UNIVERSITY OF LATVIA
FACULTY OF CHEMISTRY

THE OPTIMIZATION OF ANALYTICAL PARAMETERS FOR SCREENING AND QUANTIFICATION OF PHARMACEUTICAL RESIDUES IN THE ENVIRONMENT

DOCTORAL THESIS

ANALĪTISKO PARAMETRU OPTIMIZĀCIJA FARMACEITISKO SAVIENOJUMU NOTEIKŠANAI APKĀRTĒJĀS VIDES PARAUGOS

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The thesis is available at the Library of the University of Latvia, Raiņa Blvd. 19, Riga, Latvia.

CONTENTS

ABBREVIATIONS

AGs	Aminoglycoside antibiotics
APCI	Atmospheric-pressure chemical ionisation
APPI	Atmospheric-pressure photo ionisation
ASE	Accelerated solvent extraction
CCα	Decision limit
CCβ	Detection capability
CE	Collision energy
CNTs	Carbon nanotubes
CNTs-1	MWCNT of trademark Baytubes®
CNTs-2	MWCNTs, commercially known as TNIM4
CNTs-3	MWCNTs, functionalised with hydroxyl groups, commercially known as TNIMH4
CNTs-4	MWCNTs, functionalised with carboxyl groups, commercially known as TNIMC4
DCF	Diclofenac (an NSAID class drug)
DDD	Defined daily dose
DLLME	Dispersive liquid-liquid microextraction
dSPE	Disperse solid-phase extraction
DSTP	Dihydrostreptomycin (an aminoglycoside antibiotic)
EC	European Commission
ESI	Electrospray ionisation
FCA	Flufenamic acid (an NSAID class drug)
FNX	Flunixin (an NSAID class drug)
FT-ICR-MS	Fourier-transform ion cyclotron resonance mass spectrometry
Full-MS	Full scan
FWHM	Full width at half maximum
GC	Gas chromatography
GEN	Gentamycin (an aminoglycoside antibiotic)
HCD	Higher energy collision dissociation
HPLC	High performance liquid chromatography
IB	Ion Booster ESI source
IBP	Ibuprofen (an NSAID class drug)
KAN	Kanamycin (an aminoglycoside antibiotic)
KTP	Ketoprofen (an NSAID class drug)
LC	Liquid chromatography
LIT	Linear ion trap
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification

MAE	Microwave-assisted extraction
ME	Matrix effect
MFA	Mefenamic acid (an NSAID class drug)
MMSCC	Method matrix-matched standard calibration curve
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry or fragmentation spectra (based on context)
MXC	Meloxicam (an NSAID class drug)
N.D.	Not detected
NEO	Neomycin (an aminoglycoside antibiotic)
NFA	Niflumic acid (an NSAID class drug)
NPX	Naproxen (an NSAID class drug)
NSAIDs	Nonsteroidal anti-inflammatory drugs
Orbitrap-MS	Orbitrap mass spectrometry
PhACs	Pharmaceutically active compounds
PLE	Pressurized liquid extract
PPCPs	Pharmaceuticals and personal care products
Q	Quadrupole
QuEChERS	Sample preparation technique, the abbreviation is formed from "quick, easy, cheap, effective, rugged, and safe"
RSD	Relative standard deviation
RSDr	Repeatability
RSDwR	Within-laboratory reproducibility
SAM	Latvian State Agency of Medicines
SBSE	Stir bar sorptive extraction
SIM	Single reaction monitoring
SLE	Solid-liquid extraction
SPC	Spectinomycin (an aminoglycoside antibiotic)
SPME	Solid phase micro-extraction
STP	Streptomycin (an aminoglycoside antibiotic)
TFA	Tolfenamic acid (an NSAID class drug)
TPs	Transformation products
TOF-MS	Time-of-flight mass spectrometry
UAE	Ultrasound-assisted extraction
UPLC	Ultra-high performance liquid chromatography
VPF	Vedaprofen (an NSAID class drug)
WFD	Water Framework Directive
WW	Wastewater
WWTP	Wastewater treatment plant

ABSTRACT

The optimization of analytical parameters for screening and quantification of pharmaceutical residues in the environment. Pērkons I., supervisors Dr. Chem., Assoc. Prof. Bartkevičs V. and Dr. Chem. Pugajeva I. Doctoral thesis in analytical chemistry, 150 pages, 28 figures, 19 tables, 265 literature references, 12 annexes. In English.

The thesis presents an overview on the occurrence, fate and behaviour of pharmaceutically active compounds (PhACs) in the environment and highlights the current trends in analytical strategies for the determination of these elusive emerging pollutants, primarily focusing on issues and challenges concerning mass spectrometry (MS) based applications. Four MS-based methods have been developed for PhAC analysis in environmental matrices. In particular, two high-resolution MS (HRMS) multi-class methods based on Orbitrap-MS and Fourier-transform ion cyclotron resonance MS (FT-ICR-MS), one time-of-flight MS (TOF-MS) method for the determination of aminoglycoside antibiotics and one tandem MS (MS/MS) method for the analysis of nonsteroidal anti-inflammatory drugs. Applicability of several unconventional techniques (e.g. QuEChERS, multiwalled carbon nanotubes, mixed-mode zwitterionic-type liquid chromatography, etc.) have been explored to improve all stages of the analysis. Furthermore, a standard free suspect screening methodology was developed as a part of this thesis and used for qualitative screening of more than 500 PhACs and their transformation products in wastewater samples. Finally, the developed methods were applied to study the occurrence of PhACs in environmental samples and to investigate the fate of PhACs during advanced wastewater treatment processes (bioaugmentation and irradiation with ionizing radiation).

PHARMACEUTICAL RESIDUES, ENVIRONMENTAL POLLUTION, HIGH-RESOLUTION MASS SPECTROMETRY, TANDEM MASS SPECTROMETRY, LIQUID CHROMATOGRAPHY, AMINOGLYCOSIDE ANTIBIOTICS, NONSTEROIDAL ANTI-INFLAMMATORY DRUGS, WASTEWATER TREATMENT

ANOTĀCIJA

Analītisko parametru optimizācija farmaceitisko savienojumu noteikšanai apkārtējās vides paraugos. Pērkons I., zinātniskie vadītāji Dr. ķīm., asoc. prof. Bartkevičs V. un Dr. ķīm. Pugajeva I. Promocijas darbs, 150 lappuses, 28 attēli, 19 tabulas, 265 literatūras avoti, 12 pielikumi. Angļu valodā.

Promocijas darba literatūras apskatā apkopota patreizēji aktuālā informācija, kas skar aktīvo farmaceitiski vielu (AFV) klātbūtni apkārtējā vidē un analītiskās ķīmijas tendences šajā nozarē, īpaši akcentējot šo savienojumu noteikšanas metodoloģiju ar šķidruma hromatogrāfiju (LC) un masspektrometriju (MS). AFV noteikšanai apkārtējās vides paraugos, izstrādātas četras dažādas MS metodes. Attiecīgi, divas multi-metodes izstrādātas, izmantojot augstas izšķirtspējas MS sistēmas (HRMS) - orbitālā slazda MS (Orbitrap-MS) un Furjē transformācijas jonu ciklotrona rezonanses MS (FT-ICR-MS). Savukārt aminoglikozīdu klases antibiotiku un nesteroīdo pretiekaisuma līdzekļu noteikšanai izstrādātas metodes, kas balstās uz nolidojuma laika MS (TOF-MS) un tandēma masspektrometriju (MS/MS). Lai uzlabotu analītisko protokolu veiktspēju, darbā pārbaudīta dažādu inovatīvu tehniku pielietojamība AFV analīzē (piem. QuEChERS, oglekļa nano-caurulītes, jaukta tipa cviterjonu bāzes LC u.c.). Paralēli tradicionālajai mērķētā tipa analīzei, ar FT-ICR-MS sistēmu izstrādāta augstas veiktspējas kandidātu skrīninga metode, kas ļauj realizēt vairāk nekā 500 AFV un to transformācijas produktu analīzi notekūdeņu paraugos. Visbeidzot, visas izstrādātās metodes ir atbilstoši validētas un pielietotas praktiskas ievirzes pētījumos, lai noteiktu AFV piesārņojumu apkārtējās vides paraugos un izvērtētu šo savienojumu uzvedību notekūdeņu attīrīšanas procesos, kas balstās uz dažādiem papildapstrādes paņēmieniem, piem., bioaugmentācija un apstrāde ar jonizējošo starojumu.

