UNIVERSITAT ROVIRA I VIRGILI

# EMERGING ORGANIC CONTAMINANTS IN SEWAGE SLUDGE

**Antonio Nieto Cebrián**

**ISBN: 978-84-693-6426-0**
**Dipòsit Legal: T-1628-2010**

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# EMERGING ORGANIC CONTAMINANTS IN SEWAGE SLUDGE

Antonio Nieto Cebrián

DOCTORAL THESIS

Supervised by

Dr. Rosa Maria Marcé and Dr. Eva Pocurull

Departament de Química Analítica i Química Orgànica

UNIVERSITAT ROVIRA I VIRGILI

Tarragona

2010

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**UNIVERSITAT ROVIRA I VIRGILI**
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Química Orgànica

La Dra. ROSA M. 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: "EMERGING ORGANIC CONTAMINANTS IN SEWAGE SLUDGE", presentada per ANTONIO NIETO CEBRIÁN, per optar al grau de Doctor per la Universitat Rovira i Virgili amb menció europea, 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'esmentat doctorand.

I, per a que consti, expedim aquest certificat a Tarragona, 5 de maig de 2010.

Dra. Rosa Maria Marcé

Dra. Eva Pocurull

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Ahora que llega el final de este camino, llega el día en que me gustaría agradecer a todas las personas que me han ayudado o me han mostrado su apoyo para acabar defendiendo esta Tesis Doctoral. Siempre había dicho que mis agradecimientos no iban a ser formales y que iba a escribirlos tal y como soy. Vamos a por ello.

Primero quiero dar las gracias al Prof. Francesc Borrull, sin la aceptación del cual en el *Grup de Cromatografia i Aplicacions mediambientals*, este momento no hubiese llegado nunca.

Después a mis dos "jefas": Dr. Rosa Maria Marcé y Dr. Eva Pocurull. Muchas gracias a las dos por haber confiado en mi desde el primer momento, haberme dado consejos en momentos difíciles, las ideas brillantes cuando no se extraían los compuestos, haberme apoyado desde la distancia cuando estaba realizando mi estancia fuera (¿os acordáis cuánto me costó?) y haber sido pacientes con mi inglés hasta el último momento. Siempre os recordaré y os aseguro que he aprendido mucho trabajando con las dos.
También dar las gracias por su amabilidad y apoyo durante estos años a las Dras. Marta Calull, Carme Aguilar, Núria Fontanals y Sandra Peñalver.

I would like to thank Professor Thomas P. Knepper of the University of Applied Sciences Fresenius of Idstein (Germany) for his kind attention during my stay in his laboratory and also his scientific advice and valuable help. I would also like to thank all the members of his research group for their guidance and friendship: Manuela, Marco, Tobias, Jutta and Heike.

También agradecer a todos los compañeros del grupo que han trabajado durante mi etapa en el mismo. Han tenido que aguantar los "buenos" olores de los lodos que he analizado y con los cuales he compartido muchos "tuppers" (espero no olvidarme de nadie). Las que ya estaban: Patri, Alba, Rosa y Vanesa, las que llegaron al mismo tiempo al grupo: Marta Palomo y Anna y los que llegaron cuando yo ya estaba aquí: Irene, Laura, Noelia, Jasper, Silvia, Igor, Fernando, Jordi, Vero, Dominika, Blanca y María. Muchas gracias a todos y un trocito de esta Tesis también es vuestra. También están las incorporaciones en el grupo mas recientes a las cuales les deseo suerte: Laura, Silvia y Paula.

Y como me voy a olvidar yo de mis dos amigos dentro del grupo: Toni y Marta (¿que pensabais que me había olvidado de vosotros?. Mal pensados, jeje). Neeeen, ya sabes que al principio no nos hablamos mucho (¡eras un rancio¡), pero después no nos callábamos (jeje): muchas gracias por todo. Contigo he aprendido muchas cosas, aunque no fueran de química, en "el laboratorio de los chicos". Y Marta, la de tiempo que hemos compartido preparando patrones, optimizando gradientes o peleándonos para ver fragmentos del espectro de masas, aunque ese trabajo no era divertido

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nosotros siempre encontramos la manera de divertirnos haciéndolo. Y también hemos visitado España y Europa gracias a los congresos (el mejor ha sido el de Dresden y las "jefas" saben por que y queda entre nosotros 4). A los dos, de verdad: MUCHAS GRACIAS.

A la gente que ha estado en el grupo haciendo una estancia como Daura (mi hermana postiza de Canarias), Houda (la doctora valenciana), Arlene (la chica callada escocesa) y Núria (la chica de Girona), también gracias a vosotras esta tesis ha salido como ha salido.

Y por último a toda la gente del departamento, que nos ayudan en todo lo que pueden, como Tere, Olga, Avelina, etc.

Y ahora la parte personal:
Pues tengo que darle las gracias a tanta gente, empezando por mi familia. A mis padres, que gracias a vuestro esfuerzo, trabajo y dedicación he podido estudiar y llegar hasta donde he llegado. A mi hermana, qué te puedo decir a tí que no sepas. Gracias por estar siempre apoyándome. A vosotros tres una cosa más OS QUIERO.
Al resto de la familia, imposible de nombrar a todos, aunque sí me quiero acordar de aquellos que por desgracia no pueden ver el final de este largo camino, aunque sé que desde donde estén todos ellos también celebrarán que haya acabado. A todos MUCHAS GRACIAS.
También tengo que darle las gracias a Esther, por todo tu apoyo durante estos años, por soportar mis enfados y frustraciones cuando no salían bien las cosas y por hacerme reír incluso cuando no había muchas ganas.
Y por último a los que me conocen de verdad: Xavi, Oriol, Dani, Cris, Marta, Silvia, Eli, Lluís y Maite que me habéis animado siempre y habeis estado siempre a mi lado (aunque sea desde la distancia). En especial a Cris y Marta que hemos pasado juntos ratos buenos y menos buenos pero siempre hemos tenido tiempo para hacer algún que otro café.
Espero no haberme dejado a nadie, porque quedaría un poco feo con dos páginas de agradecimientos. A todos vosotros:

MUCHAS GRACIAS

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# 1. INTRODUCTION

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In recent years, analytical chemistry has played an important role in obtaining information about the environment. One of the main objectives of it is to develop new analytical methods to determine contaminants in different kinds of samples at low levels. A growing interest in the determination of contaminants in water (influent and effluent wastewaters, ground water, tap water or surface water), solids (sediments, soils or sewage sludge) or atmospheric samples has been proved by the growing number of publications on this topic.

Nowadays, many substances have been identified as dangerous for human health and/or the environment, and their use has been banned or regulated by different legislation in order to reduce exposure to these kinds of substances. One of the groups of contaminants widely studied in environmental samples is persistent organic contaminants. Different studies have reported the presence of contaminants such as polycyclic aromatic hydrocarbons (PAHs) or polychlorinated byphenils (PCBs) in natural waters [1,2], wastewater [3], sediments [4,5], sewage sludge [6-8] and in the atmosphere [9], among others. Additionally, a new group of environmental contaminants of interest is emerging organic contaminants.

Emerging organic contaminants include several groups of organic compounds that have been widely distributed in the environment and have attracted tremendous attention over recent decades, although they are not still regulated. This group includes pharmaceuticals, personal care products, brominated flame retardants (BFRs) and antifungals, among others. This kind of contaminant has been found for the first time in surface water [10,11]. Some preliminary data was already published in 1976 in USA and in 1985 in England but systematic research started in the 90s when German scientists published results taken from monitoring studies measuring pharmaceuticals in local rivers [12]. In the literature, different papers showed concentration of low ng/L of different emerging organic contaminants in water samples from different countries such as Spain [13,14], The United Kingdom (UK) [15,16], Germany [17] and The United States of America (USA) [18-20], among others. In the last decade, a great deal of research effort has been devoted to the identification, occurrence, fate and effects of these compounds in samples from wastewater treatment plants. In the first studies, wastewater samples were studied. For instance, Ternes [12] found different pharmaceuticals such as propranolol, carbamazepine, bezafibrate and diclofenac, among others, in influent and effluent wastewater in Germany. It is important to determine these contaminants in effluent wastewater because this water is usually discharged into rivers and these contaminants may affect the aquatic environment. Recently, the presence of these kinds of contaminants has also been studied in sewage sludge which is a more complex matrix. The determination of these contaminants in sewage sludge is also important because the sludge can be used as agricultural manure and these contaminants could contaminate ground water and be introduced into the food chain. The contamination of ground water and the re-use of sewage sludge as

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manure affect agricultural soils as Kinney *et al.* [21] showed in their study, which reported concentrations of between 285 and 540 μg/Kg of indole (a personal care product) in soils treated with sewage sludge.

This thesis focuses on the determination of two groups of emerging organic contaminants: pharmaceuticals and personal care products. As example, we will explain how pharmaceuticals are introduced into the environment. PPCPs are widespread contaminants and enter the environment from a myriad of scattered points. Patients, in the case of pharmaceuticals for human use, and animals, in the case of veterinary pharmaceuticals, are the main sources of contamination. Pharmaceuticals can be excreted as the parent compound and/or metabolites in high percentages and continuously discharged into domestic wastewater. Unwanted or expired medications are often improperly disposed of directly into wastewater. Several pharmaceuticals can therefore reach sewage treatment plants (STPs) in substantial amounts and, if they escape degradation, can remain in surface water or in sediments for a long time (Figure 1).

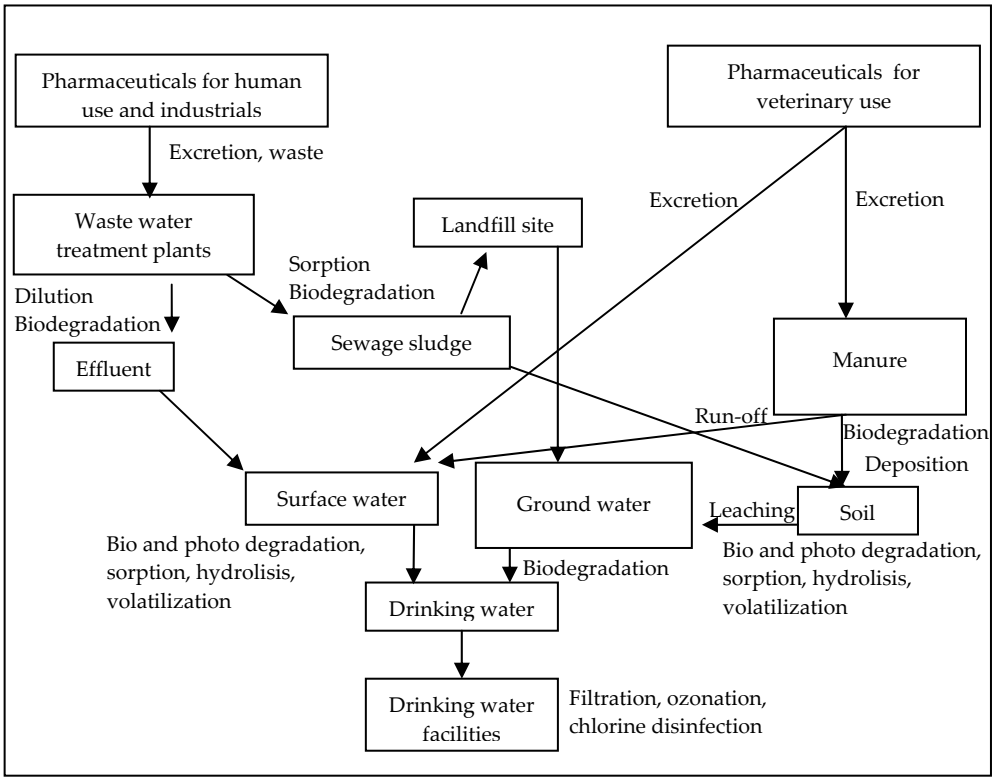


**Figure 1.** Distribution of pharmaceuticals and main transformation products in the environment.

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Due to the impact of these emerging organic contaminants in the environment, an interest in developing analytical methods to determine them in environmental samples has increased in recent years. These methods have to show both high sensitivity and selectivity because the contaminants are present at very low concentrations (levels of low ng/L in waters and low μg/Kg in sewage sludge). To achieve this objective, the methods used include an extraction and/or preconcentration step and a separation step using chromatographic techniques. In some cases, a clean-up step is needed before the chromatographic technique.

The extraction step is one of the most critical steps in the development of new analytical methods due to the complexity of the samples and the low concentration of these contaminants. Different extraction techniques have been used to extract emerging organic contaminants in solid samples. Firstly, classic techniques such as Soxhlet, ultrasonic extraction (USE) and shaking extraction have been used. Nowadays, new extraction techniques based on the use of low solvent consumption and using high temperatures and pressures have been applied. In this group, supercritical fluid extraction (SFE), microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) can be included. These techniques tend to replace the classic techniques. The extraction technique most used to extract emerging organic contaminants from water samples is solid phase extraction (SPE). In addition, other techniques such as solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) have been also used, although there is a limitation in the sorbents comercially available.

So far, some of the analytical methods reported in the literature for contaminant residue analysis are based on gas chromatography-mass spectrometry (GC-MS), which often requires derivatization of non-volatile compounds. In the last few decades, liquid chromatography-mass spectrometry (LC-MS) and LC-tandem MS have progressed impressively, both in terms of technological development and application. LC-MS-MS is indicated as the technique of choice to assay polar emerging organic contaminants and their metabolites, and is especially suitable for environmental analysis because of its selectivity and sensitivity. Another separation technique is capillary electrophoresis (CE) coupled to different detectors such as a UV detector or, as LC and GC, to mass spectrometry. However, this technique is not widely used to determine emerging organic contaminants in environmental samples mainly due to its detection limits.

Despite the fact that in the last few years some studies have focused on this topic, more studies are needed to understand the behavior of emerging organic contaminants. Therefore, a combination of different disciplines such as analytical chemistry, toxicology or engineering is required and this Thesis wants to contribute to this knowledge.

In the first part of this introduction, we will introduce the classification of different emerging organic contaminants, their principal characteristics and applications. In

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the second part of the introduction, the extraction and chromatographic techniques used to determine these contaminants in sewage sludge will be discussed.

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## 1.1. Emerging Organic Contaminants

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Emerging organic contaminants are defined as compounds that are not currently covered by existing regulations and are thought to be potential threats to environmental ecosystems and human health and safety. Over the last few decades, the adjective "emerging" has been applied to pollutants with increasing frequency [22]. They encompass a diverse group of compounds, including pharmaceuticals, personal care products (PCPs), estrogens, surfactants, perfluorinated compounds (PFCs), flame retardants, industrial additives, and gasoline additives together with their transformation products. In addition, among other compounds, new classes have been added to the list of emerging organic contaminants in the last few years, such as nanomaterials, swimming pool disinfection by-products (DBPs) and 1,4-dioxane, among others [23].

In this section, we will briefly introduce the most studied families cited above and will comment extensively on the contaminants studied in the experimental part.

### 1.1.1. Pharmaceuticals

The concern for pharmaceuticals as toxic substances in the environment and the need to assess their environmental risk has increased greatly since the early nineties. It is estimated that approximately 3,000 different substances are used as pharmaceutical ingredients, including painkillers, antiadiabetics, β-blockers, contraceptives, lipid regulators, antidepressants, impotence drugs, and so on. However, only a very small subset of these compounds has been investigated in environmental studies [24]. Table 1 shows the different groups of pharmaceuticals most studied in environmental samples and some examples of each group.

**Antibiotics**

Sulfonamides

Sulfonamides were the first chemotherapeutic agents discovered and comprise a large group of synthetic antibacterial compounds. Sulfanilamide is the basic chemical structure of various sulfonamides, which originate from various substituents in the sulfamide group. Sulfonamides act as competitive antagonists of p-aminobenzoic acid, preventing it being used to synthesize folic acid [18]. They are widely used in farm animal feedstuff and fish cultures as veterinary drugs for prophylactic and therapeutic purposes. Furthermore, sulfonamides act as growth promoting substances [25]. While this group has been used in human medicine against a wide variety of microbes, the current use is primarily in the treatment of urinary tract infections [26].

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**Table 1.** List of pharmaceuticals studied in environmental samples.

| Group | Examples |
|---|---|
| Quinolones (antibiotic) | Ciprofloxacin, ofloxacin, norfloxacin |
| Macrolides (antibiotic) | Clarithromycin, erythromycin, roxithromycin, tylosin, lincomycin, spiramycin, tilmicosin |
| Tetracyclines (antibiotic) | Oxitetracycline, 4-epitetracycline, 4-epioxitetracycline, tetracycline |
| Sulfonamides (antibiotic) | Sulfamethoxazole, sulfadiazine, sulfapyridine, sulfathiazole, sulfamethazine |
| Diuretics | Furosemide, hydrochlorthiazide |
| β-blockers | Atenolol, enalapril, metoprolol, propranolol, betaxolol |
| Gastrointestinals | Omeprazole, ranitidine |
| Psychoactive drugs | Carbamazepine, diazepam, barbital |
| Nonsteroidal anti-inflammatory drugs | Ibuprofen, diclofenac, naproxen, ketoprofen, mefenamic acid |
| Bronchodilators | Salbutamol, terbutalin |
| Lipid regulators | Bezafibrate, gemfibrozil |
| Antitumorals | Cyclophosphamide, methotrexate |
| Phosphodiesterase type V inhibitors | Sildenafil, vardenafil, tadalafil |
| Contrast products | Iopromide, iopamidol, iomeprol |
| Illicit drugs | Amphetamine, cocaine, tetrahydrocannabinol |

Tetracyclines

Tetracyclines are a group of clinically important natural products and semi-synthetic derivates characterized by broad spectrum activity against pathogenic micro-organisms including gram-positive and gram-negative bacteria [18]. They are considered as 'broad spectrum' antibiotics with a wide range of applications in therapeutics.

Tetracyclines contain an octahydronaphthacene ring skeleton, consisting of four fused rings. The structures of some tetracyclines are summarized in Figure 2. These therapeutic compounds are bacteriostatic antibiotics that act by inhibiting the formation of proteins within bacteria. These compounds have largely been used to treat general infectious diseases and as growth additives in animal feeds, although

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their veterinary use has now been abandoned in most countries because bacterial resistance to them has increased dramatically [27,28].

| Compound | 5 | 6 | 7 |
|---|---|---|---|
| Chlortetracycline | | | -Cl |
| Oxytetracycline | -OH/-H | | |
| Demeclocycline | | -OH/-H | -Cl |
| Methacycline | -OH/-H | $=CH_2$ | |
| Doxycycline | -OH/-H | $-CH_3$/-H | $-N(CH_3)_3$ |
| Minocycline | | -H/-H | |
| Tetracycline | | $-CH_3$/-OH | |

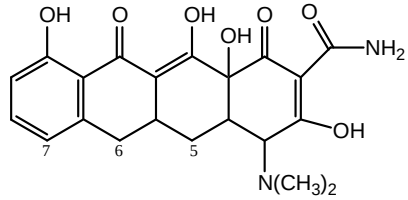


**Figure 2**. Structures of some tetracyclines.

Quinolones and fluoroquinolones

The origins of the quinolone class lie in the use of chloroquine as an antimalarial agent. A compound isolated from the commercial preparation of chloroquine proved to have antibacterial activity and was modified to produce the first marketed quinolone, nalidixic acid (patent in 1962) [29]. The essential structures of first generation quinolones and fluoroquinolones are shown in Figure 3.

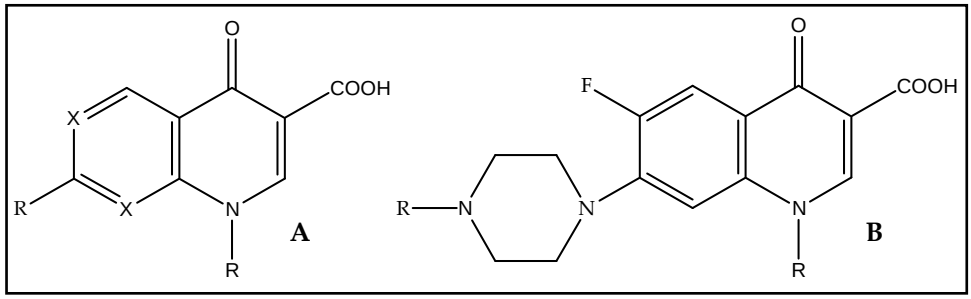


**Figure 3.** General structure of first generation quinolones (A) and fluoroquinolones (B).

Quinolones are an important group of synthetic antibiotics with bactericidal action that results from the selective inhibition of bacterial DNA synthesis. They are active against many gram-positive and gram-negative bacteria [30,31]. Numerous structurally related quinolones have been synthesized, and several of them are in routine clinical use all over the world. Their antibacterial activity is greatly increased when 6-fluoro and 7-piperazinyl groups are added to their 4-oxo-1, 4-

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dihydroquinolone skeletons. The recent introduction of fluorinated quinolones is a particularly important therapeutic advance since these agents have broad antibacterial activity and are an effective way to treat a wide variety of infectious diseases. They are widely used to treat and prevent veterinary diseases in food producing animals [32].

Attempts have been made to subdivide the quinolones on the basis of antibacterial spectrum, potency and pharmacology. Terms such as first and second generation have occasionally been used [31]. First generation quinolones refer to agents which lack good anti-gram positive activity and are used principally for human urinary tract infections, aquaculture and agriculture.

The second generation is termed fluoroquinolones piperazinyl quinolones. They are used to treat respiratory, skin and soft tissue infections, sexually transmitted diseases and urinary infections.

Examples of first generation quinolones are nalidixic acid, oxolinic and pipemedic acids whereas examples of fluoroquinolones are ciprofloxacin, enrofloxacin, norfloxacin and ofloxacin, among others.

Macrolides

The macrolides comprise a family of antibacterial agents widely used in human and veterinary medicine. Consequently, therapeutic activities on human and on farming practices can lead to the presence of such pharmaceuticals in the environment. These macrolide antibiotics are basic lipophilic molecules that consist of a macrocyclic lacton ring containing 12, 14 or 16 atoms with sugar linked via a glycosidic bond [19]. This class of antibiotic is an important alternative for patients exhibiting penicillin sensitivity and allergy [25].

The erythromycin is the most important member of this family. The presence of other macrolides such as roxithromycin, clarithromycin and tylosin, among others, has been studied in environmental samples. Macrolides possess a bacteriostatic action that inhibits protein synthesis [27].

When pharmaceuticals are determined in environmental samples, sometimes the free form is not present but degradated. For instance, Hirsch *et al.* [33] mentioned that erythromycin degradates with an apparent loss of one molecule of water when water is present at an acidic pH.

**β-Blockers**

These pharmaceuticals are the most frequently prescribed pharmaceuticals in the treatment of cardiovascular disorders such as hypertension, angina pectoris, cardiac

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arrhythmias and myocardial infarction. These pharmaceuticals are quite active. Even a small dose of the β-blockers gives sufficient blockage.

β-blockers have been added to the list of forbidden drugs by the International Olympic Committee (IOC) because they are considered to be doping agents in sports [34]. Propranolol and metoprolol belong to the category of β-blockers and, more specifically, are hydrophilic β1-receptor blocking agents. These pharmaceuticals are aminoalcohols (figure 4) and induce a chiral center. β-blockers are exceptionally toxic and most of them have a narrow therapeutic range.

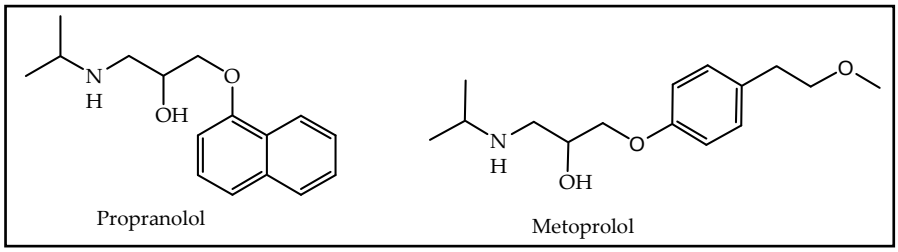


**Figure 4**. Chemical structures of main β-blockers.

## Psychoactive drugs

In this group of pharmaceuticals we can include antiepileptics, antidepressants and so on such as benzodiazepines, barbiturates, among others. Carbamazepine is an important pharmaceutical for the treatment of epilepsy, as well as having various psychotherapy applications. It is also used to treat some seizure disorders, for relief of neuralgia and for a wide variety of mental disorders [35,36]. As we mentioned before, different metabolites or degraded compounds can be found in the environment. The most important products of the metabolic pathway of carbamazepine are 10,11–dihydro-10,11-epoxycarbamazepine (CBZ-EP) and 10,11–dihydro-10,11-dihydroxicarbamazepine (CBZ-DiOH). The first one has considerable anti-convulsant activity and it also seems to cause toxic effects [37].

Benzodiazepines are psychoactive drugs whose core chemical structure is the fusion of a benzene ring and a diazepine ring. Figure 5 shows the general structure of benzodiazepines. Benzodiazepines are frequently prescribed for the pharmacotherapy of epilepsy, convulsions, and related disorders [38]. Due to their effects, benzodiazepines have become one of the most frequently prescribed and used psychoactive drugs in the world. In Spain, 68% of all pharmaceuticals consumed come from medical prescriptions. The prescription of benzodiazepines represented 1.4% of the total of medical prescriptions in 2008 [39]. This value is higher for other psychoactive drugs. For example, antidepressant pharmaceuticals represented 7% of the total of medical prescriptions.

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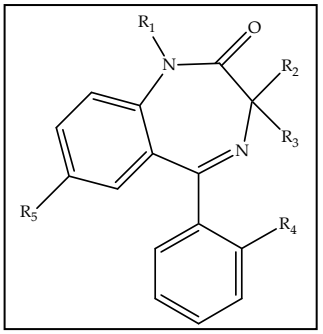


**Figure 5**. General structure of benzodiazepines.

## Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are analgesics and the most commonly used anti-inflammatory pharmaceuticals across the world nowadays. They are mainly used to treat pain, inflammation and fever in animal and human species. These pharmaceutical compounds are weak acidic compounds, due to the carboxylic groups or to the keto-enol tautomery, with pK values between 3 and 7. Many of these compounds are chiral pharmaceuticals, but often they are administered as racemates. The most popular NSAIDs are diclofenac, ibuprofen and naproxen, among others. These compounds are the most determined in different water and solid samples [14,15,40-43].

## Analgesics

Apart from the NSAIDs two important analgesics are the acetaminophen and the acetyl salicylic acid. In the list of the most used pharmaceuticals in the draft of the European Union's Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE), which only includes information from The Netherlands and Germany, the most widely consumed pharmaceuticals are acetaminophen and acetyl salicylic acid [44]. In Spain, only 2% of the total of medical prescription corresponds to this group of pharmaceuticals but most of them can be purchased without medical prescription [39].

## Lipid regulators

The most widely used lipid regulator is bezafibrate. Bezafibrate belongs to the group of fibrate pharmaceuticals, which is an important group mostly used to treat hyperlipidaemia, in which raised cholesterol levels are associated with increased

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levels of triglycerides [45]. Bezafibrate has been included in the list of the most used pharmaceuticals in Germany in the draft of CSTEE [44].

Another lipid regulator used is clofibrate, which is used for controlling high cholesterol and triacylglyceride levels in the blood. This pharmaceutical is metabolized in the human body, and most studies of environmental samples reported concentrated levels of the metabolite of clofibrate, clofibric acid [14,46]. Although bezafibrate and clofibric acid are the most determined lipid regulators in environmental samples, other lipid regulators have also been determined such as gemfibrozil, pravastatin, fenofibrate and simvastatin, among others [47-49].

**Phosphodiesterase type V inhibitors**

The phosphodiesterase type-V inhibitors are used to treat erectile dysfunction in males. There are basically three compounds: sildenafil, vardenafil and tadalafil (these compounds are the active agents of Viagra®, Levitra® and Cialis®, respectively). The physiological process of erection involves the release of nitric oxide in the corpus cavernoum of the penis, mediated by the parasympathetic nervous system. Due to the fact that the molecular structures of these pharmaceuticals are similar to that of cGMP, they act as inhibitors of cyclic guanosine monophosphate (cGMP) (specific phosphodiesterase type V) in the corpus cavernosum, resulting in more cGMP and better erections [50,51]. Although treating erectile dysfunction in males is the most common use, they have been also used for pulmonary hypertension and this is why these compounds are included in the doping list [52].

**Contrast products**

Iodinated X-ray contrast media (ICM) are the most widely used pharmaceuticals for intravascular administration. They are used in human medicine for the imaging of organs or blood vessels during diagnostic tests. They are metabolically stable in the body and are rapidly eliminated via urine and faeces. Most of these radiographic contrast media are derivatives of 2,4,6-triiodobenzoic acid with polar carboxyl and hydroxyl moieties in their chains. Some are ionic (have one or several carboxyl groups) and others are amide derivates and, as such, are neutral compounds. All have iodine atoms in the molecule because these are responsible for absorption of X-rays. Some compounds included in this group are iomeprol, iohexol and diatrizoate, among others [53,54].

**Illicit drugs**

In recent years, there has been growing interest in the determination of illicit drugs in environmental samples [55-57]. In this group of illicit drugs, different groups of

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compounds are included such as opiates, cocainics, cannabinoids and amphetamines, among others. Opiates enter the environment from both clinical and illicit uses. Spain is the fifth major opiate market in Western Europe, according to the "World Drug Report 2009" [58]. Among them, heroin still accounts for more than 70% of opiate abuse, according to a report by the United Nations Office on Drugs and Crime [56]. Methadone is the most commonly prescribed pharmaceutical for the treatment of opioid dependence and in Europe accounts for more than 90% of treatments. The most widely produced and trafficked illicit drug worldwide is cannabis. Tetrahydrocannabinol (THC) is the principal active constituent of *Cannabis sativa*. Cocaine is one of the preferred drugs of abuse. The use of cocaine has direct physiological effects, such as over-stimulation of the central nervous system, a lifetime risk of heart attack, pulmonary complications and altered serotonin levels. Another illicit drug that has central stimulant activity is amphetamine. It has been used in the treatment of obesity, narcolepsy and hypotension but now it is mainly used for its stimulant activity. Sometimes substitution treatment for opiates is used. As we mentioned before, not only is the parent compound found in the environment but also metabolites or degradation products. For instance, in this group the metabolites of THC (THC-COOH), cocaine (benzoylecgonine), morfine (acetylmorfine) and codeine (dihydrocodeine) have been found in influent and effluent wastewater samples [56].

### 1.1.2. Estrogens and their conjugates

A variety of natural compounds and anthropogenic chemicals are known or are predicted to influence the endocrine system, such as natural and synthetic hormones, and natural androgens, among others. This group of estrogens belongs to the endocrine disrupting compounds (EDCs). Apart from the estrogens in this group, there are certain synthetic and natural chemicals which also have the ability to mimic hormones, and thus are able to interfere or disrupt normal hormonal functions. Endocrine disrupting compounds are of concern due to their ecotoxicological and toxicological potencies.

Not only do 17 β-estradiol and its major metabolites (estrone and estriol) enter the aquatic ecosystem via effluents from municipal sewage treatment plants (STPs), but so do other synthetic estrogens. For example, 17 α-ethinylestradiol, one of the most common substances in the synthetic group, is widely used as a human contraceptive and/or as the active ingredient of preparations aimed at the management of menopausal and postmenopausal syndrome. This substance is also used in physiological replacement therapy in deficiency conditions, and in the treatment of prostate or breast cancer [59].

Numerous estrogenic compounds of natural and synthetic origin are excreted by human bodies and reach the aquatic environment daily via sewage treatment plants.

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Consequently domestic STPs are recognized as a main source of contamination for these contaminants. Normally, the estrogens are excreted by humans in urine or faeces in conjugate forms, for instance with a glucoronide or sulfate group linked to the H in the –OH group of the steroid ring [60]. The estrogen conjugates have low estrogenic potency compared with the parent compounds, although during treatment in a STP the free form can be reestablished [61]. The log $K_{o/w}$ of 3.1 to 4.7 indicates that estrogens are rather lipophilic and should appreciably absorb into sediment and sludge.

### 1.1.3. Personal Care Products

Apart from pharmaceuticals, another important group of contaminants included in the list of emerging organic contaminants is the group of ingredients widely known as personal care products (PCPs). In recent years there has been growing interest in the determination of this group. Several personal care product ingredients have been among the most commonly detected compounds in environmental samples. Concern about the environmental fate and potential effects of synthetic organic chemicals used in soaps, lotions, toothpaste and other personal care products continues to increase. Of particular concern are compounds that are used in large volumes, persist in the environment, bioaccumulate, or have a designed bioactivity. There are different groups of PCPs: synthetic musk fragrances, antimicrobials, sunscreen agents, insect repellents and preservatives, among others. The structures of the personal care products studied in the experimental part of this Doctoral Thesis are included in Annex 2.

**Synthetic musk fragrances**

Synthetic musk fragrances are compounds added to scent a variety of personal care products, including deodorants, shampoos and detergents. This group is divided in three different groups: the nitro musk fragrances, the polycyclic musk fragrances and the macrocyclic musk fragrances [62,63].

Nitro musk fragrances were the first to be produced and included musk xylene, musk ketone, musk ambrette, musk moskene and musk tibetene. Discussions on toxicology were raised very early on, and it was demonstrated that these compounds could be transformed in wastewater treatment plants into amino metabolites. At present, polycyclic musk fragrances are used in higher quantities than nitro musk fragrances. 1,3,4,6,7,8-hexahydro-4, 6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzapyran (HHCB) and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHNT) (known by the trade name Galaxolide® and Tonalide®, respectively) are dominating the market. The newest group of fragrances is macrocyclic musks and includes compounds such as habanolide, cyclopentadecanolide and ethylenbrassylate. These

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compounds have some advantages in comparison to the polycyclic ones (for instance, they smell more intensively) though they are currently more expensive. In addition, these compounds seem to be more easily degradable in the environment. These compounds have been determined in water and solid samples but, due to their volatility, they have also been studied in air samples [63].

**Antimicrobials**

The most widely used antimicrobials are triclosan and triclocarban due to their bactericide properties. These compounds are used in soap, toothpaste and other consumer products. Triclosan (TCS) can undergo a series of transformation reactions to produce, in some cases, more toxic and/or bio-accumulative compounds. One of the most favorable is the oxidation of TCS. This reaction leads to the formation of relatively unstable tetra and penta chlorinated diphenyl ethers, which are further decomposed into dichloro and trichlorophenols [64]. In environmental samples, some metabolites of triclosan have been determined such as methyl triclosan and three chlorinated derivates (tetra(II)triclosan, tetra(III)triclosan and pentaclosan) [65,66]. Triclocarban may have greater potential for contamination of surface water since this compound appears to be more persistent than triclosan in STPs [67]. The trichlorinated aromatic structure of triclocarban suggests potential resistance to both chemical and biological transformation processes [67].

**UV filters**

Organic ultraviolet (UV) filters are compounds used to absorb UV radiation and to protect the skin from this radiation. They are increasingly being used as a result of growing concern about UV radiation and skin cancer. Therefore there is major concern about the environmental fate and potential effect of UV filters used in beauty creams, shampoos and other personal care products, as well as those added to plastics and other materials to prevent degradation of polymers and pigments. Following research on the use and the effects of old and new formulations, the list of compounds permissible by legislation is regularly updated. The European Union (EU) currently permits 26 organic UV filters [68]. The hydrophobicity of many of these compounds indicates the potential for bioaccumulation. Among other features, UV filters must be stable on exposure to UV radiation, but recent studies [69,70] have revealed that several organic UV filters undergo degradation, mainly by photolysis, but also as consequence of reaction with chlorine and chlorinated media.

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**Insect repellents**

One of the most widely used insect repellents is N,N-diethyl-m-toluamide (DEET). DEET is classified as an indoor, residential-use pesticide by the United States Environmental Protection Agency (US EPA). From limited toxicity data, DEET is slightly toxic to aquatic invertebrates, fish and birds [76]. Another insect repellent is 1-methylpropyl 2-(2-hydroxyethyl)-1-piperidinecarboxylate (Bayrepel). This compound has a metabolite - bayrepel acid - which is a product of its oxidation. Figure 6 shows the structure of these compounds.

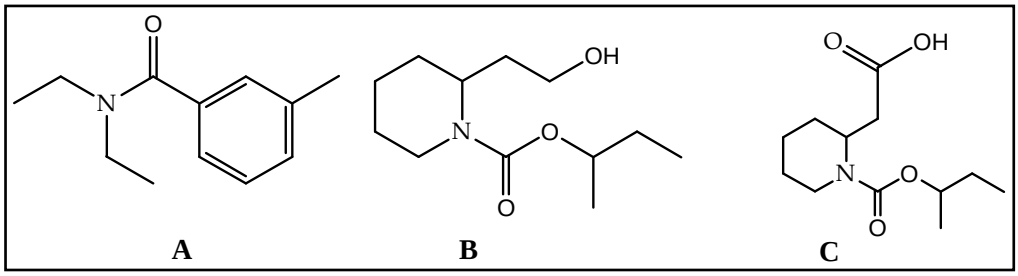


**Figure 6.** Structure of insect repellents and a metabolite. A) DEET, B) Bayrepel and C) Bayrepel acid.

**Parabens**

Parabens (p-hydroxybenzoic esters) are the most commonly used preservatives in personal care products. Parabens are also used in pharmaceuticals and food products. Different parabens have been determined in environmental samples such as methylparaben, ethylparaben, propylparaben, butylparaben and benzylparaben. The basic structure of parabens is showed in Figure 7. As some studies reported, methyl and propylparaben are the most widely used and are normally used together due to their synergistic preservative effects [72,73]. A potential link has been proposed between some types of cancer and prolonged dermal exposure to products containing paraben, for instance some deodorants [74]. However, environmental effects are supposed to be practically negligible since they are effectively removed during the treatment of urban wastewater [72,75].

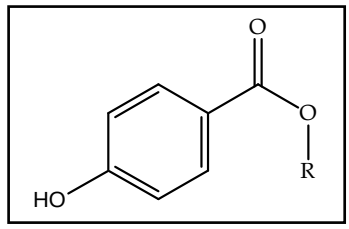


**Figure 7.** General structure of parabens.

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### 1.1.4. Other emerging organic contaminants

Recently, according to Richardson [23], Englert [76] and Farré *et al.* [77], new compounds or groups of compounds such as brominated flame retardants, perfluorinated compounds, nanomaterials, 1,4-dioxane and swimming pool disinfection by-products (DBPs) have been included in the list of emerging organic contaminants.

Brominated flame retardants are a chemically diverse class of compounds that are aromatic, aliphatic or cycloaliphatic and have different bromine contents. The most common are brominated diphenyl ethers, polybrominated biphenyls, hexabromocyclododecane and tetrabromobisphenol A. Brominated flame retardants work by releasing bromine free radicals when heated. These free radicals scavenge for other free radicals that are part of the flame propagation process. They are used in a variety of commercial applications to prevent fires starting on plastics, textiles and electronic circuits, among others [78-80].

Another example is perfluorinated compounds (perfluorooctanoic acid and perfluorooctane sulfonate are the most abundant). These compounds have been used to make stain repellents that are widely applied to fabrics and carpets, and also in the manufacture of paints and coatings, among others.

Nanomaterials can also be considered a truly emerging contaminant. Several journals have focused on the use and associated risk of nanomaterials. The term of nanomaterial was defined by the US National Nanotechnology Initiative (US NNI) as a material that has at least one dimension in the 1 to 100 nanometer range or less than 0.1 micron. They can have unique properties, including high strength thermal stability, low permeability and high conductivity. Over the past two decades, nanomaterials have emerged on the scientific front as a powerful new technology that has uses in science, engineering and medicine. They are also used in personal care products such as creams.

Interest is also increasing in 1,4-dioxane, which has been discovered to be a widespread contaminant in environmental samples, and is a probable human carcinogen. This compound is a proton acceptor and metal chelator and, as such, is used as a stabilizer in the manufacture and processing of paper, textile products and cosmetics, among others [81].

Another group of compounds included in the list of emerging organic contaminants is disinfection by-products (DBPs). Disinfection by-products are chemical, organic and inorganic substances that can form during a reaction of a disinfectant with naturally present organic matter in water and in the treatment of swimming pools. Toxicologically important DBPs include brominated, iodinated and nitrogen-containing DBPs. Brominated compounds are generally more carcinogenic than their chlorinated analogues whereas iodinated compounds are more toxic than their

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brominated analogues. Different disinfectants produce different types or amounts of disinfection by-products.

Among all the emerging organic contaminants mentioned in this introduction, we will focus the following section on the determination of pharmaceuticals and personal care products in sewage sludge.

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## 1.2. Determination of PPCPs in sewage sludge

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Pharmaceuticals and personal care products are continually released into the environment in large quantities from sources such as manufacturing residues, excretion in urine and faeces, disposal of unused pharmaceuticals and hospital and landfill disposal and subsequent leaching. The occurrence of pharmaceutical and personal care products in the environment depends on many variables. For instance, the quantity manufactured, the dosage amount, the usage frequency and metabolic pathways and the effectiveness of waste treatment.

As we mentioned before, this thesis is focused on the determination of PPCPs in sewage sludge. The high volume of sewage sludge produced nowadays is partly a consequence of the increase in the number of new STPs built to achieve the water quality standards set by the European Union (Directives 91/271/EEC and 98/15/EEC).

In general, the EU considers that re-use of sludge should be encouraged, since it represents a long-term solution, as long as the quality of the sludge re-used is compatible with public health and environmental protection requirements. The current legislation regulates the agricultural use of sewage sludge based only on the concentration of toxic heavy metals and nutrients. However, following measures that the European Commission started in 1999 [82], although emerging organic contaminants were not included - the third draft of a future Sludge Directive contained a proposal to limit values of several organic contaminants [83].

Because sewage sludge is a highly complex matrix, the determination of PPCPs in sewage sludge samples demands the use of time, labor-consuming analytical procedures and high-level analytical skills. Because of the wide variety of contaminants, their low concentrations and the composition of the matrix, the methods developed to determine these compounds in sewage sludge usually combine an efficient extraction technique and a chromatographic separation technique with selective and sensitive detection. In addition, between the extraction and separation steps, a clean-up step is sometimes also required because of the complexity of the matrix [61,84].

In the analysis of sludge, the first step is the sampling and the pre-treatment of sewage sludge. In the sampling process, it is important to consider the non-homogeneous character of the matrix. With regard to the material used for the sampling devices, stainless steel provides the best results. The samples are transported under cooled conditions to the laboratory and stored in the dark in the freezer (-18 ºC) until analysis. To enhance analyte extraction, a pre-treatment step is necessary. Basically this process consists of three different steps. The first one is the elimination of water content. There are three different methods to achieve this objective: putting the sample in room temperature with air-drying [85,86], heating the sewage sludge in an oven [87] and using the freeze dry system (lyophilization) [88]. Because of its low time-consumption and the fact that the analytes are not degraded and/or evaporated, the best option to achieve the elimination of water is

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lyophilization. The normal temperature used when the sewage sludge is dried in an oven is 100 ºC for several hours; although the time can be increased and the temperature decreased if the analytes are degraded. The room temperature system is not the best option because longer times are required. Homogenization of the sample by grinding is the second step in the pre-treatment process. And finally, the last step is to select the particle size by sieving. Generally the particle size is lower than 2 mm. Because of this pre-treatment, the concentration of analytes in sewage sludge samples is referred to as dry weight (d.w.).

After the pre-treatment process, an extraction step is applied. Trends in the extraction process include the use of less organic solvent consumption and, as a consequence, classic techniques such as Soxhlet and ultrasonic extraction (USE) are being partially replaced by recent developed techniques such as pressurized liquid extraction (PLE) and microwave-assisted extraction (MAE) that fulfill current demands for environmental analysis using high temperature and/or pressure.

Between the extraction technique and the chromatographic technique, a clean up step is sometimes necessary. The clean up is used in order to remove co-extractives. The extracts can be processed further by using techniques such as SPE [36,89,90] or gel permeation chromatography (GPC) [91-93]. SPE has been the preferred sample purification technique to clean up the extracts containing neutral and acidic pharmaceuticals because it is fast, requires a low volume of organic solvent and presents a low contamination risk. Although the clean-up is used in relevant literature, in some studies this step is avoided because it increases the time of analysis and acceptable recoveries were obtained without it [94-96].

The use of chromatographic techniques is necessary to identify and quantify PPCPs in sewage sludge. Principally, gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS-MS) are the preferred techniques to determine them.

In the following section, we will first discuss the main characteristics of chromatographic techniques and later the extraction techniques used to determine PPCPs in sewage sludge.

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### 1.2.1. Chromatographic techniques

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#### 1.2.1.1. Gas Chromatography

Gas chromatography (GC) plays an important role in the separation of volatile organic contaminants in environmental samples. GC is one of the most attractive and powerful techniques for routine analysis of some ubiquitous organic pollutants due to its good separation power. The group of PPCPs most determined in sewage sludge using GC is polycyclic and nitro musk fragrances because of the high volatility of these compounds [91,93,97,98]. However, other groups of compounds, such as UV filters and polybrominated diphenylethers (PBDEs), have also been determined by GC [92,94,99]. As an example, in the study of Plagellat *et al.* [92], four UV filters such as 3-(4-methylbenzylidene) camphor (4-MBC), octyl-methoxycinnamate (OMC), octocrylene (OC) and octyl-triazone (OT) were determined in sewage sludge from Switzerland. A group of pharmaceuticals and some personal care products were also detected in sewage sludge from The United States of America (USA) using gas chromatography as a separation technique [94].

However, the majority of pharmaceuticals lack sufficient volatility and this is not directly compatible with GC. Various groups of pharmaceuticals can be derivatized to make them suitable for GC analysis. Although such procedures may be time-consuming and can introduce errors due to side-reactions during the derivatization, they are still used widely and are well-established for routine work. Silylation is the most widely used method to derivate pharmaceuticals and personal care products with acidic groups. The authors used different agents for silylation such as a mixture of (N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)/trimethylsilylimidazole (TMSI)/dithioerytrol to determine some estrogens [61]; bis(trimethy-lsilyl)trifluoroacetamide (BSTFA)/pyridine to determine triclosan and some endocrine disrupting compounds such as nonylphenol, nonylphenol ethoxylates and bisphenol A [100] and trimethylsulfonium hydroxide (TMSH)/triethylamine (TEA) to determine some musk fragrances, plasticizers and phenolic compounds [101]. After the derivatization process, the solution was sometimes evaporated to a dry state by a gentle nitrogen stream and the residue was dissolved in an appropriate organic solvent for GC analysis.

The injector is a critical component of GC, influencing accuracy and precision. Two different injectors have been used in the determination of PPCPs in sewage sludge. The Split/Splitless injector is the most widely used, mainly in the splitless mode because of lower concentrations of the target analytes [93,98]. On the other hand, Plagellat *et al.* [92] used the PTV injector to determine four UV filters. Using the PTV injector, higher volumes of samples can be injected and, in this case, 5 μL were injected. As already known, the use of higher injection volumes involves lower limits of detection although more interferent compounds can be introduced into the system.

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Different non-polar columns have been used to separate some PPCPs. The most widely used is a column with 5% phenyl-methylpolysiloxane. This column was used to separate synthetic musk fragrances and triclosan and two related chlorophenols [91,97,98,102].

Regarding the detection step, there are a wide variety of detectors when GC is applied as a separation technique. However, due to the low concentration of the target compounds - and in order to confirm the presence of target and non-target compounds - the use of mass spectrometry [91,94,97-100] or tandem mass spectrometry [61,102] is required. These two detection systems allow both high sensitivity and selectivity. However, other detectors have also been used such as the electron capture detector (ECD) to determine permethrin, which is used as a repellent for clothing, household insect foggers and sprays, and as a body lotion for scabies control, among others [103].

Regarding the ionization mode when mass spectrometry or tandem mass spectrometry are used, two different ionization modes have been commonly applied in studies to determine PPCPs in sewage sludge: electron impact (EI) [61,98,102] and negative chemical ionization (NCI) [97,99]. EI is the most widely used ionization mode in GC-MS analysis and causes a high fragmentation of molecules whose spectral pattern is useful for identifying a sample compound. The identification of compounds comparing the mass spectrum obtained with one available in libraries is one advantage of EI because in all cases 70 eV was used as voltage. The NCI is a soft mode causing less fragmentation. This mode is highly sensitive and selective for compounds with a positive electron affinity. Although different gases can be used as reagents, methane is the most widely used.

Another important part of MS instruments is the analyzer. Two different analyzers have been commonly used for determining PPCPs in sewage sludge: the quadrupole and the ion trap.

Most authors use a quadrupole as analyzer [91-93,97,99], which operates in two different modes: full scan mode [98,100] or selected ion monitoring (SIM) [91-93,97,101]. The use of the SIM mode is useful to quantify the target analytes in samples at low concentrations. Whereas, the full-scan mode is useful for qualitative analysis.

In the case of ion trap analyzers [61,102], quantification is performed employing the selected reconstructed ion chromatograms. This achieves a similar sensitivity and maintains the qualitative information contained in the mass spectra. For instance, Morales *et al.* [102] showed that the instrumental quantification limits in the determination of triclosan and two chlorophenols are 10 times lower than the limits obtained using GC-MS detection with quadrupole analyzer. For triclosan, the instrumental limit obtained using a GC-ion trap was 0.2 ng/mL and with GC-MS with quadrupole analyzer it was 2 ng/mL. Other analyzers such as triple quadrupole,

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time of flight or quadrupole-time of flight have been used in other kinds of samples [104-106]. However, we did not find any publications regarding their use to determine PPCPs in sewage sludge samples.

Isotopically labelled compounds are best as an internal standard because environmental samples are complex mixtures. An isotopically labelled internal standard will have a similar extraction recovery, ionization response and a similar chromatographic retention time. Depending on the compounds, different internal standards have been used. In Table 2 we can see the methods used for the determination of PPCPs in sewage sludge samples using gas chromatography as a separation technique and the internal standards used.

As we can see in Table 2, in most cases isotopically labelled compounds are used when MS or MS-MS are used as detection systems. However, these compounds are rare and costly and, for this reason, compounds not present in the samples are used as internal standards.

As far as we know, multidimensional gas chromatography (GCxGC) has not yet been applied to determine PPCPs in sewage sludge despite the fact that it increases the separation capability of a chromatographic system and it has been shown to be a very suitable technique for the full separation of complex mixtures in different fields.

After the extraction technique and combining gas chromatography with mass spectrometry or tandem mass spectrometry detection, good sensitivity and selectivity are obtained and the identification and quantification of non polar compounds at trace levels are achieved. Moreover, by using an efficient derivatization step, some polar PPCPs can also be determined using this separation technique.

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**Table 2.** Methods for determining PPCPs in sewage sludge using GC.

| Group of compounds | Extraction technique | Separation technique | Internal standard | Ref. |
|---|---|---|---|---|
| Endocrine disrupting compounds | USE | GC-MS | [$^2H_{16}$] bisphenol A | [100] |
| Estrogens | USE | GC-MS-MS | 17β-estradiol acetate | [61] |
| Synthetic musks | PLE | GC-MS | Naphthalene-$D_8$ | [91] |
| Polycyclic and nitro musks | SFE | GC-MS | Anthracene-$D_{10}$ | [85] |
| Polycyclic and nitro musks | SFE | GC-MS | - | [97] |
| Organic compounds | PLE | GC-MS | Musk Xylene $D_{15}$, dibutyl phthalate-$D_4$, ethylparathion-$D_{10}$, butylbenzyl phthalate $D_4$, dioctyl phthalate-$D_4$, 4-n-nonylphenol and bisphenol A- $D_{16}$ | [101] |
| Polycyclic musks | Soxhlet | GC-MS | Naphthalene-$D_8$, acenaphthene-$D_{10}$, phenanthrene-$D_{10}$, chrysene-$D_{12}$ and perylene-$D_{12}$ | [98] |
| Polycyclic musks, UV filters and biocides | Shaking extraction | GC-MS | AHTN-$D_3$ | [93] |
| Anti-inflammatories, fragrances, preservatives, UV filters and plasticizers | Soxhlet | GC-MS | 4,4'-octobromo biphenyl | [94] |
| Pharmaceuticals, idonated contrast media and musk fragrances | USE and PLE | LC-MS-MS and GC-MS | AHTN-$D_3$ | [53] |
| Triclosan | MAE | GC-MS-MS | - | [102] |
| UV filters | Shaking extraction | GC-MS | - | [92] |
| Pharmaceuticals | MAE | GC-MS | - | [107] |
| Estrogens | PLE | GC-MS | E2-$D_4$ EE2-$D_4$ | [108] |

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### 1.2.1.2. Liquid Chromatography

Despite the undisputable merits of GC procedures for residue analysis of certain classes of pharmaceuticals and personal care products, high performance liquid chromatography (HPLC) shows much more universal applicability because most PPCPs are polar.

One of the most important parts of the liquid chromatograph is the column. In most cases, the column used to separate this kind of compound is a $C_{18}$. However, in the study of Miao *et al.* [36] to separate carbamazepine and its principal metabolites, the column of $C_8$ was used. As is very well known from Van Deemter equations, the efficiency of the chromatographic process depends on particle size, among other parameters. Small particle diameter can significantly reduce the height of the theoretical plate (HETP), which results in higher efficiency and a flatter profile of the Van Deemter curve. The particle size in the columns most widely used is between 3 and 5 μm. As already known, if the particle size is reduced, similar efficiencies are obtained with shorter columns in comparison with conventional columns. Based on this principle, a new trend in liquid chromatography is the use of ultra high performance liquid chromatography (UHPLC). Ultra Performance Liquid Chromatography (UPLC) is the trade name of Waters and Rapid Resolution Liquid Chromatography (RRLC) is the trade name of Agilent Technologies. UHPLC uses analytical columns packed with about 1.7 μm particles, which offers advantages of increased speed, improved sensitivity, selectivity and specificity compared to conventional LC analysis [109].

Up until now, only one study used UHPLC to separate PPCPs from sewage sludge. Okuda *et al.* [110] used one column of 10 cm with 1.7 μm of particle size. In this study, 61 compounds (antibiotics, analgesics, antiepileptics, blood-vessel dilators, bronchodilators and other pharmaceuticals) were separated. However, no data on the time of analysis was reported. Apart from this study, UHPLC has been used to determine pharmaceuticals and personal care products in other kinds of samples such as influent and effluent wastewater [56,111,112].

The higher back pressure resulting from the smaller particle size has limited the application of columns packed with sub-2 μm particles, primarily because UHPLC instruments are often required to perform high-speed separation on those small-particle columns. Recently new kinds of particles known as fused core particles have been commercialized. Fuse Core technology is based on a 0.5 μm outer shell of porous silica fused to a uniform and solid 1.7 μm core particle. Uniform flow channels and a reduced intra-particle flow path result in significantly less dispersion within the confines of the analytical column. The result is similar column efficiencies to columns packed with sub-2 μm particles at about half the backpressure [113]. Consequently, sub-2 μm efficiencies can be used with conventional LC equipments

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[114,115]. These columns have not been tested to separate pharmaceuticals and personal care products in sewage sludge yet, although a recent study on the comparison of fused core and conventional particles has been published on its application to a pharmacokinetic study [116]. This study showed that in a direct comparison of the 2.7 μm particle fused core column versus the 5 μm fully porous particle column, the efficiency was increased 2-3 fold, retention time was decreased 34% and a 3-4 fold increase in S/N was observed.

Another new trend in liquid chromatography columns is the use of hydrophilic interaction liquid chromatography (HILIC). These columns are used to separate very polar compounds such as peptides, carbohydrates or polar metabolites. One of the characteristics of these columns in that gradient elution is opposed to the conventional columns used in reversed phase chromatography. These columns have been used to separate some pharmaceuticals and personal care products in surface and waste water samples [115]. One example of the use of this column is the study of Gheorghe *et al.* [115], in which the separation of cocaine and its principal metabolites in waste and surface water was achieved using HILIC chromatography because the metabolites are polar. If conventional columns had been used, the separation would have been difficult to achieve.

The mobile phase in liquid chromatography is composed of two phases: one organic solvent (methanol or acetonitrile) and one aqueous phase (at a different pH). In order to obtain sufficient retention for acidic contaminants and reproducible retention times, the use of a buffer in the eluent or acidification of the mobile phase is recommended [46]. However, when mass spectrometry is used as a detection technique, the additives in the mobile phase are limited to those that are volatiles. Different additives are added to the mobile phase such as ammonium acetate [53] and formic acid [117], among others, at concentrations typically ranging from 2 to 20 mM. For example, ammonium acetate at a concentration of 5 mM (pH 5.7) was used as a modifier in the mobile phase to separate some neutral pharmaceuticals such as diazepam, carbamazepine, ifosfamide and caffeine among others [53].

It is known that temperature has an influence on liquid chromatographic separation. Recent studies show that liquid chromatography at elevated temperatures is an area of increasing interest as a means of reducing analysis time [118-120]. A few studies have demonstrated the effect of high temperatures in the separation of analytes. In the study of Yan *et al.* [119], we can see a significant improvement in the separation of alkylphenones using high flow rates and temperatures. The separation achieved in this study [119] shows that the use of high temperatures is a powerful approach to achieving separations that are as much as 50 times faster than those achieved at room temperature.

The separations at high temperatures require precise temperature control, the ability to generate a flow rate and, most importantly, a stationary phase which is stable

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enough to withstand the harsh conditions. One of the most important benefits of the use of high temperatures in the separation process is the short time of analysis. In addition, as temperature is increased, the viscosity of the eluent decreases and thus the system back pressure is greatly reduced. Another important benefit is that water can sometimes be used as the sole eluent, thereby mitigating many problems, including toxicity, flammability and costs relating to the use of organic solvent. Edge *et al.* [120] separated 10 pharmaceuticals using water as a mobile phase at 180 ºC.

Different detectors such as ultraviolet (UV) detectors, diode array detectors (DAD) [92,93] and fluorescence detector (FD) [121-123] have been used in the separation of PPCPs from sewage sludge. However, the most widely used detection system, as with gas chromatography, is mass spectrometry (MS) [122,124,125] and the tandem mass spectrometry (MS-MS) [36,47,84,89,90,117]. Different reviews have been published on the determination of pharmaceuticals and personal care products using LC-MS such as for the determination of veterinary medicines [126], pharmaceuticals [127-130] and antimicrobials [131], among others.

Plagellat *et al.* [92] and Kupper *et al.* [93] used the UV detection to determine one UV filter (Octyl-triazone (OT)). In the study of Plagellat *et al.* [92], gas chromatography was used to separate 3 UV filters (octocylene, octyl-methoxycinnamate and 3-(4-methylbenzylidene)camphor), however OT has been determined using LC-UV due to its polarity. The same method to determine OT was used in the study of Kupper *et al.* [93]. In this study, the presence of some musk fragrances, UV filters and biocides were studied. In both studies, LC-(ESI)-MS-MS in positive mode was used in order to confirm the amounts of OT in samples. In both studies, the LODs reported for OT were at levels of low μg/Kg when LC-MS-MS with a triple quadrupole analyzer was used as the separation technique.

Fluorescence detection has also been used with liquid chromatography to determine pharmaceuticals such as fluoroquinolones [122]. In the study of Ferdig *et al.* [122] MS and MS-MS were also used to confirm the presence of different fluoroquinolones. They compared the limit of quantifications (LOQs) obtained with a fluorescence detector, mass spectrometry and tandem mass spectrometry. The limits of quantification obtained with FD were one order of magnitude higher than the LOQs obtained with MS-MS.

The ionization source and the analyzer are the most important parts in the mass spectrometer. Regarding the ionization source, two different ionization interfaces are mainly used: the electrospray ionization (ESI) and the atmospheric pressure chemical ionization (APCI), working in positive or negative mode depending on the target analytes. Both interfaces operate at atmospheric pressure. Generally, the ESI mode is used for the most polar compounds and is the most extensively used for PPCPs because they exhibit higher sensitivity than in APCI. The APCI was used in the study of Ternes *et al.* [53] to determine acidic compounds such as clofibric acid, ibuprofen,

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diclofenac, bezafibrate and ketoprofen, among others. However some of these compounds have been determined using electrospray ionization [47,110] and similar limits of quantification and limits of detection were obtained. In recent years, manufacturers have developed a new ionization interface that combines both ESI and APCI modes in order to determine compounds using the best ionization conditions. In the last few years, atmospheric pressure photoionization (APPI), which is capable of ionizing non polar compounds and is possibly less susceptible to matrix effects [132], has been an emerging source. However, due to the nature of PPCPs, it has not been applied to determine these compounds.

Radjenovic *et al.* [90] developed a method for a simultaneous determination of 31 pharmaceuticals belonging to predominant therapeutic classes such as NSAIDs, analgesics, lipid regulators and antibiotics, among others. This study shows one of the most critical aspects in quantitative analysis with LC-MS or LC-MS-MS: the occurrence of matrix effect that may lead to a significant difference in the response of an analyte in a sample as compared to a pure standard solution. This matrix effect is attributed to those organic and/or inorganic components of a sample that co-elute with an analyte and interfere in the ionization process. In the study of Radjenovic *et al.* [90] to investigate the interferences of sludge matrix components, standard addition into the extraction extracts was performed. A comparison between the calibration curve made with the spiked extracts and the calibration curve of standards in water was done. In this comparison, strong MS signal suppression/enhancement effects were observed practically for all compounds using both negative or positive ionization. Different values of matrix effect for the same compound are reported in the literature due to the difference in sewage sludge samples.

