Analytical approaches for quantification of emerging micropollutants in the Belgian coastal zone

Klaas Wille

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<td>limit of quantification</td>
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<td>molecularly imprinted polymer</td>
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<td>multiple reaction monitoring</td>
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<td>MRL</td>
<td>maximum residue level</td>
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<td>MSTFA</td>
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<td>MTBSTFA</td>
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<td>Na₄EDTA</td>
<td>tetrasodium ethylenediaminetetraacetate</td>
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<td>NP</td>
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<td>NSAID</td>
<td>nonsteroidal anti-inflammatory drugs</td>
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<td>OCP</td>
<td>organochlorine pesticide</td>
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<td>ONP</td>
<td>organonitrogen pesticide</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>OP</td>
<td>octylphenol</td>
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<td>OPP</td>
<td>organophosphorus pesticide</td>
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<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
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<td>PCB</td>
<td>polychlorinated biphenyl</td>
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<td>PCP</td>
<td>personal care product</td>
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<td>PEC</td>
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<td>PEEK</td>
<td>polyether ether ketone</td>
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<td>PEVAC</td>
<td>poly(ethylene-co-vinyl acetate-co-carbon monoxide)</td>
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<td>PFBS</td>
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<td>PFC</td>
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<td>PFDoA</td>
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<td>PFDS</td>
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<td>PFHxS</td>
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<td>PFOS</td>
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<td>PFPA</td>
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<td>PFTeA</td>
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<td>PFUnA</td>
<td>perfluoroundecanoic acid</td>
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<td>predicted no effect concentration</td>
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<td>POCIS</td>
<td>polar organic chemical integrative sampler</td>
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<td>POP</td>
<td>persistent organic pollutant</td>
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<td>PPCP</td>
<td>pharmaceuticals and personal care product</td>
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<td>PTFE</td>
<td>polytetrafluoroethylene</td>
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<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<td>QqQ-MS</td>
<td>triple quadrupole mass spectrometer</td>
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<td>QSAR</td>
<td>quantitative structure–activity relationship</td>
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<td>QToF</td>
<td>quadrupole/time-of-flight MS</td>
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<td>QTRAP/QLIT</td>
<td>quadrupole/linear ion trap MS</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RC</td>
<td>regenerated cellulose</td>
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<td>RCR</td>
<td>risk characterization ratio</td>
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<td>RSD</td>
<td>relative standard deviation</td>
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<td>RYA</td>
<td>recombinant yeast assay</td>
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<td>SALDI</td>
<td>surface-assisted laser desorption ionization</td>
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<td>SBSE</td>
<td>stir-bar sorptive extraction</td>
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<td>SDME</td>
<td>single-drop micro-extraction</td>
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<td>SFE</td>
<td>supercritical fluid extraction</td>
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<td>SIM</td>
<td>selected-ion-monitoring</td>
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<td>SLE</td>
<td>solid-liquid extraction</td>
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<td>solid-phase extraction</td>
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<td>SPME</td>
<td>solid-phase microextraction</td>
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<td>SRM</td>
<td>selected reaction monitoring</td>
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<td>TBA</td>
<td>tetrabutylammonium hydrogen sulphate</td>
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<td>TD</td>
<td>thermal desorption</td>
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<tr>
<td>ToF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>TWA</td>
<td>time-weighted average</td>
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<tr>
<td>U-HPLC</td>
<td>ultra-high performance liquid chromatography</td>
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<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WFD</td>
<td>water framework directive</td>
</tr>
<tr>
<td>WWTP</td>
<td>wastewater treatment plant</td>
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CHAPTER I

GENERAL INTRODUCTION: MASS SPECTROMETRIC ANALYSIS OF EMERGING POLLUTANTS IN THE AQUATIC ENVIRONMENT

Adapted from:

1. **INTRODUCTION**

The introduction of new and more sensitive analytical equipment for the detection of chemicals in complex sample matrices on the one hand and a growing knowledge about their ecotoxicological effects on the other hand has drawn the attention to new compounds, which have heretofore been largely outside the scope of monitoring and regulation. These so-called ‘chemicals of emerging concern’ (CECs) or ‘emerging contaminants’, were previously undetected or had not been considered as a risk [1-3]. The term emerging contaminants is however somewhat ambiguous since these contaminants are not necessarily new substances [4]. CECs encompass a diverse group of compounds, including algal and cyanobacterial toxins, brominated and organophosphate flame retardants, plasticizers, hormones and other endocrine disrupting compounds (EDCs), pharmaceuticals and personal care products (PPCPs), drugs of abuse and their metabolites, disinfection by-products, organometallics, nanomaterials, polar pesticides and their degradation/ transformation products, perfluorinated compounds (PFCs), and surfactants and their metabolites [5]. Both the high environmental distribution of CECs and their potential ecotoxicological effects at very low concentrations have found increasing interest among researchers, regulating authorities and the public [2,4].

In addition, the introduction of the European Reach Legislation has drawn the attention towards the emerging more polar anthropogenic pollutants. According to Hogenboom et al. [6], REACH will drive producers to develop newly designed chemicals that will be less persistent, bioaccumulative or toxic. Generally, these newly designed chemicals could be characterized as hydrophilic compounds, which may result in higher mobilities in the aquatic environment [6]. This shift in focus from persistent organic pollutants and heavy metals towards more polar CECs, has already become apparent in the scientific literature of the last decade on environmental chemistry [5,7].

Concentrations of CECs in aquatic systems are very low, typically in the ng L\(^{-1}\) concentration level up to the low µg L\(^{-1}\) range. Relatively low concentrations of CECs may be expected to occur in biotic matrices as well. Therefore, very sensitive analytical procedures are needed to obtain sufficiently low detection limits enabling measurement of environmental concentrations. In addition, biotic samples are complex matrices containing high amounts of possible interfering compounds demanding extensive extraction and clean-up procedures to obtain extracts amenable to analysis. As a result,
the reliable quantification of CECs in both aqueous and biological samples has appeared as a huge challenge to environmental analytical chemists. The possible solutions to encounter these challenges are reviewed within this work. In this context, state-of-the-art instrumentation for sample preconcentration, analyte separation and detection was discussed. PPCPs, pesticides, estrogenic compounds, alkyphenolethoxylates (APEOs), bisphenol A (BPA) and phthalates are contaminants of particular concern, as many of them exhibit endocrine-disrupting properties [8]. Therefore, the analytical chemistry of these groups of CECs was surveyed within this review. In addition, PFCs were also considered, since these bioaccumulative chemicals are known to be abundant in the aquatic environment, where they could exert possible adverse effects on human and wildlife [9].

2. EMERGING MICROPOLLUTANTS

2.1 Pharmaceuticals

Pharmaceuticals are the active ingredients of medicinal products extensively used in human and veterinary medicine [10]. The group of pharmaceuticals include approximately 3,000 different natural or synthetic chemicals with a large variation in chemical structure, function, mode of action and behaviour [11-13]. This wide group may be classified into different therapeutic classes. The most common classes are the antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs), \( \beta \)-blockers, lipid regulators, antiepileptic drugs, \( \beta \)-2-sympathomimetics, cancer therapeutics and psychiatric drugs. Unintentionally, the universal use of pharmaceuticals turned out to a relative new environmental concern: their introduction into the aquatic environment [14]. Typically, human pharmaceuticals are excreted, either in their native form or as metabolites, followed by release into the aquatic environment through the wastewater treatment network [13,15]. In addition, improper disposal and manufacturing processes could contribute to the overall pharmaceutical load in aquatic systems as well [16,17]. Furthermore, also veterinary pharmaceuticals are introduced in huge amounts into the aquatic environment, either directly via excretion of grazing animals and fish farms, or indirectly via land application of manure [18]. Because of their continuous release into the aquatic environment, pharmaceuticals are considered as “pseudopersistent compounds” [16]. Consequently, growing public and scientific concern has arisen
regarding the occurrence and potential effects of pharmaceuticals in the aquatic environment [19]. The chemical structures of the pharmaceuticals considered in this doctoral thesis are presented in Figure I.1.
**NSAIDs**

- Salicylic acid
- Paracetamol
- Mefenamic acid
- Ketoprofen
- Carprofen
- Diclofenac

**Lipid regulators**

- Clofibric acid
- Bezafibrate
- Pravastatin

**Antibiotics**

- Sulfamethoxazole
- Trimethoprim
- Chloramphenicol
- Ofloxacin

**β-blockers**

- Propranolol
- Atenolol

**Neuroactive compounds**

- Carbamazepine

**β2-agonists**

- Salbutamol

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**Figure I.1.** Chemical structures of the targeted pharmaceuticals within this doctoral thesis.
2.2 Personal care products

Personal care products (PCPs) are generally considered together with the pharmaceuticals as the group of pharmaceuticals and personal care products (PPCPs). However, unlike pharmaceuticals, PCPs are substances intended for external use on the human body [20]. PCPs constitute a diverse group of compounds which are used in soaps, lotions, toothpaste, fragrances, sunscreens, a.o. The main classes of PCPs include ultraviolet (UV) filtering compounds, insect repellents, fragrances, disinfectants and preservatives. The chemical structures of some well-known PCPs are shown in Figure I.2.

<table>
<thead>
<tr>
<th>Musk fragrances</th>
<th>UV filtering compounds</th>
<th>Insect repellents</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHTN (7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphtalene or tonalide)</td>
<td>Benzophenone-3</td>
<td>DEET (N,N-Diethyl-m-toluamide)</td>
</tr>
<tr>
<td>Musk ketone</td>
<td>Homomenthyl salicylate</td>
<td>Icaridin</td>
</tr>
<tr>
<td>Cyclopentadecanolide</td>
<td>Octocrylene</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preservatives</th>
<th>Disinfectants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parabens (general structure)</td>
<td>Triclosan</td>
</tr>
<tr>
<td>Triclocarban</td>
<td></td>
</tr>
</tbody>
</table>

Figure I.2. Chemical structures of some environmentally important PCPs.
PCPs are generally not subjected to metabolic alterations, resulting in the release of large quantities of unaltered compounds into the environment [20]. Consequently, in accordance with the pharmaceuticals, concern about PCPs is related to their presence in the aquatic environment and their potential to elicit adverse environmental effects [21].

### 2.3 Polar pesticides

Pesticides are substances or mixtures of substances aimed at preventing, destroying, repelling or mitigating pests. They comprise a large number of compounds that belong to many chemical structurally diverse groups, which may be divided based on functional groups in their molecular structure (e.g. inorganic, organonitrogen, organohalogen or organosulfur compounds), or based on their specific biological activity towards target species (e.g. insecticides, fungicides, herbicides, acaricides, etc.). Within these different classes, herbicides are the most frequently applied pesticides followed by insecticides, fungicides and others [22-24]. Pesticides are generally utilized for agricultural practices, but also for forestry, horticulture, or amenities (including highways, airports, railways, industrial sites, parks, public spaces, and sport grounds) [25,26]. Their worldwide use resulted in an increasing concern in two areas. Firstly, there is a general concern about the possible presence of residues of pesticides and their metabolites in foods, which have, in any stage of their growth or production cycle, been treated with pesticides [22]. Secondly, land application of pesticides and subsequent drift, run-off, drainage, and leaching has lead to the contamination of the aquatic environment, with resulting potential toxic and harmful effects to non-target aquatic organisms [27,28]. A large group of pesticides are also considered as EDCs [29].

### 2.4 Endocrine disrupting compounds

According to the US Environmental Protection Agency (EPA), an environmental EDC is defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour [30]. Typical representative endocrine disruptors are natural and synthetic hormones, such as estrone (E1), 17β-estradiol (βE2), 17α-estradiol (αE2), estriol (E3), mestranol (MeEE2) and the synthetic contraceptive 17α-ethynylestradiol (EE2) [31]. The group of EDCs includes also phyto-estrogens, plant-derived estrogenic substances, which are believed to
induce sexual disruption in aquatic life, albeit with reduced potency as compared to the natural and synthetic hormones mentioned above [32]. Furthermore, a large group of man-made CECs act like EDCs as well: APEOs, metabolites and derivates used as surfactants (e.g. 4-nonylphenol); BPA which is particularly utilized in the manufacture of polycarbonate plastics and epoxy resins [31]; PPCPs (section 2.1 and 2.2), pesticides (section 2.3), phthalates (section 2.5), a.o. The chemical structures of several environmentally important EDCs are demonstrated in Figure I.3.

**Figure I.3. Chemical structures of environmentally important EDCs.**

In recent years, substantial scientific efforts have been made to evaluate the consequences of the presence of EDCs in the environment, since biological effects such as feminization, infertility and hermaphroditism in aquatic organisms and enlargement of breasts, infertility and increased rates of testicular cancer in humans have been reported [33,34].

### 2.5 Phthalates

Phthalates or di-esters of 1,2-benzenedicarboxylic acid (Figure I.3) are industrial chemicals that are mainly used as non-reactive additives in plastics, particularly polyvinyl chloride, but also in rubber, cellulose and styrene production, to improve their softness and flexibility [35]. Other applications include their use in building materials, home improvement products, PCPs, and a variety of other consumer products [36]. Phthalates are released into the environment through losses during production processes or indirectly by leaching from materials, because they are not chemically bound to the polymeric matrix [37]. Because of their properties, their high production volumes and widespread
application, phthalates are ubiquitous in the environment and have been widely detected in food, air, water, soil and sediments [35]. Within the group of phthalates, di(2-ethylhexyl)phthalate (DEHP) and n-dibutylphthalate (DBP) have been reported to be the most ubiquitous phthalates in environmental samples [38]. Since phthalates are often mentioned as suspected endocrine disruptors, increased scientific attention has been granted to these CECs [35,39].

2.6 Perfluorinated compounds

Perfluorinated compounds (PFCs) comprise a diverse group of chemicals characterized by a fluorinated hydrophobic carbon chain substituted with various hydrophilic functional groups. The hydrophobic carbon chain may vary in chain length, is fully or partially fluorinated, and may be linear or branched [40,41]. Within the group of PFCs, the perfluoroalkyl carboxylates (PFCAs) and perfluoroalkyl sulfonates (PFSAs) are the most intensively studied, with perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) as the pollutants of greatest concern [40]. Because of their chemical and thermal stability and their specific surfactant properties, PFCs are used in a great number of industrial and consumer applications and products: as additives in fire-fighting foam and food packaging, as fat and water repellents for textile, paper and leather treatment, as performance chemicals, and as polymerization aid for the production of fluorinated polymers such as polytetrafluoroethylene (PTFE) and polyvinylidene fluoride (PVDF) [40,42]. In addition, PFCs are known to be stable to heat, acids and bases, and to reducing and oxidizing agents. Consequently, PFCs have been reported to be extremely persistent environmental contaminants with bioaccumulative and toxic properties [43-45]. Therefore, the concern about the environmental fate and prevalence of PFCs has been recognized as an emerging issue in environmental chemistry. Moreover, PFOS is recently included in the Annex B list of the Stockholm Convention on Persistent Organic Pollutants (POPs), which is a global treaty to protect human health and the environment from chemicals that remain intact in the environment for long periods, become widely distributed geographically, accumulate in the fatty tissue of humans and wildlife, and have adverse effects to human health or to the environment, [325,326]. The chemical structures of the PFCs considered in this doctoral work are shown in Figure I.4.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Structural formula</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Perfluoroalkyl sulfonates</strong></td>
<td>PFASs</td>
<td></td>
<td><img src="image" alt="PFASs" /></td>
</tr>
<tr>
<td>Perfluorobutane sulfonate</td>
<td>PFBS</td>
<td>C₄F₉SO₃</td>
<td>n = 3</td>
</tr>
<tr>
<td>Perfluorohexane sulfonate</td>
<td>PFHxS</td>
<td>C₆F₁₃SO₃</td>
<td>n = 5</td>
</tr>
<tr>
<td>Perfluoroctane sulfonate</td>
<td>PFOS</td>
<td>C₈F₁₇SO₃</td>
<td>n = 7</td>
</tr>
<tr>
<td>Perfluorodecane sulfonate</td>
<td>PFDS</td>
<td>C₁₀F₂₁SO₃</td>
<td>n = 9</td>
</tr>
<tr>
<td><strong>Perfluoroalkyl carboxylic acids</strong></td>
<td>PFCAs</td>
<td></td>
<td><img src="image" alt="PFCAs" /></td>
</tr>
<tr>
<td>Perfluoropentanoic acid</td>
<td>PFPA</td>
<td>C₅HF₉O₂</td>
<td>n = 3</td>
</tr>
<tr>
<td>Perfluorohexanoic acid</td>
<td>PFHxA</td>
<td>C₆HF₁₁O₂</td>
<td>n = 4</td>
</tr>
<tr>
<td>Perfluorohexanoic acid</td>
<td>PFHpA</td>
<td>C₇HF₁₃O₂</td>
<td>n = 5</td>
</tr>
<tr>
<td>Perfluoroctanoic acid</td>
<td>PFOA</td>
<td>C₈HF₁₅O₂</td>
<td>n = 6</td>
</tr>
<tr>
<td>Perfluorononanoic acid</td>
<td>PFNA</td>
<td>C₉HF₁₇O₂</td>
<td>n = 7</td>
</tr>
<tr>
<td>Perfluorodecanoic acid</td>
<td>PFDA</td>
<td>C₁₀HF₁₉O₂</td>
<td>n = 8</td>
</tr>
<tr>
<td>Perfluoroundecanoic acid</td>
<td>PFUnA</td>
<td>C₁₁HF₂₁O₂</td>
<td>n = 9</td>
</tr>
<tr>
<td>Perfluorododecanoic acid</td>
<td>PFDaA</td>
<td>C₁₂HF₂₃O₂</td>
<td>n = 10</td>
</tr>
<tr>
<td>Perfluorotetradecanoic acid</td>
<td>PFTeA</td>
<td>C₁₄HF₂₅O₂</td>
<td>n = 12</td>
</tr>
<tr>
<td><strong>Perfluoroctane sulfonamide</strong></td>
<td>PFOSA</td>
<td>C₈H₂F₁₇SO₂N</td>
<td></td>
</tr>
</tbody>
</table>

Figure I.4. Chemical structures of the targeted PFCs within this doctoral thesis.
3. ENVIRONMENTAL CHEMISTRY

In recent years, advances in instrumentation have resulted in a significant progress in the
detection of CECs in environmental matrices. Within this review, it was not our objective
to give a complete compilation of papers dealing with analysis of CECs. Instead, it was
our purpose to discuss the current performance in quantifying CECs in the aquatic
environment and highlight some recent advances.

3.1 Pharmaceuticals

3.1.1 Water analysis

Overall, hundreds of papers have been published on pharmaceutical analysis of non-
treated and treated waters [46]. Still, it has taken until 2007 for the US EPA to publish the
EPA Method 1694 [47], a standardized methodology for the analysis of more than 70
pharmaceuticals in environmental matrices. Recently, this standard protocol was
improved by Ferrer et al. [46]. Within both methodologies, the target pharmaceuticals are
divided in several subgroups of compounds, each with their specific optimized analytical
procedure. As a result of their typical physicochemical properties and chemical structures,
this division into smaller subgroups is required. Consequently, the development of
multiclass methods, which is the general trend in recent years, demands a compromise in
the selection of experimental conditions such as sample preparation, separation and
detection [5,48]. Nevertheless, a typical procedure for the analysis of a broad group of
pharmaceuticals in aqueous matrices has been reported in literature and is shown in
Figure 1.5 [49]. This procedure included filtration and acidification for acidic
pharmaceuticals, extraction, if necessary an additional clean-up step, derivatization in
case of detection with gas chromatography (GC) and finally detection with GC or liquid
chromatography (LC) in combination with mass spectrometry (MS).
Until 2007, all studies reporting on procedures for pharmaceutical analysis have been thoroughly reviewed by Fatta et al. [49] and Kot-Wasik et al. [50]. Within recent years, there has been a tremendous progress in analytical techniques for trace analysis in environmental samples [51]. Therefore, the most recent studies on pharmaceutical analysis, from sample preparation to analyte separation and detection, are discussed below.

At first, filtration of water samples was suggested prior to the concentration procedure, to remove particulate matter and to avoid clogging of the sorbent used for solid-phase extraction (SPE) [51-53]. However, filtration removes together with suspended solids also the fraction of target compounds sorbed to particulates [54]. Therefore, it was recommended to wash the glass fiber filters with methanol after filtration [55]. Centrifugation of wastewater samples was performed as well in case of observable suspended particulate matter [56]. Before sample extraction, the pH of the samples was
adjusted or reagents may be added to optimize the extraction efficiency. Studies suggested to adjust the pH to acidic, basic or neutral conditions, depending on the analyte [47,55,57,58]. In addition, chelating agents (e.g. di- or tetrasodium ethylenediaminetetraacetate: Na₂EDTA or Na₄EDTA), quenching agents (e.g. ascorbic acid) and other preservatives could be added to samples prior to extraction [47,59-61]. Further sample preparation and clean-up is necessary for three main reasons: to remove interferences which would otherwise affect the determination of analytes, to concentrate analytes to detectable concentrations, and to perform solvent switching to the desired solvent conditions used for detection. Up to date, SPE is still the most frequently applied sample preparation technique. Using SPE, improved retention of pharmaceuticals was obtained by the development of new polymeric sorbents, mostly hydrophilic-hydrophobic balanced material. The copolymer of divinylbenzene and vinylpyrrolidone, better known as Oasis HLB, is currently the most commonly used SPE-sorbent for extraction of multiclass pharmaceuticals, next to the copolymer sorbents of Isolute ENV⁺, Strata-X and Chromabond HR-X [51,55,62]. Sorbents of sol-gels and carbon nanotubes are used less frequently [63]. SPE has generally been performed offline, thus prior to separation and detection of pharmaceuticals. Currently, on-line SPE is emerging as an effective technique, either coupled online with an LC-system or as a fully automated system [51,64-66]. According to Trenholm et al. [66], online systems provide a rapid, sensitive and robust alternative method to traditional off-line SPE, demanding smaller solvent volumes. Drawbacks of online systems are the capital cost for commercial systems, the limited sample size resulting in higher detection limits and the possible contamination of the SPE cartridge [51,62,66].

A promising extraction and clean-up technique for pharmaceuticals in environmental matrices is the use of molecularly imprinted polymers (MIPs) [62]. Due to specific cavities designed for a template molecule, MIP sorbents provide an increased selectivity and specificity for target analytes [67]. Consequently, the level of co-extracted matrix compounds is reduced, which leads to less matrix effects and a better sensitivity [68]. The use of MIPs as a selective sorbent in a SPE procedure (MISPE) has been successfully applied for several therapeutic classes [69-71]. However, the sorbent material must be custom-made and multiclass analysis is not achievable using this technique [62]. Two other approaches enabling extraction, clean-up and concentration of pharmaceuticals in aqueous samples simultaneously, are solid-phase (SPME) and liquid-phase (LPME)
microextraction. These techniques have been frequently applied for extraction of pharmaceuticals from aqueous samples [72-75]. A reduction in processing time, labour, costs and matrix effects is achieved using SPME and LPME, however, the sensitivity and precision tend not to be as good as the commonly used SPE techniques [62,72]. Stir-bar sorptive extraction (SBSE), a related technique of SPME which is usually followed by liquid or thermal desorption (LD or TD) in combination with GC, has recently been applied for detection of ibuprofen, naproxen and ketoprofen [76].

Even with advanced detectors such as high resolution or triple quadrupole mass spectrometers (QqQ-MS), good chromatographic separation is desired for the quantification of pharmaceuticals in environmental matrices down to ng L$^{-1}$ concentration levels [62]. GC is the preferred technique for separation of non-polar and volatile pharmaceuticals, while for GC-analysis of the more polar pharmaceuticals a derivatization step is required using typical derivatizing agents such as acid anhydrides, benzyl halides, alkylchloroformates, diazomethane and silylating reagents including N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) or N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) [19,51,77]. Despite the benefits of GC with respect to selectivity and sensitivity, the loss of analytes during the time-consuming derivatization process and the background noise are a matter of concern [19,62,78]. Therefore, in recent years LC has become the preferred technique for multiclass pharmaceutical analysis [19,51,62]. Current LC analysis use reversed-phase columns and mobile phases consisting of acetonitrile, methanol and water. Generally, solvent modifiers in the form of proton acceptors (e.g. ammonium acetate) and/or proton donors (e.g. formic acid) are added to the mobile phase to enhance ionisation efficiencies of basic and acidic pharmaceuticals, respectively [48]. The main negative aspect for LC analysis of pharmaceuticals in environmental matrices, is the occurrence of matrix effects. Due to co-extracted matrix constituents, the mass spectrometric analysis may suffer from signal suppression or enhancement, thereby disturbing adequate quantification [79]. Nowadays, the development of ultra-high performance liquid chromatography (U-HPLC) enabled faster separation of compounds in comparison to conventional LC, due to the use of columns packed with sub-2 µm particles. U-HPLC provides improved speed of analysis, as well as a better resolution, an increased sensitivity and a reduction of matrix effects [56,57,62]. In general, considerably improved separation of pharmaceuticals in complex matrices could be obtained using U-HPLC.
An alternative to chromatographic procedures is the use of capillary electrophoresis (CE). The importance of this technique is somewhat limited, due to the typically lower sensitivity using CE, even in combination with mass spectrometric detection techniques [80]. Nevertheless, recent studies have demonstrated the applicability of CE and its related technique based on electrokinetic supercharging (EKS) for pharmaceutical analysis [81,82].

Identification and quantification of pharmaceuticals in environmental matrices is usually performed by mass spectrometric techniques. The most common interface for pharmaceutical analysis in environmental matrices is the electrospray ionization (ESI) source [51,62,83]. In particular in case of complex environmental samples, the ESI efficiency can be affected by the co-extracted sample components, resulting in ionization suppression or enhancement effects and subsequent poor analytical accuracy and reproducibility [83]. These effects can be reduced by extensive clean-up procedures prior to LC-MS analysis, improved chromatographic separation and by dilution of the final extract. However, the most common technique is the compensation of matrix effects by the use of isotope-labelled internal standards, enabling reliable quantification [78,83]. Nevertheless, evaluation of matrix effects is usually included in the validation study of new analytical approaches for pharmaceutical analysis using ESI [46,48,52]. The second “soft” ionization technique atmospheric pressure chemical ionization (APCI) has been used less often for pharmaceutical analysis. APCI has been reported to be less susceptible to matrix effects, however, generally minor sensitivity is obtained as compared to ESI [62,83].

With respect to MS, the most commonly applied mass analyzers for pharmaceutical analysis are the QqQ mass detectors, providing precise quantification and high selectivity and sensitivity [52,60,62]. Using QqQ technology, typical limits of detection are in the low ng L⁻¹ concentration range [48,57,62,73]. Concerning the performance and confirmation criteria for residues in complex matrices, the European Union Commission Decision 2002/657/EC was established, including a system of identification points [84]. An ion (or precursor ion) contributes one point, and each multiple reaction monitoring (MRM) product ion yields 1.5 point [84]. Using QqQ, the minimum of four identification points is accomplished easily, by comparing two MRM precursor-to-product ion transitions [62]. Also ion trap mass analyzers have been reported in literature as excellent detection apparatus for pharmaceutical analysis [55,85-87]. Using ion trap instruments, a
minimum of four identification points is obtained by detection of the precursor ion, as well as at least two product ions. Recent advances in instrumentation resulted in a gain in popularity of the high-resolution full scan analysis. Time-of-flight (ToF) and Orbitrap-based MS proved to be very suitable alternatives to triple quadrupole instruments, enabling the accurate mass screening of a virtually unlimited number of analytes, being targeted as well as untargeted compounds [88]. Thanks to the high mass resolution, advantages of these instruments include increased selectivity and reduced false positives. In addition, the full scan data permits retrospective analysis of an unlimited number of pharmaceuticals [62,89]. So far, the use of single ToF and Orbitrap instruments for analysis of pharmaceuticals in environmental matrices is rather limited [89,90]. More common, the combination of two mass spectrometric techniques with complementary features has been used. These highly sophisticated MS analyzers or so-called hybrid tandem mass spectrometers constitute the latest trend in environmental chemistry, increasing the instruments versatility and the scope of the method [89]. In addition, sensitivities approaching that of QqQ systems are offered using these hybrid MS technology [62]. The hybrid systems that have been used for pharmaceutical analysis, include triple quadrupole/linear ion trap MS (QTRAP or QLIT) [59,64,72,89,91-93], quadrupole/time-of-flight MS (QToF) [94-97], and ion trap/Orbitrap MS [6]. Criteria within CD 2002/657/EC [84] related to the reliable identification and confirmation of pharmaceuticals using high-resolution MS systems are however incomplete. Therefore, additional criteria to be implemented were suggested by Nielen et al. [98]. In general, very high mass accuracy (< 5ppm) is offered by modern ToF and Orbitrap systems, providing reliable identification and quantification of pharmaceuticals in environmental matrices [90,96].

Mass spectrometric techniques are applied upon gas chromatographic separation of the pharmaceuticals as well. GC-MS is typically performed using electron impact (EI) ionization, in full scan mode for identification and in selected-ion-monitoring (SIM) mode for quantification purposes. Limits of quantification (LOQs) in the low ng L\(^{-1}\) concentration range have been obtained, and no obvious matrix effects were observed using GC-MS [53,77,78].

Less expensive detection techniques, as compared to MS, include the use of fluorescence detection (FLD), ultraviolet (UV) detection, diode array detection (DAD) and immunoanalytical techniques. Several recent studies have reported the use of these
techniques for pharmaceutical analysis in aqueous matrices [70,74,99-101]. Generally, rather low sensitivities are obtained using these techniques, limiting their use for aqueous matrices containing high amounts of pharmaceuticals such as wastewaters.

3.1.2 **Sediment analysis**

Despite the rather low lipophilicity of pharmaceuticals, interaction of the polar functional groups of pharmaceuticals with organic matter and/or minerals may result in adsorption to solids [53]. Furthermore, the application of sewage sludge as a fertilizer to agricultural land and the reuse of manure containing veterinary medicines may lead to the introduction of pharmaceuticals into the soil as well [51,102]. Therefore, in accordance with aqueous samples, the presence of pharmaceuticals in sediment, soil and sewage sludge has been studied extensively. Analytical methods for the determination of specific groups of pharmaceuticals including NSAIDs [103], anti-depressants [104], antibiotics [105] and β-blockers [106], as well as multiclass methods [92,102] have been reported in literature in recent years. The main difference with water analysis is related to the sample preparation and extraction part. Generally, pharmaceuticals are extracted from dried solid samples by conventional Soxhlet extraction [107], microwave-assisted (micellar) extraction (MA(M)E) [108,109], ultrasonic extraction [53,78,110,111], supercritical fluid extraction (SFE) [103], or pressurized liquid extraction (PLE) [92,102,112]. Nowadays, Soxhlet extraction has become less attractive due to its time- and solvent-consuming properties [51]. All five techniques were compared for the determination of four NSAIDs in river sediment [103]. Based on extraction efficiencies, average solvent consumption and extraction time, MAE was found to be the most effective extraction method. However, the most commonly used approaches seem to be ultrasonic extraction and PLE, providing good extraction efficiencies and demanding less extraction time and solvents [51,113]. Typically, mixtures of water and rather polar solvents (e.g. methanol and acetonitril) are used for achieving good recoveries during extraction [78,92,111]. An additional clean-up step by means of SPE is usually required. Finally, analysis of extracts is performed using the techniques described in the previous section [53,107,112].
3.1.3 Biota analysis

The presence of pharmaceuticals in aquatic organisms has been rarely studied, due to both the complexity of the matrix and the levels at which pharmaceuticals occur. Ramirez et al. [114] reported a screening method for the detection of 23 pharmaceuticals in fish tissue, based on extraction using a 1:1 mixture of 0.1 M aqueous acetic acid and methanol before analysis using LC-MS/MS. Another study described the use of MAME and SPE followed by UV-DAD of six pharmaceuticals in molluscs [108]. Four tetracycline antibiotics could be determined in fish muscle by SPME coupled to HPLC-photodiode array detector [115]. More recent, PLE and SPE followed by U-HPLC coupled to QqQ MS enabled the quantification of 11 pharmaceuticals in tissue of marine organisms [116]. To the best of our knowledge, no more attention has been paid to the analysis of pharmaceuticals in aquatic organisms. To obtain more information regarding their presence in marine organisms, an increasing demand exists for reliable analytical methods allowing the quantification of these micropollutants in biotic matrices [79].

3.1.4 Passive samplers

The conventional techniques of sampling, based on the collection of discrete grab or spot samples of water, are used in most aquatic monitoring programmes [117]. Although these conventional sampling techniques are very useful, generally, the determination of time-weighted average (TWA) concentrations over extended sampling periods of pollutants in the aquatic environment is not possible. As a result, the use of passive samplers, which are designed to obtain TWA concentrations, has gained in popularity. In addition, these techniques mimic biological uptake in a more straightforward manner by determining the pollution level of contaminants in relation to their freely dissolved concentration [118,119]. With respect to pharmaceuticals, the use of passive sampling devices such as polar organic chemical integrative samplers (POCISs; see Figure I.6) and Chemcatcher® passive samplers have been recently reported in literature [91,120-122]. Also poly(ethylene-co-vinyl acetate-co-carbon monoxide) (PEVAC) and polydimethylsiloxane (PDMS) has been used as passive sampling material for pharmaceuticals in the aquatic environment [90,119]. However, an important negative aspect of the passive sampler approach involves the need for laboratory calibration studies to enable correct concentration calculations [120].
After exposure, the passive sampler devices are usually extracted using polar organic solvents, such as methanol, acetone, acetonitrile, ethyl acetate and mixtures [91,119-122]. Upon evaporation to dryness, analysis could be performed by routine GC-MS or LC-MS methods [120-122]. Additionally, the use of QToF-MS, QLIT-MS and Orbitrap-MS for analysis of pharmaceuticals in passive sampler extracts has recently been reported in literature as well [90,91,119]. The suitability of high-resolution full scan analytical techniques for this application is high, since these MS instruments enable the examination of the presence in the sampler extracts of an infinite number of analytes, targeted as well as non-a priori selected pharmaceuticals.

![Diagram of POCIS](image)

**Figure I.6. Exploded view of a POCIS showing the sorbent layer contained between two membrane disks sandwiched between two support rings [324].**

### 3.2 Personal care products

In general, the methodologies for PCP analysis in environmental samples are quite similar as those described for pharmaceuticals [51]. Therefore, only a shortened discussion was provided within this study. The typical applications are discussed for each group of PCPs.

#### 3.2.1 Water analysis

##### 3.2.1.1 UV filtering compounds

UV absorbing compounds are increasingly applied as a result of a growing concern about UV radiation and skin cancer. This group includes benzophenones, salicylates,
cinnamates, camphor derivates, triazines, benzotriazoles, benzimidazole derivates, dybenzoyl methane derivates and others [123]. Sorptive extraction in combination with GC-analysis seems to be the proper analytical technology for determination of hydrophobic and volatile UV filtering compounds in water samples [51]. Recent studies have reported the use of SBSE for the extraction of PCPs from aqueous samples followed by TD-GC-MS [124,125] or LD-(U-HPLC)-MS [126]. SBSE followed by direct analysis in real-time mass spectrometry (DART-MS) provided a shorter analysis time, but detection limits are poorer and only semi-quantitative results can be obtained [127]. Other analytical approaches for PCP determination include microextraction by packed sorbent (MEPS) coupled directly to large volume injection-GC-MS [128], SPME with on-fibre derivatization by silylation followed by GC–MS/MS analysis [129] and SPE with GC-MS [130]. With respect to the benzophenones, derivatization of phenolic groups was needed to enable their detection in the low ng L⁻¹ range [129]. All these GC methods are limited to those compounds that are volatile or can be derivatized for GC determination. If multiclass determination of UV filtering compounds in environmental samples is required, (U-)HPLC coupled to QqQ-MS is usually applied after a SPE step [126,131-133].

3.2.1.2 Insect repellents

The group of insect repellents consists of chemically diverse substances [134]. N,N-diethyl-m-toluamide (DEET) and 1-piperidinecarboxylic acid 2-(2-hydroxyethyl) 1-methylpropyl ester (called Icaridin) have been reported as the most important analytes within this group [51]. These compounds have generally been analyzed with GC-MS [135]. Rodil and Moeder [134] enabled the detection of 8 insect repellents by SBSE followed by TD-GC-MS. However, several studies used SPE with subsequent LC-MS analysis as well [136,137]. Recently, SPE followed by LC–MS/MS using ESI in positive ion mode allowed the determination of 4 insect repellents obtaining limits of detection (LODs) in the low ng L⁻¹ concentration range [132].

3.2.1.3 Fragrances

The group of fragrances can be divided into nitro-aromatic musks, polycyclic musks and macrocyclic musks. Compounds of the nitro-aromatic and polycyclic musk classes are considered as ubiquitous, persistent and bioaccumulative pollutants, with the potential to generate toxicologically active compounds [138]. Recently, Bester [139] reviewed the
analytical approaches which have been used for the determination of fragrances. The use of SPE as sample preparation technique has been reported only occasionally [140,141]. Due to their hydrophobic, volatile and lipophilic properties, other extraction techniques clearly benefit above SPE [139]. Liquid/liquid extraction (LLE) has been applied for extraction of fragrances [142,143] as well as SBSE with LD [138] and microwave-assisted headspace SPME [144]. Typically, good chromatographic separation is obtained by GC, commonly followed by mass spectrometric detection [139].

3.2.1.4 Preservatives

The most common preservatives are the P-hydroxybenzoic esters or parabens, which are used in PCPs but also as preservatives in pharmaceuticals and food products. Typically, the high usage and low degradability of these compounds may lead to their prevalence in the environment, thereby exhibiting estrogenic activities [135,145]. Therefore, parabens have been typically analyzed together with other EDCs using SPE followed by LC-MS or GC-MS [146,147]. So far, little efforts have been made for the separate determination of this group of compounds in environmental matrices.

