EMERGING ORGANIC CONTAMINANTS IN AQUATIC ORGANISMS

Mireia Núñez Marcé

DOCTORAL THESIS
MIREIA NÚÑEZ MARCÉ

UNIVERSITAT
ROVIRA i VIRGILI

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DOCTORAL THESIS

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FEM CONSTAR

que la present Tesi Doctoral, que porta per títol: "EMERGING ORGANIC CONTAMINANTS IN AQUATIC ORGANISMS", presentada per MIREIA NÚÑEZ MARCÉ per optar al grau de Doctor per la Universitat Rovira i Virgili amb menció internacional, ha estat realitzada sota la nostra direcció, a l'Àrea de Química Analítica del Departament de Química Analítica i Química Orgànica d'aquesta universitat, que tots els resultats presentats són fruit d'experiències realitzades per l'esmentada doctoranda, i que compleix els requeriments per a poder optar la menció internacional.

I, per a que consti, signem aquest document a Tarragona, 31 de maig de 2017.

Dra. Eva Pocurull i Aixalà   Dra. Núria Fontanals i Torroja

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El meu sincer agraïment a totes les persones que heu col·laborat de manera directa o indirecta en l'elaboració d'aquesta tesi. Sense vosaltres no hagués estat possible. Moltes gràcies.

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ABSTRACT

Water contamination is a problem of great concern. In recent decades interest has focused on certain chemicals referred to as emerging organic contaminants (EOCs), which are not included in current monitoring programs. EOCs are a broad group of chemical substances that include different compounds widely employed in various human and industrial activities.

Analytical methods have been developed to determine the presence of EOCs in different environmental samples, mainly in water compartments. More recently, the scientific community has also focused its attention on determining the presence of these EOCs in aquatic organisms. There is concern that these chemicals could bioaccumulate in aquatic organisms and produce side effects, as well as biomagnify through the food chain.

One of the main objectives of this Doctoral Thesis was to develop new analytical methods to determine the presence of different EOCs in aquatic organisms. A second aim was to conduct an ecotoxicological study of the amphipod species *Gammarus pulex*.

From among the different EOCs we chose to work with pharmaceuticals of widespread consumption, one personal care product (triclosan), iodinated X-ray contrast media (another group of pharmaceuticals employed in diagnostic medicine), and a group of high-intensity sweeteners. It should be highlighted that there have been no previous studies that analyse these last two EOC groups (iodinated X-ray contrast media and high-intensity sweeteners) in aquatic organisms.

The analytical methods include sample treatment, separation and detection. EOCs are expected to be present in the environment in low concentrations and at trace levels in aquatic organisms. Moreover, these matrices represent an additional challenge due to their complexity as they are rich in endogenous components that could interfere with the analysis of EOCs. Therefore, special attention was paid to the treatment of samples, which included extraction and clean-up. Two different techniques for extracting the compounds selected were evaluated: pressurised liquid extraction and QuEChERS. Moreover, different clean-ups procedures for cleaning the extracts to obtain suitable samples for analysis were assessed.

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To be able to quantify the EOCs at trace level the analytical methods are based on liquid chromatography or gas chromatography in combination with mass spectrometry, as this has high selectivity, specificity and sensitivity. In the present Thesis, due to the polarity of the analytes studied, liquid chromatography was selected in all of the studies. Mass spectrometry was employed for detection, using triple quadruple and high resolution mass spectrometry (Orbitrap) as analysers.

Fish species are the organisms commonly used in studies to determine the presence of EOCs, and muscle tissue or the fish homogenate are the matrices that are generally studied. In this Doctoral Thesis different fish species were selected as study organisms for determining the presence of iodinated X-ray contrast media and high-intensity sweeteners. However, bivalves, such as mussels, are also often used in this type of study. These organisms filter large volumes of water for feeding and breathing, and can thus accumulate contaminants. Moreover, they are representative of the local conditions due to their sessile behaviour. In the present Doctoral Thesis we chose different bivalve species to study the presence of of certain widely used pharmaceuticals.

In the ecotoxicological study, the toxicity of triclosan was evaluated in the amphipod species *Gammarus pulex*, different populations that come from different environments (contaminated and uncontaminated) were evaluated in order to establish differences in triclosan sensitivity.

CHAPTER 1. INTRODUCTION

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Modern societies use a wide range of chemical substances in daily life for different purposes (human and animal health, preservation of food, absorption of UV radiation…) and most of these substances become indispensable in our everyday functions. For decades, tons of biologically active substances synthesised for use in agriculture, industry and medicine have been discharged into the environment with no regard as to the possible consequences, and this has resulted in its contamination. The problem first became noticeable at the beginning of the nineteenth century [1]. From the mid-twentieth century pollution was understood as being synonymous with the degradation of natural systems and a number of actions started to be taken, such as the purification of water so as to eliminate organic matter. In addition, problems such as eutrophication or the presence of toxicants were identified. Until the beginning of the 1990s, non-polar hazardous (toxic, persistent and bioaccumulative) compounds such as persistent organic pollutants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls and dioxins) and heavy metals were a focus of interest and awareness as priority pollutants and were therefore included in intensive monitoring programs. However, in the recent decades the development of new and more sensitive methods of analysis has made it possible to become alert to the presence of other potentially dangerous contaminants, globally referred to as emerging organic contaminants (EOCs) [1, 2].

The term EOCs refers to environmental contaminants that are unregulated substances and could potentially have adverse effects on human health and the environment. It is characteristic of these contaminants that they do not need to persist in the environment to cause negative effects, since their high transformation and removal rates are cancelled out by their continuous introduction into the environment. These contaminants are an environmental problem, and there is widespread consensus that this kind of contamination may require legislative intervention [2].

The list of EOCs includes a wide variety of everyday products with both industrial and domestic applications. They include pharmaceuticals, personal care products (PCPs), brominated flame retardants and perfluorinated compounds, among others. The term is used to cover not only these substances but also their metabolites and transformation products [3]. To date EOCs have been characterised in different aquatic environments which they enter via different pathways: effluents from wastewater treatment plants (WWTPs), septic tanks, leakage from landfills,

livestock activities including waste lagoons and application of manures to soil, aquaculture activities, hospital effluents, the subsurface storage of household and industrial waste as well as indirectly through groundwater-surface water interactions [3, 4]. Figure 1 shows the potential sources and routes of EOCs in the environment [5].

In general, there is an overall lack of information on the ecotoxicological impact of most of these EOCs in terms of their toxicity, bioaccumulation and occurrence in different environmental compartments and species. It is difficult to predict their fate in the aquatic environment, but one of the main concerns regarding the presence of EOCs is that they can bioaccumulate in non-target species and may have side effects on them. Their biomagnification through the food chain is also of concern, resulting in human and environmental health effects [6]. Some of these compounds are already reported to be endocrine-disrupting compounds (EDCs). These have the ability to interfere with the normal function of the endocrine system (the set of organs and tissues that secrete hormones), which is responsible for maintaining homeostasis and regulating the developmental processes of humans and wildlife [7].

As mentioned earlier, the analytical methods for determining EOCs in trace quantities in environmental matrices have advanced significantly over the last few decades. These advances have also enabled EOCs to be determined in biota samples. However, this is a difficult task and a major analytical challenge due to the low levels at which the target compounds are present and the high complexity of the biological matrices.

Nevertheless, examining EOCs in biota has become a very important issue because it reveals their movement within organisms and through the food chain, thereby helping us to recognise and quantify damage to organisms and their communities. When it comes to determining the effect of EOCs on aquatic organisms, it is crucial to establish and validate analytical methods that can be used to extract them from biological matrices and to determine them [8].
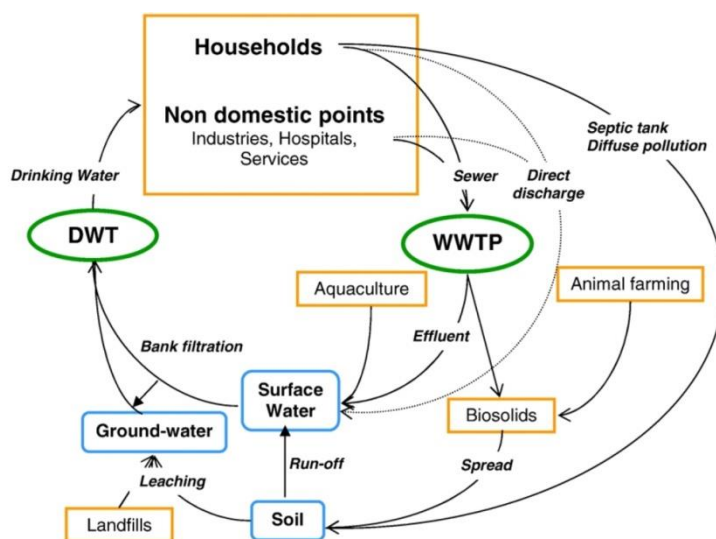
Figure 1. Origin and routes of EOCs (reprinted from Mompelat et al. [5]).

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1.1 Emerging organic contaminants

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As mentioned previously, EOCs are unregulated compounds that have the potential to produce an ecological impact as well as adverse effects on human health. They comprise a wide range of chemical compounds not included in current water treatment monitoring programs, such as pharmaceuticals, PCPs, surfactants, oestrogens, nanomaterials, swimming pool disinfection by-products, industrial additives, gasoline additives, 1,4- dioxanes, plasticisers and industrial additives. They include not only newly developed compounds but also those newly discovered in the environment. Compounds such as sucralose and other artificial sweeteners, antimony, siloxanes, musks and ionic liquids, microplastics have been added to the list of EOCs [9, 10]. Three different groups of EOCs have been studied in the present Thesis, namely pharmaceuticals, PCPs and high-intensity sweeteners. In this section, the characteristics, uses and properties of each group are described. In the following sections their occurrence in different environmental compartments is reported together with their ecotoxicological risk. In cases for which data is available in the literature, information about their occurrence in aquatic organisms is also reported.

1.1.1 Pharmaceuticals

Pharmaceuticals are extensively and increasingly being used in significant quantities worldwide in human and veterinary medicine in order to prevent, cure and treat diseases [11, 12]. These chemicals are designed to have a specific mode of action (through specific metabolic and molecular pathways) in humans and animals [6] and are often classified according to their therapeutic purpose (antibiotic, analgesic…). Table 1 shows a classification [5] in which human and animal pharmaceuticals and metabolites are categorised into 24 different classes according to their therapeutic use, based on studies into analytical developments, occurrence in aquatic compartments, their fate in the environment and their fate and elimination during wastewater and drinking water treatments. A number of compounds belonging to these classes are given as examples. The review published by Santos et al. [13] compiled data from 134 studies published between 1997 and 2009, noting that the most common therapeutic classes detected in the environment are non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics and lipid regulators.

Table 1. List of pharmaceuticals studied in environmental samples according to Montpelat et al. [5].

GROUP	EXAMPLES
Antiacid	Cimetidine, ranitidine
Antianginal	Dehydronifedipine
Antiasthmatic	Albuterol
Antibiotic	Amoxicillin, Cefuroxime, Ceftriaxone
Anticoagulant	Warfarin
Anticonvulsant	Carbamazepine, dilantin
Antidepressant, anti-anxiety, antipsychotic	Diazepam, fluoxetine, imipramine
Antidiabetic	Metformin, Glibenclamide
Antihelminthic	Ivermectin
Antifungal	Clotrimazole
Antihistamine	Diphenhydramine
Antihypertensive	Diltiazem, enalapril, valsartan
Antineoplastic	Tamoxifen, cyclophosphamide
Beta blocker	Acebutolol, atenolol, metoprolol
Birth control	Ethynylestradiol
Bronchodilator	Clenbuterol, salbutamol, terbutaline
Cardiac stimulant	Digoxin
Contrast media	Iomeprol, iohexol, iopamidol
Diuretic	Bendroflumethiazide, furosemide
Lipid regulator	Atorvastatin, bezafibrate, clofibrate
NSAID, analgesic	Diclofenac, fenoprofen, ibuprofen, naproxen
Psycho-stimulant	Caffeine, amphetamine
Vasodilatador	Pentoxifylline
Steroid hormone	Estradiol, progesterone, testosterone

The present Thesis concentrates on pharmaceuticals used in human medicine some of which are also applied in veterinary medicine, thereby focusing on environmentally important compounds belonging to different drug categories, mainly NSAIDs, lipid regulators and iodinated X-ray contrast media (ICM-XR). These classes are distinguishable by their modes of action and were chosen

because of their consumption volumes, toxicity and persistence in the environment.

According to the *Agencia Española del Medicamento* [14], from 2000 to 2012 the consumption of NSAIDs increased by 26.5%. However, since 2009 the trend has been towards a decrease in total consumption. Of NSAIDs, ibuprofen was the most widely used active substance in absolute terms and its consumption as a proportion of total NSAIDs in 2012 was 43.9%. Diclofenac was the second most commonly used NSAID over the study period, after ibuprofen. As regards the lipid regulators, this group also saw an increase in its consumption, with statins being the group most consumed. However, the consumption of fibrates has decreased with respect to the total consumption of lipid regulators.

NSAIDs are commonly used to treat inflammation and pain and to relieve fever, and are sometimes also used for the long-term treatment of rheumatic diseases. The history of these compounds goes back thousands of years to the early uses of decoctions or preparations of plants containing salicylates that were applied to treat rheumatism [15].

It was not until 1860 that salicylic acid was chemically synthesised and used as an external antiseptic, as an antipyretic and in the treatment of rheumatism [16]. In 1897 Felix Hoffman of the Bayer Company made the acetylated form of salicylic acid, acetylating the hydroxyl group on the benzene ring to form acetylsalicylic acid, a more palatable form of salicylate. This compound was given the name aspirin and its main therapeutic actions were antipyretic, anti-inflammatory and analgesic [15]. In the course of time several other drugs were discovered with similar actions to aspirin, which is why they were called "aspirin-like drugs", now known as NSAIDs. These included antipyrine, phenacetin, acetaminophen, phenylbutazone and later, indomethacin, ibuprofen and naproxen [16]. Before 1971 little was known about the mechanisms of action of NSAIDs. They produce an anti-inflammatory effect that is qualitatively and quantitatively different from that of the glucocorticosteroids and their analgesic action was of a different nature to that of the opiates. Despite the diversity of their chemical structures, all these compounds share the same therapeutic properties. They alleviate the swelling, redness and pain of inflammation, reduce a general fever and cure headaches. Moreover, to a greater or lesser extent they share a number of similar side effects.

All this makes it fairly certain that the actions of these drugs are based on a single biochemical intervention [16].

In 1971 Vane discovered the mechanism of action of aspirin and other NSAIDs. They act by inhibiting either reversibly or irreversibly one or both of the two isoforms of the cyclooxygenase enzyme (COX-1 and COX-2), which catalyse the synthesis of different prostaglandins from arachidonic acid. Classic NSAIDs inhibit both COX-1 and COX-2 to different degrees, whereas new NSAIDs act more selectively on COX-2, the inducible form responsible for inflammation reactions. Prostaglandins are known to be involved in processes such as inflammation and pain, the regulation of blood flow in the kidneys, coagulation processes and the synthesis of protective gastric mucosa. Since NSAIDs inhibit non-specific prostaglandin synthesis, most side effects, at least after long-term treatment, are related to the physiological function of prostaglandins [11].

Lipid regulators are basically two types of antilipidemic compounds: statins and fibrates. Both are used to decrease the concentration of cholesterol and fibrates, and decrease triglycerides in blood plasma too [11].

As regards fibrates, one of the first compounds to be discovered with minimal toxicity was ethyl-α-4-chlorophenoxyisobutyrate, which was given the name clofibrate. Although its mechanism of action was unknown at first, several clinical studies showed that it decreased lipid levels in hypercholesterolemic patients, mainly as the result of a reduction in the very-low-density lipoprotein (VLDL) and to a lesser extent in the low-density lipoprotein (LDL) fraction [17]. Intense research was performed in an attempt to modify clofibrate structure to identify the most potent hypolipidemic fibrates (by improving its pharmacological and pharmakinetics activities) with minimum toxicity. Of these modifications, the benzoyl derivative with a chlorine atom in position 4 yielded interesting results. This compound, known as fenofibrate significantly decreased plasma lipid concentrations in hyperlipidemic patients and included improvements compared to clofibrate [17]. In the late 1970s and early 1980s other fibrates such as gemfibrozil in the USA and bezafibrate and ciprofibrate in Europe were introduced. However, the use of fibrates was limited at that time since induction of hepatic carcinogenesis in rats and mice was demonstrated, suggesting that potential side effects could occur in humans. Eventually, it was proved that humans were resistant to these

side effects and could safely use them. Interest in fibrates reappeared in the 1990s first and foremost because their mechanism of action became known [17].

Fibrates regulate lipid metabolism. They bind specifically to peroxisome proliferator-activated receptors and, by stimulating them, activate many metabolic pathways. They increase the plasma triglyceride catabolism by inducing lipoprotein lipase gene transcription and decreasing apoC-III gene transcription. Fibrates stimulate cellular fatty acid uptake, conversion to acetyl-CoA derivatives, and catabolism by beta-oxidation pathways, which combined with a reduction in fatty acid and triglyceride synthesis, results in a decrease in VLDL production. Fibrates increase high-density lipoprotein (HDL) cholesterol by increasing apoA-I and apoA-II expression [11, 17, 18]. Although statins are now the first-line hypolipidemic compounds, fibrates are still widely prescribed [17].

ICM-XRs are a group of pharmaceuticals used in diagnostic medicine to obtain images of soft tissues such as organs and blood vessels. They are designed to absorb X-rays and create a contrast between the organ to be diagnosed and the surrounding tissue. The history of ICM-XRs dates back to 1895 and the discovery of X-rays, which made it possible to view structures that until then were only visible during surgery or autopsy. X-rays allowed the visualisation of bones. However, other parts such as organs were still not visible due to the lack of contrast with the surrounding tissues [19]. Bismuth salts and barium sulphate were the first X-ray contrast media used [19]. The element iodine was incorporated into X-ray contrast media with the compound sodium iodine, which was used for many years but, due to its toxic side effects, was displaced by organic iodine compounds. It was in 1922 that the first organic iodine compound (lipiodol) was introduced [19]. Today almost all intravascular administrations require iodinated contrast agents, and all of them are based on the tri-iodinated benzene molecule, with iodine atoms in positions 2, 4 and 6, while the other benzene positions are occupied by side chains that provide high water solubility, appropriate viscosity and low toxicity [20, 21]. The iodine is responsible for absorbing the X-ray due to its high atomic number, 53, and consequently for obtaining the radiopaque images. ICM-XRs can be classified into four categories depending on the number of triiodo-benzene rings (monomer or dimer) and their ionisation capacity [22]. Figure 2 shows the chemical structure of the tri-iodinated benzene molecule and the four categories.

Ionic monomers include different salts from diatrizoic acid. They have a carboxylic group, which in aqueous solution is dissociated to one anion and one cation. The number of free particles confers high osmolality, higher nephrotoxicity and a higher rate of adverse reactions. However, they have a less marked anticoagulant effect than non-ionic monomers [22].

Non-ionic monomers were developed with the aim of reducing the osmolality of the ionic contrast agents. The carboxyl group in the triiodobenzoic ring is substituted by a hydroxyl group. Iohexol, iomeprol, iopamidol, iopromide belong to this group [22].

The ionic dimer group includes only one commercialised active principle: ioxaglit acid. This is based on two triiodinebenzene rings and also contains a carboxyl group which is responsible for its ionisation [22].

The non-ionic dimer group is represented by just one compound: iodixanol. This has the lowest osmolality of all ICM-XRs. However, it presents a similar nephrotoxicity to other non-ionic monomers due to its high viscosity, since it is a larger molecule (dimer) [22].
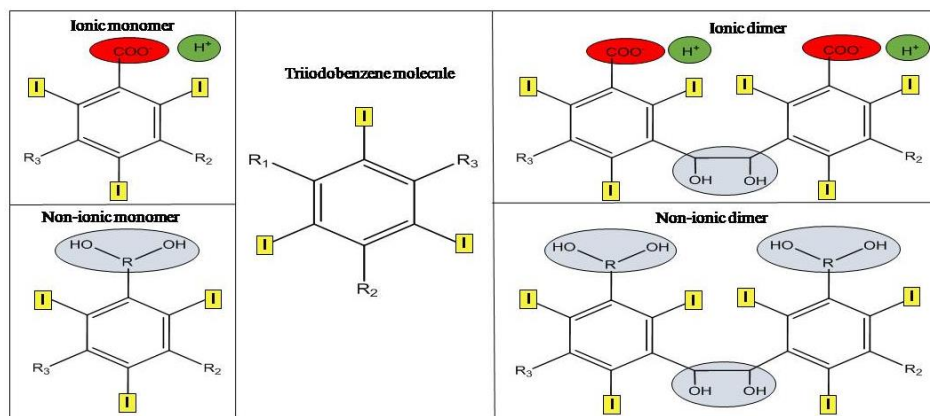
Figure 2. Classification of ICM-XR molecules (adapted from Ramírez Ribelles et al. [22]).

All have common characteristics such as high water solubility, low protein-binding capacity and non-reactive molecules. However, most of the new generation

products belong to the non-ionic monomer group. In order to obtain sufficient contrast, ICM-XRs are administered to patients either intravenously or intra-arterially in aqueous solution at a high concentration, up to 200 g/application [23]. In fact ICM-XRs are used at much higher concentrations and total doses than any other intravascular pharmaceutical. Once inside the organism, their distribution is extracellular and not organ-specific, and they do not enter cells to any significant degree. Their elimination occurs within 24 hours after administration [20, 22].

1.1.2   Personal care products

PCPs are a diverse group of organic chemicals found in different products that are widely used in daily human life, such as soaps, lotions, toothpaste, fragrances and sunscreens, to name but a few [24]. The literature classifies PCPs into five primary classes according to the areas in which they are used: disinfectants, fragrances, insect repellents, preservatives and UV filters [24, 25]. Unlike pharmaceuticals, which are intended for internal use, PCPs are externally applied to the human body and thus not subjected to metabolic alterations. Large quantities of PCPs therefore enter the environment unaltered through normal usage [25].

Of the PCPs, triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a synthetic, non-ionic, broad-spectrum antimicrobial agent that possesses mostly antibacterial but also antifungal and antiviral properties [26]. It is an off-white, odourless, tasteless, crystalline powder categorised as a halogenated aromatic hydrocarbon with phenolic, diphenyl ether and polychlorinated biphenyl substructures (see Figure 3). This means it has chemical properties related to many toxic compounds such as polychlorinated biphenyls, polybrominated diphenyl ethers, bisphenol A and dioxins [27].

Currently named Irgasan DP300 and Irgacare MP for oral applications, triclosan was first synthesised by Ciba-Geigy Co. Originally its usage was limited to health care settings and it was not until 1985 that it was introduced into toothpaste in Europe [26]. During the 1990s its usage increased greatly due to the number of products with antibacterial properties entering the consumer market, most of them containing triclosan. Today triclosan is widely used in numerous consumer products, namely toothpastes, antibacterial soaps (bars and liquids), washing-up liquids, deodorant soaps (bars and liquids), cosmetic and antiseptic products and antiperspirants/deodorants with a typical concentration in the range of 0.1-0.3% of

product weight. It is also used in other consumer products such as kitchen utensils, toys, bedding, clothes, fabrics and bin bags [27].
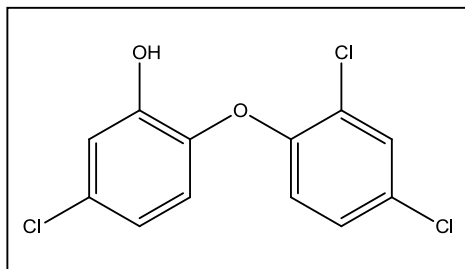
Figure 3. Chemical structure of triclosan.

Several studies on bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, have demonstrated that triclosan is bacteriostatic at low concentrations, since it inhibits fatty acid biosynthesis (impairing the production of bacterial lipids) through inhibition of the enoyl-acyl carrier protein reductase (FabI) enzyme by forming a noncovalent complex with NAD^+ in the FabI active site. As FabI is essential for normal cellular division, the result is that when triclosan-mediated FabI inhibition occurs, the cell membranes are not properly produced and bacterial proliferation stops. At higher concentrations it induces K^+ leakage, leading to membrane destabilisation and a rapid bactericidal effect [27, 28].

The American Medical Association has raised concerns about the use of triclosan in consumer products. In 2009 the American Public Health Association said it would recommend the banning of triclosan for household and non-medical uses [27]. More recently the Food and Drug Administration (FDA) released a final ruling banning the use of triclosan and 18 other antimicrobial chemicals in soaps [29].

1.1.3 High-intensity sweeteners

Another group of EOCs dealt with in this Thesis is high-intensity sweeteners. Sweeteners are defined as food additives that are used or intended to be used either to impart a sweet taste to food or as a tabletop sweetener [30]. They are classified as either high intensity or bulk (Figure 4). High-intensity sweeteners possess a

sweet taste but are noncaloric, contribute essentially no bulk to food, have greater sweetness than sugar and are therefore used at very low levels. Bulk sweeteners, on the other hand, are generally carbohydrates, providing energy (calories) and bulk to food. These have a similar sweetness to sugar and are used at comparable levels [30].

Figure 4. Classification of sweeteners (reprinted from Yebra-Biurrun [30]).

There are several different high-intensity sweeteners. Some are naturally occurring, while others are synthetic (artificial) or semisynthetic (Figure 4). The first high-intensity sweetener discovered was saccharin in 1878, followed by several others. For decades, saccharin was the most important high-intensity sweetener and its consumption increased during periods of sugar scarcity such as the World Wars, although its main consumers were diabetics. Taxes were imposed on saccharin in the early days to protect the sugar industry, but the attitude towards high-intensity sweeteners has improved since then [31]. Their main applications are no longer products specially made for consumption by diabetics or people

suffering from other metabolic disorders. Instead, high-intensity sweeteners are now used in products aimed at consumers interested in body weight management and a calorie-controlled diet [31]. The main advantages of high-intensity sweeteners are that most of them are not metabolised in the human body or are excreted without metabolic utilisation, i.e. they are noncalorific, although some, especially peptide-based sweeteners, are indeed digested and metabolised. Nevertheless, these sweeteners are virtually noncalorific, since normal use levels are extremely low and their contribution to the nutritive value of foods and beverages is insignificant compared with nutritive bulk sweeteners [31]. They have no influence on glycaemic response. Moreover, they are noncariogenic, since they do not support growth of oral cavity microorganisms [30, 31]. These characteristics make them especially helpful in the treatment of obesity, the maintenance of body weight, the management of diabetes mellitus and the prevention and reduction of dental caries [30, 32, 33].

They are mainly applied in foods and beverages for the purposes of reducing calories. For household use, table-top sweeteners such as tablets, powders, spoon-by-spoon products and liquids are consumed. Several cosmetics, especially those used for oral hygiene, also contain sweeteners to make them more pleasant for users. They are also used to mask undesired flavours in pharmaceuticals and in animal feed to ensure balanced nutrition. They are even used in tobacco and tobacco-related products [31].

As mentioned previously, high-intensity sweeteners can be of artificial (chemically synthesised), semisynthetic or natural (extracted from plants) origin. The molecular structure of the high-intensity sweeteners described in this section is detailed in Appendix II. As regards artificial sweeteners, there are three that are commonly used in the form of salt, one of these being saccharin, which is usually used as sodium or calcium salt [32]. However, sodium salt is the most frequently used form and is about 450 times sweeter than sucrose [31]. It should be noted that saccharin sweetness is accompanied by a metallic or bitter taste. In order to mask this side-taste it is often blended with other high-intensity sweeteners, especially sodium cyclamate [31]. Other artificial sweeteners used as salt are cyclamates which are salts (sodium, potassium and calcium) from cyclohexylaminosulfonic acid [31]. Sodium cyclamate is used as a non-nutritive high-intensity sweetener and the analogous calcium salt is used especially in low-sodium diets [32]. However, sodium cyclamate is the most important cyclamate high-intensity

sweetener, the second artificial sweetener discovered after saccharin and ca. 35 times sweeter than sucrose [31]. Cyclamates are suitable for all high-intensity sweeteners applications because of their good stability. Due to their bitter off-taste they are blended with saccharin [31, 32]. One final high-intensity sweetener used as salt is acesulfame–K, which is a high-intensity potassium salt sweetener of 6-methyl-1,2,3-oxathiazine-4-(3*H*)-one-2,2-dioxide and 200 times sweeter than sucrose [31]. It is heat stable, so it can be used for cooking and baking and is often blended with other high-intensity sweeteners (usually sucralose or aspartame), since it may have a bitter after-taste when used alone [32].

Aspartame is a methyl ester of the dipeptide of the amino acids L-aspartic acid and L-phenylalanine. Due to its susceptibility to hydrolytic decomposition and limited temperature stability, it cannot be used for baking or cooking. It provides energy (4 calories/g), although this nutritive value is of no practical importance. It is 180-200 times sweeter than sucrose and is also used in a variety of blends [31]. After the discovery of aspartame, intense research was performed to develop other high-intensity dipeptide sweeteners. One was alitame, which is a dipeptide of L-aspartic acid and D-alanine and a novel amine [32]. It is >2500 times sweeter than sucrose and has good high temperature stability [31]. After being readily absorbed in the gastrointestinal tract, it is rapidly metabolised and excreted [32]. Neotame is a relatively new sweetener derivative of aspartame, 6000 to 10000 times sweeter than sucrose and about 30 to 60 times sweeter than aspartame [32]. It has the intrinsic qualities of aspartame but at the same time offers additional advantages, for example increased stability in the neutral pH range. It is also suitable for baking purposes, among others [34]. It is rapidly metabolised, completely eliminated and does not accumulate in the body [32]. It can be used as a stand-alone sweetener or in blends with others sweeteners such as acesulfame-K [34].

Another artificial high-intensity sweetener is sucralose, made by the selective substitution of three hydroxyl groups of sucrose with three chlorine atoms. The resulting molecule exhibits high stability towards degradation under acidic and high temperature conditions as well as enzymatic hydrolysis. Although sucralose is made from sugar, the human body does not recognise it as such and does not metabolise it, which is why it provides no calories [32]. It is ca. 600 times sweeter than sucrose [31].

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Neohesperidin dihydrochalcone (NHDC) is a semi-synthetic sweetening molecule obtained from neohesperidine or naringin, two flavonoids present in citrus [31]. NHDC is ca. 330 times sweeter than sucrose [31]. It is limited to few applications due to its special taste, but in fairly small quantities it is blended with other high-intensity sweeteners such as acesulfame and aspartame. In addition to sweetness, it also has flavour-enhancing properties when used in a variety of foods and other products [31].

One example of a natural high-intensity sweetener is stevioside. Originally it comes from the plant *Stevia rebaudiana*, which is of South American origin but also grows in some parts of Asia. For centuries the leaves of this species have been used to sweeten foods and beverages, since they contain a number of sweet glycosides, among them stevioside and rebaudioside A. Stevioside can be isolated from the leaves of *Stevia rebaudiana* through extraction with water or water:ethanol mixtures. Stevia extracts are purified to a certain extent but further treatments are usually carried out. Depending on the processing conditions, stevioside products may contain other sweet steviol glycosides and other constituents. It is ca. 160-170 times sweeter than sucrose [31].

Another naturally occurring high-intensity sweetener is glycyrrhizic acid. This is obtained from the liquorice root of *Glycyrrhiza glabra*, which grows in Southern Europe and Asia. It is 50 times sweeter than sucrose and used as a flavouring ingredient in confectionery and pharmaceuticals [31].

Although controversy surrounds the use of sweeteners, most of them have been approved in many countries. Some, however, are restricted or even banned in some countries, as in the case of saccharin. Although it was the first high-intensity sweetener to be introduced, in the 1977 the FDA recommended that is should be banned. Today its use is restricted in several countries under an interim regulation that specifies the amount of saccharin permitted in beverages, processed food and sugar substitute. A special label is required for saccharin-sweetened products [32]. Cyclamate is approved and applied in several countries but is still banned in the USA. Glycyrrhizic acid and NHDC have also not been approved in some countries or have restricted uses [31].

1.2 Occurrence in the environment and aquatic organisms

UNIVERSITAT ROVIRA I VIRGILI
EMERGING ORGANIC CONTAMINANTS IN AQUATIC ORGANISMS
Mireia Núñez Marcé

The occurrence of EOCs in the environment has been reported in thousands of publications over recent decades, demonstrating increasing concern about them [35]. In this section the presence in aquatic organisms of the compounds described in the previous sections is summarised (when there are available data). In order to provide the environmental context, data on concentrations found in the aquatic environment are also briefly presented.

The presence of pharmaceuticals in the environment has become a subject for research over the last years. Their occurrence was first reported in the 1970s in the USA in treated wastewater, where clofibric acid, the metabolite of several fibrates such as clofibrate, was found in the range of 0.8-2 µg/L. Pharmaceuticals were subsequently detected in the 1980s in UK rivers at up to 1 µg/L [11]. Since then knowledge about the environmental occurrence of pharmaceuticals has greatly increased, especially since the mid-1990s, due to advances in and the development of new analytical methods able to determine polar compounds at trace levels [13]. The wide dissemination of pharmaceuticals at low concentrations in different aquatic compartments such as influents and effluents from WWTPs, surface waters, groundwater and drinking water is evident today, and several reviews have already been published on the subject [3, 11, 36-39].

Pharmaceuticals pass through the WWTP without being completely removed and are then continuously discharged into the environment, primarily into surface waters. Elimination rates during the WWTP process vary according to the construction and treatment technology, hydraulic retention time, season and performance of the WWTP [11]. According to Gros et al. [40] only NSAIDs showed high removal efficiencies, with the exception of diclofenac, whose removal rate varied from zero up to 100%. Gracia-Lor et al. [41] found that salicylic acid has shown similar high concentrations in influents and effluents, demonstrating low removal efficiency. It should be noted that not all the compounds show consistent behaviour during conventional treatments. In the case of ibuprofen, for example, while some studies pointed to high removal rates for this compound, others have found similar concentrations in both influent and effluent wastewaters. The removal of bezafibrate was 51%, but varied significantly between WWTPs; high removal rates were found for naproxen (81%); and ICM-XRs were not significantly eliminated. This variation in elimination rates is not surprising, since pharmaceuticals form a heterogeneous group consisting of compounds with diverse chemical properties. The efficiencies of various WWTPs

vary for the same compound due to their technology and treatment steps, and also because of other factors such as temperature and weather [11].

The widely-used NSAIDs ibuprofen, naproxen, diclofenac and some of their metabolites have often been detected in wastewater and surface water. In many countries diclofenac was frequently determined in wastewater in µg/L and in surface water at lower levels [11]. In WWTP effluents, for example, it was determined at concentrations of 1.42 µg/L [42] and 16 µg/L [43] in Belgium and Canada respectively. Diclofenac has also been found in rivers, groundwater, hospital effluents and drinking water, but at concentrations in the ng/L level [13]. However, some studies have reported higher concentrations for this compound. The highest were determined in river waters in Pakistan (4900 ng/L), probably due to the absence of advanced WWTPs in Asia [44]. In Germany, a 1030 ng/L concentration was found in river water [45]. Diclofenac has also been determined in seawater at 4 ng/L [46].

Ibuprofen has been determined in WWTP effluents at concentrations that have reached 85 µg/L in Spain [47] and 24.6 µg/L in Canada [48]. It was almost always found (≥84%) in a WWTP effluent monitoring program in the UK, with median concentrations of 3.09 µg/L and maximum values of 27.3 µg/L [49]. It was also found in river water [46, 49-52]. Kolpin et al. [53] for example, reported that ibuprofen and metabolites were present in 10% of stream water samples (in an extended monitoring study with 139 streams sampled), with maximal concentrations of 1 µg/L (median 0.2 µg/L). Ibuprofen was also found in drinking water [46] and groundwater [3]. Of the NSAIDs studied, it is one of the most frequently detected compounds in seawater [39].

Another widely reported NSAID is naproxen, which has been found in WWTP effluents in a concentration range of between 31 ng/L and 7.96 µg/L according to the compilation by Santos et al. [13]. However, in Canadian WWTP effluents median levels of 12.5 µg/L and maximal levels of up to 33.9 µg/L were encountered [48]. It has also been found in river waters at 156 ng/L [46] and 271 ng/L near a WWTP [50]. This active substance has also been detected in drinking water [13] and determined at 6 ng/L in seawater [46].

Other NSAIDs have also been found in the environment. For example, the deacylated, more active form of acetylsalicylic acid, salicylic acid, has been found

in many municipal wastewaters [41, 46, 50, 54]. Gracia-Lor et al. [41] reported concentrations of up to 276.7 and 236.1 µg/L in WWTP influent and effluent respectively. In a number of studies it has been reported in surface waters [46, 50, 54, 55]. Gros et al. [46], for instance, found a maximum concentration of 76 ng/L in river water. In a study conducted by Wille et al. [56] it was determined in over 90% of the samples analysed in Belgian coastal waters at concentrations of up to 855 ng/L [56]. According to a recent compilation of marine pharmaceutical occurrence, although ibuprofen is the NSAID most frequently detected in seawater, the highest concentrations of NSAIDs found in seawater were for ketoprofen [39]. This compound has also been determined in wastewaters and surface waters usually at ng/L level [41, 46, 50, 57]. In groundwater, the compilation of studies by Lapworth et al. [3] reported an average ketoprofen concentration of 611 ng/L.

Lipid regulators such as fibrates have been determined in several environmental samples. Bezafibrate was found in WWTP influents and effluents [41, 46, 54] in the compilation by Petrović et al. [36], with maximal concentrations of up to 4.6 µg/L (median 2.2 µg/L) being reported and further identified in surface waters at ng/L [46, 51, 52, 54, 58]. Pedrouzo et al. [54], for example, reported concentrations of up to 363 ng/L in river waters. Bezafibrate has also been found in tap water, at between 0.2 and 1.9 ng/L [52]. Wille et al. [56] also determined it below 18 ng/L in seawater.

Clofibric acid, the active metabolite from a series of widely-used blood lipid regulators such as clofibrate, etofyllin clofibrate and etofibrate [11], is one of the most frequently found and reported pharmaceuticals in monitoring studies and has a high degree of persistence [11]. Because of this persistence, it has been found in WWTP influents and effluents and in surface water [36, 51, 59, 60]. Several studies have also revealed its occurrence in groundwater. For instance, Lapworth et al. [3] reviewed concentrations of up to 7300 ng/L. It was the first pharmaceutical detected in tap water; for example Herber et al. [45] reported concentrations of up to 170 ng/L in their study, and it has also been found in seawater [61-63].

Along with other pharmaceuticals, ICM-XRs are ubiquitously distributed in the aquatic environment [64]. Due to the high doses at which they are administrated (200 g/application) and the lack of human metabolism, these compounds are found in wastewaters at µg/L level. Moreover, studies indicate that ICM-XRs cannot be

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eliminated significantly by conventional WWTPs [23]. Iopamidol has been found in municipal wastewater effluents at levels as high as 15 µg/L, and iopromide at up to 21 µg/L. Other compounds such as iomeprol and iohexol have also been determined [23]. In addition, ICM-XRs have been found in surface waters in the ng/L concentration [65]. For example, iopamidol was found in rivers and creeks in Germany (median concentration of 0.49 µg/L) and also in groundwater (0.3 µg/L) [64, 66]. However, other studies have reported higher concentrations for iopromide and diatrizoic acid [66, 67]. Iopromide was determined at 2-4 µg/L in a lake in Germany influenced by a WWTP [68], while concentrations of diatrizoic acid were found at up to 1.1 µg/L in groundwater [66], up to 4 µg/L in surface waters and up to 1.2 µg/L in drinking waters [67].

In recent years the scientific community has also become interested in the presence of these compounds in aquatic organisms. Table 2 summarises the main articles reporting the presence of pharmaceuticals in aquatic organisms, also providing the concentration (in the form of mean values or as a range). These studies include not only the compounds studied in the present Thesis but also other pharmaceuticals and their metabolites. It should be noted that only a few studies also include metabolites as target analytes. However, studies on hormones have not been included in Table 2. Although an increasing number of analytical procedures have been reported in recent years, there are still not many of them, probably because of the challenges associated with the complexity of the biological matrices [6]. To the best of our knowledge, Brooks et al. [69] conducted the first research that reported the presence of pharmaceuticals in aquatic organisms. They found fluoxetine and sertraline and their metabolites in different tissues (brain, liver and muscle) of three species of fish sampled from a stream impacted by effluent discharge. Since then other studies have been published. Freshwater organisms have been considered more often than marine organisms, since one of the most important sources exposing aquatic organisms to pharmaceuticals is wastewater effluent discharge. Moreover, most of these studies have focused on fish species as organisms of interest, with fish homogenate or muscle tissue being the commonest matrix analysed. However, some studies have also evaluated the presence of pharmaceuticals in other tissues such as the brain and the liver, since these have been described as potentially accumulating pharmaceuticals. As far as marine organisms are concerned, bivalves such as mussels are the most frequently studied because they are representative of the sampling area due to their sessile behaviour and they filter large quantities of surface waters for feeding and

breathing, which makes them particularly susceptible to environmental contaminants and therefore very useful as bioindicators for aquatic pollution monitoring [39].

While methods focusing on a single compound or compound class continue to be reported, there is evidence of an increasing emphasis on the simultaneous analysis of compounds with different physiochemical properties (multi-residue methods) in the recent literature [70-74]. Analytical methodologies that enable the simultaneous determination not only of pharmaceuticals but also other groups of EOCs or other contaminants have recently emerged. Of the analytes studied, antidepressants [69, 75-78], antibiotics [70, 73, 74, 79-81] and synthetic hormones [82-85] are the classes most frequently investigated. However, the compound most often studied and determined is carbamazepine [70-74, 77, 86-88], as can be seen in Table 2.