Different methods can be used in order to minimize this problem. Matrix effects may be compensated by using internal standards. Moreover, in studies with a long analysis time, more than one internal standard may be needed. Table 3 shows the methods developed using liquid chromatography with mass spectrometry detection and the internal standard used in each case. The ideal internal standards are those isotopically labelled when available. By using this method, a good quantification is obtained. Alternatively, the standard addition of each analyte into each sample may be used to compensate matrix effects, but this leads to a significant increase in analysis and processing time.

Apart from using internal standard or standard addition to solve the problems of matrix effect, other ways of working can reduce the matrix effect. These include the dilution of the extract if the concentration of the analytes is high, the improvement of the separation or using a pre-treatment sample between the extraction and chromatographic steps.

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**Table 3.** Methods for determining PPCPs in sewage sludge using liquid chromatography.

| Group of compounds | Extraction technique | Separation technique | Internal standard | Ref. |
|---|---|---|---|---|
| Fluoroquinolones | USE | LC-MS-MS | | [122] |
| Pharmaceuticals | USE | LC-MS-MS | | [84] |
| Estrogens | PLE | GC- MS and LC-MS-MS | BPA-$D_4$, Equilin-$D_4$, E1-3S-$D_4$, NP-$D_8$ and lanthostanol | [133] |
| Pharmaceuticals | USE | LC-MS-MS | | [110] |
| Pharmaceuticals | PLE | LC-MS-MS | 16 deuterated compounds | [134] |
| Pharmaceuticals | PLE | LC-MS-MS | $^{13}$C-phenacetin, mecroprop-$D_3$, ibuprofen-$D_3$, atenolol-$D_7$, carbamazepine-$D_{10}$, diazepam-$D_5$ and phenobarbitol-$D_3$ | [90] |
| Pharmaceuticals | PLE | LC-MS-MS | | [89] |
| Sulfonamides, Macrolides and Trimethoprim | PLE | LC-MS-MS | | [135] |
| Fluoroquinolones, sulfonamides and tetracyclines | PLE | LC-MS | | [125] |
| Carbamazepine and its metabolites | PLE | LC-MS-MS | Trimethyl $^{13}$C caffeine | [36] |
| Sulfonamides, macrolides and trimethoprim | PLE | LC-MS-MS | SMT*-phenyl-$^{13}C_6$, sulfamethoxazole-$D_4$, sulfadiazine-$D_4$, sulfathiazole-$D_4$, $N^4$-acety-lSMTX**-$D_5$ | [136] |
| Triclosan and triclocarban | PLE | LC-MS-MS | Triclosan-$^{13}C_{12}$, triclocarban-$^{13}C_{13}$ | [137] |
| Fluoroquinolones | PLE | LC-FLD | | [123] |

* Sulfamethazine
**Sulfamethoxazole

ISBN:978-84-693-6426-0/DL:T-1628-2010

The dilution of the extract minimizes the matrix effect although it has a problem of sensitivity. The improvement of the separation is important because the co-elution of the analytes may produce matrix effects although the analysis time is increased. The most used pre-treatment is solid-phase extraction and gel permeation chromatography. By using SPE, apart from the clean-up step, a preconcentration of the analytes is achieved.

As we mentioned before, the analyzer is one of the most important parts of MS instruments. Different analyzers have been used in order to determine PPCPs in sewage sludge such as quadrupole (Q) [124], ion-trap (IT) [89,122] and combinations between these analyzers such as triple quadrupole (QqQ) [47,53,84,117,135]. MS-MS is the preferred option because this technique gave us extensive information on the target analytes and increases the selectivity and sensitivity. Moreover, structural information can be obtained with this detector and more identification points are obtained using two transitions in the MRM mode.

The identification points (IP) are defined in the European Commission Decision (2002/657/EC) of 12 August 2002, "Concerning the performance of analytical methods and the interpretation of results". Different IPs, depending on the analyte, are required in order to confirm the presence of target analytes. Thus, for the confirmation of analytes listed in Group A of Annex 1 of Directive 96/23/EC (substances having anabolic effect and unauthorized substances), a minimum of 4 identification points are required and for the compounds listed in Group B of Annex 1 of the same directive (veterinary drugs and contaminants) a minimum of 3 identification points are required. Table 4 shows the number of identification points earned for liquid chromatography and mass spectrometry or tandem mass spectrometry combinations. The values of the table are included in Table 6 of the Commission Decision 2002/657/EC [138]. Although this EU Commission Decision confirms the presence of contaminants in food, this legislation is applied because of the lack of legislation concerning environmental samples.

**Table 4.** Number of identification points earned with combinations of liquid chromatography and mass spectrometry or tandem mass spectrometry.

| Technique | Number of ions | Identification points |
|---|---|---|
| LC-MS | N | N |
| LC-MS-MS | 1 precursor and 2 daughters | 4 |
| LC-MS-MS | 2 precursors each with 1 daughter | 5 |
| LC-MS-MS | 1 precursor, 1 daughter and 2 grandaughters | 5.5 |

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Using quadrupole (Q) analyzer, in order to fulfill the identification points to confirm the presence of the target analytes in the EU commission Decision 2002/657/EC [138], three different ions have to be selected for each compound: one for quantifying and other two for confirming. However, in some cases it is difficult because the molecules do not fragment enough to obtain these three ions. Apart from the ions, the relative abundance of each of these ions is also checked. The quadrupole analyzer has been used to determine different groups of PPCPs such as antibiotics, NSAIDs, and some endocrine disrupters. Although more information is obtained with full scan mode, the SIM mode is preferred because lower limits of detection are obtained.

Another analyzer used in the determination of PPCPs in sewage sludge is the ion trap. It has the advantage that it can work as MS or $MS^n$ but obviously more information is achieved when $MS^n$ is applied. Moreover, when the ion trap works as tandem mass spectrometry, lower limits of quantification are obtained in most of cases. This situation was observed in the study of Ferdig *et al.* [122]. In this study 9 fluoroquinolones were determined in different sewage sludge samples and the limits of quantification of 6 out of 9 compounds were two times lower when the ion trap works with tandem mass spectrometry. For instance, ofloxacin in SIM mode had a instrumental limit of quantification of 2.4 ng/L whereas with tandem mass spectrometry it had a instrumental limit of quantification of 1.2 ng/L. Barron *et al.* [89] determined some pharmaceuticals in sewage sludge using the ion trap as analyzer. The IT worked as $MS^n$ and the LOQs obtained for pharmaceutical compounds were lower than 580 ng/g.

Both quadrupole and ion trap analyzers have a resolution of the unit, whereas the time of flight (TOF) analyzer increases its resolution up to 10,000 times. The TOF analyzer is a powerful tool for identifying unknown compounds in complex environmental samples such as sewage sludge. This analyzer acquires the mass spectra in full scan mode. Using this analyzer we can obtain different empirical formulae and the study of fragmentation and information from the literature is required in order to identify the non target compounds. In the literature, different studies were found regarding the use of TOF in complex environmental samples. However, as far as we know, a TOF analyzer has not yet been used to determine PPCPs in sewage sludge.

As we have already mentioned, nowadays, and due to the low concentration of pharmaceuticals and personal care products in sewage sludge, tandem mass spectrometry is the preferred detection technique, with the triple quadrupole analyzer (QqQ) the most used. The MS-MS can work, among other modes, in multiple reaction monitoring (MRM) mode in order to obtain excellent sensitivity for target analytes. Confirmation of the identity of the target analytes is achieved by monitoring two characteristic precursor-product transitions, which earns four identification points and fulfils the requirements for identification and confirmation of environmental contaminants defined by EU commission Decision 2002/657/EC

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[138]. Numerous studies have shown applications in which the triple quadrupole is used as an analyzer. Two recent examples are the studies of Lillenberg *et al.* [125] and Radjenovic *et al.* [90]. Lillenberg *et al.* [125] studied the presence of fluoroquinolones, sulfonamides and tetracyclines in sewage sludge from Estonia. To extract these compounds, pressurized liquid extraction (PLE) was used as the extraction technique and, using the triple quadrupole as analyzer, the limits of quantification ranged from 0.1 ng/g (for sulfonamides) to 160 ng/g (for tetracyclines). Two transitions in the MRM mode were recommended in the EU commission Decision 2002/657/EC [138]. However, doxycycline and ofloxacin only had one transition.

Another analyzer used in hybrid mass spectrometry is quadrupole-time of flight (QTOF). Using TOF we can assign the empirical formula for each ion but by using QTOF it is easier to propose the fragmentation way of the precursor ion. Although this analyzer has been used with water samples, no studies were found concerning the determination of PPCPs in sewage sludge. Moreover, this analyzer has higher limits of quantification than a triple quadrupole analyzer, as Petrovic *et al.* [112] reported for the determination of pharmaceuticals from influent and effluent wastewater. However, by using this analyzer more structural information can be obtained and some unknown compounds can be identified.

It can be seen that, apart from musk fragrances, most PPCPs are determined by LC due to their polarity which makes this kind of chromatography more suitable. With regards to detection, the tendency is to utilize tandem mass spectrometry using different combinations of analyzers, depending on the goal. However, for target analysis, the triple quadrupole is the preferred analyzer.

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### 1.2.2. Extraction techniques

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As we mentioned above, the methods developed to determine pharmaceuticals and personal care products include an extraction technique and a chromatographic technique. Extraction techniques for solid and semi-solid samples should be exhaustive to guarantee efficient recoveries in different types of samples. The extraction step is essential in the analysis of sludge samples because of the complexity of the matrix and the low levels at which contaminants are generally present in the environment. In this step the techniques and methods applied differ. As we have already mentioned, sludge samples are one of the most complex matrices and contain a large number of organic contaminants usually present at trace levels and some of these are very strongly absorbed in the matrix. So, if the extraction is efficient in terms of sensitivity and selectivity, the chromatographic analysis will be considerably more straightforward.

Different extraction techniques have been used to determine pharmaceuticals and personal care products such as Soxhlet, shaking extraction, ultrasonic extraction, supercritical fluid extraction, microwave assisted extraction and pressurized liquid extraction. In this section, the advantages and disadvantages of each extraction technique are commented upon. We will also discuss the principal parameters optimized and the occurrence of PPCPs in sewage sludge using the different extraction techniques.

#### 1.2.2.1. Classical extraction techniques

The classical techniques are still present in most of the official methods of analysis but they are being replaced by techniques that use more complex equipment and have important advantages such as use of less solvent and time. In the group of classical extraction techniques, Soxhlet, soxtec and shaking extraction with solvents can be included.

Solvent extraction with agitation is the simplest method to extract contaminants from solid samples such as sewage sludge. A solvent is added to the dried sludge sample and they are mixed together with a mechanical shaker. Then, the mixture is usually centrifuged so that the two phases can be manually separated. Common variables that need to be optimized are the nature and volume of the solvent and stirring time.

Although the solvent volume is initially low (between 20 and 30 mL), multiple extractions are needed to increase the recoveries and, consequently, the final solvent volume increases.

An evaporation step is then required which uses a rotary evaporator or a gentle stream of nitrogen. Additionally, a clean-up step after extraction is usually necessary to reduce possible interferences. This is done using solid-phase extraction (SPE) or

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gel permeation chromatography (GPC). These steps are common in most developed methods using different extraction techniques.

Solvent extraction with agitation, widely used in the past, has recently been used to extract UV filters [92,93] and biocides [103] from sludge.

In the studies of Plagellat *et al.* [92] and Kupper *et al.* [93], four UV filters and some polycyclic musk fragrances were extracted using this extraction technique. The amount of sample was similar in both studies: 60 g in the study of Plagellat *et al.* [92] and 50 g in the study of Kupper *et al.* [93]. Although the compounds were similar, the extraction solvents were different. In the study of Plagellat *et al.* [92], three consecutive different mixtures of 20 mL were used [92] (one extraction with pentane:acetone (1:1), two more with pentane:diethyl ether (1:1), and another one with diethyl ether:dichloromethane (4:1)). In the study of Kupper *et al.* [93] three times 20 mL of hexane as solvent extraction was used. In both cases, the extracts were centrifuged and the supernatant was dried over $Na_2SO_4$. GC-MS was used for the most volatile compounds although one UV filter (OT) was determined using LC-MS-MS due to its polarity. The recoveries were similar in both studies and were higher than 75% in all cases.

Four UV filters (octyl-methoxycinnamate (OMC), 3-(4-methylbenzylidene) camphor (4-MBC), octocrylene (OC) and octyl-triazone (OT)) were found in all samples analyzed in the study of Plagellat *et al.* [92]. The OT showed the maximum concentration of 27700 μg/Kg whereas OMC showed the lowest concentration of 10 μg/Kg. In this study samples collected in winter (January) and samples collected in summer (July) were analyzed. It is expected that the amount of UV filters released to wastewater is higher during the summer due to high application rates of sunscreens related to bathing activities compared to the winter period, whilst release of UV filters originating from other applications such as cosmetics are likely to be stable throughout the year. The mean concentration in winter and summer were 1730 and 1820 μg/Kg for 4-MBC, 105 and 115 μg/Kg for OMC, 4270 and 5410 μg/Kg for OC and 3900 and 7100 μg/Kg for OT. The concentration found in the study of Kupper *et al.* [93] for OC was four times higher (20804 μg/Kg) than the concentration found in the study of Plagellat *et al.* [92]. Moreover, in the samples analyzed in the study of Kupper *et al.* [93], polycyclic musk fragrances were determined at concentrations of between 8 μg/Kg for cashmeran and 15610 μg/Kg for galaxolide.

Another classical technique used to extract pharmaceuticals and personal care products from sewage sludge is Soxhlet extraction. Soxhlet extraction was one of the preferred extraction techniques for solid samples largely because of its simplicity. It does not require complex equipment and only a few parameters have to be optimized: the nature and the volume of solvent and the extraction time.

As with all extraction techniques, the selection of solvents mainly depends on analyte polarity. For instance, in the study of Zeng *et al.* [98] a pure solvent

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(dichloromethane) was used to extract polycyclic musk fragrances [98]. In the study of Büyüksönmez *et al.* a sequentially Soxhlet extraction was achieved using a combination of pure solvents (methylene chloride, ethyl acetate and hexane) to extract some pharmaceuticals and personal care products such as anti-inflammatories, fragrances, preservatives, sunscreen agents and plasticizers, among others.

The volume of solvent depends on the amount of sample and the extraction efficiency obtained for most compounds. The volume of solvent used was between 150 and 300 mL. The amount of sample varied in each study. For example, 1 gram of sewage sludge has been used to extract polycyclic musk fragrances [98].

As we mentioned before, one of the most important parameters in the Soxhlet extraction is the extraction time. As already known, the length of extraction time of the Soxhlet method is a disadvantage because it can vary from between 6 and 24 hours [98,139].

The precision of the method varies depending on the analytes and the method used. For instance, in the determination of six musk fragrances, the RSD (%, n = 4) varied between 3.73% for galaxolide and 9.94% for cashmeran [98].

As with other extraction techniques for sewage sludge samples, a further clean-up step after the extraction step is oftenly required because of the complexity of the matrix. The most common clean-up step used is solid-phase extraction. Büyüksönmez *et al.* [94] used silica gel mixed with the extract (0.025 mg fumed silica gel for each mL of extract) in order to remove fatty acids. In most cases, activated copper was added in order to remove sulphur from the extract [98].

Closely related to Soxhlet, soxtec extraction has also been used as an extraction technique to extract estrogens from sewage sludge. The estrogens were determined alongside other endocrine disrupting compounds such as alcohol polyethoxylates, nonylphenol and octylphenol polyethoxylates. In soxtec extraction, the sample is loaded into an extraction thimble and immersed in boiling solvent. This ensures very rapid close contact between the analyte of interest and the solvent, resulting in faster extraction of the organic analytes than with the Soxhlet extraction. The thimble is then elevated above the solvent and rinsed with freshly condensed solvent. One advantage against Soxhlet is the extraction time. The extraction time using soxtec was reduced considerably using a similar amount of sample and less organic solvent volume. For instance, Jeannot *et al.* [124] spent 5 hours in the extraction process using 10 g of sewage sludge mixed with 50 mL of methanol to determine some estrogens among other endocrine disrupting compounds, with recoveries similar to those obtained with Soxhlet extraction.

Using the classical extraction techniques described in this section, different groups of pharmaceuticals and personal care products have been extracted. Zeng *et al.* [98]

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determined the presence of polycyclic musk fragrances in sewage sludge from China using Soxhlet extraction followed by GC-MS analysis. The recoveries for all the compounds were between 48 and 82%. The compound that showed the maximum concentration in the samples was galaxolide (HHCB), whose concentration was between 5.416 and 21.214 mg/Kg.

**1.2.2.2. Ultrasonic extraction**

Extraction efficiencies may be improved by using high temperatures and high pressures or, as in the case of ultrasonic extraction (USE), by using ultrasound radiation to improve the contact of the solvent with the sample. The sample is introduced to an appropriate solvent in a tube. After the extraction, a centrifugation and clean-up step are usually needed. With this technique, short extraction time and low solvent volume are required in comparison to classical techniques. As with the classic techniques, three parameters must be optimized: the nature and volume of the solvent and the extraction time.

Different extraction solvents have been used to extract pharmaceuticals and personal care products from sewage sludge using USE as the extraction technique. In most cases, combinations of different solvents have been used in order to improve the extraction efficiencies. For instance, two extractions with methanol and two additional extractions with acetone have been used to extract four estrogens [61] or different pharmaceuticals, iodinated contrast media and musk fragrances [53]. In some cases, a mixture of solvents have been used such as methanol:water with different proportions [100,110,122] or methanol:0,1M acetic acid: 5% $Na_2$-EDTA (2:1:1) [84].

Although the nature of solvent is similar to the solvent used with Soxhlet extraction, there are two differences between USE and Soxhlet extraction: the volume of the solvent and the extraction time. Table 5 summarizes the main extraction techniques for solid samples and the principal characteristics of each one. As we can see in Table 5, less solvent is used in USE in comparison to Soxhlet extraction. The volume of solvent used in USE is between 8 and 25 mL. The amount of sample when USE is used to extract PPCPs is between 20 mg and 2 g. For instance, 20 mg of sewage sludge was mixed with 8 mL of methanol:water (6:4) to extract triclosan and some endocrine disrupting compounds and the recoveries were between 51 and 102% [100]. In the study of Ferdig *et al.* [122] 1 g of sample was used to extract nine fluroquinolones from sewage sludge using 10 mL of methanol:water (3:7).

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**Table 5.** Comparison of extraction techniques for solid samples.

| | Shaking Extraction | Soxhlet | USE | SFE | MAE | PLE |
|---|---|---|---|---|---|---|
| **Selectivity** | low | low | low | high | low | low |
| **Amount of sample (g)** | 60-70 | 10-100 | 0.02-2 | 1-10 | 0.5-5 | 0.5-5 |
| **Extraction time** | 1-10 h | 6-24 h | 5-60 min | 10-60 min | 5-60 min | 3-30 min |
| **Solvent consumption (mL)** | 30-200 | 150-300 | 8-25 | 10-20 | 15-50 | 15-50 |

As it is shown in table 5 another important difference between USE and Soxhlet extraction is the extraction time. As we mentioned above, Soxhlet extraction takes between 6 and 24 hours whereas USE extraction takes only between 5 and 60 minutes depending on the target analytes. In the paper of Gatidou *et al.* [100] the different parameters that affect USE extraction were exhaustively evaluated. They studied the effect of the amount of sample, the extraction solvent, the extraction temperature and the extraction time. Regarding the extraction time, they observed that when the sewage sludge sample was extracted for 15 minutes, the recoveries ranged from 7 to 63%, indicating that the time was not enough for the sufficient extraction of the target compounds. Increasing the duration of the extraction from 15 to 30 or 45 minutes saw a significant improvement in the recoveries of the compounds. A longer extraction time (60 min) resulted in a reduction of the recoveries, probably due to the degradation of the compounds. The optimum extraction time was 30 minutes with recoveries of between 60 and 100%.

As we mentioned before, sometimes multiple extractions with fresh solvents lead to greater recoveries in reasonable extraction times [53,61]. Depending on the polarity of the analytes under study, the extraction solvents can be different in each extraction. For example, Ternes *et al.* [61] used two times methanol in the solvent extraction (4 + 3 mL) followed by two extractions with 3 mL of acetone, to extract estrone, 17β-estradiol, 17α-ethinylestradiol and mestranol from sewage sludge.

After USE extraction, two steps can be done prior to the chromatographic separation: centrifugation of the extract and a clean-up step. Centrifugation is usually used to separate the solvent and the sewage sludge. Sometimes this process takes as long as the extraction process. The clean-up step, as for other extraction techniques, can be done using SPE cartridges with $C_{18}$ as Gatidou *et al.* [100] did to determine endocrine disrupting compounds or with Strata-X as Okuda *et al.* [110] did to determine 66 pharmaceuticals and personal care products from sewage sludge. Moreover, in some

ISBN:978-84-693-6426-0/DL:T-1628-2010

cases, GPC with silica gel has been used as the clean-up step to determine a group of estrogens [61].

In this last example, Ternes *et al.* [61] determined four estrogens in two types of sewage sludge: activated and digested sludge. Estrone and 17β-estradiol showed the maximum levels in the samples (up to 37 ng/g and 49 ng/g (d.w.), respectively). 17β-estradiol was present in both activated and digested sludge at concentration levels of between 5 and 49 ng/g (d.w.). Another compound that was present in 3 out of 4 samples analyzed was 17α-ethinylestradiol at a concentration of between below the limit of quantification and 17 ng/g (d.w.). On the other hand, mestranol was always present at concentration below the limit of quantification (2 ng/g (d.w.)).

Ternes *et al.* [53] also used USE extraction to determine pharmaceuticals, iodinated contrast media and musk fragrances using GC-MS and LC-MS-MS as a separation technique depending on the volatility of the analytes. Due to their polarity, musk fragrances were determined using GC-MS and the pharmaceuticals and the iodinated contrast media using LC-MS-MS. Several environmental sludge samples were analyzed. In samples from Switzerland, tonalide and galaxolide occurred in sludge at concentrations ranging between 2.3 and 15 mg/Kg (d.w.). In most cases, concentrations of galaxolide are about two times higher than those of tonalide. In samples from Germany, tonalide and galaxolide were found in concentration levels ranging from 1.4 mg/Kg (d.w.) (tonalide) to 6.5 mg/Kg (d.w.) (galaxolide). Among the pharmaceuticals and iodinated contrast media, only diclofenac was quantified in the study above the LOQ, with concentrations ranging between 0.20 and 0.45 mg/Kg (d.w.). All the other pharmaceuticals and iodinated contrast media were not detected above the LOQ.

USE extraction has also been used to extract fluoroquinolones using the LC-MS-MS [122]. The sewage sludge samples were from Austria and they contained considerable amounts of ofloxacin (510 μg/Kg (d.w.)), norfloxacin (150 μg/Kg (d.w.)) and ciprofloxacin (230 μg/Kg (d.w.)), whereas the content of moxifloxacin was only 30 μg/Kg. Chenxi *et al.* [84] studied the presence of antibiotics in sewage sludge from The USA. In this case, the method used was USE followed by SPE and LC-MS-MS. Six antibiotics (ciprofloxacin, tetracycline, doxycycline, clarithromycin and erythromycin), one antiepileptic pharmaceutical (carbamazepine) and one antimicrobial (triclosan) were studied. All target compounds were detected in the sewage sludge analyzed. The highest concentration was 778 ng/g (d.w.) for ciprofloxacin, followed by triclosan (320 ng/g (d.w.)). Low concentration was detected for the macrolides, 3.6 ng/g (d.w.) for erythromycin-$H_2O$ and 5.2 ng/g (d.w.) for clarithromycin.

The presence of 66 pharmaceuticals was also determined in sewage sludge from Japan using USE as the extraction technique [110]. After the extraction, cleanup was conducted with solid-phase extraction and measurement was done with LC-MS-MS.

ISBN:978-84-693-6426-0/DL:T-1628-2010

The recoveries obtained using this method in primary sludge were between 40 and 130%. As many as 56 out of 66 compounds were detected and the concentrations ranged from ng/g to μg/g. The compounds that showed the highest concentrations were levofloxacin, triclosan, mefenamic acid and clarithromycin.

#### 1.2.2.3. Supercritical fluid extraction

Supercritical fluid extraction (SFE) uses a solvent in supercritical conditions. The supercritical fluids showed intermediate conditions between a gas and liquid, thus allowing penetration in different matrices and, consequently, solubilization of the analytes. The supercritical fluid most used is $CO_2$ because it has a critical temperature (Tc = 32 ºC) near to the ambient temperature and a moderate critical pressure (Pc = 72 atm). At this temperature, different thermolabile compounds can also be extracted.

SFE has been applied to extract organic contaminants from sludge samples because it overcomes some of the disadvantages of classic techniques. For instance, SFE mainly uses $CO_2$ as the extraction fluid and this is more environmentally friendly than the solvents used in other extraction techniques. However, its extraction efficiency is highly dependent on the matrix, which means that optimization procedures are tedious. The decrease of current interest in SFE is also partly due to the development of PLE and MAE, which have become widely accepted extraction techniques as we will comment below.

The most significant advantages of the SFE technique are the preconcentration effect it has, its cleanness and safety, its quantitativeness, and its simplicity and selectivity. Different parameters must be optimized when this technique is used. The most important are: pressure, temperature, modifier, extraction time, the extraction mode (dynamic, static or combination of both) and sample weight. As we mentioned above, although different extraction solvents can be used, $CO_2$ is the most common because of its reasonable critical properties.

Fluid pressure is the main parameter for extracting analytes from different matrices. The modification in the relationship between pressure and temperature can enhance the solvating power of the supercritical fluid. Cheng *et al.* [96] increased the fluid density from 0.25 to 0.6 g/mL in the extraction of one of the most abundant phthalate - DEHP. The recovery increased drastically from 15% to 92%. Thereafter, an increase in fluid density from 0.7 to 0.95 g/mL reduced DEHP recovery considerably. This can be attributed to the increase in solvating power of the fluid at higher density.

Another parameter that affects the recoveries in this extraction technique is the temperature. At constant pressure, the density of fluid decreases with an increase in temperature. The decrease in fluid viscosity increases the diffusion coefficient.

ISBN:978-84-693-6426-0/DL:T-1628-2010

Although increasing extraction temperature might accelerate the thermal desorption behavior of organics from different matrices, it can lower the fluid strength and adversely affects its ability to accept the analytes that have escaped from the matrix. However, a high extraction temperature can cause sample degradation or boiling during the extraction process, especially for volatile compounds. Different extraction temperatures have been used; for instance, 80 ºC was used to extract polycyclic and nitro musk fragrances [85,97].

The use of a modifier is a very important parameter in the optimization of SFE conditions. Cosolvents and/or modifiers are often used to enhance the solvating power, especially when pure carbon dioxide is used as an extraction fluid. Methanol is most commonly used as a modifier in the extraction of sewage sludge due to its ability to enhance the desorption rate of contaminants from the matrix. The application of the modifier mainly depends on the composition and characteristics of the sample matrix. Although methanol is the most common modifier, in the study of Lee *et al.* [97], SFE was applied to the extraction of polycyclic musk fragrances from sewage sludge and, using acetone:dichloromethane (1:1) as a modifier, the recoveries were higher than 87%. In addition to the modifier effect, when the sludge sample is wet the polarity of $CO_2$ can be enhanced during SFE. Although no studies on the influence of wet samples were published to determine pharmaceuticals, Cheng *et al.* [96] studied the influence of wet samples to extract DEHP. The recovery when the sewage sludge sample was wet was 73%, whereas, when the sample was completely dry the recovery was 100%.

Three different types of extract can be performed using SFE: static, dynamic or a combination of both modes. The static mode allows the supercritical fluid to better penetrate the matrix, while the dynamic mode continuously provides fresh $CO_2$ to the extraction cell and plays the role of carrier to replace the mixtures remaining in the extraction cell. In this extraction mode, the combination of fluid flow-rate and dynamic extraction time affects both the desorption rate and the solubility of the analytes. However, the combination extraction mode is preferred because of its complementary effects. For instance, Smyth *et al.* [85] and Lee *et al.* [97], used the combination of static and dynamic modes to extract polycyclic and nitro musk fragrances. In these cases, 2 minutes of static time, followed by 20 minutes of dynamic time using a flow of 2 mL/min, was used.

In all cases the sample weight was around 100 mg, for instance, to extract polycyclic and nitro musk fragrances [85,97]. In order to improve the contact between the sample and the fluid, the cell was filled with different inert materials such as $Na_2SO_4$ and quartz sand to extract DEHP [96] or $Na_2SO_4$ together with acid-cleaned copper powder to extract some endocrine disrupting compounds [88].

After the extraction, the analytes are collected by two different methods: retained in a solid-phase trap or in a solvent. For instance, a $C_{18}$ trap was used to retain

ISBN:978-84-693-6426-0/DL:T-1628-2010

polycyclic and nitro musk fragrances [97]. The fragrances were eluted with acetone in two aliquots of 1.5 and 1.0 mL. To extract the same musk fragrances, silica gel was also used to collect the analytes after the extraction [85]. In this case the elution was done using 10 mL of 5% acetone in hexane. The Popak-Q solid phase was used to collect DEHP [96]. The elution of DEHP from this trap was achieved using 1.5 mL of hexane. However, in the study of Meesters and Schröder [88], the solvent trap is used when the depressurized fluid is conducted to a vial containing an organic solvent such as toluene.

Only one group of PPCPs - polycyclic and nitro musk fragrances - has been extracted using SFE extraction. Lee *et al.* [97] and Smyth *et al.* [85] both studied their presence in sewage sludge from Canada. In both methods, 200 mg of sewage sludge was used as a sample and the void volume inside the thimble was reduced by a piece of glass rod of a suitable length and diameter. The extraction temperature was 80 ºC using non-modified $CO_2$. In this method 2 minutes of static time and 20 minutes of dynamic time was used and the analytes were trapped in an $C_{18}$ trap [97] or in silica gel [85]. As already mentioned, these compounds are usually determined using GC-MS due to their high volatility. In the samples analyzed by Lee *et al.* [97], the compounds that showed the highest concentrations were galaxolide and tonalide whose concentrations were between 1.34 and 20.8 mg/Kg and between 5.36 and 20.8 mg/Kg for galaxolide and tonalide, respectively. On the other hand, celestolide and phantolide were present in all samples at concentrations lower than 1 mg/Kg. Only two nitro musk fragrances were found in the sewage sludge, with concentrations between 1.8 and 422 μg/Kg and 2.4 and 347 μg/Kg for musk xylene and musk ketone, respectively.

#### 1.2.2.4. Microwave assisted extraction

In recent years, microwave assisted extraction (MAE) has caused great interest because this technique allows fast extraction with efficiencies comparable to those obtained by classic techniques. As we can see in Table 5, MAE has some advantages over the classical technique such as the short extraction time (between 5 and 60 minutes per sample) and, most importantly, the low volume of organic solvent consumed. This technique also allows some samples to be extracted simultaneously.

In comparison to the newer techniques such as PLE, the instrumental cost is lower and the experimental conditions are easy to optimize. Nowadays, this extraction technique has been applied to extract organic contaminants from solid matrices. Numerous applications of MAE have been published to determine compounds such as organochlorine pesticides [140], antimicrobials [102], surfactants [121] among others, in different kind of solid samples.

ISBN:978-84-693-6426-0/DL:T-1628-2010

MAE consists of heating the solvent in contact with the sample using microwave energy. The partition of the target analytes from the sample to the solvent depends on the temperature and the nature of the solvent. When MAE is used, the entire sample is heated simultaneously. Because of this the solvent reaches boiling temperature faster than in classic extraction techniques, so the extraction time is shorter.

The principles of microwave heating imply that the solvent must be chosen for its ability to absorb microwaves, which is defined by its dielectric constant. Non-polar solvents do not absorb microwave energy and therefore are less efficient at extraction than polar solvents. In fact, the addition of small amounts of water improves the absorption of microwave energy by non-polar organic solvents and the extraction of analytes.

Two different modes have been used in MAE to extract organic compounds from solid samples: the closed-vessel type and open-vessel type. In the closed-vessel type the temperature and pressure are controlled whereas in the open-vessel type atmospheric pressure is used during the extraction.

The closed-vessel type is the most common mode to extract these contaminants from sewage sludge. In this case, the solvent can be heated above its boiling point at atmospheric pressure. So, the extraction time and the extraction efficiencies are improved significantly. This system allows the temperature and the extraction process to be controlled, and commercial equipment can extract several samples simultaneously and use considerably less solvent. One disadvantage of this mode is that volatile compounds can be degraded because the high temperature is reached in a short time inside the cell.

The vessels used in MAE are relatively transparent to microwave radiation and are inert to the solvents used. The material in contact with the samples is usually quartz, fluoropolymer or glass.

The most important parameters that affect MAE are the nature and volume of solvent extraction, the extraction time and the microwave power.

In order to choose the appropriate solvent, different characteristics have to be studied. For instance: the ability to absorb the microwave radiation, the interactions between the solvent and the matrix and the ability to dissolve the analyte. Different mixtures of solvents have been used in MAE such as methylene chloride:methanol (2:1) to extract different PPCPs from sewage sludge such as caffeine, estradiol, ibuprofen and ketoprofen among others [107] and acetone:methanol (1:1) to extract triclosan and trichlorophenols [102].

Another important parameter related to solvent extraction is the solvent volume. The solvent volume must be enough to immerse the sample in the solvent. In the literature, the extraction cells have a volume of 100 mL and the volume of solvent is

ISBN:978-84-693-6426-0/DL:T-1628-2010

between 20 and 30 mL. For instance, 30 mL of acetone:methanol (1:1) was used to extract triclosan and chlorophenols [102]. Morales *et al.* [102] using a full factorial design, studied the effect of solvent volume. Two different volumes were tested: 15 and 30 mL, 30 mL being the optimum solvent volume to extract triclosan and tricholophenols.

As with other extraction techniques, the extraction time has a great influence on extraction efficiencies and must be considered carefully. The extraction time in microwave assisted extraction is shorter than the extraction time of classical techniques. Extraction times of between 15 and 20 minutes have been used in different studies. For example, 15 minutes of extraction time were used to extract a group of pharmaceuticals and personal care products such as caffeine, estradiol, ibuprofen and ketoprofen among others [107] and 20 minutes of extraction time were used to extract triclosan and two chlorophenols [102].

Although MAE extraction was not used to extract PPCPs in the studies of Fountoulakis *et al.* [121] and Shin *et al.* [99], we would like to mention them because they compare MAE with Soxhlet and USE [121] or with Soxhlet extraction [99] to extract PBDEs and nonylphenol and nonylphenol ethoxylates from sewage sludge. In this last study, the recovery efficiency of MAE was compared to that of conventional Soxhlet extraction by examining the recoveries of PBDEs at low and high concentrations [99]. At high levels, Soxhlet extraction methods gave more than 90% recoveries for all PBDEs, with the exception of a significantly lower recovery for BDE 209 (35%). The MAE method exhibited a significant improvement in BDE 209 recovery as well as a modest improvement for most PBDE congeners. At low levels, both extraction techniques showed roughly comparable recoveries for most PBDE congeners, ranging from 85 to 110%. Then, using MAE extraction, they saved both time (Soxhlet extraction took 16 h whereas MAE extraction took only 15 min) and solvent volume (300 mL in Soxhlet and 30 mL in MAE).

To extract different pharmaceuticals and personal care products from sewage sludge, microwave assisted extraction as an extraction technique has been used. For instance, in the study of Rice *et al.* [107], four compounds were determined using MAE/GC-MS. The sludge samples studied were from The USA and samples were collected in August and April. The conditions used to extract these compounds were as follows: 30 mL of methylene chloride:methanol (2:1) was used, the temperature was 115 ºC and the extraction time was 15 minutes. After the extraction, the sample was centrifuged for 5 minutes. Compounds quantified in these samples were ketoprofen, ibuprofen, naproxen and epicoprostanol, all of them were detected at low ng/g or μg/g (d.w.).

In samples from Spain, triclosan and two related chlorophenols - 2,4,6-trichlorophenol (TCP) and 2,4-dichlorophenol (DCP) - were determined using MAE and GC-MS-MS [102]. The method developed was applied to the analysis of several

ISBN:978-84-693-6426-0/DL:T-1628-2010

grab samples from urban STPs. The optimum conditions in MAE extraction were: 0.5 g of sample, 30 mL of acetone:methanol, and 130 ºC during 20 minutes. After the extraction step, the clean-up step consisted of a centrifugation of the sample and a SPE extraction using Oasis HLB cartridges. All compounds were detected at concentrations from low ng/g in the case of 2,4,6-TCP up to 5.4 μg/g for triclosan.

#### 1.2.2.5. Pressurized liquid extraction

In the last decade, a new extraction technique has been used to extract different pharmaceuticals and personal care products from sewage sludge. Pressurized liquid extraction (PLE) has emerged as an efficient way to increase automation, shorten processing times and reduce the amounts of solvent required to extract the target analytes because it uses high temperature and pressure. Compared to other extraction techniques, PLE provides good recoveries for most compounds and so it has currently become one of the preferred techniques.

Because PLE is the extraction technique used in this Thesis, a review of this technique has been completed and submitted to Trends in Analytical Chemistry as a revision to the literature on the extraction of pharmaceuticals and personal care products from sewage sludge. Hereafter, to avoid repetition of information, we attach a copy of the manuscript that has been accepted in this journal for publication. In this paper, we also include the most relevant results of this Doctoral Thesis because this revision was done after the experimental part of it.

UNIVERSITAT ROVIRA I VIRGILI
EMERGING ORGANIC CONTAMINANTS IN SEWAGE SLUDGE
Antonio Nieto Cebrián
ISBN:978-84-693-6426-0/DL:T-1628-2010



# PRESSURIZED LIQUID EXTRACTION: A USEFUL TECHNIQUE TO EXTRACT PHARMACEUTICALS AND PERSONAL CARE PRODUCTS FROM SEWAGE SLUDGE

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**Abstract**

In this paper we review the determination of pharmaceuticals and personal care products (PPCPs) in sewage sludge samples using pressurized liquid extraction (PLE) as an extraction technique. PLE is a sample preparation technique that is increasingly used to extract moderately volatile and non-volatile organic contaminants from solid samples. We discuss the principal parameters to be optimized in PLE such as solvent extraction, temperature, pressure and extraction time, among others. We also examine the occurrence of PPCPs in the studies where PLE is applied in sewage sludge.

**Keywords:** Pressurized liquid extraction, pharmaceuticals, personal care products, sewage sludge.

## INTRODUCTION

The occurrence of pharmaceuticals and personal care products (PPCPs) in sewage sludge samples has been the object of increasing interest in recent years, as is indicated by the growing number of scientific papers published on the subject [1].

PPCPs are included in the list of so-called "emerging organic contaminants". These pharmaceuticals and personal care products are used as ingredients in soaps, lotions, toothpaste and other products. Some PPCPs are used in large amounts which causes them to persist in the environment and to bioaccumulate. Pharmaceuticals enter sewage treatment plants (STPs) mainly by the excretion from the human body [2]. In addition, PPCPs enter STPs as a result of manufacturing processes and the disposal of expired products. Finally, these contaminants are also present in sewage sludge due to their total or partial elimination from influent wastewater during treatment in the STP.

It is important to determine the levels of these contaminants so that sewage sludge can be reused as manure in agriculture and although at present there is no regulation of organic pollutants, the use of contaminated

sewage sludge can re-introduce contaminants into the environment.

Different methods have been developed to determine PPCPs in sewage sludge, all of which consist of the extraction and the chromatographic analysis. In Figure 1, we summarize the steps of the analytical methods used. Various extraction techniques have been used such as Soxhlet extraction [3], ultrasonication extraction (USE) [4,5] and supercritical fluid extraction (SFE) [6].

In recent years, environmentally friendly extraction techniques such as microwave assisted extraction (MAE) [7-9] and pressurized liquid extraction (PLE) [10-12] have emerged as efficient ways of increasing automation, shortening the extraction time and reducing the amount of organic solvents [13].

PLE uses high pressure and temperature without reaching the critical point. The first applications of pressurized liquid extraction using sewage sludge as the matrix focused mainly on inorganic metals [14], polycyclic aromatic hydrocarbons (PAHs) [6] and polychlorinated byphenils (PCBs) [3,15]. The first paper using this technique was published in 1996 [16] and described the determination of PAHs from different solid samples. However, nowadays, different methods have been developed for determining PPCPs in sewage sludge [17] by using PLE.

Among the many advantages that PLE has over traditional techniques such as Soxhlet extraction and ultrasonication extraction are in general short extraction time, low solvent consumption and additional extract filtration, which is done by adding the inert material to the extraction cell [11,18]. The advantage of PLE over new techniques such as MAE is that PLE is not limited to those extraction solvents that can absorb microwaves. As with other extraction techniques, the main limitations of PLE are that the selectivity towards the analytes during extraction is not as high as might be desired and the fact that many interferents may be coextracted depending on the kind of sample. Another disadvantage is that the analytes are sometimes diluted, especially when a high number of cycles is used [19].

A clean-up step is sometimes needed between the PLE extraction step and the chromatographic analysis to prevent this dilution and/or to clean up the samples. The most common technique for this clean-up is solid-phase extraction (SPE) [10,11,20,21] with a variety of sorbents. In addition to cleaning, SPE also preconcetrates the analytes. Another method used to purify the extract is gel permeation chromatography [22].

As has been mentioned, a chromatographic technique is used after the clean-up step. Gas chromatography [22,23] or liquid chromatography [24,25] are used depending on the volatility of the PPCPs. Most PPCPs lack sufficient volatility which means they are not directly compatible with GC. Various

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groups of PPCPs may be derivatized to make them suited for GC analysis. This step can be drawn out and can introduce errors due to side-reactions. Despite the advantages of using GC procedures to analyze the residue of certain classes of pharmaceuticals, LC is much more applicable. Mass spectrometry detection is now the preferred detection system due to its confirmation power and to the low concentration of these contaminants in sewage sludge. Although single quadrupole instruments were successfully used when LC-MS procedures were first developed to determine residues of pharmaceuticals and personal care products [26], nowadays more complex mass analyzers such as triple quadrupole instruments are commonly employed which allow the target analytes to be unequivocally identified [10,27]. With a combination of PLE, LC and mass spectrometry or tandem mass spectrometry LODs at low μg/Kg levels of dry weight (d.w.) can be obtained [28,29].

The aim of this paper is to discuss the principal parameters for optimizing the extraction procedure and the state of the art of PLE in order to determine pharmaceuticals and personal care products in sewage sludge.

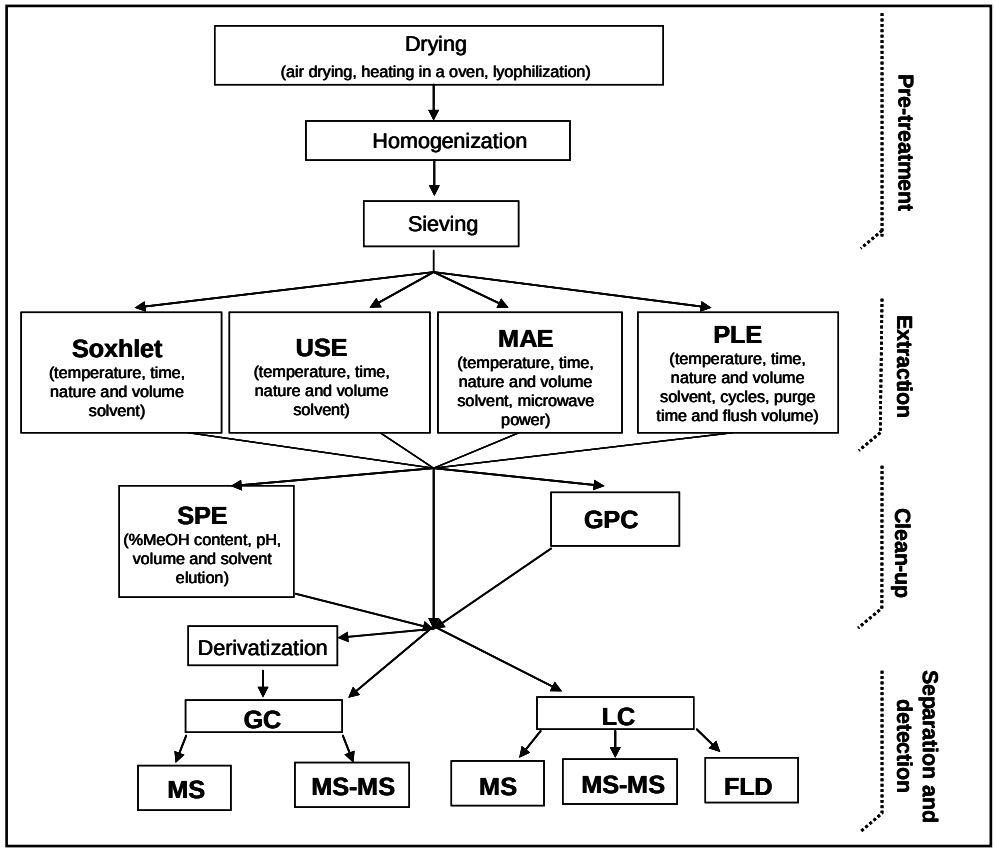


**Figure 1.** Steps in the analytical methods for determining PPCPs in sewage sludge.

ISBN:978-84-693-6426-0/DL:T-1628-2010

## 2. PLE Extraction

### 2.1 General aspects of PLE

PLE is also known as pressurized fluid extraction (PFE), enhanced solvent extraction (ESE), high pressure solvent extraction (HSPE) or, most popularly, by the Dionex trade name, accelerated solvent extraction (ASE™). PLE is regarded as a reasonably uncomplicated, exhaustive extraction technique which is easy to learn and which provides quantitative recoveries with little time spent on method development. Once the sample is introduced into the cell and mixed with the inert material, the cell is placed on the carousel. The carousel rotates the sample cell into position to transfer it to the oven chamber. The cell is then transferred to the oven and automatically sealed under pressure before being heated and pressurized. After preheating, the cell is filled with the solvent and kept in the oven at constant temperature and pressure for a user-set static time. The solvent, which contains the extracted analytes, is collected in a vial and the cell is then flushed and purged with nitrogen gas. Together these steps constitute a cycle and can be repeated several times if necessary. The total extraction time is normally between 15 and 45 minutes, although sometimes higher extraction time is necessary.

As has been mentioned, PLE works at high temperatures (usually up to 200 ºC) and high pressure (usually up to 200 bar) to quickly extract with low volumes of organic solvents and it provides similar recoveries to other techniques. Parameters that significantly affect these recoveries are the extraction solvent, the temperature, the pressure, the static extraction time, the number of cycles, and the sample weight. Other parameters such as purge time and flush volume have shown little influence on the final recoveries, so these are usually fixed. Each parameter can be optimized separately [10,11,26] or by using an experimental design [30].

### 2.2 Sample pre-treatment before PLE

A pre-treatment of the sample is needed to assure a good contact between the solvent of the extraction process and the matrix. The pre-treatment usually consists of three steps. The first involves drying the sewage sludge; that is, once the sludge has been sampled, it is usually frozen at -18 ºC until analysis. The water can be eliminated using three different strategies: air-drying [31,32], heating in an oven [6] or lyophilization [26,33]. The temperature applied to different analytes is critical. For example, if the analytes degrade when heating in an oven, a lower temperature has to be applied, which means that more time is required to dry the sample. On the other hand, if samples are lyophilized, the analytes are not evaporated or degraded and time is saved.

The second step consists of homogenization by grinding, and the

ISBN:978-84-693-6426-0/DL:T-1628-2010

third step involves sieving to ensure that the particles of the analytes are similar in size. Particles selected for PLE are usually smaller than 2 mm. Due to the pre-treatment applied, the concentrations of the target analytes are given as dry weight (d.w.).
Among all the drying treatments mentioned, the lyophilization is the most advantageous because compounds are not degradated and moreover, the drying time is shorter than in the other treatments.

**2.3 Parameters optimized in PLE**

Different parameters can be optimized in order to obtain the highest recoveries. Some of these parameters are highly influence on the recoveries. As we have mentioned before, the most importants are the extraction solvent, the temperature, the pressure, the static time and the number of cycles. On the other hand, some parameters such as purge time and flush volume are fixed in accordance with the literature because they have to be higher enough to ensure the recovery of all compounds extracted. The sample weight is another parameter that must be optimized, although this parameter depends on the kind of sample and the cell volume. In the following section we discuss each parameter and the most habitual values for each one.

**2.3.1 Extraction solvent**

As for most solid-liquid extraction techniques, the extraction solvent is one of the most important parameters to optimize in PLE. Several extraction solvents or mixture solvents can be used to ensure that the polarity of the solvent is similar to that of the target compounds. In the case of PPCPs the most common organic solvents are methanol and acetonitrile [25,27,34].
Methanol has been used to extract PPCPs in two different studies. Ternes *et al.* [34] used methanol to extract fifteen pharmaceuticals, seven iodinated contrast media and two musk fragrances from sewage sludge. In order to obtain the best recoveries, 2 cycles of 5 minutes were used and the recoveries were between 37% and 78% for acidic and neutral pharmaceuticals, between 48% and 119% for iodinated contrast media and between 78% and 109% for musk fragrances. Three other compounds extracted using methanol were the phophodiesterase type V inhibitors: sildenafil, vardenafil and tadalafil [27]. In this case, using 2 cycles of 5 minutes gave recoveries of between 45% and 103%.
In some studies, less polar solvents such as ethyl acetate and dichloromethane have been used to extract less polar compounds such as musk fragrances (among other organic compounds) [23] and antimicrobials such as triclosan and triclocarban [25]. In both studies [23,25] recoveries higher than 90% were obtained for all compounds studied.
Mixtures of water with pure solvents such as methanol and acetonitrile (among others) have also been used in

ISBN:978-84-693-6426-0/DL:T-1628-2010

order to obtain higher recoveries. Golet *et al.* [12] tested different aqueous mixtures in combination with organic solvents (acetonitrile, methanol or 2-propanol) to extract fluoroquinolones from sewage sludge. Different proportions of a mixture of water:acetonitrile were tested (1:1, 3:1, 1:3). The extraction efficiency varied slightly in terms of the aqueous/organic solvent ratio, with the highest recoveries obtained at ratio of 1:1. The mixture with acetonitrile showed recoveries 10% higher than the recoveries obtained using methanol or 2-propanol. When using water as the extraction solvent, it is important to study the water pH. Golet *et al.* [12] tested the influence of pH that ranged from acidic (pH 2) to basic (pH 11) on the extraction efficiencies of fluoroquinolones and maximum recoveries achieved at acid pH. At basic pH the recoveries were slightly higher than at neutral pH (pH 7), although this may be explain by the fact that aqueous solubility of fluroquinolones increases at extreme pHs and its minimum at neutral pHs (zwitterionic form).

One study by our group [35] determined ten pharmaceuticals in sewage sludge. We tested mixtures of water:methanol and water:acetonitrile, and observed that higher recoveries were obtained using methanol instead of acetonitrile. When the water:methanol mixture was used, water at different pHs was tested in order to obtain the maximum recoveries. Recoveries higher than 71% were obtained for all compounds except for salicylic acid (whose recovery was lower than 5%), when acidic (pH 3) water was used.

Table 1 and 2 show that, in addition to the water:methanol mixture, other mixtures have been used to extract pharmaceuticals and personal care products from sewage sludge. For example, acetone (acetone:water (3:7)) was used to extract carbamazepine and its metabolites [36] and recoveries between 80% and 93% were obtained by using 3 cycles of 5 minutes. A mixture with hexane (acetone:hexane (1:1)) was also used to extract synthetic musk fragrances [22] because these compounds are less polar than pharmaceuticals and gave recoveries of between 86% and 100%.

In studies where different groups of pharmaceuticals with different polarities were determined, different combinations of solvents in each cycle were needed. For instance, in one study our group used two different mixtures [33] and extracted a group of estrogens and their conjugates from sewage sludge. Two cycles of methanol:acetone (1:1) followed by two with methanol:water (1:1) were used as the extraction solvents because the first mixture extracted the non polar compounds and the second mixture extracted most of the polar compounds. The recoveries of estradiol 3-sulfate and estrone 3-sulfate increased by between 30% and 40% when the mixture of methanol:water (1:1) was used. The same also occurred when methanol in 2 cycles and then methanol:water (1:1)

ISBN:978-84-693-6426-0/DL:T-1628-2010

in 1 cycle were used to extract various personal care products [30].

As we can conclude from the Table 1 and 2, the solvents or mixtures of solvents used to extract pharmaceuticals and personal care products depend on the polarity of the compounds to be extracted. However, the mixture of methanol:water is the most used and gives higher recoveries for most of PPCPs, due to their high polarity

**2.3.2 Temperature and pressure**

Temperature and pressure are important parameters relating to the optimization of PLE. The temperature needed to extract PPCPs usually varies from 50 ºC to 130 ºC. Higher temperatures decrease the viscosity of liquid solvents, thus allowing better penetration of the matrix particles and enhancing extraction. In addition to reducing viscosity, high temperatures will also decrease the surface tension of the solvent, the solutes and the matrix, allowing the solvent to "wet" the sample matrix more thoroughly. Maximum temperature is limited by the degradation temperature of each analyte. However, an increase in temperature may cause some interferences to be co-extracted with target analytes [12] and the selectivity of the extraction to decrease. The most common temperature is between 80 ºC and 100 ºC; for instance, 100 ºC has been used to extract different groups of compounds such as pharmaceuticals (analgesics, vasodilatators, β-blockers and antibiotics (sulfonamides, macrolides and fluoroquinolones) among others) [11,12,21,27,34,35], and personal care products (antimicrobials, parabens and UV filters) [30].

As mentioned above, although the most commonly applied temperatures are between 80 ºC and 100 ºC, higher and lower temperatures have been used to extract different PPCPs from sewage sludge. For example, Ligon *et al.* [23] used a higher temperature (130 ºC) to extract organic phosphates, musk fragrances and plasticizers. On the other hand, Barron *et al.* [20] used 60 ºC to extract different pharmaceuticals from sewage sludge and obtained recoveries higher than 50% except for salbutamol, salicylic acid and sulfamethazine. Chu *et al.* [25] also used 60 ºC as the extraction temperature to determine two antimicrobials, and obtained recoveries of 80% and 100% for triclosan and triclocarban, respectively. Lower temperatures are commonly used to avoid analyte degradation or coextraction of interferents [11]. Accordingly, Göbel *et al.* [11] reported that increasingly darker extracts were obtained at higher extraction temperatures or pressures, which indicates that larger amounts of soluble organic matter that is also extracted when extracting sulfonamides, macrolides and trimethoprim.

Despite the influence of temperature on recoveries, few papers have studied this in depth. One exception is the study by Golet *et al.* [12] who studied the effect of temperature on

the extraction efficiency of two fluoroquinolones (ciprofloxacin and norfloxacin) by varying the temperature from 50 to 150 ºC with increments of 25 ºC. From 50 to 100 ºC the extraction efficiency increased; however, between 100 and 150 ºC the extraction efficiency remained constant, thus a temperature of 100 ºC was selected as the optimum extraction temperature. In one study by our group [33], five different temperatures (between 25 and 125 ºC) were tested to extract estrogens and their conjugates from sewage sludge. We observed that the recoveries increased by between 16% and 45% when the temperature increased from 25 to 75 ºC, and when the temperature increased from 75 to 125 ºC the recoveries decreased significantly.

When temperatures above the solvents' boiling points at atmospheric pressure are used, the pressure must be high enough to keep the solvent in the liquid state. The equipment usually has a range of pressure between 75 and 200 bar. The use of high pressure should help extract the analytes from those samples in which the analytes have become trapped in matrix pores. The pressure forces the solvent into areas of the matrices which it would not normally reach under atmospheric conditions. As Table 1 and 2 show, in most cases the pressure was set at 100 bar to extract different groups of pharmaceuticals and personal care products such as sulfonamides, macrolides and analgesics, among others. Only a few studies have found it necessary to use pressures higher than 100 bar. For instance, Osemwengie [22] used a pressure of 140 bar to extract synthetic musk fragrances. In a study by our group [30], we also used 140 bar to extract antimicrobials, parabens and UV filters from sewage sludge. However, some studies in the literature report that pressure has little effect on the extraction efficiencies [12,26]. In one of these studies [26], no significant change was recorded in the recoveries when three different pressures (60, 100 and 140 bar) were used to extract two groups of antibiotics (sulfonamides and macrolides) and other compounds such as omeprazole, trimethoprim and ranitidine. Golet *et al.* [12] observed a 10% difference in the recoveries of two fluoroquinolones (ciprofloxacin and norfloxacin) when the pressure was increased from 50 to 100 bar; when the pressure was increased from 100 to 150 bar the recoveries decreased.

The selected temperature and pressure should be high enough to extract the target analytes but low enough to do not extract interferences from the sewage sludge.

### 2.3.3 Static extraction time and number of cycles

The static extraction time and the number of cycles are related parameters which also have a strong influence on extraction efficiency. The static extraction time should be long enough to ensure contact between the analytes and the solvent. This

ISBN:978-84-693-6426-0/DL:T-1628-2010

parameter is usually set at 3 - 15 minutes to extract PPCPs in sewage sludge. The number of cycles is the number of times in which a fresh solvent gets into the cell and is in contact with the samples. This parameter is usually set from 1 to 4 when extracting PPCPs. The long exposure to the solvent allows the matrix to swell, thus improving the penetration of solvent into the sample interstices and the contact between the solvent and the analyte. On the other hand, splitting the extraction time from one into more cycles maintains a favourable solvent/sample equilibrium and thus improves partitioning into the liquid phase. As Table 1 and 2 show, different combinations of static times and numbers of cycles have been used to extract PPCPs. The most usual combination is 5 minutes of static time and 3 cycles. This combination has extracted various compounds such as several pharmaceuticals [11,21,36] and some personal care products [10,25].

Sometimes a shorter time is enough to obtain acceptable recoveries. For instance, one cycle of different static extraction times (5, 10 and 15 minutes) has been tested to extract sulfonamides, macrolides and other pharmaceuticals [26]. The highest recoveries were obtained using 1 cycle of 5 minutes, with recoveries higher than 74%, except for ranitidine, whose recovery was 54%. When a 10 minute cycle was tested, no difference from the 5 minute cycle was observed; however, when a 15 minute cycle was applied, the recoveries decreased significantly. This may be because the target analytes had degraded after being kept for longer at a high temperature. On the other hand, there are studies in the literature where a high extraction time was needed to extract certain compounds. For instance, Ligon *et al.* [23] applied a 45 minute cycle to extract musk fragrances, antimicrobials and some organic compounds used in industry, and obtained recoveries from 48% to 127%. Golet *et al.* [12] also tested different static times and numbers of cycles for extracting norfloxacin and ciprofloxacin. The total extraction time varied from 20 (4 cycles of 5 minutes) to 80 minutes (4 cycles of 20 minutes). In this study, an increase in extraction efficiency was observed (approximately 10%) when the extraction time was increased from 20 to 40 minutes, but no significant improvement was noticed when it was extended to 60 or 80 minutes. Moreover, by increasing the continuous exposure to fresh solvent, the extraction efficiency improves substantially. For example, when 2 cycles of 20 minutes were used the recoveries were 20% lower than the recoveries obtained using 4 cycles of 20 minutes. Finally, sixty minutes (4 cycles of 15 minutes) was set as the optimum static extraction time and number of cycles.

An increase in the recoveries is observed in most of cases when fresh solvent is introduced in the cell. Therefore to increase the recoveries it is better to decrease the static time and increase the number of cycles.

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ISBN:978-84-693-6426-0/DL:T-1628-2010

### 2.3.4 Sample weight

Sample weight influences the method's limits of detection because the higher the amount of sample, the lower the limits of detection that are obtained. The most common range of sample weight for determining PPCPs is between 0.2 and 5 g, depending on the volume of the cell and the kind of matrix. The equipments can usually hold different extraction cells with volumes between 1 and 66 mL.

Two important conditions must be taken into account when choosing the correct sample weight. The first is the nature of matrix; if the matrix compacts when wet, as is the case with sewage sludge and clayey soils, then a lower sample weight must be used. The second is the volume of the cell; if the cell is filled to maximum capacity with the sample, then the cell may get blocked. Although the most common cells are of 11 mL with 1 gram of sample, larger cells have also been used. For instance, up to 5 grams of sample have been used with a 33 mL cell to extract ten pharmaceutical compounds [35].

### 2.3.5 Other Parameters

As we mentioned above, there are three parameters which do not significantly affect the recoveries of the target analytes and these are fixed in accordance with the literature to ensure good extraction efficiencies. The parameters are: preheating time, flush volume and purge time. Preheating time is the time when the cell is kept in the oven at the selected temperature before the solvent is added, 5 minutes usually being enough to ensure the cell is at the fixed temperature.