FARMACEITISKIE SAVIENOJUMI, APKĀRTĒJĀS VIDES PIESĀRŅOJUMS, AUGSTAS IZŠĶIRTSPĒJAS MASSPEKTROMETRIJA, TANDĒMA MASSPEKTROMETRIJA, ŠĶIDRUMA HROMATOGRĀFIJA, AMINOGLIKOZĪDI, NESTEROĪDIE PRETIEKAISUMA LĪDZEKĻI, NOTEKŪDEŅU ATTĪRĪŠANA

INTRODUCTION

The presence of pharmaceutical residues in the environment has become a global environmental concern. Pharmaceutical products may enter the environment by several different pathways (e.g. manufacture, use and disposal) and cause potential ecotoxicological effects to both terrestrial and aquatic ecosystems [1,2]. Besides, parent compounds can undergo biotic and abiotic transformations that may lead to the formation of new substances with increased toxicity and/or mobility [3–5]. Yet, the consequences of this chronic exposure are largely unknown. Nevertheless, several prominent examples indicate that the presence of individual substances may have particularly devastating effects on some biological systems. For instance, a well-documented case is that steroidal oestrogens act as endocrine disrupting chemicals adversely affecting reproductive behaviour in fish. It should come as no surprise that pharmaceuticals can induce adverse effects on non-target organisms at environmentally relevant concentrations because they are designed to achieve optimal therapeutic function at low concentrations [6–8].

Although this topic has been studied for several decades, it remains a challenging problem that requires effective and reliable analytic procedures with sufficient sensitivity. Recent advances in mass spectrometry, especially high-resolution mass spectrometry, have accelerated this research field and provided scientists with tools that can overcome previous analytical limitations. Among them, multi-class methods are clearly in the spotlight as they provide an opportunity to analyze a wide range of pharmaceutical substances in one chromatographic run [9]. Moreover, an increasing number of methods are starting to investigate the presence of pharmaceutical residues by applying non-target and suspect screening principles that utilize compound-specific features (e.g. accurate mass, fragmentation pattern, isotopic pattern, etc.) [10,11]. Nevertheless, single-class methods are still highly relevant, because the trace level analysis of pharmaceuticals in less contaminated matrixes (e.g. groundwater, biota and surface water) requires a sensitivity that cannot be easily achieved by multi-class screening methods. Regardless of the analytical strategy used, a tailor-made approach for the development and optimization of methods is crucial to select the most suitable experimental conditions that can improve all stages of the analysis (e.g. sample preparation, chromatographic separation and mass spectrometric detection).

The practical relevance of the problem. Only a small fraction of pharmaceuticals has been extensively investigated in terms of their environmental occurrence and fate. Therefore, there is a need for reliable and sensitive methods for determining a wide range of pharmaceutically active substances in various environmental media. Sydney Brenner, 2002 Nobel laureate in medicine, once stated that “Progress in science depends on new techniques, new discoveries, and new ideas, probably in that order.” It accurately reflects the concept that novel analytical strategies pave the way for a better understanding of these emerging pollutants and their behaviour in the environment. This way, environmental analytical chemistry can provide other research fields and policy-makers with valuable information to improve prioritization, risk assessment and risk management actions. Despite the efforts made in the last two decades, there is a substantial gap between experimental evidence and policy. For instance, pharmaceutically active substances are not covered by the priority substance list established by the Directive 2013/39/EU [12], while only a dozen of them are included in the European watch list for substances of emerging concern [13]. Thus, the development of innovative methods is critical to better understand the problems associated with pharmaceutical residues in the environment. This is a key to the successful implementation of

mitigation measures and evidence-based decision making that will determine the status of the environment in the future and, hopefully, minimize the environmental impact of these elusive pollutants.

The aim of the work. Several aims were proposed during this work:

i. Developing novel mass spectrometry-based methods using different mass analysers for trace level determination of multi-class and single-class pharmaceutical residues in environmental matrices;

ii. Exploring the applicability of novel detection, sample introduction and sample preparation strategies to increase the analytical performance of developed methods;

iii. Applying the proposed methods to study the occurrence of pharmaceutical residues in the environment and to investigate advanced treatment processes for the removal of pharmaceuticals from municipal wastewater.

The approach used. The following objectives have been set to fulfil the aims of the thesis:

i. Developing four distinct analytical methods for determination pharmaceuticals in environmental matrices that rely on high-resolution mass analysers (Orbitrap-MS, FT-ICR-MS and TOF-MS) and tandem mass spectrometry.

ii. Exploring the applicability of multiwalled carbon nanotubes as an alternative dSPE sorbents for the extraction of NSAID class drugs from aqueous environmental samples.

iii. Exploring the potential to use QuEChERS approach for the extraction of multi-class pharmaceuticals from wastewater samples.

iv. Investigating the selectivity of mixed-mode zwitterionic-type liquid chromatography for the separation of polar pharmaceuticals.

v. Developing a high-resolution mass spectrometry-based method for standard free screening (i.e. suspect screening) of multi-class pharmaceuticals in wastewater.

vi. Applying the developed Orbitrap-MS method to study the removal of multi-class pharmaceuticals from wastewater through ionising radiation and bioaugmentation.

Scientific novelty.

i. Four modern analytical protocols were developed to show the versatility of different mass analysers and how each of them can provide specific benefits for the determination of multi- and single-class pharmaceuticals in environmental matrices.

ii. It was found that enhanced sensitivity can be obtained for the analysis of aminoglycoside antibiotics when high-temperature electrospray source is applied. Furthermore, three mobile phase system (water, acetonitrile and 1% formic acid in acetonitrile) can ensure satisfactory chromatographic separation of aminoglycosides without the use of ion pairing agents or other modifiers that can induce ion suppression and deteriorate instrumental sensitivity.

iii. A novel sample preparation strategy was developed for the determination of multi-class pharmaceuticals in wastewater samples that relied on freeze-drying and modified QuEChERS followed by dSPE clean-up. The method was found to produce lower matrix suppression (compared to conventional SPE approach), showed satisfactory performance and covered a wide range of analytes that can be simultaneously extracted from the sample.

iv. Several previously unreported pharmaceuticals (e.g. telmisartan, bisoprolol and amisulpride) were frequently found in wastewater samples in Latvia using the newly developed standard-free suspect screening

strategy. Moreover, this screening method was able to identify samples that displayed unique contamination patterns in terms of pharmaceutical residues, thus pointing out those WWTPs that may pose greater risks to the environment.

v. An efficient extraction method was developed for enrichment of NSAIDs using multiwalled carbon nanotubes. The results showed that high recoveries can be obtained for 11 out of 12 studied compounds (except meloxicam). Hence, indicating that this sample procedure technique can be used as a suitable substitute for conventional SPE and dSPE methodologies when analysing pharmaceuticals that can be captured via electrostatic attraction or/and van der Waals forces.

iv. Compared to other pharmaceuticals that were investigated during bioaugmentation experiments, diclofenac and ibuprofen were identified as more persistent and required longer incubation times to achieve sufficient removal efficiency during the treatment with activated sludge and activated sludge-derived bacteria/fungi. Meanwhile, macrolide antibiotics showed slower degradation rates when ionizing radiation was used as an advanced wastewater treatment strategy.

Practical application of the work. The suspect screening method can be modified and adapted to study other polar micro-pollutants, while targeted methods could be used to continuously monitor the environmental fate of pharmaceuticals in Latvia. The developed TOF-MS method is currently used for routine analysis of aminoglycoside antibiotics in various matrices in the Institute of Food Safety, Animal Health and Environment "BIOR". Meanwhile, information obtained from studying advanced treatment processes can prove useful for the improvement of current tertiary wastewater treatment practices.

Scientific publications.