Table 2 shows that the concentrations of pharmaceuticals usually ranged from non-detected up to a level of tens of ng/g, and are therefore comparatively less abundant than other contaminants such as persistent organic pollutants, which can be found at the µg/g level [6]. The highest concentrations of pharmaceuticals were found for antibiotics and NSAIDs at levels of around hundreds of ng/g. Wille et al. [70] reported concentrations of salicylic acid at up to 490 ng/g (dry weight; d.w) and paracetamol at up to 115 ng/g (d.w.) in mussel samples. Diclofenac was found at maximum concentrations of up to 103 ng/g (d.w.) in biofilm samples [87] and ibuprofen at 105.4 ng/g (wet weight; w.w.) [74] and 183 ng/g (d.w.) [89], both in macroinvertebrate samples. As for antibiotics, chlortetracycline was found in fish viscera and muscle at concentrations of 590 and 580 ng/g (d.w) respectively, and sulfadiazine was determined in fish viscera at a concentration of 190 ng/g (d.w.) [80]. Oxytetracycline and tetracycline have also been determined at up to 210 and 530 ng/g (d.w.) respectively [79]. In some studies carried out none of the analysed compounds were found when the samples were analysed [90-92], and these studies have not been included in Table 2.

Nonetheless, data on the fate of pharmaceuticals on biota suggest the existence of a threat to aquatic organisms [6].

Table 2. Presence of pharmaceuticals in aquatic organisms in ng/g (d.w.).

CLASS	COMPOUND	CONC.	ORG.	MATRIX	REF.
Antibiotic	N4-acetylsulfadiazine	ND-<LOQ	Fish	Viscera	[80]
	N4-acetylsulfamerazine	ND-20	Fish	Viscera	[80]
	Ampicillin	ND-<LOQ	Fish	Tissue	[80]
	Azithromycin	1.2-3.0	Bivalve	Homogenate	[73]
	Chlortetracycline	ND-590	Fish	Viscera	[80]
		ND-580	Fish	Tissue	[80]
	Erythromycin A	58-87	Fish		[81]
	Ofloxacin	ND-65	Mussel	Homogenate	[70]
	Oxytetracycline	140-210	Clam	Homogenate	[79]
		ND-50	Fish	Viscera	[80]
	Ronidazole	ND-1.8	Bivalve	Homogenate	[73]
	Roxithromycin	ND-<LOQ[a]	Macro.	Homogenate	[74]
	Sulfadiazine	ND-190	Fish	Viscera	[80]
		ND-50	Fish	Tissue	[80]
	Sulfamerazine	ND-70	Fish	Viscera	[80]
		ND-<LOQ	Fish	Tissue	[80]
	Sulfamethazine	ND-70	Fish	Viscera	[80]
	Sulfamethoxazole	ND-<LOQ	Bivalve	Homogenate	[73]
	Tetracycline	320-530	Clam	Homogenate	[79]
	Trimethoprim	ND-5	Macro	Homogenate	[72]
Anticoagulant	Clopidogrel	<LOQ	Fish	Homogenate	[71]
	Warfarin	ND-7	Macro	Homogenate	[72]
Anticonvulsant	Carbamazepine	ND-11	Bivalve	Homogenate	[70, 73, 86]
		17.9	Fish	Liver	[71]
		ND-6	Macro	Homogenate	[72]
		ND-1.8	Biofilm	Homogenate	[87]
		ND-0.11[a]	Fish	Liver	[77]
		ND-0.11[a]	Fish	Brain	[77]
		<LOQ-1.6[a]	Macro	Homogenate	[74]
		0.03-0.4[b]	Fish	Plasma	[77, 88]

Table 2. (Cont.)

CLASS	COMPOUND	CONC.	ORG.	MATRIX	REF.
Anticonvulsant	10,11-Epoxycarb.	ND-1.3	Bivalve	Homogenate	[73]
	2-Hydroxycarb.	ND-1.3	Bivalve	Homogenate	[73]
Antidepressant, anti-anxiety, antipsychotic	Alprazolam	ND-0.8	Bivalve	Homogenate	[73]
	Amitriptyline	ND-<LOQ[a]	Macro	Homogenate	[74]
	Amitriptyline hydrochloride	1.8	Fish	Liver	[93]
	Azaperone	ND-1.6	Bivalve	Homogenate	[73]
	Bupropion	ND-0.3	Fish	Brain	[76]
	Citalopram	0.8	Fish	Homogenate	[71]
		ND-1.9	Bivalve	Homogenate	[73]
		ND-0.2	Fish	Brain	[76]
	Desmethylsertraline	15.6	Fish	Brain	[69]
		12.94	Fish	Liver	[69]
		0.69	Fish	Muscle	[69]
	Diazepam	ND-9	Macro	Homogenate	[72]
	Fluoxetine	N.D.-79.1[a]	Fish	Homogenate	[75, 78]
		1.58	Fish	Brain	[69]
		1.34	Fish	Liver	[69]
		0.11	Fish	Muscle	[69]
		ND-1.6	Fish	Brain	[76]
	Norfluoxetine	ND-1.08[a]	Fish	Homogenate	[75]
		8.86	Fish	Brain	[69]
		10.27	Fish	Liver	[69]
		1.07	Fish	Muscle	[69]
		ND-3.6	Fish	Brain	[76]
	Norsertraline	0.57-2.8[b]	Fish	Plasma	[77]
		6.4-18[a]	Fish	Liver	[77]
		7.8-16[a]	Fish	Brain	[77]
		ND-28.9	Fish	Brain	[76]
	O-demethyl-venlafaxine	<LOQ-1.4	Bivalve	Homogenate	[73]
	Oxazepam	ND-0.96[a]	Macro	Homogenate	[74]

Table 2. (Cont.)

CLASS	COMPOUND	CONC.	ORG.	MATRIX	REF.
Antidepressant, anti-anxiety, antipsychotic	Paroxetine	ND-0.58[a]	Fish	Homogenate	[75]
		ND-0.11	Fish	Brain	[76]
	Sertraline	4.27	Fish	Brain	[69]
		3.59	Fish	Liver	[69]
		0.34	Fish	Muscle	[69]
		0.14-0.51[b]	Fish	Plasma	[77]
		1-7.9[a]	Fish	Liver	[77]
		1.0-9.2[a]	Fish	Brain	[77]
		ND-4.2	Fish	Brain	[76]
	Temazepam	ND-<LOQ	Macro	Homogenate	[72]
	Venlafaxine	ND-1.12	Fish	Brain	[76]
		2.1-2.7	Bivalve	Homogenate	[73]
		ND-43.7	Biofilm	Homogenate	[87]
		0.6	Fish	Homogenate	[71]
Antihelminthic	Crotamiton	ND- 1.5[b]	Fish	Plasma	[77]
		ND-4.3[a]	Fish	Liver	[77]
Antihistamine	Diphenhydramine	0.25-1.8[b]	Fish	Plasma	[77]
		6.5-64[a]	Fish	Liver	[77]
		6.2-17[a]	Fish	Brain	[77]
Antihypertensive	Diltiazem	ND-11.8	Biofilm	Homogenate	[87]
		ND-1.5	Bivalve	Homogenate	[73]
	Nicardipine	ND-<LOQ[a]	Macro	Homogenate	[74]
	Norverapamil	4.2-20.9	Biofilm	Homogenate	[87]
	Verapamil	11.1-21.7	Biofilm	Homogenate	[87]
Beta blocker	Atenolol	ND-<LOQ[a]	Macro	Homogenate	[74]
	Carazolol	<LOQ-3.8	Fish	Homogenate	[71]
	Propranolol	ND-63	Mussel	Homogenate	[70]
		4.2	Fish	Homogenate	[71]
	Sotalol	<LOQ	Fish	Homogenate	[71]
Bronchodilator	Salbutamol	2.6	Fish	Homogenate	[71]
Diuretic	Hydrochlorothiazide	<LOQ	Bivalve	Homogenate	[73]
Lipid regulator	Bezafibrate	ND-0.14[b]	Fish	Plasma	[77]

Table 2. (Cont.)

CLASS	COMPOUND	CONC.	ORG.	MATRIX	REF.
Lipid regulator	Gemfibrozil	ND-10.3	Biofilm	Homogenate	[87]
		0.35[b]	Fish	Plasma	[88]
NSAID, analgesic	Diclofenac	4.1-8.8	Fish	Homogenate	[71]
		<LOQ-103	Biofilm	Homogenate	[87]
		ND-12.4	Macro	Homogenate	[89]
		ND-51.5[a]	Macro	Homogenate	[74]
		0.15-1.74[b]	Fish	Plasma	[77, 88]
	Ibuprofen	ND-183	Macro	Homogenate	[89]
		0.94-3.8[b]	Fish	Plasma	[77, 88]
		ND-105.4[a]	Macro	Homogenate	[74]
	Indometacin	2.0-4.7[b]	Fish	Plasma	[77]
		1.3-5.4[a]	Fish	Liver	[77]
		0.35-2.0[a]	Fish	Brain	[77]
	Ketoprofen	ND-<LOQ[a]	Macro	Homogenate	[74]
	Mefenamic acid	0.13-0.53[b]	Fish	Plasma	[77]
		4.9- 9[a]	Fish	Liver	[77]
	Nimesulide	ND-36	Macro	Homogenate	[72]
	Paracetamol	ND-115	Mussel	Homogenate	[70]
	Phenazone	ND-<LOQ	Bivalve	Homogenate	[73]
	Salicylic acid	ND-490	Mussel	Homogenate	[70]
Stimulant	Caffeine	ND-21.4	Fish	Homogenate	[7]

ND: not detected; [a]: concentration expressed in w.w.; [b]: concentration expressed in ng/mL; Macro: Macroinvertebrate; 2-hydroxycarb: 2-hydroxycarbamazepine; 10,11-Expoxycarb: 10,11-epoxycarbamazepine.

Triclosan is considered a ubiquitous pollutant detected in all types of environmental compartments including aquatic environments (lakes and rivers), coastal and estuarine waters, WWTPs, drinking water and aquatic organisms [94].

WWTP influent concentrations of triclosan mostly range from 1.86 to 26.8 μg/L [27]. However, Kumar et al. [95] reported concentrations of up to 86.2 μg/L in WWTP influents in the USA. According to several studies the triclosan removal efficiency of WWTPs has been found to be on average 90% [27], and consequently lower concentration levels have been reported in WWTP effluents, usually ranging from 0.027 to 2.7 μg/L [27]. In one study of a Swiss WWTP, during the elimination

process 79% of triclosan was biologically degraded, 15% was sorbed to sludge and 6% left the plant in the final effluent at concentrations of between 42-213 ng/L [96]. Similar results were obtained by Bester [97]. Due to the partial removal efficiency of WWTPs, triclosan exhibits a tendency to accumulate and persist in biosolids [27]. According to an assessment performed in the USA, up to 50% of triclosan in WWTP influent remained in biosolids in WWTPs even after the activation of sludge treatment in combination with anaerobic biosolids digestion [98].

As regards surface waters, according to Montaseri et al. [99] triclosan has been identified as one of the top seven contaminants in surface waters in the USA. In a study during 1999 and 2000 on the occurrence of organic wastewater contaminants, Kolpin et al. [53] had already determined triclosan in 57.6% of the streams studied, with a median level of 140 ng/L and a maximum of 2300 ng/L. In Europe the presence of triclosan in surface waters has been investigated in several countries including Germany, Italy, Greece, Slovenia, Spain, Romania, Switzerland and the UK [94]. It was determined in Italian and Swiss lakes at concentrations of up to 14 ng/L, for example, and in another study also in Switzerland it was found in lakes and river waters at concentrations ranging from 1.4 to 74 ng/L. Maximal concentrations of up to 285 ng/L were found in Spanish rivers [94]. Triclosan has also been detected in drinking waters but at lower concentrations (near its limit of detection) [94], and concentrations of between 6.87 and 99.3 ng/L have been reported in marine water [39].

Studies from the aquatic environment have also measured triclosan in lower to higher trophic-level organisms. Several fish species have been investigated, and to a lesser extent molluscs, dolphins, algae, etc. As regards fish, several tissues and organs such as muscle, brain and gills have been evaluated.

As in the case of pharmaceuticals, early studies conducted to evaluate the presence of triclosan in aquatic organisms focused only on triclosan itself, or on other related compounds [100-103]. Recent studies, however, tend to be multiclass and include other EOCs [7, 77, 87, 88, 104, 105]. Table 3 reports the principal studies on the occurrence of triclosan in aquatic organisms.

Table 3. Presence of triclosan in aquatic organisms in ng/g (w.w).

ORG.	MATRIX	SPECIES	CONC.	REF.
Fish	Bile	*Oncorhynchus mykiss*	710-47000	[100]
		Rutilus rutilus	4400	[100]
		Perca fluviatilis	240-900	[100]
		Zoarces viviparus	440	[100]
	Plasma	*Micropterus salmoides*	3	[106]
		Esox lucius	3.1	[106]
		Morone chrysops	10.3	[106]
		Amia calva	1.9	[106]
		Lepisosteus osseus	2.8	[106]
		Pomoxis nigromaculatus	1.9	[106]
		Catostomus commersoni	2	[106]
		Cyprinus carpio	4.2	[106]
		Ictiobus cyprinellus	3.8	[106]
		Aplodinotus grunniens	5.5	[106]
		Ameiurus nebulosus	0.8	[106]
		Acipenser fulvescens	2.3	[106]
		Ictalurus punctatus	5.5	[106]
		Cyprinus carpio	11-110[b]	[77]
		Danio rerio	0.98[b]	[88]
	Muscle	*Abramis brama*	ND-3.4	[101, 107]
		Ariidae sp.	0.023	[109]
		Pomadasys sp.	0.008	[109]
		Lepomis macrochirus	17-31	[105]
	Gill	*Poecilia vivipara*	<LOQ	[108]
	Liver	*Poecilia vivipara*	<LOQ	[108]
		Cyprinus carpio	110-910	[77]
	Homogenate	*Cyprinus carpio*	ND-1.25[a]	[7]
		Gobio gobio	ND-0.62[a]	[7]
		Luciobarbus sclateri	1.98-17.41[a]	[7]
	Brain	*Cyprinus carpio*	13-88	[77]
Bivalve	Homogenate	*Modiola barbatus*	ND-2578[a]	[110]
		Mytilus galloprovincialis	ND-1385[a]	[110, 111]
		Venus gallina	ND-135[a]	[110]
		Marcia marmorata	0.72[a]	[109]
Gastropoda	Homogenate	*Helisoma trivolvis*	58.7	[103]
Biofilm	Homogenate	-	18-76.5[a]	[87]
Alga	Homogenate	*Cladophora* spp.	<LOQ-162	[102, 103]
Cetacea	Plasma	*Tursiops truncatus*	ND-0.27	[112]
	Plasma	*Orcinus orca*	9	[113]

ND: not detected; [a]: concentrations in d.w.; [b]: concentrations in ng/mL.

As for freshwater species, Adolfsson-Erici [100] analysed fish bile from rainbow trout (*Oncorhynchus mykiss*) that were caged in the receiving waters of a WWTP. The bile from individual wild fish of the species *Rutilus rutilus*, *Perca fluviatilis* and *Zoaces viviparous* caught downstream from a WWTP was also analysed. Concentrations of triclosan ranged from 710 to 47000 ng/g (w.w) for the caged organisms and from 240 ng/g to 4400 ng/g (w.w) in the wild fish. Concentrations of between 0.8 ng/g and 10.3 ng/g (w.w.) were found in the blood plasma of different freshwater fish species [106]. Mottaleb et al. [105] found concentrations of between 17 and 31 ng/g (w.w.) in the muscle tissue of *Lepomis macrochirus*, while for *Abramis brama* concentrations below its limit of quantification (LOQ) and of 3.4 ng/g (w.w.) were found by Rüdel et al. [107]. Similar results were encountered for the same species by Boehmer et al. [101]. Higher concentrations were found in the liver and brain of common carp (*Cyprinus carpio*) [77], while Venquiaruti et al. [108] detected triclosan below its LOQ in the liver and gill of *Poecilia vivipara*. Jakimska et al. [7] reported values of between 0.62 and 17.41 ng/g (d.w.) in the homogenate of different freshwater fish species. For marine fish species lower concentrations (between 0.008 and 0.023 ng/g (w.w.)) were reported [109].

As for organisms other than fish, Coogan et al. [102, 103] determined triclosan in the algae *Cladophora* spp. and in the snail *Heliosoma trivolsis* at concentrations of up to 162 ng/g and 58.7 ng/g (w.w.) respectively. Triclosan has also been determined in biofilm communities at between 18 and 76.5 ng/g (d.w.) [87]. According to the authors, the highest concentrations were found in biofilm affected by the discharge of an effluent from a WWTP and the lowest in the control site [87]. In addition, triclosan has been found in different marine bivalve species, reaching concentrations of up to 2578 ng/g (d.w.) [109-111]. Fair et al. [112] studied its presence on a higher-tropic level, specifically in the bottlenose dolphin marine mammal, with a concentration of up to 0.27 ng/g (w.w.) in plasma. Bennett et al. [113] measured triclosan among other contaminants in plasma from a captive adult female killer whale that for 20 years in captivity had been fed *Clupea pallasii*, *Mastigoteuthis flammea* and *Mallotus villosus*. The authors found concentrations of triclosan of up to 9 ng/g (w.w.) and postulated a biomagnification of this compound through the food chain.

These results show that triclosan was found not only in organisms from rivers impacted by WWTP discharge but also in organisms where no WWTP discharge

is reported, thus revealing its impact on the ecosystem. Moreover, its occurrence is not restricted to freshwater organisms, since several studies pointed to its accumulation in biota inhabiting coastal ecosystems.

Unlike the other EOCs studied in this Thesis (pharmaceuticals and PCPs), high-intensity sweeteners have only recently been considered. Since 2009 the scientific community has become more concerned about their environmental occurrence, fate and possible ecotoxicological effects and the number of publications has increased [33]. Of the high-intensity sweeteners, sucralose was the first artificial sweetener determined in WWTP effluents and surface water in Europe [114]. Today it is one of the most frequently studied sweeteners together with saccharin, cyclamate and acesulfame because of their high concentrations in the aquatic environment and their partial (saccharin and cyclamate) or limited (acesulfame and sucralose) removal in WWTPs [115]. According to Scheurer et al. [116], acesulfame and sucralose appear to be the most stable high-intensity sweeteners.

Sucralose has been determined in wastewaters in different countries including Switzerland [117], Germany [116, 118], Greece [119], the USA [120, 121], Canada [122] and Spain [123-125]. For example, it was present in WWTP influents at concentrations of between 2.0 and 9.1 µg/L [117] and between 6 and 26 µg/L [119] in Switzerland and Greece respectively. These studies found similar concentrations in effluent wastewaters [117, 119]. As regards surface waters, sucralose was found at concentration levels as high as 1 µg/L in river waters from the UK, Belgium, France, Switzerland, Italy, Spain, the Netherlands, Norway and Sweden, whereas in Germany and Eastern Europe lower concentrations were reported (100 ng/L) according to the compilation by Kokotou et al. [115]. However, Ordóñez et al. [125] reported concentrations at up to 5.3 µg/L in river waters from Spain for this compound. It has also been found in drinking water at up to 465 ng/L [126] and groundwater at up to 2.4 µg/L [120]. In addition, it has been found in coastal and marine environments [63, 127-129], with concentrations of between 6.41 and 32.3 ng/L being found in the North Sea [63], for example.

Another very persistent sweetener, acesulfame, which is also not completely removed in WWTPs, has been determined in several environmental waters [116, 117, 123, 125, 130]. For instance, it has been reported at concentrations of between 60-70 µg/L [116] and 12-43 µg/L [117] in WWTP influents from Germany and Switzerland respectively, although a maximum concentration of up to 304 µg/L

was reported in Germany [118]. As regards effluents from WWTPs, concentrations of between 14-46 µg/L in Switzerland [117] and maximal concentrations of up to 99 µg/L in Germany [118] have been reported. Acesulfame has also been found in surface waters, groundwater and drinking water. Kokotou et al. [115] compiled the results from several studies which reported concentrations from 0.01 µg/L to 2.8 µg/L in surface waters. However, the maximal concentrations (up to 53.7 µg/L) were determined in river waters in Spain by Ordóñez et al. [125]. This compound has also been determined in marine environments [63, 128] such as the North Sea, for instance, where it was found at between 0.94 and 9.7 ng/L [63].

Saccharin is another frequently studied sweetener [116, 117, 119, 123, 125] and has been found in influents from WWTPs at concentrations of between 34-50 µg/L in Germany [116] and between 3.9-18 µg/L in Switzerland [117]. According to Scheurer et al. [114], this compound is greatly eliminated in WWTPs. Kokotou et al. [119], for example, reported concentrations of saccharin of between 15-46 µg/L in WWTP influent and up to 0.3 µg/L in WWTP effluent. It was also found in surface waters at lower levels (50-150 ng/L) [115], although Ordóñez et al. [125] reported concentrations of up to 19.7 µg/L in river waters in Spain. Saccharin has also been found in groundwater at values of up to 0.26 µg/L [115] and in tap water at ng/L level [128]. In addition, it has been determined at up to 3.01 ng/L in the North Sea [63] and up to 249 ng/L in the Bohai Bay (China) [128].

Several studies have also reported the presence of cyclamate in the environment [116, 117, 123, 125, 128]. It was found at concentrations of up to 190 µg/L in WWTP influents [115]. As in the case of saccharin, cyclamate has been reported to be greatly eliminated in the WWTP process. Buerge et al. [117] for example, reported concentrations in Switzerland of between 10 and 65 µg/L in WWTP influents and between <LOD and 0.82 µg/L in effluents. It has also been reported in surface waters at the ng/L level [115], but was found at up to 15.7 µg/L in river waters in Spain [125]. Gan et al. [128] have also reported its presence in tap water (29-35 ng/L) and in the Bohai Bay in China at up to 252 ng/L.

Other high-intensity sweeteners such as aspartame, NHDC, neotame and alitame are not frequently detected [128]. They have been found at concentrations not exceeding 2 µg/L for aspartame, 0.4 µg/L for NHDC and 10 ng/L for neotame in WWTP influents. However, in the same study these compounds were not detected in WWTP effluents [131]. As regards surface waters, in the same study [131],

while acesulfame, cyclamate, sucralose, saccharin and aspartame were present in all the sampled surface waters, NHDC and neotame were less frequently determined, being present in only 27 and 16 samples out of 43 respectively, whereas the concentrations of aspartame, neotame and NHDC were 40 ng/L, 9.3 ng/L and 220 ng/L respectively [131]. In Scheurer et al. [116], although, acesulfame, saccharin, cyclamate and sucralose were determined in all the German rivers investigated, neotame, aspartame and NHDC were not detected in any river samples or in WWTP influent and effluent samples [116].

To the best of our knowledge no studies have been carried out to evaluate the occurrence of high-intensity sweeteners in aquatic organisms, presumably due to the novelty of these EOCs.

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1.3 Ecotoxicology

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Over the last 10-15 years a significant amount of research has been conducted into the risks to the environment posed by EOCs such as pharmaceuticals and PCPs. This section will present the most important toxicity studies published regarding the compounds dealt with in this Thesis.

In order to assess toxicity effects on non-target organisms, specific tests must evaluate both acute and chronic effects. In acute toxicity tests mortality is often registered, whereas in chronic toxicity tests other endpoints are usually evaluated, such as the growth index or reproduction rates. The acute effects of different trophic levels in organisms predominate over chronic effects, and bioaccumulation, probably due to the complexity of the work involved in the latter [13].

The scientific community is in broad agreement as regards the possibility that adverse effects may arise from the presence of pharmaceuticals [13]. Although pharmaceuticals are delivered at low concentrations, because of their continuous input they are considered to be pseudo-persistent contaminants. In addition, they are designed to be highly specific and are therefore extremely potent, even at very low concentrations. They are able to pass through biological membranes and reach specific cells and tissues. Since many of the biological systems targeted are common among vertebrates, fish are the most likely vertebrate organisms to be affected by pharmaceuticals in the aquatic environment. However, information on their effects in other aquatic organisms such as invertebrates and aquatic plants is limited, and the extrapolation of effects is not always possible as they have systems structures that function differently [6].

The first widely noted case of pharmaceuticals causing major ecological damage was in the early 1990s in Asia, where three vulture species declined by more than 98%. They were exposed to diclofenac through the consumption of carcasses of livestock that had been treated with this compound before death. The vultures died from kidney failure, with clinical signs of extensive visceral gout and renal damage. After these incidents diclofenac attracted much worldwide attention and became one of the most studied NSAIDs [132].

As a result of this, in 2013 diclofenac was selected for inclusion on the "EU Water Framework Directive" watch-list in order for sufficient monitoring data to be gathered for the determination of risk reduction measures. Regulatory measures

governing the use of diclofenac have been imposed by only a few countries. For example, the UK included this compound on a list of priority substances to force the water industry to seek technologies for its removal from wastewater. However, on a global scale, there is no strict legislation to control its environmental presence [44]. Recently a watch-list of substances for European Union-wide monitoring was reported in Decision 2015/495/EU, which contained 10 substances/groups of substances including the NSAID diclofenac [133].

According to the compilation of studies provided by Puckowski et al. [134], acute toxicity data for the class of NSAIDs do not generally reveal a high toxic impact on the organisms tested. For example, in tests on *Daphnia magna* in which mobility inhibition was evaluated during a 48 h exposure, the lowest median effective concentration (EC_{50}) value was for diclofenac (22.4 mg/L), followed by the mean values for aspirin and ibuprofen (88.1-108.0 mg/L) while the highest was for naproxen (166.3-174 mg/L).

Early studies conducted by Ferrari et al. [135] on bacteria, algae, microcrustaceans and fish showed relatively low toxic effects produced by diclofenac. In *Daphnia magna* at acute concentrations such as mg/L, diclofenac induced a high mortality rate. Other studies performed on the alga *Scenedesmus vacuolatus* show reproduction inhibition at a concentration of 23 mg/L, indicating no specific toxicity [13]. Studies on the fish species Japanese medaka (*Oryzias latipes*) indicate that diclofenac induced a reduction in hatchability and a delay in hatching at 1 mg/L or higher. Similar results were obtained regarding the development of zebrafish (*Danio rerio*) embryos, where delayed hatching at 1 and 2 mg/L was observed [44]. Chronic studies demonstrated that this compound induced changes in several organs (liver, kidney and gills) after 28 days' exposure to 1-5 µg/L in the species *Oncorhynchus mykiss* [136]. Similar results were obtained after 21 days' exposure to 0.5- 50 µg/L in brown trout (*Salmo trutta*) [137]. According to Lonappan et al. [44], recent studies have demonstrated that mussels are very sensitive to diclofenac, since tissue damage was already observed at the ng/L concentration level.

Another widely studied NSAID is ibuprofen. Studies on *D. magna* showed that population growth was reduced when it was exposed to concentrations of between 0-80 mg/L. Although survival was only affected at the highest level, reproduction was affected at all concentration levels and completely inhibited at the highest

[138]. The compilation by Santos et al. [13] noted that the amphipod *Gammarus pulex* showed a reduction in activity when exposed to ibuprofen concentrations ranging from 1 to 10 ng/L. Photosynthetic organisms were also affected by this compound, for example the cyanobacterium *Synechocystis* sp., which increased its growth when exposed to concentrations of ibuprofen of between 1-1000 µg/L for 5 days, whereas the growth of the plant *Lemna minor* was negatively affected after 7 days at a concentration of 1 mg/L [13]. The female *Oryzias latipes* showed several changes after being exposed to concentrations of between 1 and 100 µg/L for a period of 6 weeks, including a sharp rise in liver weight, enhanced egg production and a reduction in the number of weekly spawning events [139, 140]. In addition, a delay in the hatching time of embryos exposed to concentrations as low as 0.1 µg/L was observed [140]. *Danio rerio* exposed to ibuprofen for 21 days showed a significant decrease in egg production, reduced hatchability and increased embryo mortality at 1-10 µg/L [141].

Other studies on NSAIDs have revealed that acetylsalicylic acid affects reproduction in *D. magna* and *Daphnia longispina* at a concentration of 1.8 mg/L [142] and induces DNA damage in the mg/L range [143]. As mentioned earlier, salicylic acid, the active metabolite of acetylsalicylic acid, has been reported in the aquatic environment, but there is a lack of studies on the effects of this metabolite on aquatic organisms. Zivna et al. [144] determined that exposure to salicylic acid at different levels (from 4 to 20000 µg/L) can have effects on the early life stages of *Cyprinus carpio*. *Salmo trutta* exposed to concentrations of between 25 and 100 µg/L showed oxidative stress and non-specific histological changes in gills [145]. Environmentally relevant concentrations of this metabolite did not have negative effects on *Vibrio fischeri*, *D. magna*, *Ceriodaphnia dubia* or *Selenastrum capricornutum* [146].

According to Overturf et al. [147], NSAIDs may affect fish reproduction, but such effects would likely only occur chronically and at high environmental concentrations.

Regarding lipid regulators, the acute toxicity of clofibrate showed median lethal concentrations (LC_{50}) at values between 7.7 and 39.7 mg/L (96 h), with the fish *Gambusia holbrooki* being the most sensitive organism according to the compilation by Fent et al. [11]. As regards bezafibrate, studies on the bacterium *Vibrio fischeri*, the cyanobacterium *Anabaena* sp., the crustacean *D. magna*, the

fish fathead minnow (*Pimephales promelas*) and the hydroid *Hydra attenuata* revealed effect concentrations in the mg/L range [148-151], although the lowest observed effect concentration (LOEC) of 47 µg/L was found for *Ceriodaphnia dubia*. Studies on bivalves revealed that environmental concentrations of bezafibrate can modify fundamental cell functions in *Dreissena polymorpha* and *Mytilus galloprovincialis* and affect the larval development of the latter in the range of 10 and 1000 µg/L [152-154]. As mentioned in the previous section, clofibric acid is the main active metabolite of several fibrates and because of its high degree of persistence in the environment, has frequently been used to assess toxicity. In Ferrari et al. [135], low acute toxicity was encountered in bacteria and crustaceans along with low chronic toxicity in algae and crustacean and fish embryos. However, the study found that rotifers in chronic toxicity studies were the most sensitive organisms with a no observed effect concentration (NOEC) of 0.25 mg/L. The low toxicological potential was in agreement with other studies [155]. Meanwhile *D. magna* reproduction was affected by concentrations of between 10 µg/L and 100 µg/L in acute tests for clofibric acid, increasing the proportion of male offspring [156]. The fish species *P. promelas* also showed changes in reproductive functions in the shape of reduction in sperm count, reduced sperm motility and indications that the plasma androgen concentration was also reduced after 21 days of exposure [157]. In addition, *Oncorhynchus mykiss* showed cytological changes in gills when it was exposed for 28 days to 5 µg/L of clofibric acid [158].

As regards ICM-XRs, short-term toxicity tests have shown that iopromide has no toxic effect on bacteria (*Vibrio fischeri*, *Pseudomonas putida*), algae (*Scenedesmus subspicatus*), daphnids (*D. magna*) or fish (*Danio rerio*, *Leuciscus idus*) even at concentrations as high as 10 g/L. Moreover, in chronic toxicity tests on *D. magna*, no effect was observed at a maximum concentration of 1 g/L of the same compound [159]. According to Santos et al. [13], although it is accepted that ICM-XRs do not exhibit toxic effects at high concentration levels, additional studies should be undertaken with a view to evaluating chronic effects due to the continuous exposure of aquatic organisms to these pharmaceuticals.

Ecotoxicological data showed that mixtures have different effects to single compounds. Some examples show that a mixture of pharmaceuticals at environmentally relevant concentrations may exhibit additive effects. Acute exposure of *D. magna* to a mixture of 36 µg/L fluoxetine and 10 or 100 µg/L

clofibric acid caused significant malformation and mortality respectively, while no apparent effects for the same concentrations of individual pharmaceuticals were observed [156].

The toxicity of triclosan has been studied using several types of organism, and algal species seemed to be among the most vulnerable to its toxic effects. For example, early studies performed by Orvos et al. [160] on the alga *Scenedesmus subspicatus* encountered a 96 h biomass EC_{50} of 1.4 µg/L and a 96 h NOEC of 0.69 µg/L. Similar evidence regarding algal sensitivity was found for *Selenastrum capricornutum* [161] and the marine phytoplankton species *Dunaliella tertiolecta* [162]. A study into the short-term effects on biofilm algae and bacteria showed that environmental concentrations of triclosan caused an increase in bacterial mortality with an NOEC of 0.21 µg/L [163]. A study measuring the growth-inhibiting effect of 12 antibacterial agents indicates that triclosan is one of the most toxic for the freshwater microalga *Pseudokirchneriella subcapitata*, with an NOEC of 200 ng/L [28].

As far as invertebrate species such as *D. magna* are concerned, an EC_{50} of 390 µg/L after 48 h exposure was estimated by Orvos et al. [160]. Kim et al. [164] studied the toxicity of triclosan in another crustacean species, *Thamnocephalus platyurus*, encountering LC_{50} of 0.47 µg/L after 24 h of exposure. The LC_{50} reported after chronic exposure (10 days) to triclosan in *Chironomus tentans* and the freshwater amphipod *Hyalella azteca* was 0.4 and 0.2 mg/L respectively [165]. Canesi et al. [153] reported changes in the hemocytes of the mussel *Mytilus galloprovincialis* after 30 min exposure to triclosan. Geiss et al. [166] studied the effects of 28 days' exposure on the freshwater mollusc *Potamopyrgus antipodarum* and suggest that triclosan may cause reproductive effects at environmentally relevant concentrations and present a potential risk, since values for NOEC of 0.17 µg/L and LOEC of 0.666 µg/L were reported.

In adult fish, acute LC_{50} concentrations after 96 h of exposure to triclosan ranged from 340 µg/L in *Danio rerio* [167] and 370 µg/L in bluegill (*Lepomis macrochirus*) [160] to 1700 µg/L in *Oryzias latipes* [168]. Serious effects in the developmental stages were observed [160, 167, 169, 170]. The species *Oncorhynchus mykiss*, for example, was sensitive to triclosan in its early life stages, with significant effects on the survival rate (LOEC of 71 µg/L and NOEC of 34.1 µg/L) [160]. It also affected the mortality of embryos in *D. rerio* [167] and

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O. latipes [169]. Apart from mortality, sublethal effects have been reported for different fish species. For instance it has been demonstrated that triclosan induced delay in hatching and an increase in morphological deformities in *D. rerio* [167] and *O. latipes* [169, 170]. In addition, it induced developmental and biochemical changes resulting in altered swimming and feeding behaviours in several fish species [167, 170, 171]. Its effects were also investigated in amphibians and several studies indicate that there is evidence that triclosan affects behaviour and survivorship in tadpoles, although the effects seem to be species-specific [172]. In animal models, many lines of evidence have suggested that it has adverse effects on the endocrine function, thyroid homeostasis and antibiotic resistance [28].

As for aquatic organisms, the data clearly show that the risk from triclosan should be more related to chronic effects than acute impact. However, the sensitivity of some species even at environmental concentrations shows that ecosystems may be disturbed [94].

As regards high-intensity sweeteners, most of the studies hitherto performed to evaluate the toxicity of this group of compounds have focused on sucralose. Nevertheless, the data available so far on the environmental distribution and ecotoxicological impact of artificial sweeteners is still limited [115]. Early studies conducted in the 1980s to evaluate the toxicity of sucralose, compiled in the publication by Tollefsen et al. [173], revealed no adverse effects in acute toxicity tests on different aquatic organisms such as *D. magna*, *Lepomis macrochirus*, the green algae *Selenastrum capricornutum* and *Oncorhynchus mykiss*, and also no chronic toxicity effects on *D. magna* [173]. Nevertheless, more recent studies have performed chronic toxicity tests on different aquatic species. For example, Soh et al. [174] concluded that sucralose did not affect the uptake or growth rate of the aquatic macrophyte *Lemna gibba*. Along similar lines, Huggett et al. [175] encountered a low risk of adverse effect, since no significant reduction in survival or reproduction were observed at concentrations of up to 1800 mg/L (21 days) in *D. magna* and up to 93 mg/L (28 days) in *Americamysis bahia*. Lillicrap et al. [176] concluded that sucralose does not bioaccumulate after 48 h of exposure in aquatic organisms such as *Pseudokirchneriella subcapitata*, *D. magna* and *D. rerio*.

Stolte et al. [177] assessed the toxicity of four artificial sweeteners (acesulfame, cyclamate, saccharin and sucralose) and the natural sweetener stevioside in short-term tests on the microbial wastewater treatment community, *Scendesmus*

vacuolatus and *D. magn*a, and also in chronic tests on *Lemna minor*. The results obtained pointed to no significant effects at concentrations of up to 1000 mg/L.

Although the above-mentioned studies indicate low bioaccumulation potential and negligible acute and chronic toxicity, persistency combined with increasing usage of sucralose call for more detailed ecotoxicological assessment that include sub-lethal effects. With this in mind, Wiklund et al. [178] studied the behavioural and physiological effects of sucralose in crustaceans. Among the endpoints evaluated, altered swimming height and increased swimming speed were observed for *D. magna*, while gammarids needed more time to reach food and shelter when concentrations of sucralose were increased. The authors also agree with previous studies in which sucralose did not bioaccumulate in gammarids. In another study conducted by Hjorth et al. [179], two species of copepods, *Calanus glacialis* and *Calanus finmarchicus* were exposed to different concentrations of sucralose between 0 and 50 µg/L for a period of 96 h. Although a weak response was observed, *C. glacialis* was slightly more sensitive than *C. finmarchicus*, since its food intake increased with a higher concentration of sucralose.

A recent study saw the exposure of *Desmodesmus subspicatus*, *D. magna* and *Lemna minor* to concentrations of 100 mg/L of aspartame and saccharine. The authors found that in *L. minor* aspartame had a negative impact on the numbers and growth of fronds [180].

According to the above findings, sucralose does not alter the survival, growth and reproduction of aquatic organisms. However, changes in normal behaviour have been reported, and this should be taken into account since a linkage to higher level effects (e.g. population) is as yet unclear [173].

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1.4 Determination of emerging organic contaminants in aquatic organisms

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EOCs are a very large group of organic chemical compounds that are continually released into the environment. As mentioned before, this Thesis focuses on the determination of EOCs in aquatic organisms. Because these are highly complex matrices, determining EOCs in them calls for the use of laborious, time-consuming analytical procedures. Because of the wide variety of contaminants that comprise EOCs, their low concentrations and the composition of the matrix, the methods developed to determine these compounds in aquatic organism samples usually include an efficient extraction technique and a chromatographic separation technique with selective and sensitive detection. In addition, between the extraction and separation step, a clean-up steps is usually required because of the complexity of the matrix.

In the following sections the extraction and determination techniques most often used in analytical methods to determine EOCs in aquatic organisms are discussed. As regards extraction techniques, the next section presents a review paper in which the most recent publications are compiled by way of emphasising the new trends and advances. The paper includes other EOCs in addition to those dealt with in the present Thesis, namely other PCPs (parabens, musk fragrances and insect repellents), brominated flame retardants, perfluorinated compounds and oestrogens, because they are widely used. The section on determination techniques focuses on the groups addressed in this Thesis. As mentioned in previous sections, to the best of our knowledge no studies reporting the presence of high-intensity sweeteners in aquatic organisms have yet been published, for this reason the determination techniques reported in this section will refer to other environmental matrices such as wastewaters, surface waters and sewage sludge in which these compounds have already been determined.

1.4.1 Extraction and clean-up techniques

The extraction step is essential in analyses of aquatic organism samples because of the complexity of the matrix and the low levels at which EOCs are usually present in it. In this section a review paper is presented which has been submitted for publication to the journal Trends in Analytical Chemistry. It reviews the different extraction techniques and subsequent clean-ups employed in the determination of EOCs that have recently appeared in the most relevant publications. The extraction step is designed taking into account the type of matrix, which is usually solid, although non-solid matrices are also studied. As the methods developed in the present Thesis are for solid matrices, the review focuses mainly on this type, although a few references are also included regarding non-solid matrices such as bile and plasma. As previously mentioned, other EOC groups have been included in this paper in addition to those studied in the present Thesis. According to Bussy et al. [181] on the subject of EOCs, pharmaceuticals and EDCs in aquatic organisms have been studied most intensively and extensively over the last decade, and for this reason most of the references included in the review paper refer to these compounds.

1.4.1.1 Sample treatment for the determination of emerging organic contaminants in aquatic organisms

SAMPLE TREATMENT FOR THE DETERMINATION OF EMERGING ORGANIC CONTAMINANTS IN AQUATIC ORGANISMS

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Abstract

Chromatography coupled to mass spectrometry has become an important tool for analysing emerging organic contaminants (EOCs) in environmental samples such as aquatic organisms. Sample treatment, which includes extraction and clean-up, continues to play an important role in the analysis of complex matrices. Indeed it often becomes a bottleneck in the compromise between time and efficiency when obtaining suitable extracts for analysis. This article focuses on the state of the art in the treatment of aquatic organism samples for determining EOCs. A review is carried out of the most recent relevant publications from 2011 up to the present, in which new methods for determining EOCs in aquatic organisms were developed. The most common extraction techniques employed in these studies, such as pressurised liquid extraction, solid-liquid extraction, QuEChERS, microwave-assisted extraction and matrix solid-phase extraction along with the subsequent clean-up steps, are also examined. The most important parameters involving extraction and the clean-up step are discussed and detailed.

Keywords: emerging organic contaminants; aquatic organism; sample treatment; extraction; clean-up.

1. Introduction

In recent years the use of various chemical substances in everyday consumer products as well as in industrial processes has continued to be widespread, which means that some can be considered emerging organic contaminants (EOCs) due to their continuous release into the environment. Consequently EOCs include a huge and increasing number of chemicals found in items such as personal care products (PCPs), water disinfection by-products, nanomaterials and pharmaceuticals, among others. To date EOCs have been characterised mostly in different aquatic environments which they reach via different routes, for instance in effluents from waste water treatment plants, livestock activities and so on [1, 2]. The continuous discharge of these EOCs into the environment may lead to a degradation of ecosystems, and one of the main concerns related to their presence is that they could then bioaccumulate in non-target species and produce side effects in them [3]. Another potential concern involves possible biomagnification through the food chain, whereby they could eventually cause risks to humans. To address this issue, analytical methods need to be developed in order to obtain information about their presence in organisms (mainly in species present in the human diet) [4] and ecotoxicological studies need to be carried out to establish their potential effects in non-target species.

