmation transition against the quantification one should be within the tolerance fixed by the EU criteria [32].

Linearity was studied using standard solutions and matrix-matched calibrations by analyzing in triplicate eight concentration levels, between 1 and 1000 μg L^{-1} in the final extract, equivalent to 0.3 and 333 ng g^{-1} in soil.

The matrix effects were studied by the evaluation of signal suppression or enhancement for each pharmaceutical. The signal suppression was calculated as a percentage of the decrease or increase in signal intensity in a sample matrix versus in methanol–water (25:75, v/v). The equation used for the signal suppression calculation was (Eq. (1)):

$$\text{signal suppression (\%)} = \left[1 - \frac{Sm}{Ss}\right] \times 100 \quad (1)$$

where Sm is the slope of the calibration curve for each analyte in the sample extract (soils or sediments) spiked after extraction, and Ss is the slope in solution standard (methanol–water, 25:75, v/v) at the same concentration than the spiked sample. No pharmaceuticals were previously detected in the samples.

The extraction recoveries of the different compounds for the entire PLE–SPE–LC–ESI-MS/MS procedures were determined for soil and sediments. Soil and sediment samples were spiked with the analytes at three different concentrations: (MQL, 50 and 100 ng g^{-1}) and 33 ng g^{-1} of each ISs (volume varied between 5 and 100 μL). The solvent was removed by evaporation in a fume cupboard for 10 min. Then, the spiked samples were stirred vigorously for 30 min in order to enable better contact of analytes with the matrix. After 24-h equilibration, these samples together with the correspondent blank samples were extracted and treated by the previously described protocol. Some soil and sediment samples were left to age in the dark, at room temperature, for a period of 3 months.

The precision of the method was determined by the repeated analysis of samples of soils and sediments spiked at concentrations of 50 ng g^{-1} and calculated as the relative standard deviation (RSD, %) of measurements in quintuplicate carried out in the same day and in five non-consecutive days.

Instrumental detection limits (IDLs) and instrumental quantification limits (IQLs) were estimated by direct injection of decreasing concentrations of the standard mixture, as the amount of analyte that gave a signal-to-noise ratio of 3:1 and 10:1, respectively, in SRM mode. Method detection limits (MDLs) were confirmed by injecting seven replicated extracts of samples spiked at the estimated concentrations. Method quantification limits (MQLs) were the lower concentration that provided acceptable recovery (relative recoveries ≥70%, excepting fenofibrate and diclofenac) and precision (<20%) was tested by analyzing spiked soil and sediment samples in quintuplicate.

3. Results and discussion

3.1. Optimization of the PLE procedure

All experiments for optimizing the different steps of the method were carried out by spiking a soil sample free of contamination with a mixture of pharmaceuticals at 50 ng g^{-1}. The choice of the extraction solvent is one of the most critical parameters. Methanol (MeOH), water, combinations of both solvents at different ratios (80:20 and 50:50,v/v), acetonitrile/water 50:50(v/v), MeOH/57 mM citric acid 50:50 (v/v) and MeOH/0.1 M Na_2-EDTA 50:50 (v/v) were tested for the optimization of the PLE at different temperatures and with different sorbents. Ion complexing agent solutions, as citric acid or EDTA, frequently block the conductions and valves of the PLE system and do not improve significantly the recoveries of the analytes. Fig. 3.1A shows the recoveries obtained by extracting soil dispersed in Na_2-EDTA washed sea sand with water and mixtures of methanol-water and acetonitrile-water at 90 °C for 7 min at 500 psi and flush 100%. No great differences were observed in the recoveries provided by the different solvents. Water was selected as the best choice for its compatibility with SPE, and because it is an interesting solvent for ecological considerations.

The type of sorbent to disperse soil and sediment samples prior PLE (aluminium oxide 90, silica gel, Florisil® and sea sand) was studied. Although sea sand clearly provided the best recoveries and the coarse size of the sand grains favour the dispersion, values obtained were much lower than those reported in Fig. 3.1A ranging from 25% to 60%). An explanation for these poor recoveries is that antibacterials significantly bind to matrix components, specifically organic matter and metals. According to the literature on the subject, complexes formed between antibacterials and divalent and trivalent cations present in soil or sediment can be displaced using complexing agents [3,5,18]. As the addition of a complexing agent to the water was quite incompatible with the instruments making the method less robust and did not yield better recoveries, the washing of sea sand with it was tested.

Sea sand washed with Na_2–EDTA 0.1 M and sea sand washed with 0.1 M Na_2–EDTA–McIlvaine buffer solution (pH 4) were selected since they are the most reported complexing extractant solutions [13,16,33]. Recoveries achieved using Na_2–EDTA were slightly superior or comparables to recoveries for the McIlvaine+EDTA combination.

Other PLE extraction parameters, such as the extraction temperature (50–110 °C), number of extraction cycles (1–5), pressure (500–2500 psi), flush volume (60–120% of the extraction cell volume) and static time (3–15 min) were studied in order to select the best conditions for the analysis of the selected pharmaceuticals. Recoveries obtained are shown in Fig. S3.5, Supplementary material. The cell size of 22 and 11 mL were tested, giving best recoveries and clean extracts the 22 mL cell. The extraction temperature and the number of cycles applied were critical for improving recov- eries, while pressure had no significant influence. The increase in the flush volume and static time got better recoveries to reach their maximum at the selected values. However their effect is not as accentuated as for the other parameters. Temperature presents the most erratic effect on the analyte recoveries as previously dis- cussed [24,25]. The increase of temperature decreases significantly the dielectric constant of the water increasing the solubility of

Table 3.2
Conditions of MS/MS in PI and NI modes.

Compound	T_r (min)	CV[a] (eV)	Quantification transition[b]	CE[c] (eV)	Confirmation transition[b]	CE[c] (eV)
PI mode						
Acetaminophen	16.4	25	152→110	15	152→92.5	25
Acetaminophen-d_3	16.4	20	155→111	15	155→92.5	20
Carbamazepine	25.9	30	237→193	35	237→192	40
Carbamazepine-d_2	25.9	35	239→195	20	239→194	30
Ciprofloxacin	14.5	30	332→314	20	332→231	35
Codeine	7.4	35	300→215	25	300→199	30
Diazepam	28.9	40	285→154	25	285→193	30
Fenofibrate	36.2	25	361→233	15	361→139	30
Metoprolol	15.2	30	268→116	20	268→98	20
Norfloxacin	14.4	30	320→276	15	320→302	20
Ofloxacin	13.8	30	362→318	20	362→261	25
Oxytetracycline	15.7	25	461→426	20	461→443	10
Propanolol	18.2	30	260→116	18	260→183	20
Sulfamethoxazole	20.0	25	254→92	25	254→156	15
Tetracycline	15.0	24	445→410	20	445→427	15
Trimethoprim	11.8	40	291→123	25	291→230	25
NI mode						
Clofibric acid	8.0	20	213→127	18	213→84.5	10
Diclofenac	9.6	20	294→250	15	294→214	25
Ibuprofen	10.2	15	205→161	10	–	–
Ibuprofen-d_3	10.2	15	208→164	10	208→162	15

[a] Cone voltage.
[b] Transition = precursor ion → product ion.
[c] Collision energy.

non-polar analytes. The recoveries obtained at 70 °C were appropriate for most of analyzed compounds. The increase flush volume and the duration of the static cycles improved the recoveries. Ofloxacin, fenofibrate, codeine, trimethoprim, diazepam, metoprolol, propanolol, ibuprofen and clofibric acid gave better recoveries at temperature of 90 °C. The temperature of 110 °C provided slightly improved recoveries for ofloxacin, ciprofloxacin, metoprolol, propanolol and diclofenac but thermal degradation of some compounds can occur. The best results were obtained with the conditions reported in Section 2.3.

3.2. Optimization of isolation and pre-concentration using SPE

The performance of different types of SPE cartridges was tested, including two polymeric sorbents (Oasis HLB and Strata-X) and a strong anion exchange sorbent (Isolute SAX). The Isolute SAX car-

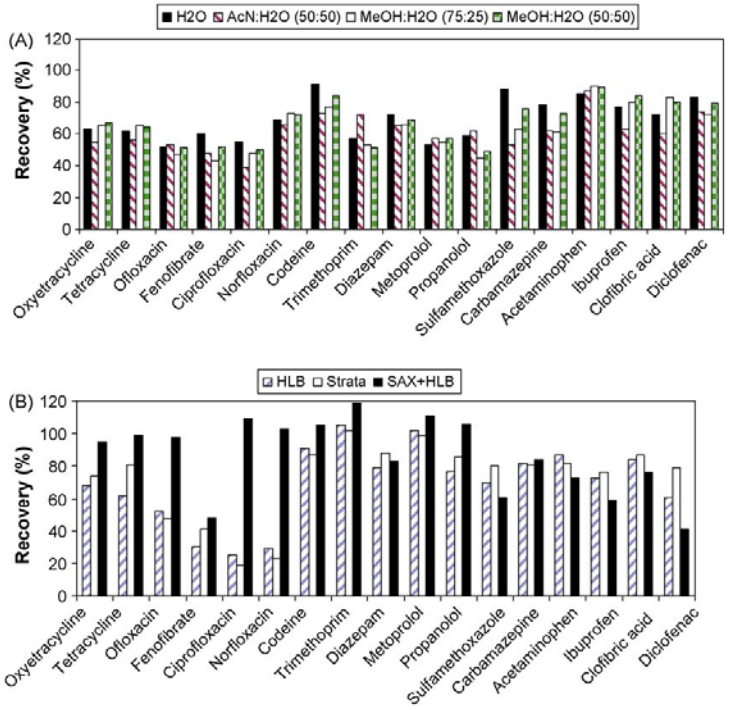


Fig. 3.1. Comparison of recoveries obtained using different (A) extraction solvents and (B) SPE cartridges. Concentration level 50 ng g^{-1}.

tridge was used combined in tandem with the OASIS HLB cartridges. The SAX column reduces matrix interferences by adsorbing anionic humic and fulvic acids from the soil extracts, avoiding contamination, blocking and overloading of the HLB sorbent. Aqueous extracts obtained from PLE have a pH of 7 and, in these conditions, pharmaceuticals are in their neutral or cationic form (see pK_a values in Table 3.1) and, consequently, they are not retained on the SAX cartridge.

The effect of the PLE extract acidification prior pass it through SPE cartridge provided recoveries between 5% and 20% lower for basic and neutral pharmaceuticals than those obtained without acidification. Nevertheless, for acidic compounds results were very similar.

As it is shown in Fig. 3.1B, the behaviour of Strata-X and Oasis HLB was very similar, achieving both recoveries better than 70%, except for ofloxacin, ciprofloxacin, norfloxacin and fenofibrate. The coupling of a previous SAX cartridge, to eliminate interfering and matrix compounds, increased the analyte's recoveries to values higher than 70%, except for fenofibrate, the recovery of which remains unchanged. This effect may due to its relatively non-polar character (it has the lowest polarity of all selected pharmaceuti- cals), therefore Oasis HLB and Strata-X cartridges were not able to retain this compound at all [34]. On the other compounds, the inclusion of the SAX cartridge only causes a marked decrease in the recovery of diclofenac (see Fig. 3.1B). This could be because at pH 7, diclofenac is in zwitterionic form keeping partly retained to the negatively charged SAX cartridge or forming complexes with the organic matter that are retained in the SAX cartridge. Between Oasis HLB and Strata-X, the former was finally selected just because the higher availability of these cartridges in the laboratory.

3.3. Optimization of LC–MS/MS

Most of the compounds showed maximum sensitivity operating in the PI mode excepting ibuprofen, diclofenac and clofibric acid that only give response in NI mode.

In PI mode, three columns (Waters Sunfire C_{18}, Waters Xterra C_{18} and Phenomenex Luna C_{18} (2)) were tested using mobile phases composed of different proportions of methanol or acetonitrile and water with different additives, such as ammonium acetate and formic acid, at various concentrations. The optimal separation of 14 compounds detected in PI mode was achieved using the Waters Sunfire column, and methanol and water, both with 0.1% formic acid, as mobile phase. Acid was used to improve ionization and sensitivity of MS detection. Fig. 3.2 depicts SRM chromatograms for the spiked soil at level 25 ng g^{-1}, illustrating the good separation and narrow peak shape obtained for the selected compounds in the PI mode. Signals of matrix components (marked in Fig. 3.2) were only observed for the transitions corresponding to acetaminophen and trimethoprim. Those matrix compounds gave peaks that were well separated from the analytes peak and did are not present in the confirmatory transition. The peak corresponding to the epimer of the tetracycline was also visible in the chromatogram. Some trans- formation of tetracycline to its epimer was always observed. This compound was quantified as the sum of both isomers. On the con- trary, oxytetracycline did not show epimerization. These results agree with previous studies [16].

In NI mode, the chromatographic separation was very troublesome, even though there were only three compounds to be detected. Six analytical columns were tested (Waters Sunfire C_{18}, Waters Xterra C_{18}, Phenomenex Luna C_{18} (2), Luna C_8, Phenomenex Gemini C_{18} and C_6-Phenyl) with different mobile phases. To illustrate the problems, Fig. 3.3 shows SRM chromatograms from an extract of a spiked sediment at 20 ng g^{-1} analyzed by NI mode, using different LC columns. In many proofs, the three analytes were too much separated even using a high percentage of acetonitrile in the mobile phase and their elution order was inverted. The most apolar compound – ibuprofen – eluted first and the most polar one – diclofenac – was the longest retained requiring more than 20 min to elute from the column and presented with broad shape unacceptable to quantify (Fig. 3.3A). This can be related to the formation of zwitterionic forms as discussed for diclofenac in the SPE optimiza- tion. In other cases, chromatographic separation was achieved, but peaks appeared with "crown" (Fig. 3.3B). The shape of these peaks did not improve significantly even adding ammonium acetate as mobile phase additive. Finally, separation was achieved (Fig. 3.3C) on the Luna C_{18} (2) with a mixture of acetonitrile/methanol (60:40, v/v), and preconditioning the column prior the next injection with acetonitrile instead of acetonitrile/methanol (60:40).

The acquisition of, at least, two transitions for reliable confirmation is possible for all pharmaceuticals, except ibuprofen that gives only one fragment with reasonable sensitivity (Table 3.2). In the PI mode, the confirmation of the compound identity by a second transition requires an additional injection due to the high number of transitions needed for the simultaneous quantification and con- firmation. The acquisition of two transitions for each compound would entail to monitor more than 28 transitions, which would reduce the number of point per peak leading to unsatisfactory peak shapes. The IDL ranged from 2 pg injected for carbamazepine, trimethoprim and fenofibrate to 34 pg for acetaminophen. The IQLs were between 7 and 114 pg injected. The second injection monitor- ing a fewer number of transitions allows to confirm the identity of pharmaceuticals at these low levels.

3.4. Validation of the method

3.4.1. Specificity and selectivity of the method

The specificity and selectivity of the method were established by the analysis of blank samples. The absence of any chromatographic peak in soil and sediment extracts, at the same retention times as target pharmaceuticals, indicated that there were not matrix compounds that might give a false positive signal in these blank samples.

3.4.2. Linearity and matrix effects

Matrix-matched calibration curves prepared in every type of sample showed good linearity between 1 and 1000 ng mL^{-1}, with correlation coefficient ≥0.993 (Table 3.3). Absolute signal suppression measured for compounds analyzed under PI conditions varied from 3% to 54% in sediments and from 0.6% to 56% in soils, as it can be seen in Table 3.4. A slight signal suppression was observed for metoprolol, codeine, trimethoprim and fenofibrate (<15% calculated using the absolute recovery). In the case of acetaminophen a little enhancement of signal was observed (≈3%) as already reported [25]. For the other compounds, higher suppression (up to 55%) was observed. For the compounds analyzed under NI conditions, suppression ranged from 19% to 34% in sediments and from 15% to 31% in sediments. The impact of the matrix effect was almost equivalent in both matrices but different for each compound. The suppression effect was only partly corrected by the addition of internal standards since matrix effects are compound dependent. However, the use of matrix-matched standards compensated quite well for the suppression effect achieving accurate quantification.

3.4.3. MDLs and MQLs

Table 3.3 also outlines MDLs for soil and sediment samples that were in the range 6–408 pg injected or 0.2–6.8 ng g^{-1} in sediments and 5–311 pg injected or 0.1–5.3 in soils. The sensitivity for sediment and soil samples was comparable. These MDLs were of the same order than those reported by Cuevas-Mestanza et al. [23] using GC–MS and Radjenović et al. [25] using LC–MS/MS and better than others reported in previous studies by LC [23,24]. Cuevas-

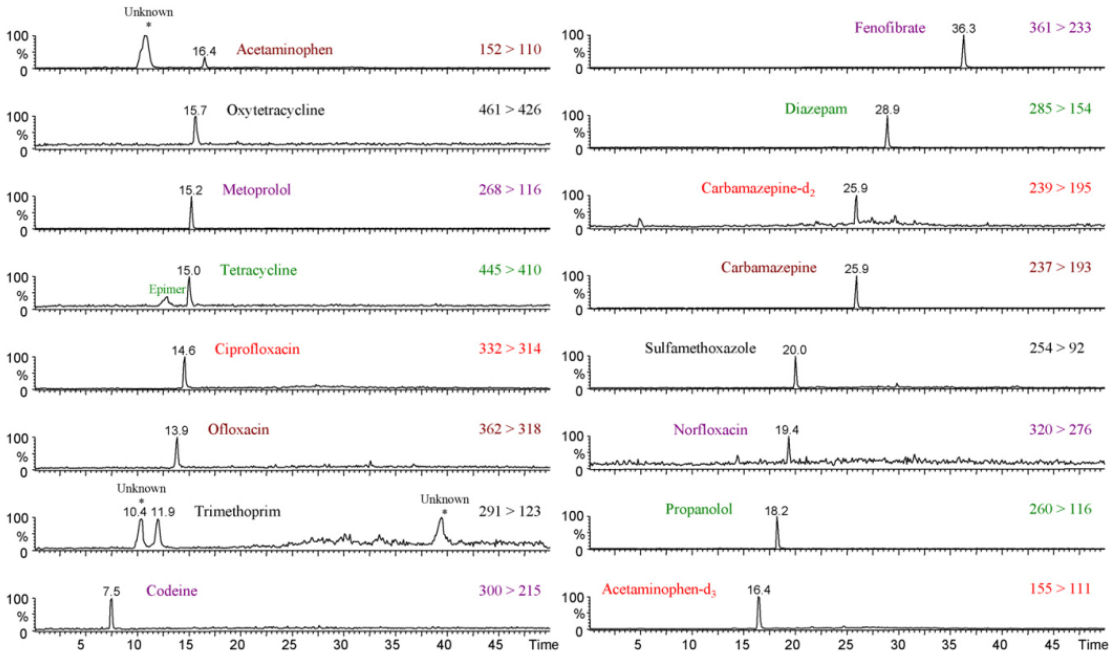


Fig. 3.2 LC–MS/MS chromatogram in PI mode obtained from an extract of soil spiked at 25 ng g^{-1} of each compound.

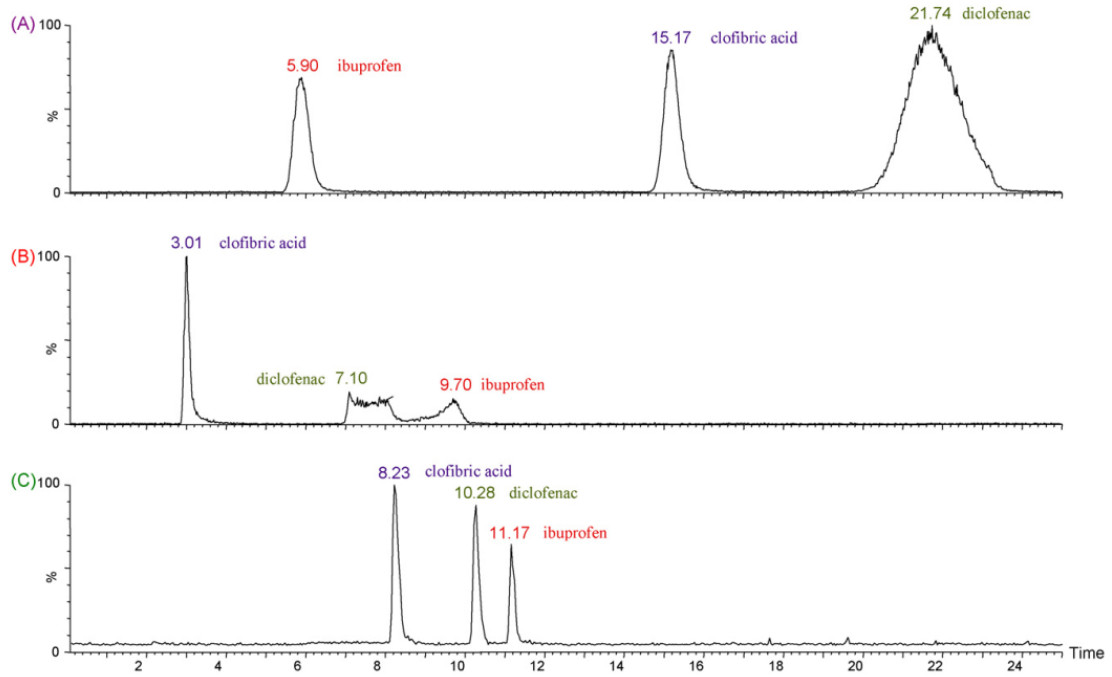


Fig. 3.3 LC–MS/MS chromatograms in NI mode obtained from an extract of soil spiked at 25 ng g^{-1} of each compound using (A) analytical column Phenomenex Luna C_{18} (2) (150 mm × 4.6 mm, 5 μm), mobile phase: water (a) and MeOH (b) and gradient from 15% to 98% b in 5 min and hold 8 min. (B) Analytical column Phenomenex C_6-Phenyl (150 mm × 2.0 mm, 3 μm), mobile phase: water (a), acetonitrile (b) and gradient from 25% to 98% b in 10 min, and (C) analytical column Phenomenex Luna C_{18} (2) (150 mm × 2.0 mm, 3 μm), mobile phase: water 5 mM ammonium formiate (a), acetonitrile/MeOH (60:40, v/v) (b), gradient from 15% to 98% b in 5 min and hold 7 min, preconditioning 15% of acetonitrile and 85% of a, 15 min.

Table 3.3
Linearity equation, method detection limits (MDL), repetitivity and reproducibility.

Compound	Equation[a]	Linearity (r^2)	MDL		Repetitivity RSD (%) ($n=5$)	Reproducibility RSD (%) ($n=5$)
			(pg injected)	(ng g^{-1})		
Sediments						
Oxytetracycline	$y = 63.655x - 383.79$	0.9998	408	6.8	7.4	13.2
Tetracycline	$y = 149.19x - 1284.2$	0.9996	354	5.9	7.9	14.5
Ofloxacin	$y = 109.52x - 1419.29$	0.9998	162	2.7	4.3	11.9
Fenofibrate	$y = 804.32x - 3202.5$	0.9994	36	0.6	3.1	4.4
Ciprofloxacin	$y = 178.92x - 993.45$	0.993	240	4.0	7.6	12.8
Norfloxacin	$y = 16.50x - 207.52$	0.9996	312	5.2	3.2	9.1
Codeine	$y = 36.841x - 85.117$	0.9991	24	0.4	3.6	5.9
Trimethoprim	$y = 197.03x - 1043$	0.998	18	0.3	6.1	8.0
Diazepam	$y = 300.58x - 631.71$	0.998	6	0.8	2.3	2.4
Metoprolol	$y = 223.06x + 2050.22$	0.9994	24	0.4	1.1	2.0
Propanolol	$y = 267.52x - 217.44$	0.998	12	1.2	0.9	2.3
Sulfamethoxazole	$y = 106.84x - 279.86$	0.998	18	0.3	1.0	1.6
Carbamazepine	$y = 218.38x - 48.63$	0.9995	12	0.2	1.2	2.8
Acetaminophen	$y = 137.23x + 241.15$	0.9993	18	0.3	1.2	3.5
Ibuprofen	$y = 48.708x - 302.94$	0.996	96	1.6	2.4	3.9
Clofibric acid	$y = 75.863x - 202.68$	0.998	30	1.5	1.7	3.2
Diclofenac	$y = 63.037x - 52.366$	0.9990	60	1.0	3.1	7.8
Soils						
Oxytetracycline	$y = 86.964x - 452.45$	0.997	311	5.3	6.9	14.1
Tetracycline	$y = 314.60x - 1322.4$	0.9992	296	4.8	7.5	14.3
Ofloxacin	$y = 197.48x - 1574.5$	0.9994	181	2.9	3.7	9.4
Fenofibrate	$y = 874.56x - 234.5$	0.9990	38	0.6	3.8	5.2
Ciprofloxacin	$y = 156.45x - 345.29$	0.998	272	4.1	7.8	13.2
Norfloxacin	$y = 34.645x - 234.25$	0.9991	280	4.7	4.2	8.0
Codeine	$y = 90.83x - 74.498$	0.9995	24	0.3	2.1	3.3
Trimethoprim	$y = 234.10x - 897.3$	0.9993	13	0.2	3.6	4.7
Diazepam	$y = 323.08x - 324.63$	0.9996	5	0.1	2.9	3.9
Metoprolol	$y = 564.30x + 434.92$	0.995	27	0.5	1.8	5.3
Propanolol	$y = 894.36x - 582.34$	0.997	22	0.4	1.2	2.1
Sulfamethoxazole	$y = 183.90x - 327.74$	0.9991	33	0.6	1.1	2.7
Carbamazepine	$y = 423.21x - 89.07$	0.9992	8	0.1	0.7	3.0
Acetaminophen	$y = 283.41x + 113.76$	0.994	12	0.1	0.8	2.8
Ibuprofen	$y = 76.740x - 45.92$	0.996	114	1.8	3.3	4.5
Clofibric acid	$y = 86.353x - 129.63$	0.998	20	0.3	1.2	2.9
Diclofenac	$y = 156.45x - 84.53$	0.9990	35	0.6	2.4	4.6

[a] Calculated as peak areas versus concentration.

Table 3.4
Percent of signal suppression of pharmaceuticals in sediments and soils spiked after extraction.

Compound	Sediments		Soils	
	Absolute	Relative[a]	Absolute	Relative[a]
Positive mode				
Oxytetracycline	18.8 ± 1.7	0.1 ± 2.3[b]	11.1 ± 1.9	0.4 ± 2.7[b]
Tetracycline	16.7 ± 5.1	−2.5 ± 1.8[b]	8.4 ± 2.9	0.5 ± 1.2[b]
Ofloxacin	41.3 ± 6.4	27.7 ± 3.1[b]	35.6 ± 4.0	26.5 ± 3.3[b]
Fenofibrate	4.5 ± 0.3	18.6 ± 1.9[c]	4.3 ± 0.5	13.7 ± 1.6[c]
Ciprofloxacin	52.0 ± 1.9	3.1 ± 0.4[c]	56.0 ± 3.3	6.2 ± 0.7[c]
Norfloxacin	54.6 ± 2.4	46.7 ± 0.7[b]	40.7 ± 3.2	38.6 ± 1.8[b]
Codeine	9.5 ± 1.0	4.9 ± 1.3[b]	9.3 ± 1.5	6.2 ± 2.1[b]
Trimethoprim	11.3 ± 4.8	6.2 ± 2.7[b]	10.1 ± 2.0	3.2 ± 1.3[b]
Diazepam	21.0 ± 0.8	7.2 ± 1.6[b]	25.4 ± 1.9	7.1 ± 3.0[b]
Metoprolol	3.1 ± 2.0	−13.8 ± 0.4[b]	0.6 ± 1.3	−5.1 ± 0.3[b]
Propanolol	21.8 ± 1.8	8.1 ± 4.5[b]	16.2 ± 1.1	7.5 ± 3.7[b]
Sulfamethoxazole	16.3 ± 0.9	1.6 ± 1.1[b]	14.8 ± 2.4	3.5 ± 2.1[b]
Carbamazepine	14.8 ± 1.7	0.0 ± 1.3[c]	8.8 ± 1.6	0.3 ± 1.9[c]
Acetaminophen	−2.6 ± 0.2	−0.5 ± 0.9[b]	−4.9 ± 1.6	0.1 ± 0.6[b]
Negative mode				
Ibuprofen	22.9 ± 1.4	−6.7 ± 0.6[d]	27.4 ± 1.0	−2.2 ± 0.9[d]
Clofibric acid	33.7 ± 1.9	12.4 ± 3.4[d]	31.4 ± 1.3	10.5 ± 2.1[d]
Diclofenac	19.3 ± 0.8	−26.8 ± 1.7[d]	15.3 ± 1.8	−13.4 ± 1.6[d]
Internal standards				
Acetaminophen-d_3	−17.2 ± 0.5	–	−15.2 ± 0.8	–
Carbamazepine-d_2	14.9 ± 1.1	–	17.6 ± 1.4	–
Ibuprofen-d_3	35.8 ± 1.7	–	29.9 ± 1.2	–

[a] Recovery relative to ISs.
[b] Acetaminophen-d_3.
[c] Carbamazepine-d_2.
[d] Ibuprofen-d_3.

Table 3.5
MQL (ng g^{-1}), recoveries (%) and RSDs at three spiking levels.

Pharmaceuticals	Soil							Sediment						
	MQL	50 ng/g			100 ng/g			MQL	50 ng/g			100 ng/g		
	MQL (ng/g)	Absolute recovery (%)	Relative recovery (%)[a]	Absolute recovery (%)	Relative recovery (%)[a]	Absolute recovery (%)	Relative recovery (%)[a]	MQL (ng/g)	Absolute recovery (%)	Relative recovery (%)[a]	Absolute recovery (%)	Relative recovery (%)[a]	Absolute recovery (%)	Relative recovery (%)[a]
Positive mode														
Oxytetracycline	18	68 ± 13	96 ± 6	63 ± 16	94 ± 13	59 ± 5	88 ± 9	23	66 ± 5	90 ± 10	63 ± 9	94 ± 6	64 ± 10	92 ± 8
Tetracycline	16	62 ± 15	98 ± 5	62 ± 10	99 ± 6	60 ± 10	95 ± 7	19	68 ± 8	88 ± 9	64 ± 8	92 ± 9	70 ± 13	97 ± 7
Ofloxacin	7	52 ± 7	100 ± 12	54 ± 17	99 ± 16	58 ± 12	99 ± 4	6	59 ± 12	96 ± 8	55 ± 10	91 ± 8	50 ± 12	101 ± 6
Fenofibrate	1.5	40 ± 7	66 ± 6	46 ± 15	64 ± 12	41 ± 9.2	59 ± 10	1.8	50 ± 5	66 ± 7	48 ± 5	67 ± 8	47 ± 14	64 ± 3
Ciprofloxacin	10	55 ± 9	86 ± 8	52 ± 16	87 ± 11	63 ± 13	91 ± 5	11	59 ± 9	84 ± 13	62 ± 11	88 ± 6	55 ± 12	83 ± 5
Norfloxacin	15	69 ± 11	71 ± 10	64 ± 12	77 ± 10	70 ± 11	83 ± 3	17	72 ± 13	74 ± 8	70 ± 6	73 ± 8	67 ± 8	74 ± 6
Codeine	1.3	91 ± 13	108 ± 6	90 ± 12	85 ± 7	94 ± 8.4	93 ± 7	1.5	98 ± 7	101 ± 4	95 ± 5	99 ± 5	99 ± 6	102 ± 4
Trimethoprim	0.9	105 ± 5	119 ± 4	95 ± 11	101 ± 4	91 ± 9.0	106 ± 8	1.2	97 ± 4	104 ± 7	95 ± 6	99 ± 11	93 ± 10	101 ± 7
Diazepam	0.25	79 ± 8	104 ± 1	79 ± 13	102 ± 10	78 ± 6.8	101 ± 6	2.3	76 ± 9	107 ± 12	79 ± 7	98 ± 5	77 ± 8	103 ± 5
Metoprolol	1	102 ± 10	81 ± 9	104 ± 12	87 ± 6	99 ± 7.5	85 ± 11	0.8	85 ± 3	92 ± 8	93 ± 8	95 ± 7	98 ± 6	99 ± 4
Propanolol	0.5	77 ± 19	96 ± 2	78 ± 16	99 ± 11	72 ± 5.1	102 ± 4	3.7	75 ± 7	90 ± 11	74 ± 11	88 ± 8	71 ± 17	81 ± 15
Sulfamethoxazole	0.9	70 ± 16	97 ± 3	76 ± 14	108 ± 9	79 ± 1.9	107 ± 13	0.9	84 ± 14	99 ± 4	87 ± 5	103 ± 14	85 ± 11	94 ± 9
Carbamazepine-d_2	–	103 ± 13	101 ± 5	102 ± 10	98 ± 4	94 ± 12	98 ± 7	–	96 ± 9	103 ± 7	91 ± 9	104 ± 17	98 ± 8	97 ± 5
Carbamazepine	0.5	82 ± 10	104 ± 6	86 ± 13	104 ± 7	85 ± 14	106 ± 12	0.5	88 ± 6	110 ± 5	88 ± 3	96 ± 7	91 ± 12	102 ± 10
Acetaminophen-d_3	–	84 ± 9	–	89 ± 11	–	87 ± 2.3	–	–	77 ± 11	–	79 ± 7	–	82 ± 8	–
Acetaminophen	0.8	87 ± 14	102 ± 9	74 ± 7	106 ± 7	82 ± 5.8	112 ± 7	0.5	72 ± 9	98 ± 8	74 ± 4	101 ± 5	72 ± 10	103 ± 6
Negative mode														
Ibuprofen	4	73 ± 8	84 ± 4	75 ± 7	86 ± 3	81 ± 9.9	89 ± 5	3.6	77 ± 11	85 ± 6	85 ± 9	93 ± 8	83 ± 18	92 ± 12
Ibuprofen-d_3	–	79 ± 13	–	61 ± 6	–	66 ± 7.2	–	–	74 ± 9	–	72 ± 6	–	70 ± 6	–
Clofibric acid	1.6	84 ± 18	102 ± 4	74 ± 11	80 ± 6	78 ± 11	91 ± 9	4.2	71 ± 9	97 ± 6	74 ± 5	95 ± 7	77 ± 10	110 ± 4
Diclofenac	3	34 ± 16	62 ± 6	37 ± 5	67 ± 2	35 ± 9.3	66 ± 12	3.7	42 ± 6	66 ± 7	37 ± 8	65 ± 12	39 ± 16	69 ± 10

[a] Recovery relative to ISs as reported in Table 3.4.

Table 3.6
Concentration (ng g^{-1}) of pharmaceuticals in different marsh areas of the Valencian Community.

Compound	Albufera sediment	Oliva-Pego sediment	Prat soil	Moros sediment	Silla soil
Oxytetracycline					
Tetracycline					
Ofloxacin	8.95	12.03		<MQL	
Fenofibrate	13.20			17.23	
Ciprofloxacin		5.95			
Norfloxacin					
Codeine	3.35				
Trimethoprim					
Diazepam	2.50	2.86	4.65	3.72	
Metoprolol	6.57				
Propanolol	1.51	2.60			
Sulfamethoxazole					
Carbamazepine	1.81	2.93	5.77	6.85	1.43
Acetaminophen	<MQL				<MQL
Ibuprofen					
Clofibric acid	<MQL	35.62			
Diclofenac					

Mestanza et al. [23] reported MDLs that varied between 4 ng g^{-1} for ibuprofen and 167 ng g^{-1} for fenofibrate. Löffler and Ternes [24] gave values of MDLs between 0.4 and 20 ng g^{-1}. As can be observed in Table 3.5, the MQLs were also at low nanogram per gram levels and ranged from 0.5 ng g^{-1} for carbamazepine acetaminophen and propanolol to 23 ng g^{-1} for oxytetracycline, which makes the method useful for the determination of low levels of pharmaceuticals in soils and sediments in real environmental samples.

3.4.4. Recovery and precision

Precision data are also listed in Table 3.3 for soil and sedi- ments. The repeatability values were in the range of 0.1–7.9% for sediments and 0.7–7.5% for soils and the reproducibility ones of 1.6–14.5% for sediments and of 2.1–14.3% for soils. These results did not show apparent differences between soil and sed- iment samples and are similar to those reported by other studies [14,16,23].

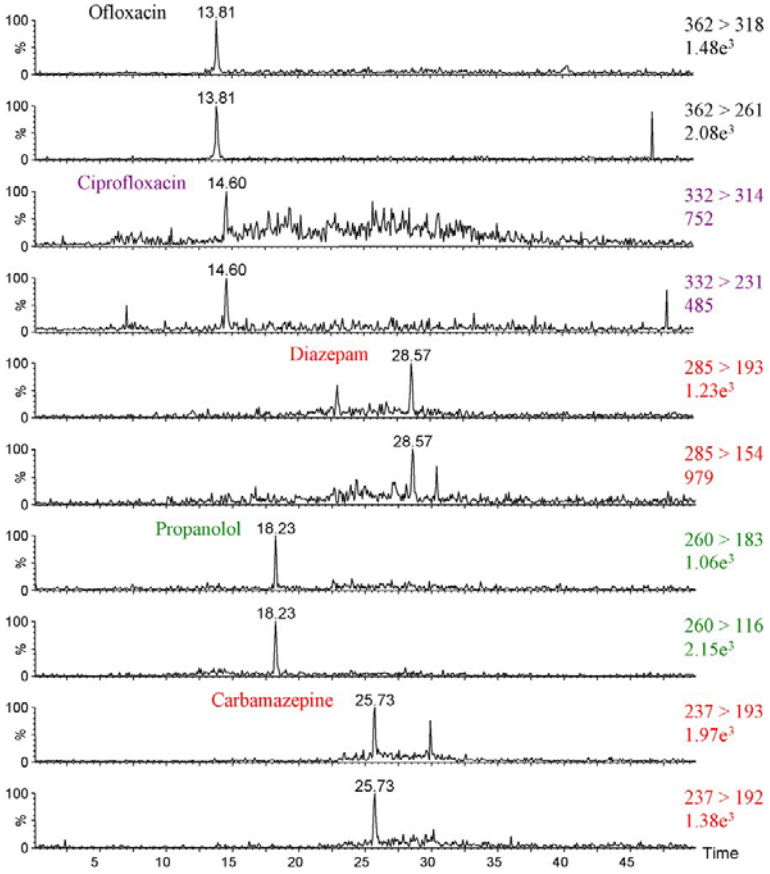


Fig. 3.4. Chromatograms showing the analysis of target pharmaceuticals in a Oliva-Pego sediment sample, including two SRM transitions for each analyte.

Due to lack of a reference material the recovery of the developed method was tested by spiking soil and sediments samples after 24 h of equilibration time. Absolute and relative recoveries were determined in soil and sediment at three concentration levels. The results of these experiments are summarized in Table 3.5. Absolute recoveries ranged from 34% to 105% with RSDs < 19% for soils and from 37% to 99% with RSDs < 17% for sediments. Rela- tive recoveries ranged from 59% to 119% with RSDs lower than 16% for soils and from 64% to 110% with RSDs lower than 17% for sediments. The recoveries varied significantly depending on com- pound but not depending on the matrix. Decreasing recoveries from aged-spiked soils for sulfonamides were recently reported [14] indicating that spiked samples can be a poor indicator of incurred samples unless they are adequately aged. To check this point, sev- eral soil and sediment samples were aged for 3 months. Absolute recoveries ranging from 32% to 106% for soils and between 42% and 98% for sediments were obtained. Comparison of the recovery obtained for each pharmaceutical in freshly spiked and aged-soils and sediments are presented in Fig. S3.6, Supplementary material. There is no evident difference between values obtained by both spiking procedures either in soil or sediment. A qualitative difference was only observed for tetracycline because the percentage of its epimer increased at the expenses of that of the tetracycline. These results demonstrated the performance of the developed extraction method to isolate analytes occurring in samples.

3.4.5. Application to samples

Table 3.6 shows the concentrations of the target pharmaceuticals detected in the contaminated samples. Of 16 samples analyzed, pharmaceuticals were detected in 5. Most detected compounds were carbamazepine (detected in all samples) and diazepam (in four samples) with concentrations between 1.4 and 6.8 ng g^{-1}. Ofloxacin, fenofibrate, ciprofloxacin, codeine, metoprolol, propanolol, acetaminophen and clofibric acid were less frequently present. Maximum concentrations were detected for clofibric acid, with average concentration of 35.6 ng g^{-1} and for fenofibrate at 17 ng g^{-1}, even through the results for fenofibrate can only be consider as semi-quantitative. A chromatogram obtained from a sediment sample taken at the Oliva-Pego marsh using both, quantification and confirmatory, SRMs for each detected analyte is shown in Fig 3.4. These preliminary date indicate that pharmaceuticals may be discharged in large amounts through wastewater effluents from human origin, arriving into natural environments. To our knowledge, these results present the first evidence of contamination of marsh areas with pharmaceuticals.

4. Conclusions

The developed method attains simultaneous extraction by PLE and pre-concentration by SPE of seventeen pharmaceuticals with a great variety of polarities and pK_a's, from soils and sediments. The use of LC–MS/MS afforded high sensitivity (MQLs in the low ng g^{-1}) and achieves unequivocal identification of these compounds. PLE followed by SAX+Oasis HLB proved efficient clean-up, yielding recovery rates for the selected compounds generally over 70%. Fenofibrate and diclofenac were the exception with recoveries up to 34%. These low recoveries only allow to obtain semi-quantitative results for these compounds.

However, in comparison with other studies, the present method achieves a significant increase in sensitivity achieving a decrease of the quantity of pharmaceuticals that could be detected in soils and sediments, being a powerful protocol to highlight pollution of pharmaceuticals in the ecosystems. The proposed analytical method also consumed very small amount of toxic chemicals and reagents (less than 11 mL of methanol per sample), with minimum waste production. It is also simple and inexpensive. Hence, it is considered to be a green analytical technique and environmental friendly method.

The application of this method to environmental samples proofs that significant amounts of acetaminophen, carbamazepine, ciprofloxacin, clofibric acid, codeine, diazepam, fenofibrate, metoprolol, ofloxacin and propanolol contaminate soils and sediments of marsh areas. According to the detected concentrations of fenofibrate in samples, more selective conditions for the analysis of this compound could be of interest (considering its non-polar character).

These data show that the proposed method is suitable for environmental monitoring and could be useful to establish the occurrence of selected human pharmaceutical compounds in soils and sediments with high content of organic matter.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.11.033.

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Supplementary material for

Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry

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Table S3.7
Physical and chemical properties of the studied soils and sediments

	Soil	Sediments
Horizon	Ap1	--
Depth (cm)	0-30	--
Water content (%)	2.69	--
Aggregate stabilty (%)	11.14	--
pH (H_20)	7.53	7.83
pH (KCl)	7.15	7.41
[b] E.C. (dS m^{-1})	2.05	
[c] CO_3 (%)	31.46	35.78
[d] O.M. (%)	2.07	16.37
N_{total} (%)	0.146	1.023
$N_{mineral}$ (cmol kg^{-1})	4.50	14.54
P_2O_5 (cmol kg^{-1})	14.74	21.93
[e] CEC (cmol kg^{-1})	15.09	
Ca^{2+} (cmol kg^{-1})	10.70	14.17
Mg^{2+} (cmol kg^{-1})	3.39	2.84
K^{+} (cmol kg^{-1})	0.50	0.44
Na^{+} (cmol kg^{-1})	0.51	0.86
Particle size distribution (%)		
< 0.002 mm	25.72	16.21
0.02-0.002 mm	8.78	
0.05-0.02 mm	19.50	
0.10-0.05 mm	7.21	
0.25-0.10 mm	18.98	
0.50-0.25 mm	4.53	
1.00-0.50 mm	1.25	
2.00-1.00 mm	0.57	
Total sand (%)	32.55	40.49
Total silt (%)	41.33	43.30

[a] Designation of soil profile horizons according FAO (1988).
[b] Electric conductivity.
[c] Total carbonate concentration.
[d] Organic matter.
[e] Cation exchange capacity

Fig S3.5
Comparison of recoveries obtained using different (A) temperatures, (B) number of cycl
(C) static time and (D) flush volume.

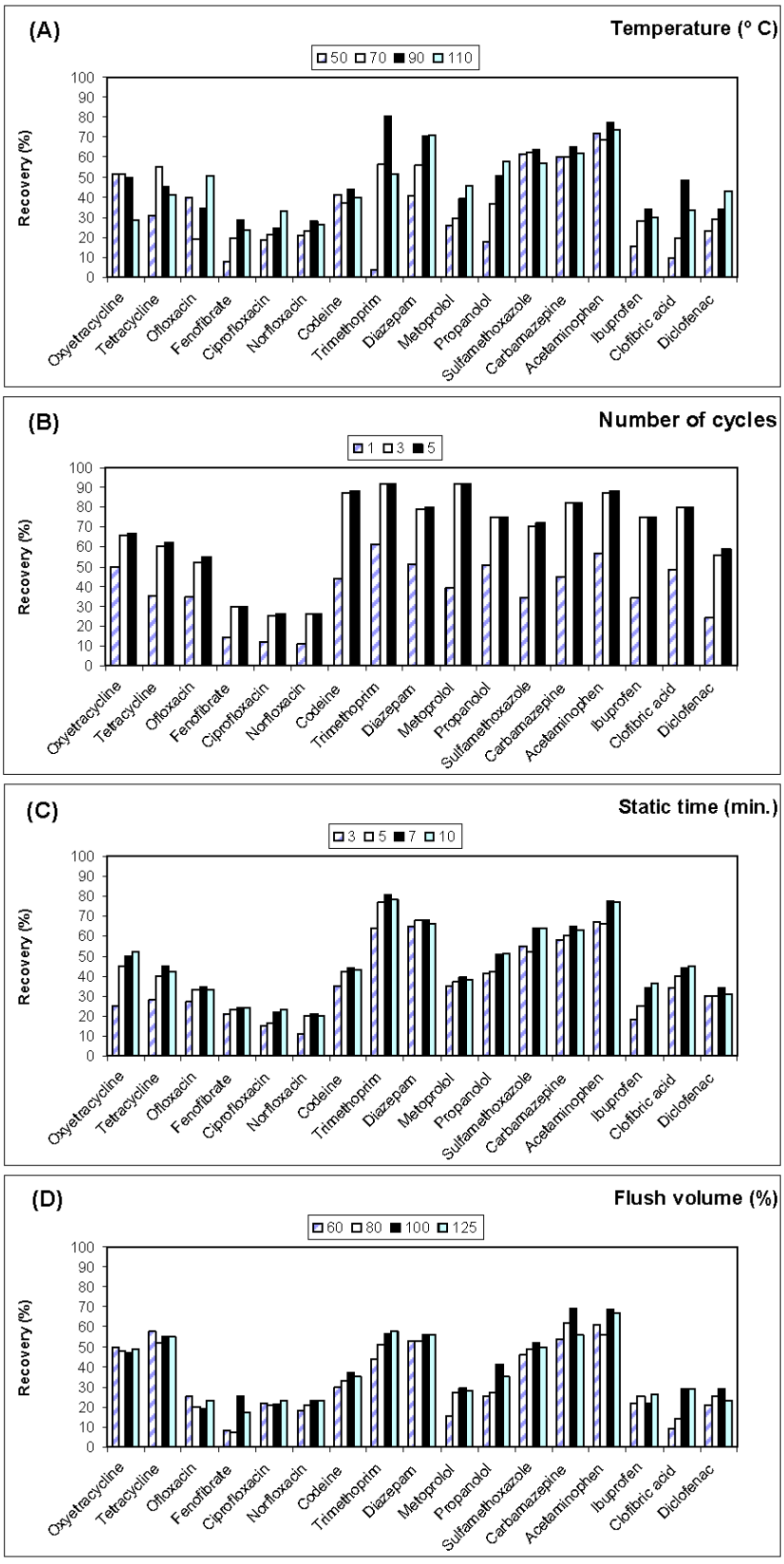
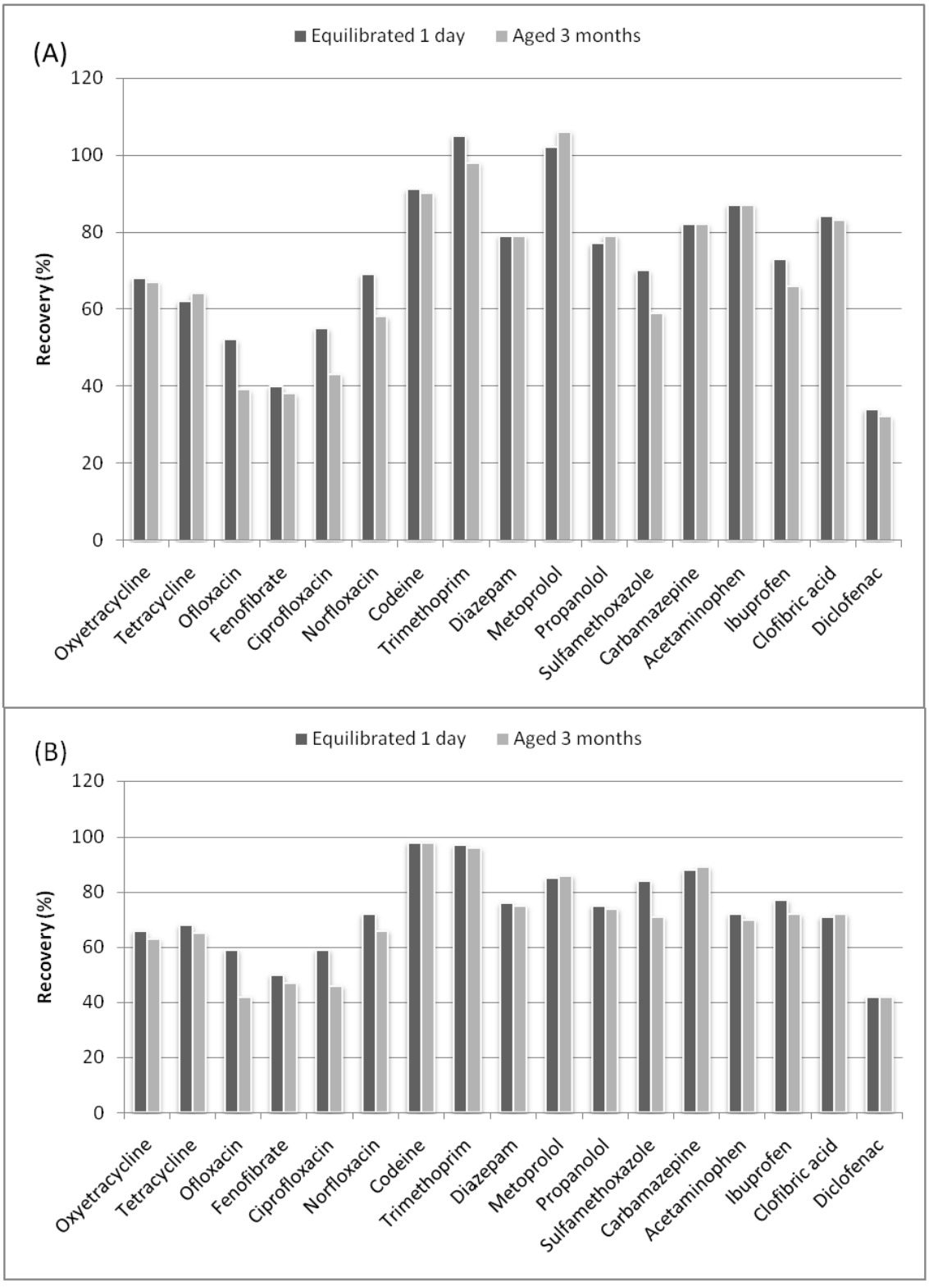


Fig.S3.6
Comparison of the recoveries obtained from soils equilibrated for 1 day and aged for 3 months (A) soils and (B) sediments



CHAPTER 4

Determination of illicit drugs in waters from L´Albufera of Valencia

Scientific publication 4:

SPE and LC-MS/MS determination of 14 illicit drugs in surface waters from the Natural Park of L'Albufera (València, Spain).

P. Vazquez-Roig, V. Andreu, C. Blasco, Y. Picó, V. Andreu

Analytical and Bioanalytical Chemistry, 397 (2010) 2851-2864.

Anal Bioanal Chem (2010) 397:2851–2864
DOI 10.1007/s00216-010-3720-x

ORIGINAL PAPER

SPE and LC-MS/MS determination of 14 illicit drugs in surface waters from the Natural Park of L'Albufera (València, Spain)

Pablo Vazquez-Roig & Vicente Andreu &
Cristina Blasco & Yolanda Picó

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Abstract A simple and robust method using solid-phase extraction (SPE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of 14 drugs of abuse and their metabolites (cocainics, amphetamine-like compounds, cannabinoids, and opiates) in surface waters has been developed. Seven SPE adsorb- ents (Oasis HLB, Oasis MCX, Oasis Wax, Supelselect HLB, Strata-X, Strata-XCW), amount of sorbent bed, water volume, and pH were investigated. The highest recoveries, as well as the simplest protocol, were obtained for Oasis HLB cartridges (6 mL/200 mg) using 250 mL of water. The proposed method was linear in a concentration range from 0.03–6 to 300–60,000 ng/L depending on the compound, with correlation coefficients higher than 0.998. Matrix effects have been studied in surface water samples, and several isotope-labeled internal standards have been evaluated as a way to compensate the signal suppression observed. Limits of detection (LODs) and quantification (LOQs) ranged from 0.01 to 1.54 ng/L and from 0.03 to 5.13 ng/L, respectively. Recoveries were 71–102% at the LOQ level and 77–104 at 50 ng/L. The intra-day and intermediate precisions were from 1% to 8% and from 2% to 11%, respectively. The present work reports for the first time the occurrence of drugs of abuse residues in surface water samples from the Natural Park of L'Albufera (Valencia, Spain). Codeine, cocaine, benzoylecgonine, ecgonine methylester, amphetamine, 3,4-methylendioxy methamphetamine, morphine, and methadone were quantified with median values of 11.10, 0.02, 5.59, 0.08, 0.21, 0.75 and 0.14 ng/L respectively, and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol was detected in one sample at levels <LOQ.

Keywords Drugs of abuse · Liquid chromatography–mass spectrometry · Triple quadrupole · Solid-phase extraction · Environmental analysis · Surface water

Introduction

The use of drugs of abuse is increasing worldwide and causes not only a well-known serious social problem but also concern as environmental emerging contaminants. Data provided by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [1] estimate that, in the last year, 22.5 million Europeans (between 15 and 64 years) smoked cannabis (22% of European adults), 12 million consumed cocaine, 11 million took amphetamines, and 9.5 millions used ecstasy. Regarding opiates, the report does not show a decline in the epidemic problems linked to heroin. The results from the survey conducted in Spain in 2007 established that 27.3% of the sample reported lifetime use of cannabis, followed by cocaine (8.3%), ecstasy (4.2%), and amphetamines (3.8%). Drugs of abuse are excreted unmetabolized or as metabolites in urine and feces, in fact, most consumed ones have been determined in the sewage system [2–15].

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Several reviews on this subject have recently appeared [2, 16–19], and it is clear that, in addition to the identification and measurement of illicit drugs in waste waters, some studies deal with their elimination rates [3–5, 20–23], but only few studies tackle their presence in treated sewage effluents [14, 20] and surface waters [8, 12, 15, 20, 24–27]. The presence of these substances is a topic of growing concern for ecological health and to estimate levels of community consumption [20, 28–30]. Several methods have been reported for drugs determination in environmental compartments [2, 16–19, 27, 31]. In particular, mass spectrometry is a powerful technique in terms of sensitivity and specificity for the detection of these drugs. Only one method developed for cocaine, benzoylecgonine and heroin employed gas chromatography–mass spectrometry (GC–MS) [3], using derivatization. Some derivatization procedures are laborious and time-consuming, or result in evaporative loss of analytes of interest. Most of the methods employed for the analysis of these drugs are based on liquid chromatography–mass spectrometry (LC-MS) [2, 16–19, 31]. Separation has been achieved with either conventional LC [4–11, 13, 15, 20, 22, 28, 29] or ultra-high performance LC (UHPLC) [12, 14, 21, 23–27, 32]. Several methods utilizing low resolution MS have been published, including: single-stage MS detection (LC-MS) in combination with reference libraries [33], ion trap [5, 6, 8, 14, 15], triple quadrupole (QqQ) [4, 7, 9, 11–13, 21–29, 32] and quadrupole-linear ion trap (QqLIT) [10, 20]. Furthermore, recently, Hogenboom et al. [34] applied the LIT Fourier Transform (FT) Orbitrap MS accurate masses screening for target and non-target analysis search of a broad range of illicit drugs and metabolites in different types of water.

Solid-phase extraction (SPE) is the selected pre-concentration technique (off- or on-line) in almost all the reported methods. To quantitatively extract the illicit drugs and to eliminate the influence of the matrix components, different types of SPE cartridges (Oasis HLB™, Oasis MCX™, Oasis MAX™, Oasis WAX™, Strata-X™, Strata-XC™, Strata-XCW™, Isolut ENV+™, Isolute C18 (EC) ™, Isolute PH™, Isolute HCX™, Bond Elut Certify™, and Chromabond Easy™) have been tested for one or two types of drugs [15, 28]. The most employed sorbents for SPE are either hydrophilic-lipophilic balanced (HLB) reversed-phases [5, 6, 8, 10–12, 20, 21, 23, 27, 32] or the mixed-mode (with a cation exchanger) modification of them [7, 11, 13, 22, 26, 28]. Recently, González-Mariño et al. [9] evaluated an amphetamine class selective molecularly imprinted polymer (MIP) commercially available as compared to Oasis HLB and Oasis MCX for the extraction and concentration of amphetamine drugs. MIP showed lower capacity as compared to Oasis sorbents. However, in this sense, SPE has not been extensively investigated yet [15].

In this work, a multi-residue method based on SPE and LC-MS/MS was developed and validated for screening and confirmation in surface waters of 14 drugs of abuse. The model drugs, which include three cocainics [benzoylecgonine (BECG), cocaine (COC), ecgonine methyl ester (ECGME)], four amphetamine-like compounds [amphetamine (AMP), methamphetamine (MAMP), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy methamphetamine (MDMA or ecstasy)], five opiates [6-acetylmorphine (6ACMOR), codeine (COD), heroin (HER), morphine (MOR), methadone (MET)], and two cannabinoids [Δ9-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-Δ9-THC (THC-COOH)], were selected to present a broad range of hydrophilicity; see log Kow values, Table 4.1. One aim of this study was to enlarge the range of tested SPE cartridges for the fourteen illicit drugs. For this, seven SPE-sorbents were compared and evaluated. Sample pH for extraction on the SPE cartridge, the amount of solid sorbent and the volume of water analyzed were studied. The target area to apply the methodology is L'Albufera Natural Park (Valencia, Spain). This park is one of the most important wetlands in Europe, not only for its biodiversity in flora and fauna but also because it is one key point for migratory birds [35]. Paradoxically, it is surrounded by towns, industries, agricultural fields, and leisure zones with numerous discotheques, as well as crossed by roads, which produce an intense pressure on the wetland [36]. L'Albufera also receives undepurated sewage waters from urban origin, together with those from water treatment plants, and the dumping of highly polluted sewage through gutters mainly under rainy weather [37]. Another aim of this work was to evaluate, for the first time, the occurrence of illicit drugs in surface waters of L'Albufera Natural Park. This data complement and enlarge the scarce studies carried out on this topic in surface waters of Spanish areas [20, 21, 23, 26, 27]

Experimental

Chemicals and standards

High purity individual standard solutions of 6ACMOR, AMP, BECG, COC, COD, ECGME, HER, MAMP, MDA, MDMA, MET, MOR and THC at 1,000 mg/L and THC-COOH at 100 mg/L, in methanol or acetonitrile were purchased from Cerilliant (Austin, TX, USA). Several deuterated compounds: benzoylecgonine-d_3 (BECG-d_3), ecgonine methyl ester-d_3 (ECGME-d_3), morphine-d_3 (MOR-d_3), methadone-d_3 (MET-d_3), amphetamine-d_6 (AMP-d_6), and 3,4-methylendioxyamphetamine-d_5 (MDA-d_5), at a concentration of 100 mg/L in methanol, also from Cerilliant, were used as surrogated standards (SSs). Individual stock solutions of each

Table 4.1 Structures, CAS Numbers and physico-chemical data of the selected drugs of abuse

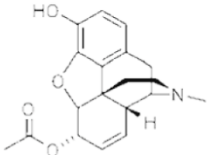
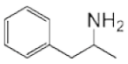
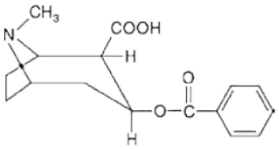
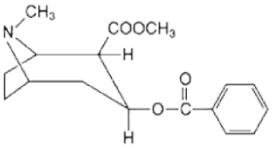
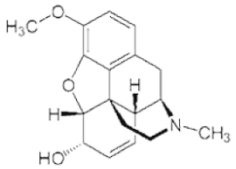
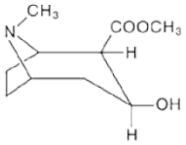
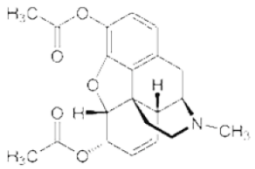
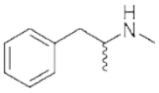
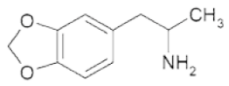
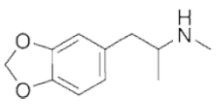
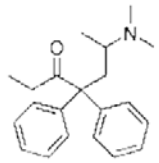
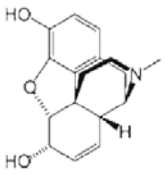
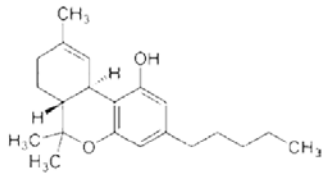
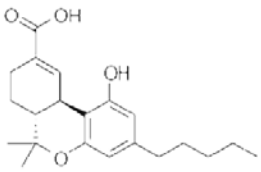
Structure	Compound	CAS Number	Empirical formula	MW	pKa	*Log* K_{ow}
	6ACMOR	2784-73-8	$C_{19}H_{21}NO_4$	327.37	---	0.42[a]
	AMP	300-62-9	$C_9H_{13}N$	135.21	10.1[b,c]	1.81[b,c]
	BECG	519-09-5	$C_{16}H_{19}NO_4$	289.13	3.2 ($pKa_{1)}$)[a] 10.1 (pKa_2)	2.72[a]
	COC	50-36-2	$C_{17}H_{21}NO_4$	303.15	8.6[b,c]	3.08[b,c]
	COD	76-57-3	$C_{18}H_{21}NO_3$	299.36	8.2[b]	1.20[b]
	ECGME	7143-09-1	$C_{10}H_{17}NO_3$	199.12	9.3 ($pKa_{1)}$)[a] 14.2 (pKa_2)	-0.23[a]
	HER	561-27-3	$C_{21}H_{23}NO_5$	369.41	7.6[a]	1.52[a]
	MAMP	537-46-2	$C_{10}H_{15}N$	149.23	9.9[d]	2.07[d]
	MDA	4764-17-4	$C_{10}H_{13}NO_2$	179.22	10.0[d]	1.59[d]
	MDMA	42542-10-9	$C_{11}H_{15}NO_2$	193.2t	10.4[d]	1.81[d]

Table 4.1 (continued)

Structure	Compound	CAS Number	Empirical formula	MW	pKa	*Log Kow*
	MET	76-99-3	$C_{21}H_{27}NO$	309.44	9.2[a]	1.49[a]
	MOR	57-27-2	$C_{17}H_{20}NO_3$	285.34	7.9-9.4[a]	0.76[a]
	THC	1972-08-3	$C_{21}H_{30}O_2$	314.47	10.6[a]	7.68[a]
	THC-COOH	56354-06-4	$C_{21}H_{28}O_4$	344.45	---	6.14[e]

[a] Data from [38]
[b] Data from [24]
[c] Data from [25]
[d] Data from [9]
[e] Data from [20]

analyte, at 50 mg/mL, were prepared every two months by dilution of the standard stock solutions in methanol–water (25:75, v/v). A mixture of the SSs at a concentration of 10 mg/L each was prepared in methanol. Working standard mixtures were also prepared at different concentrations by appropriate dilution of the individual stock solutions in methanol–water (25:75, v/v). Stock and working solutions were stored at −20 °C in the dark.

Formic acid (reagent grade), acetonitrile and methanol (gradient grade for liquid chromatography), were purchased from Merck (Darmstadt, Germany). High purity water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). Hydrochloric acid (37%), reagent grade, was purchased from Scharlau (Ferosa, Barcelona, Spain). Mobile phases were filtered through a 0.22-μm membrane polypropylene filter (Pall, MI, USA).

Oasis HLB (60 mg sorbent/3 mL and 200 mg sorbent/6 mL), Oasis MCX (60 mg sorbent/3 mL), and Oasis WAX (60 mg sorbent/3 mL) were from Waters (Milford, MA, USA). Supelselect HLB (60 mg/3 mL) were from Supelco (Bellefonte, PA, USA). Strata-X (60 mg sorbent/3 mL and 200 mg sorbent/6 mL), Strata-XC (60 mg sorbent/3 mL and 200 mg sorbent/6 mL) and Strata-XCW (60 mg sorbent/3 mL) were from Phenomenex (Torrance, CA, USA). Chromabond® SPE Vacuum Manifold with 12 ports and a self cleaning dry vacuum system™ Laboport SH (Bonsai Advanced Technologies S.L., Madrid, Spain) were used for loading the surface water samples and for drying the cartridges.