1. **Perkons, I.**; Rusko, J.; Zacs, D., Bartkevics, V. Rapid determination of pharmaceuticals in wastewater by direct infusion HRMS using target and suspect screening analysis. *Sci. Total Environ.*[1] **2021**, 755, 142688.
2. **Perkons, I**.; Pugajeva, I.; Bartkevics, V. Simultaneous Screening and Quantification of Aminoglycoside Antibiotics in Honey Using Mixed-Mode Liquid Chromatography with Quadrupole Time-of-Flight Mass Spectroscopy with Heated Electrospray Ionization. *J. Sep. Sci.*[2] **2018**, *41* (16), 3186–3194.
3. Reinholds, I.; Pugajeva, I.; **Perkons, I**.; Lundanes, E.; Rusko, J.; Kizane, G.; Nikolajeva, V.; Mutere, O.; Petrina, Z.; Baumane, L.; Bartkevics, V. Decomposition of Multi-Class Pharmaceutical Residues in Wastewater by Exposure to Ionising Radiation. *Int. J. Environ. Sci. Technol.*[3] **2017**, *14* (9).
4. Reinholds, I.; Muter, O.; Pugajeva, I.; Rusko, J.; **Perkons, I**.; Bartkevics, V. Determination of Pharmaceutical Residues and Assessment of Their Removal Efficiency at the Daugavgriva Municipal Wastewater Treatment Plant in Riga, Latvia. *Water Sci. Technol.*[4] **2017**, *75* (2).
5. Muter, O.; **Perkons, I**.; Selga, T.; Berzins, A.; Gudra, D.; Radovica-Spalvina, I.; Fridmanis, D.; Bartkevics, V. Removal of Pharmaceuticals from Municipal Wastewaters at Laboratory Scale by Treatment with Activated Sludge and Biostimulation. *Sci. Total Environ.*[1] **2017**, *584–585*.
6. Reinholds, I.; Pugajeva, I.; Zacs, D.; Lundanes, E.; Rusko, J.; **Perkons, I**.; Bartkevics, V. Determination of Acidic Non-Steroidal Anti-Inflammatory Drugs in Aquatic Samples by Liquid Chromatography-Triple Quadrupole Mass Spectrometry Combined with Carbon Nanotubes-Based Solid-Phase Extraction. *Environ. Monit. Assess.*[5] **2017**, *189* (11).
7. Pugajeva, I.; Rusko, J.; **Perkons, I**.; Lundanes, E.; Bartkevics, V. Determination of Pharmaceutical Residues in Wastewater Using High Performance Liquid Chromatography Coupled to Quadrupole-Orbitrap Mass Spectrometry. *J. Pharm. Biomed. Anal.*[6] **2017**, *133*.
8. Muter, O.; **Perkons, I**.; Svinka, V.; Svinka, R.; Bartkevics, V. Distinguishing the Roles of Carrier and Biofilm in Filtering Media for the Removal of Pharmaceutical Compounds from Wastewater. *Process Saf. Environ. Prot.*[7] **2017**, *111*.

List of conferences.

1. 78th Annual Conference of the University of Latvia, Plenary session, Riga, Latvia, 2020. **Perkons, I**.; Bartkevics, V. A non-targeted and targeted screening of human pharmaceuticals in wastewater using ultra-high resolution mass spectrometry (in book of abstracts/oral presentation).
2. 6th International Conference on Sustainable Solid Waste Management (NAXOS 2018), Naxos Island, Greece, 2018, Muter, O.; **Perkons, I**.; Bartkevics, V. Removal of pharmaceutical residues from wastewater by woodchip-derived biochar (in book of abstracts/oral presentation).

[1] *Peer reviewed journal, imprint of Elsevier (IF=6.55 (2019)), ISSN: 0048-9697*
[2] *Peer reviewed journal, imprint of Wiley-Blackwell (IF=2.88 (2019)), ISSN:* 1615-9306
[3] *Peer reviewed journal, imprint of Islamic Azad University of Research and Technology (IF=2.54 (2019)), ISSN: 1735-1472*
[4] *Peer reviewed journal, imprint of IWA Publishing (IF=1.64 (2019)), ISSN: 0273-1223*
[5] *Peer reviewed journal, imprint of Springer Nature (IF=1.90 (2019)), ISSN: 0167-6369*
[6] *Peer reviewed journal, imprint of Elsevier (IF=3.21 (2019)), ISSN: 0731-7085*
[7] *Peer reviewed journal, imprint of Institution of Chemical Engineers (IF=4.97 (2018)), ISSN: 0957-5820*

3. 75th Annual Conference of the University of Latvia, Section of Analytical Chemistry, Riga, Latvia, 2017. **Perkons, I.;** Bartkevics, V.; Pugajeva, I. Simultaneous screening and quantification of aminoglycoside residues in honey by mixed-mode hydrophilic interaction liquid chromatography coupled to time-of-flight mass spectrometry with high-temperature electrospray ionization (in book of abstracts/oral presentation);
4. 57th International Scientific Conference "Materials Science and Applied Chemistry" of Riga Technical University, Riga, Latvia, 2016. **Perkons, I**.; Reinholds, I.; Pugajeva, I.; Bartkevics, V. Determination of Streptomycin in Municipal Wastewater by Mixed-mode Hydrophilic Interaction Liquid Chromatography Coupled with Time-of-Flight Mass Spectrometry (in book of abstracts/poster session);
5. 20th International Scientific Conference EcoBalt, Tartu, Estonia, 2016. Zacs, D.; **Perkons, I**.; Viksna, A.; Bartkevics, V. Determination of Steroidal Estrogens in Tap Water Samples using Gas Chromatography-Mass Spectrometry (in book of abstracts/poster session).

1. LITERATURE REVIEW

1.1. Setting the scene: background, brief history and current state

All organisms modify their environment, and humans are no exception. As the human population has grown and the power of technology has expanded, the scope and nature of this modification have changed drastically [14]. We have mastered the art of science in order to pursue effective improvements in our daily lives. Healthcare is no exception. Our life conditions, wellbeing and life expectancy have improved substantially, while a significant decrease in disease mortality has been achieved over the past centuries. A large fraction of this can be attributed to the pharmaceutical industry.

Undoubtedly, the benefits they provide are immeasurable in the context of public health and the quality of life, but, alas, every rose has its thorn. In this case, there has been increasing awareness of the unintentional presence of pharmaceuticals in various compartments of the aquatic and terrestrial environment at concentrations capable of causing adverse effects to the surrounding flora, fauna and consequently to mankind. This has become an emerging concern because such compounds are extensively and increasingly used in human and veterinary medicine, resulting in their continuous release to the environment [1]. In 1999, Christian G. Daughton and Thomas A. Ternes published a milestone article, which established the collective term for pharmaceuticals and personal care products – "PPCP" [15]. To this day, it has accumulated over four thousand citations and remains as one of the most monumental articles in this scientific domain. In short, PPCPs is a broad term, which includes prescription and non-prescription human drugs, illegal drugs, and veterinary drugs, as well as their subsequent metabolites and conjugates, including antibiotics, hormones, anticonvulsants, antidepressants, lipid regulators, nonsteroidal anti-inflammatory drugs (NSAIDs) and other drug classes which exhibit specific therapeutic functions [2]. In addition, some disinfectants, insect repellents, preservatives, and sunscreen UV filters have also been classified as PPCPs [16].

At this moment the total amount of pharmaceutically active compounds (PhACs) which can be classified as "small molecules" and have been registered exceeds two thousand [17]. For most of them, an in-depth knowledge regarding their biochemical interactions and mechanisms of action is still decades away. Even ibuprofen, an over-the-counter painkiller, which can be found in almost every household, still draws scientific attention in this context. It is hard to comprehend how much is still "in the dark" when we look at biochemical processes which regulate interactions between drugs and human organism. Even less is known about their environmental impact and interactions with other species. Pharmacokinetic studies have long shown that many compounds, which can be classified as PhACs, display poor bioavailability. In other words, a considerable fraction of the administered dose is excreted unchanged. Taking into account this notion, one might ask, "What is the environmental fate of PhACs?" Several thousand research studies have already been conducted to provide an answer to this puzzling question. Many more will come. Yet, it was not until the late 1970s that the first research articles confirmed the presence of PhACs in the environment [18]. Superficially, a statement like this might give a false impression that the scientific community reacted sluggishly to this issue, but that is not true. The major part of this delay can be attributed to analytical procedures, namely, insufficient instrumental sensitivity and selectivity which was further amplified by laborious sample preparation protocols. 50 years ago, gas chromatography (GC) was far more superior over liquid chromatography in terms of sensitivity and separation

power. As a consequence, analyte selection was largely dependent upon their volatility or the ability to successfully derivatize them into more volatile compounds. For instance, multi-component analysis of volatile and semi-volatile organochlorine pesticides (e.g. DDT, aldrin, toxaphene, etc.) is well documented since the early 1960s [19]. However, the majority of PhACs could not be analyzed by conventional GC based techniques. In most cases, they exhibit non-volatile and relatively polar properties, which created a serious obstacle for analytical chemists at that time, because instrumental separation prior detection is crucial for successful trace level analysis. However, various research groups from environmental authorities of the United States, United Kingdom and Germany were able to overcome some of these limitations and forged the first pioneering work in this field [20–22]. This is largely attributed to advances in field desorption (FB) mass spectrometry, fast atom bombardment (FAB) mass spectrometry and, most notably, liquid chromatography. Altogether with other key instrumental discoveries, these advancements paved the way for accurate identification and quantification of many PhACs in environmental matrices [23,24]. Hence, opening the doors to future research. Several decades have passed since that moment. Research methodology has changed almost beyond recognition, but many questions remain unanswered.