The analytical procedures to determine EOCs in aquatic organisms involve sample treatment, separation and detection. Separation and detection are performed predominantly by liquid chromatography (LC) or gas chromatography (GC), usually coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS), due to the selectivity, specificity and sensitivity achieved [5-10]. Meanwhile sample treatment, which includes extraction and clean-up, is still a critical step. As the objective here is to obtain extracts suitable for quantitative analyses, extracting the target analytes and removing potential interferences are the main aims of sample treatment. This type of matrix is rich in undesired components that may not only co-extract with the analytes but also affect their response. In addition, most EOCs are commonly present in environmental samples at low concentration levels and should therefore be expected at trace levels in aquatic organisms. To overcome these problems, tedious sample treatment is usually required, which makes these studies more challenging. Fish samples and particularly muscle tissue are the most common matrices analysed

for most EOCs [5, 8]. However, other aquatic organisms are also studied, for example bivalves such as mussels.

This paper reviews the most common extraction and clean-up procedures to determine EOCs in aquatic organisms published over the last five years. Pharmaceuticals, PCPs (UV filters, insect repellents, parabens, antimicrobials and synthetic musk fragrances), brominated flame retardants such as polybrominated diphenyl ethers (PBDEs), perfluorinated compounds (PFCs) and estrogens were selected from the range of EOCs because of their widespread use. Detailed information on studies carried out before 2011 can be found in other reviews [5-10].

2. Extraction

As mentioned earlier, solid tissues are the most common matrix analysed. They are usually freeze-dried and then ground and homogenised to obtain similar-sized particles before extraction [3, 11-13]. Sometimes sodium sulphate (Na_2SO_4) is used to dry the sample instead of freeze-drying [14, 15]. Because of this pre-treatment, concentrations are usually expressed in dry weight, although the wet weight or percentage of lipid content respectively are also used when wet tissues or lipid content are measured [8].

Some studies have described enzymatic digestion [16-18] or alkaline digestion in the case of PFCs [19, 20] as being successful pre-treatments to support tissue homogenisation and achieve accurate measurements respectively.

Once the sample has been pre-treated, most of the techniques currently applied to extract EOCs from aquatic organisms are based on partitioning analytes between the sample matrix (solid) and a liquid phase, which is usually an organic solvent. On comparison with other studies covered by previous reviews it can be seen that the traditional Soxhlet extraction technique, which used to be widely used to extract different EOCs from aquatic organisms [7-9], has gradually been replaced by techniques requiring less time and less solvent, such as pressurised liquid extraction (PLE), solid-liquid extraction (SLE) and, to a lesser extent, microwave-assisted extraction (MAE), matrix solid-phase dispersion (MSPD) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe). This latter technique is now gaining in popularity and its use has spread to more EOC

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groups than shown in previous studies. All these techniques will therefore be reviewed along with their recent applications.

2.1 Solid-liquid extraction

SLE is still used today to extract EOCs from aquatic organisms due to its simplicity and the fact it requires no expensive equipment. Table 1 shows the most relevant publications from 2011 onwards. The classic technique of shaking by hand usually ensures the partitioning of the analytes between the solid matrix and the organic solvent [19] and an Ultra-Turrax device [21-23] has been used to favour homogenisation, as in the case of PFCs in mussels [21]. However, ultrasounds are generally preferred to promote contact between the matrix and the solvent [14, 16, 24, 25], becoming the extraction technique known as ultrasound-assisted solvent extraction (USE).

The efficiency of SLE depends mainly on the nature of the organic solvent used. Methanol (MeOH) is the most common solvent employed despite the different chemical nature of EOCs. It has been successful in extracting, among other compounds, UV filters [24], hormones [26], pharmaceuticals [25] and PFCs [19, 23] from various fish species and mussels. Acetonitrile (ACN) has also been used to extract a group of pharmaceuticals and personal care products (PPCPs) [16] and PFCs [21] from fish and mussels respectively. In some cases solvent mixtures have been necessary to extract analytes with a wider range of polarity. For instance, a mixture of hexane:dichloromethane (DCM) [14] was used to extract 89 EOCs including polychlorinated biphenyls (PCBs), pesticides, chlorobenzenes, brominated and chlorinated flame retardants, musk fragrances and antimicrobials from fish, clams and polychaete worms. Unfortunately one of the main drawbacks of SLE is the volume of organic solvent needed, which can be as high as in classic Soxhlet extraction, up to 150 mL even with USE, when the amount of sample extracted is about 0.1-10 g.

Extraction time is another key factor in assuring quantitative extractions. This varies from 10 to 30 minutes for most applications [14, 16, 19-21, 23-25]. In the case of USE, although ultrasound frequency can be modified to enhance extraction, this does not cause any significant decrease in extraction time. However, the centrifugation step usually needed after SLE to separate the extract causes a large increase in extraction time [16, 21, 23-25].

Table 1. Analytical methods based on SLE technique.

Matrix	Analytes	Extraction	Clean-up	Determination	Recoveries (%)	Ref.
Amphipod	29 pharmaceuticals	Agitation (0.1 g sample) ACN/ 5 min/ 2500 rpm	SPE Oasis HLB W: H_2O E: 1:1 ethyl acetate:acetone	LC-(ESI)MS/MS(QqQ)	41- 89%	[22]
Fish	17 PFCs	Mechanical agitation (1 g sample) 0.01 M KOH in MeOH/ 15 min	SPE Oasis WAX W: 20 mM $NH_4CH_3CO_2$; MeOH E: 99.5:0.5 MeOH:NH_4OH SPE Envicarb E: 80:1 MeOH:CH_3COOH	LC-(ESI)MS/MS(QqQ)	65-125%	[19]
Fish	PFCs	Agitation (2.5 g sample) 200 mM NaOH + MeOH/ 30 min + 4 M HCl/10 min/ 4000 rpm	SPE Oasis WAX W: 25 mM acetat buffer; MeOH E: MeOH (2% NH_4OH)	LC-(ESI)MS/MS(QqQ)	95-109%	[23]
Mussel	10 PFCs	Agitation (1 g sample) ACN + centrifugation 20 min/ 5000 rpm	SPE Oasis HLB W: H_2O E: MeOH	LC-(ESI)HRMS(TOF)	90-106%	[21]
Fish Crustacea Cephalopoda	UV filters and UV stabilizers	Ultrasonication (2 - 4 g sample) MeOH 3 x (15 min + 10 min/ 4000 rpm)	GPC Biobeads S-X3 E: 1:1 ethyl acetate:cyclohexane Silica gel column E: DCM:ethyl acetate (1:1)	UHPLC-(APCI)MS/MS(QqQ)	42-120%	[24]
Fish	2 pharmaceuticals	USE (1 g sample) MeOH + 0.05 M HCl 2 x (20 min/30 ºC + 15 min/3000 x g)	SPE Strata X W: H_2O E: MeOH	LC-(ESI)HRMS(Q-TOF)	40%	[25]

Table 1. (Cont.).

Matrix	Analytes	Extraction	Clean-up	Determination	Recoveries (%)	Ref.
Fish, Clam and Polychaete worms	89 compounds: (PCBs, pesticides, chlorobenzenes, BFR, CFR, musks fragrances, antimicrobials)	USE (5-10 g sample) 1:1 hexane:DCM 3 x 15 min	GPC Bio-Beads E: hexane:DCM (1:1) 1rst fraction rejected 2nd fraction divided for GC and LC: 25% LC & 75% GC passed through deactivated florisil column E: hexane:DCM	GC-(EI)MS/MS(QqQ) UHPLC-(ESI)MS/MS (QqQ)	40-119%	[14]
Fish tissues (Plasma, liver, brain)	17 PPCPs	Enzymatic digestion (acetate buffer and β-glucuronidase/sulfatase) USE (0.5 g sample; 1 mL plasma) ACN 2 x (10 min + 10 min/5000 x g) LLE 2 x (5% NaCl + 1M CH₃COOH + MTBE + 20 min shake + 10 min centrifugation 720 x g) 2 x (NaCO₃ + 20 min shake + 10 min 720 x g)	Silica gel column (3g) 1rst fraction: DCM 2nd fraction: 7:3 DCM:acetone; liver & brain pass through GPC (3:1 ciclohexane:ethyl acetate) 3rd fraction: acetone 4th fraction: 6:4 acetone:MeOH 2nd + 3rd + 4th fractions combined through SPE Oasis HLB W: H₂O (20% MeOH) E: MeOH:MTBE (7:3)	LC-(ESI)MS/MS(QqLIT)	Liver: 92-109% Brain: 88-118% Plasma: 90-110%	[16]
Fish	15 PFCs	USE (1 g sample) NaOH 0.2 M in MeOH ACN 2 x (15 min sonication and 15 min shake + HCl neutralisation + 30 min/ 9000 x g)	Hexane dSPE ENVI-Carb CH₃COOH	UHPLC-(ESI)MS/MS(QqQ)	23-149%	[20]
Fish	14 PFCs	FUSLE (0.5 g sample) 9:1 ACN: H₂O 2.5 min/ 10% irradiation power/ 0 °C	SPE Oasis WAX W: HCOOH (2%); H₂O:MeOH (95:5) E: acetone (2.5% NH₄OH)	UHPLC-(ESI)MS/MS(QqQ)	29-117%	[28]

Table 1. (Cont.).

Matrix	Analytes	Extraction	Clean-up	Determination	Recoveries (%)	Ref.
Mussel	hormones	FUSLE (0.5 g sample) MeOH 2 x (1 min + 5 min centrifugation/ 2800 x g)	Hexane SPE Oasis HLB W: H$_2$O (5% MeOH) E: MeOH	LC-(ESI)MS/MS (QqLIT)	88-100%	[26]
Mussel and fish tissues (muscle and liver)	Tricyclic antidepressants	FUSLE (0.5 g sample) 95:5 ACN:H$_2$O/ 30 s/ 10% power/ 0 °C ice-water bath	SPE Evolute-CX W: H$_2$O; MeOH E: acetone (2.5% NH$_4$OH)	LC-(ESI)MS/MS(QqQ)	Liver: 89- 109% Muscle: 94- 114% Mussel: 86-104%	[27]
Mussel and fish tissues (muscle and liver)	14 PFCs and 10 potential precursors	FUSLE (0.5 g sample) 9:1 ACN:H$_2$O/ 2.5 min/ 10% irradiation power/ 0 °C ice-water bath	SPE Evolute-WAX W: HCOOH (2%); H$_2$O:MeOH (95:5) Envi-Carb E: acetone (2.5% NH$_4$OH)	UHPLC-(ESI)MS/MS(QqQ)	Liver: 66-111% Muscle: 83-146% Mussel: 77-119%	[29]

BFR: Brominated flame retardants; CFR: chlorinated flame retardants; DCM: dichloromethane; dSPE: dispersive solid-phase extraction; E: elution; EI: electron impact; ESI: electrospray ionisation; FUSLE: focused ultrasound solid-liquid extraction ;GC: Gas chromatography; GPC: gel permeation chromatography; HRMS: high resolution mass spectrometry; LC: liquid chromatography; MS/MS: tandem mass spectrometry; PFC: perfluorinated compounds; QqLIT: hybrid triple quadrupole linear ion trap; QqQ: triple quadrupole; SPE: solid-phase extraction; TOF: time of flight; UHPLC: ultra-high performance liquid chromatography; W: wash.

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On average, centrifugation takes 10-30 minutes and the whole procedure (extraction and centrifugation) usually had to be repeated two or three times to achieve suitable results [14, 16, 20, 24, 25]. The complete extraction can therefore take more than one hour per sample. An example of this is the extraction of UV filters from different fish species [24] by USE. Peng et al. [24] applied 3 extractions of 15 min and 20 mL of MeOH (solvent) each, alternating with centrifugations of 10 minutes at 4000 rpm to achieve recoveries of between 42% and 120%.

In line with the current trend in analytical chemistry to develop environmentally-friendly methods, some authors use a closed extractor fitted with a sonic probe to perform the extraction, this being known as focused ultrasound solid-liquid extraction (FUSLE) [27]. FUSLE not only reduces the amount of organic solvent needed (5-20 mL) but also the amount of sample (0.01-1.0 g) and extraction time (from seconds to a few minutes). For instance, hormones [26] were extracted using FUSLE from 0.5 g of mussel using 2 extractions of 1 min and 10 mL of MeOH, alternating with centrifugations of 5 min at 2800 x g each, and the recoveries obtained were over 88%. FUSLE has also successfully been applied to extract PFCs [28, 29] and pharmaceuticals [27] from fish and mussels.

2.2 QuEChERS

Although originally developed to determine pesticides in fruits and vegetables [30], in recent years the QuEChERS technique has been extended to extract EOCs from different matrices such as aquatic organisms because of its simplicity and the fact it requires no expensive equipment. Table 2 shows details from recent publications that employ this extraction technique. As mentioned before, the samples are usually freeze-dried and therefore water is added (between 2 and 10 mL) to enable phase separation. The most common extracting solvent used is ACN (between 1 and 10 mL), but a mixture of ACN:MeOH (75:25) has also been used by Pereira et al. [31] because MeOH increased the extraction of quinolones and tetracyclines from fish samples. In Jakimska et al. [32], the ratio $V_{ACN}:V_{water}$ (4:1, 2:1 and 4:3) was evaluated in order to optimise the extraction efficiency of endocrine disrupting compounds (EDCs) such as preservatives, hormones and antibacterials, among others, from fish samples. The ratio 2:1 was chosen as a compromise between recoveries.

Table 2. Analytical method that employ QuEChERS as extraction technique

Matrix	Analytes	Extraction	Clean-up	Determination	Recoveries	Ref.
Mussel	2 anticonvulsants and 6 TPs	2 g sample; 10 mL H$_2$O + 10 mL ACN + QuEChERS EN method	dSPE Na$_2$SO$_4$/ PSA/ C$_{18}$ HCOOH	UHPLC-(HESI)HRMS(Orbitrap)	67- 100%	[35]
Molluscs	2 pharmaceuticals	1 organism; 250 µL ACN + 100 µL H$_2$O + QuEChERS citrate buffer	Hexane	NanoLC-(ESI) MS/MS(QqLIT)	> 85%	[38]
Benthic invertebrates	35 EOCs (pharmaceuticals and metabolites, hormones, PFCs, alkylphenols, pesticides, plasticiser)	1- 4 organisms; 500 µL ACN + 500 µL H$_2$O + QuEChERS citrate buffer	Hexane	NanoLC-(ESI)MS/MS(QqLIT)	C. riparius 38- 121% G. fossarum 50-120% P. antipodarum 47-102%	[37]
Fish	19 EDCs (triazoles, stimulants, hormones, flame retardants, plasticisers, antibacterials, preservatives)	0.5 g sample; ACN:H$_2$O (2:1) + QuEChERS AOAC method	dSPE MgSO$_4$/PSA/ C$_{18}$	UHPLC-(ESI)MS/MS(QqLIT)	C. carpio 46- 125% S. glanis 13- 109 % L. graellsii 32- 121%	[32]
Fish	32 veterinary drugs (macrolides, penicillins, quinolones, sulphonamides and tetracyclines)	5 g sample; 2 mL H$_2$O + 10 mL ACN:MeOH (75:25) QuEChERS AOAC method	Not reported	UHPLC-(ESI)MS/MS (QqQ)	69-125%	[31]
Bivalves and fish (tissues: liver, muscle and gonad)	9 synthetic musk	0.2 g of sample; 1 mL ACN + QuEChERS EN method	dSPE PSA/ C$_{18}$/ MgSO$_4$	GC-(EI)MS(Q)	46- 120%	[34]
Bivalves	7 pharmaceuticals	1 g of sample; 10 mL H$_2$O + 10 mL ACN +QuEChERS EN method	dSPE silica gel	LC-(ESI)MS/MS(QqQ)	61-95%	[36]
Fish tissues (liver and gonad)	33 EOCs (PBDEs, PCBs, musk fragrances, PAHs, pesticides, plasticiser, UV filter, alkylphenol)	5 g of sample; 5 mL ACN + Original method	dSPE 2 x (MgSO$_4$/PSA/ C$_{18}$)	GC-(EI)MS(Q)	Liver 28-108% Gonad 61-113%	[33]

dSPE: dispersive solid-phase extraction; EI: electron impact; ESI: electrospray ionisation; GC: Gas chromatography; HRMS: high resolution mass spectrometry; LC: liquid chromatography; LC: liquid chromatography; MS: mass spectrometry; MS/MS: tandem mass spectrometry; PBDE: polybrominated diphenyl ethers; PCB: Polychlorinated biphenyl; PSA: primary secondary amine; QqLIT: hybrid triple quadrupole linear ion trap; Q: quadrupole; QqQ: triple quadrupole; SPE: solid-phase extraction; TP: transformation product; UHPLC: ultra-high performance liquid chromatography. C. riparius: Chironomus riparius; G. fossarum: Gammarus fossarum; P. antipodarum: Potamopyrgus antipodarum; C. carpio: Cyprinus carpio; S. glanis: Silurus glanis; L. graellsii: Luciobarbus graellsii.

The addition of salts is required to favour phase separation between water and the organic solvent, and depending on the salts there are different QuEChERS methods: the Original Method, which uses the anhydrous $MgSO_4$ and NaCl salt composition; the European Standard Method EN 15662 (EN method), which uses citrate buffer as salts; and the AOAC Official 2007.1 Method (AOAC method), in which the acetate buffer is used. All three methods have been applied to extract EOCs from aquatic organisms.

Among other compounds, PBDEs and PCPs were extracted from different fish tissues employing the original method described by Anastassiades et al. [30], obtaining recoveries higher than 60% for most of the compounds [33]. In contrast, in the work by Jakimska et al. [32] different salt compositions were evaluated, of which the acetate buffer was the best option for extracting 19 EDCs belonging to different classes. Saraiva et al. [34] applied the EN method to extract musk from seafood, obtaining recoveries between 46%-120%. The citrate buffer was also used by Martínez-Bueno [35] to extract two anticonvulsants and six of their transformation products from mussel samples. However, in this study the $MgSO_4$ contained in the buffer was substituted for Na_2SO_4, which, according to the authors, efficiently absorbs the water. The final method provided recoveries of between 67% and 100%. The EN and the AOAC QuEChERS methods were both tested in our previous study [36] to extract seven pharmaceuticals from different bivalve species, with the EN method being the one that achieved the highest extraction recoveries.

As in the case of SLE, a miniaturisation of this technique (microQuEChERS) has also recently been applied [37, 38], in which the volumes employed were at µL level and the amount of salts was also reduced. In Berlioz-Barbier [38], for instance, fluoxetine and carbamazepine were extracted from two species of gastropod employing 100 µL of water, 250 µL of ACN and 100 mg of citrate buffer, obtaining recoveries higher than 85%.

2.3 Matrix solid-phase dispersion

Despite its advantages (simplicity, small sample size, short extraction time, less solvent than conventional techniques and no equipment required), MSPD has been less widely used than the previous techniques because the samples have to be ground up with a dispersing agent (also known as solid support) and packed

into a column. Some recent publications that employ this extraction technique can be found in Table 3.

Florisil [39], diatomaceous earth [40] and primary secondary amine (PSA) [41] are solid supports that have been used in the extraction of different flame retardants, musk fragrances and PFCs from bivalve samples. The analytes are eluted using a suitable organic solvent while interfering matrix compounds are selectively retained in the column. It should be noted that MSPD can simultaneously perform extraction and clean-up by placing a layer of co-sorbent at the bottom of the MSPD column [42]. In recent studies silica gel [40], a combination of deactivated and activated silica [39] and a combination of silica, acidified silica with 10% H_2SO_4 and deactivated Florisil with 5% water [41] have been employed for clean-up purposes. In Ziarrusta et al. [39], for example, the use of deactivated and activated silica obtained cleaner chromatograms, repetitive retention times and low %RSD (values up to 5%) in repeatability.

MSPD has been employed to extract different groups of EOCs such as musk fragrances and PBDEs [39], PFCs [40] and brominated flame retardants [41] in bivalve samples, with the recoveries obtained for most of the compounds ranging from 64% up to 126%.

2.4 Pressurised liquid extraction

In recent years PLE has expanded its field of application and, compared with previous reviews, has established itself as one of the most extensively used techniques for extracting different EOCs from aquatic organisms. Table 4 shows some examples in which PLE has been used. The main disadvantage is its high cost due to the equipment needed.

The most important parameters to be optimised are the extraction solvent followed by temperature, extraction time and number of cycles [43]. As regards extraction solvents, MeOH has been successfully applied to extract pharmaceuticals from a wide variety of aquatic organisms [11, 44, 45]. However, a high matrix effect (ME) was encountered when mussels were analysed [46] and consequently ultrapure water was used as a compromise between recoveries and ME. Poorly cleaned extracts were also encountered when MeOH was tested to extract UV filters from fish [12]. In addition, Vallecillos et al. [13] described that

Table 3. Analytical methods that employ MSPD or MAE extraction techniques.

Matrix	Analytes	Extraction	Clean-up	Determination	Recoveries	Ref.
Bivalves	8 PFCs	<u>MSPD</u> sample mixed with diatomaceous earth E: ACN	Na$_2$SO$_4$ + silica gel	LC-(ESI)MS/MS(QqQ)	Clam: 64- 114% Mussel: 97-115% Cockle: 92-126%	[40]
Bivalves	15 BFR (PBDEs among others)	<u>MSPD</u> 0.5 g sample mixed with PSA E: DCM	silica + acidified silica (10% H$_2$SO$_4$) + deactivated Florisil	GC-(CI)MS(Q)	Mussel: 99-120% Clam: 46-110% Cockle: 70- 101%	[41]
Bivalves	40 analytes (PAHS, PCBs, PBDEs, musk fragrances, pesticides)	<u>MSPD</u> 0.3 g sample mixed with Florisil E: DCM	deactivated silica + activated silica	GC-(EI)MS/MS(QqQ)	Mussel: 64-109%	[39]
Mussel	2 UV filters, 2 pharmaceuticals	MAE (3 g of sample) 1:1 acetone:heptane; Tª increased to 110ºC within 15 min and maintained for 5 min	<u>RPLC</u> Spersiorb ODS2 E: 70:30 MeOH:H$_2$O with gradient	GC-(EI)MS/MS(IT)	89-122%	[50]
Fish and mussel	11 antibiotics and metabolites	<u>MAE</u> (2 g of sample) (Proteinase-K) H$_2$O/ 5 min/ 50 W + 5 min/ centrifugation 8000 x g HCOOH + 2 x (5 mL DCM+ manual agitation)	Not reported	LC-(ESI)MS/MS(QqLIT)	Anchovy: 64-99% Wedge sole: 63- 96% Hake: 63- 99% Mussel: 61- 97%	[17]
Mussel	3 UV filters	MAE (3 g of sample) 1:1 acetone:heptane; 110ºC within 15 min.	<u>RPLC</u> Spersiorb ODS2 E: 70:30 MeOH:H$_2$O with gradient	GC-(EI)MS/MS(IT)	89-116%	[51]

BFR: brominated flame retardants; CI: chemical ionisation; DCM: dichloromethane; E: elution; EI: electron impact; ESI: electrospray ionisation; GC: Gas chromatography; GPC: gel permeation chromatography; IT: ion trap; LC: liquid chromatography; MAE: Microwave extraction; MSPD: matrix solid-phase dispersion; MS: mass spectrometry; MS/MS: tandem mass spectrometry; PAH: Polycyclic aromatic hydrocarbon; PCB: polychlorinated biphenyl; PBDE: polybrominated diphenyl ether; PFC: perfluorinated compounds; PSA: primary secondary amine; QqLIT: hybrid triple quadrupole linear ion trap; Q: quadrupole; QqQ: triple quadrupole; RPLC: reversed-phase liquid chromatography; SPE: solid-phase extraction.

fatty precipitates appeared in the PLE extracts when MeOH was tested to extract musk fragrances from fish and mussel samples. Solvent mixtures have also been employed [3, 4, 12, 19, 21, 47-49] to enhance the extraction of analytes with a wide range of polarity. For instance, 23 pharmaceuticals and some of their metabolites were extracted from different bivalve species with a mixture of MeOH:water (1:2). According to the authors, the addition of water favours the recovery of antibiotics [4]. In the work by McEneff et al. [3] another mixture, ACN:water; (3:1; v:v), was employed to extract different pharmaceuticals in mussels, and Couderc et al. [19] used a mixture of toluene:acetone (70:30) to extract PBDEs from fish samples.

Once the extraction solvent is selected, the temperature is usually optimised to increase the recoveries, bearing in mind ME [12] or avoiding analyte (e.g. pharmaceuticals) degradation [11]. For example, 100 ºC was chosen for extracting UV filters from fish using a mixture of ethyl acetate:DCM (1:1; v:v) [12].

As Table 4 shows, between 1 and 3 cycles with extraction times of between 5 and 15 min for each cycle are common values. The sample amount required is generally between 0.1 and 3 g, the most common values being 0.5 and 1 g.

When comparing PLE with other extraction techniques such as QuEChERS that do not require any equipment, Vallecillos et al. [13] reported that both extraction techniques were suitable for extracting musk fragrances from fish and mussel. However, PLE with DCM as an extracting solvent achieved lower ME than QuEChERS employing the EN method and slightly better validation parameters. In contrast, Martínez-Bueno et al. [35] studied the influence of the extraction methodology when determining two anticonvulsants and some of their transformation proucts. QuEChERS and PLE were again compared, with higher average recoveries being obtained for most of the analytes when QuEChERS was used. Moreover, QuEChERS required less solvent and sample and also a shorter extraction time. Jakimska et al. [32] also compared the same extraction techniques to determine different EDCs in fish samples, with QuEChERS being selected as the best option again, since PLE led to high ME due to the co-extraction of other matrix components that could not be removed in the clean-up step. Nevertheless, QuEChERS, PLE and USE were compared in extracting 20 pharmaceuticals in different fish species and tissues. Although the QuEChERS

Table 4. Analytical methods that employ PLE techniques.

Matrix	Analytes	Extraction	Clean-up	Determination	Recoveries	Ref.
Fish tissues (homogenate, liver and muscle)	20 pharmaceuticals	0.5- 1 g sample; MeOH/ 3 cycles/ 5 min/ 50°C	In-cell neutral alumina; GPC (LC-DAD) Acquity HSS T3 column	UHPLC-(ESI)MS/MS(QqLIT)	$L.\ graellsii$: 31- 108% $C.\ carpio$: 19- 79% $S.\ glanis$: 26- 75%	[11]
Mussel	5 pharmaceuticals	1 g sample; 3:1 ACN:H$_2$O/ 3 cycles/ 5 min/ 60°C	In-cell activated neutral alumina; SPE Strata-X cartridge W: H$_2$O E: 1:1 ethyl acetate:acetone	LC-(ESI)MS/MS(IT)	83-104%	[3]
Mussel and other bivalves	11 pharmaceuticals	1 g sample; 3:1 ACN:H$_2$O 1% HCOOH/ 3 cycles/10 min/ 100°C	In-cell neutral alumina; SPE Strata-X W: H$_2$O E: MeOH	UHPLC-(ESI)MS/MS(QqQ)	95-103%	[21]
Bivalves	23 pharmaceuticals and metabolites	0.5 g sample; 1:2 MeOH:H$_2$O/ 3 cycles/ 5 min/ 50°C	In-cell neutral alumina; SPE Oasis HLB W: H$_2$O E: MeOH	UHPLC-(ESI)MS/MS(QqLIT)	Oyster: 33-101% Clam: 30-74% Mussel: 30-116	[4]
Fish	8 UV filters	1 g sample; 1:1 ethyl acetate:DCM/ 2 cycles/ 5 min/ 100°C	In-cell Florisil; SPE Isolute C$_{18}$ E: 1:1 ethyl acetate/DCM; DCM	LC-(ESI)MS/MS(QqLIT)	36- 112%	[12]

Table 4. (Cont.).

Matrix	Analytes	Extraction	Clean-up	Determination	Recoveries	Ref.
Mussel	7 pharmaceuticals	1 g sample; H$_2$O/ 1 cycle/ 10 min/ 100ºC	SPE Oasis MAX W: H$_2$O (5% NH$_4$OH); MeOH E: MeOH (5% HCOOH)	LC-(ESI)MS/MS(QqQ)	61-90%	[46]
Fish and mussel	10 musk fragrances	0.5 g sample; DCM/ 1 cycle/ 5 min/ 60ºC	In-cell Florisil	GC-(EI)-MS/MS(IT)	Fish: 61-109% Mussel: 45-91%	[13]
Dolphin (Liver)	UV filter	1 g liver; 1:1 DCM:hexane/ 2 cycles/ 10 min/ 100ºC	Acid attack 4 x (H$_2$SO$_4$) SPE alumina E: hexane:DCM (1:2)	UHPLC-(ESI)MS/MS(QqQ)	Not reported	[48]
Fish Crustacean	4 UV filters and 4 stabilizers	1:1 hexane:DCM/ 3 cycles/ 5 min/ 100ºC	In-cell PSA GPC 2 Envirogel GPC columns (19 x 300 mm/ 19 x 150 mm) dSPE PSA	GC(EI)HRMS(TOF) LC(ESI)HRMS(Q-TOF)	46- 85%	[47]
Fish	11 pharmaceuticals	1 g sample; DCM/ 1 cycle/ 10 min/ 80ºC	SPE Oasis MCX W: ACN E: MeOH(5% NH$_4$OH)	LC-(HESI)MS/MS(QqQ)	19- 85%	[15]

Table 4. (Cont.).

Matrix	Analytes	Extraction	Clean-up	Determination	Recoveries	Ref.
Fish	18 PCBs, 7 PBDEs	1 g sample; 70:30 toluene:acetone/ 3 cycles/ 5 min/ 120ºC	Silica gel column (H$_2$ SO$_4$) activated Florisil column Florisil/Carbopack C/ Celite 545 column	GC-(EI)HRMS(Double sector)	60-120%	[19]
Clam	Tetracycline antibiotics	3 g sample; MeOH/ 1 cycle/ 15 min/ 70ºC	In cell copper (II) isonicotinate	LC-(ESI)MS/MS(QqQ)	85-94%	[44]
Molluscs	22 antibiotics	0.1 sample; MeOH/ 2 cycles/ 10 min/ 70ºC	SPE Oasis HLB W: H$_2$O E: MeOH (5% NH$_3$)	LC-(ESI)MS/MS(QqQ)	Not reported	[45]
Biofilm	13 EDCs (parabens, plasticiser, hormones, flame retardants, antibacterial) 44 pharmaceuticals	200 mg sample; Citric buffer (pH 4):ACN/ 3 cycles/ 5 min /60ºC	SPE Oasis HLB W: H$_2$O E: MeOH	UHPLC-(ESI)MS/MS(QqLIT)	24-137 %	[49]

DAD: diode array detector; DCM: dichloromethane; E:elution; EI: electron impact; ESI: electrospray ionisation; GC: Gas chromatography; GPC: gel permeation chromatography; HRMS: high resolution mass spectrometry; IT: ion trap; LC: liquid chromatography; MS/MS: tandem mass spectrometry; QqLIT: hybrid triple quadrupole linear ion trap; QqQ: triple quadrupole; Q-TOF: quadrupole time of flight; SPE: solid-phase extraction; TOF: time of flight; UHPLC: ultra-high performance liquid chromatography; W: wash. *L. graellsii: Luciobarbus graellsii; C. carpio: Cyprinus carpio; S. glanis: Silurus glanis.*

recoveries were higher than 40%, PLE was selected due to the higher recoveries of relevant compounds (such as diclofenac and propanolol), lower %RSD and fewer matrix interferences (compared with QuEChERS) [11]. In another work by the same group [49], a comparison was made between PLE and USE using the same solvent (citric buffer (pH 4):ACN; 1:1) when extracting 13 EDCs and 44 pharmaceuticals in biofilm. Despite the fact that similar recoveries were obtained, PLE was said to prevail over USE in terms of reproducibility (%RSD< 20 %), indicating greater robustness.

2.5 Microwave-assisted extraction

Although MAE has been used to extract different EOCs from different environmental matrices, it has been less widely used than PLE in the case of aquatic organisms even though both techniques require equipment. Current applications are shown in Table 3. Only a few studies have recently used this extraction technique [17, 50, 51]. It has been used to extract UV filters and pharmaceuticals from mussels [50, 51], for instance, and also to extract pharmaceuticals from fish and mussels, in which the extraction was combined with enzymatic digestion [17]. The nature of the solvent employed by the extraction is very important. It is common practice to use a binary mixture (heptane:acetone) in which only one of the solvents absorbs microwaves [50, 51]. Other important parameters affecting the extraction process are the power applied, temperature and extraction time [43]. In the work by Fernández-Torres et al. [17], for example, MAE was carried out using 2 g of mussel/fish sample with an extraction time of 5 min with 5 mL of water at 50 W to extract veterinary antibiotics from different fish species and mussels, obtaining recoveries of between 61% and 99%.

3. Clean-up

Most of the extraction techniques for aquatic organism samples are not very selective. Endogenous components present in the matrix, which can include ionic species, highly polar compounds, various organic molecules and analogous compounds or metabolites with a chemical structure close to that of the target analytes, are usually also extracted [5-8]. Therefore the removal of co-extracted

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matrix components is critical, and different clean-up procedures have been described for use during or after extraction to minimise the negative effects. The different clean-up strategies are detailed in Tables 1-4 along with the extraction techniques already described. Due to the complexity of the matrix in several studies, more than one clean-up step is usually required and different clean-up strategies are combined. Solid-phase extraction (SPE) and gel permeation chromatography (GPC) are the most common strategies.

Hexane is sometimes added to the extract prior to clean-up in order to eliminate co-extracted nonpolar and fatty compounds. This strategy has been used before SPE [26] and dispersive solid-phase extraction (dSPE) [20], and thus an already cleaner extract is contacted with the SPE sorbent. Another strategy reported in the literature is to perform an acid attack using H_2SO_4 [48]. However, sulphuric acid cannot then be employed for the analysis of certain compounds since they may be degraded [7]. Other acids (HCOOH or CH_3COOH) have also been added to the clean-up step [20, 35]; according to Martínez-Bueno [35], the addition of HCOOH favours the disruption of the compound-protein binding, which affects the recovery and ME.

3.1 Solid-phase extraction

The most commonly used clean-up strategy is SPE, which is applied after different extraction techniques for several EOCs. The main sorbents chosen for SPE are high capacity ones such as Oasis HLB [4, 16, 21, 22, 26, 45, 49] and, to a lesser extent, Strata–X [3, 21, 25]. As mentioned in previous sections, the extraction solvents employed are usually organic. This means that the extracts must be completely or partly evaporated before being loaded into the cartridge, and are then usually diluted to a certain mL up to 100 mL or 200 mL with ultrapure water and, if necessary, adjusted to a certain pH. In some studies, chelating agents are added in order to bind the residual metals present in the matrix [4, 49]. Once the extract has been loaded into the cartridges, ultrapure water is usually employed to clean the matrix [3, 4, 21, 22, 25, 45, 49], although water containing a percentage (5%-20%) of MeOH [16, 26] has also been used. MeOH is the most common solvent employed for eluting the compounds from the sorbent. Nevertheless, the addition of 5% NH_4OH in MeOH has been successfully used to elute antibiotics [45]. Other eluting solvents such as a mixture of ethyl acetate:acetone (1:1) have also been employed to elute

pharmaceuticals from Oasis HLB sorbent, when amphipod *Gammarus* sp. extract was percolated [22], and Strata-X sorbent from mussel extract [3]. A mixture of MeOH:Methyl tert-butyl ether (MTBE) (7:3) has been used to elute PCPs from different fish tissues from the Oasis HLB sorbent [16]. Isolute C_{18} is another cartridge described in the literature and supplied better results than the Oasis HLB sorbent for most lipophilic UV filters [12].

In general most studies claimed that aquatic organisms are complex matrices and require clean-up. However, after this clean-up is carried out, in some studies it is difficult to see whether it was worth it or at what level it improves recoveries or decreases ME.

More selective mixed-mode ion-exchange sorbents like Oasis MCX [15], Evolute-CX [27], Oasis MAX [46] and Oasis WAX [23, 28] have also been employed. The elution from the Oasis WAX cartridge of the target analytes was performed with 2.5% NH_4OH in MeOH or acetone. In the case of strong cation-exchangers (Oasis MCX sorbent and Evolute-CX), organic solvents (ACN and MeOH) were used to clean the matrix without losing the selectively retained analytes, and the elution took place with 5% NH_4OH in MeOH [15] or 2.5% NH_4OH in acetone [27].

Polar sorbents in the normal phase, such as alumina [48], silica gel [16, 19, 24] and Florisil [14, 18, 19] columns or cartridges with different levels of activity, are employed separately or in combination for the removal of nonpolar lipids and other nonpolar molecules [5]. However, larger volumes (up to 160 mL) of solvent are usually used in these cases in order to elute the compounds.

Apart from clean-up, SPE [18, 52] and liquid-liquid extraction (LLE) [53] have also been used to extract EOCs from non-solid matrices such as blood and bile from aquatic organisms.

3.2 Chromatographic approach

GPC is a clean-up strategy widely used for separating large molecules (e.g. lipids) on the basis of size exclusion [43]. The Bio-Beads S-X3 is a commonly employed column [24, 54]. A disadvantage of this technique is that after GPC additional steps are usually needed because of the difficulty involved in removing

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all lipids by GPC alone [14, 16, 24, 54]. In other words, after GPC the samples were passed through a silica gel column or cartridge [24, 54], a Florisil column [14], an Oasis HLB cartridge [16] or cleaned by dSPE with PSA [47]. Another disadvantage is the large volumes obtained, which makes the clean-up step tedious and increases analysis time.

In Peng et al. [24], for example, GPC was employed to clean the extract obtained from USE, which was evaporated to dryness and redissolved in ethyl acetate:cyclohexane (1:1) before being subjected to a GPC column. The analytes were eluted with the same solvent, in which the first 15 mL were rejected and the following 16 mL collected. However, some analytes still suffered from ME and for this reason the collected eluate was concentrated again for solvent exchange before further clean-up with a silica gel column, which, according to the authors, reduced the ME. In Huerta et al. [11], GPC was chosen between different strategies (SPE using Florisil cartridges and SPE using Oasis HLB followed by GPC) since lower recoveries were obtained for the other strategies, and single GPC clean-up provided satisfactory results for most of the compounds. The extract was passed through an EnviroPrep column (300 x 21.2 mm; 10 µm) coupled to a PLgel guard column using DCM:MeOH (9:1) as mobile phase. The fraction between 13.5 and 26.5 minutes was collected and a diode array detector was used to monitor the sample. In this case no further clean-up was required.

Another type of chromatography, reversed-phase liquid chromatography (RPLC), has been widely used as clean-up for the determination of UV filters [50, 51]. According to Zenker et al. [55], RPLC used as clean-up for UV filters with different properties enabled more efficient separation compared to SPE or GPC. However, for the UV filters that had similar physicochemical properties, the clean-up with GPC or SPE was very useful.

3.3 Strategies related to PLE

PLE makes it possible to perform an in-cell clean-up in which a sorbent is placed at the bottom of the extraction cell or mixed with the sample instead of the inert material in order to retain interfering substances. Alumina [3, 4, 11, 21] was the most common sorbent for the clean-up when pharmaceuticals were extracted from fish [11] and bivalves [3, 4, 21]. Other less widely used sorbents include silica gel [56], Florisil [12, 13] and PSA [47], which have been employed as in-

cell sorbents. The amount of sorbent is variable and depends on the volume of the extraction cell and ranges from 1 g to 20 g [3]. Looking at the examples detailed in Table 4, it can be seen that this strategy is almost always employed with PLE since it does not involve a lengthening of the analysis time, requires no additional parameters of the extraction to be modified and does not entail higher costs. It is usually combined with further clean-up steps such as SPE [3, 4, 12, 21] or GPC [11, 47]. This was the case in the work by Gago-Ferrero et al. [12], in which 1 g of Florisil was selected as in-cell clean-up when UV filters were determined from fish samples, since it was observed that it improved extraction efficiency and supplied a cleaner extract and a better chromatographic peak shape, although the extract required one further clean-up step with SPE. Other examples are shown in Table 4. However, in some recent studies the in-cell clean-up was the only purification step taken. Jiao et al. [44] used 3 g of copper (II) isonicotinate as in-cell sorbent for fatty samples such as clams and discovered that no further clean-up step was necessary. This was also the case in the work by Vallecillos, et al. [13], in which in-cell clean-up with Forisil was the only purification step carried out for musk fragrances in fish and mussel samples.

Another cleaning strategy made possible by PLE is on-cell clean-up, which is when a solvent with complementary properties to the one used in the extraction is passed through the sample. Although this strategy was tested, it was not included in any final method in recent publications [11].

3.4 Dispersive solid-phase extraction

This clean-up step is commonly carried out after QuEChERS extraction (see Table 2) but can also be performed after other techniques such as SLE [20]. With QuEChERS, dSPE using PSA, C_{18} and graphitised carbon black (GCB), among other sorbents, has been tested [32, 35]. However, in recent publications the most common mixture is that containing PSA and C_{18} because it removes nonpolar compounds such as lipids from fish [32-34] and bivalve [34, 35] samples. $MgSO_4$ is also added to remove excess water and improve analyte partitioning [37]. In the study by Jakimska et al. [32], after extraction different dSPEs such as $MgSO_4/PSA$, $MgSO_4/PSA/C_{18}$, $PSA/C_{18}/GCB/MgSO_4$ and $PSA/GCB/MgSO_4$ were tested, with the $MgSO_4/PSA/C_{18}$ mixture being selected since according to the authors it can be used with samples with a high lipid content and provides suitable results (recoveries higher than 50% and %RSD lower than 18%). Other

studies did not carry out a clean-up step after QuEChERS [31], while others used hexane [37, 38] to promote separation of the lipidic fraction. Nevertheless, it is always advisable to include a clean-up step after the extraction of such complex samples as aquatic organisms.