3.2.1.5 Disinfectants

The main compounds within the group of disinfectants are triclosan and triclocarban, which are often used in soap, toothpaste, and other consumer products. Especially triclosan has been frequently analysed as a marker compound in many studies. The different analytical approaches applied for this compound were reviewed by Peck [135]. Obviously, the combination of LLE or SPE with GC-MS has been routinely applied for detection of triclosan in aqueous matrices, while some studies report the use of SPE followed by LC-MS as well. Both the use of various derivatization procedures and the use of LC methods have been suggested to improve the analytical performance upon analysis of triclosan [51].

3.2.1.6 Multiclass methods

As stated before, in recent years a trend may be observed towards multi-residue methods [5]. Modern analytical equipment enables the determination of high numbers of analytes within one analytical run. With respect to PCPs, several analytical methods have been reported on the simultaneous determination of several groups of PCPs. Recently the simultaneous determination of preservatives and disinfectants in water by LC-MS/MS
was reported by Gonzalez-Marino [148], while Cuderman and Heath [149] described the determination of UV filters and antimicrobial agents or disinfectants in environmental water samples using SPE with GC-MS. Upon SPE using two polymeric cartridges (Oasis HLB and Bond Elut Plexa), a U-HPLC–(ESI)-MS/MS run of 9 minutes was developed for the simultaneous determination of 4 preservatives, 2 antimicrobial agents and 5 UV filters by Pedrouzo et al. [126]. Furthermore, analytical methods for PCPs have been regularly performed in combination with pharmaceuticals and EDCs. A selection of 9 important pharmaceuticals and PCPs were simultaneously extracted by SPE using Oasis HLB cartridges with subsequent GC-MS detection [150]. Another comprehensive approach was reported by Guitart and Readman [151], who described a method for the determination of some PPCPs, phenolic EDCs and steroids by SPE using Oasis HLB cartridges followed by GC-MS, obtaining detection limits in the low ng L⁻¹ concentration range for all compounds.

3.2.2 Sediment analysis

Several PCPs (e.g., triclosan, triclocarban, most UV-filtering compounds) show affinity to solid matrices such as sediment, SPM and sewage sludge. As a consequence, to allow a correct evaluation of the ecological impact of these substances, the evaluation of their prevalence in solid matrices is important. Therefore, several analytical approaches have been reported in recent literature. Rodil et al. [152] developed a methodology for the determination of UV filtering compounds based on the use of non-porous polymeric membranes in combination with PLE followed by detection with LC–atmospheric pressure photoionisation (APPI)–MS/MS. Fast determination of synthetic polycyclic musks in sewage sludge and sediment was enabled by microwave-assisted headspace SPME followed by GC-MS [153]. In accordance with the analysis of aqueous samples, multiresidue methods are gaining in popularity for sediment analysis as well. A new analytical strategy for the determination of UV filtering compounds, four preservatives and two antimicrobials in sewage sludge was reported by Nieto et al. [154]. The combination of PLE and U-HPLC-MS/MS resulted in detection limits below 10 ng g⁻¹. More recently, a sensitive method has been developed and validated for the determination of diverse groups of hormone-like PPCPs and steroid hormones in sewage sludge as well [155]. Sample extraction was performed by ultrasonic-assisted extraction followed by SPE and analysis with U-HPLC-MS/MS.
3.2.3 Biota analysis

It has been demonstrated that environmental exposure to pseudopersistent PCPs resulted in accumulation of the parent compounds, their metabolites, or both in tissues of aquatic organisms [156]. Balmer et al. [157] demonstrated low but detectable concentrations of UV filtering compounds in fish, obtained upon extraction of 20 g samples with dichloromethane/cyclohexane (1:1) followed by gel-permeation chromatography (GPC) and GC-MS analysis. More recently, Zenker et al. [158] reported an analytical strategy for the determination of UV filtering compounds in fish. One gram of fish tissue was suspended in 5 mL of methanol and 5 mL of acetonitrile. Next, the mixture was homogenized, centrifuged, sonicated and filtrated before LC–ESI-MS/MS analysis. Nakata et al. [159] reported an analytical method for analysis of synthetic musks in fish. Briefly, sample tissues were extracted with dichloromethane/hexane (8:1) using a Soxhlet apparatus, before GPC and GC-MS analysis. In general, analytical methods for the determination of PCPs in biotic tissue are limited in scope. However, two analytical methods have been developed for the determination of a group of ten extensively used PCPs in fish [156]. The methods consisted of extraction with acetone, clean-up with silica gel, GPC and derivatization before analysis by GC–SIM–MS or GC–MS/MS techniques.

3.3 Pesticides

Within this study, only those analytical techniques developed for environmentally prevalent more polar pesticides were considered. The chemical classification of these modern pesticides is quite complex, since they are characterized by such a variety of chemical structures and functional groups [24]. The most relevant groups of polar pesticides include the organonitrogen pesticides (ONPs) and the organophosphorus pesticides (OPPs), both covering a wide group of compounds. These pesticides are universally applied and have replaced the withdrawn group of the organochlorine pesticides (e.g. chlordane, dieldrin, DDT) [5]. In literature, numerous studies have been conducted on the analysis of pesticide-residues in environmental matrices [24]. Still, the determination of modern pesticides and their degradation products at very low concentration levels in real samples is complicated because of their polarity and thermolability. In addition, there is a need to significantly improve the sensitivity and selectivity of the analytical methodologies to meet the requirements established in Directive 98/83/EC regarding water for human consumption which has to comply with
the 100 ng L\(^{-1}\) legislation concentration for individual pesticides [160,161]. Therefore, an overview of the various approaches and recent trends used for determination of the more polar pesticides in environmental samples is given below. The pesticides considered within this doctoral work are shown in Figure I.7.

![Chemical structures of the targeted pesticides within this doctoral thesis.](image)

3.3.1 Water analysis

To remove suspended particulate matter and sediment, it was suggested to first filtrate or centrifuge water samples [162,163]. Jansson and Kreuger [162] evaluated nine different filter materials prior to analysis of 95 pesticides in surface water. Some pesticides were completely adsorbed upon filtration, while the highest recoveries were obtained using
PTFE and regenerated cellulose (RC) filters. On the other hand, as much studies reported analytical approaches without this previous treatment step [164,165]. In addition, the pH of the water samples was only rarely adjusted [162]. Regarding the extraction and enrichment of pesticides, several strategies have been reported. The use of conventional LLE has been recommended by the US EPA because of the high degree of enrichment [166,167]. The main drawbacks of LLE include low extraction efficiencies for polar compounds, the time-consuming aspect, the need for large quantities of solvents and for further clean-up, and the risk for emulsion forming during agitation [168]. To eliminate some of these inconveniences, LLE has been replaced by miniaturized liquid extraction procedures such as LPME [169], single-drop microextraction (SDME) [170] and membrane-assisted solvent extraction [171,172]. However, the most popular extraction technique is, in accordance with other polar micropollutants, the use of SPE, combining both isolation and enrichment of the target compounds [167]. A wide range of commercially available SPE sorbents have been applied, with the polymer-based OASIS HLB as the most distributed adsorbent [96,173-176]. The automated approach, namely online SPE, has been recently applied for extraction of pesticides as well [27,162,164,177]. The typical scheme of a fully automated online SPE system (with subsequent LC-MS/MS detection) is shown in Figure I.8. Commonly used online enrichment cartridges are PLRP-S columns, consisting of stable polymeric reversed phase material [27,164]. SBSE, SPME and MIPs have been employed only sporadically for pesticide extraction [161,179-182]. Finally, the use of multiwalled carbon nanotubes as solid-phase extractant have been reported once, obtaining LODs in the µg L⁻¹ range [183].
In accordance with pharmaceutical analysis, both LC and GC have been applied for the separation of pesticides. Nowadays, the tendency towards the use of more polar, less volatile and less thermostable pesticides has stimulated the application of LC in pesticide residue analysis. In addition, the introduction of U-HPLC has led to higher chromatographic resolutions and shorter analysis times [163]. On the other hand, high usage pesticides are still volatile and thermostable, and consequently GC amenable [184,185]. Therefore, GC-based analytical methods for pesticide analysis are these days still reported in literature [165,184,186].

Most recently published analytical approaches for the determination of pesticides in environmental matrices rely on detection with MS. Both APCI and ESI have been applied as ionization sources, with generally much wider applications using ESI [173,184]. Upon ionization, QqQ technology is the most frequently applied MS-tool for quantitative pesticide analysis in aqueous matrices [27,163,165,187]. The MS-MS fragmentation pattern is a powerful tool for reliable compound identification, thereby offering high sensitivity and selectivity [188]. In general, the lowest LODs, ranging from the pg L\textsuperscript{-1} to
ng L\textsuperscript{-1} concentration level, were achieved by online SPE coupled to LC–MS/MS using a QqQ instrument [27,173,189]. The combination of quadrupole with linear ion trap technology (QTRAP) has been repeatedly used as well, providing excellent sensitivity and selectivity for pesticide analysis [89,91,161,164,190]. Since QqQ and ion trap systems operate at unit resolution, their capability for analysis of non-target screening of pesticides and retrospective analysis is limited [191]. To obtain more information on the actual pesticide composition in water samples, including target as well as untargeted substances, the application of full scan high resolution MS instruments for screening purposes has gained widespread acceptance [186]. The use of ToF and QToF instruments for both pesticide screening and quantification has been frequently reported in recent literature, providing reliable accurate mass measurements and high sensitivity in full scan mode [96,175,176,182,186]. Accurate mass measurements using Orbitrap MS, whether or not in combination with a linear ion trap MS (LIT/Orbitrap), has hardly been used for pesticide analysis so far [90,192]. In addition, some detection techniques of less importance have been reported in literature: detection with UV [183], DAD and fluorescence detection [181]. The use of GC analysis with flame ionization detection (GC-FID) [193], surface-assisted laser desorption ionization MS detection (SALDI-MS) and enzyme-linked immunosorbent assay (ELISA) detection [194] have been reported in literature as well.

3.3.2 Sediment analysis

Much less efforts have been addressed for the detection of polar pesticides in sediment, soil and sewage sludge samples. In recent years, the conventional methods for pesticide extraction from solid matrices including Soxhlet or mechanical shaking, have been replaced by advanced extraction techniques such as PLE and sonication [25;195-197]. Typically, rather polar extraction solvents are used, such as acetonitrile, acetone and methanol and mixtures. Additional clean-up steps include the use of (dispersive) SPE or HPLC fractionation, obtaining extracts ready for analysis. Finally, analysis of the extracts is performed using the techniques described in the previous section.

3.3.3 Biota analysis

Very few literature reports on the detection of polar pesticides in aquatic organisms as well. Lehotay et al. [198] monitored more than 60 pesticides in oysters originating from
Chesapeake Bay (Maryland, USA). Oyster tissue was blended with acetonitrile and a series of SPE cartridges were used for clean-up. Analysis was performed using GC-MS on a quadrupole instrument in SIM mode. The obtained LODs for oyster samples were < 5 ng g\(^{-1}\) wet weight. More recently, Buisson et al. [199] described an analytical procedure for detection of pesticides in oysters as well. Oyster samples were first homogenized with an Ultra Thrax unit, then extracted by accelerated solvent extraction using acetonitrile, and finally purified on a Florisil column. Analysis was performed using LC-ESI-MS. Another study reported an analytical procedure for the determination of 29 herbicides and related metabolites in freeze-dried clams [25]. Using a mixture of 4:1 methanol/water, the analytes were extracted with ultrasonication and manual agitation. After centrifugation, removal of methanol and reconstitution in water, the extracts were cleaned and concentrated using Oasis HLB cartridges. Analysis was performed using LC-QqQ-MS, obtaining LODs for simazine, atrazine and terbuthylazine of 0.21, 0.042 and 0.012 ng g\(^{-1}\), respectively. Wille et al. [116] reported recently an analytical approach based on PLE and SPE followed by U-HPLC coupled to QqQ MS for the quantification of 14 pesticides in tissue of marine organisms, obtaining LOQs between 1 and 10 ng g\(^{-1}\).

### 3.3.4 Passive sampling

In agreement with pharmaceutical monitoring, the introduction of passive samplers has enabled the determination of the TWA pesticide concentration in aqueous matrices. Still, only few studies are available in literature regarding the use of passive sampling devices for the more polar pesticides. POCISs and Chemcatcher\(^{\circledast}\) passive sampling devices have been designed for the sampling of polar contaminants. Their use for polar pesticide sampling has been illustrated in literature recently [200-203]. In addition, PEVAC, PDMS and membrane assisted passive samplers (MAPS) have been used for pesticide sampling in the aquatic environment as well [90,119].

### 3.4 Endocrine disruptors

Many environmental pollutants act as endocrine disruptors, being regulated pollutants such as dioxins, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), as well as new emerging more polar pollutants such as estrogenic compounds, APEOs, BPA and phthalates [34]. Since the present review deals with the various approaches used for determination of emerging
pollutants, only the analytical chemistry of the emerging EDCs is reviewed below. The analytical approaches of the PPCPs (section 3.1 and 3.2) and pesticides (section 3.3), which may induce endocrine disruption as well, were discussed above.

### 3.4.1 Estrogenic compounds

The group of natural and synthetic estrogens of which E1, βE2, αE2, E3 and EE2 are important representatives, have been identified as the class with the highest EDC potential [34]. Consequently, a lot of research has been devoted to the analysis of these estrogenic compounds [204]. The recently published (beginning from 2005) analytical approaches to quantify estrogens in environmental matrices have been summarized in Table I.1 for aqueous matrices and in Table I.2 for solid matrices, as an attempt to limit the number of pages in this review. The analytical approaches for estrogenic compounds regularly include the simultaneous detection of other classes of EDCs such as PPCPs, APEOs, phthalates, BPA or phyto-estrogens.

#### 3.4.1.1 Water analysis

As can be seen from Table I.1, chemical analysis of aqueous samples generally required a pre-treatment as well as an extraction step. Sample pre-treatment typically included filtration, while pH adjustment and addition of chemical preservatives occurred only sporadically. However, filtration has not been executed in case of LLE [32,205], SBSE [206,207], SPME [208] or extraction using MIPs [209]. These latter extraction techniques have only been rarely applied, since the use of SPE for isolation and concentration of estrogens is mostly preferred. Typical SPE sorbents used for estrogens are Oasis HLB (hydrophilic-lipophilic balanced copolymer) [205,210] and octadecyl silica bonded phases [211,212]. Vulliet et al. [213] compared Strata C18-E with the styrenedivinylbenzene Strata-X for extraction of a wide group of steroids. Both sorbents exhibited comparable retention capacities towards all analytes. Extraction disks, which supply a larger contact area between the sorbent and the matrix, have been used for steroid extraction from aqueous samples as well [34,214]. Additionally, also on-line SPE using Oasis HLB [215] or PLRP-s [216] sorbents have been applied for estrogen extraction. Upon extraction, further clean-up steps using silica gel [211,217], Florisil [32,218] or NH₂ cartridges [214,219] were regularly applied.
Analysis of estrogens has been performed using LC-MS as well as GC-MS (Table I.1). Prior to GC-MS analysis, derivatization of the hydroxyl or carbonyl groups is carried out, to enhance the thermal stability and volatility of the compounds and to reduce the polarity due to decreasing dipole-dipole interactions [204]. The most common derivatization technique for estrogens consists of silylation using derivatization mixtures of MSTFA or N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) [204]. Derivatization prior to LC-MS analysis is only rarely executed, since estrogens can be analyzed directly using LC techniques [34,215]. Therefore, LC-MS based methods for detection and quantification of steroids have been increasingly applied [204]. So far, only few U-HPLC-MS methods have been reported in literature [219,220]. Recently, Kozlik et al. [212] reported the use of capillary liquid chromatography (cLC) for estrogen analysis, which is a promising approach towards green chemistry that preserves the separation advantages of classical LC. Combined with MS, sufficiently high sensitivity was obtained using this technique.

As can be deduced from Table I.1, QqQ-MS has been frequently applied upon LC separation [211,212,213,216,219]. Farré et al. [219], who compared LC-QqQ-MS, U-HPLC-ToF-MS and ELISA for estrogen analysis in water samples, concluded that the highest sensitivity was obtained after an appropriate sample pre-treatment followed by LC-QqQ-MS. On the other hand, the UPLC–QTOF-MS/MS method provided a shorter analysis time and improved selectivity for confirmation and screening purposes, while the ELISA technique could be directly applied, without previous sample extraction or clean-up, obtaining relatively low detection limits. Besides ELISA, other bioanalytical techniques such as RYA (Recombinant Yeast Assay), E-screen assay and ER-CALUX (estrogen responsive chemically activated luciferase expression) have been applied as well [205,207,220,221]. Bioanalytical techniques generally intend to determine the total estrogenic potency and are frequently complemented with chromatographic (LC or GC) analyses [34]. Finally, diode-array detectors and fluorescence detectors have only rarely been used, mainly due to their minor sensitivity and selectivity [204,206].
<table>
<thead>
<tr>
<th>Matrix</th>
<th>EDCs studied</th>
<th>Sample pre-treatment</th>
<th>Extraction method</th>
<th>Derivatization</th>
<th>Analytical method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seawater</td>
<td>E1, E2, E3, EE2, daidzein, genistein, BPA, NP, OP</td>
<td>Filtration</td>
<td>SPE HLB</td>
<td>-</td>
<td>LC-MS/MS</td>
<td>[222]</td>
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<tr>
<td>Wastewater</td>
<td>E1, E2, EE2</td>
<td>Filtration</td>
<td>Online SPE HLB</td>
<td>Dansyl chloride</td>
<td>LC-MS/MS</td>
<td>[215]</td>
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<tr>
<td>Wastewater</td>
<td>E1, E2, E3, EE2, PPCPs, other steroids</td>
<td>pH adjustment (pH 2) and filtration</td>
<td>SPE HLB</td>
<td>-</td>
<td>LC-MS/MS</td>
<td>[223]</td>
</tr>
<tr>
<td>Wastewater</td>
<td>E1, αE2, βE2, E3, EE3, other steroids</td>
<td>Filtration and pH adjustment (pH 3.4)</td>
<td>SPE Strata-X and silica clean-up</td>
<td>-</td>
<td>LC-MS</td>
<td>[217]</td>
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<tr>
<td>Surface- and groundwater</td>
<td>E1, αE2, βE2, E3, EE3, other steroids</td>
<td>Filtration and pH adjustment (pH 3)</td>
<td>SPE Strata C18-E</td>
<td>-</td>
<td>LC-MS/MS</td>
<td>[213]</td>
</tr>
<tr>
<td>Surface and wastewater</td>
<td>E1, βE2, EE2</td>
<td>Filtration (influent)</td>
<td>SPE C18 and silica gel (influent)</td>
<td>-</td>
<td>LC-MS/MS</td>
<td>[224]</td>
</tr>
<tr>
<td>Waste-, surface and drinking water</td>
<td>E1, E2, E3, EE2, DES, other steroids, PPCPs, pesticides</td>
<td>Filtration</td>
<td>Online SPE PLRP-s (cross linked styrenedivinylbenzene)</td>
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<td>LC-MS/MS</td>
<td>[216]</td>
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<tr>
<td>Surface and wastewater</td>
<td>E1, βE2, EE2, other steroids, BPA, OP, NP</td>
<td>Filtration</td>
<td>SPE C18 + silica gel (steroids) and HLB (phenols)</td>
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<td>LC-MS/MS</td>
<td>[211]</td>
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<td>-</td>
<td>MIPs</td>
<td>-</td>
<td>LC-MS</td>
<td>[209]</td>
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<td>In-tube SPME</td>
<td>-</td>
<td>LC-MS/MS</td>
<td>[208]</td>
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<td>Surface and wastewater</td>
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<td>Filtration</td>
<td>SPE HLB and Florisil clean-up</td>
<td>-</td>
<td>LC-MS/MS</td>
<td>[218]</td>
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<td>E1, αE2, βE2, E3, EE2</td>
<td>Filtration</td>
<td>Discovery DSC-18Lt column</td>
<td>-</td>
<td>cLC-MS/MS</td>
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<td>UPLC-MS/MS</td>
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<td>Waste-, surface and well water</td>
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<td>Filtration</td>
<td>SPE C18 and NH2</td>
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<td>LC-MS/MS / ELISA</td>
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<tr>
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<td>ELISA / LC-MS/MS / UPLC–QTOF</td>
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<td>LC-DAD</td>
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<td>Sample pre-treatment</td>
<td>Extraction method</td>
<td>Derivatization</td>
<td>Analytical method</td>
<td>Ref.</td>
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<tr>
<td>Wastewater</td>
<td>E1, βE2, E3, EE2</td>
<td>Addition of formaldehyde, filtration and pH adjustment (pH 3-5)</td>
<td>SPE HLB + LC-NH₂</td>
<td>MSTFA + mercaptoethanol + NH₄I (for GC)</td>
<td>GC-MS / LC-MS/MS</td>
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</tr>
<tr>
<td>Effluent and surface water</td>
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<td>Addition of formaldehyde, filtration and pH adjustment (pH 3-5)</td>
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<td>MSTFA + mercaptoethanol + NH₄I</td>
<td>GC-MS</td>
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<td>pH adjustment (pH 7)</td>
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<td>GC-MS¹</td>
<td>[214]</td>
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<td>Water</td>
<td>E1, βE2, other steroids, OP, NP, BPA, phthalates</td>
<td>Addition of sodium carbonate and acetic anhydride</td>
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<td>-</td>
<td>GC-MS</td>
<td>[207]</td>
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<td>Surface water and effluent</td>
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<td>Filtration</td>
<td>SPE HLB</td>
<td>BSTFA</td>
<td>GC-MS¹</td>
<td>[228]</td>
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<tr>
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<td>GC-HRMS (magnetic sector)</td>
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<td>MTBSTFA</td>
<td>GC-MS</td>
<td>[229]</td>
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<td>Filtration and pH adjustment (pH 2-3)</td>
<td>SPE HLB</td>
<td>BSTFA</td>
<td>GC-MS / E-screen assay</td>
<td>[230]</td>
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<td>Wastewater</td>
<td>E1, αE2, βE2, E3, EE2, MeEE2, other steroids, OP, NP, BPA, phthalates, phytoestrogens</td>
<td>Filtration in case of Yeast Assay</td>
<td>LLE with dichloromethane</td>
<td>BSTFA</td>
<td>GC-HRMS (magnetic sector)/ Recombinant Yeast Assay</td>
<td>[205]</td>
</tr>
<tr>
<td>Surface water</td>
<td>EE2</td>
<td>Filtration and pH adjustment (pH 7)</td>
<td>-</td>
<td>-</td>
<td>Immunoassay method</td>
<td>[231]</td>
</tr>
<tr>
<td>Rain, surface and wastewater</td>
<td>E1, αE2, βE2, EE2, OP, NP, BPA, phthalates</td>
<td>Filtration</td>
<td>-</td>
<td>-</td>
<td>ER-CALUX</td>
<td>[221]</td>
</tr>
</tbody>
</table>
3.4.1.2 Solid matrices and passive samplers

Estrogenic compounds are medium polar to relatively non-polar substances, with log $K_{ow}$ values in the range 2.5 to 5.3 [232]. Consequently, sorption of estrogens to suspended matter and tendency to accumulate in sediments can be expected [233]. Estrogen extraction is usually performed by use of polar or medium polar organic solvents or their mixtures [232]. Ultrasound-assisted extraction [228,232,234], PLE [226,233,235] and microwave-assisted solvent extraction (MASE) [235] have been applied to enhance the extraction efficiency and to shorten the extraction time (Table I.2). Further purification is usually needed and carried out by SPE, HPLC fractionation or GPC [33]. Few studies have reported on the analysis of estrogens in biota. Budzinski et al. [236] reported an analytical approach for estrogen analysis of fish plasma and bile, while a bioanalytical approach (ER-CALUX) was developed by Vethaak et al. [221]. Recently, several studies described the use of passive samplers for estrogenic compounds as well [230,237,238].

As shown in Table I.2, GC-MS analysis with preceding derivatization has been used more frequent as compared with LC-MS for estrogen analysis in solid samples. In addition, Hajkova et al. [232] described different GC-MS procedures for underivatized analytes as well. It was found that GC×GC hyphenated with ToF-MS provided unequivocal identification of target analytes due to a better resolution. A high resolution LC-ToF-MS method has been reported in literature as well [235].
<table>
<thead>
<tr>
<th>Matrix</th>
<th>EDCs studied</th>
<th>Sample pre-treatment</th>
<th>Extraction method</th>
<th>Derivatization</th>
<th>Analytical method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment</td>
<td>E1, αE2, βE2, E3, EE2, other steroids</td>
<td>Freeze-dried</td>
<td>Sonication with ACN/H2O, SPE EDS-1, GPC and Florisil clean-up</td>
<td>LC-MS/MS</td>
<td>[234]</td>
<td></td>
</tr>
<tr>
<td>Sediment</td>
<td>E1, βE2, EE2</td>
<td>-</td>
<td>MASE, SPE Strata X-AW and silica gel</td>
<td>LC-ToF-MS / LC-MS/MS</td>
<td>[235]</td>
<td></td>
</tr>
<tr>
<td>Sediment</td>
<td>E1, βE2, E3, NP, OP, BPA</td>
<td>Freeze-dried</td>
<td>Ultrasonic extraction with methanol, SPE HLB + HPLC fractionation</td>
<td>LC-MS/MS / YES assay and ELISA</td>
<td>[239]</td>
<td></td>
</tr>
<tr>
<td>Biosolids and sludge</td>
<td>E1, βE2, other steroids, OP, NP, BPA, phthalates</td>
<td>Addition of water, Na₂CO₃, acetic anhydride; filtration and oven-dried</td>
<td>SBSE-TD</td>
<td>-</td>
<td>GC-MS</td>
<td>[207]</td>
</tr>
<tr>
<td>Soil</td>
<td>E1, αE2, βE2, E3</td>
<td>Air dried and sieved</td>
<td>PLE (acetone or ethylacetate) + SPE C18 MSTFA:hexane (1:5)</td>
<td>GC-MS</td>
<td>[240]</td>
<td></td>
</tr>
<tr>
<td>Sediment</td>
<td>E1, βE2, E3, EE2, DES</td>
<td>Drying</td>
<td>Sonication (hexane–acetone) + SPE HLB</td>
<td>GC-MS / (GC x) GC-ToF-MS</td>
<td>[232]</td>
<td></td>
</tr>
<tr>
<td>Sludge samples</td>
<td>E1, βE2, E3, EE2</td>
<td>Freeze-dried</td>
<td>PLE with methanol:acetone (1:1) and SPE HLB + LC-NH₂ MSTFA + mercaptoethanol + NH₄I</td>
<td>GC-MS / LC-MS/MS</td>
<td>[226]</td>
<td></td>
</tr>
<tr>
<td>Suspended solids and sediment</td>
<td>E1, αE2, βE2, E3, EE2, MeEE2, NP, OP, BPA</td>
<td>Freeze-dried</td>
<td>Ultrasonic assisted extraction with methanol:acetone (1:1) and SPE Florisil</td>
<td>BSTFA</td>
<td>GC-MS</td>
<td>[228]</td>
</tr>
<tr>
<td>Sediment and suspended solids</td>
<td>E1, αE2, βE2, EE2</td>
<td>Sieved and freeze-dried</td>
<td>PLE (acetone/methanol) + HPLC fraction. MSTFA</td>
<td>GC-MS²</td>
<td>[233]</td>
<td></td>
</tr>
<tr>
<td>Fish plasma and bile</td>
<td>E1, βE2, other steroids</td>
<td>Centrifugation</td>
<td>SPE C18 and NH₂ MSTFA + mercaptoethanol + NH₄I</td>
<td>GC-MS</td>
<td>[236]</td>
<td></td>
</tr>
<tr>
<td>Sediment and biota</td>
<td>E1, αE2, βE2, EE2, OP, NP, BPA, phthalates</td>
<td>Filtration</td>
<td>-</td>
<td>ER-CALUX</td>
<td>[221]</td>
<td></td>
</tr>
<tr>
<td>Passive samplers</td>
<td>E1, βE2, EE2, BPA, PPCPs</td>
<td>-</td>
<td>POCIS</td>
<td>-</td>
<td>GC-MS</td>
<td>[237]</td>
</tr>
<tr>
<td>Passive samplers</td>
<td>E1, αE2, βE2, E3, EE2, MeEE2, BPA, OP, NP</td>
<td>-</td>
<td>POCIS</td>
<td>BSTFA</td>
<td>GC-MS</td>
<td>[238]</td>
</tr>
<tr>
<td>Wastewater</td>
<td>E1, αE2, βE2, other steroids, OP, NP, BPA, phthalates</td>
<td>-</td>
<td>Empore™ SDB-RPS BSTFA</td>
<td>GC-MS / E-screen assay</td>
<td>[230]</td>
<td></td>
</tr>
</tbody>
</table>
3.4.2 Alkylphenolethoxylates and bisphenol A

Alkylphenolethoxylates (APEOs) belong to the class of the non-ionic surfactants, which are molecules consisting of a hydrophobic, usually an alkyl or alkylaryl chain, and a hydrophilic part which can vary largely. Both the surfactants as the metabolites octylphenol (OP) and nonylphenol (NP) are relatively persistent and have been shown to cause endocrine disruption [241]. Since bisphenol A (BPA) is routinely analysed together with the APEOs (and metabolites), this well-known EDC was considered in this section as well.

3.4.2.1 Water analysis

With respect to the analysis of water samples, degradation of APEOs is expected to occur. The most common procedure for conserving APEOs is through acidification of the sample to a pH below 3 [242]. In addition, it is essential to process water samples as quickly as possible [241]. In accordance with the extraction of many of the emerging pollutants, SPE has replaced LLE and may be considered as the most abundantly applied extraction technique for APEOs and BPA. Polymer based sorbents were preferred above C18 cartridges, since both the long-chain APEOs as the metabolites OP and NP were retained. For example, Loos et al. [243] reported the use of Oasis HLB cartridges for the extraction of alkylphenols (APs), APEOs, ethoxycarboxylate metabolites and BPA. Using Oasis HLB cartridges, frequently lower recoveries were obtained for NP, a very environmentally important compound within this group because of its endocrine disrupting potency. Therefore, sequential SPE or elution using solvents with different desorption potential and polarity has been successfully applied to cover a broad range of APEOs [242,244]. Recently, an on-line column-switching SPE application was reported for the detection of BPA in water samples as well [245]. Besides, the use of more specific extraction techniques for APEOs and BPA such as SBSE, SPME, LPME and dispersive liquid-liquid microextraction (DLLME) have been reported [207,246-248]. Wang and Schnute [249] reported a U-HPLC-QTRAP method without previous sample preparation for the simultaneous quantification of NP and BPA, obtaining detection limits in the range of 0.04 to 0.057 µg L⁻¹.
Both GC-MS, with preceding derivatization, as well as LC-MS analysis have been employed for analysis of aqueous samples [249]. LC analysis is anyhow preferred for the longer chain APEO substances, since these are not volatile enough for GC-analysis. However, quantification of APEOs can be a matter of concern when using LC separation with C18 columns, since co-elution can be expected for the APEOs only differing in ethoxylate chain length [242]. In case of LC-MS, QqQ-MS has been regularly applied [245,250], while also accurate mass measurements of APEOs using QToF-MS have been reported [251]. The use of UV and fluorescence detection have been applied for the determination of BPA and NP in water as well [248,252].

3.4.2.2 Solid samples and passive samplers

The physicochemical profiles suggest that some APEOs and degradation products exert a strong affinity to SPM and organic matter. Additionally, they have the tendency to bind to sediments and to accumulate in aquatic organisms, due to their high lipophilicity and lower water solubilities [242]. Extraction from sediment can be performed by Soxhlet extraction [250], sonicated-assisted extraction [253], SFE [254], MAE [255] and PLE [256]. The latter technique offers several advantages as compared to the other methods, and was therefore found to be the main extraction technique for these compounds in sediment matrices. Further purification of the obtained extract is usually performed by SPE [242].

With respect to biota, only few studies have been conducted. Tavazzi et al. [257] described a PLE method followed by LC-MS analysis for the determination of OP, NP and BPA in fish liver. The use of passive samplers, by means of POCIS samplers, for the determination of BPA, NP and three steroids has been reported in literature as well [251].

3.5 Phthalates

Phthalates are nowadays considered as ubiquitous environmental pollutants [258]. As a result, considerable attention must be paid to the possible occurrence of sample contamination, which is a major problem throughout the analytical process. Phthalates present in laboratory equipment could contribute to sample contamination and result in systematic errors and false positives [258,259]. Consequently, very specific analytical approaches, as shortly discussed below, are required to ensure the reliability of analytical results.
3.5.1 Water analysis

Prior to analysis, extraction of phthalates from aqueous samples is needed. To this end, the use of traditional techniques including LLE [32,260] and SPE [230,260,261] have only rarely been reported. More common, extraction based on (in-tube) SPME using polymer coated fibers has been successfully applied [258,263-267]. Penalver et al. [265] compared different fibers to optimize a SPME method for the most common phthalates, obtaining the best results using the polydimethylsiloxane–divinylbenzene fiber. An important advantage of the use of SPME for phthalate extraction includes the reduced risk for secondary contamination during sample handling [266]. Other techniques such as SBSE [207,268] and LPME [269] have also been applied for extraction of phthalates, as well as DLLME [270]. Finally, a novel method, which is termed ionic liquid cold-induced aggregation dispersive liquid–liquid microextraction (IL-CIA-DLLME), has been recently applied for analysis of phthalates as well [271]. Upon sample pre-treatment, phthalates have been determined with GC-MS and LC-MS equipment [259]. LC-methods coupled to DAD or UV detectors have been applied for quantification of phthalates in water samples as well [258,262].

3.5.2 Solid matrices

Several different methods for the extraction of phthalates from sediment or sludge samples have been reported: Soxhlet extraction [37,272,273], ultrasonic assisted liquid extraction [274,275] and PLE [276]. Due to the complexity of the sample matrix, sample preparation frequently included an additional clean-up step based on SPE with C18 sorbent [275] or GPC with further fractionation using silica gel [37,272].

With respect to phthalate extraction from biota samples, recent literature is very limited. Chaler et al. [273] reported an analytical approach based on alkaline digestion with subsequent fractionation on an alumina/silica column. To the best of our knowledge, no other recent studies have examined the analysis of phthalates in aquatic organisms.

Both GC and LC have been employed for phthalate analysis in environmental matrices [273,276]. Recently, the successful application of a GC-APCI-ToF-MS method has been reported as well [274].
3.5.3 Passive samplers

The use of passive samplers consisting of styrene-divinylbenzene Empore™ SDB-RPS disks for the determination of 15 EDCs including several phthalates in wastewaters was reported by Tan et al. [230] [Table I.2]. Also semi-permeable membranes charged with Tenax TA have been applied as passive sampling devices for monitoring phthalates in the aquatic environment [277]. Prior to GC-MS analysis, the targeted phthalates were thermally desorbed from the sampler with a helium stream.

3.6 Perfluorinated compounds

The occurrence of PFCs in the aquatic system has been extensively studied in literature. Still, the quality of data obtained from analysis of PFCs in environmental matrices is a major issue of concern [278,279]. Very recent, the final report of the third interlaboratory study on PFCs in environmental matrices was published [279]. A relatively wide variance in PFC data of aqueous samples could be observed. This is probably caused by a combination of problems which are characteristic for PFC analysis, namely the occurrence of cross contamination, matrix interferences and branched isomers. These inconveniences render quantitative analysis of PFCs in environmental matrices, which is reviewed below, to a challenging task.

3.6.1 Water analysis

Several sampling and sample pre-treatment aspects have been assessed as essential for straightforward analysis of PFCs. At first, the sampling depth may affect PFC concentration results. A decreasing PFC concentration with increasing water depth has been reported by Yamashita et al. [280] and Ju et al. [281]. This can be associated with their surface-active character and, in case of ocean waters, with the global ocean circulation system [280,282]. Secondly, the sample equipment should be carefully selected to avoid contamination with and adsorption of PFCs [282]. Prior to sampling, it was suggested to rinse sample bottles using (semi-)polar solvents [5,283]. Acidification of samples for conservation purposes was discouraged to prevent volatilisation and adsorption to the sample container [284]. Besides, filtration of water samples is a matter of concern for PFC-analysis as well. The surface-active nature of these substances may result in sorption to the filter material [41]. Furthermore, several filters are sources of contamination for PFOA and perfluorononanoate (PFNA) [285]. Therefore, except in
case of water samples visibly containing particulate matter, it was suggested to avoid filtration as a sample preparation step. Schultz et al. [286] recommended centrifugation as an alternative sample clean-up step.

Generally, low concentrations (pg – ng L\(^{-1}\)) are found in water samples, requiring pre-concentration and isolation of PFCs. To this end, LLE and SPE both followed by solvent evaporation have been frequently reported in literature [287]. The common use of these techniques appeared from the third interlaboratory study on PFCs as well [279]. Within this final report, it was found that SPE was predominantly used for nearly 80% of the water samples (Figure I.9) [279]. Both techniques were compared by Gonzalez-Barreiro et al. [288]. Using LLE, the overall PFC concentration (aqueous and particulate fraction) can be determined since filtration is avoided, however, this technique is limited to long chain PFCs (C\(\geq 8\)). Apparently, SPE was best suited for PFCs with less than 10 carbon atoms, including the most important contaminants PFOS and PFOA [288]. An SPE approach enabling the determination of short as well as long chain PFCs was described by Taniyasu et al. [289]. Based on the latter publication, the ISO 25101/2006 method [290] has been established using weak anion exchange (Oasis WAX) SPE cartridges. The use of C18 and certainly Oasis HLB cartridges for PFC enrichment has been frequently reported as well [288,291-295]. Alternatively, also direct determination of PFCs from aqueous samples has been reported [286,296]. Without sample pre-treatment, PFCs were quantified using large-volume injection, thereby obtaining LOQs in the 1 ng L\(^{-1}\) range.

