UNIVERSITAT ROVIRA I VIRGILI

PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN ENVIRONMENTAL WATERS

Marta Pedrouzo Lanuza

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Marta Pedrouzo Lanuza

DOCTORAL THESIS

Supervised by

Dr. Rosa Maria Marcé and Dr. Eva Pocurull

Departament de Química Analítica i Química Orgànica

UNIVERSITAT ROVIRA I VIRGILI

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i Química Orgànica

La Dra. ROSA MARIA MARCÉ i RECASENS, Catedràtica del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili, i

La Dra. EVA POCURULL i AIXALÀ, Professora Titular del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili,

CERTIFIQUEM:
Que la present Tesi Doctoral, que porta per títol: "PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN ENVIRONMENTAL WATERS", presentada per MARTA PEDROUZO LANUZA per optar al grau de Doctor per la Universitat Rovira i Virgili, ha estat realitzada sota la nostra direcció, a l'Àrea de Química Analítica del Departament de Química Analítica i Química Orgànica d'aquesta universitat, i que tots els resultats presentats són fruit d'experiències realitzades per l'esmentada doctoranda.

I, per a que consti, expedim aquest certificat a Tarragona, 4 d'octubre de 2010.

Dra. Rosa Maria Marcé i Recasens Dra. Eva Pocurull i Aixalà

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Alguien a quien admiro, me aconsejó que entendiera la Tesis como una carrera de fondo. Hoy el tiempo pasó, y cuando soy yo quien llega a la meta recuerdo aquellas palabras con cierta nostalgia. Ahora esta etapa termina y escribiendo estas líneas me doy cuenta de lo afortunada que he sido en estos años de Tesis. En ellos he vivido un montón de cosas buenas, resumidas en todo lo aprendido y las amistades que he hecho en el camino.

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No me quiero olvidar de Daura y Houda, que llegaron a nuestro laboratorio a hacer una estancia y se llevaron mi amistad para siempre. Gracias a las dos por vuestros consejos y por los fantásticos momentos vividos.

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Esta Tesis va dedicada a todas esas personas que creyeron en mí, y que siempre tuvieron una palabra de aliento. Especialmente a quienes más quiero, mis padres y mi hermana, por apoyarme siempre en mis decisiones. Si he llegado hasta aquí es gracias a su esfuerzo y sus consejos.

Y a Enric, el mejor compañero de viaje, por estar siempre a mi lado aún estar muy lejos, por enseñarme que no hay que rendirse y darme su apoyo incondicional. Por llenar de luz mi vida…

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CHAPTER 1. INTRODUCTION

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Water is an essential element of all socio-economic development, and maintaining a safe water supply is the primary concern of any water authority. However, human activity is responsible for non-sustainable exploitation of natural resources and increasing environmental pollution. Because of this, water treatment has become one of today's most critical environmental issues. Studies of contaminant toxicity in public water systems, which may lead to adverse health effects, are needed to guide actions taken by regulatory authorities. Although many regulations are already in place to protect the public, there is a need to constantly update these, based upon the existence of new knowledge. Similarly, water treatment practices are designed to protect human health, but their performance also needs to be continually re-evaluated and optimized, based upon new research results.

In recent years, a new group of pollutants known as emerging organic contaminants has been receiving increased attention in the scientific community. Emerging organic contaminants belong to a broad category of environmental chemicals, previously unknown or unrecognized as being of concern, but which are now under increasing scrutiny. Compounds that may be emerging organic contaminants are widely used in consumer goods, serving useful everyday functions within society. However, because of their continuous input into the ecosystem they are regarded as pseudo-persistent [1]. In general, these contaminants are not regulated but in September 2009 the U.S. Environmental Protection Agency (EPA) published its final list (List-3) of unregulated emerging organic contaminants that have the potential to present health risks via drinking water exposure [2].

Although there is still an overall lack of information regarding their toxicity, bioaccumulation, or occurrence, many of these compounds are suspected to have potential effects on humans and other species. Human exposure is generally of key concern for these contaminants precisely because of their widespread applications. Some of these compounds are also reported to be endocrine disrupting compounds (EDCs). According to the EPA definition, EDCs are exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the body's natural hormones, which are responsible for the maintenance of homeostasis and the regulation of developmental processes. EDCs can include man-made chemicals such as pesticides and plasticizers, natural chemicals found in plants (phytoestrogens), pharmaceuticals, and hormones that are excreted in animal or human waste [3]. As can be seen, therefore, EDCs are not a group of substances defined by their chemical nature, but rather by their biological effects.

Richardson's review [4] has included the most well-studied emerging organic contaminant groups including, among others, pharmaceuticals and personal care products (PPCPs), brominated flame retardants (BFRs), drinking water disinfection by-products (DBPs), perfluorinated compounds such as perfluorooctanesulfonate (PFSO) and perfluorooctanoic acid (PFOA), nanomaterials, and specific compounds such as 1,4 dioxane. The widespread production and use of these emerging organic

contaminants increasingly contributes to contamination of the environment. In the following sections, some of these compounds are briefly reviewed. Since PPCPs are the primary focus of this Thesis, they are discussed in this chapter in more detail.

Brominated flame retardants have routinely been added to consumer products for several decades, in a successful effort to reduce fire-related injuries and property damage. Recently, concern regarding this emerging class of chemicals has risen following discovery of several classes of BFRs in the environment as well as in the human body [5,6].

Also, over the past decade, **nanomaterials** have become a subject of enormous interest. These materials are extremely small in size and are used in biomedical and electronic applications, food containers, and personal care products. In regard to environmental concerns, there has very recently been concern about the role of these compounds as contaminants [7,8].

In 2005 **1,4-dioxane** was identified as a chemical hazard in drinking water by the World Health Organization (WHO) [9]. It is a colorless, flammable liquid with a faint, not unpleasant odor. Among other uses, 1,4-dioxane is included as a solvent in paints, cosmetics, deodorants, detergents, and cleaning products. This chemical is a cyclic ether that is highly miscible in water. In fact, it mixes with water so readily that it can be found in groundwater. It also migrates rapidly in soil. Contamination by 1,4-dioxane can often be found in association with releases of chlorinated solvents. The EPA has listed this compound as a probable human carcinogen based upon the results of animal studies, but little information is available regarding the long-term effects of 1,4-dioxane on human health [10].

Disinfection by-products are formed by the reaction of disinfectants (chlorine, ozone, chlorine dioxide, etc.) used in the water treatment, with natural organic matter or other organic contaminants in source waters. Required disinfection practices also result in the formation of other undesirable DBPs such as haloacetic acids and trihalomethanes (chloroform, bromoform, bromodichloromethane, and dibromochoromethane) [10]. Some of these DBPs have adverse public health effects, and are therefore regulated because of their concentrations in drinking waters. However, DBPs are not regulated when chlorination is used to prevent the spread of waterborne infectious diseases in swimming pools.

Pharmaceutical and Personal Care Products are a broad group of thousands of chemicals belonging to a wide spectrum of chemical classes, and this larger group is often sub-divided into two more inclusive groups, pharmaceutically active compounds (PhACs) and personal care products (PCPs). Pharmaceutical groups include all of the prescription and over-the-counter pharmaceuticals consumed for purposes of human and veterinary health care. The term pharmaceutically active compounds (PhACs) is used to encompass all hormone-based compounds as well as all other drugs, whether therapeutic or recreational. Personal care products (PCPs)

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include many of the chemicals found in beauty and personal hygiene products such as cosmetics, fragrances, and creams applied to the skin.

After use or intake by humans (or animals in veterinary medicine), PPCPs are excreted with human or animal waste or are rinsed from the body. These are flushed down drains and into sewer systems to eventually arrive at sewage treatment plants (STPs). Current wastewater treatment methods are not efficient at removing all PPCPs and their metabolites, and therefore some are consequently released back into the aquatic environment. As seen in Figure 1, the potential sources of PPCPs in the environment include STP effluents, sludges, and illicit discharges. Studies regarding the fate of PPCPs in sewage have been conducted in numerous countries including Italy, Spain, The UK, Germany, the USA, and China [11-15]. The levels of these compounds found in effluents show that they may be only partially removed during conventional treatment, before STP-treated effluents are discharged into receiving waters, including rivers or lakes. The most serious problem is that many communities use groundwater and surface water resources for drinking water production, and these source waters may contain a significant component of this wastewater effluent [16].

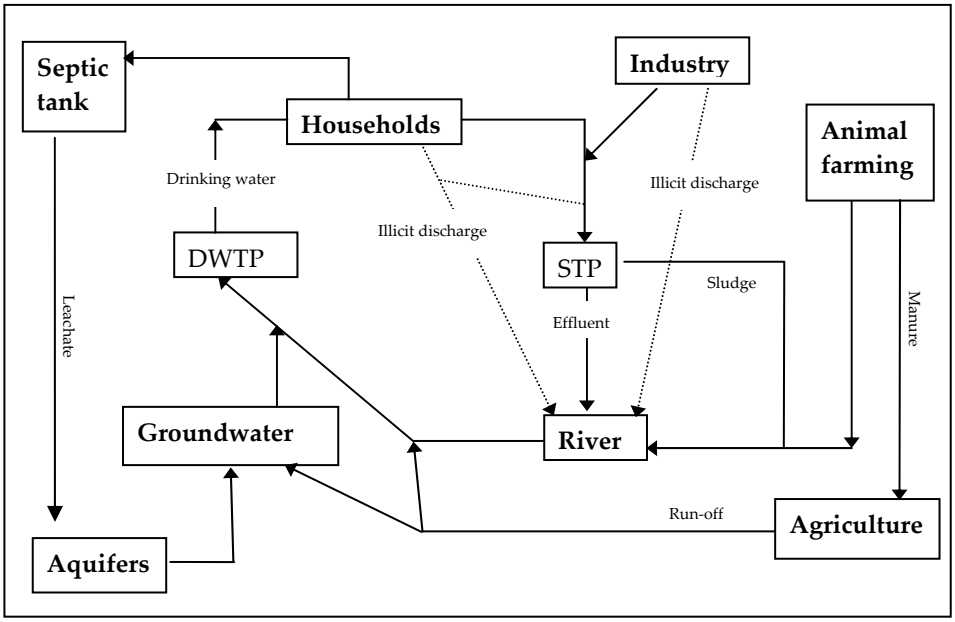

Figure 1. The water cycle.
DWTP: Drinking water treatment plant
STP: Sewage treatment plant

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Since it is known that PPCPs are present in the environment and could be causing adverse health impacts, the efficacy of various methods used to remove them from water are being studied. Consequently, while some earlier treatments remain effective today, the required degree of treatment has also increased significantly in more recent times, and additional treatment objectives have been added. Treatments must be closely linked with the water quality objectives and standards established by regional regulatory authorities. For example, to protect the environment from the adverse effects of discharges, the European Commission has adopted Council Directives 91/271/EEC and 98/15/EEC regarding urban wastewater treatment. However, specific regulations related to the presence of PPCPs in environmental waters have yet to be adopted.

Many of the new treatment technologies being developed are designed to address the health and environmental concerns raised by the findings of recent research. Although removal of PPCPs was not the initial objective of STPs, wastewater treatment facilities may be designed to include improved processes to deal with these contaminants in the future. Because newer, more sensitive techniques for detecting chemicals are now available, some previously undetected contaminants are now creating concern.

The elimination efficacy of any STP depends upon its design. Although new treatment techniques are being developed, these advances are not always immediately implemented. Typically, STPs apply primary, secondary, and usually tertiary (advanced) treatments before effluent is discharged. During sewage treatment, a redistribution of PPCPs will take place between the dissolved fraction and the solids present (primary or secondary sludge). Therefore, the release of PPCPs into the environment will occur not only with the final effluent but also with the disposal of sludge. Excess sewage sludge can be used as a fertilizer in agriculture (Figure 1). However, its safe use requires knowledge of the contaminants it may contain, and several existing studies have addressed this issue [17].

Overall, primary treatments in STPs consists of physico-chemical processes, including filtration, sedimentation, coagulation, and flocculation. Secondary treatments are designed to degrade organic matter through the use of microorganisms. Biological degradation and transformation take place aerobically through biological oxidation in activated sludge, through the use of trickling filters, or in anaerobic sludge digesters.

Tertiary treatments use physical, biological, and chemical processes to further reduce pollutants after secondary treatment. Also, the quality of effluents may be increased by reduction of odors and coloration. These treatments often include the application of chlorine and/or other chemicals to oxidize remaining organic matter. A variety of advanced treatments meant to improve removal have recently been evaluated, including advanced oxidation processes (AOPs), membrane processes (microfiltration, ultrafiltration, nanofiltration, and reverse osmosis), or adsorption

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into granular activated carbon or other materials [18,19]. Among AOPs, photodegradation by UV radiation is widely used in advanced water purification methods in order to induce photoreaction of organic pollutants. Additionally, treatment with hydrogen peroxide combined with UV radiation appears to be a very promising technique for the oxidative degradation of organic compounds [20]. Other methods also include photocatalysis with titanium dioxide, ozonation, direct and indirect photolysis, electrochemical oxidation, and ultrasonic irradiation. Caliman et al. [21] reviewed these last methods as used for removal of EDCs from water.

Several studies have reported low removal levels for PPCPs by STPs, with the consequent presence of these contaminants in effluents. Therefore, the need for new directions and considerations in wastewater treatment is evident. However, some major treatment plants are still using only limited physico-chemical processes, which unfortunately produce only undesirably low removal efficiencies for PPCPs. Careful assessments of the resulting health and environment effects, as well as community concerns about these effects, are therefore becoming increasingly important in the field of wastewater management.

As an example related to this issue, several studies conducted during the past decade regarding the presence of drugs of abuse in environmental waters have caused concern, not only in Europe but also in the USA, because the concentrations of drugs detected in environmental waters brought their levels of usage into question. Pioneering research on this subject was published by Italian researchers Zuccato et al. in 2005 [22], with levels of cocaine detected in surface and wastewaters used to estimate levels of consumption of this narcotic. Similar results reported by Kasprzyk-Hordern et al. [23], showed that one ton of cocaine enters STPs in Wales each year, with an average usage of 0.9 g/day per 1000 people in the age group 15-59. Studies like these have demonstrated that concentrations of illicit drug residues in wastewater could provide a potential opportunity to non-invasively estimate community-wide consumption of these substances. Urban wastewater entering an STP could be seen as an accessible, economical source of real-time, pooled epidemiologic information.

Because most PPCPs are not effectively removed by STPs, they enter groundwater and surface waters in the discharged wastewater effluents [24]. Studying river waters, Zuccato et al. [25] provided an evaluation of the antibiotic contamination of Italy's aqueous environment. They found that antibiotics entering STPs in northern Italy were not being efficiently removed and thus ended up in the waters receiving the effluents. Analysis of the receiving rivers confirmed that the bulk of the antibiotics causing contamination were macrolides and quinolones. The most notable results for two rivers were those for two macrolides (clarithromycin and erythromycin), which had maximum mean levels of 25.4 ng/L and 17.9 ng/L, respectively. When Kasprzyk-Hordern et al. [26] developed a multi-residue method for the determination of 28 PhACs, they found 15 of these compounds in the two

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rivers they studied in Poland and The UK. Their most significant results were those for the analgesic acetaminophen (1013−1388 ng/L) and the antidepressant tramadol (895−2108 ng/L).

Filtration of surface water and artificial recharge can guarantee a sustainable level of groundwater. Therefore, strict quality control of the waters that contribute to recharge will minimize contamination of both the groundwater and the aquifer area. Groundwater as an environmental compartment is a special type of aqueous system because of its characteristics of low residence times, low temperatures, and low degrees of dilution. These are factors that favor the presence of PhAC contaminants, and in fact most groundwater sources studied have been found to contain some of these [27]. Although there are few studies of PhACs in groundwater, Grujic et al. [28] found trimethoprim (100 ng/L), azythromycin (25−140 ng/L), and carbamazepine (6−23 ng/L) in groundwater samples from Serbia.

Because of the fact that surface water is one of the main sources of drinking water, there is a clear potential risk of drinking water contamination involving PhACs. The problem exists when conventional technologies used in drinking water treatment plants (DWTPs) are incapable of reducing the levels of trace contaminants to a point where they are no longer considered to be a potential threat to public health. Therefore, new technologies that may offer significantly improved levels of treatment need to be evaluated. While many countries use advanced technologies such as ozonation and reverse osmosis for drinking water treatment, some compounds have been shown to remain unaffected by these treatments. There is relatively little information available regarding the occurrence of PhACs in drinking water. However, some examples of existing research regarding this subject can be summarized here. For example, Benotti et al. [29] studied the occurrence of some groups of PhACs and other EDCs in post-treatment drinking water. Their most significant results were found to be those for atenolol and carbamazepine, with median values of 1.2 ng/L and 6.0 ng/L, respectively. When Huerta-Fontela [30] studied PhAC removal by a DWTP, they found mean levels of benzoylecgonine (the main metabolite of cocaine) of 45 ng/L in treated water. The authors concluded their study with a statement to the effect that since benzoylecgonine is more than 30 times less active than its parent compound, no toxic effect should be expected at the concentration levels found. A continuing problem, however, is that until now, most PPCPs lack established drinking water standards or health advisories. Furthermore, risks associated with drinking water intake also remain largely unevaluated.

With data emerging in the literature to inspire concern, it is clear that collaborative research should be undertaken to monitor high risk groups, identify pathways of exposure, and help mitigate potential adverse impacts. Contamination of water supplies is an evolving problem, and will remain an unsettled issue as long as technological change continues. Some of the contaminants now being targeted by researchers will perhaps come out with a clean slate, while others will require

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additional scrutiny. One of the hopes shared by many of today's researchers is that new science will help accelerate the process of identifying and mitigating contamination problems, before damage to either human health or the environment can occur. In any case, science and regulation must continue to evolve and change, as has been the case in the past few years, to respond to newly identified needs presented by an expanding range of chemical contaminants and our increasing scientific knowledge of them.

In light of these health and environmental concerns, a review is presented below of the most widely used analytical methods applied to this subject, as well as the occurrences of pharmaceuticals and personal care products in waters as described in the existing literature. The PhACs are discussed first, followed by the PCPs.

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1.1. PHARMACEUTICALLY ACTIVE COMPOUNDS

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The term pharmaceutically active compounds (PhACs), as mentioned above, includes medical prescription pharmaceuticals, recreational drugs of abuse, and hormone-based compounds. All these PhACs, after use, can potentially enter the environment either as parent compounds or metabolites, or conjugates of both. Because of their continuous input into the ecosystem they are regarded as pseudo-persistent. For this reason, and also because of their potential toxicity, further study of these compounds and their presence in environmental waters is needed.

In this section a brief description of the characteristics of PhACs is provided. Also, the analytical techniques most widely used for their determination are explained in detail. For purposes of discussion, these are separated into chromatographic techniques and extraction techniques, and an extensive review of studies associated with each technique is included. The most common chromatographic techniques are gas chromatography (GC) and liquid chromatography (LC), and these are frequently coupled to very sensitive detection techniques. Currently, mass spectrometry (MS) and tandem mass spectrometry (MS-MS) are the most widely used. Also, a wide variety of extraction techniques are used in advance of chromatographic analysis, such as stir bar sorptive extraction (SBSE), solid-phase microextraction (SPME), solid-phase extraction (SPE), and others.

PhAC compounds are present in the influent waters of STPs, where most of them end up being incompletely removed. New technologies are therefore being applied to improve removal processes. Below, a review is provided of the most well-studied families of PhACs found in environmental samples, including their main characteristics and some examples of the results of research regarding their presence in various types of water samples.

PHARMACEUTICALS

Pharmaceuticals are substances used in the diagnosis, treatment, alteration, or prevention of abnormal health or structural/functional conditions in the body, and there are a great number of compounds included in this group. This section includes a classification of pharmaceuticals most frequently found in waters as emerging organic contaminants. As mentioned above, both the main compounds as well as their metabolites have been studied. All of the chemical structures of the pharmaceuticals discussed in this Thesis are illustrated in Appendix II.

Among all the pharmaceuticals**, analgesics** (e.g., acetaminophen and acetylsalicylic acid) are the most commonly used agents for relieving pain. Although acetylsalicylic acid is itself not determined in samples, the presence of its metabolite (salicylic acid) has been reported in many types of waters [31]. Other analgesics including morphine, methadone, and codeine, are included in the group consisting of drugs of abuse, because of the frequency of their illicit use. Another type of heavily used

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pharmaceuticals is the **non-steroidal anti-inflammatories (NSAIDs)** (e.g., naproxen, ketoprofen, ibuprofen, diclofenac, indomethacine, and phenazone). These are pharmaceuticals with analgesic (pain reduction), anti-inflammatory (inflammation reduction), and antipyretic (temperature decreasing) effects. Although they do provide some benefits for health and well-being, these compounds need to be used carefully and with the proper dosage, because of their adverse effects on the digestive system. In terms of their chemical structure, these pharmaceuticals are weak acidic compounds, polar, and highly soluble in aqueous media. They have also been reported in environmental waters [14,32].

Lipid regulators (e.g., clofibrate, gemfibrozil, bezafibrate, and fenofibrate) can lower cholesterol and reduce the risk of cardiovascular disease by reducing lipid levels. Not only the parents but also the metabolites (clofibric acid and fenofibric acid) have been determined in environmental waters [33].

β-Blockers (e.g., atenolol, sotalol, pindolol, timolol, metoprolol, carazolol, propranolol, and betaxolol), also known as beta-adrenergic blocking agents, are drugs that block norepinephrine and epinephrine (adrenaline) from binding to β-receptors on nerves. By blocking the effects of norepinephrine and epinephrine, β-blockers reduce heart rate, reduce blood pressure by dilating blood vessels, and may constrict air passages by stimulating the muscles that surround them and causing them to contract. Studies have revealed the presence of β-blockers in environmental waters [34].

Among all the pharmaceuticals, **antibiotics**, which are used to treat infections, have attracted special interest in studies of environmental waters [35-37]. Some are used only in veterinary or human medicine, but most are used for both human and veterinary health purposes. A primary public health concern worldwide is that antibiotics present in sewage effluents may cause increased resistance in naturally occurring bacterial populations. In fact, the increase that has taken place in the number of bacterial strains resistant to multiple antibiotics has often been attributed to the irrational use of antibiotics and the increase in their discharge into wastewater.

Antibiotics are classified into macrolides, sulfonamides, quinolones, and tetracyclines, among other groups. Macrolides (e.g., azythromycin, clarithromycin, erythromycin, roxithromycin, josamycin, spyramycin, and tylosin) are widely used in the treatment of both human and veterinary diseases. These compounds are basic and lipophilic molecules consisting of a large lactone ring with amino and deoxy sugars. Macrolides have bacteriostatic action, meaning that bacterial growth and reproduction are inhibited, in contrast to bactericidal antibiotics, which directly kill bacteria. However, macrolides can also be bactericidal in high concentrations. Sulfonamides (e.g., sulfamethazine, sulfamethoxazole, sulfatiazole, sulfadiazine, and sulfaguanidine) were the first chemotherapeutic agents discovered and comprise a broad group of synthetic antibacterial compounds. They act as competitive antagonists, and have prophylactic and therapeutic purposes as well as growth-

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promoting actions [38]. They are N-substituted derivatives of sulphanilamide and show amphoteric behavior because of their aniline group, and are acidic because of the N-H bond of the sulfonamidic group. Fluoroquinolones (e.g., enrofloxacine, norfloxacine, and ciprofloxacine) are an important group of synthetic antibiotics, with bactericidal action that results from selective inhibition of bacterial DNA synthesis. Numerous structurally related quinolones have been synthetized, and several of these are in routine clinical use globally. These antimicrobials are derivatives of 3-quinolonecarboxylic acid, and are characterized by an aromatic fluorine substitution in the C-6 position. Tetracyclines (e.g., oxytetracycline, chlorotetracycline, tetracycline, doxycycline, and minocycline) are poliketides with anfoteric behavior similar to that of sulfonamides. The chemical structure of this family of antibiotics is a naftalene ring structure consisting of four fused rings. They are considered as 'broad spectrum' antibiotics and have a wide range of therapeutic applications. They are used to treat general infectious diseases and are also used as growth additives in animal feeds.

Several studies have revealed the occurrence of antibiotics in environmental waters [37,39]. Because of the presence of antibiotics in the environment, the potential exists for resistance-based selection among pathogens, and in fact, resistance genes have been detected in feedlot lagoons, STPs, river water, seawater, and groundwater [40].

Diagnostic pharmaceuticals are used during radiography to enhance the contrast between organs or vessels under examination and their surrounding tissues. These compounds are called **iodinated X-ray contrast media** (e.g., diatrizoate, iopamidol, iothalamic acid, diatrizoic acid, iohexol, iomeprol, iopromide, ioxaglic acid, and iodipamide), and they have been identified in environmental waters [41]. These substances are applied in doses up to ca. 200 g per person (corresponding to approximately 100 g of iodine), and are rapidly excreted. While they are not considered toxic to humans and wildlife, they are polar and persistent. These properties enable them to remain present in the aquatic environment and leach through the subsoil into groundwater aquifers [41]. No environmental risk is expected to derive from these tri-iodinated contrast media themselves, but their metabolites may have an ecotoxicological impact. Most of these transformation products carry free amino groups that could be mutagenic. Therefore, identification of the transformation products has become critical.

Phosphodiesterase type V inhibitors (e.g., sildenafil, tadalafil, and vardanafil), are presently undergoing clinical safety and efficacy trials, as oral agents for the treatment of male erectile dysfunction. Sildenafil, a phosphodiesterase **(**PDE) type V inhibitor, was introduced to the US market in 1998, and has been identified in environmental waters [42]. Its mode of action is based on inhibition of the breakdown of cyclic guanosine monophosphate (cGMP), increasing the efficacy of the nitric oxide (NO)/cGMP pathway. Intracellular levels of cGMP are controlled by activation of cyclic nucleotide cyclases and breakdown by phosphodiesterases.

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Specifically, there are a number of PDE isozymes that will hydrolyze cGMP to form inactive GMP, and thus cGMP levels can be raised by the use of a selective cGMP PDE inhibitor.

Psychoactive drugs (e.g., stimulants, antidepressants, antipsychotics, and anxiolytics) belong to the most widely prescribed group of pharmaceuticals. Also, the anti-epileptic carbamazepine is included in some studies as a psychoactive drug because of its use as an antidepressant [43,44]. In some cases, these psychoactive drugs are taken without a doctor's prescription in what is referred to as "nonmedical" use. There are also several pharmaceuticals classified as antidepressants, including the widely used fluoxetine and paraxetine, among others. Two families of psychoactive drugs also widely used as antidepressants are the barbiturates (e.g., pentobarbital and secobarbital) and benzodiazepines (e.g., bromazepam, diazepam, flurazepam, lorazepam, nordazepam, and tetrazepam). These are commonly used as treatments for anxiety, insomnia, or seizure disorders. With the rise in popularity of barbiturate prescriptions by those in the medical profession during the 1970s, the use of barbiturates as drugs of abuse increased as well. Barbiturates became increasingly abused to reduce anxiety, decrease inhibitions, and treat the undesirable effects of illicit drugs. Barbiturates can be extremely dangerous because they are highly addictive and can cause a life-threatening withdrawal syndrome. Barbiturate use and abuse has declined dramatically since the 1970s, mainly because a safer group of sedative-hypnotics such as the benzodiazepines are now being prescribed instead.

The most commonly used stimulant is caffeine. Caffeine can be found in some pharmaceuticals, but is also found in coffee, tea and other beverages. It is commonly found as well in environmental samples, and its high human consumption levels make this compound a reliable anthropogenic indicator. Caffeine is usually included in studies of drugs of abuse as a non-controlled drug, because of its widespread use and the high concentrations found in environmental waters [45].

Of course, the full list of pharmaceuticals currently in use is much more extensive. However, the specific types discussed above are the most commonly studied as contaminants in waters, along with other pharmaceuticals such as stomach protectors (e.g., ranitidine and omeprazole), anticancer compounds (e.g., cytarabine and iofosfamide), diuretics (e.g., furosemide and hydrochlorothiazide) and the bronchodilator salbutamol, among others [18].

HORMONES

Hormones include those of human origin such as the primary estrogenic steroids produced by all vertebrates. Steroidal hormones such as estrogens, progestogens, androgens, and corticosteroids are among the most important endocrine disrupting compounds (EDCs) with regard to their potential hazards. Natural **estrogens** (e.g.,

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estradiol (E2), estrone (E1), and estriol (E3)) are excreted by women as products of the menstrual cycle, and are excreted in urine as the conjugated molecules estrone 3-sulfate (E1-3S), estrone 3-glucoronide (E1-3G), estradiol 3-sulfate (E2-3S), 17β-estradiol 17-acetate (E2-17A), and estradiol 17-glucuronide (E2-17G) [46,47]. Synthetic estrogens including diethylstilbestrol (DSB) and 17α-ethinylestradiol (EE2) are used in oral contraceptives. In hormonal contraceptives, **progestogens** (e.g., progesterone, norethindrone, and levonorgestrel) represent the major agents designed for suppressing ovulation, and are used in combination with estrogens. **Androgens** (e.g, boldenone, epitestosterone, methyltestosterone, and nortestosterone) control developmental characteristics and have also been used as growth promoters [48].

Recent studies have reported the presence of corticosteroids as a type of steroidal hormones in environmental waters, and have indicated a need for concern [48]. **Corticosteroids** (e.g., blecomethasone, cortisone, dexamethasone, and flumethasone) are divided into glucocorticosteroids and mineralocortocosteroids, and are used in both human and veterinary therapy because of their ability to reduce inflammation and suppress allergic reactions. Moreover, corticosteroids have been used illegally as growth promoters in animal feedstocks, and the EU has banned their administration for fattening purposes as part of its 96/22/EC directive.

The presence of hormones has frequently been determined in waters, but the occurrence of their metabolites has not received the same level of attention. Moreover, information regarding the presence of androgens and corticosteroids in waters is scarce. Excessive amounts of hormones in the environment are hazardous, because they have the potential to cause adverse health effects in humans and wildlife. This is caused by interference with the normal activity of hormones involved in growth control, metabolism, and other physical functions, which is known, as previously mentioned, as the endocrine disrupting effect. Steroids of major concern include estrone and 17β-estradiol, because they can exert their physiological effects at lower concentrations than other steroids, and they can be found in the environment at concentrations above their lowest observable effect level (LOEL) for fish and plants (10 ng/L). Considerable attention has recently been paid to estrogen activity in wastewater, based upon evidence of feminization in aquatic fauna [3].

DRUGS OF ABUSE

The drugs of abuse that have been determined in environmental waters include those with the high worldwide levels of use. Some of these drugs are used with a doctor's prescription, but most are considered as "illicit" because they are used under "nonmedical" circumstances. For example, morphine, methadone, and codeine are

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therapeutic pharmaceuticals, but they can be used as drugs of abuse, and several studies have treated them as such [49,50]. Also, nicotine is not always considered a drug of abuse, but several authors include this compound in their studies of drugs of abuse because of nicotine's widespread use via tobacco. Therefore, these compounds are included in this section.

Measurement of the levels of these drugs of abuse and their metabolites in STPs can also provide a means for evaluation of societal drug-use trends [49]. Drugs of abuse can be classified as amphetamines, cocainics, opioids, cannabinoids, and methadone. Below, the main characteristics of each group are described.

Amphetamines (e.g., amphetamine, methamphetamine, and dextroamphetamine) work by increasing levels of dopamine in the brain, which is associated with pleasure, movement, and attention. The therapeutic effect of amphetamines is achieved via slow and steady increases in dopamine, which simulates the natural production of this chemical. Amphetamines are found in STPs because they are mainly excreted in the urine in unaltered forms [51]. For example, metamphetamine is excreted largely unaltered (43% of a dose) and also partially as amphetamine (4-7% of a dose). Also, methylendioxymetamphetamine (MDMA) is a ring-substituted derivative of methamphetamine that is also mainly excreted unaltered (65% of a dose) in the urine.

Cocainics are alkaloid esters extracted from the leaves of plants and include cocaine. Cocaine is used clinically as a local anesthetic and vasoconstrictor, particularly for the eyes, ears, nose, and throat. It also has powerful central nervous system effects similar to those of the amphetamines. The main metabolites of cocaine are benzoylecgonine, ecgoninemethylester, and norbenzoylecgonine [52]. According to the most recent World Drug Report produced by the United Nations Office of Drugs and Crime (UNODC), in 2009, Spain has had the highest cocaine prevalence rates in Europe for the last decade and even higher rates than the USA in recent years.

Opioids (e.g., morphine, codeine, oxycodone, and other related drugs) are commonly prescribed because of their effective analgesic and pain-relieving properties. Morphine, for example, is often used before and after surgical procedures to alleviate severe pain. Codeine, on the other hand, is often prescribed for milder pain. However, non-medical use of these drugs and their use as drugs of abuse is the main source of their presence in the environment.

Heroin is synthesized from morphine, and is one of the most dangerous drugs of abuse because it is rapidly acetylated to acetylmorphine and morphine in the liver [51]. Methadone is a synthetic opioid with uses similar to those of morphine. It also has a depressant action on the cough center and may be given to control the intractable coughing associated with terminal lung cancer. Methadone is also used in the treatment of dependence on opioid drugs. It is excreted in urine in unaltered

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form, or it may also be metabolized by N-demethylation with spontaneous cyclization of the resulting metabolite EDDP [51].

Cannabinoids (e.g., Δ^9-tetrahydrocannabinol, THC) are non-polar molecules with low solubility in water, and are therefore usually self-administered by smoking. The volatilized fractions are inhaled as a vapor and give rise to a number of physiological effects. These effects are highly dependant upon the expectations and mood of the user, the quantity consumed, and the possible presence of other drugs such as alcohol in the body. Apart from the substance's recreational uses and abuses, THC also has some medical applications. For example, its anti-emetic (inhibition of vomiting) properties are particularly useful in the treatment of cancer patients receiving chemotherapy. After being inhaled through smoking, THC is metabolized in the liver to the active metabolite 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH). This compound has been included in some studies of environmental waters, in order to evaluate levels of cannabis consumption in a local population [53]. Studies involving cannabinoids in addition to all of these families of drugs of abuse have been recently reviewed by van Nuijs et al. [49], because of their common presence in environmental waters.

Most pharmaceutically active compounds are found in environmental waters at very low concentrations (ng/L−µg/L). To successfully identify and quantify substances at these levels, appropriate extraction and chromatographic techniques are required. Therefore, the most relevant techniques used to identify and measure PhACs are next described in detail.

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1.1.1. DETERMINATION OF PHARMACEUTICALLY ACTIVE COMPOUNDS IN ENVIRONMENTAL WATERS

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As discussed above, awareness regarding the presence of pharmaceutically active compounds in the aquatic environment is rising, as research involving these emerging organic contaminants increases and analytical techniques improve. Environmental water samples contain large number of matrix components that may interfere with the determination of PhACs. Also, the analytes of interest are usually present at very low concentrations (ng/L) in environmental waters. Therefore, direct analysis may not be feasible and the samples may need to be pre-concentrated. There is no doubt that proper extraction techniques are a prerequisite for success in most analytical procedures. Furthermore, efficient chromatographic techniques that rely upon sensitive and selective detection systems are required to determine PhACs in water samples.

The low concentrations found in complex water matrices require that methods are applied using sensitive detection techniques. Most studies report the development and application of methods that allow simultaneous determination of multiple classes of compounds [54-56]. The main advantage of such approaches is that they produce broader knowledge about the presence of PhACs in the environment, while reducing costs and the time required for the analysis. However, simultaneously analyzing a wide spectrum of pharmaceuticals, all of which have different physico-chemical properties, must involve a compromise, which often cannot result in the best conditions for the study of all of the target analytes.

Several extraction techniques are used to clean up and pre-concentrate analytes of interest. The most widely used for determination of PhACs in water matrices is solid-phase extraction (SPE), which has the advantage of potentially using a wide variety of sorbents. Another current trend in the development of extraction techniques involves the concept of "green chemistry", which is concerned with reducing the large amounts of solvents used in conventional extraction techniques. Some of the techniques that have been developed to use low volumes of solvent include solid-phase microextaction (SPME), stir bar sorptive extraction (SBSE), liquid-phase microextraction (LPME), and liquid-liquid microextraction (LLME).

Not only are efficient extraction methods needed, but efficient and reliable chromatographic methods are also required for determination of the occurrence and fate of PhACs at the environmental level. Most existing analytical methods use gas chromatography (GC) or liquid chromatography (LC), and these methods are therefore reviewed in depth in this chapter. Capillary electrophoresis (CE) has also been also used in the determination of PhACs, especially NSAIDs, as reviewed by Macià et al. [57]. The low sensitivity of CE can be overcome by applying various pre-concentration strategies prior to application of electrophoretic analysis. Also, the coupling of CE and MS can be a highly effective approach for determination of PhACs in waters. Recently, Ramautar et al. [58] reviewed the applicability of two methods of different coupling, in-line and on-line SPE-CE, developed for use in complex samples such as surface waters.

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With some exceptions, PhACs tend to be polar, non-volatile, and thermally labile. For these compounds, therefore, GC without a prior derivatization step is unsuitable for their analysis. LC thus presents a great advantage here, since a derivatization step is not required. Undoubtedly however, the main advances in improving sensitivity and specificity in environmental analysis of pharmaceutical residues has been due to the application of mass spectrometry (MS) and tandem mass spectrometry (MS-MS). Specifically, MS-MS is today the technique of choice for the identification of a broad range of PhACs in environmental samples. Below, a review is presented of a selection of the most widely used methods and the new trends being applied in measurement of PhACs in environmental waters, first for chromatographic techniques (section 1.1.1.1), followed by extraction techniques (section 1.1.1.2)

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1.1.1.1. CHROMATOGRAPHIC TECHNIQUES

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As discussed above, GC and LC are the most commonly applied chromatographic techniques used for determining PhACs in waters. Therefore, a section here is dedicated to the review of some of the most relevant studies that have utilized each of these approaches.

1.1.1.1.1. Gas chromatography

Gas chromatography is a suitable technique for the determination of volatile organic PhACs from environmental waters. For analytes amenable to gas chromatography identification of non-target compounds is also sometimes feasible by GC-Ms or GC-MS-MS, as forward-search methods allow library searching to be used (e.g., the extensive library of the National Institute of Standards and Testing (NIST)).

However, use of GC-MS without previous derivatization is only suitable for non-polar, volatile and semi-volatile compounds. PhACs are mainly polar compounds, and therefore most of them require a derivatization step to decrease polarity and increase volatility, when GC is to be used as the final separation and determination technique. Derivatization of compounds containing –OH, $-CO_2H$, and $–CONH_2$ groups can be performed after extraction from water by sylilation with bis(trimethylsilyl)trifluoraocetamide (BSTFA) [59,60], N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) [56], or pentafluorobenzyl bromide (PFBBr) [61]. For example, Sebok et al. [62] reported a multi-residue method using GC-MS-MS to identify and quantify 63 water-soluble pollutants as their trimethylsilyl derivatives, including 15 pharmaceuticals and two hormones, with an analysis time of 31 minutes. Also, the determination of 18 PhACs (7 basic compounds and 11 acidic compounds) was performed using GC-MS, with a previous derivatization of the acidic drugs with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) [63]. Other authors have described studies involving PhACs that have avoided the use of laborious and time-consuming derivatization procedures. For example, Gómez et al. [64] developed a GC-MS-MS method to identify neutral and acidic pharmaceuticals in wastewater using an ion trap as analyzer, but omitting the derivatization step. Their method was considered viable for routine analysis and was applied to determination of pharmaceutical compounds in hospital effluents. A summary of GC-MS methods for the determination of PhACs in waters is provided in Table 1.

When compounds occur only in low concentrations in environmental waters, it is still necessary to perform reliable and robust trace-level determinations. One of the techniques used to enhance detection is based upon large volume injection (LVI) techniques with on-column injection or programmed temperature vaporizing (PTV) inlets. This was the approach used by Quintana et al. [56], who used LVI-GC-MS preceded by stir bar sorptive extraction, to identify a group of phenols and certain pharmaceuticals in a single analysis. In this example, LVI with a PTV large mode

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was used to attain LODs between 1 ng/L (for ketoprofen) and 800 ng/L (for salicylic acid).

In regard to the use of ionization mode, most commonly used methods rely upon electron impact (EI), although matrix effects have been reported in some cases where complex matrices have been analyzed. To overcome these drawbacks, Zhao et al. [61] developed a method using GC-MS with negative chemical ionization (NCI), using methane as the reaction gas. They identified acidic PhACs (anti-inflammatories, estrogens, and lipid regulators) in surface waters from southern China, after applying a derivatization process using pentafluorobenzyl bromide. Use of NCI coupled to GC-MS allows soft ionization with less fragmentation than EI, also providing higher sensitivity than that obtained using EI.

Table 1. Analytical methods based on GC-MS for determination of PhACs in waters.

Analytes	Chromatographic technique (analyzer)	Derivatization agents	Ref.
Hormones, anti-inflammatories, drugs of abuse	GC-MS (Q)	BSTFA	[60]
Hormones, anti-inflammatories	GC-MS (Q)	BSTFA	[59]
Lipid regulators, β-blockers, anti-inflammatories	GC-MS (Q)	MTBSTFA	[56]
Hormones	GC-MS (Q)	BSTFA	[65]
Lipid regulators, anti-inflammatories	GC-MS (Q)	PFBBr	[61]
Lipid regulators, anti-inflammatories, analgesics, caffeine, hormones	GC-MS-MS (IT)	-	[62]
Lipid regulators, anti-inflammatories	GC-MS (Q)	MTBSTFA	[66]
Lipid regulators, analgesics, hormones, antidepressants, anti-inflammatories	GCxGC (TOF)	TMSH	[67]
Anti-epileptics, anti-inflammatories, stimulants	GC-MS-MS (IT)	-	[64]
Analgesics, anti-inflammatories	GC-MS (Q)	MSTFA	[63]

TMSH: trimethylsulfonium hydroxide.

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Matamoros et al. [67] reported on an analytical procedure based upon comprehensive two-dimensional gas chromatography (GCxGC) coupled with time-of-flight mass spectrometry (TOF-MS), for the simultaneous determination of 97 organic contaminants at trace concentrations in river waters. The target analytes included 13 pharmaceuticals, and the LOQs attained ranged, for example, from 3 ng/L to 22 ng/L for the methyl ester derivatives of ibuprofen, salicylic acid, clofibric acid and estrone.

As seen in Table 1, most of the studies involving determination of PhACs using GC-MS have been performed with a single quadrupole as the analyzer. For example, Togola et al. [63] identified 18 compounds in various types of waters (waste-, surface, and drinking waters). In their study, EI mass spectra of derivatized compounds were obtained at 70 eV and were monitored in SCAN mode during preliminary analyte derivatization tests. A SIM program was designed to capture the signals from quantitative fragment ions for each target compound, as well as their surrogate standards, in order to increase detectability. Also, a single quadrupole analyzer was used to determine a group of PhACs using stir bar sorption extraction with thermal desorption in a capillary GC-MS [59]. Splitless injection was performed by ramping the PTV from 150 ºC to 300 ºC. Capillary GC was carried out on an HP-5MS fused-silica capillary column (5% diphenyl, 95% dimethylsiloxane; length 30 m; I.D. 0.25 mm; and film thickness 0.25 μm).

Another potentially effective choice for GC-MS-MS determination of PhACs is the ion trap (IT) analyzer (Table 1). The IT offers unique ion-storage capabilities that allow collection of high-sensitivity, full-scan MS spectra, with high MS resolution and the added ability to perform either MS or MS^n analysis. Sebok et al. [62] developed a multi-residue method using GC-MS-MS (IT) following solid-phase extraction. They attained LOQs lower than 4 ng/L for caffeine, ketoprofen, and diclofenac, and lower than 27 ng/L for propranolol and metoprolol. A review by Hao et al. [68] also has claimed that use of a triple quadrupole (QqQ) or IT showed great advantages for determination of PhACs in environmental waters.

1.1.1.1.2. Liquid chromatography

Liquid chromatography (LC) is a widely used technique that can overcome some of the drawbacks of GC. The polarity of most PhACs, which presents complications for the use of GC as discussed above, makes LC a relatively simple, robust, and effective technique, and often a preferable approach for determining PhACs in waters [69].

One of the most critical elements of an LC system is the column. For the analysis of PhACs, the most frequently used column types are C_{18} and C_8 [54,70]. For example, Vulliet et al. [54] used a Zorbax Eclipse XDB C_{18} (100 mm x 2.1 mm, 3.5 μm) to separate 26 steroids including natural and synthetic estrogens, in 18.6 min of

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chromatographic time. However, a current trend in LC is the effort to reduce analysis time without sacrificing separation selectivity. It is well known according to Van Deemter equations that the efficiency of the chromatographic process depends largely upon particle size, among other parameters. Small particle diameters can significantly reduce the height of the theoretical plate (HETP), which results in higher efficiency and a flatter profile in the Van Deemter curve. Along these lines, LC has unlashed into ultra-high-performance liquid chromatography (UHPLC). This is a new technology, which uses analytical columns packed with smaller-sized particles (1.7 μm in diameter vs. 4-5 μm). This development offers the advantage of increasing speed (<10 min vs. ~30-45 min), and also improved sensitivity, selectivity, and specificity when compared to conventional LC analysis. As speed and sensitivity are of critical importance in analysis of complex matrixes, applications of UHPLC are now being reported in the literature. For example, Petrovic et al. [71] developed a UHPLC-MS-MS method for the successful positive-mode determination of 23 compounds in only 14 min, about one-third of the 45-minute analysis time required by conventional LC. Furthermore, for the 12 compounds determined in negative mode, the separation was performed in only 6 min.

It has therefore been demonstrated that these sub-2 μm particles have the ability to maintain accuracy and robustness in assays, while reducing analysis time. However, since pressure increases according to the inverse square of the decreasing particle size, the use of these small particles often requires the use of specialized LC systems capable of tolerating back-pressures of up to 15,000 psi. The introduction of Fused-Core™ particles therefore has represented another useful advance, because their use eliminates the need for specialized LC instrumentation. These new Fused-Core™ particles are 2.7 μm in diameter, with a 1.7 μm solid core and a 0.5 μm porous outer shell with 9 nm pores. This configuration provides an effective combination of characteristics that allow for very rapid separations at modest pressures. Although these Fused-Core™ particle columns have been receiving ample attention in the field of pharmaceutical bioanalysis, there are still few reported applications in the analysis of environmental waters [72].

Another recent trend is that of increasing the temperature of the mobile phase above ambient temperature, which reduces chromatographic time and results in a consequent reduction in solvent use. This kind of application, known as high-temperature LC (HTLC), has recently been reviewed by Farré et al. [73] as a "green" analytical technique for determination of organic contaminants in the aquatic environment.

The main applications of LC in determination of PhACs are based upon use of reversed-phase (RP) chromatographic systems. However, for applications involving ionic and ionizable compounds and where adequate chromatographic resolution is lacking, hydrophilic interaction chromatography (HILIC) has also been introduced in recent years. One of the characteristics of HILIC columns is that they may offer an

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improvement in selectivity as compared to traditional RPLC when applied to the separation of polar compounds. On a conventional RP column, polar compounds are poorly retained, and elute early in the chromatogram along with other non-retained components of the matrix. This causes decreased ionization efficiency in the ion source. However, by using a higher proportion of organic solvent in the mobile phase, the desolvation process can be improved and sensitivity increased. Although there are still few studies where this type of chromatography has been applied to PhACs, some examples have been reported with cocaine and its metabolites, which because of their high polarity justified the application of HILIC (Table 2).

A recent study by Fontanals et al. [72] represented an initial application of on-line solid-phase extraction coupled with HILIC-MS for the determination of polar PhACs, including some drugs of abuse such as cocaine. As expected, the HILIC showed an increased sensitivity for such compounds. The method as a whole allowed LOD levels comparable to those reported for off-line SPE/HILIC-MS-MS systems using lower samples volumes. Also, Gheorghe et al. [74] determined cocaine and its principal metabolites in waste- and surface waters using HILIC-MS-MS, with their method showing increased sensitivity to the metabolite ecgonine methylester compared to RP chromatography.

As seen in Table 2, several types of detectors can be applied with LC, such as DAD, FD, or UV. For example, Turiel et al. [75] developed a method using LC-UV for determination of a group of selected fluoroquinolones with limits of detection between 8 ng/L and 20 ng/L after pre-concentration. However, most recently MS and MS-MS are being used as substitutes for these detectors because all their benefits for the identification of intermediates and unknown compounds, and for confirmation of the presence of PhACs in environmental waters.

In regard to coupling of LC and MS, three types of atmospheric pressure ionization (API) interfaces can potentially cover the entire range of polarities. These are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and the most recently developed, atmospheric pressure photoionization (APPI) [76]. The characteristic ions are the same for all three API techniques. For full-scan, positive ion mass spectra, the base peak is usually the $[M+H]^+$ ion, often along with the less abundant alkali-metal adducts $[M+Na]^+$, $[M+K]^+$. Typical volatile salts in LC-MS are ammonium acetate or formate at low mmol/L concentrations. Depending on the mobile phase composition, ions such as $[M+NH_4]^+$ may also be found. The negative ion mode typically shows the peak of the deprotonated molecule $[M-H]^-$ as the base peak.

As the softest ionization technique, ESI is the method of choice for polar to ionic compounds, whereas APCI is most effective in the determination of compounds with medium and low degrees of polarity [77]. The main drawback in the use of both of these sources is their susceptibility to the presence of other compounds in the matrix, as co-eluting compounds can cause signal enhancement or suppression. When

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analytes and other compounds in the matrix enter the ion source simultaneously, the ionization efficiency of the analyte can be influenced and a loss or increase of sensitivity may occur. This effect is greater in ESI sources, because in this technique, ions are released from electrically charged droplets. The limited amount of charge per droplet is often exhausted by the conversion of water matrix components to free ions, with no charge then remaining available for the formation of free ions of the target analytes. As such, the reproducibility and accuracy of the method can be affected.

To solve the problem of ion suppression or enhancement, an appropriate internal standard (a structurally similar unlabeled or isotopically labeled compound), having the same ionization characteristics, may be used to compensate for losses in signal intensity [78]. The use of internal standards is simple, efficient, and less time consuming than other methods, such as matrix-matched calibration. However, structurally similar, isotopically labelled compounds appropriate for use as internal standards are usually very expensive, or in some cases are not commercially available at all (e.g., for antibiotics). However, as an example of the technique's application, Gros et al. [31] found levels of ion suppression of 67–74% for some antibiotics such as azythromycin and norfloxacin, and for the β-blockers propranolol and ranitidine. They therefore used an internal standard calibration, although the compounds lacking an appropriate internal standard were quantified by dilution of the sample extracts.

Another of the most critical components of the MS system is the analyzer. Various types of analyzers have been used to determine PhACs in waters, including single quadrupole (Q) [79,80], ion trap (IT) and time-of-flight (TOF) [28]. When the benefits of individual types of these mass analyzers are compared, trap-based analyzers appear to be preferable for studying fragmentation pathways, and TOF-based analyzers are a preferred option for determination of elemental composition because of their high accuracy. LC-MS (IT) was used by Grujic et al. [28] to determine 19 pharmaceuticals from different therapeutic classes, among them sulfonamide, macrolides, and tetracyclines. TOF analyzers acquire the mass spectra in full-scan mode, and various empirical formulae can be obtained. Identification of non-target compounds requires the study of fragmentation combined with other information from the existing literature. For example, Trovó et al. [81] developed a LC-MS (TOF) method to identify six phototransformation products during sulfamethoxazole photolysis.

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Table 2. Analytical methods using LC for determination of PhACs in waters.

Analytes	Chromatographic technique (analyzer)	Ref.
Hormones	LC-UV	[82]
Hormones	LC-DAD	[83]
Hormones	LC-UV	[84]
Drugs of abuse	LC-MS-MS (QqQ)	[52]
Fluoroquinolones	LC-MS-MS (QqQ)	[70]
Sulfonamides, tetracyclines, analgesics, hormones	LC-MS-MS (QqQ)	[85]
Carbamazepine, sulfonamides, lipid regulators, anti-inflammatories	LC-MS-MS (QqQ)	[13]
Analgesics, anti-inflammatories, lipid regulators, psychiatric drugs	LC-MS-MS (QqQ)	[55]
Quinolones, sulfonamides	UHPLC-MS-MS (QqQ)	[86]
Drugs of abuse	UHPLC-MS-MS (QqQ)	[45]
Ranitidine, carbamazepine, trimethoprim, psychiatric drugs	UHPLC-MS-MS (QqQ)	[44]
Antiepileptics, lipid regulators, analgesics, β-blockers, anti-inflammatories, illicit drugs	UHPLC-MS-MS (QqQ)	[26]
Drugs of abuse	HILIC-MS-MS (QqQ)	[87]
Benzodiazepines, sulfonamides, macrolides, tetracyclines, anti-epileptics	LC-MS (IT)	[28]
β-blockers	LC-MS (QqLIT)	[34]
Analgesics, anti-inflammatories, lipid regulators, antibiotics	LC-MS-MS (QqLIT)	[31]
Macrolides	LC-MS-MS (QqLIT)	[88]
Analgesics, anti-inflammatories, lipid regulators, psychiatric drugs, anti-inflammatories	UHPLC-MS (Q-TOF)	[71]

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However, as mentioned above, PhACs tend to be found at very low concentration levels, and the dominant current trend is towards application of LC-MS-MS. Numerous studies have reported on the use of the sensitive triple quadrupole (QqQ) for determination of PhACs [13,44,55,70,85], because of its suitability for quantification. The most typical approach when working with QqQ, has been the acquisition of two or more transitions named as multiple reaction monitoring (MRM), in order to obtain excellent sensitivity for the target analytes. The most intense transition is used for quantification purposes, and the other to confirm the presence of target compounds in the sample. Besides monitoring of the MRM transitions, two other criteria must also be used for quantification in order to obtain four identification points (IP), as required by the European Commission Decision (2002/657/EC) of 12 August 2002 [89]. Although this EU Commission Decision is designed to apply to contaminants in food, it is also used for environmental samples in the current absence of more specific legislation. In order to meet these two additional criteria, first, LC retention time of the compound in the standard is compared with the retention times of compounds obtained from the samples. The retention time of the compound in the sample must be within ±2% of the retention time for the analytical standard. Secondly, the relative abundances of the two selected analyte MRM transitions in the sample must be within ±20% and ±50%, depending on the relative abundance of the two MRM ratios of the analytical standards.