4. Conclusions

Sample treatment is still the most time-consuming step in the analytical method in order to achieve extracts compatible with the detection techniques. As regards extraction techniques, although the classic Soxhlet extraction is still employed, alternative less time-consuming techniques have been widely used. SLE (another traditional technique) continues to be used due to its simplicity. PLE and QuEChERS offer the advantage of significantly reducing the amount of organic solvent consumed. Moreover, PLE offers a semi-automated extraction process and the possibility of efficient in-cell clean-up using selective sorbents. Meanwhile QuEChERS has also been noted as a powerful extraction technique and has recently been gaining in popularity. As for clean-up techniques, despite the long purification procedures that can be involved, GPC and adsorption chromatography, for example, are still widely used. In-cell clean-up in the case of PLE and dSPE in the case of QuEChERS have been described as saving time and enabling cheaper clean-ups. Some miniaturisation methods have also appeared to make sample treatment shorter and reduce solvent consumption with the aim of being more environmentally friendly while obtaining promising results.

Acknowledgments

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1.4.2 Separation and detection techniques

Due to the complexity of the samples, after the analytes are extracted, chromatographic techniques are mostly used to determine EOCs in aquatic organisms. The choice of chromatographic technique (LC or GC) is based on the nature of the analytes (physicochemical properties and thermostability) [6, 182]. These techniques at present are mainly coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) because of the selectivity, specificity and sensitivity achieved. A number of biological techniques including biosensors and immunoassays have also been used to determine EOCs such as pharmaceuticals in aquatic organisms. Despite the fact that they are not widely used, they do offer many advantages such as high sensitivity, simplicity and cost-effectiveness [6].

Although both LC and GC have been applied, most pharmaceuticals are determined by LC due to their polarity. One of the most important parts of LC is the column. In most cases, the stationary phase generally used to separate this kind of compound is a C_{18} [75, 80-82, 90, 92, 183, 184]. Some recent studies have seen the appearance of columns with sub 2 µm particle technology installed in UHPLC instruments, since the advantages of increased speed and efficiency that turns on improved sensitivity, selectivity and specificity compared with conventional LC analysis have been described [7, 71, 73, 87, 89, 185]. Martínez Bueno et al. [86], for example, compared a Zorbax XBD C_{18} with a particle size of 1.8 µm with a X-Terra C_{18} with a particle size of 3.5 µm when separating carbamazepine and oxcarbazepine and six of their transformation products in mussel samples. The main advantages the authors found when employing the 1.8 µm particle size column were increased efficiency resulting in narrow peaks, increased signal/noise ratios and the separation of isomeric compounds, as opposed to the 3.5 µm, with which an almost two-fold greater peak width was achieved. Fused core columns (particle size of 2.7 µm) are also used when no UHPLC instrument is available, achieving increased efficiency but working at low pressure [77, 88].

Some studies include a pre-column in their methods so as to prevent the rapid deterioration of the column that could occur when used with such complex samples as biota [72, 75, 80, 90, 93, 183, 186]. Chu and Metcalfe [75], for instance, used a C_{18} (4 x 2 mm) pre-column.

The mobile phase is made up of two components: an organic solvent (methanol or acetonitrile) and an aqueous component. Different additives such as formic acid, ammonium acetate or acetic acid are added to the mobile phase to adjust pH, and it is also frequent for the additives to be added not only to the aqueous component but to the organic component too [82, 86, 93, 185]. For example, formic acid at a concentration of 0.1% has been used as a modifier in the mobile phase (in both aqueous and organic components) to separate two anticonvulsants and a number of transformation products [86]. Some studies also employ mixtures of methanol and acetonitrile as an organic component [77, 88, 187].

Different detectors such as the diode array detector (DAD) [184] and the fluorescence detector [90, 92] have been used to determine of pharmaceuticals in aquatic organisms. Cueva-Mestanza et al. [184] achieved limits of detection (LODs) of between 0.03 µg/g and 0.22 µg/g for the six pharmaceuticals studied in mussel samples when 1 g of sample was analysed by microwave micellar extraction followed by LC with DAD. However, the most widely used detection systems are MS and MS/MS.

When LC is coupled with MS the most challenging part is the ionisation. Atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) are the most common interfaces, working in positive or negative mode depending on the target analytes. Generally speaking, the ESI interface is used for the most polar compounds such as pharmaceuticals and it is the most extensively used because these compounds exhibit higher sensitivity than in APCI. Moreover, only a few pharmaceuticals are efficiently ionised by APCI [6]. APCI was used, for example, in Chu and Metcalfe's study [75] to determine fluoxetine, paroxetine and the metabolite norfluoxetine in fish samples. According to the authors, the gas phase ionisation process utilised in APCI is less susceptible to the matrix effect. However, these compounds have also been determined using ESI in other studies [76, 78, 88]. The main drawback to ESI is the presence of a matrix effect, which may lead to a significant difference in the response (in the shape of signal suppression or enhancement) of an analyte in a sample as opposed to in a pure standard solution [6]. This matrix effect is attributed to those organic and/or inorganic components of a sample that coelute with an analyte and interfere in the ionisation process. This phenomenon is even more serious in the case of analytes of aquatic organism samples because of their complexity. However, APCI does

not provide suitable ionisation when the analytes have polar properties. Thus different strategies have emerged to deal with the challenging matrix effect.

Different approaches to correct this problem have been described [6]. The most popular approach to correct the matrix effect is internal standard calibration, and the ideally internal standards are those isotopically labelled standards. Using this approach achieves a good quantification. However, it is limited by availability and high cost. Chu and Metcalfe [75] employed internal standards to quantify antidepressants in fish. An alternative is the standard addition of each analyte, but this approach increases analysis time. It was employed in the work by Brooks et al. [69] to determine antidepressants in different fish tissues. A third approach consists of matrix-matched calibration curves. However, obtaining an uncontaminated matrix can in some cases be a problem. McEneff et al. [188], for example, adopted this approach instead of the internal standard, due to the cost of the latter, to determine different pharmaceuticals in mussels. Wille et al. [70] used two strategies, matrix-matched calibration curves and internal standards, to correct the matrix effect. The matrix effect can be reduced by improving the sample-preparation procedure, which includes, for example, the clean-up strategies explained in the review paper presented in the previous section, and also by improving the chromatographic separation.

Another important part of MS instruments is the analyser. Different analysers have been used to determine pharmaceuticals in aquatic organisms, including low resolution mass spectrometry analysers such as the quadrupole (Q) [81] and the ion-trap (IT) [186] and high resolution mass spectrometry analysers such as the hybrid quadrupole time of flight (Q-TOF) [189], the Orbitrap [86, 190] and the hybrid Q-Orbitrap [191]. However, the preferred analysers are the triple quadrupole (QqQ) [70, 72, 79, 93, 185, 187, 192, 193] or the hybrid triple quadrupole linear ion trap (QqLIT) [7, 12, 71, 73, 74, 77, 87-89]. The main advantage of these analysers is that they provide enhanced sensitivity and selectivity when working in multiple reaction monitoring (MRM) mode. Confirmation of the identity of the target analytes is achieved by monitoring two transitions and, in addition, the relative abundances of the specific transitions are compared with those of the standards. For example, Wille et al. [70] achieved LODs of between 1 and 10 ng/g when 1 g of mussel sample was analysed by PLE followed by SPE as the clean-up step and LC with MS/MS using QqQ.

HRMS with Orbitrap has been used to determine carbamazepine and oxcarbazepine and some of their transformation products in mussel samples at low ng/g level [86]. In the same study Orbitrap was used as a powerful tool to identify of unknown compounds, with caffeine, metoprolol, cotinine and ketoprofen being identified in the samples analysed. In another study the same authors employed the Orbitrap analyser to quantify antidepressants (venlafaxine and some of their metabolites), again in mussel samples [190]. The Q-Orbitrap was also employed by Grabicoba et al. [191] to determine seventy pharmaceuticals in two benthic organisms.

The Q-TOF analyser was employed by Valdés et al. [189] to determine carbamazepine and atenolol in fish body after being exposed under laboratory conditions.

As far as GC is concerned, this technique was used in a number of studies [69, 91, 194], for example by Brooks et al. [69] in the first biota study, where anti-depressants in different fish tissues were determined by GC coupled to a single Q with chemical ionisation (CI). Subedi et al. [91] also employed GC to determine pharmaceuticals and PCPs in fish sample. In this case GC was coupled to IT as an analyser with electronic ionisation (EI). However, in all these studies a derivatisation step was required before GC-MS analysis.

The presence of triclosan in aquatic organisms has been analysed by both LC and GC coupled to MS or MS/MS [88, 100, 101, 104, 106, 108, 195]. In early studies the analytical methods focused only on triclosan or included one or two related compounds such as methyltriclosan (its metabolite) or clorophene [101-103, 195] and were performed with GC. Apart from triclosan, more recent methods also include other PCPs and contaminants such as pharmaceuticals, with LC being the most frequently used technique [7, 77, 87, 88, 104, 108, 109, 196].

When GC is used, a derivatisation step prior to analysis is often performed in order to decrease polarity and increase the volatility of the triclosan [101, 105, 107, 110, 195, 197, 198]. Derivatisation with 2,3,4,5,6-pentafluorobenzylbromide (PFBBr), *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) or a mixture of the latter and N-trimethylsilylimidazole (TMSI) have been employed. For example, Rüdel et al. [107] reported a method using GC with previous derivatisation of triclosan with

PFBBr as the derivatising agent. Other authors have described studies involving triclosan that have avoided the use of laborious and time-consuming derivatisation procedures [91, 102, 103, 106, 112]. Coogan et al. [102] for instance, developed a GC method to identify triclosan in the algae species *Cladophora* spp. using a single Q as analyser, but with the derivatisation step omitted.

Regarding the determination step, most of the methods developed employ low resolution Q [102, 103, 105, 107, 111, 197] or IT [91, 105, 195, 198] analysers with EI [100, 105, 106, 111, 112, 195, 197, 198]. However, CI was employed by Boehmer et al. [101] and Rüdel et al. [107]. GC has also been coupled with HRMS [106, 112]. Valters et al. [106] employ a double-focusing magnetic sector to determine triclosan as well as other EOCs in fish plasma.

In recent studies that also include other compounds, triclosan has been analysed mainly by LC. In these cases the C_{18} stationary phase is the preferred option [77, 88, 104, 108, 196]. Kim et al. [104] compared two C_{18} columns with different particle sizes (2.7 and 1.8 µm). The authors explained that the Ascentis C_{18} with a particle size of 2.7 µm was selected because higher efficiencies and lower back pressure were achieved. In another study two Zorbax columns of identical size, differing only in particle size (3.5 µm and 1.8 µm), were compared. In this case, however, the 1.8 µm particle size column was selected as it provided sharper peak resolution [109]. UHPLC columns were also employed by other authors [7, 87].

As in the case of pharmaceuticals, to determine triclosan by LC the common mobile phase used methanol as the organic component or a mixture of methanol acetonitrile. The aqueous component is water or water acidified with a modifier such as ammonium acetate or acetic acid, which can also be added to the organic component [77, 108, 109].

LC has been coupled to MS/MS with the QqQ [104, 108, 109] or QqLIT [7, 77, 87, 88] analysers, in both cases using the ESI as interface, operating in negative ionisation mode to determine triclosan.

As mentioned previously, biota matrices are very complex and determination can be affected by the matrix effect. Although external calibration and matrix-matched calibration curves have been reported as being used to quantify triclosan, the

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commonest approach to correct the matrix effect is to employ internal standards [88, 101-103, 197].

As for high-intensity sweeteners, to the best of our knowledge no studies of their presence in aquatic organisms have yet been published. For this reason the instrumental techniques explained will be those employed to determine their presence in other environmental matrices because the levels in these other matrices are also expected to be low. Of artificial sweeteners, sucralose is the most frequently studied and some analytical methods still focus only on this compound [122, 126, 199-204]. Sucralose has been demonstrated to be recalcitrant in that it survives wastewater treatment technologies, and this has attracted the attention of the scientific community. However, various studies include three or four other compounds (acesulfame, saccharin, sucralose and cyclamate) [117, 120, 121, 205-207]. The simultaneous determination of more high-intensity sweeteners such as aspartame, neohesperidin dihydrochalocone, neotame and alitame in addition to those mentioned above has also been performed in several studies [116, 119, 123-125, 128, 130, 208, 209].

The technique of choice to determine high-intensity sweeteners in environmental matrices is LC coupled to MS/MS [116, 118, 119, 121, 124, 209]. However, other techniques have been employed to a lesser extent. GC was used in one study to determine sucralose in coastal and marine waters [127], but a derivatisation step was required prior to instrumental analysis, making this a very time-consuming method. Ion chromatography has also been used in one study to determine four artificial sweeteners (acesulfame, saccharin, sucralose and cyclamate) in groundwater [210]. These separation techniques were coupled with IT and QqLIT respectively [127, 210].

In the case of LC separations, most published methods use reversed-phase columns such as C_{18} [117, 118, 122, 125, 128, 130, 201, 203] and C_8 [116, 120, 206, 207]. In Scheurer et al. [116], although a Zorbax Eclipse XDB-C_8 (150 x 4.6 mm; 5 µm) column provided excellent results and was employed in their work, the authors stressed that for faster analyses a UHPLC Zorbax Eclipse XDB-C_{18} (50 x 4.6 mm; 1.8 µm) would be more suitable as it would enable the analysis time to be shortened [116]. Gan et al. [128] tested C_{18} and C_8 stationary phases with identical dimensions and chose the C_{18} column because better separation was achieved. Some authors have employed UHPLC columns instead of conventional LC

columns [126, 203, 205, 207]. For example, Perkola et al. [205] employed a UHPLC column (Acquity HSS T3 C_{18}) to determine four artificial sweeteners (sucralose, acesulfame, saccharin and cyclamate) in surface waters.

Another type of chromatography, hydrophilic interaction (HILIC), was employed by Salas et al. [123] to determine seven high-intensity sweeteners in different aqueous matrices. They compared the Atlantis bare silica and the Syncronis zwitterionic sulfoalkylbetaine stationary phases and found that the latter was more suitable since it obtained better retention and separation of the analytes studied. The authors pointed out that the organic extract obtained from the extraction could be injected directly, thus avoiding the time-consuming evaporation step [123]. HILIC was also employed by Kokotou et al. [119]. In Ordóñez et al. [125], a reversed-phase column (employing a C_{18} column) and HILIC (employing a Luna HILIC column) were compared in terms of retention mechanisms. Although for most of the compounds lower limits of quantification were achieved with HILIC, the reverse phase was selected to validate the method because of its better separation performance, lower matrix effect and better precision (lower %RSD).

With regard to the mobile phase for reversed-phase LC, water has been used as the aqueous component, acidified with acetic acid or formic acid and also with ammonium acetate (between 1 and 20 mM). Methanol has been employed as the organic component, which is used as pure solvent or acidified like the aqueous component [116-118, 121, 125]. Acetonitrile has also been used as the organic component instead of methanol but in this case it has generally been used as pure solvent or acidified with acetic acid or ammonium acetate (5 mM) like the aqueous component [122, 124, 126, 128]. As for the HILIC mobile phase, the most important parameters to be considered are the pH and ionic strength, which must be carefully adjusted as explained in the work by Salas et al. [123].

In a few studies the ion pair reagent tris(hydroxymethyl) aminomethane (TRIS) has been added to the mobile phase or postcolumn when high-intensity sweeteners are determined [116, 128] in order to increase the ionisation yield. However, Berset et al. [130] also evaluate the addition of TRIS, but in their study the sensitivity of all the compounds did not increase and it was not used.

As for detection, most of the analyses opt for MS/MS using QqQ [117-119, 121, 124, 125, 130, 205, 207]. Another analyser, QqLIT has also been employed in

some studies [116, 122, 200, 211]. As regards HRMS, TOF has been used in one study, in which aspartame, saccharin and sucralose were determined in different environmental water and beverage samples [120]. Additionally, Orbitrap was employed in one study [123] and the authors noted its capacity for reliable quantification and confirmation of the measurement of accurate masses. Batchu et al. [129] employ the Q-Orbitrap analyser in another study to determine sucralose in different aqueous samples. In Ferrer et al. [203] QqQ and Q-TOF were compared to determine sucralose, and although Q-TOF offered enhanced selectivity due to accurate mass information, QqQ provided higher sensitivity and lower LODs, which makes it more suitable for the determination of sucralose in the environment because of the low concentrations reported.

ESI is almost always employed as the interface in all LC-MS and LC-MS/MS methods. Just one study used the APCI interface [126]. High-intensity sweeteners are reported to be ionised in the negative ionisation operation mode [116, 119, 123-125, 128], although aspartame, saccharine and sucralose have also been determined in positive ionisation mode [120, 203, 206]. Ferrer et al. [203] reported a sensibility ten times higher for sucralose when ionised in positive mode than in negative mode.

To sum up the determination of pharmaceuticals and high-intensity sweeteners has been done by LC, whereas triclosan has been analysed by both types of chromatography (GC and LC). For all the analytes studied in the present Thesis the detection is mainly performed by MS/MS with QqQ or QqLIT as analysers with ESI as the ionisation source.

1.5 References

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CHAPTER 2. OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
EMERGING ORGANIC CONTAMINANTS IN AQUATIC ORGANISMS
Mireia Núñez Marcé

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The main objective of this Thesis is to determine the presence of EOCs (pharmaceuticals and high-intensity sweeteners) in aquatic organisms, as well as to study the toxicity effect of a PCP (triclosan). To achieve these objectives, different analytical methods for determining the presence of various EOCs will be developed. For this purpose, LC coupled with MS will be used and two extraction techniques (QuEChERS and PLE), along with different clean-up strategies to analyse different bivalves and fish species, will be evaluated. Then, the toxicity of triclosan for the amphipod species *Gammarus pulex* will be studied. Different populations from contaminated and uncontaminated field sites will be evaluated in order to determine differences in triclosan sensitivity.

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CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

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As mentioned in the introduction, systematic research into EOCs in aquatic environments started in the 1990s, although initial studies in this field were performed in the 70s and 80s. Since then certain compounds and groups have been extensively studied and their occurrence is well documented. However, there is an overall lack of information on the ecotoxicological impact of most of these EOCs in terms of their toxicity, bioaccumulation and occurrence in organisms living in those aquatic environments. For this reason, the scientific community's interest in the subject has increased over the last ten to fifteen years. The research in this Thesis has mainly focused on the determination of different groups of EOCs, such as pharmaceuticals, one disinfectant and a group of high-intensity sweeteners in aquatic organisms.

The present Thesis was developed within the Chromatography and Environmental Applications research group at the Universitat Rovira i Virgili, which has extensive experience in determining EOCs in different environmental matrices. Nevertheless, so far there has been little work performed to determine such compounds in aquatic organisms.

This chapter includes the experimental part of the research and the results of different studies carried out in the course of this Doctoral Thesis. These results have already been or are in the process of being published in various international scientific journals. The chapter is divided into three sections, with each section containing a brief introduction establishing the context of the research and a discussion of the most important results at the end. Although the results of the experimental research included in each section have already been discussed individually in their respective papers, the most important aspects are again briefly presented here.

In the first section, two analytical methods of determining pharmaceuticals in different species of bivalve are presented. A different extraction technique was used in each: PLE and QuEChERS. In both cases LC-MS/MS (QqQ) was employed.

In the second section two analytical methods were developed for other classes of EOCs: the first to determine a group of ICM-XR (another class of pharmaceuticals) and the second to determine high-intensity sweeteners, both in different fish species. Each method was based on PLE as the extraction technique

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and LC coupled with HRMS (Orbitrap). Both were later applied to the analysis of different fish species.

In the third section an ecotoxicological study is presented in which the toxicity of triclosan was evaluated in different populations of *Gammarus pulex*. These populations came from environments with different degrees of pollution and their resistance to triclosan was compared. This study was carried out in the Helmholtz Centre for Environmental Research-UFZ in Leipzig (Germany), during a European placement that took place during the course of the Thesis.

A list of the articles published as a result of this Thesis is included in Appendix III.

3.1. Determination of pharmaceuticals in bivalves

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The contamination of water resulting from the widespread use of pharmaceuticals has been considered in recent decades and the global occurrence of pharmaceuticals in aquatic environments has come to be seen as a problem with unknown consequences [1]. Pharmaceuticals have been determined in different aquatic environments at ng/L and µg/L [2,3]. After consumption, pharmaceuticals are excreted and continuously released into municipal sewage systems, and it is well established that most are not completely removed and are emitted after passing through WWTPs [2]. Agricultural runoff and aquaculture applications are also important sources of veterinary pharmaceuticals in aquatic systems [3]. In addition, marine ecosystems are affected by pharmaceutical contamination, especially coastal areas, which are subjected to growing pressure due to the increase in human activities [4]. Several studies have demonstrated the occurrence of these contaminants in marine and estuarine waters [5-7]. To study and evaluate the fate, the effects and the environmental and human risks they represent in aquatic ecosystems, information regarding their presence in marine organisms is urgently needed. There is a perceived need to develop analytical methods that can be applied to real complex matrices such as living organisms, thereby enabling the pharmaceuticals in them to be determined [8,9].

In order to contribute to this need, two analytical methods were developed, both focusing on the determination of pharmaceuticals in bivalves, and the results achieved are presented in this section. A group of seven pharmaceuticals of widespread consumption belonging to two different classes (non-steroidal anti-inflammatory drugs and lipid regulators) was selected. Of the different pharmaceuticals evaluated, two were studied through the presence of their metabolites. This was the case with salicylic acid (the metabolite of acetylsalicylic acid) and clofibric acid (the active metabolite from a series of widely-used lipid regulators such as clofibrate, etofyllin clofibrate and etofibrate). The molecular structure of the compounds studied is detailed in Appendix II.

Bivalves such as mussels have often been used as bioindicators for aquatic pollution monitoring. They are filter-feeding organisms, since they filter large volumes of water for feeding and breathing purposes and are therefore liable to bioaccumulate contaminants. Moreover, they are representative of the sampling area because of their sessile behaviour [4].

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As extraction techniques PLE and QuEChERS were evaluated. PLE has been established as one of the most common techniques for extracting EOCs from biota samples and in recent years has been employed in various studies to extract pharmaceuticals from aquatic organisms [8,10,11]. Meanwhile the QuEChERS extraction technique has a number of features including low solvent consumption, low extraction times and no need for expensive equipment which have led to its being used in recent years to extract EOCs from biota matrices, and some studies have also used it to extract pharmaceuticals from aquatic organisms [12,13]. In order to obtain the highest recoveries for all of the compounds, different parameters affecting PLE extraction were evaluated. In the method based on QuEChERS, the two most common QuEChERS salt buffers (citrate and acetate buffer) were tested, also with the purpose of achieving the highest extraction recoveries. In addition, different clean-up strategies for both methods were evaluated after extraction in order to clean the extract.

Due to the polarity of the analytes the separation technique applied was LC, employing an Ascentis Express C_{18} Fused-Core® column (5 cm x 4.6 mm i.d.; 2.7 μm), coupled with MS/MS for the detection, employing a QqQ as analyser with electrospray in negative ionisation mode. As mentioned in the introduction, both QqQ and QqLIT are the common analysers used to determine pharmaceuticals in aquatic organisms due to the sensitivity and selectivity achieved [8,10,13].

After optimisation, both methods were applied to determine the selected compounds in bivalves such as the mussel (*Mytilus galloprovincialis* and *Mytilus edulis*), the lagoon cockle (*Cerastoderma glaucum*), the coquina clam (*Donax trunculus*), the Manila clam (*Ruditapes philippinarum*), the striped venus clam (*Chamelea gallina*) and the sword razor clam (*Ensis* sp.).

The results of these studies have been published in the Journal of Separation Science 39 (2016) 741-747 and in Analytical and Bioanalytical Chemistry 407 (2015) 3841-3849, and are presented in the following subsections.

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3.1.1 Pressurized liquid extraction followed by liquid chromatography with tandem mass spectrometry to determine pharmaceuticals in mussels

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PRESSURIZED LIQUID EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY TO DETERMINE PHARMACEUTICALS IN MUSSELS

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Abstract

An analytical method based on pressurized liquid extraction and solid-phase extraction with a mixed-mode Oasis® MAX sorbent as clean-up, followed by liquid chromatography with electrospray ionization and tandem mass spectrometry was developed and validated for the determination of seven widely used pharmaceuticals in mussel species. The optimization of the pressurized liquid extraction and the solid-phase extraction parameters is described. The method provided extraction recoveries ranging from 61% to 90%, and limits of detection ranging from 2 ng/g to 50 ng/g (dry weight). The repeatability and reproducibility of the method, expressed as relative standard deviation, were lower than 15% and 19% respectively. The method was successfully applied to the analysis of mussel samples from different locations. The analyses showed that salicylic acid was present in mussels at concentrations up to 177 ng/g (dry weight).

Keywords: *Liquid chromatography; mussels; pharmaceuticals; pressurized liquid extraction; tandem mass spectrometry.*

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1. Introduction

The growing use and consumption of pharmaceuticals worldwide has become an environmental problem. Pharmaceuticals and their metabolites are discharged into the aquatic environment, mainly due to their incomplete removal in wastewater treatment plants [1]. Pharmaceuticals are designed to have a specific response in human or animals through target-specific metabolic and molecular pathways. They are able to pass through biological membranes by diffusion to the target cells and tissues [2]. Depending on their characteristics, they can therefore be biologically active and produce significant side effects in non-target wildlife species [3]. Some pharmaceuticals, such as diclofenac, carbamazepine, ibuprofen, propranolol, fluoxetine have been determined in different water bodies at concentrations that have been demonstrated to cause a toxic effect or that might be in the range of toxicity [1, 4]. Moreover, according to Huerta *et al.* [1], the degradation rates of these contaminants in the aquatic environment are not high enough to compensate their introduction rates, making them pseudo-persistent. For this reason, potential long-term effects of pharmaceuticals on non-target aquatic organisms should not be ignored.

Several analytical methodologies have been described for the determination of pharmaceuticals in different environments. Water bodies and sediments have been extensively studied and several reviews have been published on this issue [5-9]. In recent decades, the coastline has become a highly anthropogenic environment and, thus, a certain amount of pollution may be expected in its marine coastal waters. For this reason, the monitorization of aquatic organisms living in these environments is a pressing issue. The study of aquatic organisms represents a challenge due to the complexity of this type of matrix, as well as the low concentration levels at which pharmaceuticals are expected in this type of matrix. These are obstacles that make the study of these organisms more difficult [10]. Invertebrates and algae can be used as indicator species, as they can integrate environmental variations [1]. Mussels are feed-filter organisms and they can incorporate contaminants from the environment. The open vascular system of mussels results in direct exposure to the environment and, therefore, to the contaminants present in it [11].

Some methods have been described to determine pharmaceuticals in biota [11-18]. These methods are mainly based on liquid chromatography (LC) coupled to

mass spectrometry (MS) [1]. In addition, several extraction techniques have been employed, such as ultrasonication [19], microwave-assisted extraction [11], QuEChERS [13, 18] and pressurized liquid extraction (PLE) [12, 14, 16, 17]. In general, PLE enables rapid rates of extraction, high recoveries, low solvent consumption and short extraction time [20, 21]. The main drawback of this technique is the co-elution of interfering compounds present in the matrix, such as lipids, pigments or proteins. For this reason, PLE is often followed by a clean-up step. Adsorption columns [22] or gel permeation chromatography (GPC) [23] and also solid-phase extraction (SPE) [16] are the most common clean-up techniques employed.

The aim of this study is to develop an analytical method for the determination in mussel species of seven widely used pharmaceuticals or their metabolites, which belong to different therapeutic classes. The mussel species *Mytilus galloprovincialis* was selected to optimize and validate the method as a sentinel species. The method proposed is based on PLE, with SPE as a subsequent clean-up step using the mixed-mode anion exchange sorbent, Oasis® MAX, followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The method was applied to determine the occurrence of the selected pharmaceuticals in mussel species from different locations.

2. Materials and methods

2.1 Reagents and chemicals

The standards used were salicylic acid, clofibric acid, ketoprofen, naproxen, bezafibrate, diclofenac and ibuprofen, all of which were purchased from Sigma-Aldrich (Steinheim, Germany).

Ultrapure water was obtained using an ultrapure water purification system from Veolia Water (Sant Cugat del Vallès, Spain). Acetonitrile (ACN) and methanol (MeOH) were of HPLC grade and supplied by J.T. Baker (Deventer, The Netherlands). Acetone was also of HPLC grade and was obtained from Prolabo (Llinars del Vallès, Spain). Acetic acid (CH_3COOH), formic acid (HCOOH) and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich and nitrogen gas was sourced from Carburos Metálicos (Tarragona, Spain).

Ottawa sand was purchased from Fisher Scientific (Waltham, Massachusetts, USA) and Oasis® MAX cartridges (150 mg/6 cc) were obtained from Waters (Milford, MA, USA). Florisil and alumina were obtained from Sigma-Aldrich.

2.2 Sampling and sample preparation

Mussels (*Mytilus galloprovincialis*) from different locations, including the Ebro River Delta on the Mediterranean coast (NE Spain) and Galicia on the Atlantic coast (NW Spain), as well as the species *Mytilus edulis* from the Atlantic coast of France, were bought in the local market. In the laboratory, they were removed from the shell and homogenized in order to obtain a biotic composite which was frozen before being lyophilized using a Labconco Freezone 4.5 (Kansas City, MO, USA) freeze-drier system. After lyophilization, samples were homogenized with a mortar and then sieved (125 µm) to obtain particles of the same diameter.

2.3 Pressurized liquid extraction and solid-phase extraction clean-up

1 g of freeze-dried mussel sample was wet with acetone and spiked with the analyte mixture in order to cover the whole mussel sample and ensure interaction with the matrix. The mixture was left inside an extractor hood to allow the solvent to evaporate and it was frequently homogenized.

The extraction was performed on an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA). A cellulose filter from Teknokroma (Sant Cugat del Vallès, Spain) was placed on the bottom of an 11 mL stainless steel extraction cell. Each cell was filled with 3 g of Ottawa sand and then 1 g of freeze-dried mussel sample that had previously been mixed and homogenized with Ottawa sand, and the void volume of the cell was further covered with Ottawa sand. A cellulose filter was placed on top. Ultrapure water was chosen as the extraction solvent and the optimized extraction conditions were: preheating time 5 min, temperature 100ºC, extraction time 10 min, 1 cycle, flush volume 150%, purge time 300 s and pressure 1500 psi.

The extract obtained from the PLE (~29 mL) was cleaned up by SPE with Oasis® MAX cartridges. A vacuum pump connected to a manifold from Teknokroma was used for the SPE procedure. The cartridge was preconditioned with 5 mL of MeOH followed by 5 mL of ultrapure water and then the PLE extract was

loaded. Afterwards, the washing step consists of 3 mL of ultrapure water containing 5% NH_4OH followed by 10 mL of pure MeOH. The elution step was performed with 10 mL MeOH containing 5% HCOOH. The eluate was then evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 5 mL of 0.5% CH_3COOH in ultrapure water/ACN (70/30, v/v). The final extract was filtered using 0.2 μm PTFE syringe filters obtained from Scharlab (Sentmenat, Spain) before injection.

2.4 Liquid chromatography-tandem mass spectrometry analysis

The chromatographic instrument was an Agilent 1200 series coupled to a triple quadrupole 6410 series mass spectrometer with electrospray ionization (ESI), all from Agilent Technologies (Waldbronn, Germany).

The chromatographic column was an Ascentis Express C_{18} Fused-Core® (5 cm x 4.6 mm i.d.; 2.7 μm) from Sigma-Aldrich. The LC-MS/MS conditions were adapted from a previous work [18]. In brief, chromatographic separation was performed with a mobile phase of 0.5% CH_3COOH in ultrapure water (A) and ACN (B). The gradient started at 30% B, which was maintained for 2 min, before being raised to 39% B in 6 min, and then to 100% B in 7 min, and it was maintained at 100% B for 3 min. Subsequently, it was decreased back to the initial conditions in 2 min. The column was allowed to equilibrate under the initial conditions for 5 min between injections. The flow-rate was 0.6 mL/min, the oven temperature was 25ºC and the injection volume was 25 μL.

ESI optimal conditions were: negative ionization mode, capillary voltage 3000 V, nebulizer pressure 60 psi, drying gas (N_2) flow 7 L/min and drying gas temperature 250ºC. The MS/MS parameters were from optimized for each compound individually and were the same as [18] and summarized in supplementary information.

3. Results and discussion

3.1 Liquid chromatography-tandem mass spectrometry

The LC-MS/MS method was first evaluated in terms of linear range, limits of quantification (LOQs) and limits of detection (LODs). All of the analytes showed

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good linearity with $R^2 > 0.99$. The linear range was between 1 and 500 μg/L for diclofenac, naproxen and salicylic acid; between 1 and 250 μg/L for clofibric acid; between 0.5 and 500 μg/L for bezafibrate; and between 5 and 500 μg/L for ketoprofen and ibuprofen. LOQs were calculated as the concentration giving peak signals for which the ratio signal/noise (S/N) was ten. LODs were calculated as the concentration giving peak signal for which the $S/N \geq 3$. The LODs were between 0.1 and 0.25 μg/L for all of the compounds, apart from ketoprofen and ibuprofen, with LODs of 2.5 μg/L.

3.2 Pressurized liquid extraction

In order to obtain good extraction efficiencies several instrumental PLE parameters have to be optimized, including the following most important ones: extraction solvent, extraction temperature, extraction time and number of cycles [20]. During PLE, there are also other instrumental parameters that can be optimized, such as pressure, preheating time, flush volume and purge time. However, these parameters have been found not to have a significant effect on extraction efficiency [20]. For this reason, they were not optimized in the present study and they were set based on our previous experience [24].

For each test, a non-spiked mussel sample (blank) was analysed to subtract the signal value of analytes present in the sample. For all of the tests, 1 g of lyophilized mussel sample was spiked at 1000 ng/g (d.w.) with the studied analytes.

To optimize the extraction process, initial conditions were proposed based on common PLE conditions, which were as follows: preheating time 5 min, extraction time 10 min, extraction temperature 100ºC, pressure 1500 psi, flush volume 150%, purge time 300 s and number of cycles 1.

The first parameter optimized was the extraction solvent with the following being tested: MeOH, ACN, ultrapure water, and acidified water (0.5% CH_3COOH in ultrapure water). In order to select the most suitable solvent, the recovery of the extraction process (PLE RE) and the matrix effect (ME) were evaluated. PLE REs were calculated by comparing the peak signals of the analytes in a sample spiked before PLE with the peak signals of the analytes obtained with a sample spiked, at the same concentration, after PLE. The ME was calculated as follows:

$$ME (\%) = -[100 - (B/A*100)]$$

Where (A) is the instrumental response for standards injected directly to the LC-MS/MS and (B) is the analytes' response in a mussel extract spiked just before being injected into the LC-MS/MS. If the ME=0, no matrix effect is present. If the ME>0, there is signal enhancement and, if the ME<0, there is signal suppression.

With respect to PLE REs, as can be seen in Table 1, ACN provided lower PLE REs for the first three eluted compounds (salicylic acid, clofibric acid and ketoprofen), and acidified water gave lower PLE REs for all of the compounds, with the exception of salicylic acid and clofibric acid. MeOH provided the highest recoveries and ultrapure water was the second most efficient solvent, with just the last two eluted compounds, diclofenac and ibuprofen, displaying lower PLE REs. In terms of the ME, a high ME was observed when MeOH was used as the extraction solvent, being higher than 50% for most of the compounds. The ME is one of the main disadvantages of LC-MS and it can be especially severe at low analyte concentration levels [1]. For this reason, ultrapure water was selected as the extraction solvent, for most of the compounds it showed a similar extraction recovery to MeOH and presented a lower ME. The ME was not evaluated for ACN and acidified water because, as mentioned, their PLE REs were already lower.

Using water as the extraction solvent involves an associated difficulty. The water extract cannot be evaporated directly. Thus, one strategy is to link the aqueous PLE extract to an SPE process. The use of SPE allows an extract based on an organic solvent to be obtained that can be evaporated. It also enables the PLE extract to be cleaned in order to reduce the interfering substances. The SPE procedure is described in the following Section, since it is grouped with the clean-up strategies.

The second parameter optimized was the temperature and 40ºC, 60ºC, 80ºC, 100ºC and 120ºC were tested. It was observed that PLE REs were affected by the temperature, as they decrease as the extraction temperature drops. Table 1 also shows, as an example, the PLE REs when the temperature was set at 60ºC in comparison to when the temperature was set at 100ºC, with water as the extraction solvent. In general, all of the compounds displayed a reduction in

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terms of PLE REs when they were extracted at 60ºC. Salicylic acid was the most affected compound. In addition, when temperature was set at 120ºC, PLE REs were not increased, but ME was slightly higher (data not shown). Thus, the temperature was set at 100ºC for further experiments.

Extraction time was also evaluated between 5 and 15 minutes in increments of 5 min. In general, the PLE REs were slightly better (increase ~10%) when the extraction time was set at 10 min, and it was decided to maintain 10 min as the extraction time.

Finally, the number of cycles was also evaluated and, 2 and 3 cycles were tested. However, no increase in PLE REs was observed. For this reason, the number of cycles was set at one.

Table 1. Extraction recoveries and matrix effect using different extraction solvents and temperatures.

Compound	100ºC, 10 min						60ºC, 10 min
	ACN PLE RE (%)	H$_2$O 0.5%CH$_3$COOH PLE RE (%)	MeOH		Ultrapure water		
			PLE RE (%)	ME(%)	PLE RE (%)	ME (%)	PLE RE (%)
Salicylic acid	30	87	74	-46	90	-21	17
Clofibric acid	54	71	99	-44	84	-17	87
Ketoprofen	37	45	82	-63	78	-35	77
Naproxen	70	42	88	-69	74	-39	65
Bezafibrate	62	40	96	-68	81	-21	66
Diclofenac	73	7	82	-87	61	-38	30
Ibuprofen	78	16	89	-90	68	-33	42

%RSD (n=3)≤10%.

3.3 Clean-up

Two strategies were tested in order to reduce the ME. The first strategy involved a subsequent clean-up employing SPE because, as mentioned, the use of ultrapure water entails the use of an SPE step. The second strategy was an additional in-cell clean-up and two different sorbents were evaluated. This second strategy also involves a subsequent SPE step so that the aqueous PLE extract is converted into an organic extract that can be easily evaporated.

3.3.1. Solid-phase extraction clean-up

According to Chu and Metcalfe [16], for the determination of pharmaceuticals, Oasis® HLB has been extensively used as an SPE sorbent for cleaning up complex environmental samples prior to LC-MS/MS analysis. However, the authors postulate that, in complex samples such as biota, co-extractives present in the matrix will be retained by the sorbent and may co-elute with the target compounds in the elution process. To tackle this co-elution, in the present study, the more selective sorbent, Oasis® MAX, was tested. Oasis® MAX is based on the same polymeric structure as Oasis® HLB, and contains the quaternary amine groups on the surface, which promotes the anion-exchange interactions with the acidic compounds [25]. This is the case of the selected compounds (pK_a values for these compounds range between 3.0 and 4.9) that must be selectively retained on the Oasis® MAX sorbent by ion-exchange interactions.

Apart from the selection of Oasis® MAX, the different steps in the SPE protocol must be optimized in order to ensure the mixed-mode anion-exchange interactions between the sorbent and the target analytes.

In the present study, the different steps of the SPE clean-up were optimized using the suppliers' protocol as starting point. After loading the PLE extract (~29 mL of aqueous extract), two washing steps were performed, the first with 3 mL of 5% of NH_4OH in ultrapure water, and the second with MeOH. Different volumes (3 mL, 5 mL and 10 mL) of MeOH were tested, and 10 mL was selected as no losses of the analytes were observed. The elution step was also optimized by testing 3%, 5% and 10% of HCOOH in MeOH, with 5% being enough to elute all of the compounds. The volume of elution solvent was also assessed and 5 mL, 10 mL, 15 mL and 20 mL were tested. With 5 mL of 5% HCOOH in MeOH, all of the compounds eluted with the exception of salicylic acid (just 10% eluted) that needed a total of 10 mL of elution solvent, to achieve the largest recovery. For this reason, 10 mL was chosen as final elution volume. The elution fraction obtained was evaporated to dryness and resuspended in 10 mL of initial mobile phase composition in order to obtain a good peak shape in LC.

In order to evaluate the effectiveness of the cleaning, an aliquot of 100 µL of a 10 ppm solution was spiked to the PLE extract. The recovery of the SPE (SPE RE) was calculated for each analyte as the signal peak ratio between a mussel sample

spiked after PLE (before SPE) and the peak signal of a mussel sample spiked, at the same concentration, before injection into the LC-MS/MS system. The values of SPE REs are shown in Table 2. SPE recoveries were higher than 76% with the exception of salicylic acid, for which a recovery of 60% was recorded despite testing larger amount of elution solvent.

Moreover, apparent recoveries (App REs), which involve losses caused by any step in the whole method as they include the entire procedure (i.e. extraction, cleaning and ME), were calculated by interpolation of the signal of the analytes obtained from a mussel sample spiked at 1000 ng/g (d.w.) before PLE with an external calibration curve. As can be seen in Table 2, the App RE for most of the analytes is still low (values from 35% to 75%). Therefore, another strategy to clean up the matrix was assayed.

Table 2. Solid-phase extraction recoveries and apparent recoveries using different in-cell clean-up sorbents.

Compound	SPE+ no in-cell clean-up		SPE+ in-cell clean-up	
	SPE RE (%)	App RE (%)	Florisil App RE (%)	Alumina App RE (%)
Salicylic acid	60	53	3	29
Clofibric acid	77	75	85	80
Ketoprofen	80	60	60	63
Naproxen	95	54	57	59
Bezafibrate	76	59	49	64
Diclofenac	94	35	40	51
Ibuprofen	79	45	46	44

%RSD (n=3)≤10%.