![Figure I.9. Methods used for water extraction according to the final report of the third interlaboratory study on PFCs in environmental matrices [279].](image-url)
According to Jahnke and Berger [287], LC coupled to quadrupole MS in negative ionization mode is the preferred instrumental method for the determination of ionic PFCs in environmental matrices, including PFCAs and PFSAs. Upon LC-separation, QqQ-MS allows excellent quantification of PFCs at very low concentration levels [282]. Controversially, it has been reported that the use of MS^n (using QqQ or IT-MS) for the detection of PFSAs, including the major contaminant PFOS, is complex and less efficient [297,298]. Since these substances exhibit a very high stability even at extreme conditions (e.g., high collision energies), the use of QqQ-MS or IT-MS typically results in PFOS-fragments of [FSO₃⁻] and [SO₃⁻], with m/z ions of 99 and 80 respectively. Unfortunately, interferences that co-elute with PFOS could be observed with the same retention time and one similar transition [297,299]. Therefore, another powerful analytical approach for PFC determination involves the use of full scan high resolution accurate mass measurements. (Q)ToF-MS has been reported to be the optimal detector for quantification of PFCs, combining high selectivity with high sensitivity [300]. Recently, LC-ToF-MS was applied for the determination of 14 PFCs in surface-, sewage- and seawater with LOQs varying from 2 to 200 ng L⁻¹ [295]. In addition, high resolution MS using an Orbitrap instrument has proved his excellent applicability for PFC-determination as well, however, this has not been published in literature earlier [297]. Alternatively, the ionic PFCAs and PFSAs may be analyzed with GC as well [287]. This however needs to be preceded by a derivatization with a mixture of 2,4-difluoroaniline and N,N-dicyclohexylcarbodiimide [301] or with iso-propanol [302]. The non-ionic PFCs such as the fluorotelomer alcohols and fluoroalkyl sulphonamides are, due to their higher volatility, directly amenable to GC. Langlois et al. [302] reported a high-resolution gas chromatographic (HRGC) method for both ionic and non-ionic PFCs. In this context, the separation of PFOS-isomers was performed using the high resolution of a capillary GC-column coated with 5%-phenyl-95%-methylpolysiloxane.

In literature, specific measures have been described to overcome some characteristic difficulties during PFC-analysis. At first, background contamination in the analytical blanks is a major problem in the analysis of PFCs, especially when the low ng L⁻¹ concentration range is targeted [289,296,303]. Therefore, it was suggested to avoid the use of teflon materials throughout the extraction procedure, to clean and dry all glass material, to replace the internal parts of the HPLC system made of PTFE with stainless steel and polyether ether ketone (PEEK), and to place an additional HPLC column.
between the pump and the injector to separate sample PFCs and PFCs originating from the system [287,294,295,303]. Secondly, the limited use of mass labelled analogues to compensate for ionisation effects can contribute to the occurrence of deviating analytical results. For this reason, it is highly recommended to use an isotopically labelled internal standard for every single PFC that requires quantification [279]. Finally, the occurrence of branched isomers should be taken into consideration as well. For example, technical PFOS contains up to 30% differently branched isomers [302]. These branched isomers may have varying ionization efficiencies, resulting in a systematic quantification bias of PFCs [299]. Recently, a HPLC-MS/MS method for isomer-specific quantification of PFCs in water samples has been reported. The examination of PFC isomer profiles may be interesting to study the environmental fate of PFCs and to distinguish historical from recent contamination as well [304].

3.6.2 Solid matrices

Since the first report on the global distribution of PFOS was published in 2001, a large number of studies have been dedicated to the detection of PFCs in biota [43]. The most important techniques to extract PFCs from solid matrices include ion pair extraction (IPE) and solid-liquid extraction (SLE). Several recent studies use the IPE methods reported by Hansen et al. [305] and Giesy and Kannan [43], which consisted of ion-pairing of PFCs with tetrabutylammonium hydrogen sulphate (TBA) followed by extraction with methyl-\textit{tert}-butyl ether (MTBE) [306-308]. However, according to the recent interlaboratory study on PFCs in environmental matrices, SLE using a medium polar solvent such as methanol or acetonitrile is nowadays considered as the foremost applied approach for PFC extraction from biotic tissue (Figure I.10) [279].
Figure I.10. (Left) Methods used for extraction and (right) further clean-up of a fish sample, according to the final report of the third interlaboratory study on PFCs in environmental matrices [279].

The SLE sediment method of Powley et al. [309] and the altered version for biotic samples [310] have been regularly adopted or modified by environmental chemists [278,295]. Further clean-up of the obtained extract is usually performed by SPE or treatment with activated carbon (ENVIcarb), to reduce possible interferences [295,311,312]. This appeared from the interlaboratory study on PFCs in environmental matrices as well (Figure I.10). Another popular extraction method in this context is the approach of Berger and Hauskas [313], consisting of extraction with methanol/water (50/50; 2mM ammonium acetate) followed by filtration. Malinsky et al. [314] included a freezer-incubation step of the acetonitrile/tissue extracts to facilitate protein precipitation for improved analyte recoveries. The use of PLE for PFC extraction has been reported once in literature by Llorca et al. [315], who compared three different sample preparation techniques for PFC analysis in fish. It was found that PLE using water as extraction solvent with subsequent SPE was chosen above IPE as described by Hansen et al. [305] and alkaline digestion followed by SPE as described by Taniyasu et al. [289]. Finally, microextraction with tetrahydrofuran has been successfully applied as well for analysis of PFCs in biota [316].

In general, extraction methods similar to those for biota are used for sediment and sewage sludge samples. Bao et al. [317] performed sediment extraction with TBA and MTBE, while a SLE method using 9 mL of methanol and 10 mL of a 1% glacial acetic acid solution was applied by Gomez et al. [318]. Also Ahrens et al. [319] used methanol as extraction solvent for PFC extraction from sediments. So far, the use of PLE for sediment samples has not been reported in literature.
In recent years, PFC analysis has typically been performed using LC coupled to different mass spectrometric techniques such as ion-trap MS [311,320], tandem-MS [321], QqQ-MS [317,322], ToF-MS [295] or QTRAP-MS [314,323]. The use of U-HPLC for PFC analysis in solid matrices has been reported once in literature [318]. Also the separation and quantification of the PFC isomers has been demonstrated before [314,323]. Just as for aqueous matrices, it has been reported that correct quantification of PFCs in solid matrices is challenging [278]. Therefore, the above mentioned measures to enable accurate quantification in aqueous matrices should be taken into consideration for solid samples as well. With respect to the quantification aspect, it has been demonstrated that analytical results obtained with extracted matrix-matched calibration differ only slightly from solvent (unextracted) calibration [314]. This definitely facilitates quantification, especially when only limited clean control matrix samples are available.

3.6.3 Passive samplers

To the best of our knowledge, the use of passive samplers for PFC-monitoring in the aquatic environment has not been reported before.

4. Conceptual framework of this study

Nowadays, a growing public and scientific concern exists regarding the widespread occurrence of micropollutants in the aquatic environment. In light of the possible toxic, genotoxic and/or endocrine disrupting properties that many of these micropollutants may exert, their potential to cause adverse effects in marine organisms should not be neglected. Moreover, the ingestion of contaminated seafood has been proposed as a major source of direct human exposure to many micropollutants. Despite these facts, limited efforts have been made so far to investigate their prevalence on a quantitative basis in marine ecosystems. To study and evaluate the fate, effects, and environmental and human risks posed by these polar micropollutants in aquatic ecosystems, information regarding their presence in the marine environment is urgently needed.

Therefore, the main goal of the present study was to investigate the prevalence of targeted CECs including pharmaceuticals, PFCs and pesticides in the Belgian marine environment. This implies the need for new reliable analytical methods for their
determination in environmental matrices. Therefore, the specific objectives of this doctoral thesis were as follows:

- To develop and validate a new analytical method to enable the quantification of pharmaceuticals in aqueous samples from the Belgian coastal zone
- To develop and validate a new analytical method for the quantification of PFCs in aqueous samples from the Belgian coastal zone
- To develop new extraction procedures and analytical methods for the quantification of pharmaceuticals, pesticides and PFCs in marine biota originating from the Belgian coastal zone
- To develop and validate a new analytical method for the quantification of pharmaceuticals and pesticides in passive sampling devices deployed in the Belgian coastal zone
- To support the integrated risk assessment of CECs in the Belgian coastal zone by comparison of the obtained concentrations with regulatory standards or ecotoxicity data

This doctoral thesis consists of 4 research chapters, preceded by a general introduction to the subject in Chapter I. In this chapter, an elaborate literature review is presented of the various analytical approaches used for the determination in environmental matrices of the most important groups of CECs, including pharmaceuticals and personal care products (PPCPs), pesticides, EDCs, phthalates and PFCs. Chapters II and III describe the development and validation of new analytical methods for quantification in aqueous samples of pharmaceuticals and PFCs, respectively, as well as application thereof to water samples originating from the Belgian coastal zone. Within Chapter IV, a new method for quantification of pharmaceuticals, PFCs and pesticides in marine organisms is presented. In addition, these optimized procedures were applied to M. edulis samples originating from different locations in the Belgian coastal zone. Chapter V aimed at the development and application of a new analytical procedure for quantification of a high
number of pharmaceuticals and pesticides in passive sampling devices. Finally, in
Chapter VI, the obtained monitoring results of the CECs are discussed in terms of their
distribution in the Belgian coastal zone, bioaccumulation potential and possible
environmental risk. In addition, general conclusions are drawn and future research
recommendations are formulated.

5. REFERENCES

503.
GENERAL INTRODUCTION


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CHAPTER II

VALIDATION AND APPLICATION OF AN LC-MS/MS METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF 13 PHARMACEUTICALS IN SEAWATER

Adapted from:

CHAPTER II

VALIDATION AND APPLICATION OF AN LC-MS/MS METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF 13 PHARMACEUTICALS IN SEAWATER

Abstract

Knowledge of the presence of micropollutants such as pharmaceuticals, in coastal areas, is very limited. Therefore, the main objective of this study was to optimize and validate a new analytical method for the quantitative analysis of 13 multiclass pharmaceuticals in seawater. Target compounds included antibiotics, non-steroidal anti-inflammatory drugs, β-blockers, lipid regulators and one psychiatric drug. A combination of solid-phase extraction and liquid chromatography coupled with multiple mass spectrometry enabled their detection at the low nanogram per litre level. The limits of quantification varied between 1 and 50 ng L⁻¹ and for most components the linearities were more than 0.99 (n=7). The recoveries and precision obtained in seawater, between 95–108% and 16-27 RSD% respectively, were satisfactory. This method was applied to seawater and estuarine water samples collected in the Belgian coastal zone, to assess the prevalence of common pharmaceuticals in this marine environment. Seven pharmaceuticals, including compounds of which the presence in marine environments had not been reported earlier, were detected, with salicylic acid and carbamazepine being the most abundant, in concentrations up to 855 ng L⁻¹.
1. INTRODUCTION

Pharmaceuticals are the active ingredients of medicinal products used in human and veterinary medicine and include approximately 3,000 different compounds with a large variation in chemical structure, function and behaviour [1,2]. In Belgium, the consumption of reimbursed pharmaceuticals available at the pharmacy level ranges between 0.001 and 6 tons per year depending on the individual pharmaceutical (Table II.1) [3] (H. Beyers, personal communication). Pharmaceuticals for human use are excreted - either in their native form or as metabolites - and discharged into the sewer system [4,5]. Via sewage, pharmaceuticals reach the wastewater treatment plant (WWTP), where most pharmaceuticals are, according to current literature, not completely removed [6,7]. As a result of WWTP effluents, land application of sewage sludge, improper disposal and manufacturing processes, pharmaceuticals are introduced into natural aquatic systems [8,9]. These different pathways may result in a continuous release of pharmaceuticals into the aquatic environment. Consequently, there is growing public and scientific concern regarding the occurrence and potential effects of pharmaceuticals in the aquatic environment.
Table II.1. Physico-chemical properties of the targeted compounds [6,27,28,45] and their annual consumption at pharmacy level in 2007 in Belgium [3] (n.d. = no data).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MW (g/mol)</th>
<th>Log $K_{ow}$</th>
<th>pKa</th>
<th>Consumption (kg/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NSAIDs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>138.12</td>
<td>2.43</td>
<td>3.50</td>
<td>11</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>241.28</td>
<td>4.16</td>
<td>4.20</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>254.28</td>
<td>3.22</td>
<td>4.45</td>
<td>199</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>296.15</td>
<td>3.91</td>
<td>4.15</td>
<td>3 360</td>
</tr>
<tr>
<td><strong>Lipid regulators</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>214.65</td>
<td>2.58</td>
<td>3.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>361.83</td>
<td>3.85</td>
<td>3.60</td>
<td>176</td>
</tr>
<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>253.28</td>
<td>0.68</td>
<td>5.70</td>
<td>453</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>290.32</td>
<td>0.65</td>
<td>6.60</td>
<td>n.d.</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>323.13</td>
<td>1.14</td>
<td>5.50</td>
<td>1</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>361.37</td>
<td>0.35</td>
<td>6.20</td>
<td>322</td>
</tr>
<tr>
<td><strong>Neuroactive compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>236.27</td>
<td>2.3</td>
<td>13.90</td>
<td>6 107</td>
</tr>
<tr>
<td><strong>β-blockers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>259.34</td>
<td>3.03</td>
<td>9.49</td>
<td>2 320</td>
</tr>
<tr>
<td>Atenolol</td>
<td>266.34</td>
<td>0.46</td>
<td>9.16</td>
<td>2 232</td>
</tr>
</tbody>
</table>

As pharmaceuticals are designed to exert specific effects at low doses and to resist metabolic degradation, their possible biological activity in aquatic organisms and their persistence cannot be excluded [4,5]. Moreover, the continuous environmental input of these compounds, and resulting mixtures, may induce unnoticed adverse effects on aquatic and terrestrial organisms [10,11]. Although several studies have reported standard ecotoxicity data for pharmaceuticals [8,12,13], in general their environmental significance, pertaining to environmental effects, is largely unknown [14]. However, a preliminary risk assessment database for common pharmaceuticals with a focus on
DETECTION OF PHARMACEUTICALS IN WATER

Regulatory guidance to assess the presence of pharmaceuticals in the aquatic environment is still lacking. The Water Framework Directive (2000/60/EC) [15] and its daughter directive (2008/105/EC) [16] lay down environmental quality standards for a list of priority substances, but pharmaceuticals are not included. Furthermore, the OSPAR lists of chemicals of priority action and of substances of possible concern include, respectively, two and 19 pharmaceuticals [17]. However, these listed pharmaceuticals are used in fairly small quantities and their occurrence in the environment is limited [9]. Currently, no guidance is established for widely used and widespread occurring pharmaceuticals such as carbamazepine and diclofenac.

Nevertheless, in recent years, numerous monitoring studies have demonstrated the occurrence of pharmaceuticals in aquatic systems. They have been detected in WWTP influents and effluents and in freshwater systems in the nanogram per litre up to the microgram per litre range [18–21]. More rarely, pharmaceuticals have been detected in drinking water [22,23] and groundwater samples [21,24], mostly in the nanogram per litre range and occasionally at microgram per litre levels. In contrast to the extensive literature describing the occurrence and persistence of pharmaceuticals in freshwater systems, little attention has been paid to their prevalence and quantification in marine ecosystems [25,26].

Therefore, the main objective of this study was to develop a quantitative analytical method for pharmaceuticals in seawater. On the basis of data on the current use in Belgium (Table II.1), 13 environmentally relevant pharmaceuticals were selected from five different therapeutic classes. These included four antibiotics (sulfamethoxazole, ofloxacin, trimethoprim and chloramphenicol), four nonsteroidal anti-inflammatory drugs (NSAIDs) (mefenamic acid, diclofenac, salicylic acid, and ketoprofen), two β-blockers (propranolol and atenolol), two lipid regulators (bezafibrate and clofibrate acid) and one psychiatric drug (carbamazepine). The physicochemical properties of the pharmaceuticals considered in this study are presented in Table II.1 [6, 27, 28]. Secondly, an extensive validation study was carried out to demonstrate the applicability of this analytical approach. To this end, the method developed was applied to marine water samples taken from the North Sea and the Scheldt estuary. In this way, the presence of pharmaceuticals in the Belgian marine environment and their transfer to estuarine and marine ecosystems
was examined. This study is part of the INRAM project (www.vliz.be/projects/inram), a 4-year project that aims to use an integrated approach to assess the risks of micropollutants in the Belgian coastal zone.

2. **EXPERIMENTAL**

2.1 **Study area and sampling**

The study area is located in the three Belgian coastal harbours (Ostend, Nieuwpoort and Zeebrugge), the Scheldt estuary and the offshore coastal area of Belgium. An overview of the study area and the sampling stations is depicted in Fig. II.1. Ten sampling stations were selected in three coastal harbours; four in the harbour of Zeebrugge (ZB01–ZB04) and three in the harbours of Nieuwpoort (NP01 - NP03) and Ostend (OO02 - O04) each. In each harbour, one sampling station was representative for the major freshwater inputs into the harbour, while a second sampling location represented the water at the harbour mouth, and at least one station between these points was sampled as well. An additional station was selected at the Sluice Dock in Ostend (OO01) since at this location aquacultural activities take place. Two stations were sampled in the Scheldt estuary: one station located at the river mouth near Vlissingen, the second more upstream near Antwerp. Six sampling stations were chosen in the Belgian coastal area: three (W01–W03) were located close to the harbour mouths of Ostend, Nieuwpoort and Zeebrugge; the remaining three (W04–W06) were situated more offshore. Four sampling campaigns were carried out: in May and December 2007, April 2008 and June 2009.
2.2 Reagents and chemicals

Ketoprofen (purity 99.0%), mefenamic acid (purity better than 99.0%), carbamazepine (purity better than 99.0%), diclofenac (purity better than 99.0%), bezafibrate (purity 98.0% or better), salicylic acid (purity better than 99.0%), clofibric acid (purity 97.0%), atenolol (purity 98.0% or better), trimethoprim (purity 98.0% or better), chloramphenicol
(purity 99.0% or better), and sulfamethoxazole (purity 99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ofloxacin (purity better than 99.0%) was obtained from ICN Biomedicals (Aurora, OH, USA) and propranolol (purity better than 99.0%) was purchased from Eurogenerics (Brussels, Belgium). The synthetic isobutcar 61 [4-3(isobutylamino-2-hydroxypropoxy)-carbazole] was found to be a very suitable internal standard for this application as it corrected sufficiently for any matrix effects in the sample preparation and mass spectrometry (MS).

Acetone, methanol and formic acid were of analytical grade and were purchased from VWR (Merck, Darmstadt, Germany). High performance liquid chromatography (HPLC)-grade acetonitrile and water were obtained from VWR (Merck, Darmstadt, Germany) and Acros Organics (Fairlawn, NJ, USA), respectively. Primary stock solutions of all individual analytes were prepared in ethanol at a concentration of 1 ng μL⁻¹. Working standard mixture solutions were prepared by appropriate dilution of the stock solutions in ethanol. All solutions were stored at -20 °C in the dark.

2.3 Extraction and clean-up

The extraction technique developed is based on the method of Gómez et al. [30] and Nebot et al. [31]. Prior to extraction, the pH of the water samples was adjusted to 7.0 ± 0.5 using solutions of HCl (1 M) and NaOH (1 M). Seawater samples of 1 L were filtered through a Whatman filter paper (GF/C diameter 47 mm, particle retention 1.2 μm, Merck, Darmstadt, Germany) to avoid clogging of the sorbent. The filters were washed with 2 mL methanol to prevent loss of the compounds of interest. The methanol extract was collected and added to the filtered sample. Prior to extraction, the internal standard isobutcar 61 was added to every sample at a final concentration of 50 ng L⁻¹. Subsequently, solid-phase extraction (SPE) was carried out using Chromabond HR-X cartridges (3 mL, 200 mg, Macherey-Nagel, Düren, Germany). The cartridges were preconditioned with 5 mL methanol and 5 mL Milli-Q water. After they had been loaded with 1 L of the filtered sample pooled with the methanol from the filter washing, the cartridges were rinsed twice with 5 mL Milli-Q water. Subsequently, the cartridges were dried under a vacuum for 30 min. Elution was performed using 5 mL acetone and 2 × 5 mL methanol. Next, the extracts were dried using nitrogen and the residues were reconstituted in 300 μL acetonitrile/0.02 M formic acid (50/50) before transfer to liquid chromatography (LC)–MS vials.
2.4 Chromatographic and mass spectrometric instrumentation

Analysis was carried out using HPLC. The apparatus comprised an 1100 series quaternary gradient pump and autosampler (Hewlett Packard, Palo Alto, CA, USA). Chromatographic separation was achieved using a Nucleodur® C18 Isis HPLC column (5 μm particle size, 250 mm×4.0 mm, Macherey-Nagel, Düren, Germany). The void volume of the system was 2.5 mL. A filter (porosity 2 μm, 4 mm; Alltech, Waukegan, IL, USA) and a precolumn (Nucleodur® C18 Isis, Macherey-Nagel, Düren, Germany) were used to prevent rapid deterioration of the analytical column. The mobile phase consisted of a mixture of 0.02 M formic acid (solvent A) in water and acetonitrile (solvent B). A linear gradient of 0.3 mL min⁻¹ was used starting with a mixture of 60% solvent A and 40% solvent B for 5 min. The acetonitrile percentage was increased linearly from 40 to 100% in 20 min. Twenty microlitres was injected onto the LC-MS® system. Analytes were detected with an LCQ DECA ion trap mass spectrometer equipped with an electrospray ionization (ESI) interface (Thermo Finnigan, San Jose, CA, USA) using the optimized MS parameters described in “Results and discussion”. The optimal ionization source working parameters were as follows: sheath gas flow rate, 80 arbitrary units (a.u.); auxiliary gas, 20 a.u.; capillary temperature 350 °C; capillary voltage, -14 V; and tube lens offset, 20 V. Chromatograms and spectra were recorded and processed using Xcalibur® 2.0 (Thermo Finnigan, San Jose, CA, USA).

2.5 Validation of the method

For environmental sample analysis, rigorous validation procedures are usually not well defined. Similar to reported studies on pharmaceuticals in water [11,20,32], we validated the method developed according to Commission Decision 2002/657/EC [33] concerning the determination of analytes in products of animal origin. Also SANCO/825/00 revision 7 [34] was used as a guideline for the validation of this new analytical method. In general, validation includes the evaluation of linearity, specificity, selectivity, recovery, matrix effects and the determination of the limit of quantification (LOQ). Because the method was particularly aimed at examining marine waters, seawater was used to validate the method. On the basis of preliminary results, blank seawater samples contained low levels of some of the target analytes. The calibration curves were corrected for these concentrations. In addition, none of the pharmaceuticals were detected in reagents or Milli-Q water.
2.6 Quality assurance

Before and after analysis of a series of samples, a standard mixture (1 ng on column) of the target pharmaceuticals was injected to check the instrument parameters of the LC-ESI-MS\textsuperscript{n} system. Quality control of the method was performed by analysis of a blank sample, together with a linear calibration curve constructed using 1 L seawater samples spiked with standard solutions at seven concentrations between 1 and 1,000 ng L\textsuperscript{-1}. This was performed for every series of samples. These calibration curves were used for quantification.

3. RESULTS AND DISCUSSION

3.1 Method development

3.1.1 Extraction of the target pharmaceuticals

Irrespective of the log \( K_{\text{ow}} \) and pKa values of the substances studied (Table II.1), an extraction procedure aiming at the recovery of all target analytes in an efficient and repeatable manner was developed. To obtain a concentrated extract suitable for analysis, extraction of pharmaceuticals in water is generally executed using SPE \([20,27,31,35]\). SPE includes the extraction of the analytes by means of a solid sorbent and is usually performed off-line. Preliminary experiments were performed to evaluate the extraction efficacy of different types of SPE sorbents: OASIS HLB (3 mL, 60 mg, Waters, Milford, MA, USA), Strata-x (33 \( \mu \)m polymeric reversed phase, 6 mL, 200 mg, Phenomenex, USA), Bakerbond Speedisk extraction disk (H\textsubscript{2}O-phobic and H\textsubscript{2}O-philic, J.T. Baker, Deventer, The Netherlands) and Chromabond HR-X (3 mL, 200 mg, Macherey-Nagel, Düren, Germany) cartridges. Of all the cartridges, Chromabond\textsuperscript{®} HR-X exhibited the best performance in simultaneously retaining all the analytes at a pH of 7. Indeed, Zhang and Zhou \([36]\) demonstrated similar extraction recoveries at pH 4.2 and at pH 10.3; therefore, a pH of 7.0 was selected for the water samples to obtain an analytical compromise for the best retention for all analytes. The SPE procedure using Chromabond HR-X cartridges, which consist of a hydrophobic polystyrene-divinylbenzene copolymer, was therefore optimized. In the final extraction protocol, prior to elution, the SPE cartridges were rinsed twice with 5 mL of Milli-Q water. In marine analytical chemistry an additional advantage
of the washing step is the removal of remaining sea salt from the cartridge. Higher recovery rates were achieved by including this washing step (data not shown). Optimal elution of the pharmaceuticals was achieved using acetone followed by methanol. The most optimal elution solvent was selected on the basis of analytical characteristics such as peak area, resolution and signal-to-noise ratio.

3.1.2 Detection

Owing to the limited volatility of the pharmaceuticals, LC was the preferred chromatographic technique used in this study to achieve separation of the target analytes. Good chromatographic separation of the compounds under investigation was achieved using a C18 Isis reversed-phase LC column as the stationary phase. The retention mechanism of this column is based on steric and hydrophobic interactions and resulted in the optimized separation of all analytes. A mixture of acetonitrile and 0.1% formic acid in water proved superior as opposed to other solvent modifiers such as ammonium acetate.

Mass spectra were obtained using direct infusion of each standard in the mobile phase. The following operational parameters of the MS detector were optimized: MS ion mode, collision energy (eV), isolation width ($m/z$) and activation Q. ESI was used as the ionization source in both negative and positive ion mode by injecting the final extract twice. Detection of the negative precursor ion $[\text{M-H}]^-$ was performed for chloramphenicol, salicylic acid, bezafibrate, clofibric acid and mefenamic acid, whereas detection of the positive precursor ion $[\text{M+H}]^+$ was performed for the other compounds of interest. Precursor and product ions and collision energies are presented in Table II.2. The isolation width was set at 2.0 $m/z$, except for chloramphenicol, clofibric acid and diclofenac (3.0 $m/z$). For the activation Q, the default value of 0.25 was used, except for salicylic acid (0.35). MS$^n$ was performed for all precursor ions and allowed reliable confirmation of the target analytes. Figures II.2 and II.3 show the chromatograms and spectra obtained for the pharmaceuticals detected in positive and negative ionization mode, respectively (100 ng L$^{-1}$ spiked in seawater).
Table II.2. Precursor and product ions (m/z) and collision energy (eV) of the pharmaceuticals and internal standard (*) considered.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion</th>
<th>Product ions MS² (MS³)</th>
<th>Coll. E MS² (MS³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylic acid</td>
<td>137</td>
<td>93 (65)</td>
<td>37 (47)</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>240</td>
<td>196 (180)</td>
<td>38 (45)</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>255</td>
<td>209; 177</td>
<td>26</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>297</td>
<td>278; 250</td>
<td>30</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>213</td>
<td>127; 85</td>
<td>26</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>360</td>
<td>274 (154)</td>
<td>28 (40)</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>254</td>
<td>188; 156; 147</td>
<td>40</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>291</td>
<td>230; 258; 123</td>
<td>40</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>321</td>
<td>194; 257; 176</td>
<td>28</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>362</td>
<td>318; 344</td>
<td>45</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>237</td>
<td>194; 220; 192</td>
<td>35</td>
</tr>
<tr>
<td>Propranolol</td>
<td>260</td>
<td>183; 116; 157</td>
<td>32</td>
</tr>
<tr>
<td>Atenolol</td>
<td>267</td>
<td>225; 190; 208; 249</td>
<td>35</td>
</tr>
<tr>
<td>Isobutcar 61 (*)</td>
<td>313</td>
<td>222; 130; 196</td>
<td>32</td>
</tr>
</tbody>
</table>
Figure II.2. Chromatograms and spectra of the pharmaceuticals detected in positive ion mode (100 ng L\(^{-1}\) spiked in seawater).
3.2 Validation study

3.2.1 Identification/selectivity

Identification and confirmation of the compounds was performed according to the procedure prescribed by Commission Decision 2002/657/EC [33]. Compounds were identified on the basis of their relative retention time, which is the ratio of the retention time of the analyte to that of the internal standard. In addition, the ion ratios of the precursor and product ions in the spectrum obtained upon chromatographic analysis were taken into account when the peak in the chromatogram had a signal-to-noise ratio of at least 3:1. Commission Decision 2002/657/EC [33] also describes a system of identification points. Detection of precursor and product ions yields, respectively, one and 1.5 identification points. To obtain a minimum of four identification points, MS\textsuperscript{3} fragmentation was required for salicylic acid, bezafibrate and mefenamic acid.
3.2.2 Specificity

The specificity of our method was evaluated through the analysis of seawater samples spiked with each compound separately and of seawater samples spiked with a mixture of all compounds at a concentration of 100 ng L\(^{-1}\). The specificity of the analytical approach was confirmed since no interferences were demonstrated by using LC-MS\(^n\) as described “Experimental”. No other significant peaks with a signal-to-noise ratio of 3 or more were observed at the specific retention times of the target pharmaceuticals, indicating a high specificity of the analytical method.

3.2.3 Linearity and limit of quantification

Linearity was evaluated by seven-point calibration curves (six replicates) in seawater. Seawater samples (1 L) were spiked with a standard mixture obtaining concentrations of 1; 5; 10; 50; 100; 500 and 1,000 ng L\(^{-1}\) of the different pharmaceuticals. The mean correlation coefficients (n=6) of the calibration curves were 0.99 or higher for the target analytes, indicating good linearity in the concentration range 1–1,000 ng L\(^{-1}\) (Table II.3). This is in accordance with the correlation coefficients reported in the literature for the same pharmaceuticals in freshwater [20,30]. Despite the high salinity of our sample matrix, the linearity of the analytical method in seawater was not affected. To demonstrate the flexibility of our analytical procedure, besides seawater, calibration curves were also constructed in tap water. Correlation coefficients of 0.99 or higher were found for all compounds.

LOQs were determined using spiked matrix samples and were defined as the lowest detectable concentrations of the calibration curves with a signal-to-noise ratio of at least 10 [21,27,30,31]. The LOQs obtained for the target compounds (Table II.3) varied between 1 and 50 ng L\(^{-1}\) in seawater and were the same in tap water (chromatograms and spectra not shown). These LOQs are considered acceptable and are comparable to previously reported LOQs for the same pharmaceuticals. Indeed, similar LOQs (between 1 and 20 ng L\(^{-1}\)) were reported for bezafibrate, clofibric acid, sulfamethoxazole, carbamazepine, propranolol, chloramphenicol and thrimethoprim [32,37]. Gómez et al. [30] reported comparable LOQs for atenolol and diclofenac.
Table II.3. Validation results: limits of quantification (LOQ), correlation coefficients (R²), recovery and precision in seawater and tap water of the targeted pharmaceuticals.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOQ (ng L⁻¹)</th>
<th>R²</th>
<th>Seawater (n=42)</th>
<th>Tap water (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>5</td>
<td>≥ 0.99</td>
<td>108 27</td>
<td>98 14</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>5</td>
<td>≥ 0.99</td>
<td>95 20</td>
<td>92 14</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>50</td>
<td>≥ 0.99</td>
<td>103 18</td>
<td>109 9</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>50</td>
<td>≥ 0.99</td>
<td>101 17</td>
<td>104 14</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>5</td>
<td>≥ 0.99</td>
<td>97 18</td>
<td>94 10</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>5</td>
<td>≥ 0.99</td>
<td>98 27</td>
<td>93 11</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>10</td>
<td>≥ 0.99</td>
<td>100 19</td>
<td>106 17</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>10</td>
<td>≥ 0.99</td>
<td>108 19</td>
<td>96 12</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>5</td>
<td>≥ 0.99</td>
<td>101 17</td>
<td>102 10</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>50</td>
<td>≥ 0.99</td>
<td>97 18</td>
<td>92 14</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>5</td>
<td>≥ 0.99</td>
<td>101 19</td>
<td>100 5</td>
</tr>
<tr>
<td>Propranolol</td>
<td>1</td>
<td>≥ 0.99</td>
<td>107 18</td>
<td>94 7</td>
</tr>
<tr>
<td>Atenolol</td>
<td>50</td>
<td>≥ 0.99</td>
<td>104 16</td>
<td>105 14</td>
</tr>
</tbody>
</table>

3.2.4 Recovery and precision

Because no certified reference material was available, recoveries and intermediate precision (samples were measured on different days) were determined using seawater samples spiked with known amounts of the analytes (six replicates of seven concentrations: 1, 5, 10, 50, 100, 500 and 1,000 ng L⁻¹). The intermediate precision of the method was determined by calculating the relative standard deviation (RSD). Table II.3 summarizes the recovery and precision results. According to SANCO/825/00 revision 7 [34], typically a recovery within the range 70–110% and a repeatability of RSD≤20% are required. The mean recoveries (in the 95–108% range) were satisfactory for all the target pharmaceuticals. Zhang and Zhou [36] described the increasing extraction efficiency in
SPE of carbamazepine, sulfamethoxazole, propranolol and diclofenac owing to the increasing salt concentration. Taking this into account, we can explain the recovery rates of 100% or more of nine pharmaceuticals in this study. Except for bezafibrate and salicylic acid (RSDs of 27%), the analytical method was sufficiently precise for quantitative analysis of the pharmaceuticals (RSDs between 16 and 20%). The RSDs of bezafibrate and salicylic acid were somewhat higher at the lowest concentrations. This can be attributed to the unavailability of a representative blank sample and consequently to the variation of its presence in unspiked water samples. As can be seen from Table II.3, the method developed allows quantification of the target analytes in tap water as well. The recoveries (between 92 and 109%) and RSDs (17% or less) in tap water were satisfactory.

3.2.5 Matrix effects

Since the ESI part of the mass spectrometer may be subject to signal suppression or enhancement due to co-extracted matrix constituents [10], the validation study included the evaluation of matrix effects. Concerning the analysis of seawater samples, these matrix constituents include mainly particulate components, sea salt and other impurities. Despite the thorough optimization of our sample preparation protocol to avoid matrix effects, these were still observed following our analytical procedure. Therefore, the matrix effects were studied by comparing the calibration curves for the different compounds, in seawater and in tap water. In addition, standard mixtures in mobile phase containing concentrations equal to the spiked matrix sample concentrations were brought onto column. A signal enhancement was observed in seawater for several analytes (ofloxacin, propranolol, chloramphenicol, salicylic acid, bezafibrate, clofibric acid). On the other hand, ion suppression effects due to matrix constituents were also observed for a number of analytes (atenolol, trimethoprim, sulfamethoxazole and mefenamic acid). The influence of the matrix was negligible for carbamazepine, diclofenac and ketoprofen. No significant variations in matrix effects between the different samples were observed. The proper correction for matrix effects implies the use of one (labelled) internal standard per analyte. However, the commercial availability of reference standards is rather low, and according to the literature satisfactory results can be obtained using only one or two internal standards to correct for all compounds [11]. Therefore, with every series of
samples, calibration curves were prepared in the sample matrix to minimize the matrix effect on the quantification of the analytes.

3.3 Application to North Sea samples

The method developed was applied to water samples collected during the INRAM project (see “Study area and sampling”). Six offshore samples, 11 harbour samples and two samples from the Scheldt estuary were collected and analysed. This was repeated four times: in May and December 2007, April 2008 and June 2009. As can be deduced from Table II.4, seven different pharmaceuticals were detected. The other pharmaceuticals were not detected at any of the sampling stations. Sulfamethoxazole and trimethoprim were found up to concentrations of 96 and 29 ng L\(^{-1}\), respectively. Other antibiotics could not be detected. The widely used NSAID salicylic acid was detected very often. Salicylic acid, the deacylated, more active form of acetylsalicylic acid, was detected in more than 90% of all samples at a concentration up to 855 ng L\(^{-1}\). The \(\beta\)-blocker propranolol was found in half of the samples at levels up to 24 ng L\(^{-1}\), whereas atenolol was detected only six times up to 293 ng L\(^{-1}\). Bezafibrate was detected at concentrations below 18 ng L\(^{-1}\) and residues of the psychiatric drug carbamazepine were frequently found at levels up to 321 ng L\(^{-1}\). Salicylic acid and carbamazepine may be considered as the most relevant compounds for the North Sea and Scheldt estuary since they were detected most often and at the highest concentrations.
Table II.4. Detected concentrations (ng L⁻¹) of the pharmaceuticals of interest in water sampled in May and December 2007, April 2008 and June 2009 at the different sampling locations in the North Sea, the Scheldt estuary and Belgian harbours, (n.d. = not detected; n.m. = not measured).