Sample collection

The Natural Park of L'Albufera of Valencia (Spain) has a surface of 21,000 ha and is located 12 km south of the city of Valencia. It consists of a lagoon, wetlands around it, and the adjacent shorelines. Nowadays, fresh water flows into the lagoon through 64 spots, five of which are mouths of its hydrographical basin (Xuquer hydrological basin) and the rest are channels, which flow into L'Albufera, mainly carrying irrigation waters from the fields, as well as urban and industrial outflows. Another important aspect is the urban and industrial development, characterized by accelerated growth since the 1960s, producing a chaotic situation that impairs its control and spatial planning. This constitutes a severe problem for the lagoon, because of the high polluting capability of these industries and the lack of wastewater treatment plants. Altogether, 428,123 inhabitants and 206 industries were counted in the census of 1989, which join an unknown number of sources that are pouring untreated toxic waste to municipal sewers [36]. The Park suffers the influence of Valencia City, 12 towns and 14 municipal districts, many of them greatly increase their population in summer because of tourism.

Surface waters were collected in different points at the irrigation channels of the Natural Park area to establish only spatial variations in drugs occurrence, without taking into account temporal variations. A total of 16 samples of water were collected, the Wednesdays of the two first weeks of April 2008, covering the most important channels that flow into the lake. There were no rainfall events during the prior fortnight to the sampling. The distribution and location of the sampling points can be found in Fig. 4.1. The sample points were selected to detect changes in the concentrations of the illicit drugs, and the following were monitoring: Sampling points PM14, P1, PP8, PM11, and A2M9 were located along the Poyo Gully (also Chiva Gully or Torrente Gully). This gully runs and collects non-treated wastewater from the municipal areas of Torrente, Picaña, Paiporta, Massanassa, and Catarroja, which separates, and floods finally into the L'Albufera lake. In the same area, points PM9 and PP1W were taken from channels with water outside the influence of this gully. The other sampling points were located in the downstream of several popula- tions, such as Beniparrell, Silla, El Romani, Sollana, Sueca and Cullera, which have also contributions of wastewater treatment plants.

Samples were collected as grab samples in dark glass bottles (2.5 L) and maintained at 4 °C until their arrival at the laboratory. Once in it, samples were filtered through glass microfiber GF/A filters (Whatman, UK) prior to extraction and stored in dark bottles at −20 °C until the analysis to avoid analytes degradation [11]. Samples were extracted within 1 week.

Solid-phase extraction

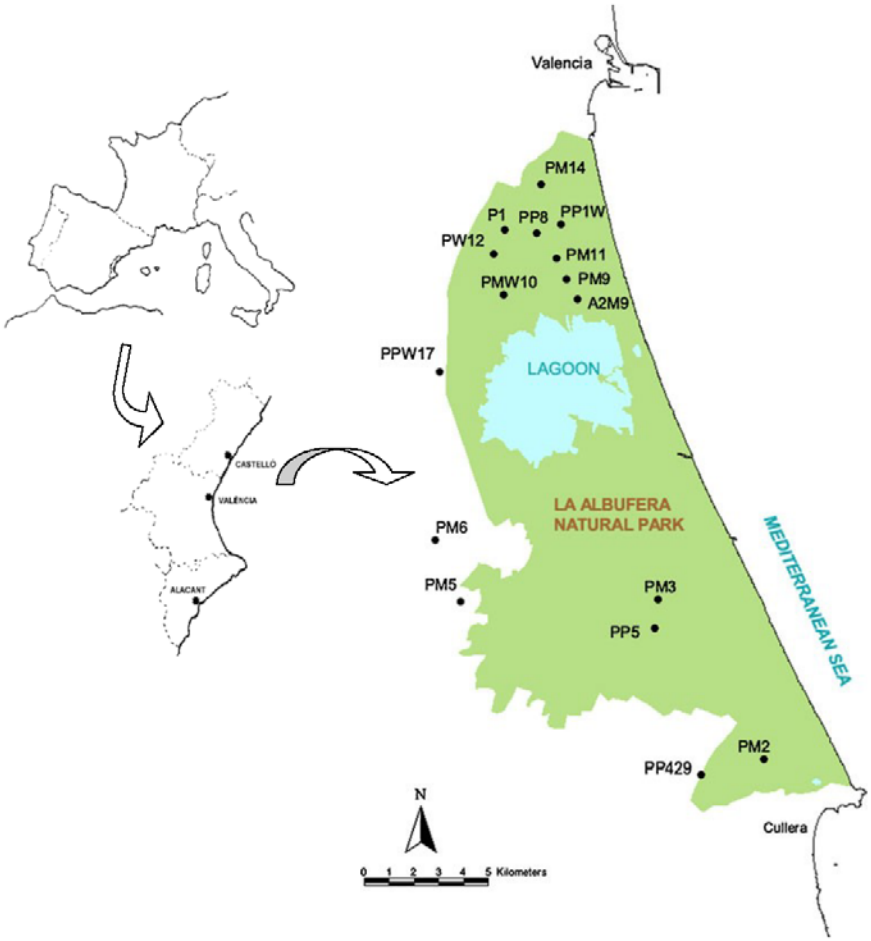
Seven SPE cartridges of three different types were tested: (1) polymeric based on HLB (Oasis, Supelselect, Strata-X), (2) mixed cation exchanger and HLB (Oasis MCX, Strata-XC, Strata-XCW), and (3) mixed anion exchanger and HLB (Oasis Wax). All cartridges were tested at three different sample pH (3, 5, and 7). Samples were brought to these pH with HCl 0.5 and/or 6 M (pH of the surface water were between 7.9 and 8.1). For Oasis HLB, Strata-X and Strata-XC 60 and 200 mg sorbent beds were checked for three different volumes of water (50, 250, and 500 mL).

Water samples were homogenized into the bottle by shaking. The exact volume of the water sample used for the extraction was transferred to a volumetric flask, adjusting the pH if required and amended with 10 μL of a 10 mg/L solution of SSs (final extract conc., 100 μg/L). Each cartridge, independently of its type and amount of phase, was conditioned with 5 mL of MeOH followed by 5 mL of Milli-Q water and 5 mL of Milli-Q water adjusted at the pH of the water (if required) using a vacuum system. Then, samples were passed through the cartridges at a flow rate of approximately 10 mL/min. After sample loading was complete, sample flasks and cartridges were rinsed with 10 mL of Milli-Q water. Cartridges were, then, dried under vacuum for 15 min, to remove excess of water. Analytes were eluted from the HLB sorbents with 6 mL of MeOH, and from the mixed-mode sorbents with 6 mL of 2% ammonia in methanol. The solvent was allowed to drip through the cartridge under gravity for 10 min. The extract was evaporated to dryness using a TurboVap [Caliper Life Science (formerly Zymark Corporation) Hopkinton, MA, USA] as proposed [26]. The water bath was set at 45 °C and the nitrogen flow at 10 psi. Once completely dried, the extract was reconstituted with 1 mL of water–methanol (75:25, v/v). In that way, an exact final volume of the extract is ensured. Prior to injection, final extract was filtered through syringe PTFE filters (0.22 μm, Analisis Vinicos, Tomelloso, Spain).

Liquid chromatography–mass spectrometry

The LC separation was performed using an Alliance 2695 HPLC separation module from Waters. A column Sunfire C_{18} (4.6×150 mm, 3.5 μm, from Waters) and a Gemini C_{18} (4.0×2.0 mm) guard cartridge (Phenomenex, Torrance, CA, USA) were used. The mobile phase combines eluent A (formic acid 0.1% in methanol) and eluent B (water 10 mM ammonium formate) in a gradient program that started at 20% A for 0.1 min, increased linearly to 90% A in 15 min, then increase to 98% A in 15 min, held for 8 min, and returned to initial conditions after 1 min followed by 11 min of equilibration time. The flow rate was 0.2 mL/min and the injection volume was 20 μL.

Fig. 4.1 Location of the sampling sites



The tandem MS analyses were performed on a Micromass Quattro triple quadrupole mass spectrometer (Manchester, UK). Instrument control, data acquisition and evaluation were done with the Masslynx NT software (v. 3.4).

In PI mode, the applied parameters were: extractor voltage 2.0 V and capillary voltage 3.0 kV; radio frequency lens, 0.5 V; electrospray source block, 125 °C; low mass (LM) 1 resolution, 12.0; high mass (HM) 1 resolution, 12.0; LM 2 resolution, 12.0; HM 2 resolution, 12.0; multiplier 650 V; desolvation temperature: 350 °C; argon collision gas 2.5×10^{-3} mbar; cone nitrogen gas flow, 50 L/h; desolvation gas: 600 L/h. The optimal quantification and confirmation transitions, and their respective cone voltages and collision energies are listed in Table 4.2.

Method validation

For each compound, the most abundant SRM transition was used for quantification while the other transition was used for confirmation. The criteria applied to confirm the identity of a suspected illicit drug were: (1) the ratio of the retention time of the analyte to that of the same analyte in standard solution should be within ±2.5% tolerance from the unit; (2) the presence of a signal at each of the two SRM transitions for the analyte; (3) the peak area ratio of the confirmation transition against the quantification one should be within the tolerance fixed by the EU criteria [32]. Because of the impossibility to obtain blanks, the samples were previously analyzed in order to determine their concentrations and PM9 and PP1W, which showed the lower concentrations (<LOQs), were selected. Positive findings were subtracted from the spiked samples.

Linearity and matrix effects were studied using standard solutions (prepared in methanol–water, 25:75) and matrix-matched calibrations (250 mL of L'Albufera Lake water were SPE, the extract was evaporated to dryness in the TurboVap and reconstituted with 1 mL volume of the standard mixture, at different concentrations). Eight concentration levels, between 0.01–1.50 ng/L and 75–15,000 ng/L (equivalent to 0.03–6 ng/L and 300–6000 ng/L in surface waters) were analyzed in triplicate.

Table 4.2 Retention times (t_r) and optimized SRM conditions used for LC-MS/MS analysis of illicit drugs in water

	t_r (min)	SRM_1 transition (Quantifier)	CV (V)	CE (V)	SRM_2 transition (Qualifier)	CV (V)	CE (V)	SRM ratio (SRM_1/SMR_2)
6-ACMOR	16.72	328>165	35	30	328>212	35	20	12.65
AMP	16.98	136>91	20	15	136>119	20	10	2.64
AMP-d_6	16.98	142>93	20	15	142>125	20	10	1.41
BECG	17.64	290>168	20	15	290>105	20	25	2.88
BECG-d_3	17.64	293>171	20	15	293>105	20	25	3.14
COC	17.78	304>182	20	15	304>82	20	25	3.58
COD	16.88	300>215	35	25	300>199	35	30	1.42
ECGME	8.62	200>82	20	20	200>182	20	15	0.64
ECGME-d_3	8.62	203>85	20	20	203>185	20	15	1.52
HER	17.25	370>165	38	45	370>268	38	30	1.53
MAMP	17.11	150>90.6	15	15	150>119	15	10	2.47
MDA	16.98	180>163	10	10	180>105	10	20	13.32
MDA-d_5	16.98	185>168	20	10	185>110	20	20	3.74
MDMA	17.11	194>163	18	10	194>105	18	20	3.06
MET	19.37	310>105	15	25	310>265	15	10	0.63
MET-d_3	19.37	313>268	20	10	313>105	20	25	1.87
MOR	15.65	286>165	30	30	286>153	30	30	1.31
MOR-d_3	15.65	289>165	35	30	289>153	35	30	1.19
THC	17.70	315>193	20	20	315>123	20	30	2.10
THC-COOH	30.78	345>327	20	15	345>299	20	15	1.65

The matrix effect was assessed by the evaluation of signal suppression or enhancement for each illicit drug in matrix extracts, and calculated as the percentage of the decrease or increase in signal intensity in the standards prepared in extracts of sample matrix (matrix-matched standards) versus those prepared in methanol–water (25:75, v/v). The equation used for this calculation was Eq. 1:

$$\text{Signal supression (\%)} = \left[1 - \frac{Sm}{Ss}\right] x100 \qquad (1)$$

(1) where Sm is the slope of the matrix-matched standards calibration curve and Ss is the slope of the methanol–water standards calibration curve.

Instrumental detection limits (IDLs) and instrumental quantification limits (IQLs) were estimated by direct injection of decreasing concentrations of the standard mixture, as the amount of analyte that gave a signal-to-noise ratio of 3:1 and 10:1, respectively, in SRM mode. Method detection limits (LODs) were confirmed by injecting seven replicated extracts of samples spiked at the estimated concentrations. Limits of quantification (LOQs) were the lower concentration that provided acceptable recovery (relative recoveries≥70%) and precision (<20%) were tested by analyzing spiked surface water samples in quintuplicate.

The method's recovery and precision were calculated by the repeated analysis of L'Albufera lake water samples spiked at two concentrations, the LOQ and 50 ng/L, and calculated as the relative standard deviation (RSD, %) of measurements in quintuplicate, (n=5) carried out in the same day and in three non-consecutive days (n=5).

Results and discussion

Liquid chromatography–mass spectrometry

The optimization of MS and MS/MS parameters was carried out by infusing individual solutions of the analytes. All drugs of abuse showed maximum sensitivity operating in the positive ionization (PI) mode, excepting the two cannabinoids that presented slightly more abundant ionization in negative (NI) mode. However, the response of these compounds in PI mode was enough to undertake environmental analysis. In the literature, determination of THC and THC-COOH has been reported, indistinctly, in both ionization modes [12, 26]. Their determination in PI mode has the advantage of attaining the simultaneous determination of all the analytes [12].

Several mobile phases, which varied in concentration and type of buffer (ammonium formate and ammonium acetate), pH (by addition of formic or acetic acids) and

organic solvent (methanol or acetonitrile) were tested. The selected mobile phase provided the highest peak areas as well as the best resolution and peak shape.

The acquisition of, at least, two transitions for reliable confirmation is possible for drugs of abuse (Table 4.2). The confirmation of the compound identity by a second transition requires an additional injection due to the high number of transitions needed for the simultaneous quantification and confirmation. The acquisition of two transitions for each compound would entail to monitor more than 34 transitions, which would reduce the number of points per peak leading to unsatisfactory peak shapes. Typical chromatograms obtained from surface water spiked at 6 ng/L of each analyte are shown Fig. 4.2. The cross-talk and ion-suppression effects due to co-elution, or almost co-elution, were checked by injection of single analyte solutions as well as standard solutions with mixtures of the analytes and evaluation of the SRM transitions signal as well as comparison of the absolute peak areas. From these experiments no "cross-talk" or ion suppression were observed. The IDL ranged from 0.03 pg injected for MET to 10.0 pg for THC-COOH. The IQLs were from 0.09 pg for MET to 30.0 pg for THC-COOH. The second injection monitoring a fewer number of transitions allows to confirm the identity of the drugs of abuse at these low levels.

Optimization of the SPE procedure

Selected cartridges with 60 mg of sorbents were tested with 50 mL of spiked water (absolute recoveries are shown in Fig. 4.3). Oasis Wax (the mixed-mode weak anionic sorbent) and Strata-XCW provided lower recoveries, whereas HLB and mixed-mode HLB-cationic exchanger cartridges pro- vided the best recoveries (33–108%). No difference in the extraction performance for the studied compounds was observed among the several trademarks of the same type of cartridges (Fig. 4.3). Sample pH for application on the SPE cartridge was studied for both, HLB and mixed-mode cation exchanger cartridges. The absolute recoveries obtained at different pH for Supelselect HLB and Oasis MCX are shown in Electronic Supplementary Material Fig. S4.5. Recoveries obtained using Supelselect HLB were not very pH sensitive, excepting ECGME (not recovered at pH 3) and AMP and MDA (higher recovered at acidic pH).

The absolute recovery of the selected drugs of abuse was determined for a series of different sample volumes (100–500 mL) spiked with 10 ng of each analyte using cartridges of 60 and 200 mg of solid sorbent. Results for 50 and 250 mL are summarized in Electronic Supplementary Material Table S4.5 for Oasis HLB and Strata-X and Strata- XCW. About a 10% decrease on the recovery was observed with the 60 mg cartridges from 50 to 250 mL water volume

Fig. 4.2 Chromatogram of the quantifier transition for each illicit drug obtained from an extract of spiked water (matrix: L'Albufera lake water and spiking level 6 ng/L) ▶

that was not for 200 mg cartridges. The maximum volume that can be passed through the 200 mg cartridge before recovery decrease is limited to 300–400 mL (data not shown) as previously described [8, 21, 24, 34]. In this study, the aspects considered to select the cartridge were the best recovery of ECGME (better with the HLB cartridges) and the simplicity of the extraction method (HLB does not require pH adjustment). In the definitive method to be validated, a water volume of 250 mL and Oasis HLB (200 mg, 6 mL) SPE cartridges were selected. These cartridges were selected because they were available in the laboratory, but any other of the HLB had given the same recoveries.

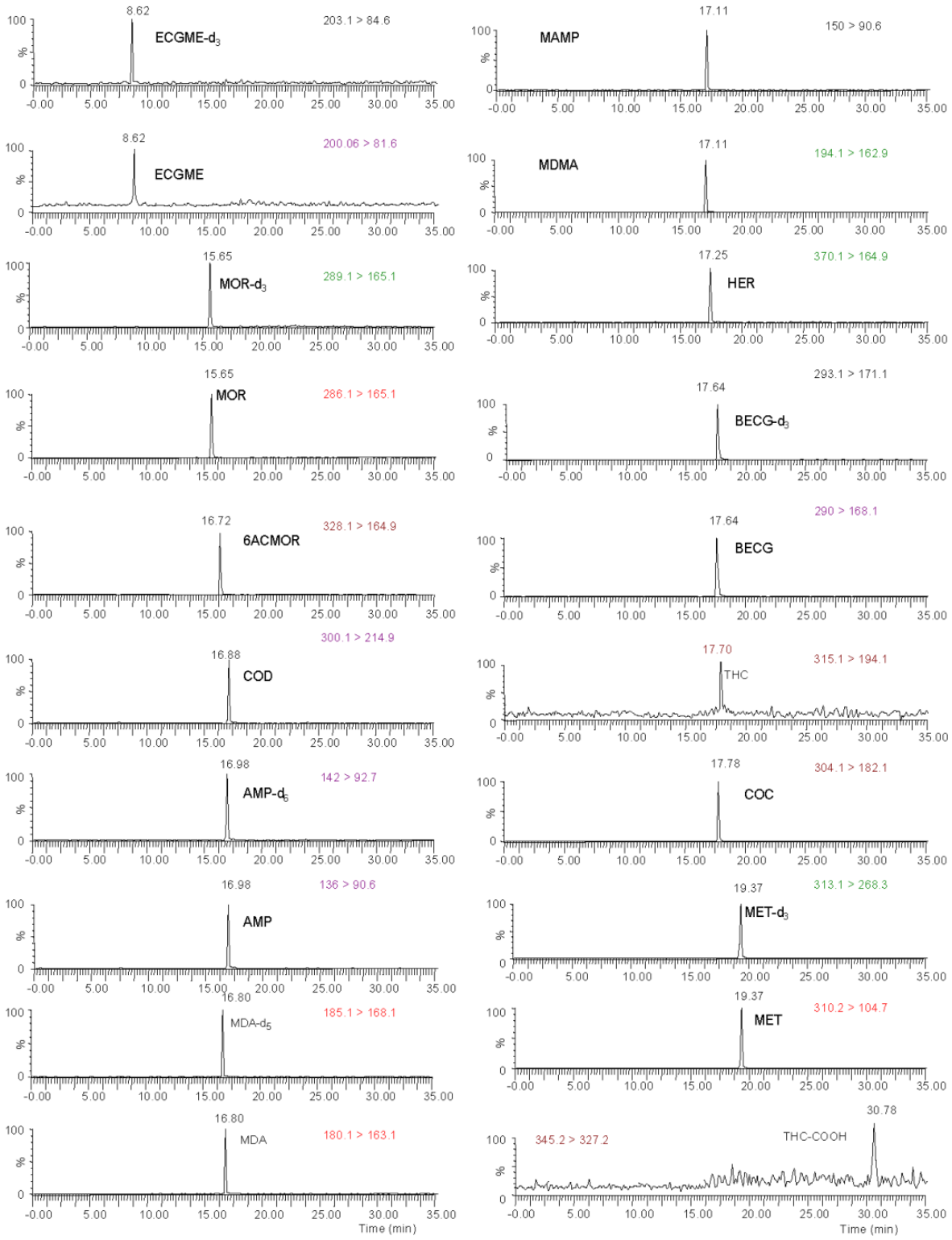
Validation of the method

Table 4.3 gives an overview of the performance of the developed method considering the following parameters: linearity, precision, accuracy, LODs, and LOQs. This Table shows the relative recovery, in which SSs were used to validate the method. The labeled analogues of some analytes were inaccessible to us because either they were not commercially available or were excessively expensive; so, COD was quantified using AMP-d_6, 6ACMOR using MOR-d_3, MDMA, MAMP, and HER using MDA-d_5, COC and THC using BECG-d_3, and THC-COOH using MET-d_3.

Matrix-matched calibration curves prepared in surface water extracts showed good linearity between 0.03–6 and 300–60,000 ng/L, with a correlation coefficient ≥ 0.998 (Table 4.3). The compensation of the matrix effect was evaluated by comparison of these curves with that obtained using methanol–water standards, as described in the "Experimental" section. The enhancement or suppression effect was only partly corrected by the addition of SSs, since matrix effects are compound dependent (see the relative signal suppression in Table 4.3 ranging, 0–20.2). However, the use of matrix- matched standards compensated for the suppression effect achieving accurate quantification.

In general, recoveries (between 71% and 121% with RSDs<18%) were satisfactory for all the compounds at both fortification levels. The worst recoveries were obtained for THC and THC-COOH (up to 60% and 67%, respectively).

The repeatability for each analyte was <8% RSD. There was no apparent difference in repeatability between the low and the high concentration levels. The intermediate precision was in all cases <12%. Only for 6ACMOR and AMP spiked at the LOQ level it was above 10%. For all the other compounds, lower RSDs were calculated. Also here, there were no differences between low and high concentrations.


8.62
ECGME-d3
203.1 > 84.6
8.62
ECGME
200.06 > 81.6
15.65
MOR-d3
289.1 > 165.1
15.65
MOR
286.1 > 165.1
16.72
6ACMOR
328.1 > 164.9
16.88
COD
300.1 > 214.9
16.98
AMP-d6
142 > 92.7
16.98
AMP
136 > 90.6
16.80
MDA-d5
185.1 > 168.1
16.80
MDA
180.1 > 163.1
17.11
MAMP
150 > 90.6
17.11
MDMA
194.1 > 162.9
17.25
HER
370.1 > 164.9
17.64
BECG-d3
293.1 > 171.1
17.64
BECG
290 > 168.1
17.70
THC
315.1 > 194.1
17.78
COC
304.1 > 182.1
19.37
MET-d3
313.1 > 268.3
19.37
MET
310.2 > 104.7
30.78
THC-COOH
345.2 > 327.2
Time (min)
Time (min)

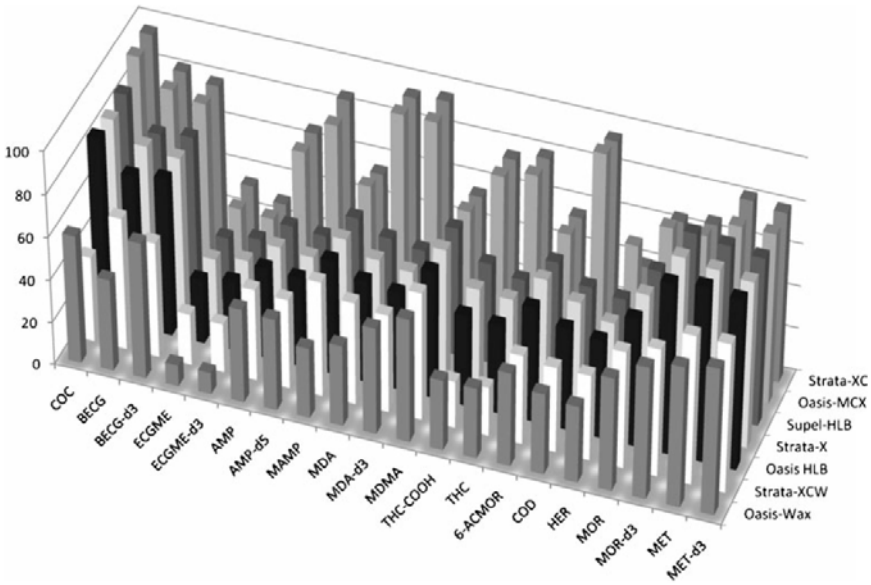


Fig. 4.3 Comparison of the absolute recovery percentage for the illicit drugs obtained by SPE with Oasis HLB, Oasis Wax, Supelselect HLB, Strata-X and Strata-XCW(matriz: L'Albufera lake water spiking level 50 ng/L and size of the cartridges 60 mg/3 mL)

Table 4.3 also outlines LODs and LOQs that were in the range of 0.01–1.54 ng/L and 0.03–5.15 ng/L. Sensitivity was of the same order as that reported by Zuccato et al. [22], Huerta-Fontela [27] and Kasprzyk-Hordern et al. [25], better than that established by Boleda et al [12, 23], Bijsma et al. [26] and Castiglioni et al. [13], and worse than that achieved by Postigo et al. [10]. These differences in sensitivity can be related to the sample volume processed, the on-line coupling of SPE to LC and the application of more sensitive mass analyzers. Results demonstrated that the method is useful for the determination of low levels of drugs of abuse environmental samples.

Table 4.3 Linearity, limits of quantification (LOQ), relative recovery (to ISs), matrix effects repeatability and reproducibility of the SPE and LC- MS/MS method

Compound	Linearity[a] range (ng/L)	Correlation coefficient (r^2)	Matrix effect (%)	LOD (ng/L)	LOQ (ng/L)	Recovery (% ± SD)		Precision (% RSDs)			
						at LOQ (n=5)	at 50ng/L (n=5)	Repeatability		Intermediate precision	
								at LOQ (n=5)	at 50ng/L (n=5)	at LOQ (n=5)	at 50ng/L (n=5)
6ACMOR	0.3–3000	0.9989	12.9	0.09	0.30	71±18	85±14	8	4	11	5
AMP	0.4–4000	0.9992	0	0.12	0.40	92±12	96±10	7	5	10	8
BECG	0.2–2000	0.9995	0	0.05	0.15	99±15	100±9	3	2	7	4
COC	0.06–600	0.9997	1.3	0.02	0.06	118±17	121±12	2	1	5	3
COD	0.03–300	0.9996	2.6	0.01	0.03	114±12	113±8	5	3	8	6
ECGME	0.35–3500	0.9995	0	0.41	1.37	91±13	96±10	4	1	5	2
HER	0.17–1700	0.9994	3.2	0.05	0.17	100±15	100±12	5	3	6	3
MAMP	0.75–7500	0.9997	4.1	0.22	0.75	117±14	113±11	4	4	6	4
MDA	1.50–15000	0.9998	0	0.41	1.37	104±16	105±10	3	1	8	7
MDMA	1.00–10000	0.9993	5.9	0.10	0.35	120±12	125±8	3	1	9	7
MET	0.03–300	0.9994	0	0.01	0.03	103±15	100±9	4	2	4	3
MOR	0.15–1500	0.9992	0	0.04	0.13	75±16	75±10	2	1	4	4
THC	6–60000	0.9989	17.2	1.22	4.07	57±14	60±11	4	3	5	7
THC-COOH	5–50000	0.9987	20.2	1.54	5.13	64±18	67±13	3	3	7	5

[a] Linearity obtained from the matrix-matched calibration curves

Application to real samples

The developed method for the determination of drugs of abuse was applied for the verification of their occurrence in superficial waters taken from L'Albufera Natural Park. Samples of surface water were collected and analyzed as described. The grab samples were taken because this is the conventional technique for environmental pollutant screening in surface water; sample is easy to collect and requires a minimum of equipment and on site time, providing a measurement of the instantaneous concentration. This approach has shown to be effective for documenting the occurrence of contaminants, particularly drugs of abuse. The main disadvantage of this sampling strategy is that samples are collected on a determined place and over a very short period of time, giving an incomplete picture of overall concentration of all pollutants and missing episodic changes on water concentration. Alternative sampling methods to palliate this disadvantage are to take composite sample or to use passive samplers. Both approaches collect the sample over a much longer period of time (24 h for composite samples or several days for passive devices), giving information on time-weighted average concentration of illicit drugs in water. However, these approaches have also limitations, such as the requirement of much more sophisticated and expensive equipment that must be placed on site for at least 24 h. Furthermore, passive samplers can only be effectively used for semi-quantitative analysis [4]. Table 4.4 summarizes the detection frequency and the levels measured in the 16 samples. All of them contained any of the drugs of abuse. Of the studied compounds, only six (ACM, HER, MAMP, MDA and THC) were not detected. The most abundant and ubiquitous compounds were COD, BECG, and its precursor COC. BECG was present in all investigated surface water samples at levels up to 78.71 ng/L (see Table 4.4). COD, MET, and MDMA were also positively identified in 37.5% of the investigated surface waters (including those samples where the compounds were detected <LOQ), with levels up to 51.62 ng/L, 0.84 ng/L, and 2.48 ng/L, respectively. The remaining detected compounds AMP, ECGME, MOR, and THC-COOH, were present in less than 12.5% of the samples, at levels up to 11.70 ng/L. The low occurrence of THC (or its metabolite THC-COOH), which according to EMCDDA is the most consumed drug in Europe and particularly in Spain, can be explained by the low tendency of these compounds to dissolve in water, since they have high partition coefficients (Kow = 5.5 – 7) and tend to sorb into sediments, suspended material or organic matter. The water sources can vary with time with regards to the concentration of drugs of abuse. Several studies [13, 27, 32] report an increase in the concentrations of drugs of abuse during the weekend, to avoid this variations samples were always taken the same day of the week.

Figure 4.4 shows selected LC-MS/MS chromatograms from a surface water sample (PM6) of the Natural Park that corroborates the excellent sensitivity of the method. Extracted ion chromatograms of both qualifier and quantifier precursor → product ion transitions with the vertical axes linked (that means relative abundances of both transitions normalized to the transition with higher response) illustrate the accurate confirmation taking into account retention time, presence of both transitions and relative abundance between them. This sample was selected as an example because it contained most of the studied abused drugs.

Average values of the 16 water samples were 11.10, 0.02, 5.59, 0.08, 0.21, 0.75, and 0.14 ng/L for COD, COC, BECG, ECGME, AMP, MDMA, MOR, and MET, respectively. These values were obtained considering that those samples with drug concentrations below LOQ were at the LOD level, and that non detected (ND) concentration of a drug was zero. However, variability of samples is high. The cumulative levels observed in the various samples collected in L'Albufera Natural Park are shown in Electronic Supplementary Material Figure S4.6. Highest total concentration of drugs of abuse and metabolites, above 120 ng/L, were found in PM 6. This sample also presented the largest number of drugs of abuse (COC, BECG. ECGME, AMP, MDMA, COD, MET, and
MOR). This fact could be due to the direct spillage of sewage water from different night clubs and discotheques of the zone in to the channel where the sample was taken. The rest of surface waters from L'Albufera Natural Park presented total levels of the target analytes below 60 ng/L (most of them below 20 ng/L) showing a fairly constant occurrence of these contaminants in the superficial waters of the natural park. In addition to PM6, five samples PM14, PP8, A2M9, P1, and PM11 are markedly more contaminated by drugs than the rest. It could be explained because all these samples were taken along the Poyo water course, which produces continuous wastewater discharges without debugging into the Albufera Lake, These non-treated wastewater samples come from several locations, which concentrates the major density of population (almost the 70% of the total population), industries, and leisure zones. It implies an increasing probability of input of these drugs in the sewage system and gutters that affects, in many cases, the irrigation channels and the marsh waters.

The observed contamination pattern and concentration of illicit drugs in surface water is comparable to those reported in similar monitoring studies carried out in surface waters from other European Countries, such as Belgium [8, 15, 25], Italy [22], Ireland [11], UK [24], Poland [24], and USA [4]. These data also match the sparse studies carried out in surface waters from Spain,

Table 4.4 Concentration levels (in ng/L) of illicit drugs in water samples from the Natural Park of L´Albufera (Valencia, Spain)

Compound	PM14	PP8	PM5	PMW 10	PM6	PPW 17	PM2	PP5	A2M9	PP 429	PW 12	P1	PM9	PP1 W	PM3	PM11
Cocainics																
COC	0.08	0.18	0.06	ND	4.43	0.08	0.25	0.08	0.031	0.12	ND	0.34	ND	ND	0.08	0.16
BECG	0.53	1.22	0.51	<LOQ	78.71	0.89	0.14	0.53	1.59	0.62	<LOQ	3.01	<LOQ	<LOQ	0.48	1.06
ECGME	ND	ND	ND	ND	1.35	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Amphetamine-like	compounds															
AMP	ND	ND	ND	ND	3.38	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MAMP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MDA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MDMA	ND	ND	ND	ND	2.48	<LOQ	ND	ND	0.14	ND	0.76	0.18	ND	ND	ND	<LOQ
Cannabinoids																
THC-COOH	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
THC	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Opiates																
6-ACMOR	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
COD	20.79	31.35	ND	ND	18.69	ND	ND	ND	35.10	ND	ND	51.62	ND	ND	ND	20.09
HER	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MOR	ND	ND	ND	ND	11.70	0.30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MET	ND	0.14	ND	ND	0.84	0.19	ND	ND	0.27	ND	ND	0.69	ND	ND	ND	<LOQ

ND non detected, <LOQ detected below the LOQ

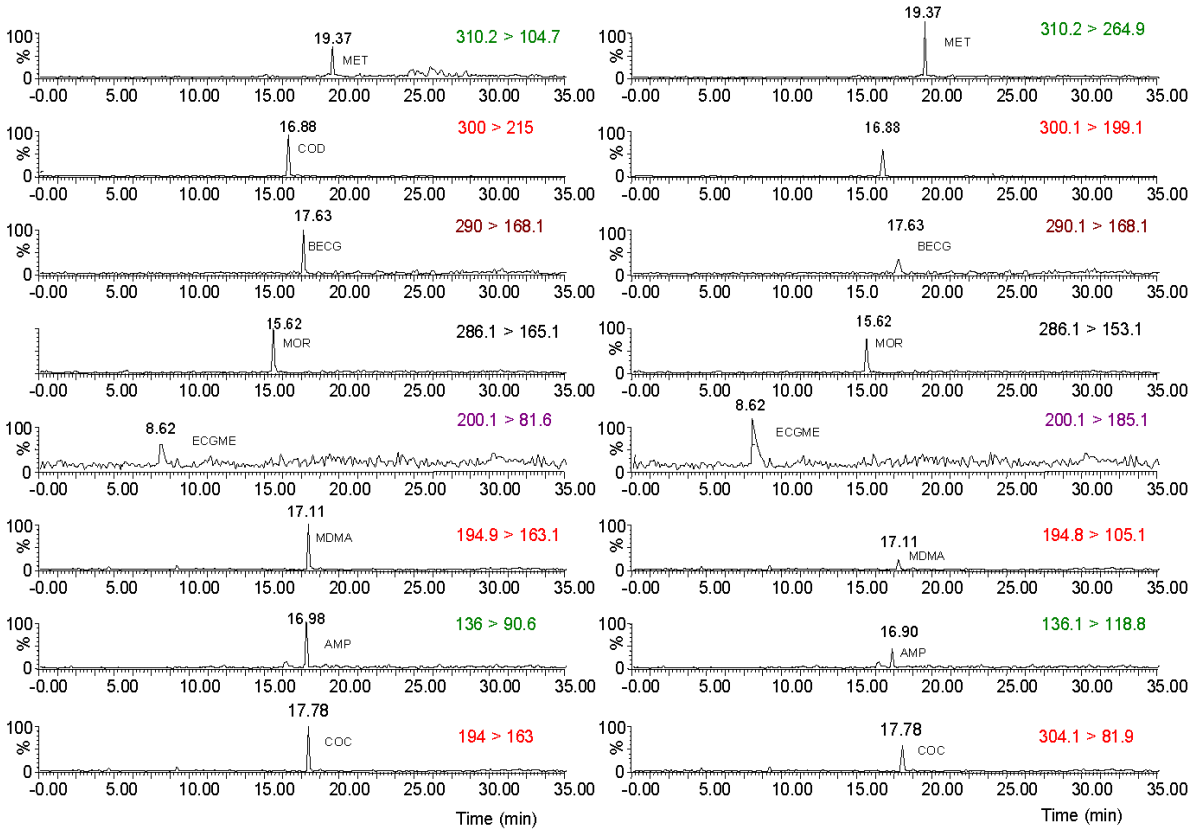


Fig. 4.4 Chromatogram obtained from a real water sample taken at PM6. Only drugs positively identified and quantified are shown

which are restricted to the Llobregat and Ebro Rivers Basins (located in Catalonia at the North of the Valencian Community) [12, 20, 21, 23, 27]. Drugs of abuse (several opiates, cocainoids, and cannnabinoids) were found in surface waters of both basins at lower nanograms per liter level. As in this study, heroin and 6-acetylmorphine were not detected in any of the samples and BECG, COC and COD were the most ubiquitous compounds. Unlike this study, THC was found in the Llobregat River basin but not in the Ebro.

BECG and COC are ubiquitous in surface waters. Based on the reported excretion values of COC, BECG, and ECGME after cocaine consumption (1–9%, 35–54%, 25–44%, respectively) and their molar mass relation, the excreted COC/BECG ratio should be between 0.02 and 1.27. In the surface waters analyzed, the median value of the COC/BECG ration was 0.12. However, in the sample PM2, the COC/BECG ration was higher than 1.00, which may indicate the direct disposal of the drug into the water. This finding has also been reported by other authors [8, 20]. The presence of ECGME in surface waters was demonstrated for the first time, even though it was found in only one sample. The presence of this compound in wastewater across Belgium has already been reported [11]. MOR and COD among the natural opiates and MET among the synthetic ones were found in some of the samples. COD and MET were the most abundant (six samples) being the COD levels the highest. MOR, found in two samples, may come from clinical use of MOR and COD but it might also come from the illicit use of HER. Finally, MDMA concentrations and frequency is higher than those of the AMP that only appeared in two samples. The ecotoxicological significance of the presence of these illicit drugs in water has not been closely studied yet [14], However, both, the health problems caused by their consumption and that most of these residues still have potent pharmacological activities, are a justifiable motive to presuppose that their regular presence in the aquatic environment will have potential implications for human health and wildlife [14, 22]. The regular presence of these substances in water samples could pose consequences for the aquatic and terrestrial organisms in an ecosystem that is the last reservoir of several autochthonous species and a key point for the migratory birds.

Conclusions

The seven SPE sorbent tested showed that appropriate absolute recoveries can be obtained for HLB (54–104%) and mixed-mode HLB cation exchanger (25–99%), after a proper optimization of conditions, such as water pH and volume and amount of sorbent. No apparent differences were observed among the different trademarks of the same type of cartridge. The use of HLB sorbent was preferred because makes the method simpler and more robust than using mixed-mode cartridges. The method proved to be sensitive enough to surface waters and wastewaters, providing high recoveries for all the analytes (71–104%) and achieving low LODs and LOQs (0.01–1.54 ng/L and 0.03–5.13 ng/L, respectively). The method was applied for the analysis of chosen drugs of abuse in surface waters from L'Albufera Natural Park (Valencia, Spain). The results confirmed the presence of these drugs in surface waters. COC and metabolites (BECG and ECGME), amphetamines (AMP and MDMA), methadone, codeine, morphine, and THC-COOH, the main metabolite of THC, were determined in water samples at levels ranging from less than 0.06 ng/L to 78.78 ng/L. The highest concentration was determined in sample PM6, which can be related to direct spillage of residual waters from a close leisure zone.

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Electronic Supplementary Material

SPE and LC-MS/MS determination of fourteen illicit drugs in surface waters from the Natural Park of L'Albufera (Valencia, Spain)

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Figure S4.5. Influence of pH adjustment, (pH 3, pH 5 and pH 7), on the absolute recoveries using (A) SupelSelect HLB SPE cartridges and (B) Strata-XC (matrix surface water from L'Albufera and spiking level 50 ng/L).

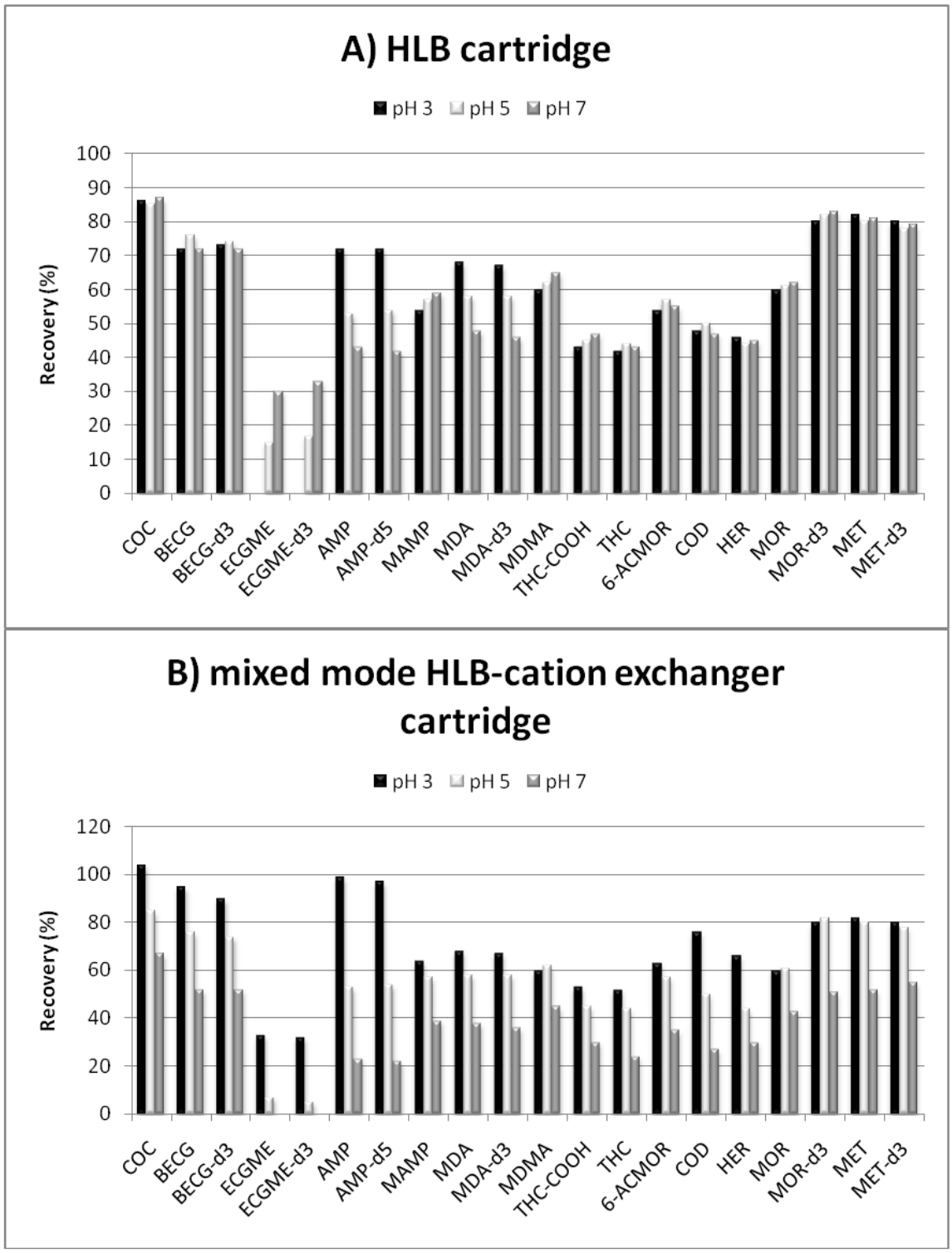
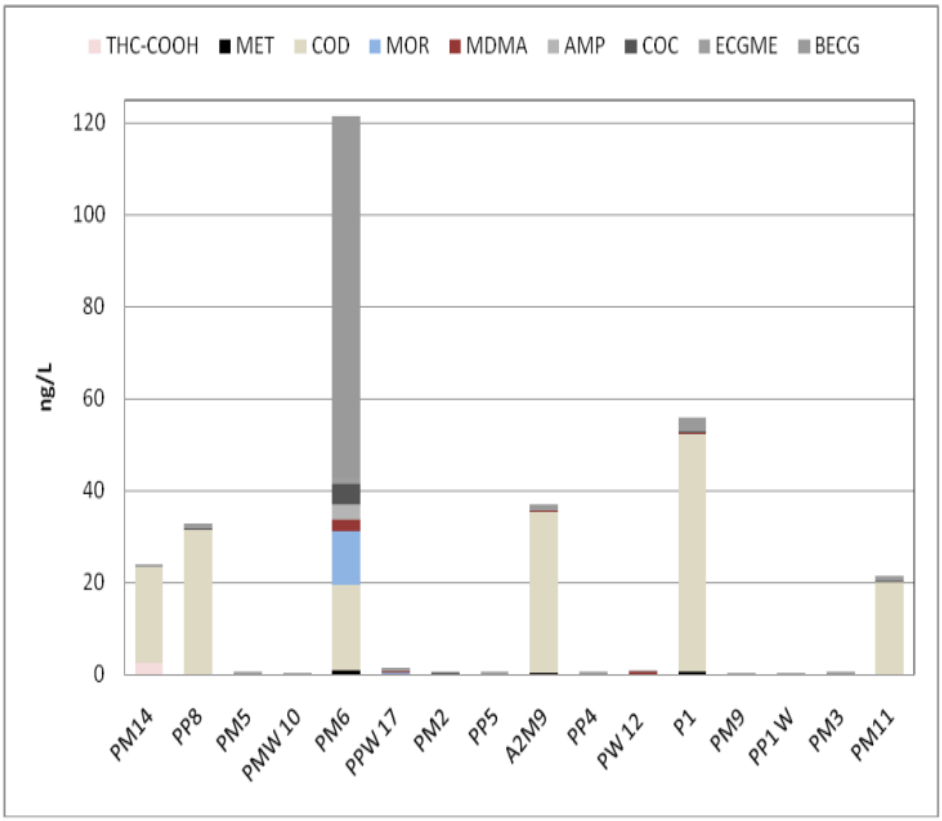


Table S4.5. Absolute recoveries (in %) for Oasis HLB, Strata X and Strata-XCW cartridges with different sorbent mass and sample volume. (matrix: L'Albufera superficial and spiking level 50 ng/L)

Compound	Oasis HLB (pH 7)				Strata-X (pH 7)				Strata-XCW (pH 2)			
	60 mg/ 3 mL		200 mg/ 6 mL		60 mg/ 3 mL		200 mg/ 6 mL		60 mg/ 3 mL		200 mg/ 6 mL	
	50 mL	250 mL	50 mL	250 mL	50 mL	250 mL	50 mL	250 mL	50 mL	250 mL	50 mL	250 mL
6ACMOR	54	50	65	63	57	45	62	57	42	34	51	48
AMP	43	35	55	50	43	31	53	43	43	32	54	49
AMP-d_5	42	38	50	51	42	30	50	42	42	35	49	44
BECG	72	66	84	79	76	64	84	76	62	53	71	69
BECG-d_3	74	67	85	80	74	62	86	74	54	36	65	68
COC	86	81	98	92	85	73	95	85	40	28	51	49
COD	48	43	60	56	50	38	56	50	40	32	49	47
ECGME	30	26	39	32	30	18	32	30	24	18	32	33
ECGME-d_3	33	27	42	37	33	21	34	33	23	12	34	35
HER	46	39	56	53	44	32	50	44	40	34	48	46
MAMP	54	44	63	59	54	42	58	54	54	45	62	58
MDA	48	41	53	48	48	36	55	48	48	40	56	53
MDMA	46	39	52	47	46	34	56	46	46	38	54	52
MET	60	54	70	60	60	48	70	62	60	53	68	69
MET-d_3	82	78	93	90	80	68	83	80	70	64	79	76
MOR	80	78	90	88	78	66	80	78	70	62	82	74
MOR-d_3	60	51	72	70	61	49	66	61	55	46	65	60
THC	80	76	94	89	82	70	85	82	60	57	73	65
THC-COOH	42	36	49	48	44	32	53	44	23	14	34	36

Figure S4.6. Cummulative levels ng/L of drugs of abuse in the surface waters of the Natural Park of L'Albufera.



CHAPTER 5

Determination of pharmaceuticals in L´Albufera of Valencia

Scientific publication 5:

Assessment of the occurrence and distribution of pharmaceuticals in a Mediterranean wetland (L'Albufera, Valencia, Spain) by LC-MS/MS

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ORIGINAL PAPER

Assessment of the occurrence and distribution of pharmaceuticals in a Mediterranean wetland (L'Albufera, Valencia, Spain) by LC-MS/MS

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Abstract The distribution of 17 pharmaceuticals between water and the solid phase (sediments and soils) was studied by utilizing solid-phase extraction (SPE) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). Two extraction procedures for soils and sediments, prior to the SPE, one based on pressurized liquid extraction (PLE) with hot water and the other on methanol/water ultrasonic extraction, were compared. Absolute recoveries were 71.2–99.3% [relative standard deviation (RSD) <21.4%)] for water, and the method detection limits (MDLs) ranged from 0.3 to 10 ng L^{-1}. Recoveries were 35.4–105.3% (RSDs <19.1%) and 42.1–97.8% (RSDs <14%) for soil and sediment samples, respectively, using PLE and 20.2–86.5% (RSDs <25.1%) and 30.3–97.4% (RSDs <19.1%) using ultrasonic extraction. Fifteen of the 17 pharmaceuticals were present in the L'Albufera water at concentrations up to 17 μg L^{-1}. Oxytetracycline and tetracycline were not detected. In sediments, only tetracycline, norfloxacin and diclofenac were not found. The other studied pharmaceuticals were present in the range from less than the method quantification limit (MQL) to 35.83 ng g^{-1}. Among the 17 target compounds, ofloxacin, ciprofloxacin, norfloxacin, trimethoprim, clofibric acid and diclofenac were not detected in soil samples. The average concentrations ranged from less than the MQL for ibuprofen to 34.91 ng g^{-1} for tetracycline. These results indicate that pharmaceuticals could survive the wastewater treatment processes, which could lead to their dissemination in water environments.

Keywords Pharmaceutical products · Surface water · Soil · Sediments · Wetlands · LC-MS/MS · Pressurized liquid extraction · Ultrasonic extraction · SPE

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Introduction

Numerous studies have evidenced the ubiquitous presence of pharmaceuticals in natural waters [1–11]. As a result of the distribution of this water between the aquatic and solid phase, the sediment and soil can be contaminated and their further migration can even lead to their infiltration into the drinking water sources [4, 5]. This phenomenon is particularly interesting in cases when the biologically treated or untreated wastewater is introduced into the aquatic ecosystems, because pharmaceuticals mainly come from human activities [12–15]. The pharmaceuticals are not necessarily persistent but they are hydrosoluble and their dumping can cause health and environmental problems that require further study [16]. This type of contamination is a growing problem that must be tackled to meet the Water Framework Directive of the European Union [17]. Thus, a better knowledge of the occurrence and fate of pharmaceuticals released into the environment will allow a proper risk assessment to be conducted for river

basins, wetlands and other related ecosystems [6, 12, 18]. In spite of the relatively high number of literature reports, the simultaneous investigation of these drugs in water, sediment and soil samples is scarce [19, 20].

Wetlands are amongst the Earth's most productive ecosystems, providing a diverse array of important ecological functions. Wetlands are fundamental to the maintenance of the water cycle because they purify and recycle water and, at the same time, capture and retain it from the rain. Wetlands are also important in the control of floods and flows, in the recharge of aquifers, in carbon sequestration, etc. [21, 22]. Among these ecosystems, the coastal wetlands present a great dynamism and biodiversity [9, 23]. Because of their open structure and relationship with the environment, coastal wetlands are usually eutrophic and rich in nutrients. These ecosystems are very fragile and are particularly sensitive to alterations in their water regime [23, 24]. In this sense, their situation becomes more critical in the Mediterranean area where the predicted climate evolution indicates a clear tendency toward rain shortage and increased temperatures [22]. Even though the importance of wetlands and the essential features that sustain have been recognized, wetland loss and degradation continues in Europe. The Water Framework Directive [17] clearly identifies the protection, recovery and conservation of these wetland zones as priority actions.

There are several methods to determine pharmaceuticals in the aquatic environment. They mainly consist of solid-phase extraction (SPE) or solid-phase microextraction (SPME) for isolation and enrichment, and liquid chromatography–mass spectrometry (LC-MS/MS) or gas chromatography–mass spectrometry (GC-MS) following derivatization for quantification [1, 7, 9–14, 25]. However, there are much fewer methods available for the extraction and quantification of pharmaceuticals at trace levels in solid matrices. The analytical procedures include extraction of the contaminants from the solid surfaces using ultrasonication, pressurized liquid extraction (PLE) and microwave-assisted solvent extraction (MAE) [2, 4, 5, 8, 24, 26, 27]. The next steps of the analytical procedure are the purification of the extracts and their determination using the same techniques described for water samples.

In the light of the above concerns, the aim of this work was to develop an analytical protocol for the sensitive determination of 17 pharmaceuticals and to apply it to the study of their spatial distribution between water, soil and sediment in water courses and channels of L'Albufera Natural Park. In this protocol, SPE was used to isolate and concentrate the chemicals from the water, followed by LC-MS/MS. For sediment and soil samples, the extraction results obtained by PLEs and ultrasonic shaking were compared. PLE conditions (dispersing agent, elution solvent, static time, number of cycles) were based on those used in a previous study [26]. The Valencia Community can be an adequate study case, where the scarcity of water and the human activity endanger the integrity and future of L'Albufera Natural Park, which is the most important wetland ecosystem of the Xuquer River Basin and one of the most significant in Spain [28, 29]. The Albufera was formed after an ancient gulf was closed off through the emergence of a coastline strip. The sandbar started to form roughly 6,000 years ago from the sediment brought down by the Turia and Xuquer rivers [18, 29–31]. The waves and the coastal currents brought the sediment along until it formed the sandbar which separated the Albufera from the Mediterranean Sea. This coastal lake is the morphological model of coastal wetland most common in the Mediterranean [18, 30]. The 17 pharmaceuticals chosen for the validation were selected as model substances based on their occurrence in wastewater and their distribution along the logarithmic octanol/water partition coefficient (log K_{ow}) scale, from log K_{ow} −0.13 to 5.19 [22]. The analytes were selected from different therapeutic classes: β-blockers (metoprolol and propanolol), antidepressants (diazepam), anti-epilectic drugs (carbamazepine), analgesics (acetaminophen and codeine), nonsteroidal anti-inflammatory drugs (ibuprofen and diclofenac), and lipid regulators (clofibric acid and fenofibrate) in addition to seven antibacterials (ciprofloxacin, norfloxacin, ofloxacin, oxytetracycline, sulfamethoxazole, tetracycline and trimethoprim).

Experimental

Chemicals and materials

All pharmaceutical standards were purchased from Sigma–Aldrich (Steinheim, Germany), except 4-epioxytetracycline that was from Acros Organics (Morris Plains, NJ, USA) and ibuprofen-d_3, acetaminophen-d_3 and carbamazepine-d_2 (internal standards, ISs) that were from CDN Isotopes (Quebec, Canada). All standards were of analytical grade (purity >97%). Stock solutions (1,000 mg L^{-1}) of each pharmaceutical were prepared in methanol with the exception of ciprofloxacin, which was prepared at 500 mg L^{-1} in water acidified with formic acid. Stock solutions were stored at −20 °C. Working solutions, at different concentrations, were prepared each 3 months by dilution of the standard stock solutions in methanol/water (25:75, v/v). A mixture of the ISs at concentrations of 10 μg mL^{-1} each was prepared in methanol and the corresponding quantity was added to water, soil and sediment samples to obtain concentrations of 50 μg L^{-1} or 50 ng g^{-1} in the final extract. Formic acid (reagent grade), acetone and dichloromethane (residue analysis) as well as acetonitrile and methanol (gradient grade for liquid chromatography) were purchased from Merck (Darmstadt, Germany). High purity water was prepared using a Milli-Q water purification

system (Millipore, Milford, MA, USA). Sea sand was from Panreac (Barcelona, Spain). Ethylenediaminetetraacetic disodium salt dihydrate (Na_2EDTA), citric acid and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Scharlau (Ferosa, Barcelona, Spain).

Na_2EDTA-washed sea sand was prepared by placing 60 g of sand into a Buchner funnel and passing 120 mL of 0.1 M Na_2EDTA through it using a vacuum. Partial drying of the sand was carried out under vacuum. Thereafter, sand was completely dried in an oven at 100 °C. pH 7.4 McIlvaine buffer was obtained by mixing 9.15 mL 0.1 M citric acid with 90.85 mL 0.2 M Na_2HPO_4.

Oasis HLB 200 mg sorbent/6 mL cartridge (Waters Corp., Milford, MA, USA) and Isolute SAX 500 mg (Symta, Madrid, Spain) were used for SPE.

Sampling area

L'Albufera was declared a Natural Park in 1986, covers an area of 210 km^2 and is located 12 km south of the city of Valencia (Spain). The park is part of the hydrographic Xuquer basin, which consists of the large (around 23 km^2) shallow (1- to 2-m depth) lagoon surrounded by rice fields (140 km^2), pine groves and dunes. This park is a place of high economic, tourist and scientific interest and it is included in the Ramsar Convention on Wetlands. The lagoon is also very important in regulating the water flow in the rice fields. The lagoon is freshwater-fed by a number of channels associated with the agricultural land uses, as well as springs, located either within the lagoon or in the surrounding marshland. At present, the water flow is controlled by a system of pumps and sluice gates at three artificial water outlets that link the lagoon to the sea, because the whole lagoon acts as a regulation reservoir in accordance with rice cultivation periods. Other serious effects are occurring in response to the industrialization of the neighbouring areas, demographic expansion in outskirt villages, tourist urbanization in coastal areas, and construction of a dense road network, which takes up over 40 ha. Wastewater from human activities is also dumped into the irrigation channels and the lagoon; the channels are designed to reuse reclaimed water for supplying the ecological flow in the wetland and the irrigation of farm areas.

Sampling was carried out in April and October 2008 at the points marked in Fig. 5.1. Sampling points were georeferenced (UTM D50). Water and sediment samples were mainly from irrigation channels, whereas soil samples were taken in the neighbouring area from the superficial horizon.

Water samples were taken from the same irrigation channels as sediments. They were obtained from the channel back or from bridges at a depth of less than 1 m (mostly 30 cm). Grab water samples (2.5 L) were collected in clean amber glass bottles. Before sample collection, each bottle was pre-rinsed with sample three times. Samples were transported in boxes packed with ice and were stored at 4 °C in a cold room upon arrival at the laboratory. They were treated within 48 h. Water samples were filtered through a Whatman GF/F glass microfiber membrane filter of 0.7 μm. Water samples had a pH ranging from 7.2 to 7.4.

Sediment samples (250 mL) were taken from irrigation channels and marshes using a Van Veen grab sampler, and transferred to polypropylene bags. Sediment samples were of pH>7.4, sandy loam texture, and with high content of calcium carbonate (>30%) and organic matter (>15%). These samples, once in the laboratory, were lyophilised (Hetosicc CD4, Birkerod, Denmark) and passed through a 2-mm-Ø sieve. The process of lyophilisation was carried out over 7 days for each sediment sample until the water content was less than 1%. Finally the lyophilised samples were stored in sealed plastic bags at 4 °C until the extraction.

Soils of this zone are developed on black and grey silts, affected intensely at the surface by the agricultural practices. The most important physical and chemical characteristics of these soils are an impermeable profile, carbonated, with hydromorphic features, and high salinity level. According to the Food and Agriculture Organization of the United Nations (FAO) classification [32], the soil comprises Calcareous gleic Fluvisol type in the saline phase, and Aplic Fluvisols. Soil samples were collected at the upper 20 cm horizon layer. Once in the laboratory, samples were dried and passed through a 2-mm-Ø sieve. The soil samples were extended in a layer of approximately 1-cm thickness on polypropylene trays and air-dried in darkness at 20 °C. Dried samples were stored in sealed plastic bags at 4 °C.

Pressurized liquid extraction of soil and sediment

The extraction method was based on a previous one developed in our laboratory [26]. Soil or sediment (3 g) were added 10 μL of a 10 ng μL^{-1} mixture of the ISs, and mixed with approximately 25 g of Na_2EDTA-washed sea sand in a mortar. This mixture was put into a 22-mL extraction cell and extracted by PLE using an ASE 200 system (Dionex, Sunnyvale, CA, USA) with hot water (90 °C) as extractant, a static period of 7 min, and a flush volume of 100% in three cycles. Pressure was set to 500 psi and purge time to 1 min. The water volume ending up in the glass vial was approximately 30 mL, using a cell size of 22 mL.

Ultrasonication extraction of soil and sediment

The PLE method was compared with an ultrasonic extraction one published previously by Blackwell et al. [33]. Briefly, soil or sediment samples (3 g) and the corresponding amount of

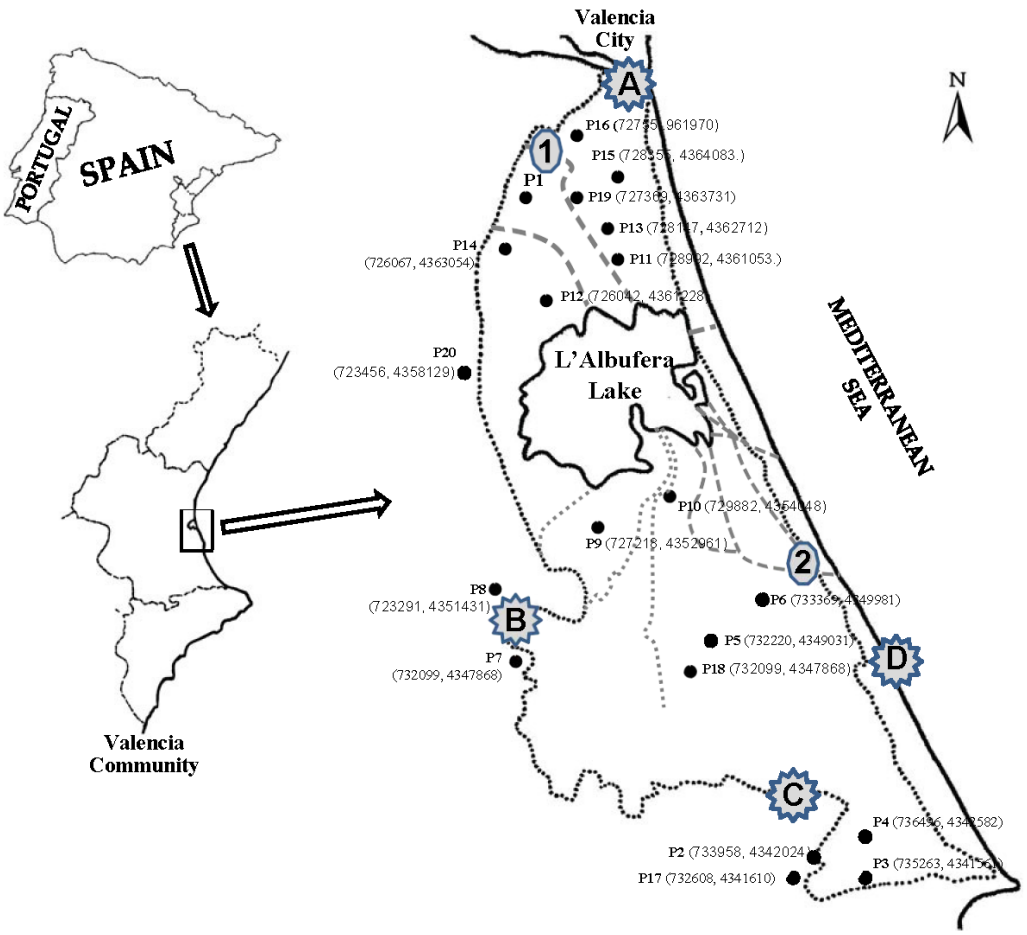


Fig. 5.1 Map with the location and georeferences of the sampling points. 1 Poyo Gully, 2 Plana Pound and Perello outflow channel. A Pinedo wastewater treatment plant (WWTP), B Albufera South WWTP, C Sueca WWTP and D El Mareny WWTP

ISs were placed into a 15-mL centrifuge tube and 5 mL extraction buffer (methanol/0.1 M Na_2EDTA/McIlvaine buffer, 50:25:25) was added. The tubes were vortex for 30 s and placed into an ultrasonic bath for 10 min before being centrifuged at approximately 1,200 g for 15 min. The supernatant was then combined and diluted to approximately 400 mL with distilled water to reduce the methanol content below 2%.

SPE for extraction of water samples and soil extracts clean-up

The process SPE/clean-up used for water samples was based on that reported by Petrovic et al. [14]. Water samples (250 mL, pH neutral) were spiked with 50 ng of surrogate/internal standards (acetaminophen-d_3, carbamazepine-d_2 and ibuprofen-d_3) and isolated using an Oasis HLB cartridge [poly(divinylbenzene-co-N-pyrrolidone)] preconditioned with 5 mL of methanol and 5 mL of Milli-Q water. Samples were passed through the cartridges at a flow rate of 10 mL min^{-1} and the cartridges were then rinsed with 5 mL of Milli-Q water and dried under vacuum for 15 min. The analytes retained were eluted with 6 mL of methanol. The extract was evaporated under a gentle stream of nitrogen and reconstituted with 1 mL methanol/water (25:75, v/v), filtered using syringe poly(tetrafluoroethylene) (PTFE) filters (0.22 μm, Analisis Vinicos, Tomelloso, Spain) and injected into the HPLC-MS/MS for analysis.

