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Development of Mass Spectrometric Methods for the determination of Emerging Pollutants in wastewater

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Ανάπτυξη Μεθόδων Φασματομετρίας Μάζας για τον προσδιορισμό Αναδυόμενων Ρύπων σε υγρά απόβλητα

ΜΠΛΕΤΣΟΥ ΑΝΝΑ

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ABSTRACT

The presence of emerging pollutants in environmental samples is unquestionable, since urban, industrial and agricultural activities release in the aquatic environment great amounts of diverse organic micropollutants over the last decade. Environmental monitoring is an urgent nowadays, as a part of a holistic environmental risk assessment. The main objective of this thesis was the development of highly sensitive methods for the determination of multi-class emerging pollutants with the use of different mass spectrometric techniques.

First, an introduction on the emerging pollutants is presented, since they encompass a diverse group of compounds, highlighting their wide-scope properties. Specific workflows and techniques for their determination are then presented, focusing in target and suspect screening by mass spectrometric techniques and especially in the development of high-resolution mass analyzers. The experimental section of the thesis consists of three parts: (i) Determination of Linear and Cyclic Siloxanes by gas chromatography hyphenated with mass spectrometry in environmental samples and evaluation of Mass Loadings and Fate in a Wastewater Treatment Plant in Greece (Chapter 3), (ii) Wide-scope quantitative target screening of 2327 emerging contaminants in wastewater samples with Ultra-Performance Liquid Chromatography Quadrupole-Time-Of-Flight Mass Spectrometry (UPLC-Q-ToF-HRMS/MS) (Chapter 4) and (iii) Suspect screening workflow for the characterization of emerging polar organic contaminants in wastewater samples with Ultra-Performance Liquid Chromatography High-Resolution Mass Spectrometry (UPLC-HRMS/MS) (Chapter 5).

It is our strong belief that these studies will constitute a step forward in environmental analysis of emerging pollutants.

SUBJECT AREA: Analytical Chemistry

KEYWORDS: Emerging pollutants, LR-MS, HR-MS, target screening, suspect screening, environmental samples

ΠΕΡΙΛΗΨΗ

Η παρουσία αναδυόμενων ρύπων σε περιβαλλοντικά δείγματα είναι αδιαμφισβήτητη, καθώς αστικές, βιομηχανικές και γεωργικές δραστηριότητες αποβάλλουν στο υδάτινο περιβάλλον μεγάλες ποσότητες διαφόρων οργανικών μικρορυπαντών, τα τελευταία χρόνια. Η παρακολούθηση και ο έλεγχος του περιβάλλοντος αποτελεί ανάγκη στις μέρες μας, σαν μέρος μιας συνολικής περιβαλλοντικής αξιολόγησης κινδύνου. Ο κύριος στόχος της παρούσας διπλωματικής διατριβής ήταν η ανάπτυξη ευαίσθητων αναλυτικών μεθοδων για το προσδιορισμό αναδυόμενων ρύπων από διάφορες κατηγορίες, με τη χρήση διαφορετικών τεχνικών φασματομετρίας μάζας.

Αρχικά παρουσιάζεται μια εκτεταμένη ανασκόπηση σχετικά με τους αναδυόμενους ρύπους, καθώς περικλείουν διάφορες ομάδες ενώσεων, τονίζοντας το εύρος των ιδιοτήτες τους. Συγκεκριμένες πορείες και τεχνικές παρουσιάζονται, στη συνέχεια, σχετικά με τον προσδιορισμό τους, στοχεύοντας κυρίως στη στοχευμένη ανάλυση και στη παρακολούθηση "'ύποπτων" ενώσεων, με τεχνικές φασματομετρίας μάζας και κυρίως στην ανάπτυξη της φασματομετρίας μάζας υψηλής διακριτικής ικανότητας. Το πειραματικό μέρος της διατριβής αποτελείται από τρία μέρη: (α) Προσδιορισμός σιλοξανίων με αέριοχρωματογραφια συζευγμένης με φασματομετρίας μαζών και εκτίμηση του φορτίου και της τύχης τους σε ένα κέντρο επεξεργασίας λυμάτων σην Ελλάδα (Κεφάλαιο 3), (β) Ποσοτική στοχευμένη ανάλυση 2327 αναδυόμενων ρύπων σε λύματα με υγροχρωματογραφία υψηλής απόδοσης συζευγμένης με φασματομετρία μάζας τεχνολογίας τετραπόλου-αναλυτή χρόνου πτήσης (Κεφάλαιο 4) και (γ) Παρακολούθηση "'ύποπτων" ενώσεων για το χαρακτηρισμό πολικών οργανικών αναδυόμενων ρύπων σε λύματα με υγροχρωματογραφία υψηλής απόδοσης συζευγμένης με φασματομετρία μάζας υψηλής διακριτικής ικανότητας.

Οι μελέτες αυτές αποτελούν ένα σημαντικό βήμα στην ανάλυση περιβαλλοντικών αναδυόμενων ρύπων.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αναλυτική Χημεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: Αναδυόμενοι Ρύποι, LR-MS, HR-MS, στοχευμένη ανάλυση, παρακολούθηση "ύποπτων ενώσεων", περιβαλλοντικά δείγματα

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PREFACE

The experimental part of this thesis was performed in two laboratories.

The first experimental part (Chapter 3) was carried out at the Wadsworth center, New York State Department of Health, and Department of Environmental Health Sciences, School of Public Health, State University of New York at Albany, U.S.A., under the supervision of Dr. Kurunthachalam Kannan and in collaboration with Dr. Nikolaos Thomaidis.

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CHAPTER 1

Emerging pollutants in environmental analysis

1.1. Introduction

Overwhelming evidence has shown that organic micropollutants have been released from urban, industrial and agricultural activities, over the last decade and nowadays are ubiquitous in the aquatic environment. These substances, referring to them as “emerging pollutants”, EPs, include a wide array of different compounds, as well as metabolites and transformation products (TPs), can be detected with highly sensitive analytical methodologies in levels of parts per trillion (ppt), even in the most complex environmental matrices. Although environmental monitoring is making great progress, many pollutants still remain undetected and thus out of water controls. Because of the vast number of possible compounds, many studies have occurred in different classes of emerging pollutants, according to priority lists established, taking into account consumption, predicted environmental concentrations, as well as ecotoxicological, pharmacological and physicochemical data. However their environmental impact (ecotoxicological and possible health risks for human) that is associated with their occurrence is still unknown. Although the reported concentrations are generally low, questions have been raised over the potential impacts of emerging pollutants in the environment on human and animal health after long-term exposure. Another fact that should be taken into consideration is that TPs may be formed in the environment and may be detected in higher concentrations than the parent compounds, or even human metabolites could be excreted from the human body [1, 2]. There is very limited knowledge on the occurrence and environmental fate of human metabolites. Additionally, the extent in which the current water and wastewater treatment infrastructures can effectively remove these compounds is also, in great extent, an unknown parameter [3]. The inefficient removal poses a serious environmental problem. Thus, it is a topic of growing interest from both research and regulatory perspectives.

1.2. Emerging Pollutants (EPs) and their transformation products (TPs)

1.2.1. Emerging Pollutants (EPs)

The term “emerging pollutants” (EPs) (or “emerging contaminants”; ECs) refers to compounds and their metabolites that are not currently covered by existing water-quality regulations, have not been often studied, overlooked, and/or are thought to be potential threats to environmental ecosystems and human health and safety. According to NORMAN, they are compounds that are not included in routine environmental monitoring programs and may be candidates for future legislation due to their adverse effects and/or persistency (http://www.norman-network.net/). Most regulating and implementation bodies, responsible for water and wastewater treatment, are working on the assumption that the so-called priority pollutants are responsible for the most significant share of environmental, human health and economic risk, even though they are representing a minor fraction of the universe of both known and yet-to be identified chemicals [4].

The EPs encompass a diverse group of compounds, including pharmaceuticals and personal care products (PPCPs), illicit drugs and drug of abuse, hormones and steroids, benzothiazoles, benzotriazoles, polychlorinated naphthalenes (PCNs), perfluorochemicals (PFCs), polychlorinated alkanes (PCAs), polydimethylsiloxanes (PDMSs), synthetic musks, quaternary ammonium compounds (QACs), bisphenol A (BPA), triclosan (TCS), triclocarban (TCC), as well as polar pesticides, veterinary products, industrial compounds/by-products, food additives and engineered nano-materials [5, 6]. Some of these groups of compounds are briefly presented subsequently.

1.2.1.1. Pharmaceutical and personal care products – illicit drugs

Pharmaceutical and personal care products (PPCPs) maintain chemical properties that can vary widely, usually containing a non-polar core with a polar functional moiety. Their transformation products may be environmentally persistent [7, 8]. For

instance, the environmental persistence of the main active metabolite of the blood lipid regulators (clofibrate, erofibrate and theofibrate), clofibric acid, is 21 years [9]. Furthermore, pharmaceuticals may have unpredicted and unknown side effects on different organisms, particularly after long-term exposure to low concentrations, as well as they may provoke bacterial resistance from the release of antibiotics to the environment. Additionally, the potential effects of metabolites, in organisms of the ecosystem, are still unknown [10]. PPCPs and their metabolites are often referred to as ‘‘effluent-derived’’ contaminants, originally present in wastewater. Antibiotics can be structurally categorized into the following groups: sulfonamides (SA), fluoroquinolones (FQ), nitroimidazoles (NI), penicillins (PE), cephalosporins (CE), tetracyclines (TC) and macrolides (MA) [7, 11]. The chemical properties of every substance determine its behavior through the wastewater treatment, the mobility, persistence and even the bioavailability in the soil matrix [7]. All antibiotics, many other pharmaceuticals and PPCPs have the tendency to persist or to be only partially degraded during treatment or even to bypass treatment altogether via sewage overflows, therefore, contributing to their load in receiving waters, many of which serve as recreational and drinking water sources [3, 7, 10, 12, 13].

One the other hand, the use of illicit drugs has gained worldwide concern due to their significant adverse impacts on human health and wellbeing of the society. Illicit drugs are those whose nonmedical use is prohibited by the international law, and mainly belong to the classes of opiates, cocaine, cannabis, amphetamines and their metabolites. The chemicals associated with these illegal laboratories including precursors and by-products as well as the synthesized drugs are often illegally buried in soil or public waste management facilities, or disposed of into sinks or toilets after which they enter the sewerage system. Research on illicit drugs has been conducted with results showing that the presence of these compounds have temporal patterns that could be used to track use, but also that many of these compounds can be attenuated by wastewater treatment plants, WWTPs. Removal of illicit drugs by wastewater treatment was generally greater than 50%, except in a WWTP that uses primary treatment [14, 15].

1.2.1.2. Steroids

Natural endogenous (17β-estradiol, estrone, estriol) and synthetic steroids (17α-ethinyloestradiol, mestranol) are excreted by humans and WWTP effluent is the primary source of synthetic steroids entering the environment. Estrogenic activity in WWTP effluents has resulted in adverse effects on environmental biota. Natural and synthetic steroids are excreted from the human body as inactive polar conjugates, but are present in sewage influent and effluent as free, active steroids. Once released from the body conjugated estrogens undergo chemical or enzymatic dissociation in bacterial sludge and reform as active estrogens [16].

1.2.1.3. Artificial sweeteners

Sucralose and other artificial sweeteners have recently been identified as persistent emerging pollutants [6]. Sucralose is a relatively new artificial sweetener. It may seem like an odd compound to include as an emerging contaminant, but it is now being found widely in environmental waters and is extremely persistent (half-life up to several years). Several research groups have reported measurements of sucralose in the environment (including river water, groundwater, and coastal waters), and research has expanded to include other artificial sweeteners, such as acesulfame, saccharin, cyclamate, and aspartame [15, 17]. Because of their recalcitrance to transformation, acesulfame and sucralose were viewed as an ideal marker for the detection of domestic wastewater in environmental waters, particularly groundwater [18, 19].

1.2.1.4. Benzotriazoles - benzothiazoles

Benzotriazoles are complexing agents that are widely used as anticorrosives. The two common forms, benzotriazole (1H-benzotriazole) and tolyltriazole (a mixture of 4- and 5-methyl-1Hbenzotriazole), are soluble in water, resistant to biodegradation, and only partially removed in wastewater treatment [15, 20]. Benzothiazoles are used as corrosion inhibitors, herbicides, antialgal agents, slimicides in paper and pulp

industry, photosensitizers, constituents of azo dyes, in de-icing/anti-icing fluids, chemotherapeutics and fungicides in lumber and leather production [21]. The benzothiazoles removal efficiencies ranged from zero to up to 80% in the conventional WWTP [22].

1.2.1.5. Perfluorochemicals

Perfluorochemicals (PFCs) are a family of anthropogenic chemicals that have been used to make products resistant to heat, oil, stains, grease and water. PFCs are persistent and widely dispersed in the environment. They are chemically unusual compounds, in that they are both hydrophobic and lipophobic, and they contain one of the strongest chemical bonds (C-F) known. The human and environmental toxicological response to such exposure is not known, but could include, among others, endocrine disruption [7, 23]. The chemical structures of PFCs make them very resistant to degradation in the environment. The two most common groups of PFCs that are measured and detected in environmental matrices are perfluoroalkyl sulphonates (PFASs) with main representative compound perfluorooctane sulphonate (PFOS) and perfluoroalkyl carboxylates (PFACs), with main representative compound perfluorooctanoic acid (PFOA). PFASs and PFACs are synthetic chemicals that do not occur naturally in the environment. Mass balance studies of PFCs at WWTP commonly report higher mass loadings of PFOA and PFOS in WWTP effluent compared to raw influent [24]. This suggests that the degradation of other fluorinated organic compounds (i.e. fluoropolymers) into PFOA and PFOS may take place during wastewater treatment [7]. In addition, perfluorooctane sulphonamide (PFOSA) has been the most frequently detected derivative of a third class, perfluorinated sulphonamides, but interestingly enough was often found below detection limits [23].

1.2.1.6. Siloxanes and musks

Siloxanes consist of a structural unit of alternating Si-O bond with organic side chains. They include cyclic and linear siloxanes and form a large group of chemicals with molecular weights from a few hundred to several hundred thousand. Siloxanes are widely used in consumer products, such as paints and cosmetics, as well as in medical products, because of their high thermal stability, smooth texture, physiologic inertness and lubricating properties. They are, in general, very persistent once released in the environment. In recent years, various studies pointed out that some siloxanes may have endocrine disrupting properties and effects on the reproduction, which may cause concern about their effect on humans and the environment [25, 26]. PDMSs have been detected in environmental samples, such as surface water, sediments and fish tissue. Cyclic siloxanes were found at greater concentrations than linear siloxanes in sediment samples. However, the summed concentrations of linear siloxanes were higher than the summed concentrations of cyclic siloxanes in sludge samples [27]. PDMSs have low ecological toxicity, which occurs at higher concentrations than those observed in the environment, and are not considered to pose an ecologically significant threat. PDMSs have very low water solubility and are primarily removed by sorption to solids during wastewater treatment. At least 94% of PDMSs are unchanged during wastewater treatment, because of high chemical and thermal stabilities. PDMSs are not toxic to wastewater microbial communities and do not affect treatment performance. PDMSs, not removed on the sludge solids, are present in wastewater treatment effluent as a component of the suspended solids. PDMSs degrade in the soil environment, as a result of abiotic processes rather than biodegradation, and have a half-life estimated to range from 4 to 28 days [7]. Synthetic musks have been used since the 1930s as fragrances in a variety of domestic and industrial products, e.g., detergent, cosmetics, shampoo, perfume, food and cigarette additives. WWTP mass balance studies indicated important removal rates [7, 28]. Musks are highly lipophilic, so they tend to accumulate in sediments, sludges, and biota.

1.2.1.7. Polybrominated diphenyl ethers - polychlorinated alkanes – polychlorinated naphthalenes

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants (BFRs) that were used in plastics, textiles, electronic circuitry, and other materials. There are 209 PBDE congeners. There is concern about their use, because of their widespread presence in the environment and in human and wildlife samples, as well as their presence in locations far from where they were produced or used.

The technical mixtures of polychlorinated alkanes (PCAs), often referred to as chlorinated paraffins (CPs), are a class of industrial chemicals, comprising of chlorinated straight-chain hydrocarbons. Trace levels may be present in treated waste effluent. PCAs are divided into three groups: shortchain PCAs (noted as sPCAs or SCCPs) comprising 10 to 13 carbon atoms, medium-chain PCAs (mPCAs or MCCPs) comprising 14 to 17 carbon atoms and long-chain PCAs (lPCAs or LCCPs) with 18 or more carbon atoms. The total number of possible congeners is unknown, but far exceeds 10,000. The concentrations of PCAs in sewage sludge, evidence of accumulation in human and environmental biota, as well as toxicity data indicate that further research is necessary to assess the risk to human health and the environment from the industrial use of this chemical group. Technical mixtures of polychlorinated naphthalenes (PCNs) have been used since the early 1900s as dielectric fluids, engine oil additives, electroplating masking compounds, wood preservatives, lubricants, and for dye production. They are also structurally similar and have similar physico-chemical properties. There are 75 PCN congeners, substituted with one to eight chlorine atoms per naphthalene molecule. PCNs are ubiquitous environmental contaminants and several PCN congeners exhibit dioxin-like toxicity [7].

1.2.1.8. Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are cationic surfactants. The molecules contain at least one hydrophobic hydrocarbon alkyl chain linked to a positively

charged nitrogen atom. The other alkyl groups are typically short-chain substituent's such as methyl or benzyl groups. Domestic use of QACs is the primary source of these compounds entering WWTPs. Removal of QACs in wastewater treatment can also be attributed to biodegradation, where degradation rates are typically reported in days or hours. WWTP discharges can result in environmental contamination of marine sediments with QACs [7, 29].

1.2.1.9. Triclosan – triclocarban - bisphenol A

TCS and TCC are antimicrobial agents widely used in personal care products, such as shampoos, soaps, deodorants, cosmetics, skin-care lotions and creams, mouth rinses, and toothpastes. Mass balance studies at WWTP show the incomplete removal of TCC and TCS during wastewater treatment. Bisphenol A is a plasticizer manufactured in high quantities and is used mainly as a monomer for the production of polycarbonate and epoxy resins, unsaturated polyester. WWTP mass balance studies have detected bisphenol A in raw water, sewage sludge and effluents. Significant reductions (up to 99%) during wastewater treatment have been reported and biodegradation is thought to be the principal removal mechanism [7, 30].

1.2.1.10. Nanomaterials

Nanomaterials are 1 to 100 nm in size and can have unique properties, including high strength, thermal stability, low permeability, and high conductivity. In the near future, nanomaterials are projected to be used in areas such as chemotherapy, drug delivery, and labeling of food pathogens. The chemical structures of nanomaterials are highly varied, including fullerenes, nanotubes, quantum dots, metal oxanes, TiO_2 nanoparticles (NPs), nanosilver, nanogold, and zerovalent iron NPs. Most nanomaterial research is centered on developing new uses for nanomaterials and new products with unique properties, but on the other side, there is also significant concern regarding nanomaterials as environmental contaminants. As such,

nanomaterials are the focus of researches, under which fate, transport, and health effects are being evaluated [15].

1.2.2. Sources, occurrence and fate

Sources of emerging pollutants in the environment that may eventually impact groundwater can be divided into point-sources and mobile sources of pollution. Point-source pollution originates from discrete locations whose inputs into aquatic systems can often be defined in a spatially discrete manner. The spatial extent or plume of pollution is therefore generally more constrained. Important examples include industrial effluents, municipal sewage treatment plants and combined sewagestorm-water overflows, resource extraction, waste disposal sites and buried septic tanks. Mobile pollution, in contrast, originates from poorly defined, diffuse sources that typically occur over broad geographical scales. Examples of mobile source pollution include agricultural runoff from bio-solids and manure sources, storm-water and urban runoff, leakage from reticulated urban sewerage systems and diffuse aerial deposition. While it is clear from literature review that the vast majority of groundwater resources do not contain emerging pollutants in concentrations that would be considered toxic and/or harmful due to natural attenuation and dilution mechanisms, there is a large variety of compounds found in groundwater, and in some cases their concentrations are significant (>100 ng L^{-1}). The combined toxicity of multiple contaminants is not well understood. In addition, there are a number of specific pollutants that have a global footprint, and are frequently detected in groundwater resources.

The most frequently reported group of compounds were pharmaceuticals, including analgesics, anti-inflammatory drugs, antibiotics, anti-epileptics (carbamazepine) and barbiturates (primidone). From a collaborative trial on Danube river, 347 compounds were detected, including carbamazepine, atrazine, sulfamethoxazole, DEET, metformin, terbuthylazine, caffeine, atrazine-desethyl, tramadol, metoprolol, terbuthylazine-desethyl, phenazone (also known as antipyrine), 4 and 5-methylbenzotriazole, atrazine-2-hydroxy, venlafaxine, isoproturon, telmisartan,

perfluorooctane sulfonate (PFOS) and metolachlor [31]. Two phenolic compounds (bisphenol A and nonylphenol (NP), both known endocrine disruptors), a flame retardant (tri(2- chloroethyl) phosphate) and the musk galaxolide were among the most frequently reported compounds [15]. Diuretics, blood lipid regulators, beta blockers, analgesics, antibiotics and fragrances are also detected frequently in WWTP samples [32]. The presence of 'free' estrogens in WWTP effluents and receiving waters is commonly reported, demonstrating that the conversion of estrogen metabolites into active forms occurs somewhere between the domestic discharge and WWTP outlet [7]. Benzotriazole, caffeine, carbamazepine, tolyltriazole, and nonylphenoxy acetic acid (NPE1C) were the most frequently detected persistent organic pollutants in European river waters [33]. Endocrine disruption compounds (EDCs), coprostanol, N,N-diethyltoluamide (DEET), caffeine and triclosan were among the most frequently detected organic wastewater contaminants in U.S. streams. DEET, Bisphenol A (BPA), tri(2-chloroethyl) phosphate, and sulfamethoxazole were among the most frequently detected organic wastewater contaminants in U.S. groundwater [34]. Accumulation of PFCs has been detected in ocean animals, such as birds and mammals, and in human tissues throughout the world [7]. The occurrence of synthetic musks in sewage sludge has been reported from Switzerland, Germany, Spain, UK, China and Hong Kong [7].

It should be noted, that effluent concentrations reported worldwide vary, probably due to differences in the regional use of the emerging contaminants and the efficiency of the wastewater treatments [35]. In wastewater treatment, two elimination processes are generally important for pharmaceutical drugs: adsorption to suspended solids (sewage sludge) and biodegradation [8]. The fate of any given contaminant in the subsurface environment will depend upon both its physicochemical properties, such as its solubility in water, K_{ow} and D_{ow} and other environmental characteristics. Indeed, the contaminant properties as well as groundwater residence time, redox conditions and total loading will be important in determining presence and persistence in the subsurface and groundwater. The main processes controlling emerging pollutants during subsurface migration are sorption, mainly to organic matter and clay minerals,

ion exchange in the soil and aquifer, and microbial degradation or transformations [15].

1.2.3. Transformation Products

Once released into the environment, EPs are subject to both biotic and abiotic transformation processes that are responsible for their transformation and/or elimination, according to their persistence, transport, and ultimate destination. Various transformations can take place, producing compounds that, to some extent, differ in their environmental behavior and ecotoxicological profile from the parent compound. Formation of transformation products (TPs) occurs mainly through oxidation, hydroxylation, hydrolysis, conjugation, cleavage, dealkylation, methylation and demethylation. The EPs and their TPs can move vertically through the soil profile to groundwater and away from the source site with mobile groundwater. They also have the potential to reach surface water when they travel laterally either as surface runoff or through subsoil tile drains, entering streams, major rivers, reservoirs, and ultimately estuaries and oceans [36].

Since there is a gap on the information on the occurrence and toxicity of TPs in the environment, we are unable to evaluate their significance in risk assessment [37, 38]. Standardized toxicity tests can provide quantitative information on the toxicity of the TP, compared to its parent compound, but these studies are limited [39-41]. In general, transformation products are less toxic and more polar than the parent compounds. However, in some cases, they may be more persistent or exhibit higher toxicity or be present at much higher concentrations [42].

Although there is legislation regulating chemicals like pesticides, veterinary drugs, persistent organic pollutants (POPs) and others, few is mentioned for their TPs. Concerns over the TPs of pesticides in plants have been expressed since 1991 (European Directive 91/414/EEC), while the term “metabolite” appears in Regulation (EC) 1107/2009 concerning the plant protection products and in Directives 2001/82/EC and 98/8/EC, concerning the veterinary medical and biocidal products, respectively. European Medicines Agency (EMEA, 2006) referred also to the need for

assessment of potential environmental risks of human medicinal products. However, in all these documents, there is no clarification on the determination, limits and toxicological effects of metabolites or TPs. In OECD guidelines, concerning the Aerobic and Anaerobic Transformation in Aquatic Sediment Systems, adopted in 2002, it is claimed that TPs detected at ≥10% of the applied radioactivity should be identified. Meanwhile, the EU Regulation 1907/2006 (REACH) requires the identification of major transformation and degradation products for the registration of the substance. In the Regulation (EC) 850/2004 on persistent organic pollutants, a reference to their transformation processes also exists.

Transformation products occurring in the environment can be classified into two main categories: biotransformation products formed by biotic or abiotic processes. The biotransformation products include human, animal and microbial metabolites in engineered and natural systems. The abiotic TPs are the outcome of hydrolysis, photolytic and photocatalytic degradation in the natural environment as well as water treatment processes, like chlorination, ozonation and advanced oxidation processes.

1.2.3.1. Transformation products formed by biotic reactions

TPs are formed by microbial activities in various natural and engineered environmental compartments such as soil, surface water or wastewater treatment. Diverse enzymatic reactions are involved in microbial transformation like oxidation (e.g., hydroxylation, N-, S-oxidation, dealkylation) and reduction (e.g., dehalogenation, nitro reduction, hydrolysis of amides and carboxyl esters). Pharmaceuticals and other chemicals consumed by humans and other mammals can be metabolized and then released into the environment as metabolites. For mammals, besides oxidation and reduction, also conjugation reactions occur with endogenous molecules such as carbohydrates, sulfate, glutathione and amino acids.

Some environmental pollutants are significantly accumulated and subsequently transformed in wildlife. In particular, aquatic organisms are considered the primary receptors, which might show qualitatively and quantitatively different metabolic

pathways compared to microbes and humans. Hence, the metabolites formed in aquatic organisms and their toxicity is of increased ecotoxicological concern.

1.2.3.1.1. Transformation products formed by microbial metabolism

Due to the increasing occurrence of emerging pollutants in wastewaters, the formation of TPs during the biological treatment and the involved mechanisms have been investigated for various classes of compounds: antibiotics[43-46], analgesics (painkillers) [47, 48], anticonvulsants [49, 50], anti-inflammatories [51-54], iodinated X-ray contrast media (ICM) [46, 55] and anti-viral/-bacterial/-fungal agents [56-59]. Most of the studies were performed in batch systems with activated sludge.

In almost all the studies, oxidative reactions such as hydroxylation, oxidation, and dealkylation were observed as the primary biotransformation mechanisms; hydroxylated metabolites were identified for triclosan [58], codeine [47], diclofenac [51, 54], sulfapyridine [44], and an UV filter [60]. In some cases, oxidation of hydroxyl groups was followed by oxidative decarboxylation, deacetylation, and dealkylation taking place at the amide moieties [55, 56, 61]. Molecules with N-, O-, or S-alkyl groups were likely transformed to dealkylated forms, as observed for naproxen [62], triclosan [57], diclofenac [51] and ICM [55, 61]. Other oxidative reactions include ring opening, oxidative deamination and oxidative dechlorination [46, 60, 63-65]. Helbling et al. intensively investigated microbial transformation of 30 xenobiotic compounds with amide groups and observed 53 TPs resulting from amide hydrolysis, N-dealkylation, hydroxylation, oxidation, dehalogenation, glutathione conjugation and many more pathways [64]. Moreover, the hydrolysis rate and the dominant reaction were related to the degree of alkyl substitution of the amide group. In other studies, predictive factors of the biotransformation reactions are investigated. Ammonia removal and amoA transcript abundance can be associated with oxidative micropollutant biotransformation reactions, without necessarily being catalyzed by ammonia monooxygenase [66].

Meanwhile, reduction reactions dominantly take place under anaerobic conditions such as reductive dechlorination of 5-chlorobenzotriazole and chlorpromazine and

dehydration and hydration of testosterone [63, 67, 68]. However, the nitro reduction of N,N-diethyl 1-4-nitrobenzamide was evident under aerobic conditions in a recent study [64].

Apart from common oxidation/ reduction, other reactions such as decarboxylation and deacetylation were reported less frequently [55, 61]. In addition, conjugation reactions such as phosphorylation, succinylation and glutathione substitution are also possible biotransformation mechanisms [46, 64].

Perfluorinated compounds (PFCs) also attract the interest of scientists concerning their degradability. There is a number of studies that are presenting the degradability and fate of the PFCs precursors such as fluorotelomer alcohols in sediments resulting in perfluorinated carboxylic acids [69, 70]. Nevertheless, no TPs of PFOA have been reported, stressing out the fact that these compounds are very stable and hardly degradable [71, 72].

1.2.3.1.2. Transformation products (metabolites) formed by human metabolism

Even though human metabolites of pharmaceuticals and personal care products (PPCPs) and their formation mechanisms have been extensively studied in pharmacology, limited information on their actual occurrence and stability in the environment is available. However, human and microbial metabolism presents partially the same metabolic reactions and thus same metabolites, which make their discrimination in environmental samples sometimes difficult. Kern et al. stated that 6 pharmaceutical TPs found in surface water samples are known human metabolites of metamizole, aminopyrine, carbamazepine and verapamil from registration files [73]. However, four out of the six metabolites were formed through epoxidation, dihydroxylation and O-demethylation which can possibly take place in microbial metabolism, as well. Likely, Perez and Barceló reported that hydroxylation products of diclofenac and aceclofenac, known as both human and microbial metabolites, were measured in wastewater samples [51]. Mass balances of influent and effluent

samples can clarify the origin of the TPs in more detail. In a tiered approach proposed by Kern et al. batch experiments with activated sludge can be used to verify the findings and quantify transformation rates [74].

For estrogenic compounds, the metabolites formed are glucuronide and sulfate conjugates and are frequently detected in untreated wastewaters [75]. However, the conjugated estrogens are vulnerable in aerobic activated sludge and end up in free estrogens after de-conjugation [76]. The de-conjugation behavior is also observed for pharmaceuticals, resulting in negative removal efficiencies [77, 78]. However, deconjugation reactions happen with different reaction rates. For instance, for estrogens sulfate conjugates are reported to be more persistent than glucuronides. In case of lamotrigine, N-glucuronide metabolite has been frequently detected in wastewater, surface water and groundwater samples, unlike the O-glucuronide, due to the difference in degradability of O- and N-glucuronide products [79].

1.2.3.1.3. Transformation products formed in wildlife

Emerging pollutants are ubiquitous in the environment due to their low degradability, and are likely to accumulate into biota (bioaccumulation) and sediments. Biotransformation in organisms, as a subsequent process of bioaccumulation, is of great interest in order to clarify the fate and toxicity of those compounds and their TPs. However, studies of drug metabolism in fish are extremely limited and the metabolic pathway and enzymes responsible for the metabolism of the drugs in fish are largely unknown [80]. Moreover, metabolites of persistent organic pollutants have been measured in various tissues (e.g., blood, blubber, fat, bird eggs) of marine organisms (including fish), but, in many cases, it is not known if the compounds formed are the result of in vivo metabolism or are bioaccumulated from the environment [81]. In a study, oxidation reactions in vitro were hindered by the increased number of bromine substituents and hydroxylated metabolites and oxidative bond cleavage products are formed in fish liver from 4,4'-dibromodiphenyl ether and tetrabromobisphenol A, but not from higher brominated flame retardants [82].

Recently, metabolites of polar organic pollutants formed in freshwater crustaceans, used in risk assessment, were identified [83, 84]. Jeon et al. found twenty five metabolites of irgarol, terbutryn, tramadol and venlafaxine in *Gammarus pulex* and *Daphnia magna*, formed via oxidation reactions including N-, O-demethylation, hydroxylation, and N-oxidation as well as glutathione conjugation [84]. This shows the relevance of metabolism also in wildlife.

1.2.3.2. Transformation products formed by abiotic processes in aquatic systems

Abiotic transformation products are formed by various water treatment processes and in the aquatic environment by hydrolysis and photolysis. Review papers provide information for already identified TPs of antibiotics and estrogens covering a wide range of abiotic processes [85, 86].

Oxidation processes such as chlorination, chloramination, ozonation, and advanced oxidation by UV/H_2O_2 treatment are the major processes used in advanced water treatment for disinfection and removal of emerging contaminants [42]. The oxidative reaction mechanisms rely often on the formation of reactive and short-lived oxygen containing intermediates such as hydroxyl radicals (•OH) [87]. Generally, the TPs formed are correlated to the conditions of the process, like the physicochemical properties of the matrix, and the specific conditions of the treatment (time, medium, etc.).

Ozone is a strong oxidant that can be used as a more selective agent for the removal of micropollutants. Ozonation may take place by the direct reaction of the ozone molecule with the target compound or by means of hydroxyl radicals produced from the decomposition of ozone in aqueous media. In practice, both direct and indirect reactions take place simultaneously. Ozone has recently been implemented as a fourth full-scale treatment step in wastewater treatment [88-90]. Next to ozonation TPs, by-products formed by oxidation of matrix components such as carcinogenic N-nitrosodimethylamine (NDMA) and bromate have to be taken into a cost-benefit

analysis of such technology [88, 89]. The degree of pesticide degradation, reaction kinetics, identity and characteristics of degradation by-products and intermediates, and possible degradation pathways through ozonation are covered and discussed in two review papers [91, 92]. An additional review on ozonation of pharmaceuticals has also showed satisfactory degradation efficiencies of a wide range of compounds in aqueous solution [93].

Chlorination is a chemical process commonly used in water treatment for disinfection. In most cases, chlorination is not applied when oxidation of organic micropollutants is the goal, because it can produce biologically active transformation products [94]. Especially when the inorganic content in the water matrix is very high, some reactive species like chloride or sulphate radicals are produced, which directly influence the formation of TPs. Chlorine radicals (Cl·) may lead to the formation of chlorinated organic compounds, which are known to be very harmful, and in some cases, able to generate persistent substances [95]. In this disinfection process, hypochlorous acid (HClO) is the main responsible reagent for pathogen destruction, but both HClO and ClO^- react with organic compounds giving addition, substitution, or oxidation products. One of the major concerns regarding the disinfection byproducts from chlorination is that NaClO is known to produce genotoxic TPs and can thus increase the acute toxicity [96].

Photochemistry represents an important degradation process, either in the environment, or as a light-related technical treatment process for advanced treatment of water. Many studies have been carried out regarding the direct and indirect photolytic or photocatalytic degradation of emerging pollutants. For pesticides, one review paper states the mineralization of a variety of pesticides by photocatalytic degradation [97], while a more recent work presents the by-products and intermediates of organophosphate pesticides by photocatalytic degradation [98]. Pharmaceuticals compounds [99-101], endocrine-disrupting compounds [100], UV filters [102], and phenol [103] have also been thoroughly surveyed for their fate, as well as for their TPs during photolysis.

1.2.4. Risk assessment - Ecotoxicological effects

Substances historically subject to regulatory oversight represent a very small subset, which is mainly a historical consequence of expediency and necessity. A major factor to consider in regulation has necessarily been the availability of suitable off-the-shelf chemical analysis technology. Although the majority of the individual chemicals released to the environment are not regulated, this does not mean that they do not pose risks. But what portion of overall risk they pose is unknown.

However it is for sure that the regulated and “controlled” chemicals are not representative of the full spectrum of known chemical stressors or the multitudes of transformation products. The multifactorial complexity faced by risk assessment includes the exposure frequency and timing, exposure duration, exposure complexity or ‘‘totality’’ (cumulative and aggregate exposure, synergism, and other multiple-stressor interactions), prior exposure history (the foundation for determining exposure ‘‘trajectory’’), or other factors including delayed-onset toxicity [4].

Current toxicity testing incorporates established test systems and traditional organisms according to guidelines and traditional end points such as mortality are assessed. Ecotoxicity testing merely provided indications of acute effects in vivo in organisms of different trophic levels after short-term exposure, and only rarely after long-term (chronic) exposures. These data are ultimately used for ecological risk assessments. Because of animal welfare and screening purposes, in vitro analyses are becoming more important, but they are not sufficient for assessing the toxicological profiles of a compound, particularly as a basis for risk analysis [8].

Tools and models are used in the hazard, exposure and risk assessment processes to evaluate both new and existing chemicals when certain data are missing. EPA's Office of Pollution Prevention and Toxics (U.S. Environmental Protection Agency OPPT) uses and promotes the use of these models, including OncoLogic™ for health hazard evaluation, and ECOSAR and AQUATOX for environmental effects and fate (http://www.epa.gov/oppt/ar/2007-2009/cross_cutting/ces.htm). Moreover, the potential risk of a substance to the environment is often characterized by

comparing the Predicted Environmental Concentration (PEC) with the Predicted No Effect Concentration (PNEC).

1.3. Identification approaches - Analytical techniques

There are various workflows in the literature for the identification of TPs, dependent indispensably on the instrumentation and the available software. The main skeleton though is summarized below and is presented in Figure 1.1:

(a) The target analysis, which is based on the determination of already known TPs; the identification is carried out with standard solutions.

(b) The suspect screening; a list of possible TPs is assembled from the literature or from prediction models and the samples are screened for those candidates.

(c) The non-target screening; the identification of novel TPs is carried out with sophisticated post-acquisition data tools and supplementary analytical techniques.

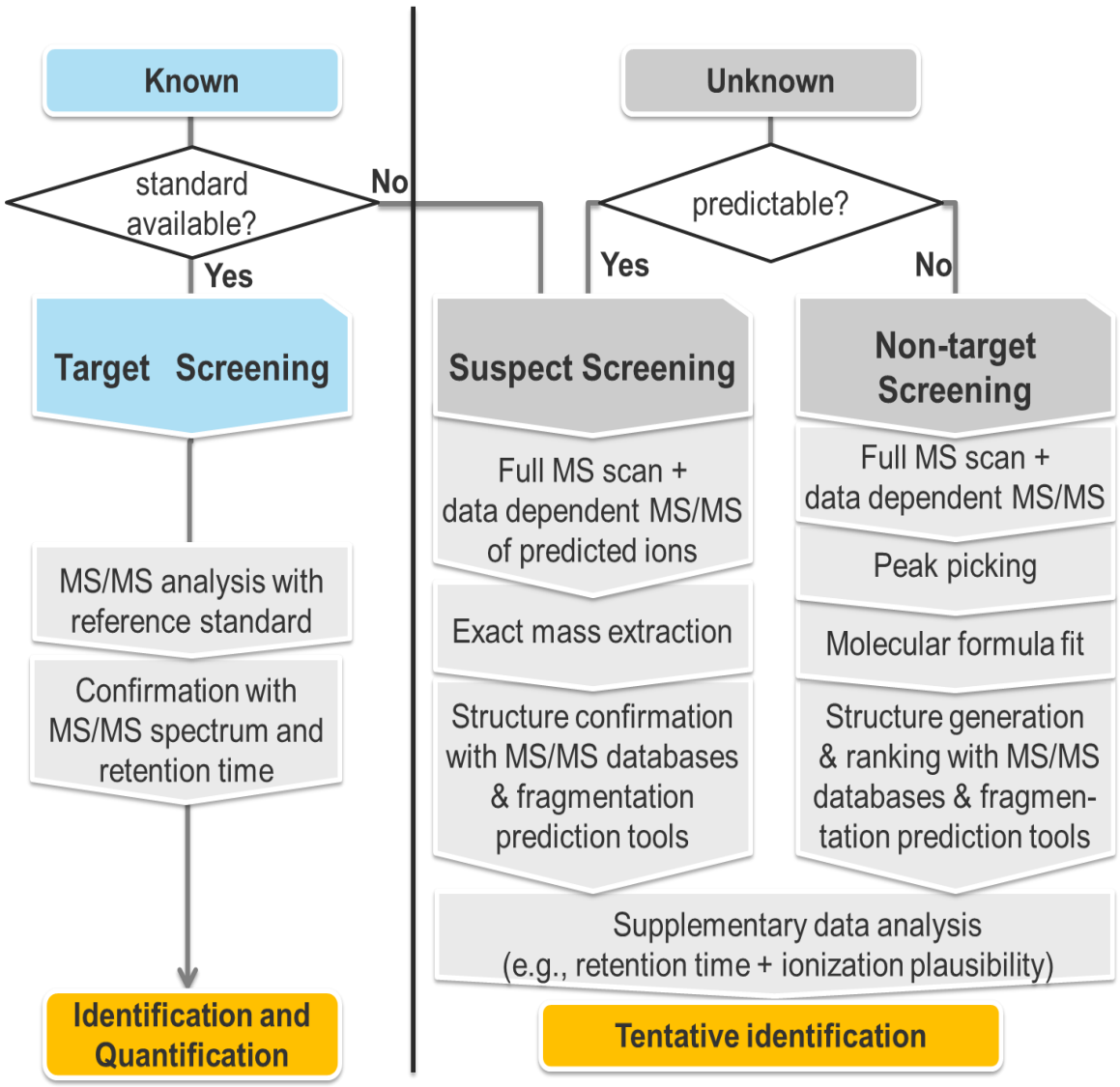


Figure 1.1 Flow chart of screening procedure of transformation products (TPs).

'Known' TPs have been confirmed or confidently identified before, other TPs are considered as 'Unknown'.

Nowadays, liquid chromatography (LC) hyphenated to mass spectrometry (MS) using a variety of mass analyzers is the technique of choice for the investigation of EPs and TPs in environmental samples. LC is a suitable chromatographic technique for polar, thermo-labile compounds, thus for the identification of transformation products, which are generally more polar than their parent molecules. Mass analyzers commonly employed are the triple quadrupole (QqQ), time-of-flight (TOF), ion-trap (IT), Orbitrap

and hybrid mass spectrometers, like quadrupole time-of-flight (Q-TOF), quadrupole-linear ion trap (Q-LIT), linear ion trap-Orbitrap or quadrupole-Orbitrap.

The development and use of powerful high resolution mass spectrometers (HR-MS) is the driving force to the development of novel analytical methodologies for the identification of TPs. Owing to their sensitivity in full-scan acquisition mode and high mass accuracy, HR-MS are suitable for both target and non-target analysis, pre- and post- acquisition processing, retrospective analysis and discovery of TPs.

As a complement to LC-MS methods, gas-chromatography-MS (GC-MS) allows to investigate GC-amenable contaminants with low polarity and/or high volatality, such as siloxanes, musks, polychlorinatedbiphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), poly-brominated diphenyl ethers (PBDEs), and certain pesticides, among others. Although single nominal analyzers like single quadrupole, ion trap or triple quadrupole can be used to this aim, HRMS is a superior technique for screening purposes. GC–HRMS has seldom been explored in environmental pollution monitoring until recently. Electron ionization (EI) source is the preferred ionization technique and the most widely applied due to its robustness, reproducibility and the existence of standardized commercial spectra libraries, which facilitates the identification of compounds. Databases are available with information for over 200,000 individual compounds, some of them already connected to software for non-target screening [104].

1.3.1. Target analysis

In target analysis, as shown in Fig.1.1, EPs and TPs are already known and standards are available, so that they can be included within a defined MS method and be monitored in routine analysis. LC hyphenated to triple quadrupole (LC-QqQ-MS/MS) is the workhorse nowadays in target analysis. The QqQ analyzer permits the application of various MS/MS modes, like product ion scan, precursor ion scan, neutral loss scan and selected reaction monitoring (SRM), which is the most predominant. SRM mode provides several advantages and interesting characteristics for target analysis such as increased selectivity, reduced interferences and high

sensitivity, which allows a robust quantification. Another important point is the possibility of diminishing the analysis time, including extraction and instrumental determination.

With the use of LC-QqQ-MS/MS, adequate results have been obtained concerning the analysis of emerging contaminants and the identification and quantification of their TPs, especially in the field of pesticides and pharmaceutical compounds, where standards are available.

In the past decades, multi-residue LC-MS methods were developed for the analysis of organic contaminants in environmental waters, such as drinking water, groundwater, surface waters, including seawater and fresh water and wastewaters [105]. The majority of these methods were aiming at the detection of pharmaceuticals [106], pesticides [107] and licit and illicit drugs [108]. Various review papers list multi-residue methods for the analysis of emerging pollutants [105, 109].

A list of various TPs of pesticides such as aldicarb, diuron, fipronil and malathion has been recorded by Martínez-Vidal et al. [107]. In 2006, Hernández et al. have developed a LC-QqQ-MS/MS method for the determination of 52 pesticides and known TPs in a MRM mode [110].

Pharmacokinetic studies and identification of human metabolites have been carried out for the majority of pharmaceutical compounds and drugs. TPs of anthelmintics, NSAIDs, phychoactive and illicit drugs and drugs of abuse have been determined in literature by LR-MS [111-114]. Another study also presents a critical review of available literature on pharmaceutical metabolites since 2009, primarily focusing on their analysis with LR-MS and toxicological significance [115].

High resolution mass spectrometry (HR-MS) on target analysis offers promising solutions to the limitations of SRM analysis which allows only monitoring of specific TPs. Virtually all compounds present in a sample can be determined simultaneously with HR-MS instruments operating in full-scan mode, making no pre-selection of compounds and associated SRM transitions necessary. Target compounds included in a database are screened in the sample based on mass accuracy, isotopic pattern, retention time and MS/MS fragments. Alternatively, hybrid instruments offer the

possibility of data-dependent MS/MS acquisition, where a MS/MS analysis is triggered if a compound from a target ion list is detected in the full scan. Moreover, HR-MS instruments have the ability to differentiate isobaric compounds with the same nominal mass but different molecular formula due to their higher resolving power [37, 116-119].

HR-MS outperforms LR-MS, regarding the level of identification of an unknown compound, since within decision 2002/657/EC, it gains more identification points and can provide mass accuracy, even in full scan mode. An ion in HR-MS gains 2 identification points, instead of 1 in LR-MS, whereas HR-MS^n transition products gain 2.5 instead of 1.5. It is clear that in HR-MS full scan mode, more than one ions are present in the mass spectra and evaluated.