<table>
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<tr>
<th>Compound</th>
<th>Sampling period</th>
<th>W01</th>
<th>W02</th>
<th>W03</th>
<th>W04</th>
<th>W05</th>
<th>S01</th>
<th>S22</th>
<th>OO1</th>
<th>OO2</th>
<th>OO3</th>
<th>OO4</th>
<th>NP1</th>
<th>NP2</th>
<th>NP3</th>
<th>ZB1</th>
<th>ZB2</th>
<th>ZB3</th>
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<tr>
<td><strong>Salicylic acid</strong></td>
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<td>May 2007</td>
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<td>126</td>
<td>26</td>
<td>53</td>
<td>65</td>
<td>18</td>
<td>n.d.</td>
<td>51</td>
<td>372</td>
<td>246</td>
<td>855</td>
<td>n.m.</td>
<td>161</td>
<td>44</td>
<td>31</td>
<td>11</td>
<td>48</td>
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<td>December 2007</td>
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<td>106</td>
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<td>68</td>
<td>59</td>
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<td>94</td>
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<td>203</td>
<td>74</td>
<td>43</td>
<td>67</td>
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<td>46</td>
<td>n.m.</td>
<td>114</td>
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<td><strong>Bezafibrate</strong></td>
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<td>n.d.</td>
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<td><strong>Trimethoprim</strong></td>
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<td><strong>Carbamazepine</strong></td>
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<td>18</td>
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<td>7</td>
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<td>5</td>
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<td>15</td>
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<td>185</td>
<td>30</td>
<td>64</td>
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<td><strong>Propranolol</strong></td>
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<td>22</td>
<td>2</td>
<td>21</td>
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<td>2</td>
<td>n.m.</td>
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<td><strong>Atenolol</strong></td>
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Many studies have reported the presence of pharmaceuticals in rivers and in influents and effluents of WWTPs [18–20,35]. The concentrations observed depend on the therapeutic class. The presence of antibiotics in surface water is generally reported in the low nanogram per litre concentration range, whereas several NSAIDs reach microgram per litre levels. The levels of salicylic acid measured in our study are within the same order of magnitude as those reported in the literature for freshwater samples. Detected concentrations of β-blockers and lipid regulators in the Belgian coastal zone are rather low in comparison with concentrations detected in surface water [18,38]. On the other hand, carbamazepine occurs at high levels and is detected very often. This can be explained by its low removal efficiency in WWTPs [21,37]. With regard to marine waters, Buser et al. [39] and Weigel et al. [25,40] reported the presence of clofibric acid and carbamazepine in the North Sea at concentrations of approximately 1 and 2 ng L⁻¹, respectively. More recent, Togola and Budzinski [41] reported higher concentrations for carbamazepine, diclofenac and ketoprofen in the Mediterranean Sea. However, in general, monitoring data for pharmaceuticals in the marine environment are rather sparse. This may reflect the absence of a method to extract and identify multiclass pharmaceuticals in marine systems [40]. In this study, a novel multiclass analytical method for pharmaceuticals was developed that clearly showed the ubiquitous character of several pharmaceutical compounds in seawater and as a result in the marine environment as well. These findings will assist to further research within the INRAM project, in which risk assessments of the pharmaceuticals detected for the marine environment will be performed.

The concentrations of some pharmaceuticals under investigation show large temporal variations. However, except for salicylic acid at W01 and W02, the same general picture of lower concentrations in the North Sea (W01 – W06) in comparison with the harbours and the Scheldt estuary could be noticed. We suggested that, owing to both dilution and degradation, there is little transport from the Scheldt estuary and the harbours to the open sea. Also previous studies on oestrogens, nonyl phenol ethoxylates, and polychlorinated biphenyls reported the limited transport from the Scheldt estuary to the North Sea [42–44]. At sampling locations OO02 and S22, target pharmaceuticals were detected most frequently and at the highest concentrations. OO02 is situated at the mouth of the river Noord-Ede and the canal Bruges-Ostend in the harbour of Ostend (in the middle of the Belgian coastal zone), whereas S22 is located in the Scheldt estuary in Antwerp. Several
WWTPs are located close to OO02 and in Flanders the effluents of more than 65 WWTPs are discharged into the Scheldt estuary. Furthermore, the WWTPs with the largest capacity are located near the Scheldt estuary: Deurne, Ghent and Antwerp (approximately 200,000 inhabitant equivalent) (Aquafin, personal communication). It may be concluded that both locations receive major inputs of contaminated industrial and domestic wastewater, probably resulting in the increased presence of the target pharmaceuticals.

4. CONCLUSION

In this study, an analytical method for the quantification of important pharmaceuticals in seawater was developed and optimized. A combination of SPE and LC-MS enabled the detection and quantification of multiclass pharmaceuticals of widely differing chemical structures in seawater at the low nanogram per litre level. The method was validated according to the laboratory quality assurance criteria developed in accordance with Commission Decision 2002/657/EC and SANCO/825/00 revision 7 [33,34]. Application of the procedure to North Sea and Scheldt estuary samples confirmed the occurrence of seven pharmaceuticals in the marine environment up to the low microgram per litre level. Frequently detected compounds were salicylic acid, carbamazepine and propranolol. Two antibiotics were detected occasionally: sulfamethoxazole and thrimethoprim. Little transport of pharmaceuticals could be observed from the Scheldt estuary and the harbours to the open sea. In general, it may be concluded that the results of this 2-year monitoring study are quite novel and may provide relevant insights into the field of pharmaceutical analysis in the marine environment.

5. REFERENCES

CHAPTER III

A VALIDATED ANALYTICAL METHOD FOR THE DETERMINATION OF PERFLUORINATED COMPOUNDS IN SURFACE-, SEA- AND SEWAGEWATER USING LIQUID CHROMATOGRAPHY COUPLED TO TIME-OF-FLIGHT MASS SPECTROMETRY

Adapted from:

CHAPTER III

A VALIDATED ANALYTICAL METHOD FOR THE DETERMINATION OF PERFLUORINATED COMPOUNDS IN SURFACE-, SEA- AND SEWAGEWATER USING LIQUID CHROMATOGRAPHY COUPLED TO TIME-OF-FLIGHT MASS SPECTROMETRY

Abstract

Perfluorinated compounds (PFCs), which are extensively used in a wide variety of applications because of their specific surfactant properties, have recently appeared as an important new class of global environmental pollutants. Quantitative analysis of PFCs in aqueous matrices remains, however, a challenging task. During this study, a new analytical method for the determination of 14 PFCs in surface-, sewage- and seawater was developed and validated. The target analytes were extracted using solid-phase extraction followed by liquid chromatography coupled to a time-of-flight mass spectrometer (LC–ToF-MS). The use of very narrow mass tolerance windows (<10 ppm) resulted in a highly selective MS-technique for the detection of PFCs in complex aqueous matrices. Validation of this analytical method in surface-, sewage and seawater resulted in limits of quantification (LOQs) varying from 2 to 200 ng L\(^{-1}\), satisfying recoveries (92-134\%) and precision (6-19 RSD\%), and good linearity (\(R^2 = 0.99\) for most analytes, n=8). Analysis of samples of the North Sea, the Scheldt estuary, and three harbours of the Belgian coastal region led to the detection of four different PFCs. Perfluorooctane sulfonate (PFOS) was found to be the most abundant PFC in levels up to 39 ng L\(^{-1}\).
1. INTRODUCTION

Perfluorinated compounds (PFCs) constitute a large group of chemicals characterized by a fully fluorinated hydrophobic carbon chain attached to various hydrophilic heads [1]. The chemical class of PFCs includes the perfluoroalkyl carboxylates, the perfluoroalkyl sulfonates, the perfluoroalkyl sulfonamides, and related products. Their chemical and thermal stability and surface tension lowering properties make them very useful for a wide variety of applications and products: as additives in fire-fighting foam and food packaging, as fat and water repellents for textile, paper and leather treatment, as performance chemicals, and as polymerization aid for the production of fluorinated polymers such as polytetrafluoroethylene (PTFE) and polyvinylidene fluoride (PVDF) [1,2]. Within the group of PFCs, perfluorooctane sulfonate (PFOS), the final degradation product of the frequently used sulfonated fluorochemicals, has been identified as the most important contaminant [2]. Moreover, PFOS fulfils the criteria of a persistent organic pollutant (POP) under the Stockholm convention [3]. As a result, EU legislation established the PFOS directive 2006/122/EC [4] which aims at ending the use of PFOS.

In general, PFCs have been reported as extremely persistent environmental contaminants with bioaccumulative and toxic properties [5,6]. Consequently, the concern about the environmental fate and prevalence of PFCs has increased in recent years. Recent monitoring studies have reported the widespread occurrence of PFCs in water [7], air [8], and biological matrices [9]. In surface water [10–12] as well as in wastewater [13,14], PFCs have generally been detected in the ng L\(^{-1}\) up to µg L\(^{-1}\) concentration range. Furthermore, PFCs have been found in seawater and open ocean waters, implying the transport of PFCs from surface water through estuaries to coastal regions and consequently to open oceans [15–17]. According to Van Leeuwen et al. [18], the quality of data obtained from analysis of PFCs in environmental matrices is a major issue of concern. The occurrence of branched isomers, matrix interferences, and cross contamination rendered quantitative analysis of PFCs in aqueous matrices a challenging task. To the best of our knowledge, the use of accurate mass high-resolution mass spectrometric techniques for the quantification of PFCs in water samples has not been reported earlier [19].

Therefore, in this study, an analytical methodology was developed using liquid chromatography (LC) coupled to a time-of-flight mass spectrometer (ToF-MS). ToF-MS
provides sensitive full scan data and allows the detection of the target PFCs by accurate mass measurements, resulting in a highly selective MS-technique. Fourteen environmentally relevant PFCs were selected, including four perfluoroalkyl sulfonates, nine perfluoroalkyl carboxylic acids and perfluorooctane sulphonamide. A validation study was carried out to demonstrate the applicability of this analytical approach. Finally, the developed method was applied to marine water samples from the North Sea and Scheldt estuary to examine the presence of PFCs in the Belgian marine environment.

2. MATERIALS AND METHODS

2.1 Study area and sampling

The study area and sampling method were already described in Chapter II, section 2.1 and Figure II.1. However, the sampling campaign for PFC-analyses of water samples was carried out in June 2009 and the water samples were stored at 4 °C in the dark without adjustment of the pH.

2.2 Reagents and chemicals

Fourteen PFCs were examined in this study: four perfluorosulfonates (potassium perfluoro-1-butane sulfonate, sodium perfluoro-1-hexane sulfonate, sodium perfluoro-1-octane sulfonate, and sodium perfluoro-1-decane sulfonate), nine perfluorocarboxylates (perfluoro-n-pentanoic acid, perfluoro-n-hexanoic acid, perfluoro-n-heptanoic acid, perfluoro-n-octanoic acid, perfluoro-n-nonanoic acid, perfluoro-n-decanoic acid, perfluoro-n-undecanoic acid, perfluoro-n-dodecanoic acid, and perfluoro-n-tetradecanoic acid) and perfluoro-1-octane sulphonamide. All analytical standards were purchased from Wellington Laboratories (Guelph, Ontario, Canada) with chemical purities of more than 98%. Six $^{13}$C-labelled internal standards were used as well: sodium perfluoro-1-[1,2,3,4-$^{13}$C$_4$] octane sulfonate, perfluoro-n-[1,2-$^{13}$C$_2$] hexanoic acid, perfluoro-n-[1,2,3,4-$^{13}$C$_4$] octanoic acid, perfluoro-n-[1,2,3,4,5-$^{13}$C$_5$] nonanoic acid, perfluoro-n-[1,2-$^{13}$C$_2$] decanoic acid, and perfluoro-n-[1,2-$^{13}$C$_2$] dodecanoic acid.

Methanol was purchased from Rathburn Chemicals (LTd Walkerburn, Scotland), while HPLC-grade water was obtained from Biosolve (Biosolve Chemicals, The Netherlands). Ammonium acetate (2.5mM) in water was obtained from dilution of LC–MS
Chromasolv® water containing 0.1% ammonium acetate (Sigma–Aldrich Labormchemikalien GmbH, Seelze). Except for PFOSA, primary stock solutions of all individual analytes were prepared in methanol at a concentration of 50 µg mL\(^{-1}\). PFOSA was purchased in nonane at the same concentration. Working standard mixture solutions were prepared by appropriate dilution of the stock solutions in methanol. All solutions were stored at −20 °C in the dark.

2.3 Extraction and clean-up

The sample preparation protocol was based on the ISO 25101/2006 method [20], which was in its turn derived from the method of Taniyasu et al. [21]. Sewage water samples and water samples, visibly containing particulate matter, were filtered through a glass fibre paper (GF 52 Ø110mm, Schleicher & Schuell, Dassel, Germany) prior to extraction. Depending on the aqueous matrix, different volumes of water were extracted. In case of surface- and sewage water, 50 mL water was extracted, while 250 mL was used for seawater samples. The \(^{13}\)C-labelled internal standards were supplemented to every sample prior to extraction to a final concentration of 100 ng L\(^{-1}\). Solid-phase extraction was carried out using Oasis HLB cartridges (6 cm\(^3\), 200 mg, Waters, Milford, MA). The cartridges were pre-conditioned with 2 mL methanol and 2 mL Biosolve water. After loading, the cartridges were rinsed with 2 mL Biosolve water for surface- and sewage water. For seawater, 3× 2 mL Biosolve water was applied. Subsequently, the cartridges were dried under vacuum for 10 min. Elution was achieved using 2× 2 mL methanol. Next, extracts were concentrated to 0.5 mL under a gentle stream of nitrogen. Finally, 0.5 mL of 2.5 mM ammonium acetate in water was added before transfer to LC–MS vials. Samples were stored at 4 °C before analysis.

2.4 Chromatographic and mass spectrometric instrumentation

The LC-apparatus comprised of a 1200 series binary gradient pump and a 1100 series autosampler (Hewlett Packard, Palo Alto, CA, USA). Chromatographic separation was achieved using a Luna® C18 (2) HPLC column (5 µm particle size, 250 mm×2.0 mm; Phenomenex Inc., Utrecht). The mobile phase consisted of a mixture of (A) 2.5 mM ammonium acetate in water and (B) methanol. A linear gradient of 0.3 mL min\(^{-1}\) was used starting with a mixture of 50% A and 50% B, increasing to 90% B in 10 min. This ratio was kept for 6 min before reversion to the initial conditions.
Analytes were detected with a time-of-flight mass spectrometer equipped with a dual electrospray ionisation interface (ESI MSD ToF, Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer was operated in the negative ion mode. Instrument parameters were: drying gas temperature of 325 °C, drying gas flow of 5 L min\(^{-1}\), nebuliser pressure of 20 psi, capillary voltage of 3500 V, and chamber voltage of 3000 V. Before analyzing a series of samples, the ToF-MS apparatus was tuned and calibrated using the ESI Tuning Mix (Agilent Technologies, Santa Clara, CA, USA). During analysis, a reference solution was pumped into the MS system at a rate of 50 µL min\(^{-1}\) using a separate sprayer connected to a 1100 series pump (Hewlett Packard, Palo Alto, CA, USA). This reference solution consisted of purine with a \(m/z\) ratio of 119.0363 and HP-0921 (hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) with a \(m/z\) ratio of 980.0164 in ACN/H\(_2\)O (95/5) (Agilent Technologies, Santa Clara, CA, USA). Accurate mass measurements could only be achieved if these reference masses were detectable. Chromatograms and spectra were recorded and processed using Agilent MassHunter Quantitative Analysis® software (Agilent Technologies, Santa Clara, CA, USA).

2.5 Validation of the method

To demonstrate the applicability of this analytical approach, a validation study was carried out. Besides the validation procedure of the accredited lab of the Flemish Environment Agency (FEA), the SANCO/2007/3131 document [22] was also used as guideline for the validation of this new analytical method. Validation comprised the assessment of specificity/selectivity, linearity, recovery, precision, and the limits of detection (LODs) and quantification (LOQs). Eight-point calibration curves were constructed in surface water (six replicates). To demonstrate the multi-matrix capacity of our analytical procedure, a limited identical validation study was performed using seawater and sewage water as well (four replicates). Representative water samples, being water from the river Kale – which is a small river near Ghent – for surface water, coastal water from the North Sea for seawater, and wastewater from industrial plants for sewage water were used to this purpose. These water samples were spiked with a standard mixture at final concentrations of 5; 7.5; 10; 20; 50; 100 and 250 ng L\(^{-1}\) and analysed as described in Sections 2.3 and 2.4. In addition, blank samples were analysed as well.
2.6 Quality assurance

Before and after analysis of a series of samples, a standard mixture (0.5 ng on column) of the targeted PFCs and the internal standards was injected to check the instrument parameters of the LC–ToF-MS system. Quality control of the method was performed by analysis of a blank sample, together with a linear calibration curve constructed using matrix samples spiked with standard solutions at seven concentration levels in the range of 5 and 250 ng L\(^{-1}\). This was performed for every series of samples. The obtained calibration curves were used for quantification.

3. RESULTS AND DISCUSSION

3.1 Background contamination

Background contamination in the analytical blanks is a major problem in the analysis of PFCs [21,23,24]. Contamination from laboratory products and instrumentations containing polytetrafluoroethylene and perfluoroalkoxy compounds, is hard to avoid. Therefore, as suggested in literature [23,25], several measures were taken to minimize this kind of contamination. Teflon materials were avoided throughout the extraction procedure. All glass material was cleaned and placed in a drying oven (400 °C) in advance. Furthermore, an additional HPLC column was placed between the pump and the injector. As a result, PFCs originating from tubing or solvents, obtained extra retention and were thus separated from the target PFCs in the sample. Thanks to the elimination of these potential sources of contamination, none of the target compounds were detected in instrumental (direct injection of the mobile phase) and procedural blanks (extracted samples of Biosolve water).

In addition, due to the ubiquitous character of PFOS, analysis of unspiked water samples, i.e. water from the river Kale for surface water, coastal water from the North Sea for seawater, and wastewater from industrial plants for sewagewater, frequently resulted in its detection in the low ng L\(^{-1}\) concentration range. For the validation of our new analytical method, the calibration curves were corrected for these concentrations.
3.2 Optimization of sample preparation

De Voogt and Sáez [25] suggested to avoid filtration because of the surface-active nature of PFCs. For this reason, filtration was only executed in case of sewagewater samples and water samples, visibly containing particulate matter, to avoid clogging of the cartridge during SPE. Preconcentration was necessary to determine low concentrations of PFCs in the water samples. To this end, solid-phase extraction is certainly the most suitable and commonly used technique [17,21,23,26,27].

Optimization of the SPE-procedure was performed by varying the sample volume, sample pH and the type of SPE cartridge. Dependent on the aqueous matrix, different sample volumes were extracted. For detection in surface- and sewagewater, a sample volume of 50 mL was found to be sufficient to meet satisfying LODs of \( \leq 10 \) ng L\(^{-1}\) for the major contaminants PFOS and PFOA. Compared to limnic systems, concentrations of most organic pollutants in the open sea are low [28]. Therefore, the sample volume was increased to 250 mL for PFC-analysis in seawater samples, resulting in LODs of \( \leq 5 \) ng L\(^{-1}\) for PFOS and PFOA (Table III.1).
Table III.1. Validation results: limits of detection (LODs) and quantification (LOQs) in ng L⁻¹, correlation coefficients (R²), recoveries (%), and intralaboratory reproducibility (RSD %) of the targeted PFCs in surface water, sea- and sewagewater. (* could not be quantified in this aqueous matrix)

| Compound | Surface water | | | Seawater | | | Sewage water | | |
|----------|--------------|------------------|------------------|------------------|------------------|------------------|
|          | LOD  | LOQ  | R² | Recovery | R.S.D. | LOD  | LOQ  | R² | Recovery | R.S.D. | LOD  | LOQ  | R² | Recovery | R.S.D. |
| PFBS     | 50   | 100  | 0.99 | 95     | 14     | 20   | 40   | 0.99 | 100     | 6     | 50   | 100  | 0.99 | 102     | 8     |
| PFHxS    | 10   | 20   | 0.99 | 92     | 10     | 1    | 2    | 0.99 | 108     | 7     | 5    | 10   | 0.99 | 99      | 9     |
| PFOS     | 7.5  | 15   | 0.99 | 92     | 14     | 1    | 2    | 0.99 | 101     | 7     | 5    | 10   | 0.99 | 96      | 16    |
| PFDS     | 20   | 40   | 0.99 | 108    | 26     | 10   | 20   | 0.99 | 96      | 18    | 10   | 20   | 0.99 | 113     | 13    |
| PFP*A*   | -    | -    | -    | -      | -      | 50   | 100  | 0.99 | 97      | 7     | 100  | 200  | 0.99 | 100     | 7     |
| PFHxA    | 50   | 100  | 0.99 | 102    | 8      | 20   | 40   | 0.99 | 103     | 12    | 50   | 100  | 0.99 | 98      | 6     |
| PFHpA    | 10   | 20   | 0.99 | 99     | 10     | 10   | 20   | 0.99 | 101     | 8     | 10   | 20   | 0.99 | 100     | 7     |
| PFOA     | 7.5  | 15   | 0.99 | 95     | 13     | 5    | 10   | 0.99 | 99      | 10    | 10   | 20   | 0.99 | 103     | 11    |
| PFNA     | 10   | 20   | 0.99 | 95     | 13     | 10   | 20   | 0.99 | 102     | 11    | 20   | 40   | 0.99 | 100     | 8     |
| PFDA     | 20   | 40   | 0.99 | 98     | 18     | 10   | 20   | 0.99 | 103     | 11    | 20   | 40   | 0.99 | 101     | 9     |
| PFUnA    | 50   | 100  | 0.98 | 103    | 16     | 10   | 20   | 0.99 | 103     | 17    | 50   | 100  | 0.99 | 99      | 12    |
| PFDoA    | 20   | 40   | 0.98 | 123    | 23     | 80   | 160  | 0.98 | 104     | 19    | 50   | 100  | 0.99 | 99      | 13    |
| PFTeA    | 100  | 200  | 0.95 | 134    | 12     | 100  | 200  | 0.96 | 100     | 18    | 100  | 200  | 0.99 | 93      | 13    |
| PFOSA    | 10   | 20   | 0.99 | 99     | 17     | 5    | 10   | 0.99 | 98      | 7     | 20   | 40   | 0.99 | 105     | 18    |
For solid-phase extraction of PFCs in water samples, the use of Oasis HLB or Oasis WAX cartridges has been reported by several studies [7,21,29–32]. Therefore, these two types of cartridges were examined within this study. The choice of the SPE-sorbent was mainly determined by the obtained recovery rates. Adjusting the sample pH to 3, Oasis WAX provided good results for the majority of the target analytes. However, using the Oasis HLB cartridges at neutral sample pH, higher recoveries for all target PFCs were obtained. Therefore, the Oasis HLB sorbent was selected for further experiments. These cartridges allow extraction of acidic, neutral, and basic analytes at neutral pH due to a combination of hydrophilic and lipophilic characteristics [33]. The Oasis HLB cartridges were rinsed with 2 mL of Biosolve water before elution (3× 2 mL for seawater samples). Washing the cartridges has been shown to remove interfering matrix components and remaining sea salt (in the case of seawater samples) from the cartridge [34,35]. Optimal elution of the PFCs was achieved using methanol.

### 3.3 LC–ToF-MS optimization

A Luna® C18 (2) HPLC column was used for chromatographic separation of the analytes. The stationary phase of this LC column consists of ultrapure metal-free silica (99.99% purity) bounded to C18-groups. The performance of the column was evaluated by the separation efficiency for the structurally related PFCs. Good chromatographic separation of the compounds under investigation was achieved using the Luna C18 (2) column. In addition, high peak efficiencies, measured as peak width at the baseline, were obtained using this column (Fig. III.1).
With respect to PFC-analysis, Berger et al. [36] compared three different MS-techniques coupled to LC: ion-trap MS, triplequadrapole MS, and ToF-MS. ToF-MS was reported to be the optimal detector for quantification of PFCs, combining high selectivity with high sensitivity. For analysis of PFCs, LC–ToF-MS has been applied as a screening [37] and confirmation [38,39] technique in biological matrices. To our knowledge, the use of ToF-MS for the quantification of PFCs in water samples has not been published earlier. The ToF-MS system used during this study, is capable of producing spectra with a mass resolution of 4000 (at m/z 200) to >10,000 (at m/z 2722) (Agilent Technologies, technical overview). According to Van der Heeft et al. [40], the mass resolving power needs to be greater than 10,000 for the entire mass range to qualify for high resolving power MS. However, Kauffmann and Butcher [41] concluded that a mass resolution of 5000–10,000 is sufficient to discriminate analytes from co-eluting sample matrix compounds. The exact masses, the mean measured masses, and the mean mass errors of the detected PFCs at LOD-level were calculated and presented in Table III.2. Except for PFPA and PFBS,
the mean mass errors were below 5 ppm. Generally, using ToF-MS with a mass resolution of 10,000 FWHM (full width at half maximum; ~5000 mass resolution), a deviation of the detected mass of 10 ppm is acceptable [42]. In conclusion, our ToF-MS application enabled the use of a very narrow mass tolerance window of ±5 ppm, thus providing a high mass accuracy.

Table III. 2. Characteristics of the PFC-analysis using a ToF-MS: theoretical masses, mean measured masses, mean mass errors, and internal standards used.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Theoretical m/z [M-H]- ion</th>
<th>Mean measured m/z</th>
<th>Mean mass error (ppm)</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFBS</td>
<td>298.9430</td>
<td>298.9459</td>
<td>6.0</td>
<td>13C4PFOS</td>
</tr>
<tr>
<td>PFHxS</td>
<td>398.9366</td>
<td>398.9375</td>
<td>2.3</td>
<td>13C4PFOS</td>
</tr>
<tr>
<td>PFOS</td>
<td>498.9302</td>
<td>498.9319</td>
<td>3.8</td>
<td>13C4PFOS</td>
</tr>
<tr>
<td>PFDS</td>
<td>598.9233</td>
<td>598.9243</td>
<td>2.0</td>
<td>13C4PFOS</td>
</tr>
<tr>
<td>PFPA</td>
<td>262.9755</td>
<td>262.9772</td>
<td>5.4</td>
<td>13C2PFHxA</td>
</tr>
<tr>
<td>PFHxA</td>
<td>312.9728</td>
<td>312.9734</td>
<td>3.0</td>
<td>13C2PFHxA</td>
</tr>
<tr>
<td>PFHpA</td>
<td>362.9696</td>
<td>362.9701</td>
<td>2.7</td>
<td>13C4 PFOA</td>
</tr>
<tr>
<td>PFOA</td>
<td>412.9664</td>
<td>412.9684</td>
<td>4.8</td>
<td>13C4 PFOA</td>
</tr>
<tr>
<td>PFNA</td>
<td>462.9632</td>
<td>462.9649</td>
<td>4.0</td>
<td>13C5PFNA</td>
</tr>
<tr>
<td>PFDA</td>
<td>512.9600</td>
<td>512.9625</td>
<td>4.8</td>
<td>13C2PFDA</td>
</tr>
<tr>
<td>PFUnA</td>
<td>562.9563</td>
<td>562.9584</td>
<td>3.8</td>
<td>13C2PFDA</td>
</tr>
<tr>
<td>PFDaA</td>
<td>612.9531</td>
<td>612.9554</td>
<td>3.7</td>
<td>13C2PFDaA</td>
</tr>
<tr>
<td>PFTeA</td>
<td>712.9467</td>
<td>712.9479</td>
<td>3.4</td>
<td>13C2PFDaA</td>
</tr>
<tr>
<td>PFOSA</td>
<td>497.9457</td>
<td>497.9467</td>
<td>2.1</td>
<td>13C4PFOS</td>
</tr>
</tbody>
</table>

The use of isotopically labelled internal standards for PFC analysis is highly recommended [43]. However, ionization suppression caused by internal standards may occur and result in lower sensitivity [43,44]. Therefore, the internal standard concentration (100 ng L\(^{-1}\)) was kept quite low. For six PFCs, the corresponding 13C-labelled internal standards were used, while the most appropriate internal standard available was used for the other compounds (Table III.2).

Analytes were identified on the basis of their relative retention time, which is the ratio of the retention time of the analyte to that of the internal standard. In addition, the accurate mass of the deprotonated molecular ions ([M–H]\(^{-}\)) in the spectrum was taken into
account when the chromatographic peak of interest had a signal-to-noise ratio of at least 3:1. As mentioned before, errors in measured masses of known compounds are generally in the range of 5–10 ppm [42,45]. Therefore, within this study, a maximum mass error of 10 ppm was allowed. Upon identification, area ratios were determined by integration of the area of an analyte under the obtained chromatograms in reference to the integrated area of the internal standard (Fig. III.1). The analyte concentrations were calculated by fitting their area ratios in an eight-point calibration curve, established by matrix samples spiked with a standard mixture obtaining concentrations in the range of 0–250 ng L$^{-1}$.

3.4 Validation study

According to Van Leeuwen et al. [18], the quality of data obtained from analysis of PFCs in environmental matrices is a major issue of concern. Therefore, a validation study was carried out to demonstrate the method’s performance. The method was evaluated for specificity/selectivity, linearity, recovery, intra-laboratory reproducibility, and limits of detection (LODs) and quantification (LOQs). Matrix-matched calibration curves, in which the analytes were spiked into the representative aqueous matrix, were used for quantification. As such, the study of matrix-induced suppression or enhancement effects could be neglected.

3.4.1 Specificity/selectivity

The specificity of our method was evaluated through the analysis of water samples spiked with each compound separately, and of water samples spiked with a mixture of all compounds at a concentration of 100 ng L$^{-1}$. The specificity of the analytical approach was confirmed since no interferences were demonstrated by using LC–ToF-MS as described above. No other significant peaks with a signal-to-noise ratio of 3 or more were observed at the specific retention times of the targeted PFCs, suggesting a high specificity of the analytical method. This was in accordance with previous reports, since excellent specificity for unequivocal compound identification is guaranteed when using a ToF-MS system [37].

3.4.2 Linearity

Linearity was evaluated in the 0–250 ng L$^{-1}$ concentration range. Eight-point calibration curves were constructed in surface water (six replicates), sea- and sewagewater (both four
replicates). The water samples were spiked with a standard mixture obtaining concentrations of 5; 7.5; 10; 20; 50; 100 and 250 ng L$^{-1}$. In addition, unspiked matrix samples were analysed as well, to check the occurrence of PFCs in blank samples. The mean regression coefficients ($R^2$) of the calibration curves were calculated by plotting area ratio versus concentration. For most target compounds, regression coefficients of 0.99 or higher were found, suggesting a good linear correlation (Fig. III.1 and Table III.1). Regression coefficients below 0.99 were found for the compounds with a longer carbon chain: $R^2$ was 0.98 for PFUnA in surface water and for PFDoA in both surface and seawater, while $R^2$ was 0.95 and 0.96 for PFTeA in surface and seawater, respectively. In addition, using the regression equation, the residuals were calculated as the difference of the obtained concentrations and the expected concentrations. Next, the obtained residuals were plotted versus the concentrations (data not shown). For all compounds, the residuals were randomly distributed, thus indicating a linear correlation in the 0–250 ng L$^{-1}$ concentration range.

3.4.3 Limits of detection (LODs) and quantification (LOQs)

Limits of detection (LODs) and quantification (LOQs) were determined based on the outcome of the eight-point calibration curves of Section 3.4.2. The concentrations of the analytes were calculated using the overall equation of the calibration curves. The LOD was defined as the higher value of the following two alternatives: (1) the lowest detectable concentration of the calibration curve with a signal-to-noise ratio of at least 3:1; (2) three times the standard deviation of the analytes concentration at the lowest detectable concentration level. The LOQs were defined as the final LOD multiplied by 2. This procedure was executed for the different aqueous matrices. The obtained LOQs of the targeted PFCs varied from 15 to 200 ng L$^{-1}$ in surface water, from 2 to 200 ng L$^{-1}$ in seawater, and from 10 to 200 ng L$^{-1}$ in sewagewater (Table III.1). Thanks to the higher sample volume, the LOQs were lower in seawater samples. Since the method was in particular optimized for the detection of PFOS and PFOA in surface water, the two major contaminants within the group of PFCs, their LOQs were the lowest: i.e. 15 ng L$^{-1}$. Generally, higher LOQs were found for the analytes with both the shortest (PFBS, PFPA) and longest carbon chain length (PFUnA, PFDoA, and PFTeA).

In general, these quantitation limits are considered acceptable and are comparable to previous reported LOQs for the same analytes. For example, Taniyasu et al. [46]
determined LODs between 4 and 60 ng L\(^{-1}\) for PFOS, PFHxS and PFBS. More sensitive methods are reported in literature as well, thanks to higher sample volumes or to the use of more sensitive mass spectrometers (triple-quadrupole mass spectrometers). The analysis of ppq (pg/L) concentrations of PFCs was reported by Yamashita et al. [15,23], Taniyasu et al. [21], and Ahrens et al. [17]. For example, the latter study reported method quantification limits of 0.004–0.367 ng L\(^{-1}\) for the same analytes. However, to obtain these LOQs, 5 L water samples were extracted, while the sample volume in this study varied between 50 and 250 mL. Once more, we would like to underline that none of the mentioned studies from the literature used accurate mass, high-resolution time-of-flight mass spectrometry to detect PFCs in aqueous matrices.

3.4.4 Recovery and precision

Since no certified reference material was available, trueness of the measurements and intra-laboratory reproducibility (samples were measured on different days and by different analysts) were assessed using blank matrix samples spiked at both the LOQ level and two times the LOQ level. This was performed in six replicates. The intra-laboratory reproducibility of the method was determined by calculating the relative standard deviation (% RSD). Table III.1 summarizes the obtained results for the different matrices. According to SANCO/2007/3131 [22], typically a recovery within the range of 70–120% and a reproducibility RSD \(\leq\)20% are required. As can be deduced from Table III.1, except for PFD\(\alpha\)A and PF\(\tau\)eA in surface water, all obtained recoveries were satisfactory. The obtained RSD values indicated satisfying precision for most analytes in the different matrices. Except for PFDS and PFD\(\alpha\)A in surface water, the analytical method was sufficiently precise for quantitative analysis of the selected PFCs in all three matrices.

3.5 Application to North Sea samples

The developed method was applied to water samples collected during the INRAM project (see Section 2.1). Six offshore samples, 11 harbour samples and 2 samples of the Scheldt estuary were collected in June 2009. As can be seen from Table III.3, four different PFCs were detected in all waters samples. The other PFCs were not detected at any of the sampling stations. PFOS was detected in every sample in levels up to 39 ng L\(^{-1}\), while PF\(\tau\)oA was found once at a concentration of 26 ng L\(^{-1}\) at sampling location S22 in
Antwerp. PFHxS and PFOA were frequently detected up to concentrations of 13 and 24 ng L$^{-1}$, respectively, both at sampling location S22 (Antwerp).
Table III.3. Detected concentrations (ng L\(^{-1}\)) of the PFCs of interest in water sampled at the different sampling locations in the Belgian coastal zone: harbour of Nieuwpoort (NP1-3), harbour of Ostend (OO1-4), harbour of Zeebrugge (ZB1-4), the North Sea (W01-W06), and the Scheldt estuary (S01: near Vlissingen, S22: near Antwerp) (n.d. = not detected).

<table>
<thead>
<tr>
<th>Sampling stations</th>
<th>W01</th>
<th>W02</th>
<th>W03</th>
<th>W04</th>
<th>W05</th>
<th>W06</th>
<th>S01</th>
<th>S22</th>
<th>NP1</th>
<th>NP2</th>
<th>NP3</th>
<th>OO1</th>
<th>OO2</th>
<th>OO3</th>
<th>OO4</th>
<th>ZB1</th>
<th>ZB2</th>
<th>ZB3</th>
<th>ZB4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFHxS</td>
<td>&lt;LOQ</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&lt;LOQ</td>
<td>n.d.</td>
<td>n.d.</td>
<td>4</td>
<td>13</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&lt;LOQ</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PFOS</td>
<td>4</td>
<td>2</td>
<td>&lt;LOQ</td>
<td>3</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>5</td>
<td>39</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>13</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
Despite the limited monitoring study, certain differences could be observed between the sampling stations in the study area. As can be seen from Table III.3, the harbour of Ostend and in particular the Scheldt estuary were most contaminated with PFCs. Analysis of the S22-sample resulted in the detection of four different PFCs, up to 39 ng L$^{-1}$. Since S22 is located in the industrial zone of Antwerp, large inputs of PFCs could be expected. Samples of the North Sea (W01–W06) were the least contaminated with PFCs. At these locations, only PFOS could be quantified in concentrations below 5 ng L$^{-1}$. These concentrations of PFOS were in the same range as those reported in previous studies of the German Bight, which is the south-eastern bight of the North Sea [17,47,48]. In accordance with Ahrens et al. [17], a decreasing contamination of PFCs with increasing distance from the coast, could be observed.

Compared to the derived PNEC values of 25 µg L$^{-1}$ for PFOS [49] and 250 µg L$^{-1}$ or 1.25 mg L$^{-1}$ for PFOA [49,50], adverse risks to aquatic organisms are not anticipated from these measured concentrations. However, the possible combined effects that the abundance of several PFCs may cause, possibly even with other micropollutants, cannot be excluded. Next to these toxicity thresholds, two studies determined a health-based guidance for PFOA in drinking water as well. According to the US Safe Drinking Water Act [51], these studies reported drinking water equivalent levels (DWEL: the lifetime exposure level of a contaminant at which adverse health effects are not anticipated to occur, assuming 100% exposure from drinking water). Tardiff et al. [52] found DWELs for PFOA ranging from 0.88 to 2.4 µg L$^{-1}$, while a guidance value of 0.040 µg L$^{-1}$ was recommended by Post [53]. The detected PFOA concentrations did also not exceed these drinking water levels.

4. CONCLUSION

A validated analytical method for the determination of 14 PFCs in surface, sewage and seawater is presented. The analytical procedure consisted of SPE applied to the water samples followed by LC–ToF-MS. The use of very narrow mass tolerance windows (<10 ppm) resulted in a highly selective MS-technique for the detection of PFCs in complex aqueous matrices. The LOQs varied between 2 and 200 ng L$^{-1}$ and recoveries obtained in surface water (92–134%) were satisfactory. Application of the method to North Sea and
Scheldt estuary samples confirmed the occurrence of several PFCs in the marine environment in levels up to 39 ng L\(^{-1}\).

5. ACKNOWLEDGEMENT

The authors would like to thank L. Raman for her practical assistance in the laboratory.