3.3.2. In-cell clean-up

In-cell clean-up with florisil and alumina was evaluated to clean the matrix. These two sorbents have been described as fat retainers [26] and used as in-cell sorbents in biotic samples [12, 26]. The PLE cell set-up was as follows: 1 g of the cleaning sorbent (florisil or alumina) was placed at the bottom of the extraction cell instead of Ottawa sand. 1 g of frozen-dried sample was then mixed with 1 g of Ottawa sand and the void volume of the cell was filled with Ottawa sand. The PLE procedure and the subsequent SPE clean-up was as in Section 3.3.1.

To evaluate if the clean-up step was effective, App REs were calculated and were compared with those obtained when no sorbent was employed and just SPE was used. In all cases, the elution fraction obtained from the SPE was evaporated to dryness and resuspended in 10 mL of the initial mobile phase composition. Table 2 shows the App REs obtained when each sorbent was added and without any sorbent being added. App REs obtained by using florisil and alumina were very similar to those found when no in-cell clean-up was applied, and even salicylic acid displayed a lower App RE in both cases. Salicylic acid may be retained by these sorbents. For this reason, in-cell clean-up was rejected as a clean-up strategy.

3.4 Method validation

The mussel species *Mytilus galloprovincialis* was selected to perform the validation. The following parameters were evaluated: App REs and ME, which were calculated as a percentage at two concentration levels. For the lowest concentration level, mussel samples were spiked at 25 ng/g (d.w.) with clofibric acid, naproxen, bezafibrate and diclofenac, and 125 ng/g (d.w.) with salicylic acid, ketoprofen and ibuprofen. This distinction was based on their different signal responses. For the highest concentration level, the same distinction between compounds was applied and samples were spiked at 50 ng/g (d.w.) and 250 ng/g (d.w.), respectively. Linear range, LODs, LOQs were evaluated as well as repeatability and reproducibility between days at two concentration levels. All of the results are shown in Table 3. In order to concentrate the extract and achieve lower LODs and LOQs, a reduction in the final volume of reconstitution was assayed, from 10 mL to 5 mL. Finally, 5 mL was chosen as the final method volume, although a reduction of App REs was observed. For salicylic acid, which was present in the blank samples, the peak area observed in the blanks was subtracted from the peak area obtained in the spiked samples.

The ME was also evaluated and all of the compounds displayed signal suppression ranging between -15% and -52%, with naproxen, ketoprofen and ibuprofen being the most affected compounds, with values of -52%, -49% and -47% respectively. The ME values found in the present study are very similar to those found in a previous work in which QuEChERS was used as extraction technique and silica gel was employed as a clean-up step when a group of pharmaceuticals were determined in bivalve samples [18].

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App REs were also calculated and they ranged between 21% and 69% at the lowest spiked level (Table 3). For the highest level, similar results were obtained (data not shown). The losses observed are mainly attributable to the ME.

Figure 1 shows a multiple reaction monitoring (MRM) chromatogram corresponding to the quantification transition for each compound, in mussel sample spiked with the analyte mixture.
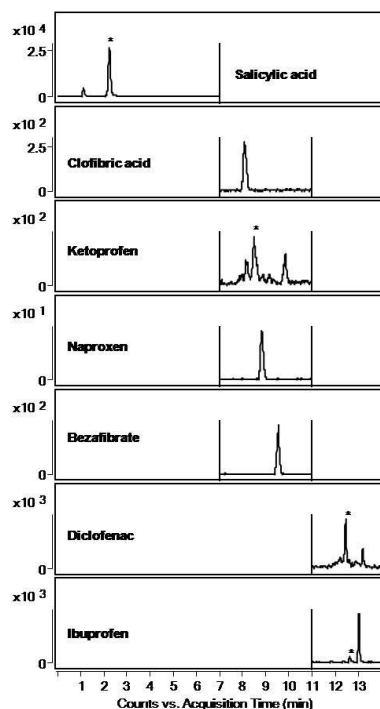

Figure 1: Multiple reaction monitoring chromatograms of the analysis of a mussel sample spiked at 50 ng/g with clofibric acid, naproxen, bezafibrate and diclofenac and 250 ng/g with salicylic acid, ketoprofen and ibuprofen. All signals correspond to the main transition.

Linear range, LOD and LOQ were obtained experimentally by spiking mussel samples at different concentration levels before extraction. Due to the high ME observed, matrix matched calibration curves were plotted for all of the compounds in order to minimize its effect, and they showed good linearity in the

range shown in Table 3, with R^2 always higher than 0.9955. LODs and LOQs were calculated following the same criteria as for the instrumental limits in Section 3.1, with the exception of salicylic acid, which was present in the blank samples, and their LOD and LOQ were estimated using an external calibration curve and its apparent recovery factor was applied. LODs were 2.5 ng/g (d.w.) for all of the compounds except for salicylic acid, which was 2 ng/g (d.w.), and for ibuprofen and ketoprofen, which were 50 ng/g (d.w.). LOQs were 12.5 ng/g (d.w.) for all of the compounds with the exception of ibuprofen and ketoprofen, which were 125 ng/g (d.w.), and salicylic acid, which was 8 ng/g (d.w.).

In a previous study performed on mussel samples [12], PLE was used as extraction technique but with MeOH as the extraction solvent and SPE with a Strata-X cartridge as a clean-up step. In this case, the recoveries ranged between 98% and 103%. However, it is not clear described which steps include these recoveries. LOQ for salicylic acid is similar to the obtained in the present study. Ramírez *et al.* [15] found similar LODs for clofibric acid and ibuprofen in fish muscle tissue, while Huerta *et al.* [14] found lower LOQ and LOD for diclofenac in different fish tissues. The results found in the present study are very similar to those found in a previous work [18] where similar compounds were analysed in bivalve samples, but using QuEChERS as the extraction technique and silica gel as a clean-up step.

Repeatability and reproducibility were evaluated using five replicate extractions (n=5) of spiked mussel sample at both concentration levels performed on the same day and different days, respectively. In both cases, the results are expressed by the percentage of relative standard deviation (%RSD). Values of repeatability were always below 15% and reproducibility values were always below 19% in the lowest level of concentration (Table 3), similar results were found at the higher concentration level.

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Table 3. Method validation data when the mussel samples were analysed by pressurized liquid extraction followed by Oasis® MAX clean-up and liquid chromatography-tandem mass spectrometry.

Compound	25 ng/g (d.w.)		Liniar range	LOD[3]	Repeatability	Reproducibility
	App RE[1] (%)	ME[2] (%)	ng/g (d.w.)	ng/g (d.w.)	(%RSD; n=5) 25 ng/g (d.w.)	(%RSD; n=5) 25 ng/g (d.w.)
Salicylic acid	69*	-15*	8- 1000	2	3*	18*
Clofibric acid	35	-32	12.5- 1000	2,5	10	12
Ketoprofen	45*	-49*	125- 1000	50	15*	17*
Naproxen	21	-52	12.5- 1000	2,5	9	19
Bezafibrate	26	-34	12.5- 1000	2,5	6	15
Diclofenac	37	-36	12.5-1000	2,5	9	15
Ibuprofen	28*	-47*	125- 1000	50	8*	10*

[1]App RE: apparent recovery
[2]ME: Matrix effect
[3]LOD: Limit of detection
*samples spiked at 125 ng/g (d.w.)

3.5 Method application

The optimized method was applied to evaluate the occurrence of the studied compounds in mussels from three different locations. The species *Mytilus galloprovincialis* from the Ebro River Delta on the Mediterranean coast (NE Spain) and Galicia on the Atlantic coast (NW Spain) and the species *Mytilus edulis* from France were selected.

Salicylic acid was found in all of the samples analysed. The concentrations found were between 87.3 ng/g (d.w.) and 177.4 ng/g (d.w.) and the lower values were found in the species *Mytilus edulis*. It should be pointed out that the other studied compounds were not found in any of the samples analysed.

Salicylic acid has been found in several studies that analyse pharmaceuticals in different water bodies [27, 28]. Some authors have already identified this compound as one of the most commonly identified pharmaceuticals in Belgium coastal waters [29]. Most of the other pharmaceuticals studied (bezafibrate, clofibric acid, diclofenac, ketoprofen) have been also detected in coastal zones [29], although they have not been detected in mussel samples.

Salicylic acid was also detected in mussel samples from Belgium, which concentrations were variable and up to 490 ng/g (d.w.) [12]. In this work [12], other pharmaceuticals such as ketoprofen, clofibric acid and diclofenac were also

evaluated. However, these compounds were not detected in any sample, as is the case in the present study. In other studies that have also determined different pharmaceuticals, such as clofibric acid and ibuprofen [15], and diclofenac [14] among others, in different fish species, only diclofenac was found at levels up to 8 ng/g [14].

4. Concluding remarks

A method was successfully developed for the determination of seven pharmaceuticals in mussels using PLE, with Oasis® MAX SPE for the clean-up step and followed by LC-(ESI)MS/MS. Different PLE parameters and clean-up strategies were evaluated in order to obtain the highest recoveries and the lowest ME. The optimal method provided ME between -15% and -52% and App REs between 21% and 69%, with LOQs ranging between 8 ng/g to 125 ng/g (d.w.). When the method was applied to analyse different species of mussels from different areas, only salicylic acid was found in all of the samples at concentrations up to 177.4 ng/g.
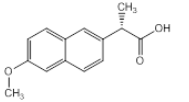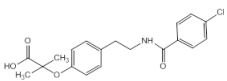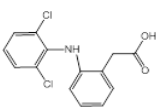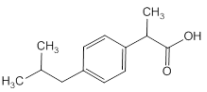
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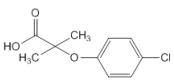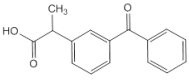
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Supporting information

Table S1. Studied compounds with their respective structures, pk$_a$, log Kow, vapour pressure, and optimal MS/MS parameters.

Compound	Structure	pk$_a$[1]	Log Kow[2]	Vapour pressure[2]	Precursor ion[3] (m/z)	Product ions[4] (m/z)	Ion ratio (%)
Salicylic acid		3.5	2.26	$8.20 \cdot 10^{-5}$	137 (80)	**93** (10) 65 (35)	7.7
Clofibric acid		3.0	2.57	$7.54 \cdot 10^{-5}$	213 (80)	**127** (10) 85 (5)	13.9
Ketoprofen		4.4	3.12	$1.46 \cdot 10^{-6}$	253 (60)	**209** (5)	
Naproxen		4.2	3.18	$1.27 \cdot 10^{-6}$	229 (60)	**169** (30) 170 (10) 185 (5)	78.7 31
Bezafibrate		3.9	4.25	$6.12 \cdot 10^{-11}$	360 (100) 360 (100) 362 (100)	**274** (10) 154 (25) 276 (10)	36.8 30.5
Diclofenac		4.1	4.51	$6.14 \cdot 10^{-8}$	294 (80) 296 (80) 294 (80)	**250** (5) 252 (5) 214 (20)	57.7 6.5
Ibuprofen		4.9	3.97	$1.86 \cdot 10^{-4}$	205 (60)	**161** (5)	

[1]pk$_a$ values were obtained using Advanced Chemistry Development (ACD/Labs Software).
2Log Kow values were obtained from PubChem.
[3]Vapour pressure expressed in mm Hg at 25ºC. Values were obtained from PubChem.
[4]In brackets the cone voltage (V) for each precursor ion is shown.
[5]In brackets the collision energy (eV) for each product ion is shown. Bold denotes the quantification ion.

3.1.2 Determination of pharmaceuticals in bivalves using QuEChERS extraction and liquid chromatography-tandem mass spectrometry

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EMERGING ORGANIC CONTAMINANTS IN AQUATIC ORGANISMS
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DETERMINATION OF PHARMACEUTICALS IN BIVALVES USING QUECHERS EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A method for the quantitative determination of seven pharmaceuticals in bivalves was developed by QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization. Both, the European Standard Method EN 15662 and the AOAC Official Method 2007.01 for QuEChERS were tested. In addition several clean-up strategies were evaluated in order to clean the matrix previous to the LC-MS/MS analyses. Dispersive solid-phase extraction with silica gel and modification of the chromatographic separation were the clean-up strategies that gave the best results.

The optimized method was validated in mussels (*Mytilus galloprovincialis*) and allowed the determination of pharmaceuticals at nanongrams per gram levels (dry weight). Limits of quantification ranged from 5 to 100 ng/g. Apparent recoveries ranged from 35% to 77%. The application of this method to bivalves revealed the presence of salicylic acid at concentrations up to 103 ng/g (d.w.).

Keywords: Pharmaceuticals; QuEChERS; liquid chromatography-tandem mass spectrometry; bivalves.

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1. Introduction

Pharmaceuticals are widely used in human as well as animal applications. They are introduced into the aquatic system due to the incapability of wastewater treatment plants to eliminate them completely and also as a consequence of agricultural runoff and aquaculture applications [1]. They are considered to be emerging organic contaminants (EOCs) and most of them are still unregulated or not commonly regulated [2]. Several studies have determined the presence of pharmaceuticals in different water bodies, such as surface [3] and ground waters [4], from nanograms per litre to micrograms per litre concentrations and in estuarine [5] and marine waters [6] at levels of nanograms per litre.

In the last few years, several studies have started to focus on the determination of EOCs in aquatic organisms. Living organisms are complex sample matrices which contain a high amount of interfering compounds that complicate the determination of the target compounds. According to the review by Huerta et al. [1] several studies describe the presence of pharmaceuticals in aquatic organisms, mainly in fish. Included among the aquatic organisms, bivalves are sessile filter-feeding organisms which interact with water and sediment and filter large volumes of water [7]. They are, therefore, particularly susceptible to environmental contaminants and are an interesting group of organisms to be monitored. In a recent publication, Martínez Bueno et al. [8] developed an analytical method for the determination of 2 anticonvulsants and some of their transformation products in mussels (*Mytilus galloprovincialis*), enabling the detection of the target compounds at low ng/g concentration levels.

The complexity of the biotic matrices normally requires time-consuming sample preparation. The most commonly used extraction techniques in biotic samples include: ultrasonication [9], rotary extraction [10], microwave-assisted micellar extraction (MAME) [7] and pressurized liquid extraction (PLE) [11]. Usually, the extraction methods based on these techniques are followed by solid-phase extraction (SPE) as a subsequent clean-up step. An alternative extraction technique is QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), which was introduced by Anastassiades et al. [12] in 2003 to determine pesticide residues in fruit and vegetables. The QuEChERS methods involve a first step based on salting-out extraction with a solvent (mainly acetonitrile) followed by dispersive SPE (dSPE). Besides its original application, this technique has already been employed

for the extraction of pharmaceuticals in different matrices such as sediments [13,14], sewage sludge [15,16], soil [17-19], blood [20-22], milk [23], molluscs [8,24] and fish [25], among others.

To determine the presence of pharmaceuticals, gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) are the most common techniques, due to their selectivity, specificity and sensitivity. In LC-MS and LC-MS/MS, atmospheric pressure ionization (API) sources are the most commonly used interfaces. In a study conducted by Schlüsener and Bester [26], the two ionization modes, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were compared for the analysis of steroid hormones in influents and effluents of sewage treatment plants and a high matrix effect was observed in the use of ESI. However, only a few pharmaceuticals can efficiently be ionized by APCI and, for this reason, most of them have to be determined using ESI [1,26].

The aim of this study was to develop a rapid method based on QuEChERS extraction followed by LC-MS/MS to determine the presence in different bivalve species of seven relevant pharmaceuticals from different therapeutic classes: salicylic acid, the metabolite of an analgesic; clofibric acid, the metabolite of a lipid regulator; bezafibrate another lipid regulator; and four non-steroidal anti-inflammatory drugs (NSAIDs) - ketoprofen, naproxen, diclofenac and ibuprofen. Of these bivalve species, the mussel *Mytilus galloprovincialis* was selected to validate the method and, later, this was applied to evaluate the occurrence of these pharmaceuticals in various bivalve species.

2. Material and methods

2.1 Reagents and chemicals

Salicylic acid, clofibric acid, ketoprofen, naproxen, bezafibrate, diclofenac and ibuprofen were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions of individual standards at 1000 mg/L were prepared in methanol and stored at 4 ºC.

The citrate buffer packet was obtained from Scharlab (Sentmenat, Spain) and contained 4 g magnesium sulphate, 1 g sodium chloride, 0.5 g sodium

hydrogencitrate sesquihydrate and 1 g sodium citrate, whereas the acetate buffer packet was obtained from Supelco (Sigma-Aldrich) and contained 6 g of magnesium sulphate and 1.5 g of sodium acetate.

Various materials for dSPE were tested: PSA/magnesium sulphate, PSA/magnesium sulphate/C_{18} and silica gel from Scharlab; PSA/magnesium sulphate/ GCB, Z- Sep$^+$, Florisil and alumina from Supelco.

Ultrapure water was obtained using an ultrapure water purification system from Veolia Water (Sant Cugat del Vallès, Spain). Acetonitrile (ACN) and methanol were of HPLC grade and supplied by Prolabo (Llinars del Vallès, Spain). Acetic acid (LC-MS grade) was purchased from Sigma-Aldrich and nitrogen gas (N_2) was sourced from Carburos Metálicos (Tarragona, Spain).

2.2 Sampling and sample pre-treatment

All bivalve species were bought in the local market including lagoon cockle (*Cerastoderma glaucum*), coquina clam (*Donax trunculus)*, manila clam (*Ruditapes philippinarum*), striped venus clam (*Chamelea gallina),* sword razor clam (*Ensis sp.*) and mussel (*Mytilus galloprovincialis*) from different locations, such as Galicia, Atlantic coast (NW, Spain) and the Ebro River Delta, Mediterranean coast (NE, Spain) and (*Mytilus edulis*) from the Atlantic coast of France.

The sample pretreatment was the same for all of the mentioned species. All organisms were removed from the shell, homogenized with a Taurus Robot 300 and the composite biotic samples obtained were frozen for 24 h before being freeze-dried with a Labconco Freezone 4.5 (Kansas city, MO, USA).

2.3 QuEChERS extraction

The European Standard Method EN 15662 was adapted to the dried matrix. One gram of frozen-dried sample was weighed in a 50 mL polypropylene centrifuge tube from Scharlab. 10 mL of water was added and the tube was shaken manually for 1 min. Then, 10 mL of ACN was added and the tube was shaken vigorously also for 1 min. Subsequently, the citrate buffer packet (EN method) was added and the tube was first shaken manually for 15 seconds and then using a vortex

(Heildolph Reax 2000) for 45 seconds. Afterwards, the tube was centrifuged for 5 min at 7000 rpm in a centrifuge from Hettich Zentrifugen (Germany). The supernatant (ACN layer) was transferred into a 15 mL centrifuge tube from Supelco containing 1 g of silica gel as a dispersive sorbent. After this step, the extract was shaken manually for 15 seconds and then using a vortex for 45 seconds, before finally being centrifuged for 5 min at 7000 rpm. 1 mL of the supernatant was transferred into a glass tube and evaporated to dryness under a gentle N_2 stream. The residue obtained was redissolved in 1 mL of 0.5% acetic acid in ultrapure water/ACN (70/30, v/v) and filtered through a 0.22 μm PTFE syringe filter from Scharlab before injection.

2.4 Liquid chromatography-tandem mass spectrometry analysis

Chromatographic analyses were performed with an Agilent 1200 series HPLC (Waldbronn, Germany) coupled to a triple quadrupole (QqQ) 6410 series MS with an ESI interface from Agilent Technologies. The HPLC system was equipped with a degasser, a binary pump, an automatic injector and a column oven, all from Agilent Technologies. The chromatographic separation was achieved with an Ascentis Express C_{18} Fused-Core® column (5 cm x 4.6 mm i.d.; 2.7 μm) from Supelco. The mobile phase was 0.5% acetic acid in ultrapure water (A) and ACN (B). The separation was performed with the following gradient: initially 30% B, which was maintained for 2 min, then raised to 39% B in 6 min and to 100% B in 17 min, which was maintained for 2 min, before finally being decreased back to initial conditions in 3 min. Between injections, the column was allowed to equilibrate under the initial conditions for 5 min. The flow-rate was 0.6 mL/min, the oven temperature was set at 25ºC and the injection volume was 25 μL.

Injections of individual standards of 1 ppm dissolved with a mobile phase composition of 50/50 (A:B) were used to optimize MS/MS parameters. For each compound, one or two precursor ion/s was/were selected and the cone voltage was then optimized for each precursor ion (Table 1). The optimized ionization source parameters were: capillary voltage of 3000 V in the negative mode, nebulizer pressure of 60 psi, drying gas (N_2) flow of 7 L/min and drying gas temperature of 250ºC. Collision energies were optimized in order to select, when possible, three characteristic multiple reaction monitoring (MRM) transitions for each compound (Table 1). Moreover, MRM ratios (the relation between the abundance of each qualifier transition and the quantifier transition) were calculated (Table 1).

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Chromatograms and spectra were recorded and processed using Agilent Mass Hunter Qualitative Analysis software.

Table 1. MRM transitions and MS/MS parameters.

Compound	C.V.[1] (V)	C.E.[2] (eV)	Precursor ion (m/z)	Product ions (m/z)	Ion ratio (%)
Salicylic acid	80	10	137 [M-H]$^-$	93 [M-H-CO$_2$]$^-$	
		35		65 [M-H-CO$_2$-CO]$^-$	7.7
Clofibric acid	80	10	213 [M-H]$^-$	127 [C$_6$H$_4$ClO]$^-$	
		5		85 [C$_4$H$_5$O$_2$]$^-$	13.9
Ketoprofen	60	5	253 [M-H]$^-$	209 [M-H-CO$_2$]$^-$	
Naproxen	60	30	229 [M-H]$^-$	169 [M-H-C$_2$H$_4$O$_2$]$^-$	
		10		170 [M-H-C$_2$H$_3$O$_2$]$^-$	78.7
		5		185 [M-H-CO$_2$]$^-$	31
Bezafibrate	100	10	360 [M-H]$^-$	274 [M-H-C$_4$H$_6$O$_2$]$^-$	
		25	360 [M-H]$^-$	154 [M-H-C$_{12}$H$_{14}$O$_3$]$^-$	36.8
		10	362 [(M+2)-H]$^-$	276 [(M+2)-H-C$_4$H$_6$O$_2$]$^-$	30.5
Diclofenac	80	5	294 [M-H]$^-$	250 [M-H-CO$_2$]$^-$	
		5	296 [(M+2)-H]$^-$	252 [(M+2)-H-CO$_2$]$^-$	57.7
		20	294 [M-H]$^-$	214 [M-H-CO$_2$-HCl]$^-$	6.5
Ibuprofen	60	5	205 [M-H]$^-$	161 [M-H-CO$_2$]$^-$	

[1] C.V.: cone voltage; [2] C.E.: collision energy

3. Results and discussion

3.1 Liquid chromatography-tandem mass spectrometry

Chromatographic separation was achieved with the following gradient: 30% B was maintained for 2 min, before being raised to 39% B in 6 min, and then to 100% B

in 7 min and it was maintained 100% B for 3 min. Subsequently, it was decreased back to the initial conditions in 2 min. However, diclofenac and ibuprofen showed a poor signal when bivalve samples were analysed and, for that reason, the gradient was slightly modified in order to improve their signals, as will be discussed in Section 3.3.2.

For MS detection with a QqQ analyser, several parameters were optimized and the optimum values are described in Section 2.4. The following ESI source parameters were optimized and the values in brackets were assayed: capillary voltage (2000-4500 V in increments of 500 V); nebulizer pressure (30, 45 and 60 psi); drying gas (N_2) flow (7, 9 and 12 L/min) and temperature (250, 300 and 350ºC). Deprotonated [M-H]⁻ molecules were selected as precursor ions for all compounds, except for bezafibrate and diclofenac, in which cases both [M-H]⁻ and [(M+2)-H]⁻ were selected as precursor ions. For both compounds the selected [M-H]⁻ gave only two product ions and selecting [(M+2)-H]⁻ as a second precursor ion, a third MRM transition could be obtained. The cone voltages tested were 60, 80, 100 and 120 V and collision energies for each precursor ion tested were between 5 and 40 eV in increments of 5 eV. For all of the compounds, three MRM transitions were monitored, with the exception of salicylic acid, clofibric acid, with which just two product ions were generated, and ketoprofen and ibuprofen, with which just one product ion could be monitored. Common fragmentation pathways are based on the loss of the carboxyl group and methyl group. The proposed formulas for the product ions obtained and their respective cone voltage and collision energies are shown in Table 1. Ion ratios detailed in Table 1 were calculated as described in Section 2.4.

3.2. QuEChERS extraction

The original QuEChERS method described by Anastassiades et al. [12] is based on the extraction with ACN and the addition of anhydrous $MgSO_4$ and NaCl, in an aqueous matrix followed by a clean-up step using PSA as dSPE. However, this method underwent several modifications in the subsequent years and other solvents, such as ethyl acetate, dichloromethane and acetone, have been used as extraction solvents. In the present study, 10 mL of water was added to the dried matrix to promote the salting-out extraction. ACN was chosen as the extraction solvent as it can be easily separated from water [27], it does not extract as much

lipophilic material, such as waxes, fat and lipophilic pigments [28], and it is the solvent of preference in the QuEChERS methodology.

With respect to the salt composition, Lehotay et al. [29] modified the original method using acetate buffer which went on to become AOAC Official Method 2007.01. The original method was also modified by using citrate buffer, being registered as European Standard Method EN 15662 [30]. The two standard methods mentioned (AOAC and EN) were tested using mussel samples.

Recoveries of the extraction process (REs) were calculated for both standard methods by comparing the peak area of spiked mussel samples at 1000 ng/g in dry weight (d.w.) and the peak area of mussel samples that were spiked after the extraction process [31]. Thus, REs show the yield of the extraction process and do not take into account any losses caused by matrix interferences. REs were very similar, with values ranging from 61% to 95% in both methodologies for all compounds, except for salicylic acid and clofibric acid, which showed recoveries around 20% higher when using citrate buffer than when using acetate buffer (data not included). For this reason, subsequent experiments were just conducted with the citrate buffer (EN method).

3.3. Clean-up strategies

One of the greatest drawbacks of LC-MS is the perturbation of the signal by co-extracted substances from the sample matrix, particularly in complex matrices such as biota [32]. For this reason several strategies were evaluated in order to clean the matrix and reduce its effect on the response.

The use of dSPE and modification of the gradient profile of the chromatographic separation were strategies that provided satisfactory results and they will be described in detail in the subsequent sections. Moreover, three other strategies were assayed although none of them provided satisfactory results. The first one was freezing out the ACN extract of QuEChERS in order to precipitate lipids, waxes, sugars and other matrix co-extractives with low solubility in ACN [33]. The second one was dilution of the ACN layer, with the dilution factors based on 0.5% acetic acid in ultrapure water/ACN at (1:1) and (2:1) being tested. However, the limits of detection (LODs) and limits of quantification (LOQs) were significantly affected since they increased as the dilution factor rose. And, finally

a pre-cleaning of the solid sample with 10 mL of hexane ultrasonicated for 10 min prior to extraction was also evaluated, without any improvement.

3.3.1. dSPE

QuEChERS method involves a second step where the extract is cleaned up by using a dSPE with the main objective to remove interfering compounds present in the matrix. Mussels are fat or lipid containing matrices, although fats are not very soluble in ACN, a certain quantity of them might co-extract, so they have to be removed prior to the final determination step [12].

PSA is used as the sorbent for dSPE in the original QuEChERS method [12] to remove various polar organic acids, polar pigments, some sugars and fatty acids [27]. Other commonly used dSPE sorbents in the QuEChERS methodology cited in the literature are C_{18}, used to remove non-polar interfering substances like lipids [27]; EnvC, for removing sterols and chlorophylls [34]; and Z- Sep$^+$ for removing fats and non-polar compounds [8]. In the present work, several commercial dSPE sorbents already combined were tested: PSA (100/600 mg PSA/MgSO$_4$), PSA/C$_{18}$ (100/600/100 mg PSA/MgSO$_4$/C$_{18}$), PSA/ EnvC (150/15/900 mg PSA/ MgSO$_4$/GCB) and Z-Sep$^+$ (500 mg). Moreover, Florisil (500 mg), alumina (1000 mg) and silica (1000 mg) were also tested.

To optimize the dSPE, several extractions of non-spiked mussel samples were conducted using the procedure described in Section 2.3, the supernatant of different extractions was mixed in order to avoid any difference in the extraction process. Later, different aliquots were spiked and transferred into 15 mL tubes containing the different sorbents mentioned above. In addition, an aliquot spiked after the extraction process without any dSPE sorbent was evaporated and reconstituted. For each compound the effectiveness of the clean-up was evaluated by comparing the signal obtained with each dSPE sorbent with the signal obtained without any dSPE sorbent. The results obtained are shown as a percentage in Fig. 1, where values higher than 100 denote an improvement in retaining interfering substances, while values below 100 denote no improvement, and thus, the clean-up procedure was not effective. As can be seen in Fig.1, none of the commercial dSPE sorbents tested resulted in an improvement in terms of retaining interfering substances and consequently increasing the signal response for all compounds and, in most cases, they even retained the target analytes. This is the case of Z-Sep$^+$,

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which was supposed to remove fats and non-polar compounds [8] but, in the present study, it completely retained four of the seven studied analytes. Only silica improved the signal response for most of the compounds and it was selected for the clean-up step. In fact, silica has been used for clean-up in different solid matrices [35].
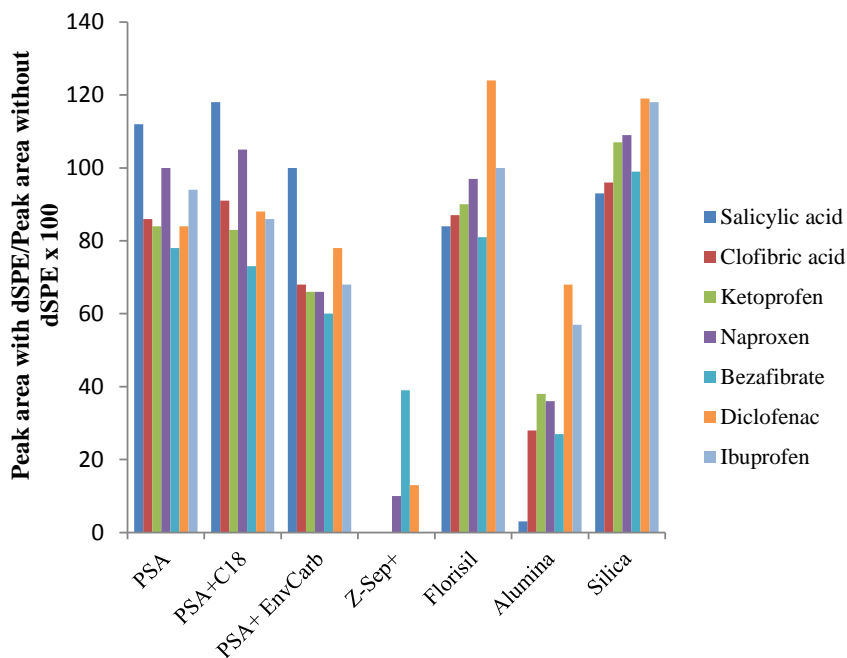

Figure 1. Peak signal ratio when different dSPE sorbents were applied in comparison to when no dSPE was applied.

3.3.2. Modifications of chromatographic conditions

Another strategy to reduce the effects of matrix compounds on the analytes response, described in the literature, is the modification of the chromatographic separation between the analytes and the co-eluting substances that interfere with the analysis [36]. According to Gosetti et al. [37] special attention should be paid towards the analytes eluting in the solvent front (highly polar and not retained compounds) or during the end of an elution gradient. With the initial gradient described in Section 3.1, chromatographic separation was achieved in 13.5 min.

However, diclofenac and ibuprofen, the last eluted compounds, showed poor signals. For this reason, analysis time was increased, with the gradient proposed in Section 2.3, where the slope of the gradient was more gradual. With this strategy, diclofenac and ibuprofen were eluted more than one minute later than with the initial gradient proposed in Section 3.1. In addition, the peak signal was slightly increased, by around 10% for these two compounds. Thus, this gradient was incorporated in the method and all of the compounds eluted in less than 14.5 min. Another strategy would be to use a longer column, however it was ruled out as the analysis time would increase.

3.4. Method validation

Once the method had been optimized, mussel *Mytilus galloprovincialis* was selected to carry out the validation study in order to demonstrate the performance of the method. Apparent recoveries (App REs), matrix effect (ME) and REs were calculated, matrix matched calibration curves were plotted for each analyte and linear ranges, (LODs) and (LOQs) were calculated. Moreover, repeatability (n=5) and reproducibility between days (n=5) were conducted. All of the results are shown in Table 2. One compound, salicylic acid, was found in blank samples. For this reason the peak area obtained in the blank was taken into account for validation. The method proved also to be applicable to the other bivalve species, providing comparable validation data.

App REs (which include the overall method) were calculated at two different concentration levels. The highest level studied was 1000 ng/g (d.w.) for all of the compounds, and the lowest level was 50 ng/g (d.w.) for clofibric acid, naproxen, bezafibrate and diclofenac, and 250 ng/g (d.w.) for salicylic acid, ketoprofen and ibuprofen. This distinction in the low concentration level was due to the difference in signal response between compounds. App REs were calculated by interpolation with an external standard calibration curve of the peak area obtained for each analyte from a sample spiked before extraction. App REs were very similar at both concentration levels. Table 2 shows the apparent recoveries for the highest level, ranging between 35% and 77%. Any loss of signal observed in the samples may be attributable to the extraction process or to ion suppression, as the App RE includes the entire method procedure.

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Another parameter assayed in the validation process was the ME, which was assessed as follows:

$$ME (\%) = -[100- (B/A*100)]$$

where (A) is the instrumental response for standards injected directly and (B) is the response of a mussel sample spiked before LC-MS/MS injection. When analytes were present in a blank sample, the peak area of the blank was subtracted from B in order to calculate the ME. If the ME = 0, no matrix effect is present, if the ME > 0, there is signal enhancement and if the ME < 0, signal suppression is present. All of the studied compounds showed signal suppression, as their ME < 0. The most affected compounds were diclofenac and ibuprofen, with values of signal suppression of 63 and 57%, respectively.

REs of the extraction process were calculated as described in Section 3.2 by comparing the instrument response from a certain amount of compound added to samples before extraction and the same amount of compound added to samples after QuEChERS extraction. REs varied between 61% and 95% which confirms, that most of the losses are due to ME.

The optimized method provided good linearity since the coefficients of determination (R^2) of the matrix matched calibration curves were acceptable for all analytes, as they were between 0.9961 and 0.9997.

LODs and LOQs were calculated as the analyte concentration that produced a peak signal of three and ten times the background noise, respectively. LOQs were considered to be the first point included in the calibration curve. For salicylic acid, that was present in the blank samples, LOD and LOQ were estimated from an external standard calibration curve using its App RE factor. LODs were between 1 and 5 ng/g (d.w.), with the exception of ibuprofen and ketoprofen, which were 50 ng/g (d.w.). LOQs were between 5 and 10 ng/g (d.w.) in all of the studied compounds except for ibuprofen and ketoprofen, which were 100 ng/g (d.w.). The present LOQs are in agreement with those found by Wille et al. [11] in a study conducted in 2011, for salicylic acid and diclofenac in mussel samples when these samples were analysed using PLE and SPE followed by ultra-high performance liquid chromatography (UHPLC)-MS/MS using QqQ as analyser. Ramírez et al. [10] reported LODs of 45.9 ng/g (d.w.) for ibuprofen and 2.69 ng/g (d.w.) for

clofibric acid in fish muscle tissue, which are also in accordance with those obtained in the present study. Huerta et al. [32] reported LOD of 0.5 ng/g (d.w.) and LOQ of 1.66 ng/g (d.w.) for diclofenac in fish homogenate, which are one order of magnitude lower than those obtained in the present study. However, these limits were achieved using a tedious method based on PLE as extraction technique and gel permeation chromatography (GPC) as a later clean-up followed by UHPLC coupled to a hybrid triple quadrupole linear ion trap mass spectrometer.

Figure 2 shows a MRM chromatogram of a mussel sample spiked at 1000 ng/g (d.w.) of each studied analyte. For each compound, the quantification transition is plotted.
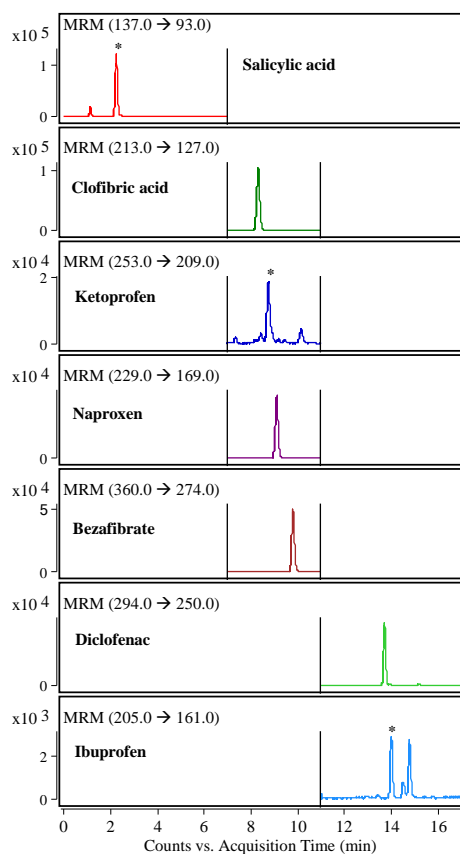
Figure 2. MRM chromatogram for each compound spiked at 1000 ng/g (d.w.). * Peak corresponding to the analyte.

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Table 2. Validation data with *Mytilus galloprovincialis* samples.

	Apparent recovery (%)	ME[1] (%)	RE[2] (%)	Linear range (ng/g)	LOD[3] (ng/g)	Repeatability (%RSD; n=5)		Reproducibility (%RSD; n=5)	
						1000 ng/g	50 ng/g	1000 ng/g	50 ng/g
Salicylic acid	46	-25	61	10- 2000	5	8	17*	15	19*
Clofibric acid	77	-11	86	10- 2000	1	3	5	10	17
Ketoprofen	48	-47	91	100- 2000	50	3	13*	6	21*
Naproxen	46	-52	95	10- 2000	2.5	2	7	6	20
Bezafibrate	48	-47	89	5- 2000	2.5	3	9	9	19
Diclofenac	35	-63	93	10- 2000	5	2	14	7	14
Ibuprofen	39	-57	90	100- 2000	50	4	8*	4	12*

Samples were spiked at 1000 ng/g (d.w.) to calculated apparent recoveries, ME and RE
[1]ME: Matrix effect; [2]RE: Recovery of the extraction process;[3]LOD: Limit of detection

Repeatability and reproducibility were calculated by means of % relative standard deviation (%RSD) at both levels of concentration used for calculating the App RE. The results, as detailed in Table 2, were always below 21%, for all of the compounds at both concentration levels.

4. Application

The method developed was applied to analyse different bivalve samples since it was tested that the validation data was similar to mussel. *Mytilus edulis* from the Atlantic coast of France and the species *Mytilus galloprovincialis* collected from two different locations the Ebro River Delta, Mediterranean coast (NE, Spain), and Galicia, Atlantic coast (NW, Spain) were bought in the local market together with lagoon cockle (*Cerastoderma glaucum*), coquina clam (*Donax trunculus*), manila clam (*Ruditapes philippinarum*), striped venus clam (*Chamelea gallina*) and sword razor clam (*Ensis sp.*) which are other widely consumed bivalve species. The confirmation criteria were: retention time, MRM transitions and ion ratios, including its % of variation accepted, as described in the European Directorate [38].

One compound, salicylic acid, was found in all of the mussel samples analysed at maximum concentrations of 103.26 ng/g (d.w.). Of the other bivalve species analysed, salicylic acid was determined in the lagoon cockle (*Cerstoderma glaucum*), coquina clam (*Donax trunculus*) and striped venus clam (*Chamelea gallina*). All of the results are shown in Table 3, where maximum concentration values from each species analysed and % of ion ratio are reported. These ion ratios are within the % of variation described by the European Directorate [38]. As an example, Fig. 3 shows two MRM chromatograms (quantification transition) where the top figure (a) refers to mussel from the Ebro River Delta and the lower one (b) belongs to the lagoon cookle (*Cerastoderma glaucum*).

Wille et. al [11] found concentrations of salicylic acid in *Mytilus edulis* in the Belgian coastal waters, in some cases similar to those found in the present study. However, in their work, they detected concentrations up to 490 ng/g, which are higher than those found in the present study. Huerta et al. [32] found diclofenac at concentrations up to 8.8 ng/g in fish homogenate in different species. This compound was not detected in the bivalve species evaluated in the present study.

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Table 3. Concentration of salicylic acid and % of ion ratio.

Species	Salicylic acid	
	Conc. (ng/g)	Ion ratio * (%)
Mytilus galloprovincialis [1]	95.88	7.3
Mytilus galloprovincialis [2]	98.47	7.4
Mytilus edulis	103.26	6.1
Cerastoderma glaucum	35.73	6.7
Donax trunculus	59.02	7.8
Chamelea gallina	65.17	8.2

[1]*Mytilus galloprovincialis* from the Ebro River Delta, Mediterranean coast (NE, Spain)
[2]*Mytilus galloprovincialis* from Galicia, Atlantic coast (NW, Spain)
*Ion ratio: Relation between the abundance of the qualifier transition (137→65) and the quantifier transition (137 →93).
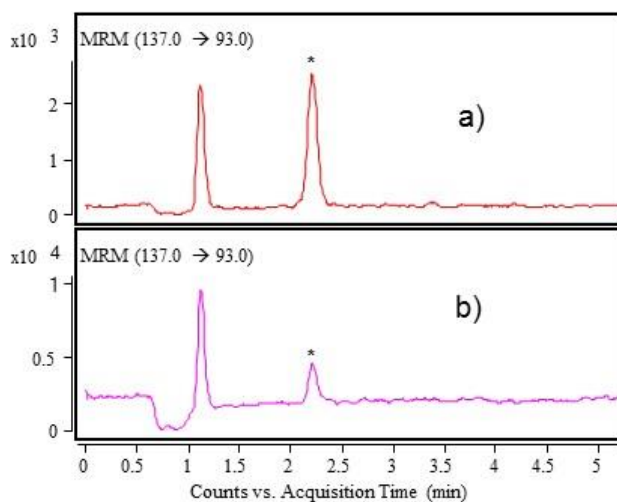
Figure 3. MRM chromatograms of: a) the mussel (*Mytilus galloprovincialis*) from the Ebro River Delta. b) the lagoon cookle (*Cerastoderma glaucum*). * Peak corresponding to salicylic acid.