Until recently, LC-MS-MS instruments with QqQ analyzers have been the most widely employed for quantitative target compound analysis. But even though the sensitivity, selectivity, and efficiency characteristics of the MRM approach are excellent, qualitative information needed to support the structural elucidation of analytes is lost [31]. Because of the highly complex nature of some samples, techniques with high resolving power are needed to provide additional structural information. To this end, hybrid mass spectrometers have been developed, which combine multiple analyzers within a single instrument and which therefore can collect more information from a given sample. There are now several types of hybrid instruments available including, for example, quadrupole-time-of-flight (Q-TOF) [43,71], quadrupole-linear ion trap (Qq-LIT) [31,77,88,90], and linear ion trap-orbitrap (LTQ-FT) [91,92]. All of these new hybrid types have been used for the determination of PhACs in environmental waters (Table 2).

Q-TOF coupled with LC has proven to be a powerful tool for the identification of trace-level constituents of complex mixtures. This was the basis of the multi-residue method developed by Petrovic et al. [71] for screening and confirmation of 29 pharmaceuticals. Hybrid Q-TOF-MS allows the measurement of all of the product ions with accurate mass data, and therefore optimal selectivity. As mentioned above, with TOF an empirical formula can be assigned for each ion, but by using Q-TOF it is easier to determine the fragmentation path of the precursor ion. This leads to a

remarkable increase in confirmatory capabilities, compared with those of QqQ instruments. Confirmation performed by Q-TOF can be considered to be unequivocal, because the risk of reporting false positives is extremely low. Other advantages of Q-TOF include high ion transmission, which results in higher MS^n sensitivity and a higher range of intensities with which accurate mass data can be acquired [91]. However, the main drawback of Q-TOF is the method's lower sensitivity compared with that of QqQ working in MRM mode [93].

While Q-TOF is most suitable for confirmation purposes, as well as for identification of metabolites, QqLIT is appropriate for both quantification and confirmation because of its ability to provide exact measurements of mass. For example, QqLIT was the technique of choice used in a study that determined 73 pharmaceutical compounds in surface waters and wastewaters [31]. Also, a group of ten sulfonamide antibiotics were determined in STP waters and river waters using QqLIT-MS [90]. In this study, sulfonamides were analyzed in ESI (PI) mode, using $[M+H]^+$ as the precursor ion in all cases. Instrumental limits of detection (ILODs) of 0.004 pg for sulfadimethoxine and 0.3 pg for sulfathiazole, sulfadiazine, sulfamethazine, and sulfamethoxazole were established. Quantification and confirmation of compounds were performed using MRM transitions. All sulfonamide antiobiotics are derivative of sulfanilamide, and show a typical fragmentation at m/z 156, which results from cleavage of the S-N bond and yields the stable sulfonilamide moiety $[M\text{-}RNH_2]^+$, which is used for purposes of quantification. The use of the linear ion trap analyzer, together with the FT Orbitrap's high resolution and accurate determinations of mass, allows high-quality, accurate MS^n spectra to be acquired.

As can be seen from the information above, the current methodological trend for determination of PhACs in waters involves the use of LC-MS-MS with various types of analyzers, selected depending upon the particular goals of the study. However, the QqQ is the preferred analyzer when a higher degree of sensitivity is required.

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1.1.1.2. EXTRACTION TECHNIQUES

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As mentioned above, analytical methods involving highly sensitive instruments are being used to determine PhACs in waters. However, their typically very low levels of concentration and the coexistence of complex matrices require extraction techniques that can selectively and efficiently pre-concentrate analytes of interest.

A variety of techniques are used to extract pharmaceutical compounds from environmental waters. Until the 1980s, the most commonly used extraction technique was liquid-liquid extraction (LLE). Recently, further efforts have been devoted to the miniaturization of LLE, in order to make the technique more versatile and effective. Reducing the use of hazardous organic solvents and processing of only small amounts of matrix are other major incentives that have motivated scientists working towards increased miniaturization. This ecologically oriented paradigm has come to be known as "green chemistry", based upon the goals of reduction of both the amounts of solvent used and the generation of by-products that may be hazardous to human health or the environment. In accordance with this paradigm, some of the main trends in new extraction techniques involve, in addition to miniaturization, automation, high-throughput performance, on-line coupling with analytical instruments, and low-cost operation with extremely low levels of solvent consumption, or even none at all [94]. Also inspired by these "green" concerns, microextraction techniques have been developed, including solid-phase microextraction (SPME), liquid-phase microextraction (LPME), microscale supported liquid membrane extraction (μ-SLME), and microscale liquid-liquid microextraction (μ-LLME). Applications of these microextraction techniques and some innovative variations of them are now being reported in the literature for determination of PhACs. For example, Hylton et al. [95] have reported on an approach that includes μ-SLME and μ-LLME for the extraction of a diverse set of antibiotics, with an extraction time of 60 min and enrichment factors as high as 2700.

Extraction methods are now receiving increased attention, and should continue to do so in the future. A critical review of modern extraction techniques has been published by Raynie [96], who makes clear the fact that meaningful advances have occurred in the development of new sorptive-based extractions. Two of these techniques are stir-bar sorptive extraction (SBSE) and the most widely used technique, solid-phase extraction (SPE). The development of various types of phases has been the main advantage that SPE has offered for achievement of increasingly efficient and selective extractions.

As a result of recent advances in extraction techniques, low concentrations of PhACs are now being detected and measured in wastewater, surface waters, and even drinking water. Below, an overview of the extraction techniques employed in the determination of PhACs is provided.

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1.1.1.2.1. Solid-phase microextraction and related techniques

SPME is a technique developed by Pawliszyn and co-workers in the early 1990s. It is based on the partition equilibrium of target analytes between a polymeric stationary phase, which uses a coated fused-silica fiber, and the sample matrix [97]. This technique is very simple, fast, easily automated, portable, and inexpensive, and also requires only small sample volumes. Extraction modes for SPME include direct immersion of the fiber and head-space analysis, and can be applied to various types of samples (liquid, gas, or solid). The extracts can be desorbed thermally for GC analysis, or by using solvents for LC analysis.

It is important to use the proper type of coating for a given application. Several coatings are commercially available for SPME analysis, including polydimethylsiloxane (PDMS), polyacrylate (PA), divinylbenzene (DVB), carboxen (CAR), carbowax (CW), and polietilenglicol (PEG). Fibers are available in varying thicknesses and with single coatings, mixtures, or co-polymers. Although these fibers are suitable for application of SPME in the determination of non-polar organic compounds, there are limitations in their application with some types of very polar compounds. Therefore, one potential challenge for SPME involves sampling and determination of polar organic compounds, although derivatization can be used to address this issue. SPME derivatization has been widely used for treatment of polar compounds in order to enhance their recovery and improve selectivity and sensitivity when the SPME method is applied. This approach makes it is possible to identify substances with poor chromatographic behavior, high levels of reactivity, or thermal instability in GC. One particularly interesting SPME approach is simultaneous derivatization and extraction, also referred to as on-fiber derivatization. In this method, the derivatizing reagent is loaded on the fiber, and the fiber is subsequently exposed to the sample. Yang et al. [65] developed a SPME on-fiber silylation technique for simultaneous extraction of some hormones and other EDCs in preparation for application of GC-MS analysis. The analytes were extracted and simultaneously converted to analogs with high levels of affinity for the coating. After SPME, the target compounds were derivatized using the head-space technique, to increase the selectivity and sensitivity of the subsequent method.

In-tube SPME is another effective sample preparation technique, based on the use of an extraction device consisting of an open, tubular fused-silica capillary with an inner surface coating. Target analytes in aqueous matrices are directly extracted and concentrated by the coating in the capillary column through repeated withdrawal and expulsion of the sample solution, and can then be directly transferred for application of LC or GC. The various procedures involved with in-tube SPME, including extraction, concentration, desorption, and injection, can be easily automated using a conventional autosampler. This method provides better accuracy, precision, and sensitivity than off-line manual techniques. Mitani et al. [70]

developed an automated, on-line, in-tube SPME LC-MS-MS method for determination of five fluoroquinolones in waters. Six different capillary columns were evaluated in terms of their extraction efficiency, with the authors concluding that the use of Carboxen 1010 PLOT gives the best results, with the highest amounts extracted.

1.1.1.2.2. Liquid-phase microextraction and related techniques

As reviewed by Sarafraz-Yazdi et al. [98], liquid-phase microextraction (LPME) was introduced in order to overcome the problems that could occur with the use of SPME fibers. Because of its advantages in terms of high enrichment, efficient sample clean-up, and low consumption of organic solvent, substantial interest has been devoted to LPME in recent years. Some applications of this method for analysis of environmental waters have been reviewed by Lee et al. [99]. In hollow-fiber membrane liquid-phase microextraction (HF-LPME), target analytes are extracted from aqueous samples and transferred into a supported liquid membrane (SLM). They are then sustained in the pores in the walls of a small porous hollow fiber. Zhang et al. [100] reported on their pioneering use of dynamic HF-LPME, combined with a port derivatization of the compounds with trimethylanilinium hydroxide (TMAH), followed by GC-MS analysis. They applied this technique to determine four pharmaceuticals (ibuprofen, ketoprofen, naproxen, and clofibric acid) in waste- and tap waters. Recovery levels were between 97% and 105%, indicating that the matrix had little, if any, significant effect.

A new direction for LPME has been developed and is referred to as electromembrane (EME). This novel system combines the technical setup used in LPME with known principles for electroextraction, which force the extraction process with an applied potential difference across the membrane. Fontana et al. [6] successfully applied EME to determine trace levels of acidic and basic PhACs in waters. Only 10 min of extraction time and 50 μL of organic solvent (octanol) were needed under ideal conditions.

Another of the main methodologies that has evolved from liquid phase microextraction, and which has received increasing attention in recent years, is dispersive liquid-liquid microextraction (DLLME). The advantages it offers include ease of operation, low cost, and a high enrichment factor. There are still few reports on studies using DLLME to determine PhACs. However, one that can be mentioned was published by Du et al. [82], who determined, for the first time, estrone and 17β-estradiol in water samples, using DLLME and LC with a variable-wavelength detector. Their recovery levels in spring, tap, and river waters ranged from 90% to 94% for estrone, and from 84% to 112% for 17β-estradiol. Their results showed that the various matrices had little effect on the efficiency of DLLME enrichment.

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1.1.1.2.3. Stir bar sorptive extraction

Stir bar sorptive extraction (SBSE) was introduced in 1999 by Pat Sandra's group, in order to overcome some of the limitations of the techniques then in existence, in particular those involving SPME and small volumes of sorptive material [101]. Their method is based on sorptive extraction, whereby the solutes are extracted into a polymer coating on a magnetic stirring rod. The extraction is controlled by the partitioning coefficient of the solutes between the polymer coating and the sample matrix, as well as by the phase ratio between the polymer coating and the sample volume. SBSE can also be applied through sampling in vapor phase (headspace), which is known as headspace sorptive extraction (HSSE) [102].

In practice, the stir bar used in SBSE is introduced into the water sample to extract the analytes into the coating polymer over a period of time. Several parameters should be optimized, such as the sample volume, agitation speed, extraction time, pH, ionic strength (NaCl), and organic modifier (MeOH). After extraction, the stir bar is removed, and the analytes are recovered by desorption, either thermal (TD) [59] or liquid (LD) [56,103]. With the thermal method, the stir bar is introduced into a glass tube, which is then placed in a thermal desorption unit installed on a GC system. In an example of this approach, Van Hoeck et al. [59] developed a multi-shot SBSE-TD-GC-MS method for the simultaneous determination of various classes of EDCs and pharmaceuticals. They carried out four different sample preparation procedures in parallel using the same water sample. The resulting stir bars were then analyzed using a single thermal desorption process followed by GC-MS.

When a liquid desorption approach is used, the stir bar is used to stir the solvent, which is suitable for the target analytes, and this extract is then injected into the LC or GC unit [83]. As an example of this approach, Almeida et al. [104] developed an SBSE–LD–LC-DAD method for the simultaneous determination of nine steroid sex hormones (estrone, 17α-estradiol, 17β-estradiol, 17α-ethynylestradiol, diethyl-stilbestrol, mestranol, progesterone, 19-norethisterone, and norgestrel) in water matrices. Assays performed on 30 mL water samples spiked at 10 μg/L levels under optimized experimental conditions, yielding recovery levels ranging from 11.1±4.9% for 17β-estradiol and 100.2±10.4% for mestranol.

Another advantage of SBSE is that is easy to apply and automate when thermal desorption is used. On the other hand, the main disadvantage is that the only commercially available fiber is based upon a single non polar polymer, polydimethylsiloxane (PDMS). To overcome this limitation, in recent years several groups have performed in-house development of more polar coatings and formats suitable for the enrichment of hydrophilic analytes with SBSE [103]. For example, Silva et al. [103] developed a novel polymeric phase based on polyurethane (PU) foams for the enrichment of polar analytes (NSAIDs and lipid regulators) in 25 mL of water samples, using LD. When compared with PDMS, recovery levels for

acetaminophen, naproxen, diclofenac, and ibuprofen were from 1% to 43% for PDMS and 45% to 91% for PU.

Bratkowska et al. [105] have recently developed a new coating of stir bar with a hydrophilic poly(vinilpyrrolidone-divinylbenzene) [poly (VPD-DVB)], a monolithic material suitable for sorptive extraction of certain PPCPs. Using this new coating, they extracted certain polar pharmaceuticals from 50 mL samples of river and sewage water, using LD with 5 mL of MeOH. When comparing the performances of the PDMS fiber and the poly (VPD-DVB) fiber, it was reported that PhACs including carbamazepine, caffeine, antipyrine, propranolol, diclofenac, and ibuprofen showed dramatically improved recovery levels. Recovery values between 42% and 110% were found for all these compounds, with the exception of caffeine (20%) and acetaminophen (9%).

Huang et al. [83] developed a method to determine six steroid hormones in wastewaters with log $K_{O/W}$ values between 2.93 and 5.38. The stir bar developed for the SBSE was coated with poly (vinylpyridine-ethylenedimethacrylate), a monolithic material. This stir bar was able to efficiently extract both polar and non polar analytes and compounds. The extraction efficiency for almost all of these compounds was greater than 90% for both influent and effluent waters, with only the hormone with the highest log $K_{O/W}$ value (5.38) showing a lower efficiency (71% in influents and 62% in effluents). Hormones were also determined using SBSE with PDMS, with recoveries between 11% for 17β-estradiol and 100% for mestranol.

Because of the previously mentioned disadvantage of this technique based upon the limitations of commercially available sorbents, the most widely used extraction technique for determination of pharmaceutical compounds continues to be SPE. It was also the technique that was preferred and primarily used for determination of PhACs in this Thesis research. Therefore, a review of the most critical aspects related to use of SPE is presented next.

1.1.1.2.4. Solid-phase extraction

Because of the wide range of commercially available sorbents, solid-phase extraction (SPE) continues to be the most commonly used technique for preparation of environmental liquid samples. SPE can be used to clean the matrix, as well as to pre-concentrate, derivatize, and store the analytes. A variety of materials are used as sorbents in SPE for extraction of PhACs. The choice of sorbent is the most critical decision in SPE, because the sorbent can influence parameters such as selectivity and capacity. Also, approaches based upon simultaneous extraction of all target analytes using multi-residue methods are widely used.

Formats including disks and plastic or glass cartridges [75,78] are commercially available. The cartridge format is the most widely used, and the most commonly

used SPE sorbents for extraction of PhACs are polymeric sorbents [31,55,106]. Conventional polymeric sorbents are usually based on styrene-divinilbenzene copolymers (St-DVB), with macroporous structure and hydrophobic characteristics. However, these are more effective in retaining non-polar compounds than polar compounds.

The hydrophilicity of a sorbent can be increased either by introducing a polar co-monomer during polymerization or by post-polymerization introduction of polar functional groups into the structure. Some typical examples of such sorbents are commercialized as Oasis HLB and Strata-X. These present a hydrophobic portion with retention mechanisms based upon hydrophobic interaction, and represent sorbents that possess enhanced selectivity for apolar and aromatic compounds. The hydrophilic portion is provided by the vinylpirrolidone group, which contains both nitrogens and carboxylic groups in its structure. Both Oasis HLB and Strata-X allow extraction of a wide range of compounds from various matrices, with highly reproducible recovery. For example, in one reported study, Strata-X was used to extract 12 PhACs from tap water, river water, seawater and effluent wastewater samples. All of the compounds showed recovery levels of 11% to 92%, with the exception of acetaminophen, which was not recovered in effluents [106]. Strata-X was also used for extraction of analytes in 3–4 L of tap water, with recovery levels of 83% for caffeine and 71–79% for E1, E2, and EE2 [107]. For PhACs, however, Oasis HLB is the most widely used sorbent [90,108]. For example, Vázquez-Roig et al. [109] extracted a group of 14 drugs of abuse from 250 mL water samples, with recovery levels between 57% and 120% of the LOQ levels.

Current research related to polymeric sorbents is focusing on the development of dual-phase or mixed-mode sorbents, in order to improve the levels of capacity and selectivity that can be obtained using a single material [110]. The main application of mixed-mode cartridges is for complex matrices, where the analytes can be retained by ionic interactions and the rest of the matrix's components by reversed phase. The elution can therefore be very selective with use of aggressive washing for removal of interfering matrix compounds.

Mixed-mode sorbents are classified as being either cationic or anionic, and can also be classified as either strong or weak in terms of ion exchange, depending on the ionic group attached to the resin. The first commercially available strong cationic sorbent was Oasis MCX, which is based on an Oasis HLB skeleton (polyvinylpyrrolidone-divinylbenzene (PVP-DVB)) and chemically modified with sulfonic groups. Later, Waters Corporation also developed a strong anionic exchange type (Oasis MAX), based upon chemical modification of the Oasis HLB using quaternary amine groups. Weak cationic (WCX) and weak anionic (WAX) versions have also been developed. Several published examples show the increasingly widespread use of these mixed-mode sorbents for extraction of PhACs. For example, Jia et al. [40] extracted tetracyclines using Oasis HLB, with the eluent then

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reconstituted in water and extracted again using a MAX sorbent. The final extract showed lower interference levels because of the MAX clean-up and as a result, no apparent matrix effects were observed. The clean-up thus reduced the matrix effect, with ion suppression values of only 5–24% reported.

Some authors have also reported on studies comparing the efficiency of several sorbents used for extraction of PhACs. For example, Grujic et al. [28] extracted antibiotics, benzodiazepines, and anti-epileptics from surface waters and groundwaters. The sorbent types they tested included one hydrophilic (Speedisk H_2O-philic DVB), one hydrophilic-lipophilic (Oasis HLB), two cationic exchanger (Supelclean LC-SCX and Oasis MCX), two St-DVB copolymer (BAKERBOND SDB-1 and Supelclean ENVI-Chrom P), two C_{18} (Speedisk octadecyl C_{18} and Supelclean ENVI-18), and one carbon cartridge (Supelclean ENVI-carb). Although some compounds showed good recovery with all of the sorbents, others were more variable, such as erythromycin, which showed good recovery only with Oasis HLB (100%), and acetaminophen, which showed the highest recovery levels with the Oasis MCX, Oasis HLB, and St-DVB sorbents.

In addition to these new mixed-mode sorbents, other options for improving extraction selectivity now include the use of immunosorbents and molecular imprinted polymers (MIPs). In immunoextraction-based techniques, SPE cartridges are filled with antibodies specific to the targeted compounds and bound onto silica-based sorbents. Since many PhACs are highly polar, immunosorbents can be very advantageous for their isolation, because the antigen-antibody interactions they rely upon are not based on the hydrophobic process [111]. MIPs, on the other hand, are synthetic polymers that are typically prepared by creating a mixture of monomers, a template, and a cross-link agent. After removal of the template by chemical reaction or extraction, binding sites are exposed, which are complementary to the template in terms of size, shape, and position of the functional groups. As a result, MIPs show highly specific recognition abilities, and allow for the selective uptake of structurally related analytes.

Some authors have reported on their application of MIPs in the determination of PhACs in waters [34,112,113]. The MIPs used may be very selective for a single compound or for a group of compounds. For example, Demeestere et al. [113] determined benzodiazepines with MISPE in river and STP water samples. They recorded extraction recovery levels higher than 70%, similar to those obtained using HLB sorbents. These results showed that paroxetine, fluoxetine, and citalopram had a high tendency to be retained on the MIP (> 68%). On the other hand, venlafaxine, trazodone, and diazepam showed recovery levels of only 6% to 38%, illustrating the selective nature of the MIP sorbent, even within a single subgroup of PhACs.

Another of the main advantages of MIPs is their effective sample clean-up capabilities, which contribute to decreased ion suppression. For example, Gros et al. [34] evaluated the advantages of MIP for extraction of β-blockers in wastewaters.

They concluded that MIPs offered a reduction of matrix effects and higher sensitivity, compared to Oasis HLB. González-Mariño et al. [114] compared three different SPE sorbents (Oasis HLB, Oasis MCX, and SupelMIP-amphetamine) when used for determination of amphetamines in wastewater. Their results showed that Oasis HLB performed poorly, because it led to interfering signals in the LC-MS-MS chromatogram for three amphetamines. With Oasis MCX, all the compounds were determined, but still showed strong signal suppression. The best performance was seen with MIPs, which rendered cleaner extracts with less matrix effects, and thus attained lower LODs (0.5–2.7 ng/L).

While the examples discussed above have all been related to the use of SPE in off-line mode, it can also be performed on-line, directly integrated into the analytical system. The advantages of the on-line approach include minimum need for sample manipulation, the potential for full automation, high throughput, ease of application, and cost efficiency. Another advantage of on-line methods is that the intermediate evaporation step typically used in off-line procedures can be avoided, with the corresponding savings in time and cost. An example of successful development of an on-line SPE method was reported by Ding et al. [88], who used their approach to determinate macrolide antibiotics. In their case, injection of only 1 mL of sample volume was sufficient to achieve LODs of 2–6 ng/L.

Postigo et al. [115] reported on a fully automated method, based upon on-line SPE with LC-MS-MS for the determination of drugs of abuse in sewage waters, including 17 compounds and metabolites belonging to the classes of amphetaminics, cannabinoids, cocainics, opiates, and lysergics. On-line SPE was performed using only 5 mL of sample, with the performance of three different cartridges evaluated: Oasis HLB, PLRP-s, and Hysphere C_{18} (EC). Although the best recoveries for cannabinoids were found with the Oasis HLB, for the rest of the compounds PLRP-s showed the best performance.

Fontanals et al. [116] developed, for the first time, an on-line SPE-LC-UV method using an in-house, mixed-mode, hypercrosslinked resin, modified with amine moieties (HXLPP-WAX). This resin was used as a weak anion-exchange sorbent, and enabled application of a washing step to remove the compounds with acidic, neutral, and basic properties. Among their group of target pharmaceuticals, the most acidic compounds (naproxen, fenoprofen, ibuprofen, and gemfibrozil) were recovered at levels close to 100%, regardless of whether the washing step was included or not. Their results were completely different for the most basic compounds (carbamazepine, metoprolol, and antipyrine), which were only recovered when the washing step was omitted, at levels of 67–92%.

Finally, another existing study has reported on the extraction of four hormones from waters (estriol, estrone, 17β-estradiol, and diethylstilbestrol), using on-line SPE-LC-UV [84]. The innovative aspect of the process in this case was the use of cigarette filters as a sorbent. When the recovery levels for river waters were compared to those

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obtained using a C_{18} sorbent, similar levels were recorded in both cases (86–105%). When Stoob et al. [117] extracted a group of sulfonamides with direct coupling of on-line SPE, using Oasis HLB, to LC-MS-MS, the recovery levels recorded were highly variable (5–104%). However, the main advantage in their case was that more than 500 samples could be analyzed with a single extraction cartridge. The costs for extraction materials were thus reduced by more than 75% compared to those of off-line SPE, where one SPE cartridge is typically used for each sample.

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1.1.2. OCCURRENCE OF PHARMACEUTICALLY ACTIVE COMPOUNDS

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Widespread occurrence of PhACs in the environment is well reported in the literature, and is recognized as an important emerging issue in the field of environmental chemistry. As discussed above, one major source of environmental PhACs is the discharge of residual PhACs and their metabolites (either in active or inactive forms) in treated wastewater effluents. Not surprisingly, a great number of PhACs have been detected in surface waters, groundwater, and even drinking water.

While the section above reviewed technical methods used to determine PhACs in water samples, this section reviews the current state of knowledge regarding the actual overall presence of residual PhACs in the environment (wastewater, surface water, groundwater and drinking water). In addition, new technologies used to remove PhACs from water are discussed.

1.1.2.1. Wastewater

As mentioned above, after their ingestion, PhACs are partially converted to metabolites and excreted (unaltered or as conjugates), to then be delivered through sewage systems to sewage treatment plants (STPs). It has been demonstrated that treatment processes currently in use are unable to completely remove these compounds, and consequently, PhACs can be introduced into the environment via STP effluents. Table 3 summarizes the results of several international studies of PhACs in STP infuents and/or effluents. For example, Zhou et al. [13] found certain PhACs including propranolol, sulfamethoxazole, carbamazepine, indomethacine, and diclofenac at concentrations from 24–2336 ng/L in influents in the UK, where carbamazepine was also measured at a level of 1662 ng/L. Slightly lower levels of carbamazepine (539 ng/L) were also found in influents from Canada [77].

When Gros et al. [31] studied the presence of 73 PhACs in wastewater influents, analgesic and anti-inflammatory compounds showed the highest concentrations, with maximum values of 18,410 ng/L (ibuprofen) and 15,600 ng/L (acetaminophen). In the existing literature, some PhACs have been shown to occur with very high ubiquity in detection samples. For example, acetaminophen, diclofenac, and naproxen showed a 100% level of positive findings in influent samples, with a maximum value of 201 μg/L for acetaminophen. As seen in Table 3, in addition to acetaminophen, acetylsalicylic acid is a heavily used pain-killer, judging by the high frequencies with which its metabolite salicylic acid is detected in influents.

As also mentioned previously, antibiotics are of special concern with regard to their ecosystem impact [86,90,118]. Watkinson et al. [118] reported the presence of several antibiotics in influents, with the maximum value recorded for cephalexin (64 μg/L), and with a ubiquity of detection of 100%.

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Table 3. Occurrence of PhACs in sewage (ng/L).

Family	Compound	Influent	Effluent	Country	Ref.
Analgesics					
	Acetaminophen	201003	n.d	Spain	[55]
		<50-19500	<50-6200	Spain	[71]
	Salicylic acid	276007	236001	Spain	[55]
Anti-inflammatories					
	Ibuprofen	7851	78	Spain	[56]
		150-960	200-750	Spain	[71]
		2600-5700		Spain	[119]
	Diclofenac	397	119	UK	[13]
			1200	Spain	[46]
		345	155	Spain	[56]
		2-43	78	Luxembourg	[85]
	Naproxen	2791	1219	Spain	[56]
		3580	720	Spain	[55]
Psychoactive drugs					
	Carbamazepine	1662	950	UK	[13]
		<100-950	<100-600	Spain	[71]
		539	453	Canada	[77]
	Diazepam	<6		Spain	[119]
Stimulants					
	Caffeine	70067	47	Canada	[77]
Lipid regulators					
	Propranolol	334	62	UK	[13]
		74-144	87-136	Spain	[34]
		<100-380	<100-520	Spain	[71]
	Metoprolol	1203-3047	79-547	Spain	[34]
	Atenolol	1740-2688	618-1370	Spain	[34]
	Trimethoprim	4300	250	Australia	[118]
		40-650	<5-230	Spain	[71]
		2038	1037	Croatia	[120]
		210	192	Canada	[77]
Hormones					
	E1		0.75	Spain	[46]
		3.9	3.0	China	[78]

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Table 3. (continued).

Hormones					
	E1		48.6	Taiwan	[121]
	EE2	49.3	46.7	China	[78]
	DSB	92	92	China	[83]
	Progesterone	90	99	China	[83]
Antibiotics					
	Chlorotetracycline	200	250	Australia	[118]
	Sulfamethoxazole	13-155	4-39	Luxembourg	[85]
		<150-960	<150-800	Spain	[71]
		4664	3176	Croatia	[120]
	Oxytetracycline	72.5	3.8	China	[40]
Drugs of abuse					
	Benzoylecgonine	545-3790	4.1-510	Spain	[122]
		1132	<0.9	Italy	[52]
		82-1898		Belgium	[74]
		2307	92	Spain	[45]
	Cocaine	195-961	1.9-31.1	Spain	[122]
		421	<1	Italy	[52]
		22-678		Belgium	[74]
		225	47	Spain	[45]
	THC-COOH	10.6-21.7	5.4-72.8	Spain	[122]
		62.7	<0.9	Italy	[52]
	Nicotine	56053	4775	Spain	[45]

As mentioned in the previous section, the most widely used form of hormonal contraception consists of a combination of steroidal hormones. As Table 3 shows, some studies have revealed that STP influents and effluents may contain similar concentrations of hormones including E1, EE2, DSB, and progesterone [78,83]. This situation needs to be taken seriously, since these hormones are considered EDCs because of their potential to produce estrogenic effects even at very low levels of concentration.

Verification of the presence of drugs of abuse in the aqueous environment is important because of two significant issues. First, the presence of these PhACs, because of their effects, might cause negative impacts on wildlife. Secondly, more comprehensive knowledge of the concentrations of such drugs in raw sewage might enable more precise estimation of their illegal usage, as proposed by Zucatto et al. [123]. Regarding drugs of abuse in STPs, there has been special attention given in the literature to the presence in influents of cocaine and its metabolite benzoylecgonine. After pioneering studies in Italy [52], several other studies have been carried out regarding this issue in Belgium [74], Spain [45,122] and so on. As Table 3 shows,

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there is a common finding in all these studies, with higher concentration values reported for the metabolite (82–3790 ng/L) than for cocaine itself (22–961 ng/L). In addition to drugs of abuse, non-controlled drugs are often included in studies of this nature, such as nicotine, which has been measured at high concentrations (56,053 ng/L) in influent samples [45].

As can be understood from these STP data, municipal and industrial wastewaters contain an abundance of various types of PhACs. These compounds pass through the wastewater treatment systems without being completely removed, and are then continuously discharged into the environment, primarily into surface waters. The concentration of these compounds in effluent waters varies among treatment plants, depending upon factors related to plant design and efficiency.

In conventional treatment systems, some compounds are degraded, others remain in aqueous phase in the effluent, and still others are adsorbed onto sediment or sludge during treatment. For example, Nieto et al. [124] indicated that some antibiotics, such as tylosin and roxithromycin, were highly adsorbed onto sludge. This also can lead to an environmental risk, because this sludge may be used as fertilizer and thereby cause potential contamination of soil and, through leaching, groundwater as well. On the other hand, some PhACs present in influent waters are not absorbed onto activated sludge or degraded in the conventional processes applied by the STPs and therefore are present in effluent waters [20,92,125]. For example, Table 3 shows that some compounds such as caffeine and acetaminophen are generally well removed. One interesting recent study by Gros et al. [126] regarding removal of PhACs, presents a new classification based on the degree of removal of these compounds by STPs. Some PhACs in fact showed the same levels of concentration both before and after treatment. Medium levels of removal (40–70%) were reported for β-blockers and receptor antagonists. Only NSAIDs showed high removal efficiencies, with the exception of diclofenac, whose removal rate varied from zero elimination up to 100%. Salicylic acid has shown similarly high concentrations in influents and effluents of 277 μg/L and 236 μg/L, respectively, demonstrating low removal efficiency [55]. Finally, not all the compounds show consistent behavior during conventional treatments (Table 3). For example, whereas Quintana et al. [56] reported high removal of ibuprofen in effluents, Petrovic et al. [71] reported largely similar concentrations of this compound in influent and effluent wastewaters.

As studies such as these demonstrate, conventional technologies require application of post-treatments to effectively remove PhACs and ensure safe effluent wastewater. The membrane bioreactor (MBR) process is an emerging, advanced wastewater treatment technology. The MBR process is a suspended-growth, activated sludge system that uses microporous membranes for solid/liquid separation, rather than secondary clarifiers. The typical arrangement involves submerged membranes in the aerated portion of the bioreactor, an anoxic zone, and internal mixed liquor recycles. Results produced by this new technology have been variable. For example,

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roxithromycin, with a moderate hydrophobic nature and a very complex chemical structure, showed low removal efficiency under a MBR treatment compared with other compounds [92]. This same study showed trimethoprim with the highest levels of removal (86–94%), largely because of its hydrophilic nature (log K_{ow}<1), basic characteristics, and reduced antibacterial potency compared to roxithromycin. Although MBR has been considered generally suitable for the treatment of wastewater, some compounds such as carbamazepine have been shown to pass through the MBR process without any reduction in concentration [127].

Another effective post-treatment is the advanced oxidation processes (AOPs). AOPs involve the application of various methods to generate the hydroxyl radical. Among these approaches, ozonation is a well-established technology for wastewater reuse purposes [88,125]. For example, ozone treatment has been reported to be an efficient removal treatment, which under conditions of excess ozone has led to 100% elimination of sulfamethoxazole, trimethoprim, and roxythromycin [92]. The poor levels of removal recorded for existing secondary treatments (e.g., naproxen at 46%) were improved with ozonation, which was also responsible for the removal of sulfonamides at levels greater than 70% [125]. The combination of ozonation and sand filtration with activated sludge treatment has been reported to produce efficient removal (>80%) of most of the PhACs studied (sulfonamides, macrolides, and trimethoprim), but with a lower removal effectively seen for carbamazepine (60%) [125]. Sui et al. [128] have reported that the double bond in the azepine ring of carbamazepine is susceptible to ozone attack. Also, certain polar compounds, including diclofenac and β-blockers such as atenolol, metoprolol, and propranolol were also susceptible to removal by the ozonation process [129].

Although improved methods are increasingly being applied in STP processing, these remain incompletely implemented. Therefore, some compounds are still present at undesirable levels in the effluent waters of some STPs. For most of the effluent samples that have been studied, concentrations are lower than those found in influents, but in one study, for example, trimethoprim was still found in 91% of the samples analyzed, with a maximum value of 0.25 μg/L [118]. In another study, however, the high influent levels of acetaminophen and caffeine were reduced in effluents to 47 ng/L and 6200 ng/L, respectively [77]. These results for effluents are in agreement with those reported by Gros et al. [31], where average levels of 3090 ng/L for ibuprofen and 276 ng/L for acetaminophen were measured. Significantly lower values were found in the USA [60], with ibuprofen measured at 341 ng/L.

Other PhACs, including carbamazepine, propranolol, most hormones, and sulfamethoxazole, showed concentrations between 150 ng/L and 800 ng/L in effluents (Table 3). In another study of hormones in effluent samples, mean concentrations of 19–26 ng/L were reported for E1, E2, and EE2 [121]. Also, several recent studies regarding drugs of abuse in STPs have been conducted, with positive findings in effluents (Table 3). For example, Postigo et al. [115] reported on these

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concentrations in four Spanish cities: Barcelona, Valencia, Benicassim, and Gandia. Almost all of their targeted drugs were present in effluents from the STPs studied, with benzoylecgonine in Gandia reported to have the highest value, 318 ng/L. Benzoylecgonine is the main urinary metabolite of cocaine, and it has been reported as present in effluents in various studies, at concentration values between <LOQ and 510 ng/L, as seen in Table 3. Zucatto et al. [130] provided an overview of the results reported by several authors, and pointed out that high levels of benzoylecgonine (1597 ng/L) and cocaine (149 ng/L) were reported in effluents from the UK. Codeine showed high levels in Switzerland (144 ng/L) and Germany (85 ng/L). The highest levels of EDDP were reported in Switzerland (73 ng/L) and Ireland (58 ng/L), whereas only 7 ng/L were reported from Spain.

1.1.2.2. Surface, ground-, and drinking water

As mentioned above, it is well known that the main source of aquatic PhAC contamination derives from their direct release in STP effluents [131]. When PhACs are not adequately removed by STPs, they can enter the aquatic system: surface waters, groundwater, and seawater. Although marine ecosystems have received little attention in regard to this issue, Wille et al. [132] recently determined 15 pharmaceuticals in the North Sea and Scheldt estuary in Belgium. Their results confirmed the presence of seven pharmaceuticals in the marine environment. The most relevant compounds in terms of frequency and concentration were salicylic acid (855 ng/L) and carbamazepine (321 ng/L).

Groundwater can become contaminated by a variety of sources, including landfills, septic systems, and agricultural fields. García-Galán et al. [133] studied the presence of 19 sulfonamides in groundwater, reporting positive results for the presence of most of these compounds, with a maximum value for sulfamethoxazole (53.90 ng/L).

In contrast to the paucity of literature describing the occurrence and persistence of PhACs in seawater and groundwater, extensive attention has been paid to the quantification of their presence in surface waters. It must be noted that 75–80% of the water in some rivers is made up of STP discharges [134]. When Zhou et al. [13] studied the impact of PhACs in river waters, they found carbamazepine at the highest concentration levels, at 46–67 ng/L upstream of the STP and 167–334 ng/L downstream of the STP. These values were in agreement with other results reported by Gros et al. [31], who found levels of carbamazepine between 1 ng/L and 60 ng/L in the Ebro River.

As seen in Table 4, PhACs such as caffeine, carbamazepine, and sulfamethoxazole are the most frequently detected PhACs in river samples. For example, Wu et al. [36] found caffeine (60–144 ng/L), salicylic acid (70–121 ng/L), and sulfamethoxazole (35–211 ng/L) in river waters from Ohio. For caffeine, a range of concentration levels

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can be found reported in the literature. For example, the maximum levels of 38–68 ng/L reported from the USA [12,44] are significantly lower than those reported by Ginebreda et al. [14] from the Llobregat River in northeastern Spain, where concentrations between 0.03 μg/L and 11.92 μg/L were recorded. Also, in a study from the Danube River, Loos et al. [15] found caffeine in 100% of the samples they analyzed, with an average level of 137 ng/L and a maximum concentration of 1467 ng/L.

Other PhACs reported with high levels of frequency have been carbamazepine and sulfamethoxazole, with maximum values of 66 ng/L and 28 ng/L, respectively [15]. According to a review by Díaz-Cruz and Barceló [27], carbamazepine (325–470 ng/L) and sulfamethoxazole (463–485 ng/L) showed the highest levels of concentration in surface water studies. Antibiotics were detected at quantifiable concentrations in more than 50% of the surface water samples studied by Watkinson et al [118]. A concentration of sulfamethoxazole as high as 1488 ng/L has even been reported from Spain's Llobregat River [90].

A seasonal study of PhACs in Spain's Henares-Jarama-Tajo river system was carried out in February, May, September, and December [135]. It was reported that almost all of the sampling points showed their maximum concentration levels in September. For example, these maximum values included 57–415 ng/L for caffeine, 25–202 ng/L for acetaminophen, 18–104 ng/L for carbamazepine, and 19–156 ng/L for diclofenac. This pattern was interpreted as being due to environmental conditions, especially rainfall, since precipitation in the watershed is concentrated between October and December.

As previously mentioned, the steroid hormones are of special concern because aquatic organisms that possess the appropriate receptors could also experience pharmaco-dynamic effects. Steroid hormone concentrations in Spain showed values of estrone between 0.75 ng/L and 1.68 ng/L in the Llobregat River basin [46]. However, in terms of estrogenic activity, levels such as these should not pose a high risk to aquatic organisms [46]. In surface waters from the Rhone-Alpes region in France, hormones were determined at concentrations between 0.3 ng/L for E1 and 11 ng/L for Levono, a synthetic progestagen. The most frequently detected compounds were the natural progestagen progesterone (1.7–4.1 ng/L), the synthetic progestagens norethindrone and levonorgestrel (2.7–11 ng/L), and testosterone and androstenedione from the natural androgens family (1.6–6.0 ng/L) [54].

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Table 4. Occurrence of PhACs (ng/L) in surface waters, groundwater, and drinking waters (ng/L).

Compound	Surface water	Ground-water	Drinking water	Country	Ref.
Analgesics					
Acetaminophen	25-202			Spain	[135]
	216-1338			UK	[26]
Salicylic acid	70-121			USA	[36]
	0.1-63.1			Spain	[135]
Stimulants					
Caffeine			220	Brazil	[107]
	13-68			USA	[12]
	12-415			Spain	[135]
	1467			Italy	[15]
			125	Spain	[30]
	23.2-38.8			USA	[44]
Psychoactive drugs					
Nordiazepam	26			Spain	[136]
Carbamazepine	325-470				
	80-3090			Spain	[14]
	82			Spain	[136]
	66			Italy	[15]
	8-130	6-23		Serbia	[28]
	9			UK	[26]
	4-5.6			USA	[44]
Anti-inflammatories					
Ibuprofen	9-2383			Luxembourg	[85]
	6.3-2784			Spain	[135]
Diclofenac	55			Luxembourg	[85]
Hormones					
E1	0.3	3.5		France	[54]
	0.90-2.9	-		USA	[12]
	66.2			Taiwan	[121]
	2			Italy	[15]
	27			Luxembourg	[85]
Progesterone			0.93	Spain	[46]
EE2		3.0		France	[54]
Estriol			11.6	Spain	[46]

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Table 4. (continued)

Antibiotics					
Ciprofloxacin	1300			Australia	[118]
Norfloxacin	13.2-18.6			France	[86]
Oxytetracycline	2.2			China	[40]
Sulfamethozaxole	30-11920			Spain	[14]
	28			Italy	[15]
	2000			Australia	[118]
	19.8-26.0			France	[86]
	1-22			Luxembourg	[85]
	71	9.9		Spain	[90]
		3.4		USA	[137]
	9			Croatia	[120]
Erythromycin		<5		USA	[137]
Trimethoprim	20-470			Spain	[14]
	150			Australia	[118]
	25	100		Serbia	[28]
Drugs of abuse					
Benzoylecgonine	1.4-346			Spain	[122]
	44-191			Belgium	[74]
	111			Spain	[45]
Cocaine	0.4-59.2			Spain	[122]
	7-26			Belgium	[74]
	10			Spain	[45]
EDDP	5.2-31.4		0.2-2	Spain	[50]
Methadone	1.9-9.4		0.1-1	Spain	[50]
Nicotine	815			Spain	[45]

A study regarding psychoactive drugs in rivers in Spain [136] has been interpreted as revealing that the use of antidepressants has dramatically increased during recent decades. Although paroxetine is one of the most commonly consumed psychoactive drugs, in the cited study it was not detected in river waters. Therefore, the authors assumed an efficient removal of this compound by STPs. However, carbamazepine was found at a maximum level of 1160 ng/L, indicating inefficient removal, as mentioned in the previous section.

The presence of drugs of abuse and metabolites has been also investigated in a variety of rivers. For example, samples from the Ebro River basin were collected from 15 different locations that were selected to represent a contamination gradient, in order to assess the efficiencies of the area's STPs [122]. The frequency of detection for cocaine, benzoylecgonine, and ephedrine was 100%, for MDMA, 64%, and for LSD, 43%. Benzoylecgonine showed the highest levels of concentration (1.4–346

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ng/L), with a median of 11.4 ng/L. Nicotine was measurable at 815 ng/L, and the rest of compounds analyzed were found at lower concentrations.

Drinking water can be obtained from various sources, including surface waters and groundwaters. Depending on the quality of the source used, a variable range of treatments are required by drinking water treatment plants (DWTPs). The effectiveness of a DWTP in eliminating or degrading contaminants depends on several factors, including the physicochemical properties of the compounds present, the overall quality of the source water, and the treatment processes applied. As an example, the efficiencies of three DWTPs in North Carolina, USA, were evaluated for their ability to remove several targeted PhACs [137]. These DWTPs used conventional processes, which included coagulation/flocculation, sedimentation, chlorination, filtration, and at one of the three, powered activated carbon adsorption, before the water was distributed to consumers. Compounds including sulfamethoxazole (28 ng/L) and erythromycin (49 ng/L) were found to be present in the source waters of these DWTPs. After treatment, only <5 ng/L of these two compounds were found to remain, respectively, indicating highly effective removal.

Standard chlorination processing for drinking water treatment may degrade estrogens inefficiently. Chen et al. [121] found that only 20–40% of estrogenic steroids were eliminated by chlorination. While they noted that conventional DWTP treatment should be able to eliminate trace levels of estrogenic steroids, the authors observed incomplete removal of the more hydrophilic compounds, especially E3.

Advanced treatments are needed when reclaimed water is to be directly used as drinking water. For example, ozone treatment has been found to effectively remove caffeine (76%), while subsequent granulated activated carbon (GAC) filtration removed cocaine (100%), MDMA (88%), benzoylecgonine (72%), and the nicotine metabolite cotinine (63%) [30]. A study published by Benotti et al. [29] concluded that the most effective treatment for the removal of PhAcs by DWTPs is oxidation using chlorine or ozone.

After DWTP treatment, drinking water is distributed and delivered in the taps of consumers. Concentrations of PhACs in drinking waters are the lowest out of the results reviewed here, and some of the relevant literature is summarized in Table 4. For example, high caffeine consumption habits have led to the compound's detection in 100% of drinking water samples reported, with a mean concentration of 0.22 μg/L. This fact, in addition to the extensive reports of caffeine in environmental samples, led Sodré et al. [107] to undertake an international evaluation of the contribution of sewage discharge to concentrations of caffeine in drinking water. They also compared their results with those of Huerta-Fontela et al. [30] from Spain, who reported a mean level of caffeine in tap water of 125 ng/L. These authors also found caffeine in 4 out of 24 treated water samples taken from a DWTP, which represented reductions in concentration of 93% [30]. As seen in Table 4, drinking water samples from Barcelona [46] were reported to be free of hormones, with the exception of one

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sample, which showed estriol at a level of 11.6 ng/L and progesterone at a level of 0.93 ng/L. According to that study's comments, these results were not thought to represent a risk of human toxicity.

As the information presented in this chapter has demonstrated, the existing literature clearly confirms the extensive presence of PhACs as contaminants in environmental waters. Water quality monitoring must continue to be used to help ensure the health and safety of consumers. Future research must also be focused on the development of improvements in the effectiveness of treatment plants.

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1.2. PERSONAL CARE PRODUCTS

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The active ingredients included in the personal care products (PCPs) intended for daily use by individuals, such as soaps, lotions, beauty aids, sunscreens, and fragrances, are typically synthetic organic compounds. These PCPs, when included together with the PhACs discussed in section 1.1, are sometimes treated as a single group referred to as pharmaceuticals and personal care products (PPCPs). PCPs are now being considered as a new group of emerging organic contaminants, and their presence in environmental waters is receiving increasing attention from the scientific community. Although significantly less literature exists regarding the study of PCPs than regarding PhACs, both of these groups of compounds have in common an overall lack of information regarding their toxicological environmental effects. By this point, however, several groups of PCPs have come to be recognized in the literature as aquatic contaminants, including organic UV filters (e.g., benzophenones), antimicrobials (e.g., triclosan), preservatives (e.g., parabens), musk fragrances (e.g., tonalide), and insect repellents (e.g., bayrepel), among others.

After being used, PCPs may be absorbed in the body and later excreted, or are more typically washed off after application. PCPs, as is also the case with PhACs, may be introduced into the aquatic environment via a number of routes, but they primarily enter through discharge in wastewater effluents. Therefore, it is reasonable to state that PCPs are continuously being introduced into the environment. Because these compounds are usually found at very low concentration levels, sensitive analytical methods are required for their determination. This requires improvements in the detection techniques applied, with extraction techniques also being an essential part of the analytical procedure. Presently, the most commonly employed analytical techniques for determining PCPs in waters are gas and liquid chromatography, both of which may be coupled to MS or tandem MS.

This section provides an up-to-date summary of our state of knowledge regarding the analytical methods best suited for determination of PCPs, and also reviews the state of existing research. An extensive review of available information regarding the occurrence of PCPs in the aquatic environment has also been compiled by the present author in a review paper already submitted for publication in the journal *Trends in Analytical Chemistry*. It was fortunately also possible to include the most relevant results found in this Thesis in that review, since experimental work had been completed prior to the article's submittal.

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1.2.1. Personal care products in environmental waters: analytical methods and occurrence

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PERSONAL CARE PRODUCTS IN ENVIRONMENTAL WATERS: ANALYTICAL METHODS AND OCCURRENCE

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Abstract

Personal care products (PCPs) are organic chemicals found in several products widely used in daily human life. These products include UV filters, preservatives, antimicrobials, musk fragrances, insect repellents and the chemicals known as siloxanes, among others. PCPs are increasingly important emerging organic contaminants due to their corroborated presence in environmental waters. We present a review of the most important extraction techniques and chromatographic techniques used to determine PCPs in waters. We also reviewed data regarding the occurrence of these compounds in different kinds of environmental waters and the removal of these compounds in sewage treatment plants.

Keywords: *environmental waters, emerging contaminants, personal care products*

INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) are included in the category known as the "emerging organic contaminants". Whereas pharmaceuticals have been extensively studied in environmental waters during the last years [1-4] and have been shown to occur widely, until recently less attention has been paid to the presence of personal care products PCPs in environmental waters. Several compounds have been included in the group of PCPs, among these are organic UV filters (e.g., benzophenones), preservatives (e.g., parabens), antimicrobials (e.g., triclosan), musk fragrances (e.g., nitro musks), insect-repellents (e.g., DEET), and siloxanes (e.g., decamethylcyclopentasiloxane (D5)).

PCPs are organic chemicals that are included in different products that are widely used in daily human life, such as lotions, gels, cosmetics and even food. Therefore, considerable amounts of PCPs are used every day. After use, they may be absorbed by the body, where they are metabolized and excreted, or washed off after application [5]. As a consequence, a significant amount of these products and their metabolites go down the

drain and reach sewage treatment plants (STPs) [6,7]. Here the PCPs are totally or partially eliminated from influent wastewater during sewage treatment, which leads to them being retained in the sewage sludge or being present in the effluents [8]. Because effluents are usually discharged into surface waters, and most PCPs are lipophilic, they can accumulate in the environment.

Some of these PCPs, such as organic UV filters and preservatives, have shown endocrine-disrupting effects [6]. Although to date there is a scarcity of data on the fate and the behaviour of these substances in the environment, several reports indicate that the presence of all these compounds in sewage and river waters is well established [9-11]. All these studies confirm that STP treatment signifycantly influences the removal of PCPs and that improved technologies are needed. Miège *et al.* [12] created a database regarding the fate of PPCPs in wastewaters and studied the removal of all these compounds. STPs with conventional activated sludge treatment showed a high removal efficiency for triclosan (>80%), whereas the removal efficiency was lower than 60% for several musk fragrances. Some authors have reported that UV-based processes (UV and UV/H_2O_2) are effective at removing several contaminants. Of these contaminants, DEET showed a high removal (>90%) when UV/H_2O_2 based process were used [13].

Determining water quality is a priority for human health, especially given the interest in reusing wastewater. Thus, selective and sensitive methods are needed to determine low levels of PCPs in environmental waters. One trend in extraction techniques is to decrease the consumption of organic solvents. For example, microextraction techniques or stir bar sorptive extraction have been used to determine organic UV filters, fragrances and preservatives [14-16]; however, the variety of polar commercial materials is limited. Solid-phase extraction (SPE) presents a wide range of sorbents and is the preferred extraction technique of most of the authors [6,17,18]. The analytical methods for detecting and quantifying PCPs in waters are generally based on gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS-MS). The decision to use either GC or LC is based on to physico-chemical properties of the target analytes. The most apolar and volatile PCPs, such as musk fragrances and siloxanes, are best determined using GC-MS and GC-MS-MS [19-21], but other PCPs have to be derivatized before GC analysis can be applied. Therefore, it is better to use LC for determining these PCPs at low environmental levels [6,17,22].

PERSONAL CARE PRODUCTS

The name PCPs is a generic term which includes all the organic compounds that are used in personal care products. All these new contaminants are revised continuously and in recent years new groups have appeared. Until now, the literature has classified PCPs into five groups according to the following areas in which they are used: organic UV filters, antimicrobials, preservatives, musk fragrances, and insect repellents. Moreover, siloxanes have also been recently classified as PCPs.
It is important to take into account not only the PCPs but also the by-products that they emit on degrading because in some cases these by-products are even more environmentally persistent [23].

Organic UV filters

Organic UV filters are very often found in sunscreen cosmetics and other personal care products as protection against UV radiation. Most of these compounds are lipophilic compounds (log K_{ow} 4-8) with conjugated aromatic rings and are relatively stable against biotic degradation [24]. Regarding the toxicity of these compounds, it has been reported that the estrogenic activity of most of the commonly used organic UV filters is in the range of other well-characterized estrogenic chemicals such as estradiol [6,10,24]. The EU cosmetics directive permits the commercial use of 26 organic UV filters. The organic UV filters most commonly found in water samples are benzophenones (BP-1, BP-3, BP-4), 2-phenylbenzimidazole-5-sulfonic acid (PBSA), 4-methyl-benzylidene camphor (4-MBC), ethylhexyl methoxycinnamate (EHMC), isoamyl methoxycinnamate (IAMC), octocrylene (OC) and octyl dimethyl-p-aminobenzoate (OD-PABA) [6,16,22].

Antimicrobials

Antimicrobials are chemicals that either kill or prevent the growth of microbes, such as bacteria, viruses, fungi or protozoa. Because of these properties, antimicrobials are widely used in personal care products. They have been receiving increasing attention because of their pronounced microbial and algal toxicity and their potential for fostering resistance. The most commonly used are triclosan (TCS) and triclocarban (TCC), the former having been reported in different kinds of waters [25,26]. Also, degradation products, such as methyl-TCS, have been studied in environmental waters [27].

Preservatives

Synthetic preservatives are widely included in the consumer personal care products. The most commonly used preservatives are parabens, such as methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB), benzylparaben (BzPB) and butylparaben (BPB). Parabens are *p*-hydroxybenzoate esters used to inhibit microbial growth and thus increase the shelf life of a wide variety

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of products (pharmaceuticals, soaps, gels, creams and food). They formulate well because they have neutral pH, no perceptible odour or taste, and do not cause discoloration or hardening. Until recently, parabens were commonly used as preservatives in cosmetics and pharmaceuticals because of their supposed low toxicity, broad spectrum of activity, inertness, worldwide regulatory acceptance, biodegradability and low cost. Nowadays, there is an increasing trend not to use parabens because of growing evidence that they are endocrine disrupters [28].

Musk Fragrances

Synthetic musk compounds are among the most important substances used in the fragrance industry. Nitro, polycyclic and macrocyclic musk fragrances are all used commercially. The European Union has decided to limit the use of nitro musk fragrances in consumer products due to toxicological concerns for humans and environment [29]. Currently, the most commonly used are polycyclic musk fragrances, which have been widely studied in environmental waters and have even been found in the Arctic sea and in lakes in the USA [30,31]. However, to date there have been no reports about the presence of macrocyclic musk fragrances in waters because the use of these substances is much more recent.

Polycyclic musks (PCMs) fragrances are a class of highly alkylated tetralin or indane substitutes. The most commonly found PCMs in water samples are galaxolide (HHCB), tonalide (AHTN), phantolide (AHDI), traesolide (ATII), cashmeran (DPMI) and celestolide (ADBI) [23,32]. The most commonly found nitro musks are musk xylene (MX), musk ketone (MK), musk ambrette (MA), musk moskene (MM) and musk tibetene (MT) [29]. Also, new fragrances that are not musk fragrances, such as OTNE ([1,2,3,4,5,6,7,8-octahydro-2,3,8, 8,-tetramethyl-2-naphtalenyl] ethan-1-one), have been introduced to the aquatic environment [19].