1.3.2. Suspect screening of EPs- prediction of TPs

Suspect screening is the technique of choice for the identification of compounds, when the confirmation of the analytes with a reference standard is not possible, but molecular formula and structure of suspected molecules can be predicted (Fig.1.1) [65, 73, 117, 118, 120].

A suspect list can be compiled from theoretical assessment based on consumption data, registered organic synthetic insecticides, fungicides, biocides and acaricides, including all major metabolites of the most commonly used insecticides and fungicides, as well as important pharmaceuticals used in the country of the study, which were not yet included in the target list [121]. Over 2000 suspect compounds were chosen in another study based on literature reports and author's knowledge on the occurrence of these compounds in water or the expectation that a compound could be of importance for the water cycle, due to its use and its physicochemical properties [122].

In suspect screening, an important step of the identification workflow is the prediction of possible TPs using computational (*in silico*) prediction tools. Commercially available or freely accessible programs have been applied in the prediction step on

environmental analysis, including the University of Minnesota Pathway Prediction System (UM-PPS) [56, 64] , CATABOL [123], PathPred [124] and Meteor [125].

The prediction system should be properly selected by considering the organism/system where TPs are formed. Meteor was built based on mammalian biotransformation reactions of common functional groups and allows prediction of the most probable transformation products, providing in parallel relevant literature references. PathPred is a multi-step reaction prediction server for biodegradation pathways of xenobiotic compounds and biosynthesis pathways of secondary metabolites. It is linked to KEGG metabolic pathway maps and it has the potential to link the prediction result to genomic information. CATABOL and UM-PPS predict microbial metabolic reactions based on biotransformation rules.

As UM-PPS is freely accessible and all applied rules are clearly assigned, it is the most common prediction tool in suspect screening and many researchers have tried to evaluate and improve its prediction power [64, 65, 73, 74]. The prediction rules behind UM-PPS are coming from the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD) and literature [126]. Since UM-BBD has integrated data generated from pure microbial cultures, the predicted pathways may not be completely appropriate for environmental systems [64]. The relatively high false positive rates of all prediction systems are of concern, since the inclusion of additional pathways increases the number of possible degradation products [127]. In UM-PPS combinatorial explosion can be limited by prioritizing the different rules using relative reasoning [128].

Suspect screening is performed by the HR-MS analysis; the exact mass for each of the predicted TPs is extracted from the chromatogram and checked by comparing with control samples. An intensity threshold value is applied to cutoff unclear spectra. The chromatographic retention time (t_R) plausibility, isotopic pattern, and ionization efficiency are used as further filters to narrow down the number of candidate peaks. Furthermore, using the MS/MS or MS^n operating mode, structures of suspected TPs are suggested based on the observed fragmentation pattern.

Depending on the above criteria, there are different identification confidence levels in HR-MS analysis of TPs. When all the above criteria are fulfilled, a probable structure is proposed based on library spectrum match or diagnostic evidence. Otherwise, tentative candidates or just unequivocal molecular formulas are the outcome of the suspect screening [129].

One approach for processing the data would be the identification of key TPs in terms of persistence over the time of the experiment. It is carried out by a data processing method which is established based on peak detection, time-trend filtration and structure assignment. Open-source software is used for peak peaking (e.g. MZmine) and processing of the chromatograms (e.g. enviMass), by noise removal and blank subtraction. Then, a meaningful time-trend is inquired and the remaining-candidate peaks are compared with a list from UM-PPS or from literature for tentative identification [130].

Another approach for suspect screening is based on the use of characteristic fragmentation undergone by emerging pollutants during MS/MS fragmentation events [73, 131]. It is based on the assumption that many TPs maintain a similar structure than the parent compound and therefore have common fragment ions. Thus, searching for specific fragment ions in MS/MS spectra throughout the chromatographic run could lead to new TPs. This is evident when applying product ion and neutral loss scans, and other techniques, such as mass defect filtering [132].

1.3.3. Non-target screening

Non-target screening implies the identification of compounds for which there is no previous knowledge available and is usually carried out after target and suspect screening. Non-target screening becomes a challenging task, but in case of TPs further information of the parent compound, like the molecular formula, the MS/MS spectrum, the retention time and other physico-chemical data may contribute for further ranking of possible structures and facilitate the identification process [84]. For non-target screening, high resolution mass spectrometry is strongly required in order

to have mass accuracy for confirmation of molecular formula and a reliable interpretation of the MS/MS spectra [118, 133, 134].

The challenge with HR instruments is the generation of massive quantities of data and subsequently their evaluation and the export of results. Moreover, their ability of operating in full scan and MS/MS mode simultaneously, provide even more data in a single run. For this reason, post-acquisition data-processing tools are necessary; computer-aided techniques provide rapid, accurate and efficient data mining. There is a number of open-source and commercial software for non-target screening, including MZmine [135], XCMS (https://xcmsonline.scripps.edu), EnviMass, Nontarget, ACD MS/Workbook Suite and vendors software like Bruker Metabolite Tools and ProfileAnalysis, Waters MassLynx and MetaboLynx, Thermo Metworks and Sieve, Applied Biosystems Data Explorer (MDS-Sciex Analyst QS) and Agilent MassHunter.

The general procedure, as shown in Fig.1.1, has several steps until it reaches the final result, which does not follow the same order in every software. The first step is always the peak picking. In this step, comparison of the sample with control or blank samples is important to exclude irrelevant peaks. The removal of noise peaks, mass recalibration and componentization of isotopes and adducts is usually carried out automatically as the next step. The assignment of the molecular formula to the accurate mass of the peak is performed using heuristic filters such as the seven golden rules of Kind and Fiehn [136]. Exploration of databases such as ChemSpider, PubChem, DAIOS database, NIST or structure generation may lead to candidate structures [137-139]. Thereby information on the parent compound (e.g. molecular formula, substructures) can help to restrict the databases search and possible structures are likely to be proposed for the compound. However, databases contain mostly only EPs but many TPs are not included yet.

Even after filtering and strict criteria and thresholds in the above parameters, the number of peaks, which correspond to non-targets can exceed the number of 1,000. It is clear that elucidation of all those peaks would demand a great amount of time and effort; prioritization of the most intense peaks is a common strategy [140].

Similar to suspect screening, the observation of the presence/absence of common characteristic ions in the fragmentation pattern of both the parent compound and the TPs, evidencing the stability/reactivity of certain parts of the molecule can be helpful [37]. For the ranking of the candidate structures, the information of MS/MS spectra has to be explored by comparing the fragmentation pattern with *in silico* mass spectral fragmentation or with spectra in libraries. There are a few databases with mass spectra, like MassBank [141] and MetLin (http://metlin.scripps.edu/index.php), however, most software usually do not take into account the fragmentation pattern. MOLGEN-MS, ACD/MS Fragmenter (www.acdlabs.com/products/adh/ms/ms_frag) and MassFrontier (www.highchem.com/index.php/massfrontier) both use fragmentation rules, whereas MetFrag offers a purely combinatorial approach based just on bond energies. Although the overall candidate ranking with MetFrag is not quite as good as that obtained with Mass Frontier and MOLGEN-MS, the scoring function used in MetFrag can improve the ranking significantly [142]. MetFusion, the newest development, combines MetFrag with spectral database searching [142]. The use of fragmentation trees as performed in SIRIUS is another approach for the structure elucidation [143]. In any case, criteria must be established for the success of the identification of the unknowns by the accuracy of the molecular ion, the isotopic fitting and the characteristic fragment ions in MS/MS mode [37].

Müller et al. proposed another approach for non-target screening, focusing on relevant compounds (features). The sample is not regarded as an isolated specimen, but rather it is evaluated in relation to a set of other samples based on considerations of e.g., their temporal, spatial, or process-related connections. This covers also the comparison of assays and controls as carried out in evaluation of many transformation experiments. The features of the sample are considered as mathematical sets and treated with statistical tools [137].

1.4. Sample pretreatment

Trace analysis of organic contaminants in environmental samples is always challenging due to the complexity and diversity of sample matrices. Matrix-dependent signal suppression or enhancement represents a major drawback in quantitative analysis of complex samples. During the last decade, a great effort has been made towards the development of more efficient extraction and clean-up approaches and the most recent tendencies are the automation through coupling of sample preparation units and detection systems (e.g. on-line SPE), the application of advanced sorbents and the application of greener approaches, such as solvent reduced techniques [144]. Central objectives of research in this area included expanding the scope of efficiently extracted contaminants, to polar compounds, mainly, reducing solvent consumption, making sample preparation more expeditious, inexpensive, and environmentally friendly and saving costs [145].

For the extraction of target analytes from complex environmental samples or/and further sample cleanup, the majority of methods involve liquid-liquid extraction (LLE) or/and solid phase extraction (SPE). Solid phase extraction (SPE) is the most preferred technique since it presents the advantages of simplicity, reproducibility, and applicability. SPE is a good way to preconcentrate water samples prior to the final determination to decrease detection limits, if necessary [146].

A wide choice of sorbents is available which rely on different mechanisms for extraction/retention of analytes. Alumina, amino or strong cation exchangers (SCX) have been proposed for ionic compounds, while C18 or polymeric sorbents, especially Hydrophilic-Liphophilic Balance (HLB) polymeric reversed phases are used for neutral or ionisable compounds working at a pH lower than the pKa of the analytes. HLB sorbent consists of a copolymer of N-vinylpyrrolidone and divinylbenzenes. The hydrophilic N-vinyl pyrrolidone increases the water wettability of the polymer and the lipophilic divinylbenzene provides the reversed-phase retention necessary to retain analytes. For compounds with varied chemical properties, mixed-mode sorbents are recommended.

For wide scope multi-residues methods, generic sample preparation is a mandatory step, in order to extract as many analytes as possible. HLB cartridges are the most common choice for pre-concentration of target analytes [118, 147, 148]. In order to ensure sufficient enrichment for a wide range of analytes from water samples, an offline solid phase extraction (SPE) method has been described by Kern et al. [73]. Samples were passed over a multi-layered cartridge containing Oasis HLB, Strata XAW, Strata XCW and Isolute ENV+ in order to enrich neutral, cationic, and anionic species of a broad range of K_{ow} values.

Liquid extraction is a very popular sample treatment technique. LE entails conventional liquid–liquid extraction (LLE) of target compounds from liquid matrices. To obtain optimal results, the extraction solvent has to be selected in such way that efficient extraction of the target compounds is obtained, whereas the extraction of matrix constituents remains limited in order to prevent excessive matrix effects (ME). The selection of the solvent therefore depends not only on the target compounds, but also on the matrix.

A number of other less common extraction techniques have been also reported, including automated solid phase extraction, on-line solid phase extraction, use of molecularly imprinted polymers (MIPs), stir bar sorptive extraction (SBSE), solid phase microextraction (SPME) and magnetic solid phase extraction through silica supported Fe_3O_4 nanoparticles [149]. These techniques present various advantages, like less contamination between samples, higher sample throughput, minimized consumption of solvents and less labour work [6, 9, 150-152].

1.5. Instrumental analysis

Both liquid (LC) and gas (GC) chromatography is used for the determination of emerging pollutants, depending on the polarity, volatility and thermal stability of the concerning compounds. Due to the polarity of most pharmaceuticals, either LC-MS, or GC-MS combined with derivatization processes, is normally used for their determination [6, 152-155].

1.5.1. Gas Chromatography (GC) -Mass Spectrometry

Gas chromatography coupled to mass spectrometry (GC-MS) is the technique most commonly employed today for the analysis of volatile organic pollutants in environmental samples. The very high number of applications is the result of the efficiency of gas chromatography separation and the good qualitative information and high sensitivity provided by mass spectrometry.

The MS fragmentation pattern can often provide unambiguous component identification by comparison with library spectra. Huge electron ionization mass spectral libraries are commercially available, such as NIST Library, which contains 250,000 spectra and the Wiley Library with 720,000 spectra with the new combined version including approximately 950,000 spectra (http://www.sisweb.com/software/ms/wiley.htm). The identification process is based on search algorithms that compare the obtained spectra with those of a library, which are generally implemented in the GC-MS instrument.

Several ionization techniques are used in GC-MS. Among them, electron ionization (EI) is the most popular because it often produces both molecular and fragment ions. In EI, gas analyte molecules are bombarded by energetic electrons (typically 70 eV), which leads to the generation of a molecular radical ion ($M^{+\cdot}$) that can subsequently generate ionized fragments. This technique generally allows for the determination of both relative molecular mass and the structure of the molecule. One important feature of electron ionization spectra is that they are highly reproducible, which means that mass spectral libraries can be used for identification of unknowns. However, in some cases, EI does not provide the sensitivity required for the analysis of very small amounts of compounds in environmental samples. This is mainly due to extensive fragmentation [156].

Mixtures to be analyzed are injected into an inert gas stream and swept into a tube packed with a solid support coated with a resolving liquid phase. The compounds most commonly analyzed by GC-MS include alkanes, polycyclic aromatic hydrocarbons (PAHs), pesticides, polychlorinated biphenyls (PCBs), as well as

endocrine disrupting chemicals [156]. For the determination siloxanes and synthetic musks in wastewater and soil samples, the method of choice is gas chromatography-mass spectrometry [26, 157].

1.5.2. Liquid Chromatography- High Resolution Mass Spectrometry (LC-HRMS)

LC-MS techniques provide a universal approach applicable to the widest number of emerging pollutants and this is the reason why they have today become the technique of choice in the field of environmental analysis. Among the different mass analyzers usually applied for target analysis, triple quadrupole (QqQ) is the most widely used for measuring and quantifying residues of EPs. However, a recent trend towards the high-resolution mass spectrometry (HR-MS; i.e. time-of-flight, TOF; Orbitrap; Fourier Transform-Ion Cyclotron Resonance, FT‑ICR) is undoubtedly observed. HRMS gives the user access to a number of diagnostic tools which were not available earlier.

High resolution mass analyzers and hybrid mass analyzers, such as Q-TOF, LIT-Orbitrap, have opened a new era in environmental analysis. Due to their high resolving power resulting in accurate mass measurements, together with the isotopic fitting information and the fragmentation pattern elucidation can provide identification with high level of confidence for target analytes. Additionally, tentative identification of suspect and unknown compounds is feasible.

The use of LC-HRMS in target analysis has some advantages derived from the full-scan operation mode of this system. Full-scan data can be reprocessed without any a priori knowledge about the presence of certain compounds; that is, no analyte-specific information is required before injecting a sample and the presence of newly identified compounds can be confirmed in previously analyzed samples simply by reprocessing the data. This retrospective analysis is the greater advantage of HR-MS target screening.

The application range of MS/MS is today extremely wide, providing different acquisition modes. It can facilitate sensitive and specific quantitative target measurements when operating in MRM mode. But also it can provide powerful untargeted approaches based on advanced scanning techniques like data-dependent or data-independent acquisitions**.**

1.5.2.1. Time of Flight (TOF) MS

The basic principle of a Q-TOF instrument is outlined in Figure 1.2

TOF resolution is directly related to the length of the flight path. As a consequence modern high resolution instruments share the characteristics of flight paths with a combined length of several meters. The introduction of a reflectron doubles the flight path and regulates the mobile energy, resulting in higher resolution. Since resolution is related to the length of flight time, TOF provides the highest resolution for relatively high m/z ion masses. Technical specifications often define resolution at such optimal m/z values. Resolving power is defined at full width at half maximum (FWHM) as m/Δm, where m is the m/z and Δm the width of the mass peak at half peak height. Orbitrap instruments produce the highest resolution for low m/z ions, which is opposite to the typical TOF performance. Off course, the price to be paid for high resolution is the number of acquired data points per time unit. Mass-resolving power in TOFMS is limited and increasing the mass-resolving power in Orbitrap-MS requires a reduced acquisition speed. Moreover in TOF instruments, the ratio of mass-to-peak width (at FWHM) is relatively constant over the entire mass range in contrast with Orbitrap analyzers.

The importance of sufficient mass resolution is that accurate and precise mass (m/z) measurements become possible. Mass-measurement uncertainty in terms of mass accuracy (i.e. average mass error) and mass precision (i.e. standard deviation on the mass error) is based on calculating the relative (ppm) or absolute (mDa) difference between the measured accurate mass and the calculated exact mass of an analyte. Both mass accuracy and precision are essential for proper measurements of

accurate mass, and pinpointing different causes of mass-measurement uncertainty can lead to improvement [105].

Hybrid tandem mass instruments, such as the Q-TOF, provide relevant structural information by obtaining product ion full spectra at accurate mass. QTOF MS/MS experiments are an excellent way of confirming potential positives, and are highly useful for elucidating the structures of unknown compounds. There are 2 main MS/MS, also reported in the literature, depending on the nature of the analysis. For target analysis, data-independent acquisition (IDA) is the most preferred one. This approach, termed MS^E (Waters) or bbCID (broad band Collision Induced Dissociation) (Brukers), involves simultaneous acquisition of accurate mass data at low and high collision energy. By applying low energy (LE) in the collision cell, no fragmentation is taking place and the information obtained is actually is full scan MS spectrum. At high collision energy (HE), fragmentation of the ions takes place and MS/MS spectra are acquired. With IDA, both molecular and fragment ion are obtained in a single acquisition without the need of pre-selection of the analytes.

On the other hand, for suspect and non-target analysis, data-dependent acquisition (DDA) is more favorable, since information over specific ions can be collected. In this case, two possibilities are available. Either, 2 injections are made, one as a survey and the next with pre-selected ions, or the determination of the candidates of interest for MS/MS information is based on predefined selection criteria. So, there is a first scan, which is processed “on-the-fly” to determine the ions that will be fragmented in a second (data-dependent) scan. The major advantage of this approach is the collection of structural information in just one injection [158].

One of the drawbacks of TOF analyzers is the possibility of the detector saturation which usually implies loss of mass accuracy. Temperature changes are responsible for small thermal expansion or contraction of the flight tube length. This is why it is very important to perform mass calibration. There are three levels of mass calibration, external, internal and lock mass calibration. External and internal calibration must include at least the mass range of interest and can be performed with the same calibrant mixture. Lock mass calibration provides an automated way of

applying the linear correction calibration to each spectrum in the analysis and it requires the presence of a continuous signal.

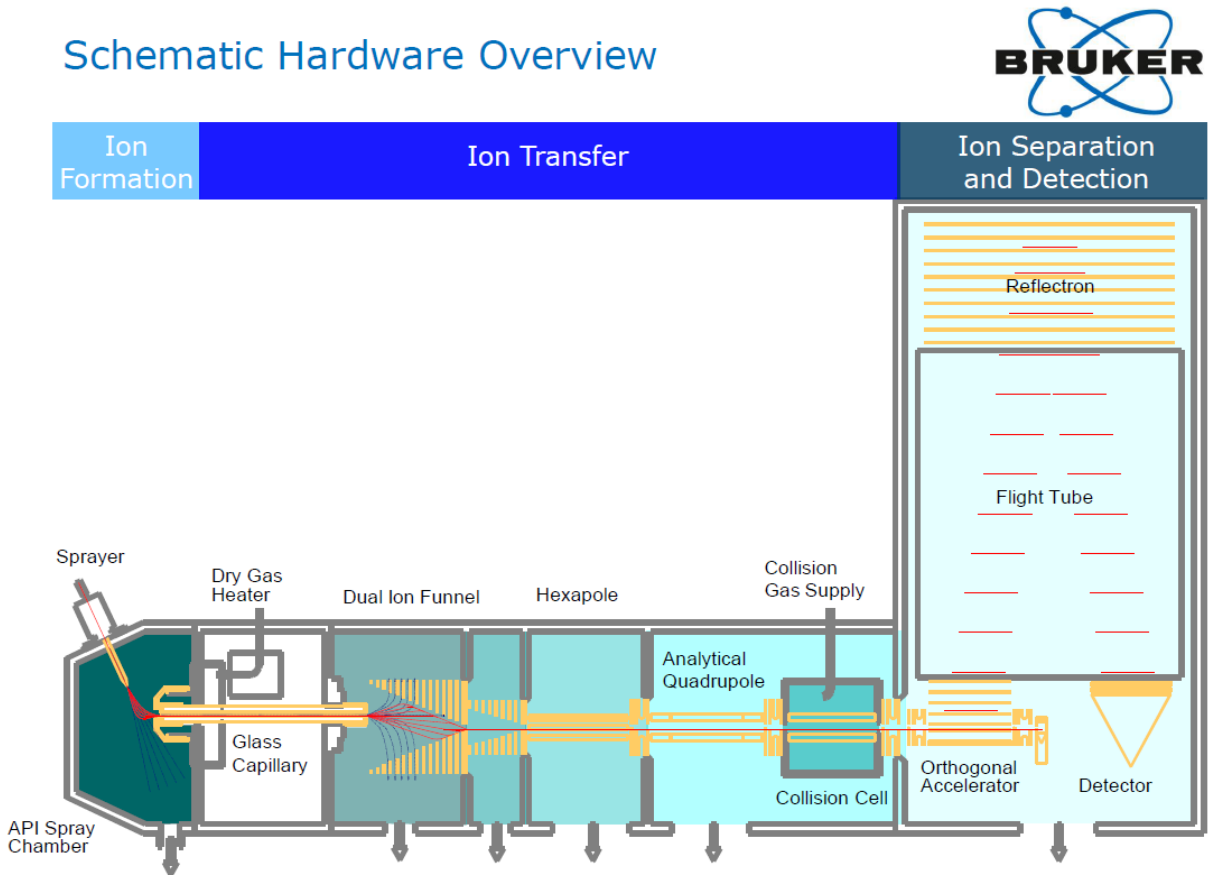


Figure 1.2 Schematic presentation of a Q-TOF instrument (maxis impact, Bruker).

Full scan HRMS data contains a wealth of information. Unfortunately, more often than not, only a fraction of the information is extracted and utilized. Existing limitations are related to hard- and software. Most post-acquisition data processing strategies are based on the high information content provided by the measured accurate masses.

HRMS is particular suited for multiresidue methods where a theoretically unlimited number of compounds can be monitored. Unfortunately, the currently available software is responsible for a number of existing bottlenecks. Current limitations are

the speed of data processing and the availability of tools for the confirmation of suspected findings. Insufficient as well is the current tool box regarding software capable in utilizing the rich information present in HRMS data (data mining).

CHAPTER 2

Scope and Objectives

2.1 The analytical problem

A huge number of articles and studies in the last years are dealing with the occurrence of organic emerging pollutants in various environmental compartments. The focus of these studies may vary, according to the scope of the study, the orientation of the scientist and inevitably the available instrumentation. They are focusing on the identification and quantitation of organic micropollutants, on their spatial or temporal distribution, their fate in environmental media, on their ecotoxicological impact or they may focus on the detection of newly introduced compounds, by developing new analytical methods, or on the identification of unknown-non target compounds.

It is obvious that the analytical problem has more than one perspectives, that need to be evaluated. The first one is to consider the magnitude of the universe of emerging pollutants. The number of chemical contaminants at trace levels can exceed those present at higher levels. As the power of analytical chemistry increases, the types of chemicals that can be detected increase, and the limits of concentration at which they can be measured are continually lowered [4]. The developments in analytical chemistry play a key role in the continuously expanding list of emerging pollutants. Under these facts, analytical methods for the determination of target compounds at very low concentration levels, as well as target analysis of hundreds of emerging pollutants by application of wide-scope screening methods is a definite need.

While the known universe of organic chemicals might seem large, the universe of potential organic chemicals (those that could possibly be synthesized and those that already exist but which have not yet been identified) is unimaginably large. This perspective may be divided into two analytical problems. Suspect screening of

chemicals that are possible to be detected in the environmental samples and non-target screening of “unknown” or “yet to be identified” compounds.

In the first case, the list of suspect compounds that may be present in the sample can be enormous, regarding the known chemicals used all over the planet, or the predicted metabolites or transformation products of all these pollutants. The analysis of so numerous analytes, with the only prior knowledge the molecular formula is unfeasible, so the point remain in choosing which compounds to incorporate in suspect screening. The important is to “screen smart, not big”, according to experience and the knowledge of the scientist.

Non-target screening is a more challenging and time-consuming task and the success is not for granted. In this case, the analyst takes greater advantage of the development of instrumentation and sophisticated software in order to find the proper workflow in order to identify compounds, previously not reported.

Keeping in mind all the above, specific strategies for target, suspect and non-target screening, together with the development of analytical techniques become a significant trend in the analysis of emerging pollutants in environmental samples. The use of HRMS analysis is the only way for monitoring samples, but improvements leading to lower detection limits for known compounds are also needed.

2.2 Research Objectives and Scope

The experimental part of the thesis is consisted of 3 studies.

In the first study, optimization and development of a validated method for the determination of linear and cyclic siloxanes was performed. The analytical technique for their determination was gas chromatography coupled with quadrupole mass spectrometry. The method was applied to wastewater (dissolved and particulate phase) and sewage sludge. Based on the volume of wastewater treated daily, mass flows of siloxanes were calculated. Further, the distribution of siloxanes between dissolved and particulate phases was determined in wastewater samples for the determination of distribution coefficients (K_d) of siloxanes. Siloxanes are compounds

that are widely used in industrial applications as well as in personal care products and biomedical devices, while 2 of them, octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5), have received considerable attention in recent years by regulatory agencies. Their analysis was a challenging task, and this is why very few data are available in the literature, concerning their occurrence and fate.

In the second study, a wide-scope screening method has been developed for the identification of 2327 emerging pollutants in wastewater samples using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). An in-house database was built with information over the retention time, qualifier ions, adducts, in-source fragments and MS/MS fragments for all the target emerging pollutants. This HRMS screening method was optimized in order to minimize the b-error (false negative results). The method was then validated for 195 representative compounds providing screening detection limit (SDL) and limit of identification (LOI), as well as decision limit (CCα) and detection capability (CCβ). Linearity, accuracy and precision and matrix effect were also evaluated. Finally, the method was applied to influent and effluent wastewater samples from a wastewater treatment plant of Athens. 371 compounds are reported in a sample with quantitation results, as well. The compounds present in the sample belong to different classes of contaminants, providing thus more “universal” information on the contamination of the wastewaters.

Finally, the third study comes as continue to the target screening of wastewater samples. This work describes the development and application of an integrated workflow based on liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometer (LC-QTOF-MS) to detect and identify suspect contaminants. Tentative identifications were based on several evaluation criteria including mass accuracy, isotopic pattern, plausibility of the chromatographic retention time and MS/MS spectral interpretation (comparisons with MS databases, *in silico* fragmentation). Moreover, new specific strategies for the identification of metabolites were applied to obtain extra confidence including the comparison of the diurnal and/or weekly concentration trends of the metabolite and the parent compound and the complimentary use of hydrophilic interaction liquid chromatography (HILIC). Thirteen out of 284 metabolites of an in-house metabolite database of

pharmaceuticals and nicotine were tentatively identified in influent samples from Athens and nine were finally confirmed with reference standards. Retrospective analysis was also performed in the samples, after non-target screening for a list of suspect surfactants.

CHAPTER 3

Determination of Linear and Cyclic Siloxanes by GC-MS in environmental samples - Mass Loading and Fate in a Wastewater Treatment Plant in Greece

3.1 Introduction

Siloxanes are polymeric organosilicon molecules that consist of a backbone of alternating silicon-oxygen [Si-O] units with organic side-chains attached to each silicon atom. Although several formulations of organosilicones are available in commerce, the most important ones are organosiloxanes (or silicones), which are oligomeric alkylsiloxanes in either cyclic or linear configurations and polymeric dimethylsiloxanes (polydimethylsiloxanes, PDMS). The oligomeric alkylsiloxanes are also known as “volatile methylsiloxanes” (VMS) and contain up to 4 [Si-O] units. PDMS has the structure $Me_3SiO(SiMe_2O)_nSiMe_3$, where n = 5-6000 [159].

The physicochemical properties of siloxanes vary, depending on their molecular weight. The very low electronegativity of Si (1.8) leads to a very polarized Si-O bond with large bond energy (108 kcal mol^{-1}) [160]. In general, siloxanes have low water solubility. Low molecular weight siloxanes, both cyclic and linear, are soluble in water on the order of a few μg L^{-1} at 25° C [161]. The water solubility decreases with increasing chain length of siloxanes. Siloxanes are characterized by their high stability, biocompatibility, surface activity, and lubricating properties [159].

Among cyclic siloxanes, octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) have received considerable attention in recent years. D4 is listed for safety assessment by the U.S. Environmental Protection Agency (EPA) in 2013-2014 and is considered to be persistent and bioaccumulative (http://www.epa.gov/oppt/existingchemicals/pubs/workplanlist.html). In toxicological studies, D4 showed weak estrogenic activity [162-164]. In short-term, controlled human exposure studies, no immunotoxic or pro-inflammatory effects of D4 were

observed [165, 166]. The Government of Canada declared that D4 does not pose a risk to human health but did recommend environmental regulatory measures (http://sehsc.americanchemistry.com/). D5 is considered to be a persistent and bioaccumulative compound [167] and has been shown to be effectively removed from the atmosphere via phototransformation [168]. In soil, D5 exhibited species- and endpoint-specific ecotoxicity [169]. Inhalation exposures of rats to D5 caused histopathological changes in the respiratory tract [170]. D5 accumulated and magnified in the aquatic food chain [171]. Cyclic siloxanes have been implicated in endocrine disruption, connective tissue disorders, adverse immunologic responses, and liver and lung damage in laboratory animal studies [172].

Siloxanes are widely used in industrial applications as well as in personal care products and biomedical devices. Personal care products and cosmetics are the most important markets for silicones, with total sales in Europe estimated at 40,000 metric tons per year for the last 25 years (http://www.silicones.eu/health-safety). In the U.S., PDMS production is approximately 99,000 tons per year [159]. In Canada, 26,657 metric tons of siloxanes were marketed in 2007-2008 (http://sehsc.americanchemistry.com/). Despite the high consumption of siloxanes, studies on environmental occurrence and fate are limited. A few studies have reported the occurrence of siloxanes in sediment and sludge [26, 173-176], water [177], indoor dust [178], personal care products [25, 179], tissues of women with breast implants [180, 181], fish and biota [174, 182, 183] and landfill gas [184].

Due to their low water solubility and high sorption coefficients, once discharged down a drain, siloxanes adsorb to particulate matter and settle down as sludge during the wastewater treatment. Nevertheless, occurrence of siloxanes in wastewater treatment plants (WWTPs) has not been studied to date. Occurrence of PDMS in aqueous-phase wastewater and sludge from eight WWTPs across North America has been shown [173]. Another study reported the concentrations of VMS and PDMS in sewage sludge from WWTPs in China [176]. Fate and removal of cyclic and linear siloxanes in WWTPs, as well as their distribution in wastewater and sludge samples, have not been investigated thus far.

3.2 Scope of the study

The objectives of this study were to investigate the occurrence and fate of five cyclic (D3-D7) and twelve linear (L3-L14) siloxanes in a WWTP in Athens, Greece. Raw influent, effluent, and sewage sludge samples were collected during seven consecutive days. A validated analytical method was applied for simultaneous determination of target compounds in wastewater (dissolved and particulate phase) and sewage sludge. Based on the volume of wastewater treated daily, mass flows of siloxanes were calculated. Further, the distribution of siloxanes between dissolved and particulate phases was determined in wastewater samples for the determination of distribution coefficients (K_d) of siloxanes. To the best of our knowledge, this is the first systematic investigation on the fate of siloxanes in a municipal WWTP.

3.3 Experimental part

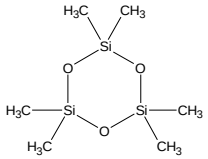
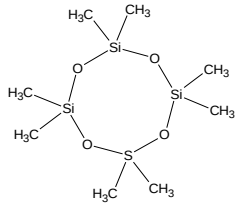
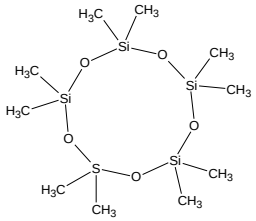
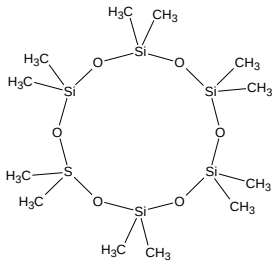
3.3.1 Chemicals and Reagents

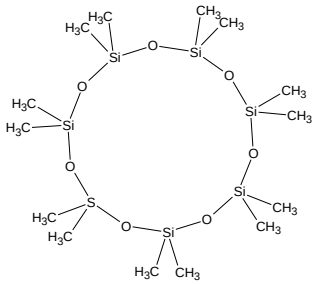
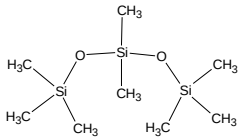
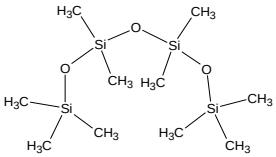
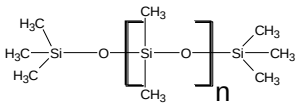
Hexamethylcyclotrisiloxane (D3), D4, D5, and dodecamethylcyclohexasilane (D6) (all >95% purity) were purchased from Tokyo Chemical Industries America (Portland, OR). PDMS 200 fluid (viscosity of 5cSt) that contained tetradecamethylcycloheptasilane (D7), linear siloxanes (L6-L7), octamethyltrisiloxane (L3) (98%), decamethyltetrasiloxane (L4) (97%), and dodecamethylpentasiloxane (L5) (97%) was purchased from Sigma-Aldrich (St. Louis, MO). Tetrakis(trimethylsiloxy)-silane (M4Q) of 97% purity was purchased from Aldrich (Milwaukee, WI) and was used as an internal standard. Information of all the analyzed siloxanes are presented in Table 3.1.

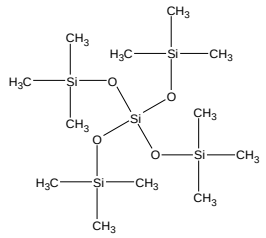
Analytical grade hexane (95% n-hexane), ethyl acetate, and sodium sulfate anhydrous were purchased from J. T. Baker (Center Valley, PA); dichloromethane was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). The composition of the PDMS mixture used in this study was identified and quantified by a gas chromatograph with flame ionization detector (GC-FID, Agilent 6890GC) and was used as a standard for the

identification of linear siloxanes and D7. Details of the composition of this PDMS mixture were described in an earlier study [179].

Table 3.1 Molecular formula, molecular weight and structures of siloxanes analyzed in this study.

Abbreviation		Analyte	Molecular Formula	Mol. Weight (g mol^{-1})	Molecular Structure
cyclic	D3	hexamethylcyclo-trisiloxane	$C_6H_{18}O_3Si_3$	222	
	D4	octamethylcyclo-tetrasilane	$C_8H_{24}O_4Si_4$	297	
	D5	decamethylcyclo-pentasilane	$C_{10}H_{30}O_5Si_5$	371	
	D6	dodecamethylcyclo-hexasilane	$C_{12}H_{36}O_6Si_6$	445	

	D7	tetradecamethylcyclo-heptasilane	$C_{14}H_{42}O_7Si_7$	519	
linear	L3	octamethyl- trisiloxane	$C_8H_{24}O2Si_3$	237	
	L4	decamethyl-tetrasiloxane	$C_{10}H_{30}O_3Si_4$	311	
	L5	dodecamethyl-pentasiloxane	$C_{12}H_{36}O_4Si_5$	385	
	L6	tetradecamethyl-hexasiloxane	$C_{14}H_{42}O_5Si_6$	459	
	L7	Polydimethylsiloxanes	$C_{16}H_{48}O_6Si_7$	533	
	L8		$C_{18}H_{54}O_7Si_8$	607	
	L9		$C_{20}H_{60}O_8Si_9$	681	
	L10		$C_{22}H_{66}O_9Si_{10}$	755	
	L11		$C_{24}H_{72}O_{10}Si_{11}$	829	
	L12		$C_{26}H_{78}O_{11}Si_{12}$	903	
	L13		$C_{28}H_{84}O_{12}Si_{13}$	977	
	L14		$C_{30}H_{90}O_{13}Si_{14}$	1051	

	M4Q	tetrakis(trimethylsiloxy)-silane	$C_{12}H_{36}O_4Si_5$	385	

3.3.2 Samples

Wastewater and sludge samples were collected from a WWTP that serves a population of 3,700,000, in Athens, Greece. The treatment process involves primary sedimentation, activated sludge process with biological nitrogen and phosphorus removal, and secondary sedimentation. The hydraulic retention time in bioreactors is 9 h, and the sludge residence time is 8 days. The sludge is managed by thickening, anaerobic digestion, and mechanical dewatering [185]. The average flow rate of sewage during the period of study was 743,193 m^3 per day, and the average production of dewatered sludge (DS) was 110,135 Kg per day.

Twenty four-hour flow-proportional composite samples of sewage influents and secondary effluents, as well as grab samples of dewatered sludge, were collected during seven consecutive days in April 2012. Additional wastewater samples from the inlet and outlet of the primary settlement tank, the inlet of the bioreactor, and sludge samples from the primary and secondary settlement tanks were collected for three consecutive days during the same period for the estimation of solid-liquid distribution coefficients (K_d) of siloxanes. All wastewater and sludge samples were collected and stored in high-density polyethylene bottles and polyethylene bags, respectively. Immediately after sampling, wastewater samples were filtered through pre-ashed glass-fiber filters (GF/F, Whatman, UK) and stored in dark at -18° C until extraction. Filters were stored at -18° C for the determination of siloxanes in particulate phase, until analysis.

3.3.3 Sample Preparation

Methods were developed, optimized, and validated for the determination of 5 cyclic and 12 linear siloxanes in dissolved and particulate phases of wastewater as well as in sludge samples.

3.3.3.1. In wastewater samples

For wastewater, 100 mL of sample were transferred into a separatory funnel, and 100 ng of internal standard (M4Q) were spiked. After 30 min of equilibration, target analytes were extracted by liquid-liquid extraction (LLE) with 50 mL of hexane followed by 25 mL mixture of hexane:dichloromethane (1:1 v/v) and then with 25 mL of mixture hexane:ethyl acetate (1:1 v/v). After each extraction, the organic layer was transferred to a flat-bottom flask and concentrated to 3-5 mL at 35° C in a rotary evaporator. Then, 0.5 mL of isooctane was added as a keeper solvent and evaporated under a gentle stream of nitrogen at room temperature. The extract was transferred into a vial that contained 0.5 mL of hexane. Samples that contained concentrations higher than the linear range of calibration were further diluted and reanalyzed. For the quantification of low molecular weight cyclic and linear siloxanes (D3-D6 and L3-L6), responses relative to the internal standard (M4Q) were used (i.e., area of sample/area of IS). For the high molecular weight siloxanes (D7 and L7-L14), quantification was based on the responses of the external calibration standards.

3.3.3.2. In sludge and particulate matter

For the analysis of sludge, 5 g of wet sample were dried and homogenized in a mortar with 25-30 g of anhydrous Na_2SO_4 and transferred into a 50 mL polypropylene tube. Then, 100 ng of internal standard (M4Q) were spiked and allowed to equilibrate for 30 min at -4° C. Next, 25 mL of hexane were added to the sample, shaken for 1 h, and centrifuged for 5 min at 5000 g. The extract was transferred to a flat-bottom flask, and the extraction was repeated twice with 25 mL of hexane:dichloromethane (1:1) followed by 25 mL of hexane:ethyl acetate (1:1). All extracts were combined, and a procedure

similar to that reported above for wastewater was followed. For the quantification of target compounds in sludge samples, an external calibration method was used.

For the analysis of target compounds in particulate matter, the extraction procedure was similar to that reported for sludge. The filters were dried in room temperature, in the dark for 48 hours. The extraction was performed with the same solvents as in the sludge but with reduced volumes of solvents, as the available mass of particulate matter was small. The extraction was carried out with 10 mL of hexane, 10 mL of hexane:dichloromethane (1:1), and 10 mL of hexane:ethyl acetate (1:1).

3.3.4 Instrumental Analysis

Analysis was performed on an Agilent 6890 gas chromatograph (GC) interfaced with an Agilent 5973 mass spectrometer (MS), and separation was achieved by an HP-5MS capillary column (30 m × 0.25 mm i.d. and 0.25 μm film thickness, Agilent). Two microliters of extract were injected, through an autosampler, in a splitless mode at 200° C, and helium was used as a carrier gas at a flow rate of 1.0 ml min^{-1}. The oven temperature was programmed from 40° C (held for 1 min); it was then increased to 220° C at a rate of 20° C min^{-1} and to 280° C at a rate of 5° C min^{-1}, with a hold time of 10 min and a post-run time of 5 min at 300° C. The MS was operated in an electron-impact ionization mode (EI) at 70 eV, and the data were acquired by selected ion monitoring (SIM). The ions were monitored, and the retention time of target compounds are presented in Table 3.2. Chromatogram of a spiked wastewater sample and a spiked sludge sample are presented in Figures 3.1 and 3.2, respectively.

Table 3.2 Retention time and monitored ions for siloxanes.

Analyte	t_R (min)	Ions	Analyte	t_R (min)	Ions
D3	3.85	207*, 96	L7	9.45	221*, 147
D4	5.31	281*	L8	10.38	221*, 147
D5	6.58	355*, 267	L9	11.37	221*, 147
D6	7.86	341*, 429	L10	12.54	221*, 147
D7	9.01	281*, 221	L11	13.92	221*, 147
L3	4.34	221*, 73	L12	15.51	221*, 147
L4	5.89	207*, 73	L13	17.25	221*, 147
L5	7.24	281*, 147	L14	19.05	221*, 147
L6	8.42	221*, 147	IS (M4Q)	6.83	281*, 369

* quantification ion

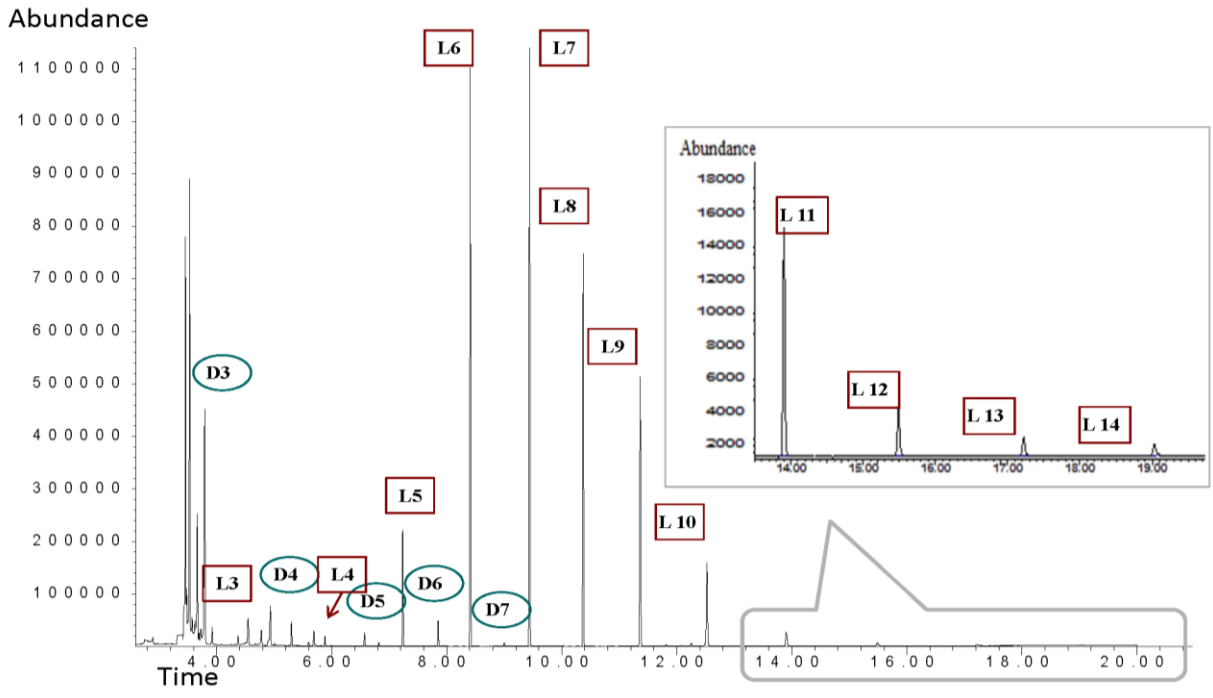


Figure 3.1 Total ion chromatogram (TIC) of a spiked wastewater sample.

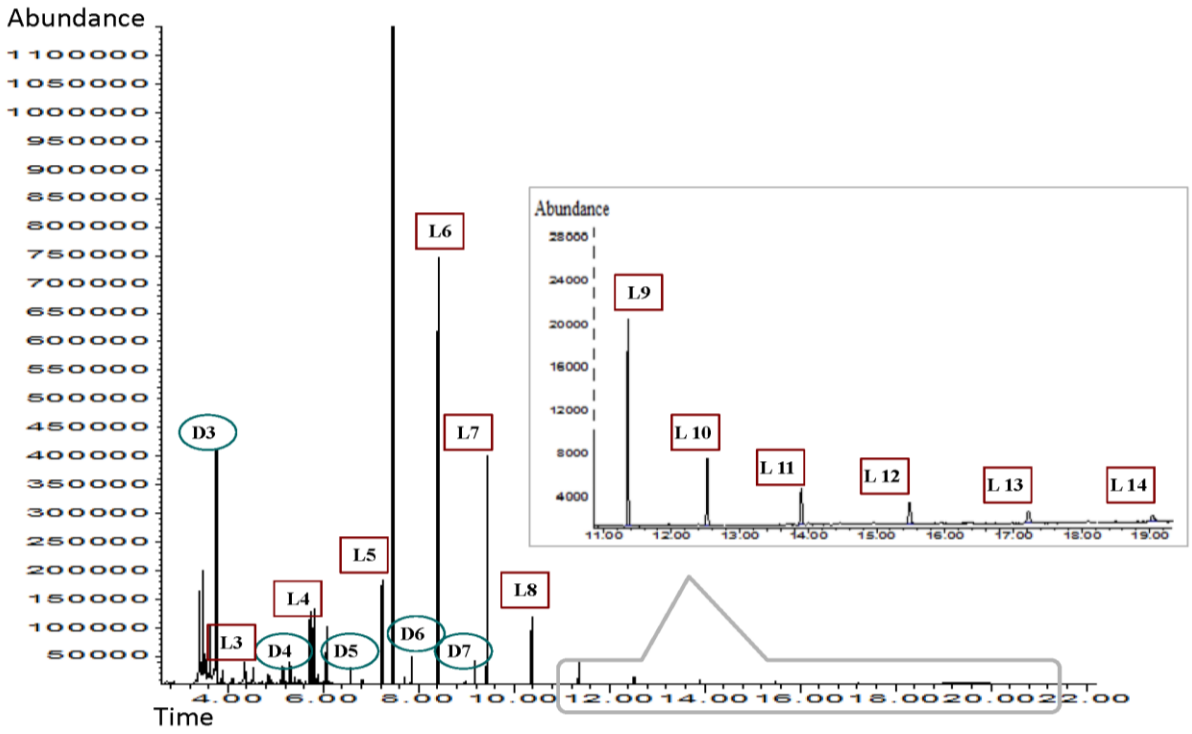


Figure 3.2 Total ion chromatogram (TIC) of spiked sludge sample.