As of now, the emerging issue of pharmaceuticals and their residues in the environment is widely acknowledged. During the past two decades, the European Commission (EC) has funded almost twenty EU-wide projects on this topic. The results of these projects have created an increasing amount of open scientific literature, expanded public awareness and allowed a gradual transformation of several solutions into real regulatory frameworks [25]. Yet, according to Directive 2013/39/EU, specific environmental quality standards (EQS) remain unimplemented for PhACs under the European Water Framework Directive [12]. Most recently, in July 2018 EC published the final report called “Options for a strategic approach to pharmaceuticals in the environment”, which carefully documents both public and stakeholder views to further strengthen the EU strategic approach to tackle PhACs in the environment. These actions are important among other things to help the EU achieve the United Nations Sustainable Development Goals, in particular goal No. 6 - "Clean Water and Sanitation" [13]. In the meantime, new studies are published nearly every day and our knowledge regarding PPCPs and PhACs significantly improves. Their ubiquitous presence and fate in the aquatic environment throughout the world have been studied extensively and our knowledge has become much more advanced. At the same time “horizon” of such pollution is rapidly expanding and new issues arise. For instance, an in-depth assessment of their metabolites and transformation products, evaluation of their persistence, bioaccumulation potential and toxicity. In the light of the above considerations, I truly hope that this dissertation will make a small contribution to the collective knowledge base, which allows us to ensure a better future for both the world and ourselves.

1.2. A brief introduction of pharmaceutically active compounds (PhACs)

The available literature still lacks a uniform classification system that can clearly define what compounds are considered as PPCPs. In this context, the majority of scientific articles devoted to PPCPs mainly focus on pharmaceutically active compounds (PhACs) and their transformation products, because they appear to pose the greatest risk. However, PPCP classification is not limited to medicinal products. In a broad sense, there is an enormous number of potential candidates that could be included. A large variety of small organic compounds (up to approximately 1000 Da) can be found in consumer goods which are designed to improve our quality of life and wellbeing. Such products are, for example, UV filters, disinfectants, cosmetics (soaps, moisturizers, lipsticks,

etc.), nutritional supplements and insect repellents. All this is just the tip of the iceberg and I do not intend to downplay the importance of other micro-pollutants. Yet, the "antagonists" of the present dissertation are medicinal products. Hence, the term "PhACs" will be used throughout this thesis to refer mainly to medical drugs and their metabolites.

In general, PhACs are an extremely diverse array of organic compounds. The main PhAC classes are antibiotics, hormones, anti-inflammatory drugs (NSAIDs), blood lipid regulators, β-blockers (beta-blockers), contrast media, antidepressants and antiepileptic drugs [16]. The following paragraphs (Section 1.2.1.-1.2.6.) are aimed to provide a condensed summary of each drug class which will be discussed in the continuous chapters of the dissertation.

1.2.1. Antibiotics

Overall, antibiotics can be considered the hot topic of PhACs. Their unintentional, yet ubiquitous presence in the environment can have a direct impact on human health through the spread of antimicrobial resistance [26]. They act against gram-positive and gram-negative bacteria through various mechanisms. In clinical applications, their classification is mostly based upon the acting mechanism, e.g. cell wall synthesis inhibitors, protein synthesis inhibitors, folic acid synthesis inhibitors, etc., however, in environmental chemistry and residue analysis it is much more convenient to categorize them by structural characteristics. The most commonly applied antibiotic classes are β-lactam antibiotics (penicillins and cephalosporins), fluoroquinolones, aminoglycosides, and macrolides [27]. Taking into account consumption rates, biodegradability potential and potential adverse effects, EC has updated Water Framework Directive's (WFD) surface water "Watch List" with the following antibiotics: ciprofloxacin, amoxicillin and macrolide antibiotics (erythromycin, clarithromycin, azithromycin).

1.2.2. NSAIDs

The NSAIDs constitute a heterogeneous group of drugs with analgesic, antipyretic and anti-inflammatory properties. From a chemical viewpoint, most of them consist of an acidic moiety attached to a planar, aromatic functionality [28]. Often colloquially referred to as "pain-killers", they are one of the most frequently used therapeutic groups, especially, aspirin, ibuprofen, paracetamol and diclofenac. In comparison to opioid analgesics, NSAIDs do not exhibit serious side effects on humans and do not induce severe addiction. Hence, many of them are available over-the-counter in many countries, including Latvia, which drastically boosts their emission rates and overall environmental footprint [29]. Although NSAIDs have been considered relatively safe, the case of diclofenac reminds us that it can be difficult to predict the eco-toxicological impact on non-mammalian vertebrates, for example, birds, fishes and amphibians [30]. Considering the contamination level of NSAIDs in aquatic compartments, aspirin, ibuprofen, ketoprofen, naproxen, paracetamol and diclofenac can be considered as the most significant ones [28]. Until recently, the latter was a part WFD's "Watch List". It was removed from the list in 2018 since sufficiently abundant data have been gathered throughout the latest monitoring survey.

1.2.3. Hormones

Another alarming class of PhACs in this context is hormones. Among the huge family of PhACs, steroid estrogens, both natural and synthetic, have attracted particular attention due to their endocrine-disrupting properties [31]. They regulate a wide range of biological functions in animals and humans. In particular, estrone (E1), 17beta-estradiol (E2) and estriol (E3) are produced naturally by all vertebrates, including our species.

Among natural estrogens, E2 is considered as the most potent compound when compared to others and is largely responsible for the development of the female reproductive system. On the other hand, 17a-ethinyl estradiol (EE2) is a synthetic estrogen which is commonly used as a contraceptive medication and displays several fold higher potency then, for example, E2 [32]. E1, E2 and EE2 are a part of WFD's "Watch List".

1.2.4. Beta-blockers and lipid regulating agents

Current statistics show a trend towards a gradual increase in the average age in Europe (population aging), while cardiovascular diseases remain the leading cause of death globally [33]. Both of these tendencies contribute significantly to the overall emissions of PhACs which are applied for the treatment of cardiovascular diseases. Yet, from an environmental perspective, these substances are frequently overlooked. In brief, there are two prominent drug classes that must be highlighted - beta-blockers and lipid regulating agents. Beta-blockers are extensively used for the treatment of high blood pressure, heart rhythm disturbances and ischaemic heart diseases. Some of them, for example, metoprolol, propranolol and nadolol, are frequently reported in biota and environmental samples. Besides, propranolol exhibits hydrophobic properties, thus it tends to bioaccumulate in marine organisms [34,35]. The second compound class is lipid regulating agents, namely, statins and fibrates. The latter, derived from fibric acid, are widely used to reduce plasma triglycerides and raise the level of high-density lipoprotein cholesterol [36]. Examples of such PhACs are clofibric acid, bezafibrate and gemfibrozil. As most of them are excreted unmodified, their fate and potential ecotoxicological risks have been studied extensively throughout the last decade. Although statins (cholesterol-reducing agents) are less frequently studied in environmental matrixes, they are one of the best-selling lipid-lowering agents and their consumption rates are on the rise [37]. Therefore, rosuvastatin and atorvastatin have attracted increased attention in recent years, because some metabolites of atorvastatin are readily hydrolysed into their original acid forms, while rosuvastatin, on the other hand, is mostly excreted unchanged [38].

1.2.5. Antidepressants

According to the Latvian State Agency of Medicines, the use of antidepressants has increased by more than 40% between 2014 and 2018 [39]. A similar trend has been observed worldwide, especially in developed countries. The selective serotonin reuptake inhibitors (SSRIs, fluoxetine, paroxetine and citalopram) are the most common class of PhACs, which can be categorized as antidepressants. Nevertheless, the serotonin-noradrenergic reuptake inhibitors, for example, venlafaxine, and the noradrenergic-dopaminergic reuptake inhibitors, such as bupropion, are also widely used in the treatment of mental disorders [4]. Recent literature has reported solid evidence that antidepressants, especially, SSRIs, can adversely impact aquatic flora and fauna. Yet, to this day, none of these compounds are a part of WFD's "Watch List".

1.2.6. Other PhACs

While these five classes of PhACs, that were discussed above, are recognized as the most relevant pollutants in aquatic compartments, there are also other troublesome compounds, which must be mentioned. For instance, the antiepileptic drug carbamazepine has been frequently detected in almost every study which investigates the occurrence of PPCPs in the environment. Why? The removal efficiency of carbamazepine during wastewater treatment processes is often negligible and it is reluctant to photo-degradation processes in surface waters. Moreover, it may bioaccumulate through the aquatic food web and has shown toxicity towards many aquatic

species including algae [40]. Also, some highly specific therapeutic classes such as iodinated X-ray contrast media and cytostatics (used in chemotherapy) can contribute to the overall pollution. Residues of these compounds are almost exclusively related to hospitals and healthcare facilities and therefore the effluents of these facilities may contain high levels of these substances. Some other notable examples are tranquilizer diazepam, the antidiabetic drug glibenclamide and proton-pump inhibitor omeprazole [41]. This list could go on for a long time. Pharmaceutical industry remains a very fast-growing sector. Year after year, new compounds emerge, that could potentially alleviate the symptoms of a certain disease or, at best, cure it. There is no doubt that human health is a priority, but as the number of PhACs increases, so do the associated risks they pose to the environment. For this reason, future research should not only focus on well-known priority PhACs but simultaneously investigate emerging substances because early assessment of potential risks is essential for proper pollution management and control.