6. REFERENCES


CHAPTER IV

DEVELOPMENT OF ANALYTICAL STRATEGIES USING U-HPLC-MS/MS AND LC-TOF-MS FOR THE QUANTIFICATION OF MICROPOLLUTANTS IN MARINE ORGANISMS

Adapted from:

CHAPTER IV

DEVELOPMENT OF ANALYTICAL STRATEGIES USING U-HPLC-MS/MS AND LC-TOF-MS FOR THE QUANTIFICATION OF MICROPOLLUTANTS IN MARINE ORGANISMS

Abstract

Organic micropollutants such as pharmaceuticals, perfluorinated compounds (PFCs), and pesticides, are important environmental contaminants. To obtain more information regarding their presence in marine organisms, an increasing demand exists for reliable analytical methods for quantification of these micropollutants in biotic matrices. Therefore, we developed extraction procedures and new analytical methods for the quantification of 14 pesticides, 10 PFCs, and 11 pharmaceuticals in tissue of marine organisms, namely blue mussels (Mytilus edulis). This paper presents these optimized analytical procedures and their application to M. edulis, deployed at five stations in the Belgian coastal zone. The methods consisted of a pressurized liquid extraction and solid-phase extraction (SPE) followed by ultra high performance liquid chromatography coupled to triple quadrupole mass spectrometry for pharmaceuticals and pesticides, and of a liquid extraction using acetonitrile and SPE, followed by liquid chromatography coupled to time-of-flight mass spectrometry for PFCs. The limits of quantification of the three newly optimized analytical procedures in M. edulis tissue varied between 0.1 and 10 ng g\(^{-1}\), and satisfactory linearities (≥0.98, n=7), recoveries (90–106%) and precision (9-26 RSD%) were obtained. Application of these methods to M. Edulis revealed the presence of five pharmaceuticals, two PFCs, and seven pesticides at levels up to 490, 5, and 60 ng g\(^{-1}\), respectively. The most prevalent micropollutants were salicylic acid, paracetamol, perfluorooctane sulfonate, chloridazon, and dichlorvos.
1. INTRODUCTION

Due to increasing anthropogenic activities and the release of various types of contaminants, marine ecosystems worldwide are subjected to a continuous pollution pressure [1]. The introduction of the European Reach Legislation has led to the development of less persistent, bioaccumulative, or toxic chemicals [2]. Generally, these newly designed chemicals could be characterized as hydrophilic compounds. As a consequence, the emerging more polar anthropogenic contaminants, such as pharmaceuticals, perfluorinated compounds (PFCs), and pesticides have recently gained more attention. Pharmaceuticals, PFCs, and pesticides are, to a large extent, dissolved in the water column. Consequently, several studies have demonstrated the occurrence of these micropollutants in marine and estuarine waters [3–7]. As such, these hydrophilic micropollutants are directly bioavailable to filter-feeding organisms including mussels and oysters. In light of the possible toxic, genotoxic and/or endocrine disrupting properties of some of these micropollutants, their potential to cause adverse effects in marine organisms should not be neglected [8,9]. Moreover, the ingestion of contaminated seafood forms a major source of human exposure to micropollutants [10–12]. To study and evaluate the fate, effects, and environmental and human risks posed by these polar micropollutants in aquatic ecosystems, information regarding their presence in marine organisms and more particular in species that are important in terms of human consumption such as mussels is urgently needed.

Biotic samples are complex matrices demanding extensive extraction and clean-up procedures to obtain extracts amenable to analysis. In addition, relatively low concentrations may be expected to occur in these matrices. As a result, the occurrence of the above-mentioned micropollutants in marine organisms has been rarely studied and an increasing demand exists for reliable analytical methods allowing the quantification of these micropollutants in biotic matrices [13]. Analytical methods for the quantification of PFCs in biotic samples have been reported in literature [14–16]. Nevertheless, within this study, the existing method of Powley et al. [17] was adapted and optimized for this application, because of the significantly different sample matrix and detection technique. Also, the study area, which is suspected to be highly polluted with PFCs [18], offered an additional motivation to include the PFCs as a target group of contaminants within this study. Until now, only few studies are available for the analysis of pharmaceuticals in marine organisms. Ramirez et al. [19] reported a screening method for the detection of 23
pharmaceuticals in fish tissue, while Cueva-Mestanza et al. [20] described an analytical method for the detection of six pharmaceuticals in mollusks. With respect to the more polar pesticides, such as atrazine, simazine, chloridazon, Carafa et al. [21] reported an analytical procedure for the detection of 29 pesticides in clams. To the best of our knowledge, little attention has been paid to the prevalence of pesticides in marine organisms—aside from the organochlorine pesticides. Because an in-depth evaluation of the presence of a wide range of pharmaceuticals and polar pesticides in this specific matrix was intended, new analytical procedures needed to be developed to enable the quantification of these compounds in a reliable and sensitive manner.

The objective of this study was to develop new extraction procedures and analytical methods for the quantification of 14 of the most intensively applied pesticides in Belgium, and 11 of most frequently used pharmaceuticals in Belgium [6] in blue mussels (Mytilus edulis). The existing analytical approach [17] for the detection of the most important PFCs in biotic samples was optimized for this specific biotic matrix as well. The analytical procedure for analysis of pharmaceuticals and pesticides consisted of a pressurized liquid extraction (PLE) and solid-phase extraction (SPE), followed by ultra high-performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-QqQ-MS/MS). For the detection of PFCs, liquid extraction, and SPE were applied followed by liquid chromatography coupled to time-of-flight mass spectrometry (LC-ToF-MS).

2. MATERIAL AND METHODS

2.1 Study area and sampling

*M. edulis* was collected in the Eastern Scheldt (ES) from subtidal plots and 50 *M. edulis* organisms were transplanted to cages deployed at different stations in the Belgian coastal zone. Two cage experiments were conducted during 2008. A long-term cage experiment ran from February till July 2008 at five stations: the marinas of Nieuwpoort (NP), Oostende (OO), and Zeebrugge (ZB2), the outport of Zeebrugge (ZB1), and one station situated in open sea at the Nieuwpoortbank (SEA; Fig. IV.1). *M. edulis* was sampled monthly to determine body concentrations of the target micropolllutants. A short-term cage experiment was set up in November 2008. Cages with *M. edulis*, also originating
from subtidal plots in the Eastern Scheldt, were deployed at the same stations, but not at the SEA-station, for 6 weeks. All cage-organisms were removed from the shell, homogenized, and these composite biotic samples were freeze-dried and stored at 4 °C prior to analysis.

Figure IV.1. Study area of the *Mytilus edulis* cage experiments in the Belgian coastal zone.

### 2.2 Reagents and chemicals

The analytical method for pharmaceutical analysis included 11 compounds. Paracetamol (99%), ketoprofen (99%), carbamazepine (>99%), diclofenac (>99%), salicylic acid (>99%), clofibric acid (97%), atenolol (≥98%), trimethoprim (≥98%), and chloramphenicol (≥99%) were purchased from Sigma-Aldrich (St-Louis, MO, USA). Ofloxacin (>99%) was obtained from ICN Biomedicals Inc. (Ohio, USA), while propranolol (>99%) was purchased from Eurogenerics (Brussel, Belgium). The synthetic isobutcar 61 (4-3(isobutylamino-2-hydroxypropoxy) carbazole) and two deuterated
pharmaceuticals, atenolol-$d_7$ ($\geq 95\%$) and salicylic acid-$d_{14}$ ($\geq 98\%$) from Toronto Research Chemicals Inc. (North York, ON, Canada), were used as internal standard.

Fourteen pesticides were included in this study. Dichlorvos ($>98\%$), dimethoate ($>99\%$), diazinon ($>98\%$), pirimicarb ($\geq 99\%$), linuron ($>99\%$), metolachlor ($\geq 98\%$), chloridazon ($\geq 99\%$), chlorpyriphos ($>99\%$), simazine ($>99\%$), isoproturon ($>99\%$), terbutylazine ($>98\%$), and diuron ($>99\%$) were obtained from Sigma-Aldrich (St-Louis, MO, USA), while atrazine ($>99\%$) and kepone ($\geq 98\%$) were purchased from Chem Service (West Hester, PA, USA). Isoproturon-$d_6$ ($>99\%$) and atrazine-$d_5$ ($>99\%$) from Sigma-Aldrich (St-Louis, MO, USA) were used as internal standard.

Ten PFCs were examined in this study: four perfluorosulfonates (potassium perfluoro-1-butane sulfonate (PFBS), sodium perfluoro-1-hexane sulfonate (PFHxS), sodium perfluoro-1-octane sulfonate (PFOS), and sodium perfluoro-1-decane sulfonate (PFDS)) and six perfluorocarboxylates (perfluoro-$n$-heptanoic acid or PFHpA, perfluoro-$n$-octanoic acid or PFOA, perfluoro-$n$-nonanoic acid or PFNA, perfluoro-$n$-decanoic acid or PFDA, perfluoro-$n$-undecanoic acid or PFUnA, and perfluoro-$n$-dodecanoic acid or PFDoA). All analytical PFC-standards were purchased from Wellington Laboratories (Guelph, Ontario, Canada) with chemical purities of more than 98%. Five $^{13}$C-labelled internal standards were used as well: sodium perfluoro-1-[1,2,3,4-$^{13}$C$_4$]octane sulfonate, perfluoro-$n$-[1,2,3,4,$^{13}$C$_4$] octanoic acid, perfluoro-$n$-[1,2,3,4,5-$^{13}$C$_5$]nonanoic acid, perfluoro-$n$-[1,2-$^{13}$C$_2$]decanoic acid, and perfluoro-$n$-[1,2-$^{13}$C$_2$]dodecanoic acid.

Analytical grade reagents were used for extraction and purification purposes, and Optima® LC-MS grade for UHPLC-MS/MS analysis. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific UK (Loughborough, UK), respectively. For LCToF-MS analysis, methanol was purchased from Rathburn Chemicals (LTd Walkerburn, Scotland), while HPLC-grade water was obtained from Biosolve (Biosolve Chemicals, The Netherlands). Ammonium acetate (2.5 mM) in water was obtained through dilution of LC-MS Chromasolv® water containing 0.1% ammonium acetate (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany). Aqueous ammonium carbonate (Merck, Darmstadt, Germany; 2 mM) and aqueous formic acid (Merck, Darmstadt, Germany; 0.08%) were prepared by appropriate dissolution or dilution in ultrapure water (Arium 611 UV system, Sartorius Stedim Biotech, Aubagne, France).
Primary stock solutions of the Pharmaceuticals and pesticides were prepared in ethanol at a concentration of 1 mg mL$^{-1}$, while methanol was used for the PFCs. Working standard mixture solutions were prepared by appropriate dilution of the stock solutions in ethanol and methanol, respectively. All solutions were stored at −20°C in the dark.

2.3 Extraction and clean-up

The sample preparation for Pharmaceuticals consisted of a PLE, which was performed on a Dionex ASE® 350 Accelerated Extractor with Solvent Controller (Dionex Corp., Sunnyvale, CA, USA). A cellulose filter (27 mm, Dionex Corp.) was placed on the bottom of a 22-mL stainless steel extraction cell. Each cell was filled with 9.5 g of aluminium oxide 90 aktiv neutral (Dionex Corp.). A mixture of 1 g of freeze-dried biotic sample with 1.5 g of diatomaceous earth (DE, ASE® Prep Diatomaceous Earth, Dionex Corp.) was placed on top of the aluminum oxide. The internal standards were added prior to extraction to a final concentration of 200 ng g$^{-1}$. A combination of acetonitrile/water (3/1) with 1% formic acid was used as the extraction solvent. Extraction was carried out at 100°C for three cycles of each 10 min. The extract (±45 mL) obtained by accelerated solvent extraction (ASE) was evaporated under nitrogen at 55°C to a final volume of 5 mL and further diluted to 100 mL with ultra-pure water. Next, SPE was carried out using Strata-X cartridges (6 mL, 200 mg, Phenomenex B.V., Utrecht, Netherlands). The cartridges were preconditioned with 5 mL of methanol and 5 mL of ultra-pure water. After loading, the cartridges were rinsed with 5 mL of ultra-pure water. Elution was performed using 2×3 mL of methanol. Finally, this eluate was evaporated under nitrogen at 55°C and reconstituted in 50 μL of acetonitrile with formic acid (0.08%) and 250 μL aqueous formic acid (0.08%). Prior to U-HPLC-MS analysis, the extracts were centrifuged at 9,000 rpm for 10 min at 4°C.

A similar combination of PLE and SPE was optimized for pesticide extraction and clean-up from biotic samples, with the following differences: the internal standards were spiked at a concentration of 100 ng g$^{-1}$, and each extraction cell was filled with 0.25 g of sample, 2 g of aluminium oxide, and 4.5 g of diatomaceous earth. Extraction was performed using 1:1 acetonitrile/methanol, at 100°C for three cycle times of 3 min. The ASE-extract obtained was evaporated to 0.5 mL before dissolving it in 10 mL of ultrapure water. SPE was carried out using Isolute ENV+ cartridges (10 mL, 200 mg, Biotage, Uppsala, Sweden). The cartridges were preconditioned with 5 mL of methanol and 5 mL of ultra-
pure water with methanol (5%). Elution was performed using 5 mL of methanol and acetonitrile. Next, the eluate was evaporated under nitrogen at 55 °C to dryness and reconstituted in 50 μL methanol and 150 μL of 2 mM aqueous ammonium carbonate. After centrifugation, the eluate was filtered using a 0.22-μm Syringe-Driven Filter Unit (Millipore, Carritwohill, Cork, Ireland).

For analysis of PFCs in biotic samples, 1 g of freeze-dried sample, spiked with the 13C-labelled internal standards at a concentration of 50 ng g⁻¹, was extracted with 10 mL of acetonitrile by homogenization with an Ultra-Turrax dispersing unit (Ika, Staufen, Germany). After centrifugation at 5,000 rpm for 20 min at 4 °C, the supernatant was reduced to 5 mL by evaporation under nitrogen at 55 °C and subsequently diluted to 100 mL with ultra-pure water. Next, SPE was carried out using OASIS HLB cartridges (6 mL, 200 mg, Waters, Milford, MA). The cartridges were preconditioned with 2 mL of methanol and 2 mL of ultrapure water. After loading, the cartridges were rinsed with 5 mL of ultra-pure water. Elution was performed using 3×2 mL of methanol and the eluates were centrifuged at 9,000 rpm for 10 min at 4 °C. Next, the eluates were evaporated to dryness under nitrogen at 55 °C. Finally, 0.25 mL of methanol and 0.25 mL of 2.5 mM aqueous ammonium acetate were added before transfer to LC-MS vials. Samples were stored at −20 °C before analysis.

2.4 Chromatography

For both the pesticides and pharmaceuticals, chromatographic separation was carried out using ultra high-performance liquid chromatography (U-HPLC). The apparatus comprised of an Accela™ High Speed LC and an Accela™ Autosampler (Thermo Scientific, San Jose, CA, USA). Chromatographic separation was achieved using a Nucleodur C18 Pyramid U-HPLC column (1.8 μm, 100×2 mm, Macherey-Nagel, Düren, Germany). For the pharmaceuticals, the mobile phase constituted of 0.08% aqueous formic acid (A), 0.08% formic acid in acetonitrile (B), and isopropanol (C). A linear gradient was used starting from 98% A and 2% B, which was held for 0.8 min. The percentage of acetonitrile was increased to 65% B in 30 s, and further to 100% B in 1 min and held for 4 min. Next, 90%B and 10%C were applied to the column for 2 min, before equilibration at initial conditions for 2 min. Pesticide separation was achieved using methanol (D) and aqueous ammonium carbonate (2 mM; E). The linear gradient started with a mixture of 98% E and 2% D for 1 min. The methanol percentage increased to 90%
DETECTION OF CECs IN BIOTA

in 30 s, and further to 100% in 3 min. Between samples, the column was allowed to equilibrate at initial conditions for 1 min.

For PFC-analysis, the LC-apparatus comprised of a 1,200 series binary gradient pump and a 1,100 series autosampler (Hewlett Packard, Palo Alto, CA, USA). Chromatographic separation was achieved using a Luna® C18 (2) HPLC column (5 μm particle size, 250×2.0 mm; Phenomenex Inc., Utrecht). The mobile phase consisted of a mixture of (F) 2.5 mM aqueous ammonium acetate and (G) methanol. A linear gradient of 0.3 mL min⁻¹ was used starting with a mixture of 50% F and 50% G, increasing to 90% G in 10 min. This ratio was kept for 6 min before reversion to the initial conditions.

2.5 Mass spectrometric detection

Detection of pharmaceuticals and pesticides was carried out using a TSQ Vantage Triple-Stage Quadrupole Mass Spectrometer (Thermo Electron) equipped with a heated electrospray ionization probe (HESI-II). The mass spectrometer operated by fast-switching between positive and negative ion mode during analysis. The parameters as presented in Table IV.1 were found to be the optimal ionization source working parameters for the respective analytes. The mass resolution at the first (Q1) and third (Q3) quadrupole was set to 0.7 Da at full width at half maximum. The cycle time was adjusted to 0.5 and 0.9 s for pharmaceutical and pesticide analysis, respectively. Argon was used as collision gas, the collision gas pressure was set at 1.5 mTorr and the chrom filter peak width at 10 s.

Table IV.1. HESI-II working parameters for ionization of the selected pharmaceuticals and pesticides.

<table>
<thead>
<tr>
<th></th>
<th>Pharmaceuticals</th>
<th>Pesticides</th>
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</thead>
<tbody>
<tr>
<td>Spray voltage (V)</td>
<td>3500</td>
<td>4000</td>
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<tr>
<td>Capillary temperature (°C)</td>
<td>270</td>
<td>315</td>
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<tr>
<td>Sheath gas pressure (arbitrary units, au)</td>
<td>25</td>
<td>25</td>
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<tr>
<td>Auxiliary gas pressure (au)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Ion sweep gas pressure (au)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vaporizer temperature (°C)</td>
<td>25</td>
<td>35</td>
</tr>
</tbody>
</table>
Perfluorinated compounds were detected with a time-of-flight mass spectrometer equipped with a dual electrospray ionization interface (ESI MSD TOF, Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer was operated in the negative ion mode. Instrument parameters were: drying gas temperature of 325 °C, drying gas flow of 5 L min$^{-1}$, nebuliser pressure of 20 psi, capillary voltage of 3,500 V, and chamber voltage of 3,000 V. Before analyzing a series of samples, the ToF-MS apparatus was tuned and calibrated using the ESI Tuning Mix (Agilent Technologies, Santa Clara, CA, USA). During analysis, a reference solution was pumped into the MS-system at a rate of 50 μL min$^{-1}$ using a separate sprayer connected to a 1100 series pump (Hewlett Packard, Palo Alto, CA, USA). This reference solution consisted of purine with a $m/z$ ratio of 119.0363 and HP-0921 (hexakis(1H,1H,3H-tetrafluoropropoxy) phosphazine) with a $m/z$ ratio of 980.0164 in ACN/H$_2$O (95/5; Agilent Technologies, Santa Clara, CA, USA). Accurate mass measurements could only be achieved if these reference masses were clearly present. Chromatograms and spectra were recorded and processed using Agilent MassHunter Quantitative Analysis® software (Agilent Technologies, Santa Clara, CA, USA).

### 2.6 Identification and quantification

The target analytes were identified based on their retention time relative to that of the internal standards. For pharmaceutical and pesticide analysis, using U-HPLC-QqQ-MS/MS in the selected reaction monitoring mode (SRM), at least two transitions were monitored. The relative abundances of these specific transitions were compared with those of the standards and both product ions were used for quantification purposes. Identification of the PFCs, using LC-ToF-MS, was performed on the basis of their accurate mass. Within this study, a maximum mass error of 10 ppm was allowed [7].

Upon identification, area ratios were determined by integration of the area of an analyte under the obtained chromatograms in reference to the integrated area of the internal standard. The analyte concentrations were calculated by fitting their area ratios in a seven-point calibration curve in tissue matrix. To this end, freeze-dried *M. Edulis* samples were spiked with a standard mixture obtaining seven final concentrations in the range of 0.1 to 250 ng g$^{-1}$ and with the appropriate concentrations of the respective internal standard mixtures.
2.7 Quality assurance

Before and after analysis of a series of samples, a standard mixture (0.1 ng on column) of the targeted analytes and the internal standards was injected to check the instrument performance of the LC-ToF-MS and U-HPLC-QqQ-MS/MS systems. Quality control of the method was performed by analysis of a blank sample, together with linear calibration curves constructed using matrix samples spiked with standard solutions at seven concentration levels ranging from 0.1 to 250 ng g\(^{-1}\). This was performed for every series of samples at least in duplicate.

3. Results and discussion

3.1 Sample preparation

3.1.1 Pharmaceuticals

Many studies describe analytical methods for the detection of pharmaceuticals in water. For marine organisms, however, only few studies are available [19,20]. Extraction of environmental matrices such as biotic tissue and sediment, is conventionally performed by means of Soxhlet extraction or sonication, demanding long extraction times and large solvent volumes [22]. In recent years, techniques such as microwave-assisted extraction (MAE) and PLE are gaining in popularity [20]. The latter extraction technique has been reported in several recent studies about the detection of pharmaceuticals in soil, sediment, and sewage sludge [23–25]. Since preliminary experiments using classical solid/liquid extraction versus PLE and MAE provided higher extraction recoveries for the target pharmaceuticals in case of PLE application, this technique was selected and further optimized for pharmaceutical extraction from biotic tissue.

To obtain the optimal extraction parameters, subsequent experiments were performed using 1 g of freeze-dried biotic tissue spiked at 250 ng g\(^{-1}\). Selection of the optimal parameters was based on the resulting peak area, signal-to-noise ratio and peak shape of each analyte upon U-HPLC-MS/MS analysis, but also on visual characteristics of the extract such as colour and turbidity.

First, different extraction solvents were tested (acetone, methanol, acetone/methanol (1:1), \(n\)-hexane, acetone:ethyl acetate (1:1), acetonitrile+1% formic acid, acetonitrile:
water (3:1)+1% formic acid). This is of crucial importance, since all pharmaceuticals of interest should be simultaneously extracted, irrespective of their chemical structure or physico-chemical properties. A mixture of acetonitrile/water (3:1) with 1% formic acid provided the best results. Second, the optimal temperature (60–100–140–180 °C), static time (3–5–10–15 min), and number of extraction cycles (1–2–3) were investigated. Three cycles of 10 min were found to be optimal for the extraction of the target pharmaceuticals. Moreover, it was found that 100 °C resulted in slightly higher recoveries compared to 60 °C or 140 °C. The flush volume, which is the amount of solvent flushed through the sample cell after extraction, was evaluated as well. Since previous studies at our laboratory showed slightly better recoveries when using a flush volume of 60%, as proposed by the manufacturer, this flush volume was further applied during this study as well [26]. Next, the addition of Al₂O₃ to the extraction cell was evaluated. Since Al₂O₃ is known to inhibit the co-extraction of lipids and other hydrophobic matrix constituents, addition of different quantities (0–4–6–9.5 g) of Al₂O₃ to the PLE cells was tested. Cleaner extracts were obtained by inserting 9.5 g of Al₂O₃ into the PLE cells. For fine powdery samples, such as freeze-dried biotic tissue, it is recommended to mix the sample with diatomaceous earth to inhibit the aggregation of the sample and to improve the solvent–matrix interactions. Therefore, 1.5 g diatomaceous earth was inserted into the extraction cell as well. The sample mass was tested by analysis of 1, 3 or 5 g of freeze-dried *M. edulis* tissue spiked at 250 ng g⁻¹. It was found that increasing the sample mass to 3 or 5 g, resulted in lower extraction efficiencies and turbid extracts. Therefore, further experiments were conducted using 1 g of tissue.

Due to the complexity of biotic samples, further cleanup was required following PLE to allow sufficiently high S/N ratios and peak resolution upon U-HPLC-MS/MS analyses. Several techniques have been described in literature for the clean-up of pharmaceuticals from complex matrices: gel permeation chromatography [27,28], solid-phase micro-extraction [29], and solid-phase extraction [23–25,30,31]. In this study, we applied the most adequate technique, namely SPE. Indeed, SPE has shown good performance in extraction of pharmaceuticals from various aqueous matrices and simultaneously allows the concentration of the sample. Several SPE-cartridges were tested: Strata-X, Chromabond HR-X (6 mL, 200 mg, Marchery-Nagel, Düren, Germany), and Oasis HLB (6 mL, 200 mg, Waters, Milford, MA). The peak areas obtained upon extraction with the three SPE-cartridges were shown in Figure IV.2. Except for ofloxacin, peak areas were
highest using the Strata-X cartridges. Based on these results, S/N ratios and the clarity of the final extract, it was decided to retain the Strata-X columns for this application.

![Figure IV.2. Peak areas of the pharmaceuticals obtained upon extraction with three different SPE cartridges.](image.png)

3.1.2 Pesticides

Pang et al. [32] determined more than 400 pesticides in grain by accelerated solvent extraction using acetonitrile as extraction solvent, followed by SPE. Besides, Carafa et al. [21] described an analytical procedure for 29 pesticides in clams. This method included extraction with ultrasonication using a mixture of methanol and water (4:1) followed by SPE using Oasis HLB cartridges. In this study, the combination of PLE and SPE was again selected, because of its excellent performance in extraction of pharmaceuticals from biotic tissue, and since PLE proved more efficient in extraction of our selected pesticides.

First, the optimal PLE-parameters were determined by analysis of a 1 g freeze-dried *M. edulis* sample spiked at 100 ng g\(^{-1}\) by using a similar approach to that described above. Some significant differences resulting in better analytical results upon U-HPLC-MS/MS
with the application of the pharmaceuticals were identified. A sample mass of 0.25 g was found to be sufficient. Two and 4.5 g Al$_2$O$_3$ and diatomaceous earth, respectively, were inserted in the cell. A mixture of methanol and acetonitrile (1:1) was found to be the optimal extraction solvent for extraction of the target pesticides at a temperature of 100 °C. An extraction time above 10 min did not significantly increase the extraction efficiency of the analytes. Three cycles of 3 min were found to be sufficient. Further clean-up was again performed by SPE. The choice of the SPE-sorbent was determined based on the recovery rates (S/N ratio and peak area) obtained and clarity of the extract. The Isolute ENV + cartridges provided the best results over Strata-X, Chromabond HR-X, and Chromabond Easy (6 mL, 200 mg, Macherey-Nagel, Düren, Germany).

3.1.3 Perfluorinated compounds

Our analytical procedure is based on the study of Powley et al. [17] on quantification of PFCs in biological samples, which is commonly used in this field [33]. The use of acetonitrile as extraction solvent was adapted from Powley et al. [17], while for clean-up and concentration of the extracts, SPE with Oasis HLB cartridges was performed. Oasis HLB cartridges have been repeatedly applied for PFC-extraction from aqueous samples [7,34,35] and their superiority above other SPE sorbents has been clearly demonstrated [7]. After SPE, clear extracts were obtained by centrifugation of the eluates.

3.2 Chromatography and mass spectrometric detection

3.2.1 Pharmaceuticals and pesticides

According to literature [36,37], LC-MS/MS is the best tool for sensitive detection of pharmaceuticals from different therapeutic classes as well as for multi-residue pesticide analysis in complex environmental matrices. Therefore, new U-HPLC-MS/MS methods were developed allowing unequivocal confirmation and quantification of the targeted pharmaceuticals and pesticides. For both groups of micropollutants, rapid chromatographic separation was achieved using a Nucleodur C18 Pyramid U-HPLC column. Based on peak intensities, areas, S/N ratios, and peak resolution of the individual analytes, this column provided better results than the Hypersil Gold (1.9 μm, 50 and 100×2.1 mm, Thermo Electron) and Acquity HSS T3 or HSS C18 (1.8 μm, 50 and 100×2.1 mm, Waters, Milford, USA) UHPLC columns. The Nucleodur C18 Pyramid also exerted a better retention for the fast-eluting pharmaceutical atenolol. Too early elution of
compounds should be avoided, in order to prevent interference with the solvent peak. To this end, the mobile phase started with a gradient of 98% 0.08% aqueous formic acid and 2% 0.08% formic acid in acetonitrile. For separation of pharmaceutical compounds using liquid chromatography, water and acetonitrile are commonly used solvents [36]. In addition, a higher ionization rate in positive ion mode may be obtained by adding formic acid to the mobile phase [36]. For 1 min, isopropanol was added to the mobile phase. The higher elution strength of isopropanol resolved the carry-over problem of ofloxacin and trimethoprim. All pesticides were separated within less than 5 min using a mobile phase consisting of aqueous ammonium carbonate (2 mM) and methanol. Methanol as eluent was preferred over acetonitrile because of its weaker elution strength, thus increasing the retention of the more polar pesticides. In line with Martins-Junior et al. [38] ammonium carbonate was selected as a buffer since it provided better chromatographic elution for polar pesticides than other additives (formic acid, acetic acid, ammonium hydroxide, ammonium acetate).

As mentioned before, we selected triple quadrupole mass spectrometry to allow reliable quantification of the selected pharmaceuticals and pesticides in extracts of tissue. At first, compound-dependent parameters were optimized by direct infusion of individual analytes (10 ng μl\(^{-1}\)) into the heated electrospray ionization source (HESI-II). Data acquisition was performed initially in full scan mode to determine an abundant precursor ion. Next, the MS/MS transitions (at least two), S-lens voltages, and collision energies were optimized for each individual compound (Table IV.2). Finally, the ionization source working parameters were optimized by direct infusion of a standard mixture (10 ng μl\(^{-1}\); Table IV.1). As such, the use of U-HPLC coupled to triple quadrupole mass spectrometry resulted in a rapid and selective multi-residue analytical method for the detection of 11 pharmaceuticals and 14 pesticides (+internal standards) in biotic extracts. The obtained SRM chromatograms of a \textit{M. edulis} sample fortified with the selected pharmaceuticals at 100 ng g\(^{-1}\) are presented in Fig. IV.3. Some minor matrix components could be noticed within the retention time windows of several pharmaceuticals. This background noise did however not affect quantification or identification, since it was chromatographically resolved from the target compounds. With respect to the targeted pesticides, no interferences were observed at their specific retention time upon analysis of \textit{M. edulis} tissue spiked with 100 ng g\(^{-1}\) (Fig. IV.4).
Table IV.2. SRM transitions, MS parameters, recoveries, and limits of quantification (LOQs) of the targeted pharmaceuticals and pesticides in *Mytilus edulis* extracts (n=21).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Int.</th>
<th>tR (min)</th>
<th>Precursor ion (m/z)</th>
<th>Product (m/z)</th>
<th>S-lens (V)</th>
<th>Collision E (eV)</th>
<th>Recovery (%)</th>
<th>LOQ (ng g&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pharmaceuticals</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atenolol</td>
<td>At</td>
<td>0.83</td>
<td>267.1 (+)</td>
<td>190.1, 145.0</td>
<td>102</td>
<td>18, 26</td>
<td>97 ± 13</td>
<td>1</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>Sal</td>
<td>2.15</td>
<td>152.0 (+)</td>
<td>110.1, 65.1</td>
<td>52</td>
<td>16, 30</td>
<td>97 ± 26</td>
<td>2.5</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Iso</td>
<td>2.25</td>
<td>291.1 (+)</td>
<td>261.1, 230.1</td>
<td>188</td>
<td>25, 23</td>
<td>101 ± 13</td>
<td>1</td>
</tr>
<tr>
<td>Propranolol</td>
<td>At</td>
<td>2.35</td>
<td>260.2 (+)</td>
<td>183.1, 116.1</td>
<td>138</td>
<td>18, 17</td>
<td>98 ± 13</td>
<td>1</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>Iso</td>
<td>2.35</td>
<td>362.1 (+)</td>
<td>318.2, 261.1</td>
<td>176</td>
<td>18, 27</td>
<td>102 ± 14</td>
<td>5</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Iso</td>
<td>2.58</td>
<td>321.0 (-)</td>
<td>257.1, 152.1</td>
<td>104</td>
<td>15, 19</td>
<td>95 ± 15</td>
<td>2.5</td>
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<tr>
<td>Carbamazepine</td>
<td>Sal</td>
<td>2.78</td>
<td>237.1 (+)</td>
<td>194.1, 193.1</td>
<td>93</td>
<td>19, 33</td>
<td>100 ± 11</td>
<td>1</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>Sal</td>
<td>2.85</td>
<td>137.0 (-)</td>
<td>93.1, 65.1</td>
<td>51</td>
<td>20, 32</td>
<td>103 ± 10</td>
<td>10</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>Sal</td>
<td>3.19</td>
<td>255.0 (+)</td>
<td>209.2, 105.0</td>
<td>295</td>
<td>14, 24</td>
<td>100 ± 12</td>
<td>5</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>Sal</td>
<td>3.46</td>
<td>213.0 (-)</td>
<td>127.1, 85.1</td>
<td>73</td>
<td>19, 13</td>
<td>100 ± 20</td>
<td>1</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>Sal</td>
<td>3.55</td>
<td>296.0 (+)</td>
<td>250.1, 214.1</td>
<td>78</td>
<td>13, 34</td>
<td>98 ± 16</td>
<td>2.5</td>
</tr>
<tr>
<td>Atenolol-d&lt;sub&gt;7&lt;/sub&gt; (At)</td>
<td>0.96</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Isobutcar 61 (Iso)</td>
<td>2.36</td>
<td>313.8 (+)</td>
<td></td>
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<tr>
<td>Salicylic acid-d&lt;sub&gt;4&lt;/sub&gt; (Sal)</td>
<td>2.84</td>
<td>141.1 (+)</td>
<td></td>
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<tr>
<td><strong>Pesticides</strong></td>
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<td></td>
</tr>
<tr>
<td>Dimethoate</td>
<td>Iso</td>
<td>2.87</td>
<td>230.0 (+)</td>
<td>199.0, 79.1</td>
<td>63</td>
<td>10, 34</td>
<td>104 ± 10</td>
<td>10</td>
</tr>
<tr>
<td>Chloridazon</td>
<td>Atr</td>
<td>2.90</td>
<td>222.0 (+)</td>
<td>104.1, 77.1</td>
<td>95</td>
<td>23, 36</td>
<td>104 ± 9</td>
<td>1</td>
</tr>
<tr>
<td>Simazine</td>
<td>Atr</td>
<td>3.11</td>
<td>202.1 (+)</td>
<td>132.1, 124.1</td>
<td>77</td>
<td>18, 18</td>
<td>100 ± 8</td>
<td>5</td>
</tr>
<tr>
<td>Pirimicarb</td>
<td>Iso</td>
<td>3.20</td>
<td>239.1 (+)</td>
<td>182.2, 72.2</td>
<td>74</td>
<td>15, 33</td>
<td>101 ± 10</td>
<td>1</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>Iso</td>
<td>3.21</td>
<td>207.1 (+)</td>
<td>72.1, 46.2</td>
<td>78</td>
<td>19, 17</td>
<td>102 ± 8</td>
<td>1</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>Iso</td>
<td>3.23</td>
<td>221.0 (+)</td>
<td>109.1, 79.1</td>
<td>81</td>
<td>19, 28</td>
<td>100 ± 10</td>
<td>1</td>
</tr>
<tr>
<td>Atrazine</td>
<td>Atr</td>
<td>3.25</td>
<td>216.1 (+)</td>
<td>174.1, 68.1</td>
<td>83</td>
<td>17, 36</td>
<td>95 ± 19</td>
<td>1</td>
</tr>
<tr>
<td>Diuron</td>
<td>Iso</td>
<td>3.25</td>
<td>233.0 (+)</td>
<td>72.1, 46.2</td>
<td>71</td>
<td>18, 16</td>
<td>103 ± 8</td>
<td>1</td>
</tr>
<tr>
<td>Linuron</td>
<td>Atr</td>
<td>3.36</td>
<td>249.0 (+)</td>
<td>182.1, 160.1</td>
<td>83</td>
<td>16, 18</td>
<td>105 ± 11</td>
<td>1</td>
</tr>
<tr>
<td>Terbutylazine</td>
<td>Atr</td>
<td>3.41</td>
<td>230.2 (+)</td>
<td>174.1, 104.1</td>
<td>70</td>
<td>18, 33</td>
<td>100 ± 8</td>
<td>1</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>Atr</td>
<td>3.61</td>
<td>284.1 (+)</td>
<td>252.2, 176.2</td>
<td>69</td>
<td>15, 24</td>
<td>101 ± 11</td>
<td>1</td>
</tr>
<tr>
<td>Diazinon</td>
<td>Iso</td>
<td>3.80</td>
<td>305.1 (+)</td>
<td>169.1, 97.0</td>
<td>87</td>
<td>20, 34</td>
<td>104 ± 17</td>
<td>1</td>
</tr>
<tr>
<td>Kepone</td>
<td>Atr</td>
<td>4.30</td>
<td>506.6 (-)</td>
<td>426.8, 424.8</td>
<td>157</td>
<td>21, 20</td>
<td>99 ± 19</td>
<td>1</td>
</tr>
<tr>
<td>Chlorpyriphos</td>
<td>Atr</td>
<td>4.64</td>
<td>349.8 (+)</td>
<td>199.9, 197.9</td>
<td>82</td>
<td>22, 21</td>
<td>98 ± 17</td>
<td>1</td>
</tr>
<tr>
<td>Isoproturon-d&lt;sub&gt;6&lt;/sub&gt; (Iso)</td>
<td>3.21</td>
<td>213.1 (+)</td>
<td></td>
<td>78.2, 52.2</td>
<td>69</td>
<td>20, 19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Atrazine-d&lt;sub&gt;5&lt;/sub&gt; (Atr)</td>
<td>3.23</td>
<td>221.1 (+)</td>
<td></td>
<td>179.1, 101.1</td>
<td>79</td>
<td>19, 27</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure IV.3. SRM chromatograms of a *Mytilus edulis* sample fortified with the target pharmaceuticals at 100 ng g⁻¹.

Figure IV.4. SRM chromatograms of a *Mytilus edulis* sample fortified with the target pesticides at 100 ng g⁻¹.
3.2.2 Perfluorinated compounds

For detection of PFCs in biotic tissue, an analytical methodology was developed using LC-ToF-MS. LC-ToFMS, which encompasses a high-resolution approach based on accurate mass measurements, has been shown superior for the detection of perfluorosulfonates as compared to tandem MS [39,40]. This specific class of PFCs, including the major contaminant PFOS, shows a very high stability even at extreme conditions (e.g., high collision energies) which makes the use of tandem MS for the detection of perfluorosulfonates very complex and less efficient [39,40]. Based on our previously developed method in aqueous matrices [7], LC-ToF-MS was found to be a highly selective MS-technique for the detection of PFCs in complex environmental matrices. Also in literature, ToF-MS has proved to be the optimum quantitative method for PFCs [41] and excellent specificity for unequivocal compound identification after a crude sample clean-up is obtained using high-resolution ToF-MS [42].