Insect repellents

Insect repellents such as N, N-diethyl-m-toluamide (DEET) piperonyl butoxide (PBO) and most recently bayrepel (1-piperidinecarboxylic acid, 2-(2-hydroxyethyl), 1-methylpropyl ester) and its degradation by-product (bayrepel acid) have been included in commercial insect repellent formulations. Little information is available on the long-term effects of these compounds in aqueous environments. However, repellents have been detected across the world in a wide range of water matrices including groundwater, surface water and drinking water [21,33-35].

Siloxanes

Siloxanes are a new group of PCPs classified by their chemical structure but not by their use. Siloxanes are polymeric organic silicones that consist of a backbone of alternating

silicon-oxigen [Si-O] units with organic side chains attached to each silicon atom. These silicone-based compounds are used in cosmetics to soften, smooth and moisten.

For the last three decades, cyclic siloxanes have been believed to be inert and have been used in several care-products including antiperspirants, skin and sun care creams, hair conditioners, colour cosmetics and particle/pigment treatment. However, recent reports suggest that cyclic siloxanes have direct or indirect toxic effects [36] and that important quantities of siloxanes have been discharged into the aquatic environment through the wastewater [37,38]. Siloxanes include octamethylcyclotetrasiloxane (D4), decamethylcyclopentasiloxane (D5), dodecamethylcyclohexasiloxane (D6) and tetradecamethylcycloheptasiloxane (D7), with D5 being the most commonly used in personal care products [36].

ANALYTICAL METHODS

PCPs are found in environmental waters at low ng/L levels, which mean that sensitive analytical methods are required. Extraction techniques are needed to clean the sample and preconcentrate the analytes.

Currently, the most commonly used technique for extracting PCPs from waters is solid-phase extraction (SPE) [6,17]. However, some studies have reported liquid-liquid extraction (LLE) [39,40]; dispersive liquid-liquid micro-extraction (DLLME) [27]; ionic liquid-based single-drop micro-extraction (IL-SDME) [41]; solid-phase micro-extraction (SPME) [14]; ultrasound-assisted emulsification-micro-extraction (USAEME) [28,42]; stir bar sorptive extraction (SBSE) [43,44] and semi-permeable membrane devices (SPMDs) [45].

To determine the presence of PCPs, both GC and LC coupled to MS and MS-MS have been used depending on the nature of the individual compound [14,28,37]. However, the most polar PCPs are determined using LC, mainly with LC-MS and LC-MS-MS [17,46,47].

Extraction techniques

Although detection techniques have improved, extraction techniques are also an important part of the analytical process and are essential for achieving reliable results and maintaining instrument performance. The main goal is the purification of the matrix to isolate the analytes of interest and remove any matrix interferents that may affect the detection system. Table 1 contains the extraction techniques used in the published methods to determine PCPs.

Although different extraction techniques have been used, SPE is the preferred technique for comprehensive chemical profiling in aqueous samples: it is well suited to extracting and concentrating many compounds that display a wide range of polarities and physicochemical properties, as is the case with PCPs.

Table 1. Analytical methods for determining PCPs in waters.

Matrix	Analytes	Extraction technique	Chromatographic technique	Ref.
River water	*UV filters and preservatives*	SPE (Oasis MCX); eluents: MeOH,MeOH (5%NH_4OH), sequential	UHPLC-MS-MS (QqQ)	[22]
River water	UV filters	SPE (Oasis HLB); eluent: MeOH	LC-MS-MS (QqQ)	[6]
River water	Triclosan and UV filters	SPE (Strata-X); eluent: ethylacetate:DCM	GC-MSD	[11]
River water	Triclosan, preservatives and UV filters	SPE (Bond Elut Plexa); eluent:MeOH:DCM	UHPLC-MS-MS (QqQ)	[17]
River water	UV filters	SBSE	GC-TD-MS	[53]
River water	*UV filters and preservatives*	SPE (Oasis MCX); eluent: MeOH, MeOH (5%NH_4OH), sequential	UHPLC-UV	[18]
River water	Antimicrobials	DLLME ($C_6H_4Cl_2$)	UHPLC-TUV	[27]
River water	Fragrances	DLLME (MeOH)	GC-MS	[57]
River water	Fragrance (OTNE)	LLE (toluene)	GC-MS	[19]
River water	*Fragrances (with pharmaceuticals)*	SPE (Strata X); eluent: ethylacetate	GCxGC-MS (TOF)	[20]
River water	*DEET*	SPE (Bond Elut); eluent: methanol:ACN	GC-MS	[21]
River water	UV filters	IL-SDME	LC-UV	[41]
River water	Fragrances	LLE (DCM and hexane)	GC-MS	[39]
River water and drinking water	Fragrances	SPE (Oasis HLB); eluent: acetone, hexane, sequential	GC-MS	[65]
River water and wastewater	UV filters	SPE (Oasis HLB; eluent: MeOH (5 mM NH_4Ac)	LC-ESI-MS-MS (QqQ)	[7]
River water and wastewater	UV filters	SPE (Oasis HLB); eluents: MeOH	LC-MS-MS (QqQ)	[6]
River water and wastewater	UV filters	SBSE	TD-GC-MS	[52]
River water and wastewater	UV filters and musks	LLE (n-hexane)	GC-MS	[40]
River water and wastewater	Antimicrobials	LLE (trichloroethane)	GC-MS-MS	[56]
River water and wastewater	*Preservatives and triclosan*	SPME (DVB/CAR/PDMS)	GC-MS-MS	[14]
River water and wastewater	UV filters	SPME (PDMS-DVB)	GC-MS-MS	[16]
River water and wastewater	UV filters, antimicrobials	SBSE	LD/LC-MS-MS (QqQ)	[44]

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Table 1. (continued).

Surface and wastewater	*UV filters and triclosan*	SPE (Oasis HLB); eluent: MeOH	LC-MS-MS	[60]
Surface and wastewater	Preservatives	USAEME; eluent: trichloroethane	GC-MS-MS (IT)	[28]
Surface and wastewater	Siloxanes	-	HS-GC/MS	[37]
Surface and wastewater	Fragrances	SPE (ENVI-18); eluent: hexane, hexane:DCM, sequential	GC-MS	[32]
Surface and wastewater	Fragrances	LLE (petroleum ether); eluent: acetone:hexane	GC-MS	[29]
Surface and wastewater	UV filters	SPE (Oasis HLB); eluent: MeOH, MeOH ($AcNH_4$), sequential	LC-MS-MS (QqQ)	[7]
Surface and wastewater	Insect repellents	SBSE	TD-GC-MS	[33]
Wastewater	Fragrances	SPE (C_{18} discs); eluent: hexane, hexane:DCM, sequential	GC-MS	[48]
Wastewater	*UV filters and instect repellents*	SPE (Strata X); eluent: ethylacetate:DCM	GC-MS (TOF)	[59]
Wastewater	Fragrances and preservatives	SPE (Strata-X)	GC-MS	[49]
wastewater	Preservatives and antimicrobials	SPE (Oasis HLB); eluent: MeOH	LC-MS-MS	[46]
Wastewater	Triclosan, preservatives and UV filters	SPE (Oasis HLB); eluent:MeOH:DCM	UHPLC-MS-MS (QqQ)	[17]
Wastewater	Triclosan	SBSE (PDMS); eluent: ACN	LD-LC-DAD	[25]
Wastewater	UV filters	SPE (C_{18}); eluent: ethylacetate: DCM (1:1)	GC-MS	[61]
Wastewater	*DEET*	SPE (Oasis HLB)	LC-MS-MS	[13]
Wastewater	*Fragrances*	USAEME; eluent: chloroform	GC-MS	[42]
Wastewater	Fragrances	SPME (CAR/PDMS)	GC-MS	[15]
Wastewater	Triclocarban	SBSE	LD/LC-MS-MS (QqQ)	[54]
Wastewater	*Triclosan, tonalide, BP-3*	SPE (Oasis HLB); eluent: MeOH	LC-MS-MS (QTrap)	[47]

In cursive the multi-residue methods.

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The SPE procedure has to be optimized in terms of the choice of SPE sorbent, sample volume and pH conditions and elution solvent. Both cartridges and discs are used in SPE (Table 1). For example, Zhang *et al.* [48] extracted a group of synthetic musk fragrances with C_{18} discs and obtained recoveries between 62% and 83%. However, the cartridge format is the most commonly used and a wide range of sorbents (e.g., hydrophilic, hydrophilic-lipohilic or the new mixed-mode polymeric sorbents) are commercially available [6,7,11,18]. For example, Strata-X (surface-modified styrene-divinil-benzene polymer) was used to extract fragrances and preservatives from 200 mL of sample obtaining recoveries higher than 70%; several elution solvents and volume solvents were also tested [49]. Lv *et al.* [32] compared the effectiveness of ENVI-18 and LC-18 at extracting a group of musk fragrances from 1 L of sample and obtained the best recoveries with ENVI-18 and the sample at pH 7.

Oasis HLB, with a hydrophilic-lipophilic balance, proved to be versatile and efficient at extracting analytes with a wide range of polarities and acid/base characters at different pHs (Table 1). For example, our group [17] used this sorbent to extract UV filters, parabens and antimicrobials from wastewater. We found recoveries of 27-89% for 100 mL of influent and 20-92% for 250 mL of effluent.

Recently, mixed-mode polymeric sorbents (anionic and cationics) have enabled highly selective extraction for acid and basic compounds. The most commonly used mixed-mode polymeric sorbent for extracting PCPs is Oasis MCX (strong cationic-exchange/reversed) because of its effectiveness at extracting the basic compounds. This new sorbent provides the dual modes of retention (ion exchange and reverse phase) and is very useful because the interactions between the analytes and the sorbent allow an exhaustive clean-up. It is known that Oasis MCX is more selective than Oasis HLB, and capable of sorpting fewer matrix components, which results in higher SPE recoveries or (more commonly) lower ion suppression in the ESI source [18]. When Kasprzyk-Hordern *et al.* [18] checked several sorbents' ability to extract preservatives and organic UV filters from waters, they obtained the best recoveries with Oasis MCX, except for BP-4, which was best extracted with Oasis HLB (94% compared to 15% with MCX).

Just as mixed-mode cartridges have increased the selectivity of SPE, so too have molecularly imprinted sorbents (MIPs), which have been used to create the so-called MISPE. At its highest point of selectivity, an MIP decreases the non-specific interactions and this can be seen in the reduced ionic suppression when working with MS. Despite all their advantages regarding selective extraction, there is little literature on MIPs and PCPs in waters. One example is that of Beltran *et al.* [50], who synthesised an MIP for benzylparaben and buthylparaben.

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The best results for 500 mL of river water were obtained with an MIP, which showed less interferences than Oasis HLB.

Microextraction techniques are based on equilibrium processes. One example is solid-phase micro-extraction (SPME), which minimizes the disadvantages of conventional techniques, such as solvent and time consumption, whilst also obtaining low limits of quantification. Several PCPs such as organic UV filters, parabens, triclosan and nitro musk fragrances are volatile compounds and can be determined with SPME-GC [14-16] because they can be easily thermally desorbed directly onto the injection port of the GC. Most studies which have used SPME to extract PCPs have used commercially available fibres to cover the lower end of the polarity scale. For example, Polo *et al.* [15] tested five fibres (polydimethylsiloxane (PDMS), polydimethylsiloxane-divinylbenzene (PDMS/DVB), carboxen-polydi-methylsiloxane (CAR/PDMS), polyacrylate (PA) and carbowax-divinylbenzene (CW/DVB)) to extract four nitro musk fragrances from waters using headspace SPME at 100 ºC. The extraction efficiency of CAR/PDMS was higher than that of the other fibres, with recoveries of 92-102% in influent waters and 96-108% in effluent waters. Although a new polar SPME fibre with a polyethylene glycol phase coating has been developed, it has not yet been applied to PCPs in waters.

SBSE is a technique based on the same principles as SPME but which has the advantage of a much larger polymer coating of polydimethylsiloxane (PDMS), thus resulting in a higher sample capacity and extraction efficiency. A thorough review of SBSE [43] shows that it has been successfully applied in the trace analysis of multitude apolar com-pounds, among them some PCPs from environmental waters. For example, Rodil *et al.* [33] used thermal desorption (TD) of the stir bar to determine for the first time eight insect repellents in waters, and obtained recoveries between 29% and 80%. When TD is not available, liquid desorption can be used. When used in combination with LC, liquid desorption can be applied to non-volatile compounds. Also, SBSE (PDMS) was used to extract insect repelling substances from water samples in 180 min of extraction time, with extraction efficiencies of between 29% and 80% [33]. As Table 1 shows, SBSE has proved a very useful technique for extracting organic UV filters [25,44,51-54]. The main drawback of SBSE is that PDMS-coated stir bars can only determine non-polar compounds, which means it is necessary consider other phases when extracting the more polar compounds. There have been few studies regarding new commercially unavailable SBSE phases [55]. However, it is important consider the relevance of these developments to extending and improving SBSE

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applications for the more polar compounds.

Among all the extraction techniques, LLE is the one that has extracted the widest range of organic pollutants from water samples. For example, Lee *et al.* [39] used LLE (100 mL of dichloromethane) to study the occurrence of synthetic musk fragrances (HHCB, AHTN, MX and MK) in waters and found recoveries of between 86 and 88% for 300 mL of tap water. In another study [40], 100 mL and 50 mL of n-hexane (two consecutive extractions) were needed to extract musk fragrances and UV filters from waters. It is well known that LLE is time-consuming, requires large volumes of organic solvents and is difficult to automate. One major trend in analytical chemistry, as previously mentioned, is to use a miniaturization technique to lower organic solvent volumes and thus reduce the environmental impact. Other typical examples of miniaturization techniques for sample preparation include dispersive liquid-liquid microextraction (DLLME), ionic liquid-based single-drop microextraction (IL-SDME) and ultrasound-assisted emulsification-microextraction (USAEME) [41,56,57]. As Table 1 shows, all of these have been applied to the extraction of PCPs from waters. In DLLME the abundant contact surface of fine droplets and analytes from the aquatic phase to the organic phase greatly enhances the extraction efficiency, thus reducing the solvent volume and the time needed. However, the main drawback of DLLME is that it uses a disperser solvent, which usually decreases the partition coefficient of analytes into the extraction solvent. Panagiotou *et al*. [57] used DLLME to determine a group of polycyclic musk fragrances (ADBI, AHMI, ATII, HHCB and AHTN) (Table 1). They found recoveries between 79% and 89% for 5 mL of river water, achieving LODs of 8-63 ng/L.

IL-SDME is used because it is simple to operate, fast, inexpensive, precise, sensitive, virtually solventless and environmentally friendly [41]. Vidal *et al.* [41] determined six organic UV filters used in cosmetics in surface waters (Table 1). The recoveries found for this kind of matrix ranged between 96% and 115% and the extraction was done only with a drop volume of 10 μL in 37 min. LODs were found between 0.06 and 3.0 μg/L, depending on the target analyte, for 20 mL of sample.

USAEME shows the advantage of using ultrasonic radiation to accelerate the mass-transfer process between two immiscible phases and therefore increase the extraction efficiency in the minimum amount of time. Regueiro *et al.* [28] applied this novel extraction technique to determine phenolic preservatives and triclosan efficiently and obtained recoveries ≥85% in only 5 min of extraction time. When the same authors determined nine musk fragrances with USAEME, they found recoveries of between 92-114% for all of them at levels of 0.1 ng/mL in surface and wastewaters (Table 1).

Chromatographic techniques

As Table 1 shows, the most commonly employed analytical methods for detecting and quantifying PCPs in waters are generally based on gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS-MS). Few papers report methods based on capillary electrophoresis (CE) because of its high LODs. However, Blanco *et al.* [58] used CE-DAD with large-volume sample stacking (LVSS) as a preconcentration technique to determine a group of parabens (methyl, ethyl, propyl, butyl and benzyl) at sub-μg/L levels. The choice of whether to use GC or LC depends on the physico-chemical properties of the target analytes. If GC is to be used, the compound must have certain characteristics such as volatilization and thermostability. Whereas siloxanes (volatile organo-silicones) and musk fragrances can be easily determined in waters by GC [15,37], other PCPs such as, preservatives and some organic UV filters need an initial derivatization step to if GC is to be successfully employed [11,14,16,53,56]. PCPs are usually derivatized by using silylating reagents such as bis(trimethylsilyl)-trifluoroacetamide (BSFTA) and N-methyl-N-trimethylsilyl-trifluoroaceta-mide (MSTFA) [16,20]. For example, in situ acetylation with acetic anhydride is one of the most common derivatization procedures for phenolic compounds such as parabens. When Regueiro *et al.* [14] determined parabens and triclosan in water samples using SPME/GC-MS-MS, they obtained LODs of 4.0-8.8 pg/mL for all the compounds except for methyl-paraben (17 pg/mL). Also, silylation can be done on-fibre after SPME, as reported by Negreira *et al.* [16] when determining phenolic UV filters in water samples.

Powerful detection systems are the best tool for achieving the LODs required in waters. Mass spectrometry with electron impact (EI) is the most commonly used technique for determining PCPs in GC-MS. Different analyzers such as ion trap (IT), quadrupole (Q) and time-of-flight (TOF) instruments have been used to determine PCPs. For example, Regueiro *et al.* [42] used an ion trap in EI positive mode to determine a group of fragrances, among other compounds. For example, Langford *et al.* [59] used GC-TOF to determine a group of organic UV filters and repellents in different kinds of waters. The mass errors were acceptable (< 2.6 mD) and LODs were in the range of 1-5 ng/L [59]. Recently, Matamoros *et al.* [20] used for the first time two dimensional gas chromatography (GCxGC) coupled with time-of-flight mass spectrometry (TOF-MS) to determine 8 PCPs, among a group of 97 organic contaminants in river waters. This is a powerful separation technique that is well-suited to complex sample characterization. The detection limits achieved for ADBI, HHCB and AHTN were in the range of 5-8 ng/L.

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Because most PCPs are polar, non-volatile and thermally labile compounds, they are best determined by LC. As Table 1 shows, LC has been used in several studies to determine UV filters, preservatives and antimicrobials [6,7,17,25,60]. Also, ultra-high-performance liquid chromatography (UHPLC) using analytical columns packed with 1.8 μm particles has recently been developed and offers increased speed, sensitivity, selectivity and specificity compared to conventional LC analysis. Several detectors have been used in LC and UHPLC to determine PCPs. For example, diode array detection (DAD) was proposed for determining triclosan in several matrixes and achieved LODs of 0.1 μg/L after SBSE with 25 mL of sample [25]. Recent advances in analytical instrumentation have made it possible to confirm the presence of a compound at very low levels using LC-MS-MS. For example, LC-Qtrap-MS was the technique of choice for determining the presence in wastewaters of several compounds, including some PCPs such as BP-3, AHTN and TCS [47]. However, the triple quadrupole (QqQ) is the commonest and most useful tool for determining PCPs in high sensitivity target analysis. By using the multiple reaction monitoring mode (MRM) to monitor two transitions between precursor and product ions, it is possible to confirm and quantify the presence of PCPs in waters at very low levels (ng/L) [6,17,22]. For example, Rodil *et al.* [60] reported a multi-residue analytical method for determining several groups of PCPs, such as triclosan, organic UV filters and insect repellents in different kinds of water. Negreira *et al.* [7] developed an SPE/LC-MS-MS method for determining six benzophenone UV filters and found LOQs between 0.4 ng/L and 8 ng/L for 500 mL of river water. In agreement with these results, our group developed a method that used SPE/UHPLC-MS-MS with a QqQ analyzer to determine organic UV filters, antimicrobials and preservatives, and obtained LOQs of 3-5 ng/L for 500 mL of river water.

In terms of LC-MS interfaces, electrospray ionization (ESI) is the most frequently used for determining PCPs. It is a soft ionization technique suitable for polar and moderately non-polar compounds. However, a critical aspect when using ESI for quantitative analysis is the occurrence of ion suppression/enhancement in complex samples [46]. This effect may cause an analyte to respond in a significantly different manner in a complex sample than in a pure standard solution. An established method for quantifying the level of ionic suppression or enhancement in environmental samples is to compare the different responses obtained for spiked extracts in different kinds of samples [18]. For example, some compounds such as BP-4 show an enhanced signal that is about 30% higher in river and treated wastewater [7]. Sometimes this effect can be corrected simply by diluting the sample [18], but this solution is not

always allowed to reach low levels. In this respect, González-Mariño *et al.* [46] used appropriated deuterated compounds as surrogate or internal standards (MPB-d_4, $^{13}C_{12}$-TCS and $^{13}C_6$-TCC) to compensate matrix effects for the analogous native analytes (MPB, TCS and TCC) in (ESI)MS-MS. This is a better method than the standard-addition method, which is more time-consuming and laborious.

OCCURRENCE OF PCPs IN ENVIRONMENTAL WATERS

In recent years, increasing attention has been paid to the occurrence of PCPs in waters, although some of them have yet to be studied in depth (Table 2). One area that requires further research is the presence of siloxanes in waters, because D5, a linear siloxane, is frequently used in several personal care products [36]. When Sparham *et al.* [37] studied the presence of D5 in effluent waters, they found levels of between 31-400 ng/L (depending on the STP process).

Another area where research is limited is the presence of insect repellents in sewage. These have been increasingly applied in order to prevent the global spread of infectious diseases by various insects. *N*,*N*-Diethyl-*m*-toluamide (DEET) was discovered in 1953 and has been superseded more recently by newer products such as 1-piperidine-carboxylic acid 2-(2-hydroxy-ethyl)-1-methylpropylester (bayrepel) and piperonyl butoxide (PBO). As Table 2 shows, high concentrations of bayrepel have been found in influent samples (3318 ng/L) [33].

Of all the PCPs that have been studied in sewage, musk fragrances and organic UV filters have received the most attention. Concentrations of fragrances in wastewater were found to reach 2300 ng/L (HHCB) in influents from China, whereas other fragrances such as DPMI, ADBI, AHMI, ATTII were not detected in either influents or effluents [48]. After STP treatments, only maximum values of 297 ng/L (HHCB) were found in effluents [48]. Smith *et al.* [29] reported an interesting study about seasonal occurrence of polycyclic and nitro musk fragrances from STPs in Canada. The lowest concentrations were found in spring for all the STPs, due to infiltration from snowmelt runoff. Values of polycyclic musk fragrances were detected at the highest concentrations, ranging from 2000 to 40000 ng/L (HHCB), 500-14000 ng/L (AHTN) and 40-2000 (ATII) in the influent samples. As Table 2 shows, nitro musk fragrances were detected at lower concentrations, and for example, MX and MK ranged from 2-13 ng/L [29].

Table 2. Occurrence of PCPs in waste and river waters (ng/L).

Compound	Influent wastewater	Effluent wastewater	River water	Country	Ref.
UV filters					
BP-1	-	-	<0.3-17	UK	[22]
	131-245	-	37	Spain	[16]
	31-148	<LOD-13	<LOD-24	Spain	[7]
BP-2	-	-	<0.5-284	UK	[22]
BP-3	31-168	16	<LOD-27	Spain	[6]
	216-462	-	52	Spain	[16]
	97-722	68-506	-	China	[61]
	-	42-54	<LOD-30	Germany	[52]
	41	19	-	Spain	[60]
	184-429	<LOD-84	<LOD-87	Spain	[7]
	11-286	20-100	-	Spain	[17]
	-	-	14	Japan	[53]
	-	42-260	17-71	Spain	[40]
	-	-	<15-44	UK	[22]
	<LOQ-904	<LOQ-121	-	Spain	[47]
BP-4	237-1481	376-1359	<LOD-849	Spain	[6]
	3565-4858	989-1947		Spain	[60]
	20-416	765-1028	20-416	Spain	[7]
	-	-	<3-371	UK	[22]
	-	-	54-220	UK	[18]
OC	36	20	-	Spain	[6]
	-	2-18	<LOD-16	Germany	[52]
	34-153	21-95	-	China	[61]
	72	-	-	Spain	[60]
	-	-	35	Slovenia	[11]
	-	15-70	13-283	Spain	[40]
OD-PABA	-	2-7	<LOD-3	Germany	[52]
	103	19	-	Spain	[17]
PBSA	181-2503	109-2679	<LOD-34	Spain	[6]
	196-342	517	-	Spain	[60]
4-MBC	-	38	5-15	Germany	[52]
	475-2128	299-1287	-	China	[61]
	41-70	25-29	-	Spain	[60]
	-	42-326	17-140	Spain	[40]
EHMC	-	11-23	<LOD-21	Germany	[52]
	54-116	30-67	-	China	[61]
	-	16-177	14-153	Spain	[40]
HMS	-	9	5	Germany	[52]
	-	-	165-345	Slovenia	[11]
IAMC	-	3	-	Germany	[52]
DHB	47-155	11	-	Spain	[17]

Table 2. (continued).

Antimicrobials					
TCS		60-719	24-157	Spain	[40]
	312	28	107	Spain	[14]
	-	-	<5-95	UK	[22]
	936	59	-	Spain	[46]
	728	74-104	-	Spain	[56]
	22-87		-	Spain	[17]
	380	70-430	-	France	[12]
	-	-	68	Slovenia	[11]
	-	-	17-29	Korea	[64]
	<LOQ-<400	<LOQ-<400	-	Portugal	[25]
	<LOQ-2417	<LOQ-512	-	Spain	[47]
TCC	5	-	-	Spain	[46]
	21-362	-	-	Spain	[17]
		48-330		USA	[54]
Preservatives					
MPB	1926-5138	MPB 1.5	2-17	Spain	[46]
	-	-	<03-400	UK	[22]
	1658-4427	1658-4427	-	Spain	[17]
	524-3259	<LOQ-112	-	Spain	[58]
	-	-	10-48	UK	[18]
EPB	198	-	-	Spain	[14]
	452-549	-	<LOQ-3	Spain	[46]
	-	-	<0.5-12	UK	[22]
	196-625	196-625	-	Spain	[17]
	-	-	4-8	UK	[18]
PPB	2640	14	13-24	Spain	[14]
	1147-1302	-	-	Spain	[46]
	-	-	<0.2-11	UK	[22]
BPB	344	-	54	Spain	[14]
Fragrances					
HHCB	-	1259-8697	44-1861	Spain	[40]
	72	65	21	Germany	[66]
	1290	212	12	China	[32]
	2300	297	-	China	[48]
	790-4443	451-1081	-	France	[12]
	-	-	7.3	USA	[65]
	-	-	1140-5740	Korea	[39]
	2893	718	-	Spain	[42]
	7750-14600	54-456	-	Canada	[29]
AHTN	-	56-981	12-194	Spain	[40]

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Table 2. (continued).

Fragrances					
	9	5	10	Germany	[66]
	378	39	8	China	[32]
	-	-	230-1130	Korea	[39]
	717	86	-	China	[48]
	210-1690	144-200	-	France	[12]
	-	-	2.8	USA	[65]
	334	99	-	Spain	[42]
	<LOQ-1932	<LOQ-315	-	Spain	[47]
	1430-8930	45-127	-	Canada	[29]
ADBI	-	11-30	-	Spain	[40]
AHMI	-	-	0.8	USA	[65]
AHMI	-	13-16	-	Spain	[40]
MX	-	59-203	10-23	Spain	[40]
	80	-	2	China	[32]
	-	-	0.15	USA	[65]
	6-15	-	-	Spain	[15]
	85-148	3-11		Canada	[29]
	-	34-218	15-40	Spain	[40]
MK	72	8	3	China	[32]
	65-203	5-46	-	Canada	[29]
	743	80	-	China	[48]
	4-6	2	-	Spain	[15]
	-	-	26-68	Korea	[64]
	113	-	-	Spain	[42]
Insect Repellents					
DEET	561	176	40-45	Spain	[33]
	-	-	64-245	Germany	[21]
	-	-	15-88	Korea	[64]
Bayrepel	3318	74	-	UK	[22]
PBO	249	3	6	UK	[22]
Siloxanes					
D5	-	31-400	<10-29	UK	[37]

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One of the most widely studied organic UV filters is BP-3, with maximum values in influent waters of between 168 ng/L and 462 ng/L (Spain) [6,16] and 722 ng/L (China) [61]. Another organic UV filter with a demonstrated presence in sewage is octocrylene with values of between 34-153 in influents and 2-70 ng/L in effluents [6,40,61].

Preservatives have been also found in sewage at high levels (Table 2). For example, several authors have reported MPB in influent waters at maximum levels of 3259 ng/L, 4427 ng/L and 5138 ng/L [17,46,58]. These levels were considerably lower in the effluent waters (1.5 ng/L) [46], which showed that these compounds were efficiently removed by the STP treatments.

One of the most commonly used antimicrobials, triclosan, was found in influent waters at levels lower than 400 ng/L [25], and at mean levels of 209 ng/L in effluents [27].

As Table 2 shows, effluent samples showed significant concentrations of PCPs. Therefore, most of these PCPs are not efficiently removed by conventional STP treatments. The main mechanisms governing the removal efficiency of PCPs are biodegradation (e.g., oxidation, hydrolysis, demethylation, cleavage of glucoronide conjugates, etc.), sorption on sludge or particulate matter (by hydrophobic or electrostatic interactions), filtration and chemical oxidation. Also, the most volatile PCPs, such as musk fragrances, can be removed by volatilization [12]. One interesting review looked at the biodegradation and removal of several PCPs [62] and concluded that biodegradation pathways need to be further researched to enable us to understand which by-products are forming and how these effect humans and the environment. Even when the parent compound cannot be detected, its transformation products may still be of concern due to their potential stability or toxicity. Sometimes, the transformation products are more lipophilic and environmentally persistent than the parent compound, as is the case with methyl-triclosan (a metabolite of TCS) [27].

Another study investigated the removal of 4 musk fragrances by various sewage treatment processes. The conventional treatment processes removed 53-56%, whereas treatments such as chlorination and UV disinfection proved ineffective [39]. A study from China into ozonation treatment [61] reported removal efficiencies ranging from 28% to 43% for 4 UV filters. When Reif *et al.* [63] studied the removal of musk fragrances in a membrane bioreactor (MBR) they found that the level achieved was only around 46-56%, which was similar to other reported removals for these compounds [39].

One of the biggest concerns about wastewater contamination is the risk of contaminants reaching surface waters. The contamination levels in surface water samples depend on several parameters such as geographical position, proximity to the wastewater discharge point and

weather conditions (rainfall). When the presence of different musk fragrances was studied, galaxolide showed the highest concentration range (between 1259-8697 ng/L) with a frequency of 100% in effluent wastewater and a mean of 440 ng/L in river waters (Table 2) [40].

As Table 2 shows, there have been several studies in different countries that have evaluated surface water contamination. For example, Kasprzyk-Hordern *et al.* [22] studied, among a list of 56 emerging organic contaminants, the presence of UV filters, preservatives and triclosan in two rivers from South Wales (UK). One of the most widely studied PCPs in waters is triclosan due to its reported presence in different kinds of waters. Matamoros *et al.* [20] found triclosan in a range of 10-161 ng/L in four rivers from NE Spain, which coincided with the results found in Korean rivers (29 ng/L) [64].

Also, benzophenones were found at maximum values of 371 ng/L (BP-4) in two rivers from the UK [22], which coincided with another study that showed levels of 416 ng/L (BP-4) in rivers from Spain (Table 2) [7]. When Kasprzyk-Hordern *et al.* [18] studied PCPs in the River Taff, the highest value was found for a group of benzophenones at 220 ng/L (BP-4) and MPB was found at levels between 10-48 ng/L. Studies by our own group [17,44] also showed the presence of BP-3 at levels of 6-28 ng/L in 3 different rivers.

As Table 2 shows, the presence of DEET has been also studied in river waters. For example, different German rivers showed maximum levels of 45 ng/L [33] and 245 ng/L [21].

Several fragrances have been also reported in this kind of matrix. River waters showed values of 1140-5740 ng/L for HHCB and 230-1130 ng/L for AHTN [39]. Other musk fragrances, such as Cashmeran, tonalide and galaxolide were found in river waters at average levels of 170 ng/L, 54 ng/L and 167 ng/L respectively in four Catalan rivers, with galaxolide being present in all the samples [20]. Also, musk ketone has been found at maximum concentrations of 68 ng/L in Korean rivers [64]. A recently developed fragrance that is not a musk fragrance, OTNE ([1,2,3,4,5,6,7,8-octahydro-2,3,8,8,tetra-methylnaphthalen-2yl] ethan-1-one) was found for the first time in German river water at levels of 10 ng/L (upstream) to 100 ng/L (mouth of the river) by Bester *et al.* [19].

The presence of PCPs has been studied not only in rivers, but also in lakes and seawater [30]. For example, Xie *et al.* [31] determined musk fragrances in the Arctic and North Sea. They studied the importance of atmospheric transport for the distribution of HHCB and AHTN.

Recently, attention has turned to the safety of tap water because of the presence of PCPs in water resources. Although research remains limited, some studies into the removal of synthetic fragrances from drinking water showed that conventional treatments were generally inefficient

[65]. Very high concentrations of musk fragrances were found in finished drinking water in Iowa (USA) with the highest concentrations being found for HHCB (2.2 ng/L and 7.8 ng/L) and AHTN (0.51 ng/L and 5.6 ng/L) [65].
Among the PCPs found in tap waters were the organic UV filters 4-MBC, OD-PABA and BP-4 at mean values of 18 ng/L, 2 ng/L and 18 ng/L, respectively [60]. However, the highest values reported in tap waters were 40 ng/L of MPB [58] and 70 ng/L of DEET [34]. Although the risk posed to humans through long-term exposure to very low concentrations of PCPs in drinking waters is unknown, pertinent EU legislation is needed [5].

CONCLUSIONS

All the information reviewed here shows that personal care products are contaminants that need to be taken seriously. An increasing number of studies indicate the presence of musk fragrances, organic UV filters, antimicrobials, preservatives, repellents and siloxanes in all kinds of water samples, even in drinking water. Since many PCPs end up in STPs, it is vital that the correct removal treatments are employed to prevent the PCPs from being discharged into environmental waters.
Several analytical methods have been developed to determine these compounds and the extraction techniques are based on minimizing steps and using less solvent. New methods with lower LODs have been developed to determine PCPs at the environmental levels (ng/L). Both GC and LC tandem mass spectrometry are the most useful for quantifying and confirming the presence of PCPs in environmental waters. Although GC-MS and GS-MS-MS are widely used, they require previous derivatization procedures before they can be applied to certain PCPs. However, LC-MS-MS working with triple quadrupole in MRM mode offers the required sensitivity without the need for any derivatization procedure. Also, new tendencies in LC have led to UHPLC, which can obtain rapid separations with good resolution.

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CHAPTER 2. OBJECTIVES

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The primary objective of the research discussed in this Doctoral Thesis is the development of analytical methods to determine pharmaceuticals and personal care products (PPCPs) in environmental waters. The compounds grouped together as PPCPs can also be divided into two sub-groups, which have been studied in this Thesis: pharmaceutically active compounds (PhACs) and personal care products (PCPs). Among the PhACs, this Doctoral Thesis is most specifically focused on pharmaceuticals, hormones, and drugs of abuse.

The performance of two types of extraction techniques was evaluated, in terms of their contribution to analytical approaches designed to attain the low detection and quantification limits required for determination of these compounds in environmental waters. All of the methods discussed are based upon liquid chromatography coupled to mass spectrometry and tandem mass spectrometry, using quadrupole or triple quadrupole analyzers.

A secondary objective of this Doctoral Thesis is to provide new information regarding the presence of PPCP contaminants in environmental waters from the region of Tarragona, through the analysis of various types of wastewater, surface water, and drinking water samples.

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CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

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As discussed in this Thesis´s Introduction, sewage treatment plants (STPs) can be ineffective at removing emerging organic contaminants from the waters they treat. Indeed, the determination of PPCPs in effluents as reported in the literature has provided abundant evidence of these current limitations. When not removed by STPs, these contaminants often enter surface waters or groundwater when effluents are discharged. Therefore, the presence of PPCPs in environmental waters has become an issue of major concern in the scientific community.

The research contained in this Thesis was carried out because of the recent interest in PPCPs in wastewater samples. River water and drinking water samples were also analyzed, because discharge of STP effluents potentially leads to contamination of these waters as well. Because PPCP compounds are often found at very low concentration levels in all of these matrices, one of the primary objectives of this Thesis was focused on the development of more sensitive analytical methods that can provide more accurate detection and quantification.

This chapter includes the experimental part and results from different studies that have been carried out through this Doctoral Thesis. These results have already been published, or are in the process of being published, in various international scientific journals, and are presented in each section here in article format. For each study or group of studies, a brief introduction is included to establish the context of the research, and the most notable results are also discussed in each section. The list of the articles published as a result of this Thesis research is included in Appendix III.

This experimental section is divided into five sub-sections. In the first, two new methods for the determination of various types of pharmaceutical compounds in waters are developed. In both of these, SPE was used as the extraction technique, and LC-MS with a single quadrupole analyzer used as the chromatographic system. These methods were applied to the analysis of waters from STPs in the cities of Tarragona and Reus, and from Catalan rivers.

In the second section, a method to determine hormones and their conjugates in waters was developed using SPE/LC-MS-MS. As in the case of pharmaceuticals, the method was then applied to determine the presence of these compounds in waters from STPs in Tarragona and Reus.

The third section involves the environmental presence of personal care products (PCPs). In these methods, the types of PCPs considered included UV filters, parabens, and antimicrobials. These were determined using SPE or stir bar sorptive extraction (SBSE) in two different methods and subsequent analysis with UHPLC-MS-MS. The methods developed and evaluated were then applied to determination of the presence of PCPs in wastewaters and river waters.

The fourth section concerns drugs of abuse. These were determined using SPE and LC-MS-MS. A new type of 'fused-core' particle column was used to avoid the high pressures associated with UHPLC. Also, several types of samples were analyzed,

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based upon the magnitude of environmental concern. In order to evaluate the efficiency of local STPs in treating this class of compounds, water samples that had received primary, secondary, and tertiary treatment were analyzed. Finally, the presence of drugs of abuse in surface water at the inlet and outlet of a drinking water treatment plant (DWTP) was studied.

In the last section, the results from a one-year study designed to monitor the presence of pharmaceuticals and hormones in STPs in the cities of Reus and Tarragona, are presented and discussed. The targeted compounds had been previously studied in the first section using LC-MS. However, for this later study, the LC-MS methods applied in the earlier studies were updated to take advantage of the availability of new LC-MS-MS methods, in order to obtain lower limits of detection and higher confirmation power. The results of the earlier studies and those using the newer equipment are also compared.

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3.1. Determination of pharmaceuticals using solid-phase extraction and liquid chromatography-mass spectrometry

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As mentioned in the Introduction, contamination of waters resulting from the widespread use of pharmaceuticals has been identified as a critical environmental issue. After consumption, pharmaceutical compounds are excreted by humans and animals as parent compounds and/or metabolites and consequently enter sewage treatment plants (STPs). However, it is well established that most compounds are not efficiently removed by conventional treatments and therefore eventually enter the environment [1,2]. Numerous studies have reported the presence of pharmaceuticals in wastewaters, surface waters, and even drinking waters [2-5]. Some of these authors have also included caffeine in their studies [4]. As discussed above, the presence of caffeine is not only due to pharmaceutical usage but also to coffee consumption. In some cases, the presence of pharmaceuticals is studied through the presence of their metabolites, such as is the case with salycilic acid, the metabolite of acetylsalycilic acid [6].

Although the potential health effects and toxicity of many pharmaceuticals remain poorly understood, concerns regarding this issue continue to arise. Particular concern has been raised by the fact that antibiotics found in sewage may cause the development of increased resistance in naturally occurring bacterial populations [7].

Numerous methods have been developed for the determination of PhACs in waters. Some of these are multi-residue analytical methods, which allow simultaneous determination of structurally diverse classes of compounds. Various techniques use solid-phase microextraction (SPME) [8] or stir bar sorptive extraction (SBSE) [9], among other approaches, for extraction of target analytes. However, solid-phase extraction (SPE) is currently the technique of choice for the determination of pharmaceutical compounds in waters [4,6,10,11]. Regarding separation techniques, some authors have reported on methods using derivatization followed by gas chromatography-mass spectrometry (GC-MS) or gas chromatography-tandem mass spectrometry (GC-MS-MS) [2,12]. However, due to the high polarity of most pharmaceuticals and hormones, liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) tend to be the preferred techniques applied [13-16].

In this section, the results two studies focused on determination of several groups of pharmaceuticals in waters are presented. In the first study presented, analgesics, anti-inflammatories, anti-ulcers, stimulants, lipid regulators, β-blockers, and anti-epileptics were the targeted groups of compounds. The second study was focused on the determination of other pharmaceuticals and antibiotics, specifically macrolides and sulfonamides. These particular compounds were chosen because they are all widely used and have been reported in the literature as present in various types of water samples. The molecular structures of all of the compounds studied are illustrated in Appendix II.

Both studies presented used SPE as the extraction technique, which is often applied because a wide range of sorbents are commercially available. Polymeric and reversed

solid-phase extraction materials, particularly hydrophilic-lipophilic balanced (HLB) [4] and mixed-mode sorbents [11], tend to be the preferred phases for extracting these compounds. In the first study, the extraction efficiency levels of three HLB sorbents (Isolut ENV+, Strata-X, and Oasis HLB) were compared.

The chromatographic technique applied was LC-MS, using a single quadrupole as analyzer, since it was the instrumentation available to our research group at the time of the study. The group worked in SIM mode and either 2 or 3 ions were monitored.

These methods were applied in order to study the presence of the pharmaceutical compounds listed above in wastewater samples from two sewage treatment plants (STPs) in the cities of Reus and Tarragona, since data regarding the presence of these compounds had never been produced for these plants. Also analyzed were samples from three rivers: the Ebre, Ter, and Llobregat, as well as tap water from the city of Tarragona.

The results of these studies have been published in the *Journal of Separation Science* 30 (2007) 297-303 and 31 (2008) 2182-2188.

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3.1.1. Pharmaceutical determination in surface and wastewaters using high-performance liquid chromatography-(electrospray) mass spectrometry

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PHARMACEUTICAL DETERMINATION IN SURFACE AND WASTE WATERS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-(ELECTROSPRAY)MASS SPECTROMETRY

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Abstract

A method was developed to determine 11 pharmaceutical compounds in water samples. The method uses solid-phase extraction (SPE) and high-performance liquid chromatography coupled to mass spectrometry (LC/MS) using electrospray ionization (ESI) in both positive and negative modes. Three different sorbents were compared for the extraction of analytes from river and wastewaters and Oasis HLB provided the best results. For the solid-phase extraction of 500 mL river water samples, the recoveries were between 41 and 101% with the exception of acetaminophen, salicylic acid and naproxen. The limits of detection (LODs) were between 3-5 ng/L for all the compounds, except naproxen which had a LOD of 15 ng/L. Acetaminophen, caffeine, carbamazepine, bezafibrate and ibuprofen were found in three of the tested river samples at ng/L levels and among them, the highest values were for caffeine and bezafibrate with 305 and 363 ng/L, respectively. For the influent and effluent water samples of the sewage treatment plant volumes of 100 and 250 mL were used, respectively, to obtain acceptable recoveries. All the compounds showed recoveries between 33-91% for effluent samples and 33-72% for influent samples, with the exception of acetaminophen, salicylic acid and bezafibrate, which had lower recoveries. The method developed enabled pharmaceuticals in the influent and effluent wastewaters to be determined in 5 campaigns carried out between February 2004 and June 2005. Several pharmaceuticals were found in the influent samples: for instance, maximum concentrations of ibuprofen and caffeine were 6 and 40 μg/L, respectively.

Keywords: *LC-MS; pharmaceuticals; river water; sewage treatment plant water; SPE*

INTRODUCTION

Large amounts of pharmaceutical compounds (e.g. anti-inflammatories, lipid regulators, antiepileptic drugs, antibiotics, etc.) are used worldwide and they may be present in surface and waste waters at ng/L levels [1-4]. They are carried to wastewater treatment plants from wastewaters and it has been shown that they are not completely removed [5]. Despite being diluted and degraded, pharmaceuticals may be hazardous because they are continuously being released [6-7]. Sensitive and specific analytical methods are required to attain detection limits in the low ng/L. Some authors have used gas chromatography-mass spectrometry (GC-MS) to determine pharmaceuticals in waters [4,8-9], but these methods are time-consuming because they often require the acidic compounds to be derivatized because of their low volatility. The applicability of capillary electrophoresis-mass spectrometry (CE-MS) has also been studied for the determination of drugs in aqueous samples [10], although it needs additional clean-up steps to minimise interferences, and the limits of detection (LODs) are not lower enough. Due to the polarity of most of the compounds, liquid chromatography-mass spectrometry (LC-MS) is the technique of choice to decrease the concentration. Methods based on LC-MS have been developed [11-14] to determine pharmaceuticals. Because of its higher selectivity, sensibility and robust detection LC-MS-MS is also reported in the literature [15-19], although it is more expensive and not always available in laboratories.

Because the concentrations of pharmaceuticals in the environmental samples are low, enrichment steps are needed. Although solid-phase microextraction (SPME) has been shown in some cases to be comparable to solid-phase extraction (SPE) for the extraction of pharmaceuticals from waters [20], SPE is widely used to determine these compounds from environmental water samples and it has been extensively applied by several authors [9,21-23]. For instance, Marchese *et al.* [24] reported a method for analysing six analgesics by SPE/LC-MS-MS in river and wastewater, with detection limits lower than 1 ng/L. Although LC-MS-MS is more sensitive and provides better confirmation than LC-MS, Farré *et al.* [14] reported a SPE/LC-MS method for determining five of these analgesics in surface water and wastewater and levels of 15-56 ng/L were detected.

The aim of the present paper is to develop a method to determine a group of 11 pharmaceuticals in surface water and wastewater. Several parameters of the method, based on SPE followed by LC-MS with electrospray ionization (ESI) interface, were optimised. The presence of these pharmaceuticals is demonstrated by analysing different kinds of waters (three river waters and wastewater). Finally, influent and effluent waste-

water from two municipal STP in Catalonia (north-east of Spain) were monitored for a period of 16 months.

EXPERIMENTAL SECTION

Reagents and standards

Acetaminophen, caffeine, metoprolol tartrate salt, propranolol hydrochloride, salicylic acid, carbamazepine, clofibric acid, naproxen, bezafibrate, diclofenac and ibuprofen were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Individual standard solutions of 1000 mg/L were prepared in MeOH:H_2O (50:50 v/v) for all the compounds except for naproxen and bezafibrate which were prepared in MeOH. A mixed standard solution of 5 mg/L was prepared weekly in MeOH:H_2O (50:50 v/v). Working standard solutions were prepared daily by dilution with MeOH:H_2O (50:50 v/v). All solutions were stored at 4 ºC.
Acetonitrile (ACN), used as the organic component of the mobile phase, and methanol (MeOH), used for the extraction procedure, were LC grade and supplied by Merck (Darmstadt, Germany) and SDS (Peypin, France), respectively. Hydrochloric acid (HCl) and acetic acid, from Prolabo (Bois, France), were used to adjust the pH of the sample and the mobile phase, respectively. Ultra-pure water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Nitrogen of 99.995% purity from Carburos Metálicos (Barcelona, Spain) was used in the extraction procedure.

Instrumentation

The chromatographic system was an HP1100 series LC-mass selective detector (Agilent Technologies, Barcelona, Spain) with an ESI interface. It was equipped with an automatic injector, a degasser, a quaternary pump, a column oven and a photodiode array detector (DAD) system. The chromatographic column was a Kromasil 100 C_{18} (25.0 x 0.46 cm) with a 5 μm particle size (Teknokroma, Barcelona, Spain).
500 mg Oasis HLB (Waters, Milford, Massachussets, USA), Isolute ENV+ (International Sorbent Technology, Mid. Glamorgan, UK), and Strata-X (Phenomenex, USA) cartridges, connected to a manifold (Teknokroma, Barcelona, Spain) and a pump as a vacuum source, were tested for the SPE procedure.

Sample preparation

The water samples were collected from the Ebro, Ter and Llobregat rivers. The wastewater samples were collected from the influent and effluent of the two domestic sewage treatment plants (STPs), which are located in two cities with populations of around 140000 inhabitants, by using pre-cleaned amber glass bottles. All samples were filtered using a 0.45 μm nylon filter (Whatman, Maidstone, UK), acidified to pH 3 (HCl) and stored at 4 ºC until analysis.

Chromatographic conditions

Since both positive and negative ionizations were needed, a binary mobile phase with two different gradient elution programs was used. Solvent A was Milli-Q water with 0.5% acetic acid (pH 2.8) and solvent B was ACN. The gradient for negative ionization mode was: 55% B, which increased to 60% in 6 min, constant for 3 min, to 80% in 12 min, to 100% in 2 min, constant for 3 min and then decreased to 55% for 3 min. The gradient for the positive ionization mode was: 18% B, which increased to 20% in 4 min, to 55% in 5 min, to 60% in 6 min, to 100% in 5 min, constant for 3 min, and finally decreased to 18% in 2 min. Temperature was kept at 30 ºC, the mobile phase flow-rate was 1 mL/min and the injection volume was 50 μL.

Table 1. Compounds studied and ions selected for quantification and confirmation in SIM mode.

Compound	m/z ions	
	Quantification	Confirmation
Acetaminophen	152 $[M+H]^+$	110 $[M-CH_3-CO+H]^+$
Caffeine	195 $[M+H]^+$	138 $[M-CH_3-N-CO+H]^+$
Metoprolol	268 $[M+H]^+$	116 [N-isopropyl-N-2 hidroxypropylamine+ $H]^+$
Propranolol	260 $[M+H]^+$	183 $[M-H_2O-C_3H_7NH]^+$
Carbamazepine	237 $[M+H]^+$	138 $[M-CO-N_2H_2-C_3H_6+H]^+$
Salicylic acid	93 $[M-H]^-$	137 $[M-H+CO_2]^-$
Bezafibrate	360 $[M-H]^-$	274 $[M-H-C_4H_6O_2]^-$
Naproxen	229 $[M-H]^-$	185 $[M-H-CO_2]^-$
Clofibric acid	213 $[M-H]^-$	127 $[M-(CH_2)_3COOH]^-$
Diclofenac	294 $[M-H]^-$	250 $[M-H-CO_2]^-$
Ibuprofen	205 $[M-H]^-$	159 $[M-H-CO_2]^-$

The mass spectrometer was acquired data simultaneously in full-scan and selected ion monitoring (SIM) modes. Different fragmentors were studied to find spectra with at least 3 ions; however, because the fragmentation of the compounds was low, the optimum conditions were performed with 2 ions. The ions selected in the SIM mode are shown in Table 1. In this acquisition mode, the most abundant ion, which corresponds to $[M-H]^+$ or $[M-H]^-$, was used for quantification and the second one was used for confirmation.

In order to find the optimum conditions for each compound in the electrospray ionization, flow injection analysis (FIA) was carried out in the positive and negative ionization modes. Finally, acetaminophen, caffeine, metoprolol, propranolol and carbamazepine were determined in the positive ionization mode while

salicylic acid, bezafibrate, clofibric acid, naproxen, diclofenac and ibuprofen were determined in the negative ionization mode. The averaged conditions for the pharmaceuticals studied were: nebulizer pressure (40 psi), drying gas flow (13 L/min), drying gas temperature (300 ºC) and capillary voltage (3000 V) for the ESI interface in positive ionization, and nebulizer pressure (30 psi), drying gas flow (12 L/min), drying gas temperature (350 ºC) and capillary voltage (3500 V) for the ESI interface in negative ionization. Fragmentation voltages were defined individually and the values used were: 40 V for naproxen, 50 V for clofibric acid and bezafibrate, 60 V for diclofenac and ibuprofen, 100 V for acetaminophen and caffeine and 125 V for salicylic acid, metoprolol, propranolol and carbamazepine.

Solid-phase extraction

Solid-phase extraction was used to preconcentrate the samples and Oasis HLB (500 mg) cartridges were selected. The sorbent was sequentially conditioned by passing 5 mL of MeOH and 2 mL of Milli-Q water and then allowed to dry for 10 min under vacuum.

The sample volumes depended on the type of water. Sample volumes of 100 and 250 mL were extracted for the influent and effluent of the STP, respectively, and 500 mL was used for river water samples. The samples were adjusted to pH 3 with HCl and passed through the cartridge at a flow rate of 10-15 mL/min. Before the elution step, the sorbent was dried under vacuum for 15 min. The retained analytes were eluted using 6 mL of MeOH. The eluate was reduced to dryness under a stream of nitrogen and the residue was redissolved with 1 mL of MeOH:H_2O (50:50).

RESULTS AND DISCUSSION

LC-(ESI)MS

The parameters that affect the performance of the ESI interface in the positive and negative ionization modes were optimized for each compound individually by FIA. To improve the sensitivity we were forced to perform two sequential injections, one in positive mode and the other in negative mode. Therefore, to reduce the time of analysis, two different elution gradients were used. Acetaminophen, caffeine, metoprolol, propranolol and carbamazepine showed better responses in the positive ionization mode and salicylic acid, bezafibrate, naproxen, clofibric acid, diclofenac and ibuprofen were determined in the negative mode. The variables optimised and the intervals tested were: nebulizer pressure (10-60 p.s.i), drying gas temperature (200-350 ºC), drying gas flow (3-13 L/min) and capillary voltage (1000-6000 V). The values that provided the best response and spectrum were selected as optimum values for each compound.

All the compounds showed good linearity by direct injection at low μg/L levels. The linear range was 2-

250 μg/L for all the compounds except acetaminophen, bezafibrate, clofibric acid, salicylic acid and ibuprofen (5-250 μg/L), diclofenac (10-250 μg/L) and naproxen (25-250 μg/L). Limits of detection, calculated as a signal-to-noise ratio of 3, were as low as 1 μg/L for all the compounds, except acetaminophen, salicylic acid, beza-fibrate, clofibric acid, diclofenac, and ibuprofen (3 μg/L) and naproxen (5 μg/L).

SPE procedure

SPE was used to reduce the limits of detection because pharmaceutical compounds are found at very low concentrations in environmental water samples.