In the case of the aqueous PLE or ultrasonic extracts, obtained from soils and sediments, clean-up was performed in the same manner as for water samples, but instead of using a unique cartridge, a SAX one (strong anion exchange medium) was placed on top of the Oasis HLB cartridge and the former was removed just before the elution of the analytes.

LC-ESI-MS/MS

In accordance with our previous study [26], the LC separation was performed using an Alliance 2695 HPLC module (Waters). In positive ion (PI) mode, a Sunfire C18 column (4.6 mm×150 mm, 3.5 μm, from Waters) and a Gemini C18 (4.0 mm×2.0 mm) guard cartridge (Phenomenex) were used. The mobile phase was eluent A (formic acid 0.1% in methanol) and eluent B (formic acid 0.1% in water) in a gradient programme that started at 20% A for 0.1 min, increased linearly to 90% A in 15 min, then increased to 98% A in 15 min, hold for 8 min, and returned to the initial conditions after 1 min followed by 11 min of equilibration time. The flow rate was 0.2 mL min^{-1}. In negative ion (NI) mode, a Luna C18 (2) column 100 Å (2.0 mm×150 mm, particle size 3 μm) and Gemini C18 (4.0×2.0 mm) guard cartridge, both from Phenomenex, were used. The mobile phase was acetonitrile/methanol (60:40, v/v) as eluent A and ammonium acetate 10 mM in water as eluent B, at a flow rate of 0.2 mL min^{-1}. The analytical column was preconditioned using 15% of acetonitrile and 85% of eluent B at the same flow rate for 11 min. A gradient programme was used as follows: 15% of eluent A for 0.1 min, followed by a linear increase to 98% in 5 min, held for 7 min. Then, a 3-min gradient returned to the preconditioning conditions 15% of acetonitrile and 85% of eluent B. The injection volume was 20 μL. The tandem MS analyses were performed on a Micromass Quattro triple quadrupole mass spectrometer (Manchester, UK). Instrument control, data acquisition and evaluation were done with the Masslynx NT software (v. 3.4). The optimal quantification and confirmation transitions, and their respective cone voltages (CV) and collision energies (CE) are listed in Table 5.1.

Method validation

Linearity was studied using standard solutions and matrix-matched calibrations by analysing in triplicate seven

Table 5.1 Retention time (T_r) of the pharmaceuticals, LC-MS/MS conditions in positive and negative ion mode and some physicochemical characteristics

Compound	T_r (min)	CV (eV)	Quantification transition	CE (eV)	Confirmation transition	CE (eV)	Log P	Log D_{ow} (pH 7.4)	pK_a
PI mode									
Acetaminophen[a]	16.50	25	152→110	15	152→92.5	25	0.46	0.34	9.38
Acetaminophen-d_3	16.40	20	155→111	15	155→92.5	20			
Carbamazepine[b]	25.92	30	237→193	35	237→192	40	2.45	2.67	13.9
Carbamazepine-d_2	25.92	35	239→195	20	239→194	30			
Ciprofloxacin[b]	14.51	30	332→314	20	332→231	35	0.28	−1.11	5.9/8.9
Codeine[a]	7.39	35	300→215	25	300→199	30	1.52	0.47	8.2
Diazepam[a]	28.88	40	285→154	25	285→193	30	2.8	2.79	3.3
Fenofibrate[b]	36.22	25	361→233	15	361→139	30	5.19	4.80	–
Metoprolol[a]	15.17	30	268→116	20	268→98	20	1.88	−0.1	9.7
Norfloxacin[a]	14.37	30	320→276	15	320→302	20	−1.0	−1.03	6.2/8.5
Ofloxacin[a]	13.77	30	362→318	20	362→261	25	−0.4	−0.44	6.05/8.2
4-Epioxytetracycline[a]	14.29	25	461→426	20	461→443	10	−1.3		
Oxytetracycline[a]	15.66	25	461→426	20	461→443	10	−1.3	−1.55	3.2/7.5/9.2
Propanolol[a]	18.20	30	260→116	18	260→183	20	3.48	1.2	9.5
Sulfamethoxazole[a]	20.00	25	254→92	25	254→156	15	0.89	0.89	1.85/5.7
4-Epitetracycline[a]	14.54	24	445→410	20	445→427	15	−1.2		
Tetracycline[a]	15.02	24	445→410	20	445→427	15	−1.2	−1.35	3.3/7.8/9.6
Trimethoprim[a]	11.81	40	291→123	25	291→230	25	0.91	0.05	6.6
NI mode									
Clofibric acid[c]	7.97	20	213→127	18	213→84.5	10	2.58	−1.36	3.46
Diclofenac[c]	9.57	20	294→250	15	294→214	25	4.51	1.26	4.15
Ibuprofen[c]	10.22	15	205→161	10	–	–	3.97	1.16	4.5
Ibuprofen-d_3	10.22	15	208→164	10	208→162	15			

[a] Related to acetamidophen-d_3 as internal standard when used

[b] Related to cabamazepine-d_2 as internal standard when used

[c] Related to ibuprofen-d_3 as internal standard when used

concentration levels, between 7.5 and 7,500 ng mL^{-1} in the final extract, equivalent to 2.5 and 2,500 ng g^{-1} in soil, and between 0.030 and 30 μg L^{-1} in water. The matrix effects were studied by comparison of the slopes of both regression equations.

The extraction recoveries of the different compounds for the entire procedures were determined for waters, soils and sediments. Samples were spiked with the analytes at three different concentrations; method quantification limits (MQLs), 0.05 and 0.5 μg L^{-1} for water; and MQLs, 5 and 50 ng g^{-1} for soils and sediments. For calculation of recoveries, the average concentrations measured in the non-spiked water samples were subtracted from the concentration values obtained for the spiked ones.

Method detection limits (MDLs) were confirmed by injecting seven replicated extracts of samples spiked at the estimated concentrations. MQLs were the lower concentration that provided acceptable recoveries (relative recoveries≥70%, excepting fenofibrate and diclofenac) and precision (<20%). It was tested by analysing spiked soil and sediment samples in quintuplicate.

Each sample was analysed in triplicate. Prior to sample analysis several tests were carried out to ensure system and laboratory performance. A calibration standard solution was used to validate calibration accuracy. The retention times of both native and labelled compounds were required to be within ±15 s of the respective retention times determined during the initial calibration. Throughout the analysis, precision and recovery were ensured. Laboratory blanks were analysed prior to each batch sample analysis consisting of 7 to 20 samples.

Results and discussion

Optimization and/or validation of the sample pre-treatment

Water samples

Oasis HLB SPE cartridges are commonly used for the analysis of pharmaceuticals in environmental matrices [1, 3, 7, 9–15]. Three parameters were optimized for the performance of the method in environmental waters: the sample extraction volume, wash volume after extraction and the elution solvent. SPE recoveries and MDLs were the criteria used to make the most appropriate choice for every parameter.

Three extraction water volumes were checked (100 mL, 250 mL and 500 mL). The extraction yield of the studied compounds is shown in the Electronic Supplementary Material Figure S5.4-A. In general, 100 and 250 mL were the volumes that provided the best recoveries with no big differences between them; 250 mL was therefore selected as the sample extraction volume because it yielded better MDLs.

Two cartridge wash volumes of water were tested (5 mL and 10 mL). Polar compounds gave better SPE recoveries with 5 mL (see Electronic Supplementary Material Figure S5.4-B). This is consistent with the fact that the solvent used for washing is water, which will elute some of the polar compounds with it. For polar compounds, the lower washing volume used was the better. For the other compounds this parameter is not so critical. Washing with 5 mL of water resulted in the best recovery for a larger number of compounds and was chosen for further analyses.

The recovery of the target compounds by SPE is highly dependent on the polarity of the eluent. Acetone, dichloromethane, acetonitrile and methanol were tested. The results (see Electronic Supplementary Material Figure S5.5-C) show that dichloromethane produced the lowest recovery for most compounds (<50%). Better recoveries were obtained with acetone and acetonitrile as the elution solvents, with most varying between 60 and 105%. The best recoveries (80– 100%) were achieved eluting with methanol. Accordingly, it was chosen as the solvent for the simultaneous extraction of the studied pharmaceuticals from water.

The method was validated and data are presented in Table 5.2. Linearity was determined using regression analysis between the area ratios and concentrations. Correlations of R^2>0.99, with the exception of ibuprofen, were obtained over a concentration range 30–30,000 ng L^{-1}. MDLs and MQLs ranged from 0.3 to 10.0 ng L^{-1} and 0.9 to 36 ng L^{-1}, respectively. The precision of the overall method was determined from five replicates. At low level, it varied by less than 20% in most cases with the exception of ibuprofen and, in high level spiked samples, it was always lower than 13%. Recoveries achieved for all target compounds ranged from 71.2 to 97.8% and from 85.2 to 98.5% at MLQs and 10 times MLQs, respectively. Oxytetracycline, 4- epioxytetracycline, tetracycline, 4-epitetracycline, metoprolol, propanol, acetaminophen and clofibric acid showed the lowest recovery rates (between 70 and 80%).

ESI-MS analysis may be subject to signal suppression or enhancement as a result of other components in the sample. Signal suppression was observed for all analytes detected. The level of suppression was greater than 10% for oxytetracycline, ofloxacin, fenofibrate, ciprofloxacin, norfloxacin, propanolol, sulfamethoxazole, carbamazepine, ibuprofen and clofibric acid, and greater than 20% for metoprolol and clofibric acid.

Soil and sediment samples

As was mentioned in the "Introduction," fewer methods have been developed for the determination of pharmaceuticals in soils and sediments [8, 24, 27, 33]

Table 5.2 Linearity and detection and quantitation limits, absolute recovery, reproducibility and matrix effect of the method used to determine pharmaceuticals in water

	MDLs	MQLs	Recoveries, % (RSD, %)		Linearity (R^2)	Matrix effect (%)
	ng/L	ng/L	At LOQ	At 10× LOQ		
Oxytetracycline	9.4	28.2	71.2 (14.1)	85.2 (10.4)	0.9987	−10.7
4-Epioxytetracycline	9.8	29.4	72.7 (12.7)	88.2 (9.7)	0.9989	−9.4
Tetracycline	10.0	30	73.5 (12.9)	85.3 (11.2)	0.9991	−8.6
4-Epitetracycline	9.8	29.4	75.6 (11.5)	87.7 (9.5)	0.9986	−7.8
Ofloxacin	8.1	24.3	86.4 (13.2)	92.5 (8.8)	0.9996	−11.9
Fenofibrate	1.8	5.4	90.3 (12.6)	97.4 (8.3)	0.9989	−12.8
Ciprofloxacin	12	36	91.2 (10.2)	93.6 (7.5)	0.9992	−12.4
Norfloxacin	9.6	28.8	90.4 (11.8)	98.5 (8.0)	0.9994	−10.5
Codeine	1.2	3.6	92.5 (13.2)	99.3 (7.9)	0.9997	−9.6
Trimethoprim	0.9	2.7	90.7 (14.9)	96.7 (8.2)	0.9997	−8.7
Diazepam	0.3	0.9	94.4 (12.8)	99.2 (7.7)	0.9996	−5.4
Metoprolol	1.2	3.6	80.1 (18.7)	93.2 (7.8)	0.9993	−25.2
Propanolol	0.6	1.8	75.8 (19.6)	94.2 (9.1)	0.9989	−18.2
Sulfamethoxazole	0.9	2.7	74.9 (10.3)	95.6 (8.9)	0.9992	−15.4
Carbamazepine	0.6	1.8	92.5 (12.3	99.3 (9.2)	0.9994	−18.9
Acetaminophen	0.9	2.7	72.8 (16.2)	81.7 (12.5)	0.9924	−4.2
Ibuprofen	4.8	14.4	97.8 (21.4)	98.3 (7.5)	0.9896	−15.4
Clofibric acid	1.5	4.5	79.2 (13.2)	85.2 (10.3)	0.9994	−12.1
Diclofenac	2.5	7.5	92.6 (11.8)	96.2 (10.9)	0.9989	−23.0

In this study, an ultrasonic-based extraction method was compared with an previously developed, very fast and simple, one-step PLE extraction with hot water (90 °C) [26], using a common clean-up procedure based on that developed to extract water samples. The performance characteristics for the majority of the 17 pharmaceuticals studied were acceptable for both methods. A comparison between the PLE and ultrasonic extraction method is illustrated in Fig. 5.2 via bar graphs for the obtained recovery data and in Table 5.3 via tabulated results for the MDLs, MQLs, matrix interferences and linearity.

Recoveries achieved at the MQLs were 35.4–105.3% for soil and 42.1–97.8% for sediments using PLE and 20.2–86.5% and 30.3–97.4% using ultrasonic extraction. These results showed a better performance of PLE in the extraction of pharmaceuticals than that of the ultrasonic extraction. Precision obtained was better using PLE method (between 5 and 19%) than using ultrasonic extraction (between 12 and 25%). MQLs of the target pharmaceuticals ranged from 0.1 ng g^{-1} (acetaminophen, carbamazepine and diazepam) to 5.3 ng g^{-1} (oxytetracycline) by PLE, and from 0.1 ng g^{-1} (acetaminophen) to 15.9 ng g^{-1} (tetracycline) by ultrasonic extraction. Limits of quantification (LOQs) were between 0.3 (trimethoprim) and 18.1 ng g^{-1} (oxytetracycline) by PLE, and between 0.5 (acetaminophen) and 48.2 ng g^{-1} (tetracycline) by ultrasonic extraction. Overall, the analytical method provided a higher LOQ for the ultrasonic extraction than for the PLE.

Matrix effects were observed and assessed for the spiked soil and sediment extracts. Soil and sediment matrix components decreased signal responses for all pharmaceuticals, excepting acetaminophen. The absolute matrix effects were −2.6 to 54.6% (suppression) for the PLE and −6.8–69.3% for the ultrasonic extraction. Relative recoveries with regards to an internal standard diminish the ion suppression. Results suggest that use of isotope-labelled internal standards is very important in reducing the matrix effects.

Summarizing, the number of pharmaceuticals with MLQs lower than 10 ng $g^{-1}$and acceptable RSDs (<20%) is slightly higher for the PLE method than the ultrasonic one. It should be kept in mind, however, that this is not only caused by the sensitivity of the LC-MS/MS detection method, which is little better for the PLE method because of the lower percentage of matrix effects, but also by the differences in recoveries and RSDs at the lowest spiked level. On the other hand, the speed and user-friendliness of the ultrasonic extraction in real practice are slightly better than for the PLE method, despite the extra centrifugation step, which can be easily performed in batch. Both methods were successfully applied in the L'Albufera Natural Park study during 2008. Figure 5.3 shows the LC-MS/MS

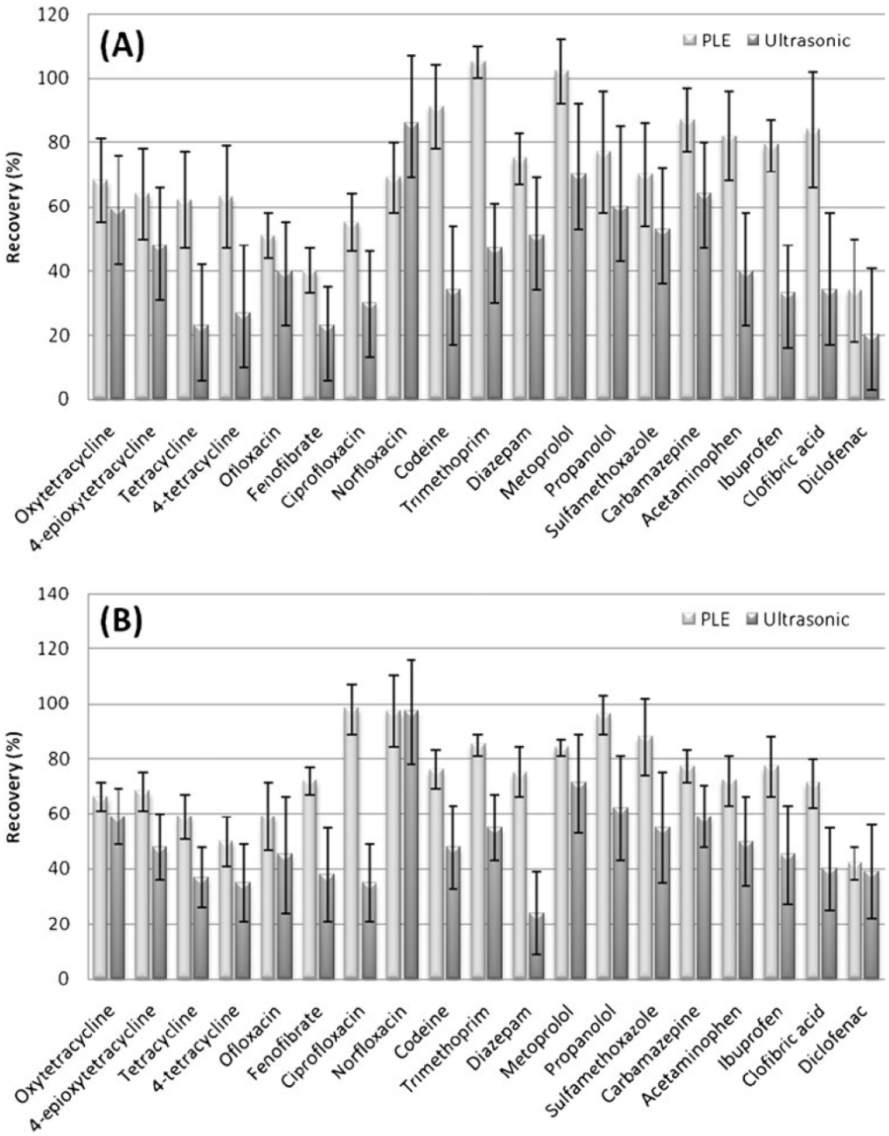


Fig. 5.2 Recoveries and RSDs obtained by ultrasonic and PLE methods at the MQLs for (A) soil samples and (B) sediment samples

chromatograms obtained from a soil sample (P10) extracted using PLE (Fig. 5.3a) or ultrasonic extraction (Fig. 5.3b). As can be observed, acetaminophen and carbamazepine were not detected using ultrasonic extraction because of the higher MDLs of this method.

Occurrence and distribution of pharmaceuticals in L'Albufera Natural Park

Occurrence of pharmaceuticals in the water samples is shown in Table 5.4, and analysis of sediments and soils extracted by PLE is shown in Tables 5.5 and 5.6. Concentrations of carbamazepine and ibuprofen in water, soil and sediment are compared in the Electronic Supplementary Material, Figures S5.6 and S5.7. Water samples taken at points P3, P6, P9, P10 and P14 suffered an unfortunate accident and could not be analysed. Among the 17 pharmaceuticals screened in surface waters from the L'Albufera Natural Park, 13 (acetaminophen, carbamazepine, ciprofloxacin, codeine, diazepam, diclofenac, metoprolol, ofloxacin, propanolol, sulfamethoxazole, ibuprofen, clofibric acid and trimethoprim) were detected (Table 5.4). Tetracycline, oxytetracycline and fenofibrate were not present in water samples but they were in soil or sediment samples, and norfloxacin was not detected in any of the samples (Tables 5.4, 5.5, 5.6).

The 15 water samples analysed were contaminated by pharmaceuticals. In these samples, carbamazepine was the

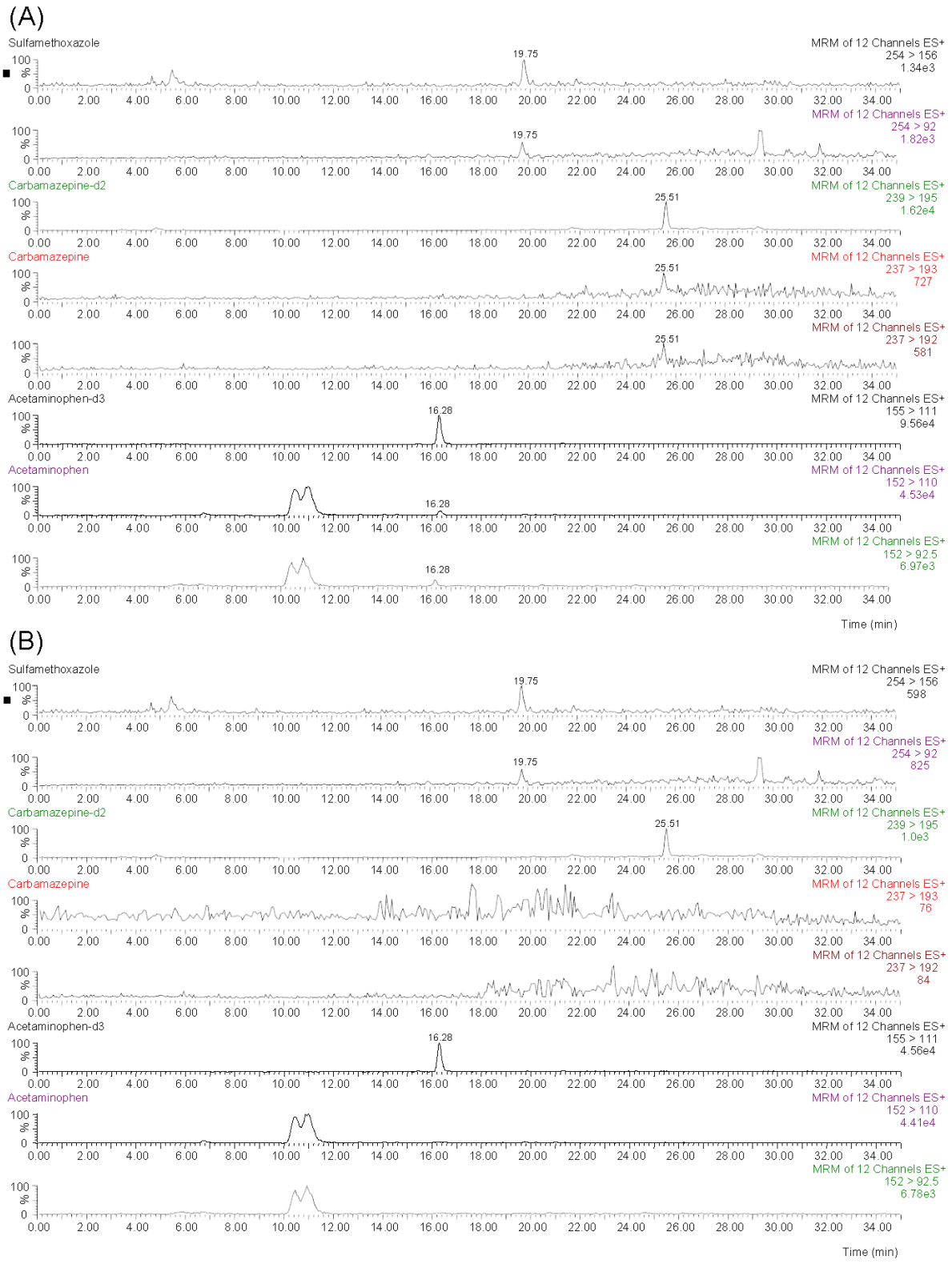
Table 5.3 MDLs, MQLs and % of matrix effect obtained using PLE and ultrasonic extraction methods for soil samples

	PLE				Ultrasonic			
	MDL ng g^{-1}	MQL ng g^{-1}	Matrix effect (%)	Linearity (R^2)	MDL ng g^{-1}	MQL ng g^{-1}	Matrix effect (%)	Linearity (R^2)
Oxytetracycline	5.3	18.1	18.8	0.9972	8.1	24.3	23.3	0.9963
4-Epioxytetracycline	5.2	15.6	17.2	0.9985	7.9	23.9	24.2	0.9962
Tetracycline	4.8	16.3	16.7	0.9992	15.9	48.2	30.4	0.9990
4-Epitetracycline	5.1	15.3	17.1	0.9990	13.2	39.0	32.9	0.9991
Ofloxacin	1.0	4.0	41.3	0.9994	4.3	13.1	57.2	0.9996
Fenofibrate	0.3	0.6	4.5	0.9990	2.0	6.0	12.1	0.9992
Ciprofloxacin	4.1	10.4	52.0	0.9987	10.5	31.5	62.5	0.9989
Norfloxacin	4.7	15.1	54.6	0.9991	5.6	17.4	69.3	0.9987
Codeine	0.3	1.3	9.5	0.9995	0.8	2.5	14.6	0.9991
Trimethoprim	0.2	0.9	11.3	0.9993	0.4	1.2	19.8	0.9990
Diazepam	0.1	0.3	21.0	0.9996	0.2	0.6	29.4	0.9996
Metoprolol	0.5	1.2	3.1	0.9954	1.2	3.8	12.9	0.9968
Propanolol	0.4	0.5	21.8	0.9969	0.8	1.6	32.4	0.9990
Sulfamethoxazole	0.3	0.9	16.3	0.9991	1.1	3.3	28.3	0.9993
Carbamazepine	0.1	0.5	14.8	0.9992	0.3	0.9	25.6	0.9994
Acetaminophen	0.1	0.8	−2.6	0.9944	0.1	0.5	−6.8	0.9942
Ibuprofen	1.8	4.3	22.9	0.9958	8.6	26.8	49.4	0.9964
Clofibric acid	0.3	1.6	33.7	0.9985	1.4	4.3	52.6	0.9989
Diclofenac	0.6	3.2	19.3	0.9990	3.0	9.2	30.6	0.9992

substance most frequently detected (14 samples, 93% of the samples) with concentrations ranging up to 31.0 ng L^{-1}. The mean concentration calculated by considering the non-detected values as zero and those of samples less than the MQL as the MQL was 9.6 ng L^{-1}. A high presence of this drug was reported by other researchers too, with mean concentrations between 1 and 794 ng L^{-1} [34–37]. Some studies confirmed that carbamazepine is not sorbed to sediments in an appreciable degree, thus it is not significantly biodegraded in wastewater treatment plants (WWTPs), and that it enters the environment in considerable amounts [38]. Acetaminophen and ibuprofen were detected with frequency lower than 66%, but at higher mean concentrations of 1,204.4 ng L^{-1} and 289.9 ng L^{-1}, respectively. For ibuprofen, a significant removal in WWTPs is reported in the literature [38], and as a result of its low distribution constant value, the removal should be based on biodegradation. Sulfamethoxazole was detected in 60% of the samples at lower mean concentration of 27.3 ng L^{-1}. This frequency of positive samples and mean concentration is similar to those reported by other authors [34], but differs from a few studies that only found some traces below the MLQ [35, 39]. Of the other pharmaceuticals, diclofenac was found in 6 samples (40%), codeine, ofloxacin and propanolol in 5 (33%), ciprofloxacin, diazepam and clofibric acid in 4 (27%), trimethoprim in 3 (20%) and metoprolol in 2 (13%) with lower mean concentrations (Table 5.4). Figure S5.5 in the Electronic Supplementary Material shows the LC-MS/ MS chromatogram obtained for water sample P14 and illustrates the good performance of the analytical method for different pharmaceuticals.

These target compounds varied spatially, being detected at higher concentrations at P1, P2, P8, P11, P13 and P19. The highest concentrations for 5 out of the 12 detected compounds in surface water were found at site P8. This sample point is located near of the Albufera South WWTP (Fig. 5.1). However, this point is not connected to the irrigation channels that gather in the wastewater coming out of the WWTP. This point is near of an industrial area with different nightclubs, and the high level of pharmaceuticals could be due to the direct spillage of sewage water in the small irrigation channels. In contrast, samples from P7, which is close to the system that drives the wastewater to the lake to maintain the ecological flow, do not show high concentrations of pharmaceuticals.

The second group of points, with high concentrations and frequency of pharmaceuticals, were the sites P1, P19, P13 and P11 (Tables 5.4, 5.5 and 5.6). These sites are mainly located parallel to the Poyo Gully, just where the pipes that carry purified water from the Pinedo WWTP to the little port of Catarroja (Portet de Catarroja) flow. This WWTP provides a constant flow of 1 m^3/s of treated water that


(A)
Sulfamethoxazole
MRM of 12 Channels ES+
254 > 156
1.34e3
19.75
MRM of 12 Channels ES+
254 > 92
1.82e3
19.75
Carbamazepine-d2
MRM of 12 Channels ES+
239 > 195
1.62e4
25.51
Carbamazepine
MRM of 12 Channels ES+
237 > 193
727
25.51
MRM of 12 Channels ES+
237 > 192
581
25.51
Acetaminophen-d3
MRM of 12 Channels ES+
155 > 111
9.56e4
16.28
Acetaminophen
MRM of 12 Channels ES+
152 > 110
4.53e4
16.28
MRM of 12 Channels ES+
152 > 92.5
6.97e3
16.28
Time (min)
(B)
Sulfamethoxazole
MRM of 12 Channels ES+
254 > 156
598
19.75
MRM of 12 Channels ES+
254 > 92
825
19.75
Carbamazepine-d2
MRM of 12 Channels ES+
239 > 195
1.0e3
25.51
Carbamazepine
MRM of 12 Channels ES+
237 > 193
76
MRM of 12 Channels ES+
237 > 192
84
Acetaminophen-d3
MRM of 12 Channels ES+
155 > 111
4.56e4
16.28
Acetaminophen
MRM of 12 Channels ES+
152 > 110
4.41e4
MRM of 12 Channels ES+
152 > 92.5
6.78e3
Time (min)

◀ Fig. 5.3 LC-MS/MS chromatograms obtained by injecting extracts of soil sample P10 (A) using PLE method and (B) ultrasonic extraction.
For concentrations see Table 5.6

arrives into L'Albufera lake through this point. L'Albufera Natural Park is the main recipient of water from the Pinedo WWTP. In particular, this WWTP injects 73 hm^3/year into the water system of L'Albufera Park irrigation network. This is the greatest single contribution to flows that the wetland receives, and it is fundamentally important for the natural ecosystem and irrigation of crops. The dissipation of pharmaceuticals through these points from the point P1, where the wastewater flows to the lake, was observed.

The other point that presents a remarkable concentration of pharmaceuticals was P2. This point is located near of Sueca WWTP. However, it is not clear why this point shows such a distribution pattern. Summarizing, higher concentrations of the detected pharmaceuticals were mainly found in the sites located near the WWTP outflows. This distribution is reasonable because the main sources of these pharmaceuticals are effluents of sewage treatment plants.

Table 5.5 outlines the concentration of pharmaceuticals in sediments. Sediments were not available at the sampling points P4, P10, P17, P18 and P20. Pharmaceuticals were not detected in the sediments taken from points P3, P5 and P7. Fourteen pharmaceuticals (acetaminophen, carbamaze- pine, ciprofloxacin, clofibric acid, codeine, diazepam, fenofibrate, ibuprofen, metoprolol, ofloxacin, oxytetracy- cline, propanolol, sulfamethoxazole, trimethoprim) were detected in the sediment samples collected from the L'Albufera Natural Park, whereas the remaining three compounds were not detected in any sample (Table 5.5). Carbamazepine was detected in 73% of the samples, followed by ofloxacin and codeine (53%), propanolol and acetaminophen (47%), and ibuprofen (40%) (Table 5.5). When considering mean concentrations in sediment (15 samples), ibuprofen was the dominating compound (6.73 ng g^{-1}), followed by ofloxacin (2.56 ng g^{-1}), codeine (2.36 ng g^{-1}) and oxytetracycline (1.88 ng g^{-1}). Oxytetracycline can bind to humic acids, proteins and organic matter as well as anionic groups in sand and soil. As a result of its high distribution constant in both sandy and loam soils, oxytetracycline is expected to show strong sorption [40].

Mean concentrations of the pharmaceuticals in sediment samples were about one thousand times lower than in water for all the target compounds, but concentration patterns remained the same, i.e. carbamazepine was the dominating

Table 5.4 Concentration of pharmaceuticals (ng L^{-1}) in waters samples from L'Albufera, Valencia, Spain

Pharmaceuticals	P1	P2	P4	P5	P7	P8	P11	P12	P13	P14	P16	P17	P18	P19	P20	Mean value[c]
Oxytetracycline[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
Tetracycline[b]	n.d.	n.d.	n.d	n.d	n.d	n.d.	n.d	n.d	n.d	n.d	n.d	n.d	n.d.	n.d.	n.d	0.0
Ofloxacin	49.3	n.d.	n.d.	n.d.	n.d.	43.9	<MQL	n.d.	n.d.	n.d.	n.d.	n.d.	<MQL	34.3	n.d.	11.7
Fenofibrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	0.0
Ciprofloxacin	14.1	n.d.	n.d.	n.d.	n.d.	30.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	20.0	6.1	4.7
Norfloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	0.0
Codeine	68.1	n.d.	n.d.	n.d.	n.d.	434.0	34.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	27.1	<MQL	37.8
Trimethoprim	53.6	n.d.	n.d.	n.d.	n.d.	32.5	40.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	8.4
Diazepam	5.6	n.d.	n.d.	n.d.	n.d.	6.3	6.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.6	n.d.	1.5
Metoprolol	5.8	n.d.	n.d.	n.d.	n.d.	n.d	5.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	0.8
Propanolol	6.8	n.d.	n.d.	n.d.	n.d.	8.4	5.9	n.d.	1.5	n.d.	n.d.	n.d.	n.d.	3.3	n.d.	1.7
Sulfamethoxazole	139.0	24.1	17.4	n.d.	n.d.	44.8	144.0	n.d.	n.d.	4.1	10.7	6.3	n.d.	18.9	n.d.	27.3
Carbamazepine	24.1	11.4	3.1	<MQL	<MQL	21.9	31.0	n.d	15.3	2.2	6.8	2.4	3.7	16.0	<MQL	9.6
Acetaminophen	n.d.	13.9	n.d.	<MQL	n.d	17,699.4	23.1	<MQL	n.d.	14.9	26.2	15.1	18.9	n.d	249.2	1,204.4
Ibuprofen	131.2	n.d.	n.d.	25.3	<MQL	3,913.7	84.3	n.d.	<MQL	n.d.	<MQL	101.4	<MQL	34.2	n.d.	289.9
Clofibric acid	n.d.	n.d.	<MQL	21.7	n.d	71.4	n.d	n.d.	n.d.	n.d.	n.d.	42.3	n.d.	n.d	n.d.	9.3
Diclofenac	125.6	n.d.	n.d.	42.6	n.d	260.9	57.6	n.d.	n.d	n.d.	n.d.	73.2	n.d.	25.3	n.d.	39.0

The most contaminated samples were P1, P8, P11 and P19

n.d. not detected

[a] Sum of oxytetracycline and 4-epioxytetracycline

[b] Sum of tetracycline and 4-epitetracycline

[c] Mean values were calculated by considering n.d. as zero and values less than MQL as the MQL

Table 5.5 Concentration of pharmaceuticals (ng g^{-1}) in sediment samples from L'Albufera, Valencia Spain

Pharmaceuticals	P1	P2	P6	P8	P9	P11	P12	P13	P14	P15	P16	P19	Mean values[c]
Oxytetracycline[a]	<MQL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.88
Tetracycline[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00
Ofloxacin	6.53	n.d.	4.12	n.d.	<MQL	3.98	n.d.	4.07	n.d.	7.05	4.25	4.36	3.91
Fenofibrate	0.81	<MQL	n.d.	n.d.	n.d.	n.d.	n.d.	<MQL	n.d.	n.d.	n.d.	n.d.	0.76
Ciprofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.21	n.d.	n.d.	0.01
Norfloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00
Codeine	6.18	n.d.	n.d.	5.96	n.d.	1.59	n.d.	5.08	<MQL	<MQL	3.38	5.96	2.36
Trimethoprim	<MQL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.09	0.45
Diazepam	1.18	n.d.	n.d.	1.43	0.95	n.d.	n.d.	1.08	<MQL	1.33	1.27	n.d.	0.54
Metoprolol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.36	n.d.	<MQL	n.d.	2.04	0.41
Propanolol	1.23	0.68	n.d.	0.90	n.d.	n.d.	n.d.	0.84	n.d.	1.64	0.65	1.11	0.47
Sulfamethoxazole	n.d.	n.d.	n.d.	n.d.	n.d.	1.59	n.d.	2.73	n.d.	n.d.	n.d.	n.d.	0.29
Carbamazepine	<MQL	0.75	0.62	1.36	n.d.	0.94	<MQL	1.14	<MQL	2.07	2.12	1.29	0.87
Acetaminophen	3.98	<MQL	n.d.	<MQL	<MQL	<MQL	1.97	<MQL	n.d.	n.d.	n.d.	n.d.	0.66
Ibuprofen	35.83	n.d.	n.d.	4.42	n.d.	n.d.	n.d.	15.57	n.d.	23.57	4.93	16.56	6.73
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.12	n.d.	n.d.	0.07
Diclofenac	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00

The most contaminated samples were P1, P8, P13 and P19

n.d. not detected

[a] Sum of oxytetracycline and 4-epioxytetracycline

[b] Sum of tetracycline and 4-epitetracycline

[c] Mean values were calculated by considering n.d. as zero and values less than MQL as the MQL. Samples P3, P4 and P7 were taken into account for the calculations

compound (Tables 5.4 and 5.5). However, diazepam and codeine occur mainly in sediments. Because biodegradation (or phototransformation) is incomplete in solid samples, important residues of fluoroquinolones can persist in agricultural soils several months after application [41]. Diclofenac, despite its high hydrophobicity, is not present in sediment and was only found in a water samples. This fact was stated by other researchers [4, 42] and occurs because diclofenac is rapidly metabolized by photodegradation and microflora of river sediments to its major metabolite 5-hydroxy-diclofenac [20, 42, 43]. Besides, in our case, this observation could also be explained because diclofenac is the compound worst extracted by the method.

In order to predict the distribution of a drug between a solid phase (sediment) and water, a number of different mechanisms involved in drug sorption have to be taken into account, the most importants are being sorption to organic matter, surface adsorption to mineral constituents, ion exchange, complex formation with metal ions such as Ca^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+} and hydrogen bonding. Most of these mechanisms are hard to calculate under the variable conditions of each particular environment; usually, only the octanol/water partition coefficient (K_{ow}) is utilized to predict the behaviour of drugs in water [44]. In this way, a compound with a high value of K_{ow} tends to accumulate in soil or sediment. By contrast, those with a low K_{ow} will tend to remain in water. Fenofibrate, the compound with the highest K_{ow} (out of those studied), is only found in sediment and soils, and not in water. But this behaviour is not replicated in the case of other compounds with high K_{ow} like diclofenac (that was only found in water), ibuprofen and propanolol. This highlights the large quantity of chemical interactions that take place, and the difficulty in establishing those most important in the behaviour of contaminants in the environment.

Table 5.6 shows that soil samples from P7, P8, P17 and P20 were not contaminated by the studied pharmaceuticals. Highest mean concentrations of the pharmaceuticals in the soil were observed in sample P13. The mean concentrations of the pharmaceuticals in soil were between 0.06 for propanolol and 2.5 ng g^{-1} for tetracycline (Table 5.6). Of all compounds, acetaminophen showed the highest concen- trations (16.05 ng g^{-1}), and carbamazepine the highest prevalence over soil locations, which reflects its high resistance to natural transformation processes such as adsorption and phototransformation. Diazepam was in six soils and in seven sediment samples. It is a lipophilic substance and showed a very low mobility in all types of soil. It can be expected that its leaching behaviour was

Table 5.6 Concentration of pharmaceuticals (ng g^{-1}) in soil samples from L'Albufera, Valencia, Spain

Pharmaceuticals	P1	P2	P3	P4	P5	P6	P9	P10	P11	P12	P13	P14	P15	P16	P18	P19	Mean values[c]
Oxytetracycline[a]	6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.30
Tetracycline[b]	n.d.	n.d.	<MQL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	34.91	2.50
Ofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00
Fenofibrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.83	n.d.	n.d.	n.d.	0.81	n.d.	0.08
Ciprofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00
Norfloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00
Codeine	<MQL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.07
Trimethoprim	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00
Diazepam	1.09	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.73	0.90	n.d.	1.19	<MQL	0.95	0.34
Metoprolol	1.66	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.75	n.d.	n.d.	n.d.	n.d.	n.d.	0.17
Propanolol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.78	n.d.	n.d.	n.d.	n.d.	0.48	0.06
Sulfamethoxazole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.63	n.d.	n.d.	4.17	n.d.	n.d.	n.d.	n.d.	n.d.	0.29
Carbamazepine	n.d.	0.77	0.58	0.87	0.69	0.52	0.65	0.52	1.38	<MQL	3.81	0.64	1.91	1.99	0.80	1.14	0.84
Acetaminophen	n.d.	<MQL	n.d.	n.d.	n.d.	n.d.	0.42	<MQL	<MQL	n.d.	16.05	0.24	n.d.	n.d.	<MQL	n.d.	1.00
Ibuprofen	<MQL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.22
Clofibric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00
Diclofenac	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00

The most contaminated samples were P10, P13 and P19

n.d. not detected

[a] Sum of oxytetracycline and 4-epioxytetracycline

[b] Sum of tetracycline and 4-epitetracycline

[c] Mean values were calculated by considering n.d. as zero and values less than MQL as the MQL. Samples P7, P8, P17 and P20 were taken into account for the calculations

mainly determined by the organic carbon content of the soils. An extensive transformation of diazepam in the soil is unlikely, because diazepam was widely stable in a water/sediment test under aerobic conditions, and transformation products might have shown certain mobility in the soil due their increased polarity [45].

Environmental implications

The environmental risks to aquatic organisms were assessed by using the mean values and worst case scenario in L'Albufera Natural Park on the basis of the risk quotients (RQ) calculated using maximum measured environmental concentrations (MECs) and predicted non-effect concentrations (PNECs) collected from the literature [46–49] (Table 5.7). It should be taken into account that the choice of data can obviously affect the outcome. Ecotoxicity data can be provided by the open scientific literature or by pharmaceutical companies. The former source is preferred because it offers lower effect values (indicating a higher risk) than the latter in a majority of the risk assessments. Only for clofibric acid and ibuprofen were the PNECs based on company-owned data. For the other PNEC values, data originating from standard tests were preferred, with long-term studies being prioritized over short-term ones. Although data from algae and fish species were indistinctly used, data from the base-set species, i.e. algae, crustaceans (Daphnia magna or Ceriodaphnia dubia) and fish, were prioritized. According to the RQ classification scheme from Hernando et al. [50], mean concentrations of ciprofloxacin, propanolol and ibuprofen and a high concentration of diclofenac could pose a low risk to the aquatic organisms (RQ between 0.1 and 1) and high concentrations of ciprofloxacin, propanolol, sulfamethoxazole and ibuprofen could pose a medium risk to the aquatic organisms (RQ between 1 and 10). These results are a good example of the interest in monitoring pharmaceuticals in the environment.

Conclusions

Two fast and efficient extraction methods for the determination of 17 pharmaceuticals in soils and sediments by LC-MS/MS analysis were optimized and validated. An ultrasonic extraction method was compared with a previously developed PLE one. The performance characteristics for the majority of the pharmaceuticals studied were acceptable in both methods. The number of pharmaceuticals with lower MQLs, higher recovery and acceptable RSDs is slightly higher for the PLE method than for the ultrasonic one. However, the speed and user-friendliness of the ultrasonic extraction method in real practice are slightly better than for the PLE. Both methods proved to be successful as a quantitative, multi-residue method for pharmaceutical residues analysis in real soil and sediment samples.

The application of the method to real samples provided evidence that L'Albufera Natural Park was contaminated by significant amounts of pharmaceuticals. Higher levels and frequency of these compounds appear in the north area of the lagoon, which is consistent with the utilization of wastewater from the Pinedo WWTP. Tetracyclines and fenofibrate are mainly accumulated in soil and sediments, and diclofenac only appears in water samples. Concentrations of pharmaceuticals in water samples are higher than those in sediment and soil samples. In the water samples, all sampling points analysed contain some of the studied drugs, with values between 2.2 ng L^{-1} and 17.7 μg L^{-1}. In sediment samples, 12 of the 16 samples have some of the studied substances, with values ranging from 0.21 to 35.8 ng g^{-1}, and in soils between 0.24 and 16.05 ng g^{-1}. The results confirmed that the method is suitable for screening

Table 5.7 Predicted no effect concentrations (PNECs), measured environmental concentrations (MECs) and risk quotients (RQs) (maximum MEC/ PNEC) of the detected pharmaceuticals in the L'Albufera Natural Park water samples

Compound	$PNEC_{water}$ (μg L^{-1})	MEC_{mean} (μg L^{-1})	$MEC_{maximum}$ (μg L^{-1})	RQ (MEC_{mean}/ PNEC)	RQ ($MEC_{maximum}$/ PNEC)
Acetaminophen	9.2[a]	1.204	17.699	0.131	1.924
Carbamazepine	0.42[b]	0.010	0.031	0.023	0.073
Ciprofloxacin	0.005[a]	0.005	0.038	0.940	7.620
Metoprolol	31[a]	0.001	0.006	0.000	0.000
Propanolol	0.005[c]	0.002	0.008	0.340	1.680
Sulfamethoxazole	0.118[a]	0.003	0.144	0.023	1.220
Tetracycline	0.09[a]	0.008	0.054	0.001	0.003
Trimethoprim	16[c]	0.009	0.071	0.002	0.017
Clofibric acid	4.2[b]	0.039	0.261	0.000	0.002
Diclofenac	0.1[d]	0.290	3.913	2.900	39.000
Ibuprofen	7.1[c]	1.204	17.699	0.170	2.492

[a] PNEC data obtained from [47]
[b] PNEC data obtained from [48]
[c] PNEC data obtained from [49]
[d] PNEC data obtained form [51]

these compounds in waters, and highlight the necessity of eliminating these pollutants in the wastewater treatment plants before their discharge into the environment.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Assessment of the occurrence and distribution of pharmaceuticals in a Mediterranean wetland (L'Albufera, Valencia, Spain) by LC-MS/MS

Pablo Vazquez-Roig • Vicente Andreu • Cristina Blasco • Yolanda Picó

Figure S5.4. Influence of different SPE parameters on the recovery of the selected pharmaceuticals from water
(A) volume of sample extracted, (B) volume of washing solvent (water) and (C) type of elution solvent.

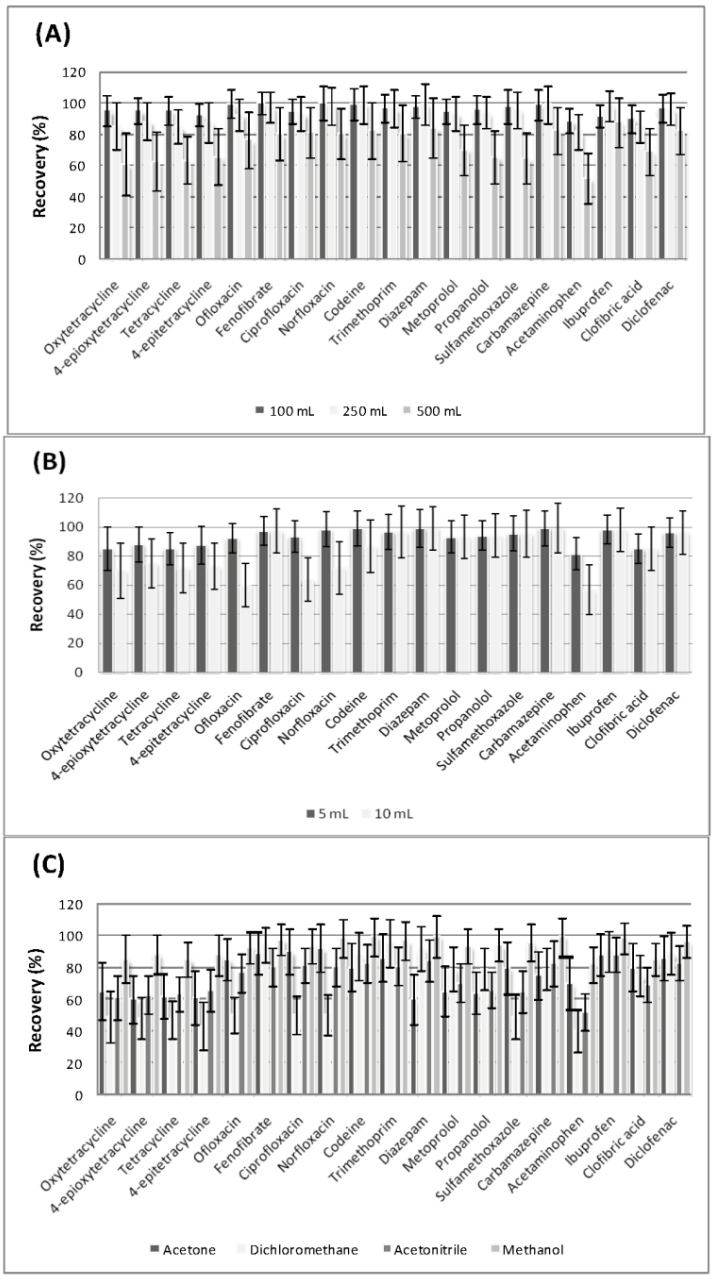


Figure S5.5. LC-MS/MS chromatogram obtained after SPE of the water sample from P14. For concentration see Table 5.4

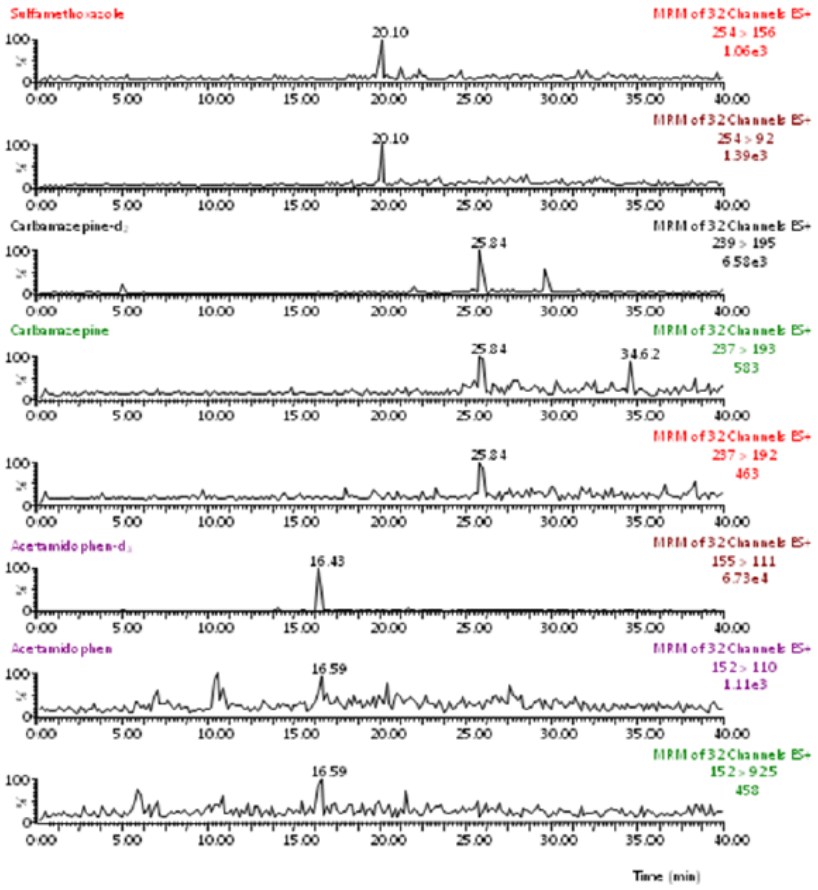


Figure S5.6. Concentration of carbamazepine found in water, sediments and soils

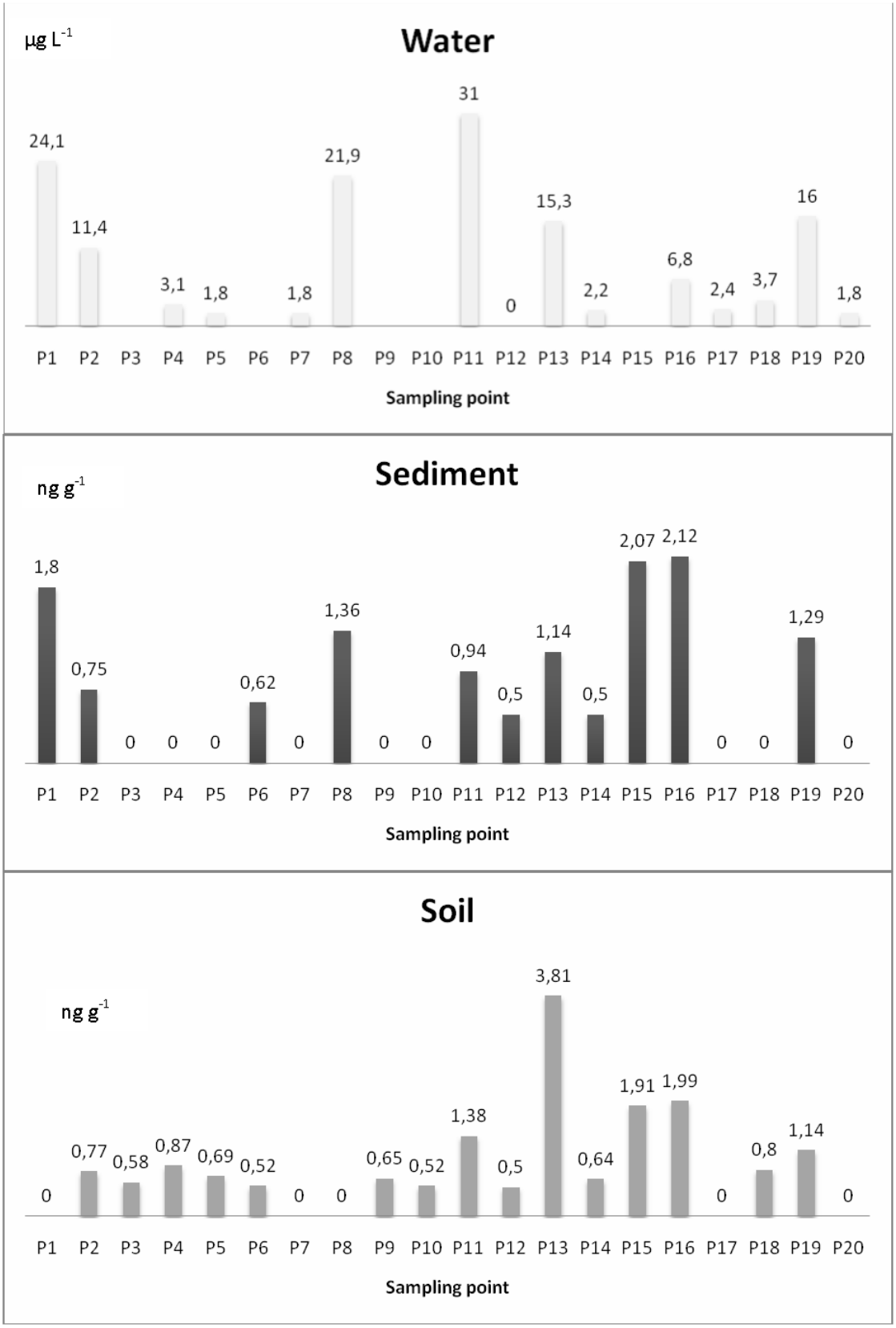
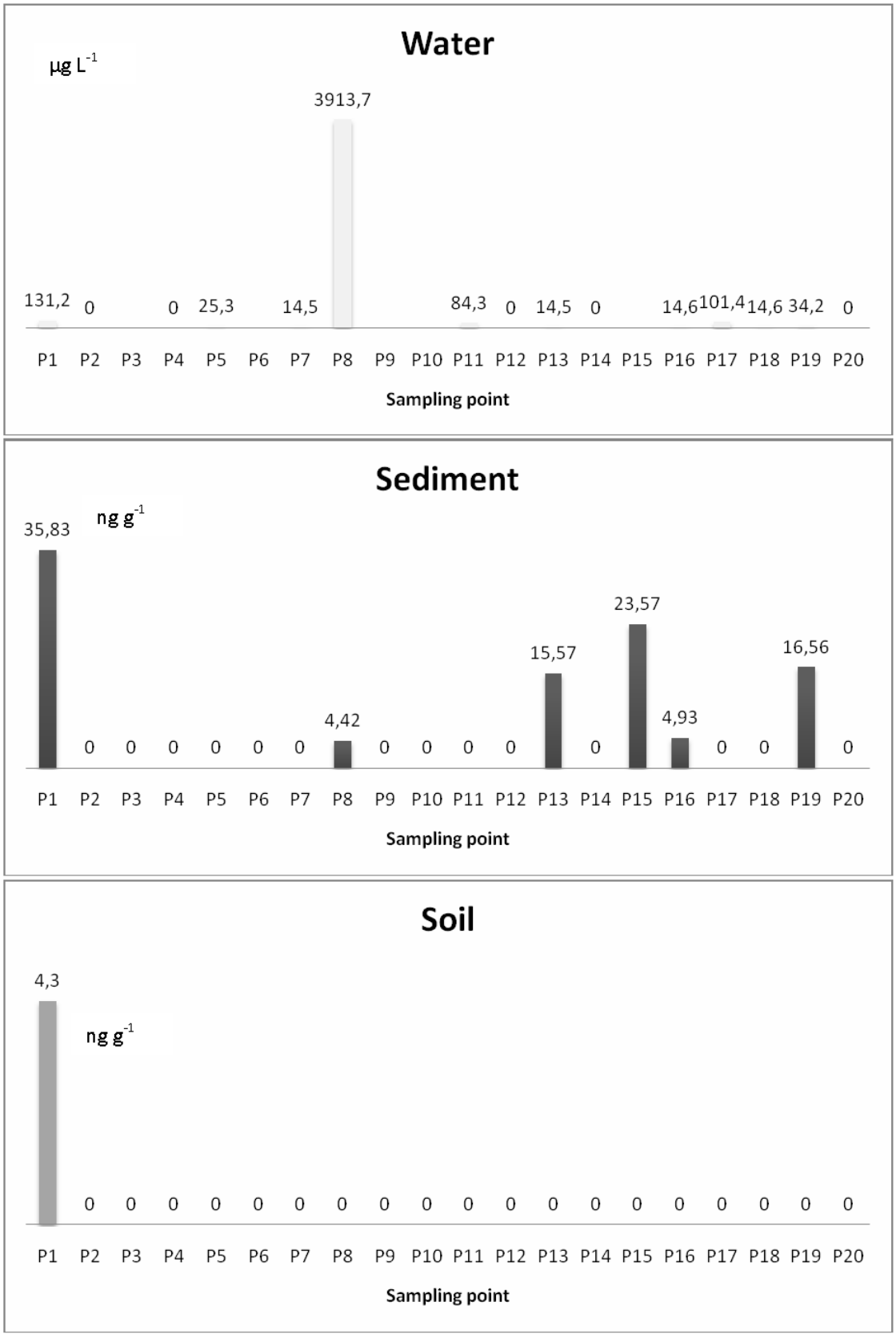


Figure S5.7. Concentration of ibuprofen found in water, sediments and soils



CHAPTER 6

Ocurrence of illicit drugs in Pego-Oliva Marsh

Scientific publication 6:

Spatial distribution of illicit drugs in surface waters of the natural park of Pego-Oliva Marsh (Valencia, Spain)

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RESEARCH ARTICLE

Spatial distribution of illicit drugs in surface waters of the natural park of Pego-Oliva Marsh (Valencia, Spain)

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Abstract
Background, aim and scope The Pego-Oliva Marsh is the second most important wetland in the Valencian Community (Spain). It is included in the RAMSAR agreement and represents one key point for migratory birds. Emerging contaminants from the human pressure, such as pharmaceuticals, illicit drugs and personal care product, are not included in the list of priority contaminants of the Water Framework Directive yet, and are neither monitored nor controlled. However, pollution of emerging contaminants can threaten the environment and even human health. In order to understand the status of the emerging contamination and recommend future rationalization of countermeasures, the occurrence of illicit drugs was investigated.
Material and methods Samples were collected at 23 sites from the main irrigation channels and the marsh. Illicit drugs were extracted using solid phase extraction and determined by liquid chromatography tandem mass spectrometry. The method detection limits ranged from 0.01 to 1.54 ng l^{-1} and the recoveries from 57% to 120%.
Results and discussion 3,4-Methylenedioxymethamphetamine, ketamine, morphine, benzoylecgonine, cocaine, methadone, 6-acetylmorphine and nor-9-carboxy-tetrahydrocannabinol were detected. The mean concentrations were 0.62, 21.33, 1.30, 1.92, 2.25, 0.32, 0.04 and 0.07 ng l^{-1}, respectively. The highest concentrations were in the north of Pego-Oliva Marsh.
Conclusions The pollution status by illicit drugs of the Pego-Oliva Marsh has been established. However, contamination levels in all the area of the natural park were low compared with those reported in other superficial waters.

Keywords Trace analysis · Illicit drugs · Pego-Oliva Marsh · Environmental analysis · Surface water

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1 Background, aim and scope

Among the most important European ecosystems are coastal wetlands, which have suffered during the last decades an increasing human pressure (Soutullo et al. 2008). This has been reflected through the intensification of agriculture and construction of infrastructures in their surroundings, or even draining part of them (Nyman et al. 2009). The Pego-Oliva Marsh Natural Park is a humid area with abundant fauna and autochthonous vegetation, situated at the extreme south of the Gulf of Valencia, in the boundary between Alicante and Valencia provinces in the Valencian Community (Spain) (Cantoral-Uriza and Sanjurjo 2010). This Natural Park, of 1,248 ha, has two main habitats, the reedbeds and the paddy fields, with smaller areas of allotments and orange groves. It is hillsided by the Segaria and Mustalla Mountains that curves around the flat

Environ Sci Pollut Res (2012) 19:971–982

area of the marsh and bounded to the West by the sandbar that separates it from the Mediterranean Sea. The marsh is located in the area of maximal rainfall of the Valencian Community, and the mountainous borders are of an intensely fractured limestone–dolomite nature, which explains the development of the exokarstic (via underground drainage dissolved through limestone) springs, which rise within the park area (Dupré et al. 1988). Furthermore, the Vedat/Bullens River, at the northern edge, collects water from the mountains and flows to the Mediterranean Sea with much of its waters entering the marsh. The Racons/Molinell River runs through the southern part of the marsh, connected by a network of irrigation channels. This marsh is included in the RAMSAR agreement, has been designated a special protection zone on birds pursuant to Directive 79/409/EEC (Council Directive 1979) and has been funded by the Life Nature programme since 1992.

During recent years, the human pressure has been increased on the marsh area. In the catchment basin of the marsh, the main economic activity is citrus cultivation, followed by vegetable cultivation and livestock. However, the existence of a settlement of 600 chalets in the Sierra de Segaria, the construction of 1,400 housings in Pego and of a golf club, hotel and equestrian centre at 500 m from the Pego-Oliva Marsh, as well as the contribution of effluents from the wastewater treatment plants, should also be mentioned. An intense effort has been, and is done to achieve a sustainable agreement and to meet standard control indexes, such as chemical or biochemical oxygen demand and nitrogen and phosphorous (Fernandez et al. 1998). However, many toxic pollutants, especially emerging contaminants coming from the human pressure, such as illicit drugs, are not listed in the Water Framework Directive yet and then, not monitored or controlled by the environmental protection departments (Blasco and Pico 2009). It may have a potential adverse effect on the water quality in the Pego-Oliva Marsh.

Illicit drugs and their metabolites have been recently recognized as environmental emerging contaminants (Postigo et al. 2008; Postigo et al. 2009; Rawls et al. 2010; Zuccato and Castiglioni 2009). Assessment of their concentrations in different environmental compartments is essential to evaluate their potential ecotoxicological effects. These compounds have also become pseudo-persistent in the environment due to their high volume of production and use and their continuous input (Berset et al. 2010). The concentration and distribution of these substances have been investigated in rivers and water bodies of several countries (Baker and Kasprzyk-Hordern 2011; Bartelt-Hunt et al. 2009; Berset et al. 2010; Bijlsma et al. 2009; Boleda et al. 2007; Boleda et al. 2009; Bones et al. 2007; Castiglioni et al. 2008; Gheorghe et al. 2008; Gonzalez-Marino et al. 2010; Huerta-Fontela et al. 2007; Huerta-Fontela et al. 2008; Kasprzyk-Hordern et al. 2007; Kasprzyk-Hordern et al. 2008; Lin et al. 2010; Postigo et al. 2010; van Nuijs et al. 2009a; van Nuijs et al. 2009b; Zuccato et al. 2008). However, few systematic studies have been reported regarding illicit drugs in Mediterranean coastal wetlands (Vazquez-Roig et al. 2010), and to our knowledge, none covers this area of study. In order to understand the pollution status of contamination by illicit drugs in the Pego-Oliva Marsh and recommend future rationalization of controlling, reducing and eliminating releases of these compounds, the concentration and distribution of 6-acethylmorphine (6-ACMOR), amphetamine (AMP), benzoylecgonine (BECG), cocaine (COC), ecgonine methyl ester (ECGME), heroin (HER), ketamine (KET) metamphetamine (MAMP), 3,4-Methylenedioxyamphetamine (MDA), 3,4-Methylenedioxymethamphetamine (MDMA), methadone (MET), morphine (MOR), Δ9-tetrahydrocannabinol (THC) and nor-9-carboxy-tetrahydrocannabinol (THC-COOH) in surface water samples of Pego-Oliva Marsh were investigated. The results of this study should be of value not only to pollution control of contaminants in the Pego-Oliva Marsh but also to guarantee the safety of drinking water.