5. Conclusions

A rapid and reproducible method was successfully developed for the quantification of seven pharmaceuticals belonging to different therapeutic classes in bivalve samples. Several strategies were evaluated in order to clean the matrix and dSPE using silica gel and modification of the chromatographic separation provided the best results. The method based on QuEChERS extraction and dSPE clean-up followed by LC-MS/MS was validated in mussels (*Mytilus galloprovincialis*). The optimum method provided App RE between 35% and 77% and LODs between 1 ng/g and 50 ng/g. The method was applied to analyse samples of different bivalve species and salicylic acid was found in mussels from three different locations and also in some of the bivalve species at ng/g (d.w.) levels.

Acknowledgments

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EMERGING ORGANIC CONTAMINANTS IN AQUATIC ORGANISMS

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3.1.3. Discussion of results

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This section presents the evaluation of two extraction techniques from aquatic organisms, namely PLE and QuEChERS. Regarding the PLE technique, as expected in line with our previous experience, the most important parameters affecting the extraction were the extraction solvent and temperature, although the extraction time also had a slight effect. Of the solvents tested, MeOH provided the highest extraction recoveries followed by ultrapure water. These results are in agreement with those from other authors who employ MeOH to extract pharmaceuticals when PLE is used [1-4]. For example, MeOH was used as the extraction solvent by Huerta et al. [1] to extract 20 pharmaceuticals and some of their metabolites, including diclofenac, from different fish tissues by PLE. In the present study, when MeOH was employed a high matrix effect was encountered (>50% for most of the compounds) and could not be reduced by the use of different clean-up strategies. Hence ultrapure water was chosen as the extraction solvent since, although the matrix effect was also present, it was less strong. Ultrapure water has been employed in other research to extract pharmaceuticals form aquatic organisms, but in those studies it was mixed with an organic solvent such as acetonitrile or methanol [5-7]. Moreover, the use of water as the extraction solvent makes it an environmentally-friendly method, which also enabled an SPE clean-up to be performed directly after extraction, avoiding the time-consuming evaporation step required if organic solvents are used. The SPE technique as a clean-up strategy has been widely employed after PLE [5-7]. Although the Oasis® HLB sorbent is the most commonly used, in the present study the mixed-mode Oasis® MAX cartridge was employed to clean the extract obtained from PLE. Another mixed-mode sorbent (the strong cation-exchange Oasis® MCX) was used by Chu and Metcalfe [2], providing selectivity for the adsorption of the basic compounds studied in their work.

As regards the QuEChERS extraction technique, with the two methods tested (EN and AOAC) similar results were obtained for all the compounds with the exception of salicylic acid and clofibric acid, for which higher recoveries were obtained when the EN method was applied. Berlioz-Barbier et al. [8] also extracted pharmaceuticals (diclofenac, ketoprofen and ibuprofen) employing the QuEChERS citrate buffer, among other EOCs. However, in their work a miniaturisation of the extraction technique was used. In the method developed in the present Thesis, different dispersive solid-phase extraction (dSPE) clean-up sorbents were tested, from which silica gel was chosen, which slightly reduces the matrix effect. In addition, during the development of the method for mussel

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samples, the chromatographic separation by means of the gradient elution profile was slightly modified to avoid the coelution of the matrix components.

Overall, both techniques are suitable for extracting the selected pharmaceuticals from bivalve samples and both supplied similar LODs. Slightly better reproducibility results were obtained with PLE than with QuEChERS. However, the QuEChERS extraction is faster and cheaper than PLE and requires no expensive equipment.

Regarding the applicability of the methods, in both cases they were applied to mussels from different locations, including the species *Mytillus galloprovincialis* and *Mytilus edulis*; in the case of the method using the QuEChERS extraction technique, this was also applied to other bivalve species such as the lagoon cockle (*Cerastoderma glaucum*), the coquina clam (*Donax trunculus*), the Manila clam (*Ruditapes philippinarum*), the striped venus clam (*Chamela gallina*) and the sword razor clam (*Ensis* sp.). Of the compounds studied, salicylic acid was found in some of the samples analysed at concentrations ranging from 36 to 177 ng/g (dry weight, d.w.). As mentioned in the papers published, this compound has previously been determined in mussel samples by Wille et al. [6]. None of the other pharmaceuticals studied has been found above its LOD. However, other studies published after the publication of the papers presented in this section or during the publication process have reported the presence of some of the compounds studied in this Thesis in aquatic organisms. For example, diclofenac was found at concentrations of up to 103 ng/g (d.w.) in biofilm [9] and also in the benthic invertebrate species *Erpobdella octoculata* at up to 33 ng/g (wet weight, w.w.). Huerta et al. [10] reported the presence of diclofenac and ibuprofen in macroinvertebrates at concentrations of up to 183 ng/g and 12.4 ng/g (d.w) respectively. In another study conducted by Berlioz-Barbier et al. [8], their presence was also reported in macroinvertebrates at up to 51.5 ng/g (w.w.) for diclofenac and at up to 105.4 ng/g (w.w.) for ibuprofen. In addition, in this study ketoprofen was detected below its limit of quantification. Chen et al. [11] also reported the presence of these two NSAIDs in fish plasma with a mean concentration of ibuprofen of 0.94 pg/µL and mean concentration of 1.74 pg/µL of diclofenac. Finally, in a study published prior to the papers presented in this section, bezafibrate was also found in fish blood plasma at a 0.14 ng/L concentration [12]. All these studies confirm the potential for the bioaccumulation of pharmaceuticals such as NSAIDs and lipid regulators in aquatic organisms.

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3.2. Determination of other emerging organic contaminants in fish samples

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This section focuses on the development of two analytical methods involving fish samples, with one method determining another group of pharmaceuticals, ICM-XR, and one determining another group of EOCs, high-intensity sweeteners, using PLE and LC coupled with HRMS in both cases. The main reason for choosing these compounds was that no previous studies of their occurrence in aquatic organisms had been carried out and therefore the results obtained would contribute to increasing current knowledge about the occurrence of EOCs in aquatic organisms.

As regards ICM-XR, a group of five compounds widely employed in diagnostic medicine which have also been determined in the aquatic environment was chosen for the study. As for the high-intensity sweeteners, eight artificial and two natural sweeteners were studied, some of which have been determined quite often in the aquatic environment and some of which have not. The molecular structure of each compound is detailed in Appendix II.

Due to the suitability of PLE and QuEChERS to extract pharmaceuticals as shown in the previous section, both techniques were evaluated again to extract ICM-XR from fish samples. PLE was selected to validate the final method due to the results achieved. The same technique was employed to extract the group of high-intensity sweeteners in fish samples. As in the paper presented in the previous section (Section 3.1.1), where PLE was employed as an extraction technique, the most important PLE parameters were optimised for each method.

As discussed in the introduction, aquatic organism samples are a very complex matrix and for this reason different clean-up approaches were evaluated for both methods in order to reduce the high matrix effect encountered [1,2]. Of these, the method developed for ICM-XR SPE employing the Oasis® MCX cartridge was selected. For the method developed for high-intensity sweeteners, PLE clean-ups were performed, conducted in the cell in two different ways: in-cell clean-up with an adsorbent that retains the interfering substances, and on-cell clean-up based on sequential extraction with two different kinds of solvent (different polarities), where the first solvent extracts the interfering substances prior to rejecting them while the second extracts the analytes [2-4].

As in the case of the previous studies, due to the polarity of the analytes, LC was employed as the separation technique coupled with HRMS with an Orbitrap as the

analyser for both methods. In order to separate the selected ICM-XR, an Ascentis Express C_{18} Fused-Core® column (5 cm x 4.6 mm i.d.; 2.7 μm) was used because of the good results obtained in previous studies [5,6], while to separate the selected high-intensity sweeteners two columns suitable for polar analytes were evaluated.

The methods developed were applied to evaluate the occurrence of the selected compounds in different fish species. According to Huerta et al. [2], the percentage of lipid content can affect the figures of merit and when higher can imply a greater matrix effect. Therefore, in the present study the lipid content of the different species was evaluated as described elsewhere [7] and the selected species were grouped according to the results obtained for their lipid content.

The results of two studies have been published in Talanta 163 (2017) 1-7 and in the Journal of Chromatography A 1479 (2017) 32–39, and are presented in the following subsections.

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3.2.1 Different sample treatments for the determination of ICM-XR in fish samples followed by LC-HRMS

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DIFFERENT SAMPLE TREATMENTS FOR THE DETERMINATION OF ICM-XR IN FISH SAMPLES FOLLOWED BY LC-HRMS

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Abstract

Iodinated X-ray contrast media (ICM-XR) are a group of pharmaceuticals widely used in medicine. Due to their low biodegradation rate, which makes their removal at wastewater treatment plants difficult, and the high doses at which they are administered, they have been detected in aquatic environments. In the present paper, a method for the quantitative determination of a group of ICM-XR in different fish species was developed and validated for the first time. Two extraction techniques were compared: pressurised liquid extraction (PLE) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), with PLE being selected, followed by liquid chromatography-high resolution mass spectrometry. In addition, several clean-up strategies were evaluated. The optimised method provided PLE recoveries ranging from 60% to 88% and limits of detection ranging from 5 ng/g to 25 ng/g (dry weight). The method was applied in order to evaluate the presence of the selected ICM-XR in different fish species.

Keywords: Iodinated X-ray contrast media; pressurised liquid extraction; high resolution mass spectrometry; matrix effect; fish samples.

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1. Introduction

Iodinated X-ray contrast media (ICM-XR) are a group of pharmaceuticals that are applied in clinical diagnosis in order to obtain radiographic images of soft tissues, such as blood vessels or organs. They are administered to patients in an aqueous solution at a high dose (200 g/application) and are designed to be inert and not to interact within the human body [1]. For this reason, they are made with structural and physicochemical features that provide them high stability, high solubility and high polarity. Consequently, they are not metabolised and are excreted through urine and faeces, within only 24 h after administration. Due to the mentioned characteristics, ICM-XR have a low biodegradation rate, which makes their removal at wastewater treatment plants (WWTPs) difficult. Therefore, they can reach other environmental compartments through effluents and the reuse of sewage sludge from WWTPs. One study conducted by Carballa *et al*. [2] investigated the behaviour of several organic contaminants through the processes of a WWTP and also their removal efficiency. They found that the ICM-XR iopromide was not removed and remained in the aqueous phase. In addition to the high concentrations at which they are administered, this fact means that these compounds can easily reach the water system and, therefore, the biota with which it is in contact. For this reason, several methods have been described to determine these compounds in environmental matrices, mainly in water bodies [1]. ICM-XR have been found in different aquatic environments, such as effluents from WWTPs at a maximum concentration of iopromide up to 20 µg/L [3], in the groundwater at a concentration of diatrizoic acid up to 1.1 µg/L [4], and in surface waters and drinking waters at low ng/L [5]. However, one study reported concentrations of diatrizoic acid up to 4 µg/L in surface waters and 1.2 µg/L in drinking waters [6].

For the determination of ICM-XR, the most commonly used analytical techniques in the literature are liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) [1]. Among the methods developed for aqueous matrices, solid-phase extraction (SPE) is the most widely used extraction technique [1]. As regards solid matrices, such as sludge, not many studies have been performed. In one study conducted by Ternes *et al*. [7], ultrasound-assisted solvent extraction (USE) was employed as the extraction technique, followed by SPE as the clean-up. In this study, none of the ICM-XR studied was detected above its limit of quantification (LOQ), which was 50 ng/g. In another study performed by Echeverría *et al*. [8], pressurised liquid extraction (PLE) was used as the extraction

technique. In this case, the ICM-XR found were also below their LOQs (25 ng/g). To date, there is no study on the bioaccumulation of these compounds in aquatic organisms. Although one study conducted in 1999 by Steger-Hartmann *et al.* [9] revealed no toxic effects produced by the administration of iopromide in short-term toxicity tests performed on bacteria, algae, crustacean and fish, as well as no long-term toxic effects on the crustacean *Daphnia magna*, it must be taken into account that continuous exposure to contaminants and their products may lead to changes over time, even though no ecotoxicological effects can be observed in acute toxicity tests [1].

Therefore, it is important to develop analytical methods to determine ICM-XR in biological matrices. In recent years, some methods to determine pharmaceuticals using fish as an indicator organism have been published [10], but none of them has focused on ICM-XR. However, this type of matrix usually involves long purification steps, which makes these studies more challenging.

The aim of this study was to develop an analytical method to determine a group of ICM-XR in different fish species. Two extraction techniques were compared: QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) and PLE. Moreover, different clean-up strategies were evaluated. The determination was performed by LC coupled with high resolution mass spectrometry (HRMS). Once the method had been developed and validated, it was applied to evaluate the occurrence of these compounds in different fish species. This was the first time that an analytical method was developed to determine these compounds in fish. Because of the high complexity of the matrix, HRMS may be advantageous for its analysis [11].

2. Experimental

2.1 Materials, reagents and standards

Iopamidol (97.5%), diatrizoic acid (92.4%), iomeprol (98.0%) iohexol (99.0%) and iopromide (97.0%) were supplied by Dr. Ehrenstofer (Augsburg, Germany) being the two latter in form of racemates. Individual stock solutions of 1000 mg/L were prepared in methanol (MeOH) and stored at -20 ºC. A mix solution of 50 mg/L in MeOH was prepared weekly and stored, also at -20 ºC. Deuterated compounds iopamidol-d8 and diatrizoic acid-d6 with an isotopic purity of 99.4%

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and 98.5%, respectively, were purchased from LGC Standards (Wesel, Germany) and were used as surrogate internal standards, which from now on they will be abbreviated as internal standards (I.S.).

The organic solvents MeOH and acetonitrile (ACN) were of HPLC grade and provided by J.T Baker (Deventer, the Netherlands). Acetone and the solvents tested for the on-cell clean-up (hexane, ethyl acetate, isooctane and dichloromethane) were also of HPLC grade and purchased from Prolabo (Llinars del Vallès, Spain). Formic acid, acetic acid, sulphuric acid and the sorbents tested for the in-cell clean-up (C_{18}, Florisil, silica and alumina) were supplied by Sigma-Aldrich (St. Louis, USA).

Ottawa sand was purchased from Fisher Scientific (Waltham, MA, USA) and Oasis® HLB cartridges (500 mg/ 6 cc) and Oasis® MCX cartridges (150 mg/ 6 cc) used in SPE were obtained from Waters (Milford, MA, USA).
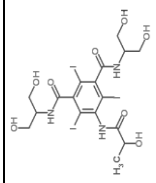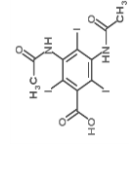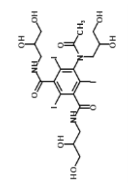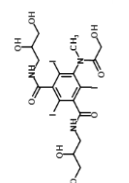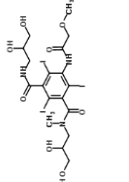
The ultrapure water was produced by an ultrapure water system from Veolia Water (Barcelona, Spain). The nitrogen gas (N_2) was obtained from Carburos Metálicos (Tarragona, Spain).

The three QuEChERS methods were evaluated. The European Standard Method EN 15662 packet was obtained from Scharlab (Sentmenat, Spain), and contained 4 g magnesium sulphate, 1 g sodium chloride, 0.5 g sodium hydrogencitrate sesquihydrate and 1 g sodium citrate. The AOAC Official Method 2007.01 packet was obtained from Waters, and contained 6 g of magnesium sulphate and 1.5 g of sodium acetate. To perform the original QuEChERS method, 4 g of anhydrous magnesium sulphate and 1 g of sodium chloride, both from Sigma-Aldrich, were mixed in the laboratory.

2.2 Sampling and sample treatment

The species *Cyprinus carpio* (common carp), *Silurus glanis* (wels catfish) and *Perca fluvialitis* (perch) were taken from the Ebro River (NE, Spain). The widely consumed marine species, *Merluccius merluccius* (European hake), *Sparus aurata* (gilt-head bream), *Mullus surmuletus* (striped red mullet), *Scomber scombrus* (Atlantic mackerel), *Thunnus thynnus* (Atlantic bluefin tuna), *Solea solea* (common sole) and *Psetta maxima* (turbot) were bought in the local market. For

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Table 1. Chemical stucture, pk$_a$, log Kow and accurate masses of the studied ICM-XR.

Compound	Chemical formula	Structure	pk$_a$[1]	log Kow[2]	Precursor ion [M+H]$^+$ (m/z)	Fragment ion (m/z)
Iopamidol	$C_{17}H_{22}I_3N_3O_8$		6.9	-2.4	777.86383	558.88678
Diatrizoic acid	$C_{11}H_9I_3N_2O_4$		1.1	1.8	614.77808	233.05626
Iohexol	$C_{19}H_{26}I_3N_3O_9$		10.6	-3.0	821.8891	803.87891
Iomeprol	$C_{17}H_{22}I_3N_3O_8$		10.6	-2.3	777.86285	686.79895
Iopromide	$C_{17}H_{24}I_3N_3O_8$		6.6	-2.1	791.87885	572.9035

[1]Values calculated using Sparc (http://archemcalc.com/sparc).
[2]Values obtained from PubChem Compound Database (pubchem.ncbi.nlm.nih.gov).

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all species, side fillets were separated and subsequently homogenised and frozen at -20 ºC for 24 h. Once frozen, the samples were lyophilised using the freeze-drying system Genevac miVac Duo Concentrator (Ipswich, Suffolk, UK).Eventually, the lyophilised samples were ground to obtain a homogeneous powder and sieved (500 µm) to obtain particles of the same size.

The percentage of lipid content of the abovementioned species was determined gravimetrically by the evaporation of the extract obtained by PLE extraction according to [12]. These PLE extractions were performed on an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA) using hexane:dichloromethane (1:1, v:v) as extraction solvent. The other extraction parameters were: pressure 1500 psi, temperature 100 ºC, preheating time 5 min, static time 10 min, number of cycles 2, purge time 90 s and flush volume 80%.

In order to optimise the extraction procedure and obtain efficient extractions, 1 g of freeze-dried fish sample was weighed and then it was wetted with acetone and, later, spiked with the analytes at the desired concentration. The mixture was homogenised and left under a hood overnight allowing the solvent to evaporate. Of the species mentioned, *Mullus surmuletus* was selected and several individuals were pooled to perform the optimisation of the method as it is one of the species with highest lipid content.

2.3 Extraction

Although the three QuEChERS methods were evaluated adapting procedures to the dry matrix, the best results were obtained by the AOAC Official Method 2007.01. To do so, 1 g of freeze-dried fish sample was weighed in a 50 mL polypropylene centrifuge tube, then 15 mL of ultrapure water was added, and the mixture was shaken manually for 1 min. Afterwards, 15 mL of ACN containing 1% acetic acid was added and it was also mixed by manual shaking for 1 min. After that, the buffer (AOAC packet) was added and the mixture was homogenised again for 15 seconds by manual shaking and for 45 seconds using a Heidolph Reax 2000 vortex. At the end, the tube was centrifuged for 5 min at 7000 rpm in a centrifuge from Hettich Zentrifugen (Germany). 1 mL of the ACN layer was transferred into a glass vial, then evaporated to dryness and re-dissolved in 1 mL of ultrapure water, which was filtered through a 0.45 µm syringe filter before being injected into the LC-HRMS system.

PLE extractions were performed with the same equipment used to determine the lipid content. A cellulose filter from Teknokroma (Sant Cugat del Vallès, Spain) was placed at the bottom of an 11 mL stainless steel cell. 3 g of Ottawa sand was placed on top, followed by 1 g of the freeze-dried sample, which had previously been mixed with 2 g of Ottawa sand. Then, Ottawa sand was added again to fill up the cell and, finally, another cellulose filter was placed on top. MeOH was used as the optimised extraction solvent and the optimal extraction parameters were: temperature 40 ºC, preheating time 5 min, static time 5 min, number of cycles 1, purge time 60 s and flush volume 50%.

The extract obtained from the PLE (~ 17 mL) was cleaned by SPE using an Oasis® MCX cartridge. In the present work, this cartridge was used to retain interfering substances instead of concentrating the extract. For this reason, the loading was collected and no elution step was performed. The SPE protocol was as follows: the cartridge was conditioned with 5 mL of ultrapure water followed by 5 mL of MeOH. Then, it was loaded with the PLE extract, which was collected in a vial and evaporated to dryness in a miVac concentrator and, finally, the dried extract was re-dissolved in 1 mL of ultrapure water that was filtered through a 0.45 µm syringe filter and injected into the LC-HRMS.

2.4 LC-(Orbitrap)HRMS analysis

Chromatographic analyses were performed with an Accela 1250 HPLC system connected to an Exactive Orbitrap™ mass spectrometer, all from Thermo Scientific (Bremen, Germany). The chromatograph was equipped with a quaternary pump (1250 bar), an Accela Autosampler automatic injector, kept at 10 ºC, and a column oven, which was maintained at 25 ºC. The interface employed was a heated electrospray ionisation (HESI-II) source, operating in positive ionisation mode. The instrument was equipped with a high energy collisional dissociation cell (HCD) in order to fragment the analytes for confirmation purposes. The chromatographic separation was performed with an Ascentis Express C_{18} Fused-Core® column (5 cm x 4.6 mm i.d.; 2.7 µm particle size) from Supelco (Sigma-Aldrich). The mobile phase composition was a mixture of ultrapure water with formic acid (pH 2.6) as solvent A and ACN as solvent B. The gradient used started with 2% B which was increased to 4% B within 2 min, and then raised to 25% B within 12 min. It was then increased to 100% B in 2 min and maintained at 100% B for 4 min. Finally, it returned to initial conditions within 2

min. The flow-rate was 0.2 mL/min and the injection volume was 25 µL. The chromatographic analysis took place within 10 min and the time between the runs was 5 min.

Optimal ionisation source parameters were: spray voltage 4.0 kV; sheath gas 60 AU (arbitrary unites); tube lens voltage 140 V; auxiliary gas 5 AU; skimmer voltage 35 V; capillary voltage 60 V; heater temperature 400 ºC; capillary temperature 280 ºC; and probe position adjustments: 0 as side to side position; D as vertical position and micrometer 0.75.

The data was acquired in one single window by continuously alternating two scan events: one without fragmentation at 50000 full width at half maximum (FWHM) resolution with an injection time of 250 ms, and one with fragmentation at 10000 FWHM with 50 ms injection time using 30 eV in the HCD. The diagnostic ions were measured for quantification (with a mass error of 5 ppm) and fragments and the corresponding ion ratios were used for confirmation purposes.

3. Results and discussion

3.1 LC-(Orbitrap)HRMS

The separation of the compounds was slightly challenging, since ICM-XR compounds are highly polar and two of them (iohexol and iopromide) present stereoisomers. For these compounds the signal of the isomers was added for quantification. The chromatographic separation described by Echeverría *et al.* [13] was used as a starting point and was slightly modified by testing different initial %B. Despite optimising the chromatographic separation (described in Section 2.4), some compounds could not be completely separated. This is the case of iohexol stereoisomer, which co-elutes with diatrizoic acid. It was not possible either to separate iohexol completely from iomeprol, although they can be distinguished by their masses. However, the final separation prevents overlapping between iopamidol and iomeprol, compounds that have the same m/z and which cannot be separated by MS.

To optimise the HRMS parameters continuous infusion of standard compounds was used, prepared with a mobile phase composition of 15% ACN and 85% water at pH 2.6 with HCOOH. The exact m/z was recorded in full scan at 50000 FWHM

for each compound in positive and negative mode. As expected, the signal obtained for all of the compounds was higher in positive mode. For all of the compounds, $[M+H]^+$ was selected for quantification (Table 1). Once the exact m/z had been recorded, each ionisation source parameter was optimised individually and a compromise was chosen for all of the compounds. For the spray voltage, values between 2 and 5 kV were evaluated, while for the capillary voltage, values from 10 to 100 V were tested. The tube lens voltage was measured between 50 and 200 V, and the skimmer voltage from 5 to 50 V. In addition, all the gas parameters and temperatures were assayed: sheath gas was evaluated between 50 and 100 AU, and the auxiliary gas from 0 to 50 AU. Capillary and heater temperature were measured between 250ºC and 450ºC. Finally, the probe position was evaluated, horizontal position (side to side) from -1 to 1, vertical position from A to D and the micrometer from 0 to 1. The optimal parameters can be found in Section 2.4.

Moreover, fragment ions for each compound were obtained for confirmation purposes. To do so, the signal intensity was monitored while applying different collision energies (from 5 to 60 eV) in the HCD. It was observed that 30 eV could be adopted as a compromise value of fragmentation for all the studied compounds, as at least one fragment ion could be obtained with the higher response. The selected fragment for each compound can also be found in Table 1. These fragments are in agreement with those reported in the literature using MS/MS [1,3,13,14]. The fragment ion from iopamidol might correspond to the cleavage of the amide bond and the loss of $C_3H_9NO_2$ and HI. The fragment ion from diatrizoic acid might correspond to the loss of 2I and HI. The fragment ion from iohexol can be assigned to the loss of a water molecule. The fragment ion from iomeprol might correspond to the loss of $C_3H_9NO_2$ due to the cleavage of the amide bond. Finally, the fragment ion from iopromide, as in the case of iopamidol, can be attributed to the loss of $C_3H_9NO_2$ and HI.

Once the LC-HRMS was optimised, instrumental limits of detection (LODs) and LOQs were experimentally evaluated (n=3). The LODs were determined in line with [11,15], when a signal intensity higher than 1×10^3 of the precursor ion was accomplished. LOQs were defined as the lowest point of the calibration curve. LODs (2 and 3 µg/L) and LOQs (5 µg/L) were achieved. The instrumental limits obtained in the present study are in accordance with those obtained with MS/MS when QqQ was employed as the analyser [13].

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3.2 Extraction

Two different techniques, QuEChERS and PLE, were evaluated to extract the five selected ICM-XR from fish. QuEChERS is a cheap technique that does not require any analytical equipment and it has been recently employed to extract pharmaceuticals from biota samples [16]. PLE has been extensively used to extract a wide range of contaminants from different solid matrices and, although it requires analytical equipment, its robustness has been demonstrated [8,17,18]. For QuEChERS extraction, three different methods were evaluated: the original QuEChERS method [19], the AOAC Official Method 2007.01 [20] and the European standard method EN 15662 [21]. To select the best QuEChERS method, apparent recoveries (App REs) were compared, which were calculated by comparing the peak signal of the analytes from samples spiked at 1500 ng/g (d.w.), before the extraction and the peak signal of the analytes in standard solutions directly injected into the LC-HRMS system. The AOAC method provided the highest App REs, which were between 11% and 48%, while they were below 14% for the other methods. Then, the extraction recoveries (REs) and the ME were also calculated for the AOAC method. REs were calculated by comparing the peak signal of the analytes in a sample spiked at 1500 ng/g (d.w.) before the extraction and the peak signal of the analytes that were spiked after extraction at the same concentration. The ME was evaluated as the formula described below, where B is the peak signal of the analytes in a sample spiked after the extraction and A is the peak signal of the analytes in standard solution directly injected into the LC-HRMS.

$$ME (\%) = -[100 - (B/A*100)]$$

When these parameters were evaluated, low REs were obtained, with values ranging from 15% to 40% with the exception of iopromide with an RE of 64%. In all the analytes, the ME was in the form of ion suppression with values ranging from 24% to 29%. The low REs were probably due to the high polarity of the analytes and they were not even improved when more polar mixtures of solvents such as MeOH/ACN were used. For this reason, QuEChERS was rejected and PLE was assayed.

According to Runnqvist *et al*. [22] and also based on our previous experience, initial conditions were fixed as: 1 g of sample, preheating time 5 min, static time 5

min, 1 cycle, extraction temperature 80 ºC, flush volume 50% and purge time 60 s.

The first parameter optimised was the extraction solvent. Water, acidified water with HCOOH (pH 2.6), MeOH, ACN, acetone and a mixture of MeOH:water (1:1; v:v) were tested. To achieve suitable conditions for the injection to LC, water extracts (17 mL) were diluted to 25 mL with ultrapure water, while extracts containing organic solvents were evaporated and re-dissolved with 25 mL of ultrapure water. Table 2 shows the PLE REs and as can be seen, MeOH showed the highest PLE REs followed by the mixture of MeOH:water (1:1; v:v). With ultrapure water diatrizoic acid displayed very low extraction, whereas, with acidified water, PLE REs were generally lower, except for this compound. ACN provided very low PLE REs, which confirms the results achieved with QuEChERS. Acetone provided similar recoveries to ACN. Moreover, the ME values obtained with MeOH were lower than those obtained with the mixture of MeOH:water (1:1; v:v). For these reasons, MeOH was selected as the extraction solvent. In addition, the organic extract of MeOH can easily be evaporated. The selection of MeOH agrees with previous studies in which the same group of ICM-XR was extracted from sewage sludge [8].

Once the solvent was chosen, the temperature was tested at 40 ºC, 60 ºC 80 ºC and 100 ºC PLE REs values were very similar at all temperatures tested, between 60% and 88%, with diatrizoic acid being the compound with the lowest PLE RE (60%), which could not be increased by varying the extraction temperature. For this reason, the extraction temperature was set at 40 ºC in order to avoid the co-extraction of interfering substances. Table 2 also details the PLE REs at 40ºC.

Afterwards, different static times (5, 10, 15 and 20 min) were assessed. Although no improvement in PLE REs was observed when the static time was increased. Special attention was paid to diatrizoic acid, whose PLE RE remained at ~60%. For this reason, it was decided to maintain the static time at 5 min.

Finally, the number of cycles (one and two cycles) was evaluated. 1 cycle was selected since very similar PLE REs were obtained in both cases. Other parameters, such as preheating time, purge time and % flush volume, are considered of minor influence on the extraction [8,18], so they were not optimised and were kept at the initial levels.

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Table 2. PLE RE and matrix effect (ME) using different extraction solvents and temperatures when the fish sample was spiked at 1500 ng/g (d.w.)

Compound	MeOH 40°C	MeOH 80°C		Water		Acidified water		MeOH/water (50:50)		ACN		Acetone	
	PLE RE (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)
Iopamidol	82	81	-40	58	-53	48	-31	67	-54	<10	-47	11	-52
Diatrizoic acid	60	55	-51	<10	-57	33	-34	58	-76	<10	-57	<10	-70
Iohexol	83	80	-45	46	-42	39	-40	63	-63	<10	-46	<10	-52
Iomeprol	79	77	-47	57	-65	49	-59	68	-68	<10	-44	10	-54
Iopromide	88	85	-42	64	-72	38	-64	63	-65	19	-41	23	-53

(%RSD (n=3) < 10%)

To sum up, the final PLE conditions consisted of 1 g of fish sample, extraction solvent MeOH, temperature 40 ºC, preheating time 5 min, static time 5 min, number of cycles 1, purge time 60 s and flush volume 50%.

3.3 Strategies to reduce the matrix effect

In general, biotic samples are rich in undesirable compounds that might interfere with the analysis. In the present study, high ion suppression was detected. For this reason, several strategies were evaluated in order to clean the matrix and reduce the interferences present in it. Two PLE clean-up strategies were tested: in-cell and on-cell clean-up. Additionally, two different SPE cartridges (Oasis® HLB and Oasis® MCX) were evaluated.

3.3.1 On-cell clean-up

One cleaning strategy that is enabled by the PLE technique is on-cell clean-up once the cell has been assembled by using an appropriate solvent previous to the extraction. Four different apolar or midpolar solvents (hexane, ethyl acetate, isooctane and dichloromethane) and ACN were evaluated. ACN was tested since our previous results obtained with both QuEChERS and PLE confirmed the low affinity of ICM-XR for ACN. The PLE conditions for the clean-up were the same as those used in [18], where interfering substances were removed from sewage sludge. None of the solvents tested resulted in a substantial improvement in terms of increasing App REs. In the case of hexane, ethyl acetate, isooctane and dichloromethane, the App RE of iopamidol was reduced between 7% and 10% approximately, and the other compounds did not show any improvement. In the case of ACN, iohexol and iopamidol showed a slight improvement, at maximum of 5%. For this reason, this strategy was rejected.

3.3.2 In-cell clean-up

Another strategy often used with the PLE technique is in-cell clean-up. Five sorbents: C_{18}, Florisil, silica, alumina and acidic silica, which preparation was adapted from [23,24], were used instead of Ottawa sand in order to perform the in-cell clean-up. As in the case of on-cell clean-up, none of the in-cell sorbents resulted in an improvement in App REs. Only the acidified silica improved the App RE of the diatrizoic acid (10% improvement). However, the App RE of iopamidol

was reduced by more than 15%. In addition, florisil slightly improved the App RE of diatrizoic acid, but less than 10%. For this reason, this strategy was also rejected.

3.3.3 Solid-phase extraction

Another strategy often used to clean complex matrices such as biota is SPE [25,26]. Two different cartridges, Oasis® HLB and Oasis® MCX, were evaluated in order to improve App RE. Oasis® HLB sorbent is a hydrophilic-lipophilic balanced reversed-phase sorbent with enhanced retention of polar analytes [13], while Oasis® MCX is a cation-exchanger sorbent based on the Oasis® HLB polymeric structure and modified with sulphonic groups, so that, ionic interactions can be established. In addition, it allows an organic solvent to be loaded, enabling the retention of the interferences in the sorbent [27].

Table 3. % App RE when different SPE sorbents were used for the clean-up and when no SPE was used. For more details see text.

Compound	App RE (%)		
	Without SPE	Oasis® HLB	Oasis® MCX
Iopamidol	30	34	35
Diatrizoic acid	7	10	16
Iohexol	20	27	33
Iomeprol	15	20	27
Iopromide	23	24	38

(%RSD (n=3) < 12%)

In order to evaluate the cartridges, App REs were also calculated and were compared with a PLE extract that was not passed through any cartridge. 1 g of sample was spiked before extraction at 500 ng/g (d.w). In the case of Oasis® HLB, the conditions proposed in [13] were used as a starting point. In brief, after conditioning the cartridge the PLE extract was loaded, which had been previously evaporated to dryness and reconstituted in 25 mL of ultrapure water adjusted at pH 3 with HCOOH. After that, 5 mL of different clean-up solvents, namely water containing 5% ACN, water at pH 3, hexane and no clean-up were evaluated. Later, the compounds were eluted using 5 mL of MeOH. Finally, the extracts were

evaporated to dryness, reconstituted in 5 mL of ultrapure water and filtered before injection.

In the case of Oasis® MCX, the PLE extract was directly loaded without prior evaporation, then the load, which contains the analytes, was collected, evaporated to dryness and reconstituted in 5 mL ultrapure water. Table 3 details the App REs for both sorbents tested as well as without SPE. As can be seen, all the App REs are slightly better for Oasis® MCX. In addition, the protocol is simpler than Oasis® HLB. For all of these reasons, Oasis® MCX sorbent was selected as a clean-up step after PLE.

3.3.4 Calibration approach

Although SPE with Oasis® MCX slightly improved the App REs, two isotopically labelled standards (iopamidol-d8 and diatrizoic acid-d6) were selected to be used as I.S. in order to compensate for the ME. The use of a higher number of isotopically labelled standards was avoided due to the high cost of them. Whereas iopamidol-d8 was used as the I.S. for iopamidol, iohexol, iomeprol and iopromide; diatrizoic acid-d6 was used as the I.S. only in the case of diatrizoic acid. The effectiveness of the deuterated compounds was evaluated by calculating the relative recoveries (REL REs) for each compound. They were calculated by the interpolation of the signal ratio (compound/deuterated compound) of a sample that had been spiked with the analytes and the deuterated compounds before PLE extraction, with a solvent calibration curve with deuterated compounds. REL REs ranged between 83% and 113%, except for iomeprol, which had a REL RE of 57%. Then, the deuterated compounds were incorporated to the method.

At the end, it was decided to concentrate the extract in order to improve the detection limits of the method, thus, the extracts were evaporated to dryness, reconstituted in 1 mL of ultrapure water and filtered before injection. At this point, it should be mentioned that, due to the low response of diatrizoic acid, it was decided to eliminate this compound as well as its corresponding I.S. (diatrizoic acid-d6) from the method and so it was excluded from validation.

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3.4 Method validation and application

Finally, the optimised method was validated in order to demonstrate its performance. The final conditions are detailed in Section 2.3 and 2.4. The species evaluated were divided into three different groups according their % lipid content, which is indicated in brackets for each species. The high lipid content group included: *Mullus surmuletus* (striped red mullet, 23%), *Scomber scombrus* (Atlantic mackerel, 21%), *Sparus aurata* (gilt-head bream, 35%) and *Psetta maxima* (turbot, 31%). The medium lipid content group included: *Cyprinus carpio* (common carp, 15%) and *Silurus glanis* (wels catfish, 12%). The low lipid content group included *Perca fluvialitis* (perch, 3%), *Thunnus thynnus* (Atlantic bluefin tuna, 2%), *Solea solea* (common sole, 5%) and *Merluccius merluccius* (European hake with 3%). From each group, one representative species was selected: *Mullus surmuletus* (high lipid content), *Cyprinus carpio* (medium lipid content) and *Thunnus thynnus* (low lipid content). Matrix-matched calibration curves with the deuterated compounds were plotted for each selected species, while LODs and REL REs were also calculated as described in Section 3.1 and 3.3.4, respectively. Moreover, blank samples were analysed in order to take into account whether any of the selected compound was present. However, none of the selected compounds was found in blank samples. All of the results can be found in Table 4. The linear range, for most of the compounds, was between 25 and 500 ng/g (d.w.) in the case of *Cyprinus carpio* and *Thunnus thynnus* and between 50 and 500 ng/g (d.w.) for *Mullus surmuletus*. LODs were 5 ng/g (d.w.) or 10 ng/g (d.w.) in all instances for *Thunnus thynnus* and *Cyprinus carpio*. In the case of *Mullus surmuletus* the LODs were higher (25 ng/g (d.w.)) since it is the species with the highest % lipid content, and therefore a higher ME. REL REs ranged from 88% to 119%. Only in the case of iomeprol they were lower, with values of 56% and 69% for *Mullus surmuletus* and *Cyprinus carpio*, respectively. Iopromide presented the highest REL RE with values up to 125% for *Thunnus thynnus*.

In addition, the repeatability and reproducibility of the method were evaluated for the three species using five replicate extractions of fish sample spiked at 100 ng/g (d.w.), performed on the same day and on different days, respectively. Both were expressed as a percentage of relative standard deviation (%RSD). Table 4 details the %RSD values obtained for the species with highest % lipid content. The values for the two other species were similar or even lower.

Table 4. Method validation data when the samples were analysed by PLE followed by Oasis® MCX clean-up and LC-HRMS

	Thunnus thynnus (low lipid content)			Cyprinus carpio (medium lipid content)			Mullus surmuletus (high lipid content)			Repeatability	Reproducibility
	Linear range (ng/g)	LOD (ng/g)	REL RE (%)	Linear range (ng/g)	LOD (ng/g)	REL RE (%)	Linear range (ng/g)	LOD (ng/g)	REL RE (%)	% RSD	% RSD
Iopamidol	25-500	10	88	25-500	10	96	50-500	25	109	4	15
Iohexol	25-500	10	106	10-500	5	89	50-500	25	95	12	14
Iomeprol	25-500	10	91	25-500	10	69	50-500	25	56	11	18
Iopromide	50-500	25	125	25-500	10	119	50-500	25	116	12	26

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An HRMS chromatogram of a fish sample (*Mullus surmuletus*) spiked at 100 ng/g is presented in Figure 1.
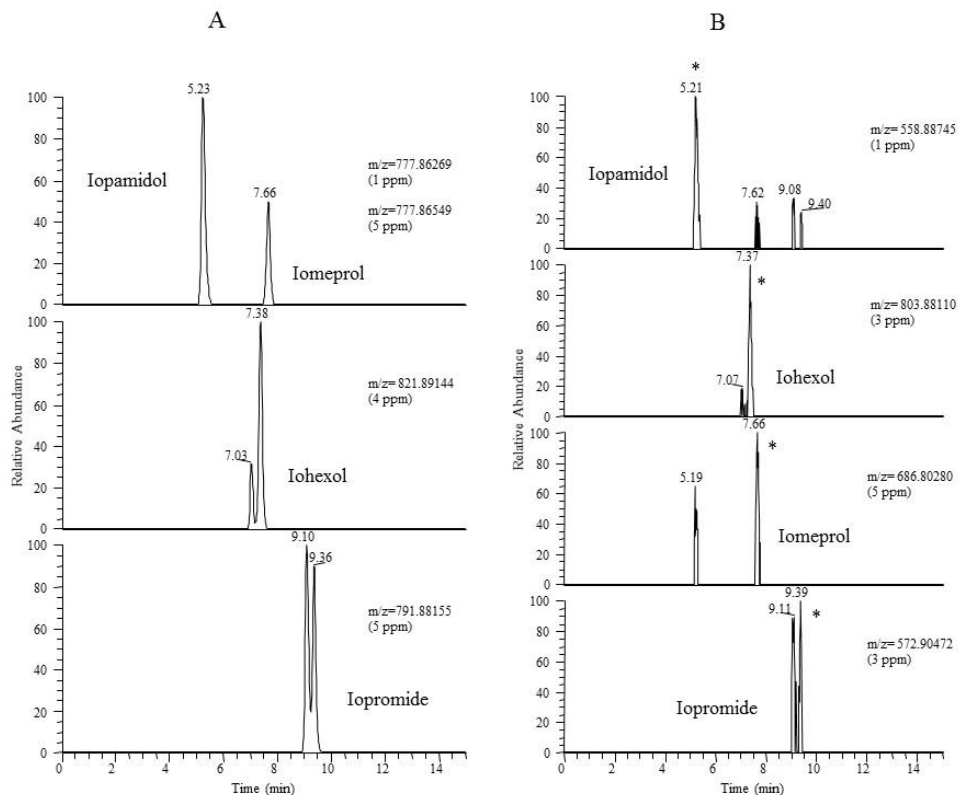
Figure 1. HRMS chromatogram and mass error in ppm of a fish sample (*Mullus surmuletus*) spiked at 100 ng/g. (A) precursor ions and (B) fragment ions.