The parameters optimized were the type of sorbent, the pH and the sample volume, the drying steps and the elution solvent and its volume. The optimization was done in triplicate using 100, 250, 500, and 1000 mL of Milli-Q water spiked to obtain a final concentration of 100 μg/L. In order to choose the best sorbent, three polymeric sorbents were tested: Isolute ENV+, Oasis HLB and Strata-X. Of the two pH sample values tested (not adjusted and pH adjusted at 3 with HCl), pH 3 provided better recoveries for all three sorbents. We studied whether the cartridges could be dried during the procedure and decided that they should be allowed to dry for 10 and 15 min between the conditioning and the sampling steps and between the sampling and elution steps, respectively. MeOH and ACN were tested for elution. Sequential volumes of 2 mL of each solvent were passed in triplicate through each cartridge. Most of the analytes under study were not quantitatively recovered using Isolut ENV+ because of its hydrophobic nature. Only paracetamol, caffeine, salicylic acid and clofibric acid showed recoveries higher than 80% with MeOH as elution solvent, so Isolut ENV+ was rejected for this study.

Strata-X and Oasis HLB have hydrophilic groups in their structure and are demonstrated to be suitable for retain polar analytes. Recoveries were good when the retained compounds were eluted with MeOH. With ACN, recoveries were lower than 50% for almost half of the analytes. To select the sample volume to be extracted, 100, 500 and 1000 mL of standard solutions in Milli-Q water were tested. The recoveries obtained for Oasis HLB and Strata-X were similar for volumes equal to or lower than 500 mL. However, acetami-nophen, and ibuprofen showed slightly lower recoveries for Strata-X with 1000 mL of sample, so Oasis HLB was finally chosen. For 1000 mL, recoveries were higher than 88% except for acetaminophen (70%).

The low recovery level found for acetaminophen was also found in other studies carried out with Oasis HLB [4]. When the sample volume was higher than 1000 mL, the recoveries decreased even with Oasis HLB. Because of the complexity of the matrix in real samples, when volumes of 1000 mL of river water were analyzed recoveries

were lower, so the volume to be extracted was reduced to 500 mL.

When influent and effluent waters from two STPs were analyzed, the sample volume had to be reduced to 100 and 250 mL for the influent and effluent samples, respectively. As Table 2 shows, the recoveries for the compounds were between 33 and 72% when influent samples were spiked at 750 ng/L. There were three exceptions: salicylic acid and bezafibrate, the recoveries of which were lower than 10%, and acetaminophen, the recovery of which was not determined because of interferences in the chromatographic peak. When 250 mL of effluent samples was spiked at 300 ng/L, the recoveries were between 50% and 91% and only salicylic acid and acetaminophen showed results lower than 37%.

Table 2. Recoveries of pharmaceuticals in influent and effluent wastewater (n=3).

Compound	%R Influent	%RSD[a)]	%R Effluent	%RSD[b)]
Acetaminophen	n.d [c)]	n.d	33	21
Caffeine	50	1	87	7
Metoprolol	72	5	77	17
Propranolol	46	2	89	13
Carbamazepine	34	26	67	10
Salicylic acid	10	26	37	30
Bezafibrate	5	38	59	20
Naproxen	50	10	50	16
Clofibric acid	33	20	54	20
Diclofenac	37	2	57	18
Ibuprofen	48	5	91	17

[a)] 100 mL spiked at 750 ng/L, [b)] 250 mL spiked at 300 ng/L, [c)] Not determined.

To check if it was necessary to analyse the samples immediately after they had been sampled, the stability of the compounds in the cartridge was also studied. Samples of 500 mL of river water were spiked with the compounds at 50 ng/L and loaded in the cartridges. Then the cartridges were stored at –23 ºC. They were analyzed after 6 and 12 months. In both cases, the recoveries found ensured their stability.

Method validation

A sample of Ebro river water was used to validate the method. When this sample was used as a blank, only caffeine was found. So, this signal was subtracted from the samples. Linearity was tested following the procedure developed in the positive and negative SIM modes and the range studied was 5-500 ng/L (Table 3). All the compounds showed r^2 >0.9925 when this type of water was used as the matrix.

Table 3. Recoveries, repeatability, reproducibility, LOD and linear range obtained with three replicates of 500 ml Ebro River water samples spiked at 100 ng/L.

Compound	%R	%RSD[a)]	%RSD[b)]	LOD (ng/L)	Linear Range (ng/L)
Acetaminophen	18	28	30	5	10-500
Caffeine	45	19	18	3	5-500
Metoprolol	68	3	4	3	5-500
Propranolol	89	3	4	3	5-500
Carbamazepine	101	4	12	3	5-500
Salicylic acid	15	5	10	5	10-500
Bezafibrate	82	11	5	5	10-500
Naproxen	25	19	29	15	50-500
Clofibric acid	61	6	24	5	10-500
Diclofenac	47	17	21	5	20-500
Ibuprofen	41	7	19	5	10-500

[a)] Repeatability.
[b)] Reproducibility between days (n=3).

Recoveries were between 41 and 101% when 500 mL spiked at 100 ng/L was analyzed except in the case of acetaminophen, naproxen and salicylic acid, for which recoveries were lower. The repeatability and reproducibility between days were determined by spiking three replicates of 500 mL of river water at 100 ng/L. As can be seen in Table 3, the results obtained, expressed as relative standard deviations (RSD) were lower than 19% for repeatability, and 29% for reproducibility between days. These values were higher only for acetaminophen. The limits of detection, calculated as a signal-to-noise ratio of 3, were 3 ng/L for caffeine, metoprolol, propranolol, and carbamazepine, and 5 ng/L for the rest of the compounds, except naproxen which had a LOD of 15 ng/L.

Application to environmental samples

Samples from three different rivers were analyzed. The rivers were particularly interesting because, after treatment, their waters go on to be consumed by humans. Several water samples were also analyzed from the influent and effluent of two STPs that receive mostly urban wastewaters and some industrial discharges. The average flow-rate of these STPs is 16000 m^3/day for STP1 and 30000 m^3/day for STP2. To confirm the presence of the compounds in these samples, two factors were considered: (1) retention time and (2) relative abundance of the two ions selected.
Peaks appeared at the same retention time as some pharmaceuticals in the river and STP chromatograms.

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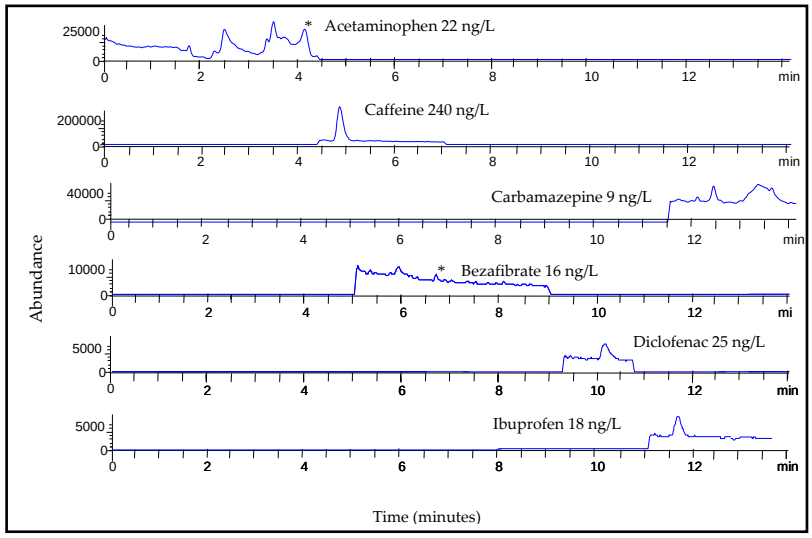

Figure 1. Extracted ion chromatograms of compounds found in a sample from the Ebro River.

Figure 1 shows the extracted ion chromatograms obtained from an Ebro river water sample. As can be seen in Table 4, acetaminophen, caffeine, carbamazepine, bezafibrate, diclofenac and ibuprofen were found in this river sample, with caffeine having the highest concentrations (240 ng/L). The other two rivers analyzed also showed high levels of caffeine, and levels of bezafibrate which in some cases were as high as 363 ng/L. Although the values of caffeine were high, it obviously did not come only from pharmaceutical compounds: coffee was also an important source.

Table 4. Concentrations (ng/L) of pharmaceuticals found in 3 river waters, %RSD (n=3) < 20.

Compound	Ebre	Llobregat	Ter
Acetaminophen	22	30	12
Caffeine	240	305	106
Metoprolol	-[a)]	14	-
Propranolol	-	-	<LOQ
Carbamazepine	9	37	19
Salicylic acid	-	19	13
Bezafibrate	16	78	363
Naproxen	-	-	-
Clofibric acid	-	11	14
Diclofenac	25	-	41
Ibuprofen	18	44	14

[a)] <LOD.

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Some authors showed ibuprofen to be one of the main pharmaceutical contaminants in ground and river water with concentrations in the ng/L [20]. For instance, Farré *et al.* [14] found maximum values of ibuprofen of 2700 ng/L in river water. In our case, however, the highest level of this compound was 44 ng/L in the Llobregat River (Table 4). All the results of the five campaigns between February 2004 and June 2005 for the influent and effluent of two STP are represented in Table 5. As can be seen, the concentrations found in effluent samples were lower than in influent samples. Because of the low recoveries of salicylic acid (10%) and bezafibrate (5%), and the fact that no recoveries were found for acetaminophen, these pharmaceuticals were not determined in the influent samples. The values of ibuprofen were higher in the influent samples of both STPs with values between 1.61-5.99 μg/L for STP1 and 1.62-3.33 μg/L for STP2. In the effluent samples, concentrations of ibuprofen were lower than 0.69 μg/L for STP1 and 0.20 μg/L for STP2.

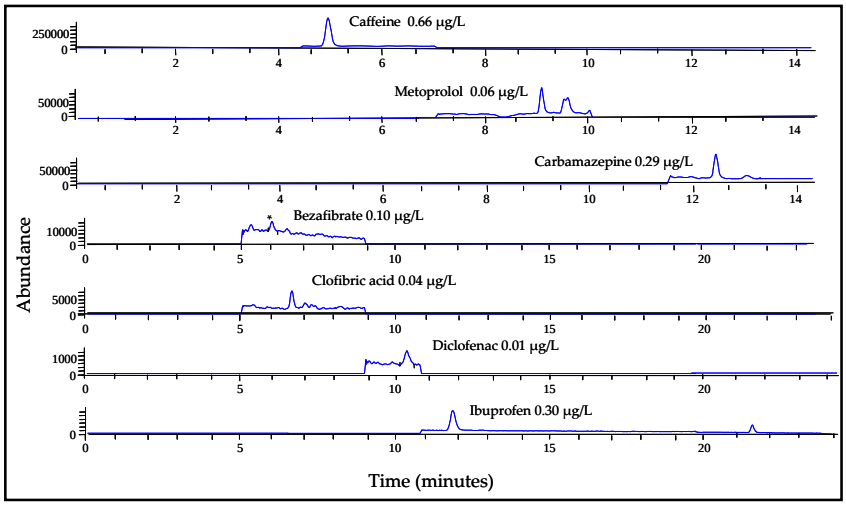

Figure 2. Extracted ion chromatograms of compounds found in a sample from a STP1 effluent.

Concentrations of caffeine in influent samples were lower than 40.12 μg/L, as can be seen in Table 5, and these high levels agree with Ternes *et al.* [15] who found caffeine values as high as 147 μg/L in raw municipal wastewater. In influent samples of STPs, Santos *et al.* [25] found maximum values of ibuprofen of 143 μg/L, and concentrations of naproxen, caffeine and carbamazepine below 16.3 μg/L. Like other authors [2,26] who have found that ibuprofen is the compound that is most removed from STPs, the results of our study showed significantly lower concentrations in effluent samples than in influent samples.

Table 5. Concentrations in the influent (Inf) and effluent (Eff) of two Catalan STP (μg/l). In all cases %RSD (n=3)< 20.

	February 04		June 04		October 04		February 05		June 05	
STP1	Inf	Eff	Inf	Eff	Inf	Eff	Inf	Eff	Inf	Eff
Acetaminophen	n.d[a)]	-[b)]	n.d	0.06	n.d	0.02	n.d	-	n.d	<LOQ[c)]
Caffeine	2.68	-	6.24	-	40.12	0.57	0.95	0.17	2.14	0.66
Metoprolol	-	0.14	0.09	-	0.07	0.04	0.07	0.11	0.04	0.06
Propranolol	0.44	0.03	-	0.02	-	-	0.15	0.01	-	<LOQ
Carbamazepine	-	0.17	0.15	0.17	0.15	0.10	0.10	0.15	0.48	0.29
Salicylic acid	n.d	0.10	n.d	-	n.d	0.19	n.d	0.06	n.d	-
Bezafibrate	n.d	0.34	n.d	0.21	n.d	0.23	n.d	0.09	n.d	0.10
Naproxen	-	-	8.62	0.12	0.65	0.42	-	0.45	0.79	-
Clofibric acid	-	-	0.04	0.03	0.03	0.12	0.14	0.02	0.12	0.04
Diclofenac	-	-	-	0.36	0.28	-	-	0.03	-	0.01
Ibuprofen	5.99	-	2.80	0.05	5.50	0.69	1.61	0.30	2.72	0.30
STP2										
Acetaminophen	n.d	-	n.d	-	n.d	0.02	n.d	-	n.d	-
Caffeine	0.42	-	3.39	-	17.05	0.02	0.49	0.21	24.20	1.01
Metoprolol	0.06	-	-	-	-	0.02	-	-	<LOQ	<LOQ
Propranolol	0.07	0.02	0.04	0.02	<LOQ	0.03	<LOQ	-	<LOQ	-
Carbamazepine	0.12	0.08	0.06	0.08	0.07	0.19	0.33	0.13	0.07	0.16
Salicylic acid	n.d	-	n.d	-	n.d	0.11	n.d	0.05	n.d	0.04
Bezafibrate	n.d	0.28	n.d	0.16	n.d	0.07	n.d	0.14	n.d	0.20
Naproxen	0.42	0.07	0.34	0.04	1.02	-	0.75	0.14	-	0.02
Clofibric acid	0.10	0.02	0.04	0.04	2.02	0.02	0.04	0.01	-	0.04
Diclofenac	0.31	0.46	0.31	0.30	-	0.10	0.55	0.02	0.12	0.12
Ibuprofen	2.86	0.20	2.95	0.02	3.33	0.13	2.59	0.07	1.62	0.14

[a)] not determined.
[b)] < limit of detection.
[c)] < limit of quantification.

On the other hand, as can be seen in Table 5, one of the lowest removal rates was observed for diclofenac and carbamazepine in agreement with other authors [26,27].

The extracted ion chromatograms for an effluent STP1 sample are shown in Figure 2. As can be seen, seven of the compounds studied were found because they had not been completely removed in the STP. The concentration of caffeine was 0.66 μg/L, and the concentration of ibuprofen and carbamazepine was 0.30 μg/L, bezafibrate was found at 0.10 μg/L and diclofenac, clofibric acid and metoprolol were found at lower concentrations.

CONCLUDING REMARKS

A method has been developed for studying the presence of 11 pharmaceutical compounds in aqueous samples using SPE followed by LC-(ESI)MS analysis. The method has been used to evaluate the presence of pharmaceuticals in wastewaters from influent and effluent STPs, and various river waters. Solid-phase extraction with Oasis HLB followed by LC-MS has been shown to be an appropriate method for the trace determination of the most widely used pharmaceuticals in water matrices. So, it is possible to preconcentrate 500 mL samples of river water, 250 mL of influent and 100 mL of effluent water from STPs. The method enabled these compounds to be determined in surface and wastewaters at levels of ng/L. Two STP were monitored for sixteen months and the presence of these compounds was also studied in three rivers. Caffeine and ibuprofen were the pharmaceuticals that were found in highest concentrations in influent samples (maximum value 40.12 and 5.99 μg/L, respectively). In river samples, caffeine showed the highest concentrations (106-305 ng/L).

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3.1.2. Simultaneous determination of macrolides, sulfonamides and other pharmaceuticals in water samples by solid-phase extraction and LC-(ESI)MS

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SIMULTANEOUS DETERMINATION OF MACROLIDES, SULFONAMIDES AND OTHER PHARMACEUTICALS IN WATER SAMPLES BY SOLID-PHASE EXTRACTION AND LC-(ESI)MS

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Abstract

This paper describes a method for determining 11 pharmaceuticals in waters by solid-phase extraction followed by liquid chromatography-(electrospray) mass spectrometry. The solid-phase extraction was carried out with Oasis HLB and the recoveries were 33-67% for 250 mL and 100 mL wastewater, 55-77% for 500 mL river water and 72-98% for 1 L tap water, with the exception of sulfamethoxazole and omeprazole which showed lower recoveries in all kinds of sample. The detection limits (LODs) in river water were of 5 ng/L for sulfadiazine, trimethoprim, sulfamethazine, sulfamethoxazole and ranitidine and 10 ng/L for the other compounds. The highest concentrations found in river waters were for sulfamethoxazole (50 ng/L). In influent wastewaters, ranitidine was the most commonly detected compound with a maximum value of 0.24 μg/L.

Keywords: *LC-MS; macrolides; omeprazole; ranitidine; SPE; sulfonamides; trimethoprim; water samples*

INTRODUCTION

Pharmaceutical compounds, included in the so-called emerging contaminants, are used so extensively in human and veterinary medicine that they have become a new environmental problem. Various studies [1-3] have shown that the currently used wastewater treatment techniques are not completely effective at removing these compounds, which include, among others, sulfonamides and macrolides, two of the most widely used groups of antibiotics in the European Union's animal husbandry [4] and human medicine [5]. Once present in effluent wastewater, these substances may enter river, sea or ground waters, thus putting drinking water supply systems at the risk. In recent years, several methods have been developed for screening and determining pharmaceuticals at trace

levels in surface water [6-8], wastewaters [9-13] and sewage sludge [14].

The prerequisite for proper risk assessment and monitoring of the quality of waste, surface and drinking waters is the availability of methods that allow these compounds to be determined at the low ng/L level (or even below it) [15-16]. The methods reported in the literature are mainly based on off-line solid-phase extraction (SPE) as a preconcentration step.

Instrumental analysis is usually performed by a gas or liquid chromatographic separation coupled to mass spectrometry (MS). Liquid chromatography (LC) is the most suitable and sensitive technique for separating a wide variety of pharmaceuticals, some of which are not amenable to gas chromatography (GC) because of their polarity. The SPE sorbents must be chosen carefully for best results. For instance, when Hilton *et al*. [17] extracted sulfamethoxazole and trimethoprim in effluent and surface waters with Strata-X, they found that recoveries were higher than with Bond Elut C_{18}. Several authors [4,7,18,19] have reported high recoveries when pharmaceuticals are extracted with the hydrophilic Oasis HLB cartridges. For instance, Abuin *et al*. [7] found recoveries between 85-115% with Oasis HLB when macrolides were extracted from 250 mL of river water.

Due to the characteristics of these compounds, SPE/LC-MS [7,19-22] and SPE/LC-MS-MS [17,18,23] have been reported as the most used techniques for determining a wide variety of pharmaceuticals. For example, Hilton and Thomas [17] developed a LC-MS/MS method for determining 13 pharmaceuticals and pharmaceutical metabolites in sewage effluents and surface waters. LODs were 10 ng/L for trimethoprim and erythromycin, and 50 ng/L for sulfamethoxazole. Cahill *et al*. [19] reported a LC-MS methodology with a method detection limit (MDL) of 14 ng/L and 23 ng/L for trimethoprim and sulfamethoxazole, respectively.

Although the electrospray ionization mode (ESI) is the preferred ionization technique, previous studies reported with LC-MS or LC-MS/MS assay with ESI were susceptible to matrix effects with ionization suppression [8,24]. An appropriate internal standard (structurally similar unlabelled standard [18,25] or isotopically labelled standard [21,26,27]) may compensate for the signal reproducibility that leads to erroneous results.

The goals of this study are as follows: (1) to develop and validate a method based on SPE/LC-MS for determining 11 pharmaceutical compounds: 5 sulfonamides, 3 macrolides, omeprazole, ranitidine and trimethoprim (2) to study ion suppression effects and to compare external and internal calibration (3) to use the method to determine pharmaceuticals in different kinds of water.

EXPERIMENTAL SECTION

Reagents and standards

All the standards: five sulfonamides (sulfamethoxazole, sulfadiazine, sulfamethazine, sulfapyridine and sulfathiazole), three macrolides (tylosin, erythromycin and roxithromycin), omeprazole, ranitidine and trimethoprim were from Sigma (St. Louis, USA), and kitasamycin hydrate, the surrogate standard (100 mg/L in acetonitrile), was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and stored at 4 ºC. Fresh stock solutions were prepared every six months. A mix of all compounds in water at a concentration of 50 mg/L was prepared weekly. Working solutions were prepared daily by diluting these solutions with water. Ultra-pure water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, EEUU), acetonitrile and methanol were HPLC grade from SDS (Peypin, France), and nitrogen was from Carburos Metálicos (Tarragona, Spain). Chlorhydric acid (HCl), sodium hydroxide (NaOH) and acetic acid from Prolabo (Bois, France) were used to adjust the pH of the sample and the mobile phase, and sodium thiosulphate 5-hydrate from Panreac (Barcelona, Spain) was used to eliminate the chlorine in tap water.

Instrumentation

The chromatographic system was an HP1100 series LC-MS selective detector (Agilent Technologies, Waldbronn, Germany) with electrospray ionization (ESI). It was equipped with an automatic injector, a degasser, a quaternary pump, and a column oven. The chromatographic column was a Kromasil 100 C_{18} (25.0 x 0.46 cm) with a particle size of 5 μm (Teknokroma, Barcelona, Spain).
A 500 mg Oasis HLB (Waters, Milford, Massachussets, USA) connected to a manifold (Teknokroma, Barcelona, Spain) and a pump as a vacuum source, was used for the SPE procedure.

Sample preparation

The water samples were collected from three Catalan rivers (the Ebro, Ter and Llobregat). The wastewater samples were collected from the influent and effluent of the two domestic sewage treatment plants (STPs), which are located in the area of Tarragona. These STPs mostly receive urban wastewaters and some industrial discharges. They are connected to similar population equivalents (around 120000) with biological oxygen demand (BOD_5) of 400 mg/L.
The average flow-rate is 30000 m^3/ day (STP1) and 16000 m^3/ day (STP2). All samples were collected by using pre-cleaned amber glass bottles, filtered using a 0.45 μm nylon filter (Whatman, Maidstone, UK), acidified

to pH 3 (HCl) and stored at 4 ºC until analysis. Before analysis, 1 g of sodium thiosulphate was added to 1 L of tap water samples (from Tarragona city) to eliminate chlorine [28]. Prior to the analysis, samples were allowed to reach room temperature and the pH was adjusted to 7 with sodium hydroxide.

Chromatographic conditions

A binary mobile phase with a gradient elution was used. Solvent A was Milli-Q water with acetic acid (pH 3) and solvent B was acetonitrile. The gradient was 10% B, which increased to 15% in 10 min, to 26% in 5 min and to 60% in 4 min; then it was increased to 100% in 4 min, kept constant for 2 min and finally returned to 10% B in 2 min. All the compounds eluted within 22 min.

In order to find the optimum conditions for each compound in the electrospray ionisation, flow injection analysis (FIA) was carried out. The average conditions selected for the optimum performance of the ESI interface in the positive mode were: nebulizer pressure 40 psi, drying gas flow-rate 12 L/min, drying gas temperature 350 ºC and capillary voltage 4000 V. Fragmentation voltages were defined individually and the values used were: 75 V for sulfamethoxazole, omeprazole and ranitidine, 100 V for sulfadiazine, sulfapyridine, sulfathiazole and sulfamethazine and 125 V for trimethoprim, erythromycin, roxithromycin and tylosin. The ions selected for quantifying the samples are shown in Table 1. In SIM mode, the most abundant ion, which is usually $[M+H]^+$, was used for quantification and the second and third most abundant were used for confirmation. Only in the case of omeprazole, was the $[M+H]^+$ ion not selected as a quantifier ion because of its low abundance. Several fragmentation voltages were studied to find spectra with three ions and, only in case of sulfamethoxazole and sulfapyridine when the fragmentation voltages (75 V and 100 V, respectively) were increased to 125 V, it was seen that the abundances of the ions showed significant differences, which could be used to confirm their presence in real samples. Under these conditions, the most abundant ions for sulfamethoxazole were 108 (100%), 156 (70%) and 254 (40%), and for sulfapyridine they were 156 (100%) 108 (80%) and 250 (50%).

Solid-phase extraction

Solid-phase extraction experiments were carried out using 500 mg Oasis HLB cartridges. These were pre-conditioned with 5 mL of MeOH followed by 2 mL of Milli-Q water. Sample volumes of 100 and 250 mL were extracted for the influent and effluent of the STP, respectively, 500 mL for river water samples, and 1000 mL for tap water.

Table 1. Compounds studied and ions selected for quantification and confirmation in SIM mode.

Compound	t_R (min)	Quantification ion (m/z)	Confirmation ion 1 (m/z)	Confirmation ion 2 (m/z)
Ranitidine	5.1	315 $[M+H]^+$	270 $[C_{11}H_{16}N_3O_3S]^+$	176 $[C_5H_{10}N_3O_2S]^+$
Sulfadiazine	8.9	251 $[M+H]^+$	156 $[C_6H_6NO_2S]^+$	108 $[C_6H_6NO]^+$
Sulfathiazole	10.1	256 $[M+H]^+$	156 $[C_6H_6NO_2S]^+$	108 $[C_6H_6NO]^+$
Sulfapyridine	10.9	250 $[M+H]^+$	156 $[C_6H_6NO_2S]^+$	108 $[C_6H_6NO]^+$
Trimethoprim	11.7	291 $[M+H]^+$	261 $[C_{13}H_{17}N_4O_2]^+$	123 $[C_5H_7N_4]^+$
Sulfamethazine	15.5	279 $[M+H]^+$	186 $[M-NC_6H_6]^+$	124 $[C_7H_{12}N_2]^+$
Omeprazole	19.8	198 $[M-H_3CO-C_7H_4N_2]^+$	346 $[M+H]^+$	151 $[C_9H_{13}NO]^+$
Sulfamethoxazole	20.0	254 $[M+H]^+$	156 $[C_6H_6NO_2S]^+$	108 $[C_6H_6NO]^+$
Erithromycin	20.8	735 $[M+H]^+$	576 $[M+H-C_8H_{15}O_3]^+$	158 $[C_8H_{16}NO_2]^+$
Kitasamycin	21.1	772 $[M+H]^+$	558 $[M-C_{11}H_{18}O_4]^+$	174 $[C_8H_{16}NO_3]^+$
Tylosin	21.5	916 $[M+H]^+$	772 $[M+H-C_7H_{12}O_3]^+$	174 $[C_8H_{14}O_4]^+$
Roxithromycin	21.8	838 $[M+H]^+$	679 $[M+H-C_8H_{15}O_3]^+$	413 $[C_{21}H_{35}NO_7]^+$

The sample adjusted to pH 7 with NaOH and spiked with the surrogate (kitasamycin) at a final concentration of 50 μg/L was passed through the cartridge at a flow rate of 10-15 mL/min. Before the elution step, the sorbent was dried under vacuum to ensure it was completely dry. The retained analytes were eluted from the cartridge with only 4 mL, which were 2 mL of MeOH and 2 mL of MeOH adjusted to basic conditions (NH_4OH 0.1%, pH 9). The eluate was concentrated under a flow of nitrogen gas to dryness and the residue was redissolved in 1 mL of H_2O (with MeOH 1%). After filtration with a 0.45 μm nylon membrane (Teknokroma, Barcelona, Spain), 50 μl of this solution was injected into the chromatographic system.

RESULTS AND DISCUSSION

LC-(ESI)MS

The parameters that affect the performance of the ESI interface in the positive ionisation mode were optimised by FIA for each compound individually. The variables optimised and the intervals tested were: nebulizer pressure (10-60 p.s.i), drying gas temperature (200-350 ºC), drying gas flow (3-13 L/min), capillary voltage (1000-6000 V) and the fragmentor voltage (50-200 V). The values that provided the best response and spectrum were selected as the optimum values for each compound (see section Chromatographic conditions).

All the compounds showed good linearity by direct injection at low μg/L levels in Milli-Q water. The linear range was 5-500 μg/L for

sulfadiazine, trimethoprim, sulfamethazine, sulfamethoxazole and ranitidine and 10-500 μg/L for the other compounds. Limits of detection, calculated as a signal-to-noise ratio of 3, were 1 μg/L for all the compounds, except for sulfapyridine, sulfathiazole, omeprazole, erythromycin, roxithromycin and tylosin (5 μg/L).

SPE procedure

Because these compounds are found in the environmental samples in low concentrations, a previous enrichment based on SPE was needed. Our previous experience [16,29] prompted us to choose Oasis HLB as the sorbent to extract them. The parameters optimised were the sample pH, the sample volume, and the elution solvent, its volume and its pH. The optimization was done in triplicate using, initially, 100 mL of Milli-Q water spiked so that the final concentration was 50 μg/L. Solution pH is expected to significantly influence extraction of the antibiotics owing the presence of acidic and basic functional groups in their structures. When different sample pH values were tested (pH 3 and 7), it was seen that the recoveries for erythromycin and omeprazole were increased when they were extracted at pH 7, in agreement with similar studies [7,23], while the recovery of the other compounds showed no strong dependence on pH. So, it was decided to adjust the sample to pH 7, prior to the extraction step. The elution of the compounds was tested with two different solvents such as MeOH [21,26] and ACN [12]. When these compounds were eluted with 5 mL of MeOH and 5 mL of ACN, the results showed that sulfonamides were well extracted with both solvents. However, the other compounds showed lower recoveries with ACN (50-63%) than with MeOH (57-81%). So MeOH was chosen as the best solvent to elute these compounds. When different media were tested (neutral, acidic and basic), it was seen that the recoveries of the macrolides and ranitidine increased when they were eluted in basic conditions. For example, omeprazole showed recoveries lower than 13% in neutral and in acidic media, and it was 69% in basic conditions, respectively, to 98% in basic conditions. After these parameters had been optimized, the volume of Milli-Q water was increased to 1000 mL and there were no significant losses in recoveries. However, when real samples were analyzed, the sample volume was considered so that the recoveries were acceptable. When tap water was analyzed, a volume of 1000 mL was used and recoveries were between 67-98%. Only sulfamethoxazole showed lower recoveries (35%). As can be seen in Table 2, when 500 mL of river water was analyzed, the recoveries were between 44% and 77%.

Table 2. Recoveries and relative standard deviations (%RSD. n=3) of selected compounds in different kinds of water.

Compound	Influent STP		Effluent STP		River water		Tap water	
	%R[a)]	%RSD	%R[b)]	%RSD	%R[c)]	%RSD	%R[d)]	%RSD
Ranitidine	65	19	63	4	71	6	98	6
Sulfadiazine	33	15	50	13	58	6	73	15
Sulfathiazole	51	3	34	3	71	4	93	11
Sulfapyridine	56	11	53	16	77	2	94	12
Trimethoprim	52	6	58	5	72	4	91	1
Sulfamethazine	62	6	67	5	74	6	96	10
Omeprazole	21	17	20	12	52	15	67	16
Sulfamethoxazole	25	19	27	17	44	20	35	20
Erithromycin	43	21	38	10	55	21	89	10
Tylosin	62	13	54	14	70	2	98	19
Roxithromycin	49	9	44	8	69	14	72	3

[a)] 100 mL spiked at 500 ng/L.
[b)] 250 mL spiked at 250 ng/L.
[c)] 500 mL spiked at 100 ng/L.
[d)] 1000 mL spiked at 50 ng/L.

When influent and effluent waters from two STPs were analyzed, the sample volume had to be reduced to 100 and 250 mL, respectively, because of the complex matrix.

The matrix effect can be a substantial drawback in the (ESI)MS, which can suppress or, less frequently, enhance the analyte signal, thus leading sometimes to erroneous results [30]. The effect of ion supression on ESI was studied by comparing the responses of series of standard solutions of analytes in Milli-Q water with those obtained from adding 125 μg/L to the extracts of 250 mL effluent sewage water samples. The results of this comparison showed differences in the concentration of sulfamethoxazole and a large degree of matrix suppression (48%). These results agree with those of other studies [11,24] in which several compounds were studied and it was seen that sulfamethoxazole and erythromycin, among other compounds, showed the effect of the complex matrix.

Although in previous studies [16,29] we did not consider it necessary to use a surrogate standard when other pharmaceuticals were determined in waters, in the present study it was decided to study the addition of the surrogate standard kitasamycin. Kitasamycin is a macrolide (which has been previously used as a surrogate [7]) and it was chosen because it elutes within the same chromatographic time frame as the analytes, responds well in the ESI (+) mode and was not present in our samples. The addition of the surrogate prior to the extraction would account for the losses during the SPE, the differences in the matrix

effect between standards and samples, and the differences in ionization or injection volumes during LC-MS analysis. As can be seen in Table 2, the recoveries of the compounds were between 33% and 65% when 100 mL of influent samples were spiked at 500 ng/L. When 250 mL of effluent samples was spiked at 250 ng/L, the recoveries were between 34% and 67%. In both cases, omeprazole and sulfamethoxazole showed the lowest recoveries with values between 20% and 27%.

Method validation

To evaluate how efficient the surrogate standard was, external and internal calibration with kitasamycin was done with both matrices (river and effluent sewage waters). When the river water sample was analyzed, only sulfamethoxazole was found and this signal was subtracted from the signal found in the spiked samples. Linearity was tested following the procedure developed in the SIM mode and the range studied was 10-200 ng/L. All the compounds showed r^2>0.9882 with both calibrations. The repeatability and reproducibility between days were determined by spiking three replicates of 500 mL of river water at 100 ng/L, and the results obtained, expressed as RSD were lower than 21% for repeatability and 23% for reproducibility. The limits of detection (LOD), calculated as a signal-to-noise ratio of 3, were 5 ng/L for sulfadiazine, trimethoprim, sulfamethoxazole and ranitidine and 10 ng/L for the other compounds. The limits of quantification (LOQ), as the concentration of the lowest point of the calibration curve, ranged from 10 to 25 ng/L. In this kind of sample, no differences were observed when a known concentration was quantified with both internal and external calibration.

When an effluent sewage water sample was analyzed, sulfametazine and sulfamethoxazole were found. These signals were subtracted from the signal found in the spiked samples. Linearity was tested following the procedure developed in the SIM mode and, in this case, the range studied was 40-1000 ng/L for sulfonamides and ranitidine and 200-1000 ng/L for macrolides, omeprazole and trimethoprim. All the compounds showed r^2>0.9814 with both calibrations. The LODs for 250 mL of effluent water, calculated as a signal-to-noise ratio of 3, were 10 ng/L for ranitidine, 20 ng/L for the 5 sulfonamide compounds and 40 ng/L for the rest. The LOQs for 250 mL of effluent water were 40 ng/L for sulfonamide compounds, with the exception of sulfatiazole, omeprazole and macrolides (200 ng/L).

The concentration of the analytes was found using both calibrations and it was seen that the results in both cases were similar for most of the compounds. However, sulfamethoxazole and erythromycin showed a lower signal while ranitidine showed an enhanced signal when an external calibration was used, because of the effect of the matrix. Therefore, we

used internal calibration for quantifying all the compounds in sewage samples.

Application to environmental samples

To demonstrate the applicability of the method, several water samples were analyzed in triplicate. Tap water samples were from Tarragona city, river water samples were from three different Catalan rivers (the Ebro, Llobregat and Ter), and wastewaters were from the influent and effluent of two STPs that receive mostly urban wastewaters.

When we analyzed 1 L of tap water, none of the analytes being studied showed any detectable amount in this kind of sample. However, the study by Ye *et al.* [31] revealed the presence of some antibiotics in drinking waters, including sulfamethoxazole at levels of 3.4 ng/L. When river samples were analyzed, it was found that the highest concentrations of our analytes were contained in the Llobregat river, as was seen in previous papers [16], because it passes through heavily industrialized zones and in a highly populated area. Concentrations of ranitidine and sulfamethoxazole were of 20 ng/L and 50 ng/L, respectively, while trimethoprim showed values <LOQ.

Table 3. Concentrations (μg/L) found in sewage samples from STP1 and STP2 using an internal calibration %RSD (n=3) < 25.

Compound	STP_1 Jul 06		STP_2 Jul 06		STP_1 Oct 06		STP_2 Oct 06	
	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.
Ranitidine	<LOQ	-	0.24	<LOQ	0.21	-	<LOQ	<LOQ
Sulfapyridine	0.20	0.15	0.80	-	<LOQ	-	2.38	-
Sulfametazine	1.82	0.06	-	-	-	-	-	-
Omeprazole	2.17	-	1.11	-	-	-	-	-
Sulfamethoxazole	-	-	1.07	-	-	-	-	-

Inf: influent sample, Eff: effluent sample.
<LOQ: below than limit of quantification.

Trimethoprim is reported to have been found at levels of 20 ng/L in river waters [11], while sulfamethoxazole was found by other authors [32] in this kind of samples at levels <20 ng/L. The extracted ion chromatograms obtained from a Llobregat river water sample can be seen in Figure 1. When the Ebro river was analyzed only roxithromycin showed levels <LOQ. When this contaminant was studied in different sites along a river in Northern colorado, maximum levels of 0.06 μg/L were found [25]. As can be seen in Table 3, most of the STPs samples contained concentrations of ranitidine ranging up to 0.24 μg/L, while high values of omeprazole (2.17 μg/L) were found in influent sewage water. This may be

because ranitidine and omeprazole are widely used for treating ulcers. High values of ranitidine were found by Castiglioni *et al*. [33] who studied 8 STPs effluent samples and the results showed values of this compound of 36-610 ng/L.

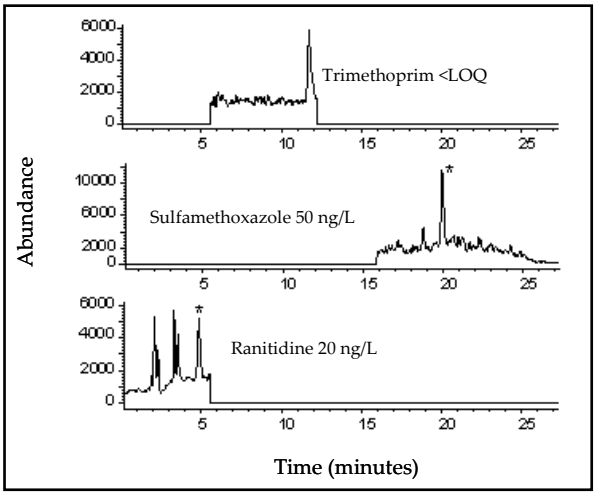

Figure 1. Extracted ion chromatograms of the compounds found in a sample from the Llobregat River.

Although some authors have shown sulfamethoxazole to be one of the most persistent compounds in waste-waters [12,34], the maximum value we found in influent samples was 1.07 μg/L, and we did not find it at all effluent samples.

For example, Carballa *et al*. [35] found levels of 674 ng/L in influent samples and Yang *et al*. [18] found maximum values of 1.09 μg/L and 0.21 μg/L in influent and effluent sewage water, respectively. Removal efficiencies of 42% and 60% have been reported for sulfamethoxazole and only 3% for trimethoprim [35,36] in STPs. As can be seen in Table 3, maximum values of sulfamethazine (1.82 μg/L) and sulfapyridine (2.38 μg/L) were found in influent samples, and the presence of sulfamethoxazole and sulfapyridine was confirmed when the voltage was increased to 125 V. Göbel *et al*. [26] reported maximum concentrations of these compounds in sewage water of 39 ng/L and 135 ng/L, respectively. We agree with these authors [26], because sulfatiazole is used exclusively in veterinary medicine, so the levels found in STPs may be lower and remain undetected in our samples. Although Roberts *et al.* [32] found 202 ng/L of erythromycin in an effluent sewage sample, and Yang *et al.* [10] showed concentrations of 0.18 μgL of tylosin in raw influent, macrolides were not found in any of the samples in this study. The extracted ion chromatogram obtained for an STP influent is shown in Figure 2.

The results showed that the compounds studied here are not found as frequently in environmental waters as other farmaceuticals from previous studies [16]. Although it has been shown that they are not very abundant in this kind of samples, it would be interesting to monitor the presence of sulfonamides, ranitidine, omeprazole and macrolides in the samples studied, due to the high concentrations found in some of them.

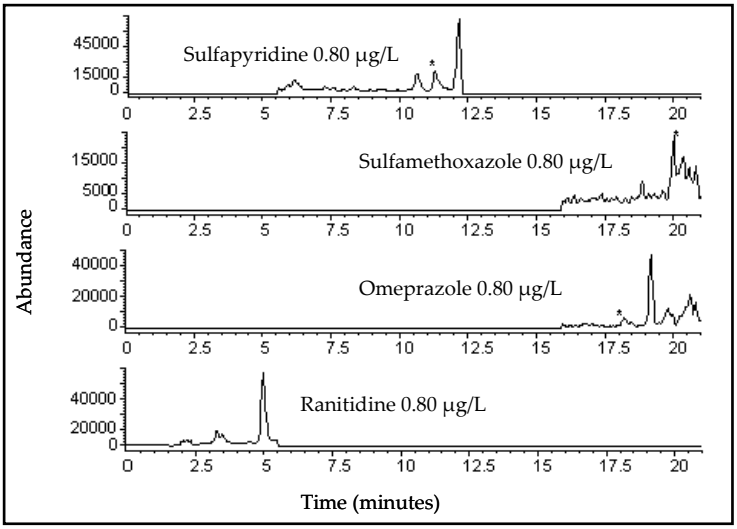

Figure 2. Extracted ion chromatograms of the compounds found in a sample from an STP2 influent in July 06.

CONCLUDING REMARKS

A method based on LC-(ESI)MS after an enrichment step by SPE (Oasis HLB) was developed to study the presence of 11 pharmaceuticals in water samples. The method was successfully applied to determine pharmaceuticals in water sources (influent and effluent STP waters, river waters, and tap water). Therefore, it is possible to extract 1000 mL of tap water samples, 500 mL of river water samples, 250 mL of effluent and 100 mL of influent water from STPs and to determine these compounds at ng/L levels. The matrix has been observed to suppress and enhance signals in the electrospray interface for some compounds in ESI mode and this was corrected by adding an appropriate surrogate standard, kitasamycin. The method was useful for checking for pharmaceuticals in STP waters and river waters and identifying the most abundant compounds. One of the river samples tested revealed concentrations of three of the analytes studied, with sulfamethoxazole at the highest concentration (50 ng/L). In sewage waters, ranitidine was the compound most commonly found, with maximum values of 0.24 μg/L in

influent samples and <LOQ in effluent samples. Higher concentrations of omeprazole (2.17 μg/L) and sulfapyridine (2.38 μg/L) were found in influent samples.

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3.1.3. Discussion of results

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Although the results of the experimental research included in this section have been discussed individually in the previously published papers cited, this section presents the most important aspects of these. As expected, after analyzing various wastewaters and river waters, the presence of most of the selected compounds was confirmed.

Two separate methods were developed for the analysis of several groups of pharmaceuticals. Both were based on solid-phase extraction and liquid chromatography coupled to mass spectrometry (SPE/LC-MS) using a single quadrupole analyzer.

To optimize the SPE procedure, the performance of three commercially available polar sorbents (Isolute ENV+, Oasis HLB, and Strata-X) was evaluated. Although all of these have hydrophilic groups in their structures (Figure 1), Oasis HLB and Strata-X, which have a vinylpirrolidone group, showed higher levels of recovery of the targeted compounds than the Isolute ENV+.

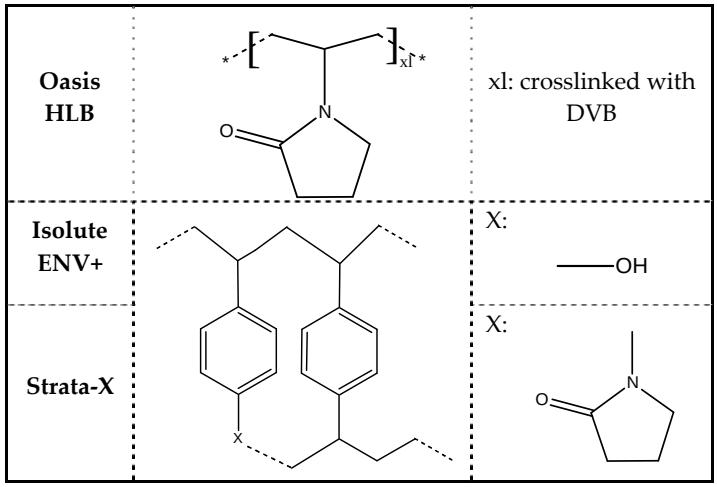

Figure 1. Structures of the sorbents studied.

When comparing the Strata-X (chemically modified with vinylpirrolidone) and Oasis HLB (a copolymer of vinylpyrrolidone and divinylbenzene) sorbents, it was found that better extraction efficiency for acetaminophen and ibuprofen was obtained when using the Oasis HLB, although results were similar using both sorbents for the rest of the compounds. Based on this comparison, Oasis HLB was chosen for use in the extractions of pharmaceuticals discussed in this section. This decision was also in agreement with the preferences of other authors [1-3], who also chose to use Oasis HLB for extraction of a similar range of pharmaceuticals.

The SPE showed that 1000 mL was usually the maximum volume that could be extracted using Milli-Q water, with this maximum volume decreasing as the matrix

analyzed became more complex. Based upon this, the volumes used in both studies were 500 mL for river water samples, 250 mL for STP effluent samples, and 100 mL for STP influent samples.

Influent samples showed the lowest levels of recovery because of the complexity of their matrices. Recovery levels of 33–72% were recorded for the targeted compounds, with the exception of omeprazole and sulfamethoxazole, for which lower values were recorded. Two compounds, salicylic acid and bezafibrate, showed recovery levels lower than 10%, and these were not quantified. Acetaminophen was also not quantified, because the shape of its peak was difficult to integrate. For effluent samples, recovery levels were in the range of 38–91%, with only salicylic acid and acetaminophen showing lower levels than these.

Low levels of recovery were attributed to ionic suppression, known to be the main drawback of the (ESI)LC-MS method. A surrogate had been used in an attempt to minimize this effect. As mentioned in the Introduction, the use of deuterate compounds is highly recommended. However, in this case its use was not possible because of both the large number of pharmaceuticals targeted and the high cost of the deuterated compounds. Instead, kitasamycin was used because of the successful results reported by Abuin et al. [4] when used in the extraction of a similar group of compounds.

As expected, the best levels of recovery were obtained with samples having the least complex matrices. When river water was subjected to extraction, recovery levels of 41–101% were obtained for all of the compounds, with the exception of acetaminophen, naproxen, and salicylic acid, which produced lower values. For extraction of antibiotics from tap water samples, it was first necessary to eliminate the chlorine that had been added during drinking water treatment and which interfered with the extraction process. Subsequently, recovery levels of 67–98% were obtained for all of the targeted compounds, with the exception of sulfamethoxazole, which showed lower values.

In the LC-MS method used, either two or three ions were monitored, depending upon the PhACs targeted. Some compounds showed a low abundance of fragments, and only two ions were monitored. The most abundant ion was used for quantification and the other for confirmation. Also, the relative abundances of the two ions were measured. Although the use of only two ions was not sufficient to allow more secure confirmations to be made, the LC-MS was the instrument available at the time this portion of the study was conducted. These limitations were later overcome when use of a QqQ instrument became available, and this equipment was used in the subsequent research that is described in the following sections.

The methods developed were applied to wastewater influent and effluent samples from two sewage treatment plants, and this research represented the first effort to determine the presence of PhACs in these STPs located in the Spanish cities of Reus

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and Tarragona. Both of these STPs use conventional activated sludge treatments. The highest concentrations of PhACs in the influents corresponded to caffeine, ibuprofen, and omeprazole, with values of 40.12 μg/L, 3.33 μg/L, and 2.17 μg/L, respectively. Other researchers later reported similar levels of such compounds from other locations. For example, Sui et al. [5] found caffeine concentrations from 3.4–6.6 μg/L in STP influent samples in Beijing. Also, a maximum ibuprofen concentration of 1.20 μg/L was reported in STP influents from Spain [6]. When García-Galán et al. [7] studied the presence of sulfonamides in STPs, a maximum sulfamethoxazole level of 302 ng/L was reported in effluents.

By comparing levels in influent samples with those in effluents, it was clear that some compounds, such as caffeine, ibuprofen, and sulfamethazine were efficiently removed by treatment. On the other hand, despite limited data, other compounds such as carbamazepine appeared to have similar concentrations in influents (0.48 μg/L) and effluents (0.29 μg/L). These results are also supported by the findings of other authors [6,8]. In one of these cases, Petrovic et al. [6] measured maximum carbamazepine levels of 0.95 μg/L (influents) and 0.60 μg/L (effluents).

In the present study, as expected, the PhAC concentrations found in samples from the three rivers were low. The highest levels recorded were 106–240 ng/L for caffeine, in agreement with other results reported in the literature [2]. However, very recently, Loos et al. [9] reported a maximum caffeine concentration of 1467 ng/L in the Danube River. In the present study, a sulfamethoxazole concentration of 50 ng/L was recorded in the Llobregat River. Subsequently, García-Galán et al. [10] reported the presence of a group of sulfonamides in the same river, and sulfamethoxazole showed the highest frequency of detection. They studied four sampling sites, where results ranged from 1.2–652 ng/L, although an outlying maximum value of 4297.3 ng/L was also reported.

The research reported here also included the first study of tap water from Tarragona, but in this effort none of the targeted compounds were detected. Other authors have reported the presence of pharmaceuticals in tap water matrices, but only because of the fact that highly sensitive instrumentation was used. For example, Ye and Weinberg [11] developed a method using LC-MS-MS with a triple quadrupole analyzer, and were able to attain very low LODs. They reported concentrations of macrolides at 1.4–4.9 ng/L and sulfamethoxazole at 3.0–3.4 ng/L in drinking water samples.

The research presented in this section was unfortunately unable to take advantage of the greater sensitivity of other types of instrumentation, in order to detect compounds that may have existed in the tap water samples. However, the successful demonstration of the presence of PhACs in sewage and river water samples should be noted, and the need for further studies focused on detection of these compounds as well as methods for their removal by STPs should also be emphasized.

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3.2. Determination of hormones by liquid chromatography-tandem mass spectrometry

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The group known as Endocrine Disruptor Chemicals (EDCs) is comprised of an extensive and expanding spectrum of compounds, as documented by various worldwide organizations including the World Health Organization (WHO), the European Union (EU), and the U.S. Environmental Protection Agency (EPA). Some of the harmful effects caused by EDCs are known to include reductions in sperm count in men; increases in the incidence of breast and prostate cancers, and decreased reproductive success and feminization of males in several wildlife species [1]. As the knowledge of EDCs increases, the list of compounds belonging to the group also expands. Among the wide range of substances with endocrine disrupting properties, the hormones are of particular interest because of their potential estrogenic potency. Moreover, some natural hormones (estriol and estrone) and synthetic hormones (ethinylestradiol) are included on the EPA's third list of candidates of contaminants in drinking water [2]. Therefore, the accurate determination of these compounds in environmental waters is crucial.

Hormones are natural and synthetic compounds included in the group known as pharmaceutically active compounds (PhACs). The study reported here was specifically focused on estrogens. Together with the progesterones, these are the hormones involved in the female menstrual cycle. Hormones are constantly excreted by humans and animals and thereby enter sewage treatment plants (STPs). When the treatments applied at these plants are not efficient at removing these compounds, hormones can become a threat to local water supply networks [3,4].

Many publications have reported the presence of hormones in wastewaters [5-7], river waters [8-10], and even drinking waters [4]. However, in most studies the presence of conjugate hormones has not been considered. For this reason, the present study was focused not only on estrogens but also on their conjugates. Many compounds are conjugated with glucuronic acid, sulphate, or acetate in order to create more polar molecules. These are then readily filtered through and excreted by the kidneys to subsequently be eliminated from the body. When metabolites arrive at the STP in influents, they may be cleaved during sewage treatment to produce non-metabolized compounds, which also increases their environmental concentration [1]. This means that effective methods of removal must target conjugates as well as free hormones.

In the analytical process, extraction techniques are first required to achieve determination of the low hormone concentration levels found in environmental waters. The most commonly used approach for this is solid-phase extraction (SPE). Based upon the results of previous experiences with extracting polar compounds (section 3.1), Oasis HLB was chosen as the sorbent. The novel aspect of this study was related to instrumentation. LC-MS had previously been used with a single quadrupole analyzer to determine estrogens [10], but this type of analyzer presents limitations when it becomes necessary to achieve very low limits of detection, and is also lacking in its ability to provide confirmation.

The most notable recent advances in terms of improving sensitivity and specificity in environmental analyses have come from application of liquid chromatography-tandem mass spectrometry (LC-MS-MS). The most commonly used analyzer for determination of target analytes is the triple quadrupole (QqQ), which is effective for determination of a broad range of compounds. As mentioned in the Introduction of this Thesis, QqQ allows for lower limits of detection and provides a greater number of the identification points (IP) used for confirmation of the presence of target analytes, when working in selected reaction monitoring (SRM) mode.

The objective of this study was the development of a method to determine 11 hormones and conjugates, including natural and synthetic estrogens. Specifically, the targeted hormones were estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), 17α-estradiol (α-E2), estriol (E3), and diethylstilbestrol (DSB). The conjugates targeted were estrone 3-sulfate (E1-3S), estrone 3-glucoronide (E1-3G), estradiol 17-glucuronide (E2-17G), 17β-estradiol 17-acetate (E2-17A) and estradiol 3-sulfate (E2-3S). The structures of all of these compounds are illustrated in Appendix II.

The method used was based upon extraction using off-line SPE followed by LC-MS-MS using a QqQ analyzer. This was the first study by our group using this type of instrumentation. The method developed was applied to the determination of hormones in sewage from two STPs in the Spanish cities of Reus and Tarragona, as well as to water from the nearby Ebro River.

The results of this study were published in *Talanta* 78 (2009) 1327-1331.

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3.2.1. Estrogens and their conjugates: determination in water samples by solid-phase extraction and liquid chromatography-tandem mass spectrometry

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ESTROGENS AND THEIR CONJUGATES: DETERMINATION IN WATER SAMPLES BY SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A sensitive method for simultaneously determining eleven free and conjugated steroid estrogens has been developed using liquid chromatography-(electrospray)triple quadrupole mass spectrometry (LC-(ESI)MS-MS) in negative mode with application to environmental aqueous matrices. Two selected reaction monitoring (SRM) transitions per compound were used, one of which was used for quantification and the second one for confirmation. The procedure includes a solid-phase extraction with an Oasis HLB solid-phase cartridge. Recoveries in 500 mL of river water spiked at 50 ng/L for sulfate estrogens and 100 ng/L for the rest of the compounds were 46-87%, except for E2, which had lower values (32%). Recoveries in wastewater were higher than 49% and 30% for all the compounds, except E2-17A and E2-17G (lower than 26% and 28%, respectively), in effluent (250 mL) and influent (100 mL), respectively. Ion supression is a well-known phenomenon when using ESI; thus its impact on method recovery made us consider this effect when quantifying our samples. Limits of detection varied from 2 ng/L to 30 ng/L in river water and 10 ng/L to 100 ng/L in sewage water. The method was used to determine the target compounds in the Ebro river water where none of the analytes were found. In effluent and infuent water samples, EE2, E1-3S and E2-3S were determined at concentration levels ranging from 35 to 160 ng/L.