2 Materials and methods

2.1 Chemicals and standards

High-purity individual standard solutions of 6-ACMOR, AMP, BECG, COC, ECGME, HER, KET, MAMP, MDA, MDMA, MET, MOR, THC at 1,000 mg l^{-1} and THC-COOH at 100 mg l^{-1} in methanol or acetonitrile were purchased from Cerilliant (Austin, TX, USA). Benzoylecgonine-d_3, ecgonine methyl ester-d_3, morphine-d_3, methadone-d_3, amphetamine-d_6 and 3,4-Methylenedioxyamphetamine-d_5, at a concentration of 100 mg l^{-1} in methanol, also from Cerilliant, were used as surrogated standards (SSs). Working standard mixtures were prepared at different concentrations by appropriate dilution of the individual stock solutions in methanol–water (25:75, v/v). Stock and working solutions were stored at −20°C in the dark.

Solvents and other chemicals used in this study were formic acid (reagent grade), acetonitrile and methanol (gradient grade for liquid chromatography), which were purchased from Merck (Darmstadt, Germany). High-purity water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). Mobile phases were filtered through a 0.22-μm membrane polypropylene filter (Pall, MI, USA). Oasis HLB cartridges (200 mg sorbent/6 mL) were from Waters (Milford, MA, USA). Chromabond® solid-phase extraction (SPE) vacuum manifold with 12 ports and a self-cleaning dry vacuum systemTM Laboport SH (Bonsai Advanced Technologies S.L., Madrid, Spain) were used for loading the surface water samples and for drying the cartridges.

2.2 Sample collection

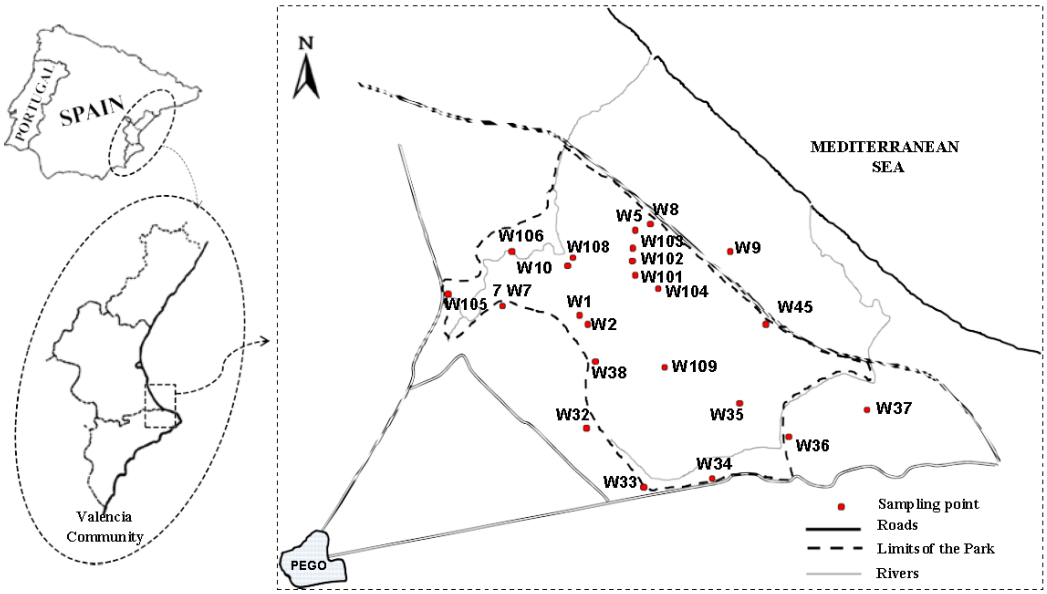
Grab surface waters were collected in 23 points of the natural park area in the 11 June 2009 to establish spatial variations in drug occurrence. Samples were taken in the most important water channels and in the marsh, as well as one sample of tap water from a chalet of the Sierra the Segaria (W35) and one spring (W1) "ullal de Bullent". There were no rainfall events during the fortnight prior to the sampling. The distribution and location of the sampling points (UTM D50) are shown in Fig. 6.1.

A homemade sampler consisted of a weighted holder equipped with a nozzle and a cap and attached to a hand-line for lowering was used to collect the water samples. A polyethylene bottle of 2.5 l was put into the sampler. Grab samples were collected by lowering the sampler to a 20–30-cm depth and collecting a sample by first opening and then closing the cap.

Before sampling, polyethylene bottles (2.5 l) were washed successively with detergent, tap water, distilled water and the sampled water. The bottles were filled to the top with the water to eliminate air bubbles and maintained at 4ºC until their arrival at the laboratory. Once in it, samples were filtered through glass microfiber GF/A filters (Whatman, UK) prior to extraction, and stored in dark bottles at −20°C until the analysis to avoid analytes degradation. The samples were extracted within 1 week.

2.3 Sample preparation

An SPE method was optimized previously by our research group (Vazquez-Roig et al. 2010). Briefly, water sample (500 mL, pH ca. 7.9–8.1) was added of the SSs (final extract concentration, 100 ng l^{-1}). Oasis HLB cartridges (6 mL, 200 mg) were preconditioned by 6 mL of methanol and 6 mL of Milli-Q water. Water samples were mixed well and trapped through the SPE tube, without any previously pH adjustment, at flow rate of 10 mL min^{-1}. Afterwards, the cartridge was washed with 6 mL of Milli-Q water and dried under vacuum for 15 min. Analytes were eluted from the HLB sorbent with 6 mL of methanol. The extract was evaporated to dryness and, finally, reconstituted with 1 mL of water–methanol (75:25, v/v). Prior to injection, final extract was filtered through syringe PTFE filters (0.22 μm, Analisis Vinicos, Tomelloso, Spain).



Sampling point	Coord X	Coord Y
W1	753935	4306819
W2	753934	4306817
W5	752699	4306105
W7	752791	4307066
W8	755291	4307954
W9	755996	4307837
W32	754086	4305048
W33	754850	4304615

Sampling point	Coord X	Coord Y
W34	755827	4304742
W35	756815	4303431
W36	756825	4305403
W37	757899	4305855
W38	754032	4306349
W45	756548	4306943
W101	754629	4307644
W102	754572	4307755

Sampling point	Coord X	Coord Y
W103	754638	4307876
W104	755050	4307399
W105	752027	4307086
W106	752953	4307739
W107	753728	4307715
W108	753738	4307740
W109	754706	4306003

Fig. 6.1 Location of the sampling sites at the Pego-Oliva Marsh in the Valencian Community (Spain)

Environ Sci Pollut Res (2012) 19:971–982

2.4 Analytical determination

The LC separation was performed using an Alliance 2695 HPLC separation module from Waters. All analytes, except THC and THC-COOH, were analyzed in positive ionization (PI) mode. In PI mode, a column Sunfire C18 (4.6×150 mm, 3.5 μm, from Waters) and a Gemini C18 (4.0×2.0 mm) guard cartridge (Phenomenex, Torrance, CA, USA) were used. The mobile phase combines eluent A (formic acid 0.1% in acetonitrile) and eluent B (water 10 mM ammonium formate) at a flow rate of 0.2 mL min^{-1} in a gradient programme that started at 2% A, increased linearly to 90% A in 15 min, then increased to 98% A in 17 min, held for 6 min, and returned to initial conditions after 1 min, followed by 11 min of equilibration.

In negative ionization (NI) mode, a column Luna C18 (2) 100Å (2.0×150 mm, 3 μm) and Gemini C18 (4.0×2.0 mm) guard cartridge, both from Phenomenex, were used. The mobile phase was composite of acetonitrile/methanol (60:40 v/v) as eluent A and ammonium acetate 10 mM in water as eluent B, at a flow rate of 0.2 mL min^{-1}. The analytical column was preconditioned using 15% of acetonitrile and 85% of eluent B at the same flow rate for 11 min. The gradient programme was 15% of eluent A for 0.1 min, followed by a linear increase to 98% in 5 min, held for 7 min. Then, a 3-min gradient returned to the preconditioning conditions 15% of acetonitrile and 85% of eluent B. The injection volume was 20 μl in both ionization modes.

The tandem MS analyses were performed on a Micromass Quattro triple quadrupole mass spectrometer (Manchester, UK). Instrument control, data acquisition and evaluation were done with the Masslynx NT software (v. 3.4).

In the PI mode, the applied parameters were extractor voltage 2.0 V, capillary voltage 3.0 kV, radio frequency lens 0.5 V; electrospray source block, 125°C; low mass (LM) 1 resolution, 12.0; high mass (HM) 1 resolution, 12.0; LM 2 resolution, 12.0; HM 2 resolution, 12.0; multiplier 650 V; desolvation temperature 350°C; argon collision gas 2.5×10^{-3} mbar; cone nitrogen gas flow, 50 lh^{-1} and desolvation gas, 600 lh^{-1}. In the NI mode, parameters were equal than in the PI mode except extractor voltage, that was 1.0 V and capillary voltage 3.2 kV. The optimal quantification and confirmation transitions and their respective cone voltages (CV) and collision energies (CE) are listed in Table 6.1.

Table 6.1 Retention times (t_r), optimized SRM transition and conditions used for LC-MS/MS analysis and ratio between product ions of the target drugs of abuse

Compound	t_r (min)	SRM_1 transition (quantifier)	CV (V)	CE (V)	SRM_2 transition (qualifier)	CV (V)	CE (V)	SRM ratio (SRM_1/SRM_2)
6-ACMOR	16.72	328>165	35	30	328>212	35	20	12.65
AMP	16.98	136>91	20	15	136>119	20	10	2.64
AMP-d_6	16.98	142>93	20	15	142>125	20	10	1.41
BECG	17.64	290>168	20	15	290>105	20	25	2.88
BECG-d_3	17.64	293>171	20	15	293>105	20	25	3.14
COC	17.78	304>182	20	15	304>82	20	25	3.58
ECGME	8.62	200>82	20	20	200>182	20	15	0.64
ECGME-d_3	8.62	203>85	20	20	203>185	20	15	1.52
HER	17.25	370>165	38	45	370>268	38	30	1.53
KET	17.11	238>125	15	25	238>179	15	15	2.64
MAMP	17.11	150>90.6	15	15	150>119	15	10	2.47
MDA	16.98	180>163	10	10	180>105	10	20	13.32
MDA-d_5	16.98	185>168	20	10	185>110	20	20	3.74
MDMA	17.11	194>163	18	10	194>105	18	20	3.06
MET	19.37	310>105	15	25	310>265	15	10	0.63
MET-d_3	19.37	313.1>268	20	10	313>105	20	25	1.87
MOR	15.65	286.1>165	30	30	286>153	30	30	1.31
MOR-d_3	15.65	289.1>165	35	30	289>153	35	30	1.19
THC	17.70	315.2>193	20	20	315>123	20	30	2.10
THC-COOH	30.78	345.2>327	20	15	345>299	20	15	1.65

SRM selected reaction monitoring, CE collision energy, CV cone voltage 6-ACMOR 6-acethylmorphine, AMP amphetamine, AMP-d_6 amphetamine-d_6, BECG benzoylecgonine, BECG-d_3 benzoylecgonine-d_3, COC cocaine, ECGME ecgonine methyl ester, ECGME-d_3 ecgonine methyl ester-d_3, HER heroin, KET ketamine, MAMP metamphetamine, MDA 3,4-methylenedioxyamphetamine, MDA-d_5 3,4-methylenedioxyamphetamine-d5, MDMA 3,4-methylenedioxymethamphetamine, MET methadone, MET-d_3 methadone-d_3, MOR morphine, MOR-d_3 morphine-d_3, THC Δ9-tetrahydrocannabinol, THC-COOH nor-9-carboxy-tetrahydrocannabinol

The most abundant characteristic selected reaction monitoring (SRM) transition was used for quantification while the second one was used for confirmation. The criteria applied to confirm the identity of a suspected illicit drug were that the retention time of the analyte in the sample could not vary more than ±1% respect to the retention time of the same analyte in standard solution, and two SRM ion precursor → ion product transitions for each analyte should be monitored. Extracted ion chromatograms (EIC) of the illicit drugs identified in the SPE extract of the water sample W8, using the quantification ion precursor → ion product transition, are presented in Fig. 6.2. Six compounds −COC, BECG, KET, MOR, MDMA and MAMP− were identified "a priori" but only the identity of five was confirmed by a second transition.

2.5 Quality control

A calibration curve (between 1 and 1,000 ng l^{-1}) was obtained using the SSs to avoid matrix effects and to ensure an adequate quantification. From those analytes without isotopically labeled analogues, quantification was made using most similar structurally chemical-deuterated compounds. Calibration curve gave correlation coefficient (r^2) higher than 0.99.

The method detection and quantification limits (MDLs and MQLs) for the target illicit drugs were calculated based on the standard deviation of the analytical results from seven parallel blank matrix samples spiked with low concentration of target standards. The analytical procedure of MDLs and MQLs was identical to the sample analysis included in the "Sample Preparation" section. The MDLs and MQLs were from 0.01 to 1.54 ng l^{-1} and from 0.03 to 5.13 ng l^{-1}, respectively. Recoveries were 57–120% from water spiked at the limits of quantification (LOQ) and 60–125% from that spiked at 50 ng l^{-1}. The RSDs were lower than 20%. The detailed results are shown in Table 6.2. These results indicated that the method was appropriate for this study.

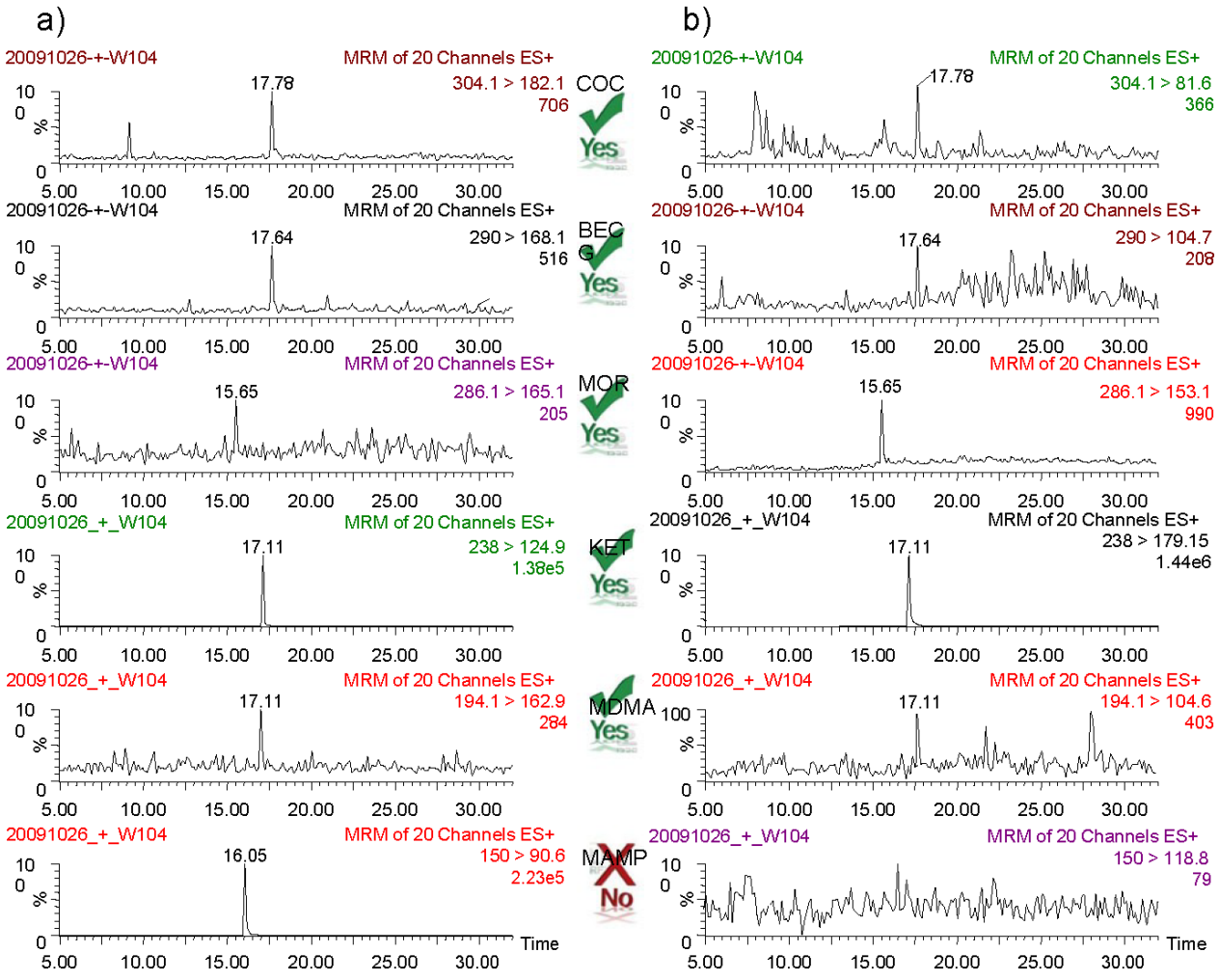


Fig. 6.2 EIC of the of the illicit drugs identified in the SPE extract of the water sample W8 (a) quantification ion precursor → ion product transition and (b) confirmation ion precursor → ion product transitions

3 Results and discussion

The concentration of illicit drugs in the Pego-Oliva Marsh and the data summary are presented in Table 6.3. All the samples analyzed were contaminated by illicit drugs. Of the studied compounds, HER, ECGME, MDA, AMP, MAMP and THC were not detected. The most occurring compounds were KET (69.5% of the samples), MDMA (74%), BECG (74%) and its precursor COC (87%). MOR (39% of the samples), MET (39%), 6-ACMOR (9%) and THC-COOH (4%) were also present. Our data were also compared with the previous studies available about some river and water bodies in Spain and other countries (Table 6.4).

The levels of COC and of its main metabolite BEGC, the most frequent illicit drugs, were up to 11.6 and 15.5 ng l^{-1}, respectively. The mean value was 2.25 ng l^{-1} for COC and 1.92 ng l^{-1} for BEGC. As can be observed comparing Tables 6.3 and 6.4, the mean level of COC in the present study was lower than those observed by other authors in surface
waters of different rivers of Europe (Boleda et al. 2007; Boleda et al. 2009; Bones et al. 2007; Zuccato et al. 2008), except for Ebro river in Spain (Postigo et al. 2010). BECG was also at lower levels than those reported in other studies (Baker and Kasprzyk-Hordern 2011; Boleda et al. 2007; Gheorghe et al. 2008; Lin et al. 2010; Postigo et al. 2010; Zuccato et al. 2008), except those performed by Bones et al. (2007) who did not find BECG in any sample and Lin et al. (2010) who determined levels below 1.6 ng l^{-1}. Pharmacokinetic studies carried out in human urine show that only 1–9% of the cocaine consumed is excreted unchanged, while 45% is excreted as BECG and 40% as ECGME (Postigo et al. 2010). Based on these values, the excreted COC/BECG ratio should range from 0.02 to 0.27 (Boleda et al. 2007). In our study, 11 of the 16 samples contained more COC than BECG. This finding is supported by earlier studies (Baker and Kasprzyk-Hordern 2011; Boleda et al. 2007; Boleda et al. 2009; Bones et al. 2007; Gheorghe et al. 2008; Lin et al. 2010; Postigo et al. 2010; Vazquez-Roig et al. 2010; Zuccato et al. 2008) that cover different usage patterns and incidence of cocaine abuse—from 0.1% in Taiwan, in the population aged 15–65 years, to 2.6% in England or 3.0% in Spain. This discrepancy between theory and results might indicate either a faster degradation route for BECG in certain environmental conditions or the direct spillage of the drug into the marsh. Bones et al. (2007) also speculate that this fact could be related to losses during the sampling filtering step. As BECG exists in the water solutions (pH 7–7.8) as a neutral zwitterionic, whereas COC is presented in cationic form, there may be a bias resulting from sorption onto particulates. ECGME, the other important human metabolite of COC, was not detected in the Pego-Oliva Marsh water. The absence of ECGME has also been reported in Belgium (Gheorghe et al. 2008) and Spain (Vazquez-Roig et al. 2010). A probably explanation is that ECGME rapidly degrades to ecgonine, which was not monitored.

Table 6.2 Limits of detection and quantification, and relative recovery (to ISs) of the SPE and LC–MS/MS method to determine the target drugs of abuse

Compound	LOD (ng l^{-1})	LOQ (ng l^{-1})	Recovery (%±SD)	
			At LOQ (n=15)	At 50 ng l^{-1} (n=15)
6-ACMOR	0.09	0.30	71±18	85±14
AMP	0.12	0.40	92±12	96±10
BECG	0.05	0.15	99±15	100±9
COC	0.02	0.06	118±17	121±12
ECGME	0.41	1.37	91±13	96±10
HER	0.05	0.17	100±15	100±12
KET	0.10	0.35	92±19	95±15
MAMP	0.22	0.75	117±14	113±11
MDA	0.41	1.37	104±16	105±10
MDMA	0.10	0.35	120±12	125±8
MET	0.01	0.03	103±15	100±9
MOR	0.04	0.13	75±16	75±10
THC	1.22	4.07	57±14	60±11
THC-COOH	1.54	5.13	64±18	67±13

LOD limits of detection, LOQ limits of quantification, 6-ACMOR 6-acethylmorphine, AMP amphetamine, BECG benzoylecgonine, COC cocaine, ECGME ecgonine methyl ester, HER heroin, KET ketamine, MAMP metamphetamine, MDA 3,4-Methylenedioxyamphetamine, MDMA 3,4-Methylenedioxymethamphetamine, MET methadone, MOR morphine, THC Δ9-tetrahydrocannabinol, THC-COOH nor-9-carboxy-tetrahydrocannabinol

Table 6.3 Presence of the studied drugs of abuse in water samples of Pego-Oliva Marsh

	MDMA	KET	MOR	MET	6-ACMOR	BECG	COC	THC-COOH
W1	0.33	N.D.	3.1	2.20	N.D.	9.1	3.2	N.D.
W2	1.07	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
W5	1.26	12.56	<LOQ	<LOQ	N.D.	2.6	7.1	N.D.
W7	1.15	9.51	N.D.	0.80	N.D.	N.D.	5.3	N.D.
W8	2.22	6.42	N.D.	N.D.	N.D.	N.D.	5.4	N.D.
W9	0.12	N.D.	N.D.	N.D.	N.D.	0.16	0.35	N.D.
W32	N.D.	12.79	N.D.	N.D.	N.D.	N.D.	0.42	N.D.
W33	<LOQ	1.96	N.D.	<LOQ	N.D.	0.32	0.38	N.D.
W34	0.30	N.D.	0.3	N.D.	N.D.	0.11	0.06	N.D.
W35	N.D.	0.89	2.0	N.D.	0.4	0.07	0.08	N.D.
W36	N.D.	0.12	N.D.	N.D.	N.D.	0.31	0.28	N.D.
W37	N.D.	0.15	N.D.	N.D.	N.D.	0.51	0.20	N.D.
W38	N.D.	1.08	N.D.	N.D.	N.D.	0.41	0.51	N.D.
W45	0.83	N.D.	N.D	N.D.	N.D.	N.D.	N.D.	N.D.
W101	<LOQ	414.92	6.8	<LOQ	N.D.	4.2	4.6	N.D.
W102	0.43	0.13	8.3	0.50	N.D.	N.D.	2.7	N.D.
W103	1.42	4.90	N.D.	<LOQ	N.D.	3.3	3.8	N.D.
W104	0.38	12.27	2.6	N.D.	N.D.	1.6	2.4	N.D.
W105	0.25	11.83	0.5	N.D.	N.D.	15.5	2.3	N.D.
W106	0.80	0.15	6.3	1.2	0.6	3.2	11.6	N.D.
W107	0.11	0.16	N.D.	N.D.	N.D.	0.18	0.21	N.D.
W108	N.D.	0.53	N.D.	N.D.	N.D.	0.41	0.76	N.D.
W109	3.40	0.21	N.D.	2.7	N.D.	2.23	N.D.	1.54
Mean value[a]	0.62	21.33	1.30	0.32	0.04	1.92	2.25	0.07
Max value	3.40	414.92	8.3	2.7	0.6	15.5	11.6	1.54

LOQ limits of quantification, N.D. non-detected MDMA 3,4-Methylenedioxymethamphetamine, KET ketamine, MOR morphine, MET methadone, 6-ACMOR 6-acethylmorphine, BECG benzoylecgonine, COC cocaine, THC-COOH nor-9-carboxy-tetrahydrocannabinol

[a] Calculated considering N.D. as zero and <LOQ as at LOD (see Table 6.2)

The new group of illicit drugs, which includes MDMA (or ecstasy) and KET, was frequently detected. The former was in 74% of analyzed samples at concentrations ranging from N.D. to 3.40 μg l^{-1}. The mean value of 0.62 ng l^{-1} was similar to that observed in the rivers of Spain and Italy (Postigo et al. 2010; Zuccato et al. 2008) and much lower than that reported in the UK rivers (Baker and Kasprzyk-Hordern 2011; Zuccato et al. 2008) and in L'Albufera wetland (Valencia, Spain) (Vazquez-Roig et al. 2010). The MDMA has analogues such as MDA and is chemically related to the amphetamines (mainly AMP and MAMP). However, the surface water of Spain, Italy, UK, Taiwan and Nebraska reportedly had important levels of MAMP, AMP and MDA (Baker and Kasprzyk-Hordern 2011; Boleda et al. 2007; Castiglioni et al. 2008; Vazquez-Roig et al. 2010; Zuccato et al. 2008).

On the contrary, levels of KET were between 0.12 and 414.92 ng l^{-1}, with a mean of 21.3 ng l^{-1}. Most of the studies carried out did not give details on this substance because it is a new illicit drug (Baker and Kasprzyk-Hordern 2011; Bones et al. 2007; Gheorghe et al. 2008; Postigo et al. 2010; Vazquez-Roig et al. 2010; Zuccato et al. 2008). The frequency, mean and maximum concentration of KET in the Pego-Oliva Marsh were higher than those reported in Spain and Taiwan (Boleda et al. 2007; Lin et al. 2010). In Spain, Huerta-Fontela et al. (2008) reported that the levels of this drug were always below the LOQ in the Llobregat River. However, in Taiwan, KET was detected in significant quantities (until 341 ng l^{-1}) in river water (Lin et al. 2010). KET is also used in human medicine and very often in veterinary medicine for its anaesthetic and analgesic effects on cats, dogs, rabbits, rats and other animals, which could be a reason for contamination in this agricultural area.

Among the opiates, MET and MOR were the most frequent. MET is the main clinical substitute for HER addicts. It was in nine samples at concentrations between N.D. and 2.7 ng l^{-1} (mean level 0.32 ng l^{-1}), which is lower than those reported in other studies (Baker and Kasprzyk-Hordern 2011; Boleda et al. 2009; Vazquez-Roig et al. 2010; Zuccato et al. 2008). MOR showed levels in the range of N.D. and

Table 6.4 Illicit drugs concentrations in other water systems (in nanograms per liter)

	Spain						Italy		UK				Taiwan		Belgium		Ireland		USA	
	Ebro River (n=28)		Llobregat River (n=16)		L'Albufera Natural Park (n=16)		Po, Lambro, Olona and Arno Rivers (n=10)		Thames River (n=5)		Major River (n=6)		Dahan and Sindian Rivers (n=11)		Grote Molenbeek, Demmer and Senette Rivers (n=3)		Boyne, Boadmeadow and Liffey rivers (n=3)		Missouri River (n=1)	
	Mean	C_{max}	Mean	C_{max}	Mean	C_{max}	Mean	C_{max}	Mean	C_{max}	Mean	C_{max}	Mean	C_{max}	Mean	C_{max}	Mean	C_{max}	Mean	C_{max}
6-ACMOR	–	–	N.D.[a]	N.D.[a]	N.D.	N.D.	<LOQ	<LOQ	<LOQ	<LOQ	–	–	N.D.	N.D.	–	–	–	–		
AMP	6.8	12.1	<LOQ[b]	<LOQ[b]	0.21	3.38	<LOQ	<LOQ	<LOQ	<LOQ	3.3	4.3	–	–	–	–	–	–	N.D.	N.D.
BECG	11.4	346	77[b]	111[b]	5.59	78.71	64.6	183	11.2	17	26.8	52.5	NA	1.6	96	191	N.D.	N.D.	–	–
COC	1.4	59.2	6[b]	10[b]	0.02	4.43	16.2	44	2.8	6	6.0	14.0	NA	0.7	15.3	26	29	33	–	–
ECGME	–	–	–	–	0.08	1.35	–	–	–	–			–	–	N.D.	N.D	–	–	–	–
HER	N.D.	N.D.	N.D.[a]	N.D.[a]	N.D.	N.D.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KET	–	–	N.D.[b]	N.D.[b]	–	–	–	–	–	–	21.3	51.0	50	341	–	–	N.D.	N.D.	–	–
MAMP	0.4	0.7	NA	NA	N.D.	N.D.	0.9	2.1	<LOQ	<LOQ	–	–	56	405	–	–	–	–	–	–
MDA	–	–	1.8[c]	4[c]	N.D.	N.D.	0.8	1.5	2.5	4	–	–	–	–	–	–	–	–	–	–
MDMA	1.0	11.8	3.7[c]	8[c]	0.75	2.48	0.8	1.7	3.6	6	8.7	24.8	–	–	–	–	N.D.	N.D	NA	62.6
MET	–	–	2.4[a]	12.0[a]	0.75	0.84	3.3	10.1	–	–	10.0	18.4	–	–	–	–	N.D.	N.D.	–	–
MOR	9.8	10.9	4.1[a]	7.0[a]	0.14	11.0	5.5	38	12.7	42	35.8	35.8	N.D.	108	–	–	N.D.	N.D.	–	–
THC	–	–	N.D.[a]	N.D.[a]	N.D.	N.D.	–	–	–	–			–	–	–	–	–	–	–	–
THC-COOH	5.5	5.5	29.9[a]	79.5[a]	<LOQ	<LOQ	0.7	3.7	0.4	1			–	–	–	–	–	–	–	–
Ref	(Postigo et al. 2010)		(Boleda et al. 2009; Huerta-Fontela et al. 2007; Huerta-Fontela et al. 2008)		(Vazquez-Roig et al. 2010)		(Zuccato et al. 2008)		(Zuccato et al. 2008)		(Baker and Kasprzyk-Hordern 2011)		(Lin et al. 2010)		(Gheorghe et al. 2008)		(Bones et al. 2007)		(Bartelt-Hunt et al. 2009)	

LOQ limits of quantification, 6-ACMOR 6-acethylmorphine, AMP amphetamine, BECG benzoylecgonine, COC cocaine, ECGME ecgonine methyl ester, HER heroin, KET ketamine, MAMP metamphetamine, MDA 3,4-Methylenedioxyamphetamine, MET methadone, MOR morphine, THC Δ9-tetrahydrocannabinol, THC-COOH nor-9-carboxy-tetrahydrocannabinol, N.D. not detected, NA data not available—not target compound in the study

[a] Data from Boleda et al. (2009)

[b] Data from Huerta-Fontela et al. (2007)

[c] Data from Huerta-Fontela et al. (2008)

978 Environ Sci Pollut Res (2012) 19:971–982

8.3 ng l^{-1} (mean 1.30 ng l^{-1}). Previous studies reported similar results (Postigo et al. 2008; Vazquez-Roig et al. 2010). MOR levels in surface waters can be attributed to its medical use or to the illicit use of HER. Only in two samples, MOR coexists with 6-ACM, a specific minority metabolite of HER in humans. However, it should also be taken into account that 6-ACMOR underwent further cleavage to MOR, which could explain better the low concentrations and frequency of 6-ACMOR compared to those of MOR than to assume the medical prescription as main source of MOR. The little frequency, or even the absence, of 6-ACM and HER has been described in several studies (Baker and Kasprzyk-Hordern 2011; Boleda et al. 2007; Boleda et al. 2009; Lin et al. 2010; Postigo et al. 2010; Vazquez-Roig et al. 2010; Zuccato et al. 2008).

The THC, principal active substance of Cannabis sativa, was not detected in any sample. This is in accordance with other studies (Boleda et al. 2009; Postigo et al. 2010; Zuccato et al. 2008) and explainable because this substance is almost completely metabolized in the human organism and is only present at traces in urine. THC-COOH, its main metabolite, was found in only one sample, at the level of 1.54 ng l^{-1}. The lower frequency of this compound compared to other illicit drugs was already noted in the Lugano, Maggiore and Varese Lakes and in the River Po in Italy (Zuccato et al. 2008). This substance presents worst recovery due to its high apolarity, which could explain the low number of positive samples.

The spatial distribution of the illicit drugs between the different points taken at the Pego-Oliva Marsh has also been studied. The cumulative results obtained are shown in Fig. 6.3. The observed contamination pattern of most illicit drugs and their metabolites is comparable to those reported in similar monitoring studies carried out in Spain (Boleda et al. 2007; Postigo et al. 2010; Vazquez-Roig et al. 2010), UK (Baker and Kasprzyk-Hordern 2011; Zuccato et al. 2008), Ireland (Bones et al. 2007), Belgium (Gheorghe et al. 2008), Taiwan (Lin et al. 2010) and Nebraska (Bartelt-Hunt et al. 2009). However, this pattern differs in the absence of amphetamine-like compounds other than MDMA. Although several towns and housing states are relatively close to this humid area, it is difficult to establish the main sources of the illicit drugs or to propose accurate back-calculations of their use at community levels. That is because the marsh receives waters from different sources including the subterranean springs and the Gallinera, Molinell, Bullent and Racons rivers, which could bring contaminated waters from long distances. Concentrations of illicit drugs determined in surface water can also be greatly affected by natural transformations (photolysis, hydrolysis, biodegradation, etc.) that decrease their levels.

The most contaminated sampling sites varied depending on the drug of abuse considered. Figure 6.4 shows the distribution of the drug of abuse in the different sampling sites. MDMA shows the maximum concentration at sampling site W109, which is located in the middle of the natural park, followed by W8, W103 and W5, which are nearest to the coast and to the area covered by hotels and discotheques. The highest concentration of KET is at the point W101. The concentration at this point is 414 ng l^{-1}, highest than any other concentration reported before. It should be mentioned that the distribution of KET is different compared with the others, which could indicate that its utilization is not only related with their use as illicit drug but also with its use as veterinary anaesthesic in livestock exploitations.

The opioids showed up at point W106, which is a source of morphine, 6-ACMOR and MET, are in the limits of the natural park and they appeared also at high concentration in the area of the reed beds. However, these opioids did not appear in the points nearest to the coast W8, W5 and W9. The cocainics appeared mainly in the points W105 and W106, which are also in the external limit of the marsh, followed by the points near the coast and located in the marsh, which also showed important concentrations.

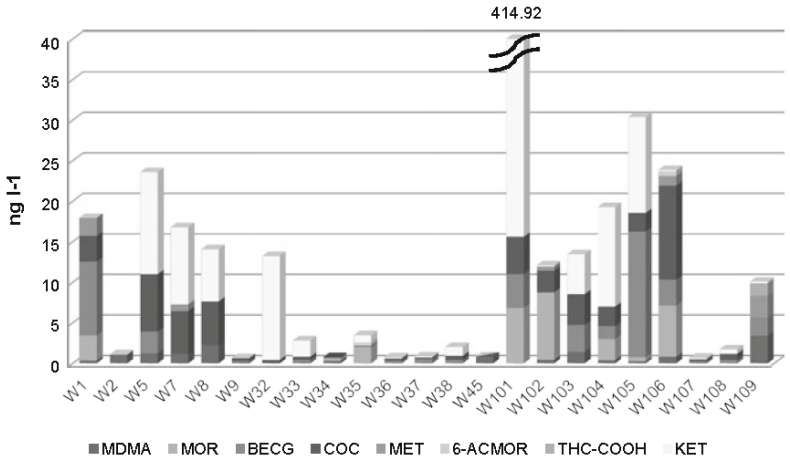


Fig. 6.3 Cumulative levels (in nanograms per liter) of illicit drugs and metabolites in surface waters of the Pego-Oliva Marsh

Environ Sci Pollut Res (2012) 19:971–982

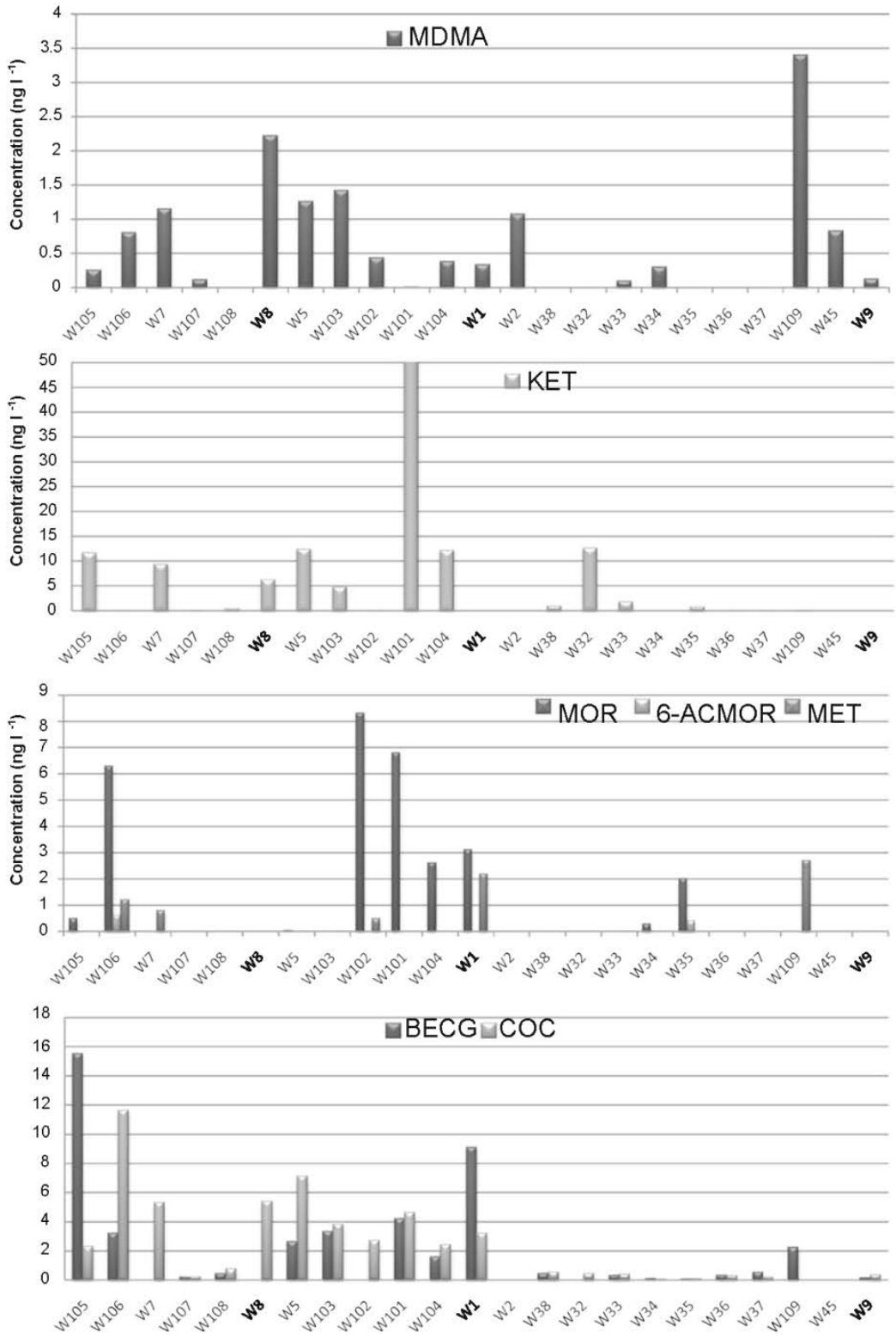


Fig. 6.4 Distribution of the illicit drugs in the different sampling points, distinguished into the different chemical classes

Information on acute and, especially chronic, toxicity of illicit drug to aquatic organisms is very scarce. To our knowledge, there are no published aquatic ecotoxicological data for illicit drugs hence the use of in silico tools to predict toxicity is a pragmatic option. ECOSAR modelling provides acute predicted non-effect environmental concentration ($PNEC_{ECO\text{-}SAR}$) data that ranges from 2.26 μg l^{-1} for methamphetamine to 9.83 μg l^{-1} for heroin but with a very high degree of uncertainty (Grung et al. 2007; Madden et al. 2009). Illicit drug concentrations measured in surface waters are generally well below the $PNEC_{ECO\text{-}SAR}$. Consequently, they would not be able to cause acute toxicity to aquatic organisms. However, illicit drugs enter the aquatic environment continuously leading to fairly constant environmental water concentrations (Grung et al. 2007). Chronic exposure to illicit drugs has the potential for numerous subtle effects, such as metabolic or reproductive changes on non-target organisms (Riehl et al. 2011; Sedore Willard et al. 2006; Stewart et al. 2011; Strehler et al. 2008). Furthermore, the potential for synergistic or additive toxicity to aquatic organisms and/or other toxic effects, not yet studied, cannot be ruled out.

4 Conclusions

Results from the monitoring performed confirmed the presence of illicit drugs. Significant levels, but lower in general than those previously reported in the literature, were found for the amphetamine-like compounds. Within the group of cocainics, COC and its metabolite BECG were confirmed to be present. MOR, 6-ACMOR and MET were detected sporadically. High levels and frequency of KET were observed. The locations with higher levels depend on the drug considered, but apparently those nearest to the coast present higher concentrations.

This study confirms the presence of some of the target compounds in concentrations that were well below to those considered sufficient to cause acute toxicity effects in aquatic organisms. However, considering the lack of knowledge on chronic and cumulative toxic effects, these concentrations could lead to a potential risk to the environment and human health. Further studies on the risk of these compounds should be undertaken.

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CHAPTER 7

Pharmaceuticals in Pego-Oliva Marsh: distribution and risk assessment

Scientific publication 7:

Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego-Oliva Marshlands (Valencia, eastern Spain)

P. Vazquez-Roig, V. Andreu, C. Blasco, Y. Picó

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Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego–Oliva Marshlands (Valencia, eastern Spain)

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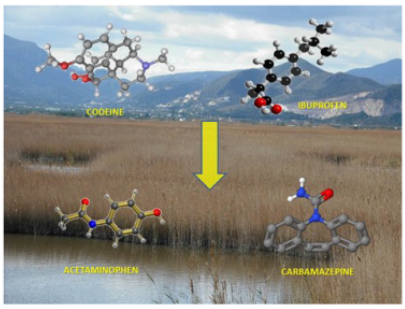
HIGHLIGHTS

- We studied the occurrence of 17 pharmaceuticals in water, sediments and soils of a Mediterranean wetland.
- The 100% of waters, 94% of sediments and 96 % of agricultural soils were polluted.
- The most frequent pharmaceuticals were codeine and ibuprofen in water and carbamazepine and acetaminophen in soil/sediment.
- Diffusion of codeine and fluoroquinolones to deeper soil horizons, was observed.
- Risk for the lower trophic levels and fishes was established.

GRAPHICAL ABSTRACT



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abstract

This study is focused on the occurrence of 17 pharmaceuticals in waters (34 samples), sediments (16 samples) and soils (23 samples, at two different depths) in a typical Mediterranean coastal wetland (Pego–Oliva marsh, Spain). Soil and sediment samples were extracted by pressurized liquid extraction (PLE). Aqueous extracts from PLE and water samples were concentrated by solid-phase extraction (SPE) and determined by liquid-chromatography tandem mass spectrometry (LC–MS/MS). Pharmaceuticals were detected in concentrations up to 112 ng/L in water samples, up to 15.1 ng/g sediments and up to 8.4 ng/g in soil. In surface waters, ibuprofen and codeine were the compounds more frequently detected (up to 59 ng/L and 63 ng/L, respectively). Ground and tap water samples analyzed were also contaminated with pharmaceuticals. The 94% of sediments and the 80% of agricultural soils were polluted (mostly by carbamazepine and acetaminophen). Diffusion of codeine and fluoroquinolones to deeper soil horizons was observed. Possible relationships between variables were established by Pearson correlations and principal components analysis (PCA). An environmental risk assessment based on the available long-term data was performed. Results showed actual risk for the lowest trophic level, and for fishes, due to the presence of fluoroquinolones and ibuprofen. Nevertheless, the presence of pharmaceuticals in the environment is not limited only to an ecological problem since contamination also affects drinking water, being a potential risk to human health.

1. Introduction

The presence of pharmaceutical residues in different environmental compartments is a growing problem of unexpected consequences, i.e. appearance of resistant bacteria as occurring in the E. coli crisis in Germany during 2011 or the decline of vulture population in India due to the bioaccumulation of diclofenac taken from carcasses of

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dead livestock (Ginebreda et al., 2010; Ratola et al., in press). In recent years, the number of pharmaceuticals detected in the environment has increased spectacularly, reaching a broad number of consumed drugs and including virtually all the existing therapeutic classes (Guillén et al., 2012-this issue). Several studies already report the contamination in effluents of wastewater treatment plants (WWTPs) (Gracia-Lor et al., 2011; Gros et al., 2006; López-Serna et al., 2012; Ratola et al., in press) and in rivers and lakes around the world (Madureira et al., 2010). However, much fewer have been addressed to evaluate the status of wetland ecosystems (Camacho-Muñoz et al., 2010; Vazquez-Roig et al., 2011), as well as to the simultaneous investigation of these compounds in water, sediments and soils (Jelic et al., 2009; Vazquez-Roig et al., 2011). The present study will contribute to the knowledge of the state and the conservation of protected wetlands and to understand the distribution of these compounds between water and solid matter, which is largely unknown.

Wetlands are one of the richest environmental areas that may include temporary marshes, lakes, reservoirs, rivers, deltas and lagoons; they are everywhere, under all climates and in every country (except in the poles). The UNEP-World Conservation Monitoring Centre has estimated that wetlands cover approximately 570,000,000 ha – roughly 6% of the Earth's land surface (Mitsch and Gosselink, 2012). In the Mediterranean, they support high concentrations of birds, mammals, reptiles, amphibians, fishes and invertebrate species, many of which are endemic to the region. They also act in controlling floods and flows, in the recharge of aquifers, in carbon sequestration, etc. Furthermore, their ecosystems assure directly the needs of mil- lions of people (i.e. rice farming areas of South East Asia, Italy, Spain, etc.). So people benefit not only from the direct resources of wetlands but also from the multiple functions and services they offer daily. Despite its importance and necessity of protection, as remarked by the Water Framework Directive (European Union, 2000), loss of water quality and extension of the wetlands are growing issues. When they are affected by human activities, particularly by those that reduce water availability and quality, their capacity to deliver ecosystem services is diminished; and this has direct and indirect effects on human health, including loss of food production, loss of livelihoods and the emergence of toxic effects (Anonymous, 2008).

In the Valencian Community region (Spain), the Pego-Oliva marsh is a typical Mediterranean coastal wetland declared as a Natural Park and included in the RAMSAR Convention (Anonymous, 2008). Unlike the L'Albufera of Valencia assessed in our previous study (Vazquez-Roig et al., 2011), this wetland, located at 70 km to South of the other, has suffered much less anthropic pressure since it is surrounded by a much smaller population (39 thousand in front of 1.5 million people of L'Albufera). The Pego-Oliva marsh stands out due to the quantity and quality of its natural freshwater supply. These characteristics make it an area of extremely rare habitats whose preservation is essential. The most important point to study pharmaceuticals in agricultural and marsh areas is the increasing use of the effluents of wastewater treatment plants for supplying the ecological flow in the wetland and for irrigation of farm areas. Since data regarding emerging contamination of Spanish aquatic systems are still scarce, it is necessary to set up surveys at different scales including these protected areas. To assess the current status of the Pego-Oliva marsh related to emerging contaminants derived from anthropic pressure, seventeen pharmaceuticals were determined in waters, soils and sediments. Pharmaceuticals were selected to cover a wide variety of therapeutic classes (β-blocker, antidepressants, anti-epileptics, analgesics, non-steroidal anti-inflammatories, lipid regulators and eight antibacterials belonging to different families), and including also those that could show ecotoxicity or long persistence in the environment.

In addition, the ecological risk associated with the occurrence of most of the pharmaceuticals in the environment is not sufficiently described and could be crucial in these natural protected areas, which have been established as one of the most useful tools for preserving large pools of biodiversity. The European Medicines Agency (EMEA), following the European Directive 2001/83/EC, has elaborated a guideline for the environmental risk assessment (ERA) of new and existing medicinal products in the environment (De Lange et al., 2006), which is based on the comparison between the predicted environmental concentrations (PEC) and the predicted no effect concentrations (PNEC) estimated from standard toxicity assays. Several studies have tested this approach in waste and surface waters describing its advantages and disadvantages (Ginebreda et al., 2010; Gros et al., 2010; Guillén et al., 2012-this issue; Hernando et al., 2006; Verlicchi et al., 2012). Our study also attempts to provide a preliminary risk assessment of the hazards that the concentrations of pharmaceuticals have detected in the aquatic environment of the Pego-Oliva marsh for the aquatic fauna.

A total of 34 water, 17 sediment and 23 soil sampling points were monitored covering the different environmental and land use (agricultural, redbird, etc.) characteristics of this wetland. We sought to understand the incoming sources, distribution and fate of these contaminants in the Pego-Oliva marsh, to determine several patterns behind the spatial distribution of these compounds and to assess the risk for the aquatic fauna on the basis of available long-term data.

2. Experimental

2.1. Sampling site

Pego-Oliva marsh is a Natural Park located in eastern Spain. It covers an area of about 1250 ha, situated in the border of two municipalities, Oliva and Pego, which were inhabited by 39,322 people (in year 2008). It is a high biodiversity area and a key point in the route of migratory birds, because of this, it has been included in the Life program of the European Union.

Three rivers surround the marsh, Bullent in the North and Racons in the South, while Revuelta river goes from North to South connecting both. At the same time, there are hundreds of irrigation channels used for agricultural purposes. This area experiences a huge variability of the precipitation regimen, with periodic floods especially in spring and autumn, and an annual total precipitation about 900 mm (Cantora Uiza and Aboal Sanjur, 2012). The human pressure in the marsh has grown in the last years, with the construction of numerous residential areas and golf clubs in the border of the marsh and the surrounding ridges. In order to minimize the human impact, several WWTPs have been built in the limits of the marsh (see Supplemental Table S7.3). The biggest one (Pego II) handles the residual waters of Pego through an aerated basin system. It has a flow of 2067 m^3/day and offers service to 5662 inhabitants.

2.2. Sampling methods

Samples were taken in June 11, 2009 to establish spatial variations in pharmaceuticals with occurrence from different points, covering all the extension of the Pego-Oliva marsh according to the distribution shown in Fig. 7l. Sampling points were geolocated (UTM 30, D50) (Supplemental Table S7.4 shows the geolocation of each sampling point together with the type of samples taken). Thirty-four water samples (pHs 7.2-8.8) were taken in clean amber glass bottles (capacity 2.5 L) from the irrigation channels, typically at 30 cm depth, and filled up to the top to eliminate air bubbles. One spring water (ullal bullent, sample W1) and one tap water from a house (sample W35) were collected as well. Water sam- ples were filtered through a Whatman GF/F glass microfiber membrane filters of 0.7 μm. Physico-chemical analysis of the samples (detailed data not shown) revealed a high salinity of the waters, reaching values up to 9438 ppm of NaCl, with an average value of 3677 ppm. The measure of the total dissolved solids (total quantity of inorganic and organic sub- stances contained in the waters) shows values up to 6530 ppm (mean value was 2670 ppm).

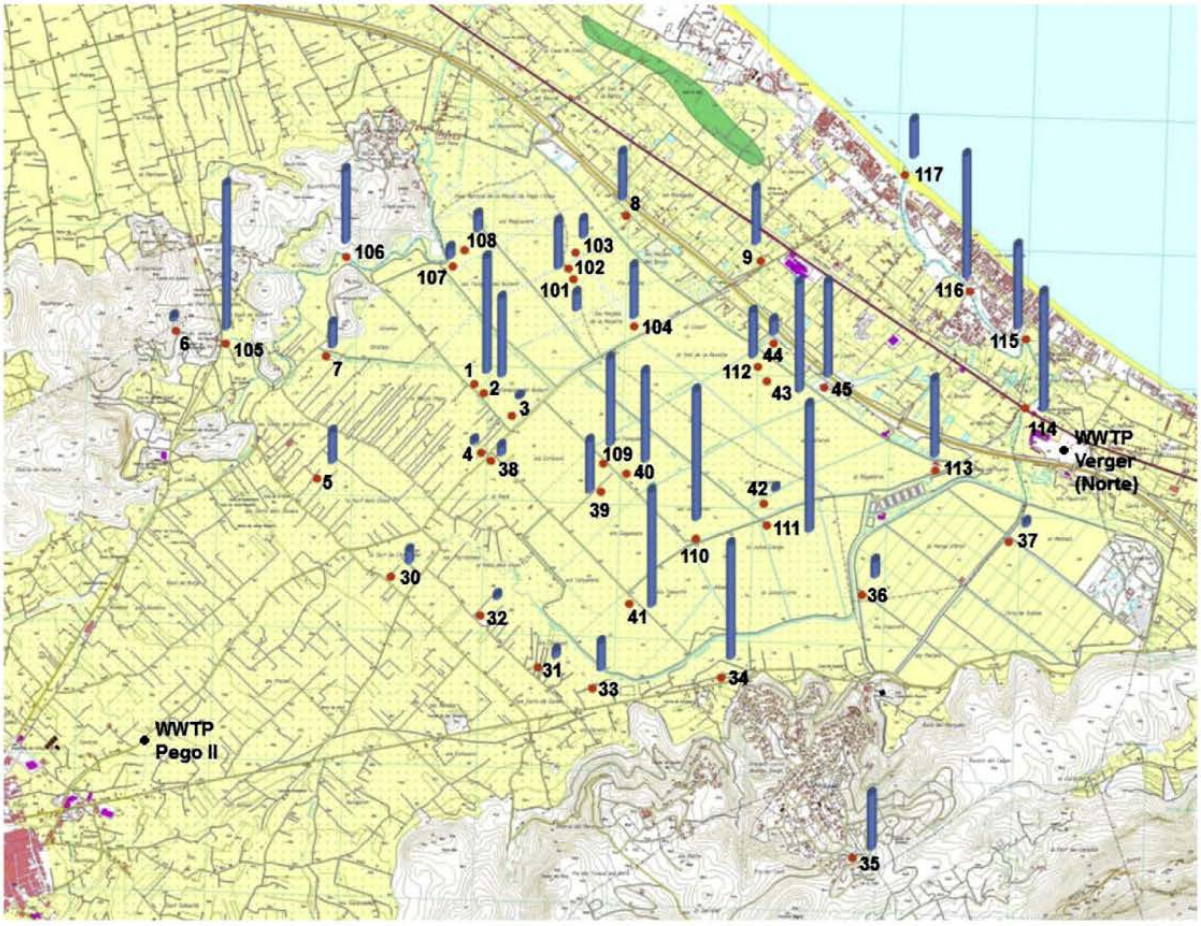

Fig. 7.1. Location of the sampling points and total concentrations of pharmaceuticals in each one of them.

Sixteen sediment samples were taken from irrigation channels and the marsh using a Van Veen grab sampler, and transferred to polypropylene bags (250 mL). Their textures were classified as sandy loamy. The sediments were taken approximately in the same place where the waters were sampled.

Soil samples of the upper 0–15 and 15–50 cm depth layers were collected. From each of the 23 sampling points (Fig. 7.1), of 16 m^2, dis- tributed at random, 5 sub-samples were taken. Once in the laborato- ry, samples were dried and passed through a 2 mm Ø sieve, and then, the sub-samples of each sampling point were homogenized to create a composite one. The soils of the area belong mainly to Calcic Fluvisols, Chromic Luvisols and Gleysols types. In general, all of them presented high carbonate content, and those taken in the marsh a high sodium absorption ratio. Mean value of organic matter content was 5.2%, with a maximum of 14.6% in sample 41B.

All samples were transported in hermetic boxes refrigerated with ice (at 4 °C) upon arrival at the laboratory and then, after the pre-treatment described below, stored in amber-polyethylene terephthalate (PET) bottles or sealed in plastic bags in the dark at −20 °C until analysis.

Waters were vacuum filtered through 0.7 μm glass fiber filters, followed by 0.45-μm nylon membrane filters (Analisis Vinicos, Tomelloso, Spain). Sediment samples were frozen, lyophilized (Hetosicc CD4, Birkerod, Denmark), pulverized, thoroughly mixed and then passed through a 2 mm Ø sieve. The process of lyophilization was carried out over 7 days for each sediment sample until the complete elimination of water. The composite soil samples were extended in a layer of approximately 1 cm thickness on polypropylene trays and air-dried in darkness at 20 °C to moisture content of approximately 3% water.

2.3. Chemicals and materials

HPLC grade acetonitrile and methanol, and reagent grade formic acid were purchased from Merck (Darmstadt, Germany). Reagent grade citric acid and disodium hydrogen phosphate (Na_2HPO_4) were from Scharlau (Ferosa, Barcelona, Spain). Ethylenediaminetetraacetic disodium salt dihydrate (Na_2-EDTA) and sea sand were from Panreac (Barcelona, Spain). Milli-Q water was prepared using a purification system (Millipore, Milford, MA, USA).

Acetaminophen (ACM), carbamazepine (CBZ), ciprofloxacin (CPX), clofibric acid (CFA), codeine (CDN), diazepam (DZM), diclofenac (DCF), fenofibrate (FNF), ibuprofen (IBP), metoprolol (MPL), norfloxacin (NFX), ofloxacin (OFX), oxytetracycline (OTY), propanolol (PRL), sulfamethoxazole (SMZ), tetracycline (TCY) and trimethoprim (TMP) were of analytical grade (purity >97%) and provided by Sigma–Aldrich (Steinheim, Germany). The epimers 4-epioxytetracycline (4-epiOTY) and 4-epitetracycline (4-epiTCY) hydrochloride were from Across Organics (Morris Plains, NJ, USA). Deuterated internal standards [ibuprofen-d_3 (IBP-d_3), acetaminophen-d_3 (ACM-d_3) and carbamazepine-d_2 (CBZ-d_2)] were from CDN isotopes (Quebec, Canada). (Table S7.5 of the Supplementary

material shows structures, therapeutic class and some properties of the studied pharmaceuticals, such as pKa, K_{ow} and D_{ow} at pHs 7 and 9).

Except for CPX, which was prepared at 500 mg/L in methanol, acidified with three drops of formic acid, all individual stock solutions were 1000 mg/L. They were stored at −18 °C in a freezer. Working standard mixtures were prepared each week by dilution of the stock solutions in methanol–water (25:75, v/v). A working mixed solution of labeled standards was prepared by dilution of individual stock solutions with methanol, and stored at −20 °C.

Hydrophilic–lipophilic-balanced reversed-phase sorbent cartridges (Oasis HLB 200 mg sorbent/6 mL from Waters Corp., Milford, MA, USA) and anion exchange cartridges (Isolute SAX 500 mg from Symta, Madrid, Spain) were used for SPE.

2.4. Analytical methods

The extraction of pharmaceuticals from water, based on solid-phase extraction (SPE), has been described elsewhere (Vazquez-Roig et al., 2011). Soil and sediment samples were extracted by pressurized liquid extraction (PLE) using a Dionex ASE200 according to a method previously developed in our laboratory (Vazquez-Roig et al., 2010). Once the aqueous extract was obtained from the ASE, clean-up was performed by SPE in the same way as for water samples, but placing an anionic exchange (SAX) cartridge on top of the HLB.

As described in our previous studies (Vazquez-Roig et al., 2010, 2011) pharmaceuticals were determined using an Alliance 2695 HPLC quaternary pump equipped with an autosampler (Waters, Milford, MA, USA), and a Micromass Quattro LC triple quadrupole mass spectrometer (Manchester, UK), working in both positive and negative ionization modes (see Supplementary material for analytical columns and mobile phases).

The injection volume and flow rate were set in both ionization modes at 20 μL and 0.2 mL min^{-1} respectively. Two selected reaction monitoring (SRM) transitions per compound as well as its ratio were utilized as confirmation criteria (Table S7.6 in the Supplemental mate- rial lists these transitions, and their respective cone voltages (CV) and collision energies (CE)).

2.5. Quality control and statistics

The method was validated according to the current standards (complete results of recoveries and detection limits are shown in Supplementary material Table S7.7). Solvent blanks containing metha- nol were prepared to run after every ten samples and after calibration curve for monitoring the instrumental background. One field and 10 laboratory blanks were analyzed for target compounds during the course of this study. Blank samples were derived from Milli-Q water and were used to determine whether sampling procedures, sampling equipment, field conditions, or sample shipment procedures intro- duced the target analytes to environmental samples (field blank) or to assess the potential for sample contamination in the laboratory (laboratory blanks). CBZ and SMZ were each detected at levels be- tween the limit of detection and of quantification in the field blank. Concentrations of pharmaceuticals in the samples were not corrected for the recovery because estimated recoveries are mostly in the range 70–120% (considered as quantitative) and they may vary among dif- ferent matrices and for different concentrations of analyte.

SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses including principal component analysis (PCA). Analysis of variance (ANOVA) and Tukey's test at $\alpha = 0.05$ were performed to detect differences in the pharmaceuticals concentrations between water, soil and sediments, and in its spatial distribution. In the cases where the homogeneity and/or normality of the data could not be assumed, the Kruskal–Wallis and Mann–Whitney non parametric test ($P \leq 0.05$) were applied.

Pearson statistical bivariate correlation analyses were applied, at 95% and 99% significance levels, between pharmaceuticals concentrations to determine possible relationships among them. When the values of a variable showed a non-normal distribution Spearman bivariate correlations were applied at the same significance levels.

Multiple stepwise regression analysis and categorical PCA were used to confirm the weight and dependence between variables, differences and identifying patterns in them and their distribution.

2.6. Risk assessment of pharmaceuticals in water

The risk assessment for the aquatic compartment has been performed according to the guidelines for the risk assessment of new and existing medicinal products in the environment (EMEA, 2012a), as well as its associated Technical Guidance Document elaborated by the EMEA (EMEA, 2012b). According to these documents, risk evaluation is performed calculating the risk quotient (RQ).

$$RQ = \frac{NEC}{PNEC}$$

PNEC is calculated by dividing the lowest short-term L(E)C50 or long-term NOEC (no-observed-effect-concentration) value available in the literature, by an assessment factor (AF). The AF is an arbitrary factor to consider the inherent uncertainty in the obtained laboratory toxicity data. If the ratio EC/PNEC is higher than one, an environmental risk could be suspected. To cover all food chain in the water, RQ is calculated at three different trophic levels of the ecosystem, algae, daphnids and fishes.

Since pharmaceuticals concentration in water is low, ecotoxicological long-term data are preferred to short-term data. However, due to the lack of long-term toxicological studies, a widespread approach is the use of data from short-term studies (EC50 or LC50) to calculate PNECs (Kim et al., 2007b; López-Serna et al., 2012; Vazquez-Roig et al., 2011).

3. Results and discussion

3.1. Levels of pharmaceuticals in the Pego–Oliva marsh

Table 7.1 outlines a summary of the maximum and mean concentra- tions found (the minimum was always below the limit of quantifica- tion), together with the frequency of each compound in the three matrices analyzed. Fig. 7.1 shows the total concentration of pharmaceu- ticals in the three matrices for each point. A complete list of the concentration of each pharmaceutical detected in each water, sediment and/or soil sample is shown in Supplemental material Tables S7.8, S7.9 and S7.10.

3.1.1. Water

All water samples contained at least one therapeutic drug (see Table S7.8 in the Supplementary information) and all the classes of therapeutic drugs were frequently found, except tetracyclines. The occurrence pattern showed the highest levels and frequency for anti-inflammatories and analgesics. IBP (74% of the samples) and CDN (59%) were the compounds more frequently detected, at concentrations between LOD and 59 ng/L (mean 16.3 ng/L) and LOD and 63 ng/L (mean 16.7 ng/L), respectively. Acetaminophen was also detected at high frequency (41% of the samples) and with the highest levels, up to 112 ng/L. In contrast, DCF was only found in 3% of the samples (even though its acidic character favors its water solubility). This behavior has been widely explained in the literature by its high environmental degradation rate. DCF is efficiently photolyzed in surface water showing a 96% of degradation over a two-week period (Brun et al., 2006; Guillén et al., 2012-this issue).

Table 7.1
Frequency, mean levels[a] and maximum values found in Pego–Oliva.

Compound	Waters			Sediment			Soil		
	Frequency (%)	Mean (SD) (ng/L)	Maximum (ng/L)	Frequency (%)	Mean (SD) (ng/g)	Maximum (ng/g)	Frequency (%)	Mean[b] (SD) (ng/g)	Maximum (ng/g)
ACM	41	7.4 (20.8)	112.2	87	2.4 (3.9)	15.1	91	0.9 (0.5)	1.8
CBZ	26	5.5 (10.9	38.8	100	0.9 (0.5)	1.7	39	0.3 (0.4)	1.5
CFA	3	0.5 (3.2)	18.4	–	<LOD	<LOD	–	<LOD	<LOD
CPX	3	1.0 (5.9)	34.6	20	1.1 (2.4)	7.3	9	0.3 (1.1)	4.6
CDN	59	16.7 (20.9)	62.7	20	0.4 (0.9)	3.6	17	0.1 (0.4)	1.7
DZM	32	1.6 (2.7)	8.6	33	0.3 (0.4)	1.2	13	0.02 (0.1)	0.3
DCF	3	0.5 (2.9)	16.9	–	<LOD	<LOD	–	<LOD	<LOD
FNF	32	4.1 (6.8)	21.4	7	1.0 (4.0)	16.1	–	<LOD	<LOD
IBP	74	16.3 (14.8)	59.0	–	<LOD	<LOD	–	<LOD	<LOD
MPL	32	3.2 (7.2)	39.3	–	<LOD	<LOD	17	0.1 (0.3)	0.3
NFX	26	8.0 (14.1)	37.2	7	0.4 (1.7)	6.8	44	1.7 (2.3)	8.4
OFX	38	11.6 (15.8)	50.2	13	0.1 (0.3)	2.7	52	1.4 (1.3)	3.3
OTY	–	<LOD	<LOD	–	<LOD	<LOD	–	<LOD	<LOD
PRL	35	1.6 (3.2)	16.6	7	0.1 (0.5)	2.1	4	0.02 (0.1)	0.4
SMZ	32	1.6 (3.8)	15.6	13	0.1 (0.3)	1.1	–	<LOD	<LOD
TCY	–	<LOD	<LOD	13	0.7 (2.0)	6.5	–	<LOD	<LOD
TMP	3	0.1 (0.5)	3.0	20	0.2 (0.5)	1.6	4	0.01 (0.03)	0.2

[a] To calculate mean, value of LOD was taken when values are <LOQ and values below LOD were considered as zeros.
[b] To calculated soil mean and maximum concentrations average values of horizons A and B were used.