Flush volume is the percentage of fresh volume introduced into the cell after the static time to drag the analytes towards the collection vial. This volume ensures that all analytes are eluted and is closely related to the final volume. Different flush volumes have been used to extract PPCPs. For example, Göbel *et al.* [11] used a flush volume of 120% to extract macrolides and sulfonamides whereas, in a previous paper by our group [26], a flush volume of only 60% was enough to push the analytes extracted out of the cell. Apart from the flush volume, the temperature and the extraction time were different in each study. Göbel *et al.* [11] used 100 ºC and 3 cycles of 5 minutes, whereas in a previous study we used 80 ºC and 1 cycle of 5 minutes. Another parameter is purge time. During this time $N_2$ goes through the circuit and sweeps the solvent along. This time varies between 30 seconds and 300 seconds. For instance, in a previous study [26], our group used 120 seconds to extract a group of antibiotics, whereas Göbel *et al.* [11] only used 60 seconds to extract the same compounds. Other purge times that have been used in studies are: 30 seconds to extract a group of pharmaceuticals and iodinated contrast media [34], 90 seconds to extract parabens, UV filters and antimicrobials [30] and 300 seconds to extract fluoroquinolones

ISBN:978-84-693-6426-0/DL:T-1628-2010

[12] and a group of 10 pharmaceuticals [35].

**2.4 Post extraction treatment**

As we mentioned in the introduction, after the extraction step a clean-up step is sometimes necessary to reduce the limit of detection and to decrease the interferences. Table 1 and 2 show the different clean-up steps used in the literature. Gel permeation chromatography columns have been used to purify PLE extracts in order to determine certain pharmaceutical and personal care products [22]. SPE has also been applied as a clean-up and preconcetration technique. Common sorbents are $C_{18}$ and Oasis HLB [10,11]. For instance, Díaz-Cruz *et al.* [10] used a mixture of acetone: methanol (1:1) to extract nine sulfonamides and two penicillins. After the PLE extraction, the extracts were evaporated under a gentle $N_2$ stream and then reconstituted in a methanolic water solution (1 mL MeOH and 10 mL $H_2O$). The reconstituted extracts were then loaded onto Oasis HLB cartridges and the cartridges were washed with 3 mL of water and eluted with 3 mL of MeOH plus 3 mL of acetone. The resulting eluates were evaporated until dryness and reconstituted with 500 μL of MeOH for the LC-MS-MS analysis.

As Table 1 shows, in most cases the mixtures of solvents have a high organic solvent content which has to be evaporated or diluted with water in order to obtain higher recoveries in the SPE extraction [24]. However this problem can not be solved solely by diluting the extracts with water, as various studies have shown [23,26,33] where low limits of detection were obtained without this step because sensitive detection systems such as mass spectrometry or tandem mass spectrometry were used.

**3. Presence of PPCPs in sewage sludge**

The different methods using PLE described in literature has enabled the determination of these PPCPs in sludge, as can be seen in Table 1, which summarizes the range of concentrations found in sewage sludge samples of pharmaceuticals, and Table 2, which summarizes the concentration found of personal care products.

Among the PPCPs, antibiotics are the most prevalent group. A wide range of synthetic antibiotics is frequently used to treat infectious diseases in human and veterinary medicine. For this purpose antibiotics are designed to act very effectively even at low doses, and in case of intra-corporal administration, to be completely excreted from the body after a short time of residence, which results in them being released into the environment. Three groups of antibiotics have received the most attention: sulfonamides, macrolides and fluoroquinolones.

As Table 1 shows, sulfamethoxazole is the most studied compound in the group of sulfonamides and shows the

highest concentrations. A maximum of 68,000 μg/Kg (d.w.) was found in the sewage sludge from a sewage treatment plant in Switzerland [24]. However, lower concentrations were found in other countries such as Germany [11] and Spain [21,26] (between 34 and 100 μg/Kg (d.w.) and up to 21 μg/Kg (d.w.) respectively). Another sulfonamide that showed high concentrations in an STP from Switzerland was sulfapyridine (28,000 μg/Kg (d.w.)) [24].

Macrolides constitute another group of antibiotics whose main application is in the treatment of upper and lower respiratory tract infections, especially as an alternative to penicillins [24]. In all studies, the preferred solvent mixture for extracting this group of compounds was methanol:water (1:1). The temperature varied between 80 and 100 ºC. The normal extraction time was 3 cycles of 5 minutes; however, in a previous study [26], we showed recoveries higher than 83% for these compounds after just 1 cycle of 5 minutes. These compounds are found at high concentrations in sewage sludge samples from different countries due to the wide use of these compounds. For instance, in Switzerland [24] concentrations of 64,000 and 67,000 μg/Kg (d.w.) were found for azythromycin and clarithromycin, respectively, and concentrations up to 4,000 μg/Kg (d.w.) were found in Spain for other macrolides such as roxithromycin, tylosin, erythromycin and azithromycin. The USE extraction has also been used in combination with LC-MS-MS to determine the macrolide group. Chenxi *et al.* [37] determined three macrolides (clindamycin, clarithromycin and erythromycin) among other pharmaceutical and personal care products. The recoveries obtained using USE as the extraction technique were similar (between 74 and 83%) to those recoveries obtained in studies combining PLE with LC-MS or LC-MS-MS [11,26]. USE extraction also needed a smaller quantity of sample (0.5 g). When USE/LC-MS-MS was applied to the sample from a sewage treatment plant in the USA, concentrations between 23.2 μg/Kg (d.w.) for clindamycin and 3.6 μg/Kg (d.w.) for erythromycin were detected.

Fluoroquinolones are highly useful antibacterial agents, particularly because of their broad spectrum of activity and good oral absorption. They are also applied in both human and veterinary medicine [12]. These compounds were present in sewage sludge from Switzerland [12] and Spain [21]. Different extraction times have been used to extract these compounds. For example, Golet *et al.* [12] used 4 cycles of 15 minutes, whereas Radjenovic *et al.* [21] used only 3 cycles of 5 minutes and recoveries in both studies were higher than 88%. Moreover, these compounds were also determined in sewage sludge using USE/LC-MS-MS [38]. Ofloxacin showed the maximum concentration (510 μg/Kg (d.w.)) and moxifloxacin showed the lowest concentration (30 μg/Kg (d.w.)) in sewage sludge from Austria. The

ISBN:978-84-693-6426-0/DL:T-1628-2010

concentration levels in Austria were between 8 and 2,370 μg/Kg (d.w.).

Another group of pharmaceuticals that are widely consumed are non-steroidal anti-inflammatory compounds (NSAIDs). Different anti-inflammatories have been determined in sewage sludge such as ibuprofen, ketoprofen diclofenac, naproxen and mefenamic acid. When these were determined in two different sewage treatment plants from Spain, ibuprofen showed the maximum concentration (548 μg/Kg (d.w.)) [21]. This result is because ibuprofen is the most widely used anti-inflammatory compound in Spain, where it requires no medical prescription. The concentrations of ibuprofen found in the Spanish studies [21,35] were higher than the concentrations in other countries such as Germany (concentration below of the limit of quantification) [39] and USA (approximately 50 μg/Kg (d.w.)) [8]. Ibuprofen is one of the NSAIDs that has been most studied using other extraction techniques such as USE [39,40], Soxhlet [41] and MAE [8] and it has yielded recoveries comparable to those obtained with PLE extraction. Mefenamic acid was also determined in sewage sludge from Spain [21] and showed the lowest concentration of the anti-inflammatory group, this being between 3.5 and 33 μg/Kg (d.w.).

The same studies also determined different analgesics. The most widely used analgesic is acetaminophen, which showed a concentration between 34 and 145 μg/Kg (d.w.) in a sewage treatment plant from Barcelona (Spain) [21]. This compound was also determined in sewage treatment plants from Tarragona (Spain), where concentrations of up to 42 μg/Kg (d.w.) were found [35].

Other pharmaceuticals which are also present in sewage sludge samples are antiepileptics and their metabolites. Carbamazepine is an important drug for the treatment of epilepsy and is also used in various psychotherapy applications. Miao *et al.* [36] found carbamazepine and some of its metabolites in sewage treatment plants from Canada at concentration levels of between 70 and 258 μg/Kg (d.w.) for carbamazepine and 1.6 and 15.4 μg/Kg (d.w.) for its metabolites. Carbamazepine was also determined in different countries such as Ireland [20] and Spain [21,35] at similar concentration levels (i.e. between 34 and 215 μg/Kg (d.w.)).

Some studies have reported information about the presence of estrogens and their conjugates in sewage sludge from different countries such as Germany [5] and Spain [33]. The log $K_{ow}$ of 3.1-4.7 indicates that estrogens are rather lipophilic and should appreciably adsorb onto sediment and sludge [5]. Estrogens of anthropogenic origin have been identified as the major contributors to endocrine-disrupting activity in the environmental samples [42].

**Table 1.** Main parameters used to determined pharmaceuticals in sewage sludge and their concentration in sewage sludge.

| Compound | Pre-treatment | PLE conditions | | | | Post-treatment | Anal. Instrum. | Recovery (%) | LODs (µg/Kg) | Concentr. in sewage sludge (µg/Kg)(d.w.) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Solvent | Temp. (ºC) | Pres. (bar) | Cycles x Static time (min) | | | | | | |
| *Sulfonamides* | | | | | | | | | | | |
| Sulfadiazine | Lyophilization | MeOH: $H_2O$ (1:1) | 80 | 100 | 1 x 5 | - | LC-MS | 76 | 7 | N.D. - <LOQ | [26] |
| | Lyophilization | MeOH: Acetone (1:1) | 75 | 100 | 3 x 5 | SPE | LC-MS-MS | 67 | 0.19 | <LOQ | [10] |
| Sulfapyridine | Lyophilization | MeOH: $H_2O$ (1:1) | 80 | 100 | 1 x 5 | - | LC-MS | 85 | 3 | N.D. - <LOQ | [26] |
| | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 64 | - | N.D. - 160 | [11] |
| | Lyophilization | MeOH: Acetone (1:1) | 75 | 100 | 3 x 5 | SPE | LC-MS-MS | 64 | 0.08 | <LOQ | [10] |
| | Dried | MeOH: $H_2O$ (1:1) | - | - | - | SPE | LC-MS-MS | - | - | 28000 | [24] |
| Sulfatiazole | Lyophilization | MeOH: $H_2O$ (1:1) | 80 | 100 | 1 x 5 | - | LC-MS | 74 | 5 | N.D. - <LOQ | [26] |
| | Lyophilization | MeOH: Acetone (1:1) | 75 | 100 | 3 x 5 | SPE | LC-MS-MS | 39 | 0.11 | <LOQ | [10] |
| Sulfamethoxazole | Lyophilization | MeOH: $H_2O$ (1:1) | 80 | 100 | 1 x 5 | - | LC-MS | 87 | 2 | N.D. - <LOQ | [26] |
| | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 86 | 3 | 0.6 – 21 | [21] |
| | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 64 | - | 34 - 100 | [11] |
| | Lyophilization | MeOH: Acetone (1:1) | 75 | 100 | 3 x 5 | SPE | LC-MS-MS | 29 | 0.06 | <LOQ | [10] |
| | Dried | MeOH: $H_2O$ (1:1) | - | - | - | SPE | LC-MS-MS | - | - | 68000 | [24] |
| Sulfamethazine | Lyophilization | MeOH: Acetone (1:1) | 75 | 100 | 3 x 5 | SPE | LC-MS-MS | 58 | 0.13 | <LOQ | [10] |
| Sulfadimethoxine | Lyophilization | MeOH: Acetone (1:1) | 75 | 100 | 3 x 5 | SPE | LC-MS-MS | 48 | 0.01 | 0.1 – 0.23 | [10] |
| *Macrolides* | | | | | | | | | | | |
| Roxithromycin | Lyophilization | MeOH: $H_2O$ (1:1) | 80 | 100 | 1 x 5 | - | LC-MS | 83 | 7 | <LOQ – 1800 | [26] |
| | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 45 | - | N.D. - 83 | [11] |
| Tylosin | Lyophilization | MeOH: $H_2O$ (1:1) | 80 | 100 | 1 x 5 | - | LC-MS | 95 | 8 | 1300-4000 | [26] |
| Erythromycin | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 46 | 10 | 34 – 111 | [21] |
| Azithromycin | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 80 | 18 | 39 – 128 | [21] |
| | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 29 | - | 5 - 158 | [11] |
| | Dried | MeOH: $H_2O$ (1:1) | - | - | - | SPE | LC-MS-MS | - | - | 64000 | [24] |

**Table 1.** Main parameters used to determined pharmaceuticals in sewage sludge and their concentration in sewage sludge (cont.).

| Compound | Pre-treatment | PLE conditions | | | | Post-treatment | Anal. Instrum. | Recovery (%) | LODs (µg/Kg) | Concentr. in sewage sludge (µg/Kg)(d.w.) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Solvent | Temp. (ºC) | Pres. (bar) | Cycles x Static time (min) | | | | | | |
| Clarithromycin | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 33 | - | 16 - 41 | [11] |
| | Dried | MeOH: $H_2O$ (1:1) | - | - | - | SPE | LC-MS-MS | - | - | 67000 | [24] |
| *Other Antibiotics* | | | | | | | | | | | |
| Norfloxacin | Dried | ACN: $H_2O$ (1:1) | 100 | 100 | 4 x 15 | MPC disk cartridge | LC-FLD | 88 | 120 | 270 – 2370 | [12] |
| Ciprofloxacin | Dried | ACN: $H_2O$ (1:1) | 100 | 100 | 4 x 15 | MPC disk cartridge | LC-FLD | 89 | 120 | 270 - 2207 | [12] |
| Ofloxacin | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 122 | 6 | 8 – 179 | [21] |
| *Anti-inflammatories* | | | | | | | | | | | |
| Ibuprofen | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 74 | 63 | <LOQ – 548 | [21] |
| | Lyophilization | MeOH: $H_2O$ (1:1) | 100 | 100 | 2 x 15 | - | LC-MS | 68 | 22* | N.D. - 99 | [35] |
| Ketoprofen | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 102 | 52 | <LOQ – 211 | [21] |
| Diclofenac | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 60 | 96 | 143 - 209 | [21] |
| | Lyophilization | MeOH: $H_2O$ (1:1) | 100 | 100 | 2 x 15 | - | LC-MS | 82 | 22* | N.D. - 42 | [35] |
| Mefenamic acid | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 20 | 4 | 3.5 – 33 | [21] |
| Naproxen | Lyophilization | MeOH: $H_2O$ (1:1) | 100 | 100 | 2 x 15 | - | LC-MS | 72 | 32* | N.D. - 242 | [35] |
| *Analgesics* | | | | | | | | | | | |
| Acetaminophen | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 44 | 4 | 34 - 145 | [21] |
| | Lyophilization | MeOH: $H_2O$ (1:1) | 100 | 100 | 2 x 15 | - | LC-MS | 109 | 22* | N.D. - 42 | [35] |
| *Antiepileptics* | | | | | | | | | | | |
| Carbamazepine | Dried | MeOH: $H_2O$ (1:1) | 60 | 100 | 2 x 5 | SPE | LC-MS-MS | 120 | 3 | 120 | [20] |
| | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 82 | 2 | 34 – 80 | [21] |
| | Lyophilization | MeOH: $H_2O$ (1:1) | 100 | 100 | 2 x 15 | - | LC-MS | 112 | 19* | N.D. - 215 | [35] |
| | Dried | Acetone: $H_2O$ (3:7) | 80 | 100 | 3 x 5 | SPE | LC-MS-MS | 93 | 0.17 | 70 - 258 | [36] |
| 2-hydroxy-carbamacepine | Dried | Acetone: $H_2O$ (3:7) | 80 | 100 | 3 x 5 | SPE | LC-MS-MS | 89 | 0.07 | 1.9 – 3.4 | [36] |

**Table 1.** Main parameters used to determined pharmaceuticals in sewage sludge and their concentration in sewage sludge (cont.).

| Compound | Pre-treatment | PLE conditions | | | | Post-treatment | Anal. Instrum. | Recovery (%) | LODs (µg/Kg) | Concentr. in sewage sludge (µg/Kg)(d.w.) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Solvent | Temp. (ºC) | Pres. (bar) | Cycles x Static time (min) | | | | | | |
| 3-hydroxy-carbamacepine | Dried | Acetone: $H_2O$ (3:7) | 80 | 100 | 3 x 5 | SPE | LC-MS-MS | 80 | 0.06 | 1.6 – 4.3 | [36] |
| 10,11dihydro-10-hydroxy-carbamacepine | Dried | Acetone: $H_2O$ (3:7) | 80 | 100 | 3 x 5 | SPE | LC-MS-MS | 90 | 0.08 | 7.5 – 15.4 | [36] |
| *Estrogens and conjugates* | | | | | | | | | | | |
| Estriol | Lyophilization | MeOH: Acetone (1:1) + MeOH: $H_2O$ (1:1) | 75 | 100 | 2 x 3<br>2 x 3 | First 2 cycles evaporated | LC-MS-MS | 84 | 26 | N.D. - 406 | [33] |
| Estrone | Lyophilization | MeOH: Acetone (1:1) + MeOH: $H_2O$ (1:1) | 75 | 100 | 2 x 3<br>2 x 3 | First 2 cycles evaporated | LC-MS-MS | 88 | 11 | N.D. - 137 | [33] |
| Diethylstilbestrol | Lyophilization | MeOH: Acetone (1:1) + MeOH: $H_2O$ (1:1) | 75 | 100 | 2 x 3<br>2 x 3 | First 2 cycles evaporated | LC-MS-MS | 81 | 12 | N.D. - 184 | [33] |
| Estradiol-3 sulfate | Lyophilization | MeOH: Acetone (1:1) + MeOH: $H_2O$ (1:1) | 75 | 100 | 2 x 3<br>2 x 3 | First 2 cycles evaporated | LC-MS-MS | 99 | 0.15 | 0.71 - 3 | [33] |
| Estrone-3 sulfate | Lyophilization | MeOH: Acetone (1:1) + MeOH: $H_2O$ (1:1) | 75 | 100 | 2 x 3<br>2 x 3 | First 2 cycles evaporated | LC-MS-MS | 100 | 0.15 | 0.64 - 7 | [33] |
| *Other pharmaceuticals* | | | | | | | | | | | |
| Warfarin | Dried | MeOH: $H_2O$ (1:1) | 60 | 100 | 2 x 5 | SPE | LC-MS-MS | 110 | 7 | 163 | [20] |
| Trimethoprim | Lyophilization | MeOH: $H_2O$ (1:1) | 80 | 100 | 1 x 5 | - | LC-MS | 77 | 2 | <LOQ | [26] |
| | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 29 | 3 | 10 – 43 | [21] |
| | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 51 | - | 13 - 133 | [11] |
| | Dried | MeOH: $H_2O$ (1:1) | - | - | - | SPE | LC-MS-MS | - | - | 41000 | [24] |
| Omeprazole | Lyophilization | MeOH: $H_2O$ (1:1) | 80 | 100 | 1 x 5 | - | LC-MS | 93 | 7 | N.D. - <LOQ | [26] |

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**Table 1.** Main parameters used to determined pharmaceuticals in sewage sludge and their concentration in sewage sludge (cont.).

| Compound | Pre-treatment | PLE conditions | | | | Post-treatment | Anal. Instrum. | Recovery (%) | LODs (µg/Kg) | Concentr. in sewage sludge (µg/Kg)(d.w.) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Solvent | Temp. (ºC) | Pres. (bar) | Cycles x Static time (min) | | | | | | |
| Famotidine | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 22 | 7 | <LOQ - 51 | [21] |
| Gemfibrozil | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 29 | 28 | 44 – 118 | [21] |
| Bezafibrate | Lyophilization | MeOH: $H_2O$ (1:1) | 100 | 100 | 2 x 15 | - | LC-MS | 92 | 25* | N.D. - 88 | [35] |
| Fluoxetine | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 30 | 4 | 72 - 123 | [21] |
| Paroxetine | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 67 | 2 | 41 – 60 | [21] |
| Loratidine | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 53 | 0.35 | 53 – 153 | [21] |
| Atenolol | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 90 | 5 | 7 – 84 | [21] |
| Propanolol | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 60 | 3 | 26 – 47 | [21] |
| Hydrochlorthiazide | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 49 | 3 | 11 – 49 | [21] |
| Glicenclamide | Dried | MeOH: $H_2O$ (1:1) | 100 | 100 | 3 x 5 | SPE | LC-MS-MS | 52 | 5 | 18 - 127 | [21] |
| Caffeine | Lyophilization | MeOH: $H_2O$ (1:1) | 100 | 100 | 2 x 15 | - | LC-MS | 106 | 22* | N.D. - 65 | [35] |
| Clofibric acid | Lyophilization | MeOH: $H_2O$ (1:1) | 100 | 100 | 2 x 15 | - | LC-MS | 81 | 28* | N.D. - 64 | [35] |
| Sildenafil | Lyophilization | MeOH | 100 | 100 | 2 x 5 | - | LC-MS-MS | 103 | 1 | 3 - 17 | [27] |
| Vardenafil | Lyophilization | MeOH | 100 | 100 | 2 x 5 | - | LC-MS-MS | 102 | 2 | N.D. – 5 | [27] |
| Tadalafil | Lyophilization | MeOH | 100 | 100 | 2 x 5 | - | LC-MS-MS | 45 | 3 | <LOQ - 12 | [27] |

Only a few studies have used PLE to extract estrogens and their conjugates from sewage sludge [33,43,44]. Two conjugates, estradiol 3-sulfate and estrone 3-sulfate, have been found in the sewage treatment plant in Tarragona at low μg/Kg concentration levels. Also, some estrogens such as estriol, estrone and diethylstilbestrol were found at higher concentrations of between 137 and 406 μg/Kg (d.w.). Muller *et al.* [43] found estrone, estradiol and ethinylestradiol at concentrations from 10 to 2 μg/Kg (d.w.). However, estriol was not detected in any sample of sewage sludge from France. Fernández *et al.* [44] analyzed different sewage sludge samples from Barcelona (Spain), and only detected estrone and estradiol in sludge at 0.056 and 0.155 μg/Kg (d.w.) respectively. These studies highlight the importance of determining the conjugates and metabolites of pharmaceuticals in sewage sludge because in some cases, metabolites contribute to endocrine-disrupting activity. The estrogens have also been determined using other extraction techniques such as USE. Ternes *et al.* [5] determined four estrogens (estrone, 17β-estradiol, 17α-ethinylestradiol and mestranol) using USE/GC-MS-MS.

To determine these analytes using GC, a derivatization step was required. Using this method, sewage sludge from Germany was analyzed and natural estrogens (estrone, 17β-estradiol) were detected up to 37 μg/Kg (d.w.) and 40 μg/Kg (d.w.), respectively. The concentration of 17α-ethinylestradiol ranged between <LOQ and 17 μg/Kg (d.w.).

Other pharmaceuticals that have also been determined in sewage sludge were β-blockers, antiulcers and lipid regulators, among others. In this group the compound that showed the maximum concentration was trimethoprim. This compound is used as a stomach protector and was found at concentration of 41,000 μg/Kg (d.w.) in sewage sludge from Switzerland [24]. In this group we included some metabolites such as clofibric acid, which is an active metabolite of clofibrate, etofibrate and etofyllinclofibrate, drugs which are used as blood lipid regulators [45,46].

Phosphodiesterase type V inhibitors such as sildenafil, tadalafil and vardenafil have also been found in sewage sludge [27]. These compounds are the active agents of Viagra®, Cialis® and Levitra®, respectively and were found in sewage treatment plants in Spain and Germany at concentration levels between 3 and 17 μg/Kg (d.w.) [27].

In recent years, illicit drugs have been determined in different environmental samples [47]; however only Kaleta *et al.* [48] have shown a method for determining residues of amphetamine in sewage sludge. This method is based on ultrasonication extraction and liquid chromatography ion-trap mass spectrometry. The concentration levels measured in samples from 12 different sewage treatment plants were between 5 and 300 μg/Kg (d.w.).

As regards PCPs, these compounds are used in large volumes, persist in

ISBN:978-84-693-6426-0/DL:T-1628-2010

the environment, bioaccumulate and are designed to have a certain bioactivity. Parabens, UV filters, antimicrobials and musks have not been extensively determined in sewage sludge using pressurized liquid extraction [22,25,30,34].

Parabens are the most common preservatives used in personal care products and are also used as preservatives in pharmaceuticals and food products. It is to be expected that parabens enter the environment because they are widely used, are resistant to antimicrobial degradation and are present in different concentrations in different kinds of samples; however, to the best of our knowledge only one study has quantified the presence of these compounds in sewage sludge [30]. Methyl and propyl parabens are the most widely used parabens and are normally used together because of their synergistic preservative effects. Concentrations of methyl and propyl parabens between 5 and 202 μg/Kg (d.w.) have been found in sewage sludge from Spain [30].

UV filters or sunscreen agents are increasingly added to cosmetics and lotions as protection against harmful UV radiation. Only one study has used PLE to determine the concentration of this group in sewage sludge. Concentration levels of octocrylene between 700 and 1,842 μg/Kg (d.w.) were found in sewage sludge from Spain [30]. Other compounds that have been determined are benzophenone-3 and octyldimethyl-p-aminobenzoic acid. In a previous study [30], we used 2 cycles of methanol and another two cycles of methanol:water (1:1) to extract these compounds from sewage sludge. In this case, the UV filters were extracted at the same time with parabens and antimicrobials. Other studies have reported information regarding the presence of these compounds in sewage sludge but this was using shaking extraction instead of PLE [49,50]. These studies obtained recoveries similar to those obtained with PLE, although they needed more time and solvent. In the study by Kupper *et al.* [49] octocrylene showed the highest concentration at 20,804 μg/Kg (d.w.). In the study by Plagellat *et al.* [50], octyltriazone showed the maximum concentration at 27,700 μg/Kg (d.w.) whereas octylmethoxycinnamate showed the lowest concentration at 10 μg/Kg (d.w.).

Triclosan and triclocarban are antimicrobial compounds used in soap, toothpaste and other consumer products.

These compounds have been determined in different kinds of samples and always show the highest concentrations of all the personal care products [7,25,30]. For example, in samples from Canada, triclocarban and triclosan showed the maximum concentration in sewage sludge using PLE extraction (5,970 and 11,550 μg/Kg (d.w.), respectively) [25]. In samples from Spain [30] triclosan also showed higher concentrations than triclo-carban. Triclosan was also determined in sewage sludge from a sewage treatment plant in the north of

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Spain using MAE as the extraction technique and GC-MS-MS [7]. The concentration ranged from 418 to 5400 μg/Kg (d.w.).

Synthetic musk fragrances are compounds added to provide scent in a variety of personal care products, including deodorant, shampoo and detergents. There are two types of synthetic musk fragrances: nitro musk fragrances and polycyclic musk fragrances. Nitro musk fragrances were the first to be produced and include musk xylene, musk ketone, musk ambrette, musk moskene and musk tibetene. In the environment, nitro substituents can be reduced to form amino metabolites of these compounds. Polycyclic musk fragrances are used in higher quantities than nitro musk fragrances. Because of their characteristics, these musk fragrances have been separated using gas chromatography. The results obtained by Ternes *et al.* [34] in Germany and Osemwengie [22] in USA show that the compounds that had the maximum concentration were galaxolide and tonalide (between 4.5 and 17.9 mg/Kg (d.w.) for galaxolide and 1,400 and 2,010 μg/Kg (d.w.) for tonalide). Polycyclic musk fragrances have been determined in sewage sludge from the USA and values below the limit of quantification were obtained. Polycyclic and nitro musk fragrances have also been determined using other extraction techniques such as Soxhlet [51], MAE [8] and SFE [31,52]. The recoveries in all cases were higher than 49%. The lowest recoveries were obtained with Soxhlet extraction, whereas the highest recoveries were obtained using SFE. In both cases the analysis was done using GC-MS. Galaxolide and tonalide were the compounds that showed the highest concentration in all studies. For instance, in sewage sludge from China the concentration for galaxolide was between 5.4 and 21.2 mg/Kg and for tonalide it was between 0.7 and 6.1 mg/Kg [51]. In this study Soxhlet extraction and GC-MS was used [51]. Lee *et al.* [52] studied the presence of seven polycyclic and nitro musks and, as other studies have shown, the concentration of galaxolide was highest in sewage sludge from Canada (between 1.34 and 20.8 mg/Kg).

Siloxanes have been widely used in consumer products such as electronics, furniture and cosmetics. Up to now only one study has determined their presence in sewage sludge samples.

Hupmann *et al.* [53] developed a method to determine the siloxanes using Soxhlet extraction and gas chromatography – flame ionization detection (GC-FID). Moreover these compounds have been determined in river water [54] and in the digestion gas of sewage sludge [55].

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**Table 2.** Main parameters used to determined personal care products in sewage sludge and their concentration in sewage sludge.

| Compound | Pre-treatment | PLE conditions | | | | Post-treatment | Anal. Instrum. | Recovery (%) | LODs (µg/Kg) | Concentr. in sewage sludge (µg/Kg)(d.w.) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Solvent | Temp. (ºC) | Pres. (bar) | Cycles x Static time (min) | | | | | | |
| *Musks fragrances* | | | | | | | | | | | |
| Galaxolide | Dried | MeOH | 100 | 100 | 2 x 5 | SPE | GC-MS | 87 | 250 | 4500 – 8500 | [34] |
| | | Hexane: Acetone (1:1) | 85 | 140 | 2x15 | GPC | GC-MS | 100 | 10 | 4940 - 17880 | [22] |
| Tonalide | Dried | MeOH | 100 | 100 | 2 x 5 | SPE | GC-MS | 78 | 250 | 1400 - 3900 | [34] |
| | | Hexane: Acetone (1:1) | 85 | 140 | 2x15 | GPC | GC-MS | 90 | 20 | 1850 – 4010 | [22] |
| Cashmeran | Dried | Hexane: Acetone (1:1) | 85 | 140 | 2x15 | GPC | GC-MS | 86 | 10 | <LOQ | [22] |
| Musk xylene | Dried | Hexane: Acetone (1:1) | 85 | 140 | 2x15 | GPC | GC-MS | 100 | 20 | <LOQ | [22] |
| Musk Ketone | Dried | Hexane: Acetone (1:1) | 85 | 140 | 2x15 | GPC | GC-MS | 92 | 30 | <LOQ | [22] |
| 2- Amino musk xylene | Dried | Hexane: Acetone (1:1) | 85 | 140 | 2x15 | GPC | GC-MS | 100 | 40 | <LOQ | [22] |
| 4- Amino musk xylene | Dried | Hexane: Acetone (1:1) | 85 | 140 | 2x15 | GPC | GC-MS | 87 | 30 | <LOQ | [22] |
| *Parabens* | | | | | | | | | | | |
| Methyl paraben | Lyophilization | MeOH + MeOH: $H_2O$ (1:1) | 100 | 140 | 2 x 5<br>2 x 5 | - | LC-MS-MS | 72 | 3 | 46 - 202 | [30] |
| Propyl paraben | Lyophilization | MeOH + MeOH: $H_2O$ (1:1) | 100 | 140 | 2 x 5<br>2 x 5 | - | LC-MS-MS | 102 | 2 | 6 - 10 | [30] |
| Benzyl paraben | Lyophilization | MeOH + MeOH: $H_2O$ (1:1) | 100 | 140 | 2 x 5<br>2 x 5 | - | LC-MS-MS | 106 | 3 | N.D. - 5 | [30] |

**Table 2.** Main parameters used to determined personal care products in sewage sludge and their concentration in sewage sludge (cont.).

| Compound | Pre-treatment | PLE conditions | | | | Post-treatment | Anal. Instrum. | Recovery (%) | LODs (µg/Kg) | Concentr. in sewage sludge (µg/Kg)(d.w.) | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Solvent | Temp. (ºC) | Pres. (bar) | Cycles x Static time (min) | | | | | | |
| *UV- filters* | | | | | | | | | | | |
| Benzophenone-3 | Lyophilization | MeOH + MeOH: $H_2O$ (1:1) | 100 | 140 | 2 x 5<br>2 x 5 | - | LC-MS-MS | 79 | 2 | 10 - 20 | [30] |
| Octocrylene | Lyophilization | MeOH + MeOH: $H_2O$ (1:1) | 100 | 140 | 2 x 5<br>2 x 5 | - | LC-MS-MS | 104 | 4 | 700 - 1842 | [30] |
| Octyldimethyl-p-aminobenzoic acid | Lyophilization | MeOH + MeOH: $H_2O$ (1:1) | 100 | 140 | 2 x 5<br>2 x 5 | - | LC-MS-MS | 108 | 2 | 132 - 170 | [30] |
| *Antimicrobials* | | | | | | | | | | | |
| Triclocarban | Dried | DCM | 60 | 100 | 3 x 5 | SPE | LC-MS-MS | 98 | 0.5 | 3050 – 5970 | [25] |
| | Lyophilization | MeOH + MeOH: $H_2O$ (1:1) | 100 | 140 | 2 x 5<br>2 x 5 | - | LC-MS-MS | 77 | 1.25 | 5 - 7 | [30] |
| Triclosan | Dried | MeOH: $H_2O$ (1:1) | 60 | 100 | 2 x 5 | SPE | LC-MS-MS | N.R. | 3 | 24600 | [20] |
| | Dried | DCM | 60 | 100 | 3 x 5 | SPE | LC-MS-MS | 97 | 5 | 620 – 11550 | [25] |
| | Lyophilization | MeOH + MeOH: $H_2O$ (1:1) | 100 | 140 | 2 x 5<br>2 x 5 | - | LC-MS-MS | 103 | 8 | 1300 - 1490 | [30] |

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## CONCLUDING REMARKS

Most recent studies have used pressurized liquid extraction to extract pharmaceuticals and personal care products from sewage sludge. PLE has many advantages over traditional extraction techniques such as low solvent consumption and, in some cases, shorter extraction time.

PLE combines good recoveries and adequate precision with rapid and selective extraction. Various methods for determining different pharmaceuticals such as anti-inflammatories, analgesics and antibiotics (among others) and personal care products such as parabens, UV filters, musk fragrances and antimicrobials have been developed. However, new methods still need to be developed to achieve lower limits of detection, to control and quantify these contaminants and their main degradation products and to identify new contaminants.

**Acknowledgements**

This study was financially supported by the General Research Board of the Spanish Ministry of Science and Technology, project CTM2008-06847-C02-01/TECNO. A.N. gratefully acknowledges the financial support of the Ministry of Science and Technology BES-2006-1332.

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## 1.3. References

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# 2. OBJECTIVE

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The main aim of the present Thesis is to develop analytical methods to determine a wide variety of emerging organic contaminants in sewage sludge such as pharmaceuticals, estrogens and personal care products. Another aim of this Thesis is to study in detail the applicability of pressurized liquid extraction to extract these contaminants from sewage sludge. Therefore, all methods are based on pressurized liquid extraction and liquid chromatography coupled to mass spectrometry or tandem mass spectrometry using three different analyzers: quadrupole, time of flight and triple quadrupole.

It is also an aim of this Thesis to provide information on the presence of these contaminants in sewage sludge, because limited information is available in current literature.

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# 3. EXPERIMENTAL PART AND DISCUSSION

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As we mentioned in the introduction, although the presence of emerging organic contaminants in water samples has been studied in some depth, information on their presence in sewage sludge is limited. For this reason, one of the objectives of this Thesis is to focus on the development of methods to determine two wide groups of emerging organic contaminants - pharmaceuticals and personal care products - in sewage sludge using pressurized liquid extraction as the extraction technique and liquid chromatography as the separation technique.

We were encouraged to do this Thesis because of the wide presence of emerging organic contaminants in influent and effluent wastewater samples. Interest in this topic by the scientific community has been growing recently. Moreover, there is little data published on the presence of most of these contaminants in sewage sludge and no data is available for the STPs studied. The results reported in this Thesis could also be considered if and when new legislation is drawn up regarding the re-use of sludge, for instance in agriculture.

This chapter includes the experimental part and the results from different studies that have been carried out through this Doctoral Thesis. These results have been published in different scientific journals and are presented in journal paper format. Previous to each study or group of them, a brief introduction is included in which the main aim is pointed out, as well as the novelty of the study when it was developed. Moreover, the most important results are briefly discussed after each section. The list of papers published as a result of this Thesis is included in Annex 3.

Then, to achieve the first objective of this thesis, in the development of analytical methods to determine some pharmaceuticals and personal care products in sewage sludge, the extraction technique selected was pressurized liquid extraction (PLE), which enables high recoveries to be obtained using high pressure and temperature. As we mentioned before, this technique has some advantages over classical extraction techniques such as low solvent consumption and short extraction time, and therefore, PLE can be qualified as more environmental friendly than classic extraction techniques. As already mentioned, we have also evaluated the application of PLE in the extraction of several groups of PPCPs from sewage sludge.

The experimental part has been divided into five sections. In the first one, we developed different analytical methods to determine several pharmaceuticals in sewage sludge. To determine them, the same extraction technique (PLE) and liquid chromatography with different mass spectrometry analyzers (Q and TOF) were used. The TOF analyzer also allowed us to identify non target analytes. The methods developed were applied to determine the presence of pharmaceuticals in sewage sludge samples from STPs in Tarragona and Reus.

In the second section, we developed a method based on PLE and LC coupled to tandem mass spectrometry to determine a group of estrogens and their conjugates.

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As in the case of pharmaceuticals, the method was applied to determine the presence of these compounds in sewage sludge samples from STPs in Tarragona and Reus.

Using the same analytical techniques as in the second section, the third section is concerned with the presence of three phosphodiesterase type-V inhibitors (sildenafil, vardenafil and tadalafil) which were studied in sewage sludge samples. Because they had not been studied in water samples, we also analyzed influent and effluent wastewater samples from STPs in Spain and Germany. This study was carried out in collaboration with Professor Thomas P. Knepper at the University of Applied Sciences Fresenius (Idstein, Germany), during my stay of four months in this university.

In the fourth section, an analytical method to determine a group of personal care products including parabens, UV filters and antimicrobials in sewage sludge was developed. In this study, the method was based on PLE and ultra high performance liquid chromatography coupled to tandem mass spectrometry (PLE/UHPLC-MS-MS).

In the last section, a monitoring study of pharmaceuticals and estrogens was carried out for one year in sewage sludge from two different sewage treatment plants in Tarragona and Reus. To do this monitoring study, the methods developed previously in the first section were adapted to liquid chromatography-tandem mass spectrometry in order to obtain lower limits of detection.

All the studies reported in this Doctoral Thesis were financially supported by the Ministry of Science and Technology (project CTM2005-01774).

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## 3.1. Determination of pharmaceuticals in sewage sludge

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ISBN:978-84-693-6426-0/DL:T-1628-2010

As mentioned in the introduction, increasing attention has recently been paid to the determination of pharmaceuticals in environmental samples. Municipal wastewater treatment plants play an important role in the life cycle of these compounds because they act as a point of source to the aquatic environment. If sorption to sewage sludge is the major removal pathway from the wastewater stream, the application of sewage sludge as soil fertilizer represents an additional entry route for pharmaceuticals into the environment. As a result of recent advances in analytical techniques, very low concentrations of pharmaceuticals are being measured in wastewater [1], surface water [2] and drinking water [3]. However, as we mentioned before, less information is available according to the presence of pharmaceuticals in sewage sludge.

The presence of pharmaceuticals in sewage sludge depends on different variables. The most important is the design of the sewage treatment plant which influences in the elimination power for each compound. If the elimination power of one compound is high, then we will expect to found it in sewage sludge due to the sorption of it in sewage sludge unless they are degraded. For instance, ibuprofen is one of the pharmaceuticals that showed a high removal in sewage treatment plants, and consequently it has been determined in sewage sludge in different studies [4,5]. On the other hand, other studies [2] have reported that the elimination power of carbamazepine in sewage treatment plants is low (an elimination power of less than 10%) but even that, this compound has been determined in sewage sludge samples [6].

According to bibliography, lipid regulators, β-blockers, analgesics, anti-inflammatories and antibiotics are the groups of pharmaceuticals most present in sewage sludge at levels of low μg/Kg or ng/Kg.

In the literature, different methods have been developed in order to determine some of these pharmaceuticals in sewage sludge using the combination of an extraction technique and a chromatographic technique. Soxhlet [7], ultrasonication extraction [8], microwave assisted extraction [9] or pressurized liquid extraction [10,11] have been used as extraction techniques. Chromatographic techniques include gas chromatography [12] or liquid chromatography [13-15] depending on the volatility and polarity of the target analytes. We have already discussed the advantages and disadvantages of these techniques in the introduction.

To begin the study of the presence of PPCPs in sewage sludge we developed different analytical methods based on pressurized liquid extraction and liquid chromatography-mass spectrometry to determine a group of pharmaceuticals. Analgesics, anti-inflammatories, β-blockers, vasodilatators, stimulants and two groups of antibiotics (sulfonamides and macrolides), among others, were selected because they are present in sewage sludge according to the few studies in literature. The structures of all compounds determined in the studies carried out during this Thesis are showed in Annex 2.

ISBN:978-84-693-6426-0/DL:T-1628-2010

Due to the polarity of the pharmaceuticals selected and the low concentration of these contaminants in sewage sludge, liquid chromatography-mass spectrometry using an electrospray ionization (ESI) interface were used for their determination after the PLE extraction. These studies were the first studies in our group where sludge samples were analyzed and PLE was used as an extraction technique.

Two different analyzers have been used in the methods described in this section: the quadrupole (sections 3.1.1 and 3.1.2) and the time of flight (section 3.1.3). First of all, we used the quadrupole analyzer because it was the MS detector available in our group. Low limits of detection were obtained using the quadrupole analyzer, although the structural information was lost when the quadrupole worked in SIM mode because only 2 or 3 ions were monitored, depending on the pharmaceuticals. However, slightly higher limits of detection in comparison to the quadrupole analyzer were obtained when the TOF analyzer was used. The TOF instrument was a powerful tool for identifying target and non-target compounds in sewage sludge, as is shown in section 3.1.3 where different non-target analytes were identified in sewage sludge samples.

The methods developed have been applied to different sewage sludge samples from two different sewage treatment plants (Tarragona and Reus). Data on the presence of pharmaceuticals were not available from these sewage treatment plants. The compounds studied were acetaminophen, caffeine, metoprolol, propranolol, carbamazepine, salicyclic acid, bezafibrate, naproxen, clofibric acid, diclofenac, ibuprofen, omeprazole, ranitidine and the sulfonamide and macrolide antibiotics.

The results obtained in these studies have been published or submitted to different analytical journals: Journal of Separation Science 30 (2007) 979-984, Journal of Chromatography A 1174 (2007) 125-131 and Analytical Methods (pending to be accepted).

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### 3.1.1. Pressurized liquid extraction of pharmaceuticals from sewage sludge

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# PRESSURIZED LIQUID EXTRACTION OF PHARMACEUTICALS FROM SEWAGE SLUDGE

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**Abstract**

A method for the quantitative determination of 10 pharmaceuticals in sewage sludge was developed by using pressurized liquid extraction (PLE) and high performance liquid chromatography–mass spectrometry with electrospray ionization (LC-(ESI)MS).
The pressurized liquid extraction was optimized with regard to solvents and operational parameters, such as temperature, pressure, extraction time and purge time. The optimum conditions were: 50 mM phosphoric acid/methanol (1:1, v/v) as the extraction solvent, temperature of 100 ºC, pressure of 100 bar, extraction time 15 minutes, 2 cycles, flush volume 150% and purge time 300 seconds.
All recoveries for pharmaceuticals were over 68% except for salicylic acid. The repeatability and reproducibility between days expressed as relative standard deviation were lower than 8% for repeatability and 10% for reproducibility. The limits of detection of all compounds were lower than 10 μg/Kg of dry weight of sewage sludge. The method was applied to determine the pharmaceuticals in sewage sludge from two domestic sewage treatment plants (STPs). The samples were collected every three months between February 2004 and June 2005. Some pharmaceuticals were determined in the samples and naproxen showed the highest value (242 μg/Kg of dry weight).

**Keywords:** Pharmaceuticals, sewage sludge samples, liquid chromatography-mass spectrometry, pressurized liquid extraction.

## INTRODUCTION

The determination of pharmaceutical compounds is an emerging issue in environmental research. There is a widespread consensus that these contaminants may require legislative intervention [1]. Many pharmaceutical compounds are highly bioactive. When present in the environment

ISBN:978-84-693-6426-0/DL:T-1628-2010

they usually occur at concentrations of a part per billion or part per trillion [1]. This group of contaminants, part of the group called new emerging contaminants, are being studied because of their potentially dangerous consequences [2]. Their main characteristic is that they do not need to persist in the environment to cause negative effects, since their high transformation and removal rates can be offset by the fact that they are continuously introduced into the environment in infiltrated water. The principal cause of their presence in the environment is excreta and the disposal of unused or expired products, but they can also be the result of manufacturing processes [2-5]. The negative effect is the risk of developing bacteria resistant to these compounds [6]; for example Ash and Iverson [7] identified bacteria resistant to sulfonamide and trimethoprim in USA rivers.

Most studies focus on liquid samples such as, drinking water [6,8], domestic wastewater [1,6,9] and wastewater from hospitals [10,11], but few studies analyse sewage sludge samples [12,13]. It is important to determine pharmaceutical compounds in sewage sludge for two reasons. The first is to evaluate the elimination power of sewage treatment plants (STPs) and the second is to determine whether sewage sludge can be used as manure.

Some methods reported in the bibliography for pharmaceutical residue analysis are based on gas chromatography-mass spectrometry (GC-MS) [2,14] but these methods normally use a derivatization step, and therefore, high performance liquid chromatography (LC) is preferred, and MS is used as detector in the majority of cases [2,10].

To extract pharmaceuticals from sludge samples, ultrasonic solvent extraction (USE) [15], microwave assistant extraction (MAE) [15] or Soxhlet extraction have been used [16]. Pressurized liquid extraction (PLE) is a newer technique which has been successfully applied to extract a variety of organic compounds from complex solid samples [17], in some cases in combination with SPE [14,15]. Some of the main criteria for an extraction technique are short extraction times combined with the possibility of automation.

Furthermore, solvent consumption should be kept low for environmental and economical reasons. The main key to shorter extraction times and reduced solvent consumption with PLE is the possibility of working at elevated temperatures above the boiling point of the solvent. Thereby the extraction process is facilitated due to increased analyte desorption and diffusion from the solid matrix [16]. For these reasons PLE seems to be more suitable and more efficient than Soxhlet [17-19]. PLE has been already applied to environmental and biological samples [18,20-22].

The aim of this paper is to use pressurized liquid extraction (PLE) and HPLC-MS with an electrospray ionisation (ESI) interface to determine a group of pharmaceuticals, including those that are most commonly found

ISBN:978-84-693-6426-0/DL:T-1628-2010

in sewage water. To demonstrate the feasibility of the method, several samples from two sewage treatment plants have been analysed.

## EXPERIMENTAL

### Materials and reagents

Caffeine, propranolol, metoprolol, carbamazepine, salicylic acid, acetaminophen, ibuprofen, bezafibrate, diclofenac and naproxen were from Sigma (St. Louis, USA), and clofibric acid was from Aldrich (Steinheim, Germany). Stock solutions of individual standards were prepared by dissolving each compound in methanol/water (1:1, v/v) at a concentration of 1000 mg/L and stored at 5 ºC.
Fresh stock solutions were prepared once a year. A mix of all compounds in methanol/water (1:1, v/v) at a concentration of 50 mg/L was prepared weekly. Working solutions were prepared daily by diluting the previous solution with methanol: water (1:1, v/v).
Ultra-pure water was obtained with a Milli-Q water purification system (18.2 MΩ·cm) (Millipore, Bedford, MA, USA), acetonitrile and methanol (HPLC-grade) were from SDS (Peypin, France), nitrogen was from Carburos Metálicos (Tarragona, Spain) and phosphoric acid was from Merck (Darmstadt, Germany).

### Sample pre-treatment

The sewage sludge samples were obtained from two STPs located in the area of Tarragona (Spain). These STPs mostly receive urban wastewaters and some industrial discharges. They are connected to similar population equivalents (around 140,000) with biological oxygen demand ($BOD_5$) of 400 mg/l. The average flow-rate is 30,000 $m^3$/day for STP1 and 16,000 $m^3$/day for STP2. The samples were collected every three months between February 2004 and June 2005. They were stored in the freezer in glass bottles.
Samples were lyophilised before analysis by the freeze dry system (Labconco, Missouri, USA). Then they were homogenized using a mortar and pestle and sieved to obtain particles with a diameter less than 125 μm.
To optimize the method, sludge sample was spiked with 160 mg/Kg of each compound, which were dissolved in acetone. After spiking, the samples were stirred intensively so that the compounds spread throughout the spiking solution in the sample and were in sufficient contact with the matrix.

### Sample extraction

Sludge samples were extracted using an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA) equipped with a 33 ml stainless steel extraction vessel. A total of 5 g of the pretreated sludge was placed in a 33 mL stainless steel extraction cell, and thoroughly mixed with aluminium oxide.

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The aluminium oxide was heated at 120 ºC in the oven for 24 hours before use.

The extracting solvent was 50 mM aqueous phosphoric acid and methanol mixture (1:1, v/v). The operating conditions were as follows: extraction temperature, 100 ºC; extraction pressure, 100 bar; preheating period, 5 min; static extraction, 15 min; final extraction volume ~ 40 mL; flush volume, 150% of the cell volume; nitrogen purge, 300 s; and number of extraction cycles, 2.

The extract was filtered with a microfilter 0.45 μm in diameter (Teknokroma, Barcelona, Spain), and then analysed by liquid chromatography.

**Chromatographic analysis**

The chromatographic instrument was an HP1100 series LC-mass selective detector (Agilent Technologies, Waldbronn, Germany) with an ESI interface and equipped with an automatic injector, a degasser, a quaternary pump, a column oven and photodiode array detection (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), and the volume injected was 50 μL. The mobile phase flow-rate was 1 ml/min and the column temperature was kept at 30 ºC.

DAD and a binary mobile phase with a gradient elution were used to optimize the extraction conditions. Solvent A was Milli-Q water with $H_3PO_4$ (pH 2.8) and solvent B was acetonitrile. The gradient was 18% B, which increased to 20% in 4 min, to 55% in 5 min, to 60% in 6 min, and kept constant for 3 min; then it was increased to 100% in 2 min, kept constant for 9 min and finally returned to 18% B in 3 min. All the compounds eluted within 22 min. The wavelengths used for the DAD were 210 nm (caffeine, propranolol, carbamazepine, salicylic acid, diclofenac and ibuprofen), 224 nm (metoprolol and clofibric acid), 230 nm (bezafibrate and naproxen) and 246 nm (acetaminophen).

Mass-spectrometry detection was used to analyse sludge samples in the same conditions as in a previous study [23]. In this case, since some compounds had to be determined in the positive ionization mode and others in the negative ionization mode, there were two gradients, one for acetaminophen, caffeine, metoprolol, propranolol and carbamazepine (positive ionization) and the other for salicylic acid, bezafibrate, naproxen, clofibric acid, diclofenac and ibuprofen (negative ionization). Both used Milli-Q water with acetic acid (pH 2.8) as solvent A and acetonitrile as solvent B. The gradient for the negative ionization mode was: 55% B, which increased to 60% in 6 min, kept constant for 3 min, increased to 80% in 12 min, then to 100% in 2 min, kept constant for 3 min, decreased to 55% in 2 min, and finally kept constant for 3 min. The gradient for the positive ionization mode was the same as the gradient for

optimizing the extraction conditions. All the compounds eluted within 30 min in the negative ionization mode and 25 min in the positive ionization mode.

The mass spectrometer simultaneously acquired data in full-scan and under selected ion monitoring (SIM); the ions that were selected for quantifying the samples appear in Table 1. 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 300 l/min, drying gas temperature 13 ºC and capillary voltage 3,000 V. In the negative mode, the average conditions were: nebulizer pressure 30 psi, drying gas flow-rate 350 l/min, drying gas temperature 12 ºC and capillary voltage 3,500 V. 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 metoprolol, propranolol, salicylic acid and carbamazepine.

## RESULTS AND DISCUSSION

### PLE optimization

In pressurized solvent extraction, 8 parameters need to be optimized: the pressure, temperature, solvent, number of cycles, static time, purge time, sample weight and flush volume. The initial conditions for the PLE optimization were selected from a previous study [17], in where fluoroquinolones in sewage sludge samples were analyzed. These conditions were: 100 bar, 100 ºC, solvent was acetonitrile:water ($H_3PO_4$ 50 mM) (1:1, v/v), 4 cycles, 15 min of static time, 300 s of purge time, 5 g of sample weight and finally 150% of

**Table 1.** Studied compounds and ions selected for quantification and confirmation in SIM mode. The abundance is in brackets.

| Compound | m/z ions | |
|---|---|---|
| | Quantification | Confirmation |
| Acetaminophen | 152 (100%) | 110 (60%) |
| Caffeine | 195 (100%) | 138 (35%) |
| Metoprolol | 268 (100%) | 116 (15%) |
| Propranolol | 260 (100%) | 183 (18%) |
| Carbamazepine | 237 (100%) | 138 (24%) |
| Salicylic acid | 93 (100%) | 137 (16%) |
| Bezafibrate | 360 (100%) | 274 (20%) |
| Naproxen | 229 (100%) | 185 (96%) |
| Clofibric acid | 213 (100%) | 127 (15%) |
| Diclofenac | 294 (100%) | 250 (30%) |
| Ibuprofen | 205 (100%) | 159 (55%) |

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flush volume. First, a blank of a sewage sludge sample was analyzed by LC-DAD under the above conditions and the chromatogram showed no peaks at the same retention time as the compounds studied. Then, the sample was spiked to optimize the PLE variables. 5 g of lyophilized sludge spiked with 160 mg/Kg of each compound was selected to perform the PLE extraction with the 33 ml cell.

The first parameter optimized was the solvent and the results are shown in Table 2. The solvent mixture selected initially did not give quantitative recoveries for most of them. Then, we tried different percentages of acetonitrile but recoveries did not improve and in fact they got worse when higher percentages were used. Quantitative recoveries were obtained for all compounds, except for salicylic acid, when methanol:water ($H_3PO_4$ 50 mM) (1:1, v/v) was used and this was the solvent mixture selected for further experiments because a change in water pH did not improve its recovery.

The second parameter optimized was the number of cycles and four consecutive simple extractions to the same sample were done. The least polar compounds were quantitatively extracted in the second cycle whereas the most polar ones were extracted in the first cycle. The extraction for salicylic acid was not quantitative when two cycles were used. For most compounds recoveries were considered negligible in the third cycle, as Figure 1 shows. For this reason two cycles were selected as optimum.

**Table 2.** PLE recoveries (n=3) by using different solvents. A= water with 50 mM $H_3PO_4$, B= water, C = water (pH = 10), D= acetonitrile, E= methanol.

| Pharmaceuticals | A:D (9:1) | A:D (1:1) | A:D (1:9) | A:E (1:1) | B:E (1:1) | C:E (1:1) |
|---|---|---|---|---|---|---|
| Acetaminophen | 28 | 30 | 17 | 111 | 76 | 77 |
| Caffeine | 29 | 24 | 11 | 109 | 78 | 79 |
| Metoprolol | 44 | 66 | 32 | 125 | 92 | 93 |
| Propranolol | 12 | 68 | 68 | 95 | 53 | 60 |
| Carbamazepine | 40 | 87 | 80 | 115 | 82 | 83 |
| Salicylic acid | 5 | 10 | 0 | 3 | 1 | 3 |
| Bezafibrate | 40 | 82 | 3 | 95 | 75 | 83 |
| Naproxen | 49 | 85 | 3 | 74 | 68 | 74 |
| Clofibric acid | 33 | 62 | 2 | 85 | 74 | 68 |
| Diclofenac | 53 | 83 | 7 | 84 | 70 | 75 |
| Ibuprofen | 64 | 82 | 6 | 71 | 75 | 70 |

The third parameter optimized was the flush volume in order to increase the preconcentration factor. However, recoveries were higher when flush volume was 150%. Thus, the initial was selected as optimum.

In order to improve the extraction of salicylic acid, purge time, pressure, temperature and static time were optimized sequentially. However, recoveries were not better when

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values other than the initial ones were used. Therefore, we decided to exclude it from this study.

Under these optimal conditions, compounds were extracted in ~ 40 mL. In order to increase the preconcentration factor and therefore decrease the limits of detection, a solid phase extraction was applied [24]. On the basis of a previous study [23], we selected 500 mg Oasis HLB cartridges (Waters) for the SPE step. This is a polymeric sorbent with hydrophilic groups in its structure which enable the polar analytes to be retained in the cartridge. The extract from PLE was diluted with water to 250 or 500 ml in order to reduce the percentage of methanol. The compounds retained in the cartridge were eluted with 6 ml of methanol [23]. However, despite the dilution, recoveries were low (all recoveries were lower than 10%) and so SPE was not applied.

## METHOD VALIDATION

When we analyzed the sewage sludge with LC-(ESI)-MS some of the compounds appeared in the chromatogram due to the higher sensibility than DAD.

For this reason the calibration curves were obtained by direct injection.

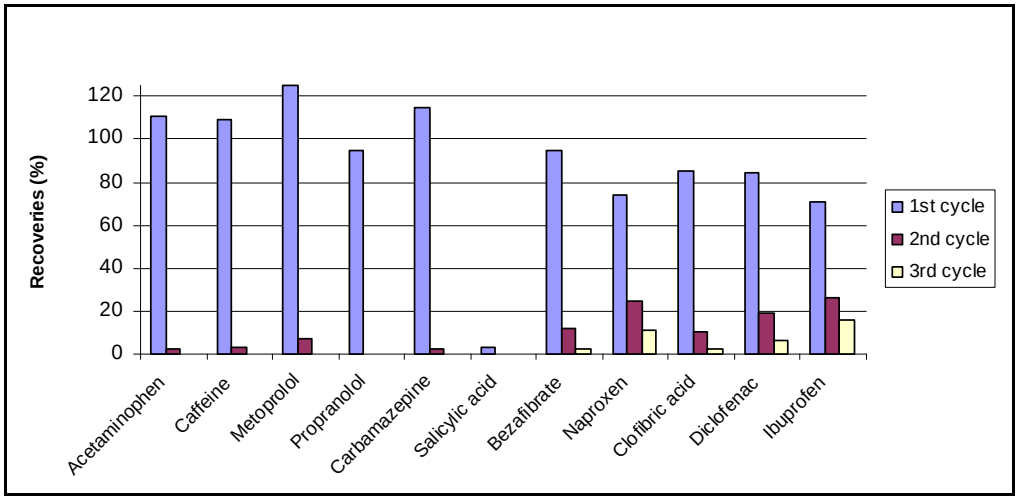


**Figure 1.** Recoveries obtained by successive extractions. Experimental conditions: 1500 psi., 100 ºC, methanol:water with 50 mM $H_3PO_4$ (1:1, v/v), 15 min of static time, 300 s of purge time and 150 % of flush volume.

The analyses were made in SIM mode, selecting the ions described in the experimental part of this article. Table 3 shows validation data obtained. To confirm the compounds, the retention time and the abundance relation were compared. Recoveries were higher than 68% for all pharmaceuticals when 5 g of sample spiked at 400 µg/Kg was analyzed.

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**Table 3.** Validation data.

| Compound | Recoveries (%) | Linear Range* (µg/l) | LOQ** (µg/Kg) | Repeatability RSD (%, n=3) | Reproducibility*** RSD (%, n=3) |
|---|---|---|---|---|---|
| Acetaminophen | 109 | 5-500 | 22 | 6 | 8 |
| Caffeine | 106 | 5-500 | 22 | 7 | 10 |
| Metoprolol | 120 | 5-500 | 19 | 6 | 8 |
| Propranolol | 93 | 5-500 | 25 | 7 | 8 |
| Carbamazepine | 112 | 5-100 | 14 | 6 | 7 |
| Bezafibrate | 92 | 5-500 | 25 | 8 | 9 |
| Naproxen | 72 | 5-500 | 32 | 7 | 10 |
| Clofibric acid | 81 | 5-500 | 28 | 6 | 8 |
| Diclofenac | 82 | 10-500 | 29 | 7 | 7 |
| Ibuprofen | 68 | 5-150 | 22 | 7 | 8 |

* Instrumental linear range
** Limit of quantification of the method
*** between days

When we analyzed sample spiked with different concentration (400 µg/Kg and 500 µg/Kg), recoveries were higher than 65% so ionic suppression was not observed. The linear range was calculated by direct injection, and linearity was good for most compounds between 5 and 500 µg/L, with determination coefficients higher than 0.9925. Repeatability and reproducibility between days, expressed as relative standard deviation (RSD), were calculated by analyzing 5 g of spiked samples at 200 µg/Kg (n=3). The results obtained showed RSDs lower than 8% for repeatability and 10% for reproducibility. Limits of detection, calculated as a signal-to-noise ratio of 3, were lower than 10 µg/kg of dry weight for all compounds. Limits of quantification (LOQ), calculated as a signal-to-noise ratio of 10 were lower than 32 µg/kg of dry weight for all compounds.

## METHOD APPLICATION

The method was applied to analyze sewage sludge samples collected every three months between February 2004 and June 2005 from two different STPs. Results are shown in Table 4.

Some trends were similar in both STPs. Metoprolol and propranolol were below the limits of detection in all the samples, probably because of their low concentration in the influent water, as was also indicated by Ternes [25] who analyzed influent water samples from a German STP. In other study by Pedrouzo *et al.* [23] metoprolol and propranolol showed low concentration in effluent and influent water in Spanish STP. Thus, these compounds were removed from Table 4.

On the other hand, caffeine, diclofenac and ibuprofen were present in most of the samples analyzed.