Good chromatographic separation of all compounds was achieved using the Luna C18 (2) LC-column and a mixture of 2.5 mM ammonium acetate in water and methanol as mobile phase. The detection of the target compounds was obtained via full scan data, from which the calculated theoretical masses of the target PFCs were extracted using very narrow mass tolerance windows. The theoretical masses, the mean measured masses, and the mass errors obtained are presented in Table IV.3. Except for PFDoA (ppm of 5.5), the obtained mean mass errors were below 5 ppm, resulting in a highly selective MS-technique for the detection of PFCs in complex biotic matrices (Fig. IV.5).
Table IV.3. Characteristics of the PFC-analysis using ToF-MS: theoretical masses, mean measured masses, mean mass errors, internal standards, recoveries, and limits of quantification (LOQs).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Theoretical m/z [M-H]⁻ ion</th>
<th>Mean measured m/z</th>
<th>Mean mass error (ppm)</th>
<th>Internal standard</th>
<th>Recovery (%) (x + RSD)</th>
<th>LOQ ng g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFHpA</td>
<td>362.9696</td>
<td>362.9705</td>
<td>3.2</td>
<td>¹³C₄ PFOA</td>
<td>103 ± 10</td>
<td>2</td>
</tr>
<tr>
<td>PFOA</td>
<td>412.9664</td>
<td>412.9680</td>
<td>4.1</td>
<td>¹³C₄ PFOA</td>
<td>100 ± 9</td>
<td>1</td>
</tr>
<tr>
<td>PFNA</td>
<td>462.9632</td>
<td>462.9648</td>
<td>4.3</td>
<td>¹³C₅PFNA</td>
<td>101 ± 15</td>
<td>2</td>
</tr>
<tr>
<td>PFDA</td>
<td>512.9600</td>
<td>512.9625</td>
<td>4.9</td>
<td>¹³C₂PFDA</td>
<td>105 ± 15</td>
<td>2</td>
</tr>
<tr>
<td>PFUnA</td>
<td>562.9563</td>
<td>562.9581</td>
<td>4.9</td>
<td>¹³C₂PFDA</td>
<td>98 ± 15</td>
<td>2</td>
</tr>
<tr>
<td>PFDoA</td>
<td>612.9531</td>
<td>612.9565</td>
<td>5.5</td>
<td>¹³C₂PFDoA</td>
<td>90 ± 17</td>
<td>5</td>
</tr>
<tr>
<td>PFBS</td>
<td>298.9430</td>
<td>298.9436</td>
<td>2.9</td>
<td>¹³C₄PFOS</td>
<td>94 ± 18</td>
<td>5</td>
</tr>
<tr>
<td>PFHxS</td>
<td>398.9366</td>
<td>398.9387</td>
<td>4.9</td>
<td>¹³C₄PFOS</td>
<td>106 ± 23</td>
<td>0.1</td>
</tr>
<tr>
<td>PFOS</td>
<td>498.9302</td>
<td>498.9317</td>
<td>3.9</td>
<td>¹³C₄PFOS</td>
<td>100 ± 16</td>
<td>0.1</td>
</tr>
<tr>
<td>PFDS</td>
<td>598.9233</td>
<td>598.9250</td>
<td>3.7</td>
<td>¹³C₄PFOS</td>
<td>96 ± 16</td>
<td>0.1</td>
</tr>
</tbody>
</table>
3.3 Method performance

The method performance of each of the three newly optimized analytical procedures was determined by constructing seven-point calibration curves in tissue matrix. To this end, freeze-dried *M. edulis* samples were used. The method proved to be applicable to pacific oysters (*Crassostrea gigas*) and brown shrimps (*Crangon crangon*) as well, and comparable limits of quantification (LOQs) were obtained upon analysis of these biotic organisms. The samples were spiked with a standard mixture at seven final concentrations between 0.1 and 250 ng g$^{-1}$ and with the appropriate concentrations of the respective internal standard mixtures. This was performed in triplicate for each application (n=21). In addition, unspiked *M. edulis* samples (n=3) were also analyzed, to check the
occurrence of the target compounds in blank samples. Based on the obtained calibration curves, the LOQ, recovery (trueness), and linearity were assessed.

Linear regression analysis was carried out by plotting the peak area ratios of the analyte against the I.S. versus the analyte concentrations. Good linearities were obtained for all analytes (regression coefficients $\geq 0.99$), except for chlorpyriphos for which $R^2$ equalled 0.98. Due to the ubiquitous character of some of the compounds, analysis of unspiked *M. edulis* samples frequently resulted in their detection in the low nanogram per gram range. The calibration curves were corrected for these concentrations. Since no certified reference material was available, the accuracy in terms of recovery of the methods was assessed using *M. edulis* samples spiked at seven concentration levels between 0.1 and 250 ng g$^{-1}$ (three replicates). According to the guidelines SANCO/10684/2009 [43] on pesticide residues analysis in food and feed, and Commission Decision 2002/657/EC [44] concerning the determination of analytes in products of animal origin, typically a recovery is required within the range of 70–120% and 80–110%, respectively. As can be deduced from Tables IV.2 and IV.3, all obtained recoveries were between 90% and 106%, indicating good accuracy for all compounds. LOQs were determined using spiked matrix samples and were defined as the lowest detectable concentrations of the calibration curve with a signal-to-noise of at least 10:1. The LOQs obtained varied between 0.1 and 10 ng g$^{-1}$. For detection of pharmaceuticals in mussel tissue, the LOQs obtained in this study are an order of magnitude lower than a previous study [20]. For detection in fish muscle tissue, Ramirez et al. [19] determined comparable values for paracetamol, atenolol, and trimethoprim, and lower LOQs for propranolol and carbamazepine. With respect to pesticide analysis, the only analogous study reported limits of detection for simazine, atrazine, and terbutylazine of 0.21, 0.042, and 0.012 ng g$^{-1}$, respectively [21]. The obtained LOQs for detection of PFCs in mussel tissue are comparable to previous reported values, yet based on wet weight sample volumes [15,45]. In general, the sensitivity of the reported methodologies is considered acceptable to good.

A well-known interference, which is associated with analysis by LC-MS, is the potential for interaction with matrix co-elutants. Due to the complexity of biotic samples, the number of co-eluting interferences and their interactions with target analytes increase [13]. To anticipate these matrix effects, quantification using matrix-matched calibration curves is suggested in literature. Besides, the use of isotopically labelled internal standards or compounds, which are structurally related with the target analytes, has also
been recommended [13,46]. In this study, both strategies to compensate for matrix effects were applied. The results obtained were thus corrected for possible matrix-induced suppression or enhancement effects, resulting in reliable analytical methods for the detection of the three groups of analytes in biotic matrices.

3.4 Application to *M. edulis* samples from the Belgian coastal zone

The developed methods were applied to *M. edulis* samples, derived from two cage experiments (see “Study area and sampling” section). Since freeze-dried samples were analyzed, the obtained results are expressed on dry weight basis in nanograms per gram. As shown in Tables IV.4 and IV.5, five different pharmaceuticals were detected in the *M. edulis* samples. The widely used non-steroidal anti-inflammatory drug (NSAID) salicylic acid was found in almost every sample in levels up to 490 ng g$^{-1}$. A second NSAID, namely paracetamol was detected less frequently at concentrations up to 115 ng g$^{-1}$. Also the β-blocker propranolol and the antibiotic ofloxacin were measured in some samples: up to 63 and 65 ng g$^{-1}$, respectively. Finally, carbamazepine was detected in concentrations ≤11 ng g$^{-1}$. Salicylic acid has been identified by Wille et al. [6] as the most prevalent pharmaceutical in water samples collected in the Belgian coastal waters, which explains the presence of this compound in the *M. edulis* samples observed in this study. No obvious temporal trends could, however, be observed during the cage experiments. Moreover, the measured concentrations of salicylic acid showed large variations over time and location.

Seven target pesticides were found in the *M. edulis* samples originating from the cage experiments. The concentrations of five pesticides (diuron, linuron, isoproturon, metolachlor, terbutylazine) were close to the limit of quantification, while the detected concentrations of chloridazon and dichlorvos were significantly higher. Chloridazon was observed at up to 16 ng g$^{-1}$ and dichlorvos was found in most samples with a maximum concentration of 60 ng g$^{-1}$. This implies that the European default maximum pesticide residue level (MRL) in foodstuffs of 10 ng g$^{-1}$ [47], was exceeded for chloridazon and dichlorvos at several stations. Carafa et al. [21] also reported the exceeding of this MRL in clams, in which up to 73 ng g$^{-1}$ of terbutylazine was retrieved.

Only two PFCs were detected in the *M. edulis* samples; PFHxS was detected only once at a concentrations of 3 ng g$^{-1}$, while PFOS was found in most samples at levels ≤5 ng g$^{-1}$. These concentrations were in the same order as those reported by So et al. [48] who found...
PFHxS and PFOS in mussel samples at levels ≤4 ng g⁻¹ in coastal waters of China and Japan. In the study by Van de Vijver et al. [18], much higher concentrations of PFOS were measured in aquatic invertebrates of the southern North Sea, which is the same study area as the present study. PFOS was measured in shrimp (C. crangon), crab (Carcinus maenas), and starfish (Asterias rubens) up to 520, 877, and 176 ng g⁻¹, respectively.
Table IV.4. Detected concentrations (ng g⁻¹ on dry weight basis) of the target micropollutants in *Mytilus edulis* deployed in a 6-month cage experiment performed at five stations in the Belgian coastal zone (n.d. = not detected; n.a. = not analyzed).

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>ES</th>
<th>ZB1</th>
<th>ZB2</th>
<th>Sea</th>
<th>NP</th>
<th>OO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Feb</td>
<td>Mar</td>
<td>Apr</td>
<td>May</td>
<td>Jun</td>
<td>Jul</td>
</tr>
<tr>
<td><strong>Pharmaceuticals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>5</td>
<td>n.d.</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>145</td>
<td>444</td>
<td>208</td>
<td>223</td>
<td>n.d.</td>
<td>172</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>1</td>
<td>11</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><strong>PFCs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFHxS</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3</td>
</tr>
<tr>
<td>PFOS</td>
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<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>4</td>
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<tr>
<td><strong>Pesticides</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>21</td>
<td>18</td>
<td>19</td>
<td>5</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Diuron</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>n.d.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Linuron</td>
<td>n.d.</td>
<td>2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Table IV.5. Detected concentrations (ng g\(^{-1}\) on dry weight basis) of the target micropollutants in *Mytilus edulis* deployed in a 6-week cage experiment performed at four stations in the Belgian coastal zone (n.d. = not detected).

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>ES</th>
<th>ZB1</th>
<th>ZB2</th>
<th>NP</th>
<th>OO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pharmaceuticals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>n.d.</td>
<td>39</td>
<td>38</td>
<td>30</td>
<td>63</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>33</td>
<td>14</td>
<td>118</td>
<td>288</td>
<td>229</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>n.d.</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>115</td>
</tr>
<tr>
<td><strong>PFCs</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFOS</td>
<td>n.d.</td>
<td>5</td>
<td>2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>25</td>
<td>7</td>
<td>8</td>
<td>60</td>
<td>8</td>
</tr>
<tr>
<td>Diuron</td>
<td>n.d.</td>
<td>1</td>
<td>1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Chloridazon</td>
<td>13</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>n.d.</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1</td>
</tr>
<tr>
<td>Terbutylazine</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

4. **Conclusions**

In this study, three separate sensitive, selective, and reliable analytical methods have been developed for the quantification of 11 pharmaceuticals, 14 pesticides, and 10 perfluorinated compounds in tissue from marine organisms. It was shown that these methods exhibited satisfactory linearities and recoveries. The LOQs varied between 0.1 and 10 ng g\(^{-1}\) for all target compounds. Application of these analytical procedures to the blue mussel (*M. edulis*) deployed at different stations in the Belgian coastal zone revealed the presence of several of the target micropollutants. Five pharmaceuticals were found in *M. edulis* samples at up to 490 ng g\(^{-1}\), two PFCs were detected at up to 5 ng g\(^{-1}\), and seven pesticides were measured at concentrations up to 60 ng g\(^{-1}\). As a consequence, the present study demonstrates that presence of micropollutants in marine ecosystems clearly affect tissue concentrations in resident marine organisms [3–7]. These findings will
contribute to the assessment of the environmental and human health risk of these emerging micropollutants.

5. ACKNOWLEDGMENTS

Mieke Naessens, Dirk Stockx, Lucie Dossche, and Vera Paltousova are greatly acknowledged for their assistance in the lab. This study is part of the INRAM project (http://www.vliz.be/projects/inram) which is funded by The Belgian Federal Science Policy Office (BELSPO). Lynn Vanhaecke is a postdoctoral fellow from the Research Foundation - Flanders (Fonds voor Wetenschappelijk Onderzoek (FWO)-Vlaanderen).

6. REFERENCES


DETECTION OF CECs IN BIOTA


CHAPTER V

RAPID QUANTIFICATION OF PHARMACEUTICALS AND PESTICIDES IN PASSIVE SAMPLERS USING ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY

Adapted from:

CHAPTER V

RAPID QUANTIFICATION OF PHARMACEUTICALS AND PESTICIDES IN PASSIVE SAMPLERS USING ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY

Abstract

The presence of both pharmaceuticals and pesticides in the aquatic environment has become a well-known environmental issue during the last decade. An increasing demand however still exists for sensitive and reliable monitoring tools for these rather polar contaminants in the marine environment. In recent years, the great potential of passive samplers or equilibrium based sampling techniques for evaluation of the fate of these contaminants has been shown in literature. Therefore, we developed a new analytical method for the quantification of a high number of pharmaceuticals and pesticides in passive sampling devices. The analytical procedure consisted of extraction using 1:1 methanol/acetonitrile followed by detection with ultra-high performance liquid chromatography coupled to high resolution and high mass accuracy Orbitrap mass spectrometry. Validation of the analytical method resulted in limits of quantification and recoveries ranging between 0.2 and 20 ng per sampler sheet and between 87.9 and 105.2%, respectively. Evaluation of the repeatability and within-laboratory reproducibility resulted in RSD values between 7 and 26%. Determination of the sampler-water partition coefficients of all compounds demonstrated that several pharmaceuticals and most pesticides exert a high affinity for the polydimethylsiloxane passive samplers. Finally, the developed analytical methods were used to measure the time-weighted average (TWA) concentrations of the targeted pollutants in passive samplers, deployed at eight stations in the Belgian coastal zone. Propranolol, carbamazepine and seven pesticides were found to be very abundant in the passive samplers. These obtained long-term and large-scale TWA concentrations will contribute in assessing the environmental and human health risk of these emerging pollutants.
1. INTRODUCTION

Large amounts of various polar anthropogenic pollutants, including pesticides and pharmaceuticals, are continuously introduced into the aquatic environment [1]. As a result, the presence of pharmaceuticals and pesticides in marine waters, typically in the lower ng L\(^{-1}\) concentration range, has been reported occasionally [2-4]. However, monitoring these pollutants in the marine environment remains an ongoing challenge within the domain of environmental chemistry [5]. Besides the determination of the concentration of these pollutants, modern monitoring techniques should also enable the evaluation of their ecotoxicological effects and the assessment of their environmental and human health risks [6]. Therefore, the determination of time-weighted average (TWA) concentrations over extended sampling periods of these pollutants in the aquatic environment has been put forward.

The most conventional screening technique involves active sampling, which is based on the collection of discrete grab or spot samples of water, and is used in most aquatic monitoring programmes [7]. To obtain long-term and large-scale TWA concentrations, a large number of samples have to be taken, which makes it an expensive and impractical technique [6,7]. Since pharmaceuticals and pesticides are mostly present at trace levels in the marine environment [2-4], large volumes of water need to be collected as well. Although these conventional sampling techniques are very useful, generally, they will not provide appropriate information for assessing the prevalence of pollutants in the marine environment on a long-term basis [8]. As a result, passive sampling techniques, which rely on the free flow of pollutants from the sampled medium to a receiving phase namely the sampling device [9], have gained in popularity, since most of the disadvantages of active sampling are avoided by this approach [6]. Additionally, passive samplers enable the discrimination between the relevant bioavailable fractions of pollutants from the total amounts present in environmental compartments [10]. In this way, passive sampling or equilibrium-based techniques mimic biological uptake in a more straightforward manner by determining the pollution level of contaminants with respect to their freely dissolved concentration [11,12]. Furthermore, passive samplers are designed to obtain TWA concentrations, providing a more complete picture of organism exposure than those concentrations measured in grab samples, certainly in cases where chemicals bioconcentrate and their environmental concentrations vary temporally [10].
With respect to pharmaceuticals and pesticides, the use of passive sampling devices such as polar organic chemical integrative samplers (POCISs) and Chemcatcher® passive samplers have been recently reported in literature [5,8,13-15]. However, the applicability of these passive sampling devices to characterize the tendency of pharmaceuticals and pesticides to bioaccumulate is limited. In addition, the quantitative aspect is still a major issue of concern, due to both the lack of calibration data to enable quantification of target analytes, as well as the missing insights in the effects of environmental conditions on the analyte uptake [14,16]. In this context, polydimethylsiloxane (PDMS) was preferred as passive sampling material for pharmaceuticals and pesticides in the marine environment in this study. So far, PDMS samplers have mainly been used for quantification of a variety of mostly hydrophobic pollutants [17]. However, Magner et al. [12] demonstrated that PDMS is suitable for mimicking biological uptake of more hydrophilic organic pollutants as well.

Detection of pharmaceuticals and pesticides in complex environmental matrices has generally been performed using liquid chromatography coupled to mass spectrometry (LC-MS/MS) [18,19]. Nevertheless, their analysis at trace concentration levels in aqueous environmental samples remains an important challenge [20]. Nowadays, advances in instrumentation have resulted in a significant progress in the detection of these pollutants in environmental matrices. At first, the use of ultra-high performance liquid chromatography (U-HPLC) enabled fast separation of compounds in comparison to conventional LC, due to the use of columns with very small particles [21]. Secondly, with respect to the mass spectrometric detection, accurate mass full scan analysis, using time-of-flight (ToF) and Orbitrap-based mass spectrometers (MS), proved to be a very suitable alternative to triple quadrupole instruments. Full scan data originating from ToF and Orbitrap instruments enable the accurate mass screening of a virtually unlimited number of analytes, targeted as well as untargeted compounds. Typically, the working resolution of an Orbitrap-MS amounts up to 100,000 at m/z 200, which is significantly higher than the resolution of a ToF-MS [22]. This high resolving power of Orbitrap-MS technology provides higher mass accuracy (<2 ppm) as compared to ToF-MS instruments (<5 ppm) [23]. Especially this high mass resolution and accuracy makes the Orbitrap MS very appropriate for the successful identification of pollutants in environmental samples containing high amounts of matrix co-extracts.
In general, the use of passive sampling devices for quantification of polar micropollutants in marine environments seems promising, however, the applicability for a wide range of compounds remains to be tested [8]. Therefore, we developed a new extraction procedure and analytical method for the quantification of the most frequently used pharmaceuticals in Belgium [4] and the most intensively applied pesticides in Belgium in PDMS passive sampling devices [24]. The method consisted of a liquid extraction using 1:1 methanol/acetonitrile followed by U-HPLC coupled to Orbitrap mass spectrometry (MS). An extensive validation study was carried out to demonstrate the applicability of this analytical approach. In addition, the sampler-water partition coefficients ($K_{sa/wa}$) of the target analytes was determined, to enable quantification of the compounds in the passive samplers. Finally, the optimized method were applied to passive samplers, deployed at several locations in the Belgian coastal zone, to study the presence of pharmaceuticals and pesticides in the Belgian marine environment.

2. MATERIAL AND METHODS

2.1 Study area and sampling

The sampler holders were deployed at eight sampling locations in the Belgian coastal zone: the marinas of Nieuwpoort (NP2), Oostende (OO2), and Zeebrugge (ZB2), the inner side of the harbour of Nieuwpoort (NP1), the outport of Zeebrugge (ZB1), and the location halfway the harbour of Oostende (OO3) were sampled. An additional location was selected at the Sluice Dock in Oostende (OO1) since at this location aquacultural activities take place. Finally, one location was situated in open sea at the Nieuwpoortbank (SEA) (Figure V.1). The samplers were deployed at 1.5 to 2 meters below surface for circa two months from May till July 2008, from March till May 2009 and from mid-July to mid-September 2010. The sampler holders were lost at the SEA-station in 2008 and 2010, at OO1 in 2009, and at OO2 in 2010.
2.2 Reagents and chemicals

The analytical method for pharmaceutical analysis included 16 substances. Paracetamol (99%), ketoprofen (99%), carbamazepine (> 99%), diclofenac (> 99%), salicylic acid (> 99%), clofibric acid (97%), atenolol (≥ 98%), trimethoprim (≥ 98%), bezafibrate (≥ 98%), sulfamethoxazole (99%), pravastatin (≥ 98%), salbutamol (99%), carprofen (> 99%) and chloramphenicol (≥ 99%) were purchased from Sigma-Aldrich (St-Louis, MO, USA). Ofloxacin (> 99%) was obtained from ICN Biomedicals Inc. (Ohio, USA), while propranolol (> 99%) was purchased from Eurogenerics (Brussel, Belgium). The $^{13}$C-labelled sulfamethoxazole-phenyl-$^{13}$C$_6$ (> 99%) from Sigma-Aldrich (St-Louis, MO, USA), two deuterated pharmaceuticals, bezafibrate-d$_6$ (> 99%) from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and salicylic acid-d$_4$ (≥ 98%) from Toronto Research Chemicals Inc. (North York, ON, Canada), were used as internal standards.

Thirteen pesticides were included in the study. Dichlorvos (> 98%), dimethoate (> 99%), pirimicarb (≥ 99%), linuron (> 99%), metolachlor (≥ 98%), chloridazon (≥ 99%),
ANALYSIS OF PASSIVE SAMPLERS

Simazine (> 99%), isoproturon (> 99%), terbutylazine (> 98%), 2,4-D (or 2,4-dichlorophenoxy acetic acid) (> 99%) and diuron (> 99%) were obtained from Sigma-Aldrich (St-Louis, MO, USA), while atrazine (> 99%) and kepone (≥ 98%) were purchased from Chem Service (West Hester, PA, USA). Isoproturon-d₆ (> 99%) and atrazine-d₅ (> 99%) from Sigma-Aldrich (St-Louis, MO, USA) were used as internal standards.

Analytical grade solvents were used for extraction and purification purposes, and Optima® LC-MS grade for U-HPLC-MS analysis. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific UK (Loughborough, UK), respectively. Aqueous formic acid (Merck, Darmstadt, Germany) and acetonitrile with formic acid (both 0.08%) were prepared by appropriate dilution of formic acid in ultra-pure water (Arium 611 UV system, Sartorius Stedim Biotech, Aubagne, France) and acetonitrile, respectively.

Primary stock solutions of the pharmaceuticals and pesticides were prepared in ethanol at a concentration of 1 µg µL⁻¹. Working standard mixture solutions were prepared by appropriate dilution of the stock solutions in ethanol. When necessary, sonication was applied to ensure the complete dissolution of the substances. All solutions were stored at -20°C in the dark.

2.3 Sampler preparation

The polydimethylsiloxane (PDMS) samplers (AlteSil Laboratory Sheet, Altec Products Ltd, Bude, United Kingdom) with a thickness of 0.5 mm, were cut into sheets of 55mm x 90mm, to obtain a total sampling surface of approximately 100 cm² and a mean mass of 3.15g. These sampler sheets were pre-cleaned for 2 h in methanol prior to use. Sampler holders made of stainless steel for mounting the passive samplers were built. The sampler sheets were fixed in such a way that they could move freely, as proposed by Smedes [25]. By this approach, the design does not limit the uptake of the target compounds. After the sampling period, the loaded sampler holders were carefully dismantled and the sheets were transferred on ice to the laboratory where they were stored in a freezer at -20 °C before analysis.
2.4 Extraction and clean-up

As proposed by Rusina [11], the surface of the sampler was cleaned with ultrapure water and wiped dry with a paper tissue before extraction. The internal standards were spiked on the surface of the samplers prior to extraction to a final concentration of 25 ng per sheet. Extraction of a sampler sheet was carried out by adding 20 mL of 1:1 acetonitrile/methanol to a 50 mL tube containing the sheet, followed by shaking this for 60 min and sonication for 60 min. The eluate was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 50 µL methanol and 150 µL of 0.08% aqueous formic acid.

2.5 Chromatography

For both the pesticides and pharmaceuticals, chromatographic separation was carried out using ultra-high performance liquid chromatography (U-HPLC). This U-HPLC-system consisted of an Accela™ high speed LC and an Accela™ autosampler and degasser (Thermo Scientific, San Jose, CA, USA). Chromatographic separation was achieved using a Nucleodur C18 Pyramid U-HPLC column (1.8 µm, 100 x 2 mm, Macherey-Nagel, Düren, Germany). For the pharmaceuticals, the mobile phase consisted of 0.08% aqueous formic acid (A) and 0.08% formic acid in acetonitrile (B). A linear gradient was used, starting from 98% A and 2% B, which was held for 0.8 min. In 30 sec the percentage of acetonitrile was increased to 65% B, which was held for 0.7 min. The percentage of acetonitrile was increased further to 100% B in 1 min and held for 2 min. Equilibration at initial conditions was done for 2.5 min. Pesticide separation was achieved using 0.08% aqueous formic acid (A) and methanol (C). The linear gradient started with a mixture of 98% A and 2% C for 1 min. The methanol percentage increased to 90% in 30 sec, and further to 100% in 3 min, which was held for 1 min. Between samples, the column was allowed to equilibrate at initial conditions for 1 min. The injection volume was 10 µl. The column oven and tray temperature were 25 °C and 15 °C, respectively.

2.6 Mass spectrometric detection

Detection of pharmaceuticals and pesticides was carried out using an Exactive™ Benchtop Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ionization probe (HESI-II). The Exactive™ is an Orbitrap-based MS, which was operated alternating from positive to negative ion mode, with both
scan types at a resolution of 50,000 at 2 Hz (2 scans per second). For the compounds of interest, a scan range of $m/z$ 80-800 was chosen. The automatic gain control (AGC) target was set at ultimate mass accuracy ($5 \times 10^5$ ions) and the maximum injection time was 500 ms. The instrumental settings were optimized to maximize the signal. The parameters as presented in Table V.1 were found to be the optimal ionization source working parameters for the respective analytes. Initial instrument calibration was done by infusing calibration mixtures for positive and negative ion mode (Thermo Fisher Scientific, San Jose, CA, USA). The positive calibration mixture included caffeine, MRFA and Ultramark® 1621, while the negative calibration solution comprised sodium dodecyl sulfate, sodium taurocholate and Ultramark® 1621. These compounds were dissolved in a mixture of acetonitrile, water and methanol, and both mixtures were infused using a Chemyx Fusion 100 syringe pump (Thermo Fisher Scientific, San Jose, CA, USA). The option of “all-ion fragmentation” using the High Energy Collision Dissociation (HCD) cell was turned off. The forevacuum, high vacuum and ultra high vacuum were maintained around 2 mbar, from $1E^{-05}$ to $3E^{-05}$, and below $8E^{-10}$ mbar, respectively. Instrument control and data processing were carried out by means of Xcalibur 2.1 and ToxID software (Thermo Electron, San Jose, CA, USA).
Table V.1. HESI-II working parameters for ionization of the selected pharmaceuticals and pesticides.

<table>
<thead>
<tr>
<th></th>
<th>Pharmaceuticals</th>
<th>Pesticides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray voltage (kV)</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Sheath gas flow rate (arbitrary units, au)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Auxiliary gas flow rate (au)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Capillary temperature (°C)</td>
<td>275</td>
<td>250</td>
</tr>
<tr>
<td>Heater temperature (°C)</td>
<td>250</td>
<td>350</td>
</tr>
<tr>
<td>Capillary voltage</td>
<td>82.5 (-30.0)</td>
<td>82.5 (-30.0)</td>
</tr>
<tr>
<td>Tube lens voltage</td>
<td>170.0 (-95.0)</td>
<td>120.0 (-95.0)</td>
</tr>
<tr>
<td>Skimmer voltage</td>
<td>20.0 (-26.0)</td>
<td>20.0 (-26.0)</td>
</tr>
</tbody>
</table>

2.7 Determination of $K_{sa/wa}$

This experimental setup was based on the study of Magner et al. [12]. Thirty-three sheets were placed in a beaker filled with 5 L of ultra-pure water under gentle stirring at 100 rpm. The water was spiked with all pharmaceuticals at a concentration of 20 µg L$^{-1}$, except propranolol and carbamazepine, which were spiked at 5 µg L$^{-1}$. The pesticides were spiked at 5 µg L$^{-1}$, apart from dichlorvos, 2,4-D, linuron and kepon, which were spiked at 20 µg L$^{-1}$. Three 1 mL water samples and three sheets were sampled after 0, 0.08, 0.75, 1, 2, 3, 5, 6, 7, 8, 9 d, respectively. After sampling, the sheets were left to dry on a paper towel and analyzed as described above. The water samples were analyzed by direct injection of 10 µl into the U-HPLC Orbitrap MS instrument, using the optimized analytical parameters. The $K_{sa/wa}$ (L kg$^{-1}$) is defined as: $K_{sa/wa} = C_{sa} / C_{wa}$ with $C_{sa}$ (g kg$^{-1}$) and $C_{wa}$ (g L$^{-1}$) as the concentrations of the analyte in the sampler and the water phase, respectively. Knowledge of the $K_{sa/wa}$ of the analytes is required to enable quantification of the compounds in the equilibrium based passive samplers. Each time water and sheets were sampled, the mean (n=3) water and sampler concentrations of the analytes were calculated. Equilibrium between the sampler and the water phase was achieved when the concentration of the analyte in the water phase remained constant throughout the experiment.

2.8 Identification and quantification

The target analytes were identified based on both their retention time relative to that of the internal standards, and their accurate mass. According to previous studies using
Orbitrap MS, a maximum mass deviation of 5 ppm was allowed within this study [20,22,26].

So far, appropriate identification criteria using these modern instruments based on high-resolution accurate mass spectrometry are incomplete in the commonly used procedure prescribed by Commission Decision 2002/657/EC [20,27]. Both the criteria concerning mass resolution and mass accuracy, as well as the system of identification points have not been fully specified for these MS systems yet. Therefore, as was suggested by several authors [21,23,28], additional criteria for the use of these accurate mass LC-MS technologies should be implemented in the standardized validation procedures. Nevertheless, using maximum mass deviations of 5 ppm, a high reliability in identification can be expected.

Upon identification, area ratios were determined by integration of the area of an analyte within the obtained chromatograms in reference to the integrated area of the internal standard. The analyte concentrations were calculated by fitting their area ratios to a ten-point calibration curve in the sample matrix. To this end, sheets were spiked with a standard mixture obtaining ten final concentrations in the range of 0.01 to 100 ng per sheet and with a final concentration of 25 ng per sheet of the internal standards.

2.9 Quality assurance

Before and after analysis of a series of samples, a standard mixture (0.1 ng on column) of the targeted analytes and the internal standards was injected to check the performance of the U-HPLC Orbitrap MS system. Quality control of the method was performed by analysis of a blank sample, together with linear calibration curves constructed using matrix samples spiked with standard solutions at ten concentration levels ranging from 0.01 to 100 ng per sheet. This was performed for every series of samples at least in duplicate.
3. RESULTS AND DISCUSSION

3.1 Extraction of the samplers: recovery optimization

3.1.1 Spiking of the samplers

Spiking of passive samplers is generally performed according to Booij et al. [29]. This method is based on equilibration of the samplers in aqueous/methanolic solutions of the compounds. However, this method failed for most pharmaceuticals and pesticides within this study, with low uptake percentages for most compounds. This can be attributed to the low sampler to water and methanol partition coefficients of polar compounds. Therefore, an alternative method was employed by directly spiking the target compounds and internal standards onto the PDMS sampler surface, and allowing the solvent carrier to volatilize [30]. This spiking method resulted in high uptake percentages of all compounds and was further applied within this study.

3.1.2 Optimization of the extraction procedure

Generally, methanol has been reported to be the appropriate extraction solvent for compounds with log $K_{ow} < 8$, with acetonitrile as a very good alternative [11]. Recently, a 3:1 mixture of acetonitrile/water with 1% formic acid and a 1:1 mixture of methanol/acetonitrile provided the best results in extracting the same pharmaceuticals and pesticides from biotic tissue, respectively [31]. Therefore, both mixtures as well as the separate solvents methanol and acetonitrile were tested as extraction solvents for the targeted compounds. In addition, different extraction volumes (20 vs. 40 mL) and extraction conditions (sampler sonication, sampler shaking or both) were tested. Therefore, sheets were spiked with the targeted compounds at three concentration levels (10, 50 and 100 ng per sheet) and mean extraction efficiencies of each analyte were calculated upon U-HPLC-MS analysis. The optimal extraction parameters were determined based on both the extraction efficiencies and the clarity of the extract. The best results were obtained by adding 20 mL of the 1:1 mixture of methanol and acetonitrile to a PDMS sheet, and allowing this to shake and sonicate, both for 60 min. The extraction efficiencies of the pharmaceuticals and pesticides ranged, respectively, between 49 and 99% and between 42 and 92%.
3.2 Chromatography and mass spectrometry detection

3.2.1 Chromatography

Recently, the development and optimization of new U-HPLC methods for rapid chromatographic separation of pharmaceuticals and pesticides for analysis of marine organisms was reported by Wille et al. [31]. The same methods were set up in front of the Orbitrap MS, allowing good separation of the targeted compounds for our application. The chromatograms obtained upon analysis of a PDMS sheet spiked at ten times the LOQ level are shown in Figures V.2 and V.3, respectively.

Figure V.2. Chromatograms of a passive sampler sheet fortified with the target pharmaceuticals at ten times the LOQ level.
3.2.2 Orbitrap MS

The excellent applicability of Orbitrap MS for metabolomic and proteomic applications has been demonstrated in literature [22,32,33]. The suitability of Orbitrap MS for the identification of a large number of pharmaceuticals in aqueous matrices, has been demonstrated as well [20]. To the best of our knowledge, the use of Orbitrap MS for the quantification of pesticides and pharmaceuticals in environmental matrices, including passive samplers, has not been reported earlier.

First, the observed masses were compared with the theoretical masses by direct infusion of individual analytes (10 ng μl⁻¹) into the heated electrospray ionization source (HESI II). Next, the ionization source working parameters for the targeted analytes were one
after the other optimized by analyzing a standard mixture (0.1 ng µL⁻¹). The optimal values of these parameters were determined based on the peak intensities, areas, S/N ratios and peak shape of the individual analytes. Since the tube lens voltage depends on the molecular structure, different values were obtained for the pharmaceuticals and pesticides in positive ion mode: 170 V and 120 V, respectively. Different temperatures for both groups were found as well (Table V.1).

Before the ions are injected into the Orbitrap, they are trapped in a curved RF-only quadrupole, the C-trap. To avoid space charging [22,34], the number of ions present in the C-trap is controlled by the use of the Automatic Gain Control (AGC). The AGC target determines the number of charges collected for every scan. Three AGC values are possible: 3 x 10⁶ ions for a high dynamic range scan, 1 x 10⁶ ions for a balanced scan and 5 x 10⁵ ions for ultimate mass accuracy. Standard mixtures of the analytes (0.1 ng µL⁻¹) were analyzed using these three possible AGC values. Based on peak shape and width, area, signal to noise ratio and mass deviation, the optimal AGC target value was found to be 5 x 10⁵ ions. The ion density in the C-trap was kept as low as possible to ensure the best resolution and mass accuracy, without a significant loss of sensitivity. Besides the AGC target, another crucial parameter using the Orbitrap MS is the mass resolution. In recent years, several studies have reported the effect of the resolving power on analytical results [22,26]. Standard mixtures of the analytes (0.1 ng µL⁻¹) were analyzed using mass resolution values varying between 10,000 and 100,000. A resolution of 50,000 at 2 Hz (2 scans per second) proved to be the best compromise between peak shape and width, mass deviation and datapoints over the chromatographic peak for this application. Therefore, a resolution of 50,000 was further applied within this study.

Identification of compounds was, together with the retention time, based on their accurate mass, i.e. by matching the theoretical mass with the observed mass. Therefore, the expected or theoretical masses of the target compounds were calculated to four decimal places, using the Xcalibur software (Tables V.2 and V.3). The mass accuracy or mass deviation was expressed in parts per million (ppm) and was defined as: 10⁶ * [(measured mass – theoretical mass) / theoretical mass]. Extracted ion chromatograms (EIC) were obtained using a 5 ppm window. The mean mass deviations of all the compounds were calculated at LOQ level (n=10) and were presented in Tables V.2 and V.3, as well as the ion mode and retention times. The mass deviations obtained were below 2 ppm for most analytes, indicating a high mass accuracy. Propranolol, isoproturon and atrazine showed
slightly higher mass deviations, while for salicylic acid a mass deviation of 4.8 was obtained. The relatively higher mass deviation of salicylic acid, the only compound with m/z ratio below 150, can be attributed to the presence of many background ions in the lower mass area [35]. The same experiences were reported in literature: mass deviations between 1 and 3 ppm for compounds with m/z ratio higher than 150, while a 5 ppm error was observed for compounds with m/z <150 [20].