The most detected antibiotics (up to 38% of the samples) were fluoroquinolones and sulphonamides. However, CFX and TMP were detected only in 3% of the samples. TCY and OTY were not present in any sample, which can be a consequence of their high tendency of binding to organic matter and metals (Gros et al., 2010; Vazquez-Roig et al., 2010).

Antidepressants (32% of samples), beta-blockers (up to 35%) and lipid regulators (up to 32%) were also frequently detected. On this last lipid regulators class, FNF was detected in 32% of the water samples whereas CFA, the clofibrate metabolite, was detected only in the 3%. This is unexpected because FNF has high $LogK_{ow}$ and is not ionizable, whereas CFA is negatively charged at water pH ($LogD_{ow}$ −0.98–−1.96). Both, FNF and CFA are fairly persistent in the environment. This difference may reflect the different usages of these lipid regulators, as well as their excretion and metabolization rates.

The concentrations detected in this marshland were lower than those found in Ebro river basin (Spain) (Gros et al., 2006), rivers in the Valencian Community (Spain) (Gracia-Lor et al., 2011), Lake Erie (USA) (Wu et al., 2009) and similar rivers in South Korea (Kim et al., 2007a). Data for comparison with other wetlands were only available for L'Albufera of Valencia (Valencia, Spain) (Vazquez-Roig et al., 2011) and the Doñana Park (Camacho-Muñoz et al., 2010). Both wetlands showed levels considerably higher than in Pego-Oliva marsh. This could be related with the size of the surrounding population. Pego-Oliva receives pharmaceuticals from the Pego II WWTP with a population served of 5600 inhabitants, while, for example L'Albufera de Valencia has contributions of pharmaceuticals mainly from Pinedo-2 WWTP with a population served of approximately 1 million inhabitants.

3.1.2. Sediment samples

To date, there is only little information about the occurrence, distribution, and effects of pharmaceuticals in sediments. In this study, all sediment samples, with the exception of the no. 45 were contaminated at levels higher than LODs. According to the equilibrium partition theory, hydrophobic chemicals are distributed between pore water and organic carbon of the sediment. Due to the polar and often ionic nature of pharmaceuticals, sorption to solid materials such as soil and sediment is not solely based on this hydrophobic partition. Rather, it is based on ionic interactions and is pH dependent and it may not be appropriate to assess their lipophilicity based only on the K_{ow} value but based on the pH-dependent n-octanol-water partition coefficient D_{ow} (Ratola et al., in press). Data on $LogK_{ow}$, $LogD_{ow}$ and pK_as are compiled in the Supplementary material (see Table S7.5).

Studying the tendency of the target compounds that remain in water or accumulate in sediments, three general tendencies could be extrapolated from the results. The presence of ACM, CBZ, CPX, TMP and TCY in a greater number of sediments than in waters (or even the absence in this latter) may indicate a tendency of this compound to be accumulated in sediments. CBZ was detected in 16 samples and ACM in 14. These compounds are not ionizable at the water pH but have low K_{ow} and relatively high water solubility. Their high prevalence was already reported in other studies (Guillén et al., 2012–this issue; López-Serna et al., 2012; Vazquez-Roig et al., 2011). A comprehensive assessment of these two compounds can only be made after elucidation the nature of sorption.

Fluoroquinolones, such as CPX, have two pKa values within pH range relevant to this study (7–9) and can be positively charged, negatively charged, zwitterionic, or uncharged (Aristilde and Sposito, 2010). Within environmentally relevant pH ranges, fluoroquinolones exist mostly as zwitterions which favor their hydrophobicity. However, interactions with other compounds can be greatly pH dependent, and fluoroquinolones complex strongly with many different cations, influencing their solubility.

Tetracyclines have three pKa values (see Supplementary material Table S7.5). Although at pH between 7 and 8 they will be negatively charged in water, their sorption to sediment is expected because of their high capacity of complexing with metals that also displace the acidic-base equilibrium (Furlong et al., 2003).

Opposite to this behavior, IBP, MPL, OFX, NFX PRL, DCF, IBP, FNF, CDN DMZ and SMZ remain in waters. In fact CFA, DCF, IBP, and MPL were not present in any sediment sample. The behavior of CFA, DCF, and IBF can be explained because they are acidic pharmaceuticals present as anionic species at pHs 8–9 and of CDN, MPL, PRL, DMZ and SMZ because they are basic pharmaceuticals that will be positively charged at pHs 8–9. Therefore, sorption to the sludge is expected to be weak due to electrostatic repulsion from the charged functional groups in the sludge. Pharmaceuticals found in the sediments could remain mainly in the pore water and not necessarily be sorbed to sediments. However, several studies report substantially higher concentrations of these pharmaceuticals than in pore water suggesting that sediments are an important reservoir for these compounds (Furlong et al., 2003; Ratola et al., in press). Depending on the $LogD_{ow}$ for each particular pharmaceutical (related to the percentage of ionic or non-ionic molecule as well as to its $LogK_{ow}$), the degree of distribution

of these compounds between water and sediment varies but is always lower than in the first group (Ratola et al., in press).

For FNF, OFX and NFX no clear conclusion can be reached. They were not detected in sediments but they are expected to adsorb to suspended solids and sediments based upon the estimated D_{ow} or the zwitterionic state. In fact, the two fluoroquinolones, OFX and NFX, were widely detected in soil samples. Degradation and biodegradation could explain this anomalous behavior. The contact between pore water and sediments is an interface where many microorganisms can be responsible of biodegradation (Ginebreda et al., 2010). Further studies in this sense are strongly required.

3.1.3. Soil samples

Presence of pharmaceuticals in soils was widespread and diffuse, probably as a consequence of the irrigation of large extensions with contaminated water. Only in samples 3A, 7B, 9A, 31A, 32B, 43A and 43B, pharmaceuticals were not detected. ACM was in 76% of the positive samples, at concentrations between LOD and 3.5 ng/L. Fluoroquinolones were detected in 28 samples, confirming the reported tendency of these compounds to remain in soils, apparently absorbed by the clay-humic complexes, bounded with clays, or complexed with organic matter (Andreu et al., 2007; Aristilde and Sposito, 2010).

CFA, DCF, FNF, IBP and tetracyclines were not detected in any sample. IBP, CFA, and DCF are anionic and highly polar remaining in water, but the FNF and tetracyclines have high $LogK_{ow}$s and could expect a tendency to accumulate in soils. CFA, DCF and both tetracyclines were almost undetectable in water samples (see Supplemental Table S7.8). FNF was present in eleven samples, and its absence in soil samples only can be explained through effective degradation and transforma- tion in its main metabolite (fenofibric acid), which was not monitored in this study. The reason for this is that FNF may not volatilize from dry soil surfaces based upon its vapor pressure and is expected to ad- sorb to it because it is non-ionizable and non-polar ($logK_{ow}$ 5.19), which also suggests that FNF is expected to have slight mobility in soil.

Fig. 7.2. shows the mean concentration of pharmaceuticals in soils at different depths. ACM, CBZ, and MPL accumulate in the superficial layer of the soil, while CDN and fluoroquinolones show a tendency to infiltrate to deeper layers. At soil pH (7-8), neutral and positively charge species will be in equilibrium for both types of compounds. Consequently, some of these substances could leach to ground water, spreading the contamination. The different behaviors of both groups of pharmaceuticals between horizons A and B are not signifi- cant from a statistical point of view, mostly because we did not find enough number of contaminated samples to obtain statistical signifi- cance. The standard deviation showed in Fig. 7.2 is very high because the values showed are the mean values for each compound in soils.

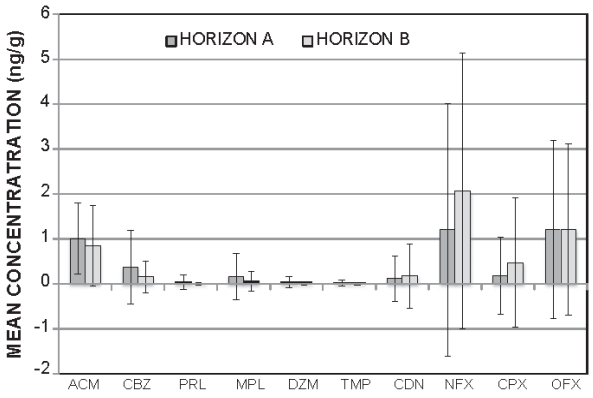

Fig. 7.2. Mean concentrations of pharmaceuticals in soils at different depths: layers A (b15 cm) and B (15–50 cm).

3.2. Spatial distribution

An irregular distribution of the pharmaceuticals can be observed in the map of Fig. 7.1 where the total concentration (sum of concentra- tions of water, soil and sediment) in each point is marked with a ver- tical bar. The total concentration between two nearby sampling points is very dependent of its conditions. Different infiltration rates in the sandy river-bed or an extra contribution or bifurcation of the channel could dilute or concentrate pharmaceuticals (changing con- centration levels) of one point related to the closer one. Because of the large number of small channels in the marsh, looking for a regular pattern in the distribution of contaminants is an arduous job.

Pego-Oliva marsh is a Natural Park conceived to keep a local sustainable economy. It is divided in two areas with different characteristics: a zone of lagoons (western part of the map of Fig. 7.1) and the rice fields. Sewage waters from nearby WWTP's are reutilized to irrigate the fields, as well as to maintain the ecological flow of Racons river. Two pumping stations avoid that waters from the lakes (reedbirds) would be mixed with those from irrigation channels (Diputacion Provincial de Alicante, 1992).

Lagoon area presents low concentrations of pharmaceuticals (sampling points 4, 5, 30-33 and 38). Higher concentrations were detected in the rice fields (central sampling points) and in the area of the Racons basin (points 111 and 113-117), but with irregular levels. Highest concentration in point 114 could be due to a discharge of the WWTP El verger in the Racons river, since this WWTP is obsolete and its effluents flow just before this sampling point (Diario informacion, 2009). Decrease in the level of point 117 might be due to a dilution with the sea water that enters in the channel.

In the northern part (points 7, 8, 101-103, 107 and 108), an area of lower concentrations could be observed. This is an agricultural area devoted to citrus orchards and rice crops. The presence of several "ullals" (spring waters), close to the Bullent river, would explain this anomaly for dilution of the drugs present in the surface waters. Sample 105 is influenced by the surrounding houses without any observable dilution effect from spring waters. In this residential area, the influence of human impact could be also observed in water from point 106. On the contrary, sample number 6, located at the top of the hill, showed markedly lower concentrations, probably because it receives less runoff water than lower parts. The presence of these anthropogenic compounds is clearly related with wastewater reuse to irrigate the crops and also with the direct disposal from residential areas close to the marsh.

In sample 35 (tap water), CDN and IBP were detected, and in sample 1 (spring water) these pharmaceuticals were also found, together with ACM and SMZ. The co-occurrence of anionic surfactants can increase the mobility of some pharmaceuticals (even those not ionizable), and depending on the pH some pharmaceuticals can be as ionic species (Cleuvers, 2003) facilitating the movement from surface to ground water, with the possibility of reaching drinking water.

3.3. Statistical analysis

The statistical analysis was mainly performed to establish whether there is a common pattern in the spatial distribution of pharmaceuticals in water, sediments and soils or whether the presence of pharmaceuticals is related to land uses.

Correlation analyses applied showed that IBP presented inverse highly significant correlations with CBZ, NFX, OFX, FNF, PRL, MPL, SMZ and DZM. CFA, DCF, CFX. TMP did not present significant correlations with the other pharmaceuticals in the analyzed water samples. IBP, NFX and OFX showed spatial dependence, and also CDN and FNF but less significant.

In the case of sediments, it has been observed that NFX, FNF, OFX and PRL showed highly significant correlations ($P \leq 0.01\%$) between them. DZM showed significant correlations ($P \leq 0.05$) with FNF,

propranolol and NFX. CFX and OFX have an inverse but significant correlation. In addition, only CFX showed spatial dependence.

In the surficial soil samples, propranolol, DZM and MPL have highly significant correlations ($P \leq 0.01$) between them, and carbamazepine and NFX show significant correlations ($P \leq 0.05$). Only MPL present a certain spatial dependence.

For sub surface samples, scarce significant correlations were observed and only appearing between CBZ and ACM. This last one showed spatial dependence at $P \leq 0.05\%$.

Categorical Principal Component Analysis (PCA) was applied to the data obtained from water, soil and sediment samples for helping to evaluate the effect of location in the study area, land uses (citrus, rice, natural zone, urban or anthropized areas) and, in the case of soils, sample depth, as grouping factors between variables. In all cases, the non-normal distribution of data variables and the high number of null or zero values (non detected pharmaceutical in the sample) have reduced the significance of this analysis, mainly in the case of the sediments and the soil sub-surface horizon where no valid results were obtained. Therefore, in the case of water samples the results only explain 55.38% of the variance. For soils, only in the surface horizons the results have certain validity, explaining 65.40% of variance.

In this way, the main effect covering the major percentage of variance was the spatial location that was always related to Dimension 1, reaching the 43.36% for waters and 44.08% in soils (Fig. 7.3). The influ- ence of land uses and/or soil horizon (Dimension 2) showed scarce effect on variables. The grouping of variables observed is confirmed by the correlation analyses and the multiple regression models obtained for the studied environmental compartments.

In the case of waters, two clear behavioral groups were observed, the first one influenced by the spatial distribution in the studied area (Dimension 1), includes OFX, FNF, PRL, MPL, DZM and SMZ; carbamazepine could be also included. The second group, more related to Dimension 2, includes acetaminophen, CDN, NFX and TMP. An extremely different behavior is observed for IBP, which is far from both groups. In this sense, the majority of pharmaceuticals, and even their highest values appear in the east of the studied area, close to the WWTP or to the urban areas near the sea. The second group of pharmaceuticals is mainly concentrated in the anthropized areas of the north-west.

PCA analysis of the soil surficial samples shows clearly two main groups, similar to that observed in water samples, one with propranolol, DZM, OFX and MPL that is related to their spatial distribution, and the second one, with low significance, includes ACM and TMP

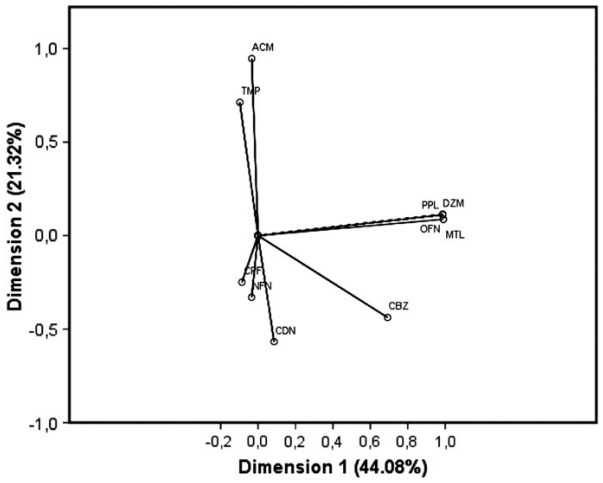

Fig. 7.3. PCA analysis with the data from the samples of the soil superficial layer.

more affected by the land use of the sampling area (Dimension 2). CDN, CFX and NFX present lower negative values in this last dimension. CBZ is not associated with any group, showing a high negative value in Dimension 1. For sub-surface soil samples and sediments PCA analysis was not significant, as explained previously.

3.4. Risk assessment of the presence of pharmaceuticals in waters

In order to perform a more realistic evaluation of hazards in the Pego–Oliva marsh, long-term data were utilized always that possible to carry out the risk assessment, with both standard and non-standard organisms. Highest concentrations of pharmaceuticals in the water samples (to set in the worst-case scenario), PNEC values (together assessment factors used) and risk quotients deemed for each analyte are shown in Table 7.2. According to these results, three drugs have a RQ higher than 1. Quinolones, CPX and OFX, showed RQ in algae of 6.9 and 3.1 respectively and 0.98 for NFX. IBP showed a RQ of 1.2 in fishes. Six week exposure assay to low concentrations of IBP resulted in changes in the pattern of reproduction of Japanese Medaka fishes (Flippin et al., 2007). Less frequent reproduction but with a higher rate of fertilized eggs was observed. In fact, IBP and other non-steroidal anti-inflammatory drugs are known to inhibit ovulation in mammals, including humans (Hernando et al., 2006).

A certain risk could be expected for those substances with a RQ between 0.1 and 1. In this group are the antibiotics SMZ, which shows risk for bacteria, ACM for the daphnid group, and DCF and PRL for fishes. In the study conducted by Hoeger et al. (2005) structural damages in organs and a reduction in the number of red blood cells were observed in trouts after 21 days of exposure to DCF, as was previously related in other experiments (Schwaiger et al., 2004). In a 4-week PRL exposure, the total number of eggs produced by Medaka fishes, and the number of viable eggs that hatched were decreased at concentrations as low as 0.5 μg/L (Huggett et al., 2002).

This risk evaluation has its limitations, such as the lack of long-term toxicological studies and the unfeasibility to carry out chronic studies during the lifespan of the organisms (especially in fishes), but on the other hand since mixture of compounds with the same pharmacological mechanism is present in waters, synergistic effects could be expected, being the real hazard greater than that calculated.

4. Conclusions

All studied pharmaceuticals except OTY were detected in waters, soils and sediments. Affinity for one or other matrix is compound dependent, varying its concentration in the function of population consumption, kinetic of degradation and physico-chemical properties. The presence of pharmaceutical compounds in surface waters of the Pego–Oliva marsh indicates the continuous discharge of these substances from the WWTPs, due to their incomplete removing efficiency for these compounds. Moreover contamination by pharmaceuticals in this coastal wetland does not only affect surface waters but also ground and tap waters. Sediment samples were contaminated with drugs detected in waters, highlighting their behavior like reservoir of hydrophobic compounds. Soils irrigated with contaminated waters have shown the presence of pharmaceuticals previously detected in surface waters. Some of these are not degraded or are retained in the rich organic matter layer of the soil and seeped into the deeper horizons, reaching potentially the ground waters.

Due to the high complexity of the Pego–Oliva marshland, general tendencies of the performance of pharmaceuticals in the environment could be envisaged but to increase its evidence more studies are required.

Hazard assessment for the aquatic environment revealed a high risk for fishes derived from the presence of IBP and fluoroquinolones. However, due to the limitations in the risk assessment, a case by case

Table 7.2
Maximum environmental concentrations (MEC) of pharmaceuticals in waters. PNEC and RQ for fish, daphnids (all species belonging to their trophic level) and algae (or bacteria) for the studied pharmaceuticals.

	MEC (ng/L)	PNEC algae (μg/L)	RQ algae	PNEC daphnid (μg/L)	RQ daphnids	PNEC fishes (μg/L)	RQ fishes
ACM	112.19	134[a,b] (Henschel et al., 1997)	0.001	0.35[a,b] (Calleja et al., 1994)	0.321	160[a,b] (Kim et al., 2007b)	0.001
CBZ	38.8	10.41[c] (Harada et al., 2008)	0.004	0.5[c] (Ferrari et al., 2004)	0.078	75.13[a,b] (Laville et al., 2004)	0.001
CPX	34.63	0.005[a,b] (Halling-Sorensen et al., 2000)	6.926	991[a] (Sanderson et al., 2003)	0.000	246,000[a] (Sanderson et al., 2003)	0.0000001[d]
CFA	18.38	1.5[c] (Ferrari et al., 2003)	0.012	2[b,e] (Ferrari et al., 2003)	0.009	86[a,b] (Henschel et al., 1997)	0.0002
CDN	62.66	238[a,b] (Ferrari et al., 2003)	0.0003	16[a] (Sanderson et al., 2003)	0.004	238[a] (Sanderson et al., 2003)	0.0003[d]
DZM	8.59	16.5[a,b] (Nunes et al., 2005)	0.001	0.48[a,b] (Calleja et al., 1994)	0.018	28[a] (Sanderson et al., 2003)	0.0003[d]
DCF	16.94	300[c] (Ferrari et al., 2003)	0.0001	100[f] (Ferrari et al., 2003)	0.0002	0.05[f] (Hoeger et al., 2005)	0.3
FNF	21.43	62.4[c] (Isidori et al., 2007)	0.027	0.78[c] (Isidori et al., 2007)	0.027	3.25[a,b] (Laville et al., 2004)	0.007
IBP	59.02	1[f] (Brun et al., 2006)	0.0001	1000[f] (Hayashi et al., 2008)	0.0001	0.05[f] (Flippin et al., 2007)	1.2
MPL	39.25	7.3[a,b] (Cleuvers, 2003)	0.005	8.8[a,b] (Huggett et al., 2002)	0.004	31[a,b] (van den Brandhof and Montforts, 2010)	0.001
NFX	37.16	0.038[a,b] (Ando et al., 2007)	0.978	1449[a] (Sanderson et al., 2003)	0.00003	14,000[a] (Sanderson et al., 2003)	0.000003[d]
OFX	50.19	0.016[a,b] (Ferrari et al., 2004)	3.137	3.13[a,b] (Isidori et al., 2005)	0.016	160[e] (Ferrari et al., 2004)	0.0003
PRL	16.64	9.4[f] (Ferrari et al., 2004)	0.002	0.9[f] (Ferrari et al., 2004)	0.018	0.025[f] (Huggett et al., 2002)	0.666
SMZ	15.58	0.0268[a,b] (Ferrari et al., 2004)	0.581	0.21[a,b] (Isidori et al., 2005)	0.074	27.35[a,b] (Laville et al., 2004)	0.001
TMP	2.97	11[a,b] (Ando et al., 2007)	0.0003	54.8[a,b] (Park and Choi, 2008)	0.0001	100[a,b] (Kim et al., 2007b)	0.00003

[a] AF=1000.
[b] Short-term data.
[c] AF=50.
[d] EC_{50} was estimated with ECOSAR.
[e] AF=100.
[f] AF=10.

review of the pharmaceuticals toxicity must be carried out to avoid unnecessary alarm. Other important and unknown issues would be to predict the impact in the human health of the regular consumption of traces of drugs in the daily drinking water.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2012.08.036.

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Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego–Oliva Marshlands (Valencia, eastern Spain)

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Supplementary material

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Table S7.3. Name, location and characteristics of the WWTPs close to Pego-Oliva Marsh.

	Pego II	Oliva-Nova (sector 2c, 2d)	Verger (Norte)	Oliva (Camping San Fernando)
Flow (m^3/day)	2067	250	200	234
Population	5662	873	160	879
2nd treatment	Aerated lagoons[a,b]	Aerated lagoons[b]	Aerated lagoons	Aerated lagoons[b]
3rd treatment	-	chlorination	-	-
Coordinates UTM 30 (ETRS 89)	X:751635 Y: 4304076	X:755633 Y: 4308371	X:758503 Y: 4306345	X:756148 Y: 4308561

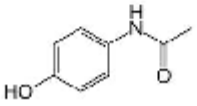
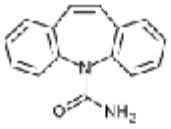
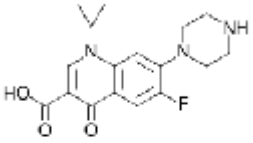
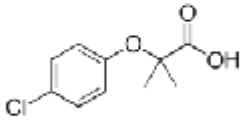
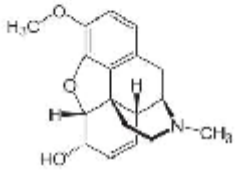
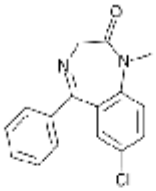
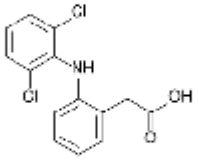
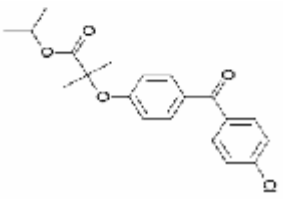
[a] Nitrogen removal
[b] Phosphorous removal

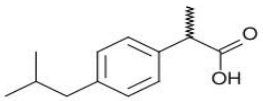
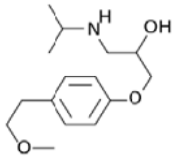
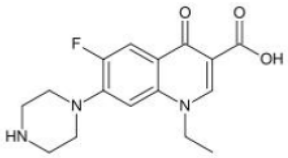
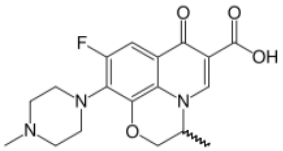
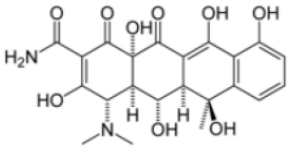
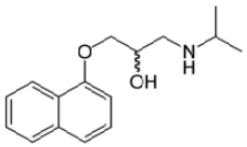
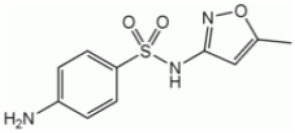
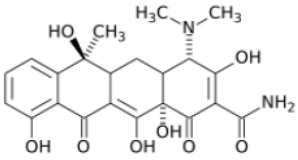
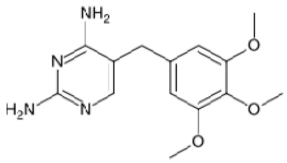
Table S7.4. Types of samples taken in each point with their georeferences (UTM 30, D50).

Sampling point	Coord X	Coord Y	Water	Sediment	Soil	
					Depth A (cm)	Depth B (cm)
1	753935	4306819	X	X	<15	
2	753934	4306817	X	X	<15	>15
3	754197	4306647			<12	>12
4	753982	4306335			<15	
5	752699	4306105	X		<12	>12
6	751666	4307115			<18	>18
7	752791	4307066	X	X	<15	>15
8	755291	4307954	X		<10	>10
9	755996	4307837	X	X	<15	>15
30	753308	4305395			<15	>15
31	754430	4304794			<15	>15
32	754086	4305048	X	X	<15	>15
33	754850	4304615	X		<15	>15
34	755827	4304742	X		<15	>15
35	756815	4303431	X			
36	756825	4305403	X	X	<15	>15
37	757899	4305855	X	X	<15	>15
38	754032	4306349	X	X	<15	>15
39	754660	4305951	X	X	<10	>10
41	755106	4305282	X		<12	>12
42	756076	4305953			<20	>20
43	756125	4306920	X	X	<15	>15
44	756082	4307302		X	<15	>15
45	756548	4306943	X	X	<15	>15
101	754629	4307644	X			
102	754572	4307755	X			
103	754638	4307876	X			
104	755050	4307399	X			
105	752027	4307086	X			
106	752953	4307739	X			
107	753728	4307715	X	X		
108	753738	4307740	X			
109	754706	4306003	X			
110	755548	4305717	X	X		
111	756060	4305905	X	X		
112	756122	4306992	X	X		
113	757438	4306340	X			
114	758005	4306801	X			
115	757818	4307352	X			
116	757561	4307678	X			
117	757053	4308431	X			

Table S7.5. Therapeutical class, chemical structure and relevant physicochemical properties of the studied compounds, including pKa, log K_{ow} and log D_{ow} at pHs 7 and 8. The D_{ow} was calculated according to the formula:

$$D_{ow} = \frac{k_{ow}}{1 + 10^{pH-pKa\ (pKb)}}$$

Compound	Therapeutical class	Structure	pKa	log K_{ow}	Log D_{ow} pH =7	Log D_{ow} pH=8
ACM	analgesic		9.38	0.49	-1.89	-0.04
CBZ	anticonvulsant		13.9	2.45	2.45	2.45
CPX	antibiotic		5.9/8.9	0.28	-0.85	-2.82
CFA	lipid regulator		3.46	2.58	-0.96	-2.96
CDN	analgesic		8.2	1.52	1.49	0.66
DZM	antidepressant		3.3	2.8	-0.90	2.80
DCF	analgesic		4.15	4.51	1.66	-0.34
FNF	lipid regulator		No ionizable groups	5.19		

IBP	anti-inflammatory		4.5	3.97	1.47	-0.53
MPL	β-blocker		9.7	1.88	1.88	1.80
NFX	antibiotic		6.22/8.51	-1.0	-1.85	-3.78
OFX	antibiotic		6.05/8.22	-0.4	-1.40	-3.35
OTY	antibiotic		3.2/7.5/8.9	-1.3		
PRL	β-blocker		9.5	3.48	0.98	2.86
SMZ	antibiotic		5.7	0.89	0.87	0.89
TCY	antibiotic		3.3/7.8/9.6	-1.2		
TMP	antibiotic		6.6	0.91	0.36	0.91

Analytical methods

Extraction of pharmaceuticals from water was based on solid-phase extraction (SPE) has been described elsewhere [Gros et al. 2006, Kasprzyk-Hordern et al. 2007, Gomez et al. 2006, Jelic et al. 2009, Jacobsen et al. 2004, Andreu et al. 2009, Loffler et al. 2003, Pérez-Carrera et al. 2010, Vazquez-Roig et al. 2010]. Briefly, 250 mL water sample spiked with deuterated interanal standard standards was passed through an HLB cartridge, then, the cartridge was rinsed with Milli-Q water and dried under vacuum for 15 min. Analytes were eluted with methanol, and the extract was evaporated to dryness and reconstituted in 1 mL of methanol/water (25:75, v/v), vortexed and filtered using a PTFE syringe filter of 0.2 μm. Vials were stored at -18 ºC until analysis.

Soil and sediment samples were extracted by pressurized liquid extraction (PLE) using a Dionex ASE200 according to a method previously developed in our laboratory [Vazquez-Roig et al. 2010]. For that, the sediment or soil was spiked with surrogate labelled standards and mixed with EDTA-washed sea sand. The mixture was placed into 22 mL extraction cells, heated to 90 ºC for three 7 min cycles using water as solvent. Once the aqueous extract was obtained from the ASE, clean-up was performed by SPE in the same way as for water samples, but placing an anionic exchange (SAX) cartridge on top of the HLB.

As described in our previous study [Vazquez-Roig et al. 2010] pharmaceuticals were determined using an Alliance 2695 HPLC quaternary pump equipped with an autosampler (Waters, Milford, MA, USA), and a Micromass Quattro LC triple–quadrupole mass spectrometer (Manchester, UK), working in both positive and negative ionization modes.

In positive mode a Sunfire C_{18} column (4.6 mm×150 mm, 3.5 µm, from Waters) and a Gemini C_{18} guard cartridge (4 mm×2 mm, Phenomenex) were used. Gradient elution was from 20% of eluent A (formic acid 0.1% in methanol) and 80% of eluent B (formic acid 0.1% in mQ water) to 90% of eluent A in 15 min, then increased to 98% A in 15 min, hold for 8 min and back to the initial conditions in 1 min, followed by 11 min of equilibration time.

In negative ion mode, a Luna C_{18} (2) column 100 Å (2 mm×150 mm, 3 µm, from Phenomenex) and the same guard cartridge as in positive mode were used. Gradient elution required three mobile phases: A (acetonitrile/methanol, 60:40, v/v), B (ammonium acetate 10 mM in water) and C (acetonitrile). Initial column conditions were 15% of eluent C and 85% of B. Then, 15% of A and 85% B increasing linearly until 98% of A in 5min, then held for 7min. At that point, in 3 min mobile phases returned linearly to the initial conditions (15% of C and 85% B) and held for 15 min.

Table S7.6. Conditions of MS/MS in positive and negative ion mode.

Compound	T_r (min.)	CV[a] (eV)	Quantification transition[b]	CE[c] (eV)	Confirmation transition[b]	[c]CE (eV)
PI mode						
ACM	16.4	25	152 → 110	15	152 → 92.5	25
ACM-d3	16.4	20	155 → 111	15	155 → 92.5	20
CBZ	25.9	30	237 → 193	35	237 → 192	40
CBZ-d2	25.9	35	239 → 195	20	239 → 194	30
CFX	14.5	30	332 → 314	20	332 → 231	35
CDN	7.4	35	300 → 215	25	300 → 199	30
DZM	28.9	40	285 → 154	25	285 → 193	30
FNF	36.2	25	361 → 233	15	361 → 139	30
MPL	15.2	30	268 → 116	20	268 → 98	20
NFX	14.4	30	320 → 276	15	320 → 302	20
OFX	13.8	30	362 → 318	20	362 → 261	25
OTY	15.7	25	461 → 426	20	461 → 443	10
PRL	18.2	30	260 → 116	18	260 → 183	20
SMZ	20.0	25	254 → 92	25	254 → 156	15
TCY	15.0	24	445 → 410	20	445 → 427	15
TMP	11.8	40	291 → 123	25	291 → 230	25
NI mode						
CFA	8.0	20	213 → 127	18	213 → 84.5	10
DCF	9.6	20	294 → 250	15	294 → 214	25
IBP	10.2	15	205 → 161	10	-	-
IBP-d3	10.2	15	208 → 164	10	208 → 162	15

[a]CV = cone voltage, [b]Transition= precursor ion → product ion, [c]CE = collision energy

Method validation

SPE was used for quantitative extraction of pharmaceuticals from waters with recoveries ranging from 82% to 99 %. Recoveries for soils and sediments, using PLE and SPE, were 71%–119% for all compounds with the exception of FNF and DCF which were of 60-70%.

The limits of detection and quantification of the method were determined on the basis of a signal-to-noise ratio of 3 and 10 respectively.

The precision (intra and inter-day) of the method was determined by the repeated analysis of soil and sediment samples spiked at concentrations of 50 ng/g and calculated as the relative standard deviation (RSD, %) of the measurements in quintuplicate. The linearity was evaluated via eight-points matrix matched calibration curves from 0.1 to 500 μg/L in the final extract, which were constructed spiking a standard solution in blank extracts of waters, soils or sediments. Each concentration level was spiked with three deuterated internal standards. Calibration curves showed correlation coefficients > 0.99, and were injected before and after each batch analysis. The matrix effect was also studied by comparing the response of matrix matched standards to that of standards prepared in methanol.

Table S7.7. LOD of the method (ng g^{-1}), relative recoveries (%) and RSD´s of various pharmaceuticals at three spiking levels.

Pharmaceuticals	Water		Sediment		Soil	
	LOD (ng/L)	Relative recovery (%)[a]	LOD (ng/g)	Relative recovery (%)[b]	LOD (ng/g)	Relative recovery (%)[c]
Positive mode						
OTY	9.4	85 ± 10	6.8	90 ± 10	5.3	96 ± 6
TCY	10	85 ± 11	5.9	88 ± 9	4.8	98 ± 5
OFX	8.1	92 ± 9	2.7	96 ± 8	2.9	100 ± 12
FNF	1.8	97 ± 8	0.6	66 ± 7	0.6	66 ± 6
CFX	12	94 ± 7	4.0	84 ± 13	4.1	86 ± 8
NFX	9.6	98 ± 8	5.2	74 ± 8	4.7	71 ± 10
CDN	1.2	99 ± 8	0.4	101 ± 4	0.3	108 ± 6
TNP	0.9	97 ± 8	0.3	104 ± 7	0.2	119 ± 4
DZM	0.3	99 ± 8	0.8	107 ± 12	0.1	104 ± 1
MPL	1.2	93 ± 8	0.4	92 ± 8	0.5	81 ± 9
PRL	0.6	94 ± 9	1.2	90 ± 11	0.4	96 ± 2
SMZ	0.9	95 ± 9	0.3	99 ± 4	0.6	97 ± 3
CBZ	0.6	99 ± 9	0.2	110 ± 5	0.1	104 ± 6
ACM	0.9	82 ± 12	0.3	98 ± 8	0.1	102 ± 9
Negative mode						
IBP	4.8	98 ± 7	1.6	85 ± 6	1.8	84 ± 4
CFA	1.5	85 ± 10	1.5	97 ± 6	0.3	102 ± 4
DCF	2.5	96 ± 11	1.0	66 ± 7	0.6	62 ± 6

[a] Recovery relative to internal standard, spiking level at 10LOQ
[b] Recovery relative to internal standard, spiking level at LOQ
[c] Recovery relative to internal standard, spiking level at LOQ

Table S7.8. Concentrations of drugs in water samples (ng/L).

	ACM	CBZ	CPX	CFA	CDN	DZM	DCF	FNF	IBP	MPL	NFX	OFX	OTY	PRL	SMZ	TCY	TMP
1	49.63				51.79				34.46						<LOQ		
2	20.43				<LOQ				27.99		29.96			16.64			
5	<LOQ								30.92								
7									27.19								
8									59.02								
9	<LOQ	19.28		18.38		6.05			<LOQ	6.69							
32																	
33									20.55								
34		38.80			<LOQ	2.59		12.49	16.64	6.46		26.37		3.31	15.58		
35					52.42				21.24								
36									21.60								
37									<LOQ								
38		31.53							<LOQ						14.89		
39	5.14	10.24			14.04			6.08	<LOQ		<LOQ	26.71		1.91			
41			34.63		25.42	6.95		17.14		6.85		47.40		2.51	3.46		
43	4.20				6.28	6.33		21.43	27.03	39.25		26.32		4.55	<LOQ		
45	7.02				36.65	3.63		6.02	19.37	6.62		26.81		2.03			
101									22.39								
102					42.41				19.16								
103									22.94								
104					50.22				18.13								
105	112.19				37.86				41.79								
106	16.27				40.68				39.06								
107									<LOQ								
108									21.61								
109	2.27	7.45			13.96	2.38		11.46		11.15	29.63	27.86		5.83	3.09		
110	5.16	24.40			21.87	5.32		17.09		7.85	37.16	27.40		6.13	2.99		
111	2.39	12.45			2.84	6.95		14.72	15.70	7.34	34.23	26.71		2.49	4.54		2.97
112									22.89			26.37					
113		8.29			13.11	1.72		6.49		3.81	37.14	28.09		1.99	<LOQ		
114	14.38				62.66						29.17	50.19					
115					9.68	4.70	16.94	8.01		4.73	32.45	27.37		1.93	2.82		
116		33.22			59.02	8.59		19.07		6.50		26.40		3.44	5.46		
117	9.38				6.87						32.25						

Table S7.9. Concentrations of drugs found in sediment samples (ng/g).

	ACM	CBZ	CPX	CFA	CDN	DZM	DCF	FNF	IBP	MPL	NFX	OFX	OTY	PRL	SMZ	TCY	TMP
1	1.59	0.36															
2	2.32	0.40				0.98									0.46		
7	<LOQ	1.69															
9	1.71	0.35			0.78												
32	<LOQ	1.57															
36	0.94	1.62															
37		1.52															
38	4.26	1.06				0.82											
39	0.87	1.44	5.62		1.48							<LOQ			1.07	6.48	
43	0.61	0.94														<LOQ	0.50
44	1.61	1.07			3.58	0.96											1.60
45																	
107	7.69	0.42															
110	15.07	0.52				<LOQ											
111		1.27	4.53			1.21		16.13			6.80	<LOQ		2.13			
112	1.94	0.89	7.25														0.92

Table S7.10. Quantification of the studied pharmaceuticals in soils (ng/g).

		ACM	CBZ	CPX	CFA	CDN	DZM	DCF	FNF	IBP	MPL	NFX	OFX	OTY	PRL	SMZ	TCY	TMP
1	**A**	1.63	2.57				0.50				2.27		6.29		0.77			
2	**A**	1.82	0.94															
	B	1.14											4.65					
3	**B**	1.36																
4	**A**			<LOQ														
5	**A**	1.78									0.81							
	B	<LOQ	0.96	5.08		<LOQ												
6	**A**	1.21					0.33											
	B					3.43												
7	**A**	1.55				0.43												
8	**A**	1.44																
	B		0.90				<LOQ											
9	**B**											8.05	4.91					
30	**A**		0.84								0.69	9.50	4.57					
	B		0.99															
31	**B**	1.24										7.39						
32	**A**	2.47																0.32
33	**A**	1.31																
	B	1.21										7.96	5.50					
34	**A**	1.26										8.15	4.21					
	B	1.15		4.93								4.77	3.72					
36	**A**	1.39																
	B	1.47																
37	**A**	1.54																
	B										0.90		3.05					
38	**A**		3.03			2.41							3.44					
	B	1.46																
39	**A**	1.30										5.28	3.10					
	B	1.53										6.33	3.06					
41	**A**	1.41											3.22					
	B	1.53											<LOQ					
42	**A**	1.65																
	B	1.46																
44	**A**		0.56															
	B	3.55										5.21						
45	**A**	1.38	0.69									4.81	3.15					
	B	1.56										4.99						

A: top 15 cm
B: 15-50 cm

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CHAPTER 8

Analysis of heavy metals in Pego-Oliva Marsh

Scientific publication 8:

Evaluation of the presence of pharmaceuticals and heavy metals in waters of a Mediterranean coastal wetland: Behavioral interrelations and the influence of the environment.

V. Andreu, E. Gimeno-García, J.A. Pascual, P. Vazquez-Roig, Y. Picó

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Title: Evaluation of the presence of pharmaceuticals and heavy metals in waters of a Mediterranean coastal wetland: Behavioral interrelations and the influence of the environment.

Article Type: Special Issue: River Basins

Keywords: Coastal wetland, heavy metals, pharmaceuticals, water, human pressure

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Abstract: The occurrence of 17 relevant pharmaceuticals and 7 heavy metals in waters of the Pego-Oliva Marsh Natural Park (Valencia Community, Spain) were monitored. This marsh has suffered continuous human pressures, such as marsh draining for agriculture, urbanization, etc. in the lasts decades. Thirty four sampling zones, covering the main land uses and water sources, were selected. Water samples were taken from the lagoon and from the most important irrigation channels. Thirty three of them were contaminated with at least one pharmaceutical. Ibuprofen and codeine were the pharmaceuticals more frequently detected, in concentrations between detection limit and a maximum of 59 ng/L and 63 ng/L, respectively.
Regarding metals, Zn showed values under the detection limit in all samples, Cd, Co, Cr, Cu, Ni and Pb appeared in concentrations lower than the WHO and EU maximum levels for drinking waters. Ni showed significant direct correlations with diazepam, norfloxacin, ofloxacin and fenofibrate, and inverse relationships with ibuprophene, at 99 and 95% of significance. Cu, Co and Cr also showed significant correlations with some of the pharmaceuticals. The influences of the water source, land uses and spatial distribution of both types of contaminants were also studied.

Evaluation of the presence of pharmaceuticals and heavy metals in waters of a Mediterranean coastal wetland: Behavioral interrelations and the influence of the environment.

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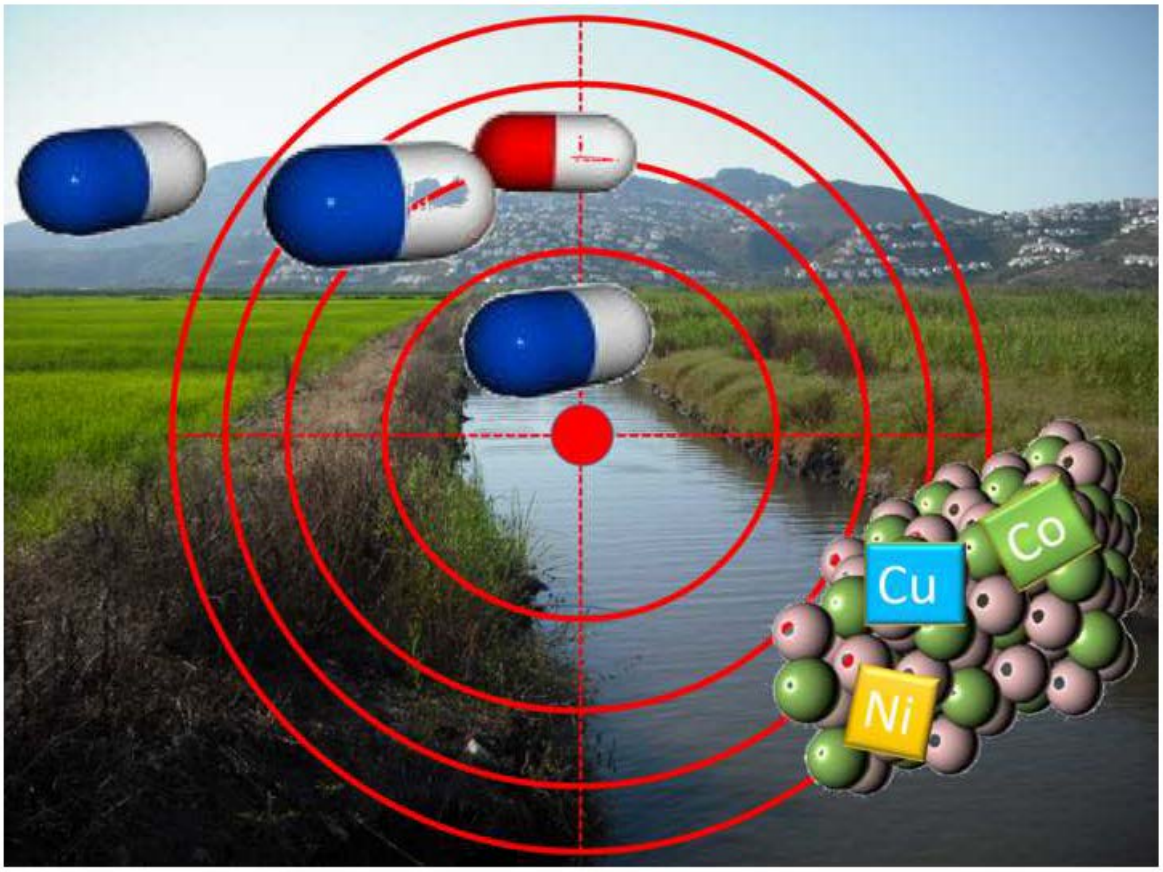
Number of words including text, figures, and table legends, but not references: **3589**

NOVELTY STATEMENT

In the last years the unexpected presence of new compounds, "emerging contaminants", in the environment has been considered as great concern. Among these compounds, pharmaceuticals are of special interest, reflecting the human fingerprint in the ecosystems.

Few studies about the presence of pharmaceuticals on coastal wetlands have been published, but even less on their interactions with other contaminants like heavy metals. This is the subject tackled by this work, which fits adequately in the scope of this Journal, showing one of the most relevant hazards for the environment, particularly for fauna, and even human health.


Co
Cu
Ni

HIGHLIGHTS

- 17 pharmaceuticals and 7 metals were studied in surface waters of a Mediterranean coastal wetland.

- Codeine, ibuprofen, and acetaminophen showed higher frequency of apparition.

- Waters from river and irrigation channels concentrate the highest values of pharmaceuticals.

- Differences according water sources and land uses were observed for all the compounds studied.

- Distance to the seashore affected the spatial distribution of pharmaceuticals and metals.

Evaluation of the presence of pharmaceuticals and heavy metals in waters of a Mediterranean coastal wetland: Behavioral interrelations and the influence of the environment.

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Abstract

The occurrence of 17 relevant pharmaceuticals and 7 heavy metals in waters of the Pego-Oliva Marsh Natural Park (Valencia Community, Spain) were monitored. This marsh has suffered continuous human pressures, such as marsh draining for agriculture, urbanization, etc. in the lasts decades. Thirty four sampling zones, covering the main land uses and water sources, were selected. Water samples were taken from the lagoon and from the most important irrigation channels. Thirty three of them were contaminated with at least one pharmaceutical. Ibuprofen and codeine were the pharmaceuticals more frequently detected, in concentrations between detection limit and a maximum of 59 ng/L and 63 ng/L, respectively.

Regarding metals, Zn showed values under the detection limit in all samples, Cd, Co, Cr, Cu, Ni and Pb appeared in concentrations lower than the WHO and EU maximum levels for drinking waters. Ni showed significant direct correlations with diazepam, norfloxacin, ofloxacin and fenofibrate, and inverse relationships with ibuprophene, at 99 and 95% of significance. Cu, Co and Cr also showed significant correlations with some of the pharmaceuticals. The influences of the water source, land uses and spatial distribution of both types of contaminants were also studied.

Keywords: Coastal wetland, heavy metals, pharmaceuticals, water, human pressure.

1. Introduction

Wetlands are amongst the Earth's most productive ecosystems. They provide a diverse array of important ecosystem services, are fundamental in the maintenance of the water cycle, and also act controlling floods and flows, in the recharge of aquifers, in carbon sequestration, etc. [1-3]. Furthermore, these areas are unique in biological diversity, constituting a great reserve of water for the planet and producing biomass and nutrients for the trophic chain [4-6].

Among these ecosystems, the coastal wetlands present the greatest dynamism and biodiversity. They are open structures, usually eutrophics and rich in nutrients [7], which show an intense relationship with the surrounding environment. In them, the rates of recycling and production of organic matter are very high. At the same time, these ecosystems shows a great fragility, being particularly sensitive to alterations in their water quality.

In the last 50 years, the increasing human pressure and the socio-economic development have caused serious damages to these ecosystems. Nowadays, the coastal strip

in most European countries is the area of most rapid growth concerning socio-economic development [8]. In particular, the Mediterranean coast of Spain, along with Ireland, has the fastest growing population in Europe, which increased up to 50 % in the past decade, with around 1.7 million houses [9,10]. This scenario has resulted in land use changes and the related hydrological alterations, with increasing problems in the management and treatment of very different type of residues (urban, agricultural, industrial, etc.).

Pharmaceuticals and heavy metals are important traces of human development in the different environmental media. The presence of pharmaceuticals has become an increasing problem for environmental sustainability, and also for human health, with consequences very scarcely known. Recent investigations show their global presence in surface waters from the low to the very low micrograms per liter concentration range [11-15]. Although these emerging contaminants have been detected in surface waters, it is not clear whether the sources are urban wastewater, animal feeding operations, industrial waste effluents, uncontrolled landfills, etc. Some pharmaceuticals have shown environmental toxicity to bacteria, algae, invertebrates, fish and other aquatic fauna [16,17].

In the same way, heavy metals can be considered as other aspect of the human fingerprint as well. Their effects on environmental and human health are well known, representing a constant threat to be controlled. However, their sources in the environment are also difficult to identify, as they could be a combination of natural and anthropogenic, both diffuse and point sources [18]. Even pharmaceuticals can contain heavy metals as inorganic impurities, being characteristic of the synthetic route of the manufacturing process [19]. The effects of all these compounds could be magnified in the wetlands.

This study is focused on the Pego-Oliva Marsh Natural Park (Valencia Community, Spain), which is characterized by a long history of human pressures, such as marsh transformations for agricultural uses, urbanization, etc. In this area, the level of 7 heavy

metals and 17 relevant pharmaceuticals, covering some of the most important therapeutic classes, was evaluated together with the possible interrelationships between them, and the possible influence of environmental factors (land uses, water sources, and distance to the seashore). Synergies between contaminants, such as pharmaceuticals and heavy metals are, until now, scarcely studied and can be relevant because of their widespread distribution not only in waters but also in soils and sediments.

2. Material and Methods

2.1. Study Area and Sampling

The Pego-Oliva marsh Natural Park has a surface of 1273 ha, and is located at the extreme South of the Gulf of Valencia in the boundary between two municipalities, Oliva and Pego (Valencia Community, Spain, 38º 52' N and 00º 03' W). The population of these towns are 39,236 (in year 2012), and can increase in more than 50,000 people during summer. The marsh is an old silted lagoon delimited, from north and south for two rivers (Racons and Bullent/Molinell) and two mountain chains (Mustalla and Segaria ridges) of karst characteristics that contribute to its water charge, and furrowed by numerous irrigation channels and ditches. At the East, a sand barrier of 9 km long separates the marsh from the sea. This marsh experiences a huge variability on the rains regime, with periodic floods especially in spring and autumn, and an annual average precipitation of 900 mm [20].

The Pego-Oliva marsh has a high biodiversity and is a key point in the route of migratory birds. Because of this, it has been included in the Life program of the European Union and in the RAMSAR Convention. Nevertheless, and besides their character of natural

protected area, in the last decades it has underwent serious changes mainly due to the human expansion and the associated need of resources and services. Therefore, parts of the marsh were drained for rice and citrus farming, and urban development, mainly in the coastal strip and the surrounding ridges. These alterations, which have affected around 70% of the wetland, have caused a remarkable decrease in the nesting of numerous species of interest. To minimize the human impact, two wastewater treatment plants (WWTPs) were built in the limits of the marsh.

Surface water samples were collected in 34 points of the Natural Park area (Figure 8.1) in June 11th of 2010. Samples were picked up in the most important water channels, rivers and springs/wells covering the study zone.

Before sampling, polyethylene bottles (2.5 L) were washed successively with detergent, tap water, distilled water and, finally, the sample water. Samples were collected as grab samples. The bottles were filled to the top with the water to eliminate air bubbles and maintained at 4 º C until their arrival at the laboratory. Once in it, samples were filtered through glass microfiber GF/A filters prior to extraction, acidified at pH < 2 and stored in dark bottles at -20 ºC until the analysis. Samples were analyzed within 1 week.

2.2. Analytical techniques

Pharmaceuticals were selected covering an important and wide variety of different therapeutic classes: β-blockers (metoprolol MPL and propranolol PRL), antidepressants (diazepam DZM), anti-epilectic drugs (carbamazepine CBZ), analgesics (acetaminophen ACM and codeine CDN), nonsteroidal anti-inflammatory drugs (ibuprofen IBF and diclofenac DCF), lipid regulators (clofibric acid CFA and fenofibrate FNF) and seven of the

most used antibacterials (ciprofloxacin CPX, norfloxacin NFX, ofloxacin OFX, Oxytetracycline OTY, sulfamethoxazole SMZ, tetracycline TCY and trimethoprim TMP).

Pharmaceutical standards were purchased from Sigma-Aldrich (Steinheim, Germany), except the epimers 4-epioxytetracycline (4-epiOTY) and 4-epitetracycline (4-epiTCY) hydrochloride that were from Across Organics (Morris Plains, NJ, USA). Deuterated internal standards [ibuprofen-d3 (IBP-d3), acetaminophen-d3 (ACM-d3) and carbamazepine-d2 (CBZ-d2)] were from CDN isotopes (Quebec, Canada). Cd, Co, Cr, Cu, Ni, Pb, and Zn Standard stock solutions were from Titrisol Merck. All standards were of analytical reagent grade (purity >97%). High purity water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA).

In the case of heavy metals extraction, all labware used was soaked in 20% (v/v) HNO3 for a minimum of 48 hours and rinsed 10 times with distilled-deionized water.

Oasis HLB 200 mg sorbent/6 mL cartridges (Waters Corp., Milford, MA, USA) were used for SPE. All reagents used were of reagent grade and gradient grade for liquid chromatography

The extraction of pharmaceuticals was based on solid-phase extraction (SPE), according [7]. The analytes were isolated by solid-phase extraction (SPE) using a combination of SAX and Oasis HLB cartridges, and then, eluted with methanol. The determination was performed by high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS), using a Micromass Quattro triple quadrupole mass spectrometer and an Alliance 2695 HPLC separation module (Manchester, UK). Instrument control, data acquisition and evaluation were done with the Masslynx NT software (v. 3.4).

Total content of seven heavy metals (Cd, Co, Cr, Cu, Ni, Pb and Zn) in the water samples were extracted by microwave acid digestion, according USEPA method 3052 [21],

and measured by Atomic Absorption Spectrometry with a Varian SpectrAA 220-FS with graphite furnace (model GTA-110).

2.3. Statistics

IBM SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses including principal component analysis (PCA). Analysis of variance (ANOVA) and Tukey's multiple range test at α=0.05 were performed to detect differences in the variables between treatments. In the cases where the homogeneity and/or normality of the data could not be assumed, the Kruskal–Wallis and Mann–Whitney non parametric test ($P \leq 0.05$) were applied.

Pearson statistical bivariate correlation analyses were applied, at 95% and 99% significance levels, between pharmaceuticals and heavy metal concentrations to determine possible relationships among them. When the values of a variable showed a non-normal distribution, Spearman bivariate correlations were applied at the same significance levels. Multiple stepwise linear regression analysis, discriminant analysis and categorical PCA were used to confirm the weight and dependence between variables, differences and identifying patterns in them.

3. Results and Discussion

The studied pharmaceuticals showed a wide distribution in the Pego-Oliva Natural Park. In fact, thirty-three of the 34 analyzed samples showed the presence of, at least, one pharmaceutical. Only sample seven, which corresponds to a well in a citrus field, did not present any of them. However, more than eight compounds were detected in a third of the

samples. CDN, IBP and ACM are the most frequently observed pharmaceuticals (Fig.8.2), appearing the first one in more than the 53% of the analyzed waters.

OTY and TCY were not detected in any sample, and CFA, DCF, TMP and CPX were detected in only one. Similar results were observed in other Mediterranean wetland [7], where tetracyclines and FNF were not detected. However, in that wetland CBZ was the most frequently detected. The highest concentrations of pharmaceuticals detected in the Pego-Oliva marsh were 112.19 µg/L for ACM, followed by 62.66 µg/L for CDN or 59.02 and 50.19 µg/L for IBP and OFX, respectively.

River and irrigation channel waters concentrate the highest values of the majority of pharmaceuticals (Fig.8.2a),mainly the lasts show these values for IBP, CFA, PRL, TMP, NFX and CPX. The presence of these pharmaceuticals, and at the levels that were detected, can imply the incidence of uncontrolled dumping or a deficient functioning of the wastewater treatment plants for their removal. In this last case, a study on 160 WWTPs effluents in the UK [22] for 70 target compounds, 16 of them pharmaceuticals, showed that in almost all cases the expected levels were surpassed. Dilutions from 10 to 100 times were needed, mainly for diclofenac and quinolones. This fact could be particularly applicable to the high frequency of IBP in the studied zone because, as is reported by the literature [23], this compound is significantly removed in the WWTPs, based in bioactive sludges.

The levels of the studied metals in the waters of the Pego-Oliva Park (Fig.8.3)were well below the limits established by national or international legislations, not only for irrigation or livestock waters [24,25] but also for drinking waters [26-28]. It is more evident for Zn, which show values < LODs. The average values of the remainder metals are in the range of 0.05-2.0 µg/L. The maximum value determined corresponded to Cu, with 19.10 µg/L, from an irrigation channel between the marsh and the orchards in the border of the

coastal strip. Overall, the metals tended to concentrate in the abandoned fields that act as interface between the lake, with the surrounding rice fields, and the citrus crops.

According with the different water sources, the levels of pharmaceuticals (Fig8.2a)did not present statistically significant differences except for CDN (95% of significance), which showed its maximum values in the river waters. The river Racons and the southern part of the studied area, at the foot of the Segaria ridge, are the zones that concentrate the major number and levels of pharmaceuticals (DCF, DZM, CDN, SMZ, PRL, TMP, NFX, CPX, OFX). It could be due to the intensive urbanization of this southern ridge and the river until its mouth. This urbanization phenomenon affects greatly the karst hydrology of the ridge through the imperviousness and sealing of its surface diminishing rainwater infiltration and reducing the aquifers recharge. On the other hand, it increases runoff coming from the washing of infrastructures (roads, buildings, etc.) and the influence of wastewaters from human population, mainly in summer.

Conversely, almost all heavy metals studied shown significant differences between water sources, except Cu and Zn (Figure8.3a).The highest values of metals appear, generally, in the lake. However, Ni presented its maximum concentrations in the river waters, with highly significant differences regarding the other sources, and Cu in the irrigation channels.

The lower mean values observed in the different land uses correspond to citrus crops, with a total average concentration of pharmaceuticals of 3.13 µg/L. Whereas, the epigraph of others (Fig.8.2b),which includes waters of urban use or influence, shows a total average value of 7.97 µg/L. On this factor, statistical significant differences were observed for PRL, CDN and NFX (Fig.8.2b).Meanwhile, for the studied metals the reedbed showed significant differences with the other uses, mainly for Cd, Cr and Ni. It has to be considered that the reedbed is a zone with strong effect of the redox processes due to the influence of the very variable water level, which is affected by the characteristic Mediterranean climate conditions.

There were not statistical significant differences between pharmaceuticals regarding the influence of the distance to the sea (Fig.8.2c).Only Diazepam (DZM) showed differences, but only significant at 90%. However, the highest total average of the pharmaceuticals content appeared in the zone close to the sea (0-2 km), with the major levels of IBP, CFA, DCF, DZM, CDN, NFX and OFX. Cd, Co, Cu and Ni showed significant differences on their concentrations regarding distance to the seashore (Fig8.3c).Generally, the higher the level of metal, the shorter the distance to the sea, except for Co and Cr that gave their major levels in the middle zone (2-3.5 km) corresponding to the marsh and rice farming (Fig 8.3c).

Even though the concentration of the studied compounds and metals could seem low, and each contaminant on its own would generally not result in acute toxicity for aquatic or other organisms [29], it has to be considered the increasing possibility of the appearance of interactions among contaminants simultaneously, which may lead to synergistic effects on fauna and humans [30,31].

Studying the possible influence among metals and pharmaceuticals, important significant correlations between Ni and IBP, DZM, NFX, OFX, TMP and DCF, and inverse relationships to IBP, at 99 and 95% of significance, were observed (Fig.8.4).The multiple stepwise regression models, which are presented in Table8.1,confirm the importance of Ni in these relationships. Cu, Co and Cr also showed significant correlations with OFX, SMZ, FNF, MPL and PRL. Graham et al. (2011) [32] who studied the apparition of antibiotic resistance gene (ARG) in bacteria in the Almendrares river (Cuba) observed that high Cu and ampicillin and tetracycline levels most often correlate with detected ARG and also for Pb, Co, Zn, and tetracycline, although in a minor extent.

The important relationship between Ni and pharmaceuticals is validated also by the PCA analysis (Fig.8.5a),in which the spatial distribution in the study area (factor 1) is the variable that explain not only this effect but also the differences to the other metals and

ibuprofen. Fig.8.5bshows PCAs for metals only. Clear differences for the behavior of Ni regarding the other metals can be observed.

The spatial distribution of the higher levels of metals and pharmaceuticals (Fig.8.6) showed a distinct distribution. Pharmaceuticals, except IBP, and Ni present equal distribution occupying, mainly, the southeastern part of the study area. This part covers a zone of the coastal sand bar and the foot of the southern ridge, highly urbanized and with an important population. The other metals were mainly in the center of the study area, which includes from the marsh, with the lake and rice fields, to the main road that limits with the coastal strip. Ibuprofen have its influence centered in the north-west of the study area, where two riding schools are located.

The spatial analysis of the data shows that the reedbed and the lake, which is strictly the marsh area, could be acting as sink and control point for the transport of pollutants, mainly of heavy metals. This is in accordance with that already reported for several natural wetlands [33-35]. These differences in the compounds distribution could be due to the characteristics of the pollutants sources, and helps to discriminate the uses of the territory and the different aspects in which the human pressure could have a bearing on the environment.

4. Conclusions

In the Pego-Oliva Marsh Natural Park, all the studied metals showed levels below those established by legislation for irrigation and drinking waters, even Zn showed values below LODs. The irrigation channels that run through the abandoned fields, which act as interface between the rice farming and the citrus crops, tends to concentrate the metals, showing their higher levels.

Pharmaceuticals appeared in all samples except in one, being codeine, ibuprofen, and acetaminophen those detected with higher frequency. Only tetracycline and oxytetracycline did not appeared in any sample. Waters from river and irrigation channels concentrate the highest values of the majority of pharmaceuticals, mainly the later showed the highest values for IBP, CFA, PRL, TMP, NFX and CPX.

Differences according water sources and land uses were observed for all the studied compounds, even though they are more noticeable for heavy metals. In addition, the distance to the seashore showed important influence in the spatial distribution of pharmaceuticals and metals in the Pego-Oliva Marsh Natural Park.

Highly significant statistical correlations were detected between Ni and ibuprofen, diazepam, norfloxacin, ofloxacin, trimethoprim and diclofenac, and inverse relationships to ibuprofen, at 99 and 95% of significance, respectively. Cr, Cu and Co also showed significant correlations with ofloxacine, simazine, fenofibrate, methoprolol and propanolol. These relationships were also observed in their spatial distribution.

Pharmaceuticals, except IBP, and Ni presented the higher levels, mainly, in the southeast of the study area. This zone covers a part of the coastal strip and the foot of the southern ridge, both highly urbanized and with an important population. Meanwhile, the remainder metals presented higher concentrations in the marsh area, and within the contact among rice fields and citrus crops. Ibuprofen was mainly detected in the north-west of the study area.

The effect of human development, in its different ways, in particularly fragile ecosystems, like the coastal wetlands, are clearly observed in the Pego-Oliva Marsh. The real importance of these impacts can be nowadays latent, but could emerge causing or having caused irreversible damages at all levels of the ecosystems, and even affecting human health.

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LEGEND OF TABLES

Table 8.1 Multiple step-wise linear regression models between the studied pharmaceuticals through heavy metals ($Y = B_0 + B_1X_1 + B_2X_2 + \ldots..$).

FIGURES CAPTIONS

Figure 8.1 Distribution of the sampling zones and location of the studied area.