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**Table 4.** Results in μg/Kg of sewage sludge samples analyzed. Relative standard deviation (%RSD) is in brackets (n= 3).

| | Febr. 04 | June 04 | October 04 | Febr. 05 | June 05 |
|---|---|---|---|---|---|
| **STP1** | **Conc** | **Conc** | **Conc** | **Conc** | **Conc** |
| Acetaminophen | n.d. | n.d. | n.d. | 34 *(5)* | n.d. |
| Caffeine | 57 *(9)* | 59 *(4)* | 57 *(13)* | 65 *(10)* | 59 *(11)* |
| Carbamazepine | n.d. | 48 | <loq | 78 *(16)* | <loq |
| Bezafibrate | 28 *(4)* | n.d. | <loq | <loq | 88 *(15)* |
| Naproxen | n.d. | 70 *(15)* | <loq | 242 *(19)* | 62 *(8)* |
| Clofibric acid | n.d. | n.d. | 28 *(9)* | n.d. | 64 *(15)* |
| Diclofenac | n.d. | 65 *(3)* | 32 *(3)* | <loq | <loq |
| Ibuprofen | 91*(10)* | 81 *(10)* | 76 *(16)* | 99 *(12)* | 70 *(13)* |
| **STP2** | | | | | |
| Acetaminophen | n.d. | n.d. | 42 *(13)* | n.d. | 32 *(2)* |
| Caffeine | <loq | 58 *(169)* | 58 *(1)* | 65 *(5)* | n.d. |
| Carbamazepine | 91 *(8)* | 215 *(4)* | 50 *(1)* | 171 *(9)* | 165 *(13)* |
| Bezafibrate | <loq | 70 *(11)* | <loq | <loq | <loq |
| Naproxen | 87 *(15)* | 59 *(16)* | n.d. | n.d. | n.d. |
| Clofibric acid | 28 *(13)* | <loq | 32 *(11)* | 33 *(14)* | 28 *(11)* |
| Diclofenac | n.d. | 58 *(12)* | <loq | 53 *(13)* | 183 *(12)* |
| Ibuprofen | n.d. | 70 *(14)* | 74 *(16)* | n.d. | 75 *(15)* |

n.d.= not detected.
<loq = limit of quantification

Ibuprofen is widely used because its medical prescription is not necessary in many countries. Caffeine not only comes from pharmaceuticals but also from the population's consumption of coffee. Other authors found high values of ibuprofen and caffeine in influent wastewater samples [23,26], and for instance, caffeine was eliminated about 99.9% in an effluent wastewater from a Canadian STP [26], so we can expect to found them in sludge.

Carbamazepine was present in all samples from STP2 and at higher concentrations than STP1. These values agree with the concentrations reported by Miao *et al.* [26] who analyzed sewage sludge samples from an STP in Canada, being its concentration 258.1 μg/Kg in untreated biosolid.

Some pharmaceuticals such as caffeine and ibuprofen showed a similar concentration throughout the period studied. Others showed different concentrations depending on the sample analyzed. However, no relationship with the season could be established. Figure 2 shows extracted ion chromatograms of a sample from STP1 collected in February 2005.

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ISBN:978-84-693-6426-0/DL:T-1628-2010

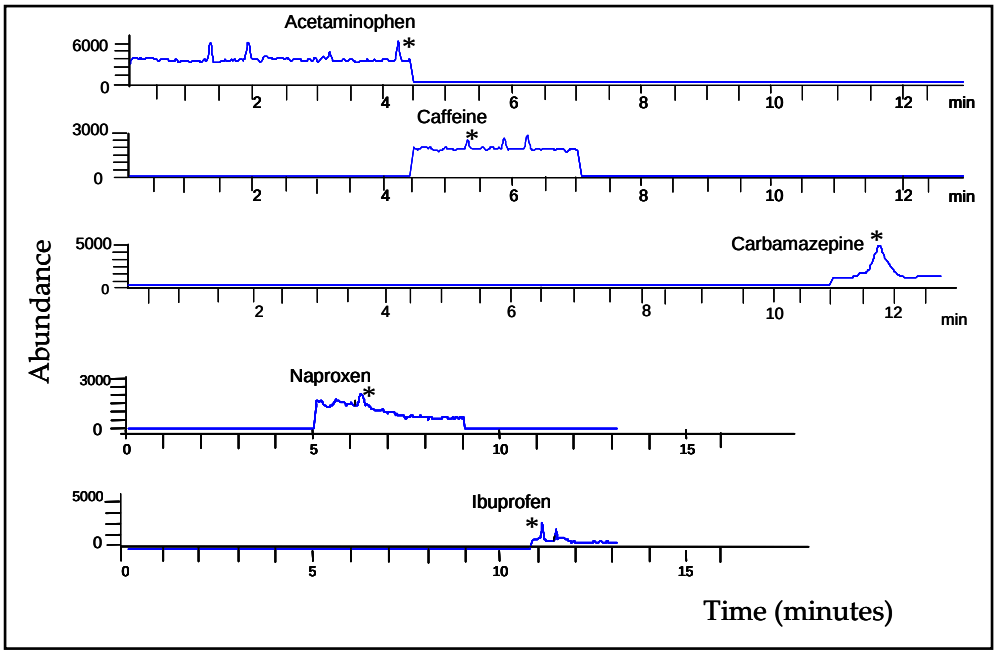


**Figure 2.** Extracted ion chromatograms of a sewage sludge sample from STP1 collected in February 2005.

## CONCLUSIONS

A method to determine a group of ten pharmaceuticals in sludge samples by PLE and LC-(ESI)MS was developed. The method determined these compounds at μg/Kg levels with good precision. Recoveries were quantitative for all compounds except for salicylic acid, which could not be extracted. The method was applied to determine pharmaceuticals in sewage sludge samples from two STPs. Carbamazepine, diclofenac, caffeine and ibuprofen were present in most of the samples analyzed at levels between 32 and 215 μg/Kg, whereas metoprolol and propranolol were not detected in most of the samples.

## Acknowledgements

This study was financially supported by the Dirección General de Investigación of the Ministery of Science and Technology, projects CTM2004-06265-C03-02 and CTM2005 -01774.

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### 3.1.2. Selective extraction of sulfonamides, macrolides and other pharmaceuticals from sewage sludge by pressurized liquid extraction

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# SELECTIVE EXTRACTION OF SULFONAMIDES, MACROLIDES AND OTHER PHARMACEUTICALS FROM SEWAGE SLUDGE BY PRESSURIZED LIQUID EXTRACTION

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**Abstract**

A method for the quantitative determination of three macrolides, five sulfonamides, ranitidine, omeprazole and trimethoprim in sewage sludge samples was developed by using pressurized liquid extraction and high performance liquid chromatography-mass spectrometry with electrospray ionization.
The extraction solvent and such operational parameters as temperature, pressure, extraction time and purge time were optimized in pressurized liquid extraction. The experimental conditions were: an extraction solvent of water (pH 3):methanol (1:1,v/v), a temperature of 80 ºC, a pressure of 100 bar, a sample weight of 5 g, an extraction time of 5 minutes, 1 cycle, a flush volume of 60% and a purge time of 120 seconds.
All recoveries were over 74%, except those for ranitidine whose value was 54%. The repeatability and reproducibility between days expressed as relative standard deviation (n= 3) were lower than 11% and 15%, respectively. The limit of detection values ranged from 2 to 11 μg/kg dry weight (d.w.). The method was applied to determine the pharmaceuticals in sewage sludge from two domestic sewage treatment plants. Roxithromycin and tylosin were determined in the samples and tylosin showed the highest value (4.0 mg/kg d.w.).

**Keywords:** Pharmaceuticals, sewage sludge samples, liquid chromatography-mass spectrometry, pressurized liquid extraction.

## INTRODUCTION

Increasing attention has recently been paid to the presence of "emerging contaminants" in the aquatic environment and wastewater treatment plants [1,2]. There is no accepted list of emerging contaminants but they are defined as "newly identified or previously unrecognised pollutants" [3] and include thousands of products used in everyday life (e.g. pharmaceuticals, personal care products, surfactants, gasoline additives, etc.).

ISBN:978-84-693-6426-0/DL:T-1628-2010

Of the pharmaceuticals, antibiotics are a particularly important group today. They are not only used in human infections but also in preventive veterinary medicine. As a consequence of their widespread use and the inappropriate treatment of human and animal excretions, antibiotic residues may be present in the environment for long periods of time [4].

Macrolide antibiotics include a family of antibacterial agents that is widely used in human and veterinary medicine [5]. The sulfonamides are the fifth most used chemotherapeutics in veterinary practice because they are cheap and have a wide spectrum of activity to prevent or treat acute and chronic bacterial infections [6]. They are also added to animal feed to promote growth [7]. Other widely used pharmaceuticals are, for instance, ranitidine and omeprazole for treating or preventing ulcers and trimethoprim for treating infections.

The presence of pharmaceuticals has been studied in many kinds of samples, but mainly in water (e.g. surface water, effluent and influent wastewater [8-11], hospital wastewater [12] and drinking water [13,14]). However, solid samples such as sewage sludge have been less studied [4,15-17].

It is important to determine pharmaceutical compounds in sewage sludge for two reasons. The first is to evaluate the elimination power of sewage treatment plants (STPs) and the second is to determine whether sewage sludge can be used as manure.

Pharmaceuticals have mainly been extracted from a solid matrix by Soxhlet extraction or sonication [9,18]. More advanced extraction techniques, such as pressurized liquid extraction (PLE) or microwave-assisted extraction (MAE), have been reported for determining a variety of pharmaceuticals [3,4,18-20]. For example, in a previous study [4] we determined β-blockers, anti-inflammatories and lipid regulators in sewage sludge, and we found such pharmaceuticals as ibuprofen, carbamazepine, acetaminophen, and caffeine, amongst others. Göbel *et al.* [15] determined some macrolides in sludge samples by PLE. In comparison to classical extraction techniques, it provided good recoveries and saved time and organic solvent.

In sewage sludges, pharmaceuticals are present in very low concentrations and coexist with a large number of potentially interfering compounds. For this reason, a clean-up step is sometimes necessary. This step can be carried out by solid-phase extraction (SPE) [3,19], or gel permeation chromatography (GPC) [21]. SPE is preferred in most instances because it is fast, requires a low volume of organic solvent and has a low contamination risk. However, some methods avoid SPE because low recoveries are obtained for some compounds [4].

Some methods based on gas chromatography with mass spectrometry detection (GC-MS) [22] have been described for determining these pharmaceutical compounds.

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However, because of the polarity of most pharmaceuticals, high performance liquid chromatography (LC) is the technique preferred. UV detection [23] and fluorescence [24] have been used but mass spectrometry detection [5,25] or tandem mass spectrometry [1,3,6] are the most suitable techniques.

The aim of the present paper is to develop a method based on pressurized liquid extraction and LC-MS with positive electrospray ionization (ESI+) to determine three macrolides (roxithromycin, erythromycin and tylosin), five sulfonamides (sulfamethoxazole, sulfapyridine, sulfadiazine, sulfamethazine, sulfatiazole), ranitidine, omeprazole and trimethoprim in sewage sludge samples. Some of these compounds have been determined in the literature, but they have never been determined all together in only one method. To demonstrate the feasibility of the method, samples from two sewage treatment plants have been analyzed.

## EXPERIMENTAL

### Materials and reagents

Sulfamethoxazole, sulfadiazine, sulfamethazine, sulfapyridine, sulfatiazole, tylosin, erythromycin, roxithromycin, omeprazole, ranitidine and trimethoprim 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 stored at 5 ºC. A mixture of all compounds in methanol at a concentration of 50 mg/L was prepared weekly. Working solutions were prepared daily by diluting this solution with water.

Ultra-pure water was obtained with a Milli-Q water purification system (18.2 MΩ·cm) (Millipore, Bedford, MA, USA). Acetonitrile and methanol (HPLC-grade) were from SDS (Peypin, France), nitrogen was from Carburos Metálicos (Tarragona, Spain) and acetic acid was from Merck (Darmstadt, Germany).

### Sample pre-treatment

The samples were collected in June 2006 and October 2006 from two STPs in the south of Catalonia. These STPs received mainly urban wastewaters and some industrial discharges.

The samples were stored in the freezer in glass bottles and lyophilized before analysis by the freeze dry system (Labconco, Kansas City Missouri, USA). Then they were homogenized using a mortar and pestle and sieved to obtain particles with a diameter less than 125 μm.

To optimize the method, sludge samples were spiked with all the compounds dissolved in acetone. They were then stirred vigorously so that the compounds were in sufficient contact with the matrix until acetone was evaporated.

### Sample extraction

Sludge samples were extracted using

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an ASE 200 pressurized liquid extraction system (Dionex, Sunnyvale, CA, USA) equipped with a 33 ml stainless steel extraction vessel. A total of 5 g of the pretreated sludge was placed in a 33 mL stainless-steel extraction cell, and thoroughly mixed with aluminium oxide, which was heated at 120 ºC in the oven for 24 hours before use.

The extracting solvent was water (pH 3):methanol (1:1, v/v). The operating conditions were as follows: extraction temperature, 80 ºC; extraction pressure, 100 bar; preheating period, 5 min; static extraction, 5 min; final extraction volume ~ 30 mL; flush volume, 60% of the cell volume; nitrogen purge, 120 s; and number of extraction cycles, 1.

The extract was filtered with a microfilter 0.45 μm pore size (Teknokroma, Barcelona, Spain), and analyzed by liquid chromatography.

**Chromatographic analysis**

The chromatographic instrument was an HP1100 series LC-mass selective detector (Agilent Technologies, Waldbronn, Germany) with an ESI interface, an automatic injector, a degasser, a quaternary pump, a column oven and photodiode array detection (DAD).

The chromatographic column was a Kromasil 100 $C_{18}$ (25.0 x 0.46 cm) with a 5 μm particle size (Teknokroma), and the volume injected was 50 μL. The mobile phase flow-rate was 1 mL/min and the column temperature was kept at 30 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 initially 10% B, which increased to 15 % in 10 min, to 26% in 5 min, to 60% in 4 min, 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. The wavelengths used were 260 nm for sulfadiazine, trimethoprim, sulfapyridine, sulfatiazole, sulfamethazine, sulfamethoxazole and omeprazole, 210 nm for erythromycin and roxithromycin, 287 nm for tylosin and 310 nm for ranitidine.

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. In this case, all compounds had to be determined in the positive ionization mode because the signals were higher. The data were acquired in selected ion monitoring (SIM) mode. Different fragmentor voltages were studied to find spectra with at least 3 ions. The ions selected for acquiring the data are shown in Table 1. The quantifier ion was selected taking into account the abundance and the ion selectivity. In most cases the quantifier ion corresponds to $[M+H]^+$, except for sulfadiazine and omeprazole.

The average conditions selected for the optimum performance of the ESI interface in the positive mode were: nebulizer pressure of 40 psi, drying

gas flow-rate of 12 l/min, drying gas temperature of 350 ºC and capillary voltage of 4,000 V. Fragmentation voltages were defined individually and the values used were: 75 V for sulfamethoxazole, omeprazole and ranitidine, 100 V for sulfadiazine, sulfapyridine, sulfatiazole, sulfamethazine and 125 V for trimethoprim, erythromycin, roxithromycin and tylosin. For additional confirmation purposes, we checked other fragmentation voltages and only sulfapyridine and sulfamethoxazole showed a significant difference in the spectrum. Therefore we used fragmentation voltages of 125 V to confirm these compounds. 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%).

**Table 1.** Studied compounds, abbreviations and ions selected for quantification and confirmation. The quantification ion is underlined. The abundances are in brackets.

| Compound | Ions | Abbreviation |
|---|---|---|
| Sulfadiazine | 251 (80%), 156 (100%), 273 (45%) | SDZ |
| Trimethoprim | 291 (100%), 261 (10%), 123 (5%) | TRI |
| Sulfapyridine | 250 (100%), 156 (70%), 108 (20%) | SPY |
| Sulfatiazole | 256 (45%), 156 (100%), 108 (20%) | STZ |
| Sulfametazine | 279 (100%), 186 (25%), 124 (15%) | SMT |
| Sulfamethoxazole | 254 (70%), 156 (100%), 108(55%) | SMX |
| Omeprazole | 198 (100%), 346 (55%), 151 (25%) | OME |
| Erythromycin | 735 (100%), 576 (60%), 158 (55%) | ERY |
| Roxithromycin | 838 (100%), 413 (80%), 679 (30%) | ROX |
| Tylosin | 916 (100%), 174 (15%), 772 (10%) | TYL |
| Ranitidine | 315 (100%), 270 (20%), 176 (10%) | RAN |

## RESULTS AND DISCUSSION

### PLE optimization

In pressurized solvent extraction, eight parameters were optimized: the pressure, temperature, solvent, number of cycles, static time, purge time, sample weight and flush volume. The initial conditions for the PLE optimization were selected from previous studies [4,15], where some pharmaceuticals had been determined in sewage sludge samples. These conditions were: 100 bar, 80 ºC, a water (pH 3):methanol solvent (1:1, v/v), 4 cycles, 15 min of static time, 120 s of purge time, 5 g of sample weight and finally 60% of flush volume.

First, a blank of a sewage sludge sample was extracted by PLE and analyzed by LC-DAD under the above conditions and the chromatogram showed no peaks at the same retention time as the compounds studied. Then, the sample was spiked to optimize the PLE variables. We selected 5 g of lyophilized sludge spiked with 100 mg/kg dry weight

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(d.w.) of each compound to perform the PLE extraction with the 33 ml cell.
The first parameter optimized was the extraction solvent and the results are shown in Table 2. The initial solvent mixture selected gave recoveries above 50% for most of them, except for erythromycin, omeprazole, sulfamethoxazole and roxithromycin. The percentages of methanol and water were varied from 0% to 100%. The recoveries for all of the compounds except sulfadiazine, sulfatiazole and trimethoprim were the highest when we used water:methanol (1:9). For sulfadiazine and sulfatiazole, recoveries were higher with water:methanol (1:1) and for trimethoprim, with water: methanol (3:1).

**Table 2**. PLE recoveries (n=3) using different solvents. A= water (pH 3), B= methanol and C= acetonitrile. For other conditions, see the text.

| Compound | A | B | A:B (3:1) | A:B (9:1) | A:B (1:1) | A:B (1:3) | A:B (1:9) | A:C (1:1) |
|---|---|---|---|---|---|---|---|---|
| SDZ | 76 | - | 66 | 53 | 77 | 45 | - | 66 |
| TRI | 47 | 42 | 71 | 54 | 60 | 34 | 60 | 55 |
| SPY | 43 | 107 | 61 | 46 | 65 | 93 | 109 | 56 |
| STZ | 56 | 16 | 76 | 62 | 81 | 95 | - | 74 |
| SMT | 85 | 119 | 92 | 80 | 89 | 97 | 119 | 90 |
| SMX | 26 | - | 27 | 17 | 15 | - | 48 | 14 |
| OME | - | 80 | 9 | - | 37 | 35 | 96 | 25 |
| ERY | - | - | 21 | 12 | - | - | 26 | - |
| ROX | - | 50 | - | 8 | - | 26 | 109 | - |
| TYL | 38 | 80 | 75 | 65 | 64 | 61 | 117 | 67 |
| RAN | 40 | 5 | 50 | 40 | 51 | - | 72 | 21 |

RSD (%)≤ 5

We attempted to improve recoveries by replacing methanol with acetonitrile but it was unsuccessful. Some of the compounds such as omeprazole and roxithromycin required a high percentage of methanol in the extraction solvent to enhance their recoveries.
Erythromycin was partially extracted only when 90% of methanol was used as the extraction solvent. This result was agree with the literature, where it is mentioned that, erythromycin degradates with an apparent loss of one molecule of water when water is present at acidic pH [26]. Because the results for each compound were best under different solvent mixtures, we decided to continue with the optimization by selecting the three solvent mixtures mentioned above. The second parameter optimized was the extraction temperature. We tested five different temperatures (25, 50, 80, 100 and 120 ºC). Table 3 shows the recovery values of all the compounds at three temperatures (50, 80 and 100 ºC); the results for the other temperatures are not shown in the table because no improvement was seen. We observed an increase in the recovery only for trimethoprim. The

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recovery was 107% when water: methanol (1:9) was used at 50 ºC, which was higher than water: methanol (3:1) at 80 ºC. Therefore we no longer considered water:methanol (3:1) as an extraction solvent. The recoveries of the other compounds did not increase significantly by changing temperatures. In both mixtures the recoveries were higher than 60% in all compounds except erythromycin and sulfamethoxazole, which gave lower recoveries with all solvents and temperatures.

Consequently we changed various parameters to improve the recoveries. We decided to continue with the optimization by selecting two temperatures and solvents: water: methanol (1:9) at 50 ºC and water: methanol (1:1) at 80 ºC.

**Table 3**. PLE recoveries using different temperatures and solvent mixtures. A= water (pH 3) and B= methanol. For other conditions, see the text.

| Compounds | 50ºC | | | 80ºC | | | 100ºC | | |
|---|---|---|---|---|---|---|---|---|---|
| | A:B (1:1) | A:B (3:1) | A:B (1:9) | A:B (1:1) | A:B (3:1) | A:B (1:9) | A:B (1:1) | A:B (3:1) | A:B (1:9) |
| SDZ | 43 | 30 | - | 77 | 66 | - | 53 | 47 | - |
| TRI | 62 | 63 | 107 | 60 | 71 | 60 | 20 | 30 | 40 |
| SPY | 43 | 42 | 76 | 65 | 61 | 109 | 54 | 47 | 65 |
| STZ | 59 | 28 | 30 | 81 | 76 | - | 64 | 28 | 44 |
| SMT | 73 | 65 | 81 | 89 | 92 | 119 | 72 | 64 | 69 |
| SMX | 39 | - | 35 | 15 | 27 | 48 | 16 | - | - |
| OME | 29 | - | 84 | 37 | 9 | 96 | 48 | - | 46 |
| ERY | 19 | 19 | 14 | - | 21 | 26 | 29 | 18 | - |
| ROX | - | 78 | 60 | - | - | 109 | 2 | 85 | 65 |
| TYL | 71 | 74 | 85 | 64 | 75 | 117 | 76 | 98 | 58 |
| RAN | 32 | 38 | 72 | 51 | 50 | 72 | 32 | 66 | 25 |

RSD (%)≤ 6 (n=3)

The third parameter optimized was extraction pressure. We tried three different values (33, 66 and 100 bar). However, recoveries did not improve when values other than the initial ones were used. Therefore, we decided to use 100 bar as the extraction pressure. In a previous study [4], we had observed the same situation and the pressure did not modify the recoveries significantly.

The fourth parameter optimized was the extraction time. Table 4 shows the results. Three different extraction times (5, 10 and 15 minutes) were tested. We observed that when water:methanol (1:1) was used and the extraction time was decreased, the recoveries increased for all compounds except sulfatiazole, ranitidine, sulfadiazine and sulfamethazine, whose recoveries were similar. The recoveries of all compounds except for erythromycin, whose problem has already been mentioned, were higher than 50% at 5 minutes. For water: methanol (1:9), the optimum extraction time was 15 minutes. We decided

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that the best mixture was water: methanol (1:1), 80 ºC and the best extraction time 5 minutes, because we saved time and energy and we obtained higher recoveries for most of them, and therefore we excluded erythromycin of the study.

**Table 4.** PLE recoveries (n=3) using different extraction times and different mixtures. A= water (pH 3) and B = methanol. For other conditions, see the text.

| Compounds | A:B (1:1) 80ºC | | | A:B (1:9) 50ºC | | |
|---|---|---|---|---|---|---|
| | 5 min | 10 min | 15 min | 5 min | 10 min | 15 min |
| SDZ | 77 | 75 | 77 | - | 6 | - |
| TRI | 82 | 80 | 60 | 72 | 46 | 107 |
| SPY | 78 | 81 | 65 | 54 | 65 | 76 |
| STZ | 64 | 67 | 81 | 5 | 12 | 30 |
| SMT | 81 | 80 | 89 | 57 | 66 | 81 |
| SMX | 64 | 66 | 15 | - | - | 35 |
| OME | 79 | 75 | 37 | 50 | 58 | 84 |
| ERY | 3 | 2 | - | 27 | 26 | 14 |
| ROX | 83 | 76 | - | 60 | 62 | 60 |
| TYL | 91 | 85 | 64 | 67 | 66 | 85 |
| RAN | 50 | 50 | 51 | 41 | 54 | 72 |

RSD (%)≤ 9

The fifth parameter optimized was the extraction cycles. We applied three consecutive simple extractions to the same sample with water:methanol (1:1) at 80 ºC and 5 minutes of static extraction. The recoveries were higher than 50% for all compounds. The recoveries were considered negligible (lower than 4%) in the second cycle. For this reason, one cycle was selected as optimum.

Purge time and flush volume were adjusted sequentially in order to check the influence on the recoveries. However, as expected, recoveries did not improve and we decided to use the initial conditions [4].

Under these optimal conditions, compounds were extracted with about 30 mL. In order to increase the preconcentration factor and therefore decrease the limits of detection, a solid-phase extraction was applied after PLE [19]. On the basis of a previous study [27], we selected 500 mg Oasis HLB cartridges (Waters) for the SPE step. Oasis HLB is a polymeric sorbent with hydrophilic groups in its structure which enables the polar analytes to be retained in the cartridge. The extract from PLE was adjusted at pH 7 and diluted with water to 250 or 500 ml in order to reduce the percentage of methanol. The compounds retained in the cartridge were eluted with 4 ml of methanol (2 ml methanol pH 8 and 2 ml methanol) and then evaporated to dryness and redissolved in 1 mL of water. However, despite the dilution, recoveries decreased and were lower than 50%. Only the recoveries of

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sulfapyridine and tylosin were higher than 68%, so SPE was not applied. In a previous paper [4] we used the SPE process to analyze some other pharmaceuticals in an attempt to reduce the limit of detection but in that case too we found similar problems.

## METHOD VALIDATION

Although LC-DAD was used for the optimization of the PLE extraction, the method was developed by liquid chromatography-electrospray ionization-mass spectrometry (LC-(ESI)MS) in order to decrease the limit of quantification and make the analysis more selective.

When the PLE extract of sewage sludge was analyzed with LC-(ESI)MS some compounds appeared in the chromatogram. For this reason the calibration curves were obtained by direct injection of standard solutions instead of analyzing a sample by the whole method developed. The acquisition was done in SIM mode, by using the ions described in Table 1. Table 5 shows the validation data obtained. To confirm the presence of the compounds, the retention times of the compounds and the relative abundance of the produced ions were compared.

Recoveries when 5 g of sample spiked at 2 mg/kg (d.w.) was analyzed were higher than 74% for all pharmaceuticals except for ranitidine (54%). To check the matrix effect, we quantified the same sample by two calibration methods: standard additions and direct injection. The result of standard additions was 1.7 mg/kg (d.w.) (n= 3) of tylosin, and the result of direct injection was 1.6 mg/kg (d.w.) (n= 3) of tylosin. We compared the calibration curves with the F-test ($\alpha$= 0.05, n= 2) and t-test ($\alpha$= 0.05, n= 2). They were comparable ($t_{calc}$= 4.228) so no matrix effect was observed.

The linear range was calculated by direct injection, and linearity was good, between 10 and 500 μg/L for most of them. Determination coefficients ($r^2$) were higher than 0.992. The precision of the method was evaluated by preparing a set of samples fortified with the analytes; the analysis was performed with three replicates. Repeatability and reproducibility between days, expressed as relative standard deviation (RSD), were calculated by analysing 5 g of spiked samples at 2 mg/kg (d.w.). The limit of quantification (LOQ), as the concentration of the lowest point of the calibration curve, ranged from 20 to 100 μg/kg (d.w.) (table 5). Limits of detection (LOD), calculated as a signal-to-noise ratio of 3, were lower than 11 μg/kg (d.w.) for all compounds.

## METHOD APPLICATION

The method was applied to analyze sewage sludge samples collected in June 2006 and October 2006 from two different STPs. The results are shown in Table 6. Sulfamethoxazole and ranitidine were excluded from the table because they were not detected in any samples.

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**Table 5.** Validation data.

| | Recoveries (%)* (n=3) | Linear range** (μg/l) | LOD*** (μg/Kg) | LOQ**** (μg/Kg) | RSD[a] (%) | RSD[b] (%) |
|---|---|---|---|---|---|---|
| SDZ | 76 | 10-500 | 7 | 100 | 5 | 9 |
| TRI | 77 | 2-500 | 2 | 20 | 0.3 | 3 |
| SPY | 85 | 5-500 | 3 | 50 | 1 | 4 |
| STZ | 74 | 10-500 | 5 | 100 | 3 | 6 |
| SMT | 87 | 2-500 | 2 | 20 | 1 | 2 |
| SMX | 81 | 5-500 | 5 | 50 | 11 | 15 |
| OME | 93 | 5-500 | 7 | 50 | 3 | 5 |
| ROX | 83 | 10-500 | 7 | 100 | 5 | 8 |
| TYL | 95 | 10-500 | 8 | 100 | 1 | 3 |
| RAN | 54 | 10-500 | 6 | 100 | 1 | 4 |

* Sample spiked at 2 mg/kg.
** Instrumental linear range
*** Limit of detection of the method
**** Limit of quantification of the method
[a] Repeatability (n=3)
[b] Reproducibility between days (n=3)

Values below the limit of quantification (<LOQ) are for peaks which were confirmed from the relative abundance of the three ions, but they could not be quantified. The results in both STPs were similar. Tylosin and roxithromycin were the only pharmaceuticals quantified in both STPs and tylosin had the highest value (1.9 mg/kg (d.w.) in STP1 and 4.0 mg/kg (d.w.) in STP2). These compounds have already been found in different kinds of samples such as water samples [8,11], surface water [20] and sewage sludge [16]. Figure 1 shows the extracted ion chromatograms of one sample from STP1 collected in June 2006. Sulfamethazine and sulfatiazole were found at concentrations below the limit of detection, probably because of their low concentration in the influent water, as was indicated by Pedrouzo *et al.* [27].

Although very few papers have determined macrolides in sewage sludge, in one of them [15] a few macrolides, sulfonamides and trimethoprim were found at levels lower than in our samples of low μg/kg (d.w.).

For instance, they found roxithromycin but at lower concentrations, between 40 and 130 μg/kg (d.w.). Sulfapyridine, sulfamethoxazole and trimethoprim were also determined in both samples analyzed at similar levels than roxithromycin. However, in another paper [16] by the same group some of these compounds were found at mg/kg (d.w.). Although only two compounds were quantified, the presence of most of the pharmaceuticals studied at concentrations below the limit of quantifications means that these compounds are present in sewage sludge and with more sensitive detectors, such as MS-MS, they could be determined.

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**Table 6.** Results in mg/Kg of sewage sludge samples analyzed. Relative standard deviation (%RSD) is in brackets (n=3).

| | STP1 | | STP2 | |
|---|---|---|---|---|
| | **June 06** | **October 06** | **June 06** | **October 06** |
| SDZ | <LOQ | <LOQ | <LOQ | - |
| TRI | <LOQ | <LOQ | <LOQ | <LOQ |
| SPY | <LOQ | - | <LOQ | <LOQ |
| STZ | <LOQ | <LOQ | <LOQ | - |
| SMT | <LOQ | - | <LOQ | <LOQ |
| OME | - | - | - | <LOQ |
| ROX | 1.8 (13) | <LOQ | 1.3 (10) | <LOQ |
| TYL | 1.9 (7) | 1.3 (5) | 4.0 (3) | 2.8 (5) |

<LOQ below of the limit of quantification, included in Table 5.
- not detected

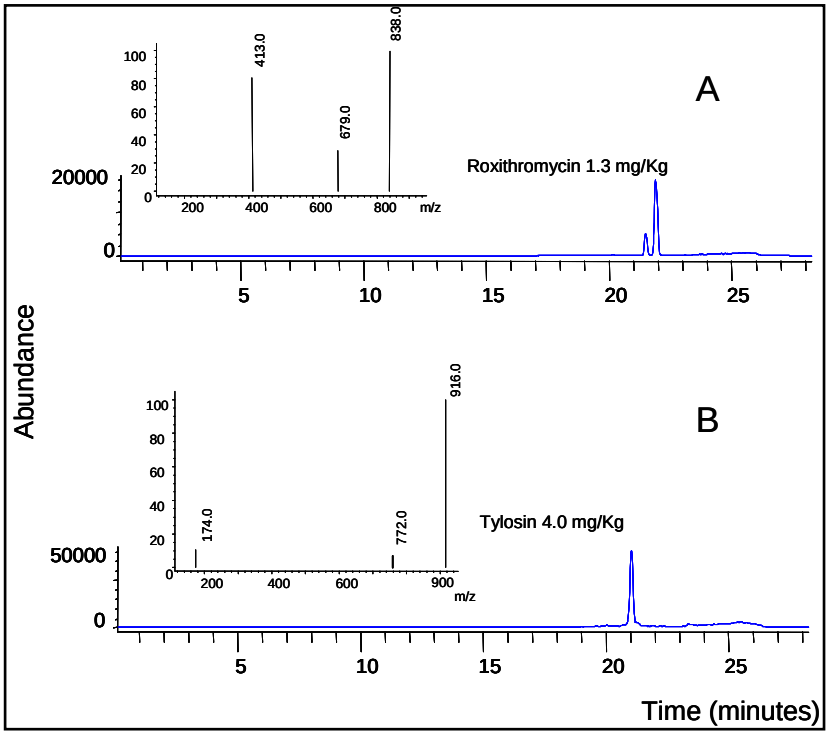


**Figure 1**. Extracted ion chromatograms and spectra of a sewage sludge sample from STP1. (A) m/z= 838. (B) m/z= 916.

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## CONCLUSIONS

Because sulfonamides and macrolides have a wide variety of different structures and physical-chemical properties, they usually require complex methods if they are to be simultaneously analyzed. A robust method was developed by using pressurized liquid extraction and LC-(ESI+)MS of sulfonamides, macrolides, ranitidine, omeprazole and trimethoprim. In the analysis of samples from two STPs two compounds (roxithromycin and tylosin) were determined in concentrations between 1.3 and 4 mg/kg (d.w.) and some others were found in several samples at levels below LOQs.

**Acknowledgements**

The authors wish to thank the personnel of the water treatment plants in Tarragona and Reus for their cooperation with all aspects of this study.
This study was financially supported by the Dirección General de Investigación of the Ministery of Science and Technology, projects CTM2004-06265-C03-02, CTM2005-01774. A. N. gratefully acknowledges the financial support of the Ministery of Science and Technology BES-2006-1332.

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### 3.1.3. Target and non-target analysis of sewage sludge using pressurized liquid extraction and liquid chromatography time-of-flight mass spectrometry

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# TARGET AND NON-TARGET ANALYSIS OF SEWAGE SLUDGE USING PRESSURIZED LIQUID EXTRACTION AND LIQUID CHROMATOGRAPHY TIME-OF-FLIGHT MASS SPECTROMETRY

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**Abstract**

This paper studies the potential of LC-TOF-MS to determine target contaminants and identify non-target contaminants from sewage sludge. A multiresidue method has been developed by using pressurized liquid extraction and liquid chromatography-mass spectrometry with time of flight analyzer (PLE/LC-TOF-MS) to determine fifteen pharmaceuticals in sewage sludge. The limits of detection and limits of quantification were between 20 and 100 μg/Kg of dry weight (d.w.) and between 50 and 250 μg/Kg (d.w.), respectively. The repeatability and reproducibility between days (n=3) were lower than 13 and 14%, respectively. Of all the pharmaceuticals determined in sewage sludge samples, tylosin showed the highest levels (954-1,081 μg/Kg (d.w.)).
The use of LC-TOF-MS also enabled non-target analytes such as alkyl ethoxylates (AEOs), nonylphenol ethoxylates (NPEOs) and phthalates to be identified in the sewage sludge samples analyzed.

**Keywords:** Liquid chromatography, multiresidue analysis, pharmaceuticals, sewage sludge, time of flight mass spectrometry.

## INTRODUCTION

In the last few years, the use of LC-TOF-MS for environmental analyses has increased. The TOF-MS system is a high-resolving power instrument capable of a resolving power of 10,000 expressed in terms of full peak width at one-half maximum (FWHM) [1]. In principle, if masses can be measured with sufficient accuracy, elemental compositions can be assigned to peaks observed during the course of an analysis. In practice, a mass measurement within 2 mDa provides a short list of elemental compositions for consideration. The risk of false-positive findings in environmental samples is significantly reduced with TOF analyzers due to their increased

ISBN:978-84-693-6426-0/DL:T-1628-2010

mass resolution and mass accuracy [2-5]. When analyzing environmental samples TOF analyzers have one additional advantage over such other analyzers as quadrupoles or triple quadrupoles: TOF analyzers work in SCAN mode, which enables the chromatograms to be reviewed or recorded so that new compounds or transformation products suspected of being present in the samples can be searched for.

Several studies have used TOF-MS to determine pharmaceuticals in a variety of environmental samples [1,5-7], but in most cases it is used only to confirm the presence of target analytes or to identify non-target analytes, not to quantify target analytes. In recent years, pharmaceuticals have received significant attention in the environmental field and this has resulted in a growing number of publications concerning their determination by LC-MS and LC-MS-MS [8-12].

Gómez *et al.* [13] and Martínez *et al.* [14] used TOF-MS to determine pharmaceuticals in water samples. Gómez *et al.* [13] used it to determine pharmaceuticals in hospital effluent wastewater, and 18 of the 20 pharmaceuticals they studied were determined at concentration levels of μg/L. Different metabolites were identified in hospital water and wastewater samples such as the metabolite of the dipyrone [13,14]. LC-TOF-MS was also used to identify metabolites and transformation products in the study by Trovo *et al.* [15] who identified nine transformation products in distilled water. The LC-TOF-MS has also been used in food analysis, for instance, macrolides, sulfonamides and anti-inflammatories, among other groups of veterinary drugs have been determined in eggs, fish and meat [7].

As has been mentioned above, TOF analysis has been used to confirm the presence of target compounds and identify non-target compounds. However, in the determination of pharmaceutical residues in environmental samples by LC-(ESI)MS, most effort so far has focused on decreasing the limit of detection (LODs) so that they can be determined at very low concentrations (most of these contaminants are present at μg/Kg or μg/L levels in the samples). Using different mass analyzers – single quadrupole (Q) [16,17] and specially triple quadrupole instruments (QqQ) [9,18] – low limits of detection can be obtained [19]. However, although these anayzers can achieve the lowest limits of detection in selected ion monitoring (SIM) and multiple reaction monitoring (MRM) modes, respectively, the structural information is reduced. On the other hand, quadrupole-time of flight (Q-TOF) analyzer keeps the structural information but it has a high cost [2].

TOF-MS and tandem mass spectrometry were compared by Labadie *et al.* [20] who determined estrogen compounds in river sediments. The instrumental detection limits achieved by LC-MS-MS were about 4-6 times lower than those achieved by LC-TOF-MS. The

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difference between the two techniques was larger when the detection limits of the method were compared: LC-MS-MS was approximately 13 times more sensitive than LC-TOF-MS with the same extraction technique. They analyzed the samples using the LC-MS-MS because LODs were lower and they were interested in quantifying the target analytes.

The main aim of the present study was to demonstrate that the new generation of LC–TOF–MS is able to perform a multiresidue analysis of pharmaceuticals of different therapeutic classes in sewage sludge. The selected compounds included antibiotics, antiepileptic agents, anti-ulceratives, analgesics and β-blockers. TOF-MS was also used to identify some non-target compounds in the samples analyzed.

## EXPERIMENTAL

### Materials and reagents

The target pharmaceuticals studied were: caffeine, propranolol, metoprolol, carbamazepine, acetaminophen, sulfamethoxazole, sulfadiazine, sulfamethazine, sulfapyridine, sulfatiazole, tylosin, erythromycin, roxithromycin, omeprazole, ranitidine and trimethoprim. All standards were from Sigma (St. Louis, USA). Stock solutions of individual standards were prepared by dissolving caffeine, propranolol, metoprolol, carbamazepine, acetaminophen in methanol:water (1:1, v/v) and the rest in methanol at a concentration of 1000 mg/L. They were stored at 5 ºC. Fresh stock solutions were prepared once a year. A mix of all compounds in methanol:water (1:1, v/v) at a concentration of 50 mg/L was prepared weekly. Working solutions were prepared daily by diluting the solution described above with methanol:water (1:1, v/v).

Ultra-pure water was obtained with a Milli-Q water purification system (18.2 MΩ·cm) (Millipore, Bedford, MA, USA). Acetonitrile and methanol (HPLC-grade) were from SDS (Peypin, France), nitrogen was from Carburos Metálicos (Tarragona, Spain) and aluminium oxide, phosphoric acid and acetic acid were from Merck (Darmstadt, Germany).

### Sample pre-treatment and PLE extraction

We collected sewage sludge samples from a conventional sewage treatment plant (STP) in Tarragona (Spain), which had activated sludge biological treatment. The STP mostly receives urban wastewaters and some industrial discharges. It is connected to population equivalents (around 140,000) that have a biological oxygen demand ($BOD_5$) of 400 mg/L and the average flow-rate is 30,000 $m^3$/day. Frozen samples were lyophilized before analysis by the freeze dry system (Labconco, Missouri, USA). Then they were homogenized using a mortar and pestle, and sieved to obtain particles with a diameter less than 125 μm.

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To extract pharmaceuticals from sewage sludge an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA) was used. The ASE 200 was equipped with 33 mL stainless steel extraction vessels.

Two extraction methods have been optimized in previous papers [16,17]. The conditions to extract caffeine, propranolol, metoprolol, carbamazepine and acetaminophen were (Method A) [16]: extraction temperature, 100 ºC; extraction pressure, 100 bar; preheating period, 5 min; static extraction time, 15 min; final extraction volume ~ 40 mL; flush volume, 150% of the cell volume; nitrogen purge, 300 s; and number of extraction cycles, 2.

For the rest of the compounds (macrolides, sulfonamides, omeprazole, ranitidine and trimethoprim) the conditions were (Method B) [17]: extraction temperature, 80 ºC; extraction pressure, 100 bar; preheating period, 5 min; static extraction time, 5 min; final extraction volume ~ 30 mL; flush volume, 60% of the cell volume; nitrogen purge, 120 s; and number of extraction cycles, 1.

In both methods, the extracting solvent was 50 mM aqueous phosphoric acid and methanol (1:1, v:v), and a total of 5 g of the pretreated sludge was placed in a 33 mL stainless steel extraction cell, and thoroughly mixed with aluminium oxide. The aluminium oxide was heated at 120 ºC in the oven for 24 hours before use [16,17].

**LC-TOF analysis**

The chromatographic instrument was an HP1200 series LC-time of flight mass spectrometer (Agilent Technologies, Waldbronn, Germany) with an electrospray ionization (ESI) interface, a manual injector, a degasser, a binary 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), and the volume injected was 20 µL. Solvent A was Milli-Q water with acetic acid (pH 2.8) and solvent B was acetonitrile. The mobile phase flow-rate was 1 mL/min and the column temperature was kept at 35 ºC. Both extracts were injected using the same gradient. Initially it was 10% B, which increased to 15% in 10 min, to 26% in 5 min, to 60% in 4 min, to 100% in 4 min, and kept constant for 22 min. Finally, it returned to 10% B in 2 min. All the target compounds eluted within the first 22 min.

The new generation of TOF-MS instruments are equipped with a dual nebulizer ion source that automatically performs accurate mass calibration by introducing the reference compounds into the solvent that exits the outlet of the chromatograph at low flow rates. The TOF-MS scanning was undertaken in an m/z range from 100 to 1,200 for all standards and samples at a scan rate of 2 cycles/s.

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**Table 1.** List of target compounds, the theoretical and experimental mass, and the average of the error calculated in the calibration curve.

| Compound | Formula | Theoretical $[M+H]^+$ | Experimental $[M+H]^+$ | Error (ppm) |
|---|---|---|---|---|
| Acetaminophen | $C_8H_9NO_2$ | 152.0712 | 152.0712 | 0.04 |
| Caffeine | $C_8H_{10}N_4O_2$ | 195.0882 | 195.0884 | 1.02 |
| Metoprolol | $C_{15}H_{25}NO_3$ | 268.1913 | 268.1916 | 1.23 |
| Propranolol | $C_{16}H_{21}NO_2$ | 260.1651 | 260.1650 | -0.21 |
| Carbamazepine | $C_{15}H_{12}N_2O$ | 237.1028 | 237.1026 | -0.79 |
| Ranitidine | $C_{13}H_{22}N_4O_3S$ | 315.1491 | 315.1492 | 0.36 |
| Sulfadiazine | $C_{10}H_{10}N_4O_2S$ | 251.0603 | 251.0601 | -0.69 |
| Sulfametazine | $C_{12}H_{14}N_4O_2S$ | 279.0916 | 279.0916 | 0.01 |
| Sulfatiazole | $C_9H_9N_3O_2S_2$ | 256.0214 | 256.0218 | 1.39 |
| Sulfamethoxazole | $C_{10}H_{11}N_3O_3S$ | 254.0599 | 254.0597 | -0.94 |
| Sulfapyridine | $C_{11}H_{11}N_3O_2S$ | 250.0650 | 250.0651 | 0.31 |
| Trimethoprim | $C_{14}H_{18}N_4O_3$ | 291.1457 | 291.1455 | -0.74 |
| Tylosin | $C_{46}H_{77}NO_{17}$ | 916.5270 | 916.5258 | -1.28 |
| Roxithromycin | $C_{41}H_{76}N_2O_{15}$ | 837.5324 | 837.5336 | 1.20 |
| Omeprazole | $C_{17}H_{19}N_3O_3S$ | 346.1225 | 346.1225 | 0.02 |

To perform the real time lock mass correction, two reference mass compounds, a lock mass solution including purine ($C_5H_4N_4$ at m/z 121.050873) and hexakis (1H, 1H, 3H (tetrafluoropentoxy)- phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$ at m/z 922.009798)) were used. The instrument provided a typical resolution of approximately 10,000 ± 500 (m/z 922). The analysis was made in positive ionization mode. The following operating conditions were used: drying gas flow 12 L/min; gas temperature 350 ºC, capillary voltage 4,000 V and nebulizer pressure 40 psi. Two different fragmentor voltages were used in each separation: 100 V (from 0 to 13 minutes) and 125 V (from 13 to 47 minutes), skimmer voltage, 65 V and the octopole RF, 250 V.

## RESULTS AND DISCUSSION

### Determination of pharmaceuticals

Because the extraction conditions used in the previous studies [16,17] were similar, we tried to extract all compounds using a single PLE method. However, when the antibiotics were extracted using Method A the recoveries decreased between 9 and 40%. On the other hand, when such pharmaceuticals as analgesics, lipid regulators, antiepileptics and β-blockers were extracted using method B the recoveries also decreased significantly. So, the compounds could not be extracted with a single PLE method and we used the two methods developed previously.

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**Table 2.** Validation data of the method.

| Compound | Linear range (μg/L)* | Recoveries (%) | RSD (%)[a] | RSD (%)[b] | LODs (μg/Kg) | LOQs (μg/Kg) |
|---|---|---|---|---|---|---|
| Acetaminophen | 25-250 | 98 | 5 | 8 | 100 | 250 |
| Caffeine | 25-250 | 99 | 8 | 11 | 100 | 250 |
| Metoprolol | 25-250 | 100 | 4 | 7 | 100 | 250 |
| Propranolol | 25-250 | 91 | 13 | 14 | 100 | 250 |
| Carbamazepine | 25-250 | 98 | 6 | 8 | 100 | 250 |
| Ranitidine | 10-500 | 56 | 12 | 15 | 50 | 100 |
| Sulfadiazine | 10-500 | 75 | 8 | 11 | 50 | 100 |
| Sulfametazine | 10-500 | 88 | 4 | 8 | 50 | 100 |
| Sulfatiazole | 25-500 | 71 | 11 | 14 | 100 | 250 |
| Sulfamethoxazole | 10-500 | 82 | 6 | 9 | 50 | 100 |
| Sulfapyridine | 5-500 | 81 | 8 | 10 | 30 | 50 |
| Trimethoprim | 5-500 | 77 | 5 | 6 | 20 | 50 |
| Tylosin | 10-500 | 91 | 9 | 11 | 50 | 100 |
| Roxithromycin | 10-500 | 84 | 4 | 7 | 50 | 100 |

* Instrumental linear range

[a] Repeatability (n=3, at 300 μg/Kg (d.w.))

[b] Reproducibility between days (n=3, at 300 μg/Kg (d.w.))

A chromatographic separation was optimized for the 15 compounds and all target compounds were eluted in 22 minutes.

Table 1 summarizes the exact and calculated mass of the target compounds and the average error obtained when the calibration curve was done by direct injection. As we can see, the error in all cases is lower than 1.39 ppm. In all cases we approximate the third decimal to draw the extract chromatogram and obtain an error lower than 2 ppm.

The instrumental linear ranges calculated by analyzing standard solutions were between 10-500 μg/L for macrolides and sulfonamides except for sulfapyridine and trimethoprim (5-500 μg/L), sulfathiazole (25-500 μg/L) and 25-250 μg/L for the other compounds.

We measured the recoveries of the analytes by spiking the sludge with the analytes. To calculate these recoveries, we subtracted the signal of the blank and then compared these signals with the signal of a standard solution. The recoveries calculated at 300 μg/Kg (d.w.) were higher than 71% except for ranitidine (56%). As expected, the recoveries were comparable with those calculated in previous studies [16,17] using different mass spectrometry analyzers and in all cases the differences were less than 10%. The repeatability and reproducibility between days expressed as the relative standard deviation (%RSD) were tested at two different concentration levels (300 and 500 μg/Kg (d.w.)), and in all cases they were less than 13% and 14%, respectively.

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**Table 3.** The LOQs obtained for the target compounds with different analyzers.

| Compound | LOQs (TOF) (µg/Kg) | LOQs (Q) (µg/Kg) | LOQs (QqQ) (µg/Kg) |
|---|---|---|---|
| Acetaminophen | 250 | 50 | 3 |
| Caffeine | 250 | 20 | 5 |
| Metoprolol | 250 | 20 | 5 |
| Propranolol | 250 | 20 | 5 |
| Carbamazepine | 250 | 20 | 3 |
| Ranitidine | 100 | 100 | 5 |
| Sulfadiazine | 100 | 100 | 10 |
| Sulfametazine | 100 | 50 | 5 |
| Sulfatiazole | 250 | 100 | 10 |
| Sulfamethoxazole | 100 | 50 | 5 |
| Sulfapyridine | 50 | 50 | 10 |
| Trimethoprim | 50 | 20 | 10 |
| Tylosin | 100 | 100 | 10 |
| Roxithromycin | 100 | 100 | 5 |

The limits of detection, calculated as a signal to noise ratio (S/N) of 3, were between 20 and 100 µg/Kg (d.w.), and the limits of quantification, calculated as S/N=10, were between 50 and 250 µg/Kg (d.w.). The validation data are summarized in Table 2.

The LOQs achieved by the quadrupole analyzer (Q) [16,17] and the triple quadrupole analyzer (QqQ) [21] in our previous studies are compared in Table 3. The LOQs obtained with the TOF analyzer were between 2 and 12 times higher than those obtained with Q, except for roxithromycin, tylosin, sulfadiazine ranitidine and sulfapyridine whose LOQs were the same with both analyzers. When the QqQ was used, the LOQs were between 5 and 80 times lower than those obtained with TOF.

The method was used to analyse sewage sludge samples collected from a sewage treatment plant in Tarragona (Spain). Table 4 summarizes the results of the samples analyzed by PLE/LC-TOF-MS.

Metoprolol, propranolol, ranitidine, sulfadiazine, sulfathiazole and sulfapyridine were excluded from the table because they were not present in any sample.

Three compounds (sulfamethoxazole, tylosin and roxythromycin) were quantified and identified. The error (expressed in ppm) obtained for the calculated mass in the samples was 1.68 ppm for sulfamethoxazole, between -1.23 and 1.57 ppm for tylosin and between 0.89 and 1.86 ppm for roxithromycin. Identification in real samples, then, was good.

The compound that was found in highest concentrations was tylosin (1,081 µg/Kg (d.w.)).

The concentrations of tylosin and roxithromycin did not vary in the samples analyzed.

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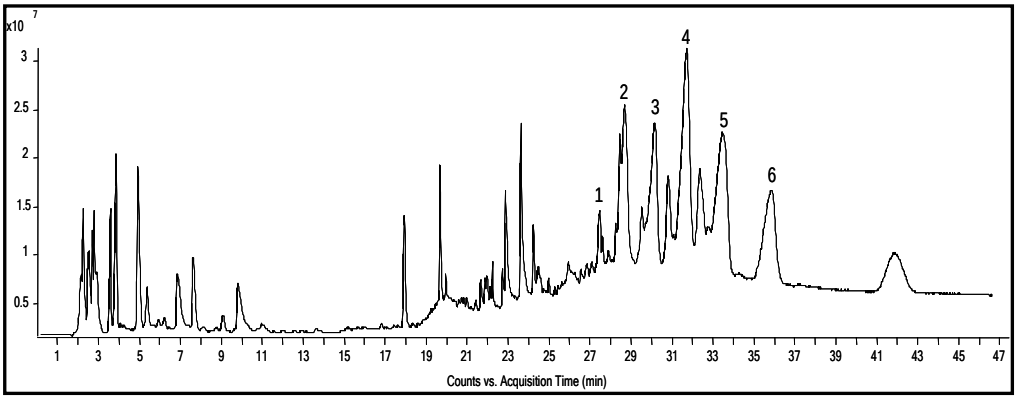


**Figure 1.** Chromatogram obtained when a sewage sludge sample was analyzed using method A of PLE and LC-TOF-MS. 1: NPEOs, 2: $C_{12}$AEOs, 3: $C_{13}$AEOs, 4: $C_{14}$AEOs, 5: $C_{15}$AEOs, 6: $C_{16}$AEOs.

**Table 4.** Concentration in μg/Kg (d.w.) of target compounds in the sewage sludge samples analyzed.

| Compound | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
|---|---|---|---|---|
| Acetaminophen | <LOQ | <LOQ | <LOQ | <LOQ |
| Caffeine | <LOQ | <LOQ | - | <LOQ |
| Carbamazepine | <LOQ | - | <LOQ | - |
| Sulfametazine | - | - | <LOQ | - |
| Sulfamethoxazole | 103 | - | - | - |
| Trimethoprim | <LOQ | <LOQ | - | - |
| Tylosin | 1057 | 1081 | 954 | 990 |
| Roxithromycin | 389 | 390 | 325 | 421 |

- < Limit of detection
RSD(%, (n=3)) < 12

Widely-used compounds such as acetaminophen and caffeine were detected in four and three samples, respectively, at concentrations below the limit of quantification.

The same samples were analyzed with QqQ in a previous study [21] and concentrations were similar. The difference in the concentration of tylosin, roxithromycin and sulfamethoxazole when TOF or QqQ were used was less than 21%. For the other compounds, when TOF analysis was used, concentrations were lower than the LODs and LOQs. However, in most cases, these compounds had been quantified by QqQ in the previous study [21].

**Identification of non-target compounds**

As mentioned above, the most important advantages of TOF analysis are the high resolution and the accurate mass measurements. Because of these advantages, we used a

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general procedure in an attempt to identify non target compounds in the samples analyzed. First of all we tried to identify compounds on the basis of their molecular weights and the possible molecular formulae in the data base; we then searched the literature to determine whether the possible compounds had already been studied. In order to confirm the presence of the compounds (when the standard is available), the retention time in the sample was compared with the retention time of the standard. The fragmentation and retention time of the standard and the sample were also compared in LC-MS-MS with a triple quadrupole analyzer working in product ion mode.

Figure 1 shows a chromatogram of one sample extracted using Method A of PLE. It shows six peaks between minute 27 and minute 36. Figure 2 shows the spectra of two of these six peaks. When we checked the spectrum of each peak, we observed the same characteristic in all of them (Figure 2); several peaks had an increment of 44 units. Each peak had a group of compounds with the same structure but with an added ethoxylation group. All the peaks contained in these chromatographic peaks are summarized in Table 5 and the mass accuracy is between -1.89 and 1.90 ppm.

The accurate masses of protonated non-target compounds retained at 27.2 min (peak 1 in Figure 1) were determined to be m/z 375.2506, 419.2778, 463.3039 and 507.3289, among others.

One possible structure for the first m/z is $C_{21}H_{36}O_4Na$, with $C_2H_4O$ being added to the other peaks. In the literature we found nonylphenol ethoxylates (NPEOs) that have this molecular weight and whose basic molecular formula is $C_{17}H_{28}O_2$ (MW 264). Some studies have reported that NPEOs are present in sewage sludge or sediments [22,23], and they are normally observed in adduct form with $Na^+$ (MW 287 when they contain one ethoxylated group). We can calculate the number of ethoxylation with equation (1).

$$\text{Number of ethoxylation} = (\text{MW our compound} - \text{MW } C_x \text{ compound}) / 44 \quad (1)$$

where MW our compound is the molecular weight obtained in the spectrum and MW $C_x$ compound is the molecular weight of the basic structure with Na, which in the case of nonylphenol is 287. This equation was used to identify the NPEOs with 2 to 9 ethoxylations.

According to the literature, AEOs are another important group of ethoxylated compounds determined in sewage sludge [24-26]. This group contains five different series of compounds, which have 12 (MW 253, $C_{14}H_{30}O_2Na$), 13 (MW 267, $C_{15}H_{32}O_2Na$), 14 (MW 281, $C_{16}H_{34}O_2Na$), 15 (MW 295, $C_{17}H_{36}O_2Na$) or 16 (MW 309, $C_{18}H_{38}O_2Na$) carbons in the alkyl chain and each compound has a different number of ethoxylations.

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To confirm whether one of these compounds is our non target peak number 2 we used equation (1) to calculate the number of ethoxylations. In this case the MW $C_x$ compound is equal to 253 for the series of 12 carbons.

One of the masses that appeared at peak number 2 is 341.2653 which corresponds to $C_{18}H_{38}O_4Na$. When we used equation (1) we observed that this mass corresponds to the compound with 2 ethoxylations in the AEOs with 12 carbons in the alkyl chain. So peak number 2 corresponds to $C_{12}$AEOs.

Following the same strategy, we determined all the series of AEOs. Hence, peak 3 corresponds to $C_{13}$AEO, peak 4 to $C_{14}$AEO, peak 5 to $C_{15}$AEO and, finally, peak 6 to $C_{16}$AEO.