***Keywords*:** *estrogens, LC-MS-MS, SPE, wastewaters*

INTRODUCTION

Recently, evidence has emerged that endocrine disrupting compounds (EDCs) can have harmful effects on aquatic organisms. Recent publications suggest that steroids may be the main source of estrogenicity in many municipal sewage treatment plants (STPs) [1]. Since the sources of natural estrogens cannot be eliminated, a number of specific treatment processes in STPs have been optimized and discussed with regard to estrogens removal [2,3]. Thus, it is also important to determine the fate and distribution of steroid conjugates in the environment since they are potential sources of active estrogens as a result of dissociation in sewage treatment plants or the input of treated wastewater directly into surface waters [4,5]. Estrogens are largely excreted from humans as conjugates, mainly glucoronides [3]. Occasionally, these conjugates can breakdown into other molecules in sewage treatment plants, resulting in the release of the active parent compound [6]. Several studies have identified the natural steroids E2 and E1 and the synthetic estrogen EE2 as the most potent estrogenic compounds in treated municipal sewage [6,7]. In a survey of effluents from German STPs, EE2 was detected above the quantification level of 1 ng/L [8] in all 20 STPs investigated. Kuch *et al.* [9] reported levels of estrone between 3 and 13 ng/L in several effluent samples in Germany.

Studies of estrogens in river waters revealed a lower concentration than in sewage waters. Research into 15 German rivers and streams showed that only estrone was present at a maximum concentration of 1.6 ng/L [10]. In various Catalan studies, estrone and estrone-3-sulfate were detected in the Llobregat river (0.68 ng/L and 0.33 ng/L, respectively) [11], and maximum levels of 0.13 μg/L of EE2 were found in the Ebro river [12].

Highly sensitive techniques are needed to detect the low contents of estrogenic compounds in the environmental samples. The most commonly used analytical technique for estrogens in the past has been gas chromatography coupled to mass spectrometry (GC-MS) [5,13,14] and tandem mass spectrometry (GC-MS-MS) [7,15], preceeding by derivatization steps. However, in recent years, separation and determination with liquid chromatography coupled to mass spectrometry (LC-MS) [14,16,17] and tandem mass spectrometry (LC-MS-MS) [4,5,18-20] has become a widely-used tool for determining estrogens in environmental samples because of its sensitivity and specificity and the fact that it does not need the derivatization step. For example, when Brossa *et al.* [12] used LC-MS for determinig free steroids in river waters they found limits of detection (LODs) between 0.002 μg/L and 0.06 μg/L per 500 mL sample. Nowadays, a triple quadrupole with multiple reaction monitoring (MRM) and selected reaction monitoring (SRM) is the most suitable tool for target

analysis of high sensitivity. This is demonstrated in several papers, such as Reddy *et al.* [4], who used tandem MS-MS to detect steroid conjugates in an MRM method in STP waters. They reported minimum detection limits (MDL) lower than 0.16 ng/L in 100 mL of influent waters.

The most extensively used method of sample preconcentration is solid-phase extraction (SPE), and the selection of the sorbent depends basically on the nature of the matrix and the properties of the analytes. For example, Kuch *et al*. [9] studied two types of sorbent (Amberlite XAD 2 and a mixture of LiChrolut EN/ Bondesil C_{18}) to determine estrogens in effluent STP waters. However, in several papers [4,21,22] Oasis HLB is the most commonly used sorbent for this kind of analytes. SPE is one of the most common techniques for pre-concentrating estrogens in waters, but some authors have also used SPME as well [23,24]. For example, Mitani *et al*. [24] used an on-line in tube SPME-LC-MS-MS system. They found recoveries of 86% (E3) in river water and levels of 35.7 pg/mL (E3) in the effluent sewage water.

The aim of this work was to develop a method to determine free estrogens and their conjugates which can be applied to a variety of water matrices and to determine the occurrence of these compounds in wastewater samples.

EXPERIMENTAL

Reagents and standards

The standards of estrone (E1), estrone 3-sulfate (E1-3S), estrone 3-glucoronide (E1-3G), 17β-estradiol (E2), estradiol 3-sulfate (E2-3S), 17β-estradiol 17-acetate (E2-17A), estradiol 17-glucuronide (E2-17G), 17α-ethinylestradiol (EE2), 17α-estradiol (α-E2), estriol (E3) and diethylstilbestrol (DSB) were from Sigma (St. Louis, USA). Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and storing it at -5 ºC. Fresh stock solutions were prepared each six months. A mix of all compounds in methanol at a concentration of 50 mg/L was prepared weekly. Working solutions were prepared daily by diluting the previous solution with water. Ultra-pure water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, EEUU), acetonitrile and methanol were HPLC grade from SDS (Peypin, France), and nitrogen was from Carburos Metálicos (Tarragona, Spain). Hydrochloric acid (HCl), sodium hydroxide (NaOH) and acetic acid from Prolabo (Bois, France) were used to adjust the pH of the sample and the mobile phase.

Sample collection

The river water samples were collected near to the mouth of the

river Ebro. The wastewater samples were collected from the influent and effluent of two Catalan domestic sewage treatment plants (STPs) with an activated sludge system. They are located in two cities, Reus and Tarragona, with populations of about 120,000 habitants. All samples were collected using pre-cleaned amber glass bottles, acidified to pH 2 (HCl) and stored at 4 ºC until analysis.

Sample extraction

Before the extraction, the sample was adjusted to pH 7 with NaOH and filtered using a 0.45-μm nylon filter (Whatman, Maidstone, UK). For each sample, a 12 mL, 500 mg Oasis HLB SPE cartridge was preconditioned by washing with 5 mL of MeOH followed by 5 mL of Milli-Q water. Sample volumes of 100 mL and 250 mL were extracted for the influent and effluent of the STP, respectively, and 500 mL for river water samples. The samples were passed through the cartridge at a flow rate of 10-15 mL/min. The analytes retained were eluted using 5 mL of MeOH (with 5% ACN). Extracts were reduced to dryness under a gentle flow of N_2 gas, using an evaporation system and final extracts were redissolved with 1 mL of MeOH:H_2O (80:20). After being filtered through 0.45 μm syringe filters (Scharlab, Barcelona, Spain), 50 μL of this solution was injected into the chromatographic system.

LC-(ESI)MS-MS

The target compounds were separated and identified using liquid chromatography-(electrospray ionization) tandem mass spectrometry in negative mode. The chromatographic instrument was an HP1200 series LC- triple quadrupole mass spectrometer from Agilent Technologies (Waldbronn, Germany) with an ESI interface, an automatic injector, a degasser, a quaternary pump and a column oven. The chromatographic column was a Kromasil 100 C_{18} (25.0 x 0.46 cm) with a 5 μm particle size (Teknokroma, Barcelona, Spain), and the volume injected was 50 μL. The mobile phase flow-rate was 1 mL/min and the column temperature was kept at 35 ºC.

A binary mobile phase with a gradient elution was used to optimize the extraction conditions. Solvent A was Milli-Q water with acetic acid (pH 2.8) and solvent B was acetonitrile. The gradient was performed as follows: 10% B, constant for 10 min, increased to 40% B for 5 min, to 60% for 10 min, to 100% B for 5 min, and then decreased to 10% B for 2 min. The system was re-equilibrated for 3 min between runs.

In order to sensitively and selectively determine the analytes, the optimization of the MS-MS parameters was carried out by flow injection analysis (FIA) for each estrogen. Experiments were performed in triplicate to be sure the observed phenomena were valid and not influenced by previous tests. Analysis was performed in the

negative ionization mode with an optimized spray potential of 3000 V, a nebulizer of 45 psi and a source temperature of 350 ºC and 12 L/min of drying gas flow. Nitrogen was used as the collision, nebulizing and desolvation gas. Selected reaction monitoring (SRM) experiments in the negative ionization mode were performed to detect ion transitions (Table 1). Product ions used for monitoring were selected on the basis of their significance in the MS-MS spectra.

Table 1. SRM settings for the studied compounds.

Compound (abbreviation)	SRM ions	Collision energy (V)
diethylstilbestrol (DSB)	**267>222**	30
	267>237	55
estrone (E1)	**269>145**	45
	269>143	45
17 ß-estradiol (E2)	**271>145**	30
	271>183	45
17α-estradiol (α E2)	**271>145**	30
	271>183	45
estriol (E3)	**287>171**	45
	287>145	45
17α-ethynilestradiol (EE2)	**295>145**	45
	295>159	30
estradiol 17-acetate (E2-17A)	**313>253**	30
	313>145	55
estrone 3-sulphate (E1-3S)	**349>269**	30
	349>113	55
estradiol 3-sulphate (E2-3S)	**351>271**	30
	351>145	55
estrone 3-glucoronide (E1-3G)	**445>269**	45
	445>113	20
estradiol 17-glucoronide (E2-17G)	447>271	30
	447>113	20

In bold the SRM transition for quantification.

RESULTS AND DISCUSSION

LC-MS-MS analysis

For the mobile phase we evaluated methanol and acetonitrile since they are the most relevant organic solvents in reversed-phase chromatography. We used the mobile phase with acetonitrile as an organic modifier because it gave the best peak shape for the estrogens, as it was also reported by Benijts *et al.* [25]. The effect of the pH of the mobile phase was investigated by injecting a working solution at various pH (3, 7

and 9). From the chromatographic point of view, we saw that acidified mobile phase (pH 3) gave the best peak shapes. Also, when pH was increased with ammonium hydroxide no significant difference was observed in the signal intensities. The SRM transitions, cone voltage and collision energy, were determined for each compound with direct injection of the standards in the MS-MS. Upon ionization, all the compounds produced negative precursor ions that were fragmented into one or more product ions. The cone voltage was 60 V for E2, αE2 and EE2, 100 V for E2-17A and 150 V for the rest of the compounds. Collision energy between 5-55 V was optimized for each analyte and the best values are shown in Table 1. For each compound, two characteristic fragmentations of the deprotonated molecular ion $[M-H]^-$ were monitored, the first and most abundant one being used for quantification, while the second one was used as a qualifier (Table 1). During the optimization experiments, eight of the eleven estrogens investigated (E1, E2, β-E2, E3, EE2, E2-17A, E1-3S and E2-3S) gave intense fragments at m/z 145 corresponded to $[C_{10}H_9O]^-$, as reported in previous studies [13,15]. In the case of steroid sulfates, product ion spectra were characterized by base peaks $[M-H-80]^-$ corresponding to the loss of SO_3. The glucoronides showed a loss of glucoronide moiety $[M-H-176]^-$.

Since the signal intensity of individual ions generally decreases as the number of ions being simultaneous scanned increases, we used time segment monitoring so that only a few of the ions were monitored within specific small time windows based on chromatographic separation. Three time windows were used: 0-17 min (E2-17G, E1-3G, E2-3S and E3), 17-22 min (E1-3S, EE, α-E2 and β-E2) and 22-36 min (E2-17A, E1 and DSB).

The compounds showed the following linear range by direct injection: 0.01-1000 μg/L for E1-3S and E2-3S, 0.05-1000 μg/L for E1-3G and E2-17G, 0.75-250 μg/L for E1 and DSB, 3-1000 μg/L for E3, 10-250 μg/L for α-E2 and E2-17 and 10-500 μg/L for E2 and EE2.

Optimization of the extraction procedure

Oasis HLB cartridges were selected because of their excellent capture capabilities for acidic and neutral analytes across a wide polarity range [26]. The effect of the sample pH on extraction efficiency was investigated using enrichment tests between pH 3 and pH 7 in 100 mL Milli-Q samples spiked at 200 ng/L. This revealed that the recoveries did not show any differences, except for E1-3S and E2-3S which were not recovered at pH 3, meaning that a neutral pH was needed. Moreover, it is reported that under neutral pH conditions, humic acids are not so well retained in the SPE sorbent, which means there are fewer interferences [11]. Another important parameter to optimize is the elution solvent. Different solvents have been used to elute these compounds such as ACN [12] or

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MeOH [4,15]. We studied the recoveries when estrogens were eluted with MeOH and acidified MeOH (with 5% acetic acid) from 100 mL of milli-Q water. The results showed that acidified MeOH could not effectively elute the sulfate estrogens. As the aim of this work was to extract all target analytes in one single step and because the recoveries obtained were good, 5 mL of MeOH were selected to elute all the compounds from the cartridge. However, from the experiments, we added 5% of ACN to MeOH to improve the recoveries in the real samples. We found that the recovery of E2-17A increased from 12% to 26% when ACN was added to the eluent in the extraction with the same volume of effluent samples.

When the volume of Milli-Q water was increased to 1000 mL the recoveries were between 79-101%. Not only was the elution solvent modified when real samples were analysed, but also the sample volume decreased so that the recoveries were acceptable. When 500 mL of river water was extracted, without evaporating the elution solvent, the recoveries were 70-108%, except for E3 and E2-17G, which had recoveries lower than 55%. These recoveries were slightly lower when the extract was evaporated to dryness. Thus, when we analysed 500 ml of river water spiked at 50 ng/L for the sulfate estrogens, and 100 ng/L for the rest of the compounds, recoveries decreased to 32-87% because of the concentration of the interferences in the matrix. The sample matrix strongly affected the recoveries in sewage water samples.

Table 2. Recoveries and relative standard deviations (%RSD, n=4) of selected compounds in different kinds of water.

Compound	Influent STP		Effluent STP		River water	
	%R[a)]	%RSD	%R[b)]	%RSD	%R[c)]	%RSD
DSB	58	4	76	5	67	7
E1	60	2	51	6	49	5
E2	53	11	61	16	32	13
α E2	30	9	49	15	76	11
E3	56	15	59	12	49	5
EE2	37	11	52	9	68	14
E2-17A	-	-	26	15	46	17
E1-3S	57	6	74	8	84	2
E2-3S	57	9	78	4	87	3
E1-3G	66	7	65	2	67	2
E2-17G	23	5	28	2	59	5

[a)] 100 mL spiked at 1000 ng/L.

[b)] 250 mL spiked at 300 ng/L.

[c)] 500 mL spiked at 50 ng/L (E1-3S and E2-3S) and 100 ng/L.

As Table 2 shows, when 100 mL of influent water was spiked at 1000 ng/L, the recoveries of the compounds were between 23% and 66%, except for E2-17A, whose recovery was very low. When 250 mL of effluent samples was spiked at 300 ng/L, the recoveries were between 28% and 76%. These recoveries agree with the results in the bibliography for most of the compounds [18,27]. For example, Gomes *et al*. [27] found recoveries between 63-72% for E1, E2, E3 and E1-3G in 100 mL of influent waters. As can be seen in Table 2, these recoveries were similar to our results (53-66%) for the same compounds in 100 mL of influent waters.

Ion suppression can be a substantial drawback with ESI [26,28] and this was evaluated when the method was developed. The differences observed in MS-MS response could be attributed to the effect of the sample matrix on the ionization efficiency. We observed a reduction in the response in the range of 10-35% when a standard of Milli-Q water was compared with the same standard in an extract of river water, and a reduction of 10-43%, when the extract was from influent sewage water. In other studies, deuterated estrogens have been added as surrogate standard to minimize the effect of the ion suppression [13,22,29,30].

Unfortunately, it is not easy to find the most suitable surrogate and they are generally very cost prohibitive. Thus, in order to select the best approach for quantifying the real samples, different calibration curves were prepared using three extracts (river, effluent and influent wastewater).

Method validation

The entire method was validated for river water and influent and effluent sewage water. In a blank of river water none of the analytes was found. Linear range was tested following the procedure developed in the negative SIM mode and the range studied with river water was 15-1500 ng/L for sulfate estrogens and 75-1500 ng/L for the rest. All the compounds showed r^2>0.992 when this type of water was used as the matrix. The repeatability and reproducibility between days were determined by spiking 500 ml of river water at 50 ng/L and 100 ng/L. The results, expressed as relative standard deviations (%RSD), were lower than 17% for repeatability (n=3), and 21% for reproducibility between days (n=4). The limits of detection, calculated as a signal-to-noise ratio of 3, were 1 ng/L for E1-3S and E2-3S, 15 ng/L for DSB, E1, E1-3G, E2-17G and 30 ng/L for the rest of the compounds. The LOQs were calculated as the concentration of the lowest point of the calibration.

To quantify the wastewater samples (both effluent and influent) two calibration curves with both kinds of samples were constructed. A blank of effluent sample did not show any of the estrogens studied. However, the chosen influent sample showed the presence of E1-3S, and the signal was subtracted to the rest of the calibration

samples. The linear range for sulfate estrogens in the effluent samples was between 30-1500 ng/L while the rest of the compounds gave linear ranges from 100 ng/L to 1500 ng/L.

The limits of detection, calculated as a signal-to-noise ratio of 3, were 10 ng/L for E1-3S and E2-3S, between 10 ng/L and 35 ng/L for DSB, E1, E1-3G, E2-17G, E3 and 70 ng/L for other compounds. Sulfate estrogens showed linear range between 50 and 1000 ng/L in 100 mL influent samples. The rest of the estrogens were tested from 150 ng/L to 1000 ng/L. The LOD and LOQ values were higher than those in pure and river water due to the complex matrices and the low sample volume stated above. The LODs were 15 ng/L (E1-3S and E2-3S), 35 ng/L (DSB, E1-3G and E2-17G), 50 ng/L (E1 and E3), and 100 ng/L for the rest of analytes.

Application to environmental samples

Although Brossa *et al.* [12] reported finding levels of 0.10 μg/L of EE2 in one sample of the Ebro river, in the present study no estrogens were detected in the same river. Other studies in different Catalan rivers found some estrogens (E1, E2, E3 and EE2) below limit of detection (<3 ng/L) [31].

The method described was applied to wastewater samples to investigate the occurrence of the estrogens in samples from two municipal STPs in Catalonia. In STP influents, DSB, E2-17A and E1-3G were never detected. As Table 3 shows, concentrations of E1, E2-17G and E3 were found below LOQ.

Table 3. Concentrations (ng/L) found in wastewaters from STP_1 and STP_2, %RSD (n=3) <15).

Compound	STP_2 March 07		STP_2 May 07		STP_1 Nov 07	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
E1	-	-	-	-	<LOQ	-
E3	<LOQ	-	<LOQ	-	-	-
EE2	154	-	-	-	-	-
E1-3S	160	35	64	<LOQ	52	<LOQ
E2-3S	76	<LOQ	-	-	<LOQ	<LOQ
E2-17G	-	-	<LOQ	-	-	-

However, EE2, E1-3S and E2-3S showed the highest concentrations in influents, with values between 52 ng/L and 160 ng/L In effluent waters only E1-3S showed the highest value (35 ng/L). The SRM chromatogram obtained for the influent sample from STP2 (March 07) is shown in Figure 1. Free estrogens have been reported to have removal efficiencies of 95% (E3), 87% (E2), and 61% (E1) [19,32]. Chen *et al*. [33] agreed with these high values of removal at 100 ng/L, but they concluded that the removal rate

was lower at higher concentrations of E3. Although we only found values <LOQ of E3 in our samples, higher levels (470 ng/L) were detected in influents, which were reduced to 99 ng/L in effluents [19]. Servos *et al*. [32] showed maximum values of 78 ng/L and 96 ng/L of E1 in influent and effluent samples, respectively, from Canadian municipal STPs.

EE2 is used in oral contraceptives and has been reported to have a high estrogenic potency [20].

Concentrations of EE2 in STPs have varied significantly in reported investigations. The maximum value for EE2 in the influents in our investigation was 154 ng/L. This is comparable with the results found by Kolpin *et al.* [34], who published a mean concentration of 73 ng/L. However, some authors have stated lower values, with Ternes *et al*. [35], for example, finding 3.3 ng/L of EE2 in influent sewage water with a removal of 67%.

In our study of influents, the conjugate of the steroid hormones E1-3S and E2-3S were found at maximal concentrations of 160 ng/L and 76 ng/L respectively. Similar results were obtained by Schlüsener and Bester [18] and Reddy *et al.* [4] who found 37 ng/L of E2-3S and 34.1 ng/L of E1-3S, respectively.

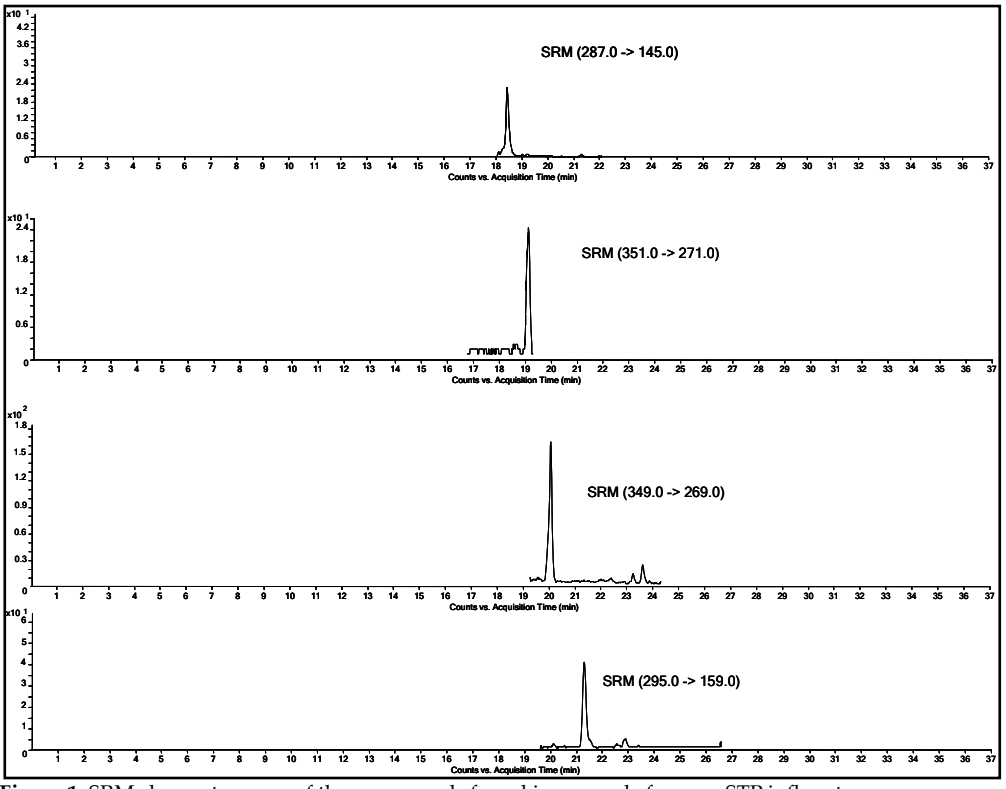

Figure 1. SRM chromatograms of the compounds found in a sample from an STP influent.

No glucoronides were detected when the STP wastewaters were analyzed. This is consistent with the well-known pathway of the deconjugation of glucoronides during the wastewater treatment process and the consequent generation of the free compounds [20]. This study has demonstrated the importance of simultaneously determining free and conjugated estrogens in very complex matrices like STP waters.

CONCLUSIONS

The analytical method based on LC-(ESI)MS-MS allows the simultaneous extraction, identification and quantification of eleven estrogens and their conjugates in water samples at low levels. Different complex matrices have been studied (influent and effluent STP waters and river water). They were enriched by SPE (Oasis HLB) in volumes of 500 mL, 250 mL and 100 mL, for river, effluent and influent water, respectively. To solve the problem of matrix effect, different calibration curves were made according to each kind of sample. The applicability of the method has been demonstrated in real samples from river water and wastewater STPs. None of these compounds were found in river water, however, in the STPs, E1-3S was the most commonly found compound, with a maximum value of 160 ng/L in an influent sample. EE2 was found at 154 ng/L and E1 and E3 were found at values <LOQ in influent samples. Some compounds such as DSB, E2, αE2 and E2-17G were not detected in any of the samples studied.

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3.2.2. Discussion of results

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As previously mentioned, this study was the first in which our group had available the use of liquid chromatography tandem mass spectrometry (LC-MS-MS) with a triple quadrupole (QqQ) analyzer. We used the QqQ analyzer in order to obtain low limits of detection, and to obtain a greater number of identification points, with two transitions in the SRM mode monitored.

Hormone compounds are generally reported in environmental waters at very low concentration levels. Therefore, very sensitive detection systems are needed to contend with these low limits of detection (LODs) and quantification (LOQs). In the previous study by our group using LC-MS and reported in Brossa et al. [1], 17-αEE2 and 17α-E2 showed LODs between 0.03 μg/L and 0.06 μg/L for 500 mL of river water samples. In the present study, we found LODs higher than expected, because the targeted compounds showed a low sensitivity to MS-MS detection. Very low LODs were only obtained for the conjugate compounds E1-3S and E2-3S (1 ng/L for 500 mL of river samples). These values increased, however, when the matrix was more complex, and 50 ng/L for 100 mL influent wastewater and 10 ng/L for effluent wastewater were recorded for 250 mL samples. The rest of the compounds showed higher LODs.

Regarding the SPE process, the performance of some sorbents such as C_{18} sorbents [2-4] and Oasis HLB [5] have been discussed for use with the extraction of hormones. Our choice was based on these results discussed in the literature as well as upon our previous experiences (section 3.1). After studying various eluate solvents, 5 mL of MeOH (5% ACN) was chosen, which gave the best recoveries for all of the compounds and for all of the matrix types. Recovery levels for 250 mL wastewater samples were between 49% and 78% for all of the compounds targeted. The only exception was for E2-17A, which showed the lowest recovery level (26%) for effluents and which was not recovered at all in influents. The glucoronide E2-17G showed a higher level of recovery in river water (59%) than in wastewater (23–28%), in all likelihood because, as may also have been the case with E2-17A, matrix effects strongly affected the recovery of hormones. As an example of an attempt to address this type of problem, Pailler et al. [5] reported the addition of 17-βestradiol-d_3 as a deuterated internal standard for the quantification of hormones, considered to be the best option to address this effect. However, in the present study another option was used, by creating three different types of calibration curves for the various matrixes (effluents, influents, and river water). Although this was a more labor-intensive solution, it also avoided the high costs associated with the deuterated compounds.

Regarding the samples analyzed, the methods applied provided a general view regarding the occurrence of hormones in wastewater influents and effluents in the two sewage treatment plants studied. Although some compounds such as E1, E3, and E2-17G showed levels less than their LOQ, maximum values were found in influent samples for E2-3S (76 ng/L), EE2 (154 ng/L), and E1-3S (160 ng/L). In a similar study from Japan [6], published after the study reported here was performed,

influent samples showed maximum levels of E1-3S (8.2 ng/L), E3-3S (21.9 ng/L), and E1-3G (3.2 ng/L), lower than those in our results.

Our study did not reveal the presence of E2 in any of the samples, in agreement with results reported by Fernández et al. [7]. However, a study from Luxemburg [5] reported maximum E2 values of 102 ng/L in STP influent and 85 ng/L in effluent.

Although, as mentioned above, the method applied in this study have shown limitations in terms of the LODs attainable for some compounds, we were for the most part successfully able to quantify the presence of estrogens and their conjugates in water samples from STPs. As an extension of these results, it is important to stress the need for the further development of new analytical methods for detecting low levels of PhACs in environmental waters. Moreover, studies indicating significant levels of toxicity for hormones and their conjugates must be taken into account in the creation of future legislation.

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3.3. Determination of personal care products by solid-phase extraction and stir bar sorptive extraction followed by ultra-high-performance liquid chromatography-tandem mass spectrometry

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Pharmaceuticals and personal care products (PPCPs) are a broad group of compounds considered as emerging organic contaminants. The results presented so far in this Thesis have been focused on the determination of the sub-group of pharmaceutically active compounds (PhACs). Therefore, to complete the study of PPCPs as a whole, in this section the results of the present study related to personal care products (PCPs) are presented and discussed.

As mentioned in the Introduction, PCPs include all of the products in daily use for purposes of bodily care, such as gels, creams, perfumes, toothpaste, etc. Chemically, PCPs include compounds such as musk fragrances, UV filters, antimicrobials, and preservatives. Recently, siloxanes have also been included in the PCPs group. Scientific interest in PCPs is due to their presence in environmental waters and their associated potential for endocrine disruption and developmental toxicity. For example, some UV filters have been shown to have estrogenic effects similar to those of the natural estrogen 17β-estradiol [1].

Because of their use in a wide variety of personal care products, these compounds can enter the environment indirectly. After being applied they tend to be rinsed off by showering, clothes washing, etc., and enter the environment via sewage treatment plants (STPs). The presence of PCPs in the environment has received less study than the presence of PhACs. However, as reviewed in section 1.2, increasing attention is being given to the environmental fate of PCPs based upon their detection in sewage waters [2-4], river waters [5,6], and even drinking waters [7,8].

The purpose of the study reported here was to develop a method for determination of different families of PCPs with a single analysis. The compounds studied were selected based upon the absence of data regarding their presence in STPs in the region of Tarragona. The importance of attaining better understanding of these compounds, as mentioned in the introduction, is based upon their widespread use, as well as the fact that some of them are considered endocrine disrupting compounds (EDCs). The studies reported here were focused on UV filters, antimicrobials, and preservatives, because most of these have been reported as present in water samples, and because they are also amenable to determination by LC analysis.

The existing literature includes studies reporting extraction of PCPs with liquid-liquid extraction (LLE) [9], dispersive liquid-liquid microextraction (DLLME) [10], ionic liquid-based single-drop microextraction (ILSDME) [11], stir bar sorptive extraction (SBSE) [12], and, most commonly, solid-phase extraction (SPE) [5,6,13,14]. In both of the original studies presented in this section, PCPs were extracted from water samples using two extraction techniques: SPE and SBSE. The advantage of SPE over SBSE is found in the wide range of commercially available sorbents. As part of this study, the extraction efficiencies of two sorbents were compared: Oasis HLB and Bond Elut Plexa. Both of these sorbents have a polar group in their structure. Bond

Elut Plexa has only recently been made commercially available, and therefore there are few studies in the literature discussing its use.

Regarding SBSE, the commercially available sorptive extraction phase is polidimethylsiloxane (PDMS). This phase is only effective for retaining compounds with low polarity. After extraction, analytes can be thermally desorbed and analyzed by on-line GC, or can be subjected to back-extraction with an organic solvent (liquid desorption) [12]. Liquid desorption with an organic solvent followed by LC was the choice of method employed in this study. All of the variables critical for an effective extraction were optimized, including extraction time, extraction temperature, choice of desorption solvent, and desorption time.

As mentioned in the Introduction, most current analytical methods for separation and detection of PCPs have used GC-MS-MS [15-17] and LC-MS-MS [5,13]. Some compounds can be determined equally well with either technique, but the most polar substances need to be derivatized before injection into GC systems. If avoidance of the derivatization step is desirable, then LC becomes the preferred analytical technique. However, interest in ultra-high-performance liquid chromatography (UHPLC) has also increased in the last few years, because it offers several clear benefits in terms of throughput, sensitivity, and resolution. This new technology uses columns with a 1.7 μm porous stationary phase, allowing increased efficiency with a shorter analysis time.

Both studies reported here were performed using UHPLC-MS-MS with a triple quadrupole analyzer and an electrospray interface. It was the first time that our group was able to take advantage of the use of UHPLC to perform rapid chromatographic analyses. In regard to the MS-MS analysis also performed, by monitoring two transitions and working in multiple reaction monitoring (MRM) mode, it was possible to confirm and quantify the presence of PCPs in environmental water samples.

The compounds targeted in the first study (section 3.3.1) were 2-phenylbenzimidazole-5-sulfonic acid (PMDSA), methylparaben (MPB), ethylparaben (EPB), benzylparaben (BPB), propyl-paraben (PPB), 2,4-dihydroxy-benzophenone (DHB), 2,2-dihydroxy-4-methoxybenzophenone (DHMB), benzophenone-3 (BP-3), triclocarban (TCC), triclosan (TCS), octocrylene (OC), and octyldimethyl-*p*-aminobenzoic acid (OD-PABA). The compounds targeted in the second study (section 3.3.2) were DHMB, BP-3, OC, OD-PABA, TCS, and TCC. The structures of all of these compounds are illustrated in Appendix II.

The methods described above were applied to wastewaters from the Spanish cities of Tarragona and Reus, was well as to samples from three nearby rivers (the Ebro, Ter, and Llobregat). These results have already been published in two papers: *J. Chromatography A* 1216 (2009) 6994-7000 and *Analytical and Bioanalytical Chemistry* 397

(2010) 2833-2839. The supplemental on-line information available for the first paper has also been included.

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3.3.1. Ultra-high-performance liquid chromatography-tandem mass spectrometry for determining the presence of eleven personal care products in surface and wastewaters

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ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR DETERMINING THE PRESENCE OF ELEVEN PERSONAL CARE PRODUCTS IN SURFACE AND WASTEWATERS

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Abstract

Personal care products (PCPs) are widely used emerging contaminants which can cause adverse environmental effects. This paper reports the development and validation of a method based on solid phase extraction (SPE) and ultra-high-performance liquid chromatography-electrospray ionization- tandem mass spectrometry (UHPLC-(ESI)MS-MS) for simultaneously determining eleven PCPs: 4 preservatives (methylparaben; ethylparaben; benzylparaben; propylparaben); 2 antimicrobial agents (triclocarban and triclosan) and 5 UV filters (2,4-dihydroxybenzophenone; 2,2-dihydroxy-4-methoxybenzophenone; benzophenone-3; octocrylene and octyldimethyl-*p*-aminobenzoic acid) in environmental waters in only 9 run minutes of chromatographic separation. The SPE was carried out with two polymeric cartridges (Oasis HLB and Bond Elut Plexa). The recoveries obtained with Bond Elut Plexa were between 69% and 101% for 500 mL of river waters, with the exception of octyldimethyl-*p*-aminobenzoic acid (46%). Limits of detection for 500 mL of river water were in the range of 1-5 ng/L. Oasis HLB was chosen for wastewater samples with recoveries between 38% and 92% (250 mL of effluents) and 36-89% (100 mL of influents). In both wastewater samples, octyldimethyl-*p*-aminobenzoic acid and methylparaben showed the lowest recoveries (20% and 27%). The method revealed benzophenone-3 as having the highest concentration levels (7 ng/L) in river waters. Most of PCPs determined were found in influent waters being methylparaben and propylparaben the ones found at highest concentration with values of 5613 ng/L and 1945 ng/L, respectively. In effluent waters, significant lower levels of some PCPs were found, being benzophenone-3 the one found at the highest concentration (100 ng/L).

***Keywords*:** *SPE, UHPLC-MS-MS, UV filters, triclosan, parabens, surface waters, wastewaters*

INTRODUCTION

Public interest in pharmaceuticals and ingredients of personal care products (PCPs) entering the environment has recently been increasing because research has shown they reach detectable and potentially harmful concentrations. PCPs, included in the so-called emerging contaminants, comprise diverse chemical substances such as fragrances, lotions, cosmetics, sun screen agents and others [1-3]. The interest in these kinds of compounds focuses on their pronounced microbial and algal toxicity and potential for fostering resistance [4] and on the fact that some PCPs (e.g., parabens, UV filters) have been suspected of being endocrine-disrupting compounds [5,6]. The main pathway through which PCPs enter the aquatic environment is from household waters that are released by sewage treatment plants (STPs) [7]. They have been found in effluent wastewaters at levels of a few μg/L [8] because conventional STPs are not designed to completely remove these pollutants. Several studies about the treatment and effective removal of personal care products have been published in recent years [9-11]. One of the most studied PCPs is triclosan because it is used as an anti-microbial agent in a large number of medical and personal hygiene products. Although it is reported that primary treatments only remove triclosan in a 32%, [12] ozonation appeared to be an effective technique for enhancing its removal [13].

A preconcentration step is needed before determining PCPs by chromatographic techniques because of the low concentration levels in the samples (ng/L and low μg/L). Some studies using solid-phase microextraction (SPME) [14,15] and stir bar sorptive extraction (SBSE) [16,17] have determined some PCPs in waters. However, solid-phase extraction (SPE) is the preferred technique for preconcentrating PCPs due to the excellent capabilities of the sorbents such as Oasis HLB [18,19], Oasis MCX [8] or Strata-X [7] to retain these compounds.

Some methods for determining PCPs include gas chromatography coupled to mass spectrometry (GC-MS) [4,16,20] and tandem mass spectrometry (GC-MS-MS) [21], but these are limited to those compound classes that are volatile or can be derivatized. Over the past 20 years, the sensitivity, specificity and reliability of liquid chromatography has advanced dramatically with liquid chromatography-mass spectrometry (LC-MS) and LC-MS-MS. The recently developed ultra-high-performance liquid chromatography (UHPLC), which uses analytical columns packed with 1.8 μm particles, offers increased speed and improved sensitivity, selectivity and specificity compared to conventional HPLC analysis [22]. Not only does UHPLC offer very low chromatographic times but it also has a better resolution and narrow peaks that help prevent the analytes from coeluting with the interferences and this can lessen the matrix effects [23].

The advances in analytical instrumentation have made it possible to confirm the presence of a compound at very low levels using liquid chromatography coupled to mass spectrometry [8]. Nowadays, the triple quadrupole (QqQ) is very common and useful tool for high sensitivity target analysis.

Monitoring two transitions between precursor and product ions working with multiple reaction monitoring mode (MRM) it is possible to confirm and quantify the presence of PCPs in waters at very low ng/L [19,24]. For example, Rodil *et al*. [19] used a QqQ to determine nine UV filters in waters and after an SPE step, they found LODs between 7 and 46 ng/L.

The goal of this paper was to develop and to validate a rapid method to determine eleven PCPs from different families in river and wastewaters. The rapid and sensitive techniques SPE/UHPLC-MS-MS allowed us to determine in a unique analysis: UV filters, preservatives and antimicrobial agents.

EXPERIMENTAL

Reagents and standards

We purchased 2-phenylbenzimidazole-5-sulfonic acid (PMDSA); methylparaben (MPB); ethylparaben (EPB); benzylparaben (BPB); propylparaben (PPB); 2,4-dihydroxybenzophenone (DHB); 2,2-dihydroxy-4-methoxybenzophenone (DHMB); benzophenone-3 (BP-3); triclocarban (TCC); triclosan (TCS); octocrylene (OC) and octyldimethyl-*p*-aminobenzoic acid (OD-PABA) from Sigma-Aldrich Chemie (Steinheim, Germany).

Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and then storing it at 4 ºC. Fresh stock solutions were prepared each six months. A mix of all compounds in water at a concentration of 50 mg/L was prepared weekly. Working solutions were prepared daily by diluting the previous solution with water.

Ultra-pure water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, EEUU); acetonitrile and methanol were HPLC grade from SDS (Peypin, France); and nitrogen was from Carburos Metálicos (Tarragona, Spain). Hydrochloric acid (HCl), sodium hydroxide (NaOH) and acetic acid from Prolabo (Bois, France) were used to adjust the pH of the sample and the mobile phase.

Sample collection

All samples were collected from Catalonia (NE Spain). The river water samples were collected from the Ebro River and Llobregat River. The wastewater samples were collected from the influent and effluent of two domestic sewage treatment plants (STPs) in two cities on the coast, with populations of about 120000 inhabitants.

All samples were collected by using pre-cleaned amber glass bottles

acidified to pH 3 (HCl) and stored at 4 ºC until analysis.

Sample extraction

Before the extraction, the sample was filtered using a 0.45-μm nylon filter (Whatman, Maidstone, UK). The cartridges used for the SPE procedure were 500 mg Oasis HLB (Waters, Milford, Massachussets, USA) and 200 mg Bond Elut Plexa (Varian, Middelburg, The Netherlands). They were connected to a manifold (Teknokroma, Barcelona, Spain) and a pump as a vacuum source.
Both cartridges, Oasis HLB and Bond Elut Plexa, were conditioned with 5 mL of MeOH and 2 mL of Milli-Q water. Sample volumes of 100 mL (influent), 250 mL (effluent) and 500 mL (river water) were extracted. River water samples were extracted with Bond Elut Plexa and sewage samples (influent and effluent) were extracted with Oasis HLB cartridges. The samples were passed through the cartridge at a flow rate of 10-15 mL/min. Then there was a clean-up step using 15% MeOH in 5 mL water solution, and afterwards the cartridge was dried for 5 min. The retained analytes were first eluted with 5 mL of MeOH and, after a completely drying; 5 mL of DCM were passed through the cartridge. Extracts were reduced under a gentle flow of N_2 gas to approximately 3-4 mL. The final extracts were diluted to 5 mL with Milli-Q water. After being filtered through 0.45 μm syringe filters (Scharlab, Barcelona, Spain), 50 μL of this solution was injected into the chromatographic system.

UHPLC-(ESI)MS-MS

Ultra-high-performance liquid chromatography-electrospray ionisation-tandem mass spectrometry, in both positive and negative modes, was used to determine the target compounds.
The chromatographic instrument was an HP 1200 liquid chromatographic system coupled to a triple quadrupole mass spectrometer from Agilent Technologies (Waldbronn, Germany) with an ESI interface, an automatic injector, a degasser, a quaternary pump and a column oven. The chromatographic column was a Zorbax Eclipse XDB C_{18} (4.6 x 50 mm) with a 1.8 μm particle size (Agilent Technologies, Waldbronn, Germany), and the volume injected was 50 μL. The mobile phase flow-rate was 0.6 mL/min and the column temperature was kept at 50 ºC.
A binary mobile phase with a gradient elution was used. Solvent A was Milli-Q water with acetic acid (pH 2.8) and solvent B was methanol. The gradient was performed as follows: 60% B increased to 100% B in 6 min, constant for 4 min and then decreased to 60% B in 3 min. The UHPLC allowed for powerful separation of the target analytes within 9 min run time.
In order to achieve sensitive and selective detection of analytes, the (ESI)MS-MS parameters were optimized by injection of each compound. Analyses were performed

in the MRM mode either in the negative or positive ionisation mode to allow the simultaneous determination of all the compounds. Nitrogen was used as collision gas. Optimized MS-MS parameters were as follows: a N_2 flow rate of 12 L/min, a spray potential of 4000 V, a nebulizer pressure of 45 psi (N_2) and a source temperature of 350 ºC.

As Table 1 shows, the cone voltage was between 80 and 200 V for all the compounds, with the exception of TCS with only 18 V. Collision energies between 5 and 30 V were optimized for each analyte and the best values are shown in Table 1. The retention time and two MRM transitions (Table 1) were compared to confirm the presence of the compounds.

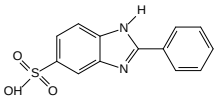
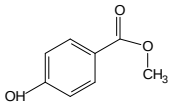
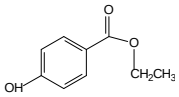
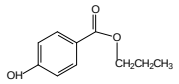
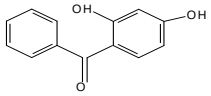
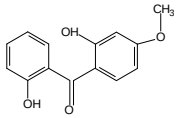
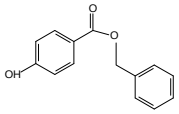
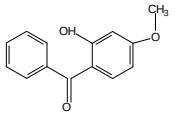
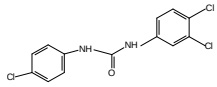
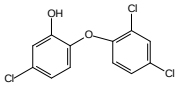
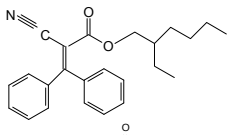
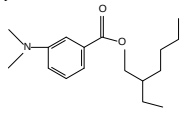
RESULTS AND DISCUSSION

UHPLC-MS-MS analysis

Methanol and acetonitrile were initially evaluated for the chromatographic separation but methanol was selected because a better peak shape was obtained. MRM transitions were determined for each compound by injection of the standards into the MS-MS. Upon ionization, all the compounds produced precursor ions that were fragmented into one or more product ions. The product ion spectra from the molecular ions of selected compounds are easily interpretable, and the main fragmentation pathways are displayed in Table 1. For each compound, two characteristic fragmentations of $[M-H]^-$ or $[M+H]^+$ were monitored, with the exception of OC, whose precursor ion was $[M+Na]^+$. The first and most abundant transition was used for quantification and the second one was used for qualification. For example, DHB and BP-3 are both UV filters with similar chemical structure which showed similar fragment ions after losing the benzene group. Thus, we could see the ion m/z 151 for BP-3 and m/z 135 for DHB. The same reasoning is given for the fragment ion $[C_6H_5O]^-$ seen in the spectrum of DHMB which has a phenol group sensitive to be lost for giving the main ion m/z 93. Table 1 also indicates that all the parabens (methyl, ethyl, propyl and benzyl) showed a fragment ion of (m/z 92) in the mass spectrum. All these parabens have a similar structure and they easily lost a methyl, ethyl, propyl and benzyl, respectively, to give the second ion. Only ethylparaben showed the transition $[M-H-CH_2CH_3]^-$ as the most abundant, whereas in the other parabens, $[C_6H_4O]^-$ was the most abundant fragment.

The TCS showed parent ions at m/z 287 and m/z 289. Both parent ions gave the same transition, leading to the chloride ion m/z 35. The cone voltage used to fragment the molecule was also much lower than it was for the other compounds. It was seen that only 18 V were enough because higher voltages decreased the response.

Table 1. Retention time, MRM conditions and proposed product ion for the determination of PCPs.

Analyte	Structure	t_R (min)	Precur. ion	Transition	Proposed product ion	CV (V)	CE (V)
PMDSA		**1.2**	**$[M+H]^+$**	**275>194**	$[M-H-SO_3]^+$	200	30
				275>211	$[M-H-SO_2]^+$	200	25
MPB		**1.5**	**$[M-H]^-$**	**151>92**	$[C_6H_4O]^-$	80	15
				151>136	$[M-H-CH_3]^-$	80	5
EPB		**1.9**	**$[M-H]^-$**	**165>136**	$[M-H-CH_2CH_3]^-$	100	15
				165>92	$[C_6H_4O]^-$	100	5
PPB		**2.5**	**$[M-H]^-$**	**179>92**	$[C_6H_4O]^-$	100	15
				179>136	$[M-H-CH_2CH_2CH_3]^-$	100	5
DHB		**2.9**	**$[M-H]^-$**	**213>135**	$[M-H-C_6H_5]^-$	130	5
				213>169	$[M-H-CH_3CHO]^-$	130	15
DHMB		**3.5**	**$[M-H]^-$**	**243>93**	$[C_6H_5O]^-$	80	15
				243>123	$[C_7H_7O_2]^-$	80	5
BPB		**3.5**	**$[M-H]^-$**	**227>92**	$[C_6H_4O]^-$	100	15
				227>136	$[M-H-C_7H_7]^-$	100	5
BP-3		**4.8**	**$[M+H]^+$**	**229>151**	$[M+H-C_6H_6]^+$	130	15
				229>105	$[C_7H_5O]^+$	130	15
TCC		**5.7**	**$[M-H]^-$**	**313>160**	$[C_6H_4NCl_2]^-$	130	5
				313>126	$[M-H-C_7H_5NOCl_2]^-$	130	15
TCS		**5.9**	**$[M-H]^-$**	**287>35**	$[Cl]^-$	18	8
				289>35	$[Cl]^-$	18	8
OC		**7.5**	**$[M+Na]^+$**	**384>272**	$[M+Na-C_8H_{16}]^+$	130	5
				384>228	$[M+Na-C_8H_{16}-CN-H_2O]^+$	130	5
OD-PABA		**8.4**	**$[M+H]^+$**	**278>166**	$[M+H-_8H_{16}]^+$	100	15
				278>151	$[M+H-C_8H_{16}-H_2O]^+$	100	15

CV: cone voltage.
CE: collision energy.

As can be seen in Table 1, there is only one compound (OC) which showed an adduct with Na^+ at m/z 384 corresponding to $[M+Na]^+$. OC and OD-PABA showed similar fragment ions and both compounds lost a fragment of C_8H_{16}. Their mass spectrum also showed the second ion (in relative abundance) due to the loss of a H_2O molecule (m/z 151) in OD-PABA, and the loss of CN^- group and the molecule of H_2O (m/z 228) in OC. Since the signal intensity of individual ions generally decreases as the number of ions being simultaneous scanned increases, time segments were monitored so that some of the analytes were monitored within specific small time windows according to their chromatographic separation. Six time windows were used in both positive (+) and negative (-) polarities as follows: (+) 0-1.3 min (PMDSA), (-) 1.3-2.3 min (MPB, EPB, PPB), (-) 2.3-4.5 min (DHB, DHMB,BPB), (+) 4.5-5.5 min (BP-3), (-) 5.5-7 (TCC, TCS), (+) 7-13 (OC, OD-PABA). As can be seen, the UHPLC allowed a chromatographic separation of the twelve initial compounds in six time windows of only 9 min.

The UHPLC-MS-MS chromatographic procedure in MRM had an excellent linear range of 0.05-500 μg/L (MPB) and 0.1-500 μg/L for the rest, except DHMB, TCS, OC and OD-PABA, which had a linear range between 0.5 μg/L and 500 μg/L, after injection of standards in Milli-Q water (r^2> 0.996) regarding all the compounds. A chromatogram of a standard of 200 ng/L was included as supplementary data. The detection limits calculated as the concentration which give a response corresponding a signal-to-noise ratio of 3, were as low as 20 ng/L for MPB and 50 ng/L for all the compounds except for DHMB, TCS, OC and OD-PABA with a LOD of 200 ng/L. The limit of quantification (LOQ), considered as the lowest concentration that can be quantified, was determined as the lowest point in the calibration curve.

Ion suppression study

It is well known that a critical aspect in quantitative analysis with ESI is the occurrence of ion suppression which may lead to a significant difference in the response of an analyte in a sample compared to a pure standard solution [25,26]. Different strategies have been proposed to minimise this effect, such as sample dilution or using internal and labelled standards [18,27], although these strategies are not useful in all cases. From our previous experience [28], we knew that ion suppression could be a big challenge. Therefore, to ensure the extraction procedure, we decided to study this effect to check the behaviour of our compounds in real samples. Because of our previous studies [25], we chose Oasis HLB as sorbent and a volume for each sample according to its complexity: 500 mL of river water, 250 mL of effluent and 100 mL of influent sewage water. After passing the samples through the cartridge, we tested the extracts in two ways to study also the action of the

evaporation step with N_2 in the effect of ion suppression. In Test 1, we evaporated the extract to dryness and reconstituted it with 5% MeOH in 1 mL of water, and in Test 2 we evaporated the extract down to 3-4 mL and diluted it with water back up to 5 mL, with a consequent less complex matrix. Both reconstituted extracts were spiked to a final concentration of 20 μg/L and the signal was compared with pure water standards. Simultaneously, the losses caused by evaporating with N_2 were evaluated with a standard in Milli-Q water and this gave satisfactory results which ruled out problems with the evaporation process. The results (%R) of both tests are shown in Table 2 and they were conclusive in helping us to decide not to evaporate until dryness.

Table 2. Study of the signal suppression effects. Values shown correspond to the recoveries.

Analyte	Test 1*			Test 2**		
	River	Effluent	Influent	River	Effluent	Influent
PMDSA	<10%	<10%	<10%	14	<10%	<10%
EPB	15	17	15	60	32	43
MPB	4	6	4	32	26	24
BPB	55	31	28	85	78	91
DHMB	58	34	34	86	76	91
DHB	66	40	49	90	89	98
PPB	37	38	43	78	81	89
BP-3	90	42	40	84	59	81
TCC	96	73	31	108	109	121
TCS	92	61	65	105	106	109
OC	40	20	9	45	40	43
OD-PABA	82	72	34	75	78	90

*Test 1: Evaporation until dryness and dissolution at 1 mL.
**Test 2: Evaporation until 3-4 mL and dissolution at 5 mL.
RSD (n=3) <10%.

Despite increasing the LODs, the SPE process become faster because we avoided the arduous task of evaporating to dryness. As can be seen in Table 2, the differences between recoveries in Test 1 and Test 2 were higher in wastewater (influent and effluent) because of the complexity of this matrix. Although we tried to avoid this effect in the evaporation step some compounds still showed a high ion suppression (> 50%) such as PMDSA, EPB, MPB, and OC. Signal suppression of MPB and EPB due to the ESI has been reported to be 48-69% in river water [8]. Therefore, we decided to assume this effect in MPB, EPB and OC. However, at this point, PMDSA was eliminated from the study because it showed the highest ion suppression effect (86-95%) in Test 2, which is in keeping

with its short retention time and probable coelution with other polar components of the matrix.

Optimization of the extraction procedure

Two different sorbents were tested to determine whether they could extract eleven PCPs in only one step. Oasis HLB and Bond Elut Plexa are both polymeric sorbents with a polar group in their structure. This property made them very suitable for extracting the selected compounds. Oasis HLB was chosen because of its demonstrated ability to retain polar compounds [25,29] thanks to a pyrrolidone group in its structure. Bond Elut Plexa, which has recently become commercially available, has a hydroxylated ligand on the surface and a narrower particle size distribution. We wanted to study the behaviour of this new sorbent, which initially seemed to have an advantage over Oasis HLB for some of our compounds. The efficiency of both sorbents was checked after the optimization of some parameters using, initially, 100 mL of Milli-Q water spiked at 1 μg/L. The sample pH was studied to ensure that it had the most suitable conditions for retaining all the analytes. The eluted extract was evaporated until 3-4 mL and reconstituted to 5 mL as was discussed in section 3.2. We could see that BP-3 was not retained in the Oasis HLB cartridge when the sample pH was in neutral conditions and it only showed a recovery of 36%. Similar behaviour was seen for DHB with recoveries of only 33%, meanwhile in acidified samples all the compounds showed recoveries higher than 67%. Therefore, samples were acidified to pH 3 prior to the extraction. Although 5 mL of MeOH were checked as elution solvent, we tried to improve the lowest recoveries of some compounds such as BP-3 (67%). Therefore, after drying the cartridge, 5 mL of DCM were added to elute the most apolar compounds and this led to a significant improvement in BP-3 recovery (99%). As the purpose of this study was to analyze very complex matrices, a clean up step of 15% MeOH in 5 mL of water solution was added before eluting the analytes, without significant losses in the recoveries. Sample volume was increased from 100 to 1000 mL to decrease the LOQs. When the sample volume was 1000 mL of Milli-Q water, the recoveries with Oasis HLB were between 77% and 101% for all the compounds, except for OC (56%). When the same study was done with Bond Elut Plexa the recoveries were even higher. Although the sorbent mass was less than half (200 mg), the recoveries were between 90% and 102%, and even OC showed increased recovery (76%).

We compared both cartridges to check the influence of the matrix in real samples (river and wastewater) and reduced the volume of the samples as the complexity of the matrix increased. For Oasis HLB, the recoveries from 500 mL of river sample were between 39% and 101%, except for MPB (25%) and EPB (22%).