Siloxanes exhibited a clear fragmentation pattern under EI mode: linear siloxanes with 7 or more Si atoms presented fragments at m/z 221 and 117, which correspond to the $[(CH_3)_3SiOSi(CH_3)_2Si(CH_3)_2]^+$ and $[(CH_3)_3SiOSi(CH_3)_2]^+$ ions, respectively. The predominant ion of cyclic siloxanes was $[M-16]^+$, which corresponds to the $M-CH_4$ group.

3.3.5 Validation

The gas chromatographic vials were capped with aluminum foil (instead of Teflon or rubber/silicon) to minimize background levels of siloxanes [25]. The analyst refrained from using hand lotions or other personal care products that contained siloxanes. Prior to instrumental analysis, hexane was injected into GC-MS, until the background levels of siloxanes became stable. Hexane also was injected after every 10 samples as a check for background contamination and carry-over contamination.

Calibration curves were prepared by injecting standard solutions in hexane and in a fortified matrix (matrix-matched standards, spiked Milli-Q water, and sludge). Repeatability and reproducibility of the method were assessed by fortification of a water and soil matrix at two different concentration levels. For water samples, D3 to D6 and L3 to L5 were spiked at 0.10 and 1.0 µg L^{-1}, and D7 and L6 to L14 were spiked at 0.72 to 9.2 µg L^{-1} and 7.25 to 920 µg L^{-1}, respectively. For sludge samples, D3 to D6 and L3 to L5 were spiked at 1.25 and 5.0 µg kg^{-1}, respectively, and D7 and L6 to L14 were spiked at 2.9 to 184 and 14.5 to 1800 µg kg^{-1}, respectively. The limit of detection (LOD) was set at a signal-to-noise ratio (S/N) of 3.3, and the limit of quantification (LOQ) was at S/N of 10 in spiked samples.

Procedural blanks were prepared for every 10 samples by passage of Milli-Q water through the entire analytical procedure. In addition, a spiked blank and a spiked matrix were analyzed with every batch of 20 samples. The procedural blanks showed pg to ng L^{-1} concentrations of D3, D4, and D5, and the concentrations found in blanks from each batch of samples were subtracted from sample values.

3.3.6 Calculation of Removal Efficiency and Distribution Coefficients

The removal efficiency of siloxanes in the WWTP was calculated from the mass flows of each compound in raw sewage (M_{inf}) and secondary effluent (M_{eff}), as shown in Equation 1:

$$Removal(\%) = \frac{(M_{inf}) - (M_{eff})}{(M_{inf})} \times 100 \quad (1)$$

The mass load of target compounds that was lost in the WWTP by all transformation processes ($W_{lost,\ STP}$) was calculated, as shown in Equation 2:

$$W_{lost,STP} = (Q_{inf} \times C_{inf}) - (Q_{eff} \times C_{eff}) - (M_{dew.sludge} \times C_{dew.sludge}) \quad (2)$$

where Q_{inf} and Q_{eff} are the flow rates of influent wastewater ($m^3\ d^{-1}$) and effluent wastewater, respectively ($m^3\ d^{-1}$), C_{inf} and C_{eff} are the total concentrations (dissolved + particulate) of the target compounds in influent and effluent wastewater (mg m^{-3}), $M_{dew.sludge}$ is the mass of dewatered sludge produced (kg dw d^{-1}), and $C_{dew.sludge}$ is the

concentration of target compounds in dewatered sludge (mg kg^{-1} dw). It should be noted that the concentrations of siloxanes in particulate matter were calculated on a volume basis (expressed as μg L^{-1}), by taking into account the concentration of suspended solids in influent and treated wastewater. Concentrations below the LOD were assigned a value equal to half the LOD [185].

Distribution coefficients (K_d) of siloxanes were estimated from concentrations measured in primary and secondary sludge, as shown in Equation 3:

$$K_d = \frac{C_s}{C_w} \quad (3)$$

where C_s is the concentration of target compound in particulate phase (ng kg^{-1}), and C_w is the concentration of target compound in dissolved phase (ng L^{-1}). For the calculation of removal efficiencies and K_d values, arithmetic means were used.

3.4 Results and Discussion

3.4.1. Validation Results

Cyclic (D3-D6) and linear siloxanes (L3-L5) showed linearity in the range of 1-100 μg L^{-1}. The regression coefficients were >0.99, as presented in Table 3.3 for wastewater and in Table 3.4 for sludge. Accuracy was determined by spiking standards in Milli-Q water and a soil matrix (as a surrogate for sludge) at six different concentrations that bracketed the calibration range. Relative overall recovery was calculated dividing the slope of the calibration curve of the spiked samples by the slope of the calibration curve of the analyte in matrix (post-extraction spikes). Recoveries of target compounds spiked into a water matrix ranged from 60.6 ± 3.7% to 134 ± 14%, and those spiked in soil ranged from 53.9 ± 7.4% to 102 ± 18% (Table 3.5).

Repeatability was estimated by the calculation of relative standard deviation (RSD) from six replicate analyses on the same day, whereas intermediate precision was calculated from six replicate analyses on two different days. The RSD for all siloxanes was <17% in water samples, with the exception of D4 and L14 (29% and 24%, respectively), and <12% in sludge spiked at two different concentrations (Table 3.6).

The LOQs for siloxanes in water ranged from 0.00011 to 0.040 µg L^{-1} and in sludge from 0.0060 to 9.9 µg kg-1 (Table 3.7).

Table 3.3 Linearity data for siloxanes in spiked wastewater samples.

Siloxanes	Use of IS	Linear range (µg L^{-1})	Slope (b ± Sb)	Intercept-y (a ± Sa)	r^2
D3	M4Q	0.01-5	5.32 ± 0.76	1.88 ± 0.44	0.96
D4	M4Q	0.01-5	9.0 ± 1.1	2.74 ± 0.66	0.97
D5	M4Q	0.01-5	3.04 ± 0.40	1.14 ± 0.23	0.97
D6	M4Q	0.01-5	7.65 ± 0.19	0.166 ± 0.091	0.997
D7	-	0.001 - 0.07	$5.1\ 10^5 \pm 2.2\ 10^4$	5570 ± 732	0.993
L3	M4Q	0.01- 5	2.797 ± 0.078	-0.009 ± 0.037	0.997
L4	M4Q	0.01- 5	3.11 ± 0.13	-0.051 ± 0.066	0.95
L5	M4Q	0.01- 5	31.09 ± 0.76	-0.26 ± 0.35	0.997
L6	M4Q	0.18 - 8.8	19.19 ± 0.23	-1.36 ± 0.95	0.9994
L7	-	0.18 - 9.2	$7.5\ 10^5 \pm 2.5\ 10^4$	$1.0\ 10^5 \pm 1.1\ 10^5$	0.996
L8	-	0.17 - 8.3	$6.0\ 10^5 \pm 1.7\ 10^4$	$5.0\ 10^4 \pm 6.7\ 10^4$	0.997
L9	-	0.14 - 7.1	$4.3\ 10^5 \pm 7.5\ 10^3$	$-3.1\ 10^4 \pm 2.5\ 10^4$	0.998
L10	-	0.11 - 5.5	$1.2\ 10^5 \pm 1.3\ 10^3$	$-1.3\ 10^4 \pm 3.4\ 10^3$	0.9995
L11	-	0.07 - 3.3	$4.5\ 10^4 \pm 7.5\ 10^2$	$-2.9\ 10^2 \pm 1.2\ 10^3$	0.998
L12	-	0.03 - 1.3	$4.3\ 10^4 \pm 1.0\ 10^3$	$7.0\ 10^2 \pm 6.4\ 10^2$	0.997
L13	-	0.01 - 0.40	$8.5\ 10^4 \pm 2.2\ 10^3$	$5.3\ 10^2 \pm 4.5\ 10^2$	0.997
L14	-	0.02 - 0.14	$1.4\ 10^5 \pm 5.4\ 10^3$	$7.3\ 10^2 \pm 4.5\ 10^2$	0.996

Table 3.4 Linearity data for siloxanes in spiked sludge samples.

Siloxanes	Use of IS	Linear range (µg kg^{-1})	Slope (b ± Sb)	Intercept-y (a ± Sa)	r^2
D3	M4Q	1 - 12.5 × 10^3	4.9 10^{-2}± 1.2 10^{-3}	-5.9 ± 4.9	0.997
D4	-	1 - 12.5 × 10^3	4.1 10^3 ± 29	1.8 10^5 ± 1.2 10^5	0.9998
D5	-	1 - 12.5 × 10^3	1.2 10^3 ± 19	1.2 10^4 ± 1.0 10^5	0.9995
D6	-	1 - 12.5 × 10^3	1.8 10^3 ± 34	2.2 10^5± 1.5 10^5	0.997
D7	-	7.25 - 145	1.8 10^3 ± 46	5.3 10^3 ± 3.4 10^3	0.998
L3	-	1 - 12.5 × 10^3	2.0 10^3 ± 13	-9.8 10^4 ± 5.6 10^4	0.9996
L4	-	1 - 12.5 × 10^3	2.3 10^3 ± 15	-2.2 10^4 ± 5.6 10^4	0.9997
L5	-	1 - 12.5 × 10^3	2.9 10^4 ± 90	7.3 10^5 ± 3.9 10^5	0.993
L6	-	17.5 - 17.6 × 10^3	3.2 10^3 ± 94	7.6 10^5 ± 6.4 10^5	0.9998
L7	-	18.5 - 18.4 × 10^3	2.2 10^3 ± 1.1 10^2	5.5 10^5 ± 7.5 10^5	0.98
L8	-	16.5 - 16.5 × 10^3	8.3 10^2 ± 42	2.0 10^5 ± 2.7 10^5	0.98
L9	-	14 - 14.1 × 10^3	3.3 10^2 ± 11	4.5 10^4 ± 6.2 10^4	0.991
L10	-	11 - 11.0 × 10^3	1.5 10^2± 7	2.9 10^4 ± 3.0 10^4	0.98
L11	-	6.5 - 6.7 × 10^3	1.7 10^2 ± 18	3.5 10^4 ± 4.6 10^4	0.92
L12	-	2.65 - 2.6 × 10^3	3.1 10^2 ± 12	1.5 10^4 ± 1.2 10^4	0.98
L13	-	2 - 790	7.6 10^2 ± 31	1.4 10^4 ± 1.0 10^4	0.99
L14		1.45 - 290	1.4 10^3 ± 63	1.3 10^4 ± 8.0 10^3	0.99

Table 3.5 Trueness data for the determination of siloxanes in wastewater and sludge.

Siloxanes	**Recovery ± SD %** (n=6 levels) **(water)**	**Recovery ± SD %** (n=6 levels) **(sludge)**	**Overall Recovery % (sludge)**
D3	60.6 ± 3.7	86 ± 16	71
D4	81 ± 17	71 ± 16	55
D5	87 ± 13	89 ± 22	62
D6	105 ± 12	102 ± 18	71
D7	134 ± 14	88 ± 12	80
L3	82.2 ± 9.4	53.9 ± 7.4	49
L4	101 ± 15	68 ± 10	58
L5	107.7 ± 5.6	72.8 ± 8.4	66
L6	80 ± 13	76.4 ± 8.7	69
L7	91 ± 13	76.8 ± 6.6	73
L8	95 ± 14	77 ± 11	67
L9	100 ± 16	78 ± 12	71
L10	115 ± 20	82.8 ± 9.0	70
L11	118 ± 19	91 ± 11	77
L12	111 ± 16	87 ± 12	72
L13	107 ± 15	93 ± 14	71
L14	101.1 ± 8.2	90 ± 13	70

Table 3.6 Precision data for the determination of siloxanes in wastewater and sludge.

	Water		**Sludge**	
Siloxanes	Repeatability – Intermediate precision %RSD		Repeatability - Intermediate precision %RSD	
	1st level	2nd level	1st level	2nd level
D3	16 - 17	5.6 - 11	7.8 - 10	6.5 - 7.6
D4	29 - 17	3.8 - 11	9.5 - 12	8.5 - 9.3
D5	4.8 - 4.7	3.1 - 7.4	5.7 - 11	4.9 - 11
D6	3.4 - 6.7	3.2 - 5.2	4.3 - 10	4.2 - 9.4
D7	4.3 - 25	3.8 - 20	8.6 - 12	4.7 - 5.6
L3	6.5 - 16	1.5 - 1.6	9.4 - 10	7.2 - 8.6
L4	2.5 - 23	2.1 - 13	6.2 - 8.7	4.5 - 6.0
L5	3.0 - 15	2.5 - 12	8.5 - 9.7	5.7 - 8.6
L6	2.5 - 9.5	4.4 - 5.1	8.5 - 12	6.0 - 6.3
L7	2.3 - 6.6	4.5 - 4.3	8.5 - 9.9	7.0 - 9.4
L8	2.8 - 3.3	5.0 - 4.5	8.7 - 11	7.5 - 11
L9	6.6 - 15	6.7 - 7.2	6.1 - 6.9	5.2 - 6.2
L10	11 - 17	11 - 17	12 - 13	4.4 - 5.9
L11	10 - 20	7.0 - 18	11 - 12	2.3 - 4.4
L12	7.1 - 13	6.9 - 13	11 - 13	2.7 - 4.9
L13	3.0 - 5.1	7.5 - 11	11 - 13	2.0 - 6.0
L14	24 - 19	8.1 - 16	11 - 13	3.9 - 5.2

Table 3.7 Method LODs and LOQs for the determination of siloxanes in wastewater and sludge.

Siloxanes	Water (µg L^{-1})		Sludge (µg kg^{-1})	
	LOD (S/N 3)	**LOQ** (S/N 10)	**LOD** (S/N 3)	**LOQ** (S/N 10)
D3	0.00010	0.00030	0.010	0.030
D4	0.000030	0.00011	0.0020	0.0060
D5	0.000060	0.00018	0.0025	0.0075
D6	0.00020	0.00060	0.0050	0.015
D7	0.00022	0.00066	0.010	0.030
L3	0.0020	0.0070	0.50	1.5
L4	0.0040	0.012	0.30	0.90
L5	0.00020	0.00060	0.020	0.060
L6	0.00041	0.0012	0.050	0.15
L7	0.00039	0.0012	0.37	1.1
L8	0.00065	0.0021	0.50	1.5
L9	0.00084	0.0029	0.85	2.5
L10	0.0019	0.0061	3.3	9.9
L11	0.0061	0.018	2.7	8.1
L12	0.013	0.040	1.3	4.0
L13	0.0081	0.024	0.79	2.4
L14	0.0072	0.022	1.4	4.4

3.4.2. Siloxanes in Wastewater Samples

Seven influent and seven effluent wastewater samples were collected during the sampling campaign in April 2012. Except for L3, all cyclic and linear siloxanes were detected in influent samples. The average total concentration of siloxanes in influent was 20.3 µg L^{-1} (Table 3.8), with linear siloxanes (75%) contributing more to the total

concentrations (Figure 3.3). D5, D6, L9, L10, and L11 were the major compounds found in influents at mean concentrations of 2.60, 1.83, 2.30, 3.20, and 4.82 μg L^{-1}, respectively. High concentrations of D5 and D6 in influents are in accordance with the consumption pattern of these compounds in Europe [174, 175, 182]. The main source of siloxanes in the WWTP is personal care products, which contain mainly D5 and D6, and the sum of the linear siloxanes [179].

Table 3.8 Concentrations of siloxanes in wastewater samples of a WWTP in Athens, Greece.

	Total Influent (μg L^{-1}) (dissolved + particulate)				**Total Effluent (μg L^{-1}) (dissolved + particulate)**			
	[N]>LOD[a]	**Mean**	**Median**	**Min-max**	**[N]>LOD**	**Mean**	**Median**	**Min-max**
D3	7	0.159	0.164	0.114 - 0.183	7	0.152	0.128	0.095 - 0.256
D4	7	0.149	0.155	0.099 - 0.187	7	0.129	0.113	0.103 - 0.197
D5	7	2.60	1.51	0.544 - 5.36	7	1.79	0.418	0.125 - 6.02
D6	7	1.83	1.70	1.16 - 3.19	7	0.026	0.020	0.002 - 0.059
D7	7	0.401	0.399	0.294 - 0.579	7	0.012	0.011	0.009 - 0.016
∑cycl.[b]		5.14				2.11		
L3	0	<LOD	<LOD	<LOD	0	<LOD	<LOD	<LOD - 0.005
L4	6	0.084	0.113	<LOD - 0.148	6	0.037	0.033	<LOD - 0.099
L5	7	0.029	0.022	0.010 - 0.067	7	0.008	0.010	0.0007 - 0.012
L6	7	0.355	0.241	0.079 - 0.968	7	0.084	0.066	0.011 - 0.163
L7	7	1.02	0.942	0.093 - 1.98	7	0.195	0.210	0.020 - 0.310
L8	7	1.89	1.67	0.440 - 3.14	7	0.225	0.240	0.019 - 0.343

L9	7	2.30	1.79	0.469 - 4.43	7	0.269	0.244	0.027 - 0.484
L10	7	3.20	3.86	1.33 - 4.89	7	0.252	0.258	0.030 - 0.500
L11	7	4.82	5.20	1.20 - 7.91	7	0.319	0.291	0.042 - 0.634
L12	7	0.895	0.858	0. 438 - 1.57	7	0.050	0.045	0.012 - 0.085
L13	7	0.416	0.510	0.137 - 0.726	7	0.021	0.018	0.007 - 0.035
L14	7	0.113	0.110	0.045 - 0.210	5	0.007	0.006	<LOD - 0.013
∑lin.[c]		15.12				1.47		
∑sil.(D/L)[d]		20.26				3.58		

[a] [N]>LOD: Number of samples with concentration higher than the method limit of detection (LOD)

[b]∑cycl.: The sum of all cyclic siloxanes D3 – D7

[c]∑lin.: The sum of all linear siloxanes L4 – L14

[d]∑sil.(D/L).: The sum of all cyclic and linear siloxanes

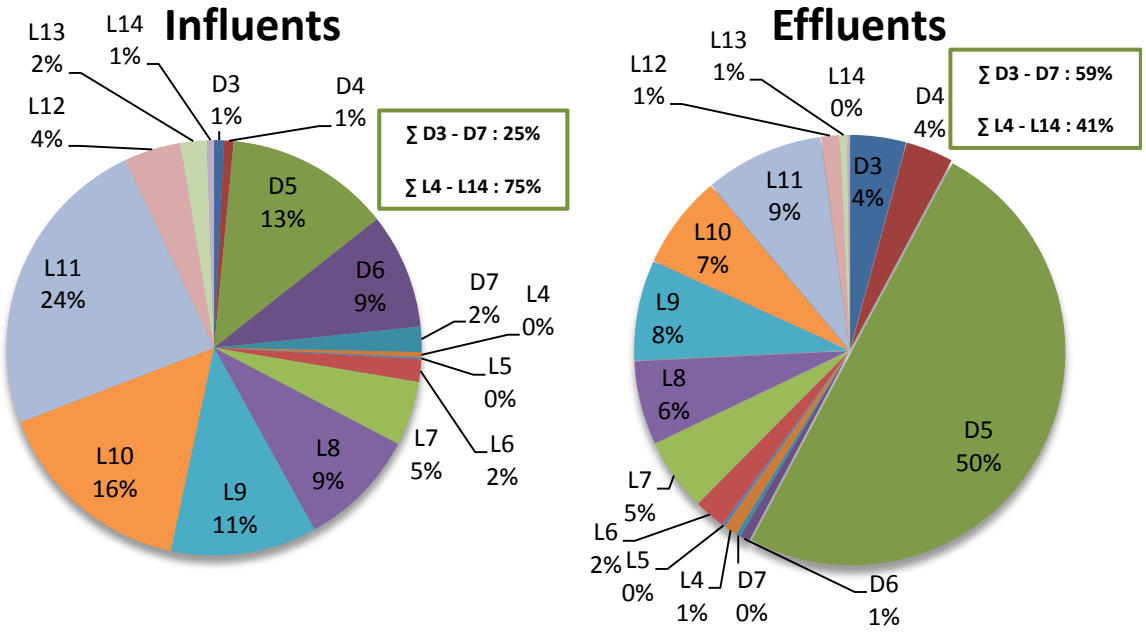


Figure 3.3 Contribution (%) of individual siloxanes to total concentrations determined in influent and effluent wastewater.

Based on the concentrations of siloxanes in influent and flow rates, daily loadings were calculated and presented in Table 3.9. The average daily loadings of siloxanes ranged from 21.7 g per day for L5 to 3.6 kg per day for L11. A total of 15.1 kg of siloxanes reach the WWTP on a daily basis, resulting in a total amount of 5.5 tons annually. By taking into consideration that this WWTP serves approximately 3.7 million residents, per capita mass loading of siloxane was calculated. For L11 and D5, respective daily loadings were estimated to be 974 and 526 mg per day per 1,000 inhabitants; a daily load of 4.1 g per 1,000 individuals was estimated for total siloxanes. To the best of our knowledge, this is the first report on daily loadings of siloxanes in WWTPs.

Table 3.9 Siloxanes mass loadings (mg per day) and normalized mass loadings (mg per day per 1000 inhabitants) in influents of a WWTP in Athens, Greece.

	Mass in ($g\ day^{-1}$)			Mass in ($mg\ day^{-1}$ /1000 inhabitants)	
	Min-max	**Mean**	**Median**	**Mean**	**Median**
D3	84.8 - 136	118	122	32	33
D4	73.6 - 144	111	116	30	31
D5	405 - 3970	1946	1122	526	303
D6	859 - 2457	1361	1267	368	342
D7	226 - 432	297	297	80	80
∑cycl.[a]		3833		1036	
L4	22.6 - 109	73.1	90.4	20	24
L5	6.99 - 51.9	21.7	16.6	5,9	4,5
L6	58.9 - 719	265	179	72	48
L7	69.0 - 1470	760	701	205	190
L8	328 - 2329	1407	1241	380	336
L9	349 - 3413	1722	1332	465	360
L10	947 - 3766	2393	2875	647	777
L11	859 - 5859	3605	3874	974	1047
L12	326 - 1166	667	639	180	173
L13	98.1 - 538	311	380	84	103
L14	33.8 - 156	84.4	81.9	22	22
∑lin.[b]		11309		3056	
∑sil.(D/L)[c]		15142		4092	

[a]∑cycl.: The sum of all cyclic siloxanes D3 – D7

[b]∑ lin.: The sum of all linear siloxanes L4 – L14

[c]∑sil.(D/L).: The sum of all cyclic and linear siloxanes

Concentrations of siloxanes in final effluents were lower than the concentrations found in influents (Table 3.8). The average total concentration of siloxanes in effluents was 3.58 μg L^{-1}. In contrast to raw wastewater, cyclic siloxanes were the major compounds in effluents, accounting for 59% of the total concentrations; linear siloxanes accounted

for 41% of the total concentrations in effluents (Figure 3.3). D5 was the major cyclic siloxane found in effluents (1.79 μg L^{-1}), whereas the concentrations of individual linear siloxanes were < 0.32 μg L^{-1}.

The distribution of siloxanes between particulate and dissolved phases showed different patterns (Figure 3.4). Cyclic siloxanes were detected mainly in the dissolved phase of influents, which suggests that adsorption of cyclic siloxanes onto the particulate matter was less significant. The distribution of linear siloxanes between dissolved and particulate phases was related to the molecular weight. As shown in Figure 3.4, linear siloxanes tend to accumulate in the particulate phase; small molecules (L5-L8) were present in the dissolved phase at 13% to 36% of the total concentrations, whereas large molecules (L9-L14) were present at 4% (on average) in the dissolved phase.

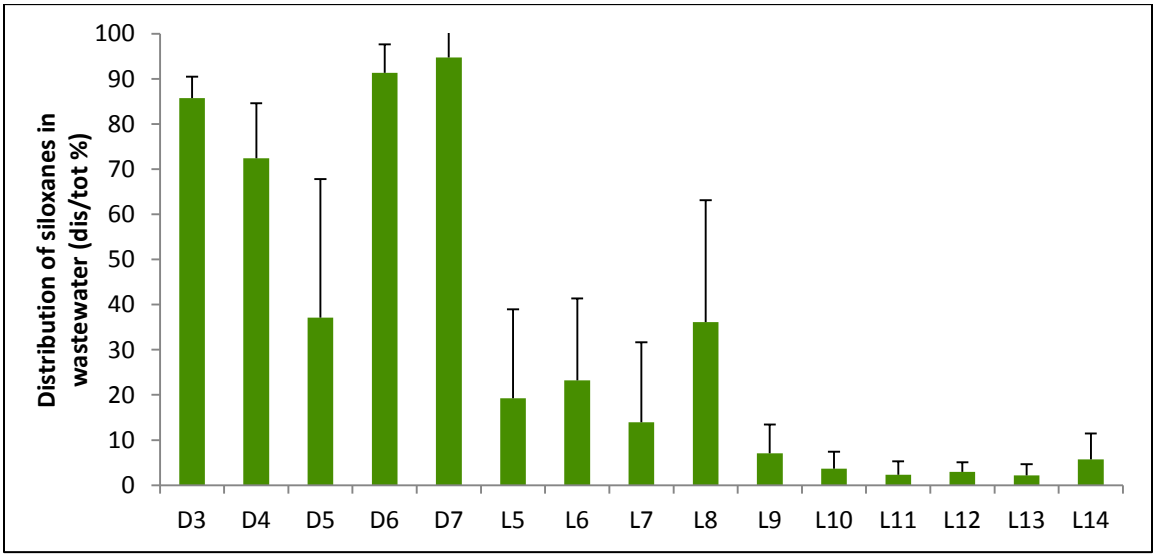


Figure 3.4 Fraction (%) of siloxanes detected in the dissolved phase of influents. L3 and L4 concentrations were below LOD in influents.

With regard to the daily variation in siloxane concentrations in wastewater, a discernible pattern was not found, although slightly higher concentrations were found in samples collected during the weekends. Concentrations of D5, D6, and L8-L10 were twofold higher in samples collected during the weekend. Concentrations of D3, D4, L6, and L7 showed little variation during the weekdays but peaked on Friday. Concentrations of

high molecular weight siloxanes (L11-L14) increased during the weekend, with a peak on Monday. The daily variations in siloxane concentrations in wastewaters may reflect the activities of individuals during the week.

3.4.3. Siloxanes in Sludge

Seven sludge samples were collected from the WWTP between April 2 and 8, 2012. The concentrations of siloxanes in sludge were calculated on a dry solids (DS) basis, and the results are reported in Table 3.10. Siloxanes were detected in all sludge samples on the order of a few to several tens of milligrams per kilogram. D5 was the major compound (mean: 15.1 mg kg^{-1}); individual linear siloxane (L7 to L11) concentrations ranged from 6.5 to 11.3 mg kg^{-1}. The mean total concentration of siloxanes in sludge was 75 mg kg^{-1}, with 72% being linear compounds. The WWTP produces 110,000 kg of dry sludge daily. This suggests that the mass loading of siloxanes in sludge is approximately 8.2 kg per day. Comparison of the concentrations of siloxanes determined in this study with those reported in an earlier study from China showed that the concentrations of all siloxanes were higher in sludge from Athens [176]. Although the concentrations were different between the two WWTPs, the percentage of cyclic to linear siloxanes was similar between the two studies.

Table 3.10 Concentrations of siloxanes in dewatered sewage sludge from a WWTP in Athens, Greece.

	[N] >LOD[a]	Mean (mg kg^{-1})	Median (mg kg^{-1})	Min-max (mg kg^{-1})
Humidity %		81	82	79 - 82
D3	7	0.009	0.025	0.007 - 0.012
D4	7	0.11	0.13	0.09 -0.13
D5	7	15.1	14.8	13.4 - 17.5
D6	7	5.03	5.00	4.73 - 5.49
D7	7	0.80	0.79	0.74 - 0.92
∑cycl.[b]		21.1		
L3	7	0.22	0.25	0.16 - 0.26
L4	7	0.056	0.067	0.050 - 0.063
L5	7	0.22	0.22	0.21 - 0.25
L6	7	3.63	3.59	3.39 - 4.07
L7	7	6.52	6.44	6.01 - 7.33
L8	7	8.51	8.43	7.90 - 9.53
L9	7	10.7	10.6	10.0 - 11.7
L10	7	11.3	11.3	10.6 - 12.4
L11	7	7.87	7.95	7.35 - 8.65
L12	7	3.38	3.40	3.16 - 3.71
L13	7	1.10	1.11	1.02 - 1.22
L14	7	0.45	0.45	0.40 - 0.49
∑lin.[c]		53.9		
∑sil.(D/L)[d]		75.0		

[a] [N]>LOD: Number of samples with concentration higher than the method limit of detection (LOD)

[b]∑cycl.: The sum of all cyclic siloxanes D3 – D7

[c]∑lin.: The sum of all linear siloxanes L4 – L14

[d]∑sil.(D/L).: The sum of all cyclic and linear siloxanes

3.4.4. Solid-Liquid Distribution Coefficients of Siloxanes

Log K_d values were calculated based on the concentrations determined in particulate and dissolved phases of samples collected from five different points in the WWTP (Table 3.11). Cyclic siloxanes exhibited lower average solid-liquid distribution coefficients than did linear siloxanes. For cyclic siloxanes, average log K_d values did not exceed 3.8 (L kg^{-1}), whereas for linear analogues, log K_d values ranged between 3.25 and 5.62 (L kg^{-1}). In general, the sorption capacities of linear siloxanes increased steadily with an increase in chain length from L5 to L9 and then decreased from L10 to L14. The K_d values of L4 and L14 were not calculated, as their concentrations in dissolved phase were below the LOD. The log K_d values determined in this study were similar to those reported in the literature (Figure 3.5) [159]. The estimated log K_d values varied only slightly among the samples taken from five different points in the WWTP (Table 3.11). Nevertheless, a slightly greater affinity of some siloxanes (D5 to D7 and L5 to L10) to secondary sludge was found, as the log K_d values were higher for these samples than in other samples. This is similar to what was observed for several other micropollutants [186-188], which may be related to sludge characteristics (e.g., carbon content), pH, ionic strength, and presence of complexing agents. Secondary sludge contains large proportions of microbial cells and exopolymeric substances produced during biological treatment.

Table 3.11 Mean solid-liquid distribution coefficients, logK_d (K_d in L kg^{-1}) of siloxanes in wastewater collected at different points in a WWTP.

	Influent primary settlement tank	Primary sludge	Effluent primary settlement tank	Influent bioreactor	Secondary sludge	Mean	Values from literature[1]
D3	2.31	1.83	3.01	2.70	2.15	2.40	2.25
D4	3.20	3.05	3.61	3.62	3.40	3.38	2.87
D5	3.58	3.64	3.15	3.74	4.42	3.71	3.30
D6	2.97	3.49	2.82	3.02	3.95	3.25	3.78
D7	3.00	3.43	3.16	3.26	3.42	3.25	NA
L4	-	-	-	-	-	-	3.5
L5	4.66	4.94	4.33	4.93	6.09	3.25	4.24
L6	5.04	5.59	5.09	5.42	5.96	4.99	4.67
L7	5.23	5.70	5.39	5.57	5.99	5.42	5.26
L8	5.31	5.75	5.43	5.64	5.97	5.58	5.79
L9	5.37	5.90	5.38	5.72	-	5.62	NA
L10	5.33	5.94	5.30	5.60	-	5.59	NA
L11	5.59	-	5.38	5.66	-	5.54	NA
L12	5.79	-	5.39	5.65	-	5.54	NA
L13	5.29	-	5.10	5.58	-	5.61	NA
L14	-	-	-	-	-	-	NA

NA: not available

K_d was not calculated, because the concentration of the compounds in the dissolved phase was <LOD

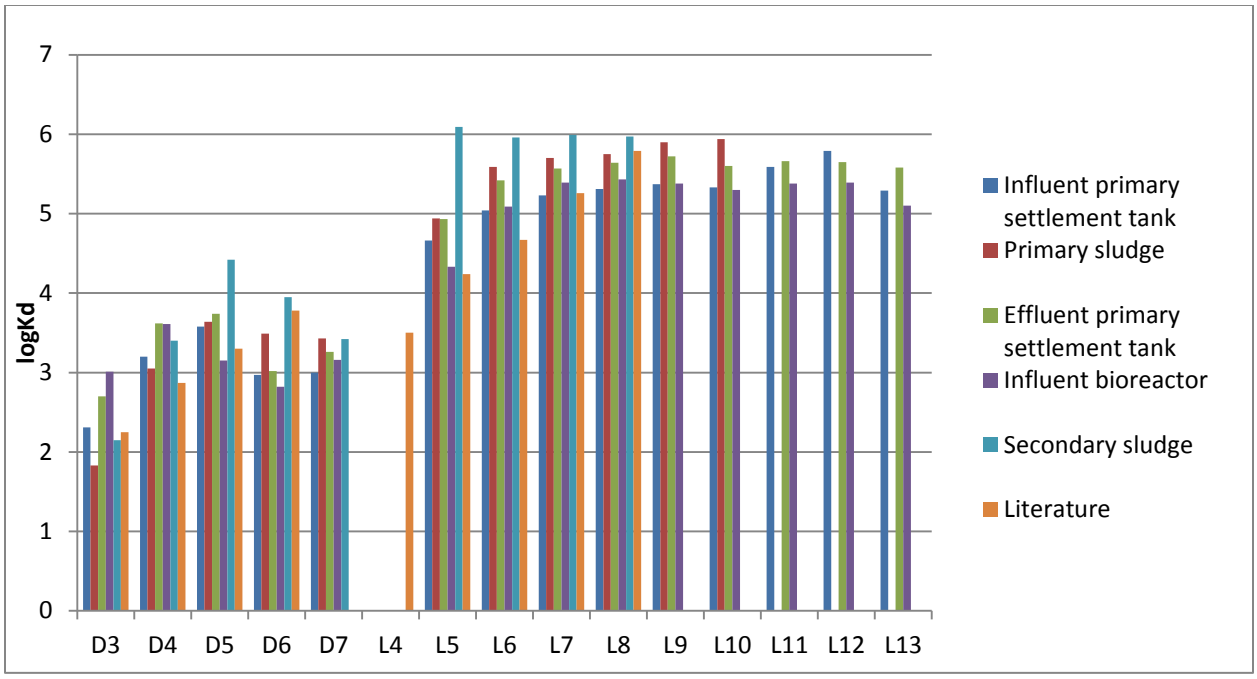


Figure 3.5 Comparison of distribution coefficients ($\log K_d$) (L kg^{-1}) for siloxanes in five types of sludge.

3.4.5. Fate of Siloxanes in WWTP

The percent removal of siloxanes during wastewater treatment was calculated using Equation (1), and the results are presented in Figure 3.6. D3 showed a small negative value (-1.9%), which suggests that this compound was not removed in the treatment process but that it was added; possible sources of addition include breakdown of larger siloxane molecules or precursor compounds, or it may be due to the fact that D3 is known to be hydrolyzed or easily volatilized. All siloxanes, except for D3, D4 and D5, showed removal rates higher than 50% in the wastewater treatment process. For 10 of the 16 compounds detected in influents, the removal efficiency was higher than 80%.

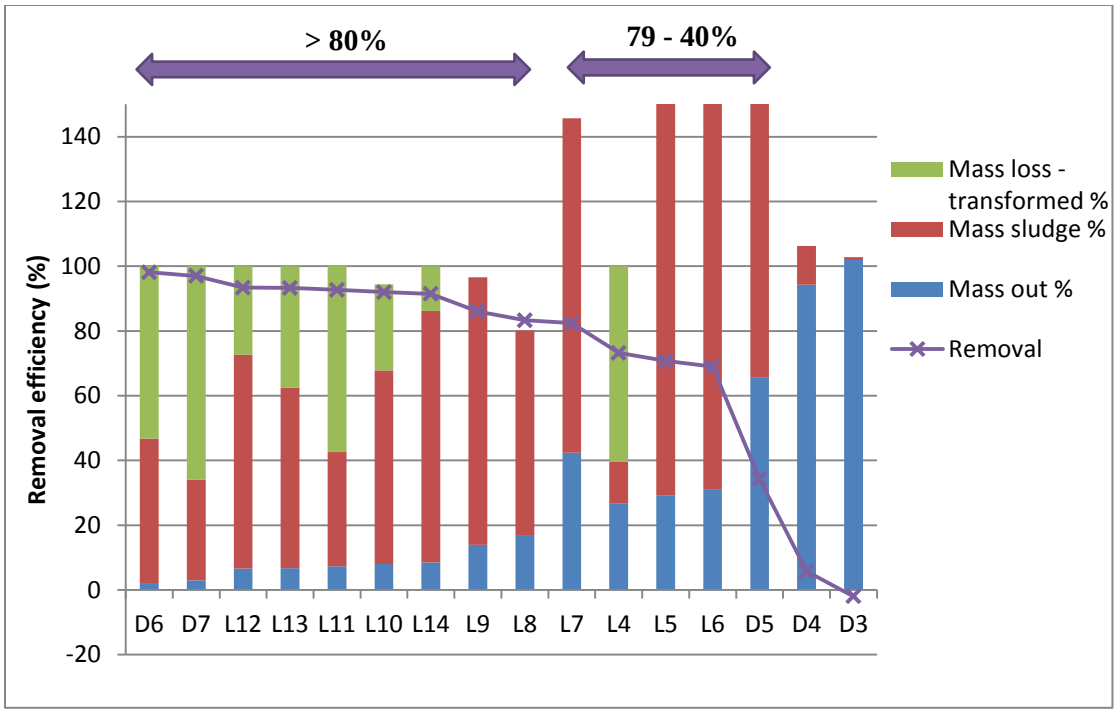


Figure 3.6 Removal efficiency (%) and fate of siloxanes during wastewater treatment.

The average removal efficiencies of cyclic and linear siloxanes were different. Regarding cyclic siloxanes, D3 and D4 were not removed; D5 showed 34.2% removal, while D6 and D7 showed the highest removal (>97%). The removal rate of linear siloxanes was between 69.0 and 93.4% (mean = 84.3%). These values are comparable to those reported by Fendinger et al., who showed a PDMS removal rate of >94% in wastewater treatment processes [173]. Among the target compounds analyzed, D6 appeared to have the highest removal rate, followed by D7, which is in agreement with the values (>93.5%) estimated by the UK Environmental Agency [171]. Based on the mass of siloxanes measured in influents, effluents, and sludge (Equation 2), it was found that, on average, 68% of total siloxanes were sorbed to sludge, and 29% were discharged as effluents. A small fraction of siloxanes was lost (e.g., volatilization), degraded, and/or transformed in the biological wastewater treatment processes.

In accordance with the distribution coefficients (Table 3.11), the removal mechanism of siloxanes in WWTPs is expected to vary, depending on the chemical structure (Figure 3.6). Sorption and accumulation to sludge was the major removal mechanism for most

of the linear siloxanes. In contrast, sorption was not significant for cyclic siloxanes, except for D5. The mass balance of D5 indicated that 66% of this compound was discharged via the effluents. The high loadings of D5 in sludge denotes an additional source during the treatment and confirms its low biodegrability [167].

Overall, a significant portion of siloxanes was lost in the WWTP (Figure 3.6). There are very few studies that contain a description of mechanisms responsible for the loss of siloxanes in the wastewater treatment processes. Polymeric and/or larger molecular weight compounds, such as PDMS, can be degraded to smaller molecular weight linear siloxanes [189]. Soil degradation studies have shown that PDMS undergoes cleavage of the siloxane backbone, forming smaller linear siloxanes or even cyclic siloxanes (at high PDMS loadings) [171]. Biodegradation of PDMS is responsible for the high mass loadings of L5, L6, and L7 estimated in the effluents (treated sludge + treated wastewater) (Figure 6).

D6 can be biodegraded to dimethylsilanediol (DMSD), which can be volatilized and/or further biodegraded [171]. Direct volatilization of D6 into the atmosphere is possible [190], which explains its loss in the WWTP. The behavior of D7 in the WWTP was similar to that of D6 (Figure 3.6). As stated above, D5 appears to be stable in the wastewater treatment process. D3 and D4 did not undergo any loss, and a slightly higher loading of D3 in effluents (102%) indicates that it was formed from the degradation of higher molecular weight siloxanes [191].

In conclusion, this is the first study to report the occurrence and fate of siloxanes in WWTPs. The loading of more than 4.0 g of linear and cyclic siloxanes per day per 1,000 inhabitants and an incomplete removal during the wastewater treatment suggest the significance of WWTPs as a source of these compounds in the aquatic environment. D5 was found at the highest concentrations in treated wastewater and dewatered sludge (1.79 μg L^{-1} and 15.1 mg kg^{-1}, respectively). The contribution of linear siloxanes to total concentrations was high in influents and in sludge (75% and 72%, respectively). However, cyclic compounds accounted for 59% of the total concentrations in effluents. The solid-liquid distribution coefficients (K_d) of siloxanes showed an affinity of siloxanes to particles, which varied depending on the structure of the compound. The removal rate

of cyclic siloxanes in the WWTP varied highly, with small molecules being not removed (D3 and D4) or removed less efficiently (35% for D5). The removal rate of linear siloxanes was between 69.0 and 93.4%, and the major removal mechanism was sorption to sludge.

CHAPTER 4

Wide-scope quantitative target screening of 2327 emerging contaminants in wastewater samples with UPLC-Q-ToF-HRMS/MS

4.1. Introduction

Contamination of water bodies has been the spotlight of scientific community concerning the preservation and sustainability of the environment. Due to the advances of analytical chemistry, a great number of regulated and non-regulated compounds are detected in various environmental samples. Emerging pollutants (EPs) is the term for compounds that are newly released in the environment or have recently been discovered by water quality controls and have yet to be studied. The EPs encompass a diverse group of compounds, for which nowadays many information are available. However, only a small proportion of the chemical compounds have been sufficiently monitored in the water bodies [192].

The determination of organic contaminants in environmental samples constitutes a great challenge, since many matrix components may interfere the analysis and mostly due to the increased number of compounds with various physico-chemical properties. The most common choice for the determination of EPs are the multi-residue methods, including however only a few hundreds of compounds. The development of high resolving power mass analyzers (HRMS) has given a more comprehensive alternative [158]. Wide-scope screening methods can detect all compounds ionized under the selected chromatographic and mass spectrometric conditions. Thus, more complete information on undesirable compounds present in the sample is feasible.

HRMS full scan acquisition technique offers the possibility of retrieving all the information concerning the analytes in post-acquisition approaches. Pre-selection of analytes is no longer necessary, while additionally retrospective analysis can provide the possibility of future evaluation of the sample, concerning other analytes. Accurate

mass measurements allow the annotation of the elemental composition for both precursor and fragment ions, as well as the isotopic information. The HRMS instruments give various possibilities regarding the operating MS/MS mode [158]. In the screening methods, data independent MS/MS mode is more commonly used. In Bruker's bbCID (broad band collision induced dissociation) or Waters MS^E mode, 2 collision energies are applying, low and high, providing MS and MS/MS spectra, respectively, at the same time [104, 122]. Also, data dependent scan mode can provide MS/MS data, only to the most abundant MS ions [148]. In that case, the first scan is defined as the survey scan, in which data are processed "on-the-fly" to determine the candidates of interest on the basis of predefined selection criteria [158]. Additionally, with the use of hybrid HRMS/MS analyzers, high-quality accurate mass MS^n spectra can be acquired [118]. Furthermore, HRMS is continually evolving, giving the possibility for quantitative analysis with constantly increasing sensitivity. Limited linear range, traditionally attributed to QTOF systems because of saturation effects, has been overcome by modern instruments [37].

The main approaches for post-acquisition data evaluation are target, suspect and non-target screening [193]. The main difference between the first approach and the latter ones is the presence of reference standard available. For a comprehensive target analysis, the reference standard is necessary in order to compare the retention time, the MS spectrum profile (precursor ion, adducts, in-source fragments), as well as the MS/MS spectrum (fragment ion and ion ration). In the literature, there are studies for target HRMS screening; More than 400 compounds were screened in water samples with a TOF analyzer [194], and 387 pesticides and pharmaceuticals were analyzed by a hybrid QTOF MS [119], while screening of 396 polar compounds was carried out by an Orbitrap analyzer [140]. The benefit of HRMS for post-acquisition evaluation of the data can pose some confusion in the terms target and suspect screening. Target screening involves identification through reference standard and in suspect screening tentative identification is made on the basis of the information provided by the technique [195]. However, there are cases in the literature that target and suspect screening are overlapped, where there is a big screening database, but the retention time is not always available. In these

workflows, once a compound has achieved some criteria set, most commonly the reference standard is purchased for its confirmation [104, 122, 147, 148]. In another study, a list of 635 chemicals with accurate mass and retention time is used for the analysis of groundwaters [196]. In this case, retention time is available for all the compounds, but a part of this list contains unidentified compound by previous analyses of the group.

A qualitative target screening can be easily performed using a customized database (with information of the retention time, molecular formula and fragmentation pattern). Due to the large size of the data acquired, sophisticated software are required to provide automated solutions in order to reduce the data evaluation time and number of false negative and false positive findings. Furthermore, according to Commission Decision 2002/657/EC and SANCO 12571/2013 confirmatory criteria were set for the identification of the analytes, in an identification-point system, which is however more low-resolution oriented. Thus, it is a clear need to set defined criteria and harmonized guidance for HRMS based identification in order to ensure reliable confirmation of the analytes [197, 198].