Figure 8.2 Mean values of the studied pharmaceuticals according water sources (A), land uses (B) and distance to the seashore (C). Different letters in each metal mean statistical significant differences between cases. No letters means no statistical differences.

Figure 8.3 Mean values of the studied heavy metals according water sources (A), land uses (B) and distance to the seashore (C). Different letters in each metal mean statistical significant differences between cases. No letters means no statistical differences.

Figure 8.4 Observed significant correlations between ofloxacin, norfloxacin and ibuprofen with the total content of Ni in the water samples.

Figure 8.5 Results of the Principal Component Analysis of the studied data. (A) Grouping of the studied pharmaceuticals and heavy metals, and (B) of the studied heavy metals only.

Figure 8.6 Zoning of the studied contaminants in the area of the Pego-Oliva natural Park.

Table 8.1

Y	B_0	*Bj*	*Xj*	R^2	*Sig.**
Ibuprophen	17,449	B_1= – 5,253 B_2= 1,902	X_1= [Ni] X_2= [Cu]	0.409	0.000
Ofloxacin	5.735	B_1= 6,689	X_1= [Ni]	0.250	0.003
Norfloxacin	1,060	B_1= 7,918	X_1= [Ni]	0.437	0.000
Diazepam	0,835	B_1= 0.901	X_1= [Ni]	0.157	0.020
Fenofibrate	4,021	B_1= 11,325 B_2= – 6,983	X_1= [Co] X_2= [Cr]	0.271	0.007
Diclofenac	-0,602	B_1= 1,257	X_1= [Ni]	0.260	0.002
Methoprolol	-0,351	B_1= 11,681	X_1= [Co]	0.474	0.002
Trimethoprim	-0,460	B_1= 0.152	X_1= [Ni]	0.603	0.001

(*) Significance at $p<0.05$.

Figure(s)

Figure 8.1

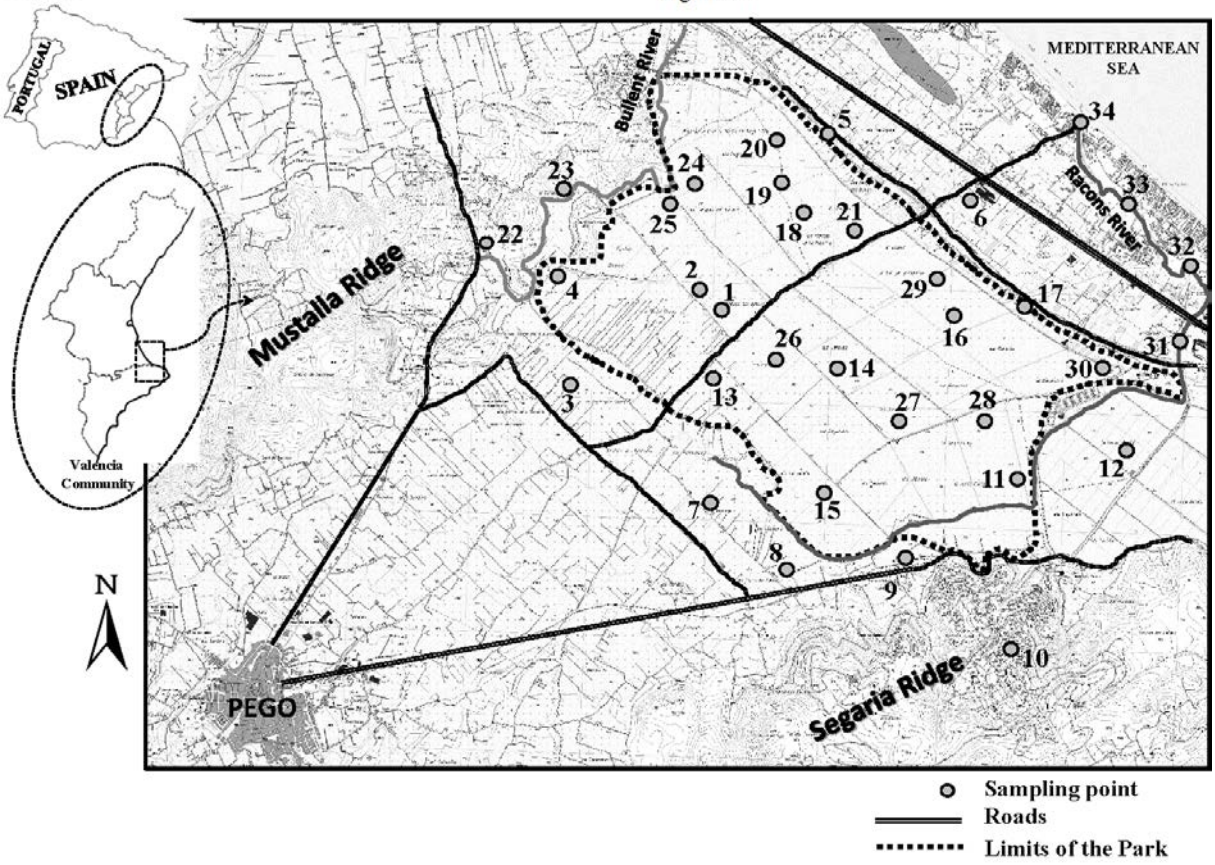


Figure 8.2

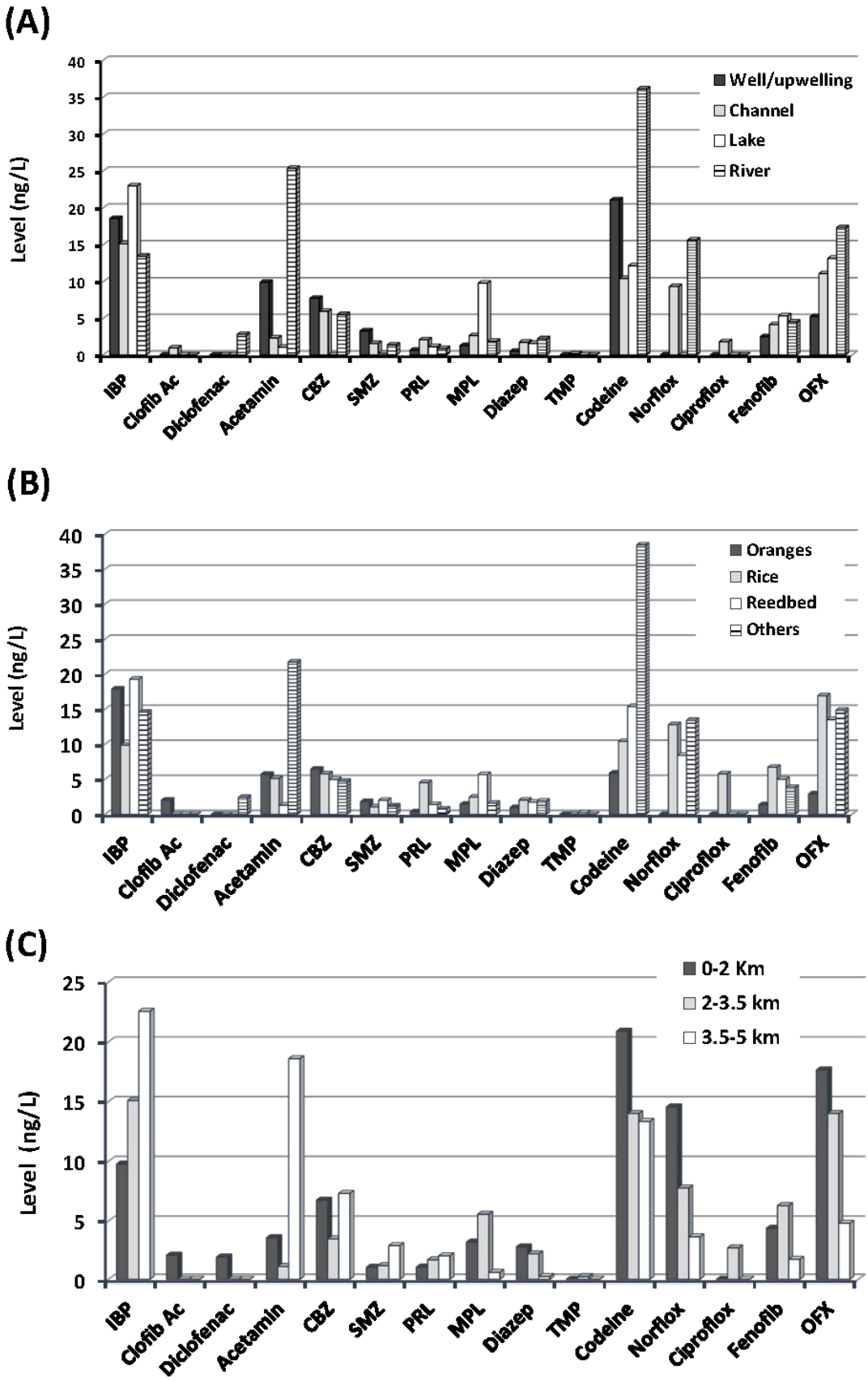

(A)
Level (ng/L)
Well/upwelling
Channel
Lake
River
IBP
Clofib Ac
Diclofenac
Acetamin
CBZ
SMZ
PRL
MPL
Diazep
TMP
Codeine
Norflox
Ciproflox
Fenofib
OFX
(B)
Level (ng/L)
Oranges
Rice
Reedbed
Others
(C)
Level (ng/L)
0-2 Km
2-3.5 km
3.5-5 km

Fig. 8.3

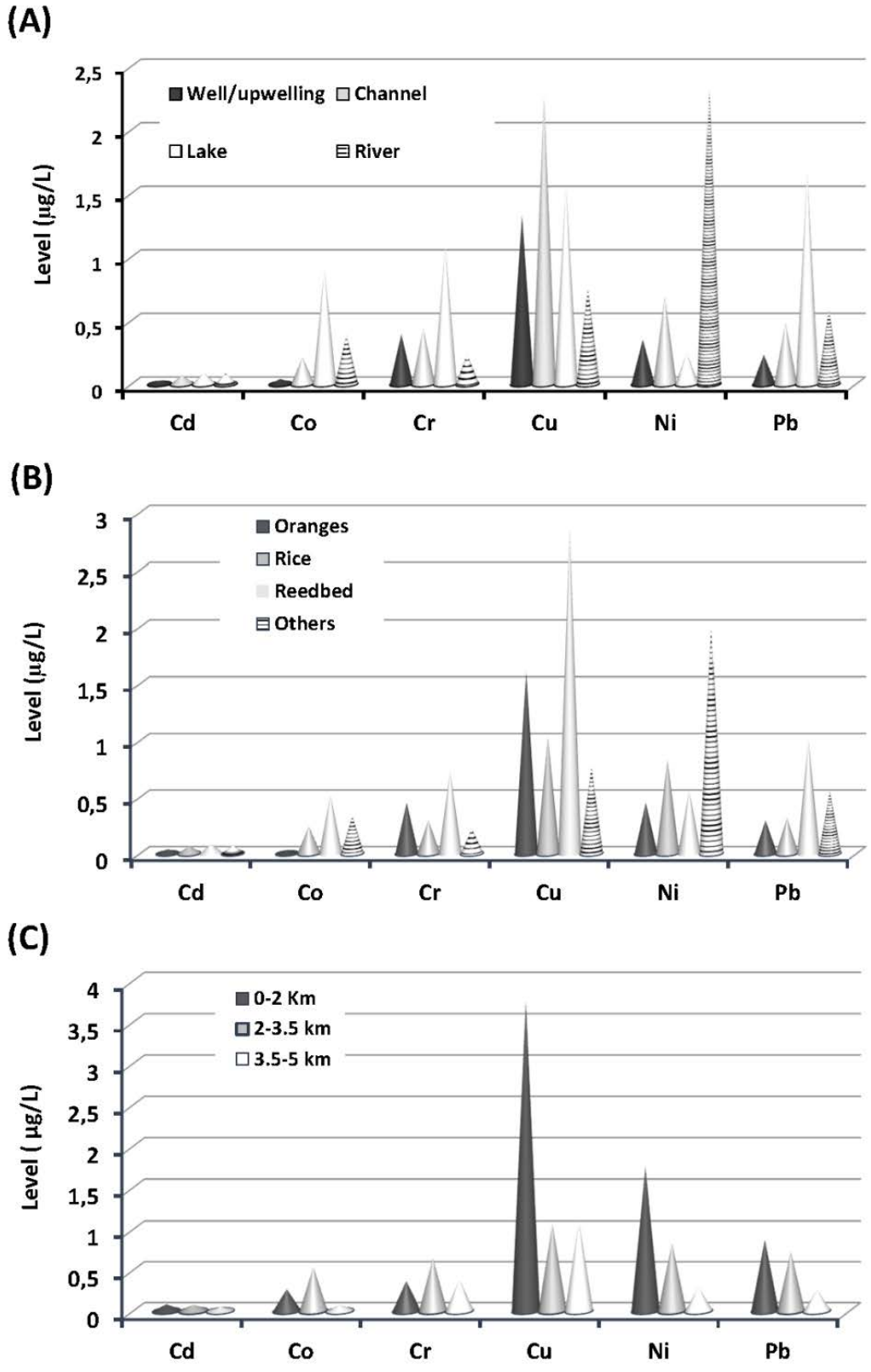

(A)
Level (µg/L)
2,5
2
1,5
1
0,5
0
Well/upwelling
Channel
Lake
River
Cd
Co
Cr
Cu
Ni
Pb
(B)
Level (µg/L)
3
2,5
2
1,5
1
0,5
0
Oranges
Rice
Reedbed
Others
Cd
Co
Cr
Cu
Ni
Pb
(C)
Level (µg/L)
4
3,5
3
2,5
2
1,5
1
0,5
0
0-2 Km
2-3.5 km
3.5-5 km
Cd
Co
Cr
Cu
Ni
Pb

Fig 8.4

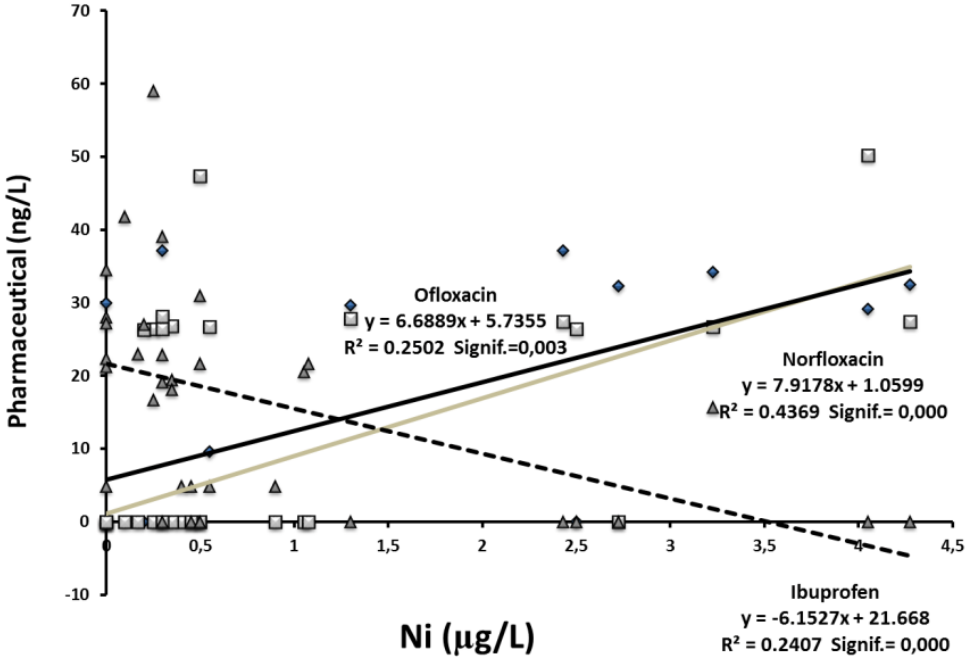
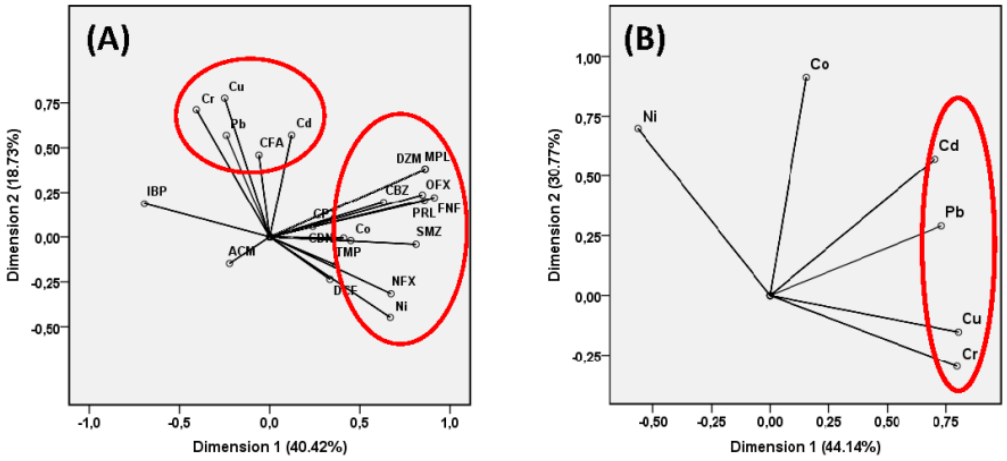

Pharmaceutical (ng/L)
Ni (µg/L)
Ofloxacin
y = 6.6889x + 5.7355
R² = 0.2502 Signif.=0,003
Norfloxacin
y = 7.9178x + 1.0599
R² = 0.4369 Signif.= 0,000
Ibuprofen
y = -6.1527x + 21.668
R² = 0.2407 Signif.= 0,000

Fig 8.5


(A)
Dimension 2 (18.73%)
Dimension 1 (40.42%)
Cu
Cr
Pb
Cd
CFA
IBP
DZM
MPL
CBZ
OFX
PRL
FNF
CIP
Co
SMZ
ENR
ACM
TMP
NFX
DFF
Ni
(B)
Dimension 2 (30.77%)
Dimension 1 (44.14%)
Co
Ni
Cd
Pb
Cu
Cr

Figure(s)

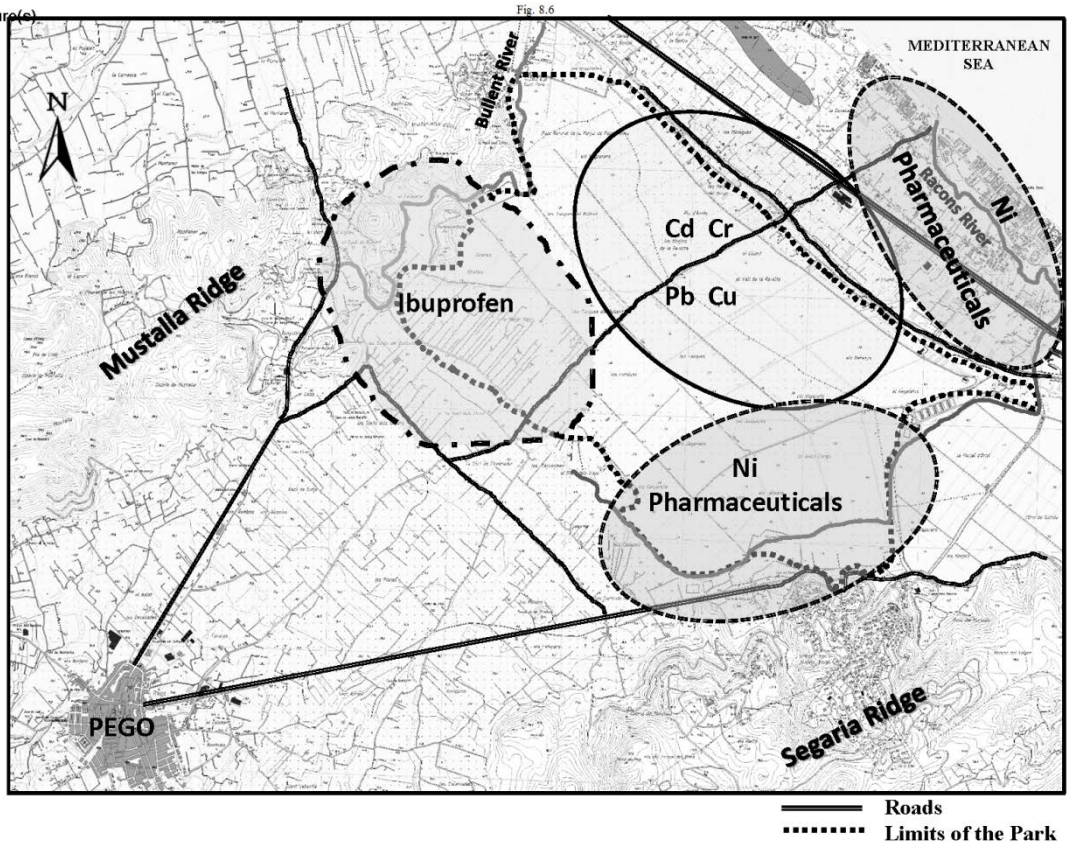


Fig. 8.6
MEDITERRANEAN SEA
N
Bullent River
Racons River
Pharmaceuticals
Ni
Cd Cr
Pb Cu
Ibuprofen
Mustalla Ridge
Ni
Pharmaceuticals
PEGO
Segaria Ridge
Roads
Limits of the Park

CHAPTER 9

Relationship between irrigation systems and levels of illicit drugs in L´Albufera of Valencia

Scientific publication 9:

Presence of Illicit Drugs in Surface Waters of Protected Natural Wetlands Connected to Traditional Irrigation Systems and Urban Areas. Management of Water Resources in Protected Areas (H. Farfán González, J.L. Corvea Porras, I. de Bustamente Gutiérrez, James.W. LaMoreaux), Environmental Earth Sciences 2013, pp 277-283 [ISBN: 978-3-642-16329-6 (Print) 978-3-642-16330-2 (Online)]

J.A. Pascual Aguilar, V. Andreu, P. Vazquez-Roig, Y. Picó

Presence of illicit drugs in surface waters of protected natural wetlands, connected to traditional irrigation systems and urban areas.

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Abstract The Mediterranean wetlands are unique in biological diversity and they offer multiple benefits constituting a great water reserve for the planet and to produce biomass and nutrients for the trophic chain. However, the increasing human impact and the socio-economic development of the last decades have provoked important losses in these ecosystems. The work has been developed in the Natural Park of La Albufera (Valencia, Spain), which includes a coastal lagoon, marshlands, dunes and pinewoods, surrounded by rice fields in its not urbanized part. In spite of this great ecological value, it suffers impacts derived from the high human and industrial occupation, and of the hydrological contributions from the connected irrigation systems. The study has been focused on the development of a combine methodology based on environmental forensics principles to identify illicit drugs and its spatial sources and implications. Results show that rather than the pattern of population distribution the traditional irrigation system connected to sewage treatment plants location is the way to introduce the illicit substances in the waters of the Natural Park.

1 Introduction

Mediterranean coastal wetlands are of great interest for their richness in biodiversity. They are also fragile systems because they are exposed to various human pressures such as farming systems (Readmand et al. 1993) and urban sprawl (Li et al. 2010), that alter their ecological and environmental conditions.

Among other emerging contaminants illicit drugs have also been detected in the aquatic environment. As for therapeutic substances, the main source of contamination for illicit drugs is human consumption. These substances are detectable in

treated water and contaminate the receiving surface waters (Zuccato and Castiglioni 2009).

Some works have study the presence of illicit substances in river systems and open water systems after passing through urban agglomerations and sewage treatment plants , although very little has been researched to analyze flow paths and water incorporation into permanent water bodies such as coastal lagoons. The aim of this works the development of an integral methodology to evaluate the presence and spatial distribution of illicit drugs in surface water of the protected Natural Park of l'Albufera de Vaelncia wetland to obtain background on how such substances travel from urban and agricultural systems to the protected area.

2 Study Area

The study has been applied to l'Albufera de Valencia Natural Park (figure9.1),located in the east of the Iberian Peninsula. The Natural Park is surrounded by a very populated hinterland, due to the influence of the City of Valencia and its metropolitan area. Major threats come from the activities developed by a population of more than 1,200,000 inhabitants. Agriculture and intensive irrigation systems developed in and out of the limits of the Natural Park are important threats to the preservation of ecosystems and water quality.

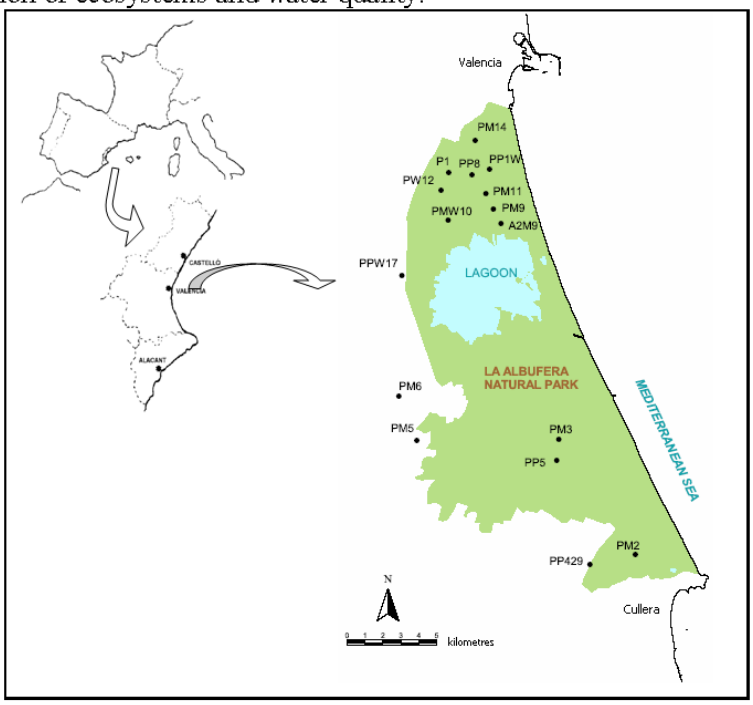

Fig.9.1 Location of the study area and sample points setting.

The present Natural Park of l'Albufera is a territory of 274.4 km^2, including its marine area. Due to secular alterations, within its limits a large proportion of the land is cover by rice fields, which occupy the primitive marshland, with only a few hectares still in their natural state (Soria et al. 2002). In the continental margins of the Natural Park, intensive irrigated agricultural gardens are also found. A shallow waters lagoon is located in the centre of the Natural Park. It is almost circular in form and is approximately, with an area of 23.7 km^2.

The hydrology of the Natural Park combines a complex human management system with natural contributions, being the water coming from the historic irrigation system the main source of water inflows to the Natural Park. There is a very dense system of overland artificial channels for irrigations with waters mainly coming from the rivers Jucar and Turia, which finally drains to the lake or directly to the sea.

3 Methodology

Methodology is based on an environmental forensics perspective integrating different sources and data formats (Taylor, 2004). It is organised around two major procedures (figure9.2): analysis of water samples and a spatial analysis with Geographical Information Systems (GIS).

Initial data consisted on (1) statistic information to municipal level on number of inhabitants and population density (inhabitants by square kilometre, h/km^2) for the year 2008 provide by the Spanish institute of Statistics; (2) an updated 2008 digital layer on land cover distribution from the CORINE project (Bossard et al. 2000); (3) a digitized map with municipal boundaries; (4) a point map with the location of Sewage waters Treatment Plants (STPs); (5) a digital layer of the traditional irrigation systems (drainage networks and areas) as stated by Hermosilla Pla (2006 and 2007), and (6) 16 water samples spatially distributed over the Natural Park collected in field work campaigns during the year 2008.

Water samples were further treated with a method using solid-phase extraction (SPE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of 14 drugs of abuse and their metabolites (cocainics, amphetamine-like compounds, cannabinoids, and opiates) (Vazquez-Roig et al. 2010).

From original tabular data and map layers GIS procedures were applied to derivate new map overlays (artificial covers such as urban and industrial surfaces) and to obtain two municipal spatial indexes, the integrate percentage of urban and industrial covers and the population density.

To identify the presence of illicit drugs in traditional irrigation systems with geographical anthropogenic origins either GIS layers and results from the illicit drugs determination with the SPE and LC-MS/MS method were compared in the GIS environment.

4 Results and discussion

Presence of illicit substances has been found in all out points analysed in the traditional irrigation water network of the Natural Park (table 9.1and figura9.2), and corroborates that urban water still incorporates emerging contaminants, including illegal, drugs after STPs treatment (Boleda et al. 2009; Postigo et al. 2010).

Most evidence has been found in cocainics COC and metabolites (BECG and ECGME), amphetamines (AMP and MDMA), methadone, codeine, morphine, and THC-COOH, the main metabolite of THC, were determined in water samples at levels ranging from less than 0.14 ng/L to 78.78 ng/L. The highest concentrations were determined in the water samples collected at sample PM6, which can be related to direct spillage of residual waters from a close leisure zone.

Table 9.1 Presence of illicit drugs (concentration levels in ng/L) in the sample points from the 1´Albufera Natural Park

	Cocainics			Amphetamine-like compounds				Cannabionids		Opiates				
	COC	BEG	ECGME	AMP	MAMP	MD	AMDMA	THC-COOH	THC	6-ACMOR	COD	HER	MOR	MET
PM14	0.08	0.53	ND	ND	ND	ND	ND	<LOQ	ND	ND	20.8	ND	ND	ND
P1	0.34	3.01	ND	ND	ND	ND	0.18	ND	ND	ND	51.6	ND	ND	0.69
PP8	0.18	1.22	ND	ND	ND	ND	ND	ND	ND	ND	31.3	ND	ND	0.14
PP1W	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PW12	ND	<LOQ	ND	ND	ND	ND	0.76	ND	ND	ND	ND	ND	ND	ND
PM11	0.16	1.06	ND	ND	ND	ND	<LOQ	ND	ND	ND	20.1	ND	ND	<LOQ
PM9	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
A2M9	0.03	1.59	ND	ND	ND	ND	0.14	ND	ND	ND	35.1	ND	ND	0.27
PMW10	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PPW17	0.08	0.89	ND	ND	ND	ND	<LOQ	ND	ND	ND	ND	ND	0.3	0.19
PM6	4.43	78.71	1.35	3.38	ND	ND	2.48	ND	ND	ND	18.69	ND	11.7	0.84
PM5	0.06	0.51	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PM3	0.08	0.48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PP5	0.08	0.53	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PM2	0.25	0.14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PP4	0.12	0.62	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

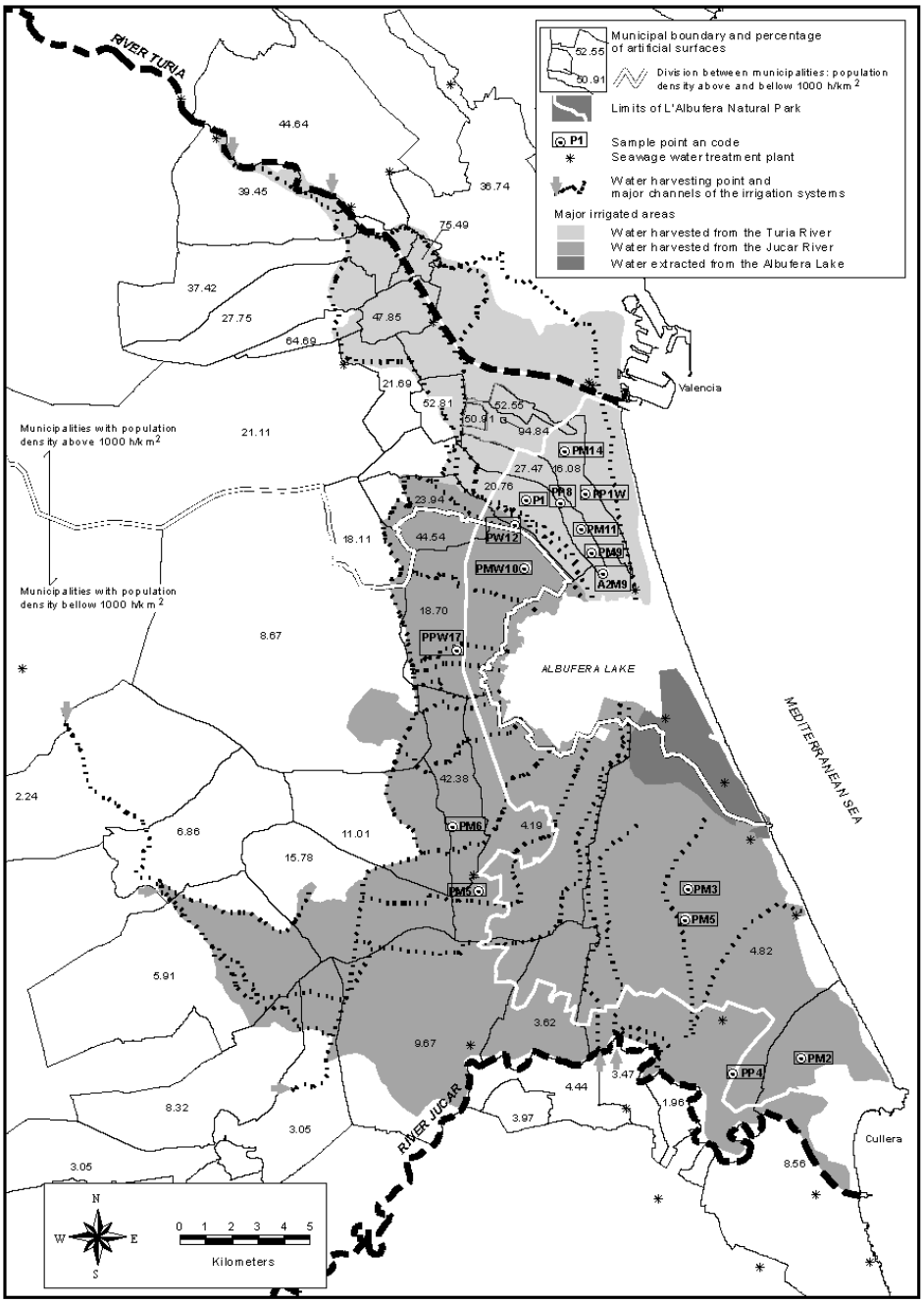

Fig.9.2 Urban and agricultural (irrigation structure) contextualization of l'Albufera Natural Park.

The geographical presence of illegal substances can be also explained by a combination of water path ways and multiuse. Residual water from urban areas, after being treated in STPs, is introduced in agricultural system for irrigation uses and finally drainage into the lake (figure9.2).Traditional irrigation systems are vehicles to supply the presence of illegal substances into the Natural Park. So far a

spatial relationship between population concentration and artificial surfaces with presence of drugs cannot be stated, although STPs drainage in the irrigation network has to be understood as the mean of illicit drugs presence in the Natural Park of l'Albufera.

5 Conclusions

The combined method using water samples and spatial analysis integrated under an environmental forensic approach has been effective to detect the presence of illicit drugs in the Natural Park o l'Albufera.

There is no a clear evidence of a geographical trend based on population density or concentration or urbanized areas, in that case further work should be done to establish the effectiveness of STPs in purifying contaminated waters with illicit substances.

The analysis has proven that the water paths of inflow waters to the Natural Park are related to the overlapping of traditional irrigation systems and networks with STPs.

Acknowledgments This work has been carried out in the scope of project HUCOMED CGL2008-01693/BTE and project GCL2007-66687-C02-01/BOS, both financed by the Spanish Ministry of Science and technology.

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CHAPTER 10

Stereoisomeric profiling of drugs of abuse and pharmaceuticals in wastewaters

Stereoisomeric profiling of drugs of abuse and pharmaceuticals in wastewaters

P. Vazquez-Roig, B. Kasprzyk-Hordern, Y. Picó

The stereoisomeric profiling of pharmaceuticals (ephedrine, norephedrine, atenolol and venlafaxine) and illicit drugs (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) and 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA)) was undertaken over a period of fourteen consequtive days in three wastewater treatment plants in the city of Valencia, Spain.

Keywords: Pharmaceuticals; Abuse Drug; Stereoisomers; Wastewater; Activated sludge; Removal efficiency.

Highlights

- Pharmaceuticals and drugs enantiomers were monitoring for 2 weeks at three WWTPs
- Selective enantiomer enrichment was observed for many target analytes
- Degradation efficiency of a WWTP is compound and enantiomer dependent
- Only MDMA and amphetamine were practically not detected in effluents

10.1 Introduction

About 56 % of pharmaceuticals and illicit drugs currently in use can be chiral. Although they are usually manufactured as racemic compounds, the stereospecificity of the human metabolism and microbial action in the waste water treatment plants (WWTPs) can degrade them enantioselectively. Since toxicity of these compounds are generally isomer-dependent [Kasprzyk-Hordern 2010a], it is important to understand the influence of the WWTPs in the selective degradation to improve their performance and protect the receiving aquatic environment. Fluoxetine, for example, is the most toxic human pharmaceutical reported so far. Its toxicity is currently assessed for the racemate. However, recent research indicates that toxic effects of fluoxetine are enantiomer dependent: S-fluoxetine is 9.4 times more toxic than R-fluoxetine. Species-specific toxic effects among enantiomers also exist; for example, *S*-(−)-propranolol has higher chronic toxicity to fathead minnows than its enantiomer, but the opposite is true in daphnids [*Nikolai et al. 2006*].

There is a lack of enantiomeric information of pharmaceuticals and illicit drugs in the environment. Although chiral HPLC methods have been extensively used for stereoisomer separation of drugs in pharmaceutical preparations [Chen et al. 2005], they are usually detected by UV detection, which it is not suitable for the typical low concentrations in the environment. Recently, new HPLC methods using chiral columns packed with antibiotics or proteins have been succesfully couple with tandem mass spectrometry to analyze drugs in WWTPs and/or surface waters [Bagnall et al. 2012, MacLeod et al. 2010, Nikolai et al. 2006]. With these type of column, several chiral pharmaceuticals and illicit drugs, among them atenolol, metoprolol, fluoxetine, venlafaxine, ibuprofen, ketoprofen, naproxen, amphetamine, methamphetamine and ephedrine have been determined in surface and wastewater individual or, up to 18 compounds, even simultaneously [MacLeod et al. 2010, Nikolai et al. 2006, Barclay et al. 2012, Hashim et al. 2011, Fono et al. 2005, Buser et al. 1999].

This paper provides useful information about the presence of 10 active substances among pharmaceuticals and illicit drugs. The enantiomeric fraction in the

influent and effluent of the WWTP was calculated and values obtained were compared among WWTPs and with those reported by other authors [MacLeod et al. 2007, Nikolai et al. 2006, Kasprzyk-Hordern et al. 2012a]. Whenever occurrence was observed, stereoselective removal (by means of the activated sludge treatment) was estimated.

This study reports for the first time a stereospecific monitoring of these substances for 2 week at three WWTPs of the Valencia City and surroundings. The environmental fate and behavior of these compounds are actually to be taken into account in order to assess possible risks for human health and water ecosystem.

10.2 Materials and methods

10.2.1 Sampling

In a sampling campaign of two weeks (April 17-May 1 of 2012), influent and effluent 24-h composite samples (time-proportional mode) were collected in three WWTPs coming from the city of Valencia (Spain) and the surrounding towns. These WWTPs have tertiary treatments but they differ in the second one: Pinedo-I has only activated sludge, Pinedo-II activated sludge and phosphorus removal, and Quart-Benager activated sludge and nitrogen removal. Pinedo-I serves to the city Valencia, Pinedo-II serves to Valencia and surrounding towns and Quart-Benager serves to towns of the industrial belt of Valencia (see operational details in Table 10.1).

Table 10.1. Operational parameters on the studied WWTPs in 2012.

Characteristics		**Pinedo-I**	**Pinedo-II**	**Quart-Benager**
Coordinates UTM (ETRS 89 zone 30N)		X:728552 Y: 4368031 Z: 5	X:728371 Y: 4368153 Z: 5	X:722456 Y: 4370419 Z: 22
Population served (thousands)		351,198	942,774	166,942
Flows	**(m^3/day)**	100602	242580	35903
Wastewater	**(% industrial/% domestic)**	0/100	0/100	60/40
Treatment		AS	AS/ N removal	AS/P removal

Average daily sewage flow (m^3/d)	106537	236,396	37,998
Designed treatment capacity (m^3/d)	124,800	200,000	60,000
Influent BOD (mg/L)	248.2	263.7	318.1

Samples were taken in 2L plastic bottles with Teflon protected caps and carried out to the laboratory for their immediately analysis. If this was not possible, the samples were frozen at−20 °C until analysis to prevent degradation of the target residues.

10.2.2 Chemicals

All reference standards (±)-amphetamine (AMP), (±)-methamphetamine (MAMP), (±)-MDA, (±)-MDMA, (±)-MDEA, (-)-ephedrine (EPH), (+)-pseudoephedrine (PEPH), (±)-norephedrine (NOR), (±)-atenolol (ATE), and (±)-venlafaxine (VEN) were purchased from LGC Standards (Teddington, UK) and Sigma-Aldrich (Gillingham, UK). The internal standard (IS) (±)-MDA-d5 was added to the samples before solid-phase extraction (SPE) and (±)amphetamine-d11, (±)-methamphetamine-d5, (±)-MDMA-d5, (±)-MDEA-d5, and (±)-atenolol-d7 before the injection.

All glassware was silanised with dimethylchlorosilane (5% DMDCS in toluene) to minimise sample loss through adsorption of basic analytes onto OH-sites present on glass surface.

10.2.3 Analytical methodology

Once in the laboratory, IS was added to the sample, filtered through a 0.45 µm glass microfiber filter and extracted following a protocol described elsewehere [Kasprzyk-Hordern et al. 2012b]. Briefly, 50 mL water was extracted using an HLB cartridge, then the cartridge was rinsed with Milli-Q water and dried under vacuum for 15 min. Cartridges containing analytes were frozen and eluted prior to the analysis with 6 mL of methanol, and the content was evaporated to dryness and reconstituted in 500 µL of methanol/water (25:75, v/v). Vials were stored at -18 ºC until analysis.
Waters ACQUITY UPLCTM system (Waters, Manchester, UK) consisting of ACQUITY UPLCTM binary solvent manager and ACQUITY UPLCTM sample manager was used for

the separation of analytes. Chiral-CBH column, 100 × 2 mm, 5 µm (Chromtech, Congleton, UK) and Chiral-CBH 10 × 2.0 mm guard column (Chromtech, Congleton, UK) were used for the separation of enantiomers of chiral pharmaceutical and illicit drugs. The separation of chiral drugs was undertaken under isocratic conditions with the usage of mobile phase (pH, 5.0) composed of 90% H2O, 10% 2-propanol and 1 mM ammonium acetate. 20 µL of the sample was injected into the system. The column was kept at 25 °C and the temperature in the sample manager was kept at 4 °C. The flow rate of mobile phase was 0.075 mL/min, which allowed for the introduction of mobile phase from LC into MS without splitting.

A TQD (triple quadrupole) mass spectrometer (Waters, Manchester, UK), equipped with an electrospray ionisation source, was used for drugs of abuse quantification. The analyses were performed in positive mode with a capillary voltage of 3 kV, a source temperature of 150 °C and a desolvation temperature of 200 °C. A cone gas flow of 0 L/h and desolvation gas flow of 450 L/h were used. Nitrogen, used as a nebulising and desolvation gas, was provided by a high purity nitrogen generator (Peak Scientific Instruments Ltd, UK). Argon (99.999%) was used as a collision gas. MassLynx 4.1 (Waters, UK) software was used to collect and analyse the obtained data. Mass spectrometry analyses were performed in the multiple reaction monitoring (MRM) mode, measuring the fragmentation of the protonated pseudo-molecular ions of each chiral drug (Table 10.2S, Supplementary Material). A dwell time of 200 ms per ion pair was used to maintain high sensitivity of the analysis and required a number of data points across the chromatographic peak. All instrumental and methods validation parameters such as: linearity and range, accuracy, precision, detection and quantification limits and calibration curve were determined. A detailed discussion of this method is presented elsewhere [Kasprzyk-Hordern et al. 2012b]. Validation parameters for this method can be found in Table 10.2S, Supplementary Material.

The relative concentration of enantiomers of chiral drugs was expressed as the enantiomeric fraction (EF) and , since we know the elution order of all enantiomers, it was calculated according with the following equation:

$$EF = \frac{(+)}{(+) + (-)}$$

where (+) and (-) are concentrations for the enantiomers based on their interaction with plane-polarized light. In the case of amphetaminic group, enantiomers were identified as R(−)- and S(+)-enantiomers. In the case of atenolol and Venlafaxine, they correspond to R(+) and S(−).

Ephedrine/pseudoephedrine has two chiral centers and as a result two pairs of enantiomers. The ephedrine enantiomers have a diastereomeric relationship with pseudoephedrine enantiomers. Therefore, for 1R,2S(−)-ephedrine and 1S,2S(+)-pseudoephedrine diastereomeric fractions (DFs) were also calculated:

$$DF = \frac{\text{ephedrine}}{\text{ephedrine} + \text{pseudoephedrine}}$$

Removal rates for each compound and WWTP were calculated from concentrations measured in the effluent and influents using this equation:

$$\%\ Removal\ Rate = 1 - \left(\frac{[Effluent]}{[Influent]}\right) * 100$$

Values <MQL were taken like the average of MDL and MQL. It is considered a 100% elimination when the analyte was detected in the influent water but not in the effluent.

10.3 Results and discussion

The quantified levels of the investigated pharmaceuticals and drugs of abuse in influent wastewaters samples analysed, are summed up in Figures 10.1, 10.2 and 10.3 for Pinedo-I, Pinedo-II and Quart-Benager WWTPs respectively.

Figure 10.1. Loads(ng/L) of drugs in influent waters from Pinedo-I WWTP in 2012.

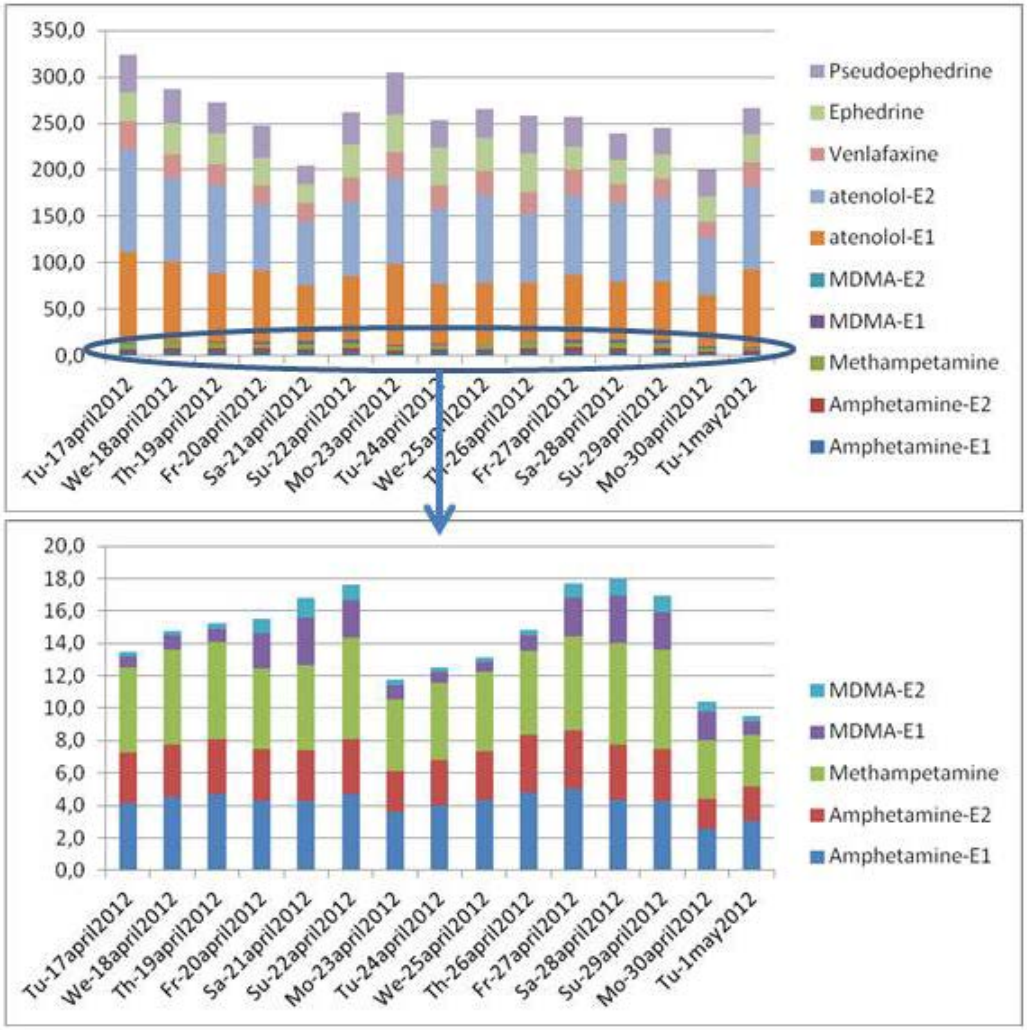


Figure 102. Loads(ng/L) of drugs in influent waters from Pinedo-II WWTP.

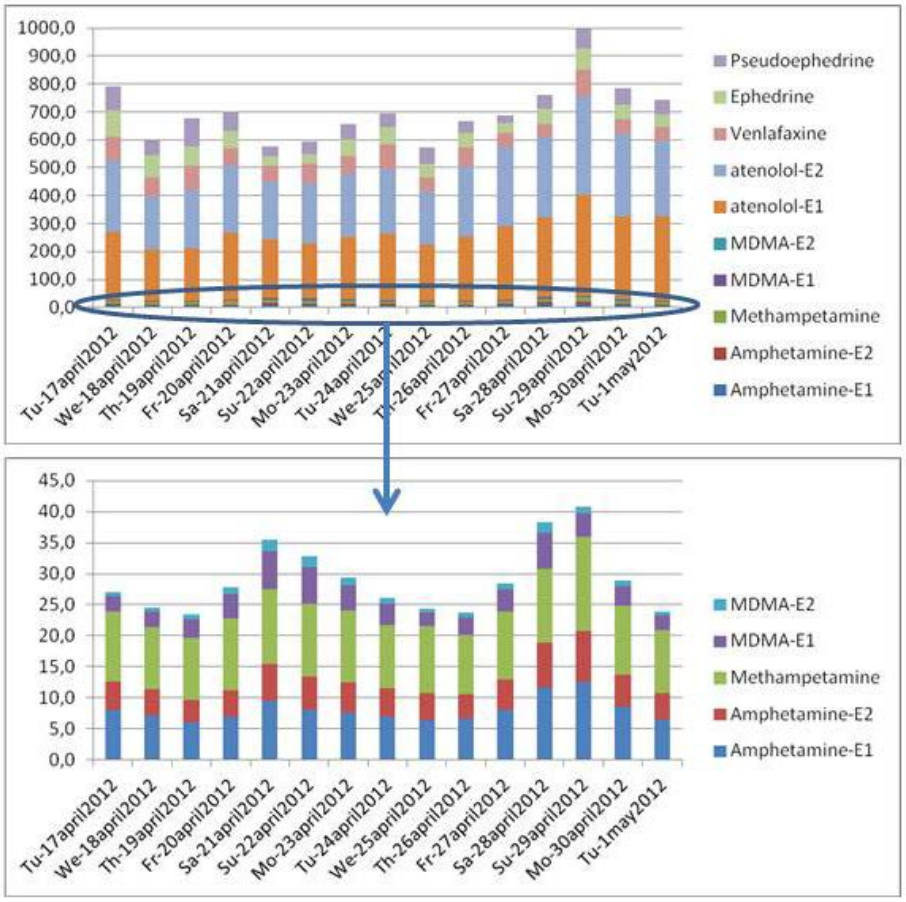
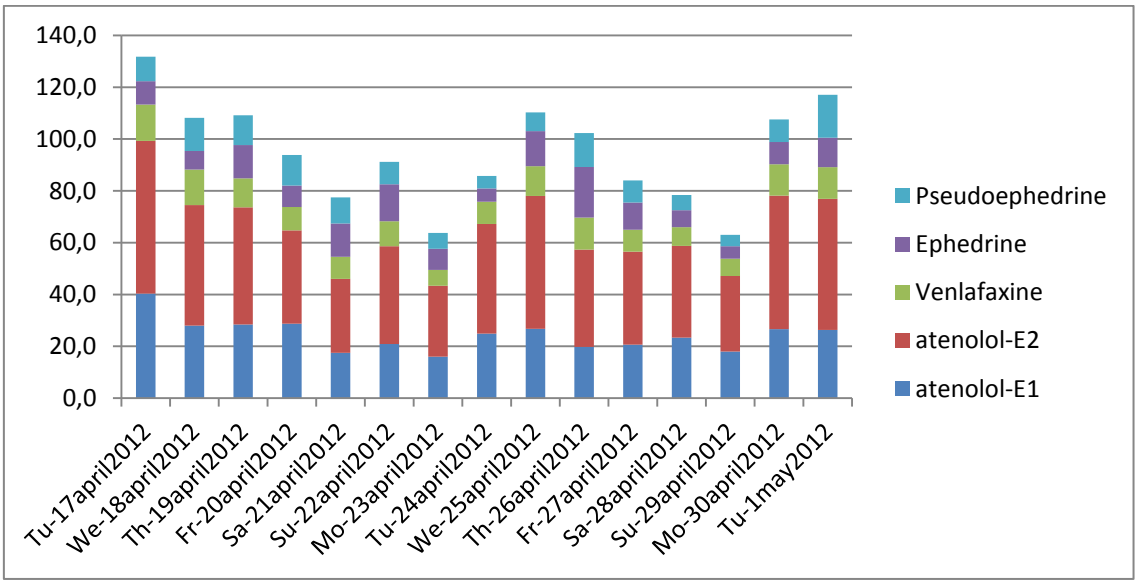


Figure 10.3. Loads (ng/L) of drugs in influent waters fromQuart-Benager WWTP.



10.3.1. Occurrence of chiral drugs in wastewaters of Valencia

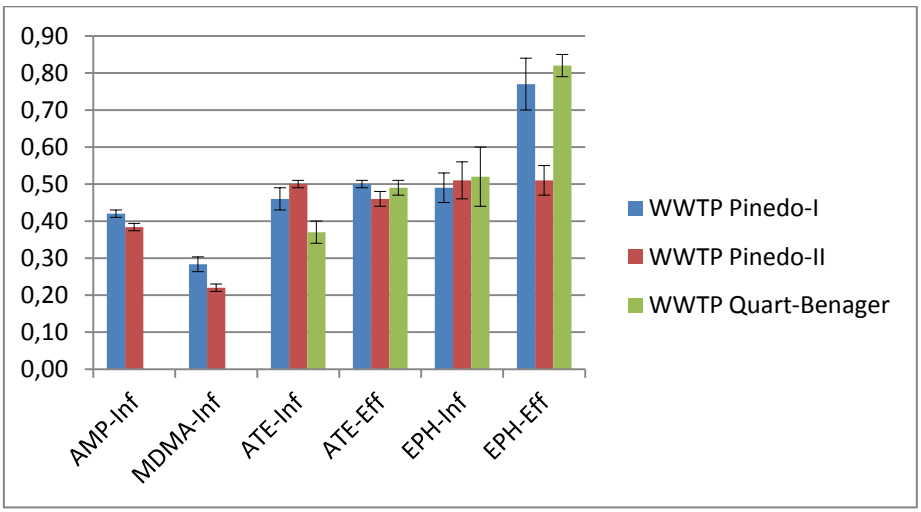
Of all compounds investigated, MDA, MDEA and norephedrine were not detected in any sample. Atenolol and venlafaxine were detected in all samples at the highest concentrations. Amphetamine, methamphetamine and MDMA were present at the lowest levels, being practically not detected in effluent samples. In general, Quart-Benager WWTP was characterized by the lowest concentrations of studied chiral drugs. It is important to note that iilegal drugs were not detected in any of the sample collected in Quart-Benager WWTP. A detailed list of the concentrations of all compounds can be consulted in Tables 1, 2 and 3 of Supplementary Material.

As it can be seen in Figures 10.1 and 10.2, corresponding to Pinedo-I and Pinedo-II WWTPs, loads of illegal drugs increased during weekend days. This effect has been also reported by other authors [Huerta-Fontanela 2007, Van Nuijs 2009, Pedrouzo 2011].

10.3.2. Enantiomeric profiling of wastewaters

Venlafaxine and methamphetamine were not correctly separated in their both isomers in the chromatographic column, thus, their enantiomeric fraction could not be calculated. This could be owing to a problem removing the matrix of the sample, since proteins on the stationary phase of the chromatographic column are very sensitive to matrix components. Enantiomeric fraction and diastereomeric fractions (in the case of ephedrine and pseudoephedrine) are showed in Fig 10.4.

Fig 10.4. Enantiomeric fractions in influent and effluent samples of the three studied WWTPs.



Except in a few cases, there is practically not inter-day variation in the value of EF for one particular drug in each WWTP. Substantial variations have been found by other authors among months and seasons [Kasprzyk-Hordern 2012b, MacLeod 2010, Nikolai 2006]. Physico-chemical properties of the water, as temperature, pH, oxygen dissolve, nitrogen and phosporus contents, etc have a direct influence in the behavoiur of biological microorganisms of the WWTPs and may have a decisive influence in the EF values [Gasser 2012]. Since these parameters are practically constant during the short time of our sampling campaing (data not shown), no high variations in EFs could be expected.

MDMA is illegally synthetized as racemate. However, humans metabolize mainly S(+)-MDMA, which leads to enrichment of MDMA with R(−) (EF<0.5). This is observed in Pinedo-I and Pinedo-II WWTPs. Amphetamine is also produce as racemate and similarly to MDMA, S(+)-amphetamine metabolizes faster than R(−)-enantiomer if administered in racemic form [Kasprzyk-Hordern et al. 2012b], and their EFs are 0,42 and 0,38 in Pinedo-I and Pinedo-II WWTPs. Unfortunately, MDMA and amphetamine were practically not detected in effluents, so the behavior of these WWTPs in enantiomeric selectivity could not be studied.

Atenolol is found in raw sample as racemate or enriched with S(−)-enantiomer (EF=0,46 and 0,37). However, during wastewater treatment enrichment of atenolol with R(+) or S(-), depending of the WWTP. In WWTP Pinedo-II, there is an enrichment with S(-), while in WWTPs of Pinedo-I and Quart-Benager there are an enrichment with R(+). Enrichment with R(+) has been previously reported by Kasprzyk-Hordern et al. (2012b), and with S(-)-atenol by Nikolai et al. *(2006)* and MacLeod et al. (2010).

Although all studied WWTPs in this work have activated sludge treatments, Pinedo-II has also biological nitrogen removal. During this removal, influent waters undergo nitrification process with different bacteria in aerobic conditions. This bacteria might favor the degradation of R(+)-enantiomer, leading to a major proportion of S(-)-propanolol. A fact which would backed this point is that nitrification process is highly favoured by higher temperatures of wastewaters [Antoniou 1990]. In WWTPs with activated sludge and nitrogen removal, this increasing in the proportion of S(-)-propanolol in summer season has been observed in two canadian WWTPs: Capital

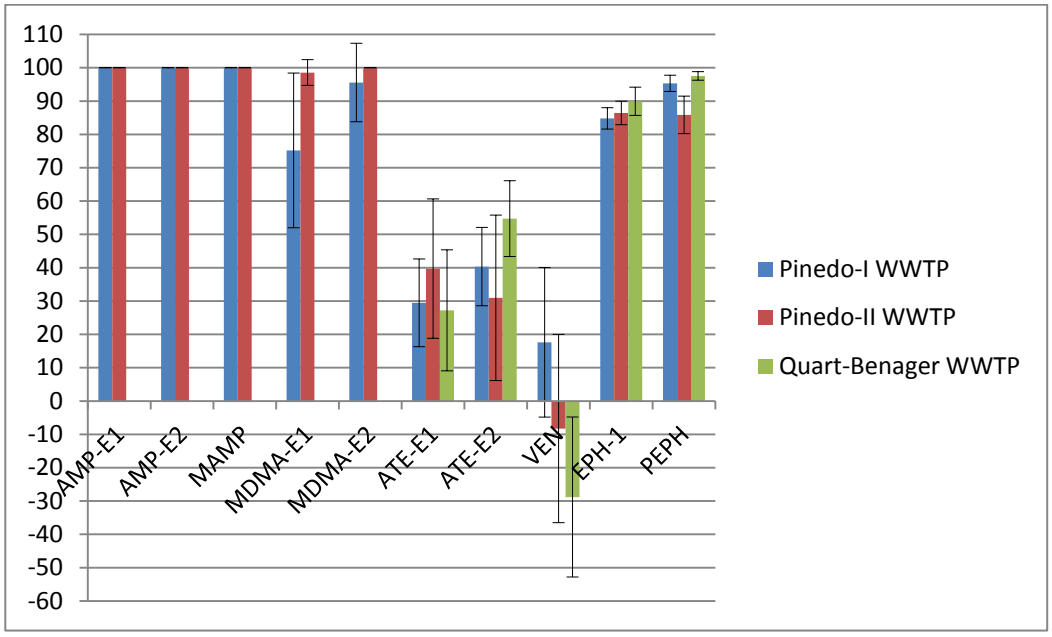
Region [MacLeod 2010] and Edmonton Gold Bar [Nikolai 2006]. However, detailed studies have to carried out to confirm this point.

In all samples, only two peaks for epedrines could be observed, corresponding to 1R,2S(−)-ephedrine and 1S,2S(+)-pseudoephedrine, coming from natural sources. Ephedrine reaches wastewater as racemate, and then it could be favoured the elimination and 1S,2S(+)-pseudoephedrine or both, exiting again as racemic substance. It seems that this enrichment with 1R,2S(−)-ephedrine occurs with high water temperatures as occurs in this study (with temperatures between 18.4 and 20.5 ºC) , while in winter is formed mainly 1S,2S(+)-pseudoephedrine [Kasprzyk-Hordern et al. 2012b].

10.3.3. Removal of chiral drugs during wastewater treatment

The average elimination rates of the compounds investigated in the different sampled WWTPs ranged from -29 to 100% (see Fig. 10.5).

Fig 10.5. Median of removal rates (%) for each compound and WWTP.



In general, degradation efficiency of a WWTP is compound and enantiomer dependent. Amphetaminic group and ephedrines are highly degradated in the three

WWTPs. Amphetamine and methamphetamine were not detected in effluent samples, so the efficiency of the three WWTPs is total. R(-)-MDMA is easier degraded than S(+)-MDMA. Pseudoehphedrine is slightly better degraded than ephedrine in WWTPs Pinedo-I and Quart-Benager, and in the same degree in Pinedo-II. Atenolol is poorly removed in the WWTP, being degradation rate different for each enantiomer and WWTP. Removal rate of S(-)-atenolol is higher than R(-)-isomer, except in WWTP Pinedo-II, as occurred with MDMA. However, these differences could not be explained with the data currently we have of it. Venlafaxine showed an anomalous behaviour. It is poorly degradated in Pinedo-I WWTP (18%), but in Pinedo-II and Quart-Benager is formed during the WWTP process. This has been also reported by other authors [Kasprzyk-Hordern et al. 2012b] and probably is due to cleavage of free venlafaxine from their glucoronide conjugated forms due to microbial processes occurring during wastewater treatment [Gasser et al. 2012]. It seems that N and P removal in Pinedo-II and Quart-Benager favor the degradation of venlafaxine metabolites.

10.3.4. Drug usage at the community level in main urban areas of Valencia

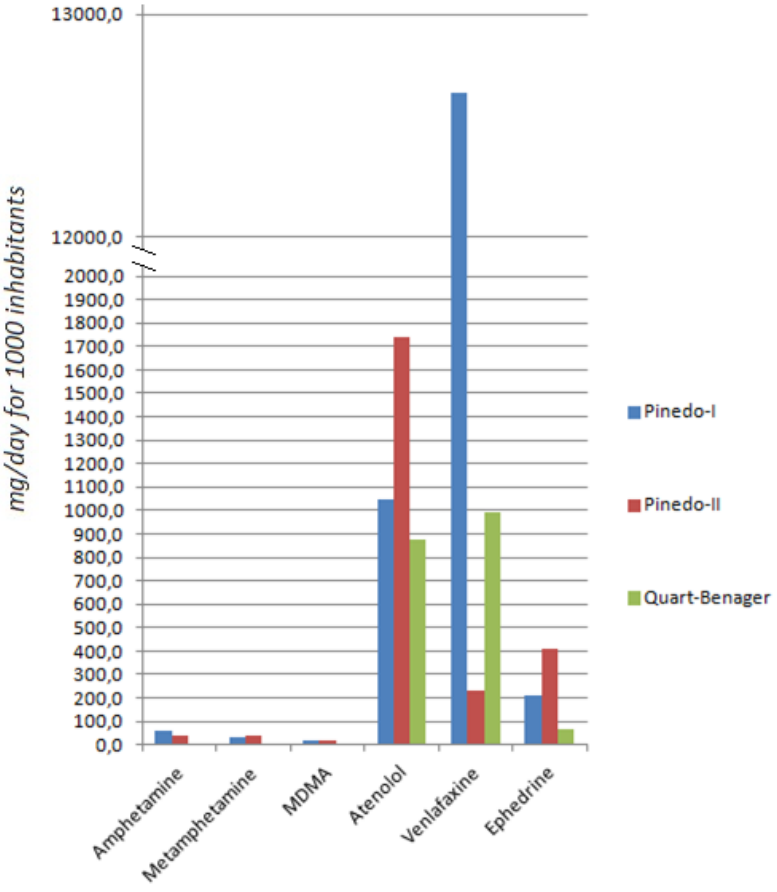
Drug usage in the Valencia city and surrounded was calculated from the drug concentrations measured at the inlets of the investigated WWTPs, by means of application of model suggested by Zuccato et al. (2008), according with the following equation:

$$Q\ (g/day/1000\ inh.) = Q_{day}/U_{ex} * M_{ratio} * N_{inh}$$

where Q_{day} is the load of the drug target residue (DTR), U_{ex} is the percentage of the DTR urinary excretion, M_{ratio} is the parent drug/DTR molar ratio and N_{inh} is the number of inhabitants provided by the WWTP staffs and corresponds to the evaluation of the population linked to the WWTPs. DTR percentages of excretion were obtained from Postigo et al. (2011) for AMP, MAMP MDMA and ephedrine, and from Lai et al. (2011) for atenolol and venlafaxine.