1.3. Consumption of PhACs in Latvia

In 2018, according to the annual consumption statistics of medicinal products in Latvia (Latvian State Agency of Medicines, SAM), two best-selling medicines were acetylsalicylic acid (aspirin) and ibuprofen [39]. Such a tendency is not surprising, because the latter is the most popular over-the-counter painkiller, while aspirin is used extensively as a preventive agent to reduce risks of heart attacks and blood clots. Both of these compounds are categorized as relatively safe and are easily metabolized and degraded in the environment. Unfortunately, the same statement can be made only for a limited number of PhACs.

To investigate the consumption of most significant PhACs in Latvia, SAM data of annual consumption were used. The statistical data on consumption from 2014 to 2018 are expressed as defined daily doses (DDD) per 1000 inhabitants in Latvia per day (DID). Such a normalized expression is handy to observe trends in the pharmaceutical sector, but it does not provide enough information regarding absolute amounts, because each PhAC has a different dose. Therefore, absolute annual consumption for selected pharmaceuticals was calculated in accordance with the current ATC/DDD classification [42]. A scenario of oral administration was selected for each defined daily dose. Demographic data were acquired from Central Statistical Bureau of Latvia [43]. A summary of the estimated annual consumption of selected PhACs and main PhAC classes/subclasses (from 2014 to 2018) is presented in Table 1 and 2, respectively. In any case, national drug consumption database may not completely reflect real-life use of PhACs, because (i) the actual prescribed dose may vary between practitioners, (ii) only oral administration dosage is taken into account in calculations and (iii) not all drugs that are sold/prescribed reach the patient's organism. Nevertheless, this information can reveal the overall consumption trends and estimate the total sold quantities (maximum scenario).

In the context of antibiotics, the current data from 2014 to 2018 shows a slight increase in total consumption (Figure 1). An almost 40% increase is observed for macrolides, lincosamides and streptogramins. On the other hand, the use aminoglycoside antibiotics is on the decline. Altogether, a slight decrease can be observed from 2017 to 2018 for almost all antimicrobials. This is most likely due to increased concerns towards antimicrobial resistanc in the European Union. Nevertheless, the overall consumption of antibiotics is higher than, for example, in 2014.

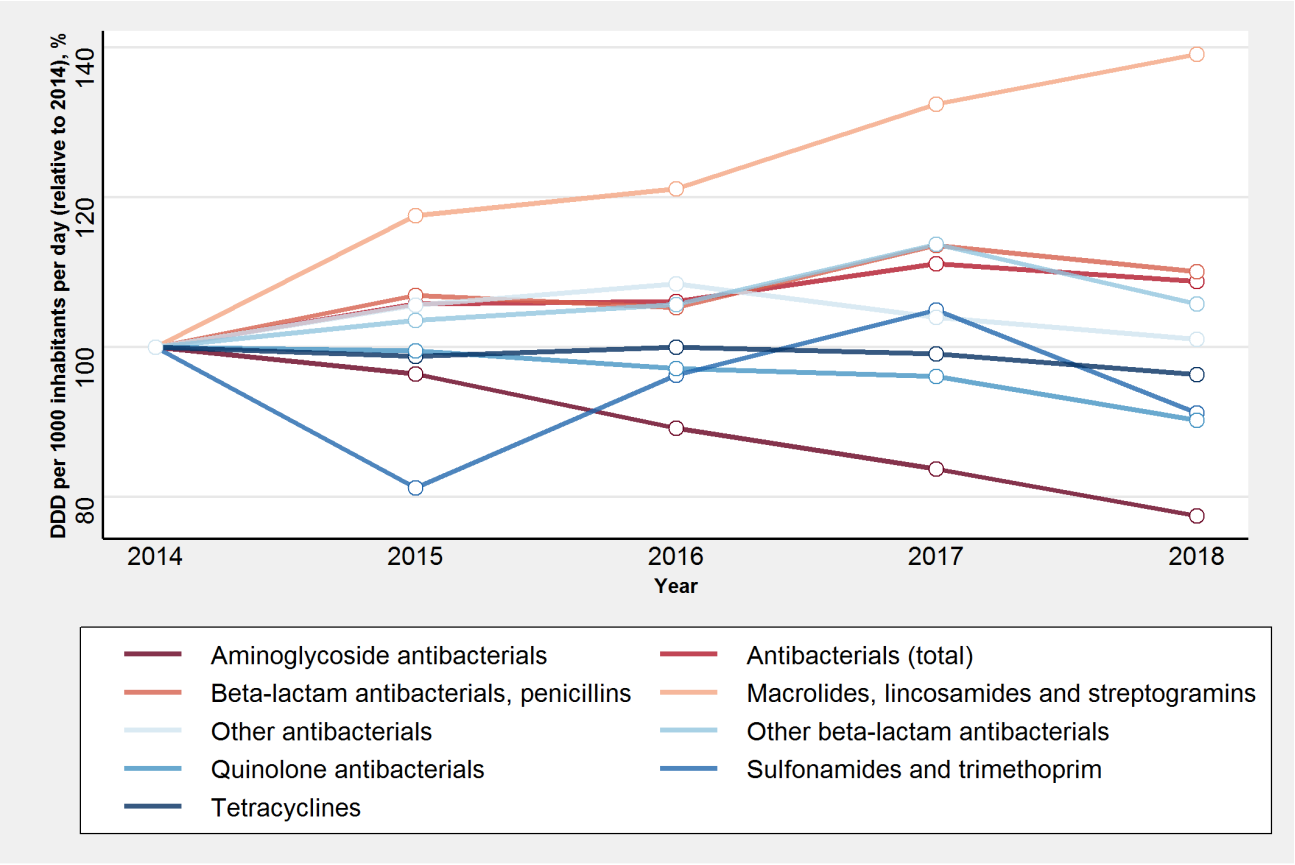


Figure 1. **Consumption trends of antibiotics in Latvia (2014 to 2018, relative to 2014)**

A similar analysis is provided in Figure 2, where annual consumption statistics for selected PhAC classes are summarized (2014-2018). Several trends can be observed. For instance, the use of hormonal medication has decreased by almost 30% since 2014. However, the data about EE2 were not available in the public database. Thus, the actual situation may differ. On the contrary, the use of antidepressants, lipid modifying agents and proton pump inhibitors is on the rise. For some compounds, such as venlafaxine (antidepressant) and rosuvastatin (lipid modifying agent), the numbers have nearly doubled since 2014. Meanwhile, the consumption of NSAIDs remains stable throughout the last five years, but it is still relatively high. Approximately 20 tons of ibuprofen and 1.5 tons of carbamazepine were consumed in 2018. This may not seem particularly noteworthy, but if we calculate the worst-case scenario that all carbamazepine equally contaminates all surface water available on Latvia (35000 million m^3 of water per year), we get a value of around 50 ng/L. Of course, this is a greatly exaggerated example, because only a limited fraction of the initial carbamazepine dose reaches the environment, yet it illustrates how crucial it is to have an effective wastewater treatment system. If we calculate the same hypothetical value for ibuprofen, which fortunately is well metabolized and efficiently removed during wastewater treatment, we get around 500 ng/L. Nevertheless, some PhACs are incredibly potent at very low doses (e.g. hormones, antidepressants). Hence, even seemingly low levels can exceed the predicted no-effect concentration and adversely impact the surrounding environment.