A quantitative target screening, however, requires greater effort. With respect to the instrumental performance of the HRMS mass analyzers and the nature of post-acquisition analysis, validation protocols and therefore quantitation are not feasible in the same way as in low resolution MS (LRMS) methods, applying to a specific number of compounds. In HRMS target screening, the list of compounds can be few thousands and this number can be continuously growing. In most of the cases, the method is evaluated only for some analytes and not for the whole list [104, 199]. Moreover, the fact that full scan acquisition mode can provide more MS/MS transitions and prevent false positive results can also contribute to the need for a HRMS oriented performance criteria. A remarkable issue in validation of HRMS methods is not only the lack of a uniform protocol to follow, but also a lack for the calculation of performance criteria [105, 200]. Recovery values are presented in the majority of studies with quantitative results [119], while screening detection limit (SDL) and limit of identification (LOI) are investigated as the main validation

parameters to estimate the threshold concentration at which detection and identification become reliable, respectively [201].

4.2. Scope of the study

The aim of this study is a comprehensive quantitative target screening of emerging pollutants in environmental samples, which involves a generic sample preparation, a UPLC-QTOF-MS/MS method and post-acquisition evaluation of the data. An in-house database was built with information of retention time, MS and MS/MS ions for 2327 compounds, including pesticides, pharmaceuticals, drugs of abuse, industrial chemicals, doping compounds, as well as some metabolites and transformation products. Optimization was performed in order to minimize false negative results and a validation protocol is proposed in order to evaluate the performance criteria of the HRMS method. The method was applied in an influent and an effluent wastewater from a wastewater treatment plant of Greece, allowing the detection and identification of 371 organic contaminants. The samples were evaluated with sophisticated software, identification points were attributed to each analyte and quantitation was also carried out.

4.3. Experimental part

4.3.1. Chemicals and Reagents

Information on the standards used for this work is provided in the Electronic Supplementary Material (Table S4.1).

All the solvents used were UPLC-MS grade. Acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany), whereas 2-propanol of LC-MS grade was from Fisher Scientific (Geel, Belgium). Distilled water was provided by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). Sodium hydroxide monohydrate (NaOH) for trace analysis ≥99.9995% and formic acid 99% were purchased from Fluka (Buchs, Switzerland).

For the sample preparation, the empty solid phase extraction polypropylene tubes (6 mL), as well as the cartridge sorbent materials Sepra ZT (Strata-X), Sepra ZT-WCX (Strata-X-CW) and ZT-WAX (Strata-X-AW) were obtained from Phenomenex (Torrance, USA). The Isolute ENV+ sorbent material and the frits (20 μm, 6 mL) were from Biotage (Ystrad Mynach, UK). Glass fiber filters (GFF, pore size 0.7 μm) used in wastewater filtration were obtained from Millipore (Cork, Ireland). Regenerated cellulose syringe filters (RC) of 15 mm diameter and 0.2 μm pore size were obtained from Phenomenex (Torrance, CA, USA).

4.3.2. Sampling & Sample Preparation

Influent and effluent wastewater samples (24-hour composite flow proportional samples) were collected from the WWTP of Athens (Greece), on the 15th of March 2014 (Saturday). Wastewater was collected in pre-cleaned high-density polyethylene (HDPE) bottles. Untreated and treated wastewaters were filtered with glass fiber filters (pore size 0.7 μm) immediately after arrival at the laboratory. Samples were stored in the dark at 4 °C until analysis.

The WWTP of Athens is designed with primary sedimentation, activated sludge process with biological nitrogen and phosphorus removal and secondary sedimentation. The estimated sewage flow for the collected samples is 720,000 m^3 day^{-1}. The closest connected household is 0.5 km and the most remote 30 km from the WWTP. The residential population connected to the WWTP based on official census, excluding commuters, is 3,700,000 and the number of people estimated based on the number of house connections is 4,562,500. The WWTP is designed to serve a population equivalent of 5,200,000 and thus is by far the largest in Greece and one of the largest in the world.

Sample extraction was carried out using a slight variation of the protocol developed by Kern et al. [73]. Sample aliquots of 100 mL were adjusted to pH 6.5, and then spiked with an internal standard mix. Solid phase extraction (SPE) was conducted using four different SPE materials simultaneously in an in-house cartridge to achieve sufficient enrichment for a very broad range of compounds (200 mg Oasis HLB, 150

mg Isolute ENV+, 100 mg Strata-X-AW and 100 mg Strata-X-CV). The cartridges were preconditioned with methanol and water and the water samples were loaded, then there was a drying step under vacuum. The elution was conducted with 4 mL of methanol/ethyl acetate (v:v 50:50) containing 2% ammonia, followed by 2 mL of methanol/ethyl acetate (v:v 50:50) containing 1.7% formic acid. Extracts were evaporated under a gentle nitrogen stream to a volume of 100 µL and then reconstituted to 0.5 mL with a final proportion of MeOH/water (v:v 1:1). Finally, the extracts were filtered through a 0.2 µm regenerated cellulose (RC) filters and were ready for injection in the chromatographic system.

4.3.3. Instrumental analysis by UPLC- Q-TOF-MS/MS

An ultrahigh-performance liquid chromatography (UHPLC) system, with a HPG-3400 pump (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany), interfaced to a QTOF mass spectrometer (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used for the screening analysis.

The chromatographic separation was performed on an Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 µm) from Thermo Fisher Scientific (Dreieich, Germany) preceded by a guard column of the same packaging material, ACQUITY UPLC BEH C18 1.7 µm, VanGuard Pre-Column, Waters (Ireland), thermostated at 30 ˚C. Mobile phase composition in Positive Ionization mode (+ESI) is (A) H_2O:MeOH (90:10) with 5 mM ammonium formate and 0.01% formic acid and (B) MeOH with 5 mM ammonium formate and 0.01% formic acid. For Negative Ionization mode (-ESI), the mobile phase is (A) H_2O:MeOH (90:10) with 5 mM ammonium acetate and (B) MeOH with 5 mM ammonium acetate.

The gradient elution program was the same for both ionization modes and applied changes in the solvent and in the flow rate. The chromatogram lasts 16 min. with 4 min of re-equilibration of the column for the next injection (Table 4.1). The injection volume was set to 5 µL.

Table 4.1 Gradient elution program of the method.

Time (min)	Flow rate (mL/min)	%A	%B
0	0.2	99	1
0.1		99	1
1	0.2		
3		61	39
14	0.4	0.1	99.9
16	0.48	0.1	99.9
16.1	0.48	99	1
19	0.48	99	1
19.1	0.2	99	1
20	0.2	99	1

The operating parameters of the electrospray ionization interface (ESI) in positive mode are: capillary voltage, 2500 V; end plate offset, 500 V; nebulizer, 2 bar; drying gas, 8 L min^{-1}; dry temperature, 200 °C; and for negative mode: capillary voltage, 3500 V; end plate offset, 500 V; nebulizer, 2 bar; drying gas, 8 L min^{-1}; dry temperature, 200 °C.

The QTOF MS system operates in broadband collision- induced dissociation (bbCID) acquisition mode and records spectra over the range m/z 50−1000, with a scan rate of 2 Hz. The Bruker bbCID mode provides MS and MS/MS spectra at the same time, while it works at two different collision energies. At low collision energy (4 eV), MS spectra were acquired and at high collision energy (25 eV), fragmentation is taking place at the collision cell resulting in MS/MS spectra.

A QTOF external calibration was daily performed with a sodium formate solution, and a segment (0.1-0.25 min) in every chromatogram was used for internal calibration, using a calibrant injection at the beginning of each run. The sodium formate calibration mixture consists of 10 mM sodium formate in a mixture of water/isopropanol (1:1). The theoretical exact masses of calibration ions with formulas $Na(NaCOOH)_{1-14}$ in the range of 50−1000 Da were used for calibration. The instrument provided a typical resolving power of 36,000−40,000 during calibration (39,274 at m/z 226.1593, 36,923 at m/z 430.9137, and 36,274 at m/z 702.8636). Mass spectra acquisition and data analysis was processed with DataAnalysis 4.1 and TargetAnalysis 1.3 and TASQ (Bruker Daltonics, Bremen, Germany).

4.4. Evaluation of the target screening results

4.4.1. Validation

4.4.1.1. Selection of analytes

A lack in the validation of screening methods is the fact that there are no clear guidelines for wide-scope methods with a high number of compounds. In such cases, it is practically infeasible to evaluate the performance criteria for all the compounds in the database. In most studies, a certain number of the database is selected as a validation set, without a reasonable pre-selection of the compounds. In this study, the validation dataset is chosen upon some rules that would guarantee its representativeness.

4.4.1.2. Optimization False Negative –False Positive results

Before performing the validation of the data, it is important to set the criteria and thresholds for the retention time tolerance, mass accuracy error and isotopic fit score. It is crucial to decide over the right values in order not to omit through the procedure truly present analytes, false negative results and, at the same time, be stringent enough to avoid a great number of false positive findings. It is obvious that in order to

increase the identification success rate, the false negative results should be as low as possible.

The aim is to investigate to which extent the peak area and intensity thresholds, the mass accuracy error, the isotopic fitting score and the retention time tolerance could be narrowed assuring a false negative rate of 5 %, or respectively a successful identification rate of 95% (the % number of compounds that were present in the sample and were identified) and at the same time minimizing the false positive findings.

Different experiments were conducted with standard solutions and spiked samples at different concentrations. The evaluation was carried out in Bruker's software TargetAnalysis, by changing the parameters for searching and scoring algorithms. First, the retention time, mass accuracy and isotopic fit thresholds were optimized in a standard solution of high concentration (100 μg/L), so that all the compounds were detected after target screening procedure, namely the successful identification rate would be 100%. Then the thresholds of peak area and intensity were optimized using different concentration's standard solutions, with purpose that successful identification rate over 95% would be achieved at the lower level (5 μg/L). The optimized parameters were also checked in spiked samples and then applied to the validation experiments and real samples.

4.4.2. Identification and Confirmation of analytes

HRMS is an excellent technique for confirmatory purposes and it constitutes a great diagnostic tool. The identification of analytes regarding accurate mass is facilitating, since mass error goes down to the ppm levels. However, there are certain specific criteria that should be met in order to confirm the presence of a compound in the sample [197].

The requirements for mass spectrometry should be according to the resolving power of the analyzer. In Decision 2002/657/EC there is an identification point system, referring also to HRMS instruments, with resolution higher than 10,000, which is

however quite obsolete nowadays. According to this, 4 identification points (IPs) are necessary for confirmation of an analyte. In HRMS, a precursor ion earns 2 IPs and a fragment ion 2.5 IPs.

The lack of confirmation requirements in environmental chemistry was first formulated in 2004 by Hernandez et al. [202]. In the Decision 2002/657/EC, IPs are attributed according to resolving power, whereas Hernandez has introduced the use of exact mass as a confirmatory tool.

Based on this Decision, a more detailed system is proposed in order to take full advantage of the capabilities of HRMS instruments (Table 4.2). Retention time and precursor ion (most abundant ion in full scan MS) earn together 2 IPs. Mass accuracy tolerance, expressed either in ppm or mDa, depends on the resolution, the type of the analyzer (TOF or Orbitrap) and the m/z. Tolerance for mass accuracy should be set after an optimization of false positive (FP) and false negative (FN) results. Isotopic fitting do certainly contain diagnostic information and earns 0.5 IP. It is a measure of the correlation between the theoretical and measured isotopic patterns of the peak. The standard deviation of the masses and intensities for all isotopic peaks provides a confidence score for the presence of the analyte. Many vendors provide a score for the isotopic fit in their software. Furthermore, fragment ions in MS/MS mode or in-source fragment ions in full scan MS earn 2.5 IPs. The ion ratio is mandatory requirement both in Decision 2002/657/EC and in Document SANCO 12571/2013, but it is not widely applied in HRMS field. Tolerance for the ion ratio is set at a universal value of ±30%, as proposed to Document SANCO 12571/2013 and also after the detailed study of Mol et al. [203]. Moreover, multiple MS or MS/MS ions are available and there is no reference on which ions to select to calculate the ion ratio. Our proposal is to follow a common line and calculate the ion ratio by dividing the response of the fragment ion (preferably from MS/MS mode) with the precursor ion (in MS mode).

Table 4.2. Proposed Identification Point system in HRMS analysis.

Requirements	Identification Points earned
Retention time + Precursor ion (mass accuracy)	2.0
Isotopic fitting (abundances and accuracy of M+1, M+2,…)	0.5
Fragment ions (mass accuracy)	2.5

4.4.2.1. Screening

The aim of a screening method is to detect and identify the presence of contaminants in the sample. The main parameters that should be studied are the minimum concentration at which identification and detection can be achieved, at a certain confidence level (usually 95%).

Two validation parameters that have been widely used are the screening detection limit (SDL) and limit of identification (LOI), which is a measure to estimate the threshold concentration at which detection and identification become reliable, respectively. The SDL is established as the lowest concentration level tested for which a compound is detected in all spiked samples, at the expected retention time and with specific mass accuracy error of the precursor ion. The LOI is established as the lowest concentration tested for which a compound is satisfactorily identified.

In our study, estimation of the SDL and LOI was performed. SDL was established as the concentration level at which the thresholds of (i) retention time and (ii) mass accuracy of the precursor ion were satisfied, while for LOI the thresholds of (i) retention time and mass accuracy of (ii) the precursor ion and (iii) fragment ion. According to the proposed IPs system, for SDL at least 2 IPs are required and for LOI at least 4 IPs.

Additionally, decision limit (CCα) and detection capability (CCβ) values were calculated from the standard addition calibration curve, according to the equations:

$\boldsymbol{CC\alpha = \frac{a}{b} + 2.33\frac{Sa}{b}}$ (at 99% confidence level) and $\boldsymbol{CC\beta = CC\alpha + 1.64\frac{Sa}{b}}$ (at 95% confidence level).

Verification of the CCβ concentration was afterwards performed through spiked samples.

4.4.2.2. Performance criteria

To further evaluate the analytical features of the method, linearity, accuracy and precision and matrix effect were evaluated. Linearity was studied in standard solutions and in spiked effluent samples. Linear dynamic range was evaluated in the range of 2.5 -1000 ng/L and regression coefficient was calculated. Method recovery was calculated by dividing the peak area of the spiked sample by the matrix-matched standard solution at 4 concentration levels. Respectively, matrix factor was estimated by dividing the peak area of matrix-matched standard solution by the peak area of the standard solution. Matrix effect is calculated by the equation: ME=(1-MF)×100. Finally, precision was calculated in terms of repeatability and presented in % RSD.

4.5. Results and Discussion

4.5.1. Selection of analytes

A selection of a representative set of compounds was performed for the optimization and validation of the screening method. The number of compounds was set at around 10% of the total database, thus the validation set contains 195 compounds, presented in Table 4.3. Moreover, these compounds were selected in order to represent all the classes of compounds in the database, like pesticides, pharmaceuticals from different categories, illicit drugs, industrial chemicals and transformation products. Another important aspect that was taken into account is the physicochemical properties of the compounds, in terms of retention time. In Figure

4.1, a distribution of retention time of the compounds of the validation dataset and the total database is presenting. In positive ionization, compounds eluted all over the chromatogram were chosen in the database, with the first compound of the data set eluted at t_R: 1.4 min. and the last at t_R: 12.4 min. The average retention time of the dataset is 7.1 ± 2.8 min, which is comparable to the values of the overall database, t_R: 7.6 ± 3.0 min. The same applied in negative ionization, with first compound eluted at t_R: 1.3 min. and the last at t_R: 13.7 min. and the average t_R: 7.8 ± 2.9, comparing to t_R: 7.7 ± 3.0 min of the overall database. The last aspect that is worth mentioning is the number of compounds in positive and in negative ionization mode. 25% of the compounds of the whole database are ionized in negative mode, that percentage in the validation data set reaches 30%, proving also the representativeness of the dataset.

Table 4.3 Compounds of the validation dataset.

	Compound name	CAS number	Molecular Formula	ESI mode	t_R	Fragm. 1	Fragm. 2	Fragm. 3
1	1-OH-Benzotriazole	2592-95-2	$C_6H_5N_3O$	(+)	3.88	91.0415	119.0478	
2	2,4-Methylenedioxy-amphetamine (MDA)	4764-17-4	$C_{10}H_{13}NO_2$	(+)	4.19	105.0699	163.0754	135.0441
3	2,4-Methylenedioxy-N-ethylamphetamine (MDEA)	82801-81-8	$C_{12}H_{17}NO_2$	(+)	4.39	163.0754	135.0441	133.0648
4	2,4-Methylenedioxy-N-methylamphetamine (MDMA)	42542-10-9	$C_{11}H_{15}NO_2$	(+)	4.18	105.0699	163.0754	135.0441
5	2-Amino-Benzothiazole	136-95-8	$C_7H_6N_2S$	(+)	5.84	124.0215	118.0525	92.0495
6	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	30223-73-5	$C_{20}H_{23}N$	(+)	6.38	249.1512	234.1277	201.1512
7	2-OH-Benzothiazole	934-34-9	C_7H_5NOS	(+)	6.53	124.0215	63.0229	90.0338
8	5-Me-Benzotriazole	136-85-6	$C_7H_7N_3$	(+)	5.83	79.0542	95.0478	105.0447
9	6-Monoacetylmorphine (6-MAM)	2784-73-8	$C_{19}H_{21}NO_4$	(+)	3.75	211.0754	268.1332	193.0648
10	7-Amino-flunitrazepam	34084-50-9	$C_{16}H_{14}FN_3O$	(+)	5.41	135.0928	256.1245	227.0979

11	8-OH-Mirtazapine	not available	$C_{17}H_{19}N_3O$	(+)	4.54	211.0866	72.0808	
12	9-OH-Risperidone	144598-75-4	$C_{23}H_{27}FN_4O_3$	(+)	5.38	207.1128		
13	Acesulfame	33665-90-6	$C_4H_5NO_4S$	(-)	2.34	82.0298	77.9655	
14	Acetamiprid	135410-20-7	$C_{10}H_{11}ClN_4$	(+)	5.17	126.0105	56.0495	90.0338
15	Albendazole	54965-21-8	$C_{12}H_{15}N_3O_2S$	(+)	9.19	234.0696	191.0148	159.0427
16	Albendazole sulfone	75184-71-3	$C_{12}H_{15}N_3O_4S$	(+)	5.86	266.0594	224.0124	
17	Alprazolam	28981-97-7	$C_{17}H_{13}ClN_4$	(+)	8.36	281.0714	274.1213	251.0371
18	Amitriptyline	50-48-6	$C_{20}H_{23}N$	(+)	8.23	233.1325	91.0452	105.0699
19	Amphetamine	300-62-9	$C_9H_{13}N$	(+)	4.19	119.0855	91.0542	65.0386
20	Arprinocid	55779-18-5	$C_{12}H_9ClFN_5$	(+)	6.7	146.0058		
21	Atenolol	29122-68-7	$C_{14}H_{22}N_2O_3$	(+)	3.09	190.0863	225.1234	145.0648
22	Atorvastatin	134523-00-5	$C_{33}H_{35}FN_2O_5$	(+)	9.96	440.2232	466.2024	
23	Atrazine	1912-24-9	$C_8H_{14}ClN_5$	(+)	8.16	174.0541	104.001	132.0323
24	Atrazine-desethyl	6190-65-4	$C_6H_{10}ClN_5$	(+)	5.73	146.0228	104.001	110.0461
25	Azithromycin	83905-01-5	$C_{38}H_{72}N_2O_{12}$	(+)	5.98	591.4215	83.04914	158.1176
26	Benzotriazole (BTR)	95-14-7	$C_6H_5N_3$	(+)	4.76	65.0386	92.0495	
27	Benzoylecgonine (BECG)	519-09-5	$C_{16}H_{19}NO_4$	(+)	4.98	168.1019	105.0335	272.1281
28	Bromazepam	1812-30-2	$C_{14}H_{10}BrN_3O$	(+)	7.28	288.0131	182.0838	209.0947
29	Bromohexine	3572-43-8	$C_{14}H_{20}Br_2N_2$	(+)	9.25	261.8861	114.1277	
30	Caffeine	58-08-2	$C_8H_{10}N_4O_2$	(+)	4.23	138.0662	110.0713	83.0604
31	Cannabidiol	13956-29-1	$C_{21}H_{30}O_2$	(-)	13.69	245.1547	179.1078	
32	Carbamazepine	298-46-4	$C_{15}H_{12}N_2O$	(+)	7.36	194.0964		
33	Carbaryl	63-25-2	$C_{12}H_{11}NO_2$	(+)	7.46	145.0648	117.0699	127.0542
34	Carprofen	53716-49-7	$C_{15}H_{12}ClNO_2$	(-)	8.94	228.0586	226.0429	
35	Cetirizine	83881-51-0	$C_{21}H_{25}N_2O_3Cl$	(+)	8.79	201.0466	166.0777	165.0699
36	Chloramphenicol	56-75-7	$C_{11}H_{12}Cl_2N_2O_5$	(-)	5.74	257.0335	152.0358	176.0358
37	Chlordiazepoxide	94-97-3	$C_{16}H_{14}ClN_3O$	(+)	8.68	282.0793	227.0496	241.0527
38	Chloro-benzotriazole	58-25-3	$C_6H_4ClN_3$	(+)	6.53	98.9996	126.0105	72.984
39	Chlorpromazine	50-53-3	$C_{17}H_{19}ClN_2S$	(+)	8.89	246.0136		
40	Chlorthalidone	77-36-1	$C_{14}H_{11}ClN_2O_4S$	(-)	5.14	189.9735	146.0248	318.9950
41	Cimetidine	51481-61-9	$C_{10}H_{16}N_6S$	(+)	3.24	159.0699	117.0481	95.0604
42	Citalopram	59729-33-8	$C_{20}H_{21}FN_2O$	(+)	6.59	262.1028	109.0454	
43	Clarithromycin	81103-11-9	$C_{38}H_{69}NO_{13}$	(+)	9.18	590.3899	158.1176	

44	Clazuril	101831-36-1	$C_{17}H_{10}Cl_2N_4O_2$	(-)	9.42	300.0101	265.0174	
45	Clobazam	22316-47-8	$C_{16}H_{13}ClN_2O_2$	(+)	8.08	259.0633	224.0944	
46	Clofibric acid	882-09-7	$C_{10}H_{11}ClO_3$	(-)	6.54	126.9956	85.0295	
47	Clomipramine	303-49-1	$C_{19}H_{23}ClN_2$	(+)	9.21	270.1044	242.0731	235.1356
48	Clopidol	2971-90-6	$C_7H_7Cl_2NO$	(+)	4.2	101.0153	86.9996	157.0289
49	Closantel	145149-50-4	$C_{22}H_{14}Cl_2I_2N_2O_2$	(-)	12.16	no fragmentation		
50	Clozapine	5786-21-0	$C_{18}H_{19}ClN_4$	(+)	7.19	296.0949	270.0793	244.0636
51	Cocaine (COC)	50-36-2	$C_{17}H_{21}NO_4$	(+)	4.84	182.1176	82.0651	150.0913
52	Codeine (COD)	76-57-3	$C_{18}H_{21}NO_3$	(+)	3.4	215.1067	243.1016	282.1489
53	Colchicine	64-86-8	$C_{22}H_{25}NO_6$	(+)	6.43	358.1649	341.1384	382.1649
54	Coumaphos	56-72-4	$C_{14}H_{16}ClO_5PS$	(+)	11.19	306.9591	334.9904	226.9923
55	Cyclamate	139-05-9	$C_6H_{13}NO_3S$	(-)	3.93	79.9574		
56	Dapsone	80-08-0	$C_{12}H_{12}N_2O_2S$	(+)	4.13	156.0114	108.0444	92.0495
57	Decoquinate	18507-89-6	$C_{24}H_{35}NO_5$	(+)	13.43	no fragmentation		
58	Diaveridine	5355-16-8	$C_{13}H_{16}N_4O_2$	(+)	3.88	245.1033	123.0665	217.1084
59	Diazepam	439-14-5	$C_{16}H_{13}ClN_2O$	(+)	9.53	257.084	154.0418	222.1151
60	Diclazuril	10320-42-0	$C_{17}H_9Cl_3N_4O_2$	(-)	10.31	333.9711	335.9672	
61	Diclofenac	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	(-)	9.22	214.0429	250.0185	130.978
				(+)	10.18	215.0496	250.0185	
62	Dimethoate	60-51-5	$C_5H_{12}NO_3PS_2$	(+)	5.23	198.9647	170.9698	124.9821
63	Dimetridazole	551-92-8	$C_5H_7N_3O_2$	(+)	4.27	95.0604	81.0447	
64	Dipyrone	50567-35-6	$C_{13}H_{17}N_3O_4S$	(-)	4.89	191.0496	175.0183	
65	Diuron	330-54-1	$C_9H_{10}Cl_2N_2O$	(+)	8.64	72.04439	105.034	
				(-)	8.51	185.9519	149.9752	159.9726
66	Doxepine	1668-19-5	$C_{19}H_{21}NO$	(+)	7.01	220.0883	235.1117	107.0491
67	Ecgonine methyl ester (EME)	7143-09-1	$C_{10}H_{17}NO_3$	(+)	1.38	82.0651	182.1176	154.0863
68	Ephedrine	299-42-3	$C_{10}H_{15}NO$	(+)	3.76	148.1121	117.0699	133.0886
69	Ethopabate	59-06-3	$C_{12}H_{15}NO_4$	(+)	6.63	206.0812	164.0706	136.0393
70	Fenbendazole	43210-67-9	$C_{15}H_{13}N_3O_2S$	(+)	10.26	268.0539	159.0427	
71	Fenoxycarb	79127-80-3	$C_{17}H_{19}NO_4$	(+)	10.59	88.03931	256.0968	116.0706
72	Fentanyl	437-38-7	$C_{22}H_{28}N_2O$	(+)	6.04	188.1434	105.0699	216.1383
73	Florfenicol	73231-34-2	$C_{12}H_{14}Cl_2FNO_4S$	(-)	4.68	185.0278	335.987	151.9675
				(+)	4.73	339.9972	241.0062	

74	Flubendazole	31430-15-6	$C_{16}H_{12}FN_3O_3$	(+)	8.65	282.0673	229.0772	
75	Fludioxonil	131341-86-1	$C_{12}H_6F_2N_2O_2$	(-)	9.43	126.0349	180.0329	181.0407
				(+)	9.48	158.0401	155.0504	185.051
76	Flunitrazepam	1622-62-4	$C_{16}H_{12}FN_3O_3$	(+)	7.83	300.0905	268.1006	286.0986
77	Flunixin	38677-85-9	$C_{14}H_{11}F_3N_2O_2$	(-)	8.34	251.0802	231.0751	211.0688
78	Fluoxetine	54910-89-3	$C_{17}H_{18}F_3NO$	(+)	8.6	148.1121		
79	Flurazepam	17617-23-1	$C_{21}H_{23}ClFN_3O$	(+)	6.61	315.0695	100.1121	288.0586
80	Furosemide	54-31-9	$C_{12}H_{11}ClN_2O_5S$	(-)	5.08	285.0106	204.9844	
81	Gemfibrozil	25812-30-0	$C_{15}H_{22}O_3$	(-)	10.86	121.0659		
				(+)	12.91	129.091		
82	Hydrochlorthiazide	58-93-5	$C_7H_8ClN_3O_4S_2$	(-)	3.36	268.9463		
83	Ibuprofen	15687-27-1	$C_{13}H_{18}O_2$	(-)	9.01	159.1179		
84	Imidacloprid	138261-41-3	$C_9H_{10}ClN_5O_2$	(+)	4.75	175.0978	209.0589	84.0808
85	Imipramine	50-49-7	$C_{19}H_{24}N_2$	(+)	8.08	236.1434	208.1121	86.0964
86	Indapamide	26807-65-8	$C_{16}H_{16}ClN_3O_3S$	(-)	6.78	188.9657	132.0329	232.9793
				(+)	6.61	132.0808	117.0573	
87	Iprodione	36734-19-7	$C_{13}H_{13}Cl_2N_3O_3$	(-)	10.42	141.067		
88	Irbesartan	138402-11-6	$C_{25}H_{28}N_6O$	(+)	9.44	207.0917	195.1492	386.2227
89	Irgarol	28159-98-0	$C_{11}H_{19}N_5S$	(+)	10.49	198.0808	125.0822	91.0324
90	Ketamine	6740-88-1	$C_{13}H_{16}ClNO$	(+)	4.61	125.0153	179.0622	207.0571
91	Ketoprofen	22071-15-4	$C_{16}H_{14}O_3$	(+)	8.53	105.0335	209.0961	
				(-)	6.77	169.0659	209.0972	197.0608
92	Lamotrigine	84057-84-1	$C_9H_7Cl_2N_5$	(+)	5.31	210.9824	166.0292	186.9824
93	Levamisol	14769-73-4	$C_{11}H_{12}N_2S$	(+)	3.68	178.0685	146.0964	123.0263
94	Levetiracetam	102767-28-2	$C_8H_{14}N_2O_2$	(+)	3.74	154.0863	126.0913	69.0335
95	Lidocaine	137-58-6	$C_{14}H_{22}N_2O$	(+)	4.54	86.0964		
96	lincomycin	154-21-2	$C_{18}H_{34}N_2O_6S$	(+)	4.08	359.2177	317.2071	126.1277
97	Lorazepam	846-49-1	$C_{15}H_{10}Cl_2N_2O_2$	(+)	8.36	275.0137	229.0527	303.0086
98	LSD	50-37-3	$C_{20}H_{25}N_3O$	(+)	5.48	223.123	281.1648	197.1073
99	Lufenuron	103055-07-8	$C_{17}H_8Cl_2F_8N_2O_3$	(+)	12.48	158.0412		
				(-)	12.35	488.9649	325.958	338.9732
100	Mebendazole	31431-39-7	$C_{16}H_{13}N_3O_3$	(+)	8.25	264.0768		
101	Mefenamic acid	61-68-7	$C_{15}H_{15}NO_2$	(+)	11.6	224.107	209.0835	

				(-)	9.21	196.1132	91.0189	180.0819
102	Meloxicam	71125-38-7	$C_{14}H_{13}N_3O_4S_2$	(-)	6.41	146.0611	113.0179	192.0125
				(+)	7.09	115.0324	141.0117	
103	Metformin	657-24-9	$C_4H_{11}N_5$	(+)	1.38	71.0604	60.057	85.0509
104	Methacrifos	62610-77-9	$C_7H_{13}O_5PS$	(+)	8.16	124.9821	209.0032	181.0083
105	Methadone (METH)	76-99-3	$C_{21}H_{27}NO$	(+)	8.04	265.1587	105.0335	223.1117
106	Methamphetamine (MA)	537-46-2	$C_{10}H_{15}N$	(+)	4.21	91.0542		
107	Metoprolol	37350-58-6	$C_{15}H_{25}NO_3$	(+)	4.93	191.1067	98.0964	116.107
108	Metronidazole	443-48-1	$C_6H_9N_3O_3$	(+)	3.58	128.0455	111.0441	82.0525
109	Midazolam	59467-70-8	$C_{18}H_{13}ClFN_3$	(+)	8.63	291.1166	244.0324	
110	Mirtazapine	61337-67-5	$C_{17}H_{19}N_3$	(+)	5.29	195.0917	209.1073	235.123
111	Monensin	17090-79-8	$C_{36}H_{62}O_{11}$	(+)	13.79	461.3262	421.2949	
112	Morantel	20574-50-9	$C_{12}H_{16}N_2S$	(+)	4.36	111.0263	123.0263	164.0528
113	Morphine (MOR)	57-27-2	$C_{17}H_{19}NO_3$	(+)	2.54	229.0859	201.091	185.0597
114	Naproxen	22204-53-1	$C_{14}H_{14}O_3$	(-)	6.74	185.0972		
				(+)	8.99	185.0961	170.0726	
115	Niflumic acid	4394-00-7	$C_{13}H_9F_3N_2O_2$	(-)	8.34	237.0645	217.0583	197.0521
				(+)	9.86	265.0583	245.0521	
116	Nitrazepam	146-22-5	$C_{15}H_{11}N_3O_3$	(+)	7.79	268.0842	236.0944	150.0447
117	Nitroxinil	1689-89-0	$C_7H_3IN_2O_3$	(-)	6.79	162.0071	126.9046	
118	Norclozapine	6104-71-8	$C_{17}H_{17}ClN_4$	(+)	7.29	296.0949	270.0793	244.0636
119	Nordiazepam	1088-11-5	$C_{15}H_{11}ClN_2O$	(+)	9.23	243.0684	208.0995	140.0242
120	Norephedrine	14838-15-4	$C_9H_{13}NO$	(+)	3.54	134.0964	117.0699	115.0542
121	Norfentanyl	1609-66-1	$C_{14}H_{20}N_2O$	(+)	4.68	150.0913	177.1386	94.0651
122	Norfluoxetine	130194-43-3	$C_{16}H_{16}F_3NO$	(+)	8.58	134.0964		
123	Norketamine	35211-10-0	$C_{12}H_{14}ClNO$	(+)	4.69	125.0153	179.0622	207.0571
124	Norsertraline	87857-41-8	$C_{16}H_{15}Cl_2N$	(+)	9.3	275.0389	158.9763	129.0699
125	Nortriptyline	72-69-5	$C_{19}H_{21}N$	(+)	8.51	105.0699	91.0452	233.1325
126	Olanzapine	132539-06-1	$C_{17}H_{20}N_4S$	(+)	4.99	256.0903	84.0808	282.1059
127	Omeprazole	73590-58-6	$C_{17}H_{19}N_3O_3S$	(+)	7.49	198.0583	151.0992	218.0144
128	o-toluenesulfonamide	88-19-7	$C_7H_9NO_2S$	(-)	4.79	no fragmentation		
129	Oxazepam	604-75-1	$C_{15}H_{11}ClN_2O_2$	(+)	8.43	269.05	241.05	
130	Oxfendazole	53716-50-0	$C_{15}H_{13}N_3O_3S$	(+)	6.66	278.0594	191.0689	284.0488

131	Oxyclozanide	2277-92-1	$C_{13}H_6Cl_5NO_3$	(-)	11.21	361.8951	175.9675	201.9468
132	Oxycodone (OC)	76-42-6	$C_{18}H_{21}NO_4$	(+)	3.61	298.1438	256.1332	241.1097
133	Paracetamol	103-90-2	$C_8H_9NO_2$	(+)	3.48	110.06	92.0495	65.0386
134	Paroxetine	61869-08-7	$C_{19}H_{20}FNO_3$	(+)	7.87	192.1183	70.0651	
135	Penconazole	66246-88-6	$C_{13}H_{15}Cl_2N_3$	(+)	10.83	70.04	158.9763	
136	Pentobarbital	5767-32-8	$C_{11}H_{18}N_2O_3$	(-)	7.69	182.1187		
137	PFDeA	335-76-2	$C_{10}F_{19}O_2H$	(-)	6.39	218.9862	468.9702	268.983
138	PFHpA	375-85-9	$C_7F_{13}O_2H$	(-)	8.79	168.9894	118.9926	
139	PFNA	375-95-1	$C_9F_{17}O_2H$	(-)	10.44	418.9734	168.9894	218.9862
140	PFOA	2395-00-8	$C_8HF_{15}O_2$	(-)	9.68	368.9766	168.9894	112.9856
141	Phenobarbital	50-06-6	$C_{12}H_{12}N_2O_3$	(-)	5.88	no fragmentation		
142	Phenytoin	57-41-0	$C_{15}H_{12}N_2O_2$	(-)	8.26	208.0768	146.0248	102.0349
143	Pioglitazone	111025-46-8	$C_{19}H_{20}N_2O_3S$	(+)	9.09	134.0934	86.0694	
144	Primidone	125-33-7	$C_{12}H_{14}N_2O_2$	(+)	5.29	162.0956		
145	Procymidone	32809-16-8	$C_{13}H_{11}Cl_2NO_2$	(-)	9.93	159.9726	95.0502	254.0145
				(+)	10.22	256.029	141.091	127.0754
146	Prometryn (Caparol)	7287-19-6	$C_{10}H_{19}N_5S$	(+)	10.06	200.0964	158.0495	116.0277
147	Propranolol	525-66-6	$C_{16}H_{21}NO_2$	(+)	6.59	183.0804	116.107	157.0648
148	Rafoxanide	22662-39-1	$C_{19}H_{11}Cl_2I_2NO_3$	(-)	12.36	344.8279		
149	Ranitidine	66357-35-5	$C_{13}H_{22}N_4O_3S$	(+)	3.14	176.0488	224.0978	130.0559
150	Rifaximin	80621-81-4	$C_{43}H_{51}N_3O_{11}$	(+)	10.06	754.3334		
151	Risperidone	106266-06-2	$C_{23}H_{27}FN_4O_2$	(+)	5.88	191.1179		
152	Ritonavir	155213-67-5	$C_{37}H_{48}N_6O_5S_2$	(+)	11.11	171.0950	426.1849	268.1478
153	Ronidazole	7681-76-7	$C_6H_8N_4O_4$	(+)	3.55	140.0455	55.0417	
154	Saccharine	6381-61-9	$C_7H_5NO_3S$	(-)	3.09	no fragmentation		
155	Salicylic acid	69-72-7	$C_7H_6O_3$	(-)	3.58	93.0346	65.0397	
156	Sertraline	79617-96-2	$C_{17}H_{17}Cl_2N$	(+)	8.94	275.0389	158.9763	
157	Simvastatin	79902-63-9	$C_{25}H_{38}O_5$	(+)	12.53	199.1481	285.185	225.1638
158	Sucralose	56038-13-2	$C_{12}H_{19}Cl_3O_8$	(-)	4.66	no fragmentation		
159	Sulfachloropyridazine	23282-55-5	$C_{10}H_9ClN_4O_2S$	(+)	4.56	156.0114	108.0444	92.0495
160	Sulfaclozine	102-65-8	$C_{10}H_9ClN_4O_2S$	(+)	5.45	219.0432	130.018	94.0651
161	Sulfadiazine	68-35-9	$C_{10}H_{10}N_4O_2S$	(+)	3.48	156.0114	108.0444	96.0556
162	Sulfadimethoxine	122-11-2	$C_{12}H_{14}N_4O_4S$	(+)	5.6	156.0114	108.0444	92.0495

163	Sulfadimidine	57-68-1	$C_{12}H_{14}N_4O_2S$	(+)	4.31	156.0114	108.0444	124.0869
164	Sulfadoxine	2447-57-6	$C_{12}H_{14}N_4O_4S$	(+)	4.75	156.0114	108.0444	140.0455
165	Sulfamerazine	127-79-7	$C_{11}H_{12}N_4O_2S$	(+)	3.95	156.0114	108.0444	92.0495
166	Sulfamethizole	144-82-1	$C_9H_{10}N_4O_2S_2$	(+)	4.21	156.0114	108.0444	92.0495
167	Sulfamethoxazole	723-46-6	$C_{10}H_{11}N_3O_3S$	(+)	4.6	156.0114	108.0444	92.0495
168	Sulfamethoxypyridazine	80-35-3	$C_{11}H_{12}N_4O_3S$	(+)	4.38	156.0114	108.0444	92.0495
169	Sulfamonomethoxine	1220-83-3	$C_{11}H_{12}N_4O_3S$	(+)	4.75	156.0114	108.0444	92.0495
170	Sulfamoxole	729-99-7	$C_{11}H_{13}N_3O_3S$	(+)	4.11	156.0114	108.0444	92.0495
171	Sulfanilamide	63-74-1	$C_6H_8N_2O_2S$	(+)	2.23	156.0114	108.0444	92.0495
172	Sulfapyridine	144-83-2	$C_{11}H_{11}N_3O_2S$	(+)	3.38	156.0114	108.0444	184.0869
173	Sulfaquinoxaline	59-40-5	$C_{14}H_{12}N_4O_2S$	(+)	5.81	156.0114	108.0444	92.0495
174	Sulfathiazole	72-14-0	$C_9H_9N_3O_2S_2$	(+)	3.63	156.0114	108.0444	92.0495
175	Sulfisoxazole	127-69-5	$C_{11}H_{13}N_3O_3S$	(+)	4.71	156.0114	108.0444	92.0495
176	Terbuthylazine	5915-41-3	$C_9H_{16}ClN_5$	(+)	9.36	174.0541	96.0556	114.1277
177	Ternidazole	1077-93-6	$C_7H_{11}N_3O_3$	(+)	4.21	128.0455	82.0525	56.0369
178	Theophyline	58-55-9	$C_7H_8N_4O_2$	(+)	3.93	124.0505	96.0556	69.0447
179	Thiabendazole	148-79-8	$C_{10}H_7N_3S$	(+)	6.15	175.0324	131.0604	92.0495
180	Thiamphenicol	847-25-6	$C_{12}H_{15}Cl_2NO_5S$	(-)	4.08	290.0259	185.0283	240.0336
181	Thiopental	59709-53-4	$C_{11}H_{18}N_2O_2S$	(-)	8.46	57.9757		
182	Tiamulin	55297-95-5	$C_{28}H_{47}NO_4S$	(+)	7.71	285.2213	192.1053	
183	Tolfenamic acid	13710-19-5	$C_{14}H_{12}ClNO_2$	(-)	10.76	216.0586		
184	Toltrazuril	69004-03-1	$C_{18}H_{14}F_3N_3O_4S$	(-)	10.97	no fragmentation		
185	Topiramate	97240-79-4	$C_{12}H_{21}NO_8S$	(+)	6.08	264.0536	127.039	
186	Tramadol	27203-92-5	$C_{16}H_{25}NO_2$	(+)	4.88	58.0651		
187	Triamterene	396-01-0	$C_{12}H_{11}N_7$	(+)	4.68	237.0883		
188	Triclabendazole	68786-66-3	$C_{14}H_9Cl_3N_2OS$	(+)	11.96	343.9339		
189	Triclosan	3380-34-5	$C_{12}H_7Cl_3O_2$	(-)	12.02	no fragmentation		
190	Trimethoprim	738-70-5	$C_{14}H_{18}N_4O_3$	(+)	4.06	261.0982	230.1176	
191	Valproic acid	99-66-1	$C_8H_{16}O_2$	(-)	7.04	no fragmentation		
192	Valsartan	137862-53-4	$C_{24}H_{29}N_5O_3$	(+)	9.21	291.1492	235.0978	207.0917
193	Vedaprofen	71109-09-6	$C_{19}H_{22}O_2$	(-)	11.24	237.1649	269.1547	225.1285
194	Venlafaxine	93413-69-5	$C_{17}H_{27}NO_2$	(+)	6.14	58.0651	121.0648	215.143
195	Δ9-Tetrahydrocannabinol (THC)	1972-08-03	$C_{21}H_{30}O_2$	(-)	12.42	245.1547	179.1078	107.0502

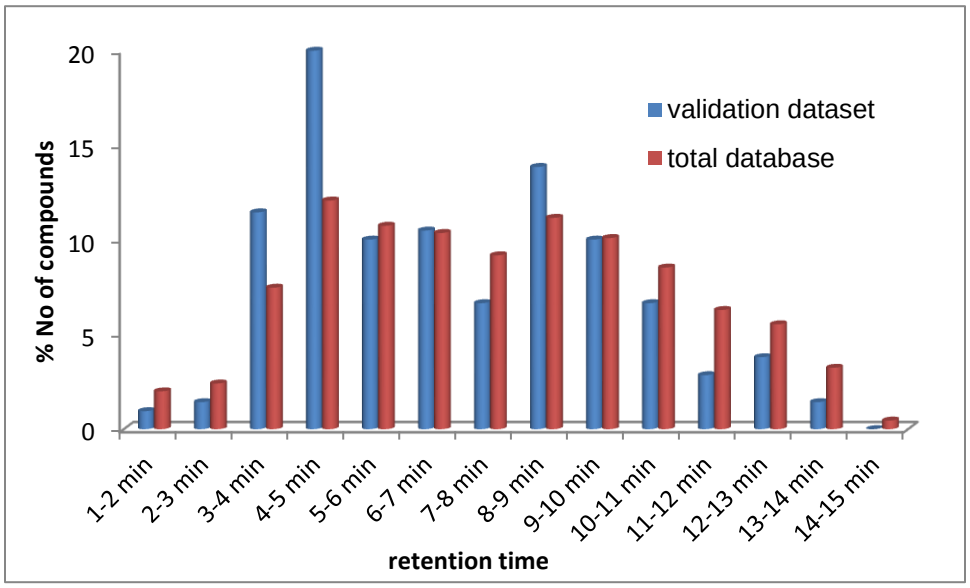


Figure 4.1 Distribution of retention times in the validation dataset and in the total database.

The only reference regarding the validation dataset exists in the guidelines for the validation of screening methods for residues of veterinary medicines (CRLs 2010), which allows validating for at least one analyte which should be selected from each known chemical class or sub-class of a multi-residue method, but nevertheless this is not advisable. It is urgent that more specific criteria should be established for the selection of the validation set, so that it can represent the whole list of compounds screened.

In suspect screening, Moschet et al. evaluated substances with $\log K_{ow}<5$, with ionizable functional groups and from different categories, covering thus a wide range of physicochemical properties and structures [199].

4.5.2. Optimization

A thorough optimization and evaluation was performed using the validation dataset in order to establish the automated filter criteria to maximize the number of target detections, while minimizing the false positive peaks. Peak picking was carried out in TargetAnalysis Bruker's software with an algorithm considering the molecular formula and the retention time, giving as a response a score for the detection of each compound. A narrow and a wide range are set for retention time, mass accuracy and isotopic fit tolerance. The wide range is in fact the values for the criteria set for the identification of a compound. By setting 2 criteria values, we can avoid manual inspection of all the compounds, and gain serious time from the data evaluation. The analytes can obtain "very good", "good" or "poor" scoring rate. When "very good" score is obtained, the compound is within very strict limits and no further evaluation is necessary. When the compound gets a "good" score, it still meets the specified criteria for the screening but manual inspection is recommended, since one parameter out of three may diverge a little. In case of "poor" rate, the compound is beyond the criteria set and is discarded.

Regarding retention time, the wide range was set at 0.4 min, so that retention time shift would not affect the results. The narrow range was tested for 0.05 min and 0.1 min, with the latter giving 14% more "very good" scoring. Mass accuracy was evaluated in terms of mDa or ppm for the absolute and the relative difference between the measured accurate mass and the calculated exact mass of an analyte, respectively. Since the resolution in ToF instruments is smaller for low m/z values, the absolute mass error was preferred, instead of the relative one ($\Delta m/m$). The narrow range was set at 2.5 mDa and the wide at 5 mDa. The isotopic fit is measured in mSigma and lower values indicate a better fit. When the thresholds for the narrow and the wide range were set to 50 and 100 mSigma respectively, 5 compounds were reported as FN. This number decreased to zero, when 100 and 200 mSigma were set as isotopic fit thresholds.

Another parameter applied during the automatic filtering is a smoothing of the chromatograms, with a Gaussian algorithm. When smoothing is applied, one less false negative result is observed.