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Therefore, we tried to improve the recoveries of these two parabens. When we did the same study with Bond Elut Plexa we realized that the recoveries of most of the analytes were higher, as can be seen in Table 3 (46-101%), particularly for MPB and EPB (88%). Both sorbents have a hydrophilic group in their structure but the different characteristics previously mentioned gives to Bond Elut Plexa more efficiency at retaining the compounds. Therefore, we chose Bond Elut Plexa to analyse river water.

Table 3. Recoveries and relative standard deviations (%RSD, n=4) of selected compounds in different kinds of water.

Analyte	Influent STP[a)]		Effluent STP[a)]		River water[b)]	
	%R[c)]	%RSD	%R[d)]	%RSD	%R[e)]	%RSD
EPB	36	3	38	12	88	9
MPB	27	3	20	12	88	11
BPB	89	2	70	2	94	2
DHMB	78	1	70	2	97	2
DHB	86	2	64	7	97	6
PPB	79	2	61	5	101	1
BP-3	59	1	56	5	70	11
TCC	39	11	67	10	69	7
TCS	85	14	92	1	89	7
OC	27	9	20	1	46	13
OD-PABA	59	5	71	7	69	6

[a)] Extraction with Oasis HLB.
[b)] Extraction with Bond Elut Plexa.
[c)] 100 ml spiked at 5 μg/L.
[d)] 250 ml spiked at 2 μg/L.
[e)] 500 ml spiked at 1 μg/L.

However, there were no significant differences in the results when we compared the effect of both sorbents in wastewaters (250 mL of effluent water and 100 mL of influent water). The results showed that both sorbents gave acceptable recoveries of the compounds. When deciding which sorbent was best for the most complex matrix, we realized that the velocity of charge in the Bond Elut Plexa cartridge was slower than in the Oasis HLB cartridge. The reason was that the particle size was different in both sorbents (60 μm for Oasis HLB and 45 μm for Bond Elut Plexa). Because it is very important to optimize the extraction procedure time, we decided to choose the Oasis HLB to extract sewage, although it is worth emphasizing that both sorbents were suitable for our compounds and the choice was only made because of the velocity in the extraction procedure.

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The recoveries for 250 mL of effluent and 100 mL of influent water were 56-92% and 39-89%, respectively, except for EPB, MPB and OC, which gave values between 20% and 38% in both cases (Table 3). A possible explanation for the low recovery of these compounds could be the fact that the matrix effect is higher in those compounds which appear at the beginning of the chromatogram.

Method validation

When a sample of river water was analysed, we only found PPB. Therefore, this signal was subtracted from the signal found in the spiked samples. The calibration curves were obtained by the whole method developed. Linear range was tested between 3 ng/L and 5000 ng/L for MPB and between 5 ng/L and 5000 ng/L for the rest of the compounds following the method developed. The precision of the method was evaluated by preparing a set of samples fortified with the analytes at levels of 100 ng/L. The repeatability (n=3) and reproducibility between days (n=3) gave results that were lower than 12% and 18% (%RSD), respectively. The LODs, calculated as previously explained, were as low as 1 ng/L for all the compounds except for BP-3 and DHMB (2 ng/L), TCS, TCC and OD-PABA (3 ng/L), and OC (4 ng/L). Limits of quantification (LOQs) were calculated as the lowest point in the calibration curve and this was 5 ng/L for all the compounds, except for MPB (3 ng/L).

Because of the presence of these PCPs in the sewage samples analyzed, we were unable to use the whole method to obtain a calibration curve in order to determine concentrations in sewage water. Therefore, the concentrations in real samples were achieved using calibration curves by injection of the standard solutions and applying the corresponding recoveries. Recoveries were checked at lower concentration (200 ng/L for influent and 100 ng/L for effluent) and results were similar to those included in the Table 3. Sewage water samples were spiked at low levels in order to determine the LOD as the concentration which give a response of signal-to-noise of 3. However, when the compounds were present in real samples, the LODs were estimated from calibration curves and the corresponding recoveries. The LODs in 250 mL of effluent waters were 3 ng/L for all the compounds except, 5 ng/L (DHMB) and 10 ng/L (TCS, TCC; OC, OD-PABA). LOQs were those which gave an instrumental response corresponding to the lowest point of the calibration curve. The LOQs were 5 ng/L for MPB, EPB, BPB, DHB, PPB and BP-3 and 20 ng/L for the rest of the compounds. The LODs in 100 mL of influent waters were 5 ng/L (MPB, EPB, BPB, DHB, PPB and BP-3), 10 ng/L (DHMB) and 20 ng/L for the rest. The LOQs were 10 ng/L for all the compounds except DHMB, TCC, TCS, OC and OD-PABA with 50 ng/L.

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Application to environmental samples

The SPE/UHPLC-MS-MS method was used to determine the presence of eleven PCPs in three kinds of matrices (river water, effluent and influent wastewater). As expected, the levels found in river waters were considerably lower than in wastewater because they become diluted when they are released into the environment [30].

MPB, DHB, PPB, and TCC were found in the Ebro and Llobregat rivers at levels lower than the limit of quantification. BP-3 was only found in the Ebro River and its concentration was 7 ng/L. Although TCS had been found in lake and river waters in previous studies at low ng/L [7,16], none sample of both rivers showed TCS.

Figure 1 shows the MRM chromatogram of a sample from the Ebro River which also shows MPB and PPB at values <LOQ.

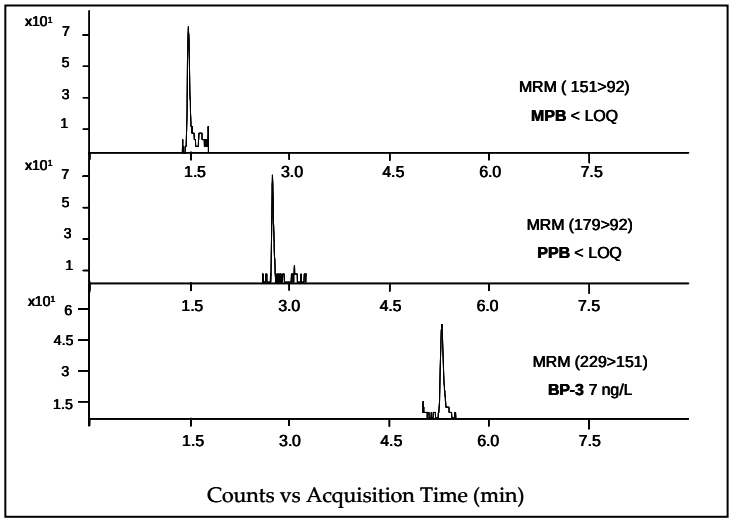

Figure 1. MRM chromatograms of a sample from the Ebro River. For conditions see the text.

To study the presence of PCPs in waters from sewage treatment plants, wastewater samples were taken during three periods of the year between the 2007 and 2008. The results in these samples are presented in Table 4. As can be seen in Table 4, EPB, MPB and PPB were the commonest compounds in the influent waters. The samples correspond to three different seasonal sets and showed some differences in the level of these analytes. For example, concentrations of EPB were found in influent waters ranging from 625 ng/L to 196 ng/L. The range for MPB was from 4427 ng/L to 1658 ng/L and for PPB was from 1945 ng/L to 77 ng/L. The highest values for EPB, MPB and PPB were in the samples taken in

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spring (Table 4), whereas the lowest values came from the samples taken in winter (Table 4). An example of a spring sample can be seen in Figure 2. Another important difference between the different sets of samples is the concentration of UV filters. For example, BP-3 was found in the three sets and its concentration was the highest in May (286 ng/l) and decreased to 11 ng/L in January. This agrees with the results reported by Rodil *et al.* [19] where the highest value of BP-3 (168 ng/L) in raw water was in July. Other UV filters found in our study were DHB (47 ng/L and 155 ng/L) and OD-PABA which was found only in one sample at 103 ng/L. The sampling area is very tourist area with warm temperatures, and this could probably be the reason why some UV filters appeared in influent waters of spring and summer.

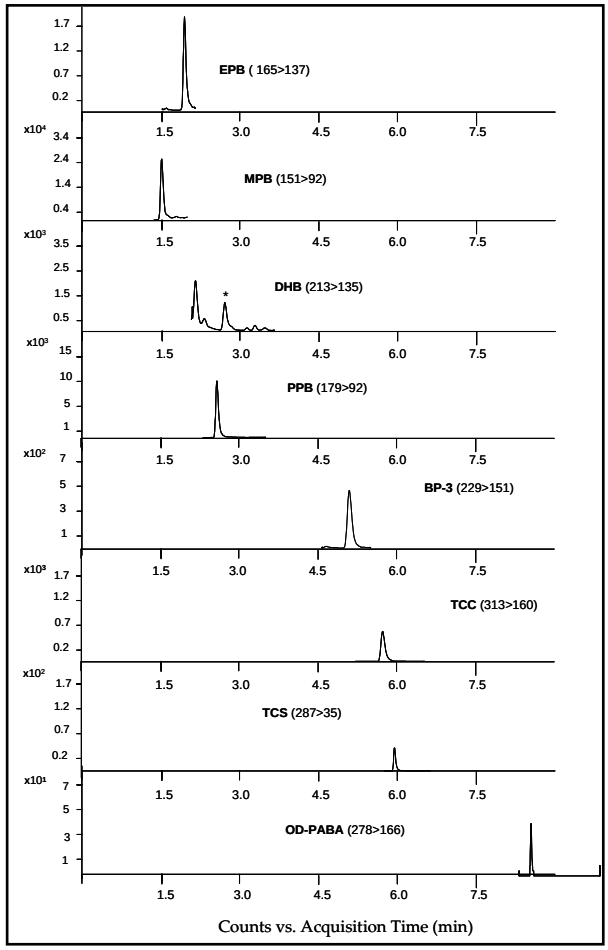

Figure 2. MRM chromatograms of a sample from an STP influent water in May 07.

Table 4. Concentrations of PCPs in wastewater samples (influent and effluent) in ng/L (n=3).

Analyte	May 07		Set 07		Jan 08	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
EPB	625	<LOD	498	<LOD	196	48
MPB	4427	<LOD	5613	<LOD	1658	<LOQ
BPB	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
DHB	155	<LOD	47	<LOD	<LOD	11
PPB	1945	24	1002	39	77	<LOD
BP-3	286	20	61	100	11	<LOD
TCC	362	<LOD	21	<LOD	<LOD	<LOD
TCS	87	<LOD	22	<LOD	<LOD	<LOD
OD-PABA	103	<LOD	<LOD	19	<LOD	<LOD

RSD<15%.

Although this is not an exhaustive study into the removal of PCPs by STP, it is shown that the tendency was for the STP process to eliminate PCPs. This statement was clearly confirmed when the high concentrations of parabens in influents were greatly reduced in effluents. One of the most commonly used PCPs is TCS and there are several studies [20,21] which show its presence in wastewater samples. For example, Kanda *et al.* [20] found levels of 3100 ng/l in influent sewage, although in the present study only 87 ng/L were detected in an influent sample. TCS is reported to be well removed during sewage treatment for activated sludge plants with measured removal rates of 95-98% [12,31] and this should ensure that no trace levels were found in effluents. This agreed with our study and no positive results for TCS and TCC were found during the effluent sampling.

CONCLUSION

A rapid method based on an SPE/UHPLC-MS-MS with triple quadrupole was developed to determine a group of emerging contaminants. These compounds were eleven representative PCPs including UV filters, parabens and antimicrobial agents. The new approach described in this paper is to determine several families of PCPs together in the same short analysis in different water matrices (river and wastewater). For the SPE, two cartridges (Oasis HLB and Bond Elut Plexa) were selected to study the extraction efficiency in the different matrices. Bond Elut Plexa was selected to extract river water because it gave the best recoveries for all the compounds. Meanwhile, Oasis HLB was chosen to extract wastewater because of its rapid extraction rate. The extract was only slightly evaporated to avoid preconcentrating the interferences that affect the ESI.

Most of the compounds showed good recoveries for river waters and

acceptable recoveries for STP waters. The UHPLC allowed a chromatographic separation of all the compounds to be obtained in only 9 minutes. The method has been proven to be linear, with LOQs in the low ng/L levels and highly selective using MRM mode. The results showed the presence of some of these compounds in river waters at very low ng/L. The highest concentrations found in influents were for MPB and PPB, (included in several commercial personal care products) with values between 77 ng/L and 5613 ng/L. Meanwhile BP-3 showed the highest concentration in effluents (100 ng/L) and river water (7 ng/L).

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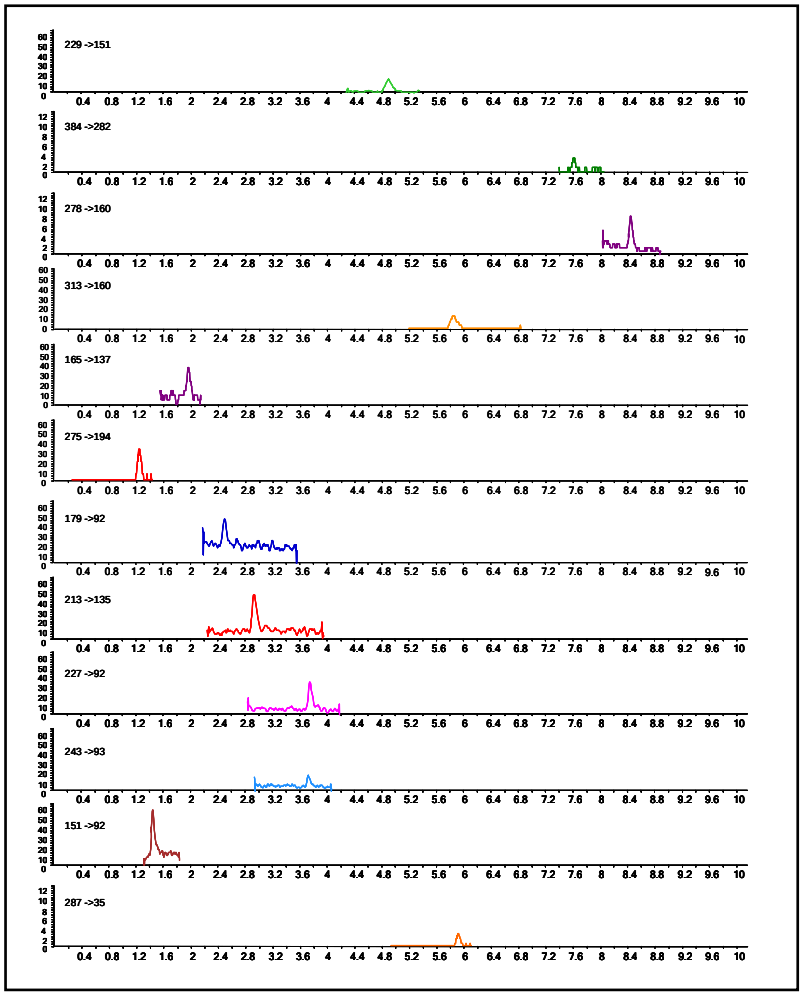
Supplementary information



MRM chromatogram of a standard of 200 ng/L.

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3.3.2. Stir bar sorptive extraction and ultra-high-performance liquid cromatography-tandem mass spectrometry for simultaneous analysis of UV filters and antimicrobial agents in water samples

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STIR BAR SORPTIVE EXTRACTION AND ULTRA-HIGH-PERFORMANCE LIQUID CROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR SIMULTANEOUS ANALYSIS OF UV FILTERS AND ANTIMICROBIAL AGENTS IN WATER SAMPLES

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Abstract

Stir bar sorptive extraction (SBSE) with liquid desorption (LD) and ultra-high performance liquid chromatography-electrospray ionization- triple quadrupole-tandem mass spectrometry (UHPLC-(ESI)MS-MS) were used for analysis of six personal care products in environmental waters: four UV filters (2,2-dihydroxy-4-methoxybenzophenone; benzophenone-3; octocrylene and octyldimethyl-*p*-aminobenzoic acid) and two antimicrobial agents (triclocarban and triclosan). Experimental conditions that affect SBSE-LD sorption efficiency (extraction time and temperature, sample pH and ionic strength) and desorption efficiency (solvent, temperature and time) were optimized. The method proved to be sensitive and 50 mL of sample was used to determine these compounds in environmental waters at trace levels. The detection limits of the analytical method were 2.5 ng/L for river water and 5-10 ng/L for effluent and influent sewage water. In river waters, benzophenone-3 was found at levels from 6 ng/L to 28 ng/L and triclosan at levels <LOQ. Benzophenone-3 was found between 75 and 127 ng/L in influent sewage, whereas concentrations of benzophenone-3 and triclosan were commonly below 25 ng/L in effluent sewage.

***Keywords**: UV filters, PCPs, UHPLC-MS-MS, SBSE, surface waters, wastewaters*

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INTRODUCTION

Antimicrobial agents and UV filters are present in personal-hygiene products such as toothpaste, soaps and sunscreen cosmetics and they are included in the emerging contaminants from so-called personal care products (PCPs). The primary route for these compounds to enter the environment is by discharge from point sources after having been washed off from skin and clothes to end up in sewage treatment plants (STPs) [1,2]. The removal of these compounds during wastewater treatment processes is not as effective as it should be. These compounds are continuously released to the environment and they may act as "pseudo-persistent compounds". As a result, aquatic organisms are exposed over their entire life cycle, and the compounds are becoming a health risk for humans [3]. Because of great concern about the effect of these compounds on the eco-system, precise data regarding their presence is required and this in turn requires high-sensitivity analytical methods. These PCPs are still being studied to determine the consequences of their continuous presence in the environmental waters [4,5].

PCPs are found in environmental waters at low concentrations (ng/L and low μg/L). Therefore, samples must be preconcentrated before their determination by chromatographic techniques. For example, LODs of 134 ng/L and 42.1 ng/L were achieved for triclosan and triclocarban respectively, using liquid-liquid microextraction (5 mL water) with liquid chromatography-ultraviolet detection [6]. Although several studies have used SPE to determine PCPs in waters [7-9], other studies have used solid phase microextraction (SPME) [10-12] and stir bar sorptive extraction (SBSE) [13,14]. SBSE is an extraction technique developed by Sandra *et al.* [15] in 1999. It is based on the same principles as those of SPME but also result in higher sensitivity because the volume of extraction phase in SBSE is 50-250 times larger than in SPME [16,17]. Polydimethylsiloxane (PDMS) is a typical coating material in commercial stir bars (Twister) and the amount used to extract environmental samples is 25-200 μL. One of the advantages of using PDMS in SBSE is that it enables predictable enrichment of non- polar compounds, although this limits the extraction of the more polar compounds [17,18]. To overcome this limitation, several authors have employed new strategies such as derivatization [17] or the use of polar polymers synthesized in-house [18] with successful results.

In methods using SBSE, thermal desorption (TD) of the analytes is usually combined with analysis by gas chromatography-mass spectrometry (GC-MS) [19,20]. For example, Kawaguchi *et al.* [20] developed derivatization in situ to determine seven benzophenone sunscreen compounds in waters with LODs of 0.5-2 ng/L.

When a thermal desorption unit is not available, liquid desorption (LD)

has been accepted as an alternative technique for determining different kinds of compounds, for example PCPs [18,21]. For example, Silva and Nogueira [21] determined triclosan by SBSE/LC-DAD and obtained LODs of 0.1 μg/L for wastewaters. To reach low levels we need improved chromatographic and detection techniques. Nowadays, increased speed and improved sensitivity is possible by use of ultra-high performance liquid chromatography (UHPLC) and tandem mass spectrometry with a triple quadrupole analyzer [7,9,22,23].

The objective of this study was to develop and validate a simple, accurate and highly sensitive method using SBSE/UHPLC-MS-MS to determine a group of PCPs in water samples. The PCPs selected are all aromatic compounds and lypophilic with octanol/water partition coefficients (log $K_{O/W}$) in the range 3.6-7.5 (Table 1). The method developed was applied to river and wastewater samples (influents and effluents).

EXPERIMENTAL

Reagents and standards

2,2-dihydroxy-4-ethoxybenzophenone (DHMB), benzophenone-3 (BP-3), triclocarban (TCC), triclosan (TCS), octocrylene (OC), and octyldimethyl-*p*-aminobenzoic acid (OD-PABA) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and storing it at 4 ºC. Fresh stock solutions were prepared every six months. A mixture of all compounds in methanol at a concentration of 50 mg/L was prepared weekly. Working solutions were prepared daily by diluting the previous solution with Milli-Q water.

Ultra-pure water was obtained with a Milli-Q water-purification system (Millipore, Bedford, MA, EEUU), acetonitrile (ACN), dichloromethane (DCM) and methanol (MeOH) were HPLC grade from SDS (Peypin, France), and nitrogen from Carburos Metálicos (Tarragona, Spain). Hydrochloric acid (HCl), sodium hydroxide (NaOH) and acetic acid from Prolabo (Bois, France) were used to adjust sample and the mobile phase pH.

Sample collection

All samples were collected from Catalonia (NE Spain). The river water samples were collected from three rivers (Ebro, Ter and Llobregat) and also from the Algars, a stream which was used as a blank. The wastewater samples were collected from the influent and effluent of the two domestic sewage treatment plants (STPs) in two cities located on the coastline, with population of about 120,000 inhabitants. All samples were collected in pre-cleaned amber glass bottles acidified to pH 3 (HCl) and stored at 4 ºC until analysis.

SBSE assays

Stir bars for sorptive extraction were obtained from Gerstel (Mulheim and der Ruhr, Germany). They consisted of a 10 mm long glass-encapsulated magnetic stir bar, externally coated with 24 μL of PDMS.

Before use, each stir bar was placed into a vial containing 1 mL ACN and magnetically stirred for 5 min. After drying with a lint-free tissue, the stir bar was placed in a glass with a 50 mL water sample at pH 5 and room temperature, and stirred at a speed of 900 rpm. After extraction for 180 min to reach equilibrium, the stir bar was removed from the sample using magnetic tweezers, rinsed with 2 mL Milli-Q water, dried with a lint-free tissue and placed in a vial containing 1 mL ACN for 15 min at 30 ºC. The stir bar was then removed by means of a cleaned magnetic rod and the stripping solvent was evaporated under a gentle stream of nitrogen. The dried residue was redissolved in 200 μL ACN:H_2O (1:1) and 50 μL of this extract was injected for UHPLC-MS-MS analysis.

After use, the stir bars were cleaned with DCM:MeOH solution (1:1, v/v) for 30 min at 30 ºC with magnetic stirring at 900 rpm. This step was repeated three times with fresh portions of the solvent mixture. After drying with a lint-free tissue, the stir bar was kept in a vial for the next analysis. Stir bars were reused at least 50 times with environmental samples. This is in agreement with the literature [16].

Instrumentation

The target compounds were determined by use of ultra-high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry. The chromatographic instrument was an Agilent 1200 series liquid chromatographic system coupled to a triple quadrupole mass spectrometer from Agilent Technologies (Waldbronn, Germany) with an ESI interface, an automatic injector, a degasser, a quaternary pump and a column oven. The chromatographic column was a Zorbax Eclipse XDB C_{18} (4.6 x 50 mm) with a 1.8 μm particle size (Agilent Technologies), and the volume injected was 50 μL. The mobile phase flow-rate was 0.6 mL/min and the column temperature was 50 ºC.

A binary mobile phase gradient was used. Solvent A was Milli-Q water with acetic acid (pH 2.8) and solvent B was MeOH. The gradient was: 60% B increased to 100% B in 6 min, constant for 4 min and then reduced to 60% B in 3 min, as in a previous paper [7]. In order to achieve sensitive and selective detection of analytes, the MS-MS conditions were optimized by direct injection of each compound. Analyses were performed either in the negative or positive ionization mode to enable the simultaneous determination of all the compounds. Nitrogen was used as collision, nebulizing and desolvation gas. MS-MS conditions for positive and negative ionizations were as follows: drying gas flow (N_2) of 12

L/min, capillary voltage of 4000 V, nebulizer pressure of 45 psi and drying gas temperature 350 ºC. The best ions were selected to obtain the MRM transitions; these are compiled in Table 1.

RESULTS AND DISCUSSION

UHPLC-MS-MS conditions

The chromatographic conditions were described in a previous paper by Pedrouzo *et al.* [7] in which some of these analytes were determined using SPE as the extraction technique. Analysis was performed either in negative or positive ionization mode to enable the simultaneous analysis of all compounds. BP-3, OC and OD-PABA were analysed in positive mode and DHMB, TCC and TCS were analysed in negative mode. Values of the cone voltage and the collision energy for each MRM transition are detailed in Table 1.

The UHPLC-MS-MS chromatographic procedure in MRM has an excellent linear range (r^2> 0.9986) in the 0.1-500 μg/L for BP-3 and TCC, and 0.3-500 μg/L for the other compounds. The detection limits calculated as the amounts for which the signal/noise ratio was 3 were 50 ng/L for BP-3 and TCC and 100-150 ng/L for the other compounds.

Table 1. Log $K_{o/w}$ and MS-MS conditions for the compounds.

Analyte	Log $K_{o/w}$[a]	MRM transition[b]	Cone voltage (V)	Collision Energy (V)
DHMB	3.93	**243>93**	80	15
		243>123	80	5
BP-3	3.64	**229>151**	130	15
		229>105	130	15
TCC	5.74	**313>160**	130	5
		313>126	130	15
TCS	5.17	**287>35**	18	8
		289>35	18	8
OC	7.52	**384>272**	130	5
		384>228	130	5
OD-PABA	6.15	**278>166**	100	15
		278>151	100	15

[a] Software calculated value, from SciFinder Scholar Database 2007: http://www.cas.org/products/sfacad.
[b] The MRM transition for quantification is in bold.

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Optimization of SBSE

The SBSE procedure was optimized using, as initial conditions, room temperature, pH 5, 50 mL sample, 2 h extraction, 600 rpm agitation and desorption with 1 mL ACN for 30 min at 50º C. Each condition was studied in detail; these included pH (2.0, 5.0, 7.0, 9.0 and 11.0), extraction time (1, 2, 3, 4, 5 and 16 h), stirring rate (600, 750 and 900 rpm), ionic strength (NaCl: 5, 10 and 15%, w/v) and organic modifier (MeOH: 5, 10 and 15%, v/v). The desorption conditions were evaluated by optimizing the desorption solvent (ACN, MeOH, DCM), agitation mode (magnetic stir and ultrasonic), desorption time (15, 30, 45 and 60 min) and desorption temperature (30 ºC, 50 ºC and 70 ºC).

The stirring rate is a very important parameter because it can affect mass transfer of the analytes towards the PDMS phase during the equilibrium process. Three levels were tested to achieve the best stirring conditions (600 rpm, 750 rpm and 900 rpm). The highest response was obtained with 900 rpm, and the speed was not increased higher than 900 rpm to ensure the integrity of the stir bar.

It was also important to determine the influence of the pH. Therefore, 50 mL Milli-Q water was placed in a glass vial and adjusted to different pH (from 2.0 to 11.0) after being spiked at 20 µg/L. As Figure 1 shows, the same response was obtained for BP-3 and DHMB at pH 7 and pH 9. For all the compounds, except OD-PABA very low extraction was achieved at pH 11. For most of them, especially OC, recovery was also low at pH 2. Although for most of the compounds the highest response was highest at pH 7, for OC and OD-PABA response was highest at pH 5. As a compromise, all the compounds were extracted at pH 5 because the difference between pH 5 and pH 7 for OC and OD-PABA was much higher.

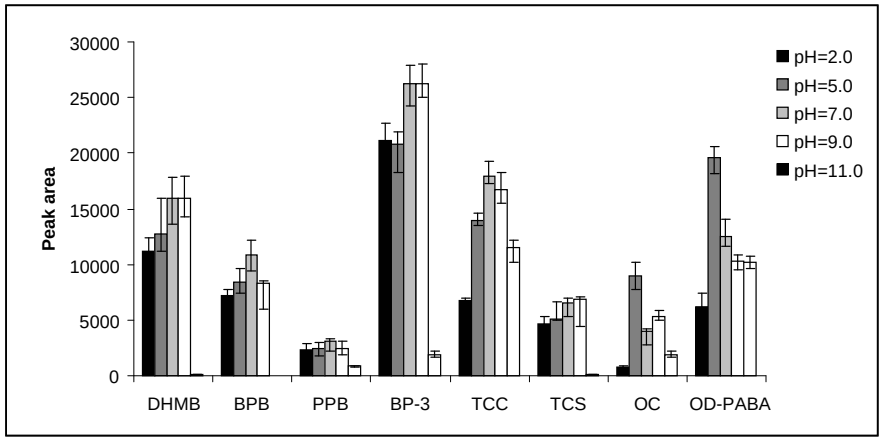

Figure 1. Effect of the pH of the sample matrix on the peak area for each compound. For conditions, see the text.

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The ionic strength of the sample was adjusted by adding NaCl from 0 to 15 % (w/v), but only for DHMB was recovery improved by addition of 10% NaCl, and that for OC and OD-PABA was substantially reduced. Consequently, the best conditions for the compounds studied were found when no salt was added.

Use of MeOH was found to minimise potential adsorption of the UV filters on glass material (wall effect) [19]. Sample solutions with different levels of MeOH were extracted under the same conditions and the extraction efficiency was compared. As Figure 2 shows, for TCC and TCS there was no difference when MeOH was added, whereas the response for BP-3 and DHMB slightly decreased when the MeOH content was increased.

However, when MeOH was 5%, the efficiency of extraction of both OC and OD-PABA improved from 19% and 22% to 49% and 61%, respectively. These results agree with those of Rodil *et al.* [19] who found that adding MeOH had a positive effect on the response of OC and OD-PABA, thus confirming that most hydrophobic compounds are adsorbed by glass. Therefore 5% MeOH was added to the samples in this study. To optimise the extraction time of the compounds, the SBSE time was varied from 120 to 300 min at 50 ºC. From Figure 3, a sampling time of 180 min was selected as a compromise between response and extraction time.

The desorption conditions were tested to ensure effective removal of the analytes from the SBSE device. Different desorption solvents were tested. Whereas MeOH and MeOH: DCM (1:1) resulted in lower extraction efficiencies for most of the compounds, ACN and MeOH:ACN (1:1) substantially improved the desorption process. The efficiencies of ACN and MeOH:ACN (1:1) were not different. So, 1 mL ACN was chosen as the desorption solvent.

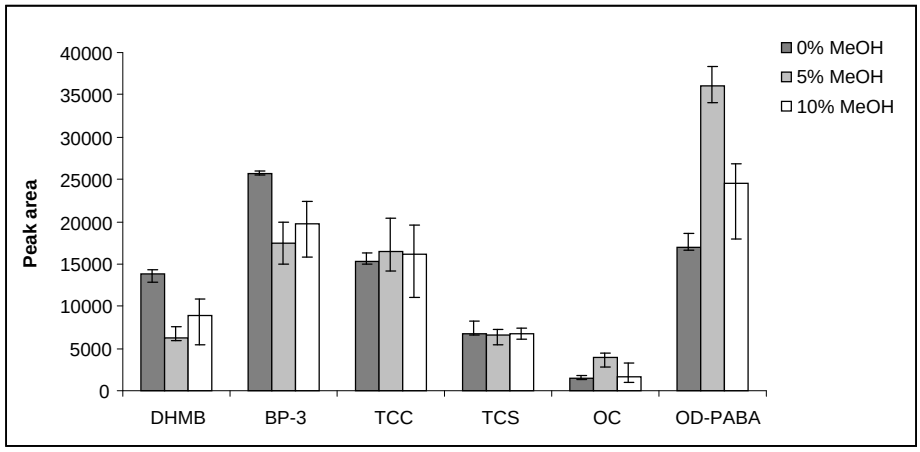

Figure 2. Effect of MeOH content of the sample on the peak area for each compound. For conditions, see the text.

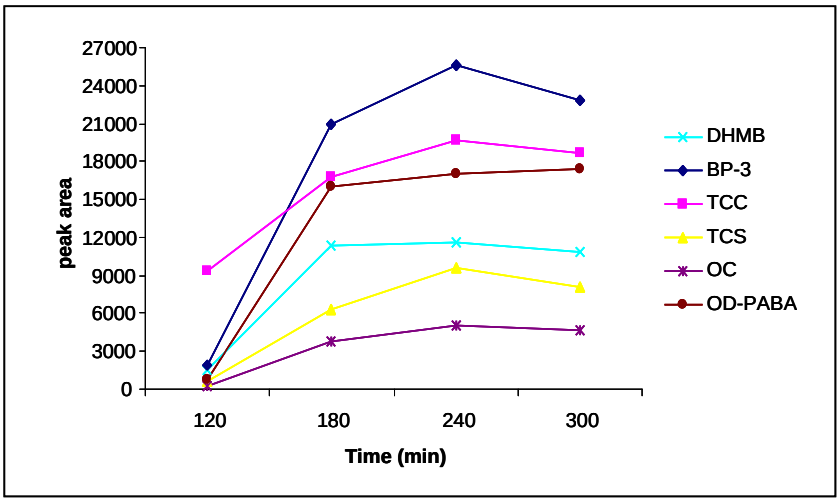

Figure 3. Effect of extraction time on the peak area for each compound. For conditions, see the text.

When we evaluated the number of desorption steps, we saw that only 1 mL was necessary and consecutive extractions did not result in any improvements.

Desorption profiles were obtained for a desorption time of 15-60 min. The results showed that 15 min was sufficient to ensure effective extraction; higher extraction times (30-60 min) did not result in an increase in the response. Temperatures from 30 ºC to 70 ºC were checked and the best extraction occurred at 30 ºC. Magnetic stirring desorption (30 ºC) was more effective than ultrasonic desorption performed in the same time (15 min, and 25 ºC of temperature). Therefore, magnetic stirring was selected for the desorption process.

The analytes that were the object of this study are found at very low levels in environmental samples, therefore an evaporation step was added. Thus, ACN solutions were evaporated until dryness and the residue was redissolved in different volumes (200 µL, 500 µL and 1000 µL) of H_2O, MeOH:H_2O and ACN:H_2O to ensure the best redissolution. According to the results obtained, the extracts were reconstituted with 200 µL ACN:H_2O (1:1), and no significant losses were observed for any analyte during the evaporation step.

Method validation

Theoretical recoveries were calculated by use of $K_{o/w}$ (Table 1) and a phase ratio (β) of 2083.3, in accordance with David and Sandra [16]. Values between 67.7% (BP-3) and 100% (OC) were calculated for all the compounds.

To investigate recoveries from environmental samples under the optimized experimental conditions (SBSE: 180 min, 900 rpm, pH 5.0, 25 ºC and desorption: 1 mL ACN, 15 min, 30 ºC), assays were performed on 50 mL of river water and sewage water

(effluent and influent).

Matrix effects are very common in LC-MS-MS techniques [7] and this possibility was studied in detail. We used three samples (river, effluent and influent) with the lowest concentrations of PCPs as a blank. A sample spiked at 200 ng/L was used to calculate the recovery from each kind of matrix by subtracting the blank responses. As Table 2 shows, SBSE obtained recoveries of 31% to 87% from river water, of 28% to 89% from the effluent and of 25 to 84% from the influent.

Table 2. Comparison of the recoveries found with a method based on SBSE[a] and SPE[b]

Analyte	River water		Effluent water		Influent water	
	%R_{SBSE}	%R_{SPE}	%R_{SBSE}	%R_{SPE}	%R_{SBSE}	%R_{SPE}
DHMB	31	97	28	70	25	78
BP-3	67	70	64	56	59	59
TCC	50	69	46	67	44	39
TCS	87	89	89	92	84	85
OC	59	46	43	20	48	27
OD-PABA	77	69	72	71	68	59

[a]SBSE conditions: 50 mL of sample spiked at 200 ng/L (n=3, %RSD<26).
[b]SPE conditions: 100 mL of influent sample spiked at 5 μg/L, 250 mL of effluent sample spiked at 2 μg/L and 500 mL of river sample spiked at 1 μg/L (See Pedrouzo *et al.* [7]).

When we compared these recoveries from real samples with those obtained from Milli-Q water (39-93%), we could see there was no significant matrix effect. The most affected compound was BP-3, whose recovery decreased from 85% (Milli-Q water) to 59-67% for the real samples. These recoveries were in the range reported with SBSE [19]. Repeatability, expressed as %RSD, was lower than 15% (n=3) and reproducibility between days was lower than 26% (n=3).

The Algars stream allowed us to construct a calibration plot without any of the target analytes in the blank sample. For the sewage waters, calibration was achieved by subtracting the signal of the analytes in the blank. The effluent blank contained low levels of TCC and the influent blank contained BP-3 and TCC. The linear range of this method was evaluated for river water (5-1000 ng/L), and effluent and influent (25-1000 ng/L) and for all these samples linearity was good ($r^2 > 0.992$). LODs of 2.5 ng/L were found for river waters and 5 ng/L for effluent and influent waters, with the exception of OC and OD-PABA (10 ng/L).

All these results were compared with those obtained in a previous study on SPE in which we used Oasis HLB and Bond Elut Plexa sorbents, which have a hydrophilic group in their structure [7].

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As Table 2 shows, recoveries were similar for the most of the compounds with both methods (SBSE and SPE). Recovery was lowest for DHMB (25% in 50 mL influent water and 28% in 50 mL effluent water) because it had the highest polarity. Recovery of OC was better with SBSE than with SPE. This fact and the decrease in the sample volume (50 mL compared with 100 mL influent and 250 mL effluent) shows that SBSE is an effective technique for determining most apolar compounds in 3 hours of extraction. Because SBSE gave us a quite selective extraction and low ion suppression, we could concentrate substantially in the evaporation step (250 times) and although SBSE used lower sample volumes, LOQs in both methods were similar.

Application to real matrices

To evaluate the applicability of this method to real matrices, river water and effluent and influent wastewater were analysed. When samples of three Catalan rivers (Ebro, Ter and Llobregat) were analysed, BP-3, TCS and OD-PABA were found at low ng/L levels (Table 3). Although only values <LOQ were found for TCS, it has been one of the most studied personal care products in environmental waters, with values, for instance, of 68 ng/L in rivers in Slovenia [24]. As Table 3 shows, BP-3 was found in all the river samples at concentrations between 6 ng/L and 28 ng/L. Similar results were found in our previous study in which we used SPE to analyze samples from the Ebro River [7]. This also agreed with the results of Negreira *et al.* [12], who found 52 ng/L of BP-3 in river watersBP-3, DHMB and TCS were the commonest compounds in the influent samples (Table 3), whereas OC (129 ng/L) only occurred in one of these samples. However, in the previous study DHMB and OC were not found in any of the samples analysed. Figure 4 shows the chromatogram obtained from an influent sample.

Table 3. Concentrations (ng/L) found in all the water samples analysed, %RSD (n=3) < 25.

Analyte	Influent wastewater				Effluent wastewater				River water		
	A	B	C	D	A	B	C	D	E	T	L
DHMB	185	-	59	<	-	55	-	-	-	-	-
BP-3	-	75	101	127	-	<	<	<	6	8	28
TCC	169	-	-	115	35	-	-	-	-	-	-
TCS	220	52	76	96	-	-	<	<	-	-	<
OC	129	-	-	-	-	<	-	-	-	-	-
OD-PABA	55	-	-	<	-	-	25	-	-	-	<

E: Ebre River; T: Ter River; L: Llobregat River
-: <LOD
<: <LOQ

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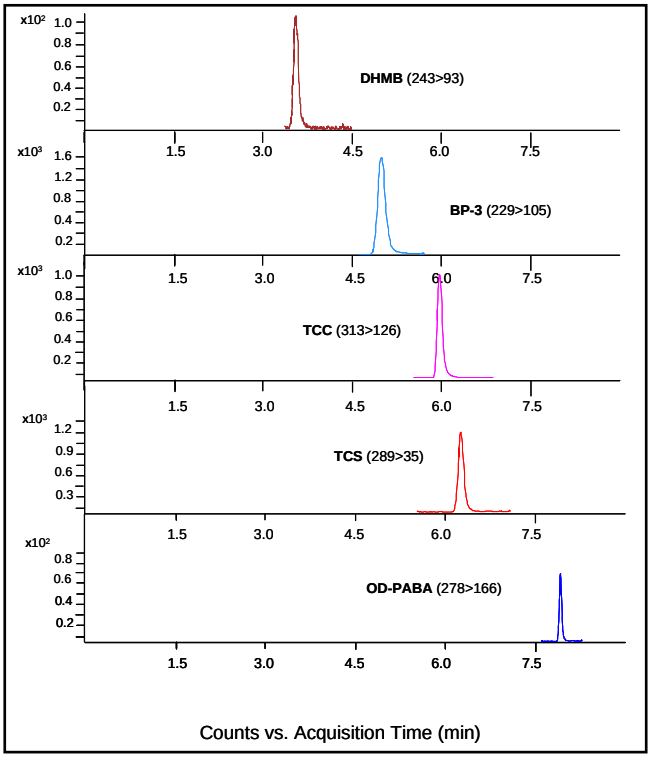

Figure 4. MRM chromatogram obtained from an influent water from an STP by SBSE-LD-LC-MS-MS, under the optimum experimental conditions.

The frequent detection of TCS and the concentration levels found in this study (between 52 ng/L and 220 ng/L) are comparable those found by Silva and Nogueira [21] who, after applying SBSE, determined TCS at levels lower than 0.4 μg/L in effluent and influent wastewater. Several papers confirm the presence of UV filters in wastewater samples, with BP-3 being one of the most commonly detected as result of its widespread use [4]. In this study, influent water contained BP-3 at concentrations from 75 ng/L to 127 ng/L in almost all the samples studied. In the previous study [7], BP-3 was, again, present in all the samples analysed, at concentrations between 11 ng/L and 286 ng/L in the influents and from <LOD to 100 ng/L in the effluents. Seasonal periods of warm weather and cold weather can be reflected in the concentrations of UV filters that are found. For example, Li *et al.* [25] found BP-3 at means of 86 ng/L-124 ng/L in February, which increased to 438-626 ng/L in July. These results were corroborated in another study [7] which found the highest concentration (286 ng/L) of BP-3 in May and the lowest concentration (11 ng/L) in January. The current study did not take into account this seasonal effect because the main purpose was developing the analytical method

rather than analyzing a large number of samples.

As with the influent samples, we studied the presence of PCPs in four effluent samples taken from two different STPs. Table 3 led us to conclude that almost all the compounds were below the LOQ (25 ng/L). Higher values were found for a few compounds, for example DHMB (55 ng/L) and TCC (35 ng/L), but only in one of the samples. Also, OD-PABA was found in one of the effluents at higher levels (25 ng/L) than those reported by Rodil *et al*. [19] who only found levels of OD-PABA between 2 and 7 ng/L in treated wastewater. Only one sample contained OC at levels below LOQ, similar to the results from our previous study [7], which agrees with the removals (higher than 89%) reported by Balmer *et al.* [26] in several STPs from Switzerland. The overall results showed that although total removals were not achieved, concentrations of PCPs in effluents were lower than in influents.

CONCLUSIONS

A group of PCPs was successfully determined using SBSE followed by desorption in a small volume of solvent then UHPLC-MS-MS analysis. The proposed method uses only 50 mL sample with a simple, easy and effective alternative methodology for determining PCPs in water matrices and the high sensitivity of the QqQ analyzer enables low LODs to be reached with this low sample volume. The novelty of this paper is to determine UV filters and antimicrobial agents in the same analysis in different water matrices (river and wastewater) with SBSE and LD/UHPLC-MS-MS. This paper shows that LD provides an alternative to thermal desorption when this is not available. A sensitive method was developed which gave LODs of 2.5 ng/L for river water and in the range 5-10 ng/L for sewage water. Recoveries from river waters (31-87%), effluent waters (28-89%), and influent waters (25-84%) were acceptable. The method enabled concentrations of BP-3 to be determined in river waters (6-28 ng/L), effluent sewage (<25 ng/L) and influent sewage (75-127 ng/L). TCS was found in all the influent samples at concentrations between 52 and 220 ng/L.

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Marta Pedrouzo Lanuza
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3.3.3. Discussion of results

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An innovative aspect of the studies discussed here was the use of UHPLC to allow a shorter chromatographic analysis time. The results were as satisfactory as expected, and the 11 targeted compounds showed a complete chromatographic separation after only 9 min. This rapid type of chromatography also offers obvious advantages in terms of reduced solvent consumption. This is especially important when the solvent used is acetonitrile, because of its current worldwide supply shortage.

Various families of widely used personal care products, including UV filters, parabens, and antimicrobials, were selected to be determined. The insect repellent DEET was also initially included. However, since this compound showed the same retention time as propylparaben but a different ionization mode, DEET was finally removed from the study. During the SPE, all of the selected compounds could be successfully extracted, except for the UV filter PMDSA, which was removed from the study because it showed the lowest levels of recovery. When we developed and applied an SBSE method using polydimethylsiloxane, we encountered a significant limitation and only the most apolar compounds could be extracted. Therefore, only the UV filters and antimicrobials were determined using SBSE method.

For the study using SPE, the extraction efficiencies of two polymeric sorbents were compared, Oasis HLB and a newly available product called Bond Elut Plexa. The choice of these two was based on previous experience as well as other results reported in the literature [1]. According to information from the manufacturer, Bond Elut Plexa has a hydroxilated ligand on its surface. When extractions were performed for 100 mL influent samples and 250 mL effluent samples, similar recovery levels were recorded with both sorbents. However, extraction with Oasis HLB was found to be preferable, because of their differences in particle size (60 μm for Oasis HLB and 45 μm for Bond Elut Plexa).

As mentioned in this Thesis's Introduction, ion suppression can be a very significant drawback in LC-(ESI)MS analysis. In the study using SPE, the role of the evaporation step in potentially increasing this effect was evaluated. Finally, it was determined that the lowest LODs could be obtained by allowing only partial evaporation of the extract. Under optimal conditions, recovery levels for all of the compounds were 27–89% for influent samples and 20–92% for effluents. Exceptions were seen with methylparaben and ethylparaben, which showed the lowest levels of recovery in both matrices (20–38%).

With 500 mL river water samples, variations were noted in the performances of the two sorbents. Bond Elut Plexa showed higher recovery levels (88%) for methylparaben and ethylparaben, compared to 22–25% with Oasis HLB. Therefore, Bond Elut Plexa was chosen for the river water analyses, and Oasis HLB for the sewage waters.

When comparing both extraction techniques, at was apparent that cleaner extracts were obtained with SBSE. Although only 50 mL of river and sewage water samples

were pre-concentrated, the clean extracts could be evaporated without drastically increasing ion suppression. Therefore, LODs could be decreased by using evaporation, with similar recovery levels as recorded with SPE for most of the targeted compounds.

The disadvantage of SBSE was that only the least polar compounds were extracted and this method could therefore not be applied to a high number of PCPs. Moreover, the optimal extraction process time was found to be 3 hours for SBSE, significantly higher than that of the SPE extraction process.

By combining SPE or SBSE extraction with UHPLC-MS-MS with a triple quadrupole analyzer, low limits of detection (LODs) were achieved. However, while river water samples showed similar LODs with the two extraction methods, (1−4 ng/L for SPE and 2.5 ng/L for SBSE), wastewater samples showed slightly LOD variations between the two techniques. Specifically, effluents showed LODs of 3−10 ng/L with SPE and 5-10 ng/L with SBSE, while influents showed LODs of 5−20 ng/L with SPE and 5−10 ng/L with SBSE.

Both methods were applied to samples from sewage treatment plants (STPs) and river waters. Maximum values of triclosan (220 ng/L) and triclocarban (169 ng/L) were found in influents when the SBSE technique was used. These values were in general agreement with the maximum values found in the same matrices with the SPE method (87 ng/L for TCS and 362 ng/L for TCC). Subsequent to the publication of the study being discussed here, other studies from Spain reported values that were in agreement with these. For example, Regueiro et al. [2] found a level of TCS of 343 ng/L in influents, and González-Mariño et al. [3] reported an even higher level in the same type of matrix (936 ng/L). Higher levels yet have been found in the USA, where TCS was found at a maximum concentration of 86,161 ng/L and TCC at 36,221 ng/L, both in influents [4]. When rivers were studied in China, TCS and TCC were found at maximum levels of 478 ng/L and 338 ng/L, respectively [5].

Both extraction methods enabled the determination of UV filters in sewage and river waters. For example, BP-3 was found at levels of 286 ng/L in influents and 6−28 ng/L in the three rivers. Another UV filter with environmental presence as reported in the literature is OC, which has been reported at levels of 72 ng/L in influents [1] and 15−70 ng/L in effluents [6]. The study reported here found OC in a single influent water sample, at a concentration of 129 ng/L (section 3.3.2).

As mentioned above, preservatives were only determined with SPE extraction method because of the polarity of the compounds. However, it is important to mention the high levels of these found in the influent samples, where methylparaben and propylparaben showed maximum concentrations of 5613 ng/L and 1945 ng/L, respectively. However, these compounds appeared to show an optimal level of removal by the STPs, as only values less than the LOQs were recorded in the effluent samples.

As demonstrated by the studies reported here, and also as reviewed in the Introduction chapter of this Thesis, PCPs are a group of emerging organic contaminants with a significant presence in environmental waters. This indicates a need for continuing study and development of effective means of control.

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3.4. Determination of drugs of abuse by solid-phase extraction and liquid chromatography-tandem mass spectrometry

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The presence of drugs of abuse in environmental waters is a critical issue for researchers. In a similar manner to that observed for the other types of compounds discussed in previous sections, drugs are excreted in unaltered form or as active metabolites after consumption, and are discharged into domestic wastewaters. Pioneering research on this subject from Italy was published in 2005 by Zucatto et al. [1], who reported on the occurrence of cocaine in the Po River. Drug residues enter sewage networks, but are not efficiently removed by STPs. As a result, these substances can often detected in effluent wastewaters [2-4], surface waters [4,5], and even drinking waters [5]. Recently, drugs of abuse have been recognized as emerging organic contaminants in the environment [6].

Studying drug levels in wastewater has also become a relatively simple and cost-effective approach to measuring community drug use levels [7,8]. For example van Nuijs et al. [9] studied spatial and temporal variations in the presence of cocaine and benzoylecgonine. They analyzed 30 wastewaters sampled at different times of the year and on two different days of the week (one weekday and one weekend day), to study cocaine consumption patterns in Belgium. Although their results showed no significant variation in terms of season of the year, constant cocaine consumption was observed during the week with peaks appearing during the weekends.

In study presented here, several drugs of abuse were used as target analytes, including cocaine and its metabolite benzoylecgonine, morphine and its metabolite 6-acetylmorphine, methadone and its metabolite EDDP, codeine and its metabolite dihidrocodeine, and the metabolite of cannabis THC-COOH. Although nicotine is not always considered as a drug of abuse, it was also included because of its high levels of consumption. The structures of all of these compounds are illustrated in Appendix II.

Although scientific interest in the environmental presence of drugs of abuse is very recent, several analytical methods can already be found reported in the literature for the determination of these substances, most of which use solid-phase extraction (SPE) as the extraction technique [3,10,11]. In the study reported here, the extraction efficiencies of several SPE sorbents were compared, including two with hydrophilic and lipophilic properties (Oasis HLB and Strata-X), and two with mixed reverse-phase cation exchange (Oasis MCX and Strata-XC). This was done in an effort to improve extraction efficiency and selectivity using these new mixed-mode sorbents, based on their potential for performing extensive clean-up and thus reducing matrix effects. Also, in order to minimize ion suppression, the samples were spiked with deuterated compounds before the SPE process was carried out.

Although González-Mariño et al. [10] have reported their development of a gas chromatography (GC) method, liquid chromatography (LC), and recently also ultra-high-performance liquid chromatography (UHPLC) [4,5,11] have been the preferred techniques used to determine drugs of abuse in water samples. To avoid the high pressures of UHPLC instruments, we used a new type of particle column based on

fused-core technology. This type of column has been developed to take advantage of the high speeds and high efficiencies offered by the use of sub-2-μm particles, but at only half of the back-pressure level. Therefore, with this type of particle column, it was possible to determine drugs of abuse with the advantages of UHPLC, but while also working at conventional LC pressures.

Clearly, determination of the low levels of drugs of abuse found in environmental waters requires the use of mass spectrometry in tandem (MS-MS). In this study, the analyzer used was a triple quadrupole, working in the selected reaction monitoring mode (SRM). We fulfil the requirement of Commission Decision 2002/657/EC [12] by using two SRM transitions and the ratio between them for a positive finding.

For the sewage treatment plant (STP) to be studied, we selected one that applied a tertiary treatment, located in a coastal city (Vila-Seca) with a large recreational park area. Samples taken after primary, secondary, and tertiary treatment were used to monitor the plant's performance over the course of a week.

In this same study, the efficiency of a drinking water treatment plant (DWTP) in removing drugs of abuse from surface waters was also studied. To do this, the levels of drugs of abuse in water samples from the Ebro River were determined, along with the presence of these after the drinking water treatment process had been applied. Although this was not an extensive study, it was sufficient to evaluate the basic levels of drugs of abuse in drinking water from this geographical area.

A paper discussing the results obtained by this study has been submitted for publication to the journal *Analytical and Bioanalytical Chemistry*.

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3.4.1. Drugs of abuse in waste- and surface waters by liquid chromatography-tandem mass spectrometry

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DRUGS OF ABUSE AND THEIR METABOLITES IN WASTE- AND SURFACE WATERS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A solid-phase extraction and liquid chromatography-tandem mass spectrometry (SPE/LC-MS-MS) method was developed and validated for the simultaneous determination of nicotine, 5 drugs of abuse (morphine, cocaine, codeine, methadone, EDDP) and 4 metabolites (dihydrocodeine, 6-acetylmorphine, 11-nor-carboxy-Δ^9-tetrahydrocannabinol and benzoylecgonine) in water samples. A Fused-Core™ particle column was used as an alternative to sub-2-μm particles in chromatographic separations to work with low backpressures and high efficiencies in short analysis times. Drugs were extracted from waste- and surface water with SPE using Oasis MCX cartridges. Electrospray (ESI) tandem MS in positive and negative mode and selected reaction monitoring were used for quantification. Calibration by linear regression analysis with deuterated internal standards was used to compensate the matrix effects. Limits of detection were found as low as 0.5-1 ng/L (surface waters) and 1-50 ng/L (wastewater). The method was applied to the analysis of different kinds of samples. Wastewater from a sewage treatment plant was collected from three sampling points (after primary, secondary and tertiary treatments) for a week. The analysis of the samples revealed a significant presence of these drugs in samples from primary treatments, where maximum concentrations of nicotine (1105 ng/L), benzoylecgonine (3336 ng/L) were found. Most of compounds showed values between<LOQ and 52 ng/L after tertiary treatment. When surface water from the Ebro River were analyzed, only benzoylecgonine showed levels between 19 and 35 ng/L.