The occurrence of the selected compounds was evaluated in different freshwater species and also in different marine species. The species *Cyprinus carpio*, *Silurus glanis* and *Perca fluvialitis* were taken from the Ebro River. The widely consumed marine species *Merluccius merluccius*, *Sparus aurata*, *Mullus surmuletus*, *Scomber scombrus*, *Thunnus thynnus*, *Solea solea* and *Psetta maxima* were bought in the local market. However, none of the ICM-XR studied was found above the respective LOD in any of the samples analysed. According to Huerta *et al.* [10] the highest levels of pharmaceuticals have been detected in tissues such as liver or

brain. For this reason, the liver of *Mullus surmuletus* was also analysed. Nevertheless, none of the studied compounds was detected above the LOD either.

In any case, as this is the first time that these ICM-XR are determined in fish sample, no data to compare whether these findings are as expected was available.

4. Conclusions

An analytical method was developed to determine a group of ICM-XR in different fish species, with PLE as extraction technique. Different approaches were conducted to reduce the high ME encountered in these samples. Of these approaches, SPE with Oasis® MCX was used as a clean-up step and calibration with isotopically labelled compounds was used to compensate this ME.

The method was validated with different fish species, according to their lipid content. The lipid content of the different species analysed slightly modified the figures of merit during the validation of the method. Thus, this content was considered during the application of the method to evaluate the occurrence of the studied compounds. Nevertheless, none of the studied ICM-XR was detected in the analysed samples.

Acknowledgments

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3.2.2 Pressurised liquid extraction and liquid chromatography-high resolution mass spectrometry to determine high-intensity sweeteners in fish samples

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PRESSURISED LIQUID EXTRACTION AND LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY TO DETERMINE HIGH-INTENSITY SWEETENERS IN FISH SAMPLES

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Abstract

An analytical method based on pressurised liquid extraction (PLE) followed by liquid chromatography-high resolution mass spectrometry (Orbitrap) was developed for the simultaneous determination of ten high-intensity sweeteners in fish samples. As the method was developed, the different PLE parameters were optimised and different clean-up strategies were evaluated, of which in-cell clean-up using alumina and on-cell clean-up with hexane were the most effective. PLE recoveries were between 43% and 94%. The method quantification limits were between 12.5 ng g^{-1} dry weight (d.w.) and 250 ng g^{-1} (d.w.) and the method detection limits between 2.5 ng g^{-1} (d.w.) and 125 ng g^{-1} (d.w.). Intra-day precision and inter-day precision were below 16% and 25%, respectively. Fish samples from different species were analysed and, saccharin was found below its method quantification limit in the species *Scomber scombrus* (Atlantic mackerel).

Keywords: sweeteners; pressurised liquid extraction; liquid chromatography-high resolution mass spectrometry; fish samples.

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1. Introduction

Recently, high-intensity sweeteners have been included in the group of emerging organic contaminants (EOCs) as, in the last few years, their widespread occurrence in the aquatic environment has been reported. They are considered extreme persistent compounds with low degradability. Most of them are not completely eliminated in wastewater treatment plants and some of them do not display environmental degradation [1]. Different studies on the issue have developed analytical methods that allow their determination in different aquatic environments [2-4]. According to Lange *et al.* [5], the sweeteners acesulfame and sucralose have been reported in the aquatic environment at concentrations higher than other EOCs, such as most pharmaceuticals and personal care products.

High-intensity sweeteners are food additives widely used as sugar substitutes in food, beverages, cosmetics, pharmaceuticals, animal feed, tobacco and tobacco-related products [6]. They can be divided into two groups: natural sweeteners, such as stevioside and glycyrrhizic acid, which are isolated from plants; and artificial ones, such as acesulfame, alitame, aspartame, cyclamate, neohesperidine dihydrochalcone, neotame, saccharin and sucralose. They are widely used due to the fact that they do not provide calories and they do not cause blood glucose levels to rise, since the insulin level is not affected, as well as being tooth-friendly [2]. For these reasons, their consumption can help to control obesity and diabetes. However, there is controversy with respect to their usage because potential health effects have been reported. Therefore, some high-intensity sweeteners have been regulated or even banned in several countries [6].

The effects of these EOCs in the ecosystem have not yet been studied in depth and data on the environmental distribution and ecotoxicological impact is still limited [1]. So far, toxicological studies have been conducted on aquatic organisms in order to evaluate the toxicity of these contaminants, due to their occurrence into the aquatic environment [7-12]. Most of these studies have focused on sucralose and they conclude that this sweetener does not alter the survival, growth or reproduction of aquatic organisms at levels above those measured is surface waters [7,12]. They also highlight that this compound may not cause toxicity to aquatic organisms at concentrations lower than 1000 mg L^{-1} [7], with this value being higher than the concentrations reported in the aquatic environment. Toxicity studies of the high-intensity sweetener sucralose on *Lemna gibba* [9], *Daphnia*

magna, *Pseudokirchneriella subcapitata* and *Danio renio* [10] revealed no toxic effects. However, one study [8] found that sucralose alters the behavioural response of *Daphnia magna* in terms of swimming and velocity, and also increases the time it takes *Gammarus* spp. to reach food and shelter. In another study [11], two copepod species were studied: *Calanus glacialis* and *Calanus finmarchicus*. In the case of *Calanus glacialis*, food intake increased when the concentration of sucralose also increased. Although negligible, acute and chronic toxicity have been reported. The most detailed assessments revealed behavioural changes that need to be taken into account, since they are modifications of the normal behaviour [8].

In order to study and evaluate the fate, effects and environmental risks posed by EOCs such as artificial sweeteners in aquatic ecosystems, information regarding their presence in aquatic organisms is urgently needed. For this purpose, analytical methods that allow their determination need to be developed. These methods have to deal with time-consuming sample preparation due to the complexity of these samples. Concerning to the extraction of EOCs from solid samples, pressurised liquid extraction (PLE) [13-15], ultrasound-assisted extraction [16,17], solid-liquid extraction [18,19] and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) [20-22] have been applied for the extraction of target compounds in fish samples. However, due to the complexity of the matrix a clean-up of the extracts is usually required in order to obtain an extract willing to be analysed. The most often purification steps applied include solid-phase extraction (SPE) [14,15,17,19], dispersive SPE [21,22] and gel permeation chromatography [13,16].

The aim of this work was the development for the first time of an analytical method for the determination of ten high-intensity sweeteners in different fish species using PLE and liquid chromatography-high resolution mass spectrometry (LC-HRMS). Finally, the method was validated and applied to the analysis of fish samples from different species.

2. Material and methods

2.1 Reagents, standards and materials,

Acesulfame-K (ACE), alitame (ALI), aspartame (ASP), cyclamate-Na (CYC), glycyrrhizic acid (GLY), neotame (NEO), neohesperidine dihydrochalcone (NHDC), saccharin-Na (SAC), stevioside (STV) and sucralose (SUC) were

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purchased from Sigma-Aldrich (St. Louis, MO, USA). All standards were of a purity higher than 96%, except for GLY (70%). Individual stock solutions of 1000 mg L^{-1} were prepared in methanol (MeOH) and stored at -20 ºC. For the preparation of the stock solution of stevioside and glycyrrhizic acid, a percentage of water (water/MeOH 5:95; v:v) was needed in order to ensure the dissolution of the solid. A mix solution of all compounds at 50 mg L^{-1} in MeOH was prepared weekly and stored also at -20 ºC.

The organic solvents MeOH, acetonitrile (ACN) and hexane were of HPLC grade and provided by J.T. Baker (Deventer, the Netherlands). Acetone was also of HPLC grade and purchased from Prolabo (Llinars del Vallès, Spain).

Formic acid (HCOOH), ammonium hydroxide (NH$_4$OH) and the sorbents tested for the in-cell clean-up (C$_{18}$, Florisil, silica and alumina) were supplied by Sigma-Aldrich. Diatomaceous earth was bought from Fisher Scientific (Waltham, MA, USA) and Oasis® HLB SPE cartridges (500 mg/6 cc) were obtained from Waters (Milford, MA, USA).

The ultrapure water was produced by ultrapure water system from Veolia Water (Sant Cugat del Vallès, Spain). The nitrogen gas (N$_2$) was obtained from Carburos Metálicos (Tarragona, Spain).

2.2 Sampling

The species *Mullus surmuletus* (striped red mullet), *Scomber scombrus* (Atlantic mackerel), *Sparus aurata* (gilt-head bream) and *Psetta maxima* (turbot) were bought in the local market, while the species *Cyprinus carpio* (common carp) and *Silurus glanis* (wels catfish) were collected from the Ebro River. Of all of the species, *Mullus surmuletus* and *Cyprinus carpio* were selected to optimise the method. For all species, the lateral fillets were separated, homogenised and frozen for 24 hours at -20 ºC. Once frozen, samples were lyophilised using the Genevac miVac Duo Concentrator freeze-drying system (Ipswich, Suffolk, UK). Then, samples were ground to obtain a homogeneous powder and sieved (500 µm) to obtain particles of similar size.

To optimise the method, the matrix was covered with acetone and then the analytes were added. The sample was periodically homogenised and the acetone was left to

evaporate overnight inside an extraction hood. This is a common procedure to enable good interaction between the analytes and the matrix [23,24].

2.3 Extraction and clean-up

To perform the extractions, an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA) was used. 11 mL extraction cells were used and mounted as follows: a cellulose filter from Teknokroma (Sant Cugat del Vallès, Spain) was placed at the bottom of the extraction cell and 3 g of alumina was added, then 1 g of sample mixed with 2 g of alumina were introduced, the void volume of the cell was filled with diatomaceous earth and, finally, another filter was placed on top.

Once the extraction cell was assembled, an on-cell clean-up using hexane was performed, for the purpose of defatting the sample, followed by the extraction of the analytes. The conditions of the on-cell clean-up can be found in [24] and the main conditions were: extraction temperature of 40 ºC at 1500 psi with a preheating time of 5 min with 2 cycles of 1 min each, a flush volume of 100% and a nitrogen purge of 360 s. For the extraction of the analytes, the optimal conditions were: MeOH:ultrapure water (1:1; v:v) as the extraction solvent, pressure of 1500 psi, preheating time of 5 min, 1 cycle, temperature of 60 ºC, extraction time of 5 min, flush volume of 50% and purge time of 300 s. The extract obtained (~17 mL) was evaporated to dryness employing a Genevac miVac Duo Concentrator, and the dried residue was reconstituted with 5 mL of MeOH:ultrapure water (1:9; v:v). The extract was then filtered through a 0.22 μm polypropylene syringe filter obtained from Serviquimia (Constantí, Spain) before injection.

2.4 Liquid chromatography-high resolution mass spectrometry

The analyses were performed on an LC system connected to an Exactive Orbitrap mass spectrometer from Thermo Scientific. The instrument was equipped with an Accela 1250 HPLC system and the interface used was a heated electrospray ionisation (HESI-II) source working in negative mode. The instrument was also equipped with a high-energy collisional dissociation cell (HCD).

In order to optimise the chromatographic separation, two columns were tested: Ascentis Express RP amide (100 x 2.1 mm i.d., 2.7 μm) from Supelco (Sigma-

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Aldrich) and Zorbax Eclipse XDB-C$_8$ (150 x 4.6 mm i.d., 5 µm) from Agilent Technologies (Santa Clara, CA, USA); being the last one selected for the present study. The optimal mobile phase was a mixture of solvent A (ultrapure water at pH 2.5 with HCOOH) and solvent B (ACN). The gradient profile started with 15% B, which was raised to 45% within 13 min and then to 100% within 2 min. Afterwards, it was maintained at 100% for 3 min and, finally, it was returned to initial conditions within 2 min. The column was allowed to stabilise for 8 min between injections. The flow-rate was 0.6 mL min^{-1}, the oven temperature was set at 25 ºC and the injection volume was 25 µL.

Optimised HRMS conditions were obtained in full scan mode at high resolution 50000 full width at half maximum (FWHM), at 200 m/z. The optimal parameters were: spray voltage of 3.5 kV; sheath gas 40 AU (arbitrary units); auxiliary gas 10 AU; tube lens voltage of -90 V; skimmer voltage of -26 V; capillary voltage of -25 V; heater temperature of 350 ºC; capillary temperature 300 ºC; and probe position adjustments: 0 as side-to-side position; C as vertical position and micrometer 0.5.

Four windows were used with different collision voltages in the HCD. In each window, two scan events were performed: one full scan at 50000 FWHM with 250 ms of injection time and with a scan range of 60-1000 m/z; and the other a fragmentation scan at 10000 FWHM with 50 ms of injection time with a scan range of 60-1000 m/z. In the first window (0 to 8.01 min) and in the third (12.01 to 14.01 min), a voltage of 20 eV in the HCD was selected. In the second (8.01 to 12.01 min) and fourth windows (14.01 to 18 min), a voltage of 40 eV in the HCD was selected. All of the selected ions can be found in Table 1.

2.5 Optimisation and validation parameters

During method optimisation various parameters were calculated as follows:

Instrumental limits of detection (ILODs) and quantification (ILOQs) were calculated by injecting standard mix solutions into the LC-HRMS. The ILOD for each compound was attributable to the concentration giving a peak signal of the precursor ion with intensity higher than $1x10^3$, in line with [25]. The ILOQ for each compound was considered the first point of the calibration curve and where the linear range started.

PLE recoveries (PLE REs) were evaluated to optimise PLE extraction. To do so, the ratio between the signal of the analytes obtained in a fish sample spiked before PLE and the signal of the analytes obtained in an extract spiked at the same concentration after PLE extraction was conducted.

Apparent recoveries (App REs) were calculated by interpolation of the signal of the analytes obtained from a fish sample spiked before PLE with an external calibration curve according to [26].

The matrix effect (ME) was calculated with the following formula:

$$ME\ (\%) = -[100 - (B/A*100)]$$

Where (A) is the instrumental response for standards injected directly to the LC-HRMS and (B) is the analytes' response in a fish extract spiked just before being injected into the LC-HRMS.

SPE recoveries (SPE REs) were calculated as the signal ratio of the analytes of a sample spiked before SPE and after SPE at the same concentration.

Linear range, method detection limits (MDLs) and method quantification limits (MQLs) were obtained experimentally by spiking fish samples at different concentrations before PLE and were calculated as ILODs and ILOQs.

Intra-day precision and inter-day precision (expressed as the % relative standard deviation) were obtained with five replicated samples performed on the same and different days, respectively. Intra-day precision was evaluated at two concentration levels (125 ng g^{-1} and 500 ng g^{-1}) and inter-day precision at 500 ng g^{-1}.

3. Results and discussion

3.1 Liquid chromatography-high resolution mass spectrometry

Two chromatographic columns (Zorbax Eclipse XDB-C$_8$ and Ascentis Express RP amide), that are suitable for the separation of high polar compounds like sweeteners were compared. It was observed that, with the Zorbax Eclipse XDB-C$_8$ column, better separation was obtained with respect to the first five eluting

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compounds (acesulfame, saccharin, cyclamate, sucralose and aspartame) and, consequently, further experiments were performed using this stationary phase, which enabled good separation in 15 min. The specific retention time of each analyte is detailed in Table 1.

Table 1. Chemical formula, retention time and accurate masses of the studied sweeteners.

Compound	t_R (min)	Formula	Precursor ion (m/z)	Fragment ion (m/z)
ACE	4,07	$C_4H_4NO_4S$	161.98621 $[M]^-$	82.02899 $[M-SO_3]^-$
SAC	4,86	$C_7H_4NO_3S$	181.99188 $[M]^-$	105.95982 $[M-C_6H_4]^-$
CYC	4,90	$C_6H_{12}NO_3S$	178.05428 $[M]^-$	79.95647 $[M-C_6H_{12}N]^-$
SUC	6,27	$C_{12}H_{19}Cl_3O_8$	395.00858 $[M-H]^-$	397.00565 $[(M+2)-H]^-$
ASP	6,42	$C_{14}H_{18}N_2O_5$	293.11542 $[M-H]^-$	200.07179 $[M-C_2H_7NO_3]^-$
ALI	7,47	$C_{14}H_{24}N_3O_4S$	330.15048 $[M-H]^-$	312.13998 $[M-H_2O]^-$
NHDC	10,30	$C_{28}H_{36}O_{15}$	611.19934 $[M-H]^-$	303.08856 $[M-C_{12}H_{20}O_9]^-$
STV	11,35	$C_{38}H_{60}O_{18}$	849.3775 $[M+HCOO]^-$	641.31903 $[M-C_6H_{10}O_5]^-$
NEO	13,06	$C_{20}H_{30}N_2O_5$	377.20935 $[M-H]^-$	200.07184 $[M-C_8H_{19}NO_3]^-$
GLY	15,06	$C_{42}H_{62}O_{16}$	821.39838 $[M-H]^-$	351.05847 $[M-C_{30}H_{46}O_4]^-$

As regards as the HRMS optimisation, in accordance with the literature, the highest sensitivity was achieved when working with ESI in negative mode [1,3,27]. The HRMS conditions described by Salas *et al.* [2] were used as a starting point, although the present study includes a higher number of sweeteners. To test these conditions, standard solutions were continuously infused together with a flow of mobile phase with 50% B. The exact m/z was recorded in a full scan at 50000 FWHM for each compound in negative mode. For all of the compounds, $[M-H]^-$ was selected for quantification, with the exception of acesulfame, saccharin and cyclamate, for which $[M]^-$ was selected, and stevioside, for which the adduct $[M+HCOO]^-$ formed due to the mobile phase was selected. Table 1 shows the selected precursor ion for each compound. The different voltages and temperatures were also optimised, and the values selected are detailed in Section 2.4.

Moreover, for confirmation purposes and to achieve an appropriate detection according to the requirements established by the guidelines of the European Directive 2002/657/CE [28], fragment ions for each compound were obtained. To do so, the signal intensity of each analyte, which had been infused individually, was monitored when different voltages (ranging from 5 to 60 eV) in the HCD were

applied. It was not possible to choose a compromise voltage for all of the compounds and, for this reason, different windows were conducted. The first window (from 0 to 8.1 min), with an HCD of 20 eV, contains the compounds acesulfame, saccharin, cyclamate, aspartme and alitame. In this window, sucralose also elutes. However, for this compound, no fragment ion was selected. Instead, due to the presence of Cl$^-$ in the molecule, two precursor ions were selected namely 395.00858 m/z and 397.00565 m/z, with 395.00858 m/z being selected for quantification and 397.00565 m/z for confirmation. The second window (from 8.1 to 12.01 min), with an HCD voltage of 40 eV, includes the compounds neohesperidine dihydrochalcone and stevioside. The third window (from 12.01 to 14 min), with an HCD of 20 eV, includes neotame and, finally, the fourth window (from 14 to 18 min), with an HCD of 40 eV, contains glycyrrhizic acid.

High-intensity sweeteners comprise different types of molecules and, for this reason, different fragmentation pathways were observed. As it is shown in Table 1, some fragments (i.e. the fragments for acesulfame, saccharin, cyclamate and alitame) are easy to explain. Nevertheless, the fragment ion 200.07179 m/z obtained for aspartame might correspond to the loss of methoxycarbonyl (CH_3OCO), amine (NH_2) and hydroxyl (OH) groups. Neohesperidine dihydrochalcone might break the molecule from the two hydroxyl substituted six atom rings through the carbon oxygen bond, giving the fragment ion 303.08856 m/z. As regards as stevioside the adduct 849.37750 m/z was selected as precursor ion, the fragment ion selected, 641.31903 m/z, might correspond to the loss of a monosaccharide. In the case of neotame, an HCD of 20 eV yielded the fragment ion of 200.07184 m/z, associated with the loss of methoxycarbonyl (CH_3OCO), 3,3-dimethyl-1-butanamine ($C_6H_{15}N$) and hydroxyl (OH) groups. Finally, the fragment ion of 351.05847 m/z of glycyrrhizic acid could be obtained by the loss of the aglycone group. It should be mentioned that the fragments from glycyrrhizic acid had a very low response, although the fragment ion 351.05847 m/z had the highest intensity. Other compounds that also displayed poor fragmentation were cyclamate and saccharin. In the case of cyclamate, for which the fragment ions described in the literature [29] when a QqQ analyser was used were 80 m/z [M-H-$C_6H_{12}N$]$^-$ and 96 m/z [M-H-C_6H_{10}]$^-$, with the highest response being recorded for 80 m/z, which was selected as the fragment ion in the present study. Moreover, the most intense fragment reported in the literature [3] for saccharin is 42 m/z, which corresponds to the [NCO]$^-$ fragment. This ion could not be monitored with an

Exactive Orbitrap mass spectrometer since the scan range starts at 50 m/z. All these fragments have previously been reported in the literature [2-4,30].

Under optimal LC-HRMS conditions, ILODs and ILOQs were determined. For most of the compounds, the ILODs were between 0.1 and 1 µg L^{-1}, with the exception of sucralose, which had an ILOD of 2.5 µg L^{-1}. For most of the compounds, the instrumental linear range started between 0.25 µg L^{-1} and 1 µg L^{-1} up to 500 µg L^{-1}, with the exception of glycyrrhizic acid, saccharin, aspartame and neotame which had a linear range between 2.5 and 500 µg L^{-1}, and sucralose ranging between 5 and 500 µg L^{-1}. The first point of each instrumental linear range was considered as the ILOQ.

3.2 Extraction

To optimise the extraction process, the species *Mullus surmuletus* (striped red mullet) was selected. In order to obtain efficient extractions, several parameters of PLE were optimised. Based on previous experience [23], initial PLE conditions were fixed as: 1 g of sample, 1500 psi, extraction temperature of 80 ºC, preheating time of 5 min, static time of 10 min, flush volume of 100%, 1 cycle and a purge time of 300 s.

The first parameter to be optimised was the extraction solvent. The solvents tested were: ACN, MeOH, ultrapure water, ultrapure water adjusted to pH 2.5 with HCl and a mixture of MeOH:ultrapure water (1:1; v:v).

PLE recoveries (PLE REs) were calculated, as described in Section 2.5, to evaluate the solvents. To do so, fish samples were spiked before PLE at 2500 ng g^{-1} (d.w.) and the signal of the analytes obtained was compared with the signal of the analytes obtained in extracts that were spiked at the same concentration after PLE. In order to obtain good peak shape, the final solution (25 mL) was a composition of ultrapure water:MeOH (9:1; v:v), similar to the initial mobile phase composition. Thus, in the case of the organic solvents, such as ACN and MeOH, the extracts were evaporated to dryness under a stream of nitrogen and the dried residue was re-dissolved to the final solution. In the case of the mixture of MeOH:ultrapure water (1:1; v:v), the PLE extract was half evaporated, assuming that all MeOH was evaporated, and then diluted to the desired composition. In the case of water as the extraction solvent, the extracts were also diluted. Figure 1 shows the PLE REs

when the abovementioned solvents were tested. Moreover, it shows the statistically significant differences between solvents when an ANOVA was performed, the LSD (Least Significant Differences) method for comparisons was employed.

As can be seen in Figure 1, good PLE REs were obtained when using ultrapure water for acesulfame, saccharin, stevioside and glycyrrhizic acid. However, with this solvent, neohesperidie dihydrochalcone (NHDC) and neotame could not be extracted. In fact, NHDC was hardly extracted with any of the solvents. With MeOH, all of the compounds were extracted with values ranging from 42% to 107%, with the exception of NHDC (10%). With the mixture of MeOH:ultrapure water (1:1; v:v), all of the compounds were extracted with values higher than 75%, with the exception of NHDC, neotame and glycyrrhizic acid, which had PLE REs of 20%, 26% and 47%, respectively. As in the case of ultrapure water, with ACN and water at pH 2.5, some compounds could not be extracted. ACN could not extract NHDC, stevioside and glycyrrhizic acid and, in the case of water at pH 2.5, NHDC and neotame were also not extracted. The mixture MeOH:ultrapure water (1:1; v:v) was chosen as the extraction solvent as it allowed all of the compounds to be extracted. In fact, the same solvent was used in our research group for extracting a group of sweeteners from sludge [29].

The second optimised parameter was the extraction temperature, which was tested at 40 ºC, 60 ºC and 80 ºC (data not shown). It was observed that the compound most affected by the variation of temperature was neotame. At 80 ºC, neotame showed a PLE RE of 26%, while at 60 ºC, it displayed a PLE RE of 86%. NHDC increased from 20% at 80 ºC to 39% at 60 ºC. Meanwhile, the PLE RE of glycyrrhizic acid slightly increased (around 8%) when the temperature increased from 40 ºC to 60 ºC. For this reason, 60 ºC was selected as the extraction temperature as a compromise.

The third parameter optimised was the extraction time and 5, 10 and 20 min were evaluated. It was observed that, with an extraction time of 5 min, there was no decrease in the PLE REs compared to 10 min and, with 20 min, there was no improvement (data not shown). For this reason, the extraction time was set at 5 min in order to make the extraction process shorter.

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Statistical significant differences at 95% confidence level.

Figure 1. PLE REs (%) using different extraction solvents when the fish sample was spiked at 2500 ng g^{-1} (d.w.). See the text for the rest of the conditions.

Other PLE parameters, such as purge time, preheating time and pressure, are considered of minor importance and they were kept at initial conditions [24,31].

The final extraction conditions were therefore 1 g of sample, MeOH:ultrapure water (1:1; v:v), 5 min extraction time, 60 ºC, 1500 psi, 50% flush volume, 1 cycle, 5 min preheating and 300 s purge time. Under these optimal conditions, the PLE REs were evaluated for *Mullus surmuletus* and *Cyprinus carpio* (Table 2). According to [13], a different % of lipid content can lead to changes in the figures of merit, and the ME is expected to be higher when the percentage of lipids increases. These two species were selected since they have different % of lipid content: 23% in the case of *Mullus surmuletus* and 15% in the case of *Cyprinus carpio* [31]. Moreover, App REs and the ME were also evaluated for both species,

as described in Section 2.5, when the samples were spiked at 2500 ng g^{-1} (d.w.). App REs (Table 2) were higher for *Cyprinus carpio* (between 118% and 45%) than for *Mullus surmuletus* (between 95% and 8%). As expected, the ME was higher for *Mullus surmuletus* and some compounds had values higher than 50% in terms of ion suppression. This was the case of aspartame (-68%), sucralose (-68%), neohesperidine dihydrochalcone (-87%), stevioside (-79%), neotame (-61%) and glycyrrhizic acid (-56%). A lower ME was observed for *Cyprinus carpio*, with a maximum value of ion suppression of -39% for neohesperidine dihydrochalcone. These results are in line with the values of % lipid content, since *Mullus surmuletus* has a higher lipid content than *Cyprinus carpio*.

Table 2. PLE REs (%), App Res (%) and ME (%) for *Mullus surmuletus* and *Cyprinus carpio* when the fish samples were spiked at 2500 ng g^{-1} (d.w.). See the text for the rest of conditions.

Compounds	*Mullus surmuletus*			*Cyprinus carpio*		
	PLE RE[a]	App RE[a]	ME[a]	PLE RE[a]	App RE[a]	ME[a]
ACE	94	95	-2	93	118	27
SAC	93	62	-37	96	106	11
CYC	92	71	-29	86	93	8
ASP	77	25	-68	69	61	-11
SUC	84	31	-68	96	76	-21
ALI	79	56	-37	78	75	-4
NHDC	46	8	-87	74	45	-39
STV	77	19	-79	88	75	-15
NEO	82	36	-61	82	74	-10
GLY	43	32	-56	69	54	-21

[a] RSD (n=3) ≤ 14.

Due to the high ME observed, particularly for the species *Mullus surmuletus*, different strategies were evaluated in order to reduce this ME and all the tests were performed with this species.

All of the strategies above were evaluated when the final volume was 25 mL. However, in order to achieve lower MDLs and MQLs, a reduction of the final volume was assayed; thus, instead of 25 mL, volumes of 10 mL and 5 mL were evaluated. Between 25 mL and 10 mL, a reduction of the App RE was observed, with saccharin and cyclamate being the compounds that showed a higher reduction

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(~20%). However, between 10 mL and 5 mL, there was almost no reduction. For this reason, 5 mL was chosen as the final reconstitution volume.

3.3 Clean-up

One strategy often used to clean the matrix is SPE. In the present study, SPE using the sorbent Oasis® HLB (lipophilic divinylbenzene-hydrophilic N-vinylpyrrolidone copolymer) was evaluated. The protocol recommended by suppliers was followed, which is summarised as follows: the cartridges were conditioned with 5 mL of MeOH and 5 mL of ultrapure water at pH 3. The PLE extract of fish sample was half evaporated, assuming that all of the MeOH was evaporated, and then the remaining aqueous extract was diluted to 25 mL with water and adjusted to pH 3 with HCOOH, before being loaded into the cartridge. A clean-up step was performed with 5 mL of a mixture of ultrapure water:MeOH (9:1; v:v) and then the cartridge was vacuum dried. The analytes were eluted with 5 mL of MeOH and the eluate was evaporated to dryness using a Genevac miVac Duo Concentrator. The dried residue was re-dissolved in 25 mL of ultrapure water:MeOH (9:1; v:v) and filtered before being injected into the LC-HRMS. SPE REs were calculated, as described in Section 2.5, to evaluate SPE without taking into account losses in other steps. Some compounds showed low SPE REs, as is the case of acesulfame, saccharin and glycyrrhizic acid, which presented SPE REs lower than 50%. For the rest of the compounds, the SPE REs were higher than 78%. Nevertheless, it was observed that the SPE did not improve the App REs.

In order to improve these low SPE REs, the SPE procedure using the Oasis® HLB sorbent was tested, as described by Arbeláez *et al.* [3] to evaluate eight of the ten sweeteners from the present study in sewage sludge. The protocol was the same as the one described by the suppliers with the exception that, in the elution step, instead of eluting with 5 mL of MeOH, the analytes were eluted with 2.5 mL of MeOH and 2.5 mL of a mixture of MeOH:NH$_4$OH (95:5; v:v), and the eluate was also evaporated to dryness and the dried residue was re-dissolved in 25 mL of ultrapure water:MeOH (9:1; v:v) before being injected into the LC-HRMS. In this case, the SPE REs increased (>70% for all of the compounds), but no improvement of the App REs was observed. As this strategy did not entail any improvement and actually lengthened the analysis time considerably, it was rejected.

Another strategy used to reduce the ME is an in-cell clean-up that was also evaluated. This step consists of the use of a sorbent inside the extraction cell in order to retain interfering compounds and obtain a cleaner extract. To do so, instead of adding diatomaceous earth at the bottom of the extraction cell, it was filled with a sorbent, and the sample was also mixed with the sorbent. Finally, the void volume of the extraction cell was filled with diatomaceous earth. The sorbents evaluated in the present study were Florisil, C_{18}, silica and alumina, all of which are often used to clean complex matrices and recommended by Dionex. Although similar results were obtained from the different sorbents tested (Figure 2), alumina was selected as the sorbent as it slightly improved the App REs of some compounds (saccharin, alitame) and it is an inexpensive material often used in biota studies [13,32].

Another strategy that was evaluated was on-cell clean-up, which consists of performing a defatting step prior to the extraction, once the extraction cell was assembled. Hexane was selected as the on-cell solvent based on the good results obtained in previous studies [24,33]. Although this step did not significantly improve the App RE of the method, an extract with a cleaner appearance was obtained, which helps to prevent the deterioration of the chromatographic column. In addition, it is a step that does not require any sample manipulation and does not involve a significant increase in the analysis time. Therefore, with the two clean-up strategies adopted, the App REs improved by 5% to 10%, with respect to those shown in Figure 2.

All of the strategies above were evaluated when the final volume was 25 mL. However, in order to achieve lower MDLs and MQLs, a reduction of the final volume was assayed; thus, instead of 25 mL, volumes of 10 mL and 5 mL were evaluated. Between 25 mL and 10 mL, a reduction of the App RE was observed, with saccharin and cyclamate being the compounds that showed a higher reduction (~20%). However, between 10 mL and 5 mL, there was almost no reduction. For this reason, 5 mL was chosen as the final reconstitution volume.

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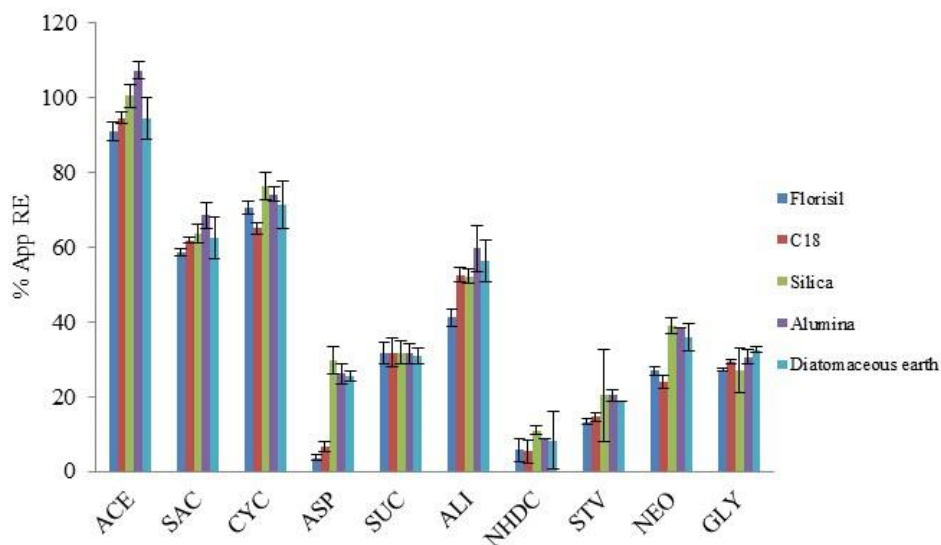
Figure 2. App REs (%) of fish samples spiked at 2500 ng g^{-1} (d.w.) when different in-cell sorbents and when no in-cell (diatomaceous earth) were used.

3.4 Method validation

The method validation was performed for the species *Mullus surmuletus* and involved the evaluation of the linear range, MDLs, MQLs, intra-day precision and inter-day precision, App REs and ME. Blank samples were evaluated in order to subtract the signal if any compound was present. However, none of the studied sweeteners was present. Moreover, some of these parameters were also evaluated for *Cyprinus carpio*. All of the validation parameters can be found in Table 3.

For the species *Mullus surmuletus*, the App REs were evaluated at two concentration levels 125 ng g^{-1} (d.w.) and 500 ng g^{-1} (d.w.), with the exception of neohesperidine dihydrochalcone, which was only evaluated at 500 ng g^{-1} (d.w.) as this compound had a low PLE RE and high ME, as well as the fact that, in the 125 ng g^{-1} (d.w.) concentration, it was below its MQL. At both levels, the App REs were very similar for all of the compounds, with values ranging from 11% to 91%. The ME was also evaluated for the highest level, with most of the compounds being subject to ion suppression, with the exception of acesulfame, which displayed ion enhancement. The most affected compounds in terms of the ME were neohesperidine dihydrochalcone and aspartame, with MEs of -93% and -89%,

respectively. The option of using internal standards to correct the high ME observed was ruled out since the selected compounds showed different responses in the LC-HRMS. In addition, they also belong to different chemical classes (sulfamates, peptides and carbohydrate derivatives) and cover a wide range of polarities that might result in different behaviour. These features mean that most likely ten isotopically labelled compounds would be needed, increasing the costs of the study.

In order to quantify the analytes, matrix-matched calibration curves were plotted. Linear range, MDLs and MQLs were obtained experimentally by spiking fish samples at different concentrations before PLE. All of the compounds showed good linearity (in the ranges shown in Table 3) with R^2 above 0.9913. For most of the compounds MQLs were between 25 ng g^{-1} (d.w.) and 50 ng g^{-1} (d.w.), with the exception of alitame and acesulfame, with a lower MQL (12.5 ng g^{-1} d.w.), and neohesperidine dihydrochalcone, with a high MQL of 250 ng g^{-1} (d.w.). As for MDLs, they ranged between 12.5 ng g^{-1} and 25 ng g^{-1} for most of the compounds, with the exception of alitame and acesulfame (2.5 ng g^{-1} d.w.) and neohesperidine dihydrochalcone (125 ng g^{-1} d.w.), as can be seen in Table 3.

Values of intra-day precision were always below 16% at both spiked levels (125 ng g^{-1} and 500 ng g^{-1} (d.w.)), as in the case of App REs neohesperidine dihydrochalcone was just evaluated at the highest level, and inter-day precision (spiked at 500 ng g^{-1}) lower than 25%.

In the case of *Cyprinus carpio*, App REs were evaluated at the highest concentration (500 ng g^{-1}), the results are detailed in Table 3. In general, they were higher than for the species *Mullus surmuletus*, as mentioned previously. The lipid content of these two species is different, with it being higher in the case of *Mullus surmuletus* (23%) than for *Cyprinus carpio* (15%). This fact means that a higher ME is observed and a lower App RE is obtained for *Mullus surmuletus*. Intra-day precision was also evaluated for *Cyprinus carpio*, ranging from 4% to 17%.

Table 3. Method validation data when the samples were analysed by PLE and LC-HRMS.

	Mullus surmuletus							Cyprinus carpio		
Compound	Linear range ng g^{-1} (d.w.)	MDL ng g^{-1} (d.w.)	125 ng g^{-1} App RE (%)	500 ng g^{-1} App RE (%)	ME (%)	Intra-day precision (%RSD; n=5)	Inter-day precision (%RSD; n=5)	App RE (%)	ME (%)	Intra-day precision (%RSD; n=5)
ACE	12.5-1000	2.5	66	91	9	4	13	122	50	7
SAC	25-1000	12.5	26	35	-54	2	14	88	19	6
CYC	25-1000	12.5	30	44	-56	4	12	72	-3	10
ASP	50-1000	25	20	11	-89	5	25	23	-59	7
SUC	50-1000	25	25	37	-58	11	16	57	-34	4
ALI	12.5-1000	2.5	40	53	-27	4	24	168	112	8
NHDC	250-1000	125	-	<10	-93	15	20	20	-70	17
STV	25-1000	12.5	13	16	-78	16	22	39	-51	10
NEO	50-1000	25	19	17	-76	11	13	38	-54	5
GLY	25-1000	12.5	26	21	-70	3	9	29	-46	7

3.5 Method applicability

As mentioned, the method was developed for the two species with different lipid content: *Mullus surmuletus* with a high lipid content and *Cyprinus carpio* with a lower lipid content. The optimised method was applied to evaluate the occurrence of the selected compounds in different fish species that had similar % of lipid content (which is indicated in brackets) to the above mentioned species. The evaluated species considered to have a high lipid content [31] were: *Mullus surmuletus* (striped red mullet, 23%), *Scomber scombrus* (Atlantic mackerel, 21%), *Sparus aurata* (gilt-head bream, 35%) and *Psetta maxima* (turbot, 31%). The species with a lower lipid content were: *Cyprinus carpio* (common carp, 15%) and *Silurus glanis* (wels catfish, 12%) [31].

The criteria to evaluate the presence of the selected compounds were the retention time, the exact mass of the precursor ion with a mass error of 5 ppm, the fragment ion and their corresponding ion ratio [21,25,34]. However, in the case of cyclamate, glycyrrhizic acid and saccharin, the presence of the fragment ion was not considered, as these compounds displayed poor fragmentation (Section 3.1). Moreover, the signals of the fragment ions were highly affected by the noise. In any case, the high confirmation capabilities of high-resolution techniques should be noted.

Among the studied compounds, saccharin was found in one of the analysed samples, in the species *Scomber scombrus* at a concentration below its MQL. Figure 3 shows the accurate mass extracted ion chromatogram of the precursor ion for saccharin in a fish sample. To the best of our knowledge, this is the first time that these compounds have been studied in aquatic organisms and, thus, it is not possible to compare the results obtained.

4. Conclusions

A PLE method followed by LC-HRMS to determine simultaneously ten high-intensity sweeteners in fish was successfully developed and validated.

PLE recoveries ranged from 43% to 94%. Several clean-up strategies were tested to reduce the high matrix effect encountered and in-cell clean-up using alumina

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combined with on-cell clean-up employing hexane were selected as the best options.

The developed method provided suitable intra-day precision (n=5) and inter-day precision (n=5) with %RSD values less than 16% and 25%, respectively.

The method was applied to determine the occurrence of the selected sweeteners in different fish species with different lipid content. Of these sweeteners, saccharin was found in one of the samples analysed, below its method quantification limit.
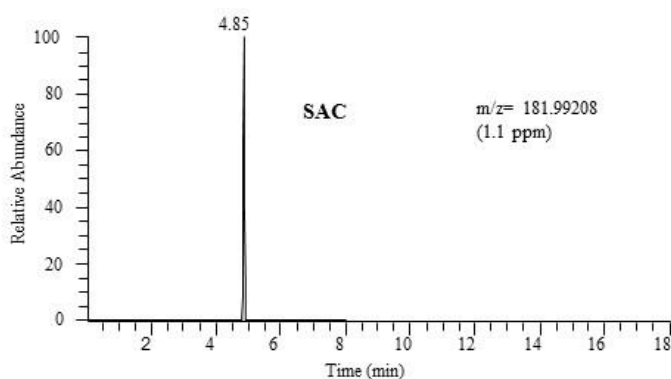
Figure 3. Accurate mass extracted ion chromatogram of the precursor ion for saccharin in a sample of the fish *Scomber scombrus*.