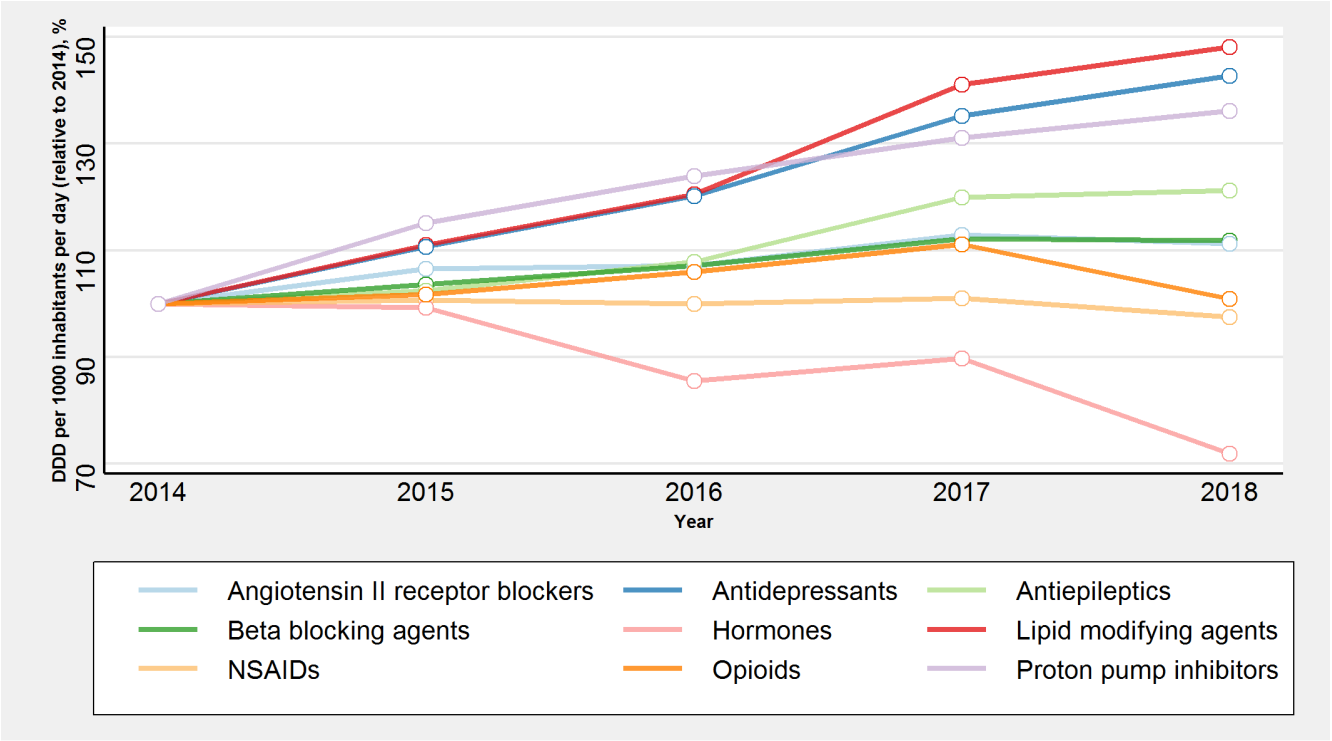


Figure 2. **Consumption trends of selected PhAC classes in Latvia (2014 to 2018, relative to 2014)**

Table 1. **Estimated annual consumption of some PhACs in Latvia from 2014 to 2018**

Class	PhAC **(Oral dose)**	Estimated annual consumption, kg				
		2014	**2015**	**2016**	**2017**	**2018**
Antibiotics	doxycycline (100 mg)	172	168	169	166	160
	amoxicillin (1500 mg)	3168	3328	3178	3323	2957
	clarithromycin (500 mg)	457	565	600	648	679
	azithromycin (300 mg)	108	117	110	125	133
	ciprofloxacin (1000 mg)	655	665	659	666	643
NSAIDs	acetylsalicylic acid (3000 mg)	146758	145076	141299	143116	134592
	paracetamol (3000 mg)	3835	4392	4417	4429	4632
	diclofenac (100 mg)	1692	1635	1581	1509	1369
	meloxicam (15 mg)	38.4	34.8	33.2	33.3	29.8
	ibuprofen (1200 mg)	19287	20065	20368	20039	20057
	naproxen (500 mg)	471	592	624	629	593
	ketoprofen (150 mg)	28.2	27.6	29.9	30.4	27.0
Hormones	estradiol (2 mg)	4.3	4.3	3.6	3.8	2.9
	estriol (2 mg)	0.3	0.3	0.3	0.3	0.3
Lipid modifying agents	atorvastatin (20 mg)	537	569	605	676	695
	rosuvastatin (10 mg)	131	157	173	215	228
	fenofibrate (200 mg)	113	123	132	144	151
Beta blocking agents	propranolol (150 mg)	38.4	37.8	35.5	35.2	32.5
	metoprolol (150 mg)	1478	1432	1378	1318	1206
	bisoprolol (10 mg)	135	135	137	139	136
	nebivolol (5 mg)	37.0	42.3	46.1	50.9	52.7

Table 1. continued

Class	PhAC **(Oral dose)**	Estimated annual consumption, kg				
		2014	**2015**	**2016**	**2017**	**2018**
Antidepressants	paroxetine (20 mg)	34.3	35.5	37.3	40.4	42.3
	escitalopram (10 mg)	21.8	24.9	25.5	30.4	32.4
	amitriptyline (75 mg)	66.8	67.6	65.9	65.4	61.6
	mirtazapine (30 mg)	15.9	17.4	20.0	22.6	23.9
	venlafaxine (100 mg)	42.5	48.2	57.3	64.1	71.0
Angiotensin II receptor blockers	losartan (50 mg)	78.6	72.6	74.4	76.0	73.4
	valsartan (80 mg)	15.0	17.9	20.6	22.4	23.9
Antiepileptics	carbamazepine (1000 mg)	1344	1351	1385	1538	1466
Opioids	tramadol (300 mg)	363	363	348	350	314
Proton pump inhibitors	omeprazole (20 mg)	321	368	384	390	398

Table 2. **Annual DDD/1000 inhabitants/day of some PhACs in Latvia from 2014 to 2018**

Class	Subclass	*Annual DDD/1000 inhabitants/day*				
		2014	**2015**	**2016**	**2017**	**2018**
Antibiotics (total)		***12.1***	***12.8***	***12.9***	***13.5***	***13.2***
	Tetracyclines	*2.3*	*2.3*	*2.3*	*2.3*	*2.3*
	Beta-lactam antibacterials and penicillins	*4.4*	*4.7*	*4.6*	*5.0*	*4.9*
	First, second and third-generation cephalosporins	*1.1*	*1.2*	*1.2*	*1.3*	*1.2*
	Macrolides, lincosamides and streptogramins	*2.0*	*2.3*	*2.4*	*2.6*	*2.8*
	Aminoglycoside antibacterials	*0.1*	*0.1*	*0.1*	*0.1*	*0.1*
	Quinolone antibacterials	*1.3*	*1.3*	*1.2*	*1.2*	*1.1*
	Other antibacterials	*0.9*	*0.9*	*1.0*	*0.9*	*0.9*
NSAIDs (total)		***128***	***129***	***128***	***129***	***125***
Hormones (total)		***3.2***	***3.2***	***2.7***	***2.9***	***2.3***
Lipid modifying agents (total)		***56.3***	***62.5***	***67.8***	***79.4***	***83.4***
Beta blocking agents (total)		***46.4***	***48.1***	***49.7***	***52.0***	***51.9***
Antidepressants (total)		***11.1***	***12.3***	***13.3***	***15.0***	***15.8***
	Selective serotonin reuptake inhibitors	*7.2*	*7.9*	*8.3*	*9.5*	*10.3*
	Other antidepressants	*2.31*	*2.77*	*3.41*	*3.81*	*3.95*
Angiotensin II receptor blockers (total)		***11.0***	***11.8***	***11.8***	***12.5***	***12.3***
Antiepileptics (total)		***8.0***	***8.2***	***8.6***	***9.6***	***9.7***
Opioids (total)		***2.6***	***2.7***	***2.8***	***2.9***	***2.7***
Proton pump inhibitors (total)		***31***	***35***	***38***	***40***	***42***

1.4. Emission sources of PhACs

The content of PhACs in the environment is related to human activities because almost all of them are synthetic products that do not occur naturally. An exception is caffeine, which is produced by several plant species. Despite this, the main source of caffeine emissions is anthropogenic and therefore it is frequently used as a chemical marker to detect domestic wastewater discharge sites. Generally, there are two types of emission sources. Point-source pollution originates from specific and discrete locations. The spatial extent of pollution is therefore

more constrained [44]. Such point-sources are municipal sewage treatment plants, waste disposal sites (e.g. landfills) [45], septic tanks (small-scale sewage treatment systems commonly observed in rural areas) and also industrial effluents (e.g. manufacturing plants, hospitals, food processing plants) [46,47]. Diffuse pollution, in contrast, is not constrained within a particular spatial point and occurs over a broad area [44]. Examples of diffuse PhAC pollution include runoff from agricultural sites, leakage from recycled sludge or bio-solids and urban runoffs [48,49]. These sources cover larger geographical scales, but, at the same time, are less intense and have lower environmental impact compared to point- source pollution. The main emission sources and environmental pathways of PhACs are summarized in Figure 3.