Keywords: drugs of abuse, tandem mass spectrometry, wastewaters, surface waters, Fused-Core™ particle

INTRODUCTION

The presence of drugs of abuse in environmental samples is becoming a matter of global concern. The source of these residues is mainly from consumers, since drugs and their metabolites are excreted through urine. Therefore, studying these levels of residues in wastewaters is an effective way to provide realistic and comparable estimates of illicit drug consumption in different communities and thus allow measures and responsibilities to be established in real time [1-3]. Most drugs and their metabolites are only partially removed during sewage treatment [4,5]. Therefore, in the last few years, large efforts have been made to improve sewage treatments, with a tertiary step designed to remove more efficiently all the organic contaminants by advanced methods (e.g., ozonation, osmosis). However, most sewage treatment plants (STPs) do not yet include these treatments due to the high cost. As a result, such substances are released into surface waters [6-8], and they can be found in finished drinking water [4,9].

When the determination of drugs of abuse was directed solely at the clinical field, these compounds were identified in urine and other biological fluids by liquid or gas chromatography, both coupled to mass spectrometry (LC-MS, GC-MS) [10-12]. Nowadays, the advances in tandem mass spectrometry (GC-MS-MS and LC-MS-MS) have made it possible to detect the presence of drugs in waters at low ng/L levels. For the first time, González-Mariño *et al*. [13] identified drugs of abuse in waters by GC-MS-MS with an ion trap, and they found similar LODs (0.8-15 ng/L) to those reported by LC-MS-MS. The main disadvantage of GC-MS is the derivatization of the analytes, which is time-consuming. Therefore, as has been reported [14], LC-MS-MS is the technique preferred by researchers because of the polarity of the drugs. The triple quadrupole (QqQ) analyzer fulfils the requirements of Commission Decision 2002/657/EC [15] using two SRM transitions and the ratio between them for a safe positive finding.

Recently, the number of studies on the determination of drugs of abuse in European wastewaters is growing with reports in Spain [16-18], Belgium [19,20] and Italy [21], all of which focused on achieving lower limits of detection and confirming the presence of these drugs. Almost all the published methods to determine drugs of abuse in waters include a sample preparation based on solid-phase extraction (SPE) [16,22,23] The wide range of SPE sorbents made this technique the most suitable to ensure the retention of the analytes of interest. Several sorbents have been checked for drugs of abuse such as Oasis HLB [22,24], Strata-X [5] and the recent appeared mixed mode sorbents such as Oasis MCX [7,16] and Strata-XC [23]. These latter sorbents offer a dual cationic-exchange/reversed phase character which could be used to improve the selectivity of the SPE

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process. SPE can be on-line coupled to LC, and Postigo *et al.* [25] determined 17 drugs and metabolites with only 5 mL of sample, saving time and cost.

Although reversed phase LC is the most used chromatographic technique for drugs of abuse [16,18], some examples were reported with hydrophilic interaction liquid chromatography (HILIC). For example, Van Nuijs *et al.* [19] evaluated the separation of 9 polar drugs of abuse with HILIC-MS-MS. The separation by HILIC improved the retention of ecgonine methyl ester, which was not retained in reversed phase.

Nowadays, ultra-high performance liquid chromatography (UHPLC) is being used more and more in the analysis of environmental waters [6,16,24] because it offers advantages over conventional LC such as the gain in separation efficiency and shorter time. However, new adapted equipment is needed, which results in a high cost. Recently, some studies have been reported with a new particle column based on Fused-Core™ technology which has been developed to provide high speed and high efficiency of sub-2-μm particles, but at half the backpressure. These columns show a very narrow size distribution and higher particle density because of the solid core. The short diffusion paths of the thin porous crust generate greatly reduced back pressures. For example, Fontanals *et al.* [26] successfully used on-line SPE-HILIC-MS with a Fused-Core™ particle column to achieve a rapid and very sensitive (low ng/L) method of determining a group of polar drugs in waters.

The aim of our study was to develop a fast method based on SPE/LC-MS-MS for the simultaneous analysis of 10 drugs and their metabolites in waters. The method was developed with a Fused-Core™ particle column to be used in conventional LC equipment. The studied compounds belong to 3 different classes of drugs of abuse: cocainics: cocaine (COC) and its metabolite benzoylecgonine (BE); opiates: morphine (MOR), 6-acetylmorphine (6AM); methadone (MTD) and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP), codeine (COD) and dihydrocodeine (DIC), cannabinoids: the metabolite 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH). Moreover, nicotine (NIC) was included in the study, as a noncontrolled drug. The developed method was applied to the analysis of wastewater in three sampling points (after primary, secondary and tertiary) from an STP, where daily samples were collected throughout one complete week. Additionally, samples from surface water at the intake of a drinking water treatment plant and after the final treatment step were analyzed.

EXPERIMENTAL

Reagents and standards

All the compounds investigated (NIC, MOR, COD, DIC, 6-AM, COC, BE, EDDP, MTD, THC-COOH) and

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deuterated analogues (EDDP-d_3, COD-d_6, MOR-d_3) were obtained from Cerilliant (Round Rock, TX, USA) as solutions in methanol or acetonitrile at a concentration of 1000 mg/L. Standard stock solutions of each compound were prepared at 100 mg/L in methanol or acetonitrile. Mixed working solutions were prepared by diluting the stock solution via appropriate dilution with water. All standard solutions were stored in amber glass bottles at -20 ºC.

Ultra-pure water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, USA), acetonitrile and methanol were HPLC grade from SDS (Peypin, France), and nitrogen was from Carburos Metálicos (Tarragona, Spain). Hydrochloric acid (HCl), sodium hydroxide (NaOH) and acetic acid from Prolabo (Bois, France) were used to adjust the pH of the sample and the mobile phase.

SPE cartridges, Oasis HLB and Oasis MCX (6 mL, 200 mg) were purchased from Waters (Mildford, MA, USA) and Strata-X and Strata-XC (6 mL, 200 mg) were purchased from Phenomenex (Le Pecq Cedex, France).

Sample collection

The wastewater samples were collected from an STP in Vila-Seca, located near the coast of Tarragona and also in a very important recreational area. Daily, three composite samples (after primary, secondary and tertiary treatments) were collected at the same time during the first week (Monday to Saturday) of November, 2009. Moreover, samples from the next Monday were analyzed to check any differences between weekends. Therefore, 7 samples were collected after the mechanical or primary treatment (1TR); 7 samples were collected after the biological treatment or secondary treatment (2TR) and 7 samples were collected after the chemical or tertiary treatment (3TR). The plant served a population of 88,000 inhabitants equivalent in the moment of the sampling campaign and the 3TR consisted in chlorination, coagulation with Al_2SO_4 and flocculation, laminar clarification and sand filtration. The average flow on a weekly basis through the plant was 18000 m^3/day (influent).

Surface water from the Ebro River was taken at the intake of a drinking water treatment plant (DWTP) and after the final treatment step. The DWTP potabilizes 4 m^3/s and supplies finished water to 71 different towns and 31 industries in its area of influence. The treatment consists of a conventional prechlorination to break-point, addition of coagulants and flocculants, sand filtration, ozonation, granular activated carbon filtration and a final postchlorination to ensure residual chlorine.

All samples (surface and wastewater) were collected by using pre-cleaned amber glass bottles acidified to pH 2 (HCl) and stored at 4 ºC until analysis (within 24 hrs) to avoid analytes degradation.

Solid-phase extraction

Prior to extraction, samples were filtered using a 0.45 μm nylon filter (Whatman, Maidstone, UK) and samples from TR1 were previously centrifuged at 9000 rpm for 15 min (Hettich Zentrifugen, Tuttlingen, Germany). Deuterated compounds (EDDP-d_3, COD-d_6, MOR-d_3) were added. Volumes of 500 mL (surface water), 250 mL (wastewater from 2TR and 3TR) and 100 mL (wastewater from 1TR) were extracted. SPE was performed with Oasis MCX cartridges that were preconditioned with 5 mL of MeOH and 2 mL of water. Subsequently, samples were passed through the cartridge at a flow rate of 10 mL/min and washed with 5 mL H_2O (2% NH_4OH). After being vacuum dried for 5 min, the cartridges were eluted with 8 mL of MeOH (5% NH_4OH). The eluates were evaporated under a nitrogen stream to dryness, re-constituted with 500 μL H_2O (1% MeOH) and filtered with 0.45 μm syringe filters (Scharlab, Barcelona, Spain). Finally, 50 μL were injected into the LC-MS-MS system.

LC-MS-MS analysis

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-(ESI)MS-MS), in both positive and negative modes, was used to determine the target compounds. The chromatographic system was an Agilent 1200 liquid chromatograph coupled to a triple quadrupole mass spectrometer from Agilent Technologies (Waldbronn, Germany) with an ESI interface, an automatic injector, a degasser, a quaternary pump and a column oven. The chromatographic column was an Ascentis Express C_{18} (4.6 x 50 mm) with a 2.7 μm particle size (Sigma-Aldrich, Madrid, Spain) and the volume injected was 50 μL. The mobile phase flow-rate was 0.4 mL/min and the column temperature was kept at 30 ºC. A maximum of 60 bars were achieved in all the run time analyses.

A binary mobile phase with a gradient elution was used. Solvent A was Milli-Q water with acetic acid (pH 2.8) and solvent B was acetonitrile. The gradient was performed as follows: 5% B increased to 15% B in 3.5 min, increased to 50% B in 2.5 min, increased to 100% B in 6 min, constant for 2 min and then decreased to 5% B in 1 min. Under these conditions, the target analytes were powerfully separated in 13 min. To obtain the best sensitivity, the run time was divided into four time windows with both positive (+) and negative (-) polarities as follows: (+) 0-3.5 min (MOR, NIC), (+) 3.5-7.5 min (6-AM, COD, DIC), (+) 7.5-10 min (MTD, COC, BE, EDDP), (-) 10-13 min (THC-COOH).

Selection of the most intense MS-MS transitions was done by infusion of the individual compounds. Analyses were performed either on the negative or positive ionization mode to allow the simultaneous determination of all the compounds. In both cases optimized MS-MS parameters were as follows: flow rate of desolvation (N_2) of 12 L/min, spray potential of 4000 V,

a nebulizer pressure (N_2) of 45 psi and a source temperature of 350 ºC. Cone voltage and collision energy were optimized with values of 60-140 V and 15-60 V, respectively. Relevant data relating to the performance of the optimized LC-MS-MS method is compiled in Table 1.

RESULTS AND DISCUSSION

LC-MS-MS conditions

A chromatographic column with Fused-Core™ particle technology was used to obtain high speed and efficiencies of sub-2 μm particles while maintaining lower back-pressures. Chromatographic separation was checked with two different mobile organic solvents (methanol and acetonitrile). An optimum mobile phase using acetonitrile gave us the satisfactory separation of all the compounds in 13 min. Although the run time was slightly higher than the times found with a sub-2 μm particle and UHPLC system [16], we obtained a method applicable to any LC equipment in an optimum run time for all the 10 compounds. Full scan mass spectra and MS-MS compound-dependent parameters (cone voltage and collision energy) were optimized by direct injection of individual solutions in Milli-Q water. For each compound, the most abundant selected reaction monitoring (SRM) transition was used for quantification whereas the second (minor transition) and the ratio between them were used to provide reliable confirmation (Table 1).

All the compounds were ionized in positive mode, with the exception of THC-COOH, due to the acidic group in its structure. This compound failed to live up to our expectations because of the high LOD. However, we included THC-COOH in the list of compounds because it could be an indicator of cannabis consumption [16]. The rest of the drugs contain an amine function which can be easily protonated during ESI process and all the compounds yielded the corresponding protonated ($[M+H]^+$) parent ion. We observed that 6-acetylmorphine (m/z 328) easily lost the acetyl group (m/z 286) and an important ion at m/z 211 has been reported due to the loss of the N-containing saturated ring [10]. This is very common for compounds with an opiate structure and may also it can be accompanied by the loss of water [10]. In our study, the fragmentation was higher and also affected the break of the rings to yield ions m/z 181 and m/z 165.

Cocaine and its metabolite benzoylecgonine lost the ring with the amine to yield two abundant ions m/z 182 (cocaine) and m/z 168 (benzoylecgonine). Both yielded ion m/z 105 which corresponds to the benzene group.

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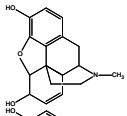
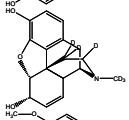
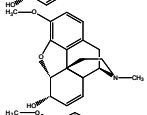
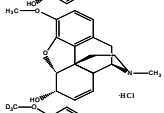
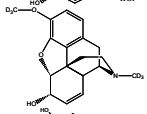
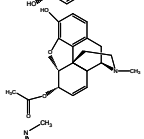
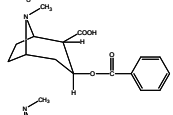
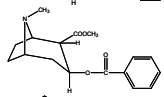
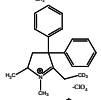
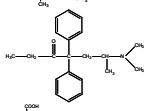
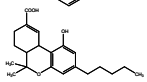
Table 1. Structures and SRM conditions for the determination of drugs of abuse.

Analyte	Structure	Precursor ion	Transition	Cone voltage (V)	Collision energy (V)	SRM2/ SRM1
NIC		**[M+H]+**	**163>130**	60	15	0.45
			163>117	60	15	
MOR		**[M+H]+**	**286>152**	100	60	0.63
			286>128	100	60	
MOR-d6		**[M+H]+**	**292>152**	60	60	0.65
			292>128	60	60	
COD		**[M+H]+**	**300>165**	100	60	0.26
			300>181	100	60	
DIC		**[M+H]+**	**302>165**	100	60	0.85
			302>141	100	60	
COD-d6		**[M+H]+**	**306>165**	100	55	0.80
			306>153	100	55	
6-AM		**[M+H]+**	**328>181**	100	45	0.25
			328>165	100	45	
BE		**[M+H]+**	**290>168**	100	15	0.05
			290>82	100	15	
COC		**[M+H]+**	**304>182**	60	15	0.06
			304>82	60	15	
EDDP		**[M+H]+**	**278>234**	140	30	0.15
			278>186	140	30	
EDDP-d3		**[M+H]+**	**281>249**	140	30	0.17
			281>234	140	30	
MTD		**[M+H]+**	**310>265**	60	15	0.19
			310>223	60	15	
THC-COOH		**[M-H]-**	**343>299**	60	15	0.29
			343>191	60	30	

In bold the SRM transition used for quantification

Methadone (m/z 310) easily lost the amine group ($HN(CH_3)_2$ and $(CH_3)_2$-$CHN(CH_3)_2$) to yield m/z 265 and m/z 223 but this did not occur in its metabolite, EDDP, due to the stability of the ring in the amine group. However, EDDP showed fragments from losses of a methyl group followed by the loss of an ethyl (m/z 234) or phenyl group (m/z 186).

Nicotine is not considered a drug of abuse but it was included in this study because the high consumption of this drug leads to a subsequent presence in environmental waters. Nicotine was the smallest molecule of all the drugs and the amine group was the most easily fragmented yielding the ion m/z 130 and m/z 117 (M-CH_3NHCH_3).

We tested the quantification of the drugs with and without surrogate. Our results confirmed that the closest surrogate to each drug was the best choice. We used three deuterated compounds (EDDP-d_3, morphine-d_3 and codeine-d_3). A higher number of deuterated drugs is used by other authors in the same analyses, including the own deuterated analyte [19,27]. However, we attempted to obtain the correct determination of all the drugs with the minimum of deuterated drugs possible due to the high cost of these compounds.

Linear range by direct injection of the standard solutions was between 0.1 μg/L and 50 μg/L for all the compounds, with the exception of THC-COOH (0.5-200 μg/L) and nicotine (0.75-50 μg/L). The method was very sensitive for all the compounds except for nicotine and THC-COOH, as reported in previous papers [16].

Optimization of the SPE step

In the initial phase of the method development, the performance of different types of SPE cartridges (Oasis HLB, Oasis MCX, Strata-X and Strata-XC) was evaluated.

Efficiencies in the recoveries were initially tested with 100 mL Milli-Q water at pH 7 and pH 3. Samples were brought to these pHs with HCl or NH_4OH. Although we did not see significant differences between acidic and neutral pH, nicotine and benzoylecgonine showed slightly better recoveries with acidic pH (pH 3) and thus was chosen. In Strata-X and Oasis HLB, elution was performed with 10 mL of MeOH, whereas in Strata-XC and Oasis MCX, the elution was performed with 8 mL MeOH (5% NH_4OH). Strata-X showed the lowest recoveries for nicotine, morphine, 6-acetylmorphine, codeine and dihydrocodeine. Oasis HLB gave similar recoveries to Oasis MCX for some drugs, but the latter improved the recoveries for codeine and dihydrocodeine with values that increased from 36% to 80% and from 39% to 85%, respectively. When we compared both cationic sorbents we realized that both gave good recoveries for all the compounds. Surprisingly, we did not obtain sharp peaks for morphine and 6-acetylmorphine with Strata-XC. Therefore, Oasis MCX was the chosen

sorbent by which to determine all the compounds. Oasis MCX retained the drugs at pH 3 because, due to the basic properties of most of them, they were protonated. The only exception was THC-COOH, whose recovery was not as good as for the rest of the compounds.

We attempted a washing step with 1 mL MeOH to remove interfering chemicals which are retained by reversed-phase interactions. However, some of our analytes such as BE, EDDP and THC-COOH were eluted with the MeOH because the interactions were not only by ion exchange mechanism but also reversed-phase. Therefore, we performed a washing step based on an aqueous solution (2% ammonium in water), without significant losses of our drugs. The eluted extracts were evaporated with N_2 and reconstituted with 500 μL water (1% MeOH).

Under the optimum conditions, the recoveries for 500 mL of Milli-Q water were between 65 and 85%.

When real samples were analyzed, sample volume had to be decreased for wastewater and recoveries shown in Table 2, were of 25-64% (100 mL of primary wastewater), 30-69% (250 mL of tertiary wastewater) and 500 mL surface water (38-77%). These values were in agreement with other studies using MCX sorbents. For example, Blijsma *et al.* [16] found values of 76% (cocaine) and 84% (benzoylecgonine), in surface waters.

Method validation

Prior to its application, the overall analytical procedure was validated for each kind of water, considering the linear range, LODs, LOQs, repeatability and reproducibility between days (Table 2).

Because the possible ion suppression in ESI-MS we ensurated an accurate quantification of the analytes in real samples by the use of deuterated internal standards, as the most commonly used method [16,25]. The most similar compound in terms of structure was checked and compared with the closest one in terms of retention time. The best choice was found using the SRM windows and using the surrogate next to each compound in the same window. Therefore, NIC and MOR were quantified using MOR-d_3, COD, DIC, 6-AM and BE using COD-d_6 and the rest of the compounds using EDDP-d_3. Before the SPE, the samples were spiked with the surrogate at a final concentration of 50, 20 and 10 μg/L, respectively. Given the complexity of the matrix, three calibration curves were used: surface waters and wastewater (from 1TR, and 3TR) with 5-8 points for each kind of matrix. Samples from 2TR were quantified with the 3TR calibration curve because of similarity in the matrix.

Samples after drinking treatment were quantified with a surface water calibration curve.

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Table 2. Validation data for all different kinds of water. Linear range (ng/L), LODs (ng/L) and %R.

Compound	Primary sewage			Tertiary sewage			Surface water		
	L. Range	LOD	%R	L. Range	LOD	%R	L. Range	LOD	%R
NIC	25-1000	10	52	10-750	2	56	5-500	1	74
MOR	10-1000	2	35	2-750	1	41	1-500	0.5	52
COD	10-1000	2	33	2-750	1	34	1-500	0.5	58
DIC	25-1000	5	41	5-750	1.5	69	1-500	0.5	76
6-AM	25-1000	5	59	5-750	1.5	61	1-500	0.5	66
BE	250-4000	-[a]	44	10-750	1	63	1-500	0.5	71
COC	125-4000	-[a]	64	2-750	1	60	1-500	0.5	57
EDDP	5-1000	2	45	2-750	1	68	1-500	0.5	77
MTD	5-500	1	48	2-750	1	51	1-500	0.5	70
THC-COOH	125-1000	50	25	20-750	5	30	10-500	1	38

-a Values not estimated due to the high levels found in the "blank" sample.
Repeatability, RSD (%, n=3)≤11%
Reproducibility between days , RSD (%, n=3)≤17%

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When some of the analytes were in the blank samples, their response was subtracted to all the calibration points. In all cases, the LOQ was used as the lowest point in the calibration curve.

As Table 2 shows, LOQs in surface water were fixed at 1 ng/L for all the compounds with the exception of NIC (5 ng/L) and THC-COOH (10 ng/L). In waters from 3TR, LOQs of 2 ng/L were found for all the compounds except 5 ng/L (DIC and 6-AM), 10 ng/L (NIC and BE) and 20 ng/L (THC-COOH). In waters from 1TR the following LOQs were found: 5 ng/L (MTD and EDDP), 10 ng/L (MOR and COD), 25 ng/L (NIC, DIC and 6-AM), 125 ng/L (COC and THC-COOH) and 250 ng/L (BE).

Limit of detection (LOD) was estimated for a signal to noise of three. For surface waters, LODs as low as 0.5 ng/L were found for all the compounds except for NIC and THC-COOH (1 ng/L). For wastewater from 3TR, LODs were 1-2 ng/L, except THC-COOH (5 ng/L). For wastewater from 1TR, LODS were 1-50 ng/L for all the compounds except for COC and BE, whose LODs could not be found because of the high levels in blank samples.

Average intensity SRM_2/SRM_1 ratios calculated from reference standard solutions (Table 1) were compared to those experimentally obtained from spiked extracts at 1-5 μg/L. Average values obtained for relative ion intensities for 1TR, 3TR and surface extracts were between 0.05 and 0.85, similar in the three kinds of samples. Tolerances were always below 23%, which fits the maximum permitted 20-50% depending on the ion intensities [15].

Application to environmental waters

As regards STPs, a total of 21 waters from three sampling points were analyzed for a week, with the exception of Sunday, but the next Monday. Most of the target analytes were found in our wastewater samples, as can be seen in Table 3.

Confirmation of positive findings was carried out by calculating the peak area ratios between the confirmation (SRM_2) and quantification (SRM_1) transitions and comparing them with the SRM_2/SRM_1 for spiked extracts in the same samples. In our study all the SRM_2/SRM_1 ratios showed a deviation ranging from 20 to 50%. Only an exception was found for THC-COOH, which tolerance was above the limits for some 1TR samples. Therefore these values were not considered as positive findings and they are not included in Table 3.

As Table 3 shows, the highest concentrations found in TR1 were for benzoylecgonine with maximum values of 2043 ng/L (weekday) and 3336 ng/L (weekend). However, cocaine, its parent compound, yielded concentrations of 160-801 ng/L (weekday) and 258-2486 ng/L (weekend) in TR1. In both cases, the first day of the monitoring showed the highest value. This could be because the selected monitoring week began after a weekend of festivities. This fact could be reflected in the results from both

Mondays, where the first was significantly different from the second one. Also, the STP was selected to be located in a very important leisure area: the coast. The high levels of both compounds are in agreement with values found by Huerta-Fontela *et al.* [24], also in Spain, with maximum concentrations of 2307 ng/L (benzoylecgonine) and 225 ng/L (cocaine). In our study, of particular note is the considerable increase of cocaine in the sample collected on Saturday (2446 ng/L), with similar values to its metabolite benzoylecgonine (2650 ng/L). When Nuijs *et al.* [20] studied temporal variations of cocaine and metabolites in different STPs in Belgium, the highest values were found on Sunday, with a maximum concentration of benzoylecgonine of 2130 ng/L, similar to our results. As the literature shows, the measurements of drugs of abuse in wastewater has become an innovative method of calculation of illicit drug consumption in local communities [28].

Despite the high levels found in 1TR, both compounds showed the greatest decrease of loads after the biological treatment. Consequently, only maximum levels of 47 ng/L and 10 ng/L were registered for benzoylecgonine and cocaine, respectively, in 2TR.

Codeine and morphine were detected at constant values in all the sampling points from 1TR and these values only decreased slightly in 2TR. For example, morphine was detected at 104-166 ng/L (1TR), 79-138 ng/L (2TR) and these values decreased to 2-53 ng/L (3TR). As can be seen in Table 3, morphine showed results higher than its metabolite, 6-acetylmorphine, which only showed maximum values of 79 ng/L in 1TR, values in agreement with Castiglioni *et al.* [7].

This secondary treatment was not entirely effective in removing the loads of codeine, and concentrations only decreased by 30-40%. This low removal figure is in agreement with Hummel *et al.* [4]. Its metabolite, dihydrocodeine, showed lower values and maximum concentrations between 9 and 14 ng/L were found in all the sampling points. Also EDDP, the primary metabolite of methadone, was ubiquitously present in the secondary treatment with values between 33 and 75 ng/L (2TR). However, EDDP was detected in only 3 samples of the 3TR point at maximum values of 8 ng/L. Although the study of Bones *et al.* [5] did not show methadone in presence of EDDP, our study showed values of methadone and EDDP in effluent samples (3TR), more in agreement with Castiglioni *et al.* [7]. To complete the study, nicotine was included as a non-controlled drug. Nicotine had been previously found by Huerta-Fontela *et al.* [17] at concentrations between 1.2-104 μg/L, in influents. We found a maximum concentration of 1105 ng/L in 1TR and only a maximum of 15 ng/L was found after tertiary treatment. As Table 3 shows, tertiary treatment registered significant removals for morphine, codeine and EDDP as concentrations decreased drastically.

Table 3. Concentrations (ng/L) found in all the sampling points from the STP during weekdays[a] and weekends[b].

Analyte	Primary sewage (1TR)		Secondary sewage (2TR)		Tertiary sewage (3TR)	
	weekday	weekend	weekday	weekend	weekday	weekend
NIC	77-171 (4)	82-1105 (3)	<LOQ (4)	<LOQ-21 (3)	<LOQ-2 (4)	<LOQ-15 (3)
MOR	104-154 (4)	141-166 (3)	79-101 (4)	103-138 (3)	5-45 (4)	2-53 (3)
COD	243-258 (4)	302-337 (3)	152-184 (4)	160-189 (3)	4-13 (4)	7-17 (3)
DIC	<LOQ (4)	<LOQ-16 (3)	8-9 (3)	5-14 (3)	<LOQ (4)	<LOQ-10 (3)
6-AM	60-79 (2)	-	9-14(2)	-	-	-
BE	1169-2043 (2)	2150-3336 (3)	14-45 (4)	15-47 (3)	9-14 (4)	1-42 (3)
COC	160-801 (4)	258-2486 (3)	<LOQ-10 (4)	2-7 (3)	<LOQ-2 (4)	<LOQ-9 (3)
MTD	12-68 (4)	43-406 (3)	22-69 (4)	11 (1)	12 (1)	-
EDDP	14-24 (4)	16-29 (3)	33-75 (4)	36-44 (3)	<LOQ-2 (4)	<LOQ-8 (3)
THC-COOH	219 (1)	144 (1)	-	-	-	-

[a]Weekday (samples collected from Tuesday to Friday (4 days)).
[b]Weekend (samples collected on Saturday and Monday (2 days)).
Note: Number of positive results is given in brackets.
<LOD

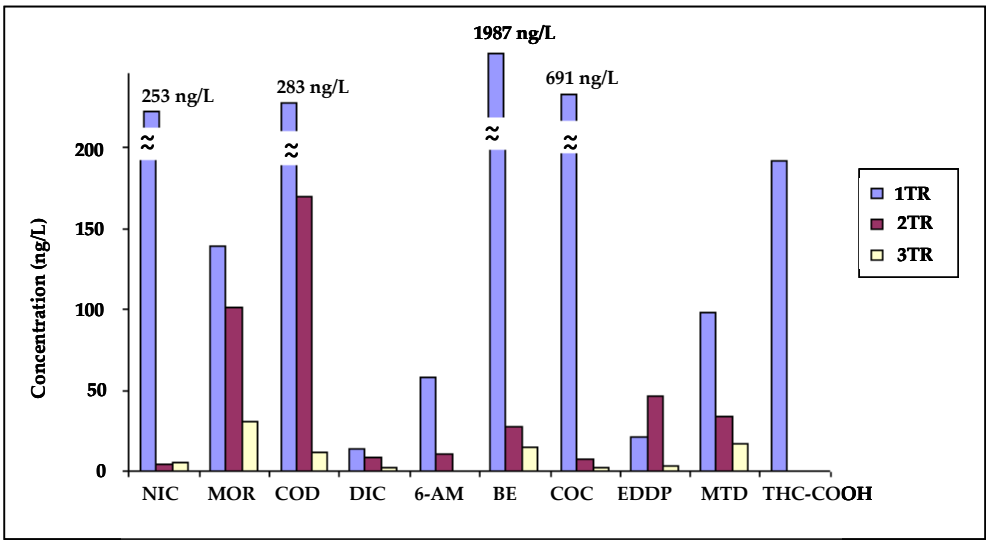

Figure 1. Mean of concentrations of drugs in the three sewage treatment points (1TR, 2TR and 3TR).

However, some of the analytes (methadone, cocaine, benzoylecgonine and nicotine) were only partially removed and low concentrations were found. Figure 1 shows the average of concentrations found for all the three sampling points. As can be seen, we found the concentrations in 1TR considerably higher than in either of the other sampling points. (2TR and 3TR). Most of the compounds showed the highest removals after 2TR, except morphine, codeine and EDDP. Moreover, the 3TR added a significant removal for all the compounds. An illustrative chromatogram of a sample from 2TR is also shown in Figure 2.

Having demonstrated the frequent presence of selected drugs in the tertiary wastewaters, we analysed surface waters from the Ebro River at the intake and outflow of a drinking water treatment plant (DWTP). Three samples were analyzed in both DWTP points and raw water showed low levels of nicotine (<LOQ) and benzoylecgonine (19 ng/L-35 ng/L). After the drinking treatment process, treated samples showed values <LOQ of benzoylecgonine. Boleda *et al.* [29] studied the occurrence of opiates and cannabinoids in surface water and finished water in the DWTP. The water treatment performed was able to completely eliminate 6 of the 8 target compounds identified at the intake of the DWTP and only methadone and EDDP were identified in the finished water (0.2 ng/L).

Conclusions

We developed a SPE/LC-MS-MS method using a Fused-Core™ particle chromatographic column for the simultaneous determination of 10 drugs of abuse with their relevant metabolites in surface and waste water. The main advantage of the

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method is that it can be used with conventional LC instrument, but with lower retention times. The analyzer was a triple quadrupole and the SRM mode yielded high selectivity and sensitivity. The matrix effect was corrected with the use of three deuterated compounds as surrogate internal standards. The SPE using Oasis MCX cartridges permitted the preconcentration of 500 mL of surface water, 250 mL wastewater (secondary and tertiary) and 100 mL of primary wastewater. The wide linear range and high sensitivity for this method facilitated its application in surface water and wastewater. The samples were analysed over a week and results were compared between days and STP sampling points.

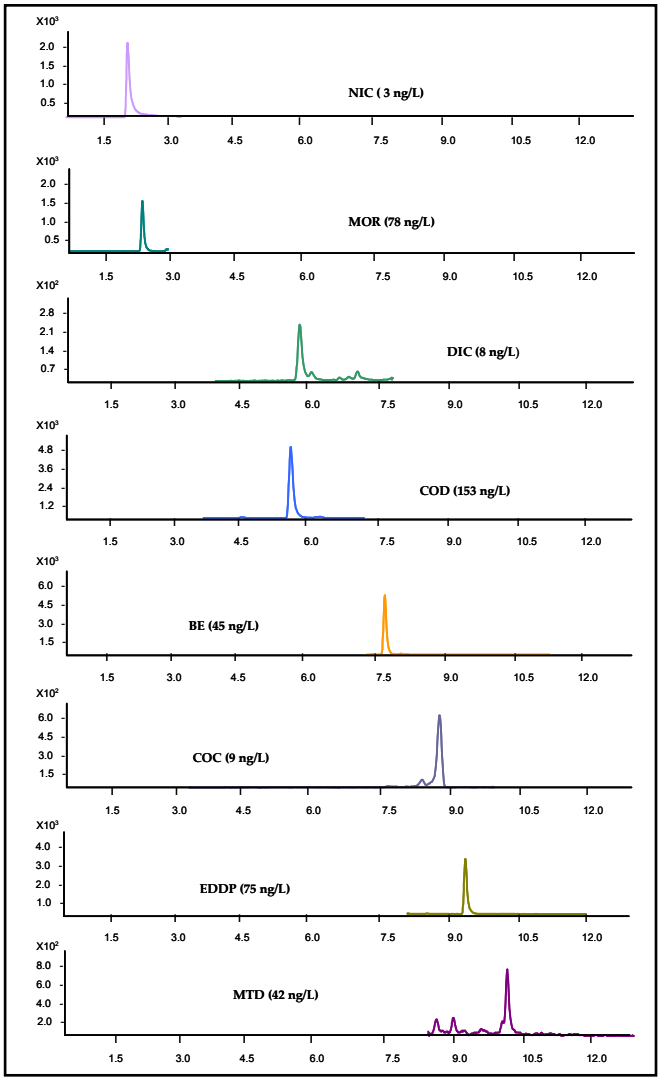

Figure 2. SRM chromatograms of a sample from 2TR.

Although the study was not so long in time, results showed an increase of drugs consumption at weekends and after a weekend of local festivities.
Concentration in the primary samples showed high and constant values of benzoylecgonine (1169-3336 ng/L) and peaks of cocaine (2486 ng/L) and nicotine (1105 ng/L). Results showed that the highest removal rate was achieved during the secondary treatment, but some compounds underwent a considerable reduction after tertiary treatment. Raw samples taken at the intake of the DWTP showed concentrations of 19-35 ng/L of benzoylecgonine and <LOQs of nicotine, while treated waters showed values <LOQ of benzoylecgonine.

Acknowledgments

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3.4.2. Discussion of results

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This study has demonstrated the presence of drugs of abuse in environmental samples, confirming the type of results that have been reported in several other studies in recent years. As mentioned previously, there is a new research trend focused on the study of the presence of drugs of abuse in wastewaters as an estimation of drug consumption levels in the population. This trend inspired the current study, which was based upon monitoring samples from a sewage treatment plant over the course of a week. Surface waters from the Ebro River were also monitored at the location of an intake site for a drinking water treatment plant (DWTP), along with post-treatment samples from the plant. The DWTP studied supplies drinking water to 65 municipalities and 33 industries, a fact that underlies the importance of the type of study performed.

We developed and applied an SPE/LC-MS-MS method with a QqQ analyzer. As recently reviewed by van Nuijs et al. [1], SPE of drugs of abuse has been performed using different sorbents. In our study, the performance of four sorbents was initially tested (Oasis HLB, Strata-X, Oasis MCX, and Strata-XC). Strata-X was removed from the study because of the low recovery levels observed for some of the targeted drugs, such as codeine, dihidrocodeine, acetylmorphine, nicotine, and morphine. When Oasis HLB and Oasis MCX were compared, Oasis MCX showed the best recovery levels for codeine and its metabolite dihidrocodeine. With Strata-XC, incorrectly-shaped peaks were obtained for morphine and its metabolite acetylmorphine. Therefore, Oasis MCX was ultimately chosen for extraction of all of the selected compounds. Oasis MCX is a strong cation-exchange type of sorbent, capable of retaining cationic analytes with dual retention modes: ion exchange and reverse phase. It allows for selective retention of analytes by ionic exchange, whereas interfering compounds are retained by reverse phase and can be eluted with a solvent for effective washing. However, since this study's analytes were found to be retained by both ionic exchange and reverse phase, the washing step applied could not be as exhaustive.

The use of a recently commercialized chromatographic column with fused-core particle technology (2.7 μm) provided good results, as expected, and successful chromatographic separation was obtained in 13 minutes. This particle size was chosen in order to increase speed while avoiding the high pressures associated with sub-2 μm particle columns. The back-pressure recorded with a flow rate of 0.4 mL/min did not exceed 65 bars, a pressure level comparable to those present in standard LC instruments. In the existing literature there are few publications involving Fused-core columns for determining drugs of abuse in waters, which is an obvious consequence of the novelty of this type of chromatographic equipment. However, Fontanals et al. [2] have reported on their application of this fused-core technology in hydrophilic interaction chromatography (HILIC), to successfully separate 8 polar PhACs (including 6 drugs of abuse) from waters in less than 13 min.

As mentioned in previous sections, mass spectrometry analysis, particularly when using electrospray ionisation (ESI), is affected by ion suppression. A surrogate had already been used in one of this Thesis's previous studies to help minimize this effect (section 3.1.2), and in the present study, deuterated surrogate compounds were used to minimize ion suppression. Specifically, three surrogates were chosen, each belonging to a different family of compounds. In this manner, the compounds from each chromatographic window were successfully quantified through use of this surrogate. Unlike in some other studies [3,4], where almost every targeted compound had its own deuterated surrogate to aid in quantification, our results were quantified with this selection of only three surrogates. However, good results were nevertheless obtained.

Certain compounds showed relatively high LOQs in the primary wastewater samples, calculated as the lowest point of the calibration curves. For example, cocaine showed a LOQ of 125 ng/L, and its metabolite benzoylecgonine showed a LOQ of 250 ng/L. These high LOQs were attributed to the high levels of cocaine and benzoylecgonine found in the sample used as a blank. For positive finding confirmation, the peak area ratios between confirmation (SRM_2) and quantification (SRM_1) transitions of the samples and comparing them with mean SRM_2/SRM_1 obtained from the spiked standards in the same sequence of samples. To consider a finding as positive, the experimental SRM_2/SRM_1 ratios should fit with those of reference standards with a maximum deviation ranging from 20 to 50% depending on the relative ion intensities. In our study, only THC-COOH showed a tolerance above the limits for some 1TR samples. Therefore these values were not considered as positive findings.

The week-long monitoring of the STP revealed some variation in levels between weekday (Tuesday to Friday) and weekend (Saturday to Monday) samples. Testing for a range of drugs and their metabolites resulted in statistically significant peaks for benzoylecgonine (3335.8 ng/L), cocaine (2486.3 ng/L), and nicotine (1105.6 ng/L) in the weekend samples. These results are in agreement with others found in the literature. For example, Bijlsma et al. [3] reported values for benzoylecgonine that varied from 4.14 μg/L (weekday) to 10.5 μg/L (weekend). Other compounds, such as THC-COOH, showed pointedly elevated weekday values.

After secondary treatment, our study revealed that some of these compounds were removed, while most of the analytes were found in lower concentrations. For example, a maximum value of only 46.7 ng/L for benzoylecgonine was recorded after secondary treatment. However, morphine (138 ng/L) and codeine (189.3 ng/L) showed similar concentrations after primary and secondary treatments.

When wastewater from tertiary treatment was analyzed, still lower concentrations were generally found. However, some compounds were not removed with increasing efficiency, with similar concentrations still found after secondary and tertiary treatment. Some compounds still present after this last treatment included

nicotine (20.5 ng/L), morphine (53.4 ng/L), benzoylecgonine (42.2 ng/L), and cocaine (9.9 ng/L).

Samples taken from the intake area of a drinking water treatment plant (DWTP) were also analyzed in this study. These were surface water samples from the Ebro River, which showed concentrations of benzoylecgonine of 19–35 ng/L. These results agree with those of Vázquez-Roig et al. [5], who found that cocaine and benzoylecgonine were ubiquitous in surface water samples from the L'Albufera Natural Park in Valencia, Spain. Their highest benzoylecgonine value was 78.71 ng/L, recorded for a sampling point related to direct spillage of residual waters from a nearby recreational area.

In regard to the removal efficiency of the DWTP for these compounds, benzoylecgonine was only found at values less than its LOQ in treated waters. These results were in agreement with those of Huerta-Fontela et al. [6], who found benzoylecgonine at mean concentrations of 45 ng/L in treated waters.

The results from this study and the others discussed, show that drugs of abuse can be considered along with other PhACs as ubiquitous contaminants, discharged into the environment from STPs. Since they also may pose risks to human health and the environment, they must also be the subject of future research regarding their detection, measurement, and elimination.

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3.5. Monitoring study of pharmaceutically active compounds using solid-phase extraction and liquid chromatography-tandem mass spectrometry

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The individual studies discussed in this Doctoral Thesis (sections 3.1 and 3.2), showed the importance of studying PhACs, because of their presence in wastewaters. Despite the environmental impacts created through the widespread use of these compounds, little information has been previously available regarding their presence in waters from our local geographical area in the South of Catalonia. Therefore, the monitoring study discussed in this section was developed, and included as a part of a project supported by the Spanish Ministry of Science and Technology (CTM 2008-06847-C02-01/TECNO). In this study, samples were taken from two STPs, in the cities of Reus and Tarragona, every two months for a period of one year. A total of 33 PhACs were monitored, which included analgesics, anti-inflammatories, β-blockers, anti-epileptics, stimulants, anti-ulcers, lipid regulators, macrolides, sulfonamides, and hormones and their conjugates.

For this study, the three previously developed methods discussed in sections 3.1 and 3.2 of this Thesis were applied. However, the methods from section 3.1 were updated to take advantage of the more recent local availability of the instrument of mass spectrometry in tandem (MS-MS) using a triple quadrupole analyzer. Therefore, the previously developed methods could be improved upon and applied to the monitoring samples included in this section´s research design.

While this study maintained the application of three independent methods to determine all the PhACs, other authors have applied multi-residue methods [1-3]. For example, Gros et al. [2] reported on their determination of 73 compounds in environmental waters using a single analysis. However, a notable disadvantage in the application of multi-residue methods is based on the significantly different physico-chemical characteristics possessed by the various analytes, which make it difficult to find the most suitable chromatographic conditions for application to all of them. Therefore, a satisfactory simultaneous analysis can only be based upon a compromise, which will favor the determination of some analytes over others.

Clearly, the use of MS-MS with a triple quadrupole analyzer has improved upon the limitations encountered when using a single quadrupole type, and it was thus expected that some compounds that had occurred only at values lower than their LODs or LOQs in the previous studies from section 3.2, could now in this study be detected and quantified. Also, improvement was expected in the confirmation of some compounds, which in the LC-MS methods were confirmed with by the use of two ions. As mentioned in the introductory chapter of this Thesis, with LC-MS-MS, and working with MRM, four identification points can be obtained, consisting of two MRM transitions, the ratio between them, and the retention time.

The results obtained in this study were for samples collected in 2007 and 2008, and these results were compared with those from the samples analyzed in the earlier 2004-2005 studies.

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These results are being published in an article accepted for publication in the journal *Water, Air & Soil Pollution*, currently in press.

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3.5.1. Presence of pharmaceuticals and hormones in waters from sewage treatment plants

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PRESENCE OF PHARMACEUTICALS AND HORMONES IN WATERS FROM SEWAGE TREATMENT PLANTS

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Abstract

This paper describes the presence of 33 pharmaceuticals and hormones in waters from two sewage treatment plants (STPs) situated in Catalonia, in northeastern Spain. The target compounds were: one psychoactive stimulant, one antiepileptic, four analgesics and non-steroidal anti-inflammatories, two lipid regulators, two anti-ulcer agents, nine antibiotics (sulfonamides and macrolides), two beta-blockers, two metabolites and eleven hormones (free and conjugates). The determination was performed using liquid chromatography-tandem mass spectrometry (LC-(ESI)MS-MS) after enrichment by solid-phase extraction (SPE) with Oasis HLB sorbent. Most of the pharmaceuticals were found in both influent and effluent samples from the two STPs. The most frequently detected were caffeine, acetaminophen, carbamazepine, diclofenac, ibuprofen, naproxen, sulfamethoxazole, sulfapyridine, sulfathiazole, ranitidine, omeprazole, estrone 3-sulphate and estradiol 17-glucuronide. Specifically, the highest concentrations found in influents were: 19850 ng/L (acetaminophen), 9945 ng/L (caffeine), 4215 ng/L (ibuprofen), 5695 ng/L (sulfamethoxazole) and 5140 ng/L (sulfathiazole). Most of the pharmaceuticals present in influent waters were found in effluents at lower concentrations. The highest concentrations in effluents were 970 ng/L (caffeine), 670 ng/L (sulfamethoxazole), 510 ng/L (bezafibrate), and 1032 ng/L (diclofenac).

Keywords: *hormones, pharmaceuticals, LC-MS-MS, SPE, wastewaters*

INTRODUCTION

Pharmaceutical compounds are considered emerging organic contaminants, which have recently attracted much attention from the scientific community. These compounds are not completely removed in wastewater treatment and they might prove to be an issue in the quality of water supplies. Not only pharmaceuticals but also some of their metabolites have been detected at low levels in sewage treatment plant effluents, river water, and also drinking water (Hernando et al., 2006; Radjenovic et al., 2008; Vanderford and Snyder, 2006; Zhao and Metcalfe, 2008). In recent years, there has been an increasing number of studies on the occurrence of pharmaceuticals in environmental waters (Gros et al., 2006; Hernandez et al., 2007). These contributions help to raise awareness of the fact that sewage treatment plants (STPs) need improved treatments. Recently, relative success has been achieved in the use of advanced technologies such as granular activated carbon, membrane technology, ozonation and ultraviolet radiation in the removal of some pharmaceuticals from water (Benítez et al., 2009; Benner and Ternes, 2009; Bolong et al., 2009). For example, oxidation with ozone (O_3) is usually applied in water treatment to remove micropollutants. Compounds with C=C groups, activated aromatic structures or heteroatoms such as nitrogen or sulfur are vulnerable to ozonation, while compounds with amide structures are ozonation resistant (Nakada et al., 2007; Zwiener, 2007). In contrast, with conventional treatment, ozonation represents a quantitative removal for certain compounds, such as diclofenac, carbamazepine and sulfamethoxazole (Zwiener, 2007).

Despite all the efforts made, pharmaceuticals are not completely removed in STPs and some of them enter the environment, either unaltered or in their main metabolite form. Studies conducted in various countries around the world (Farré et al., 2008; Khalaf et al., 2009; Wu et al., 2008) showed that non-steroidal anti-inflammatory drugs (NSAIDs) are the most frequently found in wastewaters at low μg/L, since in some countries most of them are available without the need for a prescription. Lacey et al. (2008) found salicylic acid (metabolite of acetylsalicylic acid) and ibuprofen to be the most abundant compounds of a total of 20 pharmaceuticals with maximum levels of 9.17 μg/L and 3.20 μg/L, respectively, in influent wastewaters in Ireland. A median as high as 360 ng/L of ibuprofen has been found in river water, in China (Peng et al., 2008). Not only do NSAIDs cause environmental concern, but also antibiotics are viewed as emerging environmental contaminants because of their potential adverse effects on ecosystems and human health.

Antibiotics cause ecological damage when they are released into water communities because of the potential development of antibiotic-resistant bacteria (Baquero et al., 2008;

Hernandez et al., 2007). Macrolides, sulfonamides and trimethoprim are the most frequently prescribed antibiotics for use by humans, and some of them (e.g. sulfamethazine, sulfathiazole, trimethoprim, etc.) are also used in veterinary medicine (Managaki et al., 2007). Some of these antibiotics (erythromycin, roxithromycin and tylosin) have been detected in drinking water at values lower than 5 ng/L (Ye and Weinberg, 2007).

Another group of emerging contaminants which causes concern is that of hormones, which have been identified as the greatest contributors to the endocrine-disrupting compounds (EDCs) in waters (Auriol et al., 2006; Farré et al., 2006). Particular attention has been given to the natural hormones estradiol and estrone, as well as to the synthetic estrogen 17α-ethinylestradiol, which is for being strongly estrogenic (Viglino et al., 2008; Xu et al., 2006). All of these have actually been detected in surface and ground waters at very low levels (Benotti et al., 2008; Vulliet et al., 2008). For example, estrone was detected at levels between 0.3 ng/L and 3.5 ng/L in these kinds of samples in France (Vulliet et al., 2008). When such contaminants reach the rivers, they enter the food chain and may become a potential risk to water consumed after the drinking treatment process. Some reports have identified this risk, and for example, sulfamethoxazole (< 0.25 ng/L; Vanderford and Snyder, 2006) and estriol (11.6 ng/L; Kuster et al., 2008) have been found in tap water.

Requirements of low limits of detection and the complexity of the matrix impose the use of highly sensitive and selective methods for trace determination of these compounds. Recent developments in LC-MS-MS such as triple quadrupole (QqQ), quadrupole time-of-flight (QTOF) or quadrupole ion trap (QIT) analyzers have meant that LC-MS-MS has become the most preferred technique by which to determine pharmaceuticals and hormones in multiresidue analysis (Coetsier et al., 2007). For example, Kuster et al. (2008) determined a group of hormones in river water, using LC-QqQ and they found maximum values of estriol of 11.60 ng/L.

Working with a hybrid triple quadrupole-linear ion trap mass spectrometer (QqLIT), Ding et al. (2009) achieved LODs of 2-6 ng/L in a method for the determination of macrolide antibiotics. The same detector was used by Vulliet et al. (2008) to determine estrogenic compounds in the 0.3-3.5 ng/L range in surface and ground waters, and Gros et al. (2009) determined 73 pharmaceuticals in surface and waste waters. However, triple quadrupole (QqQ) working with multiple reaction monitoring (MRM) is the most widely used technique by which to achieve low LODs in target analysis (Managaki et al., 2007).

A list of 33 target compounds (pharmaceuticals and some metabolites, hormones and conjugates) is given whereby a classification of the substances under investigation.

Pharmaceuticals were selected so as to provide a representative selection of the pharmaceuticals most used in human and veterinarian medicine and to reflect their environmental impact. Some of the selected compounds in this study were identified by the UK Environment Agency for priority investigation as a potential risk to the aquatic environment (Hilton and Thomas, 2003). The main aim of this research was to determine and evaluate the presence of these 33 compounds in two sewage water treatment plants in Catalonia to ascertain what is going to end up in rivers. The occurrence of these contaminants was studied using 7 sample sets in 2007-2008 in both STPs.

MATERIALS AND METHODS

Chemicals

The standards were purchased from Sigma-Aldrich Chemie (Steinheim, Germany): Acetaminophen (ACE), caffeine (CAF), metoprolol tartrate salt (MET), propranolol hydrochloride (PRO), salicylic acid (SAL), carbamazepine (CBZ), clofibric acid (CLO), naproxen (NPX), bezafibrate (BZF), diclofenac (DCF), ibuprofen (IBU), sulfamethoxazole (SMX), sulfadiazine (SDZ), sulfamethazine (SMZ), sulfapyridine (SPY), sulfathiazole (STZ), tylosin (TYL), erythromycin (ERY), roxithromycin (ROX), omeprazole (OMP), ranitidine (RAN), trimethoprim (TRI), estrone (E1), estrone 3-sulphate (E1-3S), estrone 3-glucoronide (E1-3G), 17β-estradiol (E2), estradiol 3-sulphate (E2-3S), 17-estradiol 17-acetate (E2-17A), estradiol 17-glucuronide (E2-17G), 17α-ethinylestradiol (EE2), 17 α -estradiol (α-E2), estriol (E3) and diethylstilbestrol (DSB). Individual standard solutions of 1000 mg/l were prepared in MeOH for all the compounds, except BZF and NPX, which were prepared in MeOH:H_2O (50:50). A mixed standard solution of 10 mg/L was prepared weekly in H_2O. All solutions were stored at 4 ºC except the hormones, which were stored at -5 ºC.
Kitasamycin hydrate (KIT), the surrogate standard (100 mg/L in acetonitrile), was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Ultra-pure water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, EEUU), acetonitrile and methanol were HPLC grade from SDS (Peypin, France), and nitrogen was from Carburos Metálicos (Tarragona, Spain). Chlorhydric acid (HCl), sodium hydroxide (NaOH) and acetic acid from Prolabo (Bois, France) were used to adjust the pH of the sample and the mobile phase.

Site and sample locations

Twenty four composite (24-h) samples from Catalonia (NE Spain), sampled from March 2007 to March 2008, were analyzed in this study. The wastewater samples were collected from the influent and effluent of two domestic sewage treatment plants (STPs) in two cities located on the coast, each with approximately 140000 inhabitants. The STPs use activated

sludge biological treatment and the biological oxygen demand (BOD_5) for influent water is about 400 mg/L. The average flow-rate was 30000 m^3/day for STP1 and 16000 m^3/day for STP2.
The study was divided into 7 sample sets but one of these 7 (November 07) was only carried out in STP2 because it was not available in STP1. All samples were collected by using pre-cleaned amber glass bottles acidified to pH 3 (HCl) and stored at -20 ºC until analysis.

Sample preparation

Solid phase extraction was used to preconcentrate the samples and Oasis HLB cartridges (500 mg, Waters, Milford, Massachussets, USA) were selected for all the compounds. Extraction methods were adapted from the previously optimized methods (Pedrouzo et al., 2008; Pedrouzo et al., 2009; Pedrouzo et al., 2007) and are explained briefly below (Method A, B, and C). In all cases, the sorbent was sequentially conditioned using 5 ml of MeOH and 2 ml of Milli-Q water and was then allowed to dry for 10 min in a vacuum. Sample volumes of 100 and 250 ml were extracted for the influent and effluent of the STP, respectively. Prior to the extraction procedure, samples were filtered using a 0.45-μm nylon filter (Whatman, Maidstone, UK) using a manifold (Teknokroma, Barcelona, Spain) and a pump as a vacuum source. They were passed through the cartridge at a flow-rate of 10-15 ml/min. The eluate was reduced to dryness under a stream of nitrogen and redissolved with 1 mL of water with a different proportion of MeOH in each case. The residue was filtered through a 0.45-μm nylon membrane (Scharlab, Barcelona, Spain) and 50 μL of this solution was injected into the chromatographic system.
In Method A, the samples were adjusted to pH 3 with HCl. The retained analytes were eluted using 6 ml of MeOH. The eluate was redissolved, after evaporation, with 1 ml of MeOH:H_2O (50:50). In Method B, the samples were adjusted to pH 7 with NaOH and spiked with the surrogate (kitasamycin). The retained analytes were eluted from the cartridge with 2 mL of MeOH and 2 mL of MeOH adjusted to basic conditions (NH_4OH 0.1%, pH 9). The eluate was reconstituted, after evaporation, with 1 mL of H_2O containing 1% MeOH. In Method C, the samples were adjusted to pH 7 with NaOH. The analytes retained were eluted using 5% ACN in 5 mL of MeOH. Extracts were redissolved, after evaporation, with 1 mL of MeOH:H_2O (80:20). All the compounds included in each method are summarized in Table 1.

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Table 1. Classification and LC-MS-MS conditions for all the studied compounds.