Table V.2. Ion mode, internal standard used, retention time, accurate mass and mean mass deviation of the targeted pharmaceuticals.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ion mode</th>
<th>Internal standard used</th>
<th>tR (min)</th>
<th>Accurate mass (m/z)</th>
<th>Mean mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salbutamol</td>
<td>+</td>
<td>Salicylic acid-d$_4$</td>
<td>2.41</td>
<td>240.1594</td>
<td>1.10</td>
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<td>Atenolol</td>
<td>+</td>
<td>Sulfamethoxazole-$_{13}$C$_6$</td>
<td>2.57</td>
<td>267.1703</td>
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<td>Ofloxacin</td>
<td>+</td>
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<td>Propranolol</td>
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<tr>
<td>Pravastatin</td>
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<td>Salicylic acid-d$_4$</td>
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<tr>
<td>Sulfamethoxazole</td>
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<td>3.03</td>
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<tr>
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<td>Clofibric acid</td>
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<td></td>
<td>3.62</td>
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### Table V.3. Ion mode, internal standard used, retention time, accurate mass and mean mass deviation of the targeted pesticides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ion mode</th>
<th>Internal standard used</th>
<th>tR (min)</th>
<th>Accurate mass (m/z)</th>
<th>Mean mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pirimicarb</td>
<td>+</td>
<td>Isoproturon-d₆</td>
<td>3.14</td>
<td>239.1503</td>
<td>0.55</td>
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<tr>
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<td>+</td>
<td>Isoproturon-d₆</td>
<td>3.18</td>
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<td>+</td>
<td>Atrazine-d₅</td>
<td>3.20</td>
<td>222.0429</td>
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</tr>
<tr>
<td>Dichlorvos</td>
<td>+</td>
<td>Atrazine-d₅</td>
<td>3.38</td>
<td>220.9532</td>
<td>1.83</td>
</tr>
<tr>
<td>Simazine</td>
<td>+</td>
<td>Isoproturon-d₆</td>
<td>3.42</td>
<td>202.0854</td>
<td>1.77</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>+</td>
<td>Isoproturon-d₆</td>
<td>3.56</td>
<td>207.1492</td>
<td>2.14</td>
</tr>
<tr>
<td>Atrazine</td>
<td>+</td>
<td>Atrazine-d₅</td>
<td>3.56</td>
<td>216.1010</td>
<td>2.30</td>
</tr>
<tr>
<td>Diuron</td>
<td>+</td>
<td>Isoproturon-d₆</td>
<td>3.60</td>
<td>233.0243</td>
<td>1.42</td>
</tr>
<tr>
<td>Terbutylazine</td>
<td>+</td>
<td>Atrazine-d₅</td>
<td>3.72</td>
<td>230.1167</td>
<td>1.93</td>
</tr>
<tr>
<td>Linuron</td>
<td>+</td>
<td>Isoproturon-d₆</td>
<td>3.72</td>
<td>249.0192</td>
<td>1.78</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>+</td>
<td>Atrazine-d₅</td>
<td>3.85</td>
<td>284.1412</td>
<td>1.82</td>
</tr>
<tr>
<td>2.4-D</td>
<td>-</td>
<td>Atrazine-d₅</td>
<td>4.16</td>
<td>218.9621</td>
<td>1.53</td>
</tr>
<tr>
<td>Kepone</td>
<td>-</td>
<td>Isoproturon-d₆</td>
<td>4.30</td>
<td>506.68260</td>
<td>0.76</td>
</tr>
<tr>
<td>Isoproturon-d₆</td>
<td>+</td>
<td></td>
<td>3.54</td>
<td>213.1869</td>
<td>1.37</td>
</tr>
<tr>
<td>Atrazine-d₅</td>
<td>+</td>
<td></td>
<td>3.56</td>
<td>221.1324</td>
<td>1.79</td>
</tr>
</tbody>
</table>

#### 3.3 Method validation

The newly developed analytical method was validated according to the criteria specified in CD 2002/657/EC [27] for quantitative confirmation as well as to the guidelines of SANCO/10684/2009 [36] on pesticide residues analysis in food and feed. In practice, validation of the method was executed by adopting the protocol proposed by Antignac et al. [37]. This protocol was tailored for validating analytical methods based on MS detection and offers a compromise between CD 2002/657/EC [27] and practical aspects and limitations related to laboratory work.

The use of isotopically labeled internal standards in MS-based chemical analysis has been highly recommended [27,38]. For the pharmaceuticals, one $^{13}$C-labelled sulfamethoxazole-phenyl-$^{13}$C₆ and two deuterated pharmaceuticals, bezafibrate-d₆ and salicylic acid-d₄, were used as internal standards. Isoproturon-d₆ and atrazine-d₅ were selected as the internal standards for the pesticides. The corresponding internal standards were used for sulfamethoxazole, bezafibrate, salicylic acid, isoproturon and atrazine, while the most appropriate internal standard available was used for the other compounds.
(Tables V.2 and V.3). These internal standards were supplemented to every sampler prior to extraction to a final concentration of 25 ng per sheet. The results obtained were thus corrected for possible matrix-induced suppression or enhancement effects.

3.3.1 Specificity

The specificity of the methods was demonstrated by analysis of blank sampler sheets (n=6) and sheets fortified with each analyte separate at their LOQ level. Sheets spiked with a mixture of all analytes at LOQ level were analyzed as well. None of the compounds were detected in the blanks. The obtained chromatograms showed a significant increase in peak area and intensity at the specific retention time of the compounds. The specificity of these analytical approaches were confirmed since no other significant peaks with a signal-to-noise ratio of 3 or more were observed at the specific retention times of the targeted pharmaceuticals and pesticides (Figures V.2 and V.3). Using Orbitrap MS, the specificity is guaranteed by the high resolving power of the instrument [22].

3.3.2 Selectivity

Analytes were identified on the basis of their relative retention time, which is the ratio of the retention time of the analyte to that of the internal standard. In addition, the accurate mass of the ions ([M−H]− or [M-H]+) in the spectrum was taken into account when the chromatographic peak of interest had a signal-to-noise ratio of at least 3:1. A maximum mass deviation of 5 ppm was allowed within this study.

3.3.3 Linearity

The linearity of the developed methods was evaluated for each target compound by preparing ten-point calibration curves (3 replicates). Blank sheets were spiked with a standard mixture obtaining concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50 and 100 ng per sampler sheet of the targeted pharmaceuticals and pesticides. Linear regression analysis was executed by plotting the peak area ratios of the analyte against the internal standard versus the analyte concentration. The mean correlation coefficients of the calibration curves were >0.99, indicating good linearity in this concentration range (Tables V.4 and V.5). Only dichlorvos, for which R² equaled 0.97, showed slightly inferior linearity.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Extraction efficiency</th>
<th>LOD</th>
<th>LOQ</th>
<th>LOQ</th>
<th>R²</th>
<th>Recovery</th>
<th>Repeatability</th>
<th>Within-lab reproducibility</th>
<th>Log $K_{ow}$</th>
<th>Ksa/wa</th>
<th>Log $K_{sa/wa}$</th>
<th>Log BAF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>ng sheet</td>
<td>ng sheet</td>
<td>ng g</td>
<td>Mean ± SD%</td>
<td>RSD%</td>
<td>RSD%</td>
<td>L kg</td>
<td>L kg</td>
<td>L kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salbutamol</td>
<td>97</td>
<td>5.0</td>
<td>10</td>
<td>3.2</td>
<td>&gt;0.99</td>
<td>102 ± 8</td>
<td>18</td>
<td>18</td>
<td>0.64</td>
<td>0.04</td>
<td>-1.42</td>
<td>-</td>
</tr>
<tr>
<td>Atenolol</td>
<td>80</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>99 ± 11</td>
<td>18</td>
<td>18</td>
<td>0.16</td>
<td>0.04</td>
<td>-1.38</td>
<td>-</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>65</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>89 ± 9</td>
<td>15</td>
<td>15</td>
<td>-0.39</td>
<td>0.21</td>
<td>-0.69</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>58</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>93 ± 11</td>
<td>17</td>
<td>18</td>
<td>0.91</td>
<td>0.68</td>
<td>-0.17</td>
<td>-</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>92</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>90 ± 7</td>
<td>15</td>
<td>16</td>
<td>0.46</td>
<td>0.05</td>
<td>-1.29</td>
<td>1.5 ± 0.6 (n=8)</td>
</tr>
<tr>
<td>Propranolol</td>
<td>49</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>94 ± 9</td>
<td>17</td>
<td>17</td>
<td>3.48</td>
<td>23.52</td>
<td>1.37</td>
<td>-</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>82</td>
<td>5.0</td>
<td>10</td>
<td>3.2</td>
<td>&gt;0.99</td>
<td>88 ± 10</td>
<td>18</td>
<td>19</td>
<td>3.10</td>
<td>0.06</td>
<td>-1.23</td>
<td>-</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>82</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>102 ± 9</td>
<td>15</td>
<td>15</td>
<td>0.89</td>
<td>0.09</td>
<td>-1.04</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>72</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>102 ± 11</td>
<td>15</td>
<td>16</td>
<td>1.14</td>
<td>0.09</td>
<td>-1.04</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>93</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>101 ± 7</td>
<td>10</td>
<td>11</td>
<td>2.45</td>
<td>30.97</td>
<td>1.48</td>
<td>-</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>98</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>102 ± 15</td>
<td>18</td>
<td>19</td>
<td>2.26</td>
<td>0.16</td>
<td>-0.79</td>
<td>-</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>75</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>93 ± 6</td>
<td>11</td>
<td>11</td>
<td>4.25</td>
<td>0.04</td>
<td>-1.41</td>
<td>-</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>72</td>
<td>5.0</td>
<td>10</td>
<td>3.2</td>
<td>&gt;0.99</td>
<td>88 ± 12</td>
<td>15</td>
<td>16</td>
<td>3.12</td>
<td>0.29</td>
<td>-0.54</td>
<td>-</td>
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<tr>
<td>Clofibric acid</td>
<td>60</td>
<td>1.0</td>
<td>2.0</td>
<td>0.6</td>
<td>&gt;0.99</td>
<td>99 ± 8</td>
<td>11</td>
<td>11</td>
<td>2.57</td>
<td>0.08</td>
<td>-1.11</td>
<td>-</td>
</tr>
<tr>
<td>Carprofen</td>
<td>75</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>93 ± 10</td>
<td>20</td>
<td>20</td>
<td>3.79</td>
<td>1.81</td>
<td>0.26</td>
<td>-</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>99</td>
<td>1.0</td>
<td>2.0</td>
<td>0.6</td>
<td>&gt;0.99</td>
<td>100 ± 9</td>
<td>17</td>
<td>20</td>
<td>4.51</td>
<td>2.40</td>
<td>0.38</td>
<td>-</td>
</tr>
</tbody>
</table>
Table V.5. Validation parameters, Log $K_{ow}$ and $K_{sa/wa}$ values of the targeted pesticides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Extraction efficiency</th>
<th>LOD</th>
<th>LOQ</th>
<th>LOQ</th>
<th>R²</th>
<th>Recovery</th>
<th>Repeatability</th>
<th>Within-lab reproducibility</th>
<th>Log $K_{ow}$</th>
<th>Log $K_{sa/wa}$</th>
<th>Log $K_{sa/wa}$</th>
<th>Log BAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pirimicarb</td>
<td>42</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>98 ± 11</td>
<td>10</td>
<td>11</td>
<td>1.70</td>
<td>356.4</td>
<td>2.52</td>
<td>-</td>
</tr>
<tr>
<td>Dimethoate</td>
<td>49</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>103 ± 14</td>
<td>13</td>
<td>13</td>
<td>0.78</td>
<td>0.5</td>
<td>-0.35</td>
<td>-</td>
</tr>
<tr>
<td>Chloridazon</td>
<td>55</td>
<td>1.0</td>
<td>2.0</td>
<td>0.6</td>
<td>&gt;0.99</td>
<td>104 ± 9</td>
<td>17</td>
<td>18</td>
<td>1.14</td>
<td>0.8</td>
<td>-0.10</td>
<td>-</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>57</td>
<td>10</td>
<td>20</td>
<td>6.3</td>
<td>0.97</td>
<td>95 ± 9</td>
<td>18</td>
<td>26</td>
<td>1.43</td>
<td>180.5</td>
<td>2.24</td>
<td>-</td>
</tr>
<tr>
<td>Simazine</td>
<td>65</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>104 ± 10</td>
<td>15</td>
<td>14</td>
<td>2.18</td>
<td>112.7</td>
<td>2.05</td>
<td>-</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>55</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>&gt;0.99</td>
<td>101 ± 7</td>
<td>9</td>
<td>10</td>
<td>2.87</td>
<td>118.2</td>
<td>2.07</td>
<td>1.1 ± 0.3 (n=8)</td>
</tr>
<tr>
<td>Atrazine</td>
<td>67</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>&gt;0.99</td>
<td>103 ± 10</td>
<td>11</td>
<td>12</td>
<td>2.61</td>
<td>162.0</td>
<td>2.20</td>
<td>-</td>
</tr>
<tr>
<td>Diuron</td>
<td>50</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>101 ± 12</td>
<td>13</td>
<td>15</td>
<td>2.68</td>
<td>138.3</td>
<td>2.13</td>
<td>1.1 ± 0.3 (n=35)</td>
</tr>
<tr>
<td>Terbutylazine</td>
<td>62</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>&gt;0.99</td>
<td>100 ± 9</td>
<td>15</td>
<td>15</td>
<td>3.21</td>
<td>332.5</td>
<td>2.40</td>
<td>0.6 ± 0.3 (n=8)</td>
</tr>
<tr>
<td>Linuron</td>
<td>69</td>
<td>5.0</td>
<td>10</td>
<td>3.2</td>
<td>&gt;0.99</td>
<td>97 ± 10</td>
<td>11</td>
<td>11</td>
<td>3.20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>49</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
<td>&gt;0.99</td>
<td>94 ± 9</td>
<td>10</td>
<td>10</td>
<td>3.13</td>
<td>2534.8</td>
<td>3.40</td>
<td>1.7 ± 0.5 (n=22)</td>
</tr>
<tr>
<td>2,4-D</td>
<td>92</td>
<td>1.0</td>
<td>2.0</td>
<td>0.6</td>
<td>&gt;0.99</td>
<td>103 ± 4</td>
<td>7</td>
<td>7</td>
<td>2.81</td>
<td>0.1</td>
<td>-1.29</td>
<td>-</td>
</tr>
<tr>
<td>Kepone</td>
<td>88</td>
<td>10</td>
<td>20</td>
<td>6.3</td>
<td>&gt;0.99</td>
<td>105 ± 9</td>
<td>19</td>
<td>20</td>
<td>5.41</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.3.4 Limit of detection and quantification

Limits of detection (LODs) and quantification (LOQs) were determined based on the outcome of the ten-point calibration curves of the previous section. The concentrations of the analytes were calculated using the overall equation of the calibration curves. The LOD was defined as the lowest detectable concentration of the calibration curve with a signal-to-noise ratio of at least 3:1. The LOQs were then determined as the final LOD multiplied by 2 [39]. The LOQs of the targeted pharmaceuticals and pesticides ranged, respectively, between 1 and 10 ng per sheet and between 0.2 and 20 ng per sheet. These LOQs are considered highly satisfactory, despite the absence of comparable data in literature. The LOQs obtained were also presented in ng per g sheet, providing values independent of the size of the sheets (Table V.4 and V.5). By calculating this, an average weight of 3.15 g (n=30) per sheet was considered.

3.3.5 Trueness

Since no certified reference material was available, trueness of the measurements was assessed by analysis of blank sheets spiked with each analyte at LOQ level, two times the LOQ level and ten times the LOQ level (recovery). This was performed in six replicates for all three concentration levels. Mean recoveries of the targeted pharmaceuticals and pesticides (n=18) varied, respectively, between 87.9 and 102.4% and between 94.2 and 105.2% (Tables V.4 and V.5). These calculated recoveries fulfill the criteria set by CD 2002/657/EC [27] and SANCO/10684/2009 [36], for which typically a recovery is required within the range of 80-110% and 70-120%, respectively.

3.3.6 Precision

Evaluation of the precision included the determination of the repeatability and the within-laboratory reproducibility of these new methods. Both validation parameters were evaluated by calculating the relative standard deviation (%RSD). To study the repeatability of the method, three series of six replicates of sheets were analyzed, and this at three concentration levels: LOQ level, two times the LOQ level and ten times the LOQ level. These analyses were carried out by the same analyst under repeatable conditions. As presented in Tables V.4 and V.5, the calculated RSD values for most compounds were below 20%, indicating good repeatability according to SANCO/2007/3131 [36]. Only
carprofen showed a slightly inferior repeatability (%RSD of 20.2). The criterion of CD 2002/657/EC [27], demanding RSD values below 15%, was achieved for half of the pharmaceuticals and most pesticides.

For evaluation of the reproducibility only the within-laboratory reproducibility was evaluated. Therefore, four series of six replicates of fortified sheets were analyzed, and this at three concentration levels: LOQ level, two times the LOQ level and ten times the LOQ level. Different analysts carried out these analyses on different days, using different spiking solutions and sampling sheets. According to CD 2002/657/EC [27] and SANCO/2007/3131 [36], typically a reproducibility RSD ≤20% is required. As can be deduced from Tables V.4 and V.5, except for carprofen and dichlorvos, all RSD values were satisfactory. The higher RSD value of dichlorvos and carprofen could be attributed to the absence of an appropriate internal standard. The use of an internal standard with a higher structural similarity could result in lower RSD values [40]. However, the commercial availability of labeled internal standards is limited and the criteria for good reproducibility were only slightly exceeded using the most appropriate internal standards.

### 3.4 Determination of $K_{sa/wa}$

A 9-d experiment was conducted, to determine the sampler-water partition coefficient ($K_{sa/wa}$). The pharmaceuticals propranolol and carbamazepine showed a high affinity for the PDMS samplers, resulting in $K_{sa/wa}$ values of 23.5 and 31.0 L kg$^{-1}$, respectively. The $K_{sa/wa}$ value obtained for carbamazepine was to a large extent in accordance with the value reported by Magner et al. [12]. For propranolol and carbamazepine, the enrichment profile is depicted in Figure V.4, using the logarithm of $K_{sa/wa}$ as y-axis. Carprofen and diclofenac showed a moderate affinity for the PDMS samplers, while for the other pharmaceuticals, the $K_{sa/wa}$ values obtained were <1 L kg$^{-1}$, indicating a lower affinity for the sampler compared to the water phase. On the other hand, the affinity for the PDMS sampler was demonstrated for most pesticides, reaching equilibrium within 3d (Figure V.4). Except for chloridazon, dimethoate and 2,4-D, all $K_{sa/wa}$ values were above 1 L kg$^{-1}$, ranging from 112.7 L kg$^{-1}$ for simazine to 2534.8 L kg$^{-1}$ for metolachlor. The $K_{sa/wa}$ value of atrazine was 162.0 L kg$^{-1}$, which is in the same order of magnitude as the partition coefficient of 153 L kg$^{-1}$ reported in literature [10]. Using this experimental setup, no $K_{sa/wa}$ values could be established for linuron and kepone. Probably, the $K_{sa/wa}$ values of both pesticides were too high. Quickly after the introduction of the pesticides,
the initial concentration of these compounds in the water phase dropped below their LOQ. This depletion phenomenon prevented the determination of $K_{sa/wa}$ for these compounds.

![Enrichment profile of the two pharmaceuticals and eight pesticides with high affinity for the PDMS passive samplers.](image)

Figure V.4. Enrichment profile of the two pharmaceuticals and eight pesticides with high affinity for the PDMS passive samplers.

The logarithmic function of the octanol-water partition coefficient of each compound (log $K_{ow}$), generally used as a criterion for hydrophobicity [41], is presented in Tables V.4 and V.5 as well [42]. Typically, the log $K_{sa/wa}$ of an analyte is lower than its log $K_{ow}$, except for pirimicarb, dichlorvos and metolachlor. However, no correlation between both partition coefficients was observed ($R^2$ of 0.097 and p-value of 0.114). Obviously, the sampler-water partitioning is not exclusively driven by the hydrophobicity of the substances, but compound-specific interactions in the sampler phase are important as well [43].

The $K_{sa/wa}$ coefficients were determined on a standardized manner, as described by Magner et al. [12], without making a distinction between different values of pH, salinity
and temperature. According to current literature [5,14,16], these environmental parameters may definitely impact the uptake of pollutants into the samplers. Therefore, an intensive separate study dealing with the effects of environmental conditions on the analyte uptake is desired, however, this was outside the scope of the present study. Consequently, further research should concentrate on improved approaches for calibration and quantification of PDMS passive samplers, thereby taking the different environmental parameters into consideration.

### 3.5 Application to passive sampler samples deployed in the Belgian coastal zone

#### 3.5.1 Targeted compounds

Passive samplers were deployed for circa two months at eight sampling locations in the Belgian coastal zone in 2008, 2009 and 2010. Compounds with $K_{sa/wa} < 1$ showed greater affinity for the water phase than for the PDMS passive sampler. As a consequence, the reliable calculation of TWA concentrations of these compounds using the samplers was inhibited [44]. Therefore, only the pharmaceuticals and pesticides with $K_{sa/wa} > 1$ were considered for quantification. First, the analytes were measured in the samplers using the optimized extraction and U-HPLC Orbitrap-MS methods as described above. Next, the concentrations of the compounds in the water phase were calculated using the following equation: $C_{wa} = C_{sa} / K_{sa/wa}$, expressed in nanograms per liter. These obtained concentrations may be considered as approximate calculated TWA concentrations, since the possible impact of the environmental conditions was not taken into consideration (see section 3.4). As can be seen from Table V.6, two pharmaceuticals were detected in all samplers: the β-blocker propranolol and the psychiatric drug carbamazepine in concentrations up to 7294 ng L$^{-1}$ and 732 ng L$^{-1}$, respectively. Propranolol and carbamazepine have been found in grab water samples collected in the same study area, in concentrations up to 24 ng L$^{-1}$ and 321 ng L$^{-1}$ [4]. Obviously, propranolol was quantified in significantly higher concentration levels using the equilibrium based passive samplers in comparison with grab water samples. A possible explanation is the decreasing hydrophilicity and thus higher affinity for the PDMS sampler of propranolol, due to the increasing salinity in the marine environment [5]. The rather high Setschenow salting-out constant of propranolol of 3.29 could significantly affect the $K_{sa/wa}$ value [45]. Assuming a salinity of 30 g L$^{-1}$, the $K_{sa/wa}$ value will increase with a factor of about fifty [46]. Much more realistic TWA concentrations, in the low ng L$^{-1}$ range, were found if this salting out
effect was taken into consideration. Carbamazepine was detected in every passive sampler as well. The calculated water concentrations were within the same order of magnitude with levels detected in grab samples. As a result, the salting-out effect was expected to be low [4,47]. Due to its high persistence, carbamazepine has been reported as an excellent tracer substance for pharmaceutical contamination [14,48]. From these results it may be concluded that the use of PDMS samplers to obtain long-term and large-scale TWA concentrations of carbamazepine, as a representative of the pharmaceuticals, could be very useful in revealing pharmaceutical contamination of the marine environment. By this approach, using the PDMS samplers, both the pollution level of hydrophobic compounds for which they were initially designed (PAHs, PCBs, …) as well as the more hydrophilic pollutants (pharmaceuticals represented by carbamazepine, pesticides, …) could be estimated simultaneously.
Table V.6. Calculated water concentrations (ng L\(^{-1}\)) of the detected pharmaceuticals and pesticides at eight stations in the Belgian coastal zone in 2008, 2009 and 2010 (n.d. = not detected).

<table>
<thead>
<tr>
<th></th>
<th>Sea</th>
<th>OO1</th>
<th>OO2</th>
<th>OO3</th>
<th>ZB1</th>
<th>ZB2</th>
<th>NP1</th>
<th>NP2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pharmaceuticals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>93</td>
<td>285</td>
<td>169</td>
<td>682</td>
<td>1,169</td>
<td>443</td>
<td>348</td>
<td>1,417</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>21</td>
<td>136</td>
<td>149</td>
<td>322</td>
<td>587</td>
<td>170</td>
<td>302</td>
<td>650</td>
</tr>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pirimicarb</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>41</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Simazine</td>
<td>n.d.</td>
<td>81</td>
<td>77</td>
<td>148</td>
<td>75</td>
<td>71</td>
<td>64</td>
<td>132</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>12</td>
<td>28</td>
<td>n.d.</td>
<td>67</td>
<td>33</td>
<td>51</td>
<td>29</td>
<td>31</td>
</tr>
<tr>
<td>Diuron</td>
<td>6</td>
<td>143</td>
<td>32</td>
<td>263</td>
<td>92</td>
<td>103</td>
<td>80</td>
<td>70</td>
</tr>
<tr>
<td>Atrazine</td>
<td>n.d.</td>
<td>31</td>
<td>13</td>
<td>56</td>
<td>42</td>
<td>34</td>
<td>33</td>
<td>19</td>
</tr>
<tr>
<td>Terbutylazine</td>
<td>3</td>
<td>94</td>
<td>95</td>
<td>355</td>
<td>63</td>
<td>283</td>
<td>62</td>
<td>220</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>1</td>
<td>19</td>
<td>4</td>
<td>104</td>
<td>7</td>
<td>49</td>
<td>5</td>
<td>21</td>
</tr>
</tbody>
</table>
As shown in Table V.6, seven pesticides were very frequently detected in the passive sampler extracts. Calculation of the TWA concentrations resulted in concentration levels of the pesticides up to 118 ng L\(^{-1}\) for pirimicarb, 164 ng L\(^{-1}\) for metolachlor, 56 ng L\(^{-1}\) for atrazine, 263 ng L\(^{-1}\) for diuron, 260 ng L\(^{-1}\) for isoproturon, 159 ng L\(^{-1}\) for simazine and 469 ng L\(^{-1}\) for terbutylazine. These values are in line with reported levels of pesticides found in traditional grab samples from the same study area: maximum detected concentrations were 77 ng L\(^{-1}\) for atrazine, 454 ng L\(^{-1}\) for diuron, 292 ng L\(^{-1}\) for isoproturon, 60 ng L\(^{-1}\) for simazine and 347 ng L\(^{-1}\) for terbutylazine [49]. According to the Water Framework Directive (2000/60/EC) [50] and its daughter directive (2008/105/EC) [51], environmental quality standards (EQSs), expressed as annual average values, were established for atrazine, diuron, isoproturon and simazine being 0.6, 0.2, 0.3 and 1 µg L\(^{-1}\), respectively. The calculated diuron concentrations exceeded these EQSs twice: at sampling locations OO2 and ZB2, both in 2008. The EQSs for the other compounds were however never exceeded. The comparison with EQSs present only a preliminary approach to characterize the environmental risks to aquatic ecosystems and organisms. To aid in assessing these risks, quantitative-structure-activity relationships (QSARs) [52-54] have recently been developed to generate screening and toxicity data. However, confirmation by direct measurements of concentrations in water is definitely required [10]. Therefore, these obtained concentration via passive samplers measurements are very useful, in particular because TWA concentrations of the relevant bioavailable fraction of the target pharmaceuticals and pesticides are provided.

Typically, the highest concentrations of the pharmaceuticals and pesticides were found at the sampling points in the harbours; more specific those of Nieuwpoort and to a lesser extent Oostende. Both locations receive major inputs of contaminated surface water, resulting in the increased presence of the targeted pharmaceuticals and pesticides. Due to both dilution and degradation effects, only few target compounds were found at the SEA-station, and this at rather low concentrations in comparison with the harbour stations.

The present study was conducted side-by-side with the recent publication results reported by Wille et al. [31] on the accumulation of the same pharmaceuticals and pesticides in marine organisms. In this way, the commonality in contaminants and concentrations accumulated by these two matrices may be determined. Bioconcentration of several pharmaceuticals in *Mytilus edulis* has been observed, including propranolol and carbamazepine, which were found to be present in the passive samplers as well. Also four
pesticides have been found both in tissue and samplers: diuron, isoproturon, terbutyazine and metolachlor. Apparently, a correlation between analyte concentrations in side-by-side exposures of biota and samplers exists for several pharmaceuticals and pesticides. The tendency of an organism to bioaccumulate is assessed by the bioaccumulation factor (BAF) which can be calculated using the following equation [55]:

$$\text{BAF} = \frac{C_{\text{biota}}}{C_{\text{water}}}$$

BAF values are expressed in L kg⁻¹, since the biota concentration ($C_{\text{biota}}$) is expressed in µg kg⁻¹ (dry weight) and the water concentration ($C_{\text{water}}$) in µg L⁻¹. The mean Log BAFs were calculated for every detected compound and are summarized in Tables 4 and 5. The obtained Log BAF values varied between 0.6 L kg⁻¹ for terbutylazine to 1.7 L kg⁻¹ for metolachlor. Comparable experimental data are not available for the target analytes, indicating the relevance of this study.

### 3.5.2 Untargeted compounds

A major advantage of the use of Orbitrap MS, is its suitability for untargeted analysis [22]. In theory, an infinite number of analytes could be screened using the high-resolution full scan data. Thus, the presence in the sampler extracts of non-a priori selected pharmaceuticals and pesticides could be examined as well. The retrospective screening of the passive sampler extracts, using a 5 ppm window, revealed the presence of two pharmaceuticals, simvastatin and fluoxetine, and one pesticide, diazinon. Since no $K_{wa}$ values were obtained for these compounds, estimation of TWA water concentrations was impossible. High affinity of simvastatin, fluoxetine and diazinon for the PDMS samplers could be expected, since their log $K_{ow}$ values amounted to 5.19, 4.05 and 3.81, respectively [42]. Only a small selection of pharmaceuticals and pesticides were screened afterwards, so it can be assumed that still other pharmaceuticals and pesticides were present in the passive sampler extracts. In conclusion, the excellent applicability of a new analytical approach to quantify a limited number of rather polar micropollutants in PDMS samplers was presented. In this context, the present study is only the initial stage of a more comprehensive study. Indeed, future research must enable the quantification of a very wide group of pharmaceuticals and pesticides in PDMS samplers by the
development of an extensive database including retention times, accurate masses and $K_{sa/wa}$ values.

4. CONCLUSIONS

PDMS passive sampling devices were evaluated as a monitoring tool for measuring the concentrations of a wide group of frequently used pharmaceuticals and intensively applied pesticides in marine waters. Therefore, a new extraction procedure using 1:1 methanol/acetonitrile was optimized and analysis was performed using ultra-high performance liquid chromatography coupled to high resolution Orbitrap-MS. Detection with the Exactive™ Orbitrap MS enabled the use of a very narrow mass tolerance window of 5 ppm, providing high mass accuracy. These analytical procedures were validated successfully according to CD 2002/657/EC [27] and SANCO/10684/2009 [36], showing their excellent performance in quantifying pharmaceuticals and pesticides in PDMS passive sampler devices. In addition, an equilibrium-experiment was performed to determine the sampler-water partition coefficient ($K_{sa/wa}$) of the target analytes. Only a limited number of pharmaceuticals showed affinity for the PDMS samplers, while for most pesticides high $K_{sa/wa}$ values were obtained. Deployment of the passive samplers at five stations in the Belgian coastal zone revealed the presence of propranolol, carbamazepine and seven pesticides. Calculation of the water concentration resulted in very high levels of propranolol up to 7 µg L$^{-1}$, which is probably an overestimation due to the salting out effect. The concentrations of the other compounds were below 750 ng L$^{-1}$. These long-term and large-scale TWA concentrations provide appropriate information for assessing the pollution level of these pollutants in the marine environment, in particular with respect to their biological uptake.

5. ACKNOWLEDGEMENTS

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ANALYSIS OF PASSIVE SAMPLERS


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CHAPTER VI

DISCUSSION AND FURTHER RESEARCH RECOMMENDATIONS
1. RESEARCH POSITION AND RELEVANCE

This doctoral thesis is embedded in the INRAM project, which stands for Integrated Risk Assessment and Monitoring of micropollutants in the Belgian coastal zone. The INRAM project aims to assess through an integrated approach the risks of micropollutants to Belgian coastal zone ecosystems and man, as schematically depicted in Figure VI.1 [1]. The objectives of this doctoral work were mainly related to the green section of the scheme, including the development of new analytical approaches for the detection/quantification of micropollutants in different environmental matrices including seawater, marine biota and passive samplers, and a preliminary exposure assessment.

As the term micropollutants includes many different groups of contaminants, the scope of this study was limited to the more polar chemicals of emerging concern (CECs) such as pharmaceuticals, polar pesticides and perfluorinated compounds (PFCs). These emerging anthropogenic contaminants have recently gained more attention due their possible toxic,
DISCUSSION AND FUTURE RESEARCH

Genotoxic and/or endocrine disrupting properties, their potential to cause adverse effects in marine organisms and their human exposure via ingestion of contaminated seafood [2-5]. To study and evaluate the fate, effects, and environmental and human risks posed by these polar micropollutants in aquatic ecosystems, information regarding their presence in the marine environment is urgently needed. Therefore, the main goal of the present thesis was to investigate the prevalence of targeted CECs in the Belgian marine environment, which implicates the need for new reliable analytical methods for the determination of the CECs in aqueous samples, biotic tissue (species that are important in terms of human consumption) and passive samplers. The main achievements of this thesis are depicted in Figure VI.2.

![Figure VI.2. Schematic overview of the main achievements within this thesis.](image)

2. THE USE OF MODERN SAMPLE EXTRACTION AND MASS SPECTROMETRY IN CEC ANALYSIS

Current literature on the determination of CECs in the aquatic environment was thoroughly reviewed in Chapter 1, thereby considering aqueous as well as solid matrices. Obviously, considerable attention was spent on the analysis of CECs in environmental matrices in the last decade. Within this field of environmental chemistry, some prominent trends could be observed. At first, the common use of GC to allow separation of the analytes has to a large
extent been replaced by the application of LC, due to the rather hydrophilic character of the CECs. However, GC has not been completely ruled out, since it is still the method of choice for separation of some typical more hydrophobic CECs such as steroids, alkylphenols, fragrances and phthalates, displaying ionisation issues in LC-MS analysis. Nevertheless, alternative LC applications have recently been reported for most of these groups as well. Secondly, a clear tendency towards multiclass methods could be noticed [6]. Recent advances in instrumentation have enabled the simultaneous detection of a large number of compounds within one analytical run. Also, the recent emergence of higher resolution LC equipment enabling the use of sub-2 µm particle sizes and high flow rates (U-HPLC) allows CECs to be resolved more easily, which results in shorter analytical run times. [7]. In addition, the use of on-line SPE is currently promoted to shorten the analysis time as well [6,8]. Finally, an additional remarkable trend is situated within the field of mass spectrometry. Obviously, a gain in popularity of high-resolution full scan analysis could be perceived. ToF and Orbitrap instruments proved to be very suitable alternatives to triple quadrupole instruments, thereby allowing the accurate mass screening of a virtually unlimited number of analytes, targeted as well as untargeted [9,10].

Despite the above-mentioned remarkable evolutions and achievements in analytical approaches, some typical problems within this domain may be listed. At first, pitfalls in the identification and quantification are still generated due to the lack of standardized criteria for the identification and confirmation of CECs in environmental matrices. Nowadays, criteria comparable to the European criteria 2002/657/EC [11] concerning the determination of analytes in products of animal origin, or to SANCO/10684/2009 [12] on pesticide residue analysis in food and feed, are missing for environmental matrices such as (sea)water and biota. For example, the evaluation of matrix effects is not always taken into consideration within validation procedures of newly developed analytical methods, while the occurrence of matrix interferences is a well-known source of false positives or negatives and erroneous quantification using LC-MS methods [13,14]. Therefore, to our opinion, there is a huge need for a standardized validation procedure for analytical methods for environmental applications.

A second issue is related to the use of modern instruments based on high-resolution accurate mass spectrometry, which in recent years have proven to be powerful screening and confirmation tools and which have been applied throughout Chapters III, IV and V. Still, appropriate identification criteria using these systems are currently incomplete in the commonly used procedure prescribed by Commission Decision 2002/657/EC [9,11]. Both the
DISCUSSION AND FUTURE RESEARCH

criteria concerning mass resolution and mass accuracy, as well as the system of identification points have not been fully specified for these MS systems yet. Therefore, as was suggested by Nielen et al. [15], additional criteria for the use of these accurate mass LC-MS technologies should be implemented in the standardized validation procedures.

Besides, several critical comments and useful recommendations concerning quantitative data obtained with new analytical methods could be enumerated. Recently, this has been demonstrated once again by Van Leeuwen et al. [16], who reported the results of an interlaboratory study on the analysis of PFCs in environmental matrices. Some typical sources that could contribute to the variance of analytical data include the occurrence of matrix effects, the limited use of mass labeled internal standards, the need for blank and recovery correction and the perceived failure in separation of isomers (especially for PFCs). However, the most important aspect is related to the fact that CECs are usually present at concentration levels close to the LODs of analytical methods, which results in less precise quantification and a higher variance. Therefore, for newly developed methods, it is definitely recommended to utilize sufficient mass labeled internal standards and to perform an extensive validation at environmentally relevant concentrations, thereby including the evaluation of matrix effects, accuracy and the natural background levels in so-called control samples. In addition, it is also suggested to participate in international interlaboratory studies or other data quality tests, which may be a good verification tool for the reliability of analytical methods.