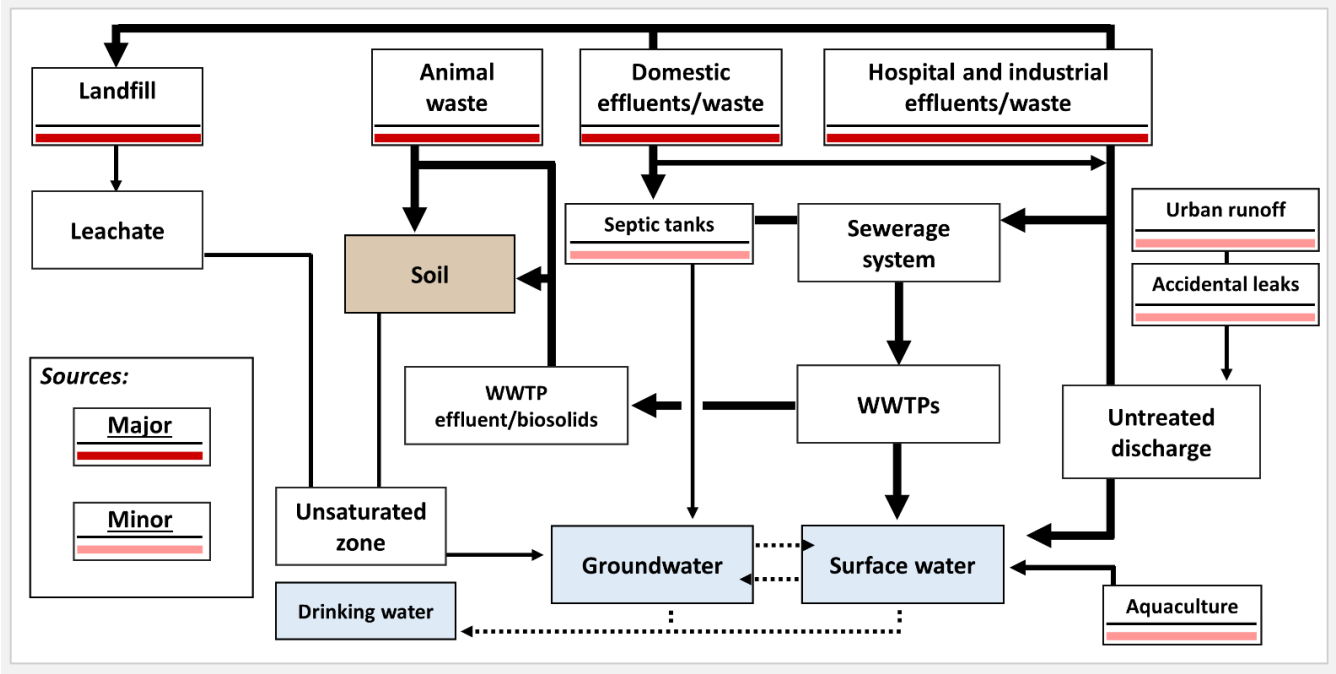


Figure 3. **Potential sources and pathways for environmental pollution by PhACs**

Domestic wastewater is considered the major source of PhACs in the aquatic environment. Typically, each household contains various emission points, which are linked to the main sewer system. Human PhACs can enter the sewer system via different routes e.g. after direct excretion from the body, when washed off (topical and transdermal administration of PhACs) and by improper disposal of medicinal products. Nevertheless, industrial effluents (e.g. effluents from healthcare facilities and pharmaceutical manufacturing sites) are of major concern, since they may discharge high concentrations of PhACs [50]. In most cases, industrial wastewater is treated on the spot by the responsible party (e.g. manufacturer or healthcare institution) before discharge into waterways or municipal sewage system, where it is mixed with domestic wastewater and consequently treated at the main wastewater treatment plant (WWTPs) [51]. In some cases, leakage from underground sewage infrastructure can be a pathway by which PhACs can bypass WWTPs and directly enter the environment. Yet, this is considered a minor pathway. Although WWTPs are well suited for removing solids and reducing the biological oxygen demand, their removal efficiency towards PhACs is limited. In some specific examples, inactive metabolites can be converted back to its initial active form during WWTP processes. Given the aforementioned reasons, WWTPs are recognized as the most serious emission source of PhACs. Nonetheless, PhAC removal rates vary significantly

depending on multiple factors, such as the physicochemical properties of individual compounds, influent load, wastewater composition, surrounding climate and, most importantly, WWTP design [52].

As mentioned before, most PhACs (antibiotics, NSAIDs, cardiovascular drugs, hormones, etc.) exhibit poor bioavailability and are not completely metabolized in human organisms. Therefore, a portion of the administered dose is excreted via urine and feces either as parent compounds or metabolites. The same applies to animals. PhACs are routinely detected in manure and manure-amended agricultural sites due to a release from livestock waste. Besides, soils irrigated with wastewater or biosolids from WWTPs can also contain PhACs and hence might reach groundwater and surface water systems. The leakage of PhACs from contaminated soils is alarming not only from the perspective of aquatic pollution but also poses risks to plant-based agricultural sectors, since the uptake of some PhACs by crop plants has been verified by several studies, indicating that contamination may migrate back to consumers [53].

Another noteworthy source of pollution is aquaculture. Marine and in-land aquaculture has been a rapidly growing sector and has become an important pillar of food production. Most livestock farming practices depend on veterinary drugs and aquaculture is no exception. They are widely used to reduce risks caused by bacteria, viruses, residual feed, and to control infectious disease outbreaks. As a consequence, veterinary drug residues can easily migrate to the surrounding environment. The situation in aquaculture is relatively unique when compared to other framing practices because the medication cannot be administered directly to each animal. It is either dissolved into the surrounding medium (water) or introduced through feed, which again rapidly dissolves in water. Hence the overall administration efficiency remains considerably low and several migration pathways of PhACs can emerge. Nevertheless, aquaculture is still considered a minor emission source of PhACs in comparison to other sources [54,55].

Lastly, agriculture is a frequently overlooked and non-conventional emission source of PhACs. For some it might come as a surprise, but PhACs, especially antibiotics, are applied in several plant-based agricultural fields and beekeeping. They are used to control bacterial diseases of plants (e.g. fire blight of pears and apples) and bees (e.g. honey bee diseases like American Foulbrood and European Foulbrood) [56,57]. Such practices are not authorized in all countries. Yet there are indications that the use of PhACs in this sector is still an issue. For instance, data provided in the Rapid Alert System for Food and Feed portal database of the European Commission on “honey and royal jelly” during 2016–2020 showed that veterinary residues have been detected in honey on five occasions. Besides, in many third world countries application of antimicrobials is still authorized for the treatment of honey bees. These considerations underlay the implication that these two sectors can contribute to the release of PhACs in the environment and pose food safety related risks.

1.5. Occurrence and environmental fate of PhACs

Pharmaceuticals are frequently detected in WWTP influents, effluents and sewage sludge. In reality, the word "frequently" would be an understatement, since PhACs and their transformation products can be found in virtually any wastewater sample of domestic origin. Moreover, they can be found at trace levels (ppt and ppb) in surface water, groundwater, sediments, soils, biota and plants [58]. As WWTPs play the most crucial role in the life cycle of PhACs and their transformation products, the opening paragraph of this section will be attributed to examine the occurrence data of PhACs in wastewater samples.

As mentioned in Section 1.4, the main transport pathway of PhACs into the environment is via WWTPs, as they cannot fully eliminate these pollutants from wastewater influents. The overall treatment process is complex and is affected by countless factors. Hence, the efficiency of different WWTPs can vary greatly. Also, the physico-chemical nature of each pollutant plays a crucial role in this process. In general, wastewater can contain up to several-fold higher PhAC concentrations compared to environmental samples. The longer the path each pollutant has to "travel" from the initial source, the higher dilution can be achieved and the expected contamination level gradually decreases. Typically, PhACs can be found in wastewater samples in concentrations up to several μg per liter. According to Kosma et al. (2010), where 11 PhACs were monitored in a WWTP in Greece for a period of one year, individual PhAC concentrations in WWTP influents and effluents ranged between 0.3 and 164.4 μg/L and 0.5 and 13.9 μg/L, respectively [59]. In a similar study by Martin et al. (2012) sixteen PhACs were evaluated in wastewater and sewage sludge samples from several WWTPs in Spain. The authors found twelve PhACs in raw wastewater with mean concentrations from 0.1 to 32 μg/L. The same compounds were also found in sewage sludge (except diclofenac). Mean concentrations in sludge ranged from 8.1 to 2206 μg/kg (dry matter) [60]. In 2012, a research group from the University of Ferrara (Italy) published an incredibly comprehensive review article, where the occurrence of more than a hundred PhACs was summarized using data from almost 264 municipal WWTPs from various locations, mostly in Europe. The main findings of this study are summarized in Table 3. In short, the authors found that there is a high spatial variability within concentrations of individual substances. To exemplify, ibuprofen, one of the most common NSAIDs, had an average concentration of 37 μg/L in untreated wastewater samples, while almost ten times lower levels (3.6 μg/L) were detected in secondary effluent. Overall, a common trend could be observed - most WWTPs allowed an efficient removal of ibuprofen (almost 90% compared to the initial amount). Conversely, data for carbamazepine were extremely scattered, making it difficult to interpret the results. Estimated removal efficiencies between studies (N=25) varied from -122% to +97%. A similar situation was also reported for other PhACs, for example, antibiotics - trimethoprim and erythromycin [61]. The main conclusion that can be drawn from this review is that there are many problematic PhACs whose removal from influents remains an issue. Besides, the exact concentrations and removal efficiencies of individual substances fluctuate greatly between different locations. Hence, a substantial amount of PhACs is discharged into surface water bodies from WWTPs and may pose a risk to aquatic life [62].