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3.2.3. Discussion of results

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Two analytical methods were developed for fish samples for which the PLE technique was selected in both studies. Although the QuEChERS extraction technique was also evaluated in the study on extracting ICM-XR, lower extraction recoveries were achieved, which is one of the reasons why PLE was directly selected for the study of high-intensity sweeteners. For both methods the most important PLE parameters were optimised in order to obtain the highest extraction recoveries. Again the extraction solvent was the most important parameter in both cases.

In both methods, after optimising the extraction technique a high matrix effect was encountered and different clean-up strategies were evaluated to eliminate or at least minimise it. In the method to evaluate the ICM-XR, Oasis® MCX was selected to retain interferences. The cartridge was not used according to the suppliers' protocol but was otherwise employed to retain interferences. With this aim in mind, instead of performing an elution step, the loading was collected with the analytes. However, the high matrix effect was still present, and for this reason it was decided to also use two internal standards to correct the matrix effect. In the method for high-intensity sweeteners, the clean-ups selected were two PLE clean-ups. Alumina was placed at the bottom of the extraction cell and also mixed with the matrix in order to retain interferences (in-cell clean-up), and before the extraction a defatting step was performed employing hexane (on-cell clean-up). In other studies in the literature both strategies have been used as clean-up in complex matrices such as biota [1-3].

The chromatographic separation for ICM-XR was rather challenging due to the high polarity of the compounds and the presence of stereoisomers, which meant that some of them could not be completely separated. In the method developed for high-intensity sweeteners, two chromatographic columns suitable for polar compounds such as high-intensity sweeteners were tested, namely the Ascentis Express RP amide (100 x 2.1 mm; 2.7 µm) and the Zorbax Eclipse XDB-C$_8$ (150 x 4.6 mm; 5 µm), with the latter being selected since better results were obtained for the first eluting compounds. When EOCs are evaluated in aquatic organisms, the most common analysers are MS/MS analysers such as QqQ or QqLIT. However, the HRMS with Orbitrap as analyser was employed in both studies presented. The same analyser has also been used recently by other authors to determine pharmaceuticals in aquatic organisms [4,5].

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When the methods were applied to evaluate the presence of the selected compounds, different fish species were examined. According to Huerta et al. [2], the highest matrix effect was encountered when high lipid content was present. The species were divided according to their lipid content, which was calculated gravimetrically [6]. The method developed for ICM-XR was applied to evaluate its occurrence in ten different species, which were divided into three categories (low, medium and high lipid content). In each category a representative species was selected and different validation parameters such as linearity, limits of detection and quantification, matrix effect and apparent recoveries were evaluated. The liver of one of the selected species was also evaluated, since according to Huerta et al. [7] the highest pharmaceutical concentrations have been reported in organs such as the liver and brain. The method developed to determine high-intensity sweeteners was applied to fewer species than the previous one. Six of the ten species examined by the ICM-XR method were evaluated here and two groups were performed (low and high lipid content). In this case, one representative species was again selected from each group and the same validation parameters evaluated.

While with the method to determine ICM-XR none of the selected compounds was found above its limit of detection, with the method for high-intensity sweeteners one compound (saccharin) was detected above its limit of detection in the species Atlantic mackerel (*Scomber scombrus*). As mentioned in the introduction, this compound has been determined in different aquatic environments including in marine waters [8,9]. To the best of our knowledge no previous research has been done to study the presence of these compounds in aquatic organisms that we could use to compare with the results achieved in the present Thesis.

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3.3. Ecotoxicological study of triclosan on *Gammarus pulex*

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As mentioned in the introduction, WWTPs effluents are sources of the continuous release of EOCs into the aquatic environment [1]. These contaminants have been determined in surface waters and influents and effluents from WWTPs at concentration levels between nanograms per liter and micrograms per liter nearly worldwide [2]. Since EOCs are not completely eliminated in WWTPs, their presence in surface waters that receive effluents from WWTPs is of recent concern.

According to Brausch et al. [3], PCPs are among the most commonly detected compounds in surface water throughout the world [3]. Different from the other group of contaminants analysed in this Thesis, such as pharmaceuticals for internal use, PCPs are intended for the external use on the human body. For this reason they are not affected by metabolic alterations and large quantities of PCPs can enter to the environment unaltered through regular usage. The presence of these contaminants may affect the species inhabiting surface waters to a different extent according to the species and the contaminant. Among PCPs one widely determined contaminant in surface waters is triclosan [3].

In this section the preliminary results of a study to evaluate the toxicity of triclosan in one species inhabiting freshwater rivers are presented. The amphipod species *Gammarus pulex* was selected because among other characteristics it is abundant in rivers and is easy to collect, handle and maintain; furthermore it is known to be sensitive to a range of stressors [4]. Different populations of *G. pulex* sampled from different field sites were evaluated in order to determine differences in triclosan sensitivity. To do this, three different groups of populations were evaluated: populations from uncontaminated sites, populations inhabiting streams impacted by the discharge of WWTPs effluents (considered contaminated sites) and populations inhabiting upstream of WWTPs.

The study presented in this section was developed during a three month European placement in the Helmholtz Centre for Environmental Research-UFZ in Leipzig (Germany) in the System Ecotoxicology Department, which has extensive experience in developing ecotoxicological studies in aquatic organisms.

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***3.3.1 Repeated toxicant exposition shapes the response of Gammarus
pulex***

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REPEATED TOXICANT EXPOSITION SHAPES THE RESPONSE OF
Gammarus pulex

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Abstract

There is a multitude of stressors contributing to the ecological deterioration of freshwater ecosystems, among them, anthropogenic contamination. One group of contaminants are personal care products (PCPs), which have been reported in surface waters worldwide. The amphipod species *Gammarus pulex* populations were sampled from contaminated and uncontaminated streams and tested for their acute and chronic toxicity of one PCP, triclosan. Acute toxicity and long term toxicity effects were evaluated in the different populations. In acute toxicity tests significant differences were observed between populations belonging to contaminated and uncontaminated environments. Hence, repeated exposure to triclosan leads to a tolerance development of field populations, but only to a limited extend.

Keywords: *Triclosan; Gammarus pulex; acute toxicity; aquatic invertebrates*

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1. Introduction

Surface waters are susceptible to environmental pollution by anthropogenic activities such as human and industrial activities. The presence of emerging organic contaminants has attracted concern of the scientific community in the last decades. One group of these contaminants are personal care products (PCPs), which includes a wide range of compounds broadly used in human daily activities. PCPs reach wastewater treatment plants (WWTPs) where they are partly eliminated during their treatment. However, due to this incomplete removal, WWTP effluents are an important and continuous source of entry of these contaminants into the aquatic environment [1]. Since the late 1990s the scientific community has focused its attention on their occurrence and several reviews have been published regarding their presence in different environments [1-3].

Among the different PCPs, triclosan is within the top ten of most commonly determined compounds [4]. This compound is a synthetic, broad-spectrum antimicrobial agent, which has also antibiotic and antimycotic properties. It is used in toothpastes, antibacterial soaps, dishwashing liquids, deodorant soaps, cosmetic and antiseptic products to name a few, but also in other items such as kitchen utensils, toys, bedding, clothes, fabrics and trash bags [5]. Since triclosan is externally applied, it is not subjected to metabolic alterations and large quantities of this compound enter to the environment unaltered through regular usage and several studies have demonstrated its widespread presence in the environment [6-9]. According to the compilation of Bedoux et al. [10] triclosan has been reported in surface waters (rivers and lakes) from several countries such as Germany, Italy, Greece, Switzerland, Spain and the UK, among others, showing concentrations of up to 285 ng/L. However, already in 2002, a study conducted by Kolpin et al. [11] identified triclosan as one of the top seven contaminants from a network of 139 streams across the USA, with a maximum concentration of 2300 ng/L. Its presence has also been reported in sediments (lake, river and other surface waters) with concentrations between 0.4 and 1329 ng/g (dry weight) [10].

Ecotoxicological studies on triclosan have been conducted in a variety of aquatic organisms, including algae, invertebrates, fishes and amphibians, to determine the potential risks for the aquatic systems. These studies highlighted its potential toxicity on highly sensitive organisms [12].

Freshwater invertebrates are susceptible of being affected by contamination and they are representative of the local conditions in freshwater ecosystems. Among these invertebrates, *Gammarus pulex* plays an important role for the freshwater food chain and for leaf litter degradation as an ecosystem function [13]. They actively participate in the fragmentation and decomposition of leaves, by the transformation of leaf material to fine particulate organic matter which provides food to collector-gatherers and filter-feeders [13]. Moreover they are the food source for other invertebrates, fish and birds. This species is widely distributed in freshwater rivers and tributaries across Europe and can be collected in large numbers using simple kick sampling techniques. They can also be kept well enough under laboratory conditions and are quite sensitive to pollutants [14]. In addition, they are purely aquatic and hence more or less bound to specific stream catchment or catchment sections. Moreover, *G. pulex* has already been used as model organism for assessing both the adverse effects and uptake potential of PCPs as well as other common pollutants [15]. Because of these attributes, it is widely used in biomonitoring studies.

An important parameter in ecotoxicology is the tolerance development of populations to contaminants, which is the ability of organisms to cope with stress, particularly the chemical stress resulting from the anthropogenic input of one or more toxic contaminants into the environment [16]. According to Amiard-Triquet et al. [16], populations of one species that were previously exposed to chemicals in their environments are less affected than individuals that were not. For example, in the work by Corcoll et al. [17] biofilms exposed to environmental concentrations of non-steroidal anti-inflammatory drugs acquired tolerance to them in later exposures. Tolerance may be achieved by many biological process responsible for physiological acclimatization or genetic adaptation [16]. In many studies, however, distinguishing between physiological acclimatization and genetic differences between individuals is not possible [18].

Therefore, the aim of the present study was to evaluate the toxicity of triclosan to the amphipod species *G. pulex*. Different populations sampled from contaminated and uncontaminated field sites were evaluated in order to determine differences in triclosan sensitivity. For this aim, LC_{50} was calculated for each population and they were grouped according their sampling sites and the different groups were compared.

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2. Materials and methods

2.1 Study area and sampling

Nine geographically distinct *G. pulex* populations were sampled in the field, which comprise three different groups of sites. The first group includes three reference sites considered uncontaminated and includes Bad Lausick (Site 1), Naunhof (Site 2) and Thümmlitzwalde (Site 3). The second group contained three sites that were sampled downstream of three different WWTPs (considered contaminated sites since organisms might be exposed to triclosan and other organic pollutants) and the last group comprised the upstream sampling of the same three WWTPs. The WWTPs sites include Hoym (Site 4 and 5), Blankenburg (Site 6 and 7) and Osterwieck (Site 8 and 9). The mentioned sites were selected according to our previous studies, since the uncontaminated sites and the WWTPs sites had been sampled in previous monitorings [19]. According to Münze et al. [19], these WWTPs are characterised by a tertiary treatment level (including nitrification denitrification and phosphorous removal). Upstream sites were considered separately since there might be diffuse and general pollution due to the surrounding land use (but not specifically to triclosan).

Adult specimens were collected in October 2015 via the kick sampling netting method. The specimens collected were transported to the laboratory in flasks containing between 500 and 1000 mL of stream water and leaves obtained from each corresponding sampling site. Water was aerated in order to provide enough oxygen to the organisms.

2.2 Acute toxicity test and long term effects

A preliminary range finding test was conducted by exposing a population from an uncontaminated site (Site 2, Naunhof) to a broad range of concentrations (control, 0.1, 0.3, 0.5, 1 mg/L), which were selected according to values in the literature for other invertebrate species such as *Daphnia magna*, *Ceriodaphnia dubia*, *Chironomus tentans* and *Hyalella Azteca* [20,21]. The range finding test was performed in order to select effective concentrations for the main acute LC_{50} test. Two stock solutions of 20000 mg/L and 40000 mg/L of triclosan were prepared in dimethyl sulfoxide (DMSO). The toxicity of this solvent was also evaluated in *G. pulex* individuals during the range finding test, where individuals were also

exposed to the maximum concentration of DMSO to which they would be exposed in the final test.

Individuals sampled on each sampling site were kept for 24 h in trays before the acute exposure assay in a climate chamber under a temperature of 15 ºC, which was the temperature of the water of the streams at the beginning of the experiment, and under a 12 h: 12 h light:dark controlled conditions. The trays where *G. pulex* were placed contained the medium from the sampling site (water and leaves), which was aerated to promote oxygen removal.

For the acute toxicity tests four different concentrations 0.25; 0.5; 0.75 and 1 mg/L were selected and they were prepared in an M7 medium. Between 10 and 15 individuals were exposed to each of the previous mentioned concentrations or used as control for each site.

The number of live/dead organisms was recorded after 24 h by gentle prodding and observation of movement of appendages. Organisms were counted as dead when none of the appendages were moving [14]. Then, the survivals were gently transferred individually into metal tea strainers, which contained a leaf of *Betula* sp. in order to provide them food. The leaves were previously weighed on analytical balance. The tea strainers containing the *G. pulex* were placed randomly in 5 outdoor mesocosm streams, with the following characteristics: length 20 m; width at water surface 0.32 (±0.03) m, average depth 0.25 (±0.11) m, discharge 160 (±9) L/min, with closed water circulation each, situated at the Helmholtz Centre for Environmental Research in Leipzig, Germany. The mortality was recorded again after 24 h (48 h after exposition).

Once the mortality had been recorded after 48 h, it was continued being evaluated every three days during the following nineteen days after exposition. At the end of the experiment mortality was recorded again.

In order to evaluate the food consumption of each surviving individual, the leaves of those individuals that survived were dried on an oven overnight and were weighed again on the analytical balance. The food consumption was calculated as follows:

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Food consumption= (initial weight-final weight) /initial weight*100

The mean consumption for each concentration on each site was calculated.

2.3 Data analysis

LC_{50} and LC_{10} values were determined after 24 h, 48 h and long-term (nineteen days after exposition) for each population. The two parameter log-logistic function (LL.2) from the drm in the R-package was selected to calculate the LC_{50} and LC_{10}. The LC_{50} and LC_{10} values for the uncontaminated sites, contaminated sites and upstream WWTPs populations were compared to determine significant differences in the tolerance to triclosan by performing pairwise t-tests in the statistical program Statgraphics (version 5.1 plus).

3. Results and discussion

3.1 Acute toxicity test

The uncontaminated sites show the highest mortality after 24 h with almost 100% of mortality on average for the highest concentration, whereas the contaminated sites present the lowest mortalities (Figure 1A). The LC_{50} value was calculated for each population as described in Section 2.3 and the value obtained for each population is plotted in Figure 1B. Mean 24 h LC_{50} for the uncontaminated populations was 0.625 mg/L, for the contaminated sites it 0.837 mg/L and for the upstream sites 0.763 mg/L. Figure 2 shows, as an example, two plots obtained with the log-logistic LL.2 function employed to calculate the LC_{50}, one from an uncontaminated site and the other from a contaminated site. As can be seen, the ratio killed/ exposed organisms was higher for the uncontaminated sites. Regarding other studies conducted on invertebrate species, Gómez-Canela [22] reported an LC_{50} value of 0.57 mg/L for *G. pulex* which is very similar to the LC_{50} value encountered in the present study on the uncontaminated sites. Kim et al. [23] reported LC_{50} values of 0.47 mg/L in the freshwater crustacean species *Thamnocephalus platyurus* after 24 h of exposition, which according to the authors is considered a sensitive species to a variety of chemical agents [23].

A

B
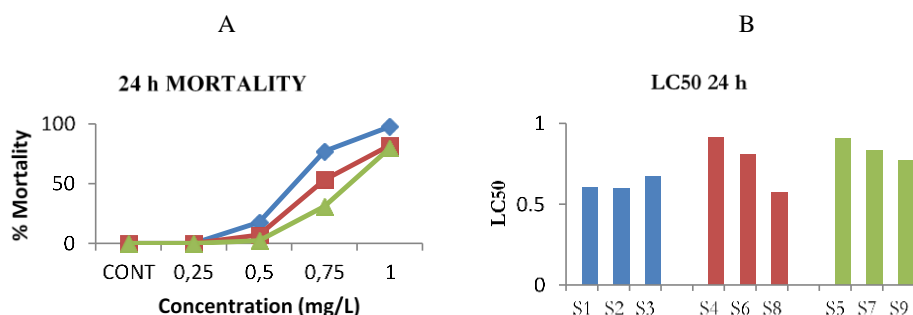
Figure 1. A. Mortality after 24 h. B. LC$_{50}$ values for gammarids from each site (blue colour uncontaminated sites; red colour upstream sites; green colour contaminated sites.
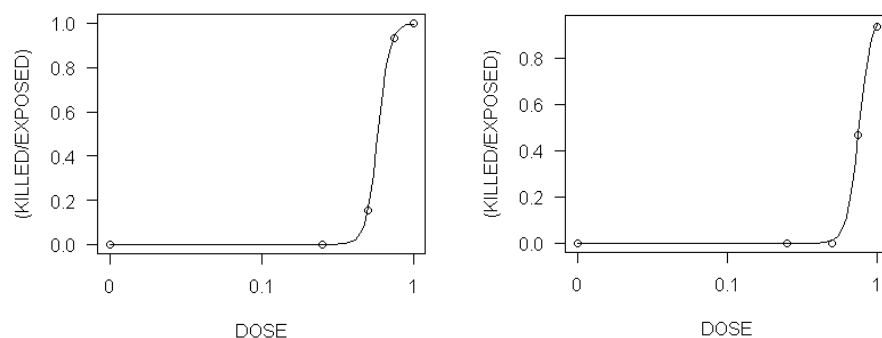
A

B
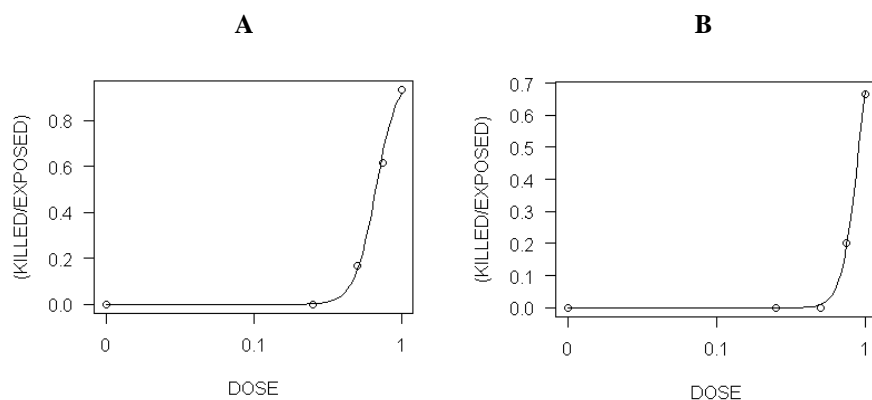
Figure 2. Plots of the log-logistic LL.2 function employed to calculate the LC$_{50}$ after 24 h of exposition to triclosan. A uncontaminated site; B contaminated site.

When LC$_{50}$ was compared through pairwise t-tests, significant differences between two groups of populations, uncontaminated and contaminated groups, were encountered (p-value<0.05). Regarding the upstream group no significant differences were found when this group was compared with the other two groups. Table 1 shows the values of the pairwise t-tests as well as the p-value obtained for each analysis. Moreover, significant differences (p-value<0.05) were also encountered between these two types of sites (contaminated and uncontaminated) for LC$_{10}$ values (data not shown), which were also calculated as detailed in Section 2.3. For acute toxicity tests after 24 h of exposition, the populations from contaminated sites were more tolerant to triclosan exposure than the upstream and uncontaminated sites.

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Regarding the 48 h mortality, the organisms that had been exposed for 24 h and survived were kept in artificial streams for further 24 h. A similar pattern as for 24 h mortality was observed, where the uncontaminated sites showed higher mortality than upstream sites and contaminated sites, being again the highest difference between the uncontaminated and contaminated groups; and the group of upstream sites an intermediate group as can be seen in Figure 3A. Consequently the values of LC_{50} were higher for contaminated sites than for uncontaminated sites. Figure 3B also shows also the LC_{50} values obtained for each population. The mean LC_{50} obtained were 0.543, 0.677, 0.7236 mg/L for the uncontaminated, upstream and contaminated sites respectively. Figure 4 shows as an example two plots obtained with the log-logistic LL.2 function employed to calculate the LC_{50} one from an uncontaminated site and the other from a contaminated site, as in the case of 24 h mortality, the ratio killed/exposed organisms for the uncontaminated sites is higher than for the uncontaminated sites.

Regarding other studies that evaluate the toxicity of triclosan to invertebrate species, Orvos et al. [20] reported values of median effective concentration (EC_{50}) after 48 h of exposition to the invertebrate species *Daphnia magna* and *Ceriodaphnia dubia* of 390 µg/L and 240 µg/L, respectively. However, the aquatic species that appeared most vulnerable to the toxic effects of triclosan were algal species. In the species *Scenedesmus subspicatus*, for example, a 96 h biomass EC_{50} of 1.4 µg/L and a 96 h no-observed effect concentration (NOEC) of 0.69 µg/L were estimated by Orvos et al. [20]. Similar sensitivity was reported by other authors for other algae species [24,25]. It is possible that current levels of triclosan in rivers and streams may surpass the NOEC for algae [12].

Table 1 shows the pairwise t-tests and the corresponding p-values obtained. As in the case of 24 h mortality among the t-tests, significant differences were encountered between the uncontaminated and contaminated groups, and no significant differences were found for any other comparisons. For acute toxicity tests after 48 h, the populations from contaminated sites continued to be more tolerant to triclosan than the uncontaminated and upstream populations.

Table 1. Pairwise t-test results with the corresponding p-value obtained for the LC_{50} values.

Sites	24 h		48 h		Long-term	
	t-student	p-value	t-student	p-value	t-student	p-value
Uncontaminated- contaminated	4.474	0.011	3.973	0.016	2.071	0.107
Uncontaminated- upstream	1.335	0.253	1.318	0.258	1.536	0.199
Contaminated- upstream	0.679	0.534	0.465	0.666	0.392	0.715
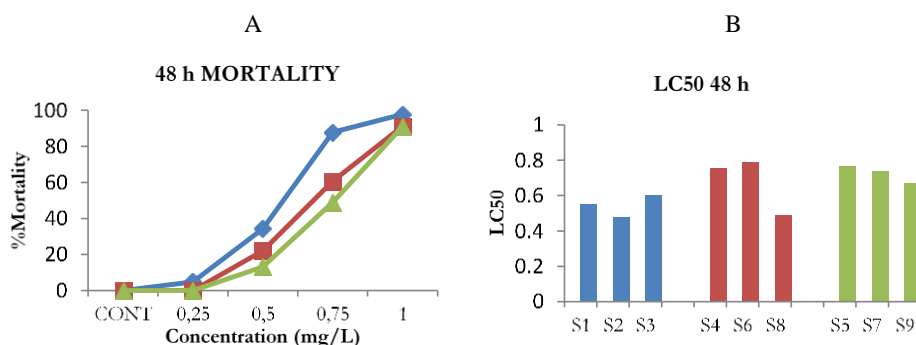
Figure 3. A. 48 h mortality. B. LC_{50} values for gammarids for each location. Blue uncontaminated sites; red colour upstream sites; green colour contaminated sites.

Figure 4. Plots of the log-logistic LL.2 function employed to calculate the LC_{50}, after 48 h. A uncontaminated site; B contaminated site.

In a study conducted by Martínez et al. [26], the toxicity of several pharmaceuticals and personal care products (PPCPs) on the freshwater rotifer *Plationus patulus* was evaluated. LC_{50} values after 48 h exposure were determined for two populations collected from different locations (one reference location and one location affected

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by wastewater) for comparison of tolerance levels between them. Although, according to the authors, the reference population was expected to be more sensitive than the population coming from a location affected by wastewater to the acute exposure of the toxicants, no consistent pattern according to the source population was observed. The reference population was more tolerant to the analgesic acetamidophenol and the stimulant caffeine, whereas it was more sensitive to fluoxetine. In another study conducted by Corcoll et al. [17], the response of biofilms from a non-polluted site to that of others downstream of WWTP to two NSAIDs (ibuprofen and diclofenac) was compared. Biofilms exposed to environmental concentrations of ibuprofen and diclofenac acquired tolerance to these NSAIDs. In our acute toxicity test significant differences in tolerance levels to triclosan were encountered between uncontaminated sites and contaminated sites.

3.2 Long term effects

Regarding the long term effects two endpoints were evaluated, the mortality and the food consumption. Regarding the long-term mortality LC_{50} was calculated for each population at the end of the experiment, and the three groups of populations were again compared by pairwise t-tests. In this case, in contrast to the results obtained in the acute effects, no significant differences (p-value>0.05) were obtained for any of the pairs compared. Table 1 shows the results of the pairwise t-tests and p-values values obtained for each test. Figure 5 shows the mean mortality for each concentration, as can be seen, for the uncontaminated and upstream sites for the highest three concentrations, mortality occurs mainly in during the first 24 and 48 hours, whereas for the contaminated sites mortality occurs during the first 6 days, especially for the 0.75 and 0.5 mg/L concentrations.
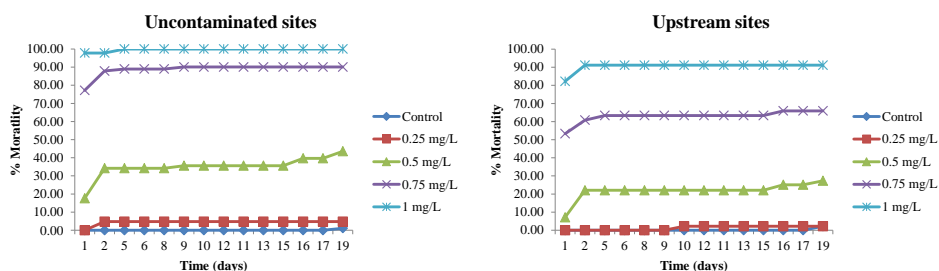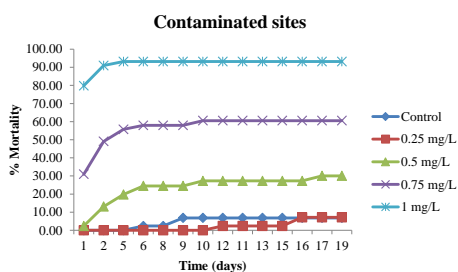
Figure 5. Long term mortality for the three groups of populations.

Regarding the food consumption, Figure 6 shows the results obtained, where the mean consumption for each group of sites on each concentration has been plotted.
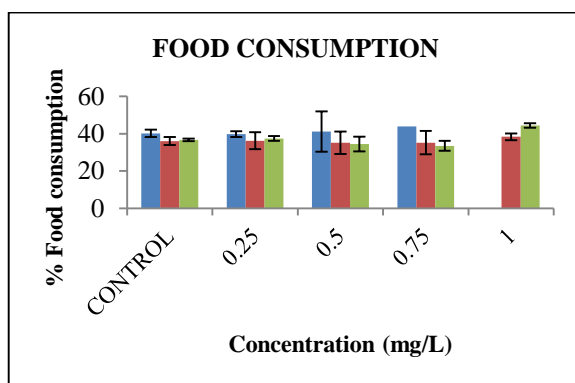
Figure 6. Food consumption: blue colour, uncontaminated sites, red colour upstream sites and green colour contaminated sites.

Although it can be seen that for the controls and the two lowest exposure concentrations a slightly higher food consumption was observed for the individuals from uncontaminated sites compared with the contaminated ones, it should be mentioned that for gammarids from uncontaminated sites, no surviving gammarids were observed for the highest concentration (1 mg/L). For the second highest concentration (0.75 mg/L), the survivals were only from one of the three uncontaminated sites.

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4. Conclusions

An ecotoxicological study was conducted to evaluate the toxicity of triclosan to the amphipod species *G. pulex*. Three groups of populations (contaminated, uncontaminated and upstream) were compared for acute and long term effects of triclosan. For acute toxicity tests after 24 h of exposition, the populations sampled from contaminated sites were more tolerant to triclosan (higher values of LC_{50} were encountered). The same trend was observed after 48 h. However, no significant differences were observed in LC_{50} values at the end of the experiment, after 19 days (long term effects). Repeated exposure leads to a tolerance development, which is, however, limited. That means also that non-target organisms are not really able to adapt to toxicant exposure.

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3.3.2. Discussion of results

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In this section the preliminary results of an ecotoxicologial study of triclosan in the amphipod species *Gammarus pulex* have been presented.

As can be seen from the results included, mortality was higher for uncontaminated sites than for upstream and contaminated sites. When LC_{50} was calculated for the three groups of sites, the lowest values were obtained for uncontaminated sites, for contaminated sites the highest, whereas upstream sites was an intermediate group with values between the other two groups. The values of LC_{50} obtained for the uncontaminated sites were similar to the value of LC_{50} reported by Gómez-Canela et al. [1] for the same species. In addition, similar values of LC_{50} after 24 h of exposition to triclosan have been reported in the literature for other invertebrates' species [2]. The values of LC_{50} obtained in the present study do not suppose a threat for this species since the concentrations found in the environment are lower; for this reason it is unlikely that triclosan will display any significant acute toxicity. However, other species such as algae and early development stages of fish present higher sensitivity and the environmental concentrations could suppose a threat for them [3].

We have also observed that in acute toxicity tests after 24 h of exposition to triclosan and after 24 h of depuration (48 h after exposition), populations belonging to contaminated sites were more tolerant to triclosan than the other populations (from uncontaminated and upstream sites), since significant differences in pair-wise t-tests were encountered between contaminated and uncontaminated sites, no significant differences were found for other comparisons.

As a preliminary results this study is of high interest, although additional sampling sites could be included and deeper statistical analyses could be performed that would contribute to more definitive conclusions.

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CHAPTER 4. CONCLUSIONS

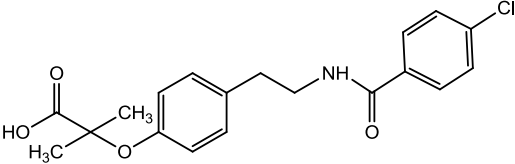EMERGING ORGANIC CONTAMINANTS IN AQUATIC ORGANISMS
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The main conclusions drawn from the studies presented in this Doctoral Thesis can be summarised as follows:

1. Different extraction techniques were successfully evaluated to extract different pharmaceuticals and a group of high-intensity sweeteners from aquatic organisms.

2. PLE was a useful technique for extracting most of the EOCs included in this Doctoral Thesis from aquatic organism samples. In addition, in one study, water was used as the extraction solvent, which makes PLE an environmentally friendly method. Moreover, water-based extracts can be directly loaded in SPE for clean-up procedure.

3. QuEChERS extraction was successfully applied to extract seven pharmaceuticals from bivalve samples, and high recoveries were obtained for most of the compounds.

4. Aquatic organism matrices are very complex and after extraction one or more clean-up steps are required to obtain suitable extracts for analysis. In addition, the lipid content of the sample can affect the figures of merit.

5. Various strategies for eliminating or reducing the matrix effect were evaluated for the different analytical methods. In the methods developed for pharmaceuticals of generalised consumption, the matrix effect was reduced in the QuEChERS method by using dSPE with silica gel as clean-up, and modifying the gradient in the chromatographic separation. In the PLE method, the clean-up consisted of SPE using an Oasis® MAX cartridge. However, none of the strategies employed was completely effective.

6. In the method for determining the presence of high-intensity sweeteners, the matrix effect was slightly reduced by using clean-ups in the cell with alumina as sorbent and hexane as solvent for defatting, whereas in the ICM-XR method, a clean-up with SPE using an Oasis® MCX cartridge was selected. Moreover, it was considered necessary to add isotopically labelled standards to correct the matrix effect.

7. Tandem mass spectrometry using the triple quadrupole mass analyser proved to be a highly sensitive tool for quantifying the target compounds. High resolution mass spectrometry with Orbitrap as the analyser was also successfully employed.

8. Among the compounds studied, salicylic acid was the only pharmaceutical of generalised consumption found in the different bivalve samples. None of the target ICM-XRs was detected above their limit of detection. The presence of saccharin in the fish species was reported for the first time in this Thesis.

9. The toxicity of triclosan was successfully evaluated by calculating the LC_{50} in different populations of *Gammarus pulex*.

10. In a short-term toxicity test the populations from uncontaminated sites had a lower tolerance to triclosan than the populations from contaminated site. Repeated exposure to toxicants leads to a tolerance development, which is, however, limited.

APPENDIX

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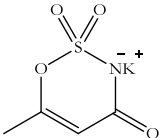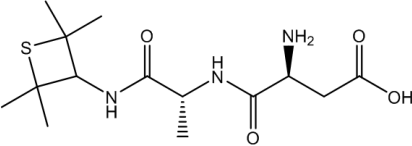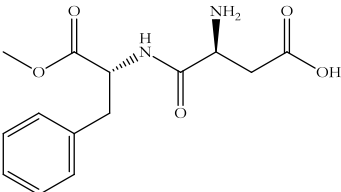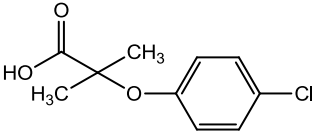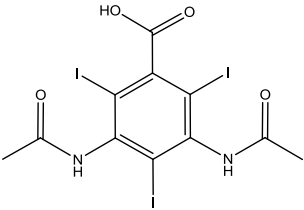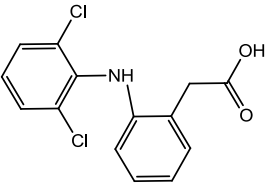
Appendix I. Abbreviations used in this Doctoral Thesis.
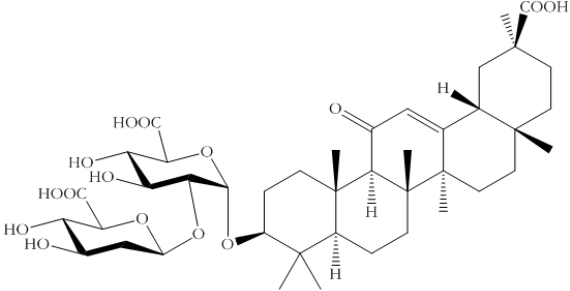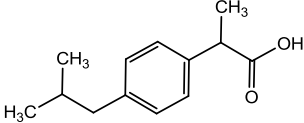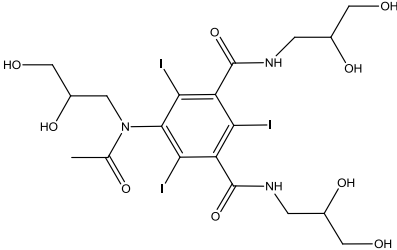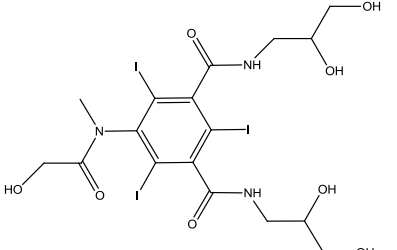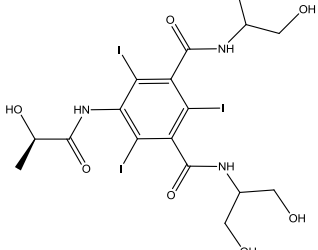
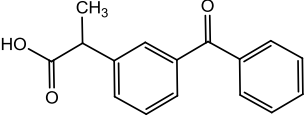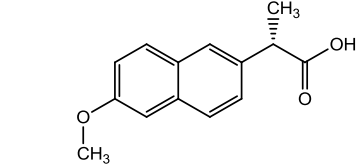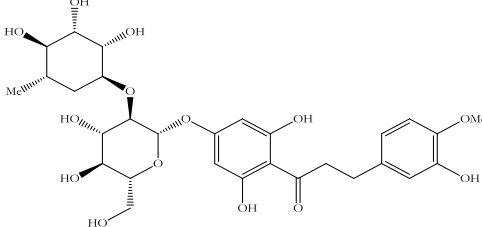|	
ACE	Acesulfame
ACN	Acetonitrile
ALI	Alitame
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
AppRE	Apparent recovery
ASP	Aspartame
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
COX	Cyclooxygenase enzyme
CYC	Cyclamate
DAD	Diode array detector
DCM	Dichloromethane
d.w.	Dry weight
dSPE	Dispersive solid-phase extraction
EC_{50}	Median effective concentration
EDC	Endocrine-disrupting compound
EI	Electron impact
EOC	Emerging organic contaminant
ESI	Electrospray ionisation
FabI	Enoyl-acyl carrier protein reductase enzyme
FDA	Food and Drug Administration
FWHM	Full width at half maximum
GC	Gas chromatography
GCB	Graphitised carbon black
GLY	Glycyrrhizic acid
HDL	High density lipoprotein
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
ICM-XR	Iodinated X-ray contrast media
IS	Internal standard
IT	Ion trap
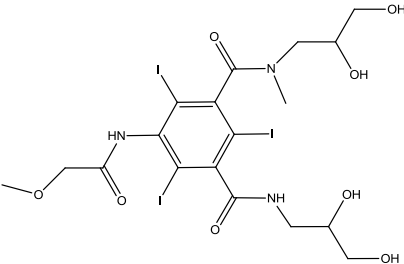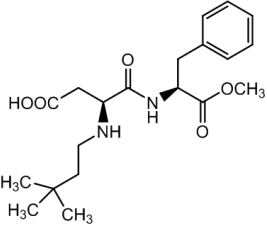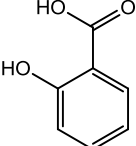
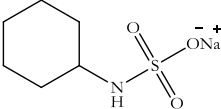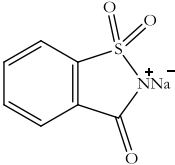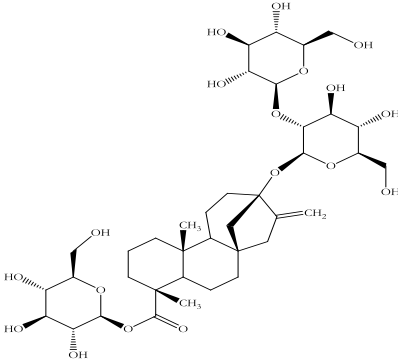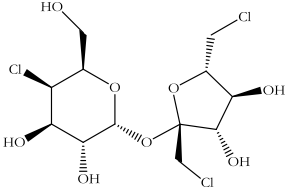|	
LC	Liquid chromatography
LC_{50}	Median lethal concentration
LDL	Low density lipoprotein
LLE	Liquid-liquid extraction
LOEC	Lowest observed effect concentration
LOD	Limit of detection
LOQ	Limit of quantification
Macro	Macroinvertebrate
MAE	Microwave assisted extraction
MAME	Microwave assisted micellar extraction
ME	Matrix effect
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBE	Methyl tert-butyl ether
MTBSTFA	*N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide
NOEC	No observed effect concentration
NEO	Neotame
ND	Not detected
NHDC	Neohesperidin dihydrochalcone
NSAID	Non steroidal anti-inflammatory drug
PCP	Personal care product
PFBBr	2,3,4,5,6-pentafluorobenzylbromide
PLE	Pressurised liquid extraction
PPCP	Pharmaceutical and personal care product
PSA	Primary secondary amine
PTFE	Polytetrafluoroethylene
Q	Quadrupole
Q-Orbitrap	Hybrid quadrupole Orbitrap
Q-TOF	Hybrid quadrupole time of flight
QqLIT	Hybride triple quadrupole linear ion trap
QqQ	Triple quadrupole
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, Safe
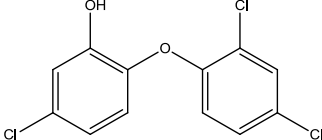
RE	Recovery
RSD	Relative standard deviation
SAC	Saccharin
SIM	Selected ion monitoring
SLE	Solid-liquid extraction
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
STV	Stevioside
SUC	Sucralose
TMSI	N-trimethylsilylimidazole
TOF	Time of flight
TRIS	Tris(hydroxymethyl) aminomethane
UHPLC	Ultra-high performance liquid chromatography
USE	Ultrasound-assisted solvent extraction
UV	Ultraviolet
VLDL	Very low density lipoprotein
w.w.	Wet weight
WWTP	Wastewater treatment plant

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

Name	Structure
Acesulfame potassium	
Alitame	
Aspartame	
Bezafibrate	
Clofibric acid	
Diatrizoic acid	
Diclofenac	

Name	Structure
Glycyrrhizic acid	
Ibuprofen	
Iohexol	
Iomeprol	
Iopamidol	

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Name	Structure
Iopromide	
Ketoprofen	
Naproxen	
Neohesperidine dihydrochalcone	
Neotame	
Salicylic acid	

Name	Structure
Sodium cyclamate	
Sodium saccharin	
Stevia	
Sucralose	
Triclosan	

Appendix III. List of publications.

M. Núñez, E. Pocurull, N. Fontanals, S. Knillmann, M. Liess, *Repeated toxicant exposition shapes the response of Gammarus pulex* Environ. Toxicol. Chem. (2017) (in preparation) (Section 3.3.1).

M. Núñez, F. Borrull, N. Fontanals, E. Pocurull, *Sample treatment for the determination of emerging organic contaminants in aquatic organisms*, Trends Anal. Chem. (2017) (submitted) (Section 1.4.1).

M. Núñez, F. Borrull, E. Pocurull, N. Fontanals, *Pressurised liquid extraction and liquid chromatography-high resolution mass spectrometry to determine high-intensity sweeteners in fish samples*, J.Chromatogr. A 1479 (2017) 32-39 (Section 3.2.2).

M. Núñez, F. Borrull, N. Fontanals, E. Pocurull, *Different sample treatments for the determination of ICM-XR in fish samples followed by LC-HRMS*, Talanta 163 (2017) 1-7 (Section 3.2.1).

M. Núñez, F. Borrull, E. Pocurull, N. Fontanals, *Pressurized liquid extraction followed by liquid chromatography with tandem mass spectrometry to determine pharmaceuticals in mussels*, J.Sep.Sci. 39 (2016) 741-747 (Section 3.1.1).

M. Núñez, F. Borrull, N. Fontanals, E. Pocurull, *Determination of pharmaceuticals in bivalves using QuEChERS extraction and liquid chromatography-tandem mass spectrometry*, Anal. Bioanal. Chem. 407 (2015) 3841- 3849 (Section 3.1.2).

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