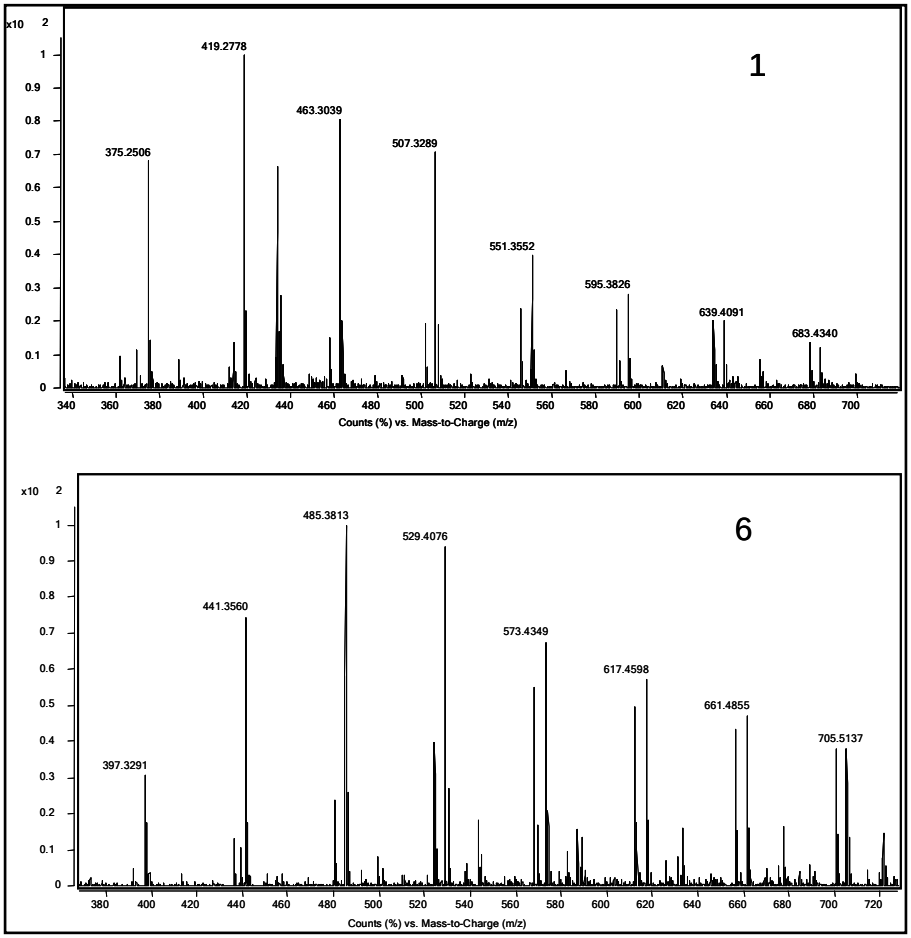


**Figure 2.** Spectrum obtained by LC-TOF-MS for peak numbers 1 and 6 in the chromatogram in Figure 1.

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**Table 5.** List of non target compounds identified. The peaks are shown in Figure 1.

| Peak | Family | Number of ethoxylations | Theoretical $[M+Na]^+$ | Experimental $[M+Na]^+$ | Error (ppm) |
|---|---|---|---|---|---|
| 1 | NPEO | 2 | 375.2511 | 375.2506 | -1.41 |
| | | 3 | 419.2773 | 419.2778 | 1.09 |
| | | 4 | 463.3036 | 463.3039 | 0.74 |
| | | 5 | 507.3298 | 507.3289 | -1.72 |
| | | 6 | 551.3560 | 551.3552 | -1.43 |
| | | 7 | 595.3822 | 595.3826 | 0.67 |
| | | 8 | 639.4084 | 639.4091 | 1.07 |
| | | 9 | 683.4346 | 683.4340 | -0.93 |
| 2 | $C_{12}$AEOs | 2 | 341.2668 | 341.2663 | -1.41 |
| | | 3 | 385.2930 | 385.2927 | -0.76 |
| | | 4 | 429.3192 | 429.3184 | -1.89 |
| | | 5 | 473.3454 | 473.3459 | 1.01 |
| | | 6 | 517.3716 | 517.3712 | -0.85 |
| | | 7 | 561.3979 | 561.3985 | 1.15 |
| | | 8 | 605.4241 | 605.4249 | 1.37 |
| | | 9 | 649.4503 | 649.4495 | -1.21 |
| 3 | $C_{13}$AEOs | 2 | 355.2824 | 355.2830 | 1.60 |
| | | 3 | 399.3086 | 399.3079 | -1.86 |
| | | 4 | 443.3349 | 443.3355 | 1.44 |
| | | 5 | 487.3611 | 487. 3620 | 1.90 |
| | | 6 | 531.3873 | 531.3883 | 1.90 |
| | | 7 | 575.4135 | 575.4130 | -0.88 |
| | | 8 | 619.4397 | 619.4386 | -1.81 |
| | | 9 | 663.4659 | 663.4667 | 1.16 |
| 4 | $C_{14}$AEOs | 2 | 369.2981 | 369.2975 | -1.57 |
| | | 3 | 413.3243 | 413.3237 | -1.44 |
| | | 4 | 457.3505 | 457.3499 | -1.33 |
| | | 5 | 501.3767 | 501.3760 | -1.44 |
| | | 6 | 545.4029 | 545.4034 | 0.84 |
| | | 7 | 589.4292 | 589.4292 | -0.60 |
| 5 | $C_{15}$AEOs | 2 | 383.3137 | 383.3132 | -1.38 |
| | | 3 | 427.3399 | 427.3392 | -1.74 |
| | | 4 | 471.3662 | 471.3667 | 1.15 |
| | | 5 | 515. 3924 | 515.3919 | -0.92 |
| | | 6 | 559.4186 | 559.4179 | -1.23 |
| | | 7 | 603.4448 | 603.4452 | 0.66 |
| | | 8 | 647.4710 | 647.4705 | -0.80 |
| | | 9 | 691.4972 | 691.4962 | -1.49 |

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**Table 5.** List of non target compounds identified. The peaks are shown in Figure 1. (Cont.)

| Peak | Family | Compound | $[M+Na]^+$ theoretical | $[M+Na]^+$ experimental | Error (ppm) |
|---|---|---|---|---|---|
| 6 | $C_{16}$AEOs | 2 | 397.3294 | 397.3291 | -0.70 |
| | | 3 | 441.3556 | 441.3560 | 0.92 |
| | | 4 | 485.3818 | 485.3813 | -1.05 |
| | | 5 | 529.4080 | 529.4076 | -0.80 |
| | | 6 | 573.4342 | 573.4349 | 1.15 |
| | | 7 | 617.4605 | 617.4598 | -1.06 |
| | | 8 | 661.4867 | 661.4855 | -1.77 |
| | | 9 | 705.5129 | 705.5137 | 1.16 |
| - | Phthalates | $C_{12}H_{14}O_4Na$ | 245.0790 | 245.0794 | 1.72 |
| | | $C_{16}H_{22}O_4Na$ | 301.1416 | 301.1412 | -1.26 |

Figure 3 shows the steps taken to identify the $C_{14}$AEOs with three ethoxylated groups. It shows the chromatogram of peak (A), the spectrum of the whole peak of $C_{14}$AEOs (B) where the difference of 44 units can be seen, the assignation of the adduct with $Na^+$ and the adduct with ammonia (C) (five units less than the adduct with $Na^+$), and finally the formulae that the software calculated (D). In order to confirm the presence of NPEOs and AEOs, we first confirmed the retention time of the standards in the LC-TOF-MS, and then studied the fragmentation of these compounds in one standard and in the samples using MS-MS with a triple quadrupole analyzer in the same chromatographic conditions. For NPEOs, the retention time and the fragmentation were the same in the standard of 200 μg/L and the samples. In the fragmentation of each compound we observed the peak corresponding to the ion $[M+Na]^+$, and the peak m/z 287 corresponding to the peak without ethoxylations.

For AEOs, the sample and a standard solution of 50 μg/L of $C_{12}$AEOs and $C_{14}$AEOs were compared (the other AEOs were not available). The retention time obtained for the standard and the sample was the same for both compounds. However, these compounds did not show any peaks other than that of the precursor ion in the spectrum so only the retention time was compared.

The bibliography reports not only ethoxylate compounds but also compounds that appear frequently in sewage sludge samples such as phthalates (see the review by Liang et al. [27]). These compounds had one characteristic ion (m/z 149) when GC with electron impact ionization was used. When an ion chromatogram of this m/z was extracted in the samples analyzed we observed that two peaks in the samples had this m/z. Ion 149 was not the only one present in the first peak (tr= 25.08 min.): ion 245 was also observed. Likewise, in the second peak (tr= 26.5 min.) ion 301 was also present. In most cases when positive ionization was carried out, the ion

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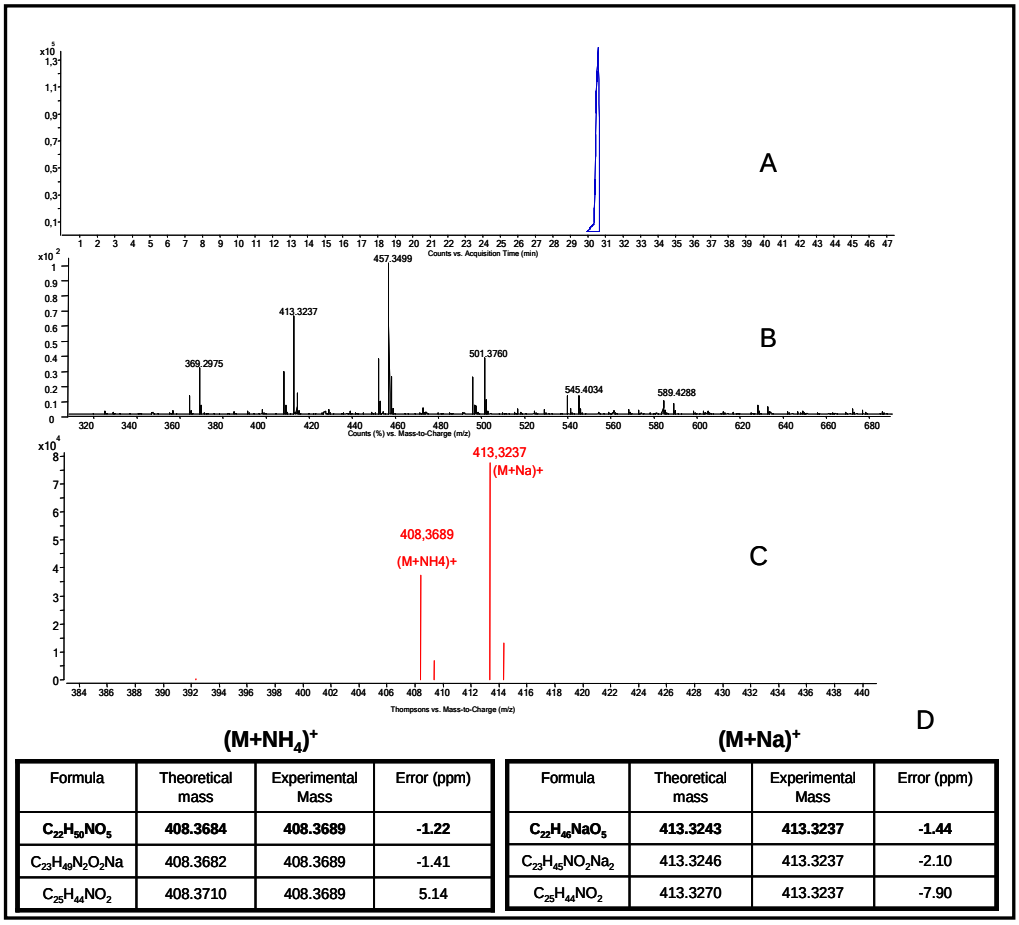
corresponded to the sodium adduct. After checking the formulae, we found that the first ion (m/z 245) corresponded to $C_{12}H_{14}O_4Na$, the formula of diethylphthalate, with an error of 0.76 ppm. The second ion (m/z 301) corresponded to $C_{16}H_{12}O_4Na$, the formula of dibutyl-phthalate, with an error of 1.02 ppm.

After the standard had been injected into the LC-TOF-MS and its retention time had been confirmed, a standard solution of both compounds was injected into the LC-MS-MS at a concentration of 0.5 mg/L to check if these peaks corresponded to these compounds.




**$(M+NH_4)^+$**

| Formula | Theoretical mass | Experimental Mass | Error (ppm) |
|---|---|---|---|
| $C_{22}H_{50}NO_5$ | **408.3684** | **408.3689** | **-1.22** |
| $C_{23}H_{49}N_2O_2Na$ | 408.3682 | 408.3689 | -1.41 |
| $C_{25}H_{44}NO_2$ | 408.3710 | 408.3689 | 5.14 |

**$(M+Na)^+$**

| Formula | Theoretical mass | Experimental Mass | Error (ppm) |
|---|---|---|---|
| $C_{22}H_{46}NaO_5$ | **413.3243** | **413.3237** | **-1.44** |
| $C_{23}H_{45}NO_2Na_2$ | 413.3246 | 413.3237 | -2.10 |
| $C_{25}H_{44}NO_2$ | 413.3270 | 413.3237 | -7.90 |

**Figure 3.** Chromatogram for the compound $C_{14}$AEOs with 3 ethoxylations (A), the spectrum of all the $C_{14}$AEOs group (B), the identification of the Na adduct and the ammonia adduct (C), and the table of results with the possible formulae (D).

The retention times and the fragmentation were the same. The ions observed for these compounds were the peak $[M+Na]^+$ and the peak m/z 149.

## CONCLUDING REMARKS

A PLE/LC-MS method for target and non target contaminants in sewage sludge samples has been developed, using a time of flight analyzer. The method enables 15 pharmaceuticals with different structures and physical-chemical properties to be extracted and provides recoveries higher than 71% for all analytes.

The compounds were determined at μg/Kg (d.w.) levels with good precision, although the LODs were higher than when other analyzers were used (Q and QqQ). The method was used to determine these compounds in sewage sludge samples. Tylosin showed the highest concentration (1081-954 μg/Kg).

The method identified 50 non-target compounds and demonstrated that LC–TOF–MS analysis is a valuable tool for unequivocally identifying pharmaceuticals and their metabolites in wastewaters. Non-target compounds such as alkyl ethoxylates, nonylphenol ethoxylates and phthalates were identified.

## Acknowledgements

The authors wish to thank the personnel of the water treatment plant in Tarragona for helping with the sampling carried out during the study.

This study was financially supported by the Dirección General de Investigación of the Ministry of Science and Technology, projects CTM2008-06847-C02-01/TECNO, CTM2005-01774. A.N. gratefully acknowledges the financial support of the Ministry of Science and Technology BES-2006-1332.

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### 3.1.4. Discussion of results

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Although the results of the experimental part of the studies of this section have been discussed individually in their respective papers, the present section discusses the most important aspects.

As we can see in the studies carried out in this section, there are some parameters in the extraction step that significantly affect the recoveries. The most important parameter is solvent extraction. The organic solvents selected to develop these studies were methanol and acetonitrile and different mixtures with water were tested due to the polarity of target analytes. In the methods developed in section 3.1.1 and 3.1.2, water:methanol (1:1) gave the highest recoveries for most of the pharmaceuticals. When acetonitrile was tested, the recoveries for some compounds such as acetaminophen, caffeine, metoprolol and ibuprofen, among others, were lower in comparison to the recoveries obtained when methanol was used. As we mentioned above, few studies have been published on the determination of pharmaceuticals in sewage sludge using PLE. After the publication of our studies, Radjenovic *et al.* [1] and Jelic *et al.* [2] corroborated that they obtained higher recoveries using the mixture of water:methanol. However they used a mixture of water:methanol (2:1) to extract analgesics, lipid regulators, antibiotics, barbiturates and diuretics, among others. Barron *et al.* [3] used the same mixture as we did (methanol:water (1:1)) to extract 27 pharmaceuticals in sewage sludge samples. Although methanol was used in most cases, acetonitrile was also used as an organic solvent in some studies. Lillenberg *et al.* [4] used a mixture of acetonitrile:water (1:1) to extract fluoroquinolones, sulfonamides and tetracyclines. The recoveries were between 52 and 92% for all compounds except for tetracycline whose recovery was 27%.

The use of fresh solvent during the extraction process also affects recoveries in pressurized liquid extraction. In the extraction of ibuprofen and diclofenac, we could see that their recoveries increased between 20 and 25% in the second cycle. For this reason, two cycles were necessary to extract these pharmaceuticals and to obtain high recoveries. The number of cycles and the static extraction time was 2 cycles of 15 minutes to extract pharmaceuticals and 1 cycle of 5 minutes to extract the antibiotics. In the studies published after our results, these variables varied between 2 cycles of 5 minutes [3] and 3 cycles of 5 minutes [1,2].

Temperature is also an important parameter. For instance, three different temperatures were tested in the extraction of sulfonamides and we could see that most compounds showed maximum recoveries at 80 ºC. Their recoveries decreased significantly at 100 ºC, due to the possible degradation of them. On the other hand, tylosin and omeprazole had a different behavior. Tylosin had similar recoveries at all temperatures tested whereas omeprazole increased its recovery when the temperature increased. So, the temperature was finally set at the compromise temperature of 80 ºC. In studies reported in literature, the temperature was fixed between 80 and 100 ºC, except in the study of Barron *et al.* [3] where the optimum

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temperature was 60 ºC. They studied at temperatures between 40 and 100 ºC and observed a negative effect of temperature above 60 ºC on the recovery of gemfibrozil, nimesulide and meclofenamic acid, among others.

Under the optimal conditions, all the recoveries were higher than 68% except for ranitidine whose recovery was 54%. Two compounds (salicylic acid and erythromycin) were not extracted, although we tested different values for each parameter. The low recovery of erythromycin is due to erythromycin degradates with an apparent loss of one molecule of water when it is present at an acidic pH [5].

A clean up step was used between extraction and chromatographic analysis in different studies in literature, with solid-phase extraction (SPE) being the preferred technique [1-3,6]. Due to the high content of methanol in the extract after the PLE extraction, in all studies the volume of methanol was reduced until 5-10% by either using a rotary vacuum, using a gentle flow of $N_2$ or diluting the extract with water up to 250 or 500 mL. Although we used the dilution strategy, the recoveries decreased for most of the compounds and SPE was not considered for our methods.

As regards the MS analyzers used, as we expected, we observed some differences in terms of sensitivity and power to identify the compounds. In Table 3.1 the limits of quantification obtained using Q and TOF analyzers are shown when the same PLE protocol was applied. The limits of quantifications achieved with TOF were between two and five times higher than the limits obtained with quadrupole, except for ranitidine, sulfadiazine, sulfapyridine, tylosin and roxythromycin, whose limits of detection were the same with both analyzers.

The methods developed were applied to analyze sewage sludge samples from two different sewage treatment plants in the area of Tarragona, Spain. Various pharmaceuticals such as caffeine, acetaminophen, bezafibrate or naproxen, among others, were quantified in the sewage sludge samples using the quadrupole analyzer. However, because of the high limits of quantification obtained using TOF analyzers, only the pharmaceuticals present at high concentrations such as antibiotics (tylosin and roxithromycin) were quantified. In most cases the concentrations found in our study were similar to those obtained in the studies published after our study [1-4].

Although TOF analyzers exhibit low sensitivity in comparison to quadrupole, it was very useful to identify non target compounds in the sewage sludge samples analyzed. Although the procedure to identify the compounds was laborious, different compounds or groups of compounds were identified such as nonylphenol ethoxylates, alquil ethoxylates and phthalates with an error in the calculated mass lower than 2 ppm in all cases. The process was laborious because after obtaining different empirical formulae in the data base, we looked for possibilities in literature and confirmed the presence of compounds injecting the standard in the LC-MS.

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**Table 3.1** Limits of quantifications obtained using pressurized liquid extraction and liquid chromatography-mass spectrometry detection with different analyzers.

| Compound | LOQs (TOF) (µg/Kg) | LOQs (Q) (µg/Kg) |
|---|---|---|
| Acetaminophen | 250 | 50 |
| Caffeine | 250 | 20 |
| Metoprolol | 250 | 20 |
| Propranolol | 250 | 20 |
| Carbamazepine | 250 | 20 |
| Ranitidine | 100 | 100 |
| Sulfadiazine | 100 | 100 |
| Sulfametazine | 100 | 50 |
| Sulfatiazole | 250 | 100 |
| Sulfamethoxazole | 100 | 50 |
| Sulfapyridine | 50 | 50 |
| Trimethoprim | 50 | 20 |
| Tylosin | 100 | 100 |
| Roxithromycin | 100 | 100 |

In literature there are different studies in which lower limits of detection using tandem mass spectrometry with different analyzers such as triple quadrupole [1] or linear ion trap [2,3] were achieved. For instance, the limits of quantification in the study of Radjenovic *et al.* [1] ranged from 0.15 to 159.8 µg/Kg (d.w.) using the triple quadrupole analyzer. At that moment, MS-MS analyzers were not available in our group, although it was used on the following sections, when our group achieved a UHPLC-QqQ instrument.

From these studies we conclude that PLE extraction is an efficient technique to extract pharmaceuticals from sewage sludge. Moreover, the presence of pharmaceuticals in sewage sludge has shown that there is a need to control them so that sludge can be reused safely.

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## 3.2. Determination of estrogens and their conjugates in sludge

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ISBN:978-84-693-6426-0/DL:T-1628-2010

ISBN:978-84-693-6426-0/DL:T-1628-2010

Considering the complexity of endocrine systems, it is not surprising that a wide range of substances cause endocrine disruption. These include both natural and synthetic chemicals. Many of these endocrine disruptor compounds are relatively persistent chemicals that are widely used in industrial and domestic activities and for specific beneficial and therapeutic purposes. The European Union has implemented the Strategy for Endocrine Disruptors [1] community. In this program, extensive work has been done towards the official designation of endocrine disrupting substances. Studies on many chemicals have been carried out and extensive lists of chemicals under evaluation for endocrine disruption effects have been drawn up. These long lists include synthetic chemicals and natural products that are commercially manufactured for a specific purpose or produced as a by product such as pesticides, polycyclic aromatic hydrocarbons, phthalate esters, bisphenol A, alkylphenols and natural and synthetic steroid sex estrogens. The present study is focused on the determination of this last group, natural and synthetic estrogens, in sewage sludge.

The applicability of estrogens as pharmaceuticals can be divided in four different groups: contraception, management of menopausal and postmenopausal syndrome, physiological replacement therapy and treatment of prostate and breast cancers. Additionally, some of these estrogens are used as growth promoters in animal farming. The consequences of the presence of estrogens in the aquatic environment are still largely unknown, but some negative impacts have been reported such as the feminization of fish, among others [2,3].

Many papers have reported the presence of various estrogens in surface waters and wastewaters [4-7]. By contrast, the presence of estrogens in sediments or sludge has been less studied than the presence of these compounds in waters. Only a few papers have reported analytical methods for these solid matrices [8-12].

In most studies, the presence of conjugate estrogens was not considered. In fact, in the third list of candidates of contaminants in drinking water from the Environmental Protection Agency (EPA) [13] only free estrogens are considered to be candidates of contaminants in drinking water. The presence of conjugate compounds in sewage sludge happens because they are excreted from human bodies in urine as conjugates and then arrive at sewage treatment plants [14,15]. In our study, estrogens and their conjugates in sewage sludge have been determined. We did this because their presence in the environment is important in that estrogen conjugates can be transformed to an active form during the sewage treatment plant process.

We also used pressurized liquid extraction, and in this case, liquid chromatography tandem mass spectrometry with a triple quadrupole analyzer was used. As we mentioned in the previous section, the LC-triple quadrupole enables lower limits of detection and more identification points (IP) to confirm the presence of target analytes [16].

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Therefore, the present study is focused on the determination of six natural and synthetic estrogens such as estrone, estriol, 17α-estradiol, 17β-estradiol, 17α-ethinylestradiol and diethylstilbestrol and five conjugates - estradiol 17-glucoronide, estrone 3-glucoronide, estrone 3-sulfate, estradiol 3-sulfate and estradiol 17-acetate - in sewage sludge.

We expected that the concentrations of estrogens and their conjugates in sewage sludge were at levels of low μg/Kg (d.w.) because their concentration in influent wastewater samples is low [4,17,18]. For example, D'Ascenzo *et al.* [18] determined concentrations up to 57 ng/L for four estrogens (estradiol, estrone, estriol and 17α-ethinylestradiol) using solid-phase extraction and liquid chromatography tandem mass spectrometry. Pedrouzo *et al.* [4] using SPE/LC-MS-MS determined six estrogens (estrone, estriol, 17 α-ethinylestradiol, and two conjugates estradiol 3-sulfate, estrone 3-sulfate and estradiol 17-glucoronide) at concentrations between below the limit of quantification and 154 ng/L in wastewater samples (influent and effluent) from the same STPs monitored in our study.

The results of this study have been published in Journal of Chromatography A 1213 (2008) 224-230.

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ISBN:978-84-693-6426-0/DL:T-1628-2010

## 3.2.1. Determination of natural and synthetic estrogens and their conjugates in sewage sludge by pressurized liquid extraction and high performance liquid chromatography-tandem mass spectrometry

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# DETERMINATION OF NATURAL AND SYNTHETIC ESTROGENS AND THEIR CONJUGATES IN SEWAGE SLUDGE BY PRESSURIZED LIQUID EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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**Abstract**

In this study we present a pressurized liquid extraction/liquid chromatography-tandem mass spectrometry (PLE/LC-MS-MS) method to determine a group of estrogens and conjugated estrogens in sewage sludge. Parameters that affect the extraction step such as extraction solvent, temperature, pressure, static extraction time, number of cycles, purge time and flush volume have been optimized. In the chromatographic separation, electrospray ionization and a triple quadrupole analyzer have been used, and the multiple reaction monitoring mode has enabled low levels of target analytes to be detected.

All recoveries were higher than 81% except for estrone 3-glucoronide and estradiol 17-glucoronide which were not extracted and consequently, they were not considered in the present study. The repeatability and reproducibility between days expressed as RSD (%) (n=3), were lower than 6% and 9%, respectively. The method developed allowed the target analytes to be quantified at low levels of μg/kg. The limits of detection were lower than 26 μg/kg of dry weight (d.w.) of sewage sludge, except for 17α-estradiol, 17β-estradiol, 17α-ethinylestradiol and estradiol 17-acetate whose values were between 150 and 175 μg/kg (d.w.). The method was applied to determine these compounds in sewage sludge from two domestic sewage treatment plants. Estrone 3-sulfate, estradiol 3-sulfate, diethylstilbestrol, estrone and estriol were determined in some samples and estriol showed the highest value (406 μg/kg d.w.).

**Keywords:** Estrogens, conjugated estrogens, sewage sludge, pressurized liquid extraction, liquid chromatography, tandem mass spectrometry.

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## INTRODUCTION

Recently, there has been a spate of interest in the presence of pharmacologically active substances. These substances are used in human medicine for diagnosis, treatment and prevention of illness. Some of these substances are excreted unmetabolized or as active metabolites, which are not completely eliminated in wastewater treatment plants and, therefore, enter to the environment [1,2].

One of these active groups are the steroid estrogens and their conjugates. Steroid estrogens of anthropogenic origin have been identified as the major contributors to endocrine-disrupting activity in both sewage water and surface waters [3]. The majority of steroid estrogens are excreted from the human body in urine as conjugates, which are largely biologically inactive. However, steroids in the free, deconjugated state have also been detected in sewage effluent [4], implying that deconjugation occurs prior to and/or during wastewater treatment. Endocrine-disrupting effects in the aquatic environment, such as feminization of male fish can probably be attributed to the presence of these compounds, amongst others, in river water [3,5]. The presence of steroid estrogens has been studied in different kinds of samples, mainly in liquid samples such as effluent and influent wastewater [1,2,4], surface water [5,6] and urine [7]. However, these compounds have been less studied in solid samples such as sediments [8-10] or sewage sludge [11-13]. Only the free form of estrogens was determined in most of these studies. The importance of controlling the presence of estrogens in this kind of matrices is because sewage sludge can be used as manure and consequently, these contaminants can find their way into the food chain.

Different techniques have been used to extract the estrogens from solid samples. The most common extraction technique is the ultrasonication (USE) [11]. Nowadays the availability of new extraction techniques such as microwave assisted extraction (MAE) [14,15] or pressurized liquid extraction (PLE) [9,16] gives us the opportunity to reduce the extraction time, the solvent consumption and to obtain better recoveries than the classical extraction techniques because these new techniques use high temperature and /or high pressure.

Gas chromatography (GC) has been used [5,10,17-19] as a separation technique but it needs a derivatization step. However, liquid chromatography (LC), which is more rugged and versatile than GC, is widely used for monitoring steroid estrogens. Due to the low concentration of estrogens in this kind of sample, LC has been coupled to more sensitive and selective detection systems such as mass spectrometry (MS) [1,4,16,20,21] or more recently tandem mass spectrometry (MS-MS) [2,6,22]. To achieve lower limits of detection in target analytes, triple quadrupole (QqQ) analyzer is the most suitable

ISBN:978-84-693-6426-0/DL:T-1628-2010

analyzer working in multiple reaction monitoring (MRM) mode [22]. However, quadrupole-time of flight (Q-TOF) analyzer [23] has also been used because it provides mass determination with higher accuracy and may provide a molecular formula but the sensitivity of Q-TOF is lower than that of QqQ analyzer.

The current paper focuses on the determination of the natural and synthetic estrogens estrone (E1), 17α–estradiol (αE2), 17β-estradiol (E2), estriol (E3), 17α–ethinylestradiol (EE2) and diethylstilbestrol (DSB) and their conjugates estradiol 17-glucoronide (E2-17G), estrone 3-glucoronide (E1-3G), estradiol 3-sulfate (E2-3S), estrone 3-sulfate (E1-3S) and estradiol 17-acetate (E2-17A) in sewage sludge. We selected these compounds according to their presence in wastewater and sludge. The main objective was to develop a method for the simultaneous determination of selected estrogens and conjugates in sewage sludge using pressurized liquid extraction and liquid chromatography-tandem mass spectrometry (PLE/LC-MS-MS) with a triple quadrupole analyzer. This method was applied to sludge samples from wastewater treatment plants (STPs).

## EXPERIMENTAL

### Materials and reagents

Estrone, 17α–estradiol, 17β-estradiol, estriol, 17α-ethinylestradiol, diethyl-stilbestrol, estradiol 17-glucoronide, estrone 3-glucoronide, estradiol 3-sulfate, estrone 3-sulfate and estradiol 17-acetate 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 stored at -15 ºC. A mixture of all compounds in methanol at a concentration of 50 mg/L was prepared weekly. Working solutions were prepared daily by diluting this solution with water.

Ultra pure water was obtained with a Milli-Q water purification system (18.2 MΩ·cm) (Millipore, Bedford, MA, USA). Acetonitrile and methanol (HPLC-grade) were from SDS (Peypin, France), nitrogen was from Carburos Metálicos (Tarragona, Spain) and acetic acid was from Merck (Darmstadt, Germany).

### Sample pretreatment

The sewage sludge samples were collected from two domestic sewage treatment plants of two cities of about 130,000 inhabitants and located in the south of Catalonia.

The sample preparation consisted of two steps. The first one was the lyophilization of frozen sewage sludge by the freeze dry system (Labconco, Missouri, USA). The second one was the homogenization of the lyophilized sample by a mortar and pestle, and sieved (125 μm) to obtained particles with the same diameters.

The spiked sewage sludge was prepared by diluting the stock

ISBN:978-84-693-6426-0/DL:T-1628-2010

mixture of standard compounds in 50 mL of acetone and mixing this with 10 g of dry sewage sludge. Subsequently, the solvent was slowly evaporated at room temperature under frequent homogenization.

**Pressurized liquid extraction**

To extract sludge samples an ASE200 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA) was used. A total of 1 g of pretreated sludge was placed in a 11 mL stainless steel extraction cell, and thoroughly mixed with aluminium oxide, which was heated at 120 ºC in the oven for 24 hours before use.

The extracting solvents were methanol:acetone (1:1) and water (pH 7):methanol (1:1). The operating conditions were: extraction temperature of 75 ºC; extraction pressure of 100 bar; preheating period of 5 min; 2 cycles of 3 minutes with methanol: acetone (1:1) followed by 2 cycles of 3 minutes with water (pH 7):methanol (1:1) as solvents, final extraction volume ~ 25 mL; flush volume of 30% of the cell volume and nitrogen purge of 120s.

The first two cycles were evaporated to dryness under a flow of nitrogen gas, and the residue was redissolved with the extract from the second two cycles.

The final extract was filtered with a microfilter 0.45 µm pore size (Teknokroma, Barcelona, Spain), and analysed by liquid chromatography-tandem mass spectrometry.

**Chromatographic analysis**

The chromatographic instrument was an HP1200 series LC- triple quadrupole tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany) with an electrospray ionization (ESI) interface, an automatic injector, a degasser, a quarternary 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 35ºC.

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 initially 10% B, kept constant for 10 min, which was increased to 40% in 5 min, to 60% in 10 min, to 100% in 5 min, kept constant for 5 min and finally returned to 10% B in 2 min. All the compounds were eluted within 31 min.

Ionization and fragmentation settings were optimized by direct injection of estrogen standard solutions. MS-MS was performed in the Multiple Reaction Monitoring (MRM) mode using ESI in the negative mode. For each compound, two characteristic fragmentations of the deprotonated molecular ion $[M-H]^-$ were monitored (Table 1), the first and most abundant one being used for quantification, while the second one was used as a qualifier. Collision energy and cone

voltage were optimized for each estrogen (Table 1). The ESI conditions were: capillary voltage 3,000 V, nebulizer 45 psi., source temperature 350 ºC and gas flow 12 L/min. Nitrogen was used as collision, nebulizing and desolvation gas. In order to maximize sensitivity, three time windows were used: 0-17 min (E2-17G, E1-3G, E2-3S and E3), 17-22 min (E1-3S, EE, αE2 and E2) and 22-36 min (E2-17A, E1 and DSB).

**Table 1.** MRM conditions used for LC-MS-MS determination of estrogens and conjugated estrogens (ESI, negative mode). Bold face ions were used to quantify.

| Compound | Abbreviation | Precursor Ion | Fragment ions | Cone Voltage (V) | Collision energy (V) |
|---|---|---|---|---|---|
| estriol | E3 | 287 | **171** | 150 | 45 |
| | | | 145 | 150 | 45 |
| estradiol 3-sulfate | E2-3S | 351 | **271** | 150 | 30 |
| | | | 145 | 150 | 55 |
| estradiol 17-glucoronide | E2-17G | 447 | 271 | 150 | 30 |
| | | | **113** | 150 | 20 |
| estrone 3-glucoronide | E1-3G | 445 | **269** | 150 | 45 |
| | | | 113 | 150 | 20 |
| estrone 3-sulfate | E1-3S | 349 | **269** | 150 | 30 |
| | | | 145 | 150 | 55 |
| 17α–estradiol | αE2 | 271 | **145** | 60 | 30 |
| | | | 183 | 60 | 45 |
| 17β-estradiol | E2 | 271 | **145** | 60 | 30 |
| | | | 183 | 60 | 45 |
| 17α-ethinylestradiol | EE2 | 295 | **145** | 60 | 45 |
| | | | 159 | 60 | 30 |
| estrone | E1 | 269 | **145** | 150 | 45 |
| | | | 143 | 150 | 55 |
| diethylstilbestrol | DSB | 267 | **222** | 150 | 30 |
| | | | 237 | 150 | 30 |
| estradiol 17-acetate | E2-17A | 313 | **253** | 100 | 30 |
| | | | 145 | 100 | 55 |

## RESULTS AND DISCUSSION

### LC-MS-MS

To optimize the separation, we tested two organic solvents (methanol and acetonitrile) and water at different pH (3, 7 and 9). A standard solution of 1 mg/L was injected and the best conditions for a good symmetric peak were obtained with acidic water (pH 3).

Although methanol is recommended for LC-MS as an organic solvent [24], we obtained better peak shape with acetonitrile.

Thus, the mobile phase was acetonitrile and acidic water.

Due to the acid characteristics of the compounds, negative ionization is the

ISBN:978-84-693-6426-0/DL:T-1628-2010

most suitable, as already reported in the literature [4,9,16]. The parameters optimized and the values tested were: gas temperature (100, 200 and 350 ºC), gas flow (8, 10, 12 mL/min), nebulizer pressure (20, 30 and 45 psi.) and capillary voltage (3,000, 4,000 and 5,000 V). The conditions which showed higher response for the majority of the compounds were: source temperature 350 ºC, gas flow 12 mL/min, nebulizer 45 psi. and capillary voltage 3,000 V.

Multiple Reaction Monitoring (MRM) was the mode used to determine these compounds because it is the most suitable for obtaining lower limits of detections for target compounds [25]. To choose the transitions in the MRM mode, different parameters were studied. The precursor and the product ions of each compound were selected. Then, different fragmentation voltages were tested (60, 80, 120, 140, 150, 160 V). In the majority of cases 150 V was the optimum value. The last parameter optimized was the collision energy; different values were tested (30, 40, 50, 55 and 60 V). In table 1 we summarize the optimum values for each condition for each compound. The optimization was done following the normal optimization procedure.

To choose the optimum two transitions for each compound, the fragmentation of these compounds was studied. The majority of the compounds have similar structures and we observed the same characteristic ions. In all cases the ion $[M-H]^-$ was selected as precursor ion. The ion m/z 145 $[C_{10}H_9O_{11}]^-$ was present in all estrogens except for glucoronide compounds and DSB. Other characteristic ions were m/z 271 and m/z 269 in the sulfate and glucoronide conjugated estrogens. These peaks correspond to the free forms of estrogens, estradiol (m/z 271) and estrone (m/z 269). The DSB showed different peaks, with m/z 237 $[M-C_2H_7]^-$ and m/z 222 $[M-C_3H_{10}]^-$ as product ions. The other transitions selected are summarized in Table 1.

To ensure the highest sensitivity of the acquisition we defined three different time windows. Firstly E2-17G, E1-3G, E2-3S and E3 were determined between 0 – 17 minutes, secondly E1-3S, EE2, α-E2 and E2 were determined between 17 – 22 minutes and finally E2-17A, E1 and DSB were determined between 22-36 minutes.

Under optimum conditions, the calibration curves obtained by direct injection of standard solutions were linear for all compounds in a wide range of concentrations, with determination coefficients ($r^2$) higher than 0.996. Significant differences in response were observed for each compound. The higher response were for sulfate and glucoronide compounds, so these compounds were the compounds which had the lowest limit of quantification 0.01 μg/L and 0.5 μg/L for sufate and glucoronide compounds, respectively. Another compound which yielded a good response was the E3, this compound had a limit of quantification of 3 μg/L. In contrast, α-E2, E2, EE2 and E2-17A were the

compounds which had the worst response and their limit of quantification by direct injection was 10 μg/L. The relative standard deviation (%RSD) in all cases was lower than 5% (n=3). The results for linear range are summarized in Table 2.

**Table 2**. Instrumental linear range and validation data for the PLE/LC-MS-MS method. For experimental data see the text.

| | Recoveries (%)[a] | Linear Range[b] (μg/L) | LODs[c] (μg/Kg) | LOQs[d] (μg/Kg) | RSD (%)[e] | RSD (%)[f] |
|---|---|---|---|---|---|---|
| E3 | 84 | 3-1000 | 26 | 75 | 5 | 7 |
| E2-3S | 99 | 0.01-1000 | 0.15 | 0.25 | 6 | 8 |
| E1-3S | 100 | 0.01-1000 | 0.15 | 0.25 | 3 | 5 |
| α-E2 | 83 | 10-250 | 150 | 250 | 5 | 7 |
| E2 | 92 | 10-500 | 150 | 250 | 5 | 8 |
| EE2 | 88 | 10-500 | 150 | 250 | 5 | 6 |
| E1 | 88 | 0.75-250 | 11 | 19 | 6 | 9 |
| DSB | 81 | 0.75-250 | 12 | 19 | 3 | 6 |
| E2-17A | 83 | 15-250 | 175 | 375 | 5 | 5 |

[a] Sample spiked at 500 μg/Kg (n=3)
[b] Instrumental linear range
[c] Limit of detection of the method
[d] Limit of quantification of the method
[e] Repeatability (n=3)
[f] Reproducibility between days (n=3)

**Pressurized liquid extraction**

To achieve fast and efficient extraction of the target compounds from a solid matrix using PLE, proper operational parameters (temperature, pressure, extraction time, number of cycles, flush volume and purge time) and an appropriate extraction solvent or mixture of solvents, with polarities closely matching that of the target compounds, should be selected.

The initial conditions were selected from previous studies, in which a few estrogenic compounds were determined from sewage sludge or sediments [9,16].

These conditions were temperature of 75 ºC, pressure of 100 bar, methanol:acetone (1:1 v/v) as extraction solvents, 5 minutes of static extraction time, 2 cycles, 120 s of purge time, 30% of flush volume and 1 g of dry sample.

First, a blank of sewage sludge was analyzed by PLE/LC-MS-MS under the above conditions and the chromatogram showed some peaks of target analytes studied at the same retention time, at concentration of low μg/Kg. Then, the extraction parameters were optimized using the recoveries of estrogenic compounds in spiked sewage sludge, and in each analysis a blank was made and the signal of the blank was subtracted. The lyophilized sludge was spiked with 500 μg/Kg (d.w.) of each

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compound to perform the PLE extraction.

The first parameter optimized was the solvent extraction, and the results are shown in Table 3.

The initial solvent selected (methanol:acetone, 1:1 v/v) gave recoveries higher than 82% except for E2-3S and E1-3S. E2-17G and E1-3G were not recovered at all. Different pure solvents (water, acetone and methanol) and binary and ternary mixtures were tested to improve the recoveries.

**Table 3.** PLE recoveries (n=3) by using different solvents. A= water (pH 7), B= methanol and C= acetone. For other conditions see the text.

| Compounds | A | B | C | A:B (1:1) | B:C (1:1) | A:B:C (1:2:1) | A:B:C (1:4.5:4.5) |
|---|---|---|---|---|---|---|---|
| E3 | - | 78 | - | 59 | 82 | 77 | 74 |
| E2-3S | 5 | 32 | 8 | 70 | 27 | 52 | 58 |
| E2-17G | - | - | - | 7 | - | - | 6 |
| E1-3G | - | - | - | 6 | - | 5 | - |
| E1-3S | 7 | 19 | - | 55 | 13 | 47 | 46 |
| α-E2 | - | 78 | - | - | 92 | 91 | 78 |
| E2 | - | 84 | - | - | 92 | 70 | 88 |
| EE2 | - | 85 | - | - | 97 | 65 | 79 |
| E1 | - | 74 | - | - | 91 | 65 | 71 |
| DSB | - | 84 | 12 | - | 91 | 17 | 51 |
| E2-17A | - | 81 | 49 | - | 99 | - | - |

RSD (%)≤ 5

The best pure solvent was methanol, the recoveries were higher than 74% except for sulfate conjugated compounds whose recoveries were 19% and 32% and glucoronide compounds which were not recovered. However, the results obtained with methanol did not improve the results obtained with the initial mixture.

Two different binary mixtures were tested. We observed that, when a mixture solvent of water:methanol (1:1, v/v) was used, only the most polar compounds were extracted and recoveries were better for E2-3S and E1-3S although glucoronide compounds were slightly extracted and the rest of the compounds were not extracted. However, they were extracted with the initial solvent.

We tried two different ternary mixtures. These ternary mixtures only improved the initial recoveries of E2-3S, and E1-3S. Similar recoveries were obtained for E3 and E2 and α-E2 in comparison with the initial conditions. However, for the most non-polar compounds worse recoveries were obtained with these ternary mixtures, even the E2-17A did not recover. The glucoronide compounds were not extracted with these ternary mixtures. The low recoveries obtained for the glucoronide compounds may be due to a strong interaction of these compounds with the sludge matrix.

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Because the result for each compound was best under different mixture solvents, we decided to continue with the optimization by selecting two different binary mixtures, methanol: acetone (1:1, v/v) and water:methanol (1:1, v/v).

The second parameter optimized was the extraction temperature. We tried five different temperatures (25, 50, 75, 100, 125 ºC). Table 4 shows the recoveries for all compounds at different temperatures and the two different solvents selected with 100 bar of pressure and 2 cycles of 5 minutes.

**Table 4.** PLE recoveries (n=3) by using different temperatures and two solvents methanol:acetone (1:1) and water:methanol (1:1). For other conditions see the text.

| | Methanol:acetone (1:1) | | | | | Water:methanol (1:1) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 25ºC | 50ºC | 75ºC | 100ºC | 125ºC | 25ºC | 50ºC | 75ºC | 100ºC | 125ºC |
| E3 | 57 | 72 | 82 | 59 | 69 | 25 | 42 | 59 | 51 | 45 |
| E2-3S | 20 | 23 | 27 | 24 | 24 | 35 | 50 | 70 | 51 | 43 |
| E2-17G | - | - | - | - | - | - | 4 | 7 | 4 | - |
| E1-3G | - | - | - | - | - | 3 | 5 | 6 | 4 | - |
| E1-3S | 5 | 10 | 13 | 12 | 12 | 10 | 45 | 55 | 51 | 38 |
| α-E2 | 67 | 84 | 92 | 68 | 68 | - | - | - | - | - |
| E2 | 66 | 81 | 92 | 72 | 69 | - | - | - | - | - |
| EE2 | 77 | 91 | 97 | 84 | 79 | - | - | - | - | - |
| E1 | 54 | 72 | 91 | 75 | 67 | - | - | - | - | - |
| DSB | 75 | 86 | 91 | 89 | 89 | - | - | - | - | - |
| E2-17A | 75 | 100 | 99 | 83 | 84 | - | - | - | - | - |

RSD (%)≤ 8

As we could see in Table 4, the best temperature was 75 ºC in both mixtures. When methanol:acetone (1:1) was used, for all compounds the recoveries were higher than 82% except for sulfate compounds, whose recoveries were 27% and 13% for E2-3S and E1-3S, respectively, and the glucoronide compounds which were not extracted. When the solvent was water:methanol (1:1) the recoveries for sulfate compounds were 70% and 55% for E2-3S and E1-3S, respectively. With this mixture the non-polar compounds were not recovered and the recoveries of glucoronide compounds were lower than 7%. Then, we did a first cycle with methanol:acetone (1:1) followed by a second cycle with water:methanol (1:1) and both extracts were mixed.

The third parameter optimized was the extraction pressure. We tried three different extraction pressures (66, 100 and 166 bar) The recoveries in the extraction at 100 bar were about 15% higher than the extraction at 66 bar and there were not differences in the analysis at 100 bar and 166 bar Therefore we selected 100 bar as an optimal extraction pressure.

The fourth and fifth parameters optimized were the static extraction time and the number of cycles, and

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they were both optimized together because these parameters are related. We tested different extraction times and numbers of cycles for both mixture solvents. Table 5 shows the recoveries obtained. Firstly, we tried one cycle of 5 minutes for each solvent and the recoveries were higher than 68% except for E2-17G and E1-3G.

**Table 5.** PLE recoveries (n=3) by using different number of cycles and extraction times. For other conditions see the text.

| | 1A+1B | 1A+2B | 2A+1B | 2A + 2B | 2C+2D | 3C+3D |
|---|---|---|---|---|---|---|
| E3 | 95 | 89 | 88 | 100 | 95 | 89 |
| E2-3S | 72 | 28 | 65 | 74 | 75 | 96 |
| E2-17G | - | 3 | - | - | 3 | 2 |
| E1-3G | 3 | 2 | 2 | 1 | 2 | 1 |
| E1-3S | 68 | 66 | 70 | 86 | 87 | 92 |
| α-E2 | 80 | 96 | 95 | 96 | 110 | 103 |
| E2 | 81 | 90 | 91 | 88 | 100 | 102 |
| EE2 | 88 | 96 | 83 | 94 | 93 | 80 |
| E1 | 85 | 80 | 84 | 91 | 98 | 100 |
| DSB | 77 | 84 | 73 | 90 | 105 | 97 |
| E2-17A | 87 | 75 | 87 | 90 | 85 | 95 |

A= methanol:acetone (1:1) and 5 minutes
B= water:methanol (1:1) and 5 minutes
C= methanol:acetone (1:1) and 3 minutes
D= water:methanol (1:1) and 3 minutes
RSD (%)≤ 10

To improve the recoveries we increased the number of cycles. As we can see in Table 5, better recoveries were obtained when 2 cycles of each mixture and five minutes were used. These recoveries increased by up to 18% for every compound except for E2-17G and E1-3G. To reduce the extraction time two and three cycles of 3 minutes of each solvent were tested and comparable recoveries were obtained. Consequently, two cycles of 3 minutes of each solvent was used as extraction time to save operation time.

The last parameter optimized was the flush volume; the recoveries for all compounds at different flush volumes of 15, 30 and 60% were tested. For most of the compounds 30% of flush volume was enough to obtain recoveries higher than 81%. The recoveries for glucoronide compounds in all cases were lower than 10% although different parameters were tested. Therefore, E2-17G and E1-3G were excluded from the study because they were not extracted in any conditions tested, probably due to the strong interaction of glucoronides with the sludge matrix. However, from another kind of matrix such as sediments these compounds were extracted from soxhlet [10] or sonication [26].

We checked 120 and 200 s as a purge time, and no differences were observed. Therefore we fixed the initial value at 120 s. As already

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mentioned in a previous study [27,28], the purge time is not an important factor in the optimization.

The final conditions for extracting the estrogens with PLE were set at 75 ºC and 100 bar with 2 cycles of methanol:acetone (1:1 v/v) followed by 2 cycles of water:methanol (1:1, v/v) applied as extraction solvents, 3 minutes of static extraction time, 120 s of purge time, 30% of flush volume and 1 gram of sample (d.w.).

To reduce the final volume of the extraction solvent we evaporated to dryness the first two cycles (methanol:acetone) and redissolved them with the extract of the second two cycles (water:methanol) and no losses were observed in the evaporation step.

Under these conditions the final solvent volume was 25 mL. Although an additional step of solid phase extraction was tested to decrease these volumes, experiments with different sorbents (Oasis HLB and Strata-X) and different dilution volumes to decrease the percentage of methanol (until 1000 mL which means 2.5% of MeOH) were not successful.

## METHOD VALIDATION

As previously stated, when the PLE extract of sewage sludge was analyzed by LC-MS-MS, some target compounds were present in the chromatogram. For this reason the calibration curves were obtained by direct injection of standard solutions instead of analyzing a sample by the whole method developed.

To confirm the presence of the compounds the retention time of the compounds, and the relationship between the two transitions shown in Table 1 were compared. Table 2 shows the validation data obtained.

Co-eluting matrix components have an influence on the ionization efficiency of the analyte and adversely affect the reproducibility and accuracy of the method, especially when external calibration curves are used for quantification. For this reason, matrix effects were checked during method development. We measured the recoveries of the analytes in a matrix extract spiked with the analytes post-extraction. When the recoveries were calculated their decrease is assumed to have been caused by matrix components in the extract. At the levels studied (500 μg/L and 250 μg/L) the matrix effect was less than 12% so matrix effect was not considered.

Recoveries when 1 g (d.w.) of sewage sludge spiked at 500 μg/Kg (d.w.) and 300 μg/Kg (d.w.) were analyzed were higher than 81% for all compounds, and the recoveries were similar in both cases. In literature these compounds were determined but using different extraction techniques. For example, in the paper by Ternes *et al.* [11] three of these compounds (E1, E2 and EE2) were determined in sewage sludge using USE/GC-MS-MS and their recoveries are similar to those obtained in the present paper. However, in this study [11] a clean-up step was necessary before the derivatization step.

In previous studies where PLE/LC-MS was selected to determine some of these compounds (E1, E2, E3, EE2 and DSB), recoveries obtained were also between 80 and 90% [9,16]. However, to the best of our knowledge, there is no information about the extraction of sulfate estrogens from sewage sludge.

The repeatability expressed as the relative standard deviation of the analysis of three samples spiked at 500 μg/Kg (d.w.), was lower than 6% (n=3). The reproducibility between days expressed as the relative standard deviation of three samples spiked at 500 μg/Kg (d.w.) and analyzed on three different days was lower than 10%. The limit of quantification (LOQ), as the concentration of the lowest point of the calibration curve, ranged from 0.25 to 375 μg/kg (d.w.). The difference in these values is due to the difference in response, as we mentioned before. Limits of detection (LOD), calculated as a signal-to-noise ratio of 3, were lower than 175 μg/kg (d.w.) for all compounds. The sulfate conjugated compounds showed the lowest limit of detection 0.15 μg/Kg.

## METHOD APPLICATION

The method developed was applied to 4 sewage sludge samples collected in May 2007 and July 2007 from two different STPs. Figure 1 shows the results of this study. α-E2, E2, EE2 and E2-17A were excluded from the figure because their concentrations were lower than the limit of quantification. It should be pointed out that these four compounds have the highest LOQ of the method.

In the samples analyzed, only two compounds appeared in all samples, E2-3S and E1-3S, at levels between 7-0.64 μg/kg (d.w.), but it should be mentioned that these compounds have a very low limit of quantification. Surprisingly, E3 only appeared in STP1 but at quite high levels such as 272 and 406 μg/kg (d.w.). However, these results are in agreement with Koh *et al.* [29] who showed a removal of E3 in sewage treatment plant of 98%, which includes the sorption in the sewage sludge and the degradation during sewage treatment. Schlüsener *et al.* [30] shows a similar situation for E3 because the levels observed in influent waters were 32-87 ng/L while in effluent water they were only 2-5.3 ng/L.

However, they did not show any difference between effluent and influent wastewater for E1-3S and E2-3S which agree also with our results. DSB only was quantified in one sample at levels of 184 μg/kg (d.w.). As regards E1, it appears in three samples at levels below 200 μg/kg (d.w.), although it is found at low ng/L level in wastewater sample [29-31]. E2 and EE2 also show low levels in wastewater [29-31] and for instance, the elimination power of EE2 during wastewater treatment was only 17% [29].

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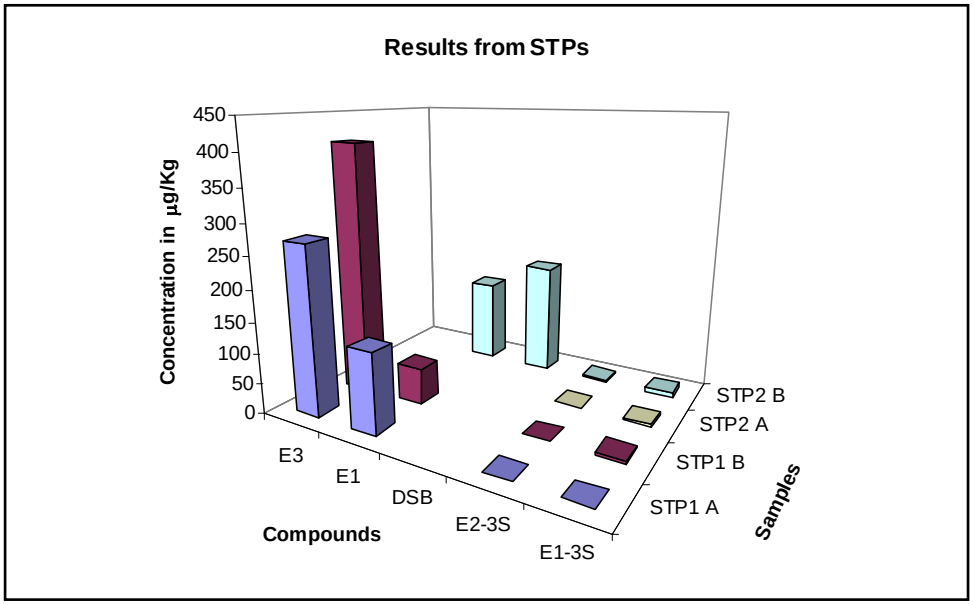


**Figure 1**. Results in µg/Kg (d.w.) of sewage sludge samples analyzed. The relative standard deviation (%RSD) in all cases was lower than 8% (n=3). For experimental conditions see text.

As an example, Figure 2 shows the MRM chromatograms of one sample from STP1 collected in May 2007 and we can see the presence of E3, E1, E2-3S and E1-3S.

## CONCLUSIONS

A selective analytical method based on PLE and LC-MS-MS has been developed for the determination of conjugated and unconjugated steroid estrogens in sewage sludge at µg/Kg (d.w.) levels and which good precision.

The high selectivity of MS-MS detection was favourable for identification and quantification. Recoveries were quantitative for all compounds except for E1-3G and E2-17G, which could not be extracted. The method was applied to determine these compounds in two STPs from Catalonia. E1-3S and E2-3S were present in all samples analyzed, E3 only appear in samples from one STP and other compounds such as α-E2, E2, EE2 and E2-17A were not present in any sample. Although some compounds have been determined in the samples, further work is needed to improve the limit of quantification of some of them in order to determine the possible low levels in the samples.

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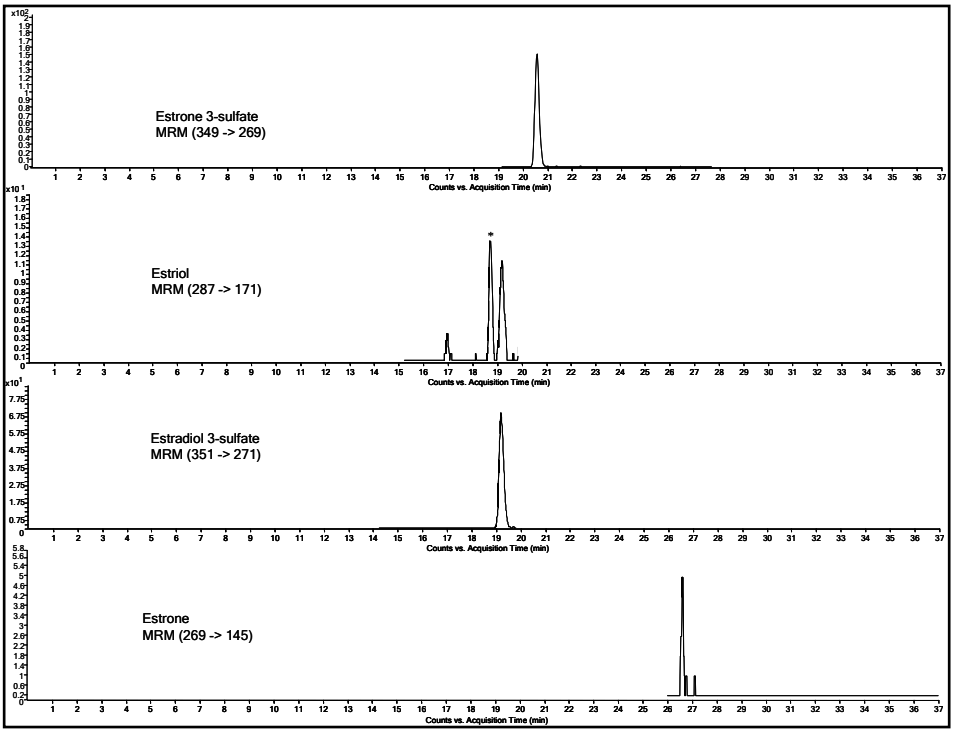


**Figure 2**. MRM chromatogram obtained by PLE/LC-MS-MS of 1 g of sewage sludge collected in STP1 in May, 2007. For experimental conditions see text.

**Acknowledgements**

The authors wish to thank the personnel of the water treatment plants in Tarragona and Reus for their cooperation with all aspects of this study.

This study was funded by the Dirección General de Investigación of the Ministry of Science and Technology, project CTM2005-01774. A.N. gratefully acknowledges the financial support of the Ministery of Science and Technology BES-2006-1332.

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### 3.2.2. Discussion of results

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In this study, as we mentioned in the introduction to this section, we used, for the first time in this Thesis, liquid chromatography tandem mass spectrometry with a triple quadrupole (QqQ) analyzer, to develop a method to determine natural and synthetic estrogens and their conjugates in sewage sludge samples using pressurized liquid extraction as the extraction technique.

We used the triple quadrupole analyzer in order to obtain low limits of detection and to achieve more identification points because two transitions in the MRM mode were monitored. When all of the ionization and fragmentation conditions were optimized, as we expected, the linear range of some of compounds (sulfate conjugate compounds, estrone and diethylstilbestrol) started at very low concentrations (lower than 0.75 μg/L). In the injection of the standards in LC-QqQ, we observed that some estrogens exhibited a wide linear range. For example, estriol had an instrumental linear range of between 3 and 1000 μg/L. On the other hand, some compounds such as 17α-estradiol, 17β-estradiol, 17α-ethinylestradiol and estradiol 17-acetate had a narrow linear range. The lowest point was between 10 and 15 μg/L and the upper limit between 250 and 500 μg/L. The LODs and LOQs of these compounds were also slightly high in comparison to other emerging contaminants because these compounds showed a low sensitivity to MS-MS detection.

In the optimization of the extraction process, we observed that solvent, temperature, the number of cycles and the static time had a great influence on the recoveries of the target analytes. This was as expected, according to our previous studies reported in section 3.1.

Regarding solvent extraction, we tested different pure solvents and binary and tertiary mixtures of water, methanol and acetone. Methanol and all mixtures with methanol extracted all of the compounds except glucoronide conjugates. Using the mixture of methanol:acetone (1:1), all of the compounds had recoveries of between 91 and 99% except sulfate and glucoronide conjugate compounds. The mixture that gave the highest recoveries for sulfate compounds was methanol:water (1:1). Our results on the extraction of free estrogens agree with the studies of Muller *et al.* [1] and Céspedes *et al.* [2]. Muller *et al.* [1] corroborated that free estrogens were extracted effectively using methanol:acetone (1:1). Moreover, parameters such as temperature or pressure were the same as in our studies. Because a unique solvent to extract all compounds was not found, the optimization was done using two mixtures: methanol:acetone (1:1) and methanol:water (1:1).

The number of cycles and the static time were optimized together because these parameters are related. After studying different extraction times (3 and 5 minutes) and different number of cycles (between 1 and 4), the best recoveries were obtained when two cycles of 3 minutes of each solvent were done. Although Muller *et al.* [1] used a different extraction time (2 cycles of 5 minutes), similar recoveries were obtained.

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Different temperatures were tested for both mixtures of methanol:acetone (1:1) and methanol:water (1:1). The best recoveries were obtained when the temperature was 75 ºC. We observed that recoveries increased between 25-75 ºC and decreased between 75-125 ºC. This was probably due to degradation of the target analytes at higher temperatures.

Finally, combining two different solvents, two cycles of methanol:acetone (1:1) followed by two cycles of methanol:water (1:1), recoveries higher than 81% were obtained except for glucoronide compounds which were not extracted (the recoveries were always lower than 10%). For this reason these compounds were excluded from the study.

With the purpose of reducing the limits of detection, two different strategies were tested. The first one was to evaporate the extract of the first two cycles (methanol:acetone (1:1)) until dryness and to redissolve it with the second extract (methanol:water (1:1)). Using this strategy, the final volume was reduced to 25 mL instead of 50 mL. The recoveries using this method were maintained. For the second strategy to reduce the limit of detection, we tested the application of a clean-up step – solid-phase extraction (SPE). Two different sorbents were tested - Oasis HLB and Strata-X. Although the percentage of the methanol volume in the extracts was reduced to less than 5% before the loading step, the recoveries decreased significantly for all compounds. Using Oasis HLB, the recoveries were between 31 and 66% and three compounds (DSB, EE2 and E2-17A) were not retained in the cartridge. When Strata-X was used, the recoveries were between 31 and 54% and four compounds (DSB, EE2, E2-17A and E2) were also not retained in the cartridge. Although these compounds were successfully extracted in wastewater samples using Oasis HLB in one study of our group [3], the clean-up step was not applied in this case because the results obtained were not good due to the presence of methanol in the extract which decreses significantly the recoveries. In the literature, another material such as Florisil was used for the clean-up step. After the publication of our study, Fernández *et al.* [4] developed a method to determine 7 free steroids and 6 conjugates. In this study they used a different extraction solvent - methanol:dichloromethane (30:70) – and used 3 cycles of 5 minutes to extract the estrogens and their conjugates. After the PLE extraction, they cleaned the extract using deactivated Florisil and obtained recoveries higher than 80% for all compounds studied.