Summarizing all the above, the criteria set for the identification of a compound are for retention time 0.4 min, for mass accuracy 5 mDa and for isotopic fit 200 mSigma. These parameters were afterwards applied at different concentration standard solutions, in order to set area and intensity thresholds that even at low concentration will give a successful identification rate of more than 95%. At concentrations 100 and 200 μg/L, none false negative result was obtained, while at 5 μg/L, the percentage reached only 2 %. Of course, it is more interesting to observe the behavior at spiked samples. At 1 μg/L and 0.5 μg/L, only 1 and 2 compounds out of 200 was reported as false negative, respectively, giving a successful identification rate of 99.3% and 98.7%. At 0.25 μg/L, 2 compounds were below their SDL and additional 2 out of 151 were reported as FN results (successful identification rate: 98.7%). At 0.05 μg/L, 5 compounds were below their SDL and 3 out of 148 were FN and at 0.025 μg/L, 13 compounds were below their SDL and 5 out of 140 were FN, providing satisfactory successful identification rate of 98% and 96.4%, respectively.

In the literature, the accurate mass scoring parameters are usually picked based on the characteristics of the instrument and the experience of the researcher [116]. Evaluation of the target screening approach was performed in comparison with a low resolution triple quadrupole routine method. Samples were analyzed by LC-HRMS and LR-QqQ-MS/MS and the detected compounds were compared, in terms of relative analyte coverage [204, 205].

In recent studies, in order to evaluate the screening workflows mainly in suspect screening, identification success rate is calculated by means of false negative and false positive results [199, 206, 207]. ‘Artificial suspect’ compounds are spiked in the sample and evaluation of the data reveals the reliability of the method at different concentrations.

The number of false positive results was extensively studied by Mol et al. in order to create an automated target screening method “fit-to-purpose”. By the term false

positives are compounds, that are reported by the software but their presence cannot be confirmed by manual verification or confirmatory analysis are included. These false positive hits obtained during screening in analysis of real samples would unnecessarily trigger follow up for full identification, quantification, and confirmation [203].

4.5.3. Screening – Calculation of CCa/CCβ & SDL/LOI

Screening detection limit (SDL) and limit of identification (LOI) were evaluated for all the compounds in the validation dataset. Starting from the lower level of concentration, 2 IPs were earned when retention time and mass accuracy tolerance were fulfilled, setting this concentration as SDL. And in case of fragment ions, additional 2 IPs were earned, indicating the concentration as LOI. The decision limit (CCα) and the detection capability (CCβ) values of the compounds of the validation dataset, as well as the IPs earned at a close concentration, are presented in Table 4.4.

In every case, at the CCβ concentration, that is calculated statistically, at least 4 IPs are earned, which can confirm the identification and detection of the compound. In nortriptyline and ibuprofen, 2.5 IPs are attributed at CCβ. Nortriptyline has low fragmentation, under the conditions applied, and no clear MS/MS spectrum is available and ibuprofen show very low sensitivity. Moreover, 9 compounds showed no fragmentation at all, under those conditions, especially in negative ionization mode, and no CCβ was available.

Table 4.4 Decision limit (CCα) and detection capability (CCβ) of validation dataset.

	Compound name	CCa (µg/L)	CCβ (µg/L)	IPs (Conc. level close to CCβ)	SDL (µg/L)	LOI (µg/L)
1	1-OH-Benzotriazole	0.11	0.16	4	0.025	0.025
2	2,4-Methylenedioxy-amphetamine (MDA)	0.06	0.09	4	0.025	0.025
3	2,4-Methylenedioxy-N-ethylamphetamine (MDEA)	0.08	0.12	4	0.025	0.025
4	2,4-Methylenedioxy-N-methylamphetamine (MDMA)	0.21	0.25	4	0.025	0.05
5	2-Amino-Benzothiazole	0.08	0.11	4	0.025	0.25
6	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	0.01	0.01	4	0.0025	0.005
7	2-OH-Benzothiazole	0.06	0.10	4	0.025	0.05
8	5-Me-Benzotriazole	0.40	0.43	4	0.025	0.025
9	6-Monoacetylmorphine (6-MAM)	0.16	0.27	4	0.025	0.25
10	7-Amino-flunitrazepam	0.08	0.12	4	0.025	0.05
11	8-OH-Mirtazapine	0.00	0.01	4	0.005	0.025
12	9-OH-Risperidone	0.0019	0.0031	4	0.0025	0.005
13	Acesulfame	high intensity in the sample				
14	Acetamiprid	0.009	0.010	4	0.0025	0.0025
15	Albendazole	0.11	0.15	4	0.025	0.025
16	Albendazole sulfone	0.04	0.06	4	0.025	0.05
17	Alprazolam	0.003	0.004	4	0.0025	0.0025
18	Amitriptyline	0.05	0.08	4	0.025	0.025
19	Amphetamine	0.011	0.014	4	0.0025	0.01
20	Arprinocid	0.029	0.046	4	0.0025	0.05
21	Atenolol	0.47	0.55	4	0.025	0.025
22	Atorvastatin	0.14	0.19	4	0.05	0.05
23	Atrazine	0.09	0.14	4	0.025	0.025
24	Atrazine-desethyl	0.09	0.13	4	0.025	0.025
25	Azithromycin	0.42	0.57	4	0.025	0.025
26	Benzotriazole (BTR)	0.38	0.39	4	0.025	0.025
27	Benzoylecgonine (BECG)	0.019	0.023	4	0.0025	0.0025
28	Bromazepam	0.047	0.075	4	0.025	0.05
29	Bromohexine	0.10	0.15	4	0.025	0.025
30	Caffeine	0.13	0.17	4	0.025	0.025
31	Cannabidiol	0.35	0.44	4	0.25	0.5
32	Carbamazepine	0.40	0.43	4	0.025	0.025
33	Carbaryl	0.08	0.13	4	0.025	0.025
34	Carprofen	0.29	0.45	4	0.25	0.5

35	Cetirizine	0.005	0.008	4	0.0025	0.0025
36	Chloramphenicol	0.01	0.01	4	0.025	0.025
37	Chlordiazepoxide	0.011	0.012	4	0.0025	0.005
38	Chloro-benzotriazole	0.07	0.10	4	0.025	0.05
39	Chlorpromazine	0.10	0.14	4	0.025	0.05
40	Chlorthalidone	0.03	0.04	4	0.0025	0.05
41	Cimetidine	0.008	0.009	4	0.0025	0.01
42	Citalopram	0.28	0.31	4	0.025	0.025
43	Clarithromycin	0.98	1.04	4	0.05	0.025
44	Clazuril	0.20	0.32	4	0.05	0.5
45	Clobazam	0.007	0.008	4	0.0025	0.0025
46	Clofibric acid	0.06	0.09	4	0.025	0.05
47	Clomipramine	0.14	0.18	4	0.025	0.25
48	Clopidol	0.01	0.02	4	0.0025	0.01
49	Closantel	0.25	0.36	2	0.25	n.f.
50	Clozapine	0.04	0.06	4	0.025	0.025
51	Cocaine (COC)	0.006	0.008	4	0.0025	0.0025
52	Codeine (COD)	0.17	0.20	4	0.025	0.05
53	Colchicine	0.004	0.006	4	0.0025	0.01
54	Coumaphos	0.009	0.015	4	0.025	0.025
55	Cyclamate	0.06	0.07	4	0.025	0.025
56	Dapsone	0.020	0.023	4	0.0025	0.025
57	Decoquinate	0.11	0.15	2	0.10	n.f.
58	Diaveridine	0.003	0.004	4	0.0025	0.005
59	Diazepam	0.004	0.006	4	0.0025	0.005
60	Diclazuril	0.09	0.13	4	0.025	0.05
61	Diclofenac	0.51	0.56	4	0.0025	0.0025
62	Dimethoate	0.06	0.09	4	0.025	0.025
63	Dimetridazole	0.07	0.07	4	0.025	0.05
64	Dipyrone	0.52	0.57	4	0.5	0.5
65	Diuron	0.02	0.04	4	0.025	0.025
66	Doxepine	0.006	0.009	4	0.0025	0.01
67	Ecgonine methyl ester (EME)	0.07	0.09	4	0.05	0.25
68	Ephedrine	0.008	0.009	4	0.0025	0.0025
69	Ethopabate	0.003	0.004	4	0.0025	0.005
70	Fenbendazole	0.06	0.08	4	0.025	0.025
71	Fenoxycarb	0.05	0.07	4	0.025	0.025
72	Fentanyl	0.011	0.013	4	0.0025	0.005
73	Florfenicol	0.020	0.033	4	0.01	0.025
74	Flubendazole	0.09	0.14	4	0.025	0.025
75	Fludioxonil	0.02	0.03	4	0.0025	0.025
76	Flunitrazepam	0.006	0.007	4	0.0025	0.01

77	Flunixin	0.01	0.01	4	0.005	0.025
78	Fluoxetine	0.07	0.11	4	0.025	0.25
79	Flurazepam	0.004	0.006	4	0.0025	0.0025
80	Furosemide	0.06	0.08	4	0.0025	0.025
81	Gemfibrozil	0.04	0.07	4	0.025	0.025
82	Hydrochlorthiazide	0.27	0.31	4	0.025	0.025
83	Ibuprofen	0.55	0.90	2	0.025	>1
84	Imidacloprid	0.04	0.05	4	0.025	0.025
85	Imipramine	0.005	0.006	4	0.0025	0.005
86	Indapamide	0.007	0.012	4	0.005	0.025
87	Iprodione	0.08	0.12	4	0.025	0.25
88	Irbesartan	0.16	0.23	4	0.0025	0.1
89	Irgarol	0.013	0.014	4	0.0025	0.0025
90	Ketamine	0.020	0.023	4	0.0025	0.005
91	Ketoprofen	0.015	0.017	4	0.0025	0.025
92	Lamotrigine	0.56	0.59	4	0.025	0.025
93	Levamisol	0.06	0.10	4	0.025	0.025
94	Levetiracetam	0.030	0.035	4	0.0025	0.0025
95	Lidocaine	0.20	0.23	4	0.025	0.025
96	lincomycin	0.017	0.020	4	0.005	0.025
97	Lorazepam	0.13	0.15	4	0.025	0.05
98	LSD	0.07	0.12	4	0.025	0.05
99	Lufenuron	0.37	0.47	4	0.25	0.5
100	Mebendazole	0.019	0.024	4	0.025	0.025
101	Mefenamic acid	0.005	0.007	4	0.0025	0.005
102	Meloxicam	0.04	0.07	4	0.01	0.025
103	Metformin	high intensity in the sample				
104	Methacrifos	0.07	0.11	4	0.025	0.025
105	Methadone (METH)	0.08	0.12	4	0.025	0.025
106	Methamphetamine (MA)	0.002	0.003	4	0.0025	0.0025
107	Metoprolol	0.52	0.60	4	0.025	0.025
108	Metronidazole	0.22	0.22	4	0.0025	0.0025
109	Midazolam	0.004	0.004	4	0.0025	0.005
110	Mirtazapine	0.11	0.15	4	0.025	0.025
111	Monensin	0.08	0.09	4	0.025	0.025
112	Morantel	0.41	0.57	4	0.05	0.25
113	Morphine (MOR)	0.06	0.07	4	0.025	0.025
114	Naproxen	0.08	0.09	4	0.0025	0.0025
115	Niflumic acid	0.20	0.23	4	0.025	0.025
116	Nitrazepam	0.021	0.035	4	0.0025	0.05
117	Nitroxinil	0.007	0.010	4	0.0025	0.025
118	Norclozapine	0.016	0.018	4	0.0025	0.025
119	Nordiazepam	0.005	0.006	4	0.0025	0.005

120	Norephedrine	0.08	0.13	4	0.025	0.025
121	Norfentanyl	0.008	0.011	4	0.0025	0.01
122	Norfluoxetine	0.45	0.65	4	0.5	1
123	Norketamine	0.012	0.015	4	0.0025	0.0025
124	Norsertraline	0.527	0.833	4	0.5	1
125	Nortriptyline	0.004	0.005	2	0.0025	0.05
126	Olanzapine	0.10	0.17	4	0.025	0.05
127	Omeprazole	0.04	0.04	4	0.0025	0.005
128	o-toluenesulfonamide	0.16	0.18	2	0.0025	n.f.
129	Oxazepam	0.10	0.11	4	0.025	0.25
130	Oxfendazole	0.04	0.06	4	0.025	0.05
131	Oxyclozanide	0.10	0.14	4	0.025	0.025
132	Oxycodone (OC)	0.003	0.004	4	0.0025	0.005
133	Paracetamol	0.08	0.11	4	0.025	0.25
134	Paroxetine	0.08	0.11	4	0.025	0.25
135	Penconazole	0.06	0.10	4	0.025	0.025
136	Pentobarbital	0.12	0.19	2	0.025	n.f.
137	PFDeA	0.52	0.62	4	0.05	0.05
138	PFHpA	0.02	0.039	4	0.005	0.025
139	PFNA	0.02	0.025	4	0.005	0.025
140	PFOA	0.003	0.004	4	0.0025	0.0025
141	Phenobarbital	0.32	0.43	2	0.025	n.f.
142	Phenytoin	0.08	0.13	4	0.025	0.25
143	Pioglitazone	0.027	0.027	4	0.025	0.025
144	Primidone	0.38	0.57	4	0.025	0.5
145	Procymidone	0.06	0.09	4	0.025	0.025
146	Prometryn (Caparol)	0.08	0.11	4	0.025	0.025
147	Propranolol	0.08	0.12	4	0.025	0.025
148	Rafoxanide	0.55	0.72	4	0.025	0.25
149	Ranitidine	0.31	0.34	4	0.025	0.025
150	Rifaximin	0.08	0.09	4	0.025	0.05
151	Risperidone	0.002	0.003	4	0.0025	0.0025
152	Ritonavir	0.25	0.40	4	0.025	0.025
153	Ronidazole	0.16	0.23	4	0.05	0.25
154	Saccharine	0.12	0.12	2	0.025	n.f.
155	Salicylic acid	0.12	0.14	4	0.025	0.025
156	Sertraline	0.12	0.18	4	0.025	0.025
157	Simvastatin	0.21	0.29	4	0.25	0.5
158	Sucralose	high intensity in the sample				
159	Sulfachloropyridazine	0.10	0.16	4	0.025	0.25
160	Sulfaclozine	0.28	0.47	4	0.025	0.25
161	Sulfadiazine	0.04	0.07	4	0.025	0.025
162	Sulfadimethoxine	0.007	0.008	4	0.0025	0.01

163	Sulfadimidine	0.008	0.010	4	0.0025	0.01
164	Sulfadoxine	0.04	0.05	4	0.025	0.025
165	Sulfamerazine	0.02	0.02	4	0.025	0.025
166	Sulfamethizole	0.04	0.07	4	0.025	0.05
167	Sulfamethoxazole	0.10	0.12	4	0.025	0.025
168	Sulfamethoxypyridazine	0.05	0.07	4	0.025	0.025
169	Sulfamonomethoxine	0.010	0.013	4	0.025	0.025
170	Sulfamoxole	0.017	0.026	4	0.025	0.05
171	Sulfanilamide	0.06	0.07	4	0.025	0.025
172	Sulfapyridine	0.06	0.09	4	0.025	0.025
173	Sulfaquinoxaline	0.05	0.08	4	0.025	0.05
174	Sulfathiazole	0.20	0.33	4	0.025	0.25
175	Sulfisoxazole	0.05	0.07	4	0.025	0.025
176	Terbuthylazine	0.04	0.06	4	0.025	0.025
177	Ternidazole	0.017	0.023	4	0.0025	0.025
178	Theophyline	0.14	0.19	4	0.05	0.25
179	Thiabendazole	0.004	0.005	4	0.0025	0.0025
180	Thiamphenicol	0.19	0.32	4	0.025	0.5
181	Thiopental	0.14	0.20	4	0.025	0.25
182	Tiamulin	0.03	0.04	4	0.0025	0.0025
183	Tolfenamic acid	0.15	0.25	4	0.025	0.25
184	Toltrazuril	0.09	0.14	2	0.025	n.f.
185	Topiramate	0.17	0.19	4	0.01	0.025
186	Tramadol	0.61	0.65	4	0.025	0.025
187	Triamterene	0.019	0.028	4	0.005	0.05
188	Triclabendazole	0.40	0.53	4	0.25	1
189	Triclosan	0.35	0.45	2	0.25	n.f.
190	Trimethoprim	0.10	0.14	4	0.025	0.05
191	Valproic acid	0.41	0.44	2	0.025	n.f.
192	Valsartan	0.75	0.80	4	0.025	0.025
193	Vedaprofen	0.11	0.14	4	0.05	0.25
194	Venlafaxine	0.46	0.48	4	0.025	0.025
195	Δ9-Tetrahydrocannabinol (THC)	0.03	0.06	4	0.025	0.05

n.f.: no fragmentation occurred in bbCID spectra under the conditions set.

Although, the SDL and LOI could be misleading to be equivalent to CCβ, there is a difference. LOI is a level of concentration, preselected, at which an analyte can be identified, while CCβ represent a statistical evaluation of the concentration at which an analyte can be identified with a beta-error 5%.

In the literature, the most widely used validation parameters are the screening detection limit (SDL) and limit of identification (LOI) [104, 200, 203]. The identification criterion is the presence of two ions, the precursor and a fragment, at the expected retention time [201]. CCβ values were calculated in a wide-range HRMS screening method for 87 banned veterinary drugs in biological samples [208].

Since there is not specific guideline for the performance criteria of screening HRMS methods, many scientists are based mostly in Document SANCO 12571/2013, Decision 2002/657/EC and in CRL 2010 in order to plan a validation protocol [200, 203].

According to Document SANCO 12571/2013, the SDL of the qualitative screening method is the lowest level at which an analyte has been detected with an acceptable false-negative rate of 5%. For analytes that have not been included in the initial method validation, the confidence level of detection at a certain residue level will not be known. Consequently analytes outside of the scope of validation can be detected using the method, but no SDL can be specified.

Whereas, according to guidelines for the validation of screening methods for residues of veterinary medicines (CRLs 2010), which supplements Commission Decision 2002/657/EC, CCβ is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of β (i.e. false compliant rate), which for screening tests should be < 5%. Moreover, according to Directive 2002/657/EC, for screening methods, the estimation of detection capability CCβ is mandatory. Vergeynst et al. propose in an extensive study, CCα and CCβ as a measure of detection in HRMS measurements [209].

4.5.4. Performance Criteria-Validation results

4.5.4.1. Linearity

Linearity was studied in solvent, in matrix extracts and in spiked samples for the 195 compounds of the validation dataset. The linear dynamic range was evaluated in standard solution at 8 concentration levels, ranging from 0.5 µg/L to 200 µg/L. In spiked samples, linearity was studied at 9 concentration levels, from 0.0025 µg/L to 1 µg/L. The linear range, the slope (b) and the correlation coefficients (r^2) of the calibration curve and the standard addition curve are presented in Table 4.5.

Table 4.5 Validation results- Linearity.

		standard addition curve			standard solution curve		
	Compound name	**Linear range (µg/L)**	**b (slope)**	**r^2**	**Linear range (µg/L)**	**b (slope)**	**r^2**
1	1-OH-Benzotriazole	0.025-1	139389	0.992	100-200	251	0.995
2	2,4-Methylenedioxy-amphetamine (MDA)	0.025-1	265484	0.996	10-200	10432	0.988
3	2,4-Methylenedioxy-N-ethylamphetamine (MDEA)	0.025-1	2145429	0.990	5-200	20060	0.992
4	2,4-Methylenedioxy-N-methylamphetamine (MDMA)	0.025-1	47025	0.993	10-200	6927	0.996
5	2-Amino-Benzothiazole	0.025-1	200120	0.995	5-200	8651	0.98
6	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	0.0025-1	4456697	0.991	0.5-200	46533	0.990
7	2-OH-Benzothiazole	0.025-1	794006	0.994	5-200	16518	0.98
8	5-Me-Benzotriazole	0.025-0.5	2563823	0.94	10-200	315466	0.991
9	6-Monoacetylmorphine (6-MAM)	0.025-1	747406	0.991	5-200	8685	0.98
10	7-Amino-flunitrazepam	0.025-1	1193428	0.990	5-200	17985	0.98
11	8-OH-Mirtazapine	0.005-1	674331	0.92	0.5-200	12891	0.992
12	9-OH-Risperidone	0.0025-1	1114650	0.992	0.5-200	17247	0.993
13	Acesulfame	high intensity in the sample			5-200	1446	0.99997
14	Acetamiprid	0.0025-0.5	661819	0.995	0.5-5	8192	0.997
15	Albendazole	0.025-1	133739	0.991	5-200	4057	0.996
16	Albendazole sulfone	0.025-1	183957	0.998	5-200	5859	0.989
17	Alprazolam	0.0025-1	1224590	0.997	0.5-200	14037	0.996
18	Amitriptyline	0.025-1	2140946	0.99	5-200	19380	0.994

19	Amphetamine	0.0025-1	504746	0.99	0.5-200	5556	0.993
20	Arprinocid	0.0025-1	133236	0.996	0.5-200	6130	0.991
21	Atenolol	0.025-1	830430	0.97	5-200	14733	0.998
22	Atorvastatin	0.05-1	112654	0.98	5-200	674	0.990
23	Atrazine	0.025-1	668032	0.990	10-200	81095	0.99
24	Atrazine-desethyl	0.025-1	302741	0.992	10-200	33373	0.992
25	Azithromycin	0.025-1	96154	0.975513	5-200	1053	0.9990
26	Benzotriazole (BTR)	0.025-1	188954	0.9997	10-200	19019	0.98
27	Benzoylecgonine (BECG)	0.0025-1	1011704	0.990	0.5-200	14228	0.995
28	Bromazepam	0.025-1	62417	0.997	2-200	1360	0.995
29	Bromohexine	0.025-1	136872	0.99	2-200	2449	0.990
30	Caffeine	0.025-1	120547	0.996	5-200	1283	0.994
31	Cannabidiol	0.25-1	23825	0.992	5-200	642	0.995
32	Carbamazepine	0.025-1	407339	0.995	5-200	1871	0.990
33	Carbaryl	0.025-1	103199	0.990	10-200	14682	0.995
34	Carprofen	0.25-1	8736	0.98	50-200	946	0.97
35	Cetirizine	0.0025-0.5	771602	0.96	0.5-5	5560	0.990
36	Chloramphenicol	0.0025-1	326032	0.996	0.5-200	12327	0.992
37	Chlordiazepoxide	0.0025-1	343187	0.994	0.5-200	4554	0.99990
38	Chloro-benzotriazole	0.025-1	77662	0.994	2-200	1228	0.998
39	Chlorpromazine	0.025-1	1015397	0.993	0.5-200	11156	0.999
40	Chlorthalidone	0.0025-0.5	22623	0.99	0.2-50	8652	0.991
41	Cimetidine	0.0025-1	614113	0.997	0.5-200	12329	0.995
42	Citalopram	0.025-1	378473	0.995	5-200	10598	0.99
43	Clarithromycin	0.05-1	680408	0.980	5-200	8434	0.96
44	Clazuril	0.05-1	19758	0.999	5-200	3185	0.994
45	Clobazam	0.0025-1	331552	0.996	0.5-200	3942	0.998
46	Clofibric acid	0.025-1	25110	0.997	5-200	8153	0.998
47	Clomipramine	0.025-1	1455164	0.99	5-200	15730	0.995
48	Clopidol	0.0025-1	369605	0.991	0.5-200	5127	0.999
49	Closantel	0.25-1	4543	0.990	5-200	6415	0.995
50	Clozapine	0.025-1	193170	0.999	5-200	13522	0.998
51	Cocaine (COC)	0.0025-1	744353	0.999	0.5-200	15206	0.994
52	Codeine (COD)	0.025-1	764278	0.996	5-200	13197	0.9992
53	Colchicine	0.0025-1	939962	0.991	0.5-200	10860	0.9997
54	Coumaphos	0.025-1	383720	0.991	0.5-200	4722	0.996
55	Cyclamate	0.025-1	47762	0.997	5-200	3276	0.998
56	Dapsone	0.0025-1	317558	0.994	0.5-200	10672	0.998
57	Decoquinate	0.25-1	67683	0.998	5-200	1197	0.992
58	Diaveridine	0.0025-1	1115074	0.999	5-200	5564	0.99
59	Diazepam	0.0025-1	835941	0.995	0.5-200	15130	0.996
60	Diclazuril	0.025-1	42329	0.993	5-200	3428	0.991
61	Diclofenac	0.0025-1	62616	0.97	5-200	5134	0.990

62	Dimethoate	0.025-1	301266	0.994	10-200	27113	0.998
63	Dimetridazole	0.025-1	304499	0.994	0.5-200	1738	0.998
64	Dipyrone	0.5-1	1798	0.996	10-200	961	0.98
65	Diuron	0.025-1	549227	0.999	5-200	4726	0.996
66	Doxepine	0.0025-1	870483	0.990	0.5-200	15960	0.993
67	Ecgonine methyl ester (EME)	0.05-1	586498	0.997	5-200	15931	0.99
68	Ephedrine	0.0025-1	7237024	0.993	0.5-200	59638	0.97
69	Ethopabate	0.0025-1	401406	0.996	0.5-200	6385	0.992
70	Fenbendazole	0.025-1	121617	0.995	0.5-200	3447	0.992
71	Fenoxycarb	0.025-1	199838	0.997	10-200	22141	0.991
72	Fentanyl	0.0025-1	1195523	0.99	0.5-200	17380	0.993
73	Florfenicol	0.01-1	183255	0.997	0.5-200	1965	0.9998
74	Flubendazole	0.025-1	249602	0.991	5-200	2240	0.999
75	Fludioxonil	0.0025-1	1902620	0.9993	5-200	12991	0.998
76	Flunitrazepam	0.0025-1	239256	0.997	0.5-200	3873	0.99
77	Flunixin	0.005-1	472667	0.999	0.5-200	4273	0.997
78	Fluoxetine	0.025-1	201474	0.993	5-200	15641	0.996
79	Flurazepam	0.0025-1	681478	0.998	0.5-200	10936	0.99
80	Furosemide	0.0025-1	27650	0.990	5-200	1118	0.991
81	Gemfibrozil	0.025-1	79406	0.99	5-200	1445	0.997
82	Hydrochlorthiazide	0.025-1	86975	0.98	5-200	2142	0.997
83	Ibuprofen	0.025-1	6930	0.995	10-200	828	0.999
84	Imidacloprid	0.025-1	219850	0.998	5-200	16614	0.998
85	Imipramine	0.0025-1	988205	0.989	0.5-200	16695	0.993
86	Indapamide	0.005-1	154530	0.998	0.5-200	2405	0.998
87	Iprodione	0.025-1	70176	0.993	5-200	8683	0.998
88	Irbesartan	0.0025-0.5	254905	0.93	0.5-5	8051	0.995
89	Irgarol	0.0025-0.5	2351176	0.97	0.5-5	45062	0.992
90	Ketamine	0.0025-1	1131005	0.993	0.5-200	12732	0.990
91	Ketoprofen	0.0025-1	196690	0.997	5-200	16107	0.993
92	Lamotrigine	0.025-1	134545	0.996	5-200	8124	0.98
93	Levamisol	0.025-1	551050	0.992	5-200	11187	0.995
94	Levetiracetam	0.0025-1	193822	0.996	0.5-200	17513	0.991
95	Lidocaine	0.025-1	732526	0.995	5-200	24978	0.99
96	lincomycin	0.005-1	347789	0.994	0.5-200	3896	0.997
97	Lorazepam	0.025-1	39651	0.993	50-200	8429	0.991
98	LSD	0.025-1	413280	0.990	5-200	20229	0.99
99	Lufenuron	0.25-1	11662	0.97	5-200	2883	0.997
100	Mebendazole	0.025-1	235524	0.997	0.5-200	3363	0.999
101	Mefenamic acid	0.0025-1	58933	0.991	5-200	1005	0.9988
102	Meloxicam	0.01-1	260420	0.998	0.5-200	2787	0.99
103	Metformin	high intensity in the sample			5-200	10239	0.9996
104	Methacrifos	0.025-1	25020	0.990	10-200	12922	0.997

105	Methadone (METH)	0.025-1	501481	0.992	5-200	20095	0.99
106	Methamphetamine (MA)	0.0025-1	1634741	0.997	0.5-200	14929	0.995
107	Metoprolol	0.025-1	1479026	0.93	5-200	13858	0.993
108	Metronidazole	0.025-1	211133	0.998	1-200	1608	0.992
109	Midazolam	0.0025-1	3788777	0.994	0.5-200	24216	0.98
110	Mirtazapine	0.025-1	1220819	0.99	5-200	17261	0.98
111	Monensin	0.05-1	60642	0.99995	5-200	9865	0.98
112	Morantel	0.25-1	201593	0.97	5-200	56274	0.998
113	Morphine (MOR)	0.025-1	152841	0.999	5-200	7789	0.999
114	Naproxen	0.0025-1	64489	0.997	2-200	5397	0.998
115	Niflumic acid	0.025-1	367597	0.994	0.5-200	2584	0.990
116	Nitrazepam	0.0025-1	599604	0.998	2-200	18072	0.990
117	Nitroxinil	0.0025-1	958876	0.998	0.5-200	7756	0.993
118	Norclozapine	0.0025-1	165678	0.990	0.5-200	7478	0.990
119	Nordiazepam	0.0025-1	512374	0.999	0.5-200	5521	0.99
120	Norephedrine	0.025-1	772685	0.99	5-200	10198	0.990
121	Norfentanyl	0.0025-1	598435	0.995	0.5-200	31007	0.99
122	Norfluoxetine	0.5-1	23378	0.980	1-5	2460	0.992
123	Norketamine	0.0025-0.5	571128	0.9996	0.5-200	7247	0.990
124	Norsertraline	0.5-1	2338	0.991	50-200	388	0.99992
125	Nortriptyline	0.0025-1	1571982	0.997	0.5-200	17993	0.991
126	Olanzapine	0.025-1	336879	0.99	5-200	9988	0.998
127	Omeprazole	0.0025-1	174622	0.997	0.5-100	4430	0.980
128	o-toluenesulfonamide	0.0025-0.5	15990	0.95	0.5-0.1	312	0.990
129	Oxazepam	0.025-1	39044	0.997	50-200	351	0.9998
130	Oxfendazole	0.025-1	152011	0.97	0.5-200	3479	0.999
131	Oxyclozanide	0.025-1	291584	0.990	0.5-200	3656	0.994
132	Oxycodone (OC)	0.0025-1	697274	0.997	0.5-200	9108	0.992
133	Paracetamol	0.025-1	295483	0.997	5-200	2785	0.999
134	Paroxetine	0.025-1	274674	0.992	5-200	15264	0.997
135	Penconazole	0.025-1	159299	0.991	10-200	22706	0.990
136	Pentobarbital	0.025-1	30859	0.995	5-200	3168	0.998
137	PFDeA	0.05-1	1414	0.93	50-200	1356	0.991
138	PFHpA	0.005-1	453511	0.993	2-200	2318	0.994
139	PFNA	0.005-1	613565	0.996	0.5-200	3438	0.995
140	PFOA	0.0025-1	675348	0.995	1-200	3720	0.996
141	Phenobarbital	0.025-1	19475	0.993	5-200	155	0.992
142	Phenytoin	0.025-1	27679	0.991	5-200	216	0.997
143	Pioglitazone	0.025-1	1698680	1.00	0.5-5	12028	0.990
144	Primidone	0.025-1	80139	0.995	5-200	967	0.98
145	Procymidone	0.025-1	13377	0.990	5-200	886	0.997
146	Prometryn (Caparol)	0.025-1	662462	0.995	10-200	54532	0.970
147	Propranolol	0.025-1	308080	0.993	5-200	11020	0.995

148	Rafoxanide	0.025-1	2012	0.973	5-200	6802	0.98
149	Ranitidine	0.025-1	702433	0.98	5-200	11618	0.994
150	Rifaximin	0.025-1	285727	0.97	10-200	32249	0.98
151	Risperidone	0.0025-1	5637537	0.996	0.5-200	53194	0.994
152	Ritonavir	0.025-0.5	267295	0.994	0.5-5	24277	0.993
153	Ronidazole	0.05-1	51755	0.98	5-200	3619	0.99
154	Saccharine	0.025-1	187317	0.992	1-200	1168	0.9997
155	Salicylic acid	0.025-1	212824	0.98	5-200	1309	0.998
156	Sertraline	0.025-1	335512	0.99	5-200	6723	0.997
157	Simvastatin	0.25-1	15084	0.994	5-200	1584	0.989
158	Sucralose	0.25-1	18973	0.9	10-200	978	0.998
159	Sulfachloropyridazine	0.025-1	152368	0.994	5-200	1053	0.993
160	Sulfaclozine	0.025-1	36646	0.991	5-200	961	0.996
161	Sulfadiazine	0.025-1	292519	0.99	5-200	3049	0.992
162	Sulfadimethoxine	0.0025-1	565131	0.999	0.5-200	4076	0.993
163	Sulfadimidine	0.0025-0.5	667656	0.995	1-200	5927	0.993
164	Sulfadoxine	0.025-0.5	1138679	0.998	5-200	5372	0.99
165	Sulfamerazine	0.025-1	518926	0.998	5-200	4703	0.998
166	Sulfamethizole	0.025-1	90875	0.992	5-200	1931	0.99
167	Sulfamethoxazole	0.025-1	376646	0.991	5-200	2674	0.991
168	Sulfamethoxypyridazine	0.025-1	299030	0.992	5-200	9743	0.98
169	Sulfamonomethoxine	0.025-1	469582	0.999	5-200	3874	0.98
170	Sulfamoxole	0.025-1	256988	0.999	5-200	4436	0.994
171	Sulfanilamide	0.005-1	26652	0.993	5-200	8262	0.991
172	Sulfapyridine	0.025-1	679829	0.992	5-200	4389	0.991
173	Sulfaquinoxaline	0.025-1	142802	0.996	5-200	1070	0.995
174	Sulfathiazole	0.025-1	145907	0.997	5-200	1696	0.991
175	Sulfisoxazole	0.025-1	123928	0.998	5-200	1580	0.990
176	Terbuthylazine	0.025-1	310095	0.994	10-200	8457	0.996
177	Ternidazole	0.0025-1	198352	0.9991	0.5-200	1234	0.995
178	Theophyline	0.05-1	54039	0.992	5-200	9636	0.990
179	Thiabendazole	0.0025-1	1052781	0.990	0.5-200	12724	0.998
180	Thiamphenicol	0.025-1	66162	0.997	5-200	2600	0.995
181	Thiopental	0.025-1	15899	0.98	5-200	5548	0.991
182	Tiamulin	0.0025-1	1201337	0.996	5-200	1246	0.99
183	Tolfenamic acid	0.025-1	113276	0.996	2-200	1192	0.990
184	Toltrazuril	0.025-1	60659	0.992	5-200	8431	0.98
185	Topiramate	0.01-1	264848	0.980	5-200	2188	0.998
186	Tramadol	0.025-1	1001022	0.993	5-200	14339	0.98
187	Triamterene	0.005-0.5	764416	0.999	0.5-200	11640	0.996
188	Triclabendazole	0.25-1	75028	0.97	5-200	1117	0.993
189	Triclosan	0.25-1	99858	0.992	0.5-200	2066	0.997
190	Trimethoprim	0.025-1	905659	0.991	5-200	11992	0.995

191	Valproic acid	0.025-1	32958	0.982	5-200	9249	0.991
192	Valsartan	0.025-1	177654	0.95	5-200	642	0.991
193	Vedaprofen	0.05-1	8130	0.9995	5-200	555	0.9996
194	Venlafaxine	0.025-1	588777	0.990	5-200	20887	0.99
195	Δ9-Tetrahydrocannabinol (THC)	0.025-1	169640	0.995	5-200	1401	0.9994

4.5.4.2. Recovery- Matrix Effect- Repeatability

Method precision data were estimated by the determination of repeatability values. Repeatability was calculated in %RSD and was estimated from the analysis of spiked samples, where six replicates were analyzed at three (3) fortification level (1, 0.01, 0.005 μg/L). % RSD was below 20% in all cases, except bromohexine and methcriphos, which showed decreased sensitivity and amphetamine at the lowest concentration level (0.005 μg/L).

Fortified samples, standards in solvent, and standards in matrix were analyzed in order to evaluate the effectiveness of the extraction procedure and the matrix effects. Recovery experiments were performed at four (4) fortification levels (0.5, 0.25, 0.05 and 0.025 μg/L). Satisfying recoveries (50-120%) can be observed for the majority of the compounds. Matrix effect was calculated at 5 levels of concentrations (0.5, 0.25, 0.05, 0.025 and 0.01 μg/L), according to the equation:

$$Matrix\ effect\ \% = (Matrix\ Factor - 1) \times 100$$

$$Matrix\ Factor = \frac{Peak\ Area_{matrix-matched}}{Peak\ Area_{std}}$$

The results for repeatability, recoveries and matrix effect are presented in total in Table 4.6.

Table 4.6 Validation results- Performance criteria.

		% Recovery				% RSD			% ME				
	(µg/L)	0.5	0.25	0.05	0.025	1	0.01	0.05	0.5	0.25	0.05	0.025	0.01
1	1-OH-Benzotriazole	119				24			-82				
2	2,4-Methylenedioxy-amphetamine (MDA)	113	148	106		7.6			-91	-95	-93		
3	2,4-Methylenedioxy-N-ethylamphetamine (MDEA)	70	87			27			-91	-94	-57		
4	2,4-Methylenedioxy-N-methylamphetamine (MDMA)	125	124	97		13			-41				
5	2-Amino-Benzothiazole	73	105			32			-94	-92			
6	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	121	96	125	99	0.38	4.7	14	-86	-97	-97	-36	-35
7	2-OH-Benzothiazole	132				16			-90	-96			
8	5-Me-Benzotriazole	139	128	107		13			-75				
9	6-Monoacetylmorphine (6-MAM)	141	190			20			-92	-92			
10	7-Amino-flunitrazepam	109	152	147		12			-89	-92	-92		
11	8-OH-Mirtazapine	157			51	7.6	8.2	15	-34			-13	-17
12	9-OH-Risperidone	111	156	103	71	9.7	18	19	-92		-90	-81	-68
13	Acesulfame	89	77	156	110	9.7		11					
14	Acetamiprid				112		5.9	11				-9	17
15	Albendazole	79	193	141		12			-81	-95	-93		
16	Albendazole sulfone	120				11			-79				
17	Alprazolam	134	152	130	68	9.8	16	19	-51	-45	-41	4	-15
18	Amitriptyline	95	177	72		18			-88	-95	-51		
19	Amphetamine	61	96		55	10	16	33	-22	-34	-38	20	-11
20	Arprinocid	67	122	149	69	9.6	13	20	-58	-56	-43	-10	11
21	Atenolol	78	146	122		14			-76	-82	-34		
22	Atorvastatin	41				18			-71				
23	Atrazine	118	132			9.3			-89				
24	Atrazine-desethyl	153	221			8.3			-88				
25	Azithromycin	102	111			20			-36	-33			
26	Benzotriazole (BTR)	131	131	107		13			-86				
27	Benzoylecgonine (BECG)	102	177	127	111	11	14	13	-70	-62	-56	-22	-56
28	Bromazepam	133	237	105		17			-67	-78		-17	-29
29	Bromohexine	23	50			47			-81	-63	-42	-18	-25
30	Caffeine	101	144	102		10			-41	-34	10		
31	Cannabidiol	69	84			9.0			-6	-30	-62		

32	Carbamazepine	111	116	107		8.8			-10	-4	6		
33	Carbaryl	75	105	113		19			-78				
34	Carprofen	66	103			13			-30	-46			
35	Cetirizine				79		13	12				56	28
36	Chloramphenicol	84	100	68	77	9.1		15	-28	-56	-62	-2	-25
37	Chlordiazepoxide	130	125	135	89	5.5	7.2	16	-88	-95	-88	-13	-3
38	Chloro-benzotriazole	88	136	89		11			-27			-51	
39	Chlorpromazine	21	115			20			-74	-59		-1	-7
40	Chlorthalidone				161	5.6	6.4	22				41	
41	Cimetidine	56	334		79				-44	-68		-19	-7
42	Citalopram	136	127	98		9.2			-80	-46	-57		
43	Clarithromycin	108	104	53		13			-55	-67	-65		
44	Clazuril	87	83	84		8.6			-49	-46	-63		
45	Clobazam	113	170	137	81	7.7	9.1	12	-60	-46	-74	-40	-37
46	Clofibric acid	118	109	110		7.0			-32	-36	-48		
47	Clomipramine	99	131	105		16			-83	-78	-56		
48	Clopidol	77	114	171	124	8.7	13	17.3	-87	-94	-93	-51	-94
49	Closantel	29	45			22			-25	-50	-94		
50	Clozapine	102	133	144		17			-95	-96	-80		
51	Cocaine (COC)	115	231	62	88	7.8	12	14	-28	-53	-49	-32	-36
52	Codeine (COD)	111	171	74		17			-89	-90	-48		
53	Colchicine	89			70	12	17	18	-79	-68		-18	-8
54	Coumaphos	63		148	30	11	18		-69	-45	-32	-37	-46
55	Cyclamate	107	83	162		11			-15	13	-33		
56	Dapsone	61	144		88	11	7.6	9.3	-58	-47		-17	-28
57	Decoquinate	80				18			-12			-22	
58	Diaveridine	81			112	9.8	12	19	-59	-40		11	-18
59	Diazepam	127	147	117	96	8.0	13	16	-36	-53	-22	9	-19
60	Diclazuril	71	80	100		15			-47	-71	-83		
61	Diclofenac	106	104	106	77	8.5	13	15	75	52	73	99	
62	Dimethoate	128	126	129		12			-94				
63	Dimetridazole	99	131	66	82	8.0	4.4	8.1	-13	-23	-34	-16	13
64	Dipyrone	128				12			-80	-47			
65	Diuron	99	101	133		4.3			-31	-31	-58		
66	Doxepine	104	140	75	72	11	18	18	-59	-75	-72	-12	-16
67	Ecgonine methyl ester (EME)	82				11			-93				
68	Ephedrine	90	110	82	92	3.8	4.3	16.0	-56	-85	-70	-7	-31
69	Ethopabate	79	131	43	85	4.2	11	13	-32	-66	-83	2	-48
70	Fenbendazole	64	142	182		20			-75	-53	-63	-5	-38
71	Fenoxycarb	142	197	165		15			-77				
72	Fentanyl	114	143	122	89	8	12	13	-80	-45	-75	-17	-17
73	Florfenicol	69	99	142	145	6.9		19	-35	-55	-73	-22	-15

74	Flubendazole	110	174			18			-86	-93			
75	Fludioxonil	91	79	87		14		19	-24	-26	-52		
76	Flunitrazepam	148	132	70	69	7.8	13	16	-38	-64	-49	3	-18
77	Flunixin	86	91	132	78	7.7		20	-44	-52	-83	-23	-2
78	Fluoxetine	77	136	68		20			-90	-95	-47		
79	Flurazepam	122	159	110	59	11	16	19	-50	-45	-42	1	-4
80	Furosemide	83	74	120	160	12		19	28	106	104		
81	Gemfibrozil	80	94	93		8.5			-26	-21	-63		
82	Hydrochlorthiazide	94	124	137	103	9.6		17					
83	Ibuprofen	146				19			-13				
84	Imidacloprid	97	90	74		2.3			-79	-78	-69		
85	Imipramine	93	119	63	65	4.9	12	19	-29	-55	-26	17	-6
86	Indapamide	100	121	69	78	5.1	13	17	-70	-79	-66	-14	-14
87	Iprodione	78	65	89		16			-40	-31	-57		
88	Irbesartan				144		3.5	14				109	115
89	Irgarol				78		5.1	6.3				13	54
90	Ketamine	126	239	98	114	4.7	12	13	-26	-28	-33	-33	-6
91	Ketoprofen	106	110	95	93	5.7	13	11	-48	-51	-28	-12	
92	Lamotrigine	127	128	112		12			-83	-66	-20		
93	Levamisol	115	181	97		16			-81	-92	-77		
94	Levetiracetam	138	122		116	11	13	15	-53	-31		-81	-57
95	Lidocaine	136	154	97		9.4			-84	-67	-77		
96	lincomycin	90			132	7.6	16	18	-86			-57	-10
97	Lorazepam	78	115	138		19			-9	48			
98	LSD	103				9.2			-89				
99	Lufenuron	4				33			-50	-68	-90		
100	Mebendazole	80	307			18			-22	-55		6	-18
101	Mefenamic acid	89	93	136		13		15	-38	-30	-21		
102	Meloxicam	61	100	72	79	3.8			-39	-64	-53	-38	-11
103	Metformin	90	34	76		12			-37	-61	-54		
104	Methacrifos	7	17			**39**			-75				
105	Methadone (METH)	115	239	132		12			-88	-92	-49		
106	Methamphetamine (MA)	103	186	118	89	9.0	13	18	-39	-24	-40	-31	9
107	Metoprolol	100	97	107		13			-78	-75	-30		
108	Metronidazole	99	142		113	3	7	10	-57	-46	-32	-34	-1
109	Midazolam	124	199	145	58	11	10	13	-67	-44	-76	13	-51
110	Mirtazapine	119	146	80		19			-85	-80	-50		
111	Monensin	80				19			3				
112	Morantel	62	69			22			-78	-91			
113	Morphine (MOR)	276	217	154		10			-96	-97	-85		
114	Naproxen	103	116	98	142	2.4	11	18	-27				
115	Niflumic acid	97	149	96	120	8.3		14	-11	1	17	44	68

116	Nitrazepam	116	197	97	52	17			-82	-48	-64	-40	-18
117	Nitroxinil	73	86	104	123	6.7		12	-28	-41	-43	19	-40
118	Norclozapine	72	167	29	57	4.4	19	20	-52	-64	-71	-10	-26
119	Nordiazepam	128	183	136	80	8.4	12	18	-85	-70	-56	-58	-36
120	Norephedrine	63	126	49		21			-86	-88	-61		
121	Norfentanyl	97	131	110	61	13	13	17	-20	-24	-14	-4	4
122	Norfluoxetine	47					8.7					13	15
123	Norketamine	104			85	15	18	18	-55		-65	-21	-12
124	Norsertraline	84				19			-84				
125	Nortriptyline	78	163	103	64	5.0	4.5	21.0	-59	-52	-34	-7	-36
126	Olanzapine	82			116	24			-96				
127	Omeprazole	70	107	162	46	9.7		8.3	-69	-50	-51	-10	-13
128	o-toluenesulfonamide				98			15			65		
129	Oxazepam	103				22			-61				
130	Oxfendazole	84	313			11			-53	-36		7	-33
131	Oxyclozanide	34	26	114		14			-15	-26	-28	25	41
132	Oxycodone (OC)	106	147		113	19	18	20	-67	-59		-18	-11
133	Paracetamol	109				15			-84				
134	Paroxetine	58				17			-94				
135	Penconazole	133	181	77		8.6			-82				
136	Pentobarbital	123	123	133		4.5			-23	-42	-76		
137	PFDeA	137				8.2			14				
138	PFHpA	116	110	123	94	0.88		19	-16	-12	-44	-27	-5
139	PFNA	102	75	89	59	8.3		19	11	-8	-16	30	27
140	PFOA	111	92	103	88	6.6		21	-14	-4	-22	4	12
141	Phenobarbital	94	124	216		5.5			-2	-33	-8		
142	Phenytoin	100	89	158	71	9.8			-38	-43	-16	-12	
143	Pioglitazone				60			8.7				0	30
144	Primidone	159	134	101		9.9			23	39	32		
145	Procymidone	62	97			11			-87	-87			
146	Prometryn (Caparol)	121	147	99		8.1			-90				
147	Propranolol	77	146	130		11			-85	-93	-91		
148	Rafoxanide	1				15			-10	-35	-90		
149	Ranitidine	58	116	127		20			-76	-82	-62		
150	Rifaximin	92	61			18			-61				
151	Risperidone	115	159	134	67	10	11	13	-45	-36	-54	-17	-16
152	Ritonavir				57		12					-27	
153	Ronidazole	87	129			17			-48				
154	Saccharine	104	94	148	125	5.9		8.0	-12	-2	0	15	12
155	Salicylic acid	100	95	132	132	13		16	-23	-1	-32		
156	Sertraline	60	57	72		28			-90	-94	-85		
157	Simvastatin	68	89			26			-74	-93			
158	Sucralose	114	109	137	113	5.3		17					

159	Sulfachloropyridazine	79	224	228		7.6			-56	-90	-95		
160	Sulfaclozine	69	260			18			-50	-85			
161	Sulfadiazine	89	266	114		12			-73	-93	-90		
162	Sulfadimethoxine	80	172	182	63	8.0	19	25	-73	-24	-65	-47	-49
163	Sulfadimidine	69	143		111	13	13	18	-53	-55		-21	-83
164	Sulfadoxine	74	111	210		12			-66	-95	-95		
165	Sulfamerazine	74	158	144		14			-75	-95	-93		
166	Sulfamethizole	72	161	158		15			-55	-91	-91		
167	Sulfamethoxazole	47	96	174		6.2			-59	-75	-75		
168	Sulfamethoxypyridazine	65	137			21			16	-28			
169	Sulfamonomethoxine	79	149			7.8			-50	-92			
170	Sulfamoxole	37	145			13			-79	-96			
171	Sulfanilamide	146	121			17		19	-60	-41			
172	Sulfapyridine	78	129	109		8.4			-69	-90	-89		
173	Sulfaquinoxaline	92	150	133		12			-57	-89	-87		
174	Sulfathiazole	80	105	127		16			-71	-95	-93		
175	Sulfisoxazole	77	118			20			-63	-92			
176	Terbuthylazine	114	159	89		7.6			-86				
177	Ternidazole	83	113		121	3.7	7.5	14	-48	-79		-15	-26
178	Theophyline	91				18			-60				
179	Thiabendazole	82	112	161	48	8.5	12	14	-83	-85	-65	-6	-26
180	Thiamphenicol	77	117	133		8.2			-47	-66	-78		
181	Thiopental	49				23			-32	-70			
182	Tiamulin	69	108			11			-1	-19			-51
183	Tolfenamic acid	61	63	94	111	5.9			-41	-57	-58	17	-16
184	Toltrazuril	67	54	76		14			-27	-12	-69	-4	
185	Topiramate	148	128	136	93	15	17	19	-74	-60	-31	-15	
186	Tramadol	64	118	105		13			-23	1	-66		
187	Triamterene	61		112	70	27			-24		-35	-17	19
188	Triclabendazole	21				36			-69	-91	-84		
189	Triclosan	24	15	61		17			-47	-49	-72		
190	Trimethoprim	106		159		8.3			-85		-64		
191	Valproic acid	97	111	168	152	1.6		16	51	54	39	36	20
192	Valsartan	131	99	104		17			46	55	35		
193	Vedaprofen	122	146			3.5			-50	-69			
194	Venlafaxine		143	102		7.5			-75	-45	-29		
195	Δ9-Tetrahydro-cannabinol (THC)	82	144			14			-12	-32	-56		

4.5.5. Identification and Confirmation of analytes

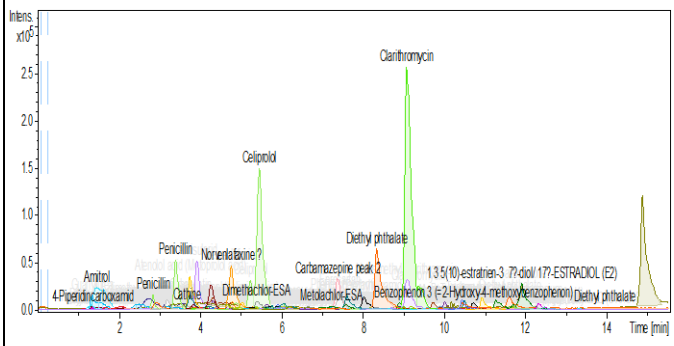
Wastewater samples were analyzed and evaluated and confirmation of the analytes detected is attributed based on the identification points (IPs), as described in Table 1. In Fig 4.2, a chromatogram of influent and effluent wastewater is presented with all the analytes detected. From the chromatograms, it is obvious that the majority of the compounds are detected in positive ionization mode, where higher intensities are observed (at least one magnitude higher). We should also note that in influent wastewater, metformin was deselected intentionally from the chromatogram, since its intensity is one magnitude higher than the rest of the compounds in positive ionization.