Fig. 10.6 shows drug consumption estimations for each drug and WWTP investigated.

Fig 10.6. Average daily consumption of the investigated drugs, expressed as mg/day for 1000 inhabitants.



As it can be seen, prescription drugs are vastly consumed comparing with illicit drugs. Among them, ephedrine is markedly more consumed than the rest ones. MDMA is highly consumpted, above other cities of Europe [Thomas et. 2012] and only exceeded by Antwerp (Belgium), London (E), Amsterdam, Eindhoven and Utrecht (Netherlands). Amphetamine and methamphetamine are consumed above the majority of European cities, being below than the cities previously named, Helsinky and Turku (Finland), Budweis (Czech republic) and Oslo (Norway), which presented loads of methamphetamine until 350 mg/day/1000 inhab.

Excretion rate of venlafaxine in the urine is only 4.7% of the intake doses, being the majority excreted as the metabolite O-demethyl-venlafaxine [Howell et al. 1993]. It would result in a substantial overestimation of the population, thus being inappropriate for consumption estimation.

10.4 Conclusions

The quantified levels of the investigated pharmaceuticals and drugs of abuse in the wastewater samples indicate that Atenolol and Venlafaxine were detected in all samples and presented the highest concentrations. Except ephedrine, drugs present very low levels in raw wastewaters, being practically not detected in effluent samples.

The elimination of the target compounds depends on each WWTP and substance. Drugs of abuse are highly degraded in the three WWTPs, while Atenolol is poorly removed in the WWTP. Venlafaxine can be formed or degraded in the WWTP, depending of the treatment technology used.

In general, Valencia shows a level of consumption above many European cities, but below of the big cities of Netherlands, Finland and Belgium.

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Table S10.2. Optimised MRM conditions for the analysis of chiral drugs by HPLC-MS/MS

Analyte	CV/CE	MRM1 (quantification)	CV/CE	MRM2 (confirmation)
R/*S*(±)-Amphetamine	18/8	136.16 > 119.10	18/16	136.16 > 91.10
1R,*2S*(−)-Ephedrine *1S*,*2S*(+)-Pseudoephedrine	23/12	166.09 > 148.10	23/21	166.09 > 133.00
R/*S*(±)-Norephedrine	21/11	152.10 > 134.10	21/19	152.10 > 117.10
R/*S*(±)-MDA	21/11	180.03 > 163.10	21/22	180.03 > 105.10
R/*S*(±)-MDMA	24/13	194.09 > 163.10	24/24	194.09 > 105.10
R/*S*(±)-Methamphetamine	24/19	150.20 > 91.10	24/10	150.20 > 119.05
R/*S*(±)-MDEA	28/13	208.09 > 163.10	28/27	208.09 > 105.10
R/*S*(±)-Atenolol	34/25	267.23 > 74.00	34/25	267.23 > 145.00
R/*S*(±)-Venlafaxine	27/12	278.15 > 260.10	27/32	278.15 > 121.00
R/*S*(±)-Atenolol-d7	34/25	274.30 > 79.05	–	–
R/*S*(±)-Amphetamine-d11	18/8	147.16 > 130.10	–	–
R/*S*(±)-MDEA-d5	28/13	213.09 > 163.00	–	–
R/*S*(±)-MDA-d5	21/11	185.09 > 168.10	–	–
R/*S*(±)-MDMA-d5	26/13	199.1 > 165.10	–	–
R/*S*(±)-Methamphetamine-d5	24/19	155.2 > 92.3	–	–

CV-cone voltage [V]; CE-collision energy [eV].

Table S10.3. Average concentrations (ng/L) of drugs in Pinedo-I WWTP.

Day		AMP-E1	AMP-E2	MAMP	MDMA-E1	MDMA-E2	ATE-E1	ATE-E2	VEN	EPH-E1	PEPH-E2
17april2012	Influent	33.5	25.3	42.5	5.4	2.1	795.6	896.9	243.5	243.2	327.8
	Effluent						385.1	406	163.8	45.1	14.7
18april2012	Influent	36.6	26	47.9	6.9	2.5	698.1	726.2	209.1	275.8	294
	Effluent				3.4		521.7	510.9	191.2	45.8	21.1
19april2012	Influent	43.4	32.2	56.0	7.4	3.3	683.3	882.3	212.0	311.3	316.8
	Effluent						483.0	508.9	156.0	43.7	12.9
20april2012	Influent	44.7	33.9	52.4	22.5	9.4	795.4	757.6	213.2	304.6	372.5
	Effluent				6.4		620.6	595.0	198.7	40.3	11.5
21april2012	Influent	48.9	35.7	60.2	34.0	13.8	676.8	781.9	233.8	228.3	240.1
	Effluent				17.72		444.3	416.6	237.0	45.1	10.2
22april2012	Influent	42.3	31.6	56.9	20.8	8.7	617.8	731.8	231.9	332.2	306.7
	Effluent				6.8		401.7	428.4	163.2	56.8	16.8
23april2012	Influent	30.5	21.5	38.0	7.6	2.6	735.8	791.7	240.6	342.0	387.2
	Effluent						626.0	623.8	177.8	60.0	22.0
24april2012	Influent	33.1	23.4	39.9	5.9	2.2	538.0	677.7	203.5	338.4	248.7
	Effluent						439.6	429.1	292.7	46.6	24.8
25april2012	Influent	37.8	26.8	42.9	5.2	2.3	572.4	824.7	225.7	320.8	264.2
	Effluent						318.9	346.5	113.4	46.5	23.4
26april2012	Influent	45.2	32.7	48.4	8.7	3.5	589.5	697.7	216.0	393.7	378.1
	Effluent						371.0	381.3	157.9	41.3	10.9
27april2012	Influent	45.5	33.1	52.5	21.6	7.9	622.5	787.3	250.8	220.8	292.4
	Effluent				6.79		620.6	595.0	234.0	43.9	6.8
28april2012	Influent	48.0	37.3	69.6	31.9	11	681.1	928.5	228.0	292.7	309.6
	Effluent				15.41	3.45	419.8	420.7	139.7	42.4	16.1
29april2012	Influent	41.1	30.6	58.8	22.3	9.7	595.9	858.2	206.0	248.0	277.2
	Effluent				14.54		483.4	487.4	204.6	42.2	5.0
30april2012	Influent	30.4	21.4	42.1	20.2	7.6	646.7	713.6	206.6	311.5	334.2
	Effluent				7.8	2.69	451.2	431.6	152.2	37.9	11.5
1may2012	Influent	29.8	20.5	31.5	7.6	3.2	809.2	870.2	264.3	290.3	281.2
	Effluent						470.6	474.8	185.7	26.7	5.1

Table S10.4. Concentrations (ng/L) of drugs in Pinedo-II WWTP.

Day		AMP-E1	AMP-E2	MAMP	MDMA-E1	MDMA-E2	ATE-E1	ATE-E2	VEN	EPH-E1	PEPH-E2
17april2012	Inf.	30.7	17.8	42.7	9.4	2.6	934.0	973.2	316.9	356.0	341.3
	Eff.						431.8	544.1	249.3	25.0	26.3
18april2012	Inf.	31.4	18.6	43.4	11.1	3.0	801.7	824.0	303.6	348.1	234.6
	Eff.						385.8	486.9	207.2	30.4	25.6
19april2012	Inf.	27.6	16.8	45.7	13.9	3.8	858.3	965.3	387.5	327.5	464.3
	Eff.						632.2	719.9	266.9	33.0	25.5
20april2012	Inf.	33.1	20.5	54.3	18.8	4.9	1137.3	1145.6	287.1	294.6	336.9
	Eff.						871.8	880.2	390.3	34.5	31.4
21april2012	Inf.	46.0	28.1	58.3	29.8	8.5	995.7	1015.4	254.5	178.7	158.6
	Eff.				2.9		566.0	570.0	330.9	26.0	35.6
22april2012	Inf.	36.2	24.2	52.6	26.5	7.6	877.5	962.4	314.8	166.4	199.6
	Eff.						395.6	414.8	308.7	27.6	29.0
23april2012	Inf.	31.5	19.6	47.3	17.2	5.1	918.3	928.5	256.6	24.13	231.0
	Eff.						657.4	699.0	354.3	35.2	35.4
24april2012	Inf.	28.5	18.7	41.9	13.5	4.2	967.2	941.5	363.9	265.1	195.6
	Eff.						945.9	1083.4	347.7	40.8	48.4
25april2012	Inf.	26.6	18.1	45.4	9.3	2.7	843.9	801.3	193.4	213.5	235.0
	Eff.						903.0	1032.6	293.6	32.1	29.7
26april2012	Inf.	30.5	18.5	43.8	12.5	3.7	1059.2	1152.3	310.6	241.6	185.9
	Eff.						464.4	509.8	230.0	28.2	22.6
27april2012	Inf.	36.3	21.3	49.0	15.9	4.2	1163.8	1275.3	212.5	150.1	136.3
	Eff.						649.6	739.6	257.2	31.0	32.1
28april2012	Inf.	50.9	31.6	52.4	25.1	7.3	1248.5	1263.7	195.8	237.1	214.1
	Eff.				3.1		532.0	698.5	262.4	32.7	26.4
29april2012	Inf.	39.9	26.0	48.5	11.8	3.5	1152.3	1127.0	299.8	243.7	212.0
	Eff.						497.6	640.8	258.6	28.2	33.3
30april2012	Inf.	33.5	20.3	43.7	12.3	3.8	1163.1	1157.6	201.2	208.4	231.9
	Eff.						451.0	588.7	240.4	30.4	25.9
1may2012	Inf.	29.0	18.5	44.5	10.3	2.9	1322.3	1191.1	218.7	196.0	226.7
	Eff.						754.4	1021.2	271.0	34.7	32.6

Table S10.5. Concentrations (ng/L) of drugs in Quart-Benager WWTP.

Day		AMP-E1	AMP-E2	MAMP	MDMA-E1	MDMA-E2	ATE-E1	ATE-E2	VEN	EPH	PEPH
17april2012	Inf.						778.0	1136.5	270.6	174.3	182.9
	Eff.						430.7	458.3	321.2	22.1	6.9
18april2012	Inf.						613.4	1018.9	301.3	156.4	281.0
	Eff.						343.5	390.4	334.5	22.3	5
19april2012	Inf.						650.3	1034.5	255.9	294.7	263.0
	Eff.						508.4	517.6	367.9	25.6	5.1
20april2012	Inf.						949.3	1189.9	297.3	274.4	390.0
	Eff.						673.5	789.1	317.3	24.3	4.7
21april2012	Inf.						658.0	1075.7	323.0	483.1	381.3
	Eff.						415.6	454.9	305.8	20.7	4.9
22april2012	Inf.						606.6	1095.4	278.4	414.3	251.4
	Eff.						471.4	419.5	331.0	24.3	4.8
23april2012	Inf.						541.5	928.4	207.6	276.9	207.1
	Eff.						377.2	414.7	354.1	22.5	5.4
24april2012	Inf.						558.3	947.5	192.7	113.6	109.3
	Eff.						410.2	401.4	304.9	23.6	5.0
25april2012	Inf.						559.4	1072.3	240.2	283.5	150.5
	Eff.						558.5	584.0	358.5	25.6	8.6
26april2012	Inf.						481.2	913.7	300.4	475.1	318.6
	Eff.						562.4	605.5	314.7	24.8	5.8
27april2012	Inf.						684.9	1190.5	282.8	347.9	282.7
	Eff.						579.6	640.9	296.3	26.1	5.7
28april2012	Inf.						782.4	1188.2	243.4	218.3	197.3
	Eff.						406.5	395.8	372.0	23.3	4.3
29april2012	Inf.						584.9	946.2	216.8	156.8	141.4
	Eff.						434.3	443.8	331.4	23.1	4.2
30april2012	Inf.						634.8	1228.6	288.8	204.3	207.5
	Eff.						457.8	444.8	386.5	23.1	4.5
1may2012	Inf.						630.7	1212.8	293.2	271.0	398.0
	Eff.						302.3	312.7	326.2	25.4	5.2

SUMMARY OF RESULTS AND DISCUSSION

1. Development of analytical methodologies

1.1 Liquid chromatography tandem mass spectrometry determination

For the separation of legal and illegal drugs (LIDs) in environmental samples, GC and LC have been commonly reported. Since LIDs have usually a polar character, LC is more suitable for their analysis. Besides, some pharmaceuticals are thermolabiles, which may produce degradation and loss of signal response with GC. LC-MS/MS with electrospray ionization (ESI) was chosen as the technique for identification, quantification, and confirmation of all contaminants analyzed during this thesis. Mass spectrometry was operated in selected reaction monitoring (SRM) mode because using this mode, LODs and the chemical background noise produced by sample matrix is reduced. Even if matrix components co-eluted with analytes, they have different product ions that allow their distinction, providing a very good selectivity. To achieve an unequivocal confirmation of the substances, together with the retention time of the analyte in LC, two transitions were monitored for each analyte, obtaining the four identification points as required in the Commission Decision 2002/657/EC [European Union 2002] for confirmation of banned substances. Specific transitions chose for the analysis of tetracyclines (TCs), pharmaceuticals and illegal drugs and their retention times are showed in tables 2.1 3.2 and 4.2 respectively. In the case of TCs, chemical transformation to their 4-epimers may occur in tetracycline, oxytetracycline and chlortetracycline. These epimers share the same transitions with their isomeric compounds, differing only in their retention times (see table 2.1). This remarks the interest in a good chromatographic separation.

The optimization of MS and MS/MS parameters was carried out by infusing individual solutions of the analytes. Most of the compounds were analyzed in positive ionization (PI) mode. However, few compounds show more sensitive response in negative mode (NI). This is the case of cannabinoids (THC and THC-COOH), ibuprofen, diclofenac and clofibric acid. The response of cannabinoids in PI mode can be enough sensitive to undertake environmental analysis allowing to determine them together with the other abuse drugs. Ibuprofen only gives one transition with reasonable

sensitivity (see table 3.2), as reported by other authors [Jelic et al. 2009, Ferrer et al. 2010]. In negative ionization mode, the chromatographic separation of pharmaceuticals was very problematic despite the low number of compounds involved. These compounds have very different polarities (see values of Log K_{ow} in table 3.1). Diclofenac, the most non-polar compound, was strongly retained in the chromatographic column (see Fig.3.3 A). Besides, it showed a broad shape, being unacceptable to quantify. With other columns, the peaks appeared with crown (Fig.3.3 B), even adding a modifier, as ammonium acetate, in the mobile phase. Finally, separation was achieved changing the solvent utilized in the preconditioning of the column, using only acetonitrile instead of the mixture acetonitrile/methanol employed during the chromatographic separation (Fig.3.3 C).

One of the most problematic issues related with the use of ESI-MS analysis is the signal suppression or enhancement as a result of the other matrix components present in the sample. In water, signal suppression was observed for all the pharmaceuticals detected (see table 5.2). The level of suppression was greater than 10% for oxytetracycline, ofloxacin, fenofibrate, ciprofloxacin, norfloxacin, propanolol, sulfamethoxazole, carbamazepine, ibuprofen and clofibric acid, and greater than 20% for metoprolol and clofibric acid. In the pharmaceutical analysis of soil and sediments, absolute signal suppression measured for compounds analyzed under PI conditions varied from 3% to 54% in sediments and from 0.6% to 56% in soils, as it can be seen in Table 3.4. A slightly signal suppression was observed for metoprolol, codeine, trimethoprim and fenofibrate (<15% calculated using the absolute recovery). In the case of acetaminophen a little enhancement of signal was observed (≈3%) as already reported [Radjenovic et al. 2009]. For the other compounds, higher suppression (up to 55%) was observed. For the compounds analyzed under NI conditions, suppression ranged from 19% to 34% in sediments and from 15% to 31% in soils. The suppression effect was only partly corrected by the addition of internal standards since matrix effects are compound dependent.

In illicit drugs, relative signal suppression in water sample analysis ranged between 0 and 20% (see Table 4.3). Same as pharmaceuticals analysis, internal standards could not correct totally this effect. However, the use of matrix-matched

standards compensated quite well for the suppression effect attaining accurate quantification.

1.2 Solid sample extraction

Pharmaceuticals were extracted from sediments and soils by means of pressurized liquid extraction (PLE). In this technique, solid sample is dispersed with some agents (as diathomeus earth, silica gel, etc) and put it in a stainless steel cell. System use pressure, temperatures and several static extractions, collecting all fractions in a vial. It has the advantage of being fully automated, allowing up to 24 samples carried out without the attention of the analyst and using a minimal quantity of solvent per sample. PLE method developed for extraction of multi-class analysis of pharmaceuticals in soil and sediment was compared with an ultrasonic extraction published previously by Blackwell et al. [Blackwell et al. 2004]. This method is fast and simple, being also commonly utilized in pharmaceutical analysis [Hu et al. 2012**,** Xu et al. 2008, Minten et al. 2011].

1.2.1. PLE extraction for tetracyclines

Tetracyclines (TCs) strongly interact with di- and trivalent metals in the clay mineral interlayers or with hydroxyl groups at the surface of the soil particles. To avoid these undesirable effects and facilitate the extraction, soil was homogenizated with EDTA washed sand as dispersing agent, in order to chelate metals present in the sample. Different sorbents (alumina, Florisil® and basic and neutral silica) were tested, giving lowest recoveries, except silica, which provides similar results to sand. Other PLE parameters, as sample weight, solvents, pressure, temperature and number of solvent cycles were optimized. Water was selected as extractant solvent. The possible interconversion between TCs and their 4-epimeric forms with the high temperatures of the extraction procedure was checked by spiking different soil samples only with the TCs (without the epimer standards) but was not observed (see Fig. 2.3). After SPE purification and preconcentration, recoveries were in the range of 71 to 96%.

These results were compared with two published methods based on PLE, recently reported by Jacobsen et al. [Jacobsen et al. 2004] and O'Connor et al. [O'Connor et al. 2007] (see Fig 2.1). All of them provided acceptable and similar recovery levels of the studied TCs. The main advantages of the proposed procedure for the TCs extraction from soils are that it only uses water as extractant and the stability of the compounds through the extraction procedure. O'Connor et al. [2007] established that the percent recoveries of the optimized PLE method depended on soil type, and more specifically, on clay content. In the present study, the method was applied only to one type of soil because it is representative of more than 50% of the Spanish agricultural soils with 20% of clay (see characterics of the soil in Table S2.5). LODs and LOQs were in the range 1-3 and 3-10 µg/kg, respectively, which covers the expected range for environmental soil samples (see Table 2.2).

1.2.2. PLE extraction for multi-class pharmaceuticals

Once confirmed the goodness of the PLE method to TCs, it was extended to determine seventeen commonly used pharmaceuticals in soils and sediments. Solid sample was also grinded and homogenizated with EDTA washed sand, to improve the extraction recovery of FQs and TCs.

Methanol (MeOH), water, combinations of both solvents at different ratios (80:20 and 50:50, v/v), acetonitrile/water 50:50 (v/v), MeOH/57mM citric acid 50:50 (v/v) and MeOH/0.1M Na_2–EDTA 50:50 (v/v) were tested for the optimization of the PLE with different sorbents. Fig. 3.1 shows the recoveries obtained by extracting soil dispersed in Na_2–EDTA washed sea sand with water and mixtures of methanol–water and acetonitrile–water at 90 °C for 7 min at 500 psi and flush 100%. No great differences were observed in the recoveries provided by the different solvents. Water was selected as the best choice for its compatibility with SPE, and because it is an interesting solvent for ecological considerations.

As it can be seen in Fig. S3.5, extraction temperature has a great importance in the recovery efficiencies of some compounds, providing higher recoveries 90 °C and 110 °C. However, certain degradation at 110 °C was observed for some compounds, as

oxytetracycline, trimethoprim and clofibric acid. Remaining time of the sample in contact with the solvent has a relatively few importance in recoveries. From 5 min, a higher time does not led to higher recoveries. This is also the case of a high number of cycles. Three cycles increase considerably the extraction efficiency comparing with one, but five practically achieve same results than three, indicating that sample was almost exhausted. Flush volume (volume of fresh solvent, expressed as % of the cell volume) above 80% had little influence on recoveries, and pressure do not afect them.

In the optimized method, sample was introduced in stainless steel cell of 22 mL and heated to 90 °C with a static period of 7 min and extracted by a flush volume of 100% in three cycles using water as a solvent under a pressure of 500 psi. The water volume ending up in the glass vial was approximately 30 mL, which was concentrated and cleaned-up means of SPE.

Method detection (MDLs) and quantification (MQLs) limits ranged from 0.1 to 6.8 ng g^{-1} and from 0.25 to 23 ng g^{-1}, respectively. Relative recoveries ranged from 59% to 119% for soils and from 64% to 110% for sediments. Some soil and sediment samples were left to age in the dark, at room temperature, for a period of three months to check if recoveries were maintained or decreased (comparison is presented in Fig S3.6). There is no evident difference between values obtained by both spiking procedures either in soil or sediment. A qualitative difference was only observed for tetracycline because the percentage of its epimer increased at the expenses of that of the tetracycline.

PLE method was compared with an ultrasonic-based extraction method developed by Blackwell et al. [2004]. The performance characteristics for the majority of the 17 pharmaceuticals studied were acceptable for both methods. Comparison between both methods is illustrated in Fig. 5.2 via bar graphs for the obtained recovery data and in Table 5.3 via tabulated results for the MDLs, MQLs, matrix interferences and linearity. Overall, the analytical method provided a higher LOQ for ultrasonic extraction than for PLE. Absolute matrix effects were −2.6 to 54.6% for the PLE and between −6.8 and 69.3% for the ultrasonic extraction (positive number means suppression). The number of pharmaceuticals with MLQs lower than 10 ng/g and acceptable RSDs (<20%) is slightly higher for the PLE method than the ultrasonic one.

However, this is not only caused by the sensitivity of the LC-MS/MS detection method, which is slightly better for the PLE method because of the lower percentage of matrix effects, but also by the differences in recoveries. On the other hand, the speed and user-friendliness of the ultrasonic extraction in real practice are slenderly better than for the PLE method, despite the extra centrifugation step, which can be easily performed in batch. Figure 5.3 shows the LC-MS/MS chromatograms obtained from a soil sample (P10) extracted using PLE (Fig. 5.3a) or ultrasonic extraction (Fig. 5.3b). As can be observed, acetaminophen and carbamazepine were not detected using ultrasonic extraction because of the higher MDLs of this method.

1.3 SPE isolation of waters and PLE extracts from solid samples

Solid-phase extraction (SPE) is the selected technique for preconcentration of analytes and elimination of the matrix components, which might cause undesirable effects in the determination by LC-MS/MS, as a decrease of analytes ionizated or a high background noise. Different types of SPE cartridges are available, but for LIDs, Oasis HLB (polymeric sorbent) has turn into the cartridge of reference.

1.3.1 SPE for pharmaceuticals

Since dozens of methods were published before the beginning of this thesis to analyze pharmaceuticals in waters, the process SPE/clean-up used for water samples was based on that reported by Petrovic et al. [2006]. Briefly, 250 mL of water samples (pH neutral) were spiked with surrogate/internal standards, passed through an Oasis HLB cartridge and the cartridges were then rinsed with 5 mL of Milli-Q water. Analytes retained on the cartridge were eluted with methanol. The extract was evaporated under a gentle stream of nitrogen, reconstituted, and injected into the HPLC-MS/MS for analysis. Three parameters were optimized: the sample extraction volume, wash volume after extraction and the elution solvent. Three extraction water volumes were checked: 100 mL, 250 mL and 500 mL (see Fig. S5.4-A). 100 and 250 mL provided similar recoveries, but 250 mL was selected because it yielded better MDLs. Two cartridge wash volumes of water were tested (5 mL and 10 mL). For polar compounds,

the lower washing volume used was the better (see Fig. S5.4-B). This is consistent with the fact that the solvent used for washing is water, which will elute some of the polar compounds with it. The recovery of the target compounds by SPE is highly dependent on the polarity of the eluent. Acetone, dichloromethane, acetonitrile and methanol were tested. The results (see Fig. S5.4-C) show that dichloromethane produced the lowest recovery for most compounds (<50%). Better recoveries were obtained with acetone and acetonitrile as the elution solvents, with most varying between 60 and 105%. The best recoveries (80-100%) were achieved eluting with methanol. The method was validated and data are presented in Table 5.2. Linearity was determined using regression analysis between the area ratios and concentrations, giving correlations of R^2>0.99 for all compounds except for ibuprofen. MDLs and MQLs ranged from 0.3 to 10.0 ng L^{-1} and 0.9 to 36 ng L^{-1}, respectively. Recoveries achieved for all target compounds ranged from 71.2 to 97.8% and from 85.2 to 98.5% at MLQs and 10 times MLQs, respectively.

In the case of the aqueous PLE or ultrasonic extracts obtained after extraction from soils and sediments, clean-up was performed as for water samples, but instead of using a unique cartridge, a SAX one (strong anion exchange) was placed on top of the Oasis HLB cartridge. The former was removed just before the elution of the analytes. The SAX cartridge reduces matrix interferences by adsorbing anionic humic and fulvic acids from the soil extracts, avoiding contamination, blocking and overloading of the HLB sorbent. Aqueous extracts obtained from PLE have a pH of 7 and, in these conditions, pharmaceuticals are in their neutral or cationic form (see pKa values in Table 3.1) and, consequently, they are not retained on the SAX cartridge. The inclusion of the SAX cartridge only causes a marked decrease in the recovery of diclofenac (see Fig. 3.1-B). This could be because at pH 7, diclofenac is in zwitterionic form keeping partly retained to the negatively charged SAX cartridge or forming complexes with the organic matter that are retained in the SAX cartridge. In general, the coupling of the SAX cartridge increased the analyte's recoveries to values higher than 70%, except for fenofibrate, which remains unchanged. This effect may due to its relatively non-polar character (it has the lowest polarity of all selected pharmaceuticals), not being fully retained in the Oasis HLB [Stolker et al. 2004].

Performance of Oasis HLB was compared with other polymeric cartridge, Strata-X. As it is shown in Fig 3.1-B, their behaviors were very similar, achieving both recoveries better than 70%, except for ofloxacin, ciprofloxacin, norfloxacin and fenofibrate.

1.3.2 SPE for illicit drugs

A SPE method to analyze fourteen drugs of abuse, including three cocainics [benzoylecgonine (BECG), cocaine (COC), ecgonine methyl ester (ECGME)], four amphetaminics [amphetamine (AMP), methamphetamine (MAMP), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA or ecstasy)], five opiates [6-acetylmorphine (6ACMOR), codeine (COD), heroin (HER), morphine (MOR), methadone (MET)], and two cannabinoids [Δ^9-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-Δ^9-THC (THC-COOH)], in surface waters was developed and validated. Selected drugs presented a broad range of hydrophilicity (see log Kow values in Table 4.1). Performance of seven SPE cartridges (belonging to three different types) were compared and evaluated:

· (1) polymeric based on HLB (Oasis, Supelselect, Strata-X),

· (2) mixed cation exchanger and HLB (Oasis MCX, Strata-XC, Strata-XCW)

· (3) mixed anion exchanger and HLB (Oasis Wax).

Selected cartridges with 60 mg of sorbents were tested with 50 mL of spiked water (absolute recoveries are shown in Fig. 4.3). Oasis Wax (the mixed-mode weak anionic sorbent) and Strata-XCW provided lower recoveries, whereas HLB and mixed-mode HLB-cationic exchanger cartridges provided the best recoveries (33–108%). No difference in the extraction performance for the studied compounds was observed among the several trademarks of the same type of cartridges (Fig. 4.3).

Sample pH for application on the SPE cartridge was studied for both, Supelselect HLB and Strata-XC (mixed-mode cation exchanger) cartridges. The absolute recoveries obtained at different pH are shown in Figure S4.5. Recoveries obtained using Supelselect HLB were not very pH sensitive, except for ecgonine methyl ester (not recovered at pH 3), amphetamine and MDA (higher recovered at acidic pH).

The absolute recovery of the selected drugs of abuse was determined for a series of different sample volumes (50 and 250 mL) spiked with 10 ng of each analyte using cartridges of 60 and 200 mg of solid sorbent (see Table S4.5 for Oasis HLB and Strata-X and Strata-XCW). About a 10% decrease on the recovery was observed with the 60 mg cartridges from 50 to 250 mL water volume that was not for 200 mg cartridges.

Finally, Oasis HLB was selected because the best recovery of ECGME and the simplicity of the extraction method (HLB does not require pH adjustment). In the definitive method to be validated, a water volume of 250 mL and Oasis HLB (200 mg, 6 mL) SPE cartridges were selected.

An overview of the performance of the developed method is given in Table 4.3 considering the following parameters: linearity, precision, accuracy, LODs, and LOQs. Matrix-matched calibration curves prepared in surface water extracts showed good linearity with a correlation coefficient≥0.998. In general, recoveries (between 71% and 121% with RSDs<18%) were satisfactory for all the compounds. The worst recoveries were obtained for THC and THC-COOH (up to 60% and 67%, respectively). LODs and LOQs that were in the range of 0.01–1.54 ng/L and 0.03–5.15 ng/L.

2. Monitoring and risk assessment in L´Albufera of Valencia

2.1 Ocurrence of illegal drugs: levels and spatial distribution

A total of 16 samples of surface waters were collected on April 2008, covering the most important irrigation channels that flow into the lake of the Natural Park to establish spatial variations in drug occurrence. There were no rainfall events during the prior fortnight to the sampling. The distribution and location of the sampling points can be found in Fig. 4.1. Sampling points PM14, P1, PP8, PM11, and A2M9 were located along the Poyo Gully (also Chiva Gully or Torrente Gully). This gully runs and collects non-treated wastewater from the municipal areas of Torrente, Picaña,

Paiporta, Massanassa, and Catarroja, which floods finally into the L'Albufera lagoon. In the same area, points PM9 and PP1W were taken from channels with water outside the influence of this gully. The other sampling points were located in the downstream of several populations, such as Beniparrell, Silla, El Romani, Sollana, Sueca and Cullera, which have also contributions of wastewater treatment plants.

Table 4.4 summarizes the detection frequency and the levels measured in the 16 samples. All of them contained any of the drugs of abuse. Of the studied compounds, only six (6-acetylmorphine, heroin, methamphetamine, MDA and THC) were not detected. The most abundant and ubiquitous compounds were codeine, benzoylecgonine, and its precursor cocaine. Benzoylecgonine was present in all investigated surface water samples at levels up to 78.71 ng/L (see Table 4.4). Codeine, methadone, and MDMA were also positively identified in 37.5% of the investigated surface waters (including those samples where the compounds were detected <LOQ), with levels up to 51.62 ng/L, 0.84 ng/L, and 2.48 ng/L, respectively. The remaining detected compounds amphetamine, ecgonine methyl ester, morphine and THC-COOH, were present in less than 12.5% of the samples, at levels up to 11.70 ng/L. The low occurrence of THC (and its metabolite THC-COOH), which according to *European Monitoring Centre for Drugs and Drugs Addiction* is the most consumed drug in Europe and particularly in Spain, can be explained by the low tendency of these compounds to dissolve in water, since they have high partition coefficients (Log K_{ow}= 5,5-7) and tend to sorb into sediments, suspended material or organic matter.

The cumulative levels observed in the various samples collected in L'Albufera Natural Park are shown in Figure S4.6. Highest total concentration of drugs of abuse and metabolites, above 120 ng/L, were found in PM 6. This sample also presented the largest number of drugs of abuse (cocaine, benzoylecgonine, ecgonine methyl ester, amphetamine, MDMA, codeine, methadone, and morphine). This fact could be due to the direct spillage of sewage water from different night clubs and discotheques of the zone into the channel where the sample was taken. Figure 4.4 shows selected LC-MS/MS chromatograms from this surface water sample (PM6), presenting both quantification and confirmation transitions. The rest of surface waters from L'Albufera Natural Park presented total levels of the target analytes below 60 ng/L (most of them

below 20 ng/L) showing a fairly constant occurrence of these contaminants in the superficial waters of the natural park. In addition to PM6, five samples PM14, PP8, A2M9, P1, and PM11 are markedly more contaminated by drugs than the rest. It could be explained because all these samples were taken along the Poyo water course, which produces continuous wastewater discharges without debugging into the Albufera Lake. These non-treated wastewater samples come from several locations, which concentrates the major density of population (almost the 70% of the total population), industries, and leisure zones. It implies an increasing probability of input of these drugs in the sewage system and gutters that affects, in many cases, the irrigation channels and the marsh waters.

The observed contamination pattern and concentration of illicit drugs in surface water is comparable to those reported in similar monitoring studies carried out in surface waters from other European Countries, such as Belgium [Van Nuijs et al. 2009a, Gheorghe et al. 2008, Kasprzyk-Hordern et al. 2008a], Italy [Zuccato et al. 2008], Ireland [van Nuijs et al. 2009b], UK [Kasprzyk-Hordern et al. 2007], Poland [Kasprzyk-Hordern et al. 2007], and USA [Bartelt-Hunt et al. 2009]. These data also match the sparse studies carried out in surface waters from Spain, which are restricted to the Llobregat and Ebro Rivers Basins (located in Catalonia at the North of the Valencian Community) [Boleda et al. 2007, Postigo et al. 2010, Huerta-Fontela et al. 2008, Boleda et al.2009, Huerta-Fontela et al. 2007].

Based on the reported excretion values of cocaine, benzoylecgonine and ecgonine methyl ester after cocaine consumption (1–9%, 35–54%, 25–44%, respectively) and their molar mass relation, the excreted cocaine/benzoylecgonine ratio should be between 0.02 and 0.27. In the surface waters analyzed, the median value of the cocaine/benzoylecgonine ration was 0.12. However, in the sample PM2, the cocaine/benzoylecgonine ratio was higher than 1.00, which may indicate the direct disposal of the drug into the water. This finding has also been reported by other authors [Van Nuijs et al. 2009a, Postigo et al. 2010]. The presence of ecgonine methyl ester in surface waters was demonstrated for the first time, even though it was found in only one sample. The presence of this compound in wastewater across Belgium has already been reported [Van Nuijs et al. 2009b]. Morphine and codeine among the

natural opiates and methadone among the synthetic ones were found in some of the samples (Table 4.4). Codeine and methadone were the most abundant (six samples) being the codeine levels the highest ones. Morphine, found in two samples, may come from clinical use of morphine and codeine but it might also come from the illicit use of heroin. Finally, MDMA concentrations and frequency is higher than those of the amphetamine, which only appeared in one sample.

2.1.1 Spatial analysis with Geographical Information Systems (GIS).

To obtain a background on how such substances travel from urban and agricultural systems to the protected area, results from the illicit drugs determination were compared in the GIS environment.

Initial data consisted on (1) statistic information to municipal level on number of inhabitants and population density (inhabitants by square kilometre, h/km^2) for the year 2008 provide by the Spanish institute of Statistics; (2) an updated 2008 digital layer on land cover distribution from the CORINE project [Bossard et al. 2000]; (3) a digitized map with municipal boundaries; (4) a point map with the location of wastewater treatment plants (WWTPs); (5) a digital layer of the traditional irrigation systems (drainage networks and areas) as stated by Hermosilla Pla (2006 and 2007), and (6) 16 water samples spatially distributed over the Natural Park collected in field work campaigns during the year 2008 (see previous section 2.1).

From original tabular data and map layers GIS procedures were applied to derivate new map overlays (artificial covers such as urban and industrial surfaces) and to obtain two municipal spatial indexes, the integrate percentage of urban and industrial covers and the population density.

The geographical presence of illegal substances can be also explained by a combination of water path ways and multiuse. Residual water from urban areas, after being treated in WWTPs, is introduced in agricultural system for irrigation uses and finally drainage into the lake (figure 9.2). Traditional irrigation systems are vehicles to supply the presence of illegal substances into the Natural Park. So far a spatial relationship between population concentration and artificial surfaces with presence of

drugs cannot be stated, WWTPs drainage in the irrigation network has to be understood as the mean of illicit drugs presence in the Natural Park of l'Albufera.

2.2 Ocurrence of pharmaceuticals: levels and implications for the aquatic fauna

Sampling was carried out in April and October 2008 at the points marked in Fig. 5.1, together with their georeferences (UTM D50). Water and sediment samples were mainly from irrigation channels, whereas soil samples were taken in the neighbouring area from the superficial horizon.

Occurrence of pharmaceuticals in the water samples is shown in Table 5.4, and analysis of sediments and soils extracted by PLE is shown in Tables 5.5 and 5.6. Concentrations of carbamazepine and ibuprofen in water, soil and sediment are compared in the Figures S5.6 and S5.7. Water samples taken at points P3, P6, P9, P10 and P14 suffered an unfortunate accident and could not be analysed. Among the seventeen pharmaceuticals screened in surface waters from the L'Albufera Natural Park, thirteen (acetaminophen, carbamazepine, ciprofloxacin, codeine, diazepam, diclofenac, metoprolol, ofloxacin, propanolol, sulfamethoxazole, ibuprofen, clofibric acid and trimethoprim) were detected (Table 5.4). Tetracycline, oxytetracycline and fenofibrate were not present in water samples but they were in soil or sediment samples, and norfloxacin was not detected in any of the samples (Tables 5.4, 5.5, 5.6).

The fifteen water samples analysed were contaminated by pharmaceuticals. In these samples, carbamazepine was the substance most frequently detected (93% of the samples) with concentrations ranging up to 31.0 ng/L. A high presence of this drug was reported by other researchers too, with mean concentrations between 1 and 794 ng/L [Kim et al. 2007c, Gros et al. 2006, Moldovan et al. 2006, Conley et al. 2008]. Some studies confirmed that carbamazepine is not sorbed to sediments in an appreciable degree, thus it is not significantly biodegraded in wastewater treatment plants (WWTPs), and that it enters the environment in considerable amounts [Ternes et al. 2004]. Acetaminophen and ibuprofen were detected with frequency lower than 66%, but at higher mean concentrations of 1,204.4 ng/L and 289.9 ng/L, respectively. For ibuprofen, a significant removal in WWTPs is reported in the literature [Ternes et

al. 2004], and as a result of its low distribution constant value, the removal should be based on biodegradation. Sulfamethoxazole was detected in 60% of the samples at lower mean concentration of 27.3 ng/L. This frequency of positive samples and mean concentration is similar to those reported by other authors [Kim et al. 2007c], but differs from a few studies that only found some traces below the MLQ [Gros et al. 2006, Kasprzyk-Hordern et al. 2008b]. Of the other pharmaceuticals, diclofenac was found in six samples (40%), codeine, ofloxacin and propanolol in five (33%), ciprofloxacin, diazepam and clofibric acid in four (27%), trimethoprim in three (20%) and metoprolol in two (13%) with lower mean concentrations (Table 5.4). Figure S5.5 shows the LC-MS/MS chromatogram obtained for water sample P14 and illustrates the good performance of the analytical method for different pharmaceuticals.

These target compounds varied spatially, being detected at higher concentrations at P1, P2, P8, P11, P13 and P19. The highest concentrations for five out of the twelve detected compounds in surface water were found at site P8. This sample point is located near of the Albufera South WWTP (Fig. 5.1). However, this point is not connected to the irrigation channels that gather in the wastewater coming out of the WWTP. The high level of pharmaceuticals could be due to the direct dumping of sewage water in the small irrigation channels. In contrast, samples from P7, which is close to the system that drives the wastewater to the lake to maintain the ecological flow, do not show high concentrations of pharmaceuticals.

The second group of points, with high concentrations and frequency of pharmaceuticals, were the sites P1, P19, P13 and P11. These sites are mainly located parallel to the Poyo Gully, just where the pipes that carry purified water from the Pinedo WWTP to the little port of Catarroja (Portet de Catarroja) flow. This WWTP provides a constant flow of 1 m^3/s of treated water that arrives into L'Albufera lake through this point. L'Albufera Natural Park is the main recipient of water from the Pinedo WWTP. In particular, this WWTP injects 73 hm^3/year into the water system of L'Albufera Park irrigation network. This is the greatest single contribution to flows that the wetland receives, and it is fundamentally important for the natural ecosystem and irrigation of crops. The dissipation of pharmaceuticals through these points from the point P1, where the wastewater flows to the lake, was observed. Summarizing, highest

concentrations of the detected pharmaceuticals were mainly found in the sites located near the WWTP outflows. This distribution is reasonable because the main sources of these pharmaceuticals are effluents of sewage treatment plants.

Table 5.5 outlines the concentration of pharmaceuticals in sediments. Sediments were not available at the sampling points P4, P10, P17, P18 and P20. Pharmaceuticals were not detected in the sediments taken from points P3, P5 and P7.

Carbamazepine was detected in 73% of the samples, followed by ofloxacin and codeine (53%), propanolol and acetaminophen (47%), and ibuprofen (40%). When considering mean concentrations in sediment (15 samples), ibuprofen was the dominating compound (6.73 ng/g), followed by ofloxacin (2.56 ng/g), codeine (2.36 ng/g) and oxytetracycline (1.88 ng/g). As a result of the high distribution constant of oxytetracycline in both sandy and loam soils, it is expected to show strong sorption [Loke et al. 2002].

Mean concentrations of the pharmaceuticals in sediment samples were about one thousand times lower than in water for all the target compounds, but concentration patterns remained the same, i.e. carbamazepine was the dominating compound (Tables 5.4 and 5.5). However, diazepam and codeine occur mainly in sediments. Diclofenac, despite its high hydrophobicity, is not present in sediment and was only found in water samples. This fact was stated by other researchers [Pérez-Carrera et al. 2010, Buser et al. 1998] and occurs because diclofenac is rapidly metabolized by photodegradation and microflora of river sediments to its major metabolite 5-hydroxy-diclofenac [Varga et al 2010, Buser et al. 1998, Gröning et al. 2007]. Besides, in our case, this observation could also be explained because diclofenac is the compound worst extracted by the method.

In order to predict the distribution of a drug between a solid phase (sediment) and water, a number of different mechanisms involved in drug sorption have to be taken into account, such as sorption to organic matter, surface adsorption to mineral constituents, ion exchange, complex formation with metal ions and hydrogen bonding. However, most of these mechanisms are hard to calculate and usually, only the octanol/water partition coefficient (K_{ow}) is utilized to predict the behavior of drugs in water [Diaz-Cruz et al. 2003]. In this way, a compound with a high value of K_{ow} tends to

accumulate in soil or sediment. Fenofibrate, the compound with the highest K_{ow} (out of those studied), is only found in sediment and soils, and not in water. But this behaviour is not replicated in the case of other compounds with high K_{ow} like diclofenac (that was only found in water), ibuprofen and propanolol.

Table 5.6 shows that soil samples from P7, P8, P17 and P20 were not contaminated by the studied pharmaceuticals. Highest mean concentrations of the pharmaceuticals in the soil were observed in sample P13. The mean concentrations of the pharmaceuticals in soil were between 0.06 for propanolol and 2.5 ng/g for tetracycline. Of all compounds, acetaminophen showed the highest concentrations (16.05 ng/g), and carbamazepine the highest prevalence over soil locations, which reflects its high resistance to natural transformation processes such as adsorption and phototransformation. Diazepam was in six soils and in seven sediment samples. It is a lipophilic substance and showed a very low mobility in all types of soil. It can be expected that its leaching behaviour was mainly determined by the organic carbon content of the soils. An extensive transformation of diazepam in the soil is unlikely, because diazepam is widely stable in a water/sediment test under aerobic conditions, and transformation products might have shown certain mobility in the soil due their increased polarity [Oppel et al. 2004].

2.2.1 Environmental implications

The environmental risks to aquatic organisms were assessed by using the mean values and worst case scenario in L'Albufera Natural Park on the basis of the risk quotients (RQ) calculated using maximum measured environmental concentrations (MECs) of pharmaceuticals and predicted non-effect concentrations (PNECs) collected from the literature [46–49] (Table 5.7). Ecotoxicity data can be provided by the open scientific literature or by pharmaceutical companies. Only for clofibric acid and ibuprofen were the PNECs based on company-owned data. For the other PNEC values, data originating from standard tests were preferred, with long-term studies being prioritized over short-term ones. Although data from algae and fish species were indistinctly used, data from the baseset species, i.e. algae, crustaceans (Daphnia

magna or Ceriodaphnia dubia) and fish, were prioritized. According to the RQ classification scheme from Hernando et al. (2006), mean concentrations of acetaminophen, ciprofloxacin, propanolol and ibuprofen could pose a low risk to the aquatic organisms (RQ between 0.1 and 1). Mean concentrations of diclofenac and high concentrations of ciprofloxacin, propanolol, sulfamethoxazole and ibuprofen could pose a medium risk to the aquatic organisms (RQ higher than 1).

3. Monitoring and risk assessment in Pego-Oliva Marsh

3.1 Ocurrence of illegal drugs in waters of the Marsh

Surface waters were collected in Twenty-three points of the natural park area on June 11th, 2009 to establish spatial variations in drug occurrence. Samples were taken in the most important water channels and in the marsh, as well as one sample of tap water from a chalet of the Sierra the Segaria (W35) and one spring water (W1) “ullal de Bullent”. There were no rainfall events during the fortnight prior to the sampling. The distribution and location of the sampling points (UTM D50) are shown in Fig. 6.1. The concentration of illicit drugs in the Pego-Oliva Marsh and the data summary are presented in Table 6.3.

All the samples analyzed were contaminated by illicit drugs. Heroin, ecgonine methyl ester, MDA, amphetamine, metamphetamine and THC were not detected in any sample. The most frequently detected compounds were benzoylecgonine (74%) and its precursor cocaine (87%), MDMA (74%), ketamine (69.5% of the samples), morphine (39% of the samples), methadone (39%), 6-acethylmorphine (9%) and THC-COOH (4%). Our data were also compared with the previous studies available about some river and water bodies in Spain and other countries (Table 6.4). The levels of cocaine and of its main metabolite benzoylecgonine were up to 11.6 and 15.5 ng/L respectively. The mean value was 2.25 ng/L for cocaine and 1.92 ng/L for benzoylecgonine. As can be observed comparing Tables 6.3 and 6.4, the mean level of

cocaine in the present study was lower than those observed by other authors in surface waters of different rivers of Europe. Benzoylecgonine was also at lower levels than those reported in other studies [Baker and Kasprzyk-Hordern 2011; Boleda et al. 2007; Gheorghe et al. 2008; Lin et al. 2010; Postigo et al. 2010; Zuccato et al. 2008].

Pharmacokinetic studies carried out in human urine show that only 1-9% of the cocaine consumed is excreted unchanged, while 45% is excreted as benzoylecgonine and 40% as ecgonine methyl ester [Postigo et al. 2010]. Based on these values, the excreted cocaine/benzoylecgonine ratio should range from 0.02 to 0.27 [Boleda et al. 2007]. In our study, 11 of the 16 samples contained more cocaine than benzoylecgonine. This finding is supported by earlier studies [Baker and Kasprzyk-Hordern 2011; Boleda et al. 2007; Boleda et al. 2009; Bones et al. 2007; Gheorghe et al. 2008; Lin et al. 2010; Postigo et al. 2010; Vazquez-Roig et al. 2010a; Zuccato et al. 2008]. This discrepancy between theory and results might indicate either a faster degradation route for benzoylecgonine in certain environmental conditions or the direct spillage of the drug into the marsh. Bones et al. (2007) also speculate that this fact could be related to losses during the sampling filtering step. As benzoylecgonine exists in the water solutions (pH 7–7.8) as a neutral zwitterionic, whereas cocaine is presented in cationic form, there may be a bias resulting from sorption onto particulates. Ecgonine methyl ester, the other important human metabolite of cocaine, was not detected in the Pego-Oliva Marsh water. A probably explanation is that ecgonine methyl ester rapidly degrades to ecgonine, which was not monitored.

The new group of illicit drugs, which includes MDMA (or ecstasy) and ketamine, was frequently detected. The former was in 74% of analyzed samples at concentrations up to 3.40 ng/L. The mean value of 0.62 ng/L was similar to that observed in the rivers of Spain and Italy [Postigo et al. 2010; Zuccato et al. 2008] and much lower than that reported in the UK rivers [Baker and Kasprzyk-Hordern 2011; Zuccato et al. 2008] and in L'Albufera wetland (Valencia, Spain) [Vazquez-Roig et al. 2010a]. The MDMA has analogues such as MDA and is chemically related to the amphetamines (mainly amphetamine and metamphetamine). Surface waters of Spain, Italy, UK, Taiwan and Nebraska had reportedly important levels of metamphetamine,

amphetamine and MDA [Baker and Kasprzyk-Hordern 2011; Boleda et al. 2007; Castiglioni et al. 2008; Vazquez-Roig et al. 2010a; Zuccato et al. 2008].

Ketamine is also used in human medicine and very often in veterinary medicine for its anaesthetic and analgesic effects on cats, dogs, rabbits, rats and other animals, which could be a reason for contamination in this agricultural area. It has been poorly monitored in the world, because it is a new illicit drug. Its levels were between 0.12 and 414.92 ng/L, with a mean of 21.3 ng/L. The frequency, mean and maximum concentration of ketamine in the Pego-Oliva Marsh were higher than those reported in Spain and Taiwan [Boleda et al. 2007; Lin et al. 2010]. In Spain, Huerta-Fontela et al. (2008) reported that the levels of this drug were always below the LOQ in the Llobregat River. However, in Taiwan, ketamine was detected in significant quantities (until 341 ng/L) in river water [Lin et al. 2010].

Among the opiates, methadone and morphine were the most frequent. Methadone is the main clinical substitute for heroin addicts. It was in nine samples at concentrations between below limit of detection and 2.7 ng/L (mean level 0.32 ng/L), which is lower than those reported in other studies [Baker and Kasprzyk-Hordern 2011; Boleda et al. 2009; Vazquez-Roig et al. 2010a; Zuccato et al. 2008]. Morphine showed levels in the range of limit of detection and 8.3 ng/L (mean 1.30 ng/L). Previous studies reported similar results [Postigo et al. 2008; Vazquez-Roig et al. 2010a]. Morphine levels in surface waters can be attributed to its medical use or to the illicit use of heroin. Only in two samples, morphine coexists with 6-acethylmorphine, a specific minority metabolite of heroin in humans. However, it should also be taken into account that 6-acethylmorphine underwent further cleavage to morphine, which could explain better the low concentrations and frequency of 6-acethylmorphine compared to those of morphine than to assume the medical prescription as main source of morphine. The little frequency, or even the absence, of 6-acethylmorphine and heroin has been described in several studies [Baker and Kasprzyk-Hordern 2011; Boleda et al. 2007; Boleda et al. 2009; Lin et al. 2010; Postigo et al. 2010; Vazquez-Roig et al. 2010a; Zuccato et al. 2008].

THC, principal active substance of *Cannabis sativa*, was not detected in any sample. This is in accordance with other studies [Boleda et al. 2009; Postigo et al.

2010; Zuccato et al. 2008] and explainable because this substance is almost completely metabolized in the human organism and is only present at traces in urine. THC-COOH, its main metabolite, was found in only one sample, at the level of 1.54 ng/L. The lower frequency of this compound compared to other illicit drugs was already noted in the in Italy [Zuccato et al. 2008]. This substance presents worst recovery due to its high apolarity, which could explain the low number of positive samples.

The spatial distribution of the illicit drugs between the different points taken at the Pego-Oliva Marsh has also been studied. The cumulative results obtained are shown in Fig. 6.3. The observed contamination pattern of most illicit drugs and their metabolites is comparable to those reported in similar monitoring studies carried out in Spain [Boleda et al. 2007; Postigo et al. 2010; Vazquez-Roig et al. 2010a], UK [Baker and Kasprzyk-Hordern 2011; Zuccato et al. 2008], Ireland [Bones et al. 2007], Belgium [Gheorghe et al. 2008], Taiwan [Lin et al. 2010] and USA [Bartelt-Hunt et al. 2009]. However, this pattern differs in the absence of amphetamine-like compounds other than MDMA. Although several towns and housing states are relatively close to this humid area, it is difficult to establish the main sources of the illicit drugs or to propose accurate back-calculations of their use at community levels. That is because the marsh receives waters from different sources including the subterranean springs and the Gallinera, Molinell, Bullent and Racons rivers, which could bring contaminated waters from long distances. Concentrations of illicit drugs determined in surface water can also be greatly affected by natural transformations (photolysis, hydrolysis, biodegradation, etc.) that decrease their levels. The most contaminated sampling sites varied depending on the drug of abuse considered. Figure 6.4 shows the distribution of the drug of abuse in the different sampling sites. MDMA shows the maximum concentration at sampling site W109, which is located in the middle of the natural park, followed by W8, W103 and W5, which are nearest to the coast and to the area covered by hotels and discotheques. The highest concentration of ketamine is at point W101. The concentration at this point is 414 ng/L, highest than any other concentration reported before. It should be mentioned that the distribution of ketamine is different compared with the others, which could indicate that its utilization is not only related with their use as illicit drug but also with its use as

veterinary anaesthesic in livestock exploitations. The opioids showed up at point W106, which is a source of morphine, 6-acethylmorphine and methadone, are in the limits of the natural park and they appeared also at high concentration in the area of the reed beds. However, these opioids did not appear in the points nearest to the coast W8, W5 and W9. The cocainics appeared mainly in the points W105 and W106, which are also in the external limit of the marsh, followed by the points near the coast and located in the marsh, which also showed important concentrations.

Information on acute and, especially chronic, toxicity of illicit drug to aquatic organisms is very scarce. To our knowledge, there are no published aquatic ecotoxicological data for illicit drugs hence the use of in silico tools to predict toxicity is a pragmatic option. ECOSAR modelling provides acute predicted non-effect environmental concentration (PNECECO-SAR) data that ranges from 2.26 μg/L for methamphetamine to 9.83 μg/L for heroin but with a very high degree of uncertainty [Grung et al. 2007; Madden et al. 2009]. Illicit drug concentrations measured in surface waters are generally well below the PNECECO-SAR. Consequently, they would not be able to cause acute toxicity to aquatic organisms. However, illicit drugs enter the aquatic environment continuously leading to fairly constant environmental water concentrations [Grung et al. 2007]. Chronic exposure to illicit drugs has the potential for numerous subtle effects, such as metabolic or reproductive changes on nontarget organisms [Riehl et al. 2011; Sedore Willard et al. 2006; Stewart et al. 2011; Strehler et al. 2008]. Furthermore, the potential for synergistic or additive toxicity to aquatic organisms and/or other toxic effects, not yet studied, cannot be ruled out.

3.2 Ocurrence of pharmaceuticals and risk assessment

Samples were taken in June 11, 2009 to establish spatial variations in pharmaceuticals with occurrence from different points, covering all the extension of the Pego-Oliva marsh according to the distribution shown in Fig. 7.1. Sampling points were geolocated (UTM30, D50) (Table S7.4 shows the geolocation of each sampling point together with the type of samples taken). Thirty-four water samples (pH 7.2–8.8) were taken from the irrigation channels. One spring water (ullal bullent, sample W1)

and one tap water from a house (sample W35) were collected as well. Physico-chemical analysis of the samples (detailed data not shown) revealed a high salinity of the waters, reaching values up to 9438 ppm of NaCl, with an average value of 3677 ppm. The measure of the total dissolved solids (total quantity of inorganic and organic substances contained in the waters) shows values up to 6530 ppm (mean value was 2670 ppm).

Sixteen sediment were taken from irrigation channels approximately in the same place where the waters were sampled. Their textures were classified as sandy loamy.

Twenty three soil samples of the upper 0–15 and 15–50 cm depth layers were collected (Fig. 7.1). The soils of the area belong mainly to Calcic Fluvisols, Chromic Luvisols and Gleysols types. Mean value of organic matter content was 5.2%, with a maximum of 14.6% in sample 41B.

Table 7.1 outlines a summary of the maximum and mean concentrations found (the minimum was always below the limit of quantification), together with the frequency of each compound in the three matrices analyzed. Fig. 7.1 shows the total concentration of pharmaceuticals in the three matrices for each point. A complete list of the concentration of each pharmaceutical detected in each water, sediment and/or soil sample is shown in Tables S7.8, S7.9 and S7.10.

3.2.1. Water samples

All water samples contained at least one therapeutic drug (see Table S7.8). The occurrence pattern showed the highest levels and frequency for anti-inflammatories and analgesics. Ibuprofen (74% of the samples) and codeine (59%) were the compounds more frequently detected, at concentrations between LOD and 59 ng/L (mean 16.3 ng/L) and LOD and 63 ng/L (mean 16.7 ng/L), respectively. Acetaminophen was also detected at high frequency (41% of the samples) and with the highest levels, up to 112 ng/L. In contrast, diclofenac was only found in 3% of the samples (even though its acidic character favors its water solubility). This behavior has been widely explained in the literature by its high environmental degradation rate. Diclofenac is

efficiently photolyzed in surface water showing a 96% of degradation over a two-week period [Brun et al. 2006, Guillén et al. 2012]. The most detected antibiotics (up to 38% of the samples) were fluoroquinolones and sulphonamides. However, ciprofloxacin and trimethoprim were detected only in 3% of the samples. Tetracycline and oxytetracycline were not present in any sample, which can be a consequence of their high tendency of binding to organic matter and metals [Gros et al. 2010, Vazquez-Roig et al. 2010b].

Antidepressants (32% of samples), β-blockers (up to 35%) and lipid regulators (up to 32%) were also frequently detected. On this last lipid regulators class, fenofibrate was detected in 32% of the water samples whereas clofibric acid, the clofibrate metabolite, was detected only in the 3%. This is unexpected because fenofibrate has a high Log K_{ow} and is not ionizable, whereas clofibric acid is negatively charged at water pH (Log D_{ow} −0.98−1.96). Both, fenofibrate and clofibric acid are fairly persistent in the environment. This difference may reflect the different usages of these lipid regulators, as well as their excretion and metabolization rates.

The concentrations detected in this marshland were lower than those found in Ebro river basin (Spain) [Gros et al. 2006], rivers in the Valencian Community (Spain) [Gracia-Lor et al. 2011], Lake Erie (USA) [Wu et al. 2009] and similar rivers in South Korea [Kim et al. 2007a]. Data for comparison with other wetlands were only available for L'Albufera of Valencia (Valencia, Spain) [Vazquez-Roig et al. 2011] and the Doñana Park [Camacho-Muñoz et al. 2010]. Both wetlands showed levels considerably higher than in Pego-Oliva marsh. This could be related with the size of the surrounding population. Pego-Oliva receives pharmaceuticals from the Pego WWTP with a population served of 5600 inhabitants, while, for example L'Albufera de Valencia has contributions of pharmaceuticals mainly from Pinedo-2 WWTP with a population served of approximately 1 million inhabitants.

3.2.2. Sediment samples

In this study, all sediment samples, with the exception of the no. 45 were contaminated at levels higher than LODs (see Table S7.9). Due to the polar and often

ionic nature of pharmaceuticals, sorption to solid materials such as soil and sediment is based on ionic interactions and is pH dependent. It may not be appropriate to assess their lipophilicity based only on the K_{ow} value but based on the pH-dependent n-octanol-water partition coefficient D_{ow} [Ratola et al. 2012]. Data on Log K_{ow}, Log D_{ow} and pK_as are compiled in the Table S7.5).

Studying the tendency of the target compounds that remain in water or accumulate in sediments, three general tendencies could be extrapolated from the results. The presence of acetaminophen, carbamazepine, ciprofloxacin, trimethoprim and tetracycline in a greater number of sediments than in waters (or even the absence in this latter) may indicate a tendency of these compounds to be accumulated in sediments. Carbamazepine was detected in 16 samples and acetaminophen in 14. These compounds are not ionizable at the water pH but have low K_{ow} and relatively high water solubility. Their high prevalence was already reported in other studies [Guillén et al. 2012, López-Serna et al. 2012b, Vazquez-Roig et al. 2011]. Fluoroquinolones, such as ciprofloxacin, have two pK_a values within pH range relevant to this study (7–9) and exist mostly as zwitterions which favor their hydrophobicity [Aristilde and Sposito 2010]. Besides, fluoroquinolones complex strongly with many different cations, influencing their solubility. Tetracyclines have three pK_a values (see S7.5). Although at pH between 7 and 8 they will be negatively charged in water, their sorption to sediment is expected because of their high capacity of complexing with metals that also displace the acidic-base equilibrium [Furlong et al. 2003]. Opposite to this behavior, ibuprofen, metoprolol, ofloxacin, norfloxacin, popranolol, diclofenac, ibuprofen, fenofibrate, codeine, diazepam and sulfamethoxazole remain in waters. In fact clofibric acid, diclofenac, ibuprofen, and metoprolol were not present in any sediment sample. The behavior of clofibric acid, diclofenac, and ibuprofen can be explained because they are acidic pharmaceuticals present as anionic species at pHs 8-9 and of codeine, metoprolol, propanolol, diazepam and sulfamethoxazole because they are basic pharmaceuticals that will be positively charged at pHs 8-9. Therefore, sorption to the sludge is expected to be weak due to electrostatic repulsion from the charged functional groups in the sludge. Pharmaceuticals found in the sediments could remain mainly in the pore water and not necessarily be sorbed to sediments. However,

several studies report substantially higher concentrations of these pharmaceuticals in sediments than in pore water suggesting that sediments are an important reservoir for these compounds [Furlong et al. 2003, Ratola et al. 2012].

For fenofibrate, ofloxacin and norfloxacin no clear conclusion can be reached. They were not detected in sediments but they are expected to adsorb to suspended solids and sediments based upon the estimated D_{ow} or the zwitterionic state. In fact, the two fluoroquinolones, ofloxacin and norfloxacin, were widely detected in soil samples. Degradation and biodegradation could explain this anomalous behavior. The contact between pore water and sediments is an interface where many microorganisms can be responsible of biodegradation [Ginebreda et al. 2010]. Further studies in this sense are strongly required.

3.2.3. Soil samples

Presence of pharmaceuticals in soils was widespread and diffuse, probably as a consequence of the irrigation of large extensions with contaminated water. Only in samples 3A, 7B, 9A, 31A, 32B, 43A and 43B, pharmaceuticals were not detected. Acetaminophen was in 76% of the positive samples, at concentrations between LOD and 3.5 ng/g (see table S7.10). Fluoroquinolones were detected in Twenty-eight samples, confirming the reported tendency of these compounds to remain in soils, apparently absorbed by the clay–humic complexes, bounded with clays, or complexed with organic matter [Andreu et al. 2007, Aristilde and Sposito 2010]. Clofibric acid, diclofenac, fenofibrate, ibuprofen and tetracyclines were not detected in any sample. Ibuprofen, clofibric acid and diclofenac are anionic and highly polar remaining in water, but the fenofibrate and tetracyclines have high Log K_{ow}s and could expect a tendency to accumulate in soils. Fenofibrate was present in eleven samples, and its absence in soil samples only can be explained through effective degradation and transformation in its main metabolite (fenofibric acid), which was not monitored in this study.

Fig. 7.2. shows the mean concentration of pharmaceuticals in soils at different depths. Acetaminophen, carbamazepine, and metoprolol accumulate in the superficial layer of the soil, while codeine and fluoroquinolones show a tendency to infiltrate to

deeper layers. At soil pH (7-8), neutral and positively charge species will be in equilibrium for both types of compounds. Consequently, some of these substances could leach to ground water, spreading the contamination.

3.2.4 Spatial distribution

An irregular distribution of the pharmaceuticals can be observed in the map of Fig. 7.1 where the total concentration (sum of concentrations of water, soil and sediment) in each point is marked with a vertical bar. The total concentration between two nearby sampling points is very dependent of its conditions. Different infiltration rates in the sandy river-bed or an extra contribution or bifurcation of the channel could dilute or concentrate pharmaceuticals (changing concentration levels) of one point related to the closer one. Because of the large number of small channels in the marsh, looking for a regular pattern in the distribution of contaminants is an arduous job.

Pego-Oliva marsh is divided in two areas with different characteristics: a zone of lagoons (western part of the map of Fig. 7.1) and the rice fields. Sewage waters from nearby WWTP's are reutilized to irrigate the fields. Two pumping stations avoid that waters from the lakes (reedbirds) would be mixed with those from irrigation channels [Diputacion Provincial de Alicante, 1992]. Lagoon area presents low concentrations of pharmaceuticals (sampling points 4, 5, 30–33 and 38). Higher concentrations were detected in the rice fields (central sampling points) and in the area of the Racons basin (points 111 and 113–117), but with irregular levels. Highest concentration in point 114 could be due to a discharge of the WWTP El verger in the Racons river, since this WWTP is obsolete and its effluents flow just before this sampling point [Diario informacion, 2009]. Decrease in the levels of point 117 might be due to a dilution with the sea water that enters in the channel.

In the northern part (points 7, 8, 101–103, 107 and 108), an area of lower concentrations could be observed. This is an agricultural area devoted to citrus orchards and rice crops. The presence of several “ullals” (spring waters), close to the Bullent river, would explain this anomaly for dilution of the drugs present in the surface waters. Sample 105 is influenced by the surrounding houses without any

observable dilution effect from spring waters. In this residential area, the influence of human impact could be also observed in water from point 106. On the contrary, sample number 6, located at the top of the hill, showed markedly lower concentrations, probably because it receives less runoff water than lower parts. The presence of these anthropogenic compounds is clearly related with wastewater reuse to irrigate the crops and also with the direct disposal from residential areas close to the marsh. In sample 35 (tap water), codeine and ibuprofen were detected, and in sample1 (spring water) these pharmaceuticals were also found, together with acetaminophen and sulfamethoxazole.