Regarding the samples analyzed in the sewage treatment plants of Tarragona and Reus, three estrogens and two conjugates were found in the samples analyzed. Some differences were observed in the occurrence of these compounds in both STPs. For instance, estriol was found only in the sewage treatment plant from Reus, whereas diethylstilbestrol was only found in the STP from Tarragona. Other compounds such as estrone and the two sulfate conjugates were found at similar levels in both STPs. Fernández *et al.* [4] also found estrogens in sewage sludge but identified E1 and E2 at

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lower concentrations than in our study (0.056 and 0.155 μg/Kg (d.w.) for E1 and E2, respectively).

Although, as we mentioned before, the method had a limitation in the LOD of some compounds, we were able to quantify the presence of estrogens and their conjugates in sewage sludge. Due to the presence of these endocrine disruptors in sewage sludge, the effect on the environment of these compounds, and because sewage sludge can be used as manure in agriculture, estrogens and their conjugates should be taken into account when future legislation is drawn up.

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## 3.3. Determination of phosphodiesterase type V inhibitors in sewage sludge and waste water samples

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To extend the number of pharmaceutical compounds studied in this Thesis, a group of three phosphodiesterase type-V inhibitors was included. Although other compounds are included in this group, the most common pharmaceuticals are sildenafil, vardenafil and tadalafil. This study was started in the University of Applied Sciences Fresenius (Idstein, Germany) during my stay of four months in Professor Thomas P. Knepper's group.

Sildenafil, vardenafil and tadalafil are the active agents of Viagra®, Levitra® and Cialis®, respectively. All of these pharmaceuticals are used to treat erectile dysfunction in males. The physiological process of erection involves the release of nitric oxide in the corpus cavernosum of the penis, mediated by the parasympathetic nervous system. Nitric oxide binds to the receptors of the enzyme guanylate cyclase, which results in increased levels of cyclic guanosine monophosphate (cGMP) leading to smooth muscle relaxation of helicine arteries followed by increased blood flow and hence an erection. The pharmaceuticals mentioned are potent inhibitors of phosphodiesterase type-V (PDE-V), which is responsible for degradation of cGMP. The molecular structures of these drugs are similar to that of cGMP, in that they act as competitive binding agents of PDE-5 in the corpus cavernosum, resulting in more cGMP and better erections. However, these compounds are also used for treating pulmonary hypertension, Raynaud's phenomenon, altitude sickness and Duchenne/Becker muscular dystrophy [1-3]. In recent years, a residue of sildenafil was found in different athletes during anti-doping tests and it is now included on the list of doping substances [4].

These compounds have been determined in different kinds of samples such as herbal dietary supplements and plasma, among others [5-7] but not in the environmental samples. Moreover, not only sewage sludge samples were analyzed but also influent and effluent wastewater samples.

Solid-phase extraction (SPE) is the preferred technique to extract contaminants from water samples [8-10] and therefore we decided to apply SPE to develop a method to determine three compounds in influent and effluent wastewater. Moreover, a concentration and a clean-up step were simultaneously done when using the SPE technique. For the sewage sludge samples, the extraction technique was pressurized liquid extraction (PLE). After the extraction techniques, the samples were analyzed using liquid chromatography–tandem mass spectrometry (LC-MS-MS).

In this study, two different analyzers were used: the triple quadrupole for the samples from Tarragona, and the linear ion trap for the analysis carried out in Germany. In both cases, the analyzers worked in multiple reaction monitoring mode (MRM).

The methods developed were applied to samples from Germany and Spain. Eight grab effluent wastewater samples from Germany were analyzed. The effluent samples were divided in two groups: samples from STPs located in major cities

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(Frankfurt, Darmstadt, Giessen, Wiesbaden) and samples from spas (Bad Orb, Bad Homburg von der Höhe and Bad Nauheim).
Moreover, the STP from Tarragona was studied in more detail. Therefore samples were collected every two months during the year 2008 and the presence of sildenafil, vardenafil and tadalafil was determined in influent and effluent waste water samples and sewage sludge samples during the year 2008. Using all of this data, the approximate removal efficiency of the STP in Tarragona was calculated for the three compounds.
In order to study the possible degradation metabolites of sildenafil, the fixed bed bioreactor (FBBR) experiment was carried out. A biologically active fixed bed bioreactor operating under aerobic conditions was used for investigating the primary degradation of the target analytes and the pathways of their breakdown products. The test device benefits from its simple experimental set-up, ease of operation and the fact it can provide aqueous samples suitable for direct injection into liquid chromatography. A glass column filled with glass beads forms the main part of an FBBR (see Fig 3.1) which enables microorganisms to accumulate on the surface. The experimental set-up was based on a mixed microbial community from a natural setting other than isolated cultures, as this guaranteed a high environmental relevance of the outcomes.

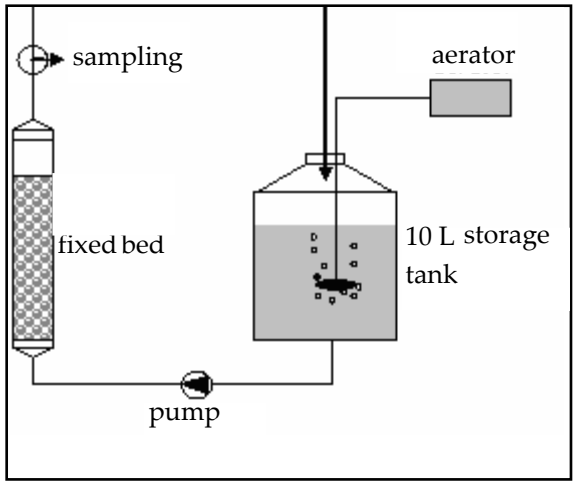


**Figure 3.1.** Schematic set-up of the FBBR.

The water phase circulated with a flow rate of 17 mL/min in a close loop and a membrane pump aereated the water in the storage tank. Samples were taken via a three-way valve. The FFBR experiments were set up in the dark to avoid photodegradation. In Professor T.P. Knepper's group, the FBBR had been applied in different studies such as the degradation of fluorosurfactants and barbiturates [11,12]. In our experiment we observed that the concentration of sildenafil in the

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FBBR did not vary during 30 days in effluent water and 17 days in surface water, so possible degradation products were not observed. Although negative results were obtained with the FBBR, we included in the MRM method a transition of the main metabolite of sildenafil, desmethylsildenafil (461 to 283), as reported in previous studies [6,13]. However, the metabolite was not present in any sample analyzed. Thus, the results obtained in the degradation studies were not included in the paper.

This study has been published in Water Research 44 (2010) 1607-1615. The supplementary information available on-line is also included.

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### 3.3.1. Phosphodiesterase type V inhibitors: Occurrence and fate in wastewater and sewage sludge

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# PHOSPHODIESTERASE TYPE V INHIBITORS: OCURRENCE AND FATE IN WASTEWATER AND SEWAGE SLUDGE

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**Abstract**

The contamination of wastewater and sewage sludge has been examined for three phosphodiesterase type V inhibitors sildenafil, vardenafil and tadalafil, active agents of Viagra®, Levitra® and Cialis®, respectively. Sensitive quantification methods based on solid-phase extraction (SPE) and pressurized liquid extraction (PLE) followed by high performance liquid chromatography - tandem mass spectrometry (LC-MS/MS) have been developed to determine these compounds in wastewater and sewage sludge.

Effluent water of nine sewage treatment plants (STP) has been analyzed to asses the impact of the phosphodiesterase type V inhibitors on the environment. One municipal STP (Tarragona, Spain) has been thoroughly studied over the year 2008 (i) with respect to the distribution of these compounds among influent and sewage sludge and (ii) the elimination efficiency.

The developed methods allowed quantification at trace concentrations. Sildenafil was present in all investigated samples at the low ng/L and ng/g range, respectively. Tadalafil was not detected or below the limit of detection (LOQ) in effluent water taken in Spain but in sewage sludge (12 ng/g - < LOQ). Vardenafil was present only in one sludge sample and between 5 ng/g and < LOQ in effluent water. The overall removal efficiency of the STP in Tarragona (Spain) is 68%, 69% and 80% for sildenafil, tadalafil and vardenafil, respectively. This study shows for the first time the determination of these compounds in wastewater and sewage sludge.

**Keywords:** Sildenafil, viagra, effluent wastewater, tandem mass spectrometry.

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## INTRODUCTION

Sewage water contains various pollutants, including a broad spectrum of pharmaceutically active compounds that can be discharged into the environment via the sewage treatment plant (STP) effluent water

ISBN:978-84-693-6426-0/DL:T-1628-2010

(Daughton and Ternes, 1999). Besides, reports about stimulating drugs like cocaine and amphetamines, eg. 3,4-methylenedioxy- N-methylamphetamine (MDMA) in natural waters (Castiglioni *et al.*, 2006; Boleda *et al.*, 2007; Huerta-Fontela *et al.*, 2007) have attracted attention to the scientific community. The consumed amount of stimulating drugs is usually higher than legally prescribed quantities. Another class of drugs which are often illegally acquired are the active agents of Viagra®, Levitra® and Cialis® namely sildenafil citrate, vardenafil and tadalafil, respectively. They are used in therapies for erectile dysfunction acting as a selective inhibitor of phosphodiesterase type V which cleaves cyclic guanosine monophosphate (cGMP) (Berzas et al., 2002; Daraghmeh et al., 2001). Viagra®, or sildenafil citrate, was devised to treat pulmonary hypertension, or high blood pressure in arteries of the lungs. The drug works by suppressing the enzyme that controls blood flow, allowing the vessels to relax and widen. The same mechanism facilitates blood flow into the penis of impotent men. In the case of athletes, increased cardiac output and more efficient transport of oxygenated blood to the muscles can enhance endurance. For that reason, sildenafil has been recommended to be listed as a doping substance (Longman, 2008).

Until now, no information can be found in literature concerning the concentration of phosphodiestarase type V inhibitors in environmental samples. However, these compounds have been determined in dietary supplements for male sexual potency (Zhu *et al.*, 2005), in pharmaceutical samples (Berzas *et al.*, 2002; Abad-Elbary *et al.*, 2004) and in biological samples such as plasma (Cooper *et al.*, 1997; Eerkes *et al.*, 2002; Al-Ghazawi *et al.*, 2007; Kim *et al.*, 2003). Sildenafil is readily absorbed and metabolized in the liver to desmethylsildenafil by cytochrome P450 3A4 (Eerkes *et al.*, 2002). Several authors (Cooper *et al.*, 1997; Eerkes *et al.*, 2002; Al-Ghazawi *et al.*, 2007; Kim *et al.*, 2003) provide information about this principal metabolite, which is present at 30 - 40% of the parent drug concentration in plasma after single oral dosing (Cooper *et al.*, 1997).

For the determination of sildenafil in biological tissues and in dietary human supplements, gas chromatography (GC) has been applied (Berzas *et al.*, 2002), but the polarity of sildenafil, vardenafil and tadalafil, favours high performance liquid chromatography (HPLC) (Zhu *et al.*, 2005; Cooper *et al.*, 1997; Eerkes *et al.*, 2002) as separation technique. For detection, tandem mass spectrometry is most appropriate since it exhibits both, high sensitivity and selectivity.

Since the concentrations of the target analytes in environmental samples are expected to be in the low ng/L range, an efficient sample preparation to extract the analytes from the respective matrix is required. For aqueous samples solid-phase extraction (SPE) is widely used and has already been applied to extract

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sildenafil from rat serum (Guermouche and Bensalah, 2006). For solid samples pressurized liquid extraction (PLE) has successfully used to determine pharmaceutical compounds in sewage sludge (Nieto *et al.*, 2008; Chu and Metcalfe, 2007; Nieto *et al.*, 2009).

The aim of this work was to develop a method to determine the phosphordiesterase type V inhibitors sildenafil, tadalafil and vardenafil in influent and effluent wastewater as well as in sewage sludge.

For sample preparation SPE and PLE have been optimized and applied prior analytical detection with HPLC - tandem mass spectrometry. The analytical method was applied to determine these compounds in grab effluent wastewater samples from different STPs in Germany and Spain (n= 9). The STP in Tarragona (Spain) has been studied thoroughly. Influent and effluent wastewater as well as sewage sludge have been analyzed in order to assess the elimination efficiency on the one hand, and the contribution of STPs to the contamination of the aquatic environment with these phosphodiesterase type V inhibitors on the other hand.

## EXPERIMENTAL

### Reagent and standard

All solvents were HPLC-grade. Hexane, acetonitrile, acetic acid and aluminum oxide were obtained from Merck/ VWR-International (Darmstadt, Germany), methanol was from Merck/VWR-International and SDS (Peypin, France), respectively. Acetone and dichloromethane were from SDS (Peypin, France), nitrogen was purchased from Carburos Metálicos (Tarragona, Spain).

SPE cartridges (Oasis-HLB 60 mg) were from Waters (Eschborn, Germany). Fluazifop-butyl served as an internal standard (IS) and was supplied by Sigma Aldrich (Gilingham, UK). Sildenafil, vardenafil and tadalafil were pharmaceutically grade in tablet form with concentrations of 50 mg of sildenafil and 10 mg of tadalafil and vardenafil.

Stock solutions were prepared by dissolving each compound in methanol at a concentration of 1 g/L and stored at -20 ºC. Working solutions were prepared weekly by diluting this solution with acetone. Ultra pure water was obtained with a Milli-Q water purification system (18.2 MΩ·cm) (Millipore, Bedford, MA, USA).

### Sampling

In order to check the approximate concentration of sildenafil in the effluent water, grab samples (n = 8) have been investigated from the STP of Beuerbach (Germany).

Further, nine municipal STPs were sampled. They were divided in two groups: (i) major cities (Frankfurt/Main, Darmstadt, Giessen and Wiesbaden in Germany, and Tarragona and Reus in Spain) and (ii) spas in Germany (Bad Orb, Bad

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Homburg von der Höhe and Bad Nauheim).

The STP of Tarragona has been thoroughly investigated. Twenty four hours composite influent and effluent samples as well as sludge samples were taken every two month over the year 2008 and have been analyzed for the target compounds. This STP receives mostly urban wastewaters and some industrial discharges (population equivalent approx. 140,000; $BOD_5$ = 400 mg/L). Aqueous samples were adjusted to pH 2 and stored at -25 °C before sample preparation. The average flow (calculated for 2008) was 529,000 $m^3$ per month for influent water and 516,000 $m^3$ per month for effluent water. The annual production of sewage sludge (dry weight, d.w.) in 2008 was 1915 t.

**Sample preparation**

For SPE, two different materials were tested to extract the target analytes from water, Waters Oasis-HLB and Waters Oasis-MCX. The extracted volume was 200 mL for effluent water and 100 mL for influent water. Elution of the analytes from Oasis-HLB was done with 1.5 mL acetone:ethylacetate (1:1, v:v); this step was repeated twice. When Oasis-MCX was used, the elution was carried out with 3 x 1.5 mL acetone.

The final preconcentration step was based on Oasis-HLB. The sorbent was sequentially conditioned with 2 mL hexane, 6 mL methanol and 10 mL pristine groundwater (Niedern-hausen, Germany).

After filtration 20 ng of the IS fluazifop-butyl was added (β= 1 ng/μL in acetone) which was selected because of its structural similarity, similar retention in HPLC like the target analytes. $C_{13}$ labeled standards would have been optimal to compensate analytical errors but were not available for sildenafil, vardenafil or tadalafil. The samples passed through the SPE cartridge at a flow rate of 10 – 15 mL/min. Before elution, the SPE-sorbents were dried with passing nitrogen for 40 min. After elution, the extracts were evaporated to dryness under a gentle stream of nitrogen and the residue was redisolved in 1 mL of the mobile phase (acetonitrile:water (1:1, v:v) + 5 mmol ammonium acetate).

PLE was performed using the ASE 200 (Accelerated Solvent Extraction, Dionex, Sunnyvale, CA, USA). The sewage sludge samples were homogenized, frozen, lyophilized using the freeze-dry system (Labconco, Kansas City, MO, USA), sieved through a 125 μm screen and stored in a closed flask at room temperature. The sludge sample was loaded into an 11 mL stainless steel extraction cell. Before loading the sample, a glass fiber filter was placed in the outlet of the cell, followed by a 1 g layer of aluminium oxide, which was heated at 120 ºC for 24 h before use. One gram of dried sewage sludge was then placed into the extraction cell and carefully mixed and filled

ISBN:978-84-693-6426-0/DL:T-1628-2010

with aluminium oxide. No filter was placed at the top of the extraction cell.
The following parameters have been optimized for the extraction: solvent, temperature, pressure, static extraction time and flush volume. The initial conditions were: a temperature of 75 ºC, a pressure of 100 bar, 1 cycle of 5 minutes of static time, 60% of flush volume and 60 s of purge time. For this procedure 1 g of sewage sludge (d.w.) was spiked with the target analytes at 25 ng/g and 50 ng of IS.
The optimization of each parameter was done one-by-one, different adjustments were tested and the conditions giving the highest extraction yield were fixed (see Table 1). Four different pure solvents and three different binary mixtures have been used for the extraction. Five different temperatures (25, 50, 75, 100 and 125 ºC) and three different pressures (60, 100 and 140 bar) were tested. The static extraction time has been tested in combination with varying the number of cycles. Table 1 summarizes the obtained results and reflects the final PLE method which was as follows: methanol was used as extraction solvent at a temperature of 100 ºC, a pressure of 140 bar, two cycles of 5 min of static extraction time and a flush volume of 60%. Finally, the cell was purged with nitrogen for 60 seconds. The final extracts were filtered with a microfilter 0.45 μm pore size (Teknokroma, Barcelona, Spain) and analysed with LC-MS-MS.

**Liquid chromatography - tandem mass spectrometry**

Analysis of the target analytes in grab effluent samples was performed with LC-MS-MS. The chromatographic instrumental setup consisted of two Series 200 Micro Pumps, a Series 200 Vacuum Degasser and a Series 200 Autosampler (Perkin Elmer, Norwalk CT, USA). Separation was achieved on a HALO $C_{18}$ column (Advanced Material Technology, Wilmington, USA). This system was coupled to a 3200 QTrap (Applied Biosystems, Foster City, CA, USA).
Analysis of the target compounds in influent, effluent and sewage sludge samples of the STP in Tarragona was done on an HP1200 series LC-triple QqQ tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany).
The LC-MS-MS systems were operated in multiple-reaction monitoring (MRM) mode. For each compound, two transitions of the protonated molecular ion $[M+H]^+$ were monitored (Table 2).
Compound dependent parameters for the MS detection and the respective transitions are listed in Table 2. Further information about the chromatographic separation and instrumental characteristics can be gathered from the supplement material.

**Method validation**

The linear range was studied using effluent water (200 mL), influent water (100 mL) and sewage sludge

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(1g, d.w.) from Tarragona (Spain) spiked at concentrations between 1 – 250 ng/L and 2 – 200 ng/g, respectively with sildenafil, tadalafil and vardenafil (7 data points). Background contamination was subtracted from each calibration point.

The recoveries were calculated using 200 mL effluent water, 100 mL influent water and 1 g of dried sewage sludge. In each case, the target analytes were spiked at 25 ng/L, 50 ng/L and 25 ng/g, respectively (n = 2). After sample preparation and analysis with LC-MS-MS the signal intensity was compared to the signal obtained from a standard of same concentration in pure solvent (eluent A).

To calculate the signal to noise ratio (S/N) the lowest point of the calibration curve was used. The S/N = 3 and S/N =10 were used to determine the limit of detection (LOD) and the limit of quantification (LOQ), respectively.

The repeatability and reproducibility were determined by measuring a spiked extract (25 ng/L for water samples and 25 ng/g for sewage sludge) three times in a row, and on three subsequent days, respectively. The repeatability and reproducibility is expressed as the relative standard deviation (RSD [%]).

## RESULTS AND DISCUSSION

### Method Optimization

For SPE, two different materials have been tested for extraction of the target analytes from aqueous samples. Best recoveries were obtained with Oasis-HLB whereas extraction of sildenafil, vardenafil and tadalafil with Oasis-MCX was poor (<20%). In order to obtain fast and efficient extraction of phosphodiesterase type V inhibitors from sewage sludge using PLE, several parameters have been optimized (Table 1).

Apart from the respective spiked sample a blank has been analyzed in each case, too. The obtained signal was then subtracted from the signal of the spiked sample. This procedure had to be done since there was no sewage sludge not contaminated with at least one of the target analytes. Table 1 summarized the recoveries obtained for the target analytes with each extraction solvent. The highest recoveries were obtained when methanol was used. Recoveries increased when the temperature increased, only fluazifop-butyl (IS) decreased at 125 °C. For tadalafil the extraction efficiency was not temperature dependent. Finally 100 °C were set since the extracts were less brownish compared to those which were gained at 125 °C.

The results obtained when the static time and the number of cycles were optimized are summarized in Table 1. One cycle of 5 minutes was enough to obtain total extraction of sildenafil and vardenafil. However, more time was necessary to extract tadalafil and fluazifop-butyl. The highest recoveries for tadalafil and fluazifop-butyl were obtained when 10 minutes of extraction time was applied. Further, 2 cycles of 5 minutes were more

efficient than 1 cycle of 10 minutes. The highest tested pressure (140 bar) and a flush volume of 60% gave the best results.

The final optimum conditions to extract the phosphodiesterase type V inhibitors were: methanol as extraction solvent, 100 ºC, 140 bar, 2 cycles of 5 minutes and 60% of flush volume.

**Table 1.** Recoveries obtained during the optimization of the PLE method.

| Parameters | Sildenafil | Tadalafil | Vardenafil | Fluazifop-butyl (IS) |
|---|---|---|---|---|
| *Solvent* | | | | |
| ➢ **Methanol** | **76** | **29** | **78** | **95** |
| Acetone | 8 | 11 | 19 | 64 |
| DCM | 6 | 13 | 22 | 37 |
| Water | 0 | 0 | 0 | 0 |
| Water:Methanol (1:1, v:v) | 0 | 37 | 0 | 94 |
| Methanol:DCM (1:1, v:v) | 76 | 26 | 68 | 61 |
| Methanol:Acetone (1:1, v:v) | 65 | 21 | 77 | 62 |
| *Temperature [°C]* | | | | |
| 25 | 49 | 30 | 50 | 82 |
| 50 | 65 | 33 | 68 | 69 |
| 75 | 76 | 29 | 78 | 95 |
| ➢ **100** | **90** | **31** | **98** | **81** |
| 125 | 100 | 30 | 98 | 52 |
| *Pressure [bar]* | | | | |
| 60 bar | 93 | 28 | 95 | 80 |
| 100 bar | 90 | 33 | 97 | 81 |
| ➢ **140 bar** | **90** | **49** | **97** | **88** |
| *Static extraction time (min)* | | | | |
| 5 (1 cycle) | 79 | 49 | 82 | 95 |
| 9 (3 cycles each 3 min ) | 92 | 19 | 98 | 84 |
| 10 (1 cycle) | 96 | 53 | 98 | 79 |
| ➢ **10 (2 cycles each 5 min)** | **102** | **59** | **102** | **94** |
| 15 (1 cycle) | 102 | 35 | 96 | 74 |
| 15 (3 cycles each 5min) | 106 | 42 | 105 | 95 |
| 20 (2 cycles each 10 min) | 97 | 37 | 91 | 69 |
| *Flush volume (%)* | | | | |
| ➢ **60%** | **102** | **59** | **102** | **94** |
| 90% | 107 | 45 | 104 | 78 |

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**Analytical method to determine phoshodiesterase type V inhibitors by LC-MS-MS**

The fragmentation patterns obtained for vardenafil and sildenafil after (+)ESI and collision induced dissociation (CID) show similarities due to related structures (Figure 1a/b).

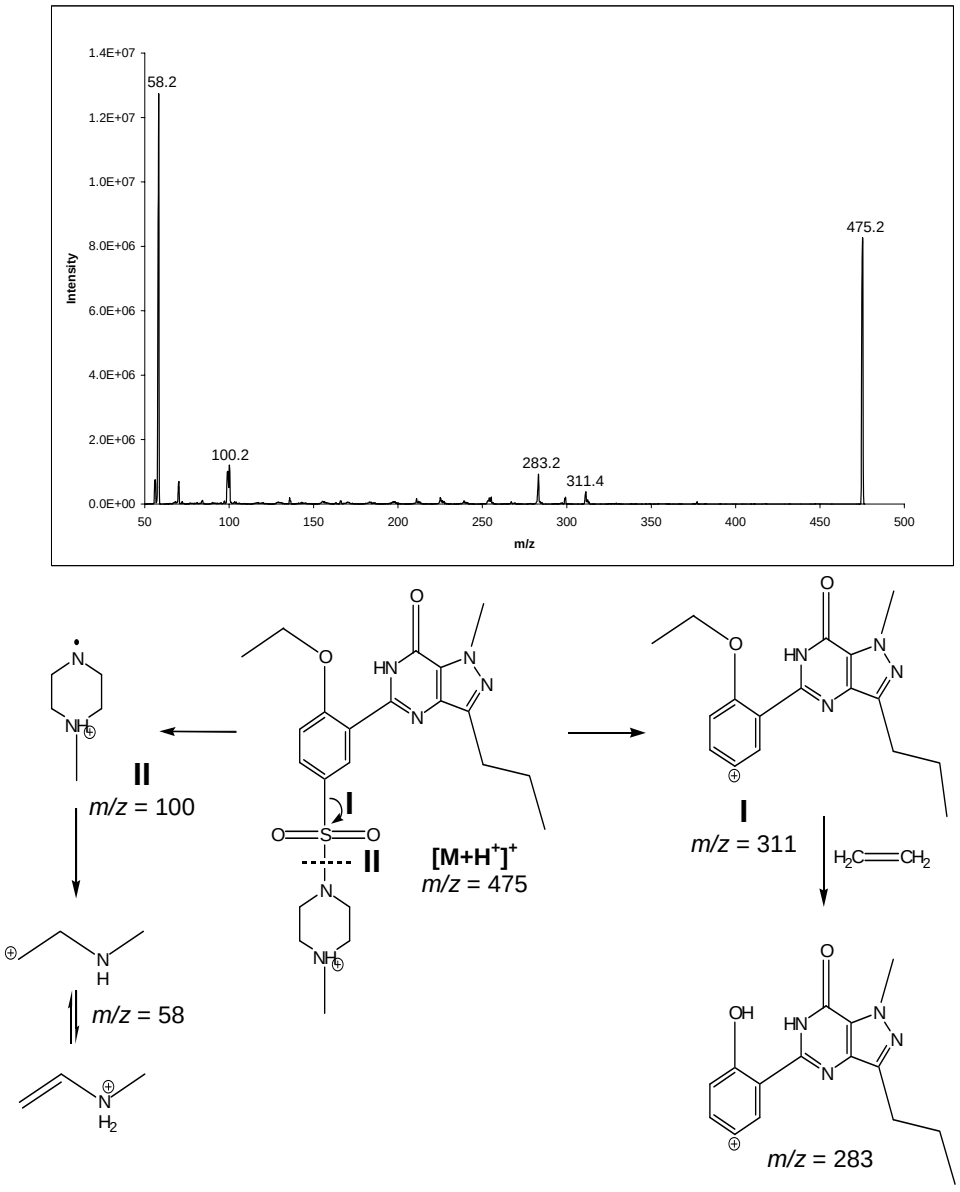


**Figure 1a**. Product ion scan of sildenafil after (+)ESI and collision induced dissociation (CID) and the proposed structures of the formed fragment ions.

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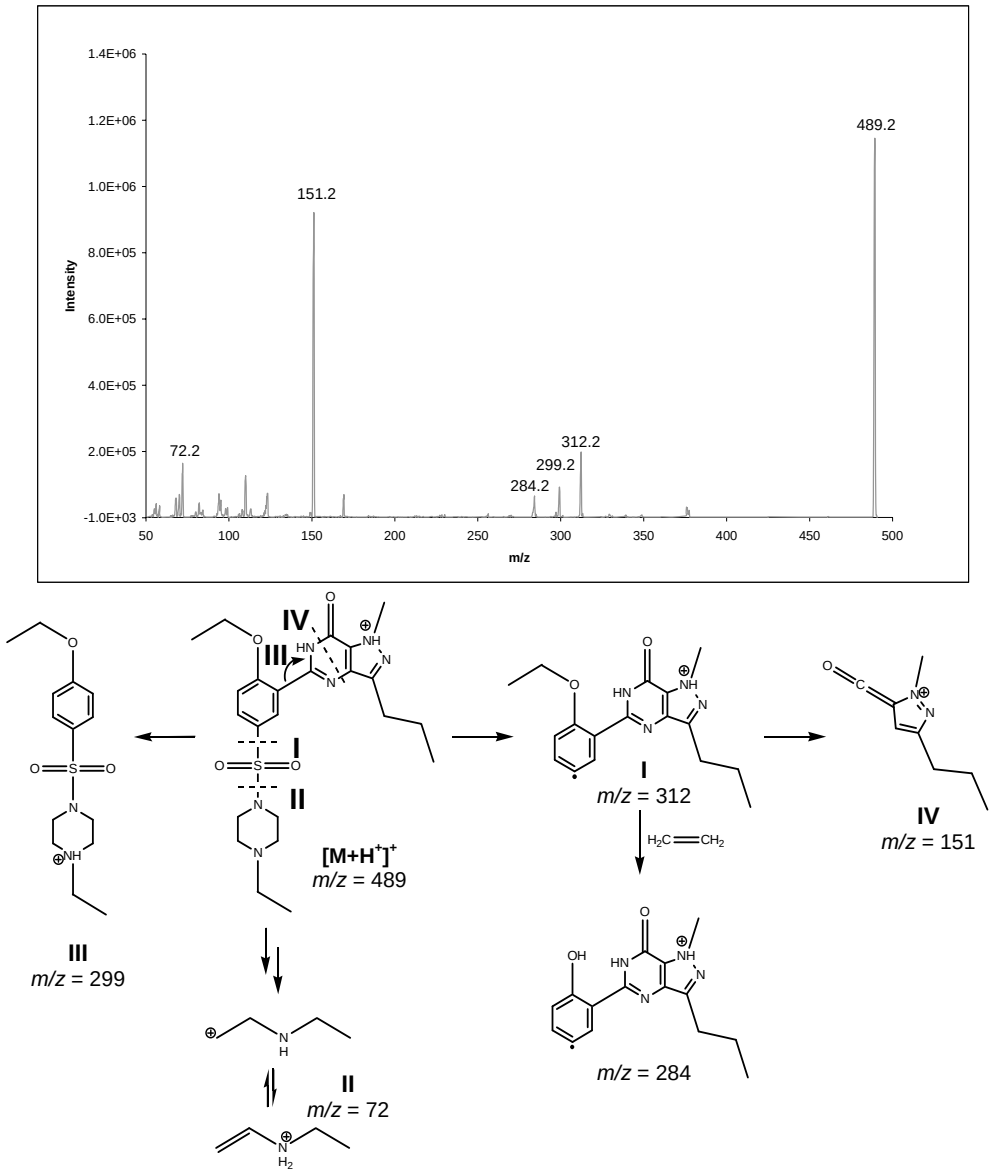


**Figure 1b**. Product ion scan of vardenafil after (+)ESI and collision induced dissociation (CID) and the proposed structures of the formed fragment ions.

Zhu *et al.* (Zhu *et al.*, 2005) determined these compounds with LC - single quadrupole MS and yielded for both compounds the fragments m/z 283 and m/z 311. However, by utilizing LC-MS-MS in our case following collision induced dissociation fragmentation (CID) different fragments of the molecular ion ($[M+H]^+$) were obtained. We found

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m/z 283 and m/z 311 as well as m/z 100 and m/z 58 for sildenafil (Figure 1a), and m/z 284, m/z 299 and m/z 312 as well as m/z 151 and m/z 72 for vardenafil (Figure 1b). The transition of m/z 475 ($[M+H]^+$) to m/z 283 for sildenafil after (+)ESI-MS-MS has also been reported by Eerkes *et al.* (Eerkes *et al.*, 2002).

The mass spectra obtained after (+)ESI of sildenafil and vardenafil were quite different despite their similar structures presumably resulting from different protonation sites. A protonation of a methylated than a protonation of an ethylated cyclic amino group seems to be preferred.

The individual fragmentation patterns can be explained if the protonation of sildenafil is assumed to be at the piperazine moiety and cleaves the fragment m/z 100 after CID. The according fragment for vardenafil should appear at m/z 114 but has not been observed. For this compound the protonation probably takes place at the pyrazol moiety.

Therefore the fragment at m/z 151 can be formed (Figure 1b). Cleavage at the sulfono group yields m/z 311 for sildenafil and m/z 312 for vardenafil. Both show an ethylene loss (delta 28 amu) resulting in the fragments at m/z 283 and m/z 284, respectively. The CID fragments observed did not vary applying different collision voltages as well as different mass spectrometers. The same fragments were observed for sildenafil and vardenafil when LC-triple QqQ tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany) with an ESI interface was used applying different collision energies (5 - 45 V). Tadalafil shows a strong fragmentation pattern with the most abundant ions m/z 268 and m/z 204. Since the structure is different from sildenafil and vardenafil, no similarities are observed.

## METHOD VALIDATION

The dynamic range of sildenafil, tadalafil and vardenafil spiked in effluent, influent and sewage sludge was determined. After applying the optimized analytical methods based on SPE or PLE prior LC-MS-MS detection, a linear regression of the calibration curve was obtained in the range of 2.5 ng/L - 250 ng/L in aqueous samples and 5 –200 ng/g in sewage sludge (Table 2).

Acceptable determination coefficients ($R^2$>0.9937, 7 data points) were obtained for all analytes. Sildenafil and vardenafil co-eluted due to their very similar structures. However, employing tandem mass spectrometry allowed simultaneous quantification in the given dynamic range.

For calibration the IS fluazifop-butyl was used. The relative standard deviation (%RSD) of its signal intensity in all analyzed influent water samples, effluent water samples and sewage sludge samples plus the according calibration points was 11, 8 and 15%, respectively. The recovery rates of the analytes were calculated for each matrix. Background contamination was subtracted from the obtained signals.

**Table 2.** MS and validation parameters of the analytical methods for sildenafil, tadalafil, vardenafil and fluazifop-butyl (IS).

| | **Sildenafil** | **Tadalafil** | **Vardenafil** | **Fluazifop-butyl (IS)** |
|---|---|---|---|---|
| ***MS parameter transition*** | **475 > 58**<br>475 > 100 | **390 > 268**<br>390 > 204 | **489 > 151**<br>489 > 312 | **384 > 282**<br>384 > 328 |
| Cone Voltage [V] *(QqQ)* | 160 | 130 | 200 | 130 |
| Collision Energy [V] *(QqQ)* | 30 | 10 | 40 | 15 |
| | 40 | 5 | 45 | 20 |
| Collision Energy [V] *(LIT)* | 41 | 17 | 63 | 25 |
| | 63 | 75 | 53 | 25 |
| Declustering potential [V] *(LIT)* | 71 | 26 | 81 | 50 |
| Entrance potential [V] *(LIT)* | 4.5 | 4 | 4 | 10 |
| CEP [V] *(LIT)* | 18 | 20 | 16 | 21 |
| Cell exit potential [V] *(LIT)* | 3 | 4 | 4 | 2 |
| ***Recovery [%]*** | | | | |
| Sludge (spiked at 25 ng/g d.w.) | 103 | 45 | 102 | 94 |
| Influent (spiked at 50 ng/L) | 68 | 80 | 78 | 69 |
| Effluent (spiked at 25 ng/L) | 78 | 91 | 92 | 72 |
| Surface water (spiked at 25 ng/L) | 94 | 92 | 102 | n.m. |
| ***Linear range*** | | | | |
| Sludge [ng/g] | 5 – 150 | 5 – 200 | 5 – 100 | - |
| Influent wastewater [ng/L] | 4 - 150 | 10 - 200 | 8 - 250 | |
| Effluent wastewater [ng/L] | 2.5 – 200 | 2.5 – 250 | 2.5 – 250 | - |
| ***Limit of detection*** | | | | |
| Sludge [ng/g] | 1 | 3 | 2 | - |
| Influent [ng/L] | 1 | 5 | 2 | - |
| Effluent [ng/L] | 1 | 1.5 | 1 | - |
| ***Limit of quantification*** | | | | |
| Sludge [ng/g] | 3 | 5 | 5 | - |
| Influent [ng/L] | 2.5 | 6 | 4 | - |
| Effluent [ng/L] | 3 | 2.5 | 2.5 | - |
| ***Repeatability in RDS [%, n=3])*** | | | | |
| Sludge | 8 | 8 | 10 | - |
| Influent | 9 | 12 | 7 | - |
| Effluent | 5 | 11 | 6 | - |
| ***Reproducibility between days in RDS [%, n=3]*** | | | | |
| Sludge | 12 | 13 | 16 | - |
| Influent | 12 | 17 | 8 | - |
| Effluent | 8 | 15 | 8 | - |

The recovery rates for the three compounds lay between 45 % and 103 % (Table 2). The LOQ was defined as a signal to noise ratio (S/N) of minimum 10 and the LOD were defined as minimum S/N = 3. The

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LOQs and LODs obtained are summarized in Table 2.
The repeatability and reproducibility between days (n=3) were lower than 12% and 17%, respectively.
In-depth elucidation of the matrix effects during ESI and verification of the assembled analytical methods can be gathered from the supplementary material.

**METHOD APPLICATION**

**Analysis of STP effluent wastewater**

In order to check the approximate concentration of sildenafil in effluent water, several samples have been investigated from the STP of Beuerbach (Germany). An external calibration was applied and approximately 2 ng/L (mean value) were detected in the analyzed samples (n = 8 samples). The subsequent sampled STP in Germany and Spain (effluent water) all showed contamination with the phosphor-diesterase type V inhibitors. The obtained quantification results are summarized in Figure 2. Values below the LOQ are expressed as 0.5*LOQ. Sildenafil was present in 100% of all analysed effluent water samples at levels between 6 and 18 ng/L. A maximum value of 18 ng/L was determined in the STP effluent from Bad Homburg (spa in Germany). The minimum value of 6 ng/L was detected in samples from Darmstadt (Germany). There were no significant differences in sildenafil concentrations between the samples from Spain and Germany. Compared with tadalafil and vardenafil, sildenafil was always detected at higher concentrations (except in the case of Wiesbaden where tadalafil was most abundant). Tadalafil was quantified in all samples from Germany at levels between 3 and 8 ng/L. All over, the concentration of tadalafil was higher in Germany than in Spain. Vardenafil was present in all samples from Germany but below the LOQ in samples from Spain.
Figure 2 shows that the STP effluent samples from the spas showed higher concentrations of sildenafil and it can be seen that the sum of all three compounds is higher in spas than in urban STP.

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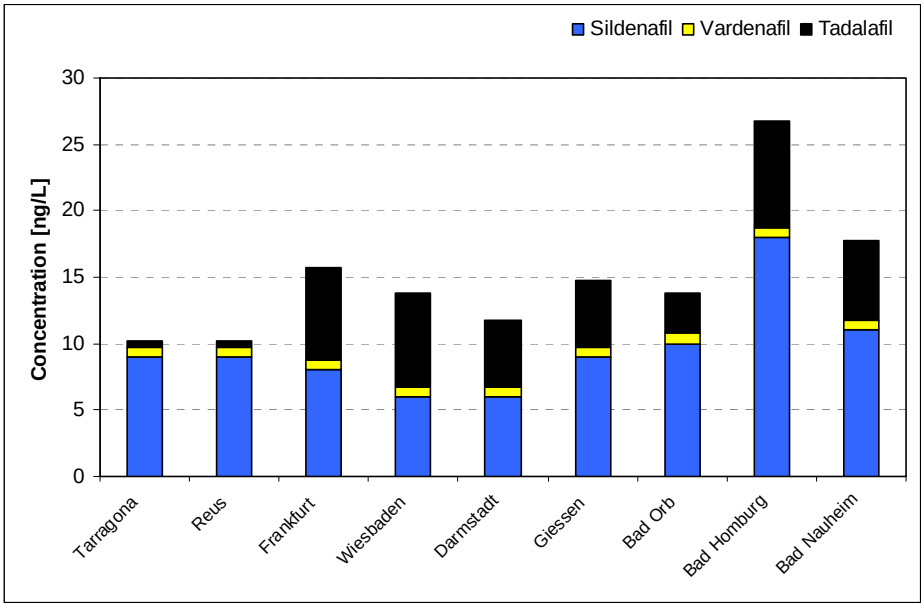


**Figure 2**. Quantification results of STP effluent grab samples from Germany and Spain.

**Presence of sildenafil, tadalafil and vardenafil in the STP of Tarragona**

Sewage sludge and wastewater influent and effluent samples of the municipal STP in Tarragona were analyzed over the year 2008 (Figure 3). Sildenafil was the most abundant compound in all three compartments with mean concentrations of 32 ng/L, 8 ng/L and 8 ng/g in influent, effluent and sewage sludge, respectively.

Maximum concentrations for sildenafil and vardenafil can be observed in July and for tadalafil in May. The higher population may be one reason for that since Tarragona is the summer residence for many tourists. The annual amount of incoming sewage water is 6350,000 $m^3$, i.e. 202 g sildenafil enter the STP. With the effluent water 51 g (25%) are released into the environment. In the sewage sludge (annual production is 1915,017 t (d.w.)) 15 g (7.3%) of sildenafil can be recovered. According to that, the removal rate (adsorption plus degradation) for sildenafil is approx. 68% in the STP Tarragona.

The principal metabolite of sildenafil (UK-103,320) was not detected in any screened effluent water sample (n=8). The applied transition in order to search for the metabolite was m/z 461 to m/z 283, as different authors have described in the literature (Eerkes *et al.*, 2002; Kim *et al.*, 2003).

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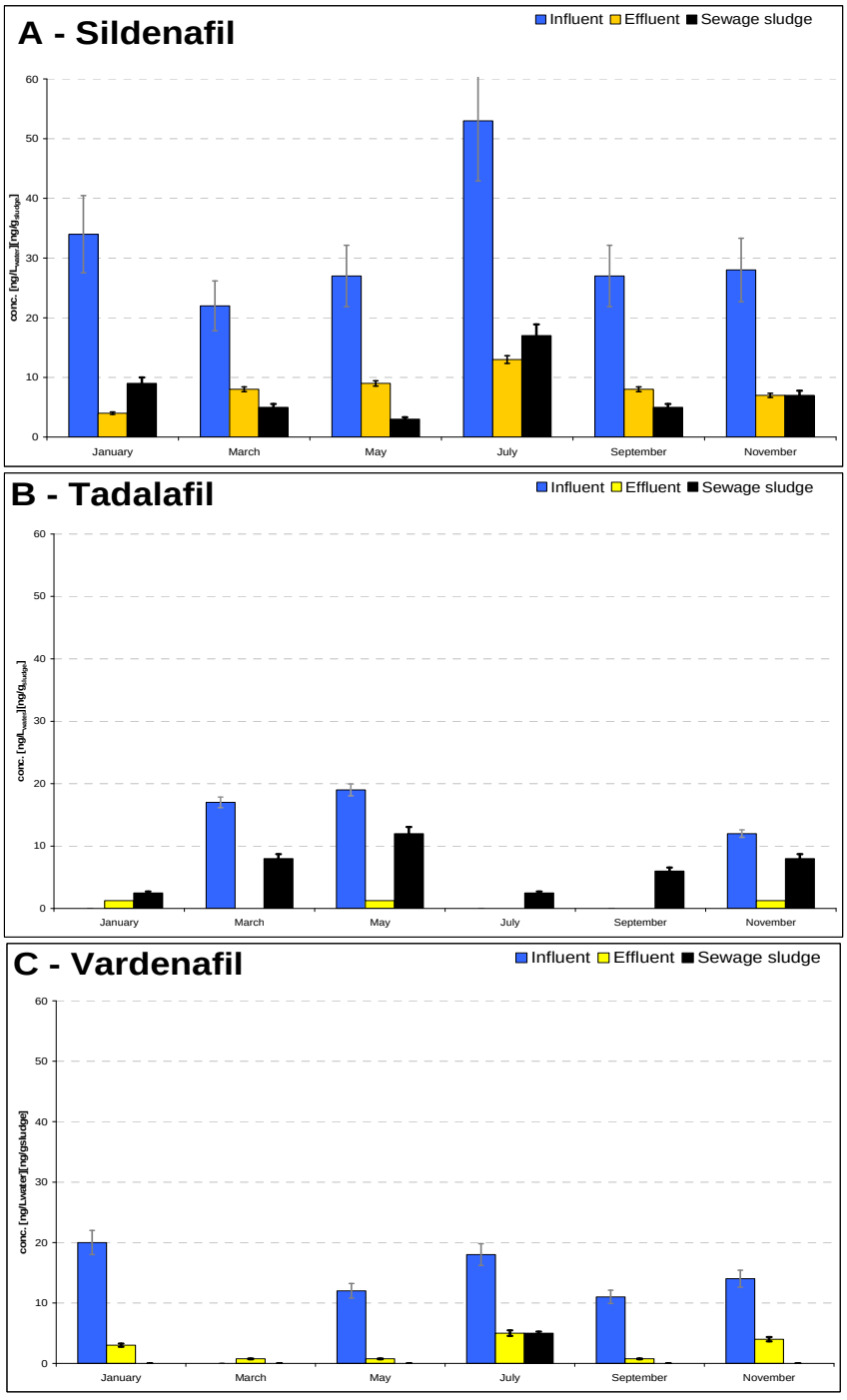


**Figure 3.** Quantification results of sildenafil, vardenafil and tadalafil in influent and effluent wastewater and sludge samples from the STP Tarragona (Spain).

For tadalafil and vardenafil the removal rate in the STP Tarragona is approx. 69% and 80%, respectively.
We deduce that these compounds are not completely removed in STPs. On average 26% of the influent mass concentration of sildenafil were found in the effluent water (20 and 19% for vardenafil and tadalafil, respectively).

## CONCLUSIONS

This study shows for the first time the determination of sildenafil, vardenafil and tadalafil in wastewater and sewage sludge. Since the elimination of the investigated phosphodiesterase type V inhibitors is not complete as demonstrated on the basis of the studied STP in Tarragona, such compounds are carried into the aquatic environment with the effluent water or with sewage sludge.
One hundred percent of the investigated samples were contaminated with at least one of the studied compounds. Up to now it is not known at what concentration these compounds adversely affect aquatic organisms.
Phosphodiesterase type V cleaves the cGMP which is a second messenger and responsible for the intracellular signal transduction in many different organisms. Therefore, the impact of sildenafil, vardenafil and tadalafil requires further research.
Inclusion of these compounds in monitoring campaigns is recommended.

**Acknowledgements**

This work was supported financially by the European Commission (6th Framework program, Project Aqua Terra GOCE-CT-2005-505428, www.eu-aquaterra.de <http://www.eu-aquaterra.de/>) and the Hessian Ministry for Environment, Rural Area and Consumer Protection.
This study was also financially supported by the Dirección General de Investigación of the Ministry of Science and Technology, project CTM2008-06847-C02-01/TECNO. A.N. gratefully acknowledges the financial support of the Ministry of Science and Technology BES-2006-1332.

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**Supplementary Information**

**Analytical conditions for the analysis of sildenafil, vardenafil and tadalafil**

The chromatographic instrumental setup consisted of two Series 200 Micro Pumps, a Series 200 Vacuum Degasser and a Series 200 Autosampler (Perkin Elmer, Norwalk CT, USA). Separation was achieved on a HALO $C_{18}$ column, 5 x 0.21 cm, particle size 2.7 μm (Advanced Material Technology, Wilmington, USA).
A binary mobile phase gradient was used. Eluent A was 5 mM ammonium acetate in water:acetontrile (95:5, v:v) and eluent B was 5 mM ammonium acetate in acetontrile:water (80:20, v:v). The flow rate was set to 0.3 mL $min^{-1}$ and the injection volume was 50 μL. The initial conditions of the gradient program were 20% B, kept constant for 1 min, then increased to 100% in 7 min, maintained for 5 min and returned to 20% B within 2 min. The column as finally reequilibrated for 5 min.
One of the mass spectrometer used was a 3200 Q Trap (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ionspray interface. The system was operated in multiple-reaction monitoring (MRM) mode. For each compound, two characteristic fragmentations of the protonated molecular ion $[M+H]^+$ were monitored (Table 2). The most abundant transition was used for quantification, while the second most abundant was used as a qualifier. A positive ionization voltage of 5500 kV was applied to the ESI needle. Nitrogen was used as nebulizer gas (55 psi), turbo gas (65 psi), curtain gas (25 psi) and CAD gas (5 arbitrary units on a scale of 1 to 12). The temperature of the turbo gas was maintained at 550 ºC. Compound-dependent parameters (Table 2) were optimized by direct infusion of a solution (100 ng $mL^{-1}$ in eluent A/eluent B in equal parts) of the individual analytes and the internal standard, respectively. Optimization was carried out automatically with the 'Quantitative Optimization' tool included in the Analyst Software (Version 1.4.2, Applied Biosystems). The principal metabolite of sildenafil was sought by applying an MRM experiment with the transitions m/z 461 to m/z 283 in different effluent water samples.
Analysis of the target compounds in influent, effluent and sewage sludge samples of the STP in Tarragona was done on an HP1200 series LC-triple QqQ tandem mass spectrometer (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 (5.0 x 0.46 cm) 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 kept at 50 ºC. The same eluent and gradient program as described before has been applied.
Detection of the analytes was performed in the MRM mode after positive ionization. Fragmentation voltage and collision energy were optimized for each compound (Table 2). ESI conditions for the positive mode were: capillary voltage 4,000 V, nebulizer gas ($N_2$) 45 psi, source temperature 350 ºC, gas flow ($N_2$) 12 L/min.

ISBN:978-84-693-6426-0/DL:T-1628-2010

Nitrogen was used as collision gas. In order to maximize sensitivity, two time windows were used: 0-6 min for sildenafil and vardenafil and 6-14 min for tadalafil and fluazifop butyl (IS).

**Investigation of the matrix effects in ESI-LC-MS-MS**

One disadvantage in ESI-MS quantitative analysis is what is known as matrix effect. It occurs because the ESI source is highly susceptible to other components present in the matrix, which may result in a signal suppression or enhancement leading to erroneous results. In order to evaluate the degree of ion suppression different experiments were carried out with different matrices.
The matrix effect observed with each target analyte was calculated as the percentage of decrease in signal intensity in a sample matrix versus a pure solvent, using the equation 1.

Equation (1): Matrix effect (%) = $(1 - \left[\frac{(I_s - I_x)}{I_{MeOH}}\right])x100$

Where $I_s$ is the area of each peak in the spiked matrix, $I_x$ is the area in the unspiked matrix and $I_{MeOH}$ is the area of each analyte in the standard in MeOH at the same concentration level than the corresponding spiked sample. Influent water has been analyzed at two different concentration levels (10 and 25 ng/L). Tadalafil showed the highest matrix effect (87%) whereas for vardenafil and tadalafil were lower 31 and 51%, respectively. At both concentrations the difference in the matrix effect was less than 5%.
For effluent water the same procedure was done. The matrix effect was lower than in influent water and similar for both tested concentrations (10 and 25 ng/L) - 53% for tadalafil, 21% for vardenafil and 30% for sildenafil.
The matrix effect in sewage sludge was also checked. In this case the extract of sewage sludge sample (25 mL) was spiked at 12.5 and 37.5 ng. Differences between each spiked levels was observed. When the extract spiked at 37.5 ng was analyzed the matrix effect of vardenafil and sildenafil was lower than 1% whereas for tadalafil was 30%. However, when the extract spiked at 12.5 ng was analyzed, the matrix effect of vardenafil was the same, but for sildenafil and tadalafil increased until 30 and 92%, respectively.
The IS fluazifop buthyl is able to compensate the observed matrix effects. The following tables provides the obtained quantification results when influent and effluent waste water and sewage sludge was spiked at 10 ng with all three compounds. In the influent water 8 – 8.4 ng, in effluent water 8.5 – 11 ng and in sewage sludge 9.3 – 11.7 ng of the spiked 10 ng could be recovered with the analytical methods.

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**Addition of 10 ng in influent and effluent waste water and sewage sludge**

INFLUENT WASTE WATER

| | Sildenafil | Vardenafil | Tadalafil | Fluazifop butyl |
|---|---|---|---|---|
| Area of blank | 315 | 98 | 13 | 1225 |
| Area of spiked | 408 | 179 | 13 | 1187 |
| Concentration in the blank (ng/L) | 28.5 | 14.8 | 9.7 | - |
| Concentration in the addition (ng/L) | 36.9 | 23.1 | 17.7 | - |
| Recovery (%) | 84 | 83 | 80 | - |

EFFLUENT WASTE WATER

| | Sildenafil | Vardenafil | Tadalafil | Fluazifop butyl |
|---|---|---|---|---|
| Area of blank | 165 | 61 | 0 | 1678 |
| Area of spiked | 385 | 265 | 24 | 1728 |
| Concentration in the blank (ng/L) | 6.2 | 2.7 | 0 | - |
| Concentration in the addition (ng/L) | 14.7 | 11.6 | 11.1 | - |
| Recovery (%) | 85 | 88 | 111 | - |

SEWAGE SLUDGE

| | Sildenafil | Vardenafil | Tadalafil | Fluazifop butyl |
|---|---|---|---|---|
| Area of blank | 64 | 0 | 4 | 258 |
| Area of spiked | 170 | 54 | 19 | 234 |
| Concentration in the blank (ng/g) | 7.4 | 0 | 1.9 | - |
| Concentration in the addition (ng/g) | 19.1 | 9.3 | 11.7 | - |
| Recovery (%) | 117 | 93 | 98 | - |

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### 3.3.2. Results and discussion

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This study demonstrated for the first time the presence of three phosphodiesterase type-V inhibitors in environmental samples such as influent and effluent waste water and sewage sludge. The methods used to determine them were based on solid-phase extraction (SPE) for water samples and pressurized liquid extraction (PLE) for sewage sludge samples, prior to liquid chromatography–tandem mass spectrometry analysis.

Regarding the extraction of these compounds from water samples, two different sorbents were tested: Oasis HLB and Oasis MCX. Better results were obtained with Oasis HLB and recoveries in surface water were around 90%. To extract these compounds from sewage sludge samples, pressurized liquid extraction was used as the extraction technique. The optimization of PLE parameters was done parameter by parameter, as in previous studies.

During the optimization process, we observed the great influence of some parameters such as the solvent, the temperature and the extraction time. The solvent that gave higher recoveries was methanol (between 29% and 95%). We observed that temperature influenced the recoveries greatly. In all cases the recoveries increased around 40% when the temperature increased between 25 ºC - 100 ºC. As we observed in our previous studies, the introduction of fresh solvent increased the recoveries of all compounds when the extraction time and the number of cycles were studied.

As can be seen in the supplementary information, two different LC systems were used. We used LC with a column of 5 cm and 2.7 μm of particle size, coupled to tandem mass spectrometry with a QTrap analyzer to determine the presence of phosphodiesterase type-V inhibitors in effluent waste water samples in Germany. Ultra high performance liquid chromatography (UHPLC) (the column had a particle size of 1.8 μm), coupled to tandem mass spectrometry with triple quadrupole was used to analyze samples of sewage sludge and influent and effluent water from Tarragona. All the conditions were the same in both LC systems except for the temperature (in UHPLC it was 50 ºC in LC 30 ºC) and the flow (in UHPLC it was 0.6 mL/min in LC 0.3 mL/min). Because only three compounds were separated, the advantages of using UHPLC were not clearly observed. The retention time was reduced by only 3 minutes because, by using the column of 2.7 μm, the separation was quite short.

Using both of the LC systems, sildenafil and vardenafil co-eluted due to the similarities in their structures, although by using tandem mass spectrometry detection we were able to identify and quantify each compound. As we mentioned before, although two different analyzers were used - the QTrap and the triple quadrupole - no difference was observed in terms of sensitivity. Although we expected lower limits of detection using QqQ, the LODs were similar and both analyzers had the same linear ranges by direct injection. Moreover, the mass spectra obtained for the compounds were the same for both analyzers. In section 3.2 of the paper, mass spectra were extensively discussed.

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The presence of sildenafil was confirmed in all samples analyzed and the total amount of phosphodiesterase type-V inhibitors in the effluent waste varied between 10 and 27 ng/L. Vardenafil and tadalafil were also found in Germany. However, in Spain these compounds were found at lower levels than in Germany because sildenafil is more extensively used in Spain than the other two. The concentrations found of these phosphodiesterase type-V inhibitors in all samples were at levels of low ng/L or μg/Kg for water and sludge samples, respectively.

In the study of the sewage treatment plant in Tarragona, we observed that sildenafil showed the maximum concentration in samples taken in July (52 ng/L, 14 ng/L and 18 μg/Kg (d.w.), for influent water, effluent water and sludge, respectively). In all the samples sildenafil showed higher concentrations than tadalafil or vardenafil.

In this study, as we mentioned in the introduction, we could also calculate approximate removal rates (adsorption plus degradation) for these compounds in the sewage treatment plant in Tarragona. The approximately calculated removals were 68% for sildenafil, 69% for tadalafil and 80% for vardenafil.

This study confirms that the removal of pharmaceuticals in sewage treatment plants was partial and that their determination in effluent wastewater water samples is important because, in most cases, STPs discharge effluent water into rivers and sludge can be reused as manure.

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## 3.4. Determination of personal care products in sewage sludge

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ISBN:978-84-693-6426-0/DL:T-1628-2010

ISBN:978-84-693-6426-0/DL:T-1628-2010

In this section, to extend the number of emerging organic contaminants studied in this Thesis, we present a method to determine a group of personal care products including UV-filters, preservatives and antimicrobials.

Concern about the environmental fate and potential effects of synthetic organic chemicals used in soaps, lotions, toothpaste and other personal care products continues to increase. Of particular concern are compounds that are used in large volumes, persist in the environment, bioaccumulate or have a designed bioactivity.

As we mentioned in the introduction, personal care products (PCPs) comprise diverse chemical substances such as musk fragrances, UV filters, preservatives and antimicrobials, among others. The main pathway through which PCPs enter the aquatic environment is from household waters that are released by sewage treatment plants (STPs) [1,2].

Previous studies demonstrated the presence of some of these emerging contaminants in sewage sludge. For example, in the study of Chu *et al.* [3] different sewage sludge samples from Canada were analyzed using PLE/SPE/LC-MS-MS and they found concentrations of low μg/Kg of triclosan and triclocarban, which are antimicrobials. Another group of personal care products determined in sewage sludge was musk fragrances, as Ternes *et al.* [4] and Osemwengie [5] showed in their studies. These studies showed that these musk fragrances are present at higher concentrations than antimicrobials. Due to their volatility, these compounds are determined using gas chromatography as a separation technique. For instance, Ternes *et al.* [4] found concentrations of between 1.4 and 15 mg/Kg in sewage sludge from Switzerland using PLE/SPE/GC-MS. UV-filters have also been determined in sewage sludge samples. These compounds have showed concentrations of between low μg/Kg and low mg/Kg. For example, in the study of Plagellat *et al.* [6], a maximum concentration of 27700 μg/Kg (d.w.) of octyl-triazone and 18740 μg/Kg (d.w.) of octocrylene was determined by using shaking extraction and GC-MS. As far as we know, no information about parabens and these two UV filters in sewage sludge has been reported in the literature.

The purpose of this study is to develop a method to determine in a unique analysis a wide variety of PCPs belonging to different groups. The compounds were selected because no data was available on their presence in sewage treatment plants from the Tarragona area and they have not been determined in a unique analysis. In the literature, different studies have shown the presence of some of these groups in environmental samples. This is extremely important because some PCPs (parabens and UV-filters, among others) are considered endocrine disruptor compounds and represent human and/or environmental risk. The compounds determined were: four parabens, two antimicrobials and six UV filters.

Pressurized liquid extraction was used as the extraction technique due to the high efficiencies obtained by this technique in the extraction of pharmaceuticals in our

ISBN:978-84-693-6426-0/DL:T-1628-2010

previous studies, the efficiencies reported in the literature for some personal care products and also because it is one of the objectives of this Thesis. In this study, the solvent was optimized by testing different pure solvents and binary and tertiary mixtures, as in previous studies. Other important parameters such as temperature, pressure, extraction time and the number of cycles were optimized using a fractional experimental design $2^{6-2}$. Using this experimental design, we fixed two values of each parameter and the influence of each one was analyzed using the Pareto Charts. With this experimental design we can reduce the number of experiments to achieve the best conditions [7].

As regards LC separation, we used UHPLC for the first time in our group. As we mentioned in the introduction, UHPLC allows increased speed and improved sensitivity, selectivity and specificity compared to conventional LC analysis [8]. 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 which can lessen the matrix effects [9].

From the limited data in the literature, we expect that personal care products are present in sewage sludge at concentrations of low μg/Kg. In order to achieve these levels, a sensitive detector is needed. In our study, tandem mass spectrometry with a triple quadrupole analyzer was used. By monitoring two transitions between precursor and product ions and working with the multiple reaction monitoring mode (MRM), it is possible to confirm and quantify the presence of PCPs in sewage sludge at very low concentrations.
The method developed was applied to determine the presence of personal care products in different samples from the STP in Tarragona.
The results obtained in this study have been published in Journal of Chromatography A 1216 (2009) 5619-5625.