Group	Compound	Method	MRM Transitions	Cone Voltage (V)	Collision energy (V)	Ion mode
Analgesics/ Antiinflammmatories	Acetaminophen	A	152→93	100	25	PI
			152→110		15	
	Naproxen	A	229→140	50	30	NI
			229→185		5	
	Salicylic acid	A	137→93	75	15	NI
			137→65		30	
	Diclofenac	A	294→214	75	20	NI
			294→250		10	
	Ibuprofen	A	205→161	75	5	PI
Psychoative estimulants	Caffeine	A	195→110	125	25	PI
			195→138		15	
Anti-epileptics	Carbamazepine	A	237→179	150	35	PI
			237→193		35	
Lipid regulators	Clofibric acid	A	213→85	50	5	NI
			213→127		10	
	Bezafibrate	A	360→154	100	30	NI
			360→274		15	
B-blockers	Metoprolol	A	268→116	125	15	PI
			268→159		20	
	Propranolol	A	260→116	125	15	PI
			260→183		15	
Antibiotics	Trimethoprim	B	291→145	125	35	PI
			291→249		20	
Sulfonamide antibiotics	Sulfamethoxazole	B	254→108	100	20	PI
			254→156		10	
	Sulfapyridine	B	250→108	75	25	PI
			250→156		15	
	Sulfadiazine	B	251→108	75	25	PI
			251→156		10	
	Sulfamethazine	B	279→124	100	25	PI
			279→186		15	
	Sulfathiazole	B	256→108	75	20	PI
			256→156		10	
Macrolide antibiotics	Tylosin	B	916→174	150	35	PI
			916→772		30	
	Erythromycin	B	735→158	150	30	PI
			735→576		30	
	Roxithromycin	B	838→679	175	15	PI
			838→158		30	
Antiulcer agents	Omeprazole	B	346→151	75	15	PI
			346→198		10	
	Ranitidine	B	315→176	100	15	PI
			315→270		10	

Table 1. (Continued).

Group	Compound	Method	MRM Transitions	Cone Voltage (V)	Collision energy (V)	Ion mode
Hormones	E1	C	269→145	150	45	NI
			269→143		55	
	E2	C	271→145	60	30	NI
			271→183		45	
	EE2	C	295→145	60	45	NI
			295→159		30	
	α-E2	C	271→145	60	30	NI
			271→183		45	
	E3	C	287→171	150	45	NI
			287→145		45	
	DSB	C	267→222	150	30	NI
			267→237		55	
Conjugate hormones	E1-3S	C	349→269	150	30	NI
			349→145		55	
	E1-3G	C	445→269	150	45	NI
			445→113		20	
	E2-3S	C	351→271	150	30	NI
			351→145		55	
	E2-17A	C	313→253	100	30	NI
			313→145		55	
	E2-17G	C	447→271	150	30	NI
			447→113		20	

Description of the methods A: Pedrouzo et al. (2007), B: Pedrouzo et al. (2008), C: Pedrouzo et al. (2009)

Chromatographic analysis

The LC-MS-MS chromatographic analysis was conducted on an Agilent 1200 series LC and a 6400 series triple quadrupole mass spectrometer from Agilent Technologies (Waldbronn, Germany) using an electrospray (ESI) interface, an automatic injector, a degasser, a quaternary pump and a column oven.

The chromatographic column was a Kromasil 100 C_{18} (25.0 x 0.46 cm) with a 5 μm particle size (Teknokroma, Barcelona, Spain), and the volume injected was 50 μL. A binary mobile phase with a gradient elution was used. For all the analysis, solvent A was Milli-Q water with acetic acid (pH 3) and solvent B was acetonitrile. Nitrogen was used as the nebulizing gas in both negative and positive ionization modes. Optimization of parameters and fragment ions for the different methods (Method A, B, and C) was performed by flow injection analysis (FIA) of each compound. These are compiled in Table 1.

Method A

The chromatographic conditions were described in Pedrouzo et al. (2007), where a quadrupole was used as detection system. In this study, we determined these compounds by LC-MS-MS and lower concentrations and confirmation data were obtained.

Since both positive and negative ionizations were needed, two different gradient elution programs were used. The gradient for negative ionization mode was: 55% B, which was increased to 60% in 6 min, kept constant for 3 min, increased to 80% in 12 min, to 100% in 2 min, kept constant for 3 min and then decreased to 55% for 3 min. The gradient for the positive ionization mode was: 18% B, which increased to 20% in 4 min, to 55% in 5 min, to 60% in 6 min, to 100% in 5 min, remained constant for 3 min, and finally decreased to 18% in 2 min. Temperature was kept at 30 ºC, the mobile phase flow-rate was 1 mL/min and the injection volume was 50 μL. For ESI in negative mode: nebuliser pressure (30 psi), drying gas flow-rate temperature (350 ºC), capillary voltage (3500 V) and drying gas flow-rate (12 L/min). Values of the cone voltage and the collision energy for each MRM transition are specified in Table 1. It is noteworthy that two MRM transitions were achieved for all the compounds except for ibuprofen (Table 1). Although the identity of ibuprofen could not be confirmed because the MS-MS spectrum contained one diagnostic ion only, we decided not to exclude this compound from the study because its high consumption, thus, concentrations are given.

Method B

The chromatographic separation was described in Pedrouzo et al. (2008). The gradient was 10% B, which was increased to 15% in 10 min, to 26% in 5 min and to 60% in 4 min; then it was increased to 100% in 4 min, kept constant for 2 min and finally returned to 10% B in 2 min. All the compounds eluted within 22 min.
We used a LC-MS-MS, as described above. Parameters for ESI positive ionization were nebulizer pressure (40 psi), drying gas flow-rate (12 L/min), drying gas temperature (350 ºC), and capillary voltage (4000 V). As can be seen in Table 1, values of cone voltage were between 75 V and 175 V and collision energy were between 10 V and 35 V. Product ions used for monitoring were selected on the basis of the MS-MS spectra.
The optimization of the MS-MS parameters was performed by injecting each compound. Parameters for ESI positive ionization were nebuliser pressure (40 psi), drying gas Tª (300 ºC), capillary voltage (3000 V) and drying gas flow-rate (13 L/min).

Method C

A LC-MS-MS method for simultaneous determination of hormones and their conjugates was applied as described in detail by Pedrouzo et al. (2009). A binary mobile phase with a gradient elution was used to optimize the extraction conditions. Solvent A was Milli-Q water with acetic acid (pH=2.8) and solvent B was acetonitrile. The gradient was performed as follows: 10% B, kept constant for 10 min, increased to 40% B for 5 min, to 60% for 10 min, to 100% B for 5 min, and then decreased to 10% B for 2 min.

The system was re-equilibrated for 3 min between injections. MS-MS analysis was performed in the negative ionization mode with an optimized spray potential of 3000 V, a nebulizer of 45 psi and a source temperature of 350 ºC and 12 L/min of drying gas flow-rate. Nitrogen was used as the collision, nebulizing and desolvation gas.

RESULTS AND DISCUSSION

Quantification and method validation

Recovery values for effluent matrix are given in Table 2 and as it was expected, they were similar to the recoveries found in the previous studies. Also, comparing with the LC-MS method, the MS-MS method allowed us to calculate the recovery for acetaminophen (Method A) and it was 45% (RSD<19%), in influent waters. However, because of the low recoveries of salicylic acid (metabolite of acetylsalicylic acid), bezafibrate and E2-17A, the concentrations were not determined in influents. Influent samples were quantified with a Milli-Q calibration curve and recoveries for each compound were applied.

Calibration curves of the entire method were used for quantification of effluent samples. Only compounds from Method B required a surrogate standard to quantify the samples. The use of a surrogate (kitasamycin) was justified by the high level of ion suppression of the matrices. The linear range for effluent samples is shown in Table 2.

Limits of quantification (LOQs) were calculated as the lowest point of the calibration curve, this being higher in influents (10-200 ng/L) than in effluents (3-100 ng/L) because of the different sample volume. Limits of detection (LODs) were set as the concentration at which the s/n ratio was 3 (Table 2).

Occurrence of pharmaceuticals and hormones in STPs

As Table 3 and Table 4 show, several families of pharmaceuticals and hormones were found in the sewage waters from the two STPs.

Confirmation criteria applied to the target compounds were the following: presence of two characteristic MRM transitions at the correct retention time and the correct ratio between product ions (except for ibuprofen with only one MRM transition). Caffeine is one of the most wides-pread pharmaceuticals and values of 950-9945 ng/L were determined in influent from both STPs, as noted herein and in agreement with the literature (Nikolaou et al., 2007). Due to the high level found in influents, the samples were diluted to be quantified. Even higher concen-trations of caffeine had been found in previous papers (Pedrouzo et al., 2007), with maximum values of 40 μg/L in influents. This concurred with Huerta-Fontela et al. (2008), who reported, also in Spain, concentrations of caffeine up to 209 μg/L and 24 μg/L in 40 studied influent samples.

Table 2. Validation data for all the compounds.

Compound	Recoveries[a] (%R)		LODs (ng/L)		Linear range (ng/L)
	Influent	Effluent	Influent	Effluent	Effluent
Acetaminophen	45	49	5	2	5-500
Caffeine	55	80	8	3	5-1000
Metoprolol	75	75	8	3	5-400
Propranolol	50	85	8	3	5-1000
Carbamazepine	35	65	5	2	5-1000
Salicylic acid	-	40	5	2	5-400
Bezafibrate	-	67	3	1	3-2000
Naproxen	50	55	15	7	10-2000
Clofibric acid	37	57	5	2	5-2000
Diclofenac	38	52	8	2	5-2000
Ibuprofen	52	89	15	7	10-2000
Ranitidine	65	68	8	2	5-2000
Trimethoprim	58	63	15	10	25-2000
Sulfamethazine	62	72	15	10	25-2000
Sulfathiazole	51	58	15	10	25-2000
Sulfapyridine	57	62	15	10	25-1000
Sulfadiazine	43	47	15	5	10-2000
Tylosin	61	55	25	15	25-2000
Erythromycin	42	44	25	15	25-2000
Roxithromycin	52	48	10	2	5-2000
Omeprazole	20	22	10	2	5-1000
Sulfamethoxazole	25	29	20	10	25-2000
E1	60	51	50	25	100-1500
E2	53	61	100	70	100-1500
EE2	37	52	100	70	100-1500
α-E2	30	49	100	70	100-1500
E3	56	59	50	25	100-1500
DSB	58	76	50	25	100-1500
E1-3S	57	74	15	10	30-1500
E1-3G	66	65	50	25	100-1500
E2-3S	57	78	15	10	30-1500
E2-17A	-	25	100	70	100-1500
E2-17G	23	28	50	25	100-1500

%RSD<19, (n=3)
"-" recoveries<10%

Table 3. Concentrations (ng/L) found in STP1, RSD<15% (n=3).

Compound	March 07		May 07		July 07		Set 07		Jan 08		March 08	
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Acetaminophen	9792	9	11250	-	8652	-	12247	30	7665	<LOQ	19640	<LOQ
Caffeine	9945	19	6540	882	4655	580	5154	672	8296	34	8750	375
Metoprolol	<LOQ	-	<LOQ	-	-	-	73	-	<LOQ	-	-	-
Propranolol	34	-	20	-	-	-	<LOQ	-	-	150	<LOQ	10
Carbamazepine	90	76	252	110	250	8	322	52	105	48	308	52
Salicylic acid	n.m	13	n.m	68	n.m	182	n.m	9	n.m	134	n.m	15
Bezafibrate	n.m	385	n.m	302	n.m	289	n.m	225	n.m	220	n.m	283
Naproxen	235	15	1160	241	360	155	639	38	220	39	685	30
Diclofenac	145	243	374	427	133	1032	415	303	270	146	520	368
Ibuprofen	532	30	3615	190	2215	<LOQ	1175	137	172	<LOQ	3062	81
Ranitidine	85	406	870	120	320	-	320	12	<LOQ	60	504	70
Sulfathiazole	1870	161	965	-	1326	205	1340	<LOQ	3165	-	<LOQ	-
Sulfapyridine	1445	538	73	165	<LOQ	-	310	<LOQ	730	152	380	-
Sulfamethoxazole	3210	670	2490	110	1140	<LOQ	1195	45	2985	420	453	44
E2-17G	376	-	<LOQ	-	615	-	415	-	316	-	<LOQ	-
E1-3S	<LOQ	35	320	<LOQ	<LOQ	<LOQ	-	255	<LOQ	-	<LOQ	-
E2-17A	n.m	<LOQ	n.m	-	n.m	176	n.m	166	n.m	-	n.m	85

n.m: not measured
- <LOD

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Table 4. Concentrations (ng/L) found in STP2, RSD<17% (n=3).

Compound	March 07		May 07		July 07		Set 07		Nov 07		Jan 08		March 08	
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Acetaminophen	19250	-	17655	-	13300	<LOQ	19150	-	19850	-	11470	<LOQ	9150	-
Caffeine	3435	10	3365	970	1360	120	3790	<LOQ	7514	205	950	243	6380	170
Metoprolol	15	<LOQ	40	-	30	-	<LOQ	-	<LOQ	-	-	-	<LOQ	-
Propranolol	22	-	20	-	<LOQ	<LOQ	<LOQ	15	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Carbamazepine	55	95	405	170	22	<LOQ	20	60	205	70	44	65	85	40
Salicylic acid	n.m	125	n.m	9	n.m	115	n.m	50	n.m	80	n.m	20	-	200
Bezafibrate	n.m	510	n.m	340	n.m	100	n.m	200	n.m	260	n.m	140	n.m	300
Naproxen	1300	395	605	260	330	30	2280	85	1100	80	550	220	840	691
Diclofenac	315	474	275	505	160	260	410	130	350	870	<LOQ	160	265	520
Ibuprofen	1405	325	4215	495	1100	<LOQ	3580	-	1795	15	660	120	3140	955
Ranitidine	20	-	650	-	270	-	730	-	2175	<LOQ	<LOQ	<LOQ	350	355
Sulfametazine	55	-	-	-	50	<LOQ	<LOQ	<LOQ	<LOQ	-	-	<LOQ	<LOQ	-
Sulfathiazole	975	-	30	-	5140	<LOQ	870	<LOQ	560	-	157	-	2235	-
Sulfapyridine	-	-	165	-	<LOQ	<LOQ	360	-	2475	<LOQ	<LOQ	-	195	<LOQ
Sulfamethoxazole	1715	515	1935	<LOQ	2665	410	3395	<LOQ	5695	75	1030	<LOQ	3460	<LOQ
E2-17G	-	-	<LOQ	-	225	<LOQ	-	<LOQ	-	-	220	<LOQ	-	-
E1-3S	160	52	640	<LOQ	143	<LOQ	-	<LOQ	520	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
17α-EE2	154	-	-	-	-	-	<LOQ	-	-	-	-	-	<LOQ	-
E2-17A	n.m	-	n.m	-	n.m	135	n.m	<LOQ	n.m	-	n.m	<LOQ	n.m	-

n.m: not measured
- <LOD

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This level of caffeine is not only due to the amount present in pharmaceuticals, but also to its presence in some products such as coffee, tea, chocolate or sports drinks, among others. This widespread use makes that caffeine was even detected in river waters. Three catalan rivers showed values between 106-305 ng/L (Pedrouzo et al., 2007) and Conley et al. (2008) found levels between 23.2-38.8 ng/L in the Tennessee River with frequency of 100% in the samples analyzed. The widespread use of non-prescription consumption analgesics was corroborated because of the high levels of acetaminophen found in the influents under study, with a maximum concentration of 19850 ng/L (sample diluted to be quantified). Although influent samples could not be quantified, values between 9 and 200 ng/L of salicylic acid were found in effluents. These results concurs with Spongberg and Witter (2008), who found levels between 0.4 μg/L and 8 μg/L in influents and also Lacey et al. (2008) found levels of 0.3-9.1 μg/L of this compound in influents and <0.1 μg/L in effluents.

Nonsteroidal anti-inflammatory drugs (NSAIDs) were also found in waters. As can be seen in Tables 3 and 4, the highest levels correspond to ibuprofen with a maximum concentration of 4215 ng/L in influents (STP2, May 2007). However, these high concentrations decreased to a maximum of 955 ng/L, in effluent samples. An example of an effluent chromatogram is shown in Figure 1.

Another NSAID, such as naproxen was found at levels between 220-1160 ng/L in influents from STP1 and 330-2280 ng/L in influents from STP2. These values for NSAIDs agree with the results reported by Farré et al. (2008b). High levels of antibiotics were also found in both STPs. This is the case of sulfamethoxazole, which was found in both influents at values between 453 ng/L and 5695 ng/L. This concurs with several studies (Díaz-Cruz et al., 2008; Göbel et al., 2004), which declared sulfamethoxazole to be one of the highest pharmaceutical found in environmental waters. For example, Göbel et al. (2004) found maximum concentrations of 641 ng/L in influent wastewaters. Karthikeyan and Meyer (2006) found trimethoprim and sulfamethoxazole in wastewaters with values of frequency of 70% and 80%, respectively. In our study, only one influent sample revealed concentrations of trimethoprim (505 ng/L) and data for this pharmaceutical was excluded from Tables 3 and 4. However, Vanderford and Snyder (2006) reported levels of trimethoprim of 1140 ng/L in influent and lower than 0.50 ng/L in effluent. Antibiotics like tylosin, erythromycin and roxithromycin belong to the family of macrolides and they are used in both veterinary and human medicine. Our study only showed some values <LOQ and they are also not shown in Tables 3 and 4. However, these antibiotics were found in influent waters by Göbel et al. (2004) at values between 44 ng/L and 67 ng/L (erythromycin) and 22 ng/L-30 ng/L (roxithromycin).

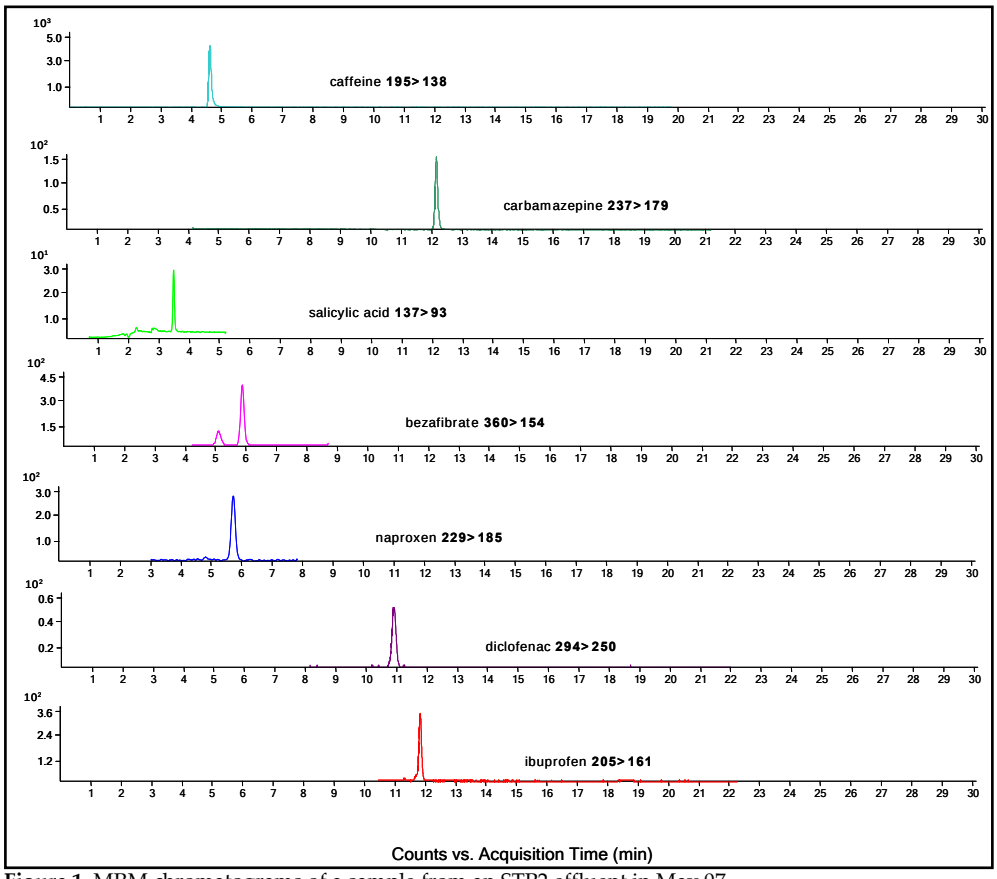

Figure 1. MRM chromatograms of a sample from an STP2 effluent in May 07.

Pharmaceuticals showed similar behaviour in both STPs, but certain compounds showed some differences. Such is the case of sulfamethazine, an antibiotic used in veterinary medicine. Possibly because of the proximity of some veterinary industries to the area, STP2 showed concentrations of 55 ng/L (influents) and <LOQ (effluents), whereas STP1 only showed values <LOQ in some influent samples (data not shown in Table 3).

Carbamazepine, an anti-epileptic, was found in all the influent waters at concentrations between 20 and 405 ng/L. This compound showed low removal and sometimes, similar values could be seen in effluents and influents (Tables 3 and 4). β-blockers are used particularly for the management of cardiac arrhythmias. In this study, two β-blockers (metoprolol and propranolol) were found at concentrations up to 73 ng/L and 34 ng/L respectively, in influent waters, but higher values were found by Gros et al. (2008). In their study of eight β-blockers, values of metoprolol (2408 ng/L) and propranolol (117 ng/L) in influents were reduced to 375 ng/L and 104 ng/L, respectively, in effluents. Bezafibrate, a blood lipid

regulator, which had been detected in levels up to μg/L in sewage water by Heberer et al. (2002), showed maximum concentrations of 510 ng/L in effluents. Ranitidine and omeprazole are pharmaceuticals used as anti-ulcer agents. In our study, ranitidine was present in all the influent samples with values between 20 and 2175 ng/L. However, the presence of omeprazole was not so evident and only values <LOQ were found in a few samples (data not shown). Other compounds with concentrations lower than LOQ were clofibric acid (metabolite of clofibrate) and some hormones (E2, E3, E2-3S, E1, E1-3G and DSB) and consequently, these results were omitted from the Tables 3 and 4. Hormones are removed via degradation process, and although a considerable amount is adsorbed to sludge (Nieto et al., 2008), some of the compounds remain still soluble in the effluents. Although we could not determine some of these hormones, because of the LODs, 17β-estradiol was detected in influents in a range of concentration between 49.4 ng/L and 93.3 ng/L (Hernando et al., 2004), and Farré et al. (2006) reported concentrations of estrone between 7.9 ng/L (effluents) and 13.9 ng/L, (influent) waters.

When Viglino et al. (2008) studied the occurrence of synthetic and natural hormones in influents, they found the highest values for estriol (125 ng/L) and 17α-ethinylestradiol (243 ng/L), while estrone was detected at trace levels. Lower values of the synthetic estrogen 17α-ethinylestradiol were found by Farré et al. (2006) at levels below LOD (<2 ng/L), in influent waters. In our study, only a few influent samples showed 17α-ethinylestradiol, with a maximum concentration of 154 ng/L, and it was not detected in effluent samples (Table 4).

It is known that hormones are typically excreted by mammals in the conjugate, inactive form (glucoronide or sulphate conjugates) (Kvanli et al., 2008; Servos et al., 2005). Although glucoronides were not found in the earlier paper by our group (Pedrouzo et al., 2009), in this study our LOQs were lower and both STPs showed concentrations in similar concentrations of estradiol 17-glucoronide (<LOQ-615 ng/L) in influents. In effluents, only STP2 showed values of estradiol 17-glucoronide below LOQ, whereas in STP1 it was not detected. Other conjugates yielding positive results, as was reported in our previous paper (Pedrouzo et al., 2009), are sulfate conjugates with the highest concentration of estrone 3-sulphate (640 ng/L in influents and 52 ng/L in effluents). Some samples showed values <LOQ of estradiol 3-sulphate in influents and effluents (data not shown in Tables 3 and 4). However, the conjugate E2-17A showed maximum values of 176 ng/L in effluents, whereas its presence was not measured in influents because of the low recovery.

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Removal of pharmaceuticals and hormones in STPs

Although not as many samples were studied as to conclude a complete study of removals of pharmaceuticals in STPs, we could estimate the behaviour of these pharmaceuticals in STPs (influents and effluents). As can be seen in Figure 2, in general effluent samples showed values of pharmaceuticals at lower concentration than influent samples. There are several compounds, such as diclofenac and carbamazepine which are not well removed in the STPs studied, which concurs with the literature (Gagnoc and Lajeunesse, 2008; Tixier et al., 2003; Zhou et al., 2009).

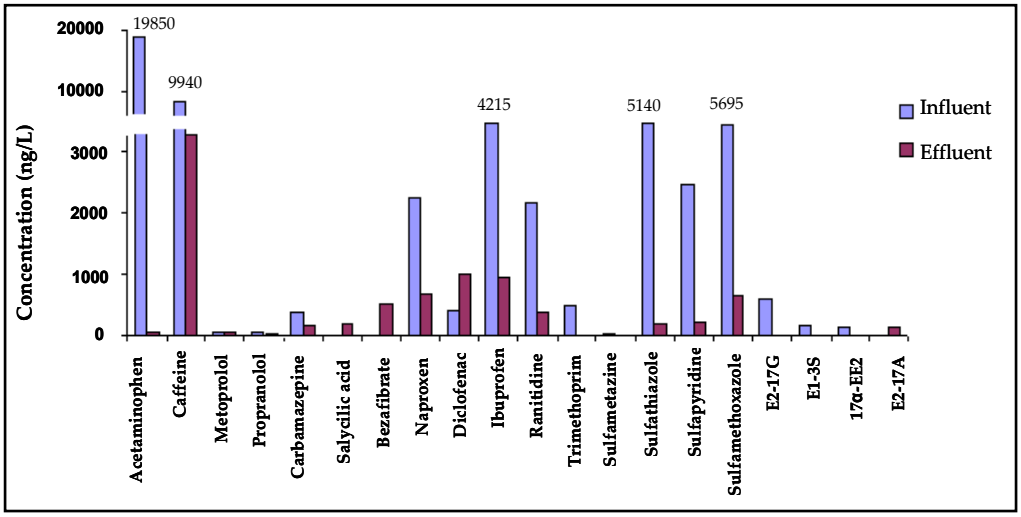

Figure 2. Maximum concentrations found in influents and effluents from both STPs.

In our study, diclofenac was present in the effluent samples at maximum levels of 1032 ng/L (Table 3). Comparing the results from influent and effluent, we assumed an incomplete removal for this compound. We conclude that some sulfonamides are well removed with values between 70 and 85% for sulfamethoxazole, sulfapyridine and sulfathiazole. The highest removals were acetaminophen (almost 100%), caffeine (>85%), and ibuprofen (>80%), in agreement with Gagnon and Lajeneusse (2008) who reported a removal of ibuprofen higher than 95%. Also most of our samples revealed an efficient removal of ranitidine (>90%) in STP1 and slightly lower in STP2. But, in some cases higher concentrations were found in effluent than in influent, in agreement with Farré et al. (2008a).

As regards sulfamethoxazole, significant lower removals were found in the literature (24%) (Ternes et al., 2007). Not only did these authors study sulfamethoxazole, but

also its metabolite (N^4-acetyl-sulfamethoxazole) to avoid the underestimation of removal rates. However, as it was previously mentioned, this study showed higher removals for this compound.

The present study shows the removals in STPs with conventional treatments. However, removal rates published in the literature vary greatly depending on the treatment facilities and the nature of the contaminant. For example, Gebhardt and Schroeder (2007) stated that the oxidation methods using O_3/UV and H_2O_2/UV successfully led to the complete elimination of persistent and hardly eliminable pharmaceutical compounds such as carbamazepine, diclofenac and clofibric acid. The study reported by Nakada et al. (2007) demonstrated that the efficiencies of removal during advanced treatment (filtration and ozonation) of secondary effluent gave efficient removals of sulfonamides, macrolides and trimethoprim (> 90%), the ozonation being the greatest contributor. After prechlorination and sand filtration, estrone and estrone-3-sulfate were found to have been completely removed from waters (Rodríguez-Mozaz et al., 2004).

Although there is an increasing focus on the use of advanced post-treatment units in STPs (e.g, ozone, advanced oxidation process) to enhance the removals of these contaminants, most conventional STPs do not have these high-cost treatment processes. The incomplete removal of these compounds in STPs is an evident environmental problem with adverse impact in surface waters that represents a matter of concern.

CONCLUSIONS

Several groups of pharmaceuticals and metabolites (caffeine, analgesics, anti-inflammatories, lipid regulators, ß-blockers, antiepileptics, antibiotics) and some hormones (free and conjugates) were the subject of the monitoring program carried out in two sewage treatment plants in Catalonia. Three different methods were successfully applied for the appropriate determination of pharmaceuticals at low levels in the samples. In influent waters, the dominant compounds that were found were acetaminophen (7665 ng/L-19850 ng/L) and caffeine (950 ng/L-9945 ng/L). Residual amounts of these compounds were observed in effluent samples, but only caffeine showed the highest values with a maximum of 970 ng/L. The estimation of the removal in these STPs was also discussed. The lowest removals were seen for some pharmaceuticals, such as carbamazepine and diclofenac. However, acetaminophen was removed almost at 100%, ibuprofen > 80%, caffeine over 85%, sulfathiazole, sulfapyridine and sulfamethoxazole between 70 and 85%.

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3.5.2. Discussion of results

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As described above, this monitoring study applied three previously developed methods, but with all of these updated to use LC-MS-MS with a QqQ analyzer. Confirmation of the presence of a given compound was based upon monitoring of two transitions between precursor and product ions, working in multiple reaction monitoring (MRM) mode. Other criteria used were the MRM ratio (calculated as the ratio between the abundances measured during both transitions) and the retention time. In combination these represented four identification points (IPs) according to the specifications of EU Commission Decision 2002/657/EC [1]. As mentioned, the confirmation power of the compounds with respect to the previous LC-MS studies was thus increased, since earlier only two ions were monitored. However, one exception in this study was ibuprofen, for which only one transition was used. As expected, these MRM methods also decreased the limits of detection (LODs) and quantification (LOQs). Table 3.2 presents a comparison of the LOQs obtained with both analyzers.

Table 3.1. Limits of quantification obtained with single quadrupole and triple quadrupole analyzers.

Compound	LOQs (Q) (ng/L)	LOQs (QqQ) (ng/L)
Acetaminophen	20	5
Caffeine	10	5
Metoprolol	10	5
Propranolol	10	5
Carbamazepine	10	5
Salycilic acid	20	5
Bezafibrate	20	3
Naproxen	100	10
Diclofenac	40	5
Clofibric acid	20	5
Ibuprofen	20	10
Ranitidine	40	5
Omeprazole	200	5
Trimethoprim	200	25
Sulfamethoxazole	40	25
Sulfapyridine	40	25
Sulfadiazine	40	10
Sulfamethazine	40	25
Sulfathiazole	200	25
Tylosin	200	25
Roxithromycin	200	5
Erythromycin	200	25

Values measured using 250 mL effluent samples.

As it was expected, lower LOQs could be obtained by using the QqQ analyzer. Therefore, more compounds were quantified in the monitoring study than in the study using a single quadrupole. As discussed in section 3.2, hormones were not very sensitive to the LC-MS-MS analysis, and LOQs for these compounds remained higher than expected. However, with the MRM transitions it was possible to quantify acetaminophen, which could not previously be quantified in influent samples using the LC-MS method, because the SIM chromatogram peak had a shape that was difficult to integrate. However, in this study, acetaminophen was one of the analytes found to have the highest concentrations in influents from both STPs, with a maximum level of 19,850 ng/L. Other compounds found to have high concentration levels in influents included ibuprofen (4,215 ng/L), caffeine (9,940 ng/L), and sulfamethoxazole (5695 ng/L). In effluent samples, these high levels found in influents decreased. As such, maximum values were measured of 30 ng/L (acetaminophen), 955 ng/L (ibuprofen), 970 ng/L (caffeine), and 670 ng/L (sulfamethoxazole). All these values were in general agreement with the results of the previous studies detailed in sections 3.1 and 3.2. However, comparison of the sulfonamide compounds does also reveal some notable differences between the studies. For example, although sulfathiazole was not detected at all in the previous study, a maximum level of 5,140 ng/L was recorded in this monitoring study. However, none of the more recent results for sulfamethazine were as high as the 1,820 ng/L recorded in the study from section 3.2.

When comparing these results with those reported in the literature for other effluent studies, it was found that other reported values for ibuprofen ranged from 106.7 ng/L [2] up to a maximum value of 20 μg/L [3]. For the sulfonamide antibiotics, however, this study's results for sulfamethoxazole showed the highest concentration (5695 ng/L), exceeding the values found in the literature. For example, García-Galán et al. [4] found sulfamethoxazole in four STPs along the Ebro River basin at levels between 12.4 ng/L and 302 ng/L.

Some compounds, including salycilic acid, bezafibrate, and the hormone E2-17A, could not be quantified in influent samples because of their low levels of SPE recovery. However, these compounds have been reported in influents by other researchers. For example, Gracia-Lor et al. [5], using SPE/UHPLC-MS-MS analysis, reported a maximum influent level of salycilic acid of 276.7 μg/L and effluent level of 23.61 μg/L.

This monitoring study also provided useful data regarding the efficiency of both STPs in removing PhACs. There are several existing studies involving removal of these compounds by STPs, which have established that some PhACs are not efficiently removed by conventional activated-sludge treatments [6]. However, the results of the study reported here were mixed, showing good removal efficiency for some compounds including caffeine, acetaminophen, ibuprofen, and some sulfonamides, while low levels of removal were recorded for carbamazepine and

diclofenac. These results were in agreement with those of Zhou et al. [7], who reported a removal efficiency range of 43−54% for carbamazepine. As discussed above, when some compounds are not efficiently removed by STPs, they can enter surface waters with the discharge of effluents. This has been reported for rivers in Madrid in the case of carbamazepine, where a maximum level of 1160 ng/L has been reported.

While most northern European STPs include tertiary wastewater treatments, in Spain these are being introduced slowly. Therefore, only primary and secondary treatments are performed, with the second based upon conventional activated sludge methods. Consequently, there is a need to assess the limitations of current wastewater treatment processes, as well as to evaluate which operational parameters most strongly affect the efficiency of pharmaceutical removal [6]. As Rosal et al. [8] have reported, for example, ozonation seems to be an efficient treatment for removal of some PhACs, including carbamazepine, diclofenac, and β-blockers.

Although the presence of most of these PhACs is not the subject of current legislation, some of them are included on the recently created third list of contaminant candidates in drinking water issued in October, 2009 by the EPA. Therefore, it is important that removal treatments be evaluated in order to prevent or minimize the discharge of PhACs into the environment. Meanwhile, ongoing in-depth studies of the presence of PhACs in the aquatic environment are needed, as well as further assessment of the environmental risks these compounds may create.

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CHAPTER 4. CONCLUSIONS

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The major conclusions that can be drawn from the studies presented in this Doctoral Thesis can be summarized as follows:

1. The methods developed in this Doctoral Thesis to determine pharmaceutically active compounds and personal care products attained, for most of the compounds, limits of quantification low enough for their determination in wastewaters, surface waters, and even drinking waters.

2. Solid-phase extraction (SPE) is a useful technique for extraction of PPCPs from waters, with high recoveries in most cases. The choice of sorbent is critical, because sorbent performance is strongly influenced by the characteristics of the compounds targeted. Here, two commercially available polymeric sorbents, Oasis HLB and Bond Elut Plexa, were used to extract a wide range of PPCPs. Also, the new mixed-mode sorbent Oasis MCX was successfully applied to the extraction of cationic compounds including a group of drugs of abuse.

3. Stir bar sorptive extraction (SBSE) using the commercially available sorbent polydimethylsiloxane (PDMS) was also found to be a useful technique for extraction of some personal care products. However, PDMS showed clear limitations for use with the most polar compounds, and therefore this sorbent was only applied to extraction of personal care products with the lowest levels of polarity.

4. Ultra-high-performance liquid chromatography (UHPLC) is also a promising technique, which allows for good separation of analytes with a short analysis time. UHPLC was successfully applied to separate 11 personal care product compounds in 9 minutes.

5. A new fused-core particle column was successfully applied to separate a group of drugs of abuse and their metabolites. The main advantage of this column was the fact that it permitted a short analysis time while using a conventional liquid chromatography instrument.

6. Although mass spectrometry with a single quadrupole analyzer allowed the determination of some pharmaceutically active compounds in water samples, tandem mass spectrometry with a triple quadrupole analyzer provided more identification points and lower limits of detection. Therefore LC-MS-MS is concluded to be the most preferable technique for determination of PPCPs in environmental waters.

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7. Various groups of pharmaceutically active compounds (pharmaceuticals, hormones, and drugs of abuse) were determined in a range of water sample types. The highest concentrations found in influent wastewaters were recorded for acetaminophen (19,640 ng/L), caffeine (9945 ng/L), ibuprofen (4,215 ng/L), sulfamethoxazole (5,695 ng/L), cocaine (2,486 ng/L), and benzoylecgonine (3,336 ng/L). Lower values were found in effluents for most of the compounds, and river samples showed maximum levels of caffeine (305 ng/L) and sulfamethoxazole (50 ng/L).

8. The high levels of personal care products recorded reflect the widespread use of compounds such as the preservatives methylparaben (5,613 ng/L) and propylparaben (1945 ng/L), which showed the highest maximum values in influents. Ethylparaben showed the highest maximum value in effluents (48 ng/L), whereas the UV filter BP-3 showed the highest level recorded in river waters (28 ng/L).

9. The efficiency of three sewage treatment plants in Catalonia, (Reus, Tarragona, and Vila-Seca), in terms of removal of PPCPs, was evaluated for the first time. Results confirmed that the studied STPs did not show an efficient removal of PPCPs. The compounds showing the lowest levels of removal were carbamazepine, diclofenac, codeine, morphine, and EDDP. The highest levels of removal were recorded for acetaminophen, ibuprofen, most UV filters, parabens, cocaine, benzoylecgonine, and nicotine.

10. Because of increasing recent interest in the presence of drugs of abuse in environmental waters, the efficiency of a drinking water treatment plant (DWTP) in terms of removal of these compounds was evaluated. Results showed efficient removal levels for all of the compounds studied, and only benzoylecgonine was found at levels above its LOQ in the treated water.

11. The studies presented in this Doctoral Thesis have further demonstrated the presence of pharmaceutically active compounds and personal care products in environmental waters. Because many of the environmental risks associated with these compounds remain poorly understood, further studies of their toxicity in the aquatic ecosystem are essential for development of appropriate new legislation.

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Appendix

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Appendix I. Abbreviations used in this Doctoral Thesis.

ACN	Acetonitrile
AHTN	Tonalide
AOPs	Advanced oxidation processes
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure ionization
BFRs	Brominated flame retardants
BSFTA	Bis(trimethylsilyl)-trifluoroacetamide
CE	Capillary electrophoresis
cGMP	Cyclic guanosine monophosphate
GC	Gas chromatography
D5	Decamethylcyclopentasiloxane
DAD	Diode array detection
DBPs	Disinfection by-products
DCM	Dichloromethane
DEET	N,N-diethyl-m-toluamide
DEHP	Di(2-ethylhexyl)phatalate
DLLME	Dispersive liquid-liquid microextraction
DWTP	Drinking water treatment plant
ECD	Electron capture detector
EDCs	Endocrine disrupting compounds
EDDP	1,5-dimethyl-3,3-diphenylpyrrolidine
EI	Electron impact
EME	Ecgonine methylester
EPA	Environmental protection agency
ESI	Electrospray ionization
EU	European Union
FD	Fluorescence detection
GAC	Granulated activated carbon
HF-LPME	Hollow-fiber membrane liquid-phase microextraction
HHCB	Galaxolide
HILIC	Hydrophilic interaction chromatography
ICM	Iodinated X-ray contrast media
ILODs	Instrumental limits of detection
IL-SDME	Ionic liquid-based single-drop microextraction
IP	Identification point
IT	Ion trap
LC	Liquid chromatography
LD	Liquid desorption
LIT	Linear ion trap
LLME	Liquid-liquid microextraction

LOD	Limit of detection
LOEL	Lowest observable effect level
LOQ	Limit of quantification
LPM	Liquid-phase microextraction
LSD	Lysergic acid diethylamide
LVI	Large volume injection
4-MBC	3-(4-methyl)benzylidene
MBR	Membrane bioreactor
MDMA	3,4-Methylenedioxymethamphetamine
ME	Membrane extraction
MeOH	Methanol
MIPs	Molecular imprinted polymers
MISPE	Molecularly imprinted solid-phase extraction
MRM	Monitoring reaction mode
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
NCI	Negative chemical ionization
NIST	Institute of Standards and Testing
NP	Normal phase
NSAIDs	Non steroideal anti-inflammatories drugs
OC	Octocrylene
PCPs	Personal care products
PDMS	Polydimethylsiloxane
PhACs	Pharmaceutically active compounds
PPCPs	Pharmaceuticals and personal care products
PTV	Programmed temperature-vaporising
PVP-DVB	Polyvinylpyrrolidone-divinylbenzene
Q	Quadrupole
QqQ	Triple quadrupole
RP	Reversed phase
SBSE	Stir bar sorptive extraction
SIM	Selected ion monitoring
μ-SLME	Microscale supported liquid membrane extraction
SPE	Solid-phase extraction
SPMD	Semi-permeable membrane devices
SPME	Solid-phase microextraction
St-DVB	Styrene-divinilbenzene
STP	Sewage treatment plant
TCC	Triclocarban
THC	Tetrahydrocannabinol
THC-COOH	11-*nor*-9-carboxy-Δ^9-tetrahydrocannabinol

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TCS	Triclosan
TD	Thermal desorption
TOF	Time-of-flight
UHPLC	Ultra-high-performance liquid chromatography
UNODC	United Nations Office of Drugs and Crime
USAEME	Ultrasound-assisted emulsification microextraction
UV	Ultraviolet
VPD-DVB	Vinilpyrrolidone-divinylbenzene

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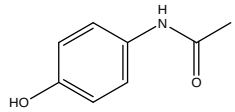
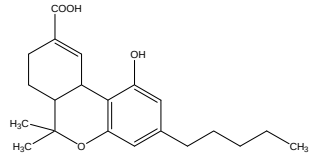
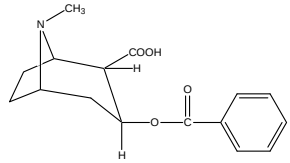
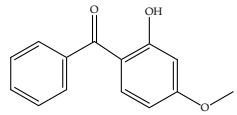
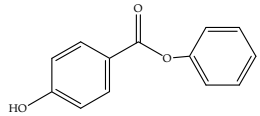
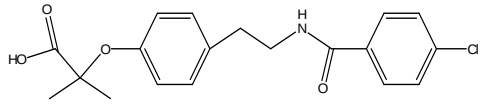
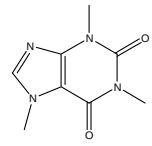
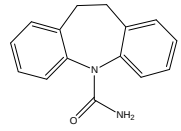
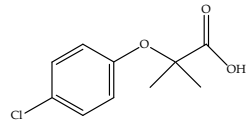
Appendix II. Name and structure of the compounds determined in the present Doctoral Thesis.

Name	Structure
Acetaminophen (analgesic)	
6-acetylmorphine (metabolite of morphine)	
Benzoylecgonine (metabolite of cocaine)	
Benzophenone-3 (UV filter)	
Benzylparaben (preservative)	
Bezafibrate (lipid regulator)	
Caffeine (stimulant)	
Carbamazepine (psychiatric drug)	
Clofibric acid (metabolite of clofibrate)	

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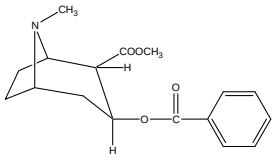
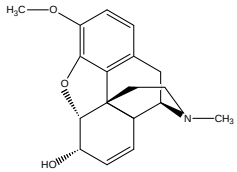
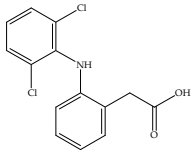
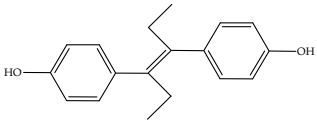
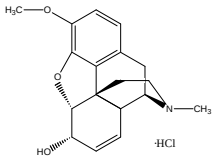
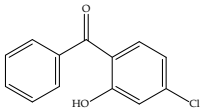
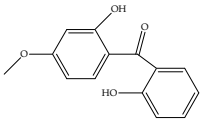
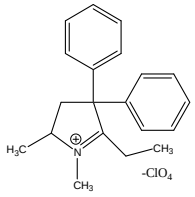
Name	Structure
Cocaine (drug of abuse)	
Codeine (drug of abuse)	
Diclofenac (anti-inflammatory)	
Diethylstilbestrol (hormone)	
Dihydrocodeine (metabolite of codeine)	
2,4-Dihydroxy benzophenone (UV filter)	
2,2′-Dihydroxy 4-methoxy-benzophenone (UV filter)	
EDDP (drug of abuse)	

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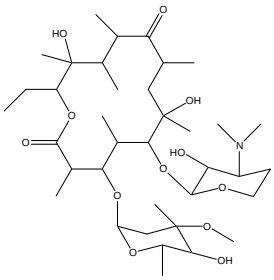
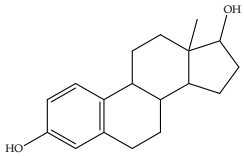
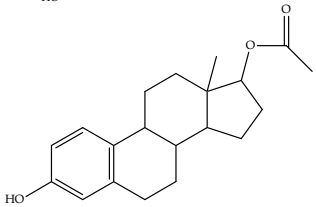
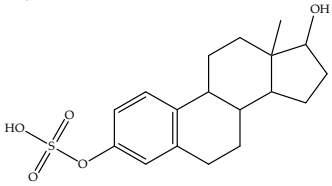
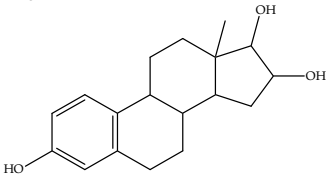
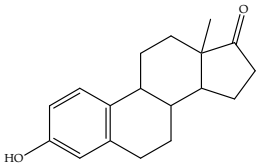
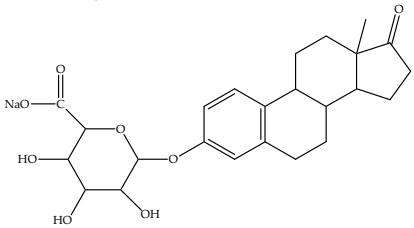
Name	Structure
Erythromycin (antibiotic)	
Estradiol (hormone)	
Estradiol 17-acetate (conjugated hormone)	
Estradiol 3-sulfate (conjugated hormone)	
Estriol (hormone)	
Estrone (hormone)	
Estrone 3-glucuronide (conjugated hormone)	

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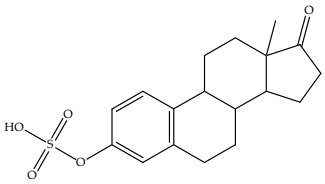
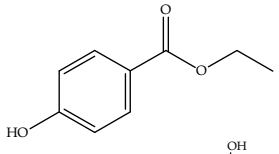
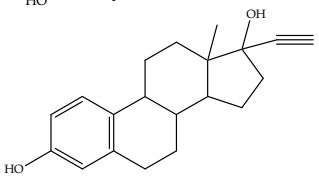
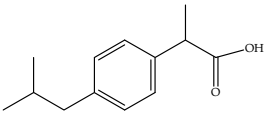
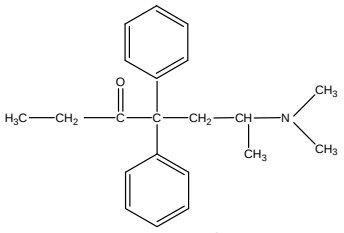
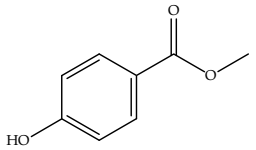
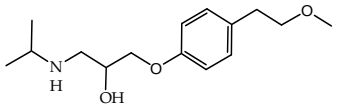
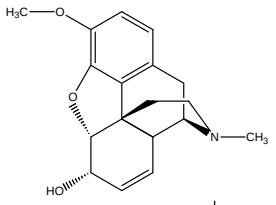
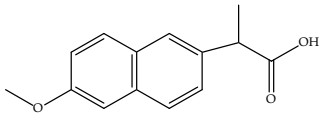
Name	Structure
Estrone 3-sulfate (conjugated hormone)	
Ethylparaben (preservative)	
17α-ethinyl estradiol (hormone)	
Ibuprofen (anti-inflammtory)	
Methadone (drug of abuse)	
Methylparaben (preservative)	
Metoprolol (β-blocker)	
Morphine (drug of abuse)	
Naproxen (anti-inflammatory)	

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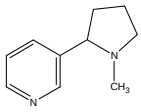
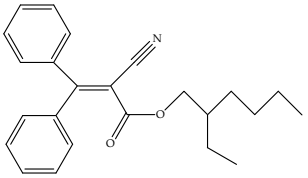
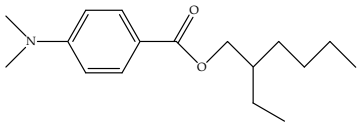
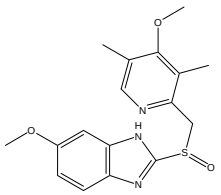
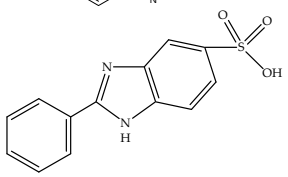
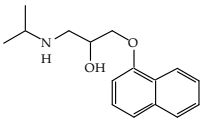
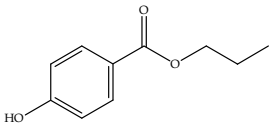
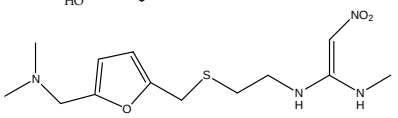
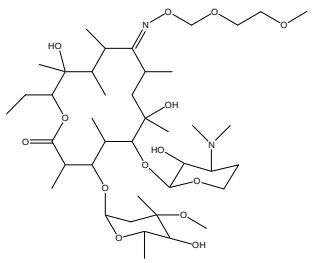
Name	Structure
Nicotine (noncontrolled drug)	
Octocrylene (UV filter)	
Octyldimethyl-p-aminobenzoic acid (UV filter)	
Omeprazole (stomach protector)	
2-Phenylbenzimidazole 5-sulfonic acid (UV filter)	
Propranolol (β-blocker)	
Propylparaben (preservative)	
Ranitidine (stomach protector)	
Roxithromycin (antibiotic)	

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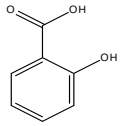
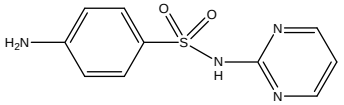
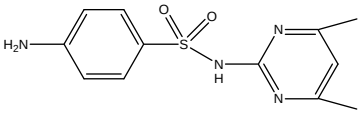
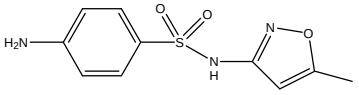
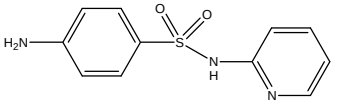
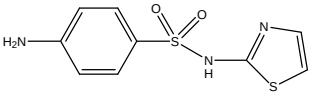
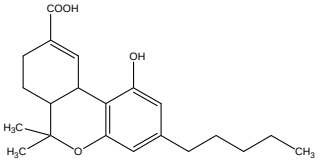
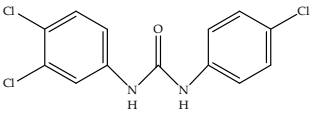
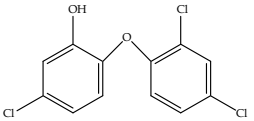
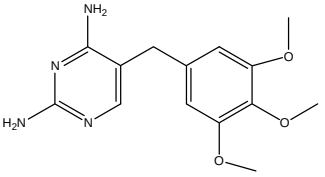
Name	Structure
Salicylic acid (metabolite of acetylsalicylic acid)	
Sulfadiazine (antibiotic)	
Sulfametazine (antibiotic)	
Sulfamethoxazole (antibiotic)	
Sulfapyridine (antibiotic)	
Sulfatiazole (antibiotic)	
THC-COOH (metabolite of THC)	
Triclocarban (antimicrobial)	
Triclosan (antimicrobial)	
Trimethoprim (antibiotic)	

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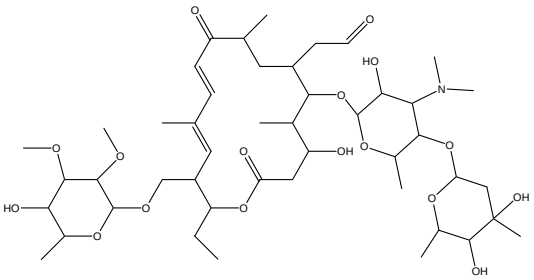
Name	Structure
Tylosin (antibiotic)	

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Appendix III. List of publications

M. Pedrouzo, S. Reverté, F. Borrull, E. Pocurull, R.M Marcé, *"Pharmaceutical determination in surface and wastewaters using high-performance liquid chromatography-(electrospray)-mass spectrometry.*" J. Sep. Sci., 30 (2007) 297-303 (section 3.1.1).

M. Pedrouzo, F. Borrull, R.M. Marcé, E. Pocurull, "*Simultaneous determination of macrolides, sulfonamides and other pharmaceuticals in water samples by solid-phase extraction and LC-(ESI)MS"* J. Sep. Sci., 31 (2008) 2182-2188 (section 3.1.2).

M. Pedrouzo, F. Borrull, E. Pocurull, R.M Marcé *"Estrogens and their conjugates: determination in water samples by solid-phase extraction and liquid chromatography-tandem mass spectrometry."* Talanta, 78 (2009) 1327-1331 (section 3.2.1).

M. Pedrouzo, F. Borrull, R.M. Marcé, E. Pocurull *"Ultra-high-performance liquid chromatography-tandem mass spectrometry for determining the presence of eleven personal care products in surface and wastewaters."* J. Chromatogr. A, 1216 (2009) 6994-7000 (section 3.3.1).

M. Pedrouzo, F. Borrull, R.M. Marcé, E. Pocurull, *"Stir bar sorptive extraction and ultra-high-performance liquid cromatography-tandem mass spectrometry for simultaneous determination of UV filters and antimicrobial agents in water samples."* Anal. Bioanal. Chem., 397 (2010) 2833-2839 (section 3.3.2).

M. Pedrouzo, F. Borrull, E. Pocurull, R.M. Marcé, *"Presence of pharmaceuticals and hormones in waters from sewage treatment plants."* Water Air & Soil Pollut., (2010) DOI:10.1007/s11270-010-0585-8 (section 3.5.1).

M. Pedrouzo, F. Borrull, E. Pocurull, R.M Marcé, *"Drugs of abuse and their metabolites in waste-, and surface waters by liquid chromatography-tandem mass spectrometry."* Anal. Bioanal. Chem., (2010) (submitted) (section 3.4.1).

M. Pedrouzo, F. Borrull, R.M. Marcé, E. Pocurull, *"Personal care products in environmental waters: analytical methods and occurrence."* Trends Anal. Chem., (2010) (submitted) (section 1.2.1).

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