More specifically, in Fig 4.3, the identification of ephedrine in influent wastewater is presented in detail. The extracted ion chromatograms of ephedrine and of the 2 fragments have the same chromatographic picture in the spiked sample and in the sample. Additionally to mass accuracy error (0.2 ppm, 0.0 mDa) and isotopic fitting (16 mSigma), an in-source fragment ($C10H14N^+$) is observed in the MS spectrum and 2 bbCID fragments ($C10H14N^+$ and $C19H9^+$) in the MS/MS spectrum with the same ion ratios with the spiked sample. According to the identification point system proposed, 2 IPs are attributed for the mass accuracy, 0.5 for the isotopic fitting and the 2 fragments earn 2.5 IPs each, in total 7.5 IPs for the identification of ephedrine.

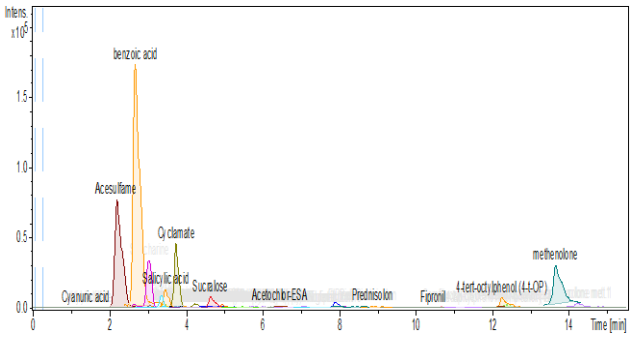
On the contrary, irbesartan in effluent wastewater (Fig. 4.4) has earned only 2 IPs, since no fragments were observed in the bbCID spectrum. Mass accuracy was 1.1 ppm/ 0.5 mDa, but no isotopic fitting score was extracted because of the low intensity of the analyte.

1a) Influent wastewater

Positive ESI

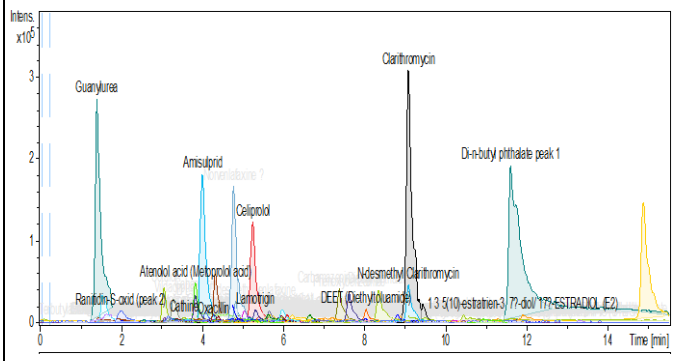


Negative ESI



1b) Effluent wastewater

Positive ESI



Negative ESI

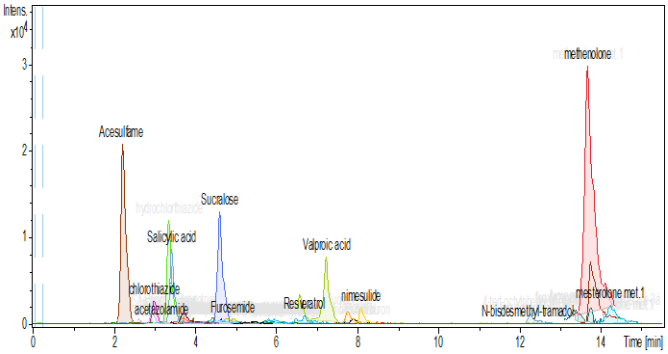


Figure 4.2. Chromograms of the analytes in the samples (m/z ± 0.002 mDa).

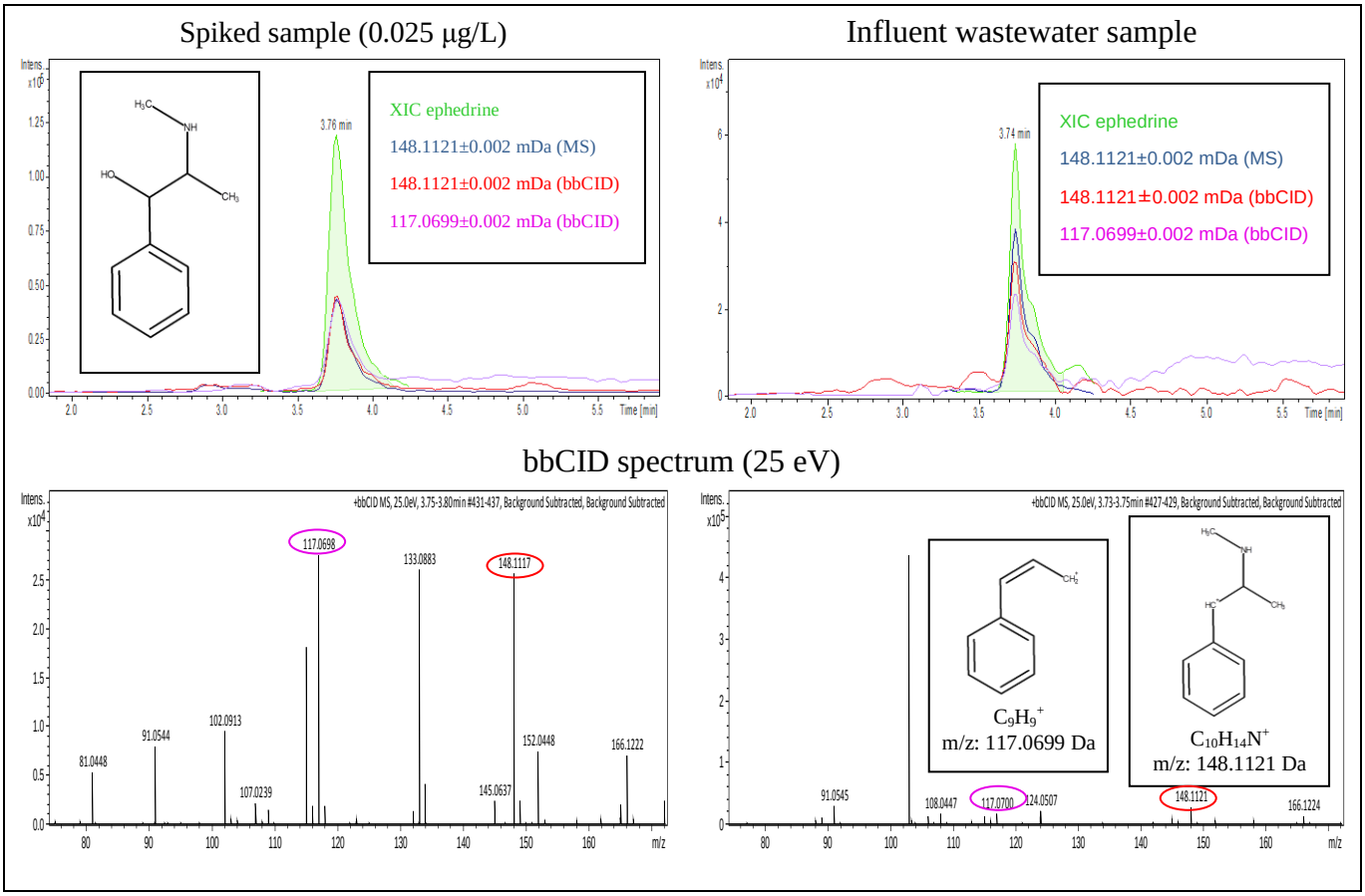


Figure 4.3. Identification of Ephedrine (7.5 IPs).

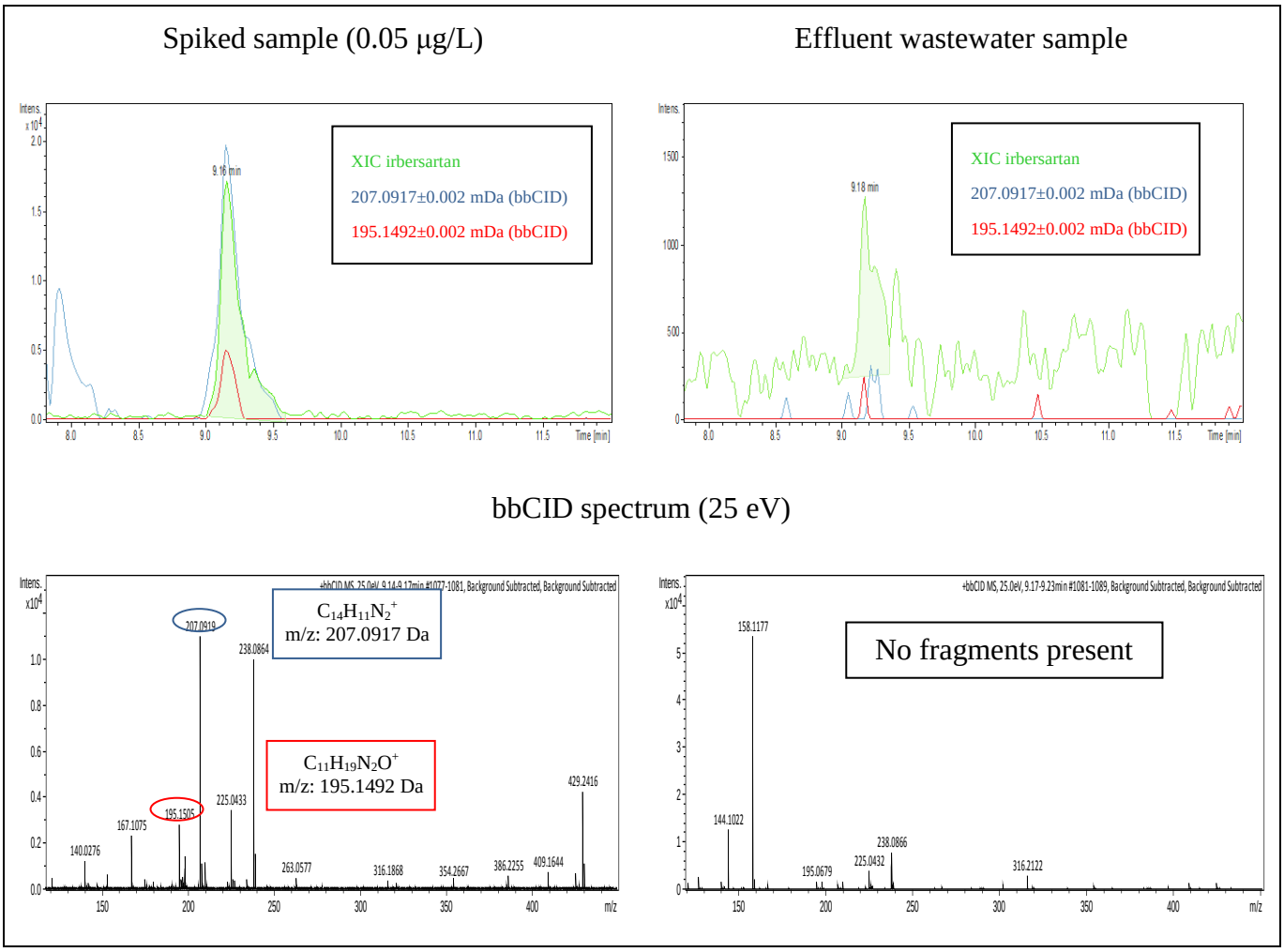


Figure 4.4. Screening of Irbesartan (2.0 IPs).

4.5.6. Screening of influent & effluent wastewater

The optimized and validated method was applied to influent and effluent wastewater samples of the same day from a wastewater treatment plant of Athens. For the identification of the compound, studying the MS spectrum, the retention time, mass accuracy criteria should be met, gaining thus 2 IPs. When studying the MS/MS spectrum, if fragments are also available, additional 2.5 IPs are earned. For screening of the compounds, at least 2 (≥ 2) IPs are required, and for identification at least 4 (≥ 4) IPs. In Table 4.7, all the detected analytes in influent and effluent are presenting, accompanied by the identification points earned in every sample.

In total, 371 compounds were detected in the samples; 338 in influent wastewater and 301 in effluent wastewater. In influent wastewater, 219 compounds were identified, earning at least 2 IPs, and the rest 188 were identified with additional MS/MS information. 61 pesticides were detected, 205 drugs, including pharmaceuticals, illicit and drugs of abuse, 4 sweeteners, 10 perfluorinated compounds (PFCs), 8 aminoacids, 47 transformation products and other chemicals. In effluent wastewater, 192 compounds were identified and the 109 were identified. 51 pesticides were present, 191 drugs, 4 sweeteners, 11 PFCs, 4 aminoacids, 49 transformation products and other chemicals. It is worth mentioning that more TPs are detected in effluent wastewater, because they are formed through the wastewater procedure in the plant.

To the authors' knowledge, this is the first study reported the presence of 371 organic micropollutants in wastewaters, belonging in various classes. 66 pesticides, belonging to different classes were detected in total. 29 stimulants, most of them being amphetamine derivatives and 9 sympathomimetics (ephedrine derivatives) are reported. Moreover 9 anesthetics, closely related to lidocaine, which is well reported in the literature, are present in the samples. Various other drugs are identified, categorized as drugs against high blood pressure, cardiovascular diseases, diuretics, anti-diabetics, antiviral, anti-histamin, etc. This categorization, as presented in Table 4.7 can provide a valuable and holistic information for the consumption of drugs in the area of Athens.

From the target "well known compounds" reported in the literature, tramadol is not identified in the wastewater samples analyzed. Instead, the authors claim that O-desmethyl-venlafaxine is present in the samples. They are isobaric substances, with the same molecular formula and very close retention times. MS/MS fragmentation and experiment with spiking the sample with the compounds led to the conclusion that O-desmethyl-venlafaxine is the analyte eluted.

347 target compounds have been reported in a collaborative trial on water analysis, organized by NORMAN Association, where 18 laboratories took part, employing LC and GC techniques [31]. 141 compounds are reported from Moschet et al. in 8 river

waters [210]. In a screening list of 2188 potential suspects, 55 compounds were finally identified on surface waters [122]. Another study reports 122 emerging contaminants detected in 33 water samples, but it is not clarified whether the identification is based on reference standards or the compounds are tentatively identified [104].

Table 4.7. Quantitative results of wastewater samples.

	Compound Name	CAS number	influent wastewater		effluent wastewater	
			IPs	C (µg/L)	IPs	C (µg/L)
	Pesticides					
1	Acetochlor	34256-82-1	2.5	0.0003	2.5	0.0002
2	Dimethachlor	50563-36-5	2	0.07	n.d.	
3	Dimethachlor-ESA	-	2.5	2.0	2.5	1.1
4	Dimethachlor-OXA	1086384-49-7	2.5	0.54	2	0.09
5	Metolachlor	51218-45-2	5	0.002	5	0.007
6	Metolachlor-ESA	171118-09-5	2.5	0.43	2.5	0.05
7	Diuron	330-54-1	≥5	0.011	≥5	0.02
8	Fenuron	101-42-8	n.d.		2	0.088
9	Difenoxuron	14214-32-5	2.5	0.02	2.5	0.19
10	Diflubenzuron	35367-38-5	2.5	0.005	n.d.	
11	Fluometuron	2164-17-2	≥5	0.58	≥5	13.56
12	Metobromuron	3060-89-7	2	0.004	n.d.	
13	Dimethoate	60-51-5	2	0.03	2	0.04
14	Fludioxonil	131341-86-1	2.5	0.005	2	0.004
15	Cyprodinil	121552-61-2	n.d.		2	0.002
16	Flutolanil	66332-96-5	2	0.01	2	0.01
17	Fipronil	120068-37-3	≥5	0.02	≥5	0.01
18	Fipronil sulfone	120068-36-2	2.5	0.0012	2.5	0.002
19	Imidacloprid	138261-41-3	2	0.02	5	0.12
20	Terbutryn	886-50-0	2	0.003	2.5	0.0006
21	Prometryn (Caparol)	7287-19-6	2	0.04	2	0.04
22	Thiodicarb	59669-26-0	2	0.009	2.5	0.04
23	Propamocarb	24579-73-5	2.5	0.010	2.5	0.004
24	Dioxacarb	6988-21-2	2	0.004	n.d.	
25	Isoprocarb	2631-40-5	2.5	0.003	n.d.	
26	Iprovalicarb	140923-17-7	n.d.		2	0.02
27	Methiocarb (Mercaptodimethur)	2032-65-7	2	0.01	n.d.	
28	Propham	122-42-9	2.5	0.05	2.5	0.08
29	Temephos	3383-96-8	2.5	0.45	n.d.	
30	Pirimiphos-methyl	29232-93-7	2	0.01	4.5	0.02

31	Monocrotophos	6923-22-4	2	0.01	n.d.	
32	Carbendazim	10605-21-7	2	0.01	2	0.02
33	Carbofuran-3-hydroxy	16655-82-6	5	0.02	2.5	0.007
34	Chlormequat	7003-89-6	2.5	0.02	4.5	0.007
35	Napropamide	15299-99-7	2	0.007	2	0.02
36	Climbazole	38083-17-9	5	0.15	≥5	0.19
37	Difenoconazole	119446-68-3	2	0.01	n.d.	
38	Penconazole	66246-88-6	2	0.03	≥5	0.09
39	Cyproconazole	94361-06-5	n.d.		2	0.02
40	Fluconazole	86386-73-4	≥5	0.09	≥5	0.75
41	Thiabendazole	148-79-8	2	0.010	2.5	0.01
42	Atrazine	1912-24-9	2	0.03	n.d.	
43	Atrazine-desisopropyl	1007-28-9	2.5	0.16	2.5	0.39
44	Simazine	122-34-9	2	0.05	2.5	0.11
45	Azoxystrobin	131860-33-8	≥5	0.60	≥5	1.9
46	Azoxystrobin acid	1185255-09-7	5	0.04	≥5	0.09
47	Dalapon	75-99-0	2.5	0.01	n.d.	
48	Dazomet	533-74-4	2.5	0.05	2.5	0.11
49	Dikegulac	18467-77-1	2	0.0003	n.d.	
50	Famoxadone	131807-57-3	2	0.0006	n.d.	
51	Imazapyr	81334-34-1	2	0.01	2.5	0.01
52	Methoxyfenozide	161050-58-4	4.5	0.21	≥5	0.94
53	N-2,4-Dimethylphenylformamide (DMF. Metabolite Amitraz)	60397-77-5	2.5	0.008	2	0.0004
54	Naptalam (N-1-Naphthylphthalamicacid)	132-66-1	n.d.		2.5	0.06
55	Thiamethoxam	153719-23-4	2.5	0.0006	5	0.01
56	Cycloheximide	66-81-9	2.5	0.11	n.d.	
57	Carboxin	5234-68-4	2.5	0.0009	n.d.	
58	Oxycarboxin	5259-88-1	2.5	0.01	2	0.011
59	Picaridin (Icaridin)	119515-38-7	2	0.03	2	0.07
60	DEET (Diethyltoluamide)	134-62-3	5	0.07	5	0.02
61	Metalaxyl	57837-19-1	≥5	0.003	≥5	0.08
62	Amitrole	61-82-5	2.5	1.17	2	0.04
63	Dinoterb	1420-07-1	5	0.03	2.5	0.01
64	Fluazifop	69335-91-7	2.5	0.02	2.5	0.05
65	Propoxur	114-26-1	≥5	0.003	≥5	0.003
66	Piperonyl butoxide	51-03-6	≥5	0.11	2	0.003
	Opiates, opioids					
67	Morphine	57-27-2	≥5	0.64	2	0.0012
68	Normorphine	466-97-7	2.5	0.02	n.d.	
69	Methadone (METH)	76-99-33	2	0.08	2.5	0.04
70	Codeine (COD)	76-57-3	2	0.04	5	0.44

71	Norcodeine	467-15-2	n.d.		2.5	0.08
72	EDDP	30223-73-5	2	0.12	2	0.10
73	Hydrocodone	125-29-1	n.d.		2.5	0.02
	Stimulants- Amphetamins					
74	Cocaine (COC)	50-36-2	5	0.11	n.d.	
75	Benzoylecgonine (BECG)	519-09-5	≥5	0.30	2	0.05
76	Ecgonine methyl ester (EME)	7143-09-01	2	0.11	n.d.	
77	Amphetamine	300-62-9	≥5	0.27	n.d.	
78	Methamphetamine (MA)	537-46-2	2.5	0.07	n.d.	
79	Dimethylamphetamine	1009-69-4	2.5	0.12	2.5	0.95
80	Ethylamphetamine	457-87-4	2	0.18	2.5	1.3
81	3,4-methylenedioxy-amphetamine (MDA)	4764-17-4	≥5	2.1	2	0.22
82	3,4-methylenedioxy-N-methylamphetamine (MDMA)	42542-10-9	2	0.16	2	0.09
83	PMMA (para-Methoxy-N-methylamphetamine)	3398-68-3	5	6.1	5	19
84	Metaraminol (3,β-dihydroxyamphetamine)	337376-15-5	2.5	1.2	n.d.	
85	Pholedrine (p-hydroxy-methylamphetamine)	6114-26-7	n.d.		2.5	1.1
86	4-methyl-2-hexanamine	105-41-9	2	0.59	2	0.11
87	Mephentermine	100-92-5	2	0.74	2.5	0.97
88	Phenelzine	51-71-8	2	3.6	2.5	0.75
89	Pyrovalerone	3563-49-3	n.d.		2.5	0.31
90	Phendimetrazine	17140-98-6	2	0.46	2	0.26
91	Midodrine	133163-28-7	2	0.20	2.5	1.08
92	Heptaminol	372-66-7	2.5	0.49	2.5	0.64
93	Cathine/ Norpseudoephedrine	492-39-7	2.5	0.12	2.5	0.05
94	Nikethamide	59-26-7	n.d.		2.5	0.72
95	Pemoline	2152-34-3	2	0.05	n.d.	
96	Aminorex	2207-50-3	2	0.43	2	2.23
97	Dimefline	1165-48-6	n.d.		2.5	0.46
98	Ethamivan	304-84-7	n.d.		≥5	0.48
99	TMA (trimethoxyamphtamine)	1082-23-1	2	0.26	2	0.26
100	3,4-DMA (dimethoxyamphtamine)	120-26-3	2	0.22	2	0.17
101	4-Methyl-pyrrolidino-propiophenone (MPPP)	28117-80-8	2	0.01	2	0.01
102	2 C-D (2,5-dimethoxy-4-methylphenethylamine)	24333-19-5	2	3.41	2.5	1.41
	Sympathomimetics					

103	Ephedrine	299-42-3	≥5	0.34	≥5	0.03
104	Norephedrine	492-41-1	2.5	0.82	≥5	0.22
105	Etafedrine	48141-64-6	2.5	0.02	2.5	0.12
106	Metanephrine	5001-33-2	2.5	0.005	n.d.	
107	Phenylephrine	1416-03-1	n.d.		2	0.01
108	Apophedrin (Phenylethanolamine)	7568-93-6	5	0.07	5	0.03
109	Isoetharine	7279-75-6	2.5	0.01	2.5	0.03
110	Methoxamine	337376-15-5	2	0.00	2.5	0.02
111	Nylidrin	447-41-6	2.5	0.00	2	0.01
	Hallucinogenic (cannabinoids)					
112	Δ9-Tetrahydrocannabinol (THC)	1972-08-3	n.d.		2	0.01
	Benzodiazepines tranquilizers					
113	Alprazolam	92623-85-3	2	0.03	≥5	0.04
114	Clobazam	22316-47-8	2	0.009	2	0.001
115	Diazepam	439-14-5	2.5	0.04	2.5	0.04
116	Nordiazepam	1088-11-5	2.5	0.008	2.5	0.009
117	7-amino-flunitrazepam	34084-50-9	2	0.03	2	0.04
118	Lorazepam	846-49-1	n.d.		2.5	0.01
119	Midazolam	59467-70-8	n.d.		2	0.02
120	Temazepam	846-50-4	≥5	0.03	5	0.03
121	Oxazepam	604-75-1	2.5	0.01	2.5	0.02
	Barbiturates					
122	Phenobarbital	50-06-6	2	0.01	2.5	0.02
123	Primidone	125-33-7	n.d.		2.5	0.10
124	Bemegride	64-65-3	2.5	0.68	2	0.05
	Antipsychotics					
125	Clozapine	5786-21-0	2	0.15	2	0.08
126	Quetiapine	111974-69-7	≥5	0.02	2	0.01
127	Amisulpride	71675-85-9	≥5	0.07	≥5	0.07
128	Amisulpride-N-Oxide	71675-85-9	2	0.004	5	0.01
129	Sulpiride	15676-16-1	≥5	0.04	≥5	0.08
130	Haloperidol	52-86-8	2.5	0.0001	≥5	0.0005
131	Risperidone	106266-06-2	n.d.		2	0.003
132	Paliperidone (9-OH-Risperidone)	147687-18-1	n.d.		2	0.01
133	Buspirone	36505-84-7	≥5	0.02	n.d.	
134	Levomepromazine sulfoxide	7052-08-6	2	0.09	2.5	0.09
	Antiepileptic					
135	Carbamazepine	298-46-4	5	0.61	≥5	1.7
136	Carbamazepine-10,11-epoxid	36507-30-9	5	0.19	4.5	0.25

137	10-Hydroxy-carbamazepine	29331-92-8	5	0.42	5	1.00
138	Oxcarbazepine	28721-07-5	5	0.04	5	0.05
139	Topiramate	97240-79-4	5	0.40	≥5	0.61
140	Lamotrigine	84057-84-1	≥5	0.06	2.5	1.6
141	Levetiracetam	102767-28-2	≥5	0.59	2.5	0.12
142	Valproic acid	99-66-1	2.5	25	2.5	0.18
143	Phenytoin	57-41-0	n.d.		2	0.03
	Antidepressants					
144	Amitriptyline	50-48-6	≥5	0.23	5	0.11
145	Nortriptyline	894-71-3	2	0.004	2	0.01
146	Doxepine	1668-19-5	2	0.05	2	0.04
147	Mirtazapine	61337-67-5	≥5	0.33	≥5	0.49
148	8-OH-Mirtazapine	-	2.5	0.09	n.d.	
149	Desmethyl mirtazapine	61337-68-6	2	0.03	2	0.04
150	Maprotiline	10262-69-8	2	0.01	n.d.	
	SSRIs (serotonin replacing inhibitors)					
151	Citalopram	59729-33-8	≥5	1.0	≥5	1.0
152	Norcitalopram	144025-14-9	≥5	0.23	≥5	0.32
153	Sertraline	79617-96-2	2	0.10	2.5	0.02
154	Fluoxetine	54910-89-3	2.5	0.10	2.5	0.07
	SNRIs (serotonin-norepinephrine reuptake inhibitors)					
155	Duloxetine	116539-59-4	n.d.		2.5	0.005
156	Venlafaxine	93413-69-5	≥5	0.92	≥5	2.0
157	Venlafaxine-N-oxide	1094598-37-4	2	0.01	2.5	0.06
158	N,O- bisdesmethyl-venlafaxine	135308-74-6	5	0.12	5	0.16
159	N-Desmethylvenlafaxine	149289-30-5	4.5	1.9	5	6.5
160	O-desmethylvenlafaxine	93413-62-8	≥5	0.89	≥5	1.1
	Anesthetics					
161	Benzocaine	94-09-7	2	0.01	n.d.	
162	Bupivacaine	38396-39-3	2	0.004	n.d.	
163	Lidocaine	137-58-6	2.5	0.17	2.5	0.69
164	Mepivacaine	96-88-8	2.5		n.d.	
165	Prilocaine	721-50-6	2	0.005	2.5	0.006
166	Procaine	59-46-1	2.5	0.002	2.5	0.006
167	para-fluorofentanyl	90736-23-5	2	0.002	2.5	0.004
168	Norfentanyl	1609-66-1	n.d.		2.5	0.001
	Antiviral drugs					
169	Amantadine	768-94-5	5	0.06	5	0.09
170	Atazanavir	198904-31-3	2.5	0.02	2.5	0.05
171	Darunavir	206361-99-1	5	0.15	5	0.10
172	Ritonavir	155213-67-5	≥5	0.014	2.5	0.009

173	Emtricitabine	143491-57-0	5	0.33	5	0.15
174	Tenofovir	147127-20-6	2.5	0.30	n.d.	
	Hypertension- diuretic drug					
175	Aliskiren	173334-57-1	≥5	0.27	≥5	0.25
176	Valsartan	137862-53-4	≥5	0.66	5	0.92
177	Candesartan	139481-59-7	2	0.29	2	0.42
178	Telmisartan	144701-48-4	2.5	0.22	5	0.18
179	Verapamil	52-53-9	2.5	0.02	2	0.02
180	D617 (met. of verapamil)	34245-14-2	5	0.07	5	0.10
181	Eprosartan	133040-01-4	5	0.84	2.5	0.22
182	Irbesartan	138402-11-6	2	0.40	2	0.40
183	Diltiazem	42399-41-7	5	0.10	5	0.07
184	Nordiltiazem	-	≥5	0.03	2.5	0.02
185	Deacetyldiltiazem	42399-40-6	5	0.13	5	0.28
186	Phenoxybenzamine	59-96-1	2.5	0.41	2.5	0.45
187	Furosemide	54-31-9	≥5	0.03	2.5	0.03
188	Hydrochlorothiazide	58-93-5	≥5	0.28	≥5	0.32
189	Bendroflumethiazide	73-48-3	2.5	0.01	n.d.	
190	Acetazolamide	59-66-5	5	0.03	5	0.004
191	Amiloride	2016-88-8	2	0.05	2.5	0.03
192	Chlorthalidone	77-36-1	n.d.		2	0.008
	Antidiabetic drugs					
193	Sitagliptin	486460-32-6	≥5	0.48	n.d.	
194	Vildagliptin	274901-16-5	5	0.29	5	0.51
195	Pioglitazone	111025-46-8	2	0.004	2	0.004
196	Lacosamide	175481-36-4	4.5	0.02	5	0.04
197	Nateglinide	105816-04-4	2	0.005	n.d.	
198	Metformin	657-24-9	≥5	93	≥5	35
199	Guanylurea	926-72-7	2	0.74	≥5	5.0
	Antihistamine					
200	Hydroxyzine	68-88-2	2.5	0.004	n.d.	
201	Cetirizine	83881-52-1	≥5	0.14	5	0.18
202	Chlorpheniramine	132-22-9	2.5	0.01	2.5	0.008
203	Crotamiton	483-63-6	2.5	0.01	5	0.01
204	Diphenhydramine	58-73-1	≥5	0.04	≥5	0.04
205	Orphenadrine	83-98-7	≥5	0.05	≥5	0.04
206	Norорphenadrine (Tofenacin, Elamol)	15301-93-6	2	0.007	2.5	0.01
	Antiulcer					
207	Cimetidine	51481-61-9	2	0.07	2.5	0.50
208	Ranitidine	66357-35-5	≥5	2.6	≥5	1.0
209	Ranitidine-S-oxide	73851-70-4	2	0.17	2.5	0.11
	Cardiovascular diseases-intravascular					

210	Rosuvastatin	287714-41-4	2.5	0.17	2	0.13
211	Atorvastatin	134523-00-5	≥5	1.5	n.d.	
212	Gemfibrozil	25812-30-0	≥5	0.24	2	0.05
213	Fenofibric acid	49562-28-9	≥5	0.61	2	0.30
214	Propafenone	54063-53-5	≥5	0.56	≥5	0.53
215	Iopromide	73334-07-3	≥5	1.6	≥5	0.94
216	Clopidogrel Carboxylic acid	144457-28-3	≥5	0.60	≥5	0.56
	CNS stimulants					
217	Caffeine	58-08-2	≥5	9.6	5	3.0
218	Paraxanthin (1,7-dimethylxanthine)	611-59-6	≥5	5.9	≥5	0.92
219	Theophylline (1,3-dimethylxanthine)	58-55-9	≥5	2.0	n.d.	
220	Pentoxyfylline	*6493-05-06	5	0.64	n.d.	
221	Nicotine	54-11-5	≥5	13.0	≥5	0.93
222	Cotinine	486-56-6	5	9.1	5	0.54
223	Hydroxycotinine	34834-67-8	≥5	11.8	2.5	0.07
	Analgesics-NSAIDs					
224	O-N-bisdesmethyltramadol	-	2.5	0.02	2.5	0.02
225	O-desmethyltramadol	73986-53-5	2.5	0.03	5	0.01
226	N-desmethyltramadol	75377-45-6	2.5	0.01	2.5	0.01
227	Tramadol-N-oxide	147441-56-3	2	0.10	2.5	0.12
228	Salicylic acid	69-72-7	5	5.4	≥5	0.14
229	Paracetamol	103-90-2	≥5	4.8	2.5	0.14
230	4-Acetamidoantipyrine	83-15-8	5	0.07	5	0.09
231	4-Formylaminoantipyrine	1672-58-8	2.5	0.02	≥5	0.03
232	Isopyrin (4-Isopropyl-aminoantipyrine)	3615-24-5	n.d.		2.5	0.02
233	Meptazinol	54340-58-8	n.d.		2.5	0.003
234	Pethidine	57-42-1	2	0.001	2.5	0.003
235	Salicylamide	65-45-2	2	0.01	2.5	0.11
236	Diclofenac	15307-86-5	2.5	0.08	n.d.	
237	Fenbufen	36330-85-5	≥5	1.7	5	0.39
238	Fenoprofen	29679-58-1	2.5	5.1	2.5	2.0
239	Flufenamic acid	530-78-9	2.5	0.02	2.5	0.03
240	Flurbiprofen	51543-39-6	2	0.48	n.d.	
241	Ibuprofen	15687-27-1	≥5	1.1	n.d.	
242	Indoprofen	31842-01-0	2	0.22	n.d.	
243	Ketoprofen	22071-15-4	2.5	0.11	n.d.	
244	Meclofenamic Acid	644-62-2	2.5	0.02	n.d.	
245	Mefenamic acid	61-68-7	5	0.51	5	0.05
246	Naproxen	22204-53-1	5	0.93	2	0.05
247	Niflumic acid	4394-00-7	≥5	0.14	≥5	0.27

248	Nimesulide	51803-78-2	n.d.		2.5	0.098
249	Sulindac	38194-50-2	2	0.002	n.d.	
250	Oxaprozin	21256-18-8	2.5	0.44	n.d.	
251	Antipyrine /Phenazone	60-80-0	n.d.		2	0.05
	beta-blockers					
252	Albuterol	18559-94-9	2	0.005	2.5	0.02
253	Atenolol	29122-68-7	≥5	1.65	≥5	1.07
254	Atenolol acid (Metoprolol acid)	63659-18-7	5	0.47	≥5	0.12
255	Betaxolol	63659-18-7	2	0.008	n.d.	
256	Bisoprolol	66722-44-9	≥5	0.03	≥5	0.07
257	Carteolol	51781-06-7	2.5	0.002	n.d.	
258	Celiprolol	56980-93-9	≥5	0.42	≥5	0.33
259	Metoprolol	37350-58-6	5	0.81	≥5	1.3
260	Pindolol	13523-86-9	2	0.001	n.d.	
261	Propranolol	525-66-6	5	0.13	2.5	0.21
262	Salbutamol	18559-94-9	2.5	1.2	2.5	0.72
263	Sotalol	3930-20-9	5	0.43	5	0.55
264	Esmolol	103598-03-4	2.5	0.002	2.5	0.01
	Antibiotics					
265	Azithromycin	83905-01-5	2.5	0.03	≥5	0.06
266	Roxithromycin	80214-83-1	5	0.02	2	0.03
267	Clarithromycin	81103-11-9	≥5	2.7	≥5	2.4
268	N-desmethyl Clarithromycin	101666-68-6	≥5	0.72	5	0.93
269	Sulfadiazine	68-35-9	5	0.04	5	0.02
270	N4-Acetylsulfadiazine	127-74-2	2.5	0.07	2.5	0.46
271	Sulfadimidine	57-68-1	2	0.0008	5	0.005
272	N4-Acetylsulfamethazine (N4-Acetylsulfadimidine)	100-90-3	2	0.03	2.5	0.08
273	Sulfamethoxazole	723-46-6	5	0.09	≥5	0.36
274	N4-Acetyl-sulfamethoxazole	21312-10-7	2.5	0.03	2	0.02
275	Sulfapyridine	144-83-2	≥5	0.03	≥5	0.06
276	Trimethoprim	738-70-5	5	0.06	≥5	0.41
277	Linezolid	165800-03-3	2	0.03	2.5	0.06
278	Metronidazole	443-48-1	2.5	0.17	5	0.13
279	Ternidazol	1077-93-6	2	0.03	n.d.	
280	Nigericin	28380-24-7	2	0.41	2.5	0.84
281	Levamisole	14769-73-4	2	0.06	≥5	0.10
	Antibacterial - veterinary drugs					
282	Enrofloxacin	93106-60-6	2.5	0.02	n.d.	
283	Marbofloxacin	115550-35-1	2	0.05	n.d.	
284	Triclocarban	101-20-2	2	0.11	2.5	0.35

285	Triclosan	3380-34-5	2	0.08	2.5	0.08
286	Decoquinate	18507-89-6	n.d.		2	0.099
	Anticonvulsant					
287	Pregabalin	148553-50-8	5	0.68	5	0.45
288	Gabapentin	60142-96-3	≥5	0.79	≥5	0.24
289	Warfarin	81-81-2	2.5	1.9	n.d.	
	Chemotherapeutic-anti-cancer drugs					
290	Cytarabin	147-94-4	2	0.90	n.d.	
291	Ifosfamide	3778-73-2	2.5	0.10	2.5	0.28
292	Cyclophosphamide	50-18-0	2.5	0.009	2.5	0.03
	Other drugs					
293	Memantine	19982-08-2	2.5	0.04	2.5	0.06
294	Acamprosate	77337-76-9	2	0.52	2	0.84
295	Fluocinolone acetonide	67-73-2	n.d.		2.5	0.01
296	Benserazide	14919-77-8	2	0.48	2	1.47
297	Benzamidine	618-39-3	2.5	0.70	2	0.65
298	Dextromethorphan	125-71-3	n.d.		2	0.0017
299	Vigabatrin	60643-86-9	2	0.18	n.d.	
300	Guaifenesin	93-14-1	2.5	0.56	n.d.	
301	Piracetam	7491-74-9	2.5	0.03	2.5	0.33
	Steroids					
302	17β-Estardiol (E2)	50-28-2	2.5	0.59	2.5	0.60
303	Prednisolone	50-24-8	2.5	2.2	n.d.	
304	Drostanolone metabolite	-	2.5	0.02	2.5	0.03
305	Mesterolone metabolite	-	2.5	0.07	2.5	0.13
306	Progesterone	57-83-0	2.5	1.71	n.d.	
307	19-Norandrosterone	1225-01-0	n.d.		2.5	1.04
308	allo-THF (Allotetrahydrocortisol)	302-91-0	2.5	1.47	n.d.	
309	THE (Tetrahydrocortisone)	200-161-9	≥5	0.98	n.d.	
310	THF (Tetrahydrocortisol)	53-02-1	2.5	2.8	n.d.	
	PFCs					
311	PFBuS	375-73-5	2.5	0.007	5	0.006
312	PFDeA	335-76-2	5	0.05	2	0.04
313	PFHpA	375-85-9	2	0.006	2	0.006
314	PFHps	335-77-3	2.5	0.0007	2	0.0005
315	PFHxA	307-24-4	2	0.002	5	0.004
316	PFHxS	355-46-4	2.5	0.005	2	0.004
317	PFNA	375-95-1	2	0.010	2	0.01
318	PFOA	2395-00-8	≥5	0.008	5	0.006
319	PFOS	1763-23-1	2.5	0.03	2.5	0.004
320	PFPeA	2706-90-3	2	0.002	4.5	0.002
321	PFUnA	2058-94-8	n.d.		2	0.0003

	Sweeteners					
322	Acesulfame	33665-90-6	≥5	1.9	≥5	0.64
323	Cyclamate	139-05-9	5	24	2.5	1.0
324	Saccharine	81-07-2	2.5	3.1	2.5	0.011
325	Sucralose	56038-13-2	2.5	0.60	2.5	0.98
	Industrial Chemicals					
326	Benzotriazole (BTR)	95-14-7	2	0.04	5	0.49
327	1-Hydroxy-Benzotriazole	2592-95-2	2	0.17	2.5	0.16
328	4-Hydroxy-Benzotriazole	26725-51-9	2	0.27	2.5	0.64
329	4-Me-Benzotriazole/ 5-Me Benzotriazole	29385-43-1	2	0.0009	≥5	1.2
330	Benzothiazole (BTH)	95-16-9	2.5	0.05	2.5	0.07
331	2-Amino-Benzothiazole	136-95-8	≥5	0.07	≥5	0.09
332	2-Me-S-Benzothiazole	615-22-5	≥5	0.06	≥5	0.03
333	2-OH-Benzothiazole	934-34-9	2	0.15	2.5	0.21
334	4-tert-octylphenol (4-t-OP)	27193-28-8	≥5	1.2	≥5	0.51
335	4-nonylphenol (4-NP)	104-40-5	≥5	0.07	2	0.03
336	4-Nonylphenol-mono-ethoxylate	104-35-8	2	0.01	2	0.004
337	Melamine	108-78-1	2	0.65	2	0.66
338	Bisphenol A	80-05-7	5	0.03	n.d.	
339	Benzenesulfonamide	98-10-2	2.5	0.12	2	0.07
340	o-toluenesulfonamide	88-19-7	2	0.13	2	0.09
341	Galaxolidone	-	2.5	0.59	2.5	0.60
342	Diethyl phthalate	84-66-2	5	2.0	5	1.8
343	Dimethyl phthalate	131-11-3	2.5	0.02	2.5	0.02
344	Di-n-butyl phthalate	84-74-2	5	0.33	5	1.1
345	Benzyl butyl phthalate	85-68-7	≥5	0.05	≥5	0.08
346	Triethylphosphate	78-40-0	2.5	0.05	5	0.05
347	Triphenyl phosphate (TPP)	115-86-6	5	0.05	5	0.12
348	Benzophenon 3 (2-Hydroxy-4-methoxybenzophenon)	131-57-7	2.5	1.5	n.d.	0.20
349	Prolinamide	51-06-9	2	4.1	2	1.5
350	Benzoic acid	65-85-0	2.5	49	2.5	29
351	2-Aminobenzimidazole	934-32-7	n.d.		2.5	0.13
352	Tributylamine	102-82-9	2.5	0.008	2.5	0.01
353	Benzyl-dimethyl-dodecylammonium	139-07-1	2	0.07	n.d.	
354	Didecyldimethylammonium (DADMAC (C10:C10))	2390-68-3	2	0.02	2	0.01
355	2-Aminoheptane	123-82-0	5	0.64	2	0.16
356	4-Piperidin carboxamide	39546-32-2	2	1.0	2	0.95
357	Ethyl sulfate	540-82-9	2.5	3.6	2	1.3

	Aminoacids - Naturally occurring compounds					
358	Alanine (Ala)	56-41-7	2.5	11	n.d.	
359	g-Aminobutiric acid (GABA)	56-12-2	2.5	7.2	2.5	13
360	Glutamic acid (Glu)	56-86-0	2	13	2	4.5
361	Methionine (Met)	63-68-3	2.5	2.3	2	0.06
362	Proline (Pro)	147-85-3	2	7.8	n.d.	
363	Serine (Ser)	56-45-1	2	2.8	n.d.	
364	Valine (Val)	72-18-4	≥5	59	2	48
365	Leucine (Leu)	328-39-2	2.5	12	n.d.	
366	Adenosine	58-61-7	≥5	0.61	2.5	0.58
367	Resveratrol	501-36-0	2.5	0.11	2.5	0.12
368	1,4-butanediol (1,4 BD)	110-63-4	2	0.0006	n.d.	
369	2-Phenylphenol	90-43-7	2.5	0.09	n.d.	
370	Dimethylaniline	95-68-1	2.5	0.02	n.d.	
371	2-Phenethylamine	64-04-0	2.5	0.09	2	0.12

4.5.7. Quantitation of analytes

Quantification of the analytes was performed with comparison of the peak areas in the sample with those in a spiked sample. The concentrations of the analytes in the spiked sample ranged depending on the estimated concentration of every analyte.