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### 3.4.1. Determination of personal care products in sewage sludge by pressurized liquid extraction and ultra high performance liquid chromatography-tandem mass spectrometry

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# DETERMINATION OF PERSONAL CARE PRODUCTS IN SEWAGE SLUDGE BY PRESSURIZED LIQUID EXTRACTION AND ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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**Abstract**

This paper describes a method for the determination of a group of personal care products including four UV filters, four preservatives and two antimicrobials in sewage sludge. The method combines pressurized liquid extraction and ultra high performance liquid chromatography-tandem mass spectrometry. Most of the parameters that affect the extraction step such as temperature, pressure, static extraction time, number of cycles, purge time and flush volume were optimized using a fractional experimental design. In the chromatographic step, the compounds were detected by using tandem mass spectrometry with a triple quadrupole analyzer with electrospray ionization in positive and negative modes. The use of small diameter particles (1.8 μm) in the chromatographic column allowed the compounds to be eluted in 9 minutes. The entire process took a total of 39 minutes.
All recoveries were higher than 72% except for 2,4-dihydroxybenzophenone (a UV filter), whose recovery was 30%. The repeatability and reproducibility between days expressed as RSD (%) (n = 3) were less than 8% and 13%, respectively. The LODs and LOQs were lower than 8 μg/kg and 12.5 μg/kg of dry weight (d.w.), respectively. When the method was applied to determine the compounds in sewage sludge from a domestic sewage treatment plant, triclosan (an antimicrobial) and octocrylene (a UV filter) showed the highest levels, 1490 μg/kg (d.w.) and 1842 μg/kg (d.w.), respectively. This paper describes for the first time the determination of parabens and two UV filter (octyldimethyl-p-aminobenzoic acid and benzophenone-3) in sewage sludge.

**Keywords:** Parabens, antimicrobials, ultra-violet filters, sewage sludge, pressurized liquid extraction, ultra high performance liquid chromatography, tandem mass spectrometry.

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## INTRODUCTION

The presence of pharmaceuticals and personal care products (PPCPs) in environmental samples is a topic of increasing interest. PPCPs are widely used around the world as a means of protecting and improving human and animal health. There is growing concern that these compounds pass through sewage treatment plants and enter the environment [1]. Of particular concern are compounds that are used in large volumes, persist in the environment, bioaccumulate, or have designed bioactivity [2].

Within the PPCP category, personal care products (PCPs) have been the focus of study less frequently than pharmaceuticals. Among PCPs, triclosan and triclocarban are antimicrobial compounds used in soap, toothpaste, and other consumer products. Triclosan is also used as a biocide in sportswear, footwear, carpets, plastic toys, and kitchenware [3].

Another group of PCPs are UV filters. Sunscreen agents (UV filters) are chemical compounds that mitigate the deleterious effects of sunlight and are used in a variety of cosmetics, specifically in those designed for sun protection such as sunscreen creams, lotions and sprays [4,5].

Another group of PCPs is made up of parabens, which are the most common preservatives used in personal care products. Parabens are also used as preservatives and bactericides in pharmaceuticals and food products. Methyl paraben and propyl paraben are the most widely used and are normally used together due to their synergistic preservative effects [6-8].

In recent years, the levels and consequences of PCPs in different environmental waters has been the subject of several studies; however, only a few PCPs have been determined in sewage sludge [9,10] and therefore there is little information available regarding their presence in these types of samples.

Some UV filters have been determined by gas chromatography-mass spectrometry (GC-MS) [10,11] and parabens and triclosan by gas chromatography-tandem mass spectrometry (GC-MS-MS) [3], but a derivatization step was necessary in all cases.

To determine most PCPs, the best option is liquid chromatography coupled with mass spectrometry (LC-MS) due to the polarity of PCPs and the low concentrations at which these compounds have been found in environmental samples [12-14]. Nowadays, the application of advanced LC-tandem MS to environmental analyses has allowed a broad range of compounds to be determined and has thus permitted the comprehensive assessment of environmental contaminants.

Different analyzers have been used, but the triple quadrupole analyzer is the most suitable to obtain low detection limits for the target analytes.

Ultra high performance liquid chromatography (UHPLC) also known as Rapid resolution liquid chromatography (RRLC) (trade name

ISBN:978-84-693-6426-0/DL:T-1628-2010

of Agilent Technologies) or ultra performance liquid chromatography (UPLC) (trade name of Waters) allows the possibility of extending the usefulness of this widely used separation technique. UHPLC uses analytical columns packed with 1.8 μm particles, which offers the advantages of increasing speed, improving sensitivity, selectivity and specificity compared to conventional LC analysis. The higher efficiency of small particles enables shorter columns to be used, reducing analysis time and solvent consumption.

Up to now, UHPLC has not been extensively applied, but as a method of determining PCPs, it has been used in a few studies [15]. The lower degree of band broadening in UHPLC also benefits mass spectrometric detection, concentrating the analyte at the peak center and thereby increasing response. Thus, methods using UHPLC in conjunction with mass spectrometry (MS) offer improved performance for quantitative analyses over existing liquid chromatography-tandem mass spectrometry methods [16].

Due to the low concentration of PCPs in environmental samples, several techniques have been used to extract some of these compounds from sludge and sediments, including soxhlet extraction [17], pressurized liquid extraction [10,12,18] and microwave-assisted extraction [19].

The focus of this paper is the development of a method for determining a set of personal care products from different groups in sewage sludge in a single analysis. Four preservatives (methyl paraben, ethyl paraben, propyl paraben and benzyl paraben), six UV filters (octyldimethyl-p-aminobenzoic acid, benzophenone-3, 2,4-dihydroxybenzophenone, 2,2'-dihydroxy-4-methoxybenzophenone, octocrylene and 2-phenylbenzimidazole-5-sulfonic acid) and two antimicrobials (triclosan and triclocarban) have been determined in sewage sludge using pressurized liquid extraction followed by ultra high performance liquid chromatography-tandem mass spectrometry. To the best of our knowledge this is the first time that parabens and two UV-filters studied have been determined in sewage sludge.

## EXPERIMENTAL

### Materials and reagents

Four preservatives (methyl paraben, ethyl paraben, propyl paraben and benzyl paraben), six UV filters (octyldimethyl-p-aminobenzoic acid, benzophenone-3, 2,4-dihydroxybenzophenone, 2,2'-dihydroxy-4-methoxybenzophenone, octocrylene, and 2-phenylbenzimidazole-5-sulfonic acid) and two antimicrobials (triclosan and triclocarban) were provided by Sigma (St. Louis, MO, USA).

The abbreviations are summarized in Table 1. Stock solutions of individual standards were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L and storing them at 5 ºC. A mixture of all compounds in methanol at a

ISBN:978-84-693-6426-0/DL:T-1628-2010

concentration of 100 mg/L was prepared weekly. Working solutions were prepared daily.
Ultra pure water was obtained with a Milli-Q water purification system (18.2 MΩ·cm) (Millipore, Bedford, MA, USA). Acetone, acetonitrile, dichloromethane and methanol (HPLC-grade) were acquired from SDS (Peypin, France), nitrogen was from Carburos Metálicos (Tarragona, Spain) and acetic acid and aluminium oxide were from Merck (Darmstadt, Germany).
In the PLE optimization, the data analysis was performed using Statgraphics1 Plus version 5.1 software (Manugistics, Inc., Rockville, MD, USA).

**Sample pre-treatment**

The sewage sludge samples were homogenized, frozen, lyophilized using the freeze-dry system (Labconco, Kansas City, MO, USA), sieved through a 125 μm screen and stored in a closed flask at room temperature.
To optimize the extraction procedure, fractions of the sample (10 g) were placed in different beakers, completely covered with acetone (50 mL) and spiked with the analytes at different levels. The acetone was left to evaporate at room temperature until the sludges were dry (3 hours). The mixture was stirred frequently in order to obtain a homogenous material.

**Pressurized liquid extraction**

Extraction was done with an ASE 200 pressurized liquid extractor (Dionex, Sunnyvale, CA, USA) equipped with 11 mL capacity stainless-steel cells. One cellulose filter followed by 1 g of aluminum oxide was placed at the bottom of each cell. After loading the corresponding amount of aluminum oxide and 1 g of sample, the remaining volume in the cell was filled with aluminum oxide. The aluminum oxide was heated to 120 ºC in the oven for 24 hours before use. Extraction time, extraction temperature, number of cycles, pressure, flush volume and purge time were optimized using a fractional factorial experimental design ($2^{6-2}$) after the different solvents being tested. Experiments to optimize the extraction procedure were performed by the extraction of 1 g of a sewage sludge sample spiked at 100 μg/Kg (d.w.).
The extracting solvents were methanol and a mixture of water (pH 7) and methanol (1:1). The operating conditions were: extraction temperature of 100 ºC; extraction pressure of 140 bar; preheating period of 5 minutes; 2 cycles of 5 minutes with methanol followed by 2 cycles of 5 minutes with water (pH 7): methanol (1:1), final extraction volume ~ 25 mL; flush volume of 30% of the cell volume and nitrogen purge of 90 s.
The extract was filtered with a microfilter with a pore size of 0.45 μm (Teknokroma, Barcelona, Spain), and analyzed by ultra high performance

liquid chromatography-tandem mass spectrometry.

**UHPLC-MS-MS analysis**

The chromatographic instrument was an HP1200 liquid chromatograph-triple quadrupole tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany) with electro-spray ionization (ESI), an automatic injector, a degasser, a quaternary pump and a column oven. The chromatographic column was a Zorbax (5.0 x 0.46 cm) 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 kept at 50 ºC.

We used a binary mobile phase with a gradient elution. Solvent A was Milli-Q water with acetic acid (pH 3) and solvent B was methanol. The gradient was initially 60% B, which increased to 100% in 6 min, kept constant for 4 min and finally returned to 60% B in 3 min. All the compounds eluted within 9 min.

Ionization and fragmentation settings were optimized by direct injection of standard solutions. The parameters optimized for electrospray ionization were: gas temperature (100, 200 and 350 ºC), gas flow (8, 10, 12 L/min), nebulizer pressure (20, 30 and 45 psi.) and capillary voltage (3000, 4000 and 5000 V). These ESI conditions were obtained as a compromise using the optimum values for the majority of the compounds. The optimum ESI conditions for the positive and negative mode were: capillary voltage 4,000 V, nebulizer gas ($N_2$) 45 psi, source temperature 350 ºC, gas flow ($N_2$) 12 L/min. Nitrogen was used as collision gas, and MS-MS was performed in the Multiple Reaction Monitoring (MRM) mode. In order to maximize sensitivity, six time windows were used and a different ionization mode was used in each window. The time windows and the ionization mode are detailed in Table 1.

For each compound, two characteristic fragments of the deprotonated $[M-H]^-$ or protonated molecular ion $[M+H]^+$, depending on the ionization mode, were monitored (Table 1). The ion $[M+Na]^+$ was monitored for OC due to the higher response compared to $[M+H]^+$. For TCS the ions were two isotopic peaks of trichlorinated species. The most abundant transition was used for quantification, while the second most abundant was used as a qualifier. Fragmentation voltage and collision energy were optimized for each compound (Table 1).

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**Table 1.** MRM conditions used for UHPLC-MS-MS determination of personal care products. Bold face transitions were used to quantify.

| Compound | Abbr. | Trans. | Frag. voltage (V) | Coll. Ener. (V) | Ioniz. mode | Time window (min) |
|---|---|---|---|---|---|---|
| 2-Phenylbenzimidazole-5-sulfonic acid | PMDSA | **275<194** | 200 | 30 | Pos. | 0 – 1.3 |
| | | 275<211 | 200 | 25 | | |
| Methyl paraben | MPB | **151<92** | 80 | 15 | Neg. | 1.3 – 2.3 |
| | | 151<136 | 80 | 5 | | |
| Ethyl paraben | EPB | **165<136** | 100 | 15 | Neg. | |
| | | 165<92 | 100 | 5 | | |
| Propyl paraben | PPB | **179<92** | 100 | 15 | Neg. | 2.3 – 4.5 |
| | | 179<136 | 100 | 5 | | |
| 2,4-Dihydroxy-benzophenone | DHB | **213<135** | 130 | 15 | Neg. | |
| | | 213<169 | 130 | 15 | | |
| Benzyl paraben | BPB | **227<92** | 100 | 15 | Neg. | |
| | | 227<136 | 100 | 5 | | |
| 2,2'-dihydroxy-4-methoxybenzophenone | DHMB | **243<93** | 80 | 15 | Neg. | |
| | | 243<123 | 80 | 5 | | |
| Benzophenone-3 | BP-3 | **229<151** | 130 | 15 | Pos. | 4.5 – 5.5 |
| | | 229<105 | 130 | 15 | | |
| Triclocarban | TCB | **313<160** | 130 | 5 | Neg. | 5.5 - 7 |
| | | 313<126 | 130 | 15 | | |
| Triclosan | TCS | **287<35** | 18 | 8 | Neg. | |
| | | 289<35 | 18 | 8 | | |
| Octocrylene | OC | **384<272** | 130 | 5 | Pos. | 7 - 13 |
| | | 384<228 | 130 | 5 | | |
| Octyldimethyl-p-aminobenzoic acid | ODPABA | **278<166** | 100 | 15 | Pos. | |
| | | 278<151 | 100 | 15 | | |

## RESULTS AND DISCUSSION

### Optimization of UHPLC-MS-MS

Two organic solvents, methanol and acetonitrile, and water at different pH (3, 6 and 8) were tested to optimize the gradient separation and the conditions in the MS-MS detector. After injecting a standard solution of 500 µg/L, the best conditions for obtaining a good separation and symmetric peaks were found with acidic water (pH 3, acetic acid) and methanol.

Different parameters were tested to optimize the conditions for MS-MS, the first of which were the electrospray ionization conditions. We tested both ionization modes (positive and negative).

To increase the sensitivity of the acquisition and to obtain the chromatogram combining both ionization modes, we defined six different time windows, as described in Table 1.

Fragmentation voltage and collision energy were tested in order to select the transitions in the MRM mode.

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Table 1 summarizes the optimum values of these parameters for each compound. Triclosan has a fragmentation voltage of 18 V because when this value was increased the response decreased. The higher fragmentation voltage used with PMDSA resulted in the best response.
The same product ions were observed in all parabens. The first was m/z 92, which corresponds to the loss of the $CO_2$ group and the methyl, ethyl, propyl or benzyl group depending on the compound. The other product ion in parabens was m/z 136, which corresponds to the loss of methyl, ethyl, propyl or benzyl. In some UV filters we observed the loss of 77, which corresponds to $[M\text{-}C_6H_5]^+$. This situation appears in BP-3 (229<151) and DHB (213<135). In TCS both transitions have the product ion m/z 35, which corresponds to $Cl^{35}$. The transitions selected are summarized in Table 1.
Linear range was tested by injection of standards at concentration levels ranging from 0.01 to 500 µg/L and the determination coefficient ($r^2$) values were higher than 0.996 for all compounds.

**Pressurized liquid extraction**

Initial conditions were selected from previous studies, where some UV filters or antimicrobials were determined in sewage sludge or sediments [10,18,20]. The initial conditions were: temperature of 75 ºC, pressure of 100 bar, 5 minutes of static extraction time, one cycle, 60 s of purge time, 60% of flush volume and 1 gram of dry sample.
Different blanks of sewage sludge were analyzed by PLE/UHPLC-MS-MS under the initial conditions and the chromatograms showed some peaks of target analytes at the same retention time. In each experiment a blank and spiked samples were analyzed and the signal of the blank was subtracted.
Different pure and binary mixture solvents were tested to optimize the extraction solvent. The recoveries obtained when different pure solvents and mixtures of solvents were tested are shown in Table 2. Six different pure solvents were tested: methanol, dichloromethane, acetone, water, acetonitrile and ethyl acetate. The results were different for each solvent because of the different characteristics of the compounds. As expected, the most non-polar compounds, TCB, TCS, OC and ODPABA had better recoveries when organic solvents were used as the extraction solvent. MPB, EPB and PPB had the highest recoveries when water was used. PMDSA, BPB, DHB, and BP-3 were not extracted with any pure solvent. In general, higher recoveries were obtained with methanol, with eight of the 12 compounds extracted, although some of them had very low recoveries.

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**Table 2.** PLE recoveries (n = 3) using different solvents. A= water (pH 7), B= methanol, C= acetone, D= dichloromethane, E= acetonitrile and F= ethyl acetate. For other conditions see the text.

| Compounds | A | B | C | D | E | F | A:B (1:3) | A:B (1:1) | B:D (1:1) |
|---|---|---|---|---|---|---|---|---|---|
| PMDSA | 3 | - | - | - | - | - | 3 | - | - |
| MPB | 39 | 19 | - | - | - | 7 | 7 | 14 | - |
| EPB | 72 | 18 | - | - | - | 40 | 24 | 58 | 9 |
| PPB | 60 | 15 | - | 1 | - | 51 | 1 | 50 | 6 |
| DHB | - | - | - | - | - | - | - | - | - |
| BPB | - | - | - | - | 5 | 15 | - | 7 | 12 |
| DHMB | 13 | 12 | - | - | - | - | - | - | - |
| BP-3 | - | - | - | - | - | 7 | - | 16 | - |
| TCB | - | 53 | - | 30 | 54 | - | - | - | 33 |
| TCS | - | 100 | 20 | 40 | 98 | - | - | - | 100 |
| OC | - | 100 | 70 | 65 | 100 | 22 | 10 | 34 | 99 |
| ODPABA | - | 100 | 20 | 41 | 86 | 9 | 8 | 16 | 86 |

RSD (%)≤ 8

Therefore, we tested three different binary mixtures, as shown in Table 2, water:methanol (1:1), water:methanol (1:3) and methanol:dichloromethane (1:1). Higher recoveries were obtained when methanol was used as extraction solvent for all compounds except for MPB and EPB whose recoveries were higher when water:methanol (1:1) was used.

The binary mixture that provided the highest recoveries was water: methanol (1:1), although the recoveries were less than 58%. To check if this solvent allowed higher recoveries to be obtained, different numbers of cycles were tested. As shown in Table 3, no differences were found when the number of cycles increased to four. Although the recoveries were not high for any of the compounds, water: methanol (1:1) proved the best binary mixture to extract parabens, and methanol to extract most non-polar compounds. We therefore decided to test one cycle of methanol followed by one cycle of water:methanol (1:1).

The extraction of these compounds using methanol and water:methanol (1:1) was tested with one and two cycles of each solvent. Table 3 shows that higher recoveries were obtained with two cycles of each solvent. When three cycles of each solvent were tested the recoveries were similar to the extraction obtained with two. PMDSA and DHMB were not extracted under these conditions.

To find the conditions for fast and efficient extraction of the target compounds from a solid matrix using PLE, a fractional factorial design was chosen to investigate the influence of temperature, pressure, extraction time, purge time, flush volume and number of cycles on extraction efficiency.

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**Table 3.** PLE recoveries (n = 3) using different cycles and different solvents. A= water (pH 7), B= methanol. For other conditions see the text.

| | A:B (1:1) 1 cycle | A:B (1:1) 2 cycle | A:B (1:1) 4 cycle | B 1 cycle + A:B (1:1) 1 cycle | B 2 cycles + A:B (1:1) 2 cycles |
|---|---|---|---|---|---|
| PMDSA | - | - | - | - | - |
| MPB | 14 | 20 | 28 | 34 | 52 |
| EPB | 58 | 66 | 51 | 34 | 40 |
| PPB | 50 | 69 | 63 | 100 | 100 |
| DHB | - | - | - | - | 15 |
| BPB | 7 | 3 | 29 | 86 | 100 |
| DHMB | - | - | - | - | - |
| BP-3 | 16 | 15 | 23 | 7 | 38 |
| TCB | - | - | - | 35 | 35 |
| TCS | - | - | - | 100 | 100 |
| OC | 34 | 40 | 50 | 100 | 100 |
| ODPABA | 16 | 13 | 19 | 86 | 100 |

RSD (%)≤ 9

The combination of methanol followed by water:methanol (1:1) was selected as the optimum solvent for our experimental design.

The experimental design selected was a $2^{6-2}$ fractional factorial design. In this design the confounding factors were purge time and flush volume. These factors were considered as confounding factors because, as found in previous studies [21,22], they do not significantly affect recoveries. Purge time was confounded with the interaction of temperature, pressure and extraction time and flush volume was confounded with the interaction of pressure, extraction time and number of cycles.

In the experimental domain we fixed two values for each parameter, one for the low level and another for the high level. These values were for temperature (50 and 100 ºC), pressure (60 and 140 bar), extraction time (5 and 15 minutes), cycles (2 and 3), flush volume (30 and 90%) and purge time (30 and 90 seconds). The Statgraphics statistical package was used to generate the experimental matrix and to calculate the standardized main effects of the factors considered. Pareto charts were used to identify the most influential factors. We considered individual recoveries and the average of all recoveries. For example, Figure 1 shows the Pareto chart for the average of all recoveries and exhibits information similar to that in the Pareto chart for each compound analyzed separately. It should be mentioned that we cannot analyze the Pareto chart for four compounds because all the experiments have the same recoveries, 0% (PMDSA and DHMB) or 100% (PBP and TCS). For the rest of the compounds, in most cases, temperature was the most important parameter in the extraction of these compounds. The recoveries

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were higher when the temperature was at the highest level (100 ºC). The extraction time was the second most important parameter in seven of eight compounds and the recoveries were higher when the extraction time was at the lowest level (5 minutes).

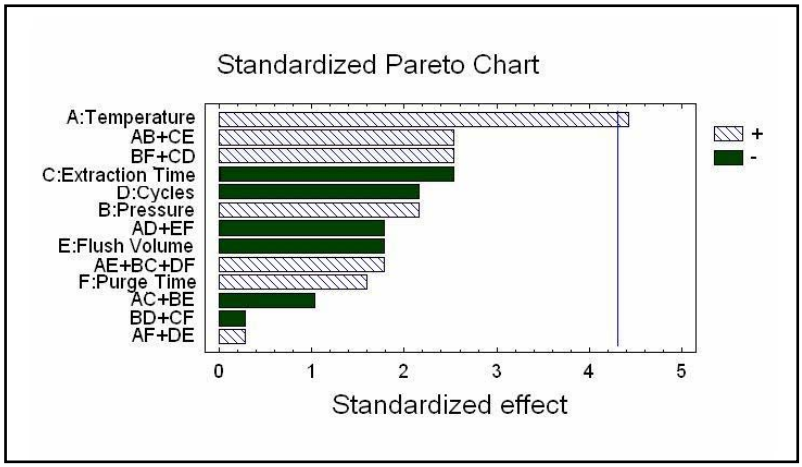


**Figure 1.** Standardized Pareto chart obtained from the response of the average of all recoveries in the fractional factorial design.

**Table 4.** PLE recoveries obtained with the $2^2$ experimental design. For other conditions see the text.

| **Experiment** | **1** | **2** | **3** | **4** |
|---|---|---|---|---|
| **Temperature (ºC)** | **100** | **125** | **100** | **125** |
| **Extraction time (min)** | **3** | **3** | **5** | **5** |
| PMDSA | 0 | 0 | 0 | 0 |
| MPB | 42 | 100 | 72 | 100 |
| EPB | 73 | 96 | 80 | 100 |
| PPB | 103 | 102 | 102 | 104 |
| DHB | 13 | 30 | 30 | 27 |
| BPB | 104 | 104 | 106 | 102 |
| DHMB | 0 | 5 | 4 | 0 |
| BP-3 | 35 | 60 | 79 | 61 |
| TCB | 70 | 50 | 77 | 65 |
| TCS | 102 | 100 | 103 | 104 |
| OC | 52 | 61 | 104 | 70 |
| ODPABA | 74 | 73 | 108 | 73 |

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To confirm whether these were the optimum values, we decided to test another high temperature (125 ºC) and another short extraction time (3 min) using a $2^2$ full factorial design. The factors and their levels were for temperature (100 and 125 ºC) and for extraction time (three and five minutes).

The other conditions were fixed depending on the values obtained in the previous Pareto chart from the fractional factorial design. These conditions were two cycles, pressure of 140 bar, flush volume of 30% of the volume of the cell and a purge time of 90 seconds. The recoveries obtained in the full factorial design are summarized in Table 4, which shows that the highest recoveries for most compounds were obtained in experiment 3 (100 ºC and 5 minutes).

In some cases, the recoveries in experiments 1 and 2 (extraction time of three minutes) were lower than when the extraction time was five minutes. At five minutes, when the temperature increased, the recoveries of MPB and EPB increased to 100%, however, the recoveries of BP-3, TCB, OC and ODPABA decreased. As shown in Table 4, PMDSA and DHMB were not extracted and were therefore excluded from this study. In the end, the optimum conditions for extracting personal care products with PLE were set at 100 ºC and 100 bar with two cycles of methanol followed by two cycles of water:methanol (1:1, v/v), five minutes of static extraction time, 90 s of purge time and 30% flush volume. All recoveries exceeded 72% except in the case of DHB, whose recovery was 30%. A pre-concentration step before the LC analysis was not necessary because a low method detection limit was obtained, thereby saving time, energy and also minimizing matrix effect in the LC-MS-MS. Applying the entire method (extraction and chromatographic process) took only 39 minutes.

## METHOD VALIDATION

As mentioned above, some target analytes were found in the sample when a PLE extract of different blanks of sewage sludge was analyzed by UHPLC-MS-MS. For this reason the calibration curves were obtained by injection of standard solutions instead of by analyzing a sample using the entire method. Table 5 shows the validation data obtained. The identification and confirmation criteria for the analysis of our target analytes were based on a series of Commission Decision [23]. This decision provides rules for the analytical methods to be used in analyzing the presence of residues in products of animal origin. To confirm the presence of the compounds, the retention time of the compounds and relationship between the two transitions were compared. For instance, for methyl paraben the second transition was 36% of the first transition in the standard injection and in the samples this value was between 30% and 38%.

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**Table 5 .** Validation data (n = 3).

| | Recoveries (%)[a] | Linear Range (µg/L) [b] | LODs (µg/Kg) [c] | LOQs (µg/Kg) [d] | RSD (%)[e] | RSD (%)[f] |
|---|---|---|---|---|---|---|
| MPB | 72 | 250-0.25 | 3 | 6.3 | 4 | 5 |
| EPB | 80 | 250-0.1 | 1.75 | 2.5 | 3 | 5 |
| PPB | 102 | 250-0.1 | 1.75 | 2.5 | 2 | 7 |
| DHB | 30 | 250-0.1 | 2 | 2.5 | 6 | 10 |
| BPB | 106 | 250-0.25 | 3 | 6.3 | 2 | 5 |
| BP-3 | 79 | 250-0.1 | 1.5 | 2.5 | 1 | 1 |
| TCB | 77 | 250-0.1 | 1.25 | 2.5 | 4 | 8 |
| TCS | 103 | 250-0.5 | 8 | 12.5 | 8 | 13 |
| OC | 104 | 500-0.25 | 3.5 | 6.3 | 3 | 8 |
| ODPABA | 108 | 500-0.1 | 1.75 | 2.5 | 3 | 4 |

[a] Sample spiked at 25 µg/Kg (d.w.)
[b] Instrumental linear range
[c] Limit of detection of the method
[d] Limit of quantification of the method
[e] Repeatability (n = 3)
[f] Reproducibility between days (n = 3)

A difference of less than ± 20% was considered acceptable according to EU directive. The matrix effects were checked while the method was being developed. We measured the recoveries of the analytes by spiking the matrix extract with them. To calculate these recoveries, we subtracted the signal of the blank and then we compared these signals with the signal of a standard solution. A decrease was assumed to be caused by matrix components in the extract. At the levels studied (100 µg/Kg (d.w.) and 25 µg/Kg (d.w.)) the matrix effect was less than 15% and was therefore not considered.

The recoveries were also determined at 25 µg/Kg (d.w.), with a difference of less than 5% respects to the recoveries obtained at 100 µg/Kg (d.w.).

Our recoveries were consistent with those found in other studies in which UV filters and antimicrobials were determined separately. For example, studies that used PLE or shaking extraction to extract UV filters in sewage sludge and sediments reported similar recoveries to those obtained by our method for OC, ODPABA and BP-3 [9,10,24]. To our knowledge, there are no other studies in the literature concerning the determination of parabens in sewage sludge using PLE as an extraction technique. However, some studies have reported recoveries in indoor dust of over 70% for all parabens when PLE and matrix solid-phase dispersion were used as extraction techniques [3,20]. Antimicrobials (TCS and TCB) are often studied together with other groups of compounds and the recoveries are always higher than 80% in different kinds of samples such as sewage sludge [18], sediments [12] or indoor dust [3,20]. When TCS was analyzed using an MAE with GC-MS-

ISBN:978-84-693-6426-0/DL:T-1628-2010

MS [19], recoveries of more than 81% were obtained in sewage sludge and sediments. Therefore, our study is relevant in that good recoveries were obtained using the same extraction process for groups of compounds which have different characteristics.

The precision of the method was evaluated by extracting consecutive extraction of 3 spiked samples at 25 μg/Kg (d.w.) (within day repeatability) and three spiked sludge samples at 25 μg/Kg (d.w.) in three days (reproducibility between days). The relative standard deviation varied between 1 and 8% and 1 and 13%, respectively.

The limit of quantification (LOQ), calculated as the concentration of the lowest point of the calibration curve, ranged from 2.5 to 12.5 μg/kg (d.w.). The LOQ for triclosan is almost one order of magnitude higher than other compounds, as in other studies reported [18]. The lower sensitivity of determination for triclosan is due to the poor yield of the product ion (m/z 35) relative to the transition monitored for other compounds. The limits of detection (LODs) were defined for a ratio signal to noise of 3 for all compounds and were lower than 8 μg/kg (d.w.). The LODs and LOQs obtained in our study were comparable to and slightly better than those obtained in other studies using different detection systems and extraction techniques. Antimicrobial compounds (TCS and TCB) were determined in sewage sludge using PLE/LC-MS-MS with a triple quadrupole analyzer with detection limits of 1.5 and 0.2 μg/kg (d.w.) for TCS and TCB, respectively [18].

To our knowledge, only the OC (UV filter) has been determined in sewage sludge, but using a shaking extraction process and GC-MS [9]. In that study, the LOD was 6 μg/kg (d.w.) – a slightly higher result than our LOD for OC (3.5 μg/kg (d.w.)). But there are studies in which UV filters were determined in other kind of samples. For instance, when the UV filters ODPABA, OC and BP-3 were determined in lake sediments using PLE/GC-MS, the detection limits were between 2 and 6 μg/kg (d.w.) [10]; however in the literature no data for these compounds using LC-MS-MS were found.

**METHOD APPLICATION**

The method developed was applied to three sewage sludge samples collected in January, July and September of 2007 from a STP in the city of Tarragona. Table 6 shows the results of this study. EPB and DHB were excluded from the table because they did not appear in any samples.

The levels of some compounds were similar in all samples throughout the year. For instance, PPB showed consistent levels of between 6 and 10 μg/Kg (d.w.). Lee *et al.* [25] found the highest levels of MPB and PPB in influent and effluent water, but in the literature we could not find studies reporting the concentrations of parabens in sewage sludge samples. In our case the parabens MPB and PPB were present in all samples at

levels between 6 and 202 μg/Kg (d.w.). TCB and TCS also showed similar concentrations in all samples: between 1300 and 1490 μg/Kg (d.w.) and between 5 and 7 μg/Kg (d.w.), respectively. Metcalfe *et al.* [18] found similar concentrations in municipal biosolids for triclosan and even higher concentrations of TCB. When TCS was studied in different kinds of sludges (primary, biological or disinfected) using a MAE/GC-MS-MS, concentrations between 418 and 5400 μg/Kg (d.w.) were found in a STP in Spain [19]. One of the UV filters (OC) showed twice the concentration in the sample collected in summer. In winter, OC was detected at 700 μg/Kg (d.w.) while in summer the levels increased to 1800 μg/Kg (d.w.). Although we have only a few samples, this result may be explained by the fact that OC is used in sun blocks to protect the skin and its use in the tourist area of Tarragona increases in summer.

**Table 6.** Results in μg/Kg (d.w.) of sewage sludge samples analyzed. Relative standard deviation (%RSD) is in brackets (n = 3).

| | **January 2007** | **July 2007** | **September 2007** |
|---|---|---|---|
| MPB | 202 (5) | 80 (4) | 46 (5) |
| PPB | 7 (9) | 10 (5) | 6 (5) |
| BPB | 5 (4) | - | - |
| BP-3 | 20 (3) | 20 (4) | 10 (4) |
| TCB | 7 (2) | 6 (3) | 5 (2) |
| TCS | 1490 (8) | 1300 (7) | 1328 (5) |
| OC | 700 (4) | 1800 (9) | 1842 (9) |
| ODPABA | 132 (5) | 153 (5) | 170 (4) |

The concentration of 1800 μg/Kg (d.w.) was low in comparison with that found in the study of Plagellat *et al.* [9] where higher concentrations (between 320 and 18740 μg/Kg (d.w.)) were detected in different sewage sludges from Switzerland. The different design of the sewage treatment plant may be one of the reasons of this difference. Two of the UV filters studied (OC and BP-3) were also found in lake sediments in Germany at levels between 60 to 90 μg/Kg (d.w.) [10]. UV filters have even been found in fish samples at levels of a few μg/Kg of OC and BP-3 [24,26]. The high concentration in fish results is obviously due to the presence of these compounds in water and to the bioaccumulation potential of the lipophilic UV-filters. This occurs because the elimination power of STPs is not 100%, as is confirmed by Balmer *et al.* [24] who found an

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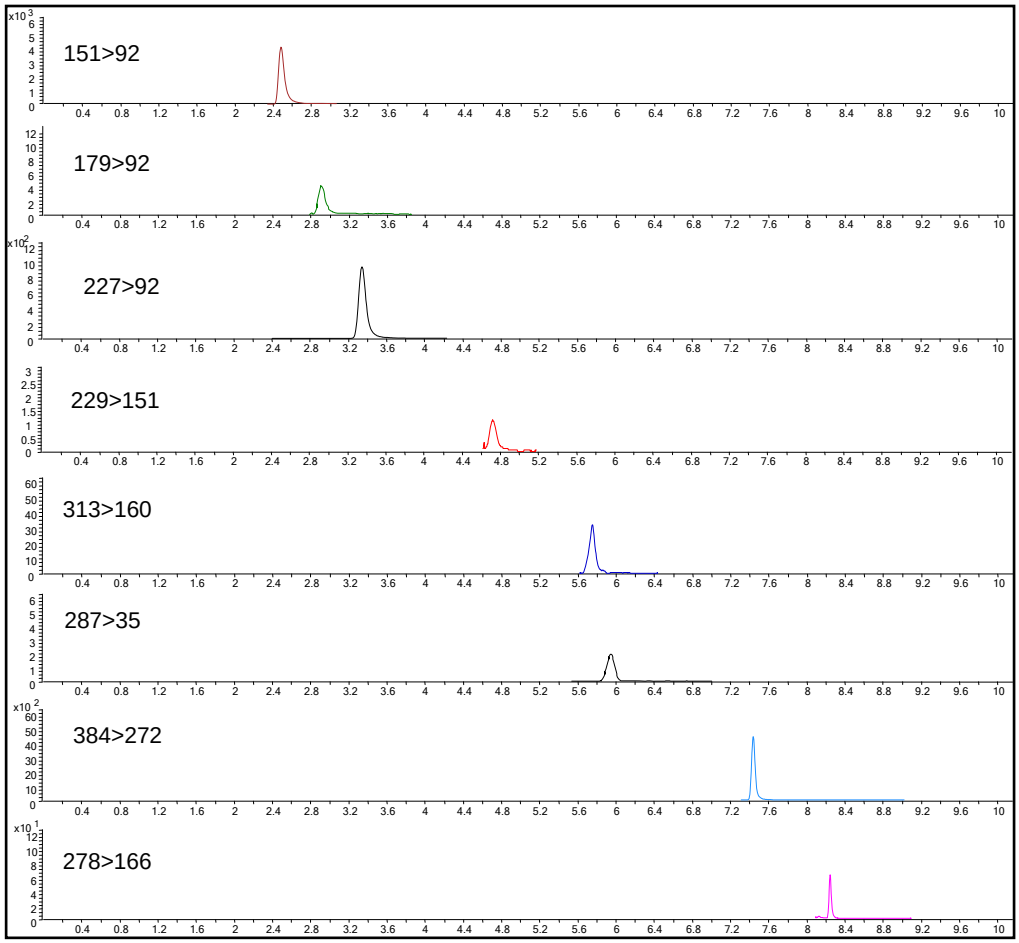


**Figure 2.** MRM chromatogram obtained by PLE/UHPLC-MS-MS of 1 g of sewage sludge collected in July 2007. For experimental conditions see text.

elimination power of between 68% and 99% for BP-3 and OC. As an example, Figure 2 shows the MRM chromatograms of a sample collected in July 2007.

## CONCLUSIONS

The suitability of the PLE technique with UHPLC-MS-MS with triple quadrupole analyzer for the determination of preservatives, UV filters and antimicrobials all together in a single analysis of sewage sludge has been demonstrated for the first time. The method can be qualified as a rapid method, with 30 minutes of extraction time and 9 minutes of separation analysis.

The limit of detection and limit of quantification were lower than 8 µg/Kg (d.w.) and 12.5 µg/Kg (d.w.), respectively. In the analysis of three different samples from a STP, eight

ISBN:978-84-693-6426-0/DL:T-1628-2010

different target analytes were determined. The UV filter, OC, showed the highest concentration in the summer sample (1842 μg/Kg (d.w.)) and triclosan showed the highest concentration in the winter sample (1490 μg/Kg (d.w.)). For the first time, this paper demonstrated the presence of two UV filters (ODPABA and BP-3) and parabens in sewage sludge samples.

**Acknowledgements**

The authors wish to thank the personnel of the water treatment plant in Tarragona for their cooperation in all aspects of this study.
This study was financially supported by the Dirección General de Investigación of the Ministry of Science and Technology, project CTM2008-06847-C02-01/TECNO. A.N. gratefully acknowledges the financial support of the Ministry of Science and Technology BES-2006-1332.

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### 3.4.2. Results and discussion

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From an analytical point of view, the most relevant aspect of this study was the use of ultra high performance liquid chromatography-tandem mass spectrometry to separate 11 personal care products and the use of a factorial experimental design to optimize the PLE conditions.

In this case, the UHPLC system using a Zorbax column (5.0 x 0.46 cm, 1.8 μm of particle size) was used for chromatographic separation. Methanol and acetonitrile were evaluated initially for the chromatographic separation but methanol was selected because a better peak shape was obtained. By using this type of column, analysis time is shorter than with conventional LC analysis. In our case, we achieved a good separation of 11 personal care products with different psychico-chemical properties in 9 minutes. Consequently, the consumption of organic solvent is lower than in conventional LC which makes the analysis cheaper. Nowadays, this characteristic is noteworthy when acetonitrile must be used due to the shortage of this solvent.

The influence of solvent on the extraction technique has been also demonstrated, as in the previous studies. When pure solvents were tested, the most polar compounds were extracted with water, and the less polar compounds were extracted using methanol as a solvent. After pure solvents, mixtures of methanol:water were tested and recoveries were slightly lower than the recoveries obtained by using methanol and water separately. To check if the recoveries using this solvent could be improved, we tested a different number of cycles, an important parameter to be optimized in PLE.

After testing a different number of cycles and two solvents, the recoveries were lower than 69%. So, as in other studies, we decided to mix both solvents in order to obtain higher recoveries. In this study we also confirmed that the introduction of fresh solvent increased the recoveries.

The use of a factorial experimental design enables us to reduce the experimental work in the optimization process. For this reason, the rest of the parameters of the pressurized liquid extraction were optimized using a fractional experimental design $2^{6-2}$. The analysis of the results using the Pareto Chart confirmed some characteristics that had been observed in previous studies. For instance, temperature is the factor that most significantly affects the recoveries. In this case, the recoveries increased when the temperature increased. The results also showed that purge time and flush volume had less influence on the recoveries. To confirm whether these values were the optimum, we used a full factorial design $2^2$ fixing all parameters except the two most important parameters obtained in the fractional experimental design: the temperature and the extraction time.

Under optimum conditions, the recoveries were higher than 72%, except for DHB whose recovery was 30%. Two compounds (DHMB and PMDSA) had to be excluded from the study because they did not extract with any condition. The recoveries found

ISBN:978-84-693-6426-0/DL:T-1628-2010

in our study were consistent with those found in other studies in which UV filters and antimicrobials were determined. For instance, triclocarban and triclosan were determined in sewage sludge using PLE and LC-MS-MS and recoveries of 98% were obtained for both compounds [1]. Using other extraction techniques such as microwave assisted extraction (MAE), triclosan was also determined and recoveries of between 80 and 100% were obtained from sludge and sediments [2]. Some UV filters were determined in sewage sludge (such as 3-(4-methylbenzylidene) camphor, octyl-mehoxycinnamate, octocrylene and octyl-triazone) using shaking extraction and GC analysis except for OT which, due to its high molecular weight, was determined by LC-MS-MS. In this study, the recoveries for all UV filters were higher than 75% [3].

Combining PLE and UHPLC-MS-MS with a triple quadrupole analyzer, low limits of detection were obtained and most of the compounds were quantified in the samples. The compounds that showed the highest concentrations were the antimicrobial triclosan (between 1300 and 1490 μg/Kg (d.w.)) and the UV filter octocrylene (between 700 and 1842 μg/Kg (d.w.)). We also reported, for the first time, the presence of parabens and two UV filters (ODPABA and BP-3) in sewage sludge samples. We found methyl paraben, propyl paraben and benzyl paraben at concentrations of between 5 and 202 μg/Kg (d.w.) and ODPABA and BP-3 between 10 and 170 μg/Kg (d.w.).

This study showed the presence of personal care products in sewage sludge. Moreover, parabens and two UV filters were determined for the first time in sewage sludge. As we mentioned before, the parabens and UV filters are considered endocrine disrupting compounds and, consequently, these compounds should be taken into account in the list of possible contaminants.

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## 3.5. Monitoring study of pharmaceuticals in sewage sludge

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So far we have developed several analytical methods which have enabled us to demonstrate the presence of a wide group of PPCPs in sewage sludge samples. In this chapter we have included a monitoring study of 20 pharmaceutical compounds and 9 estrogens and their conjugates in sewage sludge samples from two different sewage treatment plants. The samples were collected every two months for one year. The 20 pharmaceuticals belong to different groups determined in previous studies such as anti-inflammatories, lipid regulators, β-blockers, stimulants, antiulcers, analgesics, antiepileptics and antibiotics, such as macrolides and sulfonamides.

In the monitoring study, we used the methods developed in sections 3.1 and 3.2 of the experimental part of this Doctoral Thesis. In the methods described in section 3.1, we used mass spectrometry with a quadrupole analyzer for different groups of pharmaceuticals such as anti-inflammatories, lipid regulators, β-blockers, stimulants, antiulceratives, analgesics and antiepileptics, among others. However, when this monitoring study was carried out we had the opportunity of adapting the method to analyze extract of PLE using liquid chromatography-tandem mass spectrometry with a triple quadrupole analyzer. One of the most important advantages of using the triple quadrupole as an analyzer in this study is that some compounds such as metoprolol, propranolol and the sulfonamides group, among others, could be detected and quantified in most of the samples analyzed because the limits of detections achieved with this analyzer were significantly lower. In the study in section 3.1, these compounds were not detected or were detected below the limit of quantification. Moreover, using this analyzer in MRM mode, a better confirmation of the target analytes was done because two transitions were monitored for all compounds.

In order to simplify the extraction process, we tried to use a unique extraction method for all the pharmaceuticals. However, this was not possible because different extraction solvents or temperatures were necessary for group of compounds. Finally, to extract pharmaceuticals and estrogens and their conjugates from sewage sludge, we used the methods previously developed, so that three different extraction methods and three chromatographic analysis methods were applied to determine 29 compounds.

In this monitoring study we analyzed sewage sludge samples from two different sewage treatment plants. The STPs are located in the area of Tarragona (NW of Spain). Both STPs belong to cities that have similar populations (around 140,000 habitants). The importance of this study is that it showed for the first time the presence of pharmaceuticals in sewage sludge from the two biggest STPs in the area of Tarragona. In a previous study (section 3.1.1), only eleven pharmaceuticals were monitored. The results obtained in this study, which corresponded to samples taken in 2007 and 2008, were compared with the results of 2004 and 2005.

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The paper including the results of this study has been accepted for publication in Environmental Toxicology and Chemistry. Moreover, the supplementary information available on-line has been also included.

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### 3.5.1. Occurrence of pharmaceuticals and hormones in sewage sludge

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ISBN:978-84-693-6426-0/DL:T-1628-2010

# OCCURRENCE OF PHARMACEUTICALS AND HORMONES IN SEWAGE SLUDGE

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**Abstract**

The present study evaluates the presence of 9 estrogens and their conjugates and 20 pharmaceuticals such as anti-inflammatories, lipid regulators and antibiotics among others in sewage sludge from two sewage treatment plants (STPs) in the Tarragona area (Spain) for the period March 2007 until March 2008. Target analytes have been determined using different methods involving pressurized liquid extraction and liquid chromatography (electrospray ionization) tandem mass spectrometry (PLE/LC-(ESI)MS-MS).

Most of the pharmaceuticals and estrogens were found at low μg/Kg dry weight (d.w.) levels in the sewage sludge samples analyzed. Some compounds were present in all samples such as acetaminophen, caffeine, carbamazepine and ibuprofen, among others. Other compounds, such as estriol only was determined in the STP of Reus. The compounds that showed the highest concentration in both STPs were roxithromycin and tylosin (1446 and 1958 μg/Kg (d.w.), respectively). The presence of these compounds in sewage sludge demonstrated that they are partially or totally removed from the influent wastewater by sorption into the sewage sludge.

**Keywords:** Pharmaceuticals, estrogens, monitoring, sewage sludge, liquid chromatography, tandem mass spectrometry.

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## INTRODUCTION

The Municipal wastewaters contain a wide variety of contaminants of which the active ingredients of pharmaceuticals and estrogens are important groups. These compounds are included in the so called emerging organic contaminants and one important characteristic of them is that they do not need to persist in the environment to potentially cause negative effects; that is, although their rates of transformation and removal are high, this is offset by their continuous introduction into the environment [1,2].

There are different groups of pharmaceutical compounds such as anti-inflammatories, lipid regulators, anti-ulcers drugs and antibiotics, among others.

ISBN:978-84-693-6426-0/DL:T-1628-2010

Pharmaceuticals and estrogens vary considerably in their molecular structures and physicochemical properties. Some of them are hydrophobic, but most of them are completely water soluble at environmental levels.

Pharmaceuticals and estrogens are not completely assimilated by the human body; for instance, according to Hirsch *et al.* [3] more than 60% of roxythromycin, erythromycin and trimethoprim are excreted unmetabolized. Sewage treatment plants do not always eliminate them effectively, although various designs have been used. Some studies reported different concentration levels in effluent wastewater [4-7]. Pharmaceuticals and estrogens are present in sewage sludge because they have been partially or totally sorpted from the influent wastewater samples.

Determining the presence of these contaminants in sewage sludge is important because sewage sludge can be used as manure, with the result that the contaminants may be introduced in the food chain [8-10].

The low concentration of pharmaceuticals and estrogens in sewage sludge means that extraction and separation techniques are necessary for their determination. Different extraction techniques have been used to extract them, but nowadays microwave assisted extraction (MAE) [11,12] and pressurized liquid extraction (PLE) [13-15] are the preferred extraction techniques.

Liquid chromatography coupled with tandem mass spectrometry has been the most widely chosen technique for separating and determining pharmaceuticals and estrogens according to their polarity [16]. Triple quadrupole analyzer (QqQ) is the most commonly used analyzer for target analysis; although there are studies that use different analyzers such as ion-trap [12,17], quadrupole-time of flight (QTOF) [18,19] or quadrupole-ion trap (QTrap) [20].

Recently, there has been a spate of interest in the presence of pharmaceuticals in environmental samples [4,5,21]. There is extensive literature about the determination of pharmaceuticals and estrogens in water samples such as wastewater or surface water; however, the presence of these contaminants in sewage sludge has received little attention to date [21]. There are a few studies which report concentrations of pharmaceuticals at levels of low μg/Kg in sewage sludge from different countries such as Spain [15,22], Switzerland [23,24] and Germany [13,25,26], among others. The most studied group of pharmaceuticals in sewage sludge is antibiotics, which can include different classes such as sulfonamides and macrolides. These classes have been determined in sewage sludge samples from Germany [25] and Switzerland [13]. Concentrations between 5 and 160 μg/Kg (d.w.) were found for sulfapyridine, sulfamethoxazole, trimethoprim and roxythromycin in Germany [25]. However, higher concentrations of sulfapyridine, sulfamethoxazole, trimethoprim, azythromycin and clarithromycin were found in Switzerland,

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at levels between 28 and 68 mg/Kg (d.w.) [13]. Miao *et al.* [27] found carbamazepine at a concentration of 69.6 μg/Kg (d.w.) and some metabolites at concentrations between 1.9 and 7.5 μg/Kg (d.w.) in sewage sludge from Canada.

Estrogens and their conjugates have been less studied than pharmaceuticals and only a few studies are found in the literature [12,22] regarding the determination of these compounds in sewage sludge. Ternes *et al.* [12] detected up to 37 μg/Kg (d.w.) and up to 49 μg/Kg (d.w.) of the natural estrogens estrone and 17β-estradiol respectively. Mestranol was detected below the limit of quantification in all samples, whereas 17α-ethinylestradiol showed a concentration ranging from below the limit of quantification to 17 μg/Kg (d.w.). In addition to free estrogens, in a previous paper [22] we determined two conjugates (estradiol 3-sufate and estrone 3-sulfate) at a concentration between 0.64 and 7 μg/Kg (d.w.).

The objective of the present study is to monitor twenty pharmaceuticals, and nine estrogens and their conjugates in sewage sludge from two different sewage treatment plants in the south of Catalonia for the period from March 2007 to March 2008. The group of pharmaceuticals monitored includes anti-inflamatories, lipid regulators, β-blockers, stimulants, antiulcer drugs, analgesics, anti-epileptics and antibiotics such as macrolides and sulfonamides.

## EXPERIMENTAL

### Materials and reagents

The compounds studied are listed in **Table 1** according their therapeutic category. All standards were from Sigma (St. Louis, USA) except clofibric acid from Aldrich (Steinheim, Germany). Stock solutions of individual standards were prepared by dissolving caffeine, propranolol, metoprolol, carbamazepine, acetaminophen, ibuprofen, bezafibrate, diclofenac, naproxen and clofibric acid in methanol:water (1:1, v/v) and dissolving the reamining compounds in methanol at a concentration of 1000 mg/L. The stock solutions were then stored at 5 ºC. The hormones and their conjugates were stored at -15 ºC due to their low stability. Fresh stock solutions were prepared once a year. Standard solutions in methanol:water (1:1, v/v) at a concentration of 50 mg/L were prepared weekly. Working solutions were prepared daily by diluting the previous solution with methanol:water (1:1, v/v).

Ultra-pure water was obtained with a Milli-Q water purification system (18.2 MΩ·cm) (Millipore, Bedford, MA, USA), acetonitrile and methanol (HPLC-grade) were from SDS (Peypin, France), nitrogen was from Carburos Metálicos (Tarragona, Spain) and phosphoric acid and acetic acid were from Merck (Darmstadt, Germany).

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**Table 1.** List of compounds studied.

| |
|---|
| **Antibiotics** |
| Sulfadiazine |
| Sulfatiazole |
| Sulfapyridine |
| Sulfamethoxazole |
| Sulfamethazine |
| Erythromycin |
| Tylosin |
| Roxithromycin |
| **Non-steroidal anti-inflammatory drugs** |
| Ibuprofen |
| Naproxen |
| Diclofenac |
| **Estrogens and their conjugates** |
| Estriol |
| Estradiol 3-sulfate |
| Estrone 3-sulfate |
| 17β-estradiol |
| 17α-ethinylestradiol |
| Estrone |
| Diethylstilbestrol |
| Estradiol 17-acetate |
| 17α-estradiol |
| **Anti-ulceratives** |
| Omeprazole |
| Ranitidine |
| Trimethoprim |
| **β-blockers** |
| Metoprolol |
| Propranolol |
| **Analgesics** |
| Acetaminophen |
| **Stimulants** |
| Caffeine |
| **Antiepileptics** |
| Carbamazepine |
| **Lipid regulators** |
| Bezafibrate |
| Clofibric acid |

## Sampling and sample pre-treatment

We collected sewage sludge samples from two conventional sewage treatment plants (STPs) that use activated sludge biological treatment and are located in Tarragona (STP1) and Reus (STP2) (Catalonia, Spain).

These STPs mostly receive urban wastewaters and some industrial discharges. Each one serves a similar number of people (around 140,000) with a biological oxygen demand ($BOD_5$) for influent water of 400 mg/L. The average flow-rate is 30,000 $m^3$/day for Tarragona and 16,000 $m^3$/day for Reus. The sewage sludge samples corresponded to a mix of primary and secondary sewage, which was anaerobically digested and then dehydrated using press filters. The samples were collected every two months between March 2007 and March 2008 using glass dark bottles and they were stored in a freezer (-15 ºC) until they were analyzed (less than 1 month).

Samples were lyophilized before being analyzed using the freeze dry system (Labconco, Missouri, USA). They were then homogenized using a mortar and pestle and sieved to obtain particles with a diameter less than 125 μm.

## PLE extraction

To extract the pharmaceuticals from sewage sludge, three methods, previously developed, were used that employed pressurized liquid extraction using an accelerated solvent extraction (ASE) 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA). Depending on the method, the ASE 200 was equipped with 33 or 11 ml stainless steel extraction vessels. In all cases, the samples were mixed with aluminium oxide (previously heated

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at 120 ºC for 24 h and the extracts were filtered with a 0.45 μm diameter microfilter (Teknokroma, Barcelona, Spain), and then analyzed using liquid chromatography coupled with tandem mass spectrometry.
Method A was used to extract anti-inflammatories, β-blockers, lipid regulators, analgesics and other substances such as caffeine and clofibric acid (metabolite of clofibrate) [14]. Method B was used to extract macrolides and sulfonamides [15] and Method C was used to extract estrogens and their conjugates [22]. Method conditions are included as supplementary information section.

**LC-MS-MS**

The chromatographic instrument was an HP1200 LC-triple quadrupole tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany) with an electrospray ionization (ESI) interface, an automatic injector, a degasser, a quaternary pump and a column oven. The chromatographic column was a Kromasil 100 $C_{18}$ (25 x 0.46 cm) with a 5 μm particle size (Teknokroma, Barcelona, Spain), and the volume injected was 50 μL. Solvent A was Milli-Q water with acetic acid (pH 2.8) and solvent B was acetonitrile. The mobile phase flow-rate was 1 mL/min.
Method A and B were developed in a previous paper [14,15] using a single quadrupole mass spectrometer, and in this study they have been adapted to a tandem mass spectrometer using a triple quadrupole analyzer. MS-MS was performed in the Multiple Reaction Monitoring (MRM) mode using ESI in the negative and positive mode. The chromatographic and MS-MS conditions are included in the supplementary information.

**RESULTS AND DISCUSSION**

**Table 2** and **Table 3** show the results of the sewage sludge samples collected every two months between March 2007 and March 2008 from two different STPs located in the area of Tarragona and Reus. No sample was collected from STP1 in November because there were problems in the sewage treatment plant.
Metoprolol, sulfadiazine, estriol and estradiol 17-acetate are excluded from Table 2 (which shows the results for STP1) because these compounds appeared in only a few samples and always at a concentration lower than the limit of quantification. For the same reason, clofibric acid, ranitidine, sulfadiazine and estradiol-17-acetate were excluded from the Table 3 (which shows the results for STP2).
Antibiotics were the pharmaceuticals that showed the highest concentration in both STPs. Among them, roxythromycin and tylosin showed the highest concentrations ranging from 337 to 1446 μg/Kg (d.w.) and 1074 to 1958 μg/Kg (d.w.), respectively. In a previous paper [15] we found similar concentrations for roxithromycin and slightly higher concentrations for tylosin in sewage sludge samples from the same STPs at levels up to 2 mg/Kg (d.w.) for

ISBN:978-84-693-6426-0/DL:T-1628-2010

roxithromycin and 4 mg/Kg (d.w.) for tylosin. Sulfonamides had not been quantified in the previous study [15] because higher limits of detection were obtained with the quadrupole analyzer. In the present study, some sulfonamides appeared in all samples during monitoring; these were sulfathiazole and sulfapyridine in STP1, and sulfamethazine in STP2. Two different studies by Göbel *et al.* [13,25] determined different sulfonamides and trimethoprim in sewage sludge from different sewage treatment plants. One of these [13] used PLE/LC-MS-MS to study sewage sludge samples from Switzerland, and they found concentrations of between 28 and 68 mg/Kg for some pharmaceuticals such as sulfapyridine, sulfamethoxazole and trimethoprim. However, lower concentrations of sulfapyridine, sulfamethoxazole, trimethoprim and roxithromycin (between 13 and 160 μg/Kg (d.w.)) were found in sewage sludge from Germany and Switzerland [25]. The concentrations found in our study were similar to the second study because we found less than 178 μg/Kg (d.w.) for the sulfonamide group.

Some compounds such as acetaminophen, caffeine, carbamazepine and ibuprofen appeared in all samples analyzed from both STPs. This is because they are used in high quantities; in Spain for instance, ibuprofen can be bought without medical prescription.

The concentration range of ibuprofen was between 24 and 114 μg/Kg (d.w.) in both STPs. Ibuprofen was also determined in other studies; for example Radjenovic *et al.* [28] obtained higher concentrations (between 299 and 548 μg/Kg (d.w.)) in sewage sludge samples from Barcelona. They also identified concentrations of acetaminophen in sludge from Barcelona [28], these being between 33 and 145 μg/Kg (d.w.). In our study, the range of concentrations was greater, being 13 and 419 μg/Kg (d.w.). The maximum concentration of caffeine was 74 μg/Kg (d.w.). 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.

The presence of pharmaceuticals and estrogens in sewage sludge is due to their sorption in sewage sludge. The quantity of each compound that can be eliminated, via degradation or sorption in sludge, depends on the design of the sewage treatment plant. Different studies of influent and effluent waste water samples [29-31] reported that sewage treatment plants are able to eliminate (sorption + degradation) around 60% of acetaminophen, caffeine, ibuprofen, naproxen and sulfamethoxazole, among others.