3.2.5. Statistical analysis

Categorical Principal Component Analysis (PCA) was applied to the data obtained from water, soil and sediment samples for helping to evaluate the effect of location in the study area, land uses (citrus, rice, natural zone, urban or anthropized areas) and, in the case of soils, sample depth, as grouping factors between variables. Therefore, in the case of water samples the results only explain 55.38% of the variance. For soils, only in the surface horizons the results have certain validity, explaining 65.40% of variance. In this way, the main effect covering the major percentage of variance was the spatial location that was always related to Dimension 1, reaching the 43.36% for waters and 44.08% in soils (Fig. 7.3). For sub-surface soil samples and sediments PCA analysis was not significant.

In the case of waters, two clear behavioral groups were observed, the first one influenced by the spatial distribution in the studied area (Dimension 1), includes ofloxacin, fenofibrate, propanolol, metoprolol, diazepam and sulfamethoxazole; carbamazepine could be also included. The second group, more related to Dimension 2, includes acetaminophen, codeine, norfloxacin and trimethoprim. An extremely different behavior is observed for ibuprofen, which is far from both groups. In this sense, the majority of pharmaceuticals, and even their highest values appear in the east of the studied area, close to the WWTP or to the urban areas near the sea. The

second group of pharmaceuticals is mainly concentrated in the anthropized areas of the north-west.

PCA analysis of the soil surficial samples shows clearly two main groups, similar to that observed in water samples, one with propranolol, diazepam, ofloxacin and metoprolol that is related to their spatial distribution, and the second one, with low significance, includes acetaminophen and trimethoprim more affected by the land use of the sampling area (Dimension 2). Carbamazepine is not associated with any group, showing a high negative value in Dimension 1.

3.2.6 Risk assessment of the presence of pharmaceuticals in waters

In order to perform a more realistic evaluation of hazards in the Pego-Oliva marsh, long-term data were utilized always that possible to carry out the risk assessment, with both standard and non-standard organisms. Highest concentrations of pharmaceuticals in the water samples (to set in the worst-case scenario), PNEC values (together assessment factors used) and risk quotients (RQ) deemed for each analyte are shown in Table 7.2. According to these results, three drugs have a RQ higher than 1. Quinolones, ciprofloxacin and ofloxacin, showed RQ in algae of 6.9 and 3.1 respectively and 0.98 for NFX. Ibuprofen showed a RQ of 1.2 in fishes. Six week exposure assay to low concentrations of IBP resulted in changes in the pattern of reproduction of Japanese Medaka fishes [Flippin et al. 2007]. Less frequent reproduction but with a higher rate of fertilized eggs was observed. In fact, ibuprofen and other non-steroidal anti-inflammatory drugs are known to inhibit ovulation in mammals, including humans [Hernando et al. 2006].

A certain risk could be expected for those substances with a RQ between 0.1 and 1. In this group are the antibiotics sulfamethoxazole, which shows risk for bacteria, acetaminophen for the daphnid group, and diclofenac and propanolol for fishes. In the study conducted by Hoeger et al. (2005) structural damages in organs and a reduction in the number of red blood cells were observed in trouts after 21 days of exposure to diclofenac, as was previously related in other experiments [Schwaiger et al. 2004]. In a 4-week propanolol exposure, the total number of eggs produced by Medaka fishes,

and the number of viable eggs that hatched were decreased at concentrations as low as 0.5 µg/L [Huggett et al. 2002].

3.3 Ocurrence of heavy metals in water samples

Total content of seven heavy metals (Cd, Co, Cr, Cu, Ni, Pb and Zn) in 34 water samples taken were extracted by microwave acid digestion, according USEPA method 3052 [EPA 1996], and measured by Atomic Absorption Spectrometry with a Varian SpectrAA 220-FS with graphite furnace (model GTA-110).

The levels of the studied metals in the waters of the Pego-Oliva Park (Fig. 8.3) were well below the limits established by national or international legislations, not only for irrigation or livestock waters [Ayers et al. 1994, WHO 2006] but also for drinking waters [Council 1998, Decreto 2003, WHO 2008]. It is more evident for Zn, which show values <LODs. The average values of the remainder metals are in the range of 0.05-2.0 µg/L. The maximum value determined corresponded to Cu, with 19.10 µg/L, from an irrigation channel between the marsh and the orchards in the border of the coastal strip. Overall, the metals tended to concentrate in the abandoned fields that act as interface between the lake, with the surrounding rice fields, and the citrus crops.

Almost all heavy metals studied shown significant differences between water sources, except Cu and Zn (Figure 8.3a). The highest values of metals appear, generally, in the lake. However, Ni presented its maximum concentrations in the river waters, with highly significant differences regarding the other sources, and Cu in the irrigation channels. Meanwhile, for the studied metals the reedbed showed significant differences with the other uses, mainly for Cd, Cr and Ni. It has to be considered that the reedbed is a zone with strong effect of the redox processes due to the influence of the very variable water level, which is affected by the characteristic Mediterranean climate conditions.

3.3.1. Statistical analysis and relationship with pharmaceuticals

Statistical analyses including principal component analysis (PCA), analysis of variance (ANOVA) and Tukey's multiple range test at α=0.05 were performed. Pearson statistical bivariate correlation analyses were applied, at 95% and 99% significance levels, between pharmaceuticals and heavy metal concentrations to determine possible relationships among them.

Cd, Co, Cu and Ni showed significant differences on their concentrations regarding distance to the seashore (Fig 8.3c). Generally, the higher the level of metal, the shorter the distance to the sea, except for Co and Cr that gave their major levels in the middle zone (2-3.5 km) corresponding to the marsh and rice farming (Fig 8.3c).

Studying the possible influence among metals and pharmaceuticals, important significant correlations between Ni and ibuprofen, diazepam, norfloxacin, ofloxacin, trimethoprim and diclofenac, and inverse relationships to ibuprofen, at 99 and 95% of significance, were observed (Fig. 8.4). The multiple stepwise regression models, which are presented in Table 8.1, confirm the importance of Ni in these relationships. Cu, Co and Cr also showed significant correlations with ofloxacin, sulfamethoxazole, fenofibrate, metoprolol and propanolol. Graham et al. (2011) who studied the apparition of antibiotic resistance gene (ARG) in bacteria in the Almendrares river (Cuba) observed that high Cu and ampicillin and tetracycline levels most often correlate with detected ARG and also for Pb, Co, Zn, and tetracycline, although in a minor extent.

The important relationship between Ni and pharmaceuticals is validated also by the PCA analysis (Fig. 8.5a), in which the spatial distribution in the study area (factor 1) is the variable that explain not only this effect but also the differences to the other metals and ibuprofen. Fig. 8.5b shows PCAs f1 or metals only. Clear differences for the behavior of Ni regarding the other metals can be observed.

The spatial distribution of the higher levels of metals and pharmaceuticals (Fig. 8.6) showed a distinct distribution. Pharmaceuticals, except ibuprofen, and Ni present equal distribution occupying, mainly, the southeastern part of the study area. This part covers a zone of the coastal sand bar and the foot of the southern ridge, highly urbanized and with an important population. The other metals were mainly in the

center of the study area, which includes from the marsh, with the lake and rice fields, to the main road that limits with the coastal strip. Ibuprofen has its influence centered in the north-west of the study area, where two riding schools are located.

The spatial analysis of the data shows that the reedbed and the lake, which is strictly the marsh area, could be acting as sink and control point for the transport of pollutants, mainly of heavy metals. This is in accordance with that already reported for several natural wetlands [Mitsch et al. 1989, Verhoeven et al. 2006, Mander et al. 2009]. These differences in the compounds distribution could be due to the characteristics of the pollutants sources, and helps to discriminate the uses of the territory and the different aspects in which the human pressure could have a bearing on the environment.

CONCLUSIONS

According to the objectives of the present doctoral thesis, the research carried out and the results described in the previous chapters, the following conclusions can be outlined.

1. Simultaneous extraction of tetracyclines from soils by hot-water PLE, cleanup and preconcentration by SPE, and analysis by LC-ESI-MS/MS was successful. It provides similar results to other methods, but reducing the use of organic solvents and the attention of the analyst.

2. The method was effectively extended to seventeen pharmaceuticals, with great variety of polarities and pKa´s, which were simultaneously extracted from soils and sediments by PLE followed clean-up with SAX + Oasis HLB. Recovery rates for the selected compounds were higher than 70%, with the exception of fenofibrate (40%) and diclofenac (34%). The results for these compounds can only be considered semi quantitative.

3. The comparison of this method with other based on ultrasonic extraction developed by Blackwell et al. (2004) showed that the performance characteristics for the majority of the studied pharmaceuticals studied were appropriate and equivalent by both methods. However, the number of pharmaceuticals with lower limit of quantification, higher recovery and acceptable relative standard deviation was slightly higher for the pressurized liquid extraction method than for the ultrasonic extraction.

4. A SPE method to analyze drugs of abuse in surface waters was developed. After comparison of different SPE cartridges, Oasis HLB was chosen for its superiority in the compound recoveries (71–104%). Low limits of detection (0.01-1.54 ng/L) were achieved.

5. The application of this method to L'Albufera Natural Park and the Pego-Oliva marsh confirmed the presence of illicit drugs. Cocaine and metabolites, amphetamine and ecstasy, methadone, codeine, morphine, and 11-nor-9-

carboxy-Δ^9-tetrahydrocannabinol (main metabolite of cannabis) were quantified in L´Albufera water samples at levels ranging from less than 0.06 ng/L to 78.78 ng/L. Cocaine, benzoylecgonine and amphetamine-like compounds were also present in the Oliva Pego marsh but at low concentration than in L'Albufera. In the marsh, morphine, 6-acetylmorphine and methadone were detected sporadically. However, high levels and frequency of ketamine were observed.

6. Concentrations of illicit drugs were combined with the results of the spatial analysis with Geographical Information Systems (GIS) taking L'Albufera Natural Park as study case and confirming that the traditional irrigation system connected to sewage treatment plants could be a source of illicit substances to the waters of the Natural Park.

7. Water, soil and sediment samples of L'Albufera Natural Park were analyzed to monitor pharmaceuticals. Higher levels and frequency appear in the north area of the lagoon, where the higher human pressure is located. Concentrations of pharmaceuticals were higher in water than in sediments or soils. In the water samples, all sampling points analysed contain some of the studied pharmaceuticals, with values between 2.2 ng/L and 17.7 μg/L. In sediment samples, 12 of the 16 samples have some of the studied substances, with values ranging from 0.21 to 35.8 ng/g, and in soils between 0.24 and 16.05 ng/g.

8. Risk assessment for the aquatic fauna of the L'Albufera water samples was performed calculating the hazard quotients at mean and maximum concentration of each pharmaceutical. Mean concentration of diclofenac determined in all the Natural Park denotes a high risk to the aquatic organisms. In the most contaminated points, acetaminophen, ciprofloxacin, propanolol, diclofenac, sulfamethoxazole and ibuprofen could also pose a high risk to the aquatic organisms.

9. A similar study was conducted in Pego-Oliva Marsh, where all selected pharmaceuticals, excepting oxytetracycline were also detected. Contamination by pharmaceuticals not only affects surface waters but also ground and tap waters. Sediment samples were contaminated with substances detected in waters, highlighting their behavior like reservoir of hydrophobic compounds. Soils irrigated with contaminated waters have also shown the presence of pharmaceuticals previously detected in surface waters. Some of these are seeped into the deeper horizons, reaching the ground waters.

10. The risk assessment performed for the aquatic environment revealed that ibuprofen presents a high risk for fishes and fluoroquinolones for algae. The concentrations of diclofenac and propranolol involve a medium risk for fish, and acetaminophen and sulfamethoxazole for daphnids and algae, respectively.

11. Furthermore, in this marsh, possible relation between pharmaceutcals detected and heavy metals (Cd, Co, Cr, Cu, Ni and Pb) was studied, showing a strong correlation between Ni and the presence of pharmaceuticals.

12. Of all compounds investigated in wastewater samples from Valencia, MDA, MDEA and norephedrine were not detected. Atenolol and venlafaxine were detected in all samples at the highest concentrations. Amphetamine, methamphetamine and MDMA presented the lowest levels, being practically not detected in effluent samples. Loads of illegal drugs increased during weekend days. The stereospecific degradation of LIDs depends on the characteristics of each wastewater treatment plant and substance.

13. The presence of pharmaceutical and illicit drugs in surface waters indicates the continuous discharge of these substances from the wastewater treatment plants, due to their incomplete removing efficiency for these compounds. The monitoring of these emerging compounds is extremely important to assure the quality of the aquatic fauna, but also our drinking water.

RESUMEN EN CASTELLANO

El rápido incremento de la población urbana ha traído asociado una intensa presión en el medioambiente circundante. Actualmente, la franja costera en la mayoría de países europeos es el área que ha experimentado un mayor desarrollo socioeconómico. Algunas pinceladas obtenidas de distintas fuentes dan una idea global de la importancia del crecimiento demográfico de estas zonas. Hoy en día, 3 mil millones de personas –alrededor de la mitad de la población mundial- viven a menos de 200 kilómetros de la costa. Dos quintas partes de las ciudades con poblaciones de entre 1 y 10 millones están localizadas cerca de la costa. En Bélgica, Portugal y España, la densidad de población en los 10 kilómetros más próximos a la línea costera es el doble que en las zonas del interior. Alrededor de 70 de los 455 millones de ciudadanos de la Unión Europea (incluyendo los nuevos Estados miembros), esto es, el 16% de la población vive en municipios costeros, aunque la costa representa sólo el 11% del total de la superficie de la Unión Europea. La costa mediterránea de España, junto con la de Irlanda, sufre el crecimiento poblacional más rápido de Europa, incrementándose más del 50% en la última década. Además 1,7 millones de casas en España, la mayoría localizada a lo larga de la costa, son segundas residencias.

Durante muchos años, la gestión de estas áreas ha sido focalizada en satisfacer las necesidades de la creciente población, en detrimento de los ecosistemas. En estos últimos años, los contaminantes emergentes, como medicamentos y drogas de abuso, han centrado la atención de los científicos, ya que son buenos indicadores de éste desarrollo antropogénico, en cuanto que sus concentraciones aumentan a la par que la población. En el medio ambiente, éstas sustancias y sus efectos tóxicos potenciales son un área prioritaria de investigación dentro de la química medioambiental [Richardson et al. 2011]. Un mejor conocimiento de la incidencia y el destino final de los medicamentos vertidos al medioambiente ayudará a una correcta evaluación del riesgo en las cuencas hidrográficas, humedales y otros ecosistemas relacionados. La Fig. A1 muestra como estos contaminantes llegan al medioambiente, principalmente a través de su excreción por la orina de humanos y animales, y de los tratamientos realizados en acuicultura. Los medicamentos y drogas de abuso son ubicuos en efluentes de depuradoras así como en aguas superficiales y potables [Ferrer et al. 2010, Pailler et al. 2009].

Las aguas residuales son reconocidas mundialmente como una de las principales fuentes de contaminación medioambiental, incrementándose los vertidos durante las tres últimas décadas. En muchos casos, estas aguas residuales no son procesadas apropiadamente, bien por una falta de plantas depuradoras o por una sobresaturación de las mismas. Esta última situación es típica en la Comunidad Valenciana (España), donde hay un enorme incremento de la población durante el verano debido al turismo. Además, esta temporada coincide con una época de escasez de lluvias, que pone en jaque la calidad del agua. Un volumen de agua insuficiente hace la dilución y la canalización de estos contaminantes más difícil [Ginebreda et al. 2010) y como consecuencia de esto, los contaminantes llegan a las aguas naturales, suponiendo una amenaza para el medio acuático.

Esta circunstancia es particularmente critica en las capas estáticas de agua en lagos y humedales que cubren el litoral valenciano, ya que estas áreas protegidas juegan un papel importante como reserva de agua, en el control de las inundaciones, recarga de acuíferos, etc. [EEA 2012]. Además, estos humedales protegidos son hábitats de una gran biodiversidad, siendo puntos clave para las aves migratorias. El incremento de la presión humana y el desarrollo socio-económico han causado, durante el siglo XX, la desaparición de la mitad de los humedales del mundo, amenazando seriamente la futura conservación de estos ecosistemas.

Según pudimos comprobar al comenzar ésta tesis, la incidencia de estos contaminantes en humedales naturales no había sido estudiada con anterioridad ni tampoco se había procedido a la determinación simultánea de estas sustancias en los tres compartimentos medioambientales: agua, suelo y sedimento; solamente unos pocos estudios analizaban simultáneamente agua y sedimentos [Yang et al. 2011, Silva et al. 2011, Kim et al. 2007a] o agua y suelos [Tso et al. 2011, Raich-Montiu et al. 2007]. Al abordar esta tesis, ya existía un cierto número de publicaciones sobre la determinación de medicamentos y drogas en el agua [Kasprzyk-Hordern et al. 2008a, González-Mariño et al. 2010, López-Serna et al. 2013]. Los métodos propuestos se basaban en la extracción en fase sólida (SPE) o microextracción en fase sólida (SPME) para el enriquecimiento de los analitos, y cuantificación mediante cromatografía líquida acoplada a la espectrometría de masas en tándem (LC-MS/MS) o derivatización

seguida de cromatografía de gases acoplada a la espectrometría de masas (GC-MS). Sin embargo, los métodos para la extracción y cuantificación de medicamentos a niveles traza en matrices sólidas (ej. suelos y sedimentos) eran escasos. Una posible razón para la falta de métodos analíticos para suelos y sedimentos es la complejidad de sus interacciones con medicamentos o drogas, las cuales pueden tener, dependiendo del pH, carga neutra, catiónica, aniónica o anfótera. Por tanto, sus propiedades físico-químicas tales como Log K_{ow}, absorción y adsorción a sólidos o su degradación puede cambiar con el pH. Más aún, las interacciones específicas (intercambio catiónico, puentes catiónicos, complexación superficial, quelación de metales, etc.) pueden enlazar estos contaminantes con la materia orgánica y arcillas, haciendo de los sedimentos y/o suelos un depósito de estos compuestos (ej.: fluoroquinolonas y tetraciclinas) y dificultando enormemente su extracción. Esto corrobora la necesidad de desarrollar nuevos métodos analíticos para estudiar éstas matrices.

Teniendo en cuenta ésta situación, el objetivo global de ésta tesis doctoral fue dar una visión general de la calidad de las aguas de algunos parques naturales de la Comunidad Valenciana, tratando de descubrir si la presencia de medicamentos y drogas supone un riesgo para la fauna acuática de estas áreas protegidas.

Por tanto, la metodología que se uso en la elaboración de esta tesis fue:

1. Desarrollo y validación de metodologías analíticas basada en PLE y extracción en fase sólida seguida de cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS) para la determinación de medicamentos de distintas clases terapéuticas y drogas de abuso en muestras de agua, suelo y sedimento.

2. Estudiar las concentraciones, distribución y destino de estos contaminantes antropogénicos en humedales costeros mediterráneos de la Comunidad Valenciana (L´Albufera de Valencia y el Marjal de Pego-Oliva), tratando de determinar el origen y si hay algún patrón detrás de distribución espacial de estos compuestos.

3. Evaluar la amenaza que suponen estos compuestos para la fauna acuática, basado en los datos toxicológicos disponibles (determinados en laboratorios o estimados por ordenador) de medicamentos y drogas. Los organismos diana pertenecían a los tres diferentes niveles de la cadena trófica, para obtener una imagen completa del potencial impacto de estas sustancias para el medio acuático.

Para esto, la tesis ha sido dividida en diez capítulos. El capítulo 1 es una introducción general, que trata varios aspectos. Primeramente se considera el estado actual de la metodología analítica, dando una visión global de las técnicas más novedosas y ventajosas para el análisis de medicamentos y drogas en matrices medioambientales sólidas y líquidas, discutiendo los factores a tener en cuenta cuando se crean y optimizan estos métodos. Se dan ejemplos de las principales aplicaciones, y se detallan las perspectivas de futuro. Para concluir este capítulo, se esboza la importancia de los medicamentos y drogas como contaminantes medioambientales haciendo hincapié en los estudios de toxicidad, evaluación del riesgo y legislación aplicable.

Los capítulos del 2 al 10 presentan el trabajo experimental realizado durante el doctorado, recopilado en forma de publicaciones, el cual fue planificado y diseñado para conseguir los objetivos propuestos.

En el capítulo 2, se desarrolla y valida un método para determinar simultáneamente cuatro tetraciclinas (TC, OTC, CTC y DC) en suelos, usando extracción con PLE y SPE seguido de LC-MS/MS con triple cuadrupolo (QqQ). Este fue el punto de inicio de esta tesis porque las tetraciclinas están fuertemente enlazadas al suelo ya que tienen varios grupos funcionales ionizables a pHs medioambientales típicos [Jacobsen et al. 2004]. Pueden existir como cationes, aniones, o iones mixtos, lo cual complica la predicción de su comportamiento frente a la adsorción-absorción, disponibilidad y transporte. El método fue aplicado a muestras de suelos de diferentes localizaciones y despúes de diferentes tratamientos con lodos de depuradora. Esta fue la primera vez que se detectaron residuos de tetraciclinas en suelos agrícolas españoles.

En el capítulo 3, se desarrolla y optimiza el método desarrollado en el capítulo anterior para la determinación de 17 medicamentos (β-bloqueantes, antidepresivos, antiepilépticos, analgésicos, antinflamatorios no esteroideos, reguladores de lípidos y antibióticos) en suelos y sedimentos.

En el capítulo 4, se desarrolló un método para analizar 14 drogas y algunos de sus metabolitos en aguas. Un objetivo de éste estudio fue ampliar los tipos de sorbentes sólidos que se utilizan para la extracción. Para ello se evaluaron 7 fases sólidas de distinta composición y distinta marca comercial. Se valoraron entre otros

parámetros, el pH de la muestra para la extracción, la cantidad de sorbente sólido y el volumen de agua. Otro objetivo de este trabajo fue evaluar, por primera vez, la incidencia de drogas en aguas superficiales del parque natural de L´Albufera. Estos datos complementan y amplían los escasos estudios llevados a cabo en este area y en aguas superficiales de otras zonas de nuestro país.

En éste parque natural, se implantó un programa de monitorización para conocer la incidencia de medicamentos en el mismo. Los resultados se presentan en el capítulo 5, que estudia la distribución espacial de medicamentos en aguas, suelos y sedimentos de los canales de L´Albufera y sus alrededores. En este protocolo, se utilizó SPE para aislar y concentrar los contaminantes del agua, seguido de LC-MS/MS mientras que para suelos y sedimentos, los resultados de la extracción obtenidos mediante PLE fueron comparados con otro método basado en la extracción por ultrasonidos. Las condiciones de la PLE (agente dispersante, disolvente de elución, tiempo de permanencia, número de ciclos) se basaron en aquellos utilizados en capítulo anterior.

En el capítulo 6, se monitorizo la presencia de drogas en aguas del Marjal de Pego-Oliva para entender su estado de contaminación y recomendar medidas para el control, reducción y eliminación de los vertidos de estos compuestos. Los resultados de este estudio son de valor no solo para el control de la polución en el Marjal, sino también para garantizar la seguridad del agua potable.

En el capítulo 7 se monitorizaron medicamentos en el Marjal, y se llevó a cabo una evaluación del riesgo derivado de la presencia de medicamentos en sus aguas. El estudio cubrió un total de 34 aguas, 17 sedimentos y 23 suelos, cubriendo los diferentes usos medioambientales del terreno (agricultura, anidamiento de aves, etc.), característicos de este humedal. El estudio intenta establecer el origen de estos contaminantes, su distribución y destino en el Marjal de Pego-Oliva, para determinar su patrón de distribución espacial y evaluar el riesgo para el medioambiente acuático en base a los datos toxicológicos crónicos disponibles.

En el capítulo 8 se analizaron metales pesados en aguas del Marjal de Pego-Oliva, correlacionando su presencia con las concentraciones de medicamentos en los mismos puntos de muestreo. Sinergias entre contaminantes, tales como

medicamentos y metales pesados han sido, hasta el momento, escasamente estudiados y pueden ser relevantes por su amplia distribución, no solamente en aguas sino también en suelos y sedimentos.

El capítulo 9 está focalizado en el desarrollo de una metodología combinada basada en principios forenses medioambientales para identificar drogas y determinar espacialmente su origen e implicaciones. Para ello se trató de identificar un patrón en función de la distribución de la población, los sistemas de riego tradicionales y su conexión con plantas depuradoras de aguas como vehículo para la introducción de sustancias ilícitas en el parque natural de L´Albufera.

Finalmente, el capítulo 10 fue desarrollado en la Universidad de Bath, y se focaliza en ampliar el conocimiento de estos contaminantes en su origen, los vertidos de depuradora, a través de su cantidad y proporción entre sus isómeros. Para ello se analizaron mediante cromatografía quiral aquellos compuestos que tienen enantiómeros, es decir anfetamina, metanfetamina y efedrina. La obtención del perfil enantiomérico demuestra ser una valiosa prueba para verificar si la presencia de estos compuestos proviene de su uso como estupefacientes o si proviene del metabolismo de otras drogas de abuso.

De acuerdo a los objetivos de la presente tesis doctoral, la investigación llevada a cabo y los resultados descritos en los capítulos previos, se concluye lo siguiente:

1. La extracción simultanea de tetraciclinas de suelos por medio de Extracción Liquida Presurizada con agua a alta temperatura, preconcentración y purificación por medio de Extracción en Fase Sólida y determinación por cromatografía liquida acoplada a la espectrometría de masas en tándem masas resultó apropiada y oportuna. Este método proporciono resultados similares a otros ya descritos en la literatura, pero reduciendo el uso de disolvente orgánicos y la atención del analista.

2. El método se amplió con elevada efectividad a la determinación de diecisiete medicamentos, con una gran variedad de polaridades y propiedades acido-base, que se extrajeron simultáneamente de suelos y sedimentos por

Extracción Liquida Presurizada seguida de purificación con cartuchos SAX y Oasis HLB. Las recuperación fueron mayores del 70% excepto para el fenofibrato (40%) y el diclofenaco (34%), para los cuales sólo se pudieron obtener resultados semicuantitativos.

3. El método desarrollado se comparó con un método de extracción por ultrasonidos desarrollado por Blackwell et al. (2004), mostrando que las características de ambos métodos para la mayoría de medicamentos fueron satisfactorias, sin embargo el método de Extracción con Líquidos Presurizados proporciono mayores recuperaciones y menores límites de cuantificación que el método de ultrasonidos.

4. Se desarrolló un método de Extracción en Fase Sólida para analizar drogas de abuso en aguas. Después de la comparación de diferentes cartuchos, los Oasis HLB fueron elegidos por proporcionar mejores recuperaciones (71–104%) y limites de detección bajos, entre 0.01 y 1.54 ng/L.

5. El método desarrollado se aplicó al análisis de las aguas del Parque Natural de L'Albufera (Valencia, España). Cocaína y sus metabolitos, anfetamina, éxtasis, metadona, codeína, morfina y 11-nor-9-carboxi-Δ^9-tetrahidrocanabinol (metabolito principal del cannabis) se cuantificaron en aguas de L'Albufera a concentraciones entre 0.06 ng/L y 78.78 ng/L. Cocaína, benzoilecgonina y derivados de la anfetamina también estaban presentes en el Marjal de Pego-Oliva pero a concentraciones inferiores a las halladas en L'Albufera. En el marjal, morfina, 6-acetilmorfina y metadona se detectaron esporádicamente y en cambio se observó una elevada frecuencia y altos niveles de ketamina.

6. Las concentraciones de drogas de abuso determinadas en el Parque Natural de L'Albufera se combinaron con los resultados del análisis espacial con el Sistema de Información Geográfica (SIG) confirmándose que los sistemas de riego

tradicionales conectados a las plantas de tratamiento residual es la vía de llegada al medioambiente de estos compuestos.

7. Se monitorizo la presencia de fármacos en muestras de aguas, suelos y sedimentos del parque natural de L'Albufera. Los mayores niveles y frecuencia de estos compuestos se detectó en el área norte del lago donde la densidad de población es mayor. Las concentraciones en agua fueron más bajas que en suelos y sedimentos. En las muestras de agua, todos los puntos del muestreo contenían algunos de los medicamentos estudiados, con valores comprendidos entre 2.2 ng/L y 17.7 µg/L. En muestras de sedimentos, 12 de las 16 muestras tomadas estaban contaminadas por alguna de las sustancias estudiadas, con valores entre 0.21 y 35.8 ng/g, y en suelos entre 0.24 y 16.05 ng/g.

8. Con los resultados del estudio de medicamentos en L'Albufera se hizo una evaluación del riesgo que supone la presencia de estos compuestos para la fauna acuática. En los puntos más contaminados, acetaminofen, ciprofloxacino, propanolol, diclofenaco, sulfametoxazol e ibuprofeno podrían presentar un riesgo alto para los organismos acuáticos. Teniendo en cuenta los valores medios de las concentraciones en todos los puntos, sólo el diclofenaco posé un riesgo alto para la vida acuática.

9. Un estudio similar se realizo en el marjal de Pego-Oliva donde, a excepción de la tetraciclina, se detectaron también todos los medicamentos seleccionados. La contaminación por medicamentos no afecta solamente a aguas superficiales, sino también a las aguas subterráneas y al agua potable del grifo. Las muestras de sedimentos estaban contaminadas con sustancias detectadas en aguas, poniendo de manifiesto su comportamiento como reservorio de compuestos hidrofóbicos. Los suelos regados con aguas contaminadas también mostraron la presencia de medicamentos detectados previamente en las aguas. Algunos de estos compuestos son filtrados a capas más profundas del suelo, alcanzando las aguas subterráneas.

10. La evaluación del riesgo que estas concentraciones de medicamentos poseen para el medio acuático revela alto riesgo para los peces por la presencia de ibuprofeno, y para algas por fluoroquinolonas. Diclofenaco y propanolol suponen un riesgo medio para peces, acetaminofén para dáfnidos, y sulfametoxazol para algas.

11. Además en este Marjal de Pego-Oliva, se estudió la posible relación entre medicamentos y metales pesados (Cd, Co, Cr, Cu, Ni y Pb), mostrando una fuerte correlación entre el níquel y los medicamentos y la presencia de fármacos a mayores concentraciones en las zonas costeras.

12. De todas las sustancias analizadas en estaciones depuradoras de aguas residuales de Valencia, no se detectaron MDA, MDEA y norefredrina. Atenolol y venlafaxina fueron detectados en todas las muestras y con las concentraciones más altas. Anfetamina, metamfetamina y MDMA presentaron los niveles más bajos, siendo practicamente no detectados a la salida de las depuradoras. Hay un incremento del consumo durante el fin de semana. La degradacion estereoespecífica de drogas y medicamentos depende de cada sustancia y de las características de cada depuradora.

13. La presencia de medicamentos y drogas en aguas superficiales indican la continua descarga de estas sustancias desde las depuradoras, debido a una incompleta eliminación de estos compuestos. La monitorización de estos compuestos emergentes es extremadamente importante para asegurar la calidad de las aguas y la seguridad de la fauna acuática, pero también de nuestra agua potable.

RESUM EN VALENCIÀ

El ràpid increment de la població urbana ha portat associat una intensa pressió en el mediambient circumdant. Actualment, la franja costanera en la majoria de països europeus és l'àrea que ha experimentat un major desenvolupament socioeconòmic. Algunes pinzellades obtingudes de distintes fonts donen una idea global de la importància del creixement demogràfic d'estes zones. Hui en dia, 3 mil milions de persones -al voltant de la mitat de la població mundial- viuen a menys de 200 quilòmetres de la costa. Dos cinquenes parts de les ciutats amb poblacions d'entre 1 i 10 milions estan localitzades prop de la costa. A Bèlgica, Portugal i Espanya, la densitat de població en els 10 quilòmetres més pròxims a la línia costanera és el doble que en les zones de l'interior. Al voltant de 70 dels 455 milions de ciutadans de la Unió Europea (incloent els nous Estats membres), açò és, el 16% de la població viu en municipis costaners, encara que la costa representa només el 11% del total de la superfície de la Unió Europea. La costa mediterrània d'Espanya, junt amb la d'Irlanda, patix el creixement poblacional més ràpid d'Europa, incrementant-se més del 50% en l'última dècada. A més 1,7 milions de cases a Espanya, la majoria localitzada a llarg de la costa, són segones residències.

Durant molts anys, la gestió d'estes àrees s'ha focalitzat a satisfir les necessitats de la creixent població, en detriment dels ecosistemes. En estos últims anys, els contaminants emergents, com a medicaments i drogues d'abús, han centrat l'atenció dels científics, perquè són bons indicadors d'este desenvolupament antropogènic, ja que les seues concentracions augmenten al mateix temps que la població. En el medi ambient, estes substàncies i els seus efectes tòxics potencials són una àrea prioritària d'investigació dins de la química mediambiental [Richardson et al.. 2011]. Un millor coneixement de la incidència i el destí final dels medicaments abocats al medi ambient ajudarà a una correcta avaluació del risc en les conques hidrogràfiques, aiguamolls i altres ecosistemes relacionats. La Fig. A1 mostra com estos contaminants arriben al mediambient, principalment a través de la seua excreció per l'orina d'humans i animals, i dels tractaments realitzats en aqüicultura. Els medicaments i drogues d'abús són ubics en efluents de depuradores així com en aigües superficials i potables [Ferrer et al. 2010, Pailler et al. 2009]. Les aigües residuals són reconegudes mundialment com una de les principals fonts de contaminació mediambiental, havent-se incrementats els

abocaments durant les tres últimes dècades. En molts casos, estes aigües residuals no són processades apropiadament, bé per una falta de estacions depuradores o per una sobresaturació de les mateixes. Esta última situació és típica a la Comunitat Valenciana (Espanya), on hi ha un enorme increment de la població durant l'estiu a causa del turisme. A més, esta temporada coincidix amb una època d'escassetat de pluges, que posa en escac la qualitat de l'aigua. Un volum d'aigua insuficient fa la dilució i la canalització d'estos contaminants més difícil [Ginebreda et al. 2010) i com a conseqüència d'açò, els contaminants arriben a les aigües naturals, suposant una amenaça per al medi aquàtic.

Esta circumstància és particularment critica en les capes estàtiques d'aigua en llacs i aiguamolls que cobrixen el litoral valencià, ja que estes àrees protegides juguen un paper important com a reserva d'aigua, en el control de les inundacions, recarrega d'aqüífers, etc. [EEA 2012]. A més, estos aiguamolls protegits són un hàbitat d'una gran biodiversitat, sent punts clau per a les aus migratòries. L'increment de la pressió humana i el desenvolupament socioeconòmic han causat, durant el segle XX, la desaparició de la mitat dels aiguamolls del món, amenaçant seriosament la futura conservació d'estos ecosistemes.

Segons vam poder comprovar al començar esta tesi, la incidència d'estos contaminants en aiguamolls naturals no havia sigut estudiada amb anterioritat ni tampoc s'havia procedit la determinació simultània d'estes substàncies en els tres compartiments mediambientals: aigua, sòl i sediment, i només uns pocs estudis analitzaven simultàniament aigua i sediments [Yang et al. 2011, Silva et al. 2011, Kim et al. 2007a] o aigua i sòls [Tso et al. 2011, Raich-Montiu et al. 2007]. A l'abordar esta tesi, ja existien un cert nombre de publicacions sobre la determinació de medicaments i drogues en l'aigua [Kasprzyk-Hordern et al. 2008a, González-Mariño et al. 2010, López-Serna et al. 2013]. Els mètodes proposats es basaven en l'extracció en fase sòlida (SPE) o microextracció en fase sòlida (SPME) per a l'enriquiment de l'anàlit, i quantificació per mitjà de cromatografia líquida acoblada a l'espectrometria de masses en tàndem (LC-MS/MS) o derivatització seguida de cromatografia de gasos acoblada a l'espectrometria de masses (GC-MS). No obstant això, els mètodes per a l'extracció i quantificació de medicaments a nivells traces en matrius sòlides (ex. sòls i sediments)

eren escassos. Una possible raó per a la falta de mètodes analítics per a sòls i sediments és la complexitat de les seues interaccions amb medicaments o drogues, les quals poden tindre, depenent del pH, càrrega neutra, catiònica, aniónica o amfòtera. Per tant, les seues propietats fisicoquímiques com ara Log Kow, absorció i adsorció a sòlids o la seua degradació pot canviar amb el pH. Més encara, les interaccions específiques (intercanvi catiònic, ponts catiònics, complexació superficial, quelació de metalls, etc.) poden enllaçar estos contaminants amb la matèria orgànica i argiles, fent dels sediments y/o sòls un depòsit d'estos compostos (ex.: fluorquinolones i tetraciclines) i dificultant la seua extracció. Açò corrobora la necessitat de desenvolupar nous mètodes analítics per a estudiar estes matrius. Tenint en compte esta situació, l'objectiu global d'esta tesi doctoral va ser donar una visió general de la qualitat de les aigües d'alguns parcs naturals de la Comunitat Valenciana, tractant de descobrir si la presència de medicaments i drogues suposa un risc per a la fauna aquàtica d'estes àrees protegides.

Per tant, la metodologia que s'usa en l'elaboració d'esta tesi va ser:

1. Desenrotllament i validació de metodologies analítiques basada en PLE i extracció en fase sòlida seguida de LC-MS/MS per a la determinació de medicaments de distintes classes terapèutiques i drogues de d'abús en mostres d'aigua, sòl i sediment.

2. Estudiar les concentracions, distribució i destí d'estos contaminants antropogènics en aiguamolls costaners mediterranis de la Comunitat Valenciana (L´Albufera de València i la Marjal de Pego-Oliva) , tractant de determinar l'origen i si hi ha algun patró darrere de distribució espacial d'estos compostos.

3. Avaluar l'amenaça que suposen estos compostos per a la fauna aquàtica, basat en les dades toxicològiques disponibles (determinats en laboratoris o estimats per ordinador) de medicaments i drogues. Els organismes diana pertanyien als tres diferents nivells de la cadena tròfica, per a obtindré una imatge completa del potencial impacte d'estes substàncies per al medi aquàtic.

Per a açò, la tesi ha sigut dividida en deu capítols.

El capítol 1 és una introducció general, que tracta diversos aspectes. Primerament es considera l'estat actual de la metodologia analítica, donant una visió

global de les tècniques més noves i avantatjoses per a l'anàlisi de medicaments i drogues en matrius mediambientals sòlides i líquides, discutint els factors a tindre en compte quan es creguen i optimitzen estos mètodes. Es donen exemples de les principals aplicacions, i es detallen les perspectives de futur. Per a concloure este capítol, s'esbossa la importància dels medicaments i drogues com contaminants mediambientals fent insistència en els estudis de toxicitat, avaluació del risc i legislació aplicable.

Els capítols del 2 al 10 presenten el treball experimental realitzat durant el doctorat, recopilat en forma de publicacions, el qual va ser planificat i dissenyat per a aconseguir els objectius proposats.

En el capítol 2, es desenvolupa i valida un mètode per a determinar simultàniament quatre tetraciclines (tetraciclina, oxitetraciclina, clortetraciclina i doxiciclina) en sòls, usant extracció amb PLE i SPE seguit de LC-MS/MS amb triple quadrupol (QqQ) . Este va ser el punt d'inici d'esta tesi perquè les tetraciclines estan fortament enllaçades al sòl ja que tenen diversos grups funcionals ionitzables a pHs mediambientals típics [Jacobsen et al. 2004]. Poden existir com a cations, anions, o ions mixtos, la qual cosa complica la predicció del seu comportament enfront de l'adsorció-absorció, disponibilitat i transport. El mètode va ser aplicat a mostres de sòls de diferents localitzacions i després de diferents tractaments amb fangs de depuradora. Esta va ser la primera vegada que es van detectar residus de tetraciclines en sòls agrícoles espanyols.

En el capítol 3, es desenrotlla i optimitza el mètode desenrotllat en el capítol anterior per a la determinació de 17 medicaments (β-bloquiants, antidepressius, antiepilèptics, analgèsics, antinflamatoriis no esteroideos, reguladors de lípids i antibiòtics) en sòls i sediments.

En el capítol 4, es va desenrotllar un mètode per a analitzar 14 drogues i alguns dels seus metabòlits en aigües. Un objectiu d'este estudi va ser ampliar els tipus de sorbents sòlids que s'utilitzen per a l'extracció. Per a això es van avaluar 7 fases sòlides de distinta composició i distinta marca comercial. Es van valorar entre altres paràmetres, el pH de la mostra per a l'extracció, la quantitat de sorbent sòlid i el volum d'aigua. Un altre objectiu d'este treball va ser avaluar, per primera vegada, la

incidència de drogues en aigües superficials del parc natural de L´Albufera. Estes dades complementen i amplien els escassos estudis duts a terme en este tema i aigües superficials d'altres zones del nostre país.

En este parc natural, s'implanta un programa de monitorització per a conéixer la incidència de medicaments en el mateix. Els resultats es presenten en el capítol 5, que estudia la distribució espacial de medicaments en aigües, sòls i sediments dels canals de L´Albufera i els seus voltants. En este protocol, es va utilitzar SPE per a aïllar i concentrar els contaminants de l'aigua, seguit de LC-MS/MS mentres que per a aigües, sòls i sediments, els resultats de l'extracció obtinguts per mitjà de PLE van ser comparats amb un altre mètode basat en l'extracció per ultrasons. Les condicions de la PLE (agent dispersant, dissolvent d'elució, temps de permanència, nombre de cicles) es van basar en aquells utilitzats en capítol anterior. En el capítol 6, es monitoritza la presència de drogues en aigües de la Marjal de Pego-Oliva per a entendre el seu estat de contaminació i recomanar mesures per al control, reducció i eliminació dels abocaments d'estos compostos. Els resultats d'este estudi són de valor no sols per al control de la pol·lució en la Marjal, sinó també per a garantir la seguretat de l'aigua potable.

En el capítol 7, es van monitoritzar medicaments en la Marjal, i es va dur a terme una avaluació del risc derivat de la presència de medicaments en les seues aigües. L'estudi va cobrir un total de 34 aigües, 17 sediments i 23 sòls, cobrint els diferents usos mediambientals del terreny (agricultura, nidificació d'aus, etc.), característics d'este aiguamoll. L'estudi intenta establir l'origen d'estos contaminants, la seua distribució i destí en la Marjal de Pego-Oliva, per a determinar el seu patró de distribució espacial i avaluar el risc per al mediambient aquàtic basant-se en les dades toxicològiques crònics disponibles.

En el capítol 8 es van analitzar metalls pesats en aigües de la Marjal de Pego-Oliva, correlacionant la seua presència amb les concentracions de medicaments en els mateixos punts de mostratge. Sinergies entre contaminants, com ara medicaments i metalls pesants han sigut, fins al moment, escassament estudiats i poden ser rellevants per la seua àmplia distribució, no sols en aigües sinó també en sòls i sediments.

El capítol 9 està focalitzat en el desenrotllament d'una metodologia combinada basada en principis forenses mediambientals per a identificar drogues i determinar espacialment el seu origen i implicacions. Per a això es va tractar d'identificar un patró en funció de la distribució de la població, els sistemes de reg tradicionals i la seua connexió plantes depuradores d'aigües com a vehicle per a la introducció de substàncies il·lícites en el parc natural de L´Albufera.

Finalment, el capítol 10 (desenvolupat en la Universitat de Bath), està enfocat a entendre el comportament dels medicaments i drogues en les plantes de tractament d'aigües residuals a través del seu anàlisi quiral. El mesurament dels nivells d'aquestes substàncies, abans i després del tractament, tenen un valor incalculable en la comprovació de la contribució de les aigües residuals en la contaminació dels parcs naturals, així com la possible formació d'algunes substàncies per degradació dels seus metabòlits conjugats a les plantes de tractament. A més, el perfil enantiomèric dels LIDs ens permet saber si les plantes de tractament estan degradant-los enantioselectivament, ja que la toxicitat d'aquests compostos és diferent generalment entre els isòmers.

D'acord amb els objectius de la present tesi doctoral, la investigació duta a terme i els resultats descrits en els capítols previs, es conclou el següent:

1. L'extracció simultanieja de tetraciclines de sòls per Extracció Liquida Pressuritzada amb aigua a alta temperatura, preconcentració i purificació per mitjà d'Extracció en Fase Sòlida i determinació per cromatografia liquida acoblada a l'espectrometria de masses en tàndem masses va resultar apropiada i oportuna. Este mètode proporciona resultats semblants a altres ja descrits en la literatura, però reduint l'ús de dissolvent orgànics i l'atenció de l'analista.
2. El mètode es va ampliar amb elevada efectivitat a la determinació de dèsset medicaments, amb una gran varietat de polaritats i propietats acido-base, que es van extraure simultàniament de sòls i sediments per Extracció Liquida Pressuritzada seguida de purificació amb cartutxos SAX i Oasi HLB. Les recuperació van ser majors del 70% excepte per al fenofibrato (40%) i el diclofenaco (34%) , per als quals només es van poder obtindre resultats semiquantitatius.

3. El mètode desenvolupat es va comparar amb un mètode d'extracció per ultrasons desenrotllat per Blackwell et al. (2004), mostrant que les característiques d'ambdós mètodes per a la majoria de medicaments van ser satisfactòries, no obstant això el mètode d'Extracció amb Líquids Pressuritzats proporciona majors recuperacions i menors límits de quantificació que el mètode d'ultrasons.
4. Es va desenvolupar un mètode d'Extracció en Fase Sòlida per a analitzar drogues d'abús en aigües. Després de la comparació de diferents cartutxos, els Oasis HLB van ser triats per proporcionar millors recuperacions (71-104%) i limites de detecció baixos, entre 0.01 i 1.54 ng/L.
5. El mètode desenvolupat es va aplicar a l'anàlisi de les aigües del Parc Natural de L'Albufera (València, Espanya) . Els resultats van confirmar la presència de cocaïna i els seus metabòlits, amfetamina, èxtasi, metadona, codeïna, morfina i 11-nor-9-carboxi-Δ^9-tetrahidrocanabinol (metabòlit principal del cànnabis) es van quantificar en mostres d'aigües de L'Albufera a nivells en el rang de 0.06 ng/L a 78.78 ng/L. Cocaïna, benzoilecgonina i derivats de l'amfetamina també estaven presents en la Marjal de Pego-Oliva però a concentracions inferiors a les trobades en L'Albufera. En la marjal, morfina, 6-acetilmorfina i metadona es van detectar esporàdicament i en canvi es va observar una elevada freqüència i alts nivells de ketamina.
6. Les concentracions de drogues d'abús determinades en el Parc Natural de L'Albufera es van combinar amb els resultats de l'anàlisi espacial amb el Sistema d'Informació Geogràfica (SIG) confirmant-se que els sistemes de reg tradicionals connectats a les plantes de tractament residual és la via d'arribada al mediambient d'estos compostos.
7. Es monitoritza la presència de fàrmacs en mostres d'aigües, sòls i sediments del parc natural de L'Albufera. Els majors nivells i freqüència d'estos compostos es va detectar en l'àrea nord del llac on la densitat de població és major. Les concentracions en aigua van ser més altes que en sòls i sediments. En les mostres d'aigua, tots els punts del mostratge contenien alguns dels medicaments estudiats, amb valors compresos entre 2.2 ng/L i 17.7 µg/L. En

mostres de sediments, 12 de les 16 mostres preses estaven contaminades per alguna de les substàncies estudiades, amb valors entre 0.21 i 35.8 ng/g.

8. Amb els resultats es va fer una avaluació del risc que té la presència d'estos compostos en L'Albufera per a la fauna aquàtica. En els punts més contaminats, acetaminofen, ciprofloxacin, propanolol, diclofenac, sulfametoxazol i ibuprofé podrien presentar un risc alt per als organismes aquàtics. Tenint en compte els valors mitjans de les concentracions en tots els punts, només el diclofenac va suposar un risc alt per a la vida aquàtica.
9. Un estudi semblant es realitza a la marjal de Pego-Oliva on, a excepció de la tetraciclina, es van detectar també tots els medicaments seleccionats. La contaminació per medicaments no afecta només aigües superficials, sinó també a les aigües subterrànies i a l'aigua potable de l'aixeta. Les mostres de sediments estaven contaminades amb substàncies detectades en aigües, posant de manifest el seu comportament com a reservori de compostos hidrofòbics. Els sòls regats amb aigües contaminades també també mostraren la presència de medicaments detectats prèviament en les aigües. Alguns d'estos compostos són filtrats a capes més profundes del sòl, aconseguint les aigües subterrànies.
10. L'avaluació del risc que estes concentracions de medicaments posseïxen per al medi aquàtic revela alt risc per als peixos per la presència d'ibuprofé, i per a algues per fluoroquinolones. Diclofenac i propanolol suposen un risc mitjà per a peixos, acetaminofén per a dáfnids, i sulfametoxazol per a algues.
11. A més en esta Marjal de Pego-Oliva, es va estudiar la possible relació entre medicaments i metalls pesants (Cd, Co, Cr, Cu, Ni i Pb) , mostrant una forta correlació entre el níquel i els medicaments i la presència de fàrmacs a majors concentracions en les zones costaneres.
12. De totes les substàncies analitzades en estacions depuradores d'aigües residuals de València, no es van detectar MDA, MDEA i norefredrina. Atenolol i venlafaxina van ser detectats en totes les mostres i amb les concentracions més altes. Anfetamina, metamfetamina i MDMA van presentar els nivells més baixos, sent *practicamente no detectats a l'eixida de les depuradores. Hi ha un

increment del consum durant el cap de setmana. La degradació estereoespecífica de drogues i medicaments depèn de cada substància i de les característiques de cada depuradora.

13. La presència de medicaments i drogues en aigües superficials indiquen la contínua descàrrega d'estes substàncies des de les depuradores, a causa d'una incompleta eliminació d'estos compostos. La monitorització d'estos compostos emergents és extremadament important per a assegurar la qualitat de les aigües i la seguretat de la fauna aquàtica, però també de la nostra aigua potable.

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INDEXES

INDEX I: List of tables

CHAPTER 1: Introduction

Table 1.1 Overview of off-line SPE methods applied to LIDs in waters. 22

Table 1.2 Overview of the on-line methods applied to pharmaceuticals in waters. 23

Table 1.3 Main methods utilized for the analysis of LIDs in solid samples. 24

Table 1.4 Mean concentration (ng/L) of pharmaceuticals monitored in wetlands and other protected areas. 29

CHAPTER 2: Analysis of tetracylines in soils

Table 2.1 LC-MS/MS conditions for confirming and quantifying the selected tetracyclines in soil samples 50

Table 2.2 Linear regression parameters of TCs from soil calibration curves ranging from 10 to 500 µg kg^{-1} (six points, triplicate analyses), LODs, and LOQs 53

Table 2.3 Recoveries and the relative standard deviations of the studied TCs in soil spiked at different concentrations 54

Table 2.4 Results of the investigation of 100 samples collected of the Ap horizon (0-20 cm) from 50 fields located in the South surrounding of Valencia city in February 2007 54

Table S2.5 Physico-chemical characteristics of the soil samples 58

CHAPTER 3: Analysis of pharmaceuticals in soils and sediments

Table 3.1 Therapeutical classes, chemical structures and relevant physicochemical properties of the studied compounds. 67

Table 3.2 Conditions of MS/MS in PI and NI modes. 70

Table 3.3 Linearity equation, method detection limits (MDL), repetitivity and reproducibility. 73

Table 3.4 Percent of signal suppression of pharmaceuticals in sediments and soils spiked after extraction. 73

Table 3.5 MQL (ng g^{-1}), recoveries (%) and RSDs at three spiking levels. 74

Table 3.6 Concentration (ng g^{-1}) of pharmaceuticals in different marsh areas of the Valencian Community..... 75

Table S3.7 Physical and chemical properties of the studied soils and sediments. 79

CHAPTER 4: Determination of illicit drugs in water from L´Albufera of Valencia

Table 4.1 Structures, CAS Numbers and physico-chemical data of the selected drugs of abuse 89

Table 4.2 Retention times (tr) and optimized SRM conditions used for LC-MS/MS analysis of illicit drugs in water 93

Table 4.3 Linearity, limits of quantification (LOQ), relative recovery (to ISs), matrix effects repeatability and reproducibility of the SPE and LCMS/MS method 96

Table 4.4 Concentration levels (in ng/L) of illicit drugs in water samples from the Natural Park of L´Albufera (Valencia, Spain) 98

Table S4.5 Absolute recoveries (in %) for Oasis HLB, Strata X and Strata-XCW cartridges with different sorbent mass and sample volume (matrix: L'Albufera superficial water and spiking level 50 ng/L)..... 103

CHAPTER 5: Determination of pharmaceuticals in L´Albufera of Valencia

Table 5.1 Retention time (Tr) of the pharmaceuticals, LC-MS/MS conditions in positive and negative ion mode and some physicochemical characteristics 113

Table 5.2 Linearity and detection and quantitation limits, absolute recovery, reproducibility and matrix effect of the method used to determine pharmaceuticals in water 115

Table 5.3 MDLs, MQLs and % of matrix effect obtained using PLE and ultrasonic extraction methods for soil samples 117

Table 5.4 Concentration of pharmaceuticals (ng L^{-1}) in waters samples from L'Albufera, Valencia, Spain..... 119

Table 5.5 Concentration of pharmaceuticals (ng g^{-1}) in sediment samples from L'Albufera, Valencia Spain 120

Table 5.6 Concentration of pharmaceuticals (ng g^{-1}) in soil samples from L'Albufera, Valencia, Spain..... 121

Table 5.7 Predicted no effect concentrations (PNECs), measured environmental concentrations (MECs) and risk quotients (RQs) (maximum MEC/ PNEC) of the detected pharmaceuticals in the L'Albufera Natural Park water samples..... 122

CHAPTER 6: Ocurrence of illicit drugs in Pego-Oliva Marsh

Table 6.1 Retention times, optimized SRM transition and conditions used for LC-MS/MS analysis and ratio between product ions of the target drugs of abuse 138

Table 6.2 Limits of detection and quantification, and relative recovery (to ISs) of the SPE and LC–MS/MS method to determine the target drugs of abuse..... 140

Table 6.3 Presence of the studied drugs of abuse in water samples of Pego-Oliva Marsh 141

Table 6.4 Illicit drugs concentrations in other water systems (in nanograms per liter) . 142

CHAPTER 7: Pharmaceuticals in Pego-Oliva Marsh: distribution and risk assessment

Table 7.1 Frequency, mean levels and maximum values found in Pego–Oliva. 157

Table 7.2 Maximum environmental concentrations (MEC) of pharmaceuticals in waters. PNEC and RQ for fish, daphnids (all species belonging to their trophic level) and algae (or bacteria) for the studied pharmaceuticals. 160

Table S7.3 Name, location and characteristics of the WWTPs close to Pego-Oliva Marsh 163

Table S7.4 Types of samples taken in each point with their georeferences (UTM 30, D50) 164

Table S7.5 Therapeutical class, chemical structure and relevant physicochemical properties of the studied compounds, including pKa, log K_{ow} and log D_{ow} at pHs 7 and 8. The D_{ow} was calculated according to the formula: 165

Table S7.6 Conditions of MS/MS in positive and negative ion mode. 168

Table S7.7 LOD of the method (ng g^{-1}), relative recoveries (%) and RSD´s of various pharmaceuticals at three spiking levels. 170

Table S7.8 Concentrations of drugs in water samples (ng/L) 171

Table S7.9 Concentrations of drugs found in sediment samples (ng/g). 172

Table S7.10 Quantification of the studied pharmaceuticals in soils (ng/g) 172

CHAPTER 8: Analysis of heavy metals in Pego-Oliva Marsh

Table 8.1 Multiple step-wise linear regression models between the studied pharmaceuticals through heavy metals (Y = B0 + B1X1 + B2X2 + …..) 202

CHAPTER 9: Relationship between irrigation systems and levels of illicit drugs in L´Albufera of Valencia

Table 9.1 Presence of illicit drugs (concentration levels in ng/L) in the sample points from the l´Albufera Natural Park 216

CHAPTER 10: Stereoisomeric profiling of drugs of abuse and pharmaceuticals in wastewaters

Table 10.1 Operational parameters on the studied WWTPs in 2012 225

Table S10.2 Optimised MRM conditions for the analysis of chiral drugs by HPLC-MS/MS. 240

Table S10.3 Average concentrations (ng/L) of drugs in Pinedo-I WWTP in 2012 241

Table S10.4 Concentrations (ng/L) of drugs in Pinedo-II WWTP 242

Table S10.5 Concentrations (ng/L) of drugs in Quart-Benager WWTP 243

INDEX II: List of figures

AIMS AND SCOPE

Figure A1 Sources and pathways of pharmaceuticals in the environment 8

CHAPTER 1: Introduction

Figure 1.1 Properties of the families of compounds studied in this review: a) Pka and b) partition coefficient octanol/water (expressed as Log P). 20

Figure 1.2 Chromatograms in positive ESI mode of a standard mixture of pharmaceuticals, A)137 transition analyzed in less than 5 min with UPLC (Ref. [15], with permission), and B) 146 transitions in 26 min with HPLC (Ref. [25], with permission). 28

Figure 1.3 Product ion mass spectrum of desmethylazithromycin, reported for the first time in the environment, as reported by Terzic et al. [63]. 29

CHAPTER 2: Analysis of tetracylines in soils

Figure 2.1 Efficiency of the PLE system using different extractants. Soil samples were spiked at 50 μg kg^{-1}, and the extracts were analyzed by LC-ESI-MS/MS without further cleanup step 51

Figure 2.2 Efficiency on the SPE cleanup using different SPE platforms at two different concentrations, 1.25 and 12.5 μg l^{-1} 51

Figure 2.3 LC-MS/MS chromatograms of soil spiked at 10 μg kg^{-1} of each tetracycline including the internal standard. A) TIC of the ten precursor → product ion transitions reported in Table 2.1 , B) mass chromatogram for DC (445 → 428 transition), C) mass chromatogram for TC (445 → 427 transition), D) mass chromatogram for OTC (461 → 443 transition), and E) mass chromatogram for CTC (479 → 444 transition) 52

Figure 2.4 LC-MS/MS chromatograms of soil spiked at 10 μg kg^{-1} of each tetracycline epimer and the internal standard. A) TIC of the ten precursor → product ion transitions reported in Table 2.1, B) mass chromatogram for e-TC (445 → 427

transition), C) mass chromatogram for e-OTC (461 → 443 transition), and D) mass chromatogram for e-CTC (479 → 444 transition) 53

Figure 2.5 LC-MS/MS chromatogram corresponding to sample 1b 55

CHAPTER 3: Analysis of pharmaceuticals in soils and sediments

Figure 3.1 Comparison of recoveries obtained using different (A) extraction solvents and (B) SPE cartridges. Concentration level 50 ng g^{-1}. 70

Figure 3.2 LC–MS/MS chromatogram in PI mode obtained from an extract of soil spiked at 25 ng g^{-1} of each compound..... 72

Figure 3.3 LC–MS/MS chromatograms in NI mode obtained from an extract of soil spiked at 25 ng g^{-1} of each compound using (A) analytical column Phenomenex Luna C18 (2) (150mm×4.6mm, 5µm), mobile phase: water (a) and MeOH (b) and gradient from 15% to 98% b in 5 min and hold 8min. (B) Analytical column Phenomenex C6-Phenyl (150mm×2.0mm, 3µm), mobile phase: water (a), acetonitrile (b) and gradient from 25% to 98% b in 10 min, and (C) analytical column Phenomenex Luna C18 (2)(150mm×2.0mm, 3µm), mobile phase: water 5mM ammonium formiate (a), acetonitrile/MeOH (60:40, v/v) (b), gradient from 15% to 98% b in 5 min and hold 7min, preconditioning 15% of acetonitrile and 85% of a, 15 min. 72

Figure 3.4 Chromatograms showing the analysis of target pharmaceuticals in a Oliva-Pego sediment sample, including two SRM transitions for each analyte. 75

Figure S3.5 Comparison of recoveries obtained using different (A) temperatures, (B) number of cycles, (C) static time and (D) flush volume..... 80

Figure S3.6 Comparison of the recoveries obtained from soils equilibrated for 1 day and aged for 3 months (A) soils and (B) sediments..... 81

CHAPTER 4: Determination of illicit drugs in water from L´Albufera of Valencia

Figure 4.1 Location of the sampling sites 92

Figure 4.2 Chromatogram of the quantifier transition for each illicit drug obtained from an extract of spiked water (matrix: L'Albufera lake water and spiking level 6 ng/L)..... 95

Figure 4.3 Comparison of the absolute recovery percentage for the illicit drugs obtained by SPE with Oasis HLB, Oasis Wax, Supelselect HLB, Strata-X and Strata-XCW(matriz: L'Albufera lake water spiking level 50 ng/L and size of the cartridges 60 mg/3 mL) 96

Figure 4.4 Chromatogram obtained from a real water sample taken at PM6. Only drugs positively identified and quantified are shown 99

Figure S4.5 Influence of pH adjustment, (pH 3, pH 5 and pH 7), on the absolute recoveries using (A) SupelSelect HLB SPE cartridges and (B) Strata-XC (matrix surface water from L'Albufera and spiking level 50 ng/L). 102

Figure S4.6 Cummulative levels ng/L of drugs of abuse in the surface waters of the Natural Park of L'Albufera. 103

CHAPTER 5: Determination of pharmaceuticals in L´Albufera of Valencia

Figure 5.1 Map with the location and georeferences of the sampling points. 1 Poyo Gully, 2 Plana Pound and Perello outflow channel. A) Pinedo wastewater treatment plant (WWTP), B) Albufera South WWTP, C) Sueca WWTP and D) El Mareny WWTP 112

Figure 5.2 Recoveries and RSDs obtained by ultrasonic and PLE methods at the MQLs for (A) soil samples and (B) sediment samples 116

Figure 5.3 LC-MS/MS chromatograms obtained by injecting extracts of soil sample P10 (A) using PLE method and (B) ultrasonic extraction. For concentrations see Table 5.6 118

Figure S5.4 Influence of different SPE parameters on the recovery of the selected pharmaceuticals from water (A) volume of sample extracted, (B) volume of washing solvent (water) and (C) type of elution solvent 125

Figure S5.5 LC-MS/MS chromatogram obtained after SPE of the water sample from P14. For concentration see Table 5.4 126

Figure S5.6 Concentration of carbamazepine found in water, sediments and soils..... 127

Figure S5.7 Concentration of ibuprofen found in water, sediments and soils 128

CHAPTER 6: Ocurrence of illicit drugs in Pego-Oliva Marsh

Figure 6.1 Location of the sampling sites at the Pego-Oliva Marsh in the Valencian Community (Spain) 137

Figure 6.2 EIC of the of the illicit drugs identified in the SPE extract of the water sample W8 (a) quantification ion precursor → ion product transition and (b) confirmation ion precursor → ion product transitions 139

Figure 6.3 Cumulative levels (in nanograms per liter) of illicit drugs and metabolites in surface waters of the Pego-Oliva Marsh 143

Figure 6.4 Distribution of the illicit drugs in the different sampling points, distinguished into the different chemical classes 144

CHAPTER 7: Pharmaceuticals in Pego-Oliva Marsh: distribution and risk assessment

Figure 7.1 Location of the sampling points and total concentrations of pharmaceuticals in each one of them. 155

Figure 7.2 Mean concentrations of pharmaceuticals in soils at different depths: layers A (<15 cm) and B (15–50 cm). 158

Figure 7.3 PCA analysis with the data from the samples of the soil superficial layer. 159

CHAPTER 8: Analysis of heavy metals in Pego-Oliva Marsh

Figure 8.1 Distribution of the sampling zones and location of the studied area 203

Figure 8.2 Mean values of the studied pharmaceuticals according water sources (A), land uses (B) and distance to the seashore (C). Different letters in each metal mean statistical significant differences between cases. No letters means no statistical differences. 204

Figure 8.3 Mean values of the studied heavy metals according water sources (A), land uses (B) and distance to the seashore (C). Different letters in each metal mean statistical significant differences between cases. No letters means no statistical differences. 205

Figure 8.4 Observed significant correlations between ofloxacin, norfloxacin and ibuprofen with the total content of Ni in the water samples. 206

Figure 8.5 Results of the Principal Component Analysis of the studied data. (A) Grouping of the studied pharmaceuticals and heavy metals, and (B) of the studied heavy metals only 206

Figure 8.6 Zoning of the studied contaminants in the area of the Pego-Oliva natural Park 207

CHAPTER 9: Relationship between irrigation systems and levels of illicit drugs in L´Albufera of Valencia

Figure 9.1 Location of the study area and sample points setting. 214

Figure 9.2 Urban and agricultural (irrigation structure) contextualization of l'Albufera Natural Park. 217

CHAPTER 10: Stereoisomeric profiling of drugs of abuse and pharmaceuticals in wastewaters

Figure 10.1 Loads (ng/L) of drugs in influent waters from Pinedo-I WWTP in 2012..... 229

Figure 10.2 Loads(ng/L) of drugs in influent waters from Pinedo-II WWTP 230

Figure 10.3 Loads (ng/L) of drugs in influent waters fromQuart-Benager WWTP 230

Figure 10.4 Enantiomeric fractions in influent and effluent samples of the three studied WWTPs 231

Figure 10.5 Median of removal rates (%) for each compound and WWTP 233

Figure 10.6 Average daily consumption of the investigated drugs, expressed as mg/day for 1000 inhabitants. 235