Another important shortcoming within the domain of environmental chemistry, is the current lack of comparability of analytical methods and results. In literature, widely differing analytical methodologies have been used for obtaining concentrations of CECs in environmental matrices. Therefore, to our opinion, there is a huge need for standardized ‘state-of-the-art’ methods for the different groups of CECs. In Chapter I of this doctoral work, the EPA Method 1694 [17] and the ISO 25101/2009 [18] method were mentioned as standardized methodologies for analysis of pharmaceuticals and PFCs in water samples, respectively. Besides, EPA methods for analysis of different groups of pesticides are also available [19]. However, these standardized methods are only rarely applied in recent monitoring studies. We believe that the development of advanced up-to-date standardized methods and the widespread application thereof, would result in reliable and particularly comparable monitoring data.
3. DISTRIBUTION OF CECs IN THE MARINE ENVIRONMENT

3.1 Pharmaceuticals

In general, research towards the fate and the presence of CECs in the marine environment is sparse, certainly in contrast to the great efforts that have been made to study occurrence of CECs in freshwater systems. This has been pointed out in Table VI.1 in which a compilation of all marine monitoring data available in literature for the pharmaceuticals and pesticides included within this thesis have been summarized. Acetylsalicylic acid was included in this list as well, since fast hydrolysis to salicylic acid is expected. With an exception for the pharmaceutical levels obtained by Togola and Budzinski [20], typical concentrations in marine waters are below 200 ng L\(^{-1}\) (Table VI.1). Comparable pharmaceutical concentrations were found in this thesis (Chapter II), with somewhat higher levels for the NSAID salicylic acid (up to 855 ng L\(^{-1}\)), the β-blocker atenolol (up to 293 ng L\(^{-1}\)) and the psychiatric drug carbamazepine (up to 321 ng L\(^{-1}\)). Also propranolol, bezafibrate and sulfamethoxazole have been detected in water samples from the Belgian coastal zone. To the best of our knowledge, the presence of atenolol, propranolol, and bezafibrate in the marine environment have not been reported earlier. Concomitantly, the abundance of salicylic acid in water samples was reflected to the mussel tissue concentrations as well, which amounted up to 490 ng g\(^{-1}\) (Chapter IV). Bioconcentration has also been observed for propranolol and carbamazepine, however, lower concentrations were found in the marine biota for these two compounds. Comparison of the obtained pharmaceutical concentrations in biotic tissue with literature data is not straightforward, since analogues monitoring data are very sparse. Only for carbamazepine a concentration level of 0.83 to 1.44 ng g\(^{-1}\) (wet weight) in fish tissue has been reported [37]. Two other pharmaceuticals, ofloxacin and paracetamol, could be quantified in mussel tissue as well, in spite of their absence in water samples. This discrepancy could be attributed to the fact that traditional grab water sampling has been carried out. Grab water samples are taken at only a specific time and location, and may not necessarily be representative of pollutant levels at other times [38]. Therefore, the use of passive samplers for pharmaceutical monitoring in marine waters was investigated in Chapter V. By means of passive sampling, a direct estimation of the freely dissolved concentration of CECs is obtained, which enables the prediction of biological uptake in a more straightforward manner as compared to conventional grab sampling.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ng L⁻¹)</th>
<th>Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pharmaceuticals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>&lt; 8000 / &lt; 1.6 – 85.8</td>
<td>Mediterranean Sea / Seine estuary</td>
<td>20/21</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>&lt; 42 / 1.8 – 82.7 / 2</td>
<td>Mediterranean Sea / Seine estuary / North Sea</td>
<td>20/21/22</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>0.5 – 7.8 / 18 / &lt; LOQ – 1.35</td>
<td>North Sea / Elbe estuary / North Sea</td>
<td>25/26/26</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>&lt; 1100 / 7.1 – 172.5 / 6.2</td>
<td>Mediterranean Sea / Seine estuary / Elbe estuary</td>
<td>20/21/26</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>&lt; 2.4 – 33.2</td>
<td>Seine estuary</td>
<td>21</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>200000</td>
<td>Mediterranean Sea</td>
<td>20</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>7</td>
<td>Baltic Sea</td>
<td>24</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>0.03^</td>
<td>Mediterranean Sea</td>
<td>27</td>
</tr>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>0.2 – 1.5^ / 2.35 – 8.18 / 1.27 – 5.93 / &lt; LOQ – 30</td>
<td>Mediterranean Sea / Po delta / Adriatic Sea / Bay of Veys</td>
<td>27/28/28/29</td>
</tr>
<tr>
<td>Chloridazon</td>
<td>&lt; 50 / 19 – 432 / &lt; LOD – 736 / &lt; 420</td>
<td>Vilaine estuary / Chesapeake Bay / Scheldt estuary / Scheldt estuary</td>
<td>30/31/32/33</td>
</tr>
<tr>
<td>Clopyrifos</td>
<td>&lt; LOD – 101.48 / &lt; LOD – 40.59</td>
<td>Po delta / Adriatic Sea</td>
<td>28/28</td>
</tr>
<tr>
<td>Diazinon</td>
<td>&lt; LOD – 3.1</td>
<td>Chesapeake Bay</td>
<td>31</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>&lt; LOD – 56 / &lt; 50</td>
<td>Chesapeake Bay / Scheldt estuary</td>
<td>31/33</td>
</tr>
<tr>
<td>Diuron</td>
<td>0.4 – 2.5^ / &lt; LOD – 40.78 / 1.65 – 25.32</td>
<td>Mediterranean Sea / Po delta / Adriatic Sea</td>
<td>27/28/28</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>&lt; LOD – 32.08 / &lt; LOD – 0.53 / &lt; LOQ – 42 / &lt; 50</td>
<td>Po delta / Adriatic Sea / Bay of Veys / Vilaine estuary</td>
<td>28/28/29/30</td>
</tr>
<tr>
<td>Linuron</td>
<td>&lt; LOD – 30.79 / &lt; LOD – 1.08</td>
<td>Po delta / Adriatic Sea</td>
<td>28/28</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>0.3 / &lt; LOD – 171.53 / &lt; LOD – 59.29 / &lt; 100</td>
<td>North Sea / Po delta / Adriatic Sea / Vilaine estuary</td>
<td>22/28/28/30</td>
</tr>
<tr>
<td>Pirimicarb</td>
<td>0.7</td>
<td>North Sea</td>
<td>36</td>
</tr>
<tr>
<td>Simazine</td>
<td>0.1 – 0.9^ / 1.45 – 24.13 / 1.83 – 25.96 /</td>
<td>Mediterranean Sea / Po delta / Adriatic Sea</td>
<td>27/28/28</td>
</tr>
<tr>
<td>Terbutylazine</td>
<td>0.7 / 0.35 – 6.94 / 0.57 – 234.50</td>
<td>North Sea / Po delta / Adriatic Sea</td>
<td>22/28/28</td>
</tr>
<tr>
<td></td>
<td>&lt; LOQ – 261 / &lt; LOD – 52,000 / &lt; LOQ – 0.83</td>
<td>Scheldt estuary / Huelva estuary / North sea</td>
<td>32/34/36</td>
</tr>
</tbody>
</table>

^ values obtained by passive samplers; ^ values obtained by both grab and passive sampling
However, only a minority of the targeted pharmaceuticals showed affinity for the PDMS passive sampling material, probably due to their low hydrophobicity. Only four pharmaceuticals including carbamazepine, propranolol, carprofen and diclofenac were considered for quantification in the samplers, since their predicted $K_{sa/wa}$ coefficients were $>1$. For the other pharmaceuticals, which have been found in mussel tissue, such as salicylic acid, paracetamol and ofloxacin, the use of more polar passive samplers is desired. Regarding carbamazepine, its ubiquitous character was confirmed using the PDMS passive samplers as described in Chapter V, obtaining slightly higher concentrations as compared with the levels in the grab samples. For propranolol, excessively high concentrations (up to 7.3 µg L$^{-1}$) were quantified using the passive sampling devices. This was ascribed to a possible salting out effect on propranolol due to the salinity of the seawater, while $K_{sa/wa}$ was determined in pure water. In addition, it may be assumed that other differences in environmental conditions (water flow rate, temperature, pH, and biofouling) between calibration of $K_{sa/wa}$ in the laboratory and deployment of the samplers in marine waters, may affect the final time-weighted average concentrations of pharmaceuticals or CECs in general [38]. In conclusion, it seems that pharmaceuticals are omnipresent in the Belgian coastal zone, despite the efforts made in wastewater treatment plants to remove these micropollutants.

### 3.2 Pesticides

Besides pharmaceuticals, this thesis described the monitoring of a selection of rather polar pesticides in the marine environment as well, thereby using biotic tissue (Chapter IV) and passive sampling devices (Chapter V). Analysis of mussel tissue revealed the presence of 7 pesticides, from which only two pesticides were present above 2 ng g$^{-1}$: dichlorvos and chloridazon. Apparently, dichlorvos has the tendency to bioconcentrate in mussel tissue (up to 60 ng g$^{-1}$), while a decrease in acetylcholinesterase (AChE) activity in bivalves using laboratory assays caused by dichlorvos has been reported in literature [39,40]. Therefore, further research is definitely recommended to examine possible toxic effects caused by exposure to the measured dichlorvos levels. Despite their presence in biota, dichlorvos and chloridazon were not detected in passive samplers. In contrast to chloridazon ($K_{sa/wa} = 0.79$ L kg$^{-1}$), dichlorvos exhibited a high affinity for the sampler compared to the water phase, obtaining a $K_{sa/wa}$ value of 181 L kg$^{-1}$. However, the absence of dichlorvos could probably be attributed to its minor sensitivity obtained upon
optimization of the analytical procedure, achieving an LOQ of 20 ng per sheet while most pesticides could be quantified ≤ 2 ng per sheet.

Seven pesticides were very frequently detected in the passive sampler extracts: pirimicarb, simazine, isoproturon, diruon, atrazine, terbutylazine and metolachlor. Generally, the time-weighted average passive sampler concentrations for the targeted pesticides were below 500 ng L⁻¹, with noticeably higher levels for diuron and certainly terbutylazine as compared to the other pesticides. As can be seen from Table VI.1, atrazine, diuron, metolachlor, simazine and terbutylazine have been frequently detected in marine waters before. Comparison with literature concentration data is complicated, since a wide range of concentrations could be observed in the various marine waters. Leaving a few outliers out of consideration, typical pesticide concentrations in marine waters are below 1 µg L⁻¹.

3.3 Perfluorinated compounds

With respect to PFCs, relatively more data on their presence in marine environments have been reported. Concentrations of PFOA and PFOS, which are considered as the two major contaminants within the group of the PFCs, are usually around tens of pg to a few ng L⁻¹ in ocean and coastal seawaters [41]. The concentrations of the detected PFCs (PFOS, PFOA, PFHxS and PFOSA) within our PFC-monitoring study (Chapter III) were in the same concentration order, except for the S22 and OO2 sample, in which higher levels were measured. Since S22 is located in the industrial zone of Antwerp, large inputs of PFCs could be expected. With respect to the marine tissue, PFOA could not be detected while PFOS was found in most mussel samples. In general, PFOS was found to be very abundant in the Belgian marine environment, however, in considerably low concentrations.

3.4 Distribution in the Belgian coastal zone

In general, a decreasing contamination of the micropollutants with increasing distance from the coast could be observed. The highest micropollutant concentrations were found at the harbour locations and in the Scheldt estuary (sample location S22). The industrial zone of Antwerp and the major inputs of contaminated industrial and domestic wastewater rendered the Scheldt estuary into a highly polluted marine environment. Rather high levels of the micropollutants were quantified in the coastal harbours as well,
probably due to the discharge of high volumes of polluted surface water into the harbours. From the data obtained for pharmaceuticals, pesticides as well as PFCs, generally lower concentrations in the North Sea could be noticed. To our opinion, both dilution and degradation processes resulted in little transport of micropollutants from the Scheldt estuary and the harbours towards the open sea.

4. BIOACCUMULATION FACTOR

Chapter IV has demonstrated that aquatic organisms can accumulate micropollutants which are present in the surrounding aquatic environment. Apparently, some chemicals such as carbamazepine and diuron were found only at low levels in biotic tissues, whereas others such as salicylic acid and dichlorvos were found up to substantially higher concentrations. Bioconcentration or bioaccumulation is the increase in concentration of a substance in or on an organism, relative to the concentration of the substance in the surrounding medium [42]. The tendency of the organism to bioaccumulate is assessed by the bioaccumulation factor (BAF) which can be calculated using the following equation [43,44]:

\[
BAF = \frac{C_{\text{biota}}}{C_{\text{water}}}
\]

BAF values are expressed in L kg\(^{-1}\), since the biota concentration (\(C_{\text{biota}}\)) is expressed in \(\mu\text{g kg}^{-1}\) (dry weight) and the water concentration (\(C_{\text{water}}\)) in \(\mu\text{g L}^{-1}\). The mean Log BAFs were calculated for every detected compound and are summarized in Table VI.2. The obtained Log BAF values varied between 0.6 L kg\(^{-1}\) for terbutylazine to 3.7 L kg\(^{-1}\) for propranolol, while for PFOS the Log BAF amounted to 2.8 L kg\(^{-1}\). Comparable experimental data are available for PFOS: Log BAF values of 3.3 and 4.5 were obtained for eel and lake trout respectively, however, expressed on a wet weight basis [43,44]. Obviously, the BAF values differed considerably by using grab sample data or passive sampler data for carbamazepine and propranolol. This can be attributed to the fact that higher water concentrations were obtained by using the passive sampling devices, especially for propranolol. Both the Log \(K_{\text{ow}}\) and the Log \(K_{\text{sa/wa}}\) values are presented in Table VI.2 as well. The Log \(K_{\text{ow}}\) is commonly used as an indication of a chemical's potential for bioconcentration by aquatic organisms [42]. Hydrophilic or polar micropollutants (\(\log K_{\text{ow}} < 2.5\)) will stay particularly in the water phase, while hydrophobic or non-polar substances (\(\log K_{\text{ow}} > 4\)) will partition to solids such as
sediments, suspended matter or biota. Depending on the compound, medium hydrophilic micropollutants with Log \( K_{ow} \) between 2 and 4 will adsorb to solids or stay in the aqueous phase [45]. No correlations between the Log BAF values and the Log \( K_{ow} \) coefficients (\( R^2 \) of 0.063 and p-value of 0.548) nor between the Log BAF values and the Log \( K_{sa/wa} \) coefficients (\( R^2 \) of 0.391 and p-value of 0.133) could be observed. Apparently, bioaccumulation is not exclusively driven by the hydrophobicity of substances, which turns prediction of partitioning of CECs into different parts of the aquatic environment into a difficult task. For this reason, information regarding their presence in both the aquatic phase and biotic tissue is required, which is provided within this thesis.

Table VI.2. Bioaccumulation factors (BAFs), Log \( K_{ow} \) [46] and Log \( K_{sa/wa} \) values of the detected CECs in water and biota

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log BAF</th>
<th>Log ( K_{sa/wa} )</th>
<th>Log ( K_{ow} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L kg(^{-1})</td>
<td>L kg(^{-1})</td>
<td></td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>2.1 ± 0.4 (n=48)</td>
<td>1.48</td>
<td>2.45</td>
</tr>
<tr>
<td></td>
<td>1.1 ± 0.4 (n=32) *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>3.7 ± 0.4 (n=9)</td>
<td>1.37</td>
<td>3.48</td>
</tr>
<tr>
<td></td>
<td>1.5 ± 0.6 (n=8)  *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>3.1 ± 0.5 (n=65)</td>
<td>-0.79</td>
<td>2.26</td>
</tr>
<tr>
<td>Diuron</td>
<td>1.1 ± 0.3 (n=35) *</td>
<td>2.13</td>
<td>2.68</td>
</tr>
<tr>
<td>Isoproturon</td>
<td>1.1 ± 0.3 (n=8)  *</td>
<td>2.07</td>
<td>2.87</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>1.7 ± 0.5 (n=22) *</td>
<td>3.40</td>
<td>3.13</td>
</tr>
<tr>
<td>Terbutylazine</td>
<td>0.6 ± 0.3 (n=8)  *</td>
<td>2.40</td>
<td>3.21</td>
</tr>
<tr>
<td>PFOS</td>
<td>2.8 ± 0.3 (n=14)</td>
<td>-</td>
<td>6.28</td>
</tr>
</tbody>
</table>

* obtained with passive sampler data

Besides the ecological relevance of BAFs, their determination is also important for regulatory purposes. Nowadays, the European regulation on chemical substances REACH requires bioconcentration factors (BCFs), which can be considered as BAFs obtained on a standardized manner [42], for all compounds. However, the experimental determination of BCFs is time-consuming, complicated, expensive and moreover, calculating these for the many thousands of chemical substances of interest is simply not possible [47]. Therefore, in recent years, models based on quantitative structure–activity relationships
(QSARs) have been developed to predict the partitioning of pollutants in biotic tissue [48-50]. With respect to CECs, modeling QSARs may certainly form the subject for further research, to enable accurate estimations of the accumulation and toxicity caused by CECs in organisms, thereby reducing the experimental variability as much as possible.

5. **RISK ASSESSMENT**

The risks for the aquatic environment, associated with the measured water concentrations of the targeted CECs within this thesis, have been assessed by using the regulatory guidance of the Water Framework Directive (2000/60/EC) [51] and its daughter directive (2008/105/EC) [52]. In this way, it was observed that the Environmental Quality Standard (EQS) of diuron was exceeded at two sampling locations (OO2 and ZB2) (Chapter V). However, this preliminary risk assessment is limited to the priority substances listed in the WFD guidance, such as the pesticides atrazine, diuron, isoproturon and simazine. Although PFOS is included in the WFD list of possible priority substances or priority hazardous substances, no EQS for PFOS has been established. Consequently, the preliminary risk assessment for PFOS was restricted to a comparison with the Predicted No Effect Concentration (PNEC) value of 25 µg L\(^{-1}\) [53]. Obviously, no adverse risks to aquatic organisms may be anticipated. Also the drinking water equivalent levels (DWELs) reported in literature of 0.04 – 2.4 µg L\(^{-1}\) for PFOA and the provisional health advisory developed by the US EPA for PFOA (400 ng L\(^{-1}\)) and PFOS (200 ng L\(^{-1}\)) were not exceeded [54-57]. In addition, no guidance is currently established for widely used and widespread occurring pharmaceuticals such as carbamazepine and salicylic acid. The risk assessment of pharmaceuticals is usually carried out by comparison of the Predicted Environmental Concentration (PEC) with a PNEC value and/or by calculation of the risk characterization ratio (RCR = PEC / PNEC). The obtained pharmaceutical monitoring data within this thesis did not pose an immediate substantial risk to marine life in the Belgian coastal zone, since all RCRs were far below 1 [58].

The above mentioned assessments, based on acute ecotoxicity data, present only a preliminary approach to characterize the environmental risks to aquatic ecosystems and organisms for the CECs detected within this thesis. In order to perform a more comprehensive risk assessment, more information on the chronic, mixture and mechanism specific toxic effects of CECs is needed. Furthermore, the measurements of
the body burdens of CECs in species that are important in terms of human consumption such as mussels (Chapter IV), are very useful in estimating the human exposure and dietary intake of these CECs. It was observed that the European default maximum residue level (MRL) for pesticides in foodstuffs of 10 ng g\(^{-1}\) [59] was exceeded for chloridazon and dichlorvos at several stations. Consequently, these findings will contribute to the evaluation of the human risks posed by these emerging micropollutants.

6. FURTHER RESEARCH RECOMMENDATIONS

In addition to the previous remarks, some final research recommendations can be stated. Several pharmaceuticals, including widely used active substances such as carbamazepine and diclofenac, usually undergo only limited degradation in conventional WWTPs [60]. Both their polar nature and the low concentrations at which they occur in WWTPs render their efficient removal a challenge [61]. Rather low elimination rates were reported for other CECs as well, including surfactant degradation products, PPCPs and polar pesticides [62]. Consequently, these substances pass through WWTPs unaltered before they find their way into receiving waters or land by the application of sewage sludge [63]. It is clear that a general removal strategy, which can tackle a broad spectrum of these recalcitrant micropollutants at both extremely low and very high concentrations, is required. Innovative strategies such as ozonation, advanced oxidation processes and bio-Pd technology have been suggested to obtain successful removal of micropollutants, and should be further explored [64,65]. Additionally, if degradation of parent compounds by microbial activity occurs, metabolic intermediates are formed, which could also be toxic to aquatic organisms. For this reason, modern monitoring and toxicity studies certainly should also include metabolites of CECs as well.

In 2006, the EU legislation established the PFOS directive 2006/122/EC [66], which aims at ending the use of PFOS. In addition, it was recommended to voluntarily reduce the use of PFOA as well [67]. Consequently, alternatives and substitute materials were introduced, which still offer the needed functionality, but lack the toxicity and bioaccumulation potential of PFOS, PFOA and the longer chain PFCs in general. Typical substitutes include new and short-chain PFCs (< eight carbons) and their mixtures. However, these “new” PFC substances could contribute significantly to the total PFC level in waters, since their removal from drinking water by common treatment techniques
has been proven to be difficult [67]. In addition, it has been reported that concentrations of short-chain PFCs with mildly phytotoxic properties, such as trifluoroacetic acid (TFA), could amount to harmful levels in ecosystems [68]. Therefore, more toxicological information on these short chain PFCs is needed, to assess their environmental impact. Besides, future environmental monitoring studies should certainly include these short-chain PFCs.

In the light of REACH, routine measurements of CECs are required in order to ensure that the quality of the aquatic environment is maintained [69]. Therefore, cost-effective and user-friendly monitoring strategies such as passive sampling devices are needed. Although passive samplers constitute an emerging alternative or complement to conventional grab sampling, their usage for the polar CECs is still rather limited. Passive sampling devices such as polar organic chemical integrative samplers (POCISs) and Chemcatcher® samplers have been recently applied for pharmaceutical and pesticide monitoring [70,71]. However, the quantitative aspect is still a major issue of concern, due to both the lack of calibration data to enable quantification of the target analytes, as well as the missing insights in the effects of environmental conditions on the analyte uptake. Therefore, further research should concentrate on the use of passive samplers as an efficient alternative to active water sampling of CECs, and more specific on the approaches for calibration and quantification thereof.

This thesis is a comprehensive evaluation study of the presence of a wide variety of emerging micropollutants in the Belgian marine environment. New data of CECs in water, biotic tissue and passive samplers are provided, thereby using newly developed analytical methods employing modern and sophisticated analytical equipment. The obtained findings will surely contribute to an integrated assessment of the environmental and human health risk of these emerging micropollutants, however, much additional research is still needed.
7. REFERENCES

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DISCUSSION AND FUTURE RESEARCH

SUMMARY

In the last decade, there has been a growing public and scientific concern regarding the occurrence and potential harmful effects of chemicals of emerging concern (CECs) in the aquatic environment. However, little attention has been paid so far to their prevalence and quantification in marine ecosystems. To study and evaluate the fate, effects, and environmental and human risks posed by these emerging contaminants, information regarding their presence in marine waters and organisms is urgently needed. Therefore, the research within this doctoral thesis aimed at the determination of different groups of CECs, including pharmaceuticals, perfluorinated compounds (PFCs) and pesticides, in environmental matrices. To this end, new analytical methods employing modern and sophisticated equipment were developed, validated and applied to samples originating from the Belgian coastal zone.

In **Chapter I**, a comprehensive overview is given of the analytical chemistry of the most environmentally important groups of CECs, including the pharmaceuticals and personal care products (PPCPs), pesticides, steroid hormones, PFCs, alkyphenolethoxylates, bisphenol A and phthalates. Due to both the typically very low concentrations at which CECs occur and the fact that environmental samples are complex matrices demanding extensive extraction and clean-up procedures, very specific and sensitive analytical procedures are needed. In this context, state-of-the-art instrumentation for sample preconcentration, analyte separation and detection was discussed. Several prominent trends could be observed: the common use of LC to allow separation of the CECs instead of GC, the development and application of multiclass methods, and a gain in popularity of high-resolution full scan analysis, combined with the trend towards the use of sub-2 µm particle sizes and high flow rates (U-HPLC). Overall, due to the recent advances in instrumentation, a significant progress in the analytical chemistry of CECs in environmental matrices could be noticed.

The objective of **Chapter II** was to develop, optimize and validate a new analytical method for the quantitative analysis of 13 multiclass pharmaceuticals in seawater. Using a
combination of solid-phase extraction (SPE) and liquid chromatography coupled to multiple mass spectrometry (LC-MS/MS), limits of quantification between 1 and 50 ng L\(^{-1}\) could be obtained. Validation according to Commission Decision 2002/657/EC resulted for most pharmaceuticals in linearitys > 0.99 and recoveries between 95–108%. Application of this method to water samples from the Belgian coastal zone, revealed the presence of seven pharmaceuticals. Salicylic acid and carbamazepine were the most abundant pollutants, in concentrations up to 855 ng L\(^{-1}\).

The development, validation and application of a new analytical method for the determination of 14 PFCs in surface-, sewage- and seawater was described in Chapter III. The occurrence of branched isomers, matrix interferences and cross contamination rendered quantitative analysis of PFCs in aqueous matrices a challenging task. However, the use of SPE followed by LC coupled to time-of-flight mass spectrometry (LC–ToF-MS) using very narrow mass tolerance windows (<10 ppm) resulted in a highly selective analytical approach for the detection of PFCs in complex aqueous matrices. Validation of this new method in surface-, sewage- and seawater resulted in LOQs ranging from 2 to 200 ng L\(^{-1}\), satisfying recoveries (92–134%) and good linearities (R\(^2\)=0.99 for most analytes). Analysis of North Sea and Scheldt estuary samples confirmed the presence of several PFCs, with the main contaminant perfluorooctane sulfonate (PFOS) detected in concentrations up to 39 ng L\(^{-1}\).

In Chapter IV, the occurrence of 14 pesticides, 10 PFCs, and 11 pharmaceuticals in freeze-dried tissue of blue mussels (Mytilus edulis) is described. Therefore, new methods were presented consisting of pressurized liquid extraction and SPE followed by ultra high-performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-QqQ-MS/MS) for pharmaceuticals and pesticides. For PFCs, a liquid extraction using acetonitrile and SPE followed by LC-ToF-MS was applied. Evaluation of the method performance resulted in LOQs in M. edulis tissue between 0.1 and 10 ng g\(^{-1}\), and satisfactory linearities (≥0.98) and recoveries (90–106%) were obtained. Five pharmaceuticals, two PFCs, and seven pesticides at levels up to 490, 5, and 60 ng g\(^{-1}\), respectively, were detected in the M. edulis samples originating from cage experiments at
different locations in the Belgian coastal zone. The most prevalent micropollutants were salicylic acid, paracetamol, PFOS, chloridazon and dichlorvos.

The use of passive samplers as an efficient alternative to active water and biological sampling of CECs is presented in Chapter V. A new analytical method was optimized for the quantification of a high number of pharmaceuticals and pesticides in passive sampling devices. The analytical procedure consisted of extraction using 1:1 methanol/acetonitrile followed by detection with U-HPLC coupled to high resolution and high mass accuracy Orbitrap MS. The LOQs obtained were between 0.2 and 20 ng per sampler sheet and the recoveries ranged from 88 to 105%. In addition, the sampler-water partition coefficients of all compounds were determined. It was found that few pharmaceuticals and most pesticides exerted a high affinity for the polydimethylsiloxane passive samplers. This was confirmed by measuring the time-weighted average concentrations of the targeted pollutants in passive samplers, deployed at eight stations in the Belgian coastal zone. Propranolol, carbamazepine and seven pesticides were found to be very abundant in the passive samplers.

Finally, in Chapter VI, general conclusions of this doctoral work and further research perspectives were formulated. The major goal of this doctoral thesis was the development of new analytical approaches for quantification of emerging micropollutants in the Belgian coastal zone. The obtained concentrations of the targeted micropollutants in marine waters, biota and passive samplers were discussed in terms of their distribution in the marine environment. In addition, the bioaccumulation potential of the measured micropollutants was determined and a preliminary risk assessment based on acute ecotoxicity data and regulatory standards was provided. Still, more information on both the accumulation potential of CECs and their chronic, mixture and mechanism specific toxic effects is needed, to enable the integrated assessment of the environmental and human health risk. Within this view, modeling QSARs may certainly form the subject for further research.
SAMENVATTING

De laatste 10 jaar is er zowel vanuit maatschappelijke als wetenschappelijke hoek een toenemende bezorgdheid merkbaar omtrent de aanwezigheid en de potentiële effecten van bepaalde opkomende contaminanten in het aquatisch milieu. Evenwel werd de aanwezigheid en de kwantificering van deze vervuilende stoffen in het mariene milieu tot nu toe slechts in beperkte mate bestudeerd. Om een juiste inschatting te kunnen maken van de verspreiding, effecten en risico’s voor mens en milieu veroorzaakt door deze polluenten, is er duidelijk meer informatie nodig omtrent hun aanwezigheid in mariene wateren en organismen. Het huidige doctoraatsonderzoek kadert binnen deze problematiek en heeft als algemeen doel het bepalen van verschillende groepen van opkomende contaminanten waaronder farmaceuticals, pesticiden en perfluorverbindingen in diverse milieumatrices. Daartoe werden nieuwe analysemethoden ontwikkeld, gevalideerd en toegepast op stalen afkomstig van verschillende plaatsen aan de Belgische kust, daarbij gebruik makend van moderne en gesofisticeerde analytische apparatuur.

In Hoofdstuk I wordt een uitgebreid literatuuroverzicht gegeven van de recente analytische methodiek voor de detectie van de belangrijkste groepen van opkomende contaminanten. Daartoe behoren de farmaceuticals en persoonlijke verzorgingsproducten, de pesticiden, de steroïden, de perfluorverbindingen, de alkylphenolethoxylaten, bisfenol A en de ftalaten. Deze stoffen komen typisch in heel lage concentraties voor in het aquatisch milieu en bovendien gaat het om heel complexe matrices waaruit de doelstoffen moeten geïsoleerd worden. Om deze redenen is een grondige staalvoorbereiding, een geschikte extractiemethode en heel specifieke en gevoelige analytische apparatuur essentieel. In dit opzicht werd de huidige stand van zaken besproken van de mogelijke methoden en apparatuur om de doelstoffen te extraheren, opconcentreren, scheiden en detecteren in diverse aquatische milieumatrices. Enkele trends die naar voorkomen zijn de volgende: het toenemend gebruik van vloeistofchromatografie (LC) in plaats van gaschromatografie (GC), de ontwikkeling van methoden die toepasbaar zijn voor verschillende groepen van contaminanten tegelijk, en het toenemend gebruik van hoge resolutie massaspectrometrie (MS), en dit alles in combinatie met het toenemend gebruik van ultrahoge druk vloeistofchromatografie. Over het algemeen kan men stellen dat door
de recente ontwikkelingen op vlak van instrumentatie er een enorme vooruitgang is geboekt in dit onderzoeksdomein.

Het doel van Hoofdstuk II bestond erin om een nieuwe analysemethode te ontwikkelen, optimaliseren en valideren voor de kwantitatieve bepaling in zeewater van 13 farmaceuticals uit verschillende therapeutische klassen. De combinatie van vaste-fase extractie en LC gekoppeld aan ion trap MS resulteerde in kwantificeringsgrenzen tussen 1 en 50 ng L\(^{-1}\). Validatie volgens de Europese criteria (2002/657/EC) toonde aan dat een lineaire (R\(^2\) > 0.99) en accurate (95–108%) methode werd bekomen. Toepassing van deze nieuwe methode op waterstalen afkomstig uit het studiegebied van de Belgische kustzone toonde de aanwezigheid aan van 7 farmaceuticals. Salicylzuur en carbamazepine werden hierbij het vaakst en in de hoogste concentraties teruggevonden, meer bepaald tot een concentratie van 855 ng L\(^{-1}\).

De ontwikkeling, validatie en toepassing van een nieuwe analysemethode voor de bepaling van 14 perfluorverbindingen in oppervlakte-, afval- en zeewater is beschreven in Hoofdstuk III. De aanwezigheid van vertakte isomeren en het mogelijk optreden van matrixinterferenties en contaminatie maakt van de kwantificering van perfluorverbindingen in waterige matrices een complexe taak. Door gebruik te maken van vaste-fase extractie gevolgd door LC gekoppeld aan een ‘time-of-flight’ MS met een lage massa-afwijkingsgrens (< 10 ppm), werd een heel selectieve methode bekomen voor deze toepassing. Validatie van deze methode resulteerde in kwantificeringslimieten tussen 2 en 200 ng L\(^{-1}\), een geschikte accuraatheid (92-134%) en lineariteit (R\(^2\) = 0.99 voor de meeste van de doelstoffen). Analyse van stalen afkomstig van de Belgische kustzone bevestigde de aanwezigheid van verschillende perfluorverbindingen, waarbij PFOS teruggevonden werd in de hoogste concentraties tot 39 ng L\(^{-1}\).

In Hoofdstuk IV werd de aanwezigheid van 14 pesticiden, 10 perfluorverbindingen en 11 farmaceuticals in weefsel van de blauwe mossel (*Mytilus edulis*) bestudeerd. Daartoe werden nieuwe methoden opgesteld die voor de bepaling van farmaceuticals en pesticiden bestaan uit vloeistofextractie onder verhoogde druk en temperatuur, gevolgd door vaste-fase extractie en detectie met behulp van ultra-hoge druk vloeistofchromatografie en
triple quadrupole massaspectrometrie (U-HPLC-QqQ-MS/MS). Voor de perfluorverbindingen werd gebruik gemaakt van vloeistofextractie met acetonitrile gevolgd door vaste-fase extractie en LC-ToF-MS detectie. Evaluatie van de prestatiecriteria van deze methode resulteerde in kwantificeringsgrenzen tussen 0.1 en 10 ng g\(^{-1}\), adequate lineairiteiten (R\(^2\) > 0.98) en terugvindingen tussen 90 en 106%. Vijf farmaceuticals, 2 perfluorverbindingen en 7 pesticiden in respectievelijke concentraties tot 490, 5 en 60 ng g\(^{-1}\), werden gedetecteerd in de *M. Edulis* stalen afkomstig van kooiexperimenten uitgevoerd op verschillende plaatsen in de Belgische kustzone. De meest voorkomende polluenten waren salicylzuur, paracetamol, PFOS, chloridazon en dichloorkos. Het gebruik van passieve bemonsteringstechnieken als alternatief voor actieve bemonstering van de opkomende contaminanten in water en biota werd besproken in *Hoofdstuk V*. Daartoe werd een nieuwe analysemethode ontwikkeld voor de kwantificering van een groot aantal farmaceuticals en pesticiden in passieve bemonsteringssystemen. Deze analysemethode bestond uit extractie met een mengsel van methanol en acetonitrile (50/50) gevolgd door detectie met behulp van U-HPLC gekoppeld aan hoge resolutie en accurate massa analyse met behulp van Orbitrap MS. Dit resulteerde in kwantificeringsgrenzen tussen 0.2 en 20 ng per sampler sheet en terugvindingen tussen 88 en 105%. Daarnaast werden de partitiecoëfficiënten tussen water en het passief bemonsteringssysteem bepaald voor alle componenten. Enkele farmaceuticals en de meeste pesticiden vertoonden een hoge affiniteit voor de passieve bemonsteringssystemen bestaande uit polydimethylsiloxaan. Dit werd bevestigd bij het bepalen van de tijdsgewogen gemiddelde concentraties van de polluenten in de passieve bemonsteringssystemen die werden geplaatst op 8 verschillende locaties in de Belgische kustzone. Hierbij werden zowel de farmaceuticals propranolol en carbamazepine als zeven pesticiden vaak gedetecteerd in de passieve bemonsteringssystemen. Tenslotte werden in *Hoofdstuk VI* de algemene besluiten van deze thesis geformuleerd, alsook enkele toekomstige onderzoeksmogelijkheden. Het hoofddoel van deze doctoraatstudie bestond in de ontwikkeling van nieuwe analysemethoden om kwantificering van de opkomende micropolluenten in de Belgische kustzone mogelijk te
SAMENVATTING

maken. De gemeten concentraties van de micropolluenten werden in dit deel besproken
op vlak van hun verspreiding in het mariene milieu. Ook werd hun vermogen tot bio-
accumulatie bepaald en werd een beperkte risico-inschatting uitgevoerd op basis van
korte-termijn ecotoxiciteitsdata en standaardwaarden afkomstig uit de wetgeving. Om tot
een geïntegreerde risico-inschatting voor mens en milieu te komen, is er duidelijk nog
nood aan meer informatie omtrent hun bio-accumulatie potentieel alsook aan chronische-,
mengsel- en mechanismespecifieke toxiciteitsdata. In dit opzicht vormt de verdere
ontwikkeling van de QSAR-technologie een zeer interessant onderzoeksperspectief.
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Personalia

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Education

1996-2002  Sciences-Mathematics + 2u Latin
           Stella Matutinacollege, Lede
2002-2007  Master of Bioscience Engineering: Environmental Technology
           Faculty of Bioscience Engineering, Ghent University
           Thesis: Co-metabolic biodegradation of 17α-ethinyloestradiol
           (LabMET, Ghent University).
2007-2011  PhD research in Veterinary Sciences
           Laboratory of Chemical Analysis
Faculty of Veterinary Medicine, Ghent University

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2007-2011 Completion of the doctoral training programme of the doctoral schools of Life Sciences and Medicine

Professional activities during PhD research

2008-2010 Close collaboration with the Flemish Environment Agency (FEA), Laboratory for Analysis of Organic Micropollutants, Ghent.

Development of new analytical procedures for detection of perfluorinated compounds in environmental matrices.

2009-2010 Tutor of Master student in Veterinary Science.

2010 Tutor assistant for the practical theory and exercise session on the primary components of the course ‘Food and Environmental Chemistry’ at the Faculty of Veterinary Science, Ghent University.

Specialist studies during PhD research


2009 Advanced Academic English for doctoral schools of Life Sciences and Medicine: writing skills (course), Ghent University.

2009 TSQ Vantage (triple-quadrupole MS) training course by ThermoFisher Scientific. Laboratory of Chemical Analysis, Ghent University.
2010  Exactive (Orbitrap-MS) training course by ThermoFisher Scientific. Laboratory of Chemical Analysis, Ghent University.

2010  Effective scientific communication for doctoral schools of Life Sciences and Medicine (course), Ghent University.

**Scientific publications**


K. Verheyden, H. Noppe, J. Vanden Bussche, **K. Wille**, K.M. Bekaert, L. De Boever, J. Van Acker, C.R. Janssen, H.F. De Brabander, L. Vanhaecke, Characterisation of steroids in wooden crates of veal calves by accelerated solvent extraction (ASE (R)) and ultra-


Abstracts


of pharmaceuticals in marine waters. VLIZ Young Scientists’ Day, Bruges, Belgium, 29 February 2008.


Conferences, workshops, seminars and research visits

✓ Tenth International Symposium on Advances in Extraction Techniques and Hyphenated Chromatographic Analyzers (HTC-10), Bruges, Belgium, 28/01/08 – 30/01/08. Poster presentation.
✓ VLIZ Young Scientists’ Day, Bruges, Belgium, 6 March 2009. Poster presentation.
✓ Dionex workshop: sample preparation and extraction, Antwerp, Belgium, 17 September 2008.
✓ Symposium: 75 jaar diergeneeskunde aan de Ugent, Merelbeke, Belgium, 19 March 2009. Poster presentation.

Recent Developments in the Environmental Chemistry of Per and Polyfluorinated Compounds (PFCs), Workshop ICCE 2009, Stockholm University, Sweden, 14 June 2009.


BelTox annual scientific meeting, Louvain-la-Neuve, Belgium, 25 November 2010. Poster presentation.

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✓ Online Enrichment Environmental LC – MS Meeting, Flemish Environment Agency (FEA), Ghent, Belgium, 25 May 2011.

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Klaas
Analytical approaches for quantification of emerging micropollutants in the Belgian coastal zone

Klaas Wille

2011