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**Table 2**. Results of sludge samples of STP1 (µg/Kg (d.w.)).

| Compound | March 07 | May 07 | July 07 | September 07 | January 08 | March 08 | Maximum | Minimum |
|---|---|---|---|---|---|---|---|---|
| Acetaminophen | 174 | 264 | 233 | 181 | 362 | 419 | 419 | 64 |
| Caffeine | 74 | 62 | <LOQ | 72 | 43 | 66 | 74 | <LOQ |
| Propranolol | <LOQ | 9 | <LOQ | 11 | 26 | 22 | 26 | <LOQ |
| Carbamazepine | 34 | 23 | 42 | 12 | 41 | 15 | 42 | 12 |
| Bezafibrate | <LOQ | 9 | <LOQ | <LOQ | <LOQ | <LOQ | 9 | <LOQ |
| Naproxen | 4 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 4 | <LOQ |
| Clofibric acid | - | 7 | 10 | - | - | - | 10 | 7 |
| Diclofenac | 28 | 38 | 26 | <LOQ | 83 | 12 | 83 | <LOQ |
| Ibuprofen | 63 | 60 | 71 | 114 | 78 | 44 | 114 | 44 |
| Ranitidine | <LOQ | 2 | 3 | <LOQ | <LOQ | <LOQ | 3 | <LOQ |
| Sulfatiazole | 29 | 24 | <LOQ | <LOQ | 23 | 37 | 37 | <LOQ |
| Sulfapyridine | 19 | 38 | 7 | 31 | 19 | 8 | 38 | 7 |
| Trimethoprim | <LOQ | 14 | - | <LOQ | 17 | - | 17 | <LOQ |
| Sulfamethazine | - | <LOQ | 15 | - | 9 | - | 15 | <LOQ |
| Omeprazole | <LOQ | 1 | <LOQ | <LOQ | <LOQ | <LOQ | 1 | <LOQ |
| Sulfamethoxazole | 112 | 19 | <LOQ | <LOQ | 65 | 25 | 112 | <LOQ |
| Tylosin | 1337 | 1316 | 1074 | 1263 | 1453 | 1305 | 1453 | 1074 |
| Roxithromycin | 337 | 386 | 349 | 482 | 614 | 578 | 614 | 337 |
| Estradiol 3-sulfate | 3 | 2 | 3 | 20 | 28 | 25 | 28 | 2 |
| Estrone 3-sulfate | 28 | 4 | 7 | 18 | <LOQ | <LOQ | 28 | <LOQ |
| 17$\alpha$-ethinylestradiol | - | - | - | <LOQ | <LOQ | 313 | 313 | <LOQ |
| Estrone | 72 | <LOQ | 137 | <LOQ | 56 | 53 | 137 | <LOQ |
| Diethylstilbestrol | 37 | 25 | 184 | <LOQ | <LOQ | <LOQ | 184 | <LOQ |

Relative standard deviation (RSD)(%, n=3)<11
-: < limit of detection
<LOQ: below to the limit of quantification

**Table 3**. Results of sludge samples of STP2 (µg/Kg (d.w.)).

| Compound | March 07 | May 07 | July 07 | September 07 | November 07 | January 08 | March 08 | Maximum | Minimum |
|---|---|---|---|---|---|---|---|---|---|
| Acetaminophen | 49 | 13 | 39 | 153 | 150 | 39 | 57 | 153 | 13 |
| Caffeine | 53 | 45 | 54 | 63 | 44 | 64 | 45 | 64 | 44 |
| Metoprolol | <LOQ | 12 | <LOQ | <LOQ | 21 | <LOQ | <LOQ | 21 | <LOQ |
| Propranolol | 13 | 12 | 15 | <LOQ | <LOQ | <LOQ | <LOQ | 15 | <LOQ |
| Carbamazepine | 21 | 42 | 21 | 22 | 11 | 32 | 30 | 42 | 11 |
| Bezafibrate | - | <LOQ | - | - | 13 | <LOQ | <LOQ | 13 | <LOQ |
| Naproxen | <LOQ | 57 | <LOQ | <LOQ | <LOQ | 13 | | 57 | <LOQ |
| Diclofenac | 13 | <LOQ | 42 | 55 | 30 | 19 | 87 | 87 | <LOQ |
| Ibuprofen | 51 | 63 | 50 | 41 | 76 | 45 | 24 | 76 | 24 |
| Sulfatiazole | 63 | 19 | 27 | 39 | 41 | 26 | <LOQ | 63 | <LOQ |
| Sulfapyridine | 16 | <LOQ | - | - | 16 | 20 | 5 | 20 | <LOQ |
| Trimethoprim | 2 | - | 2 | <LOQ | <LOQ | <LOQ | - | 2 | <LOQ |
| Sulfamethazine | 10 | 14 | 26 | 11 | 20 | 12 | <LOQ | 26 | <LOQ |
| Omeprazole | <LOQ | 23 | - | <LOQ | - | <LOQ | <LOQ | 23 | <LOQ |
| Sulfamethoxazole | 128 | <LOQ | 18 | 18 | - | 178 | <LOQ | 178 | <LOQ |
| Tylosin | 1958 | 1526 | 1674 | 1874 | 1737 | 1516 | 1674 | 1958 | 1516 |
| Roxithromycin | 1446 | 1181 | 1373 | 1193 | 1265 | 1048 | 1205 | 1446 | 1048 |
| Estriol | 223 | 272 | 406 | 163 | 104 | <LOQ | 113 | 406 | <LOQ |
| Estradiol 3-sulfate | 189 | 0.79 | 0.71 | 28 | 88 | <LOQ | 115 | 189 | <LOQ |
| Estrone 3-sulfate | 37 | 0.64 | 4 | <LOQ | 22 | 14 | 26 | 37 | <LOQ |
| 17$\alpha$-ethinylestradiol | 290 | - | - | <LOQ | 213 | - | - | 290 | <LOQ |
| Estrone | 113 | 129 | 60 | 56 | 43 | 60 | <LOQ | 129 | <LOQ |
| Diethylstilbestrol | <LOQ | 34 | - | <LOQ | 43 | 37 | <LOQ | 43 | <LOQ |

RSD(%, n=3)<12
-: < LOD

ISBN:978-84-693-6426-0/DL:T-1628-2010

On the other hand, another study showed that the removal rate in different sewage treatment plants for some compounds such as carbamazepine was lower than 10% [32-34]. Although this low elimination rate, we determined concentrations between 11 and 42 μg/Kg of carbamazepine in samples from both STPs. Our results agree with other studies in which carbamazepine has been determined. Barron *et al.* [17] used PLE/LC-MS-MS to determine carbamazepine in sewage sludge from Ireland at a concentration of 120 μg/Kg (d.w.). Radjenovic *et al.* [28] found carbamazepine at a concentration between 72 and 123 μg/Kg (d.w.). In sewage sludge from Canada, Miao *et al.* [27] determined carbamazepine at concentrations between 70 and 258 μg/Kg (d.w.) and different metabolites of carbamazepine such as 2- hydroxylcarbamazepine, 3-hydroxycarbamazepine and dihydroxycarbamazepine at lower concentrations between 1.6 and 15.4 μg/Kg (d.w.).

Our study also determined levels of estrogens and their conjugates. In general, this group is present in sewage sludge at very low concentrations. The estrogen with the highest concentration was estriol which was found at a concentration of 406 μg/Kg (d.w.) in the sample taken in July from STP2. Estradiol 17-acetate was not quantified in any sample probably because it had the highest limit of quantification (375 μg/Kg (d.w.)) On the other hand, we observed two estrogens in all samples, namely estradiol 3- sulfate and estrone and the levels ranged from below the limit of quantification to 189 μg/Kg (d.w.). As in a previous study [22], estriol was not found in the samples from the STP1; however, as we mentioned above, in the samples from STP2 estriol had a maximum concentration of 406 μg/Kg (d.w.). Ternes *et al.* [12] determined some estrogens in sewage sludge using GC-MS-MS and ultrasonication extraction (USE) as the extraction technique. They found estrone at a concentration between 16 and 37 μg/Kg (d.w.) whereas we found it at a higher concentration (up to 137 μg/Kg (d.w.)). The conjugate sulfate estrogens have received less attention. To our knowledge, they have only been studied in the last paper that we published [22]. In the present study, estradiol 3-sulfate and estrone 3-sulfate were found at concentration between 3 and 189 μg/Kg (d.w.), whereas in our previous study [22], they were found at concentrations lower than 7 μg/Kg (d.w.).

Table 2 and 3 show that the concentrations of only three pharmaceuticals (diclofenac in STP1 and acetaminophen and ibuprofen in STP2) were higher in winter samples. This may occur because the consumption of these compounds is higher in winter than in summer. On the other hand, we are unable to explain why some compounds showed different concentrations during the monitoring campaign, for instance sulfamethoxazole in STP1 or estrone in STP2.

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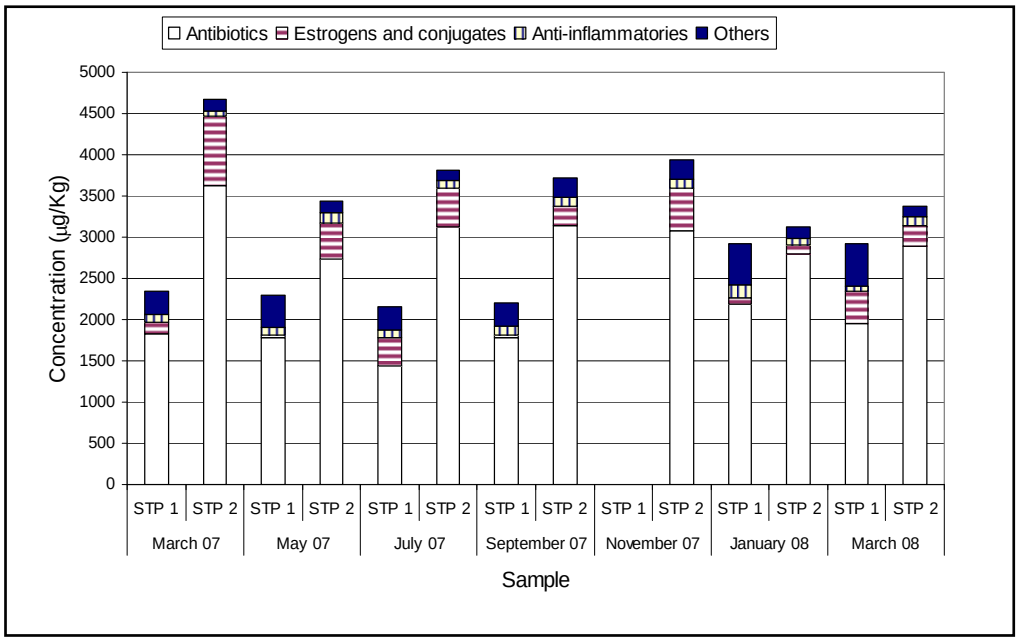


**Figure 1.** Concentration in µg/Kg of four groups (antibiotics, estrogens and their conjugates, anti-inflammatories and others) found in the samples analyzed from sewage treatment plants of Tarragona (STP1) and Reus (STP2).

The compounds studied were divided into four different groups in order to evaluate the concentration of each group of pharmaceuticals in the samples analyzed (see **Fig. 1**). These groups were: antibiotics, estrogens and their conjugates, anti-inflammatories, and others. In this last group we included the anti-ulceratives, antiepileptics, analgesics, stimulants, β-blockers and lipid regulators. In all cases, the samples from STP2 showed higher levels of pharmaceuticals than those from STP1, mostly two times higher. This may be explained by the dilution effect because the average flow rate for STP1 (Tarragona) is about two times larger than for STP2 (Reus). We can observe the highest amount of pharmaceuticals and estrogens (4675 µg/Kg) in the sample from March 2007. The concentration of antibiotics was always higher in the samples from STP2, and this was mainly because of the high concentrations of roxithromycin and tylosin. The average concentration of antibiotics in the samples from STP1 was 1820 µg/Kg and in the samples from STP2 was 3054 µg/Kg. In all samples, the concentration of antibiotics was always between 70% and 90% of the total amount of pharmaceuticals and estrogens found.
The concentrations of estrogens and their conjugates were higher in the STP2 than in the STP1 in all cases except in the samples taken in March 08. This is because of the high

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concentration of estriol found in the STP2, as was previously mentioned. On the other hand, the concentration of substances from the "others" group was higher in the samples from STP1 than in the samples from the STP2. The concentration of this group was between 276 and 522 μg/Kg in STP1 and between 124 and 239 μg/Kg in STP2. In **Figure 1** we can see similar concentrations of anti-inflammatories in all samples, these being between 56 and 161 μg/Kg in the samples from STP1 and between 64 and 120 μg/Kg in the samples from STP2. The highest concentration of anti-inflamatories was found in the sample taken in January 2008. This situation may be caused by the high consumption of these kinds of pharmaceuticals during the winter.

## CONCLUSIONS

The concentrations of 9 estrogens and their conjugated and 20 pharmaceuticals in sewage sludge samples from Tarragona and Reus (Spain) ranged between 2 and 1958 μg/Kg (d.w.). Their presence in sewage sludge means that they are completely or partially sorpted in sewage sludge.
Some compounds, such as acetaminophen, carbamazepine and ibuprofen, among others, were found in all samples. In both STPs, the highest concentration was that of tylosin, this being 1,453 and 1,958 μg/Kg (d.w.) in STP1 and STP2, respectively.

**Acknowledgements**

The authors wish to thank the personnel of the water treatment plants in Tarragona and Reus for their cooperation.
The present study was funded by the Dirección General de Investigación of the Spanish Ministry of Science and Technology, projects CTM2005-01774 and CTM2008-06847-C02-01/TECNO. A.N. gratefully acknowledges the financial support of the Spanish Ministry of Science and Technology BES-2006-1332.

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**Supplementary Information**

**1. PLE extraction and LC-MS-MS analysis.**

Three different methods have been used to determine the pharmaceuticals present in sewage sludge samples. Regarding to the extraction conditions used to extract the compounds three different methods, previously developed, have been used. **Table 1s** summarize the PLE extraction conditions.

Methods A and B were developed in previous papers [14,15] using a single quadrupole mass spectrometer, and in the present study they have been adapted to a tandem mass spectrometer in order to achieve lower limits of detection. Because some compounds in Method A had to be determined in the positive ionization mode and others in the negative ionization mode, there were two gradients, one for acetaminophen, caffeine, metoprolol, propranolol and carbamazepine (positive ionization) and another for bezafibrate, naproxen, clofibric acid, diclofenac and ibuprofen (negative ionization). One gradient was used in both Methods B and C because the same ionization mode was the optimum for all the compounds. The LC conditions are also summarized in **Table 1s**.

Ionization and fragmentation settings were optimized by injecting standard solutions. MS-MS was performed in the Multiple Reaction Monitoring (MRM) mode using ESI in the negative and positive mode. The fragmentation ions, the cone voltage and the collision cell of each compound were summarized in **Table 2s**. Two characteristic fragmentations of the deprotonated molecular ion $[M-H]^-$ or protonated molecular ion $[M+H]^+$ were monitored for each compound, the first being most abundant and used for quantification, while the second one was used as a qualifier. Cone voltage and collision energy were optimized for each compound. In all cases, nitrogen was used as the collision, nebulizing and desolvation gas. The average conditions selected for the optimum performance of the ESI interface are summarized in **Table 1s**. Method A used two time windows in positive mode: 0-7 min (acetaminophen and caffeine) and 7-32 min (metoprolol, propranolol and carbamazepine). It also used two time windows in the negative mode: 0-9.5 min (bezafibrate, naproxen and clofibric acid) and 9.5-25 min (diclofenac and ibuprofen).

Method B used four time windows in the positive ionization mode: 0-10 min (ranitidine and sulfadiazine), 10-18 min (sulfathiazole, sulfapyridine, trimethoprim and sulfamethazine), 18-20.5 min (omeprazole, sulfamethoxazole, erythromycin and tylosin) and 20.5-27 min (roxithromycin). The time windows used for Method C are reported in a previous paper [22].

**2. Matrix effect.**

Co-eluting matrix components influence the electrospray ionization efficiency of the analyte and adversely affect the reproducibility and accuracy of the method, especially when external calibration curves are used for quantification. For this reason, matrix effects were checked during method development. The matrix effect

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was checked at two different concentrations (300 μg/Kg and 500 μg/Kg (d.w.) for estrogens and 35 μg/Kg and 75 μg/Kg (d.w.) for the rest) using the same methodology described in previous studies [14,15,22]. The matrix effect observed with each target analyte was calculated as the percentage of decrease or enhance in signal intensity in a sample matrix versus a pure solvent. The results are according to those one obtained in previous studies [14,15,22]. **Table 3s** list the matrix effect calculated for each compound. As we can see in the **Table 4s**, in most of cases we observed enhancement of the signal, and in all cases the matrix effect was lower than ± 20%. The low matrix effect calculated can be due to the diluted extract obtained in the PLE therefore the matrix effect was considered negligible.

**3. Method validation.**

**Table 4s** summarizes the linear range obtained by injecting standards. The determination coefficients ($r^2$) were higher than 0.995 for all compounds.

When 5 g (d.w.) of sewage sludge spiked at 50 μg/Kg for method A and B and 1 g (d.w.) spiked at 300 μg/Kg for method C, the recoveries were higher than 70% (RSD < 9%, n = 3) for all compounds except ranitidine, whose recovery was 60%, and, as we expected, no significant differences were observed compared with previous studies [14,15,22].

**Table 3s** also summarizes the limits of detection (LOD) and the limits of quantification (LOQ) of the methods. The LODs that were calculated as a signal-to-noise ratio of 3 were between 0.15 and 175 μg/Kg (d.w.). The sulfate conjugated compounds showed the lowest limit of detection 0.15 μg/Kg (d.w.). The LOQ, as the concentration of the lowest point of the calibration curve, ranged from 0.25 to 375 μg/kg (d.w.). As we expected, the LODs and LOQs obtained with QqQ were lower than those obtained with Q in previous papers [14,15]. For instance, the LOD for omeprazole with LC-MS was 7 μg/Kg (d.w.) and the LOD with LC-MS-MS was 0.5 μg/Kg (d.w.). Also, bezafibrate had a LOQ of 25 μg/Kg (d.w.) with LC-MS and this limit was reduced until 1 μg/Kg (d.w.) with LC-MS-MS.

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**Table 1s.** PLE extraction conditions, LC gradients and ESI conditions for methods A, B and C.

| METHOD | PLE | HPLC | | | | ESI | Ref. |
|---|---|---|---|---|---|---|---|
| | | Neg. Ionizat. | | Pos. Ionizat. | | | |
| | | t (min) | % ACN | t (min) | % ACN | | |
| **A** | **Solvent**: 50 mM aqueous phosphoric acid:methanol (1:1)<br>**Temperature:** 100 ºC<br>**Pressure:** 100 bar<br>**Preheating time:** 5 min<br>**Static Time:** 15 min<br>**Number of cycles:** 2<br>**Flush volume:** 150%<br>**Purge Time:** 300 s<br>**Sample weight:** 5 g (d.w.)<br>**Cell:** 33 mL | 0 | 55 | 0 | 18 | Positive Ionization<br>**Nebulizer pressure:** 40 psi<br>**Drying gas flow-rate:** 13 L/min<br>**Drying gas temperature:** 300 ºC<br>**Capillary Voltage:** 3000 V<br>Negative Ionization<br>**Nebulizer pressure:** 30 psi<br>**Drying gas flow-rate:** 12 L/min<br>**Drying gas temperature:** 350 ºC<br>**Capillary Voltage:** 3500 V | [14] |
| | | 6 | 60 | 4 | 20 | | |
| | | 9 | 60 | 9 | 55 | | |
| | | 21 | 80 | 15 | 60 | | |
| | | 23 | 100 | 18 | 60 | | |
| | | 26 | 100 | 20 | 100 | | |
| | | 28 | 55 | 29 | 100 | | |
| | | | | 32 | 18 | | |
| **B** | **Solvent**: 50 mM aqueous phosphoric acid:methanol (1:1)<br>**Temperature:** 80 ºC<br>**Pressure:** 100 bar<br>**Preheating time:** 5 min<br>**Static Time:** 5 min<br>**Number of cycles:** 1<br>**Flush volume:** 60%<br>**Purge Time:** 120 s<br>**Sample weight:** 5 g (d.w.)<br>**Cell:** 33 mL | t (min) | % ACN | | | Positive Ionization<br>**Nebulizer pressure:** 40 psi<br>**Drying gas flow-rate:** 12 L/min<br>**Drying gas temperature:** 350 ºC<br>**Capillary Voltage:** 4000 V | [15] |
| | | 0 | 10 | | | | |
| | | 10 | 15 | | | | |
| | | 15 | 26 | | | | |
| | | 19 | 60 | | | | |
| | | 23 | 100 | | | | |
| | | 25 | 100 | | | | |
| | | 27 | 10 | | | | |
| **C** | **Solvent**: methanol:acetone (1:1) (A) + water:methanol (1:1) (B)<br>**Temperature:** 75 ºC<br>**Pressure:** 100 bar<br>**Preheating time:** 5 min<br>**Static Time:** 3 min<br>**Number of cycles:** 2 (A) + 2 (B)<br>**Flush volume:** 30%<br>**Purge Time:** 120 s<br>**Sample weight:** 1 g (d.w.)<br>**Cell:** 11 mL | t (min) | % ACN | | | Negative Ionization<br>**Nebulizer pressure:** 45 psi<br>**Drying gas flow-rate:** 12 L/min<br>**Drying gas temperature:** 350 ºC<br>**Capillary Voltage:** 3000 V | [22] |
| | | 0 | 10 | | | | |
| | | 10 | 10 | | | | |
| | | 15 | 40 | | | | |
| | | 25 | 60 | | | | |
| | | 30 | 100 | | | | |
| | | 35 | 100 | | | | |
| | | 37 | 10 | | | | |

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**Table 2s**. MRM conditions used for LC-MS-MS determination of target analytes. Bold face transitions were used for quantification.

| Compound | Ionization mode | Cone Voltage (V) | Collision energy (V) | Transitions |
|---|---|---|---|---|
| Acetaminophen | PI | 100 | 25 | 152<93 |
| | | | 15 | **152<110** |
| Caffeine | PI | 125 | 25 | 195<110 |
| | | | 15 | **195<138** |
| Metoprolol | PI | 125 | 15 | **268<116** |
| | | | 20 | 268<159 |
| Propranolol | PI | 125 | 15 | **260<116** |
| | | | 15 | 260<183 |
| Carbamazepine | PI | 150 | 35 | **237<179** |
| | | | 35 | 237<193 |
| Salicylic acid | NI | 75 | 15 | **137>93** |
| | | | 30 | 137<65 |
| Bezafibrate | NI | 100 | 30 | **360<154** |
| | | | 15 | 360<274 |
| Naproxen | NI | 50 | 30 | 229<140 |
| | | | 5 | **229<185** |
| Clofibric acid | NI | 50 | 5 | 213<85 |
| | | | 10 | **213<127** |
| Diclofenac | NI | 75 | 20 | 294<214 |
| | | | 10 | **294<250** |
| Ibuprofen | NI | 75 | 5 | **205<161** |
| Ranitidine | PI | 100 | 15 | 315<176 |
| | | | 10 | **315<270** |
| Sulfadiazine | PI | 75 | 25 | **251<108** |
| | | | 10 | 251<156 |
| Sulfatiazole | PI | 75 | 20 | **256<108** |
| | | | 10 | 256<156 |
| Sulfapyridine | PI | 75 | 25 | **250<108** |
| | | | 15 | 250<156 |
| Trimethoprim | PI | 125 | 35 | **291<145** |
| | | | 20 | 291<249 |
| Sulfamethazine | PI | 100 | 25 | **279<124** |
| | | | 15 | 279<186 |
| Omeprazole | PI | 75 | 15 | **346<151** |
| | | | 10 | 346<198 |
| Sulfamethoxazole | PI | 100 | 20 | **254<108** |
| | | | 10 | 254<156 |

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**Table 2s**. MRM conditions used for LC-MS-MS determination of target analytes. Bold face transitions were used for quantification (Cont.).

| | | | | |
|---|---|---|---|---|
| Erythromycin | PI | 150 | 30 | **735<158** |
| | | | 30 | 735<576 |
| Tylosin | PI | 150 | 35 | **916<174** |
| | | | 30 | 916<772 |
| Roxithromycin | PI | 175 | 15 | 838<679 |
| | | | 30 | **838<158** |
| Estriol | NI | 150 | 45 | **287<171** |
| | | | 45 | 287<145 |
| Estradiol 3-sulfate | NI | 150 | 30 | **351<271** |
| | | | 55 | 351<145 |
| Estradiol 17-glucoronide | NI | 150 | 30 | 447<271 |
| | | | 20 | **447<113** |
| Estrone 3-glucoronide | NI | 150 | 45 | **445<269** |
| | | | 20 | 445<113 |
| Estrone 3-sulfate | NI | 150 | 30 | **349<269** |
| | | | 55 | 349<145 |
| 17α-estradiol | NI | 60 | 30 | **271<145** |
| | | | 45 | 271<183 |
| 17β-estradiol | NI | 60 | 30 | **271<145** |
| | | | 45 | 271<183 |
| 17α-ethinyl-estradiol | NI | 60 | 45 | **295<145** |
| | | | 30 | 295<159 |
| Estrone | NI | 150 | 45 | **269<145** |
| | | | 55 | 269<143 |
| Diethylstilbestrol | NI | 150 | 30 | **267<222** |
| | | | 30 | 267<237 |
| Estradiol 17-acetate | NI | 100 | 30 | **313<253** |
| | | | 55 | 313<145 |

PI: Positive ionization
NI: Negative ionization

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**Table 3s.** Information about the matrix effect for each compound.

| Compound | Matrix effect (%) |
|---|---|
| Acetaminophen | 20 |
| Caffeine | 18 |
| Metoprolol | -11 |
| Propranolol | 15 |
| Carbamazepine | -17 |
| Bezafibrate | 14 |
| Naproxen | 18 |
| Clofibric acid | 11 |
| Diclofenac | 8 |
| Ibuprofen | 12 |
| Ranitidine | 18 |
| Sulfadiazine | 14 |
| Sulfatiazole | 16 |
| Sulfapyridine | 15 |
| Trimethoprim | 13 |
| Sulfamethazine | 13 |
| Omeprazole | 7 |
| Sulfamethoxazole | 19 |
| Tylosin | 5 |
| Roxithromycin | 10 |
| Estriol | 11 |
| Estradiol 3-sulfate | -18 |
| Estrone 3-sulfate | -15 |
| 17α-estradiol | 20 |
| 17β-estradiol | 18 |
| 17α-ethinylestradiol | -11 |
| Estrone | 8 |
| Diethylstilbestrol | 12 |
| Estradiol 17-acetate | 15 |

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**Table 4s**. Validation data.

| Compound | Recovery (%)* | Linear Range (µg/L)** | LODs (µg/Kg) | LOQs (µg/Kg) |
|---|---|---|---|---|
| Acetaminophen | 105 | 0.3-100 | 1 | 3 |
| Caffeine | 98 | 0.5-100 | 3 | 5 |
| Metoprolol | 104 | 0.5-100 | 3 | 5 |
| Propranolol | 95 | 0.5-250 | 1 | 5 |
| Carbamazepine | 98 | 0.3-250 | 1 | 3 |
| Bezafibrate | 93 | 0.1-500 | 0.25 | 1 |
| Naproxen | 75 | 1-500 | 3 | 10 |
| Clofibric acid | 79 | 0.5-500 | 1 | 5 |
| Diclofenac | 78 | 0.5-500 | 1 | 5 |
| Ibuprofen | 70 | 1-500 | 3 | 10 |
| Ranitidine | 60 | 0.5-500 | 0.5 | 5 |
| Sulfadiazine | 78 | 1-500 | 3 | 10 |
| Sulfatiazole | 81 | 1-500 | 3 | 10 |
| Sulfapyridine | 80 | 1-100 | 3 | 10 |
| Trimethoprim | 75 | 1-500 | 2 | 10 |
| Sulfamethazine | 87 | 0.5-500 | 1 | 5 |
| Omeprazole | 95 | 0.5-300 | 0.5 | 5 |
| Sulfamethoxazole | 75 | 0.5-500 | 1 | 5 |
| Tylosin | 98 | 1-500 | 3 | 10 |
| Roxithromycin | 80 | 0.5-500 | 1 | 5 |
| Estriol | 84 | 3-1000 | 26 | 75 |
| Estradiol 3-sulfate | 99 | 0.01-1000 | 0.15 | 0.25 |
| Estrone 3-sulfate | 100 | 0.01-1000 | 150 | 0.25 |
| 17α-estradiol | 83 | 10-250 | 150 | 250 |
| 17β-estradiol | 92 | 10-500 | 150 | 250 |
| 17α-ethinylestradiol | 88 | 10-500 | 150 | 250 |
| Estrone | 88 | 0.75-250 | 11 | 19 |
| Diethylstilbestrol | 81 | 0.75-250 | 12 | 19 |
| Estradiol 17-acetate | 83 | 15-250 | 175 | 375 |

RSD(%, $n$=3) < 9

* Recoveries obtained when 5 g (d.w.) of sewage sludge spiked at 50 µg/Kg for method A and B and 1 g (d.w.) spiked at 300 µg/Kg for method C

** Instrumental Linear Range

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### 3.5.2. Results and discussion

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Regarding the analytical methods, the methods described in sections 3.1.1 and 3.1.2 were successfully adapted to LC-MS-MS in order to achieve lower limits of detection. In Table 3.2, we include the limits of quantifications obtained for these compounds using the quadrupole and the triple quadrupole analyzers. As we expected, the use of a triple quadrupole analyzer enabled lower limits of detection and quantification obtained in comparison with the limits of quantification obtained using the quadrupole analyzer in the first studies. Moreover, by using the triple quadrupole analyzer, we obtained more confirmation power because two transitions were studied for each compound.

**Table 3.2.** Limits of quantifications obtained in the studies with quadrupole and triple quadrupole analyzers.

| Compound | LOQs (Q) (µg/Kg) | LOQs (QqQ) (µg/Kg) |
|---|---|---|
| Acetaminophen | 50 | 3 |
| Caffeine | 20 | 5 |
| Metoprolol | 20 | 5 |
| Propranolol | 20 | 5 |
| Carbamazepine | 20 | 3 |
| Ranitidine | 100 | 5 |
| Sulfadiazine | 100 | 10 |
| Sulfamethazine | 50 | 5 |
| Sulfatiazole | 100 | 10 |
| Sulfamethoxazole | 50 | 5 |
| Bezafibrate | 25 | 1 |
| Naproxen | 32 | 10 |
| Clofibric acid | 28 | 5 |
| Diclofenac | 29 | 5 |
| Ibuprofen | 22 | 10 |
| Sulfapyridine | 50 | 10 |
| Trimethoprim | 20 | 10 |
| Tylosin | 100 | 10 |
| Roxithromycin | 100 | 5 |

Due to the advantage of using the QqQ analyzer, more compounds were quantified in the monitoring study than in the study using the quadrupole analyzer. For example, all of the compounds belonging to the sulfonamides group determined in section 3.1.2 had a concentration below the limit of quantification. However, in this study different sulfonamides were quantified. For example, sulfapyridine showed a concentration of between 7 and 38 µg/Kg (d.w.) in STP 1, and sulfatiazole showed a concentration of between below the limit of quantification and 63 µg/Kg (d.w.) in STP 2.

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In the study in section 3.1.1, we carried out a monitoring study of pharmaceuticals from the same STPs from February 2004 to June 2005. If we compare the results, we can observe some similarities and differences. As we mentioned before for the sulfonamides group and due to the use of the triple quadrupole analyzer, metoprolol and propranolol were quantified, whereas in the previous study these compounds were not detected in the samples. The levels found for acetaminophen were higher in the present study (maximum 419 μg/Kg (d.w.)) than in the previous study (maximum 42 μg/Kg (d.w.)). On the other hand, the concentration of carbamazepine was lower in the latest study. Moreover, the same concentration levels were found for caffeine and bezafibrate in both monitoring studies.

In this Doctoral Thesis we have demonstrated the presence of different pharmaceuticals and personal care products included in the list of so-called "emerging organic contaminants". The presence of these contaminants is not currently legislated against. Only the Environmental Protection Agency (EPA) defines the third contaminant candidates list in drinking water in October 2009. This list includes some compounds studied in this Doctoral Thesis such as E1, E2, E3, EE2 and erythromycin.

Apart from the compounds studied in this Thesis, there are some other emerging organic contaminants which have been scarcely studied in sludge such as illicit drugs and siloxanes, among others. We consider that it is important to confirm the presence of the emerging organic contaminants in manure produced from sewage sludge because it is possible that they entering the food chain.

Moreover, the results presented in this Thesis are an important contribution to the knowledge of the presence of emerging organic contaminants in sewage sludge which, we hope, will be taken take into account when further legislation is drawn up.

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# 4. CONCLUSIONS

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The conclusions drawn from the studies of the present Thesis can be summarized as follows:

1. The methods developed in this Thesis to determine pharmaceuticals, estrogens and personal care products showed, for most of the compounds, limits of quantifications low enough for their determination in sewage sludge samples.

2. Pressurized liquid extraction (PLE) is a useful technique to extract pharmaceuticals and personal care products from sewage sludge with high recoveries. However, some compounds such as salicylic acid, erythromycin, estradiol 17-glucoronide, estrone 3-glucoronide and 2-phenylbenzimidalzole 5-sulfonic acid were not extracted using PLE.

3. The parameters of PLE that significantly affect the recoveries are solvent extraction, temperature, static extraction time and the number of cycles. The selection of the solvent is very critical because it strongly depends on the characteristics of the analytes. Therefore, to extract a group of contaminants sometimes different solvents must be used, either in a mixture or in succcesive extractions.

4. The experimental design is shown to be a useful tool to optimize the different parameters affecting the recoveries while minimizing the amount of experiments to perform. The fractional factorial design has been successfully applied to optimize the pressurized liquid extraction conditions.

5. Ultra high performance liquid chromatography allows the possibility of extending the usefulness of this widely-used separation technique. This separation technique has been applied to separate eleven personal care products such as parabens, UV filters and antimicrobials. Short analysis time (only 9 minutes to separate 11 personal care products) and low consumption of the solvent were observed in the method developed.

6. Although mass spectrometry with a quadrupole enabled some contaminants to be determined in sewage sludge, using tandem mass spectrometry with a triple quadrupole analyzer meant lower limits of detection, more information on the fragmentation of the target analytes and more identification points to be obtained.

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7. Using the TOF analyzer, the presence of non-target compounds was studied and we could confirm the presence of different surfactants such as nonylphenol ethoxylates, alcohol ethoxylates and phthalates in sewage sludge samples. The LC-MS with a time of flight analyzer is a powerful tool for screening analysis. However, the limits of detection obtained using the TOF analyzer were slightly higher in comparison to a quadrupole analyzer.

8. A unique method to determine a group of PCPs including parabens, antimicrobials and UV filters has been developed. Among this group of compounds, the UV filter OC and the antimicrobial triclosan showed the highest concentration in the sewage sludge samples analyzed (1842 μg/Kg (d.w) and 1490 μg/Kg (d.w.), respectively).

9. Our studies demonstrated for the first time the presence of parabens, two UV filters and estrogen conjugates in sewage sludge.

10. We also demonstrated for the first time the presence of phosphodiesterase type V inhibitors not only in sewage sludge but also in wastewater samples.

11. Of the different groups of pharmaceuticals included in the studies of this Doctoral Thesis, such as anti-inflammatories, antiepileptic drugs and antibiotics, among others, the pharmaceuticals that showed maximum concentration in the sewage sludge samples analyzed were the antibiotics roxythromycin and tylosin (up to 4 mg/Kg).

12. Our studies demonstrated the presence of pharmaceuticals and personal care products in environmental samples such as sewage sludge. The presence of these contaminants in the environment may have negative effects and, for this reason, it is important that these contaminants are taken into account when drawing up further legislation.

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# ANNEXS

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**Annex I.** Abbreviations used in this Doctoral Thesis.

| | |
|---|---|
| ACN | Acetonitrile |
| AHNT | Tonalide |
| APCI | Atmospheric pressure chemical ionization |
| BFRs | Brominated flame retardants |
| CE | Capillary electrophoresis |
| cGMP | Cyclo guanosine monophosphate |
| GC | Gas chromatography |
| GPC | Gel permeation chromatography |
| DAD | Diode array detector |
| DBPs | Disinfection by-products |
| DCM | Dichloromethane |
| DEET | N,N-diethyl-m-toluamide |
| DEHP | Di(2-ethylhexyl)phthalate |
| ECD | Electron capture dectector |
| EDCs | Endocrine disrupting compounds |
| EI | Electron impact |
| ESI | Electrospray ionization |
| FD | Fluorescence detection |
| GPC | Gel permeation chromatography |
| HETP | Height of the theoretical plate |
| HHCB | Galaxolide |
| ICM | Iodinated X-ray contrast media |
| LC | Liquid chromatography |
| LIT | Linear ion trap |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| MAE | Microwave assisted extraction |
| 4-MBC | 3-(4-methyl)benzylidene |
| MeOH | Methanol |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS-MS | Tandem mass spectrometry |
| NCI | Negative chemical ionization |
| NSAIDs | Non steroideal anti-inflammatories drugs |
| OC | Octocrylene |
| OMC | Octyl-methoxycinnamate |
| OT | Octyl triazone |
| PAHs | Polycyclic aromatic hydrocarbons |
| PBDEs | polybrominated diphenil ethers |
| PCBs | Polychlorobyphenyls |

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| | |
|---|---|
| PFCs | Perfluorinated compounds |
| PLE | Pressurized liquid extraction |
| PPCPs | Pharmaceuticals and personal care products |
| PCPs | Personal care products |
| Q | Quadrupole |
| QqQ | Triple quadrupole |
| SBSE | Stir bar sorptive extraction |
| SFE | Supercritical fluid extraction |
| SIM | Selected ion monitoring |
| SPE | Solid-phase extraction |
| SPME | Solid-phase microextraction |
| STPs | Sewage treatment plants |
| TCH | Tetrahydrocannabinoid |
| THC-COOH | Tetrahydrocannabinoid acid |
| TCP | Trichlorophenol |
| TCS | Triclosan |
| TOF | Time of flight |
| UHPLC | Ultra high performance liquid chromatography |
| USE | Ultrasonic extraction |
| US EPA | United State Environmental Protection Agency |
| US NNI | US National Nanotechnology Initiative |
| UV | Ultraviolet |

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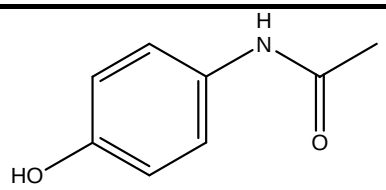
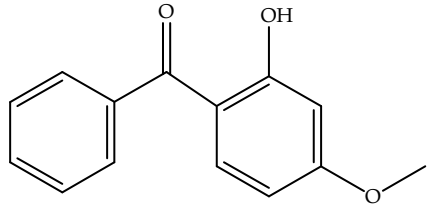
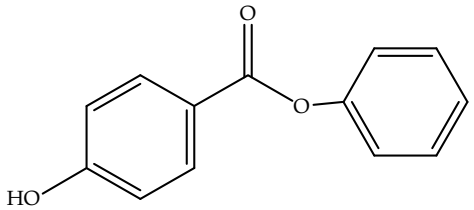
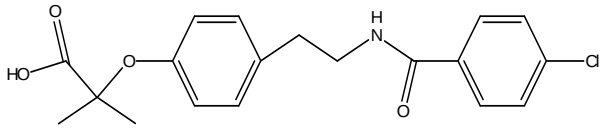
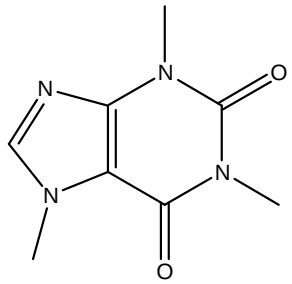
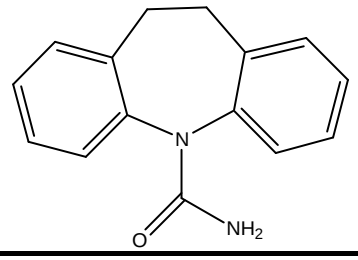
**Annex II.** Structure of the compounds determined in the present Doctoral Thesis.

| Name | Structure |
|---|---|
| Acetaminophen (analgesic) |  |
| Benzophenone-3 (UV filter) |  |
| Benzyl paraben (preservative) |  |
| Bezafibrate (lipid regulator) |  |
| Caffeine (stimulant) |  |
| Carbamazepine (psychoactive drug) |  |

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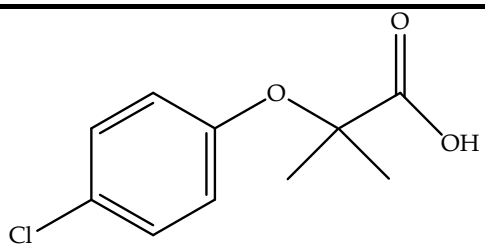
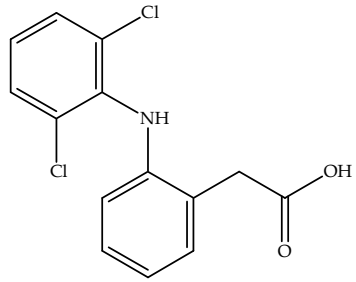
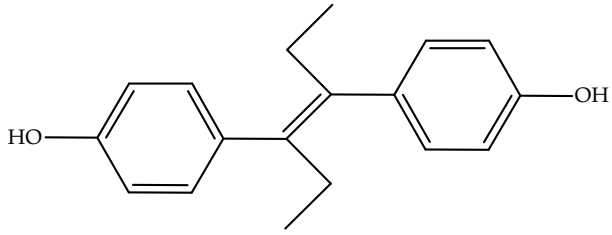
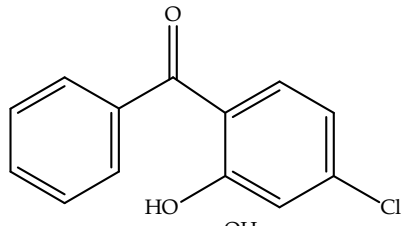
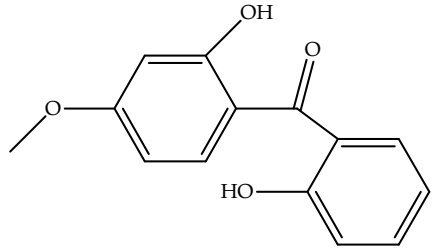
| Name | Structure |
|---|---|
| Clofibric acid (metabolite of clofibrate (lipid regulator)) |  |
| Diclofenac (anti-inflammatory) |  |
| Diethylstilbestrol (estrogen) |  |
| 2,4-Dihydroxy benzophenone (UV filter) |  |
| 2,2′-Dihydroxy 4-methoxybenzophenone (UV filter) |  |

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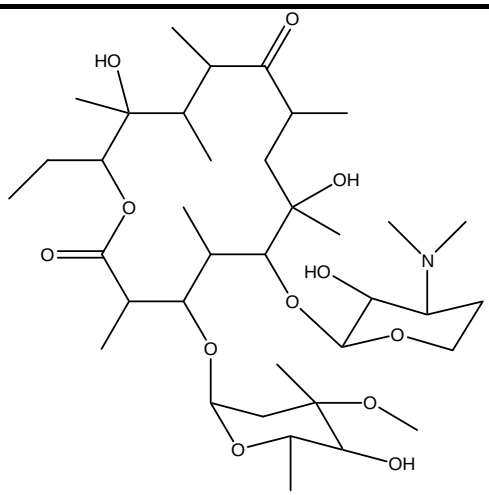
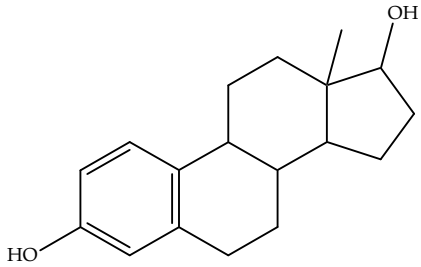
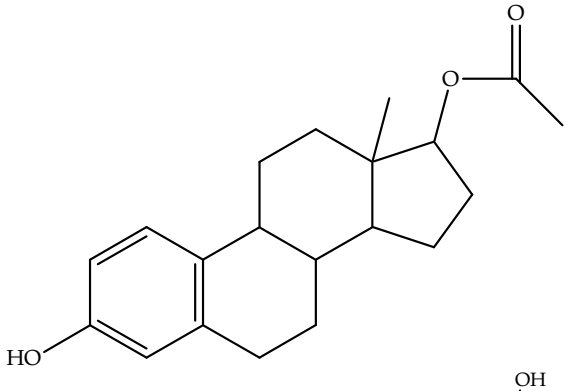
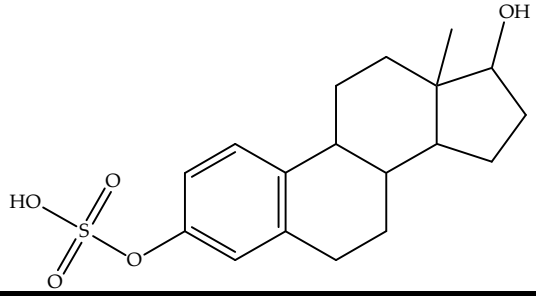
| Name | Structure |
|---|---|
| Erythromycin (macrolide antiobitic) |  |
| Estradiol (estrogen) |  |
| Estradiol 17-acetate (conjugated estrogen) |  |
| Estradiol 3-sulfate (conjugated estrogen) |  |

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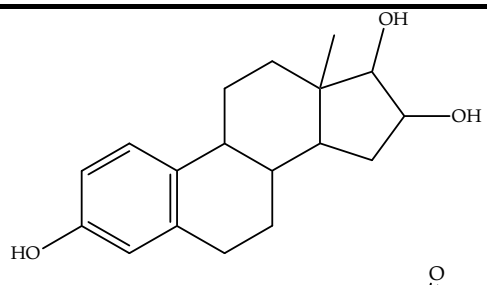
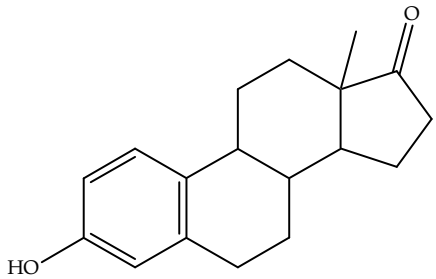
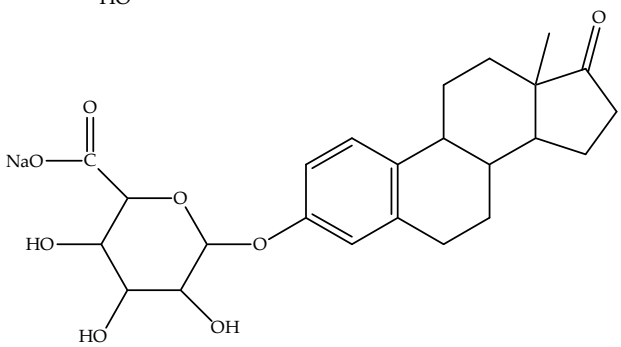
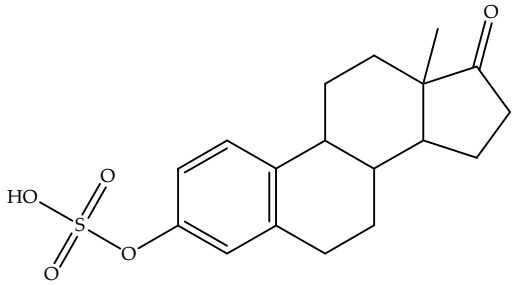
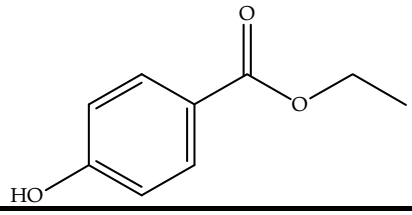
| Name | Structure |
|---|---|
| Estriol (estrogen) |  |
| Estrone (estrogen) |  |
| Estrone 3-glucuronide (conjugated estrogen) |  |
| Estrone 3-sulfate (conjugated estrogen) |  |
| Ethyl paraben (preservative) |  |

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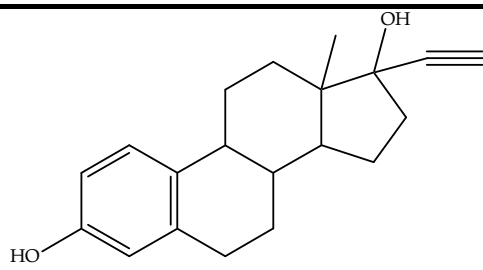
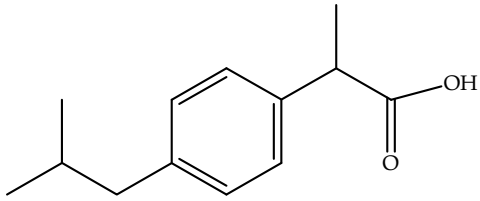
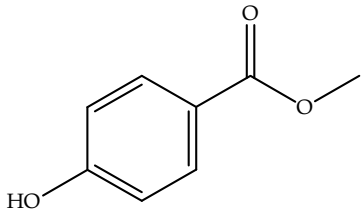
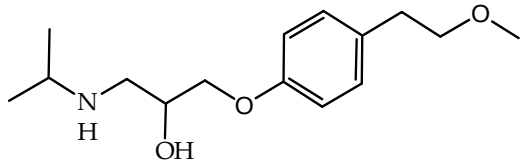
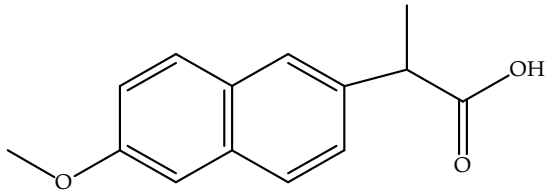
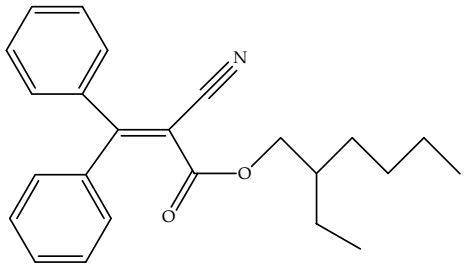
| Name | Structure |
|---|---|
| 17α-ethinyl estradiol (estrogen) |  |
| Ibuprofen (anti-inflammatory) |  |
| Methyl paraben (preservative) |  |
| Metoprolol (β-blockers) |  |
| Naproxen (anti-inflammatory) |  |
| Octocrylene (UV filter) |  |

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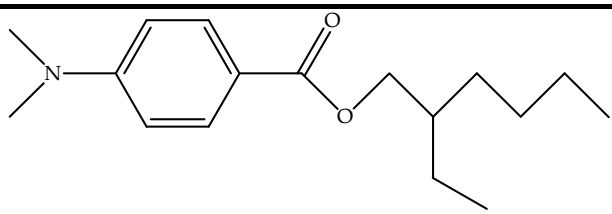
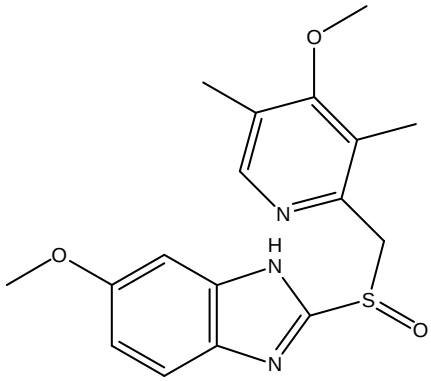
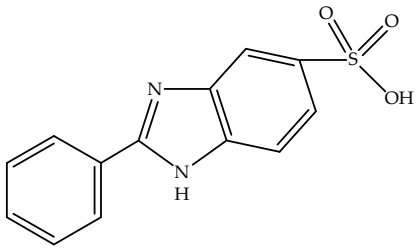
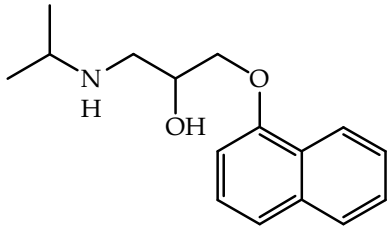
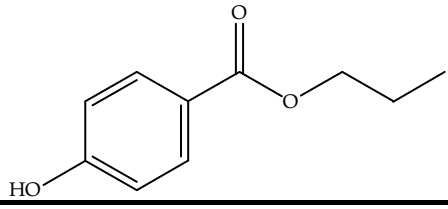
| Name | Structure |
|---|---|
| Octyldimethyl p-aminobenzoic acid (UV-filter) |  |
| Omeprazole (stomach protector) |  |
| 2-Phenyl-benzimidazole 5-sulfonic acid (UV filter) |  |
| Propranolol (β-blockers) |  |
| Propyl paraben (preservative) |  |

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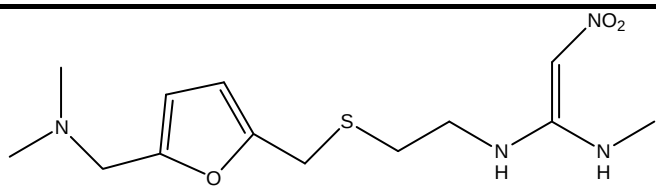
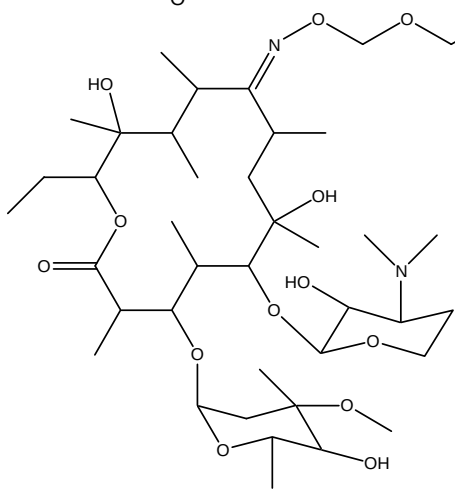
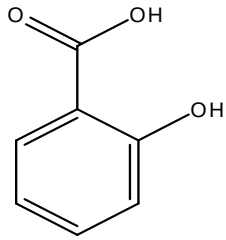
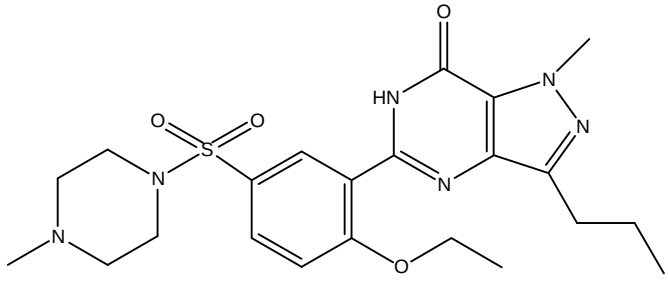
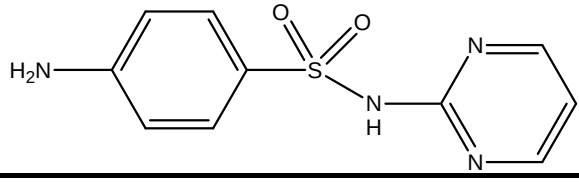
| Name | Structure |
|---|---|
| Ranitidine (stomach protector) |  |
| Roxithromycin (macrolide antibiotic) |  |
| Salicylic acid (metabolite of acetyl salicylic acid (analgesic)) |  |
| Sildenafil (phosphodiesterase type V inhibitor) |  |
| Sulfadiazine (sulfonamide antibiotic) |  |

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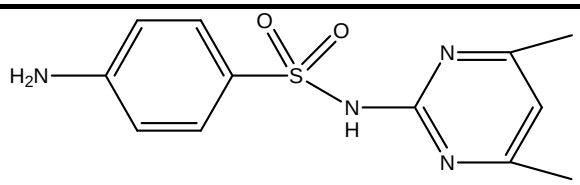
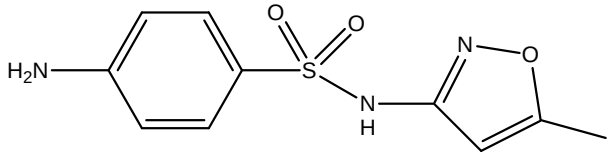
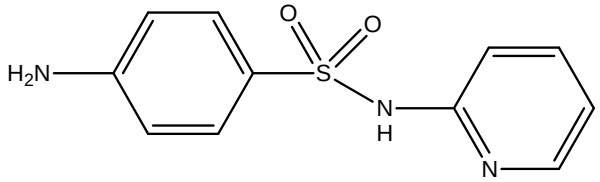
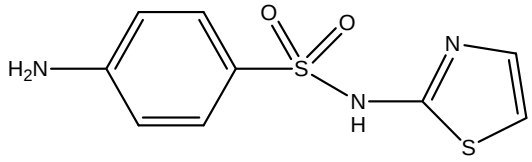
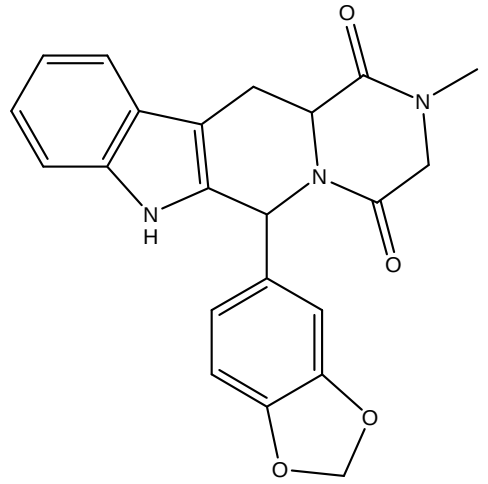
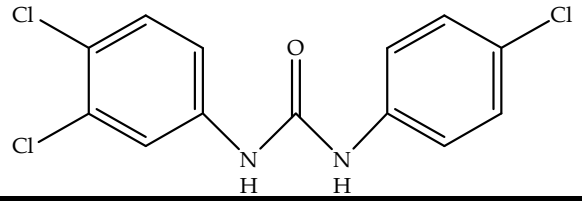
| Name | Structure |
|---|---|
| Sulfametazine (sulfonamide antibiotic) |  |
| Sulfamethoxazole (sulfonamide antibiotic) |  |
| Sulfapyridine (sulfonamide antibiotic) |  |
| Sulfatiazole (sulfonamide antibiotic) |  |
| Tadalafil (phosphodiesterase type V inhibitor) |  |
| Triclocarban (antimicrobial) |  |

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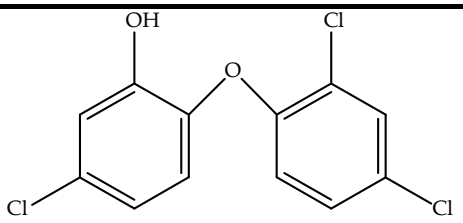
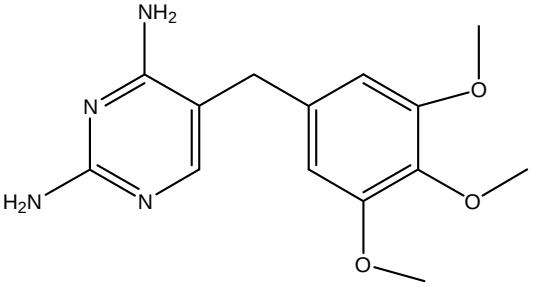
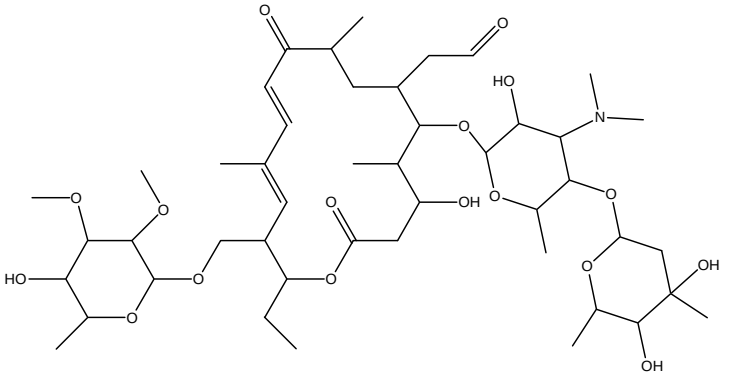
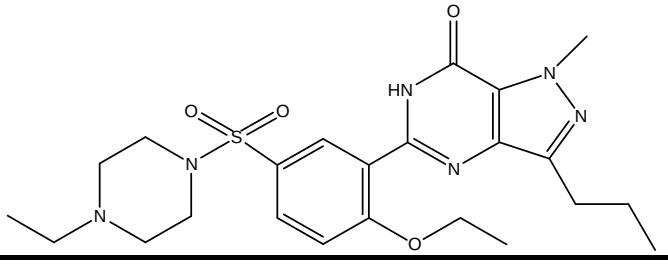
| Name | Structure |
|---|---|
| Triclosan (antimicriobial) |  |
| Trimethoprim (antibiotic) |  |
| Tylosin (macrolide antibiotic) |  |
| Vardenafil (phosphodiesterase type V inhibitor) |  |

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**Annex III.** List of publications obtained in this Doctoral Thesis, included in the introduction and the experimental part, which are published or submitted for publication in different scientific journals.

- A. Nieto, F. Borrull, E. Pocurull, R.M. Marcé, *Pressurized liquid extraction: A useful technique to extract pharmaceuticals and personal care products from sewage sludge,* Trends Anal. Chem. (accepted) (section 1.2.2.5).

- A. Nieto, F. Borrull, E. Pocurull, R.M. Marcé, *Pressurized liquid extraction of pharmaceuticals from sewage sludge,* J. Sep. Sci., 30 (2007) 979-984 (section 3.1.1).

- A. Nieto, F. Borrull, R.M. Marcé, E. Pocurull, *Selective extraction of sulfonamides, macrolides and other pharmaceuticals from sewage sludge by pressurized liquid extraction,* J. Chromatogr. A, 1174 (2007) 125-131 (section 3.1.2).

- A. Nieto, F. Borrull, R.M. Marcé, E. Pocurull, *Multiresidue analysis of sewage sludge using pressurized liquid extraction and liquid chromatography time of flight mass spectrometry,* Analytical Methods (2010) submitted section (3.1.3).

- A. Nieto, F. Borrull, E. Pocurull, R.M. Marcé, *Determination of natural and synthetic estrogens and their conjugates in sewage sludge by pressurized liquid extraction and liquid chromatography-tandem mass spectrometry,* J. Chromatogr. A, 1213 (2008) 224-230 (section 3.2.1).

- A. Nieto, M. Peschka, F. Borrull, E. Pocurull, R.M. Marcé, T.P. Knepper, *Phosphodiesterase type V inhibitors: occurrence and fate in wastewater and sewage sludge*, Water Res. 44 (2010) 1607-1615 (section 3.3.1).

- A. Nieto, F. Borrull, R.M. Marcé, E. Pocurull, *Determination of personal care products in sewage sludge by pressurized liquid extraction and ultra high performance liquid chromatography-tandem mass spectrometry,* J. Chromatogr. A, 1216 (2009) 5619-5625 (section 3.4.1).

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- A. Nieto, F. Borrull, E. Pocurull, R.M. Marcé, *Occurrence of pharmaceuticals in sewage sludge,* Environmental Toxicology and Chemistry (accepted) (section 3.5.1)

Apart from the publications included in the Thesis, a chapter for a book and another review have been published by invitation:

- A. Nieto, F. Borrull, R.M. Marcé, E. Pocurull, *Pressurized liquid extraction of contaminants from environmental samples,* Current Anal. Chem., 4 (2008) 157-167.

- E. Pocurull, A. Nieto, R.M. Marcé, "*Organic contaminants in sewage sludge: determination and occurrence"*, in "Sludge: Types, Treatment Processes and Disposal.". Nova Science Publishers. 2009. ISBN 978-1-160741-842-9.