As shown in Table 4.7, the concentrations of the analytes range in influent from 93 µg/L (metformin) to 0.14 ng/L (haloperidol) and in effluent from 48 µg/L (valine) to 0.22 ng/L (acetochlor). In Figure 4.5, the distribution of the concentrations of the analytes from the sub-ng level until some mg is presented. Apart from metformin, also valproic acid and caffeine are the most abundant drugs in influent wastewater. In effluent, metformin together with its metabolite gualynurea and N-desmethylvenlafaxine present the higher concentrations. For pesticides, most abundant compounds were fluometuron, azoxystrobin and a metabolite of dimethachlor, both in influent and effluent wastewaters. Sweeteners are also present in high concentration (0.6 µg/L sucralose to 24 µg/L cyclamate), but they are removed more than 60 % during the treatment. Benzoic acid is the most abundant from the rest of the chemicals, at concentrations 49 µg/L and 29 µg/L in influent and

effluent wastewater, respectively. It is mainly consumed, at a big extend, in the production of other chemicals. An interesting chemical that is present in high concentrations is ethyl sulfate, which is a minor metabolite of human after alcohol consumption [211]. Aminoacids are not emerging pollutants, but they are present in wastewater in very high concentrations. In influent, the concentrations are above 2 μg/L for the aminoacids detected and while significant removal is observed, in effluent, valine and g-aminobutiric acid (GABA) are at concentrations over 10 μg/L.

In an overview of the literature from 2005, Hernandez at al. reported that most methods in environmental analysis focus on the quantification of a limited number of contaminants (fewer than 100 compounds) [158]. Quantification results in HRMS screening methods are reported in effluent wastewater, where 15 out of 98 target analytes were detected and quantified, among them PFCs, pharmaceuticals and pesticides [212]. In lake sediments, 13 out of 180 target compounds were detected with concentration ranging from pg/gdw to ng/gdw [121, 213]. 23 compounds were also detected and quantified in effluent wastewaters and river waters, with concentration, comparable to our study, in the ng/L to mg/L level [119].

Removal rates were also calculated. More than half (53.5%) of the compounds are removed during the wastewater treatment. 66 compounds are satisfactorily removed (>80%) while more than 30% of the analytes are formed during the treatment. 16 compounds are only detected in effluent wastewater, among them 4 pesticides and 5 TPs.

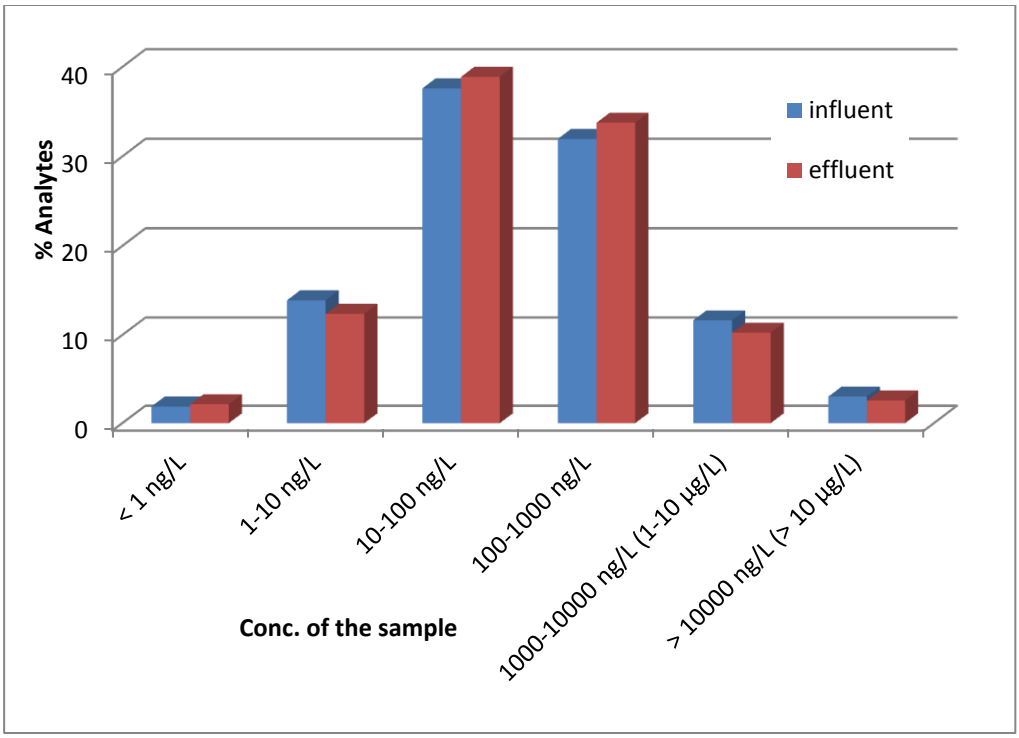


Figure 4.5. Distribution of the concentrations of the analytes in the samples

CHAPTER 5

Suspect screening workflow for the characterization of emerging polar organic contaminants in wastewater samples with UPLC-HRMS/MS

5.1. Introduction

The chemical pollutants that are regulated under international legislation represent a very small fraction of the universe of chemicals that occur in the environment as a result of human activities. Most regulatory bodies responsible for water and wastewater treatment still work on the assumption that a few well-known compounds such as the priority pollutants of the water framework directive are responsible for the most significant environmental, human health and economic risks, even though they only represent a tiny fraction of both known and yet-to-be identified chemicals [4].

Numerous organic contaminants are present in wastewater including many emerging pollutants, related transformation products (TPs) [140, 212] and human metabolites. If removal is incomplete in the wastewater treatment plant (WWTP), these (mostly) synthetic organic chemicals are released into the aquatic environment, resulting in a major source of contamination. Thus, the fate of emerging pollutants and their TPs in WWTPs is of paramount environmental importance.

Only a relatively small proportion of these organic contaminants can be determined using the existing target screening methods, where the chemicals to be analyzed are selected in advance. This can result in bias due to the focus on preselected analytes, such that large classes of potential chemical stressors cannot be considered. Therefore, one of the hottest trends in environmental analysis is the use of high resolution mass spectrometry (HR-MS) coupled with liquid chromatography (LC) to screen samples for suspect pollutants. In suspect screening, the name and the molecular formula are known but no reference standard is available. One step further, in non-target screening, no preselection of pollutants is performed [193]. To

obtain a holistic view of risk, target-based environmental monitoring needs to be accompanied by suspect or even non-target analysis.

Different comprehensive and semi-automated strategies (generally) combining target and suspect screening have been developed in the last few years. These approaches, based on LC-HRMS, allow the evaluation of the potential presence of a larger number of substances without necessarily purchasing the standards for all of them. Instead, purchase and confirmation can be restricted to substances which there are solid evidence that they may be present in the samples. The validation and application of suspect screening methods have been carried out focusing on specific categories of substances including pesticides [199], pharmaceuticals [207], iodinated contrast media photodegradation products [214] and transformation products [73] in surface or natural waters, as well as pesticides and pharmaceuticals in effluent wastewater [117]. Other studies considered a wide scope of suspects [104, 140, 212, 213, 215]. These strategies led to the detection of some TPs in the environment for the first time.

Schymanski et al. [140] and Hug et al. [212] performed target, suspect and non-target screening using a LTQ Orbitrap, with both studies successfully identifying suspect and non-target compounds in wastewater, including confirmation with reference standards in some cases. In general, however, the identification of suspect or unknown compounds is s a very difficult and time consuming task with no guarantee of success [120] and this was reflected in the suspect and non-target screening results of 17 groups performing LC-MS on a surface water sample in a recent collaborative trial [31]. With the increasing interest in suspect and non-target workflows, there is also a need to communicate the confidence in the identifications in a way that reflects the evidence available [129].

5.2. Scope of this study

None of the aforementioned studies focused on the analysis of influent wastewaters (IWW). The analysis of micropollutants present in influents provides valuable information about patterns of human consumption of several substances (e.g

pharmaceuticals, drugs of abuse). Here, the analysis of human metabolites is of paramount importance. Thus, the main objectives of the present work were (i) the development of additional strategies to support identification of suspect organic compounds and (ii) the actual identification of these substances in wastewater using an integrated workflow based on liquid chromatography - quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS).

An in-house database with eight high consumption pharmaceuticals (amisulpride, atenolol, clarithromycin, metformin, niflumic acid, ranitidine, tramadol and venlafaxine), plus nicotine and their related human metabolites (284 suspects in total) was developed, based on a metabolite prediction software and pharmacokinetic literature. Apart from the usual criteria used in suspect analysis (e.g. mass accuracy or isotopic fit) new specific strategies were applied in the tentative identification in order to increase the identification confidence of these compounds. One investigated hypothesis was that both the parent compounds and their related metabolites follow similar diurnal or/and weekly concentration trends in influents. The complementary use of HILIC was also investigated as well as the comparison of the spectra obtained for a given substance in ESI (+) and ESI (-), when possible, and retention time plausibility, using an in-house developed QSRR prediction model. Moreover, since a high proportion of surfactants were observed among the tentatively identified non-target substances, a retrospective suspect screening was performed for these compounds.

5.3. Experimental part

5.3.1. Chemicals and Reagents

Chemicals and reagents are described in detail in section 4.3.1, in target screening chapter.

5.3.2. Substance selection for the suspect screening

For the evaluation of the suspect screening workflow, 173 reference standards were used (Table 5.1). All pharmaceuticals were of high purity grade (>90 %) and were purchased from Sigma–Aldrich (Athens, Greece) and LGC Promochem (Molsheim, France) with the exception of sulfadoxine and sulfaclozine, which were donated by the National Laboratory of Residue Analysis of Food of Animal Origin of the Hellenic Ministry of Rural Development and Food. Regarding psychotropic and illicit drugs, all the compounds were of high purity (98 %). Solutions or solids were purchased from LGC Promochem (Molsheim, France) with the exception of topiramate and lamotrigine, which were obtained from Glenmark (Mahwah, NJ, USA) and Sigma–Aldrich Chemie GmbH (Steinheim, Germany), respectively. 1H-benzotriazole (99%) and 2-OH-benzothiazole (98%) were purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). 5-Me-1H-benzotriazole (98%) and 2-amino- benzothiazole (97%) were purchased from Acros Organics (Morris Plains, NJ) and 1-OH-benzotriazole (≥ 98%) and chlorobenzotriazole (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). All the additional analytes were purchased from Sigma-Aldrich (Steinheim, Germany).

Table 5.1 List of artificial suspect compounds.

	Compound	Molecular Formula	CAS number
1	1-OH-Benzotriazole	$C_6H_5N_3O$	2592-95-2
2	2-Amino-Benzothiazole	$C_7H_6N_2S$	136-95-8
3	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	$C_{20}H_{23}N$	30223-73-5
4	2-OH-Benzothiazole	C_7H_5NOS	934-34-9
5	3,4-methylenedioxyamphetamine	$C_{10}H_{13}NO_2$	4764-17-4
6	3,4-methylenedioxy-N-ethylamphetamine	$C_{12}H_{17}NO_2$	82801-81-8
7	3,4-methylenedioxy-N-methylamphetamine	$C_{11}H_{15}NO_2$	42542-10-9
8	5-Me-Benzotriazole	$C_7H_7N_3$	136-85-6
9	6-Monoacetylmorphine	$C_{19}H_{21}NO_4$	2784-73-8
10	7-amino-flunitrazepam	$C_{16}H_{14}FN_3O$	34084-50-9
11	8-OH-Mirtazapine	$C_{17}H_{19}N_3O$	*not available*
12	9-OH-Risperidone	$C_{23}H_{27}FN_4O_3$	144598-75-4
13	Albendazole	$C_{12}H_{15}N_3O_2S$	54965-21-8
14	Albendazole sulfone	$C_{12}H_{15}N_3O_4S$	75184-71-3

15	Alprazolam	$C_{17}H_{13}ClN_4$	28981-97-7
16	Amitriptyline	$C_{20}H_{23}N$	50-48-6
17	Amphetamine	$C_9H_{13}N$	300-62-9
18	Arprinocid	$C_{12}H_9ClFN_5$	55779-18-5
19	Atenolol	$C_{14}H_{22}N_2O_3$	29122-68-7
20	Atorvastatin	$C_{33}H_{35}FN_2O_5$	134523-00-5
21	Atrazine	$C_8H_{14}ClN_5$	1912-24-9
22	Atrazine-desethyl	$C_6H_{10}ClN_5$	6190-65-4
23	Benzotriazole	$C_6H_5N_3$	95-14-7
24	Benzoylecgonine	$C_{16}H_{19}NO_4$	519-09-5
25	Bromazepam	$C_{14}H_{10}BrN_3O$	1812-30-2
26	Bromohexine	$C_{14}H_{20}Br_2N_2$	3572-43-8
27	Caffeine	$C_8H_{10}N_4O_2$	58-08-2
28	Carbamazepine	$C_{15}H_{12}N_2O$	298-46-4
29	Carbaryl	$C_{12}H_{11}NO_2$	63-25-2
30	Carprofen	$C_{15}H_{12}ClNO_2$	53716-49-7
31	Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	56-75-7
32	Chloro-benzotriazole	$C_6H_4ClN_3$	94-97-3
33	Chlorpromazine	$C_{17}H_{19}ClN_2S$	50-53-3
34	Chlordiazepoxide	$C_{16}H_{14}ClN_3O$	58-25-3
35	Cimetidine	$C_{10}H_{16}N_6S$	51481-61-9
36	Citalopram	$C_{20}H_{21}FN_2O$	59729-33-8
37	Clarithromycin	$C_{38}H_{69}NO_{13}$	81103-11-9
38	Clazuril	$C_{17}H_{10}Cl_2N_4O_2$	101831-36-1
39	Clobazam	$C_{16}H_{13}ClN_2O_2$	22316-47-8
40	Clofibric acid	$C_{10}H_{11}ClO_3$	882-09-7
41	Clomipramine	$C_{19}H_{23}ClN_2$	303-49-1
42	Clopidol	$C_7H_7Cl_2NO$	2971-90-6
43	Closantel	$C_{22}H_{14}Cl_2I_2N_2O_2$	145149-50-4
44	Clozapine	$C_{18}H_{19}ClN_4$	5786-21-0
45	Cocaine	$C_{17}H_{21}NO_4$	50-36-2
46	Codeine	$C_{18}H_{21}NO_3$	76-57-3
47	Colchicine	$C_{22}H_{25}NO_6$	64-86-8
48	Coumaphos	$C_{14}H_{16}ClO_5PS$	56-72-4
49	Cyclamate	$C_6H_{13}NO_3S$	139-05-9
50	Dapsone	$C_{12}H_{12}N_2O_2S$	80-08-0
51	Decoquinate	$C_{24}H_{35}NO_5$	18507-89-6
52	Diaveridine	$C_{13}H_{16}N_4O_2$	5355-16-8
53	Diazepam	$C_{16}H_{13}ClN_2O$	439-14-5
54	Diclazuril	$C_{17}H_9Cl_3N_4O_2$	10320-42-0
55	Diclofenac	$C_{14}H_{11}Cl_2NO_2$	15307-86-5
56	Dimethoate	$C_5H_{12}NO_3PS_2$	60-51-5
57	Dimetridazole	$C_5H_7N_3O_2$	551-92-8

58	Diuron	$C_9H_{10}Cl_2N_2O$	330-54-1
59	Doxepin	$C_{19}H_{21}NO$	1668-19-5
60	Ecgonine methyl ester (EME)	$C_{10}H_{17}NO_3$	7143-09-1
61	Ephedrine	$C_{10}H_{15}NO$	299-42-3
62	Ethopabate	$C_{12}H_{15}NO_4$	59-06-3
63	Fenbendazole	$C_{15}H_{13}N_3O_2S$	43210-67-9
64	Fenoxycarb	$C_{17}H_{19}NO_4$	79127-80-3
65	Fentanyl	$C_{22}H_{28}N_2O$	437-38-7
66	Florfenicol	$C_{12}H_{14}Cl_2FNO_4S$	73231-34-2
67	Flubendazole	$C_{16}H_{12}FN_3O_3$	31430-15-6
68	Fludioxonil	$C_{12}H_6F_2N_2O_2$	131341-86-1
69	Flunitrazepam	$C_{16}H_{12}FN_3O_3$	1622-62-4
70	Flunixin	$C_{14}H_{11}F_3N_2O_2$	38677-85-9
71	Fluoxetine	$C_{17}H_{18}F_3NO$	54910-89-3
72	Flurazepam	$C_{21}H_{23}ClFN_3O$	17617-23-1
73	Furosemide	$C_{12}H_{11}ClN_2O_5S$	54-31-9
74	Gemfibrozil	$C_{15}H_{22}O_3$	25812-30-0
75	Hydrochlorthiazide	$C_7H_8ClN_3O_4S_2$	58-93-5
76	Ibuprofen	$C_{13}H_{18}O_2$	15687-27-1
77	Imidacloprid	$C_9H_{10}ClN_5O_2$	138261-41-3
78	Imipramine	$C_{19}H_{24}N_2$	50-49-7
79	Indapamide	$C_{16}H_{16}ClN_3O_3S$	26807-65-8
80	Iprodione	$C_{13}H_{13}Cl_2N_3O_3$	36734-19-7
81	Ketamine	$C_{13}H_{16}ClNO$	6740-88-1
82	Ketoprofen	$C_{16}H_{14}O_3$	22071-15-4
83	Lamotrigine	$C_9H_7Cl_2N_5$	84057-84-1
84	Levamisol	$C_{11}H_{12}N_2S$	14769-73-4
85	Levetiracetam	$C_8H_{14}N_2O_2$	102767-28-2
86	Lidocaine	$C_{14}H_{22}N_2O$	137-58-6
87	Lincomycin	$C_{18}H_{34}N_2O_6S$	154-21-2
88	Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	846-49-1
89	Lysergic acid dethylamide (LSD)	$C_{20}H_{25}N_3O$	50-37-3
90	Mebendazole	$C_{16}H_{13}N_3O_3$	31431-39-7
91	Mefenamic acid	$C_{15}H_{15}NO_2$	61-68-7
92	Meloxicam	$C_{14}H_{13}N_3O_4S_2$	71125-38-7
93	Metformin	$C_4H_{11}N_5$	657-24-9
94	Methacrifos	$C_7H_{13}O_5PS$	62610-77-9
95	Methadone	$C_{21}H_{27}NO$	76-99-3
96	Methamphetamine	$C_{10}H_{15}N$	537-46-2
97	Metoprolol	$C_{15}H_{25}NO_3$	37350-58-6
98	Metronidazol	$C_6H_9N_3O_3$	443-48-1
99	Midazolam	$C_{18}H_{13}ClFN_3$	59467-70-8
100	Mirtazapine	$C_{17}H_{19}N_3$	61337-67-5

101	Morantel	$C_{12}H_{16}N_2S$	20574-50-9
102	Morphine	$C_{17}H_{19}NO_3$	57-27-2
103	Naproxen	$C_{14}H_{14}O_3$	22204-53-1
104	Niflumic acid	$C_{13}H_9F_3N_2O_2$	4394-00-7
105	Nitrazepam	$C_{15}H_{11}N_3O_3$	146-22-5
106	Nitroxinil	$C_7H_3IN_2O_3$	1689-89-0
107	Norclozapine	$C_{17}H_{17}ClN_4$	6104-71-8
108	Nordiazepam	$C_{15}H_{11}ClN_2O$	1088-11-5
109	Norephedrine	$C_9H_{13}NO$	14838-15-4
110	Norfentanyl	$C_{14}H_20N_2O$	1609-66-1
111	Norketamine	$C_{12}H_{14}ClNO$	35211-10-0
112	Nortriptyline	$C_{19}H_{21}N$	72-69-5
113	Olanzapine	$C_{17}H_{20}N_4S$	132539-06-1
114	Oxazepam	$C_{15}H_{11}ClN_2O_2$	604-75-1
115	Oxfendazole	$C_{15}H_{13}N_3O_3S$	53716-50-0
116	Oxyclozanide	$C_{13}H_6Cl_5NO_3$	2277-92-1
117	Oxycodone	$C_{18}H_{21}NO_4$	76-42-6
118	Paracetamol	$C_8H_9NO_2$	103-90-2
119	Paroxetine	$C_{19}H_{20}FNO_3$	61869-08-7
120	Penconazole	$C_{13}H_{15}Cl_2N_3$	66246-88-6
121	Pentobarbital	$C_{11}H_{18}N_2O_3$	5767-32-8
122	Perfluoroheptanoic acid (PFHpA)	$C_7F_{13}O_2H$	375-85-9
123	Perfluorononanoic acid (PFNA)	$C_9F_{17}O_2H$	375-95-1
124	Perfluorooctanoic acid (PFOA)	$C_8F_{15}O_2H$	2395-00-8
125	Phenobarbital	$C_{12}H_{12}N_2O_3$	50-06-6
126	Phenytoin	$C_{15}H_{12}N_2O_2$	57-41-0
127	Primidone	$C_{12}H_{14}N_2O_2$	125-33-7
128	Procymidone	$C1_3H_{11}Cl_2NO_2$	32809-16-8
129	Prometryn (Caparol)	$C_{10}H_{19}N_5S$	7287-19-6
130	Propranolol	$C_{16}H_{21}NO_2$	525-66-6
131	Ranitidine	$C_{13}H_22N_4O_3S$	66357-35-5
132	Risperidone	$C_{23}H_{27}FN_4O_2$	106266-06-2
133	Ronidazole	$C_6H_8N_4O_4$	7681-76-7
134	Saccharine	$C_7H_5NO_3S$	6381-61-9
135	Salicylic acid	$C_7H_6O_3$	69-72-7
136	Sertraline	$C_{17}H_{17}Cl_2N$	79617-96-2
137	Sucralose	$C_{12}H_{19}Cl_3O_8$	56038-13-2
138	Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	23282-55-5
139	Sulfaclozine	$C_{10}H_9ClN_4O_2S$	102-65-8
140	Sulfadiazine	$C_{10}H_{10}N_4O_2S$	68-35-9
141	Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	122-11-2
142	Sulfadimidine	$C_{12}H_{14}N4O_2S$	57-68-1
143	Sulfadoxine	$C_{12}H_{14}N_4O_4S$	2447-57-6

144	Sulfamerazine	$C_{11}H_{12}N_4O_2S$	127-79-7
145	Sulfamethizole	$C_9H_{10}N_4O_2S_2$	144-82-1
146	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	723-46-6
147	Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	80-35-3
148	Sulfamonomethoxine	$C_{11}H_{12}N_4O_3S$	1220-83-3
149	Sulfamoxole	$C_{11}H_{13}N_3O_3S$	729-99-7
150	Sulfanilamide	$C_6H_8N_2O_2S$	63-74-1
151	Sulfapyridine	$C_{11}H_{11}N_3O_2S$	144-83-2
152	Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	59-40-5
153	Sulfathiazole	$C_9H_9N_3O_2S_2$	72-14-0
154	Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	127-69-5
155	Terbuthylazine	$C_9H_{16}ClN_5$	5915-41-3
156	Ternidazole	$C_7H_{11}N_3O_3$	1077-93-6
157	Theophylline	$C_7H_8N_4O_2$	58-55-9
158	Thiabendazole	$C_{10}H_7N_3S$	148-79-8
159	Thiamphenicol	$C1_2H_{15}Cl_2NO_5S$	847-25-6
160	Thiopental	$C_{11}H_{18}N_2O_2S$	59709-53-4
161	Tiamulin	$C_{28}H_{47}NO_4S$	55297-95-5
162	Tolfenamic acid	$C_{14}H_{12}ClNO_2$	13710-19-5
163	Toltrazuril	$C_{18}H_{14}F_3N_3O_4S$	69004-03-1
164	Topiramate	$C_{12}H_{21}NO_8S$	97240-79-4
165	Tramadol	$C_{16}H_{25}NO_2$	27203-92-5
166	Triamterene	$C_{12}H_{11}N_7$	396-01-0
167	Triclabendazole	$C_{14}H_9Cl_3N_2OS$	68786-66-3
168	Triclosan	$C_{12}H_7Cl_3O_2$	3380-34-5
169	Trimethoprim	$C_{14}H_{18}N_4O_3$	738-70-5
170	Valproic acid	$C_8H_{16}O_2$	99-66-1
171	Valsartan	$C_{24}H_{29}N_5O_3$	137862-53-4
172	Vedaprofen	$C_{19}H_{22}O_2$	71109-09-6
173	Venlafaxine	$C_{17}H_{27}NO_2$	93413-69-5

For the confirmation of suspect compounds, amisulpride-N-oxide, atenolol acid, N-desmethyl-clarithromycin, cotinine, nor-cotinine, ranitidine-S-oxide and guanylurea were provided by Eawag.

22 internal standards (IS) were used during the analysis of wastewater samples at a concentration of 1 µg L-1. The list of IS includes diuron-d6, atrazine-d5, sulfadimidine-d4, sulfadimethoxine-d4, sulfadiazine-d4, meloxicam-d3, flunixin-d3, olaquindox-d4, phenylbutazone-C13, MPFOA, acesulfame-d4, sucralose-d6, which

were purchased from Sigma-Aldrich (Steinheim, Germany) and morphine-d3, codeine-d6, cocaine-d3, diazepam-d5, THC-d3, THCA-d3, LSD-d3, ketamine-d4, oxazepam-d5, and MDMA-d5 which were obtained from LGC Promochem (Molsheim, France).

5.3.3. Samples and sample preparation

Suspect screening was performed in influent and effluent wastewater samples (24-hour composite flow proportional samples) that were collected from the WWTP of Athens (Greece) on the 15th of March 2014 (Saturday). Additionally, 2-hour flow proportional influent samples were also collected during the same day every two hours. Wastewater was collected in pre-cleaned high-density polyethylene (HDPE) bottles. Untreated and treated wastewaters were filtered with glass fiber filters (pore size 0.7 μm) immediately after arrival at the laboratory. Samples were stored in the dark at -18 °C until analysis.

Details regarding the WWTP of Athens are presented in detail in section 4.3.2, in target screening chapter.

Sample preparation is described in section 4.3.2, in target screening chapter.

5.3.4. Instrumental analysis

Analysis was carried out using a UHPLC/QTOF-MS system, equipped with a UHPLC apparatus (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany), consisting of a solvent rack degasser, auto-sampler, a binary pump with solvent selection valve and a column oven coupled to the QTOF-MS mass analyzer (Maxis Impact, Bruker Daltonics, Bremen, Germany).

In suspect screening, reverse-phase (RP) and HILIC chromatographic separation were used for the identification of suspect compounds.

In RP, the chromatographic separation is the same as in target screening analysis (see section 4.3.3).

In hydrophilic interaction liquid chromatography (HILIC), separation is performed on an ACQUITY UPLC BEH Amide column (2.1 × 100 mm, 1.7 µm) from Waters (Dublin, Ireland) preceded by a guard column of the same packaging material, kept at 40 ˚C. For (+) ESI, the aqueous phase consists of H_2O with 1 mM ammonium formate and 0.01% formic acid and the organic phase was ACN/$H_2$0 95/5 with 1 mM ammonium formate and 0.01% formic acid. For (-) ESI, the aqueous phase consists of H_2O with 10 mM ammonium formate and the organic phase was ACN/$H_2$0 95/5 with 10 mM ammonium formate. The adopted elution gradient, for both ionization modes, starts with 100% of organic phase and keeps stable for 2 minutes, decreasing to 5 % in 10 min, and keeps constant for the following 5 min. The initial conditions are restored within 0.1 min and let to re-equilibrate for 8 min. The flow rate is 0.2 mL min^{-1} and the injection volume is set to 5 µL.

The QTOF system was equipped with an electrospray ionization interface (ESI), operating in positive and negative mode, with the following operation parameters: capillary voltage 2500 V (+) ESI and 3500 (-) ESI; end plate offset, 500 V; nebulizer pressure 2 bar; drying gas 8 L min^{-1} and gas temperature 200 °C.

Full scan mass spectra were recorded over the range 50-1000 m/z with a scan rate of 2 spectra s^{-1}. MS/MS experiments were conducted using AutoMS acquisition mode (data-dependent). The collision energy applied was set to predefined values, according to the mass and the charge state of every ion. A QTOF external calibration was performed daily with the manufacturer's solution, as described previously. The instrument provided a typical resolving power (FWHM) between 36,000-40,000 at m/z 226.1593, 430.9137 and 702.8636. Mass spectra acquisition and data analysis was processed with DataAnalysis 4.1 and TargetAnalysis 1.3 (Bruker Daltonics, Bremen, Germany).

5.3.5. Processing of suspect substances

5.3.5.1. Suspect screening workflow

The suspect peak lists were obtained by using the function Find Compounds–Chromatogram (TargetAnalysis, Bruker Daltonics, Bremen, Germany), which creates the base peak chromatograms for the masses that accomplish thresholds of intensity previously selected, excluding the isotopic peaks. The tentative identification of these compounds was based on the evaluation of different criteria which are summarized in the flow chart presented in Figure 5.1. The criteria included (1) the subtraction of the compounds present in the processed method-procedural blanks and the application of peak area and intensity thresholds, (2) the application of mass accuracy threshold of 2 mDa and 5 ppm on the monoisotopic peaks, (3) the application of isotopic fitting threshold (≤100 mSigma), (4) the evaluation of the peak score, considering only peaks with the Peak Area/ Peak Intensity ratio greater than 4. Peak Area / Peak Intensity ratio is the threshold of peaks' (a)symmetry during the evaluation of the peak score for all the artificial suspect compounds. The range was 4-38 and as the mean peak score ± 3SD (conf. level 99%) is 20±12, the preferable peak score should be between 8-32. Then, (5) the evaluation of the chromatographic retention time plausibility, using an in-house QSRR retention time prediction model (CRTPM) is performed 19. The predicted t_R was considered to match if it was within ±3δ (standardized residual) of the measured value, as this covers 99.7% of normally distributed data. For most retention times, this is approximately equivalent to ± 2 min. Next step is the (6) evaluation of the presence of characteristics adducts: $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ in positive mode, and $[M-H]^-$, $[M-H_2O-H]^-$, $[M+Cl]^-$ and $[M+HCOOH-H]^-$ in negative mode and (7) MS/MS spectral interpretation, including comparisons with spectra from the MassBank database [141] and NIST 2014 library. Spectral similarity values were calculated with the OrgMassSpecR package in R language (http://CRAN.R-project.org/package=OrgMassSpecR) [216]; a threshold of 0.7 was used. Moreover other strategies, described in results and discussion section, were applied in order to increase the identification confidence. For the tentatively

identified compounds that were commercially available, the corresponding standard was purchased in order to confirm the identity of the substance (Level 1).

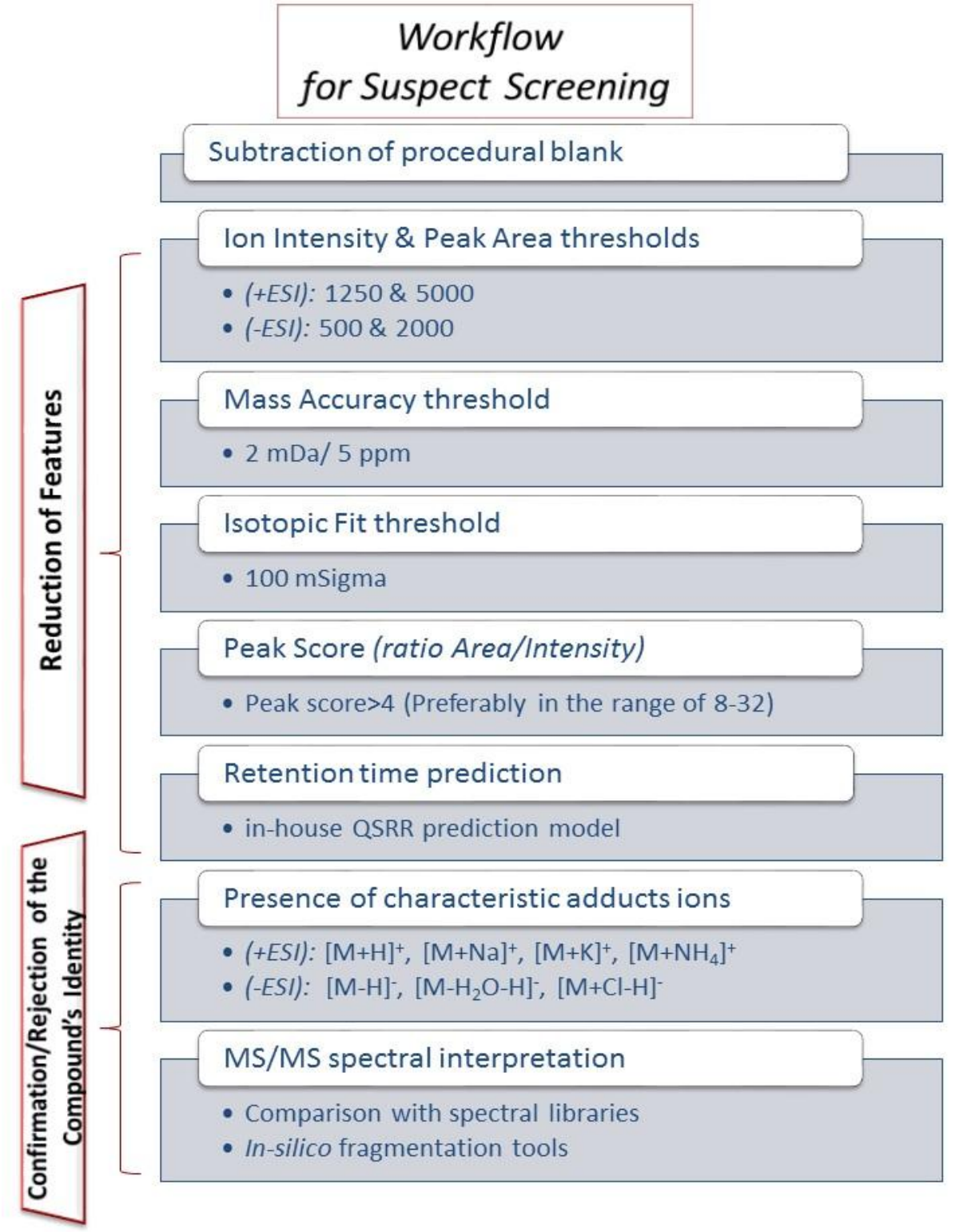


Figure 5.1 Suspect screening flow chart.

5.3.5.2. List of suspect substances

Firstly, a validation of the suspect screening method was performed using known target compounds (Table 5.1) to determine the successful identification rate. Secondly, a suspect database (hereafter “metabolite suspects”) was created for 9 substances and their related human metabolites. Eight high consumption pharmaceuticals (amisulpride, atenolol, clarithromycin, metformin, niflumic acid, ranitidine, tramadol and venlafaxine) and nicotine were selected. The related metabolites were obtained by reviewing the literature [74, 153, 217] and by using the MetabolitePredict software from Bruker (Version 2.0, Bruker Daltonics, Bremen, Germany). The full list, including the molecular formulas and the SMILES code (http://www.daylight.com/dayhtml/doc/theory/theory.smiles.html) (where possible) is presented in the Electronic Supplementary Material (Table S5.1). For metabolites with several possible substitution isomers, the number of structural isomers of the suspects is indicated and the SMILES code covers only one of these cases, the most likely substance, according to my knowledge. When a molecular formula was detected during suspect screening, all possible isomers were considered for the evaluation of the identity of the compound.

Finally, an additional suspect screening was performed retrospectively, as it became evident that surfactant peaks dominated the non-target results, that are not presented in the present thesis. The suspect surfactant list (Schymanski et al. [140]) included Linear Alkylbenzyl Sulfonates (LAS), SulfoPhenyl Alkyl Carboxylic acids (SPACs), SulfoPhenyl Alkyl Di-Carboxylic acids (SPADCs), Di-Alkyl Tetralin Sulfonates (DATS), Sulfo-Tetralin Alkyl Carboxylic acids (STACs), Sulfo-Tetralin Alkyl Di-Carboxylic acids (STADCs), Alkyl Sulfates (AS), Alkyl Ethoxy Sulfates (AES), Secondary Alkyl Sulfonates (SAS), and 15 NonylPhenol EthOxylate (NPEO) sulfates (NPEO-S). PEGs and AGNs were not included in the retrospective screening as they were studied in detail using the non-target approach. This list is hereafter called “suspect surfactants” in this manuscript.

5.3.5.3. Communication of the levels of confidence

The system presented by Schymanski et al. to communicate the level of confidence achieved in the identification of the detected compounds was used [129]. Level 1 corresponds to confirmed structures where a reference standard is available, level 2 to probable structures, level 3 for tentative candidate(s), level 4 to unequivocal molecular formulas and level 5 to exact mass(es) of interest, as presented in Figure 5.2.

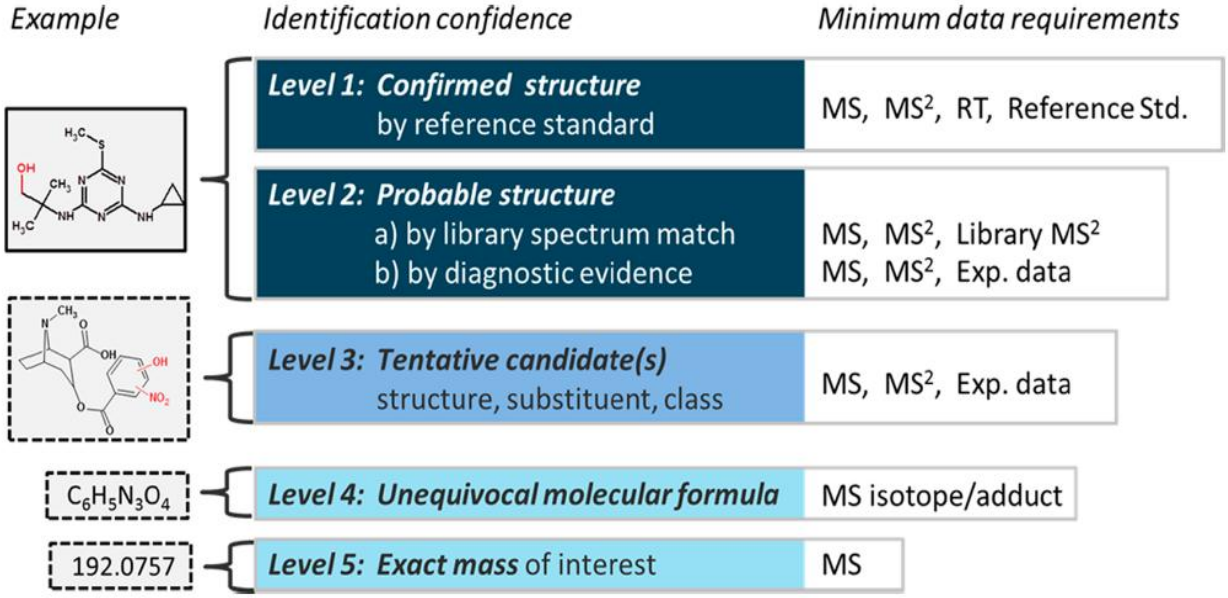


Figure 5.2 Identification levels [129].

5.4. Results and Discussion

5.4.1. Optimization and evaluation of the suspect screening approach

The suspect screening workflow was firstly evaluated with the 173 target compounds, applied as artificial suspects. The evaluation was carried out using TargetAnalysis (Bruker). The only a priori information was the exact mass of the protonated ion in (+) ESI, $[M+H]^+$, and the deprotonated ion in (-) ESI, [M-H]-, calculated from the chemical formula.

Different parameters were optimized to obtain an acceptable percentage of false negative results while minimizing the number of false positive results. First, the peak area and intensity thresholds were optimized using real wastewater spiked at 1 µg L^{-1} with the aim of succeeding false negative rate <5%. These values (area, 5000 (+) ESI & 2000 (-) ESI; intensity, 1250 (+) ESI & 500 (-) ESI were further applied to lower concentrations. Results are shown graphically in Figure 5.3. It can be observed that 94% of the compounds were retained at 0.05 µg L^{-1}. Then, mass accuracy (2 mDa and 5ppm) and isotopic fit (100 mSigma) thresholds were also applied. It can be observed that the false negative rate was below 5% for both of these parameters. The combination of the aforementioned filters led to an acceptable false negative rate of 10% at 0.05 µg L-1 as shown in Figure 5.3 (red line).

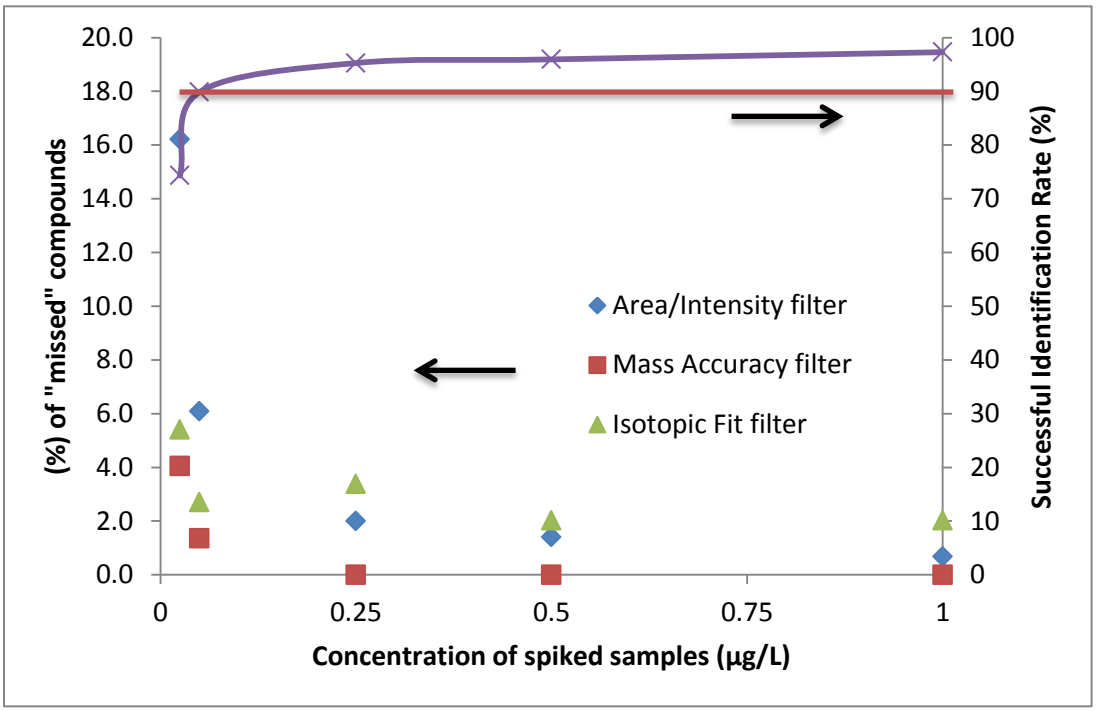


Figure 5.3 Successful identification rate of suspect screening by applying the screening filters at different concentrations of artificial suspect compounds in spiked effluent samples.

The reduction of features (false positives) due to the applied filters described in this section is summarized in Table 5.2. The cumulative reduction of hits ranged from

44% to 74% for the different evaluated concentrations. Further steps for the reduction of false positives will be discussed in the next section.

Table 5.2 Reduction of false positives after the application of the suspect screening filters at different concentrations of artificial suspect compounds in spiked effluent samples.

C (µg/L)	Total hits after the application of Area & Intensity filter	Hits after the application of Mass Accuracy filter	Hits after the application of Isotopic Fit filter	**(%) Cumulative Reduction of hits***
1	405	343	227	**44** (15[a], 34[b])
0.5	467	386	218	**53** (17[a], 44[b])
0.25	471	382	223	**53** (19[a], 42[b])
0.05	477	365	183	**62** (23[a], 50[b])
0.025	370	276	95	**74** (25[a], 66[b])

[a]*Reduction of hits (%) due to mass accuracy filter;*

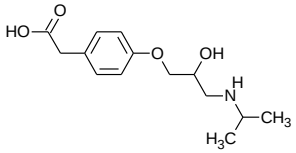
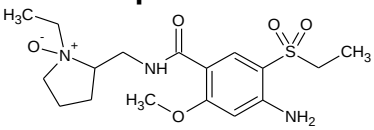
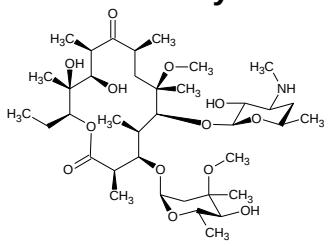
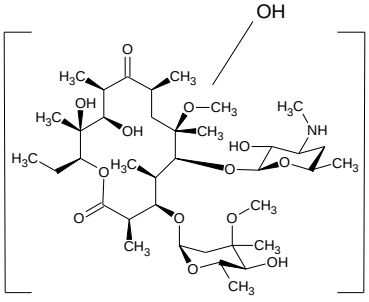
[b]*Reduction of hits (%) due to isotopic fit filter.*

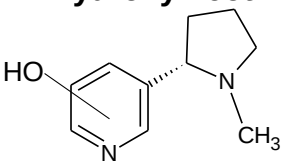
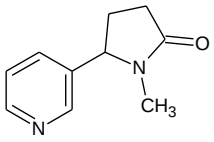
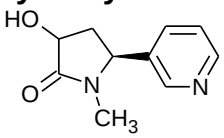
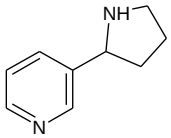
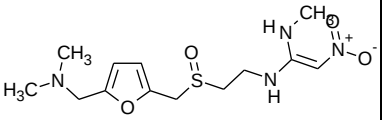
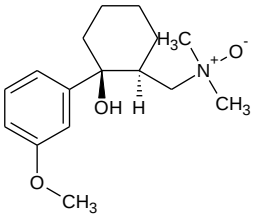
5.4.2. Suspect screening of wastewater samples for metabolites of pharmaceuticals

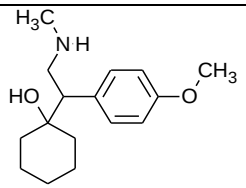
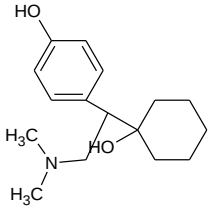
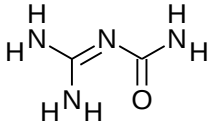
Suspect screening of the influent wastewater (IWW) sample using the “metabolite suspects” yielded 1660 hits in (+) ESI and 864 in (-) ESI applying only the accuracy threshold (2 mDa). After the application of steps 1-5 above (Figure 5.1), this number decreased to 79 hits in (+) ESI, corresponding to 37 compounds and 71 hits in (-) ESI, corresponding to 21 substances. The 37 and 21 substances remaining in both polarities, were then investigated closer based on steps 6 and 7 (presence of characteristic adducts and spectral interpretation). After the evaluation of all steps, 13 suspect compounds, only in (+) ESI, were tentatively identified that fulfilled all the criteria set. None of the evaluated substances was tentatively identified in (-) ESI, most probably due to the lower sensitivity on this operational mode. The identified

suspects corresponded to all but one of the selected parent compounds (niflumic acid) and are given in Table 5.3.

Table 5.3 Details on the 13 identified suspect metabolites.

Name, Structure and Formula	Parent	Exp. t_R (Pred. t_R)[a]	Additional Evidence[b]	Level
Atenolol acid  $C_{14}H_{21}NO_4$	*Atenolol*	5.1 (5.5)	-Similarity 0.92 with MassBank record EA069710[c]. -Confirmation with reference standard	1
Amisulpride-N-oxide  $C_{17}H_{27}N_3O_5S_1$	*Amisulpride*	5.6 (6.5)	- Confirmation with reference standard	1
N-desmethyl clarithromycin  $C_{37}H_{67}NO_{13}$	*Clarithro-mycin*	10.1 (9.6)	-Intra-day trend consistent with the parent compound[d] -Intra-week trend consistent with the parent compound[e] -Confirmation with reference standard	1
Hydroxyclarithromycin  $C_{38}H_{69}NO_{14}$	*Clarithro-mycin*	8.5 (9.2)	-Intra-day trend consistent with the parent compound[d] -Intra-week trend consistent with the parent compound[e]	3

Hydroxynicotine  $C_{10}H_{14}N_2O_2$	*Nicotine*	3.1 (2.3)	-	3
Cotinine  $C_{10}H_{12}N_2O$	*Nicotine*	4.9 (5.1)	-Similarity 0.99 with MassBank record WA000998[c] -Confirmation with reference standard	1
Hydroxycotinine  $C_{10}H_{12}N_2O_2$	*Nicotine*	3.9 (4.0)	- HILIC/RP elution supports properties[f] -Confirmation with reference standard	1
Nornicotine  $C_9H_{12}N_2$	*Nicotine*	2.5 (2.6)	-Similarity 0.77 with NIST record 1185301[b] -Feasible t_R[c]	2b
Ranitidine-S-oxide  $C_{13}H_{22}N_4O_4S_1$	*Ranitidine*	2.0 (2.9)	-Intra-week trend consistent with the parent compound[e] -Confirmation with reference standard	1
Tramadol-N-oxide  $C_{16}H_{25}NO_3$	*Tramadol*	6.0 (8.0)	-Intra-week trend consistent with the parent compound[e]	2b
N-desmethyl venlafaxine	*Venlafaxine*	7.4 (6.3)	-Similarity 0.96 with MassBank record EA103410[c] -Intra-day trend consistent with the	1

 $C_{16}H_{25}NO_2$			parent compound[d] -Intra-week trend consistent with the parent compound[e] -Confirmation with reference standard	
O-desmethyl venlafaxine  $C_{16}H_{25}NO_2$	*Venlafaxine*	6.0 (6.4)	-Similarity 0.98 with MassBank record EA105304[c] -Intra-day trend consistent with the parent compound[d] -Intra-week trend consistent with the parent compound[e] -Confirmation with reference standard	1
Guanylurea  $C_2H_6N_4O$	*Metformin*	1.3 (1.2)	- HILIC/RP elution supports properties[f] -Confirmation with reference standard	1

[a]All the compounds presented in this table showed feasible chromatographic retention times according to the model;

[b]Additional evidence apart from the visual evaluation of the MS/MS spectra and other previously discussed thresholds;

[c]Calculated similarity between the experimental spectra and the one obtained in the MS/MS database;

[d]By evaluating 2-hours composite samples collected every two hours during 24 hours;

[e]By evaluating 7 24-h composite samples corresponding to 7 consecutive days.

[f]Plausible elution times according to the physicochemical properties and identical MS/MS spectra.

Mass spectra were available for several of these compounds, allowing the assignment of a confidence level of 2a initially where the measured spectra matched the database spectra. As an example, Figure 5.4 shows the suspect compound cotinine, a metabolite of nicotine detected at high intensity in IWW and at low concentrations in EWW. A clear MS/MS spectrum was obtained in ESI(+) and matched well with the MassBank spectrum WA000998 (score = 0.99); thus level 2a could be reached. A commercial standard of cotinine was purchased and the identity

of the compound was confirmed via appropriate MS, MS/MS and t_R matching, reaching finally Level 1. Mass spectral matching led also to the identification with confidence level 2b of other suspect compounds such as atenolol acid, N-desmethyl venlafaxine, O-desmethyl venlafaxine and nornicotine.

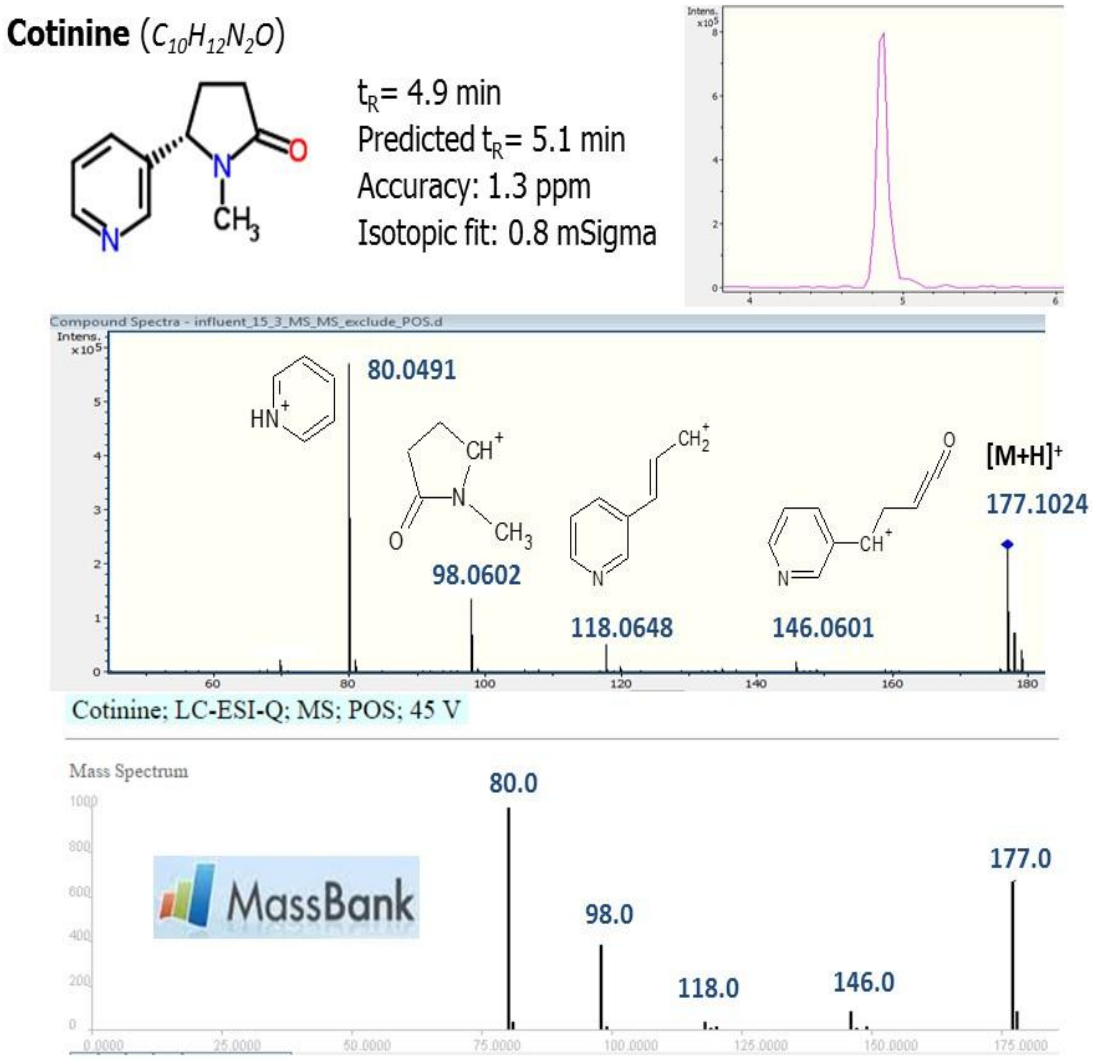


Figure 5.4 Identification of the suspect compound cotinine.

Where mass spectra were not available in libraries, other evidence was pursued to increase the confidence of the suspect identification. The spectrum of the tentatively identified metabolite hydroxycotinine (speculated to be 3- or 4-hydroxy-1-methyl-5-(3-pyridinyl)-2-pyrrolidinone) according to the fragments shown in Figure 5.5, had some peaks in common with the confirmed suspect cotinine (m/z 80.0493, 118.0647). Other distinct fragments (e.g. m/z 98.0602 and 146.0601) were no longer present and instead m/z 134.0591 and 149.0699 indicated that the hydroxylation occurred on the pyridinyl group, but two possible candidates remained, resulting in a Level 3. The measured t_R in RP was also within the range of the predicted values for this substance and the measured t_R in HILIC is plausible according to its physicochemical properties. The identity of 3-hydroxycotinine was confirmed through the purchase and analysis of the standard for this compound, reaching level 1.

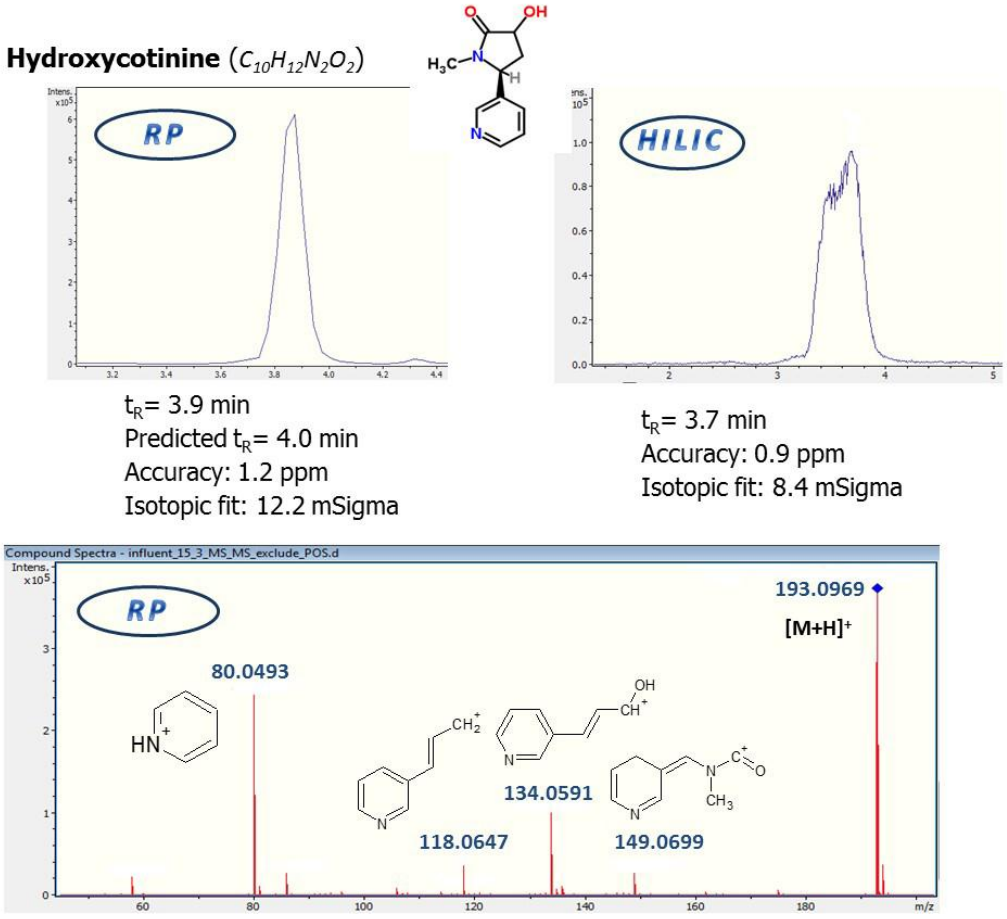


Figure 5.5 Identification of the suspect compound hydroxycotinine.

The complementary nature of HILIC and RP elution as well as the presence in influent and effluent samples was exploited in the identification of guanylurea, a metabolite of metformin. While guanylurea was only present at low levels in the influent (data not shown), it was present at much higher levels in the effluents at intensities higher than the parent metformin (Figure 5.6), demonstrating its formation during wastewater treatment. Guanylurea was detected at a low t_R (1.3 min) in the RP column and at 6.3 minutes in HILIC, in line with its physicochemical properties. As the presence of the peak at m/z 60.0552 is also present in the metformin MS/MS spectrum, the standard of guanylurea was purchased and the identification was confirmed to achieve Level 1.

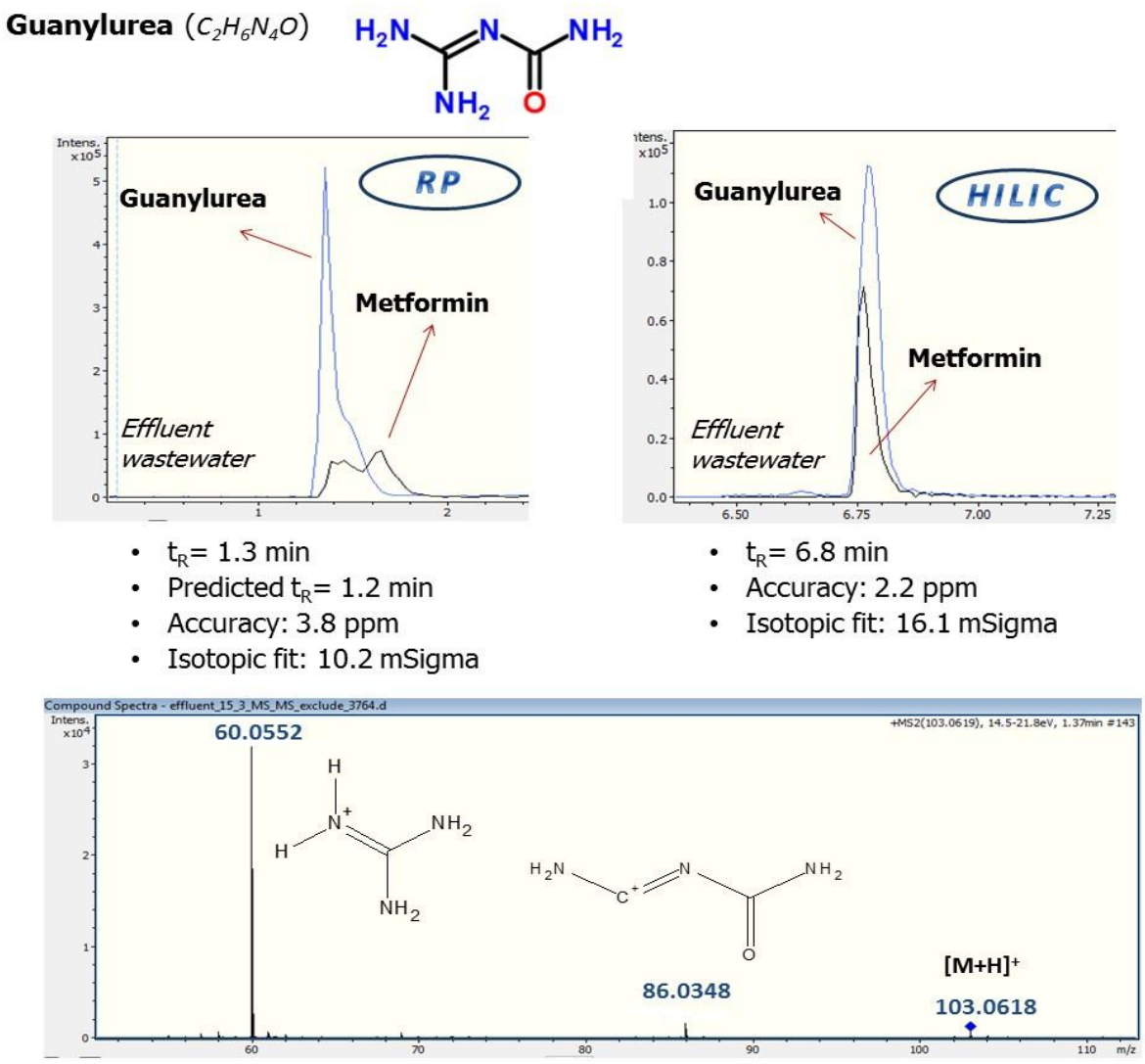


Figure 5.6 Identification of the suspect compound guanylurea.

The intra-day concentration profile of parent and metabolites can also be used to provide additional evidence for the identification, as indicated for clarithromycin and two related metabolites, N-desmethyl clarithromycin and hydroxyclarithromycin, in Figure 5.7. These two metabolites showed plausible t_R according to the model and also clear and comprehensive MS/MS spectra that fit with the proposed structures. As the concentration in IWW was measured every two hours, it can be observed that the profiles are identical for the metabolites and for clarithromycin. The identity of N-desmethyl clarithromycin was confirmed with the use of a commercial standard reaching level 1, while the hydroxylated metabolite remains at Level 3.

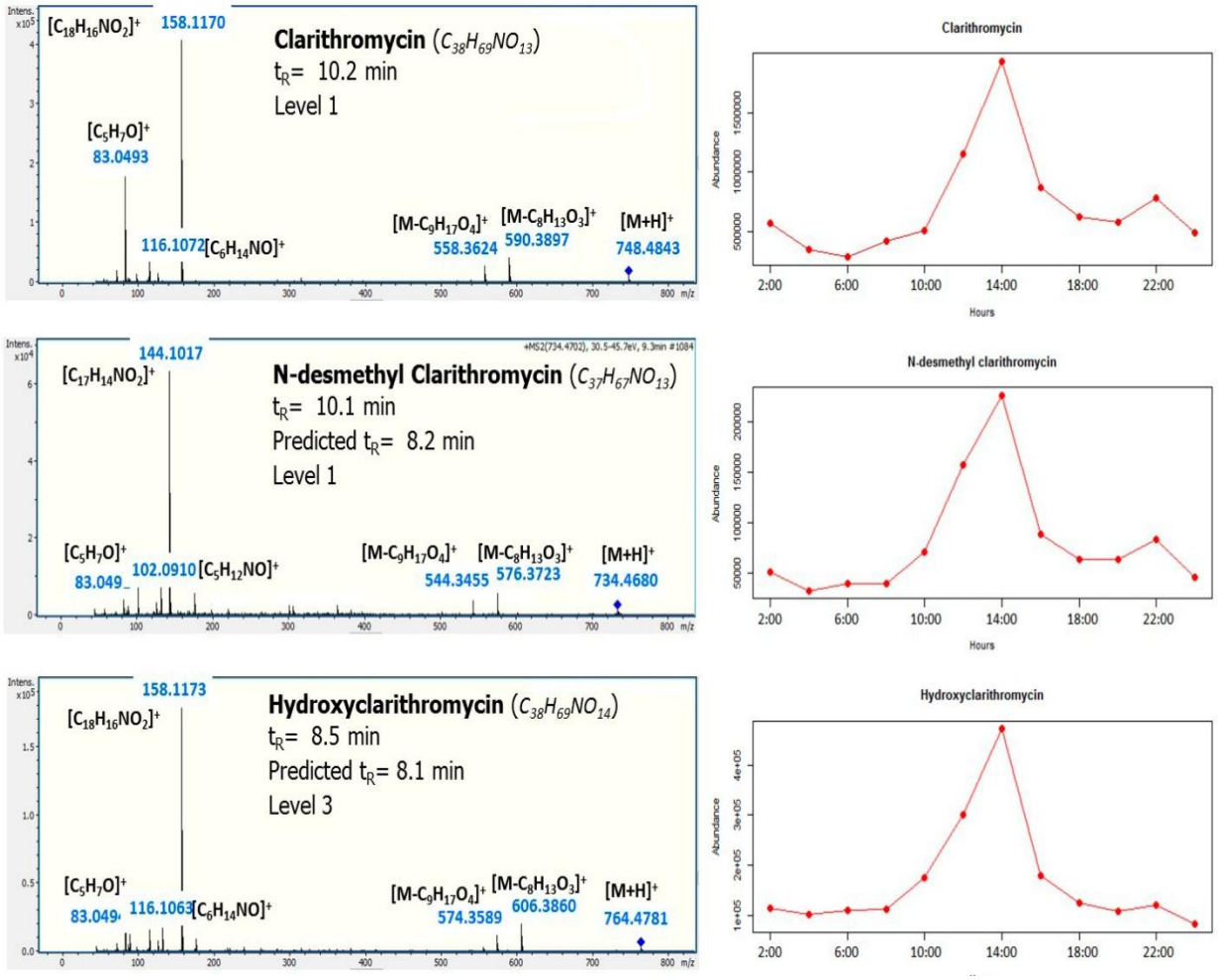


Figure 5.7 MS/MS spectra and intra-day concentration profiles of clarithromycin and its metabolites N-desmethyl clarithromycin and hydroxyclarithromycin, in influent wastewater samples.

This procedure was also used in the case of venlafaxine and its two metabolites N-desmethyl venlafaxine and O-desmethyl venlafaxine (final level 1), as it is shown in Figure 5.8(A). An excellent interrelation in the intra-day profiles among parent/metabolites was observed. A good intra-day interrelation between the two confirmed metabolites cotinine and hydroxycotinine (Figure 5.8(B)) was also observed. In this case, the profiles of the metabolites did not match well the profile of the parent compound, nicotine. This indicates that this new strategy can provide valuable additional evidences for the identification, but the results should be interpreted with caution. The absence of interrelation does not imply a false positive result.

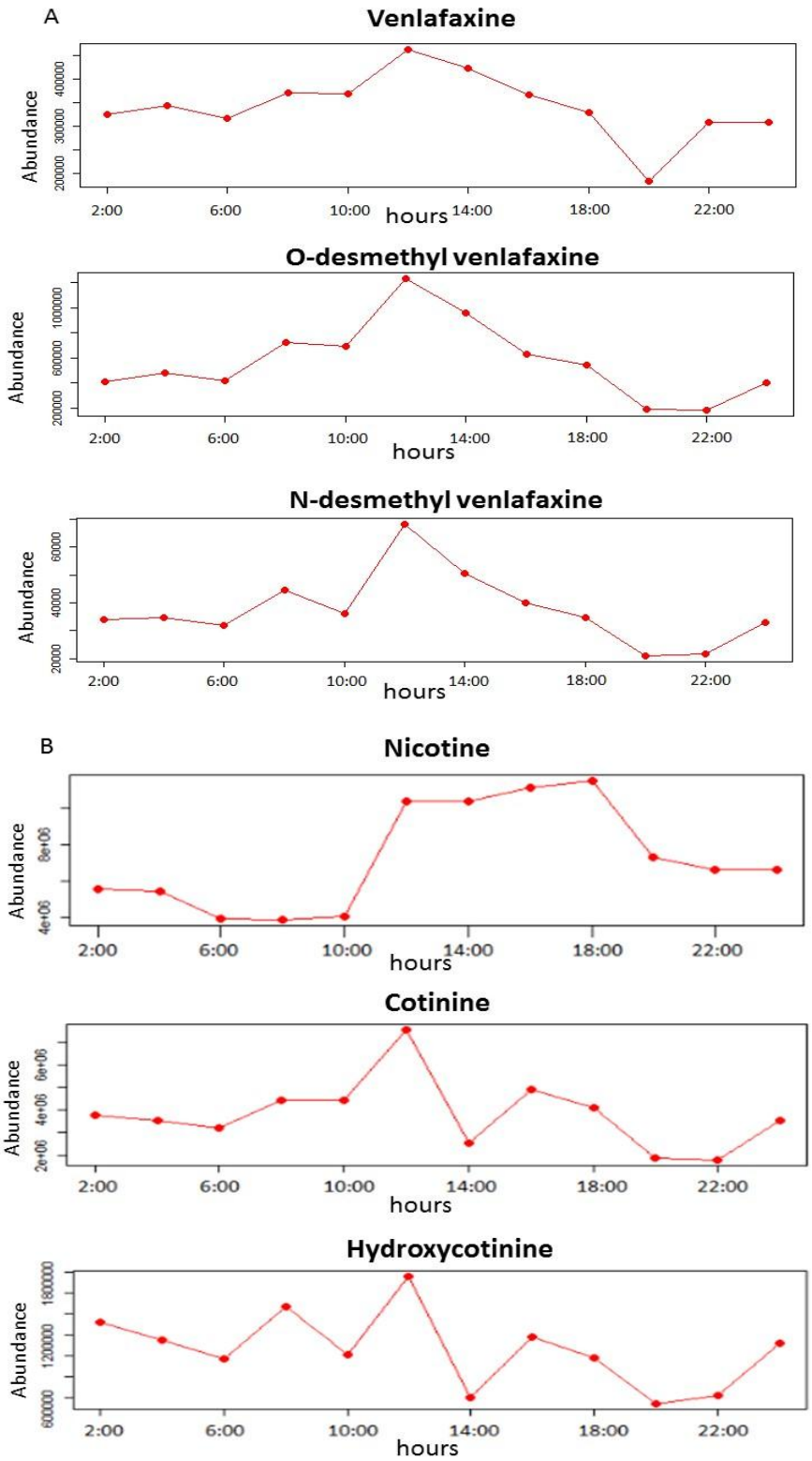


Figure 5.8 Intra-day concentration profiles of (A) venlafaxine and related metabolites and (B) nicotine and related metabolites, in influent wastewater samples.

Similar conclusions were reached from the comparison of the intra-week concentration profiles among parent compounds and related metabolites. Very similar profiles were observed for the aforementioned metabolites of clarithromycin and venlafaxine and their corresponding parent compounds during 7 consecutive days (Figure 5.9(A) & (B)). Also good interrelations were found for the metabolites ranitidine-S-oxide and tramadol-N-oxide with their parent compounds. However, poor correlations were found for the rest of investigated compounds (some of them confirmed), showing that this strategy may not always provide supporting evidence.

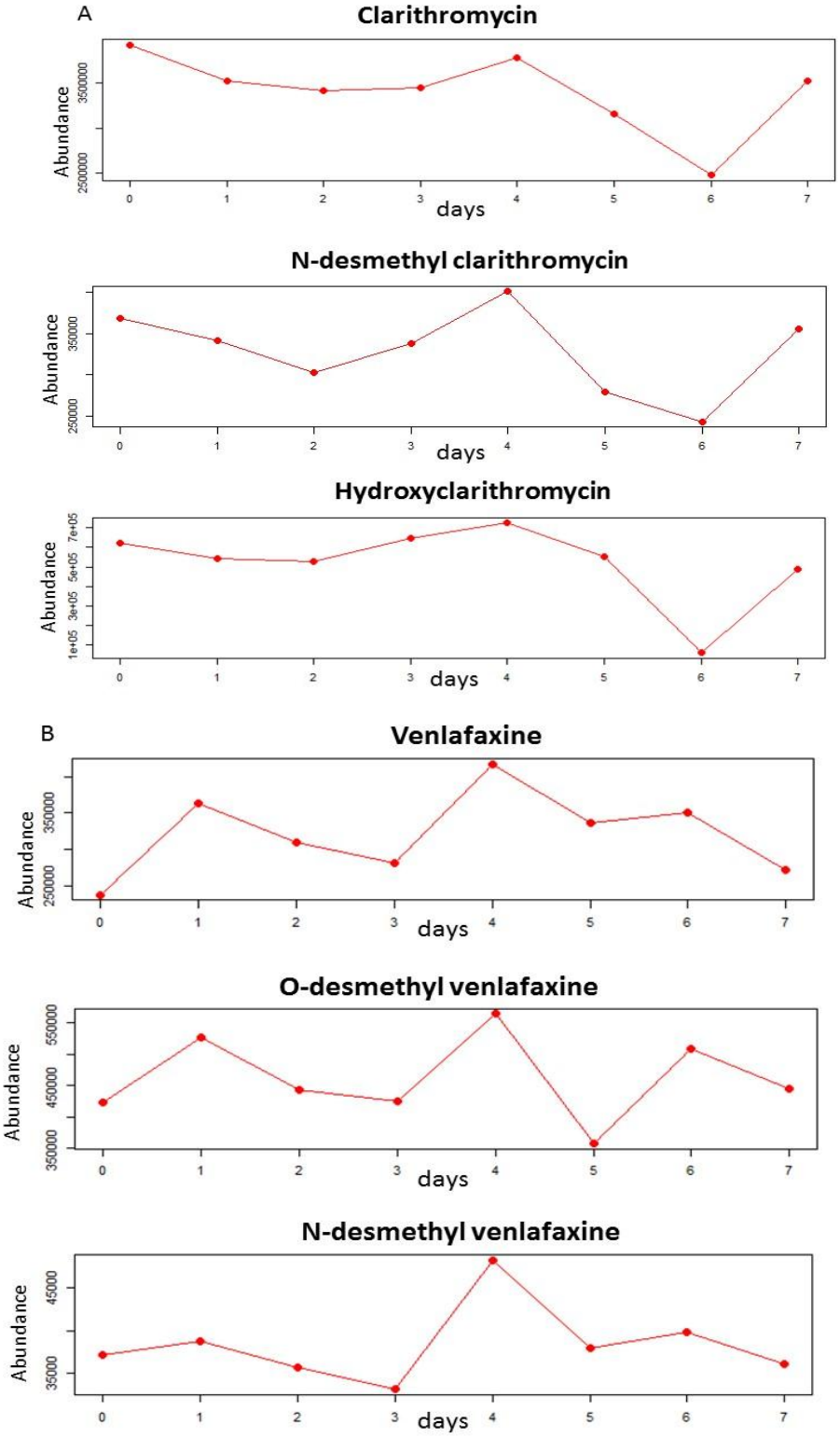


Figure 5.9 Intra-week concentration profiles of (A) clarithromycin and related metabolites and (B) venlafaxine and related metabolites.

In the end, 9 of the 13 tentative candidates (amisulpride-N-oxide, atenolol acid, N-desmethyl clarithromycin, cotinine, nor-cotinine, ranitidine-S-oxide, N-desmethyl-venlafaxine, O-desmethyl-venlafaxine and guanylurea) were confirmed with a commercial standard to reach Level 1, while the rest remained tentative as shown in Table 5.3.

5.4.3. Retrospective suspect screening of surfactants

As a high number of tentatively identified surfactant substances of different types were among the most intense peaks in non-target screening, the presence of surfactants was studied in detail through retrospective suspect screening using the surfactants list described above.

After applying the aforementioned thresholds of peak area and intensity, mass accuracy, isotopic fitting and peak score, 110 suspects remained (88 in (-) ESI and 22 in (+) ESI) and were further evaluated. Plausible t_R times among the homologue series, in RP and in HILIC mode, along with the evaluation of the MS/MS spectra were the main criteria used for the tentative identification of the suspects. This information along with the names, molecular formulas and exact masses is summarized in the Electronc Supplementary Material (Table S5.2).

In total, 82 substances out of 398 suspects were tentatively identified. 38 out of these 82 compounds were tentatively identified with MS/MS evidence and 44 without MS/MS evidence, but with additional information (plausible t_R and chromatographic peak shape among the homologue series), supporting their presence. 19 substances were rejected on the basis that either the t_R or MS/MS did not match or simply due to the absence of evidence supporting their presence. An example can be found in SPCs, the type of surfactants which showed the highest intensity along with LAS. Ten of these substances, from C4-SPAC to C13-SPAC, were tentatively identified. Figure 5.10(A) shows how t_R increased constantly when increasing the length of the alkyl chain. Peak shapes were also consistent among the homologue series. Moreover, plausible MS/MS spectra were observed, including the characteristics

fragments m/z = 79.9574 (SO_3^-) and m/z = 183.0121 ($C_8H_7SO_3^-$), as it is exemplified in Figure 5.10(B) for the compound C8-SPAC.

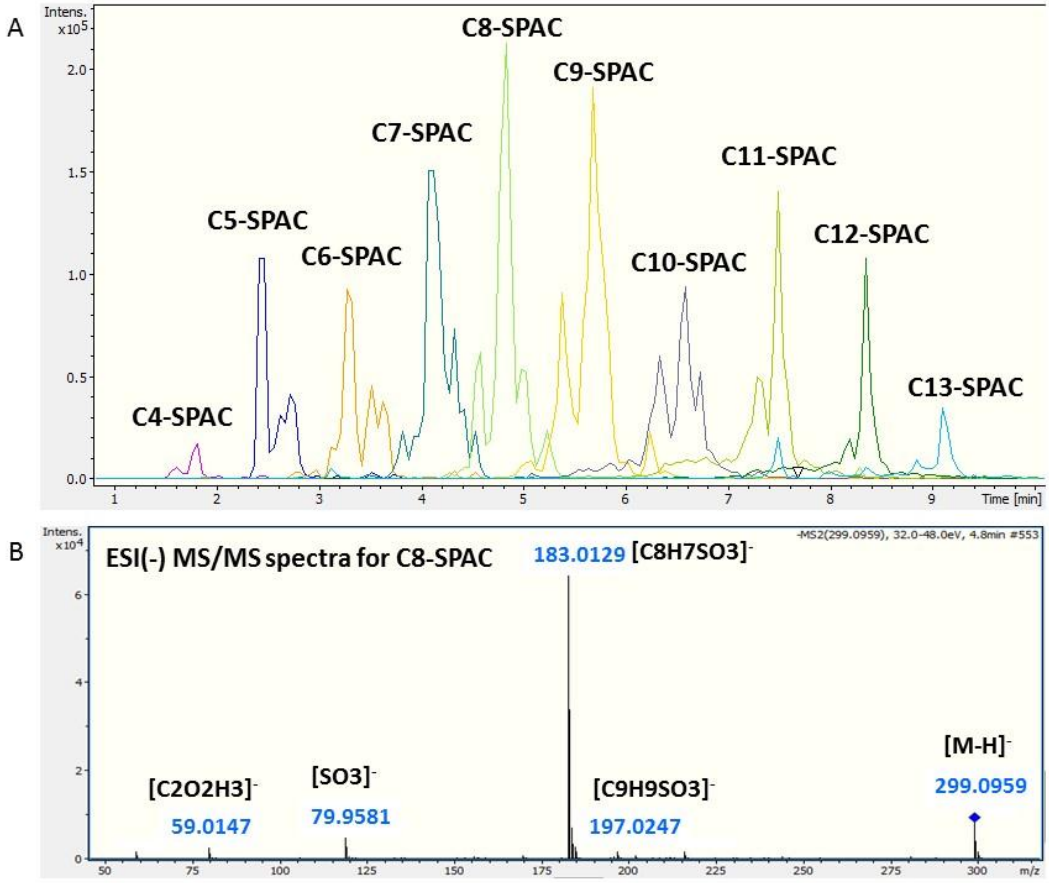


Figure 5.10 (A) Extracted ion chromatograms for the detected SPAC surfactants. (B) MS/MS spectra for the compound C8-SPAC.

Spectra for some of these substances are available in MassBank stemming from the previous study [140] and showed a good fit compared with those obtained experimentally (e.g. C8-SPAC, similarity 0.978 with spectrum ETS00018). This clearly shows the benefits of sharing also suspect spectra in public libraries. Other groups of surfactants were identified similarly, including DATS (7 substances tentatively identified), LAS (4 substances tentatively identified), AS (4 substances tentatively identified), AEOs (13 substances tentatively identified) or DEAs (3 substances tentatively identified). However, there were other classes of surfactants for which MS/MS spectra could not be obtained due to the low intensity of the peaks.

It was observed (and it seems consistent) that the less transformed surfactants (e.g. DATS), were detected in higher number and higher intensity than their related TPs (e.g. STACs and STADCs), since the evaluated matrix was IWW. The opposite trend was observed in a previous study performed with EWW [140], showing that the comparison between these two matrices may provide additional evidence in the tentative identification of surfactants. In these cases the tentative identification was based on chromatographic criteria. An example of this type can be found in AES. Figure 5.11(A) summarized the specific case for the surfactants of the type C13-AES. Seven of these substances were identified based on the increasing t_R (from 13.2 to 13.9 min) when increasing the length of the alkyl chain, along with consistent peak shapes. A plausible chromatographic behavior was also observed for these compounds in HILIC mode, showing t_R from 1.2 to 1.4 min, as it is shown in Figure 5.11(B).

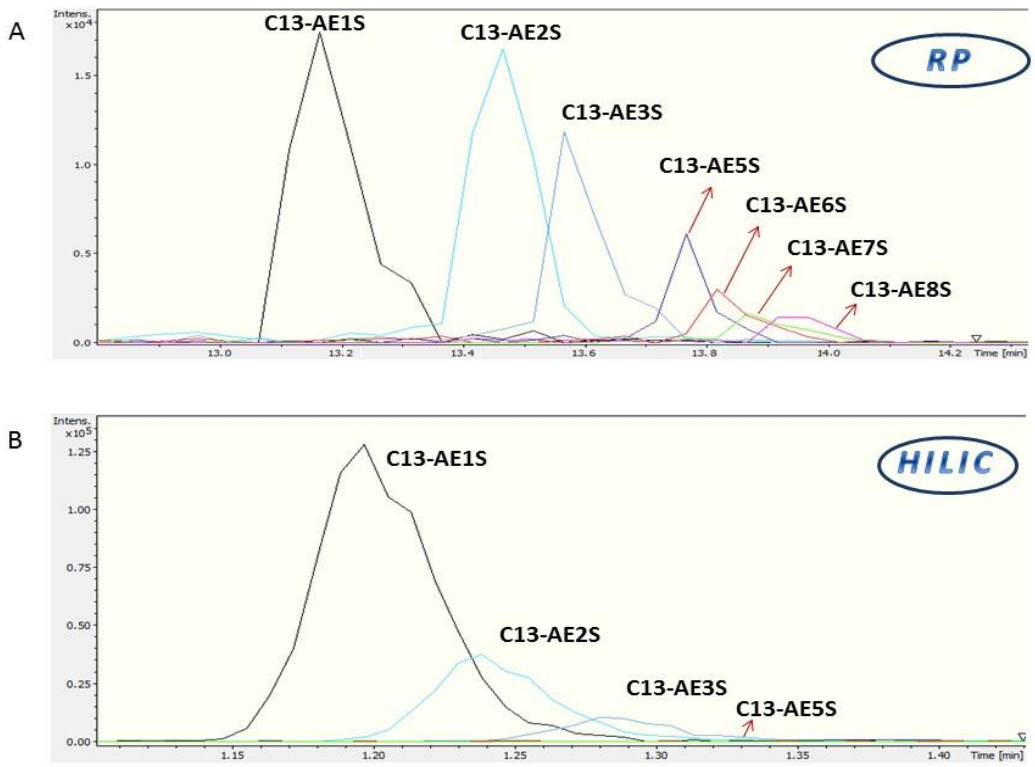


Figure 5.11 (A) Extracted ion chromatograms for the detected C13-AES surfactants in RP mode. (B) Extracted ion chromatograms for the detected C13-AES surfactants in HILIC mode.

The observed t_R in both RP and HILIC were also plausible when compared with those obtained for C12-AES, C14-AES and C16-AES. Although the identification confidence is not as high as in the cases where MS/MS data are available, these results are supported by the fact that previous studies also detected these substances and a similar chromatographic behavior was reported [140, 218].

In view of these results, it seems that the new strategies applied here provide valuable additional evidence for the identification of suspect and unknown compounds in environmental samples. The comparison of the daily and/or weekly concentration trends may help increase the identification confidence. Consistent with previous studies, a high proportion of the peaks detected at the highest intensity corresponded to surfactants and the identification of the surfactant series GES is reported for the first time. The usefulness of retrospective analysis is clear as these GES were found subsequently in samples from another European geographical area.

CHAPTER 6

Conclusions

In the field of environmental analysis, there is a great development during the last decade, especially with the contribution of HRMS. However, specific methods for the study of a group of analytes that have not been thoroughly studied before is definitely a need. Specific and sensitive methods based on either LR or HR mass spectrometry can provide valuable information over the occurance, fate and distribution of analytes in the environment.

Moreover, through wide-scope target screening by HRMS, we have detected and quantified numerous analytes that are not part of any environmental quality control. This is an indication of the importance of databases with many analytes for wide-scope target screening.

Additionally, restrospective analysis of the samples is the greater advantage of HRMS methods, in order to look back in previous samples for new or recently released or reported compounds.

Suspect Screening is a methodology for the identification of analytes based on some knowledge, for a specific purpose and can give valuable information for metabolites, TPs or a specific group of compounds. Although, there is still a need for better and more complete compound databases, mass libraries and software for *in-silico* fragmentation, retention time prediction tools and metabolism prediction tools.

As for Non-target Screening, it is an essential final step for a comprehensive environmental analysis, since the majority of the compounds in the samples remain still unknown.

ABBREVIATIONS AND ACRONYMS

ACN	Acetonitrile
bbCID	broad band Collision Induced Dissociation
CCα	decision limit
CCβ	detection capability
ECs	Emerging Contaminants
EI	Electron ionization
EPs	Emerging Pollutants
ESI	electrospray ionization interface
EWW	effluent wastewaters
FN	false negative
FP	false positive
FWHM	Full Width at Half maximum
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HR-MS	high resolution mass spectrometers
ICM	iodinated X-ray contrast media
IDA	data-independent acquisition
IPs	identification points
IT	ion-trap
IWW	influent wastewaters

LC	Liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
LOI	limit of identification
LOQ	limit of quantification
LR-MS	Low Resolution Mass Spectrometry
ME	Matrix Effect
MeOH	methanol
MS	mass spectrometry
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAHs	polycyclic aromatic hydrocarbons
PCAs	polychlorinated alkanes
PCNs	polychlorinated naphthalenes
PDMSs	polydimethylsiloxanes
PEC	Predicted Environmental Concentration
PFCs	perfluorochemicals
PNEC	Predicted No Effect Concentration
POPs	persistent organic pollutants
PPCPs	pharmaceuticals and personal care products
QqQ	Triple quadrupole
QSRR	Quantitative structure–retention relationship
Q-TOF	quadrupole time-of-flight

RP	Reversed phase
SDL	screening detection limit
SIM	selected ion monitoring
SPE	solid phase extraction
TOF	time-of-flight
TPs	Transformation Products
UM-PPS	University of Minnesota- pathway prediction system
WWTP	Wastewater treatment plant

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