Table 3. **Summary on PhACs found in influents and effluents from 264 WWTPs**

PhAC class	Untreated wastewater (influent)		Treated wastewater (secondary biological effluent)	
	Concentration range, µg/L	**PhACs of concern**	**Concentration range, µg/L**	**PhACs of concern**
NSAIDs	Min:0.0016 Max:373	Ibuprofen^{AB}, diclofenac^{A}, naproxen^{A}, ketoprofen^{A}, Acetaminophen^{B}, tramadol^{B}	Min:0.001 Max:57	Ibuprofen^{AB}, diclofenac^{A}, naproxen^{A}, ketoprofen^{A}, acetaminophen^{A}, tramadol^{B}, dipyrone^{B}
Antibiotics	Min:0.001 Max:32	trimethoprim^{A}, sulfamethoxazole^{A}, erythromycin^{A}, ciprofloxacin^{A}, ofloxacin^{B}, sulfadiazine^{B}, sulfapyridine^{B}, cefalexim^{B}	Min:0.001 Max:6.7	trimethoprim^{A}, sulfamethoxazole^{A}, erythromycin^{A}, ciprofloxacin^{A}, norfloxacin^{A}, ciprofloxacin^{B}, erythromycin^{B}, roxithromycin^{B}, ofloxacin^{B}
Beta-blockers	Min:0.006 Max: 25	atenolol^{AB}, metoprolol^{A}, propranolol^{A}	Min:0.005 Max:73	atenolol^{AB}, metoprolol^{A}, propranolol^{A}
Lipid regulators	Min:0.001 Max:30	bezafibrate^{AB}, gemfibrozil^{A}, clofibric acid^{A}	Min:0.0015 Max:80	gemfibrozil^{A}, bezafibrate^{A}, clofibric acid^{A}, fenofibric acid^{B}
Psychiatric drugs	Min:0.0025 Max:25	carbamazepine^{A}, fluoxetine^{A}, diazepam^{B}, gabapentin^{B}, amitriptyline^{B}	Min:0.001 Max:20	carbamazepine^{A}, diazepam carbamazepine^{B}, gabapentin^{B}
Hormones	Min:0.002 Max:3	estradiol^{AB}, estrone^{A}, ethinylestradiol^{A}, cimetidine^{A}	Min:0.002 Max:0.11	cimetidine^{AB}, estrone^{A}, estradiol^{A}, ethinylestradiol^{A}
Diuretics	Min:0.004 Max:1.8	Hydrochlorothiazide^{AB}, furosemide^{AB}	Min:0.0025 Max:11	Hydrochlorothiazide^{AB}, furosemide^{AB}
PhAC^{A} – PhACs which are most frequently found in wastewater samples; PhAC^{B} – PhACs with the highest average concentration in wastewater samples.				

In a study by Vulliet et al. (2011), the occurrence of 52 substances was investigated in surface and groundwater samples from France. Reported results indicate that residues of PhACs were found in all samples, regardless of the sample origin or sampling season. The most frequently detected compounds were salicylic acid, acetaminophen and carbamazepine. High detection frequency of the first two PhACs can be attributed to their high consumption. Alternatively, carbamazepine consumption rates are at least a fold lower, but has been frequently found everywhere due to its persistence. Nonetheless, the presented data suggest that PhACs are less prevalent in in groundwater as compared to surface water [63]. But again, groundwater contamination can achieve high levels in highly urbanized areas. To give an example, a study carried out by López-Serna et al. (2013) investigated 95 PhACs and various PhAC transformation products in urban groundwater underlying the metropolis of Barcelona (Spain). The PhAC concentrations reported in groundwater occasionally exceeded the ones observed in nearby surface waters. One of the most alarming findings of this study was that antibiotics were found in high frequency (reaching sub µg/L levels), while transformation products of corresponding parent

compounds were found in lower concentrations [64]. Such observation is not a coincidence, because results agree with the findings of other studies that antibiotics account for a significant fraction of total contamination [65].

It should not be forgotten that the marine environment can be affected by PPCPs, especially PhACs. In many occasions, WWTPs are geographically located close to seas and oceans. The Baltic Sea ecosystem is particularly sensitive to PhACs, because of its relatively low biodiversity and slow water exchange rates. Thus, many pollutants, especially the persistent ones, can remain in Baltic Sea for a considerably longer period compared to other marine waters. In 2017, United Nations Educational, Scientific and Cultural Organization (UNESCO) and the Baltic Marine Environment Protection Commission (HELCOM) published a status report on pharmaceuticals in the Baltic Sea region, which is the most comprehensive data source regarding Baltic Sea region. The report includes data on 167 PhACs from several thousand environmental samples throughout the region. Data were reported by Denmark, Estonia, Finland, Germany, Poland, Russia and Sweden. Unfortunately, Latvia did not participate in this project and no information is available regarding the Gulf of Riga. The reported information shows that the most frequently detected substances in the Baltic Sea belong to the therapeutic groups of NSAIDs, cardiovascular agents, and central nervous system agents. The presence of NSAIDs was found in all compartments of the Baltic Sea environment and, without much surprise, the most commonly detected NSAIDs were diclofenac, ibuprofen and paracetamol. In the context of antibiotics, sulfamethoxazole was detected most often (9% from all surface water samples), having a median concentration of about 16 ng/L. A higher detection frequency was observed for cardiovascular agents. Namely, metoprolol and bisoprolol were found in 16% and 23% of water samples, respectively. While carbamazepine, which has already earned a bad reputation, was detected in more than 60% of the reported water samples, with levels up to 73 ng/L. The reported data clearly shows that there is an emerging problem in Baltic Sea region regarding the undesirable presence of PhACs in the aquatic environment [66]. Besides, this is just the tip of an iceberg. The authors of the HELCOM report warn that caution must be applied as these findings may underestimate the true extent of PhAC pollution as some of the analytical protocols used to obtain the data may have had inadequate sensitivity [64].

PhACs used in livestock treatment may enter the environment from manure when it is applied to farmlands. Hence, veterinary drug residues can reach the upper soil layer and migrate to groundwater. Martínez-Carballo et al. (2007) investigated several classes of antibiotics (tetracyclines, sulfonamides, trimethoprim, and fluoroquinolones) in manure samples and soils fertilized with manure. Results revealed that manure can contain high levels of selected compounds, e.g. chlortetracycline and tetracycline was detected in swine manure at concentrations up to 46000 ng/g and 23000 ng/g (dry weight), respectively. Nevertheless, only chlortetracycline was frequently found in soil samples. Despite the high initial residue content in manure, these results indicate that abiotic (hydrolysis, oxidation, photo-degradation, etc.) and biotic degradation processes occur during the transport of PhACs from manure to soil and also in the soil itself [67]. Other studies have likewise demonstrated that PhACs are extensively transformed by soil chemical and microbiological processes. For instance, Solliec et al. (2017) examined the fate of tetracycline antibiotics in soils, drainage waters and swine manure. A gradual decrease of concentration between these three sample types was found. Namely, the observed concentration in drainage waters was found to be almost 3-fold lower than in manure, while the concentration in soil barely exceeded several ng/g. That said, transformation products of tetracycline antibiotics were found in concentrations higher than the parent compound. However, the transformation pathway of PhACs is complex and could result in other bioactive

compounds, which can exhibit potentially higher toxicity than their parent [68]. Generally, migration of PhACs to the surrounding environment due to application of animal manure and sewage sludge to farmlands is much slower than, for example, direct WWTP effluent discharge into surface waters. Direct contact with aquatic and terrestrial species, in this case, is more limited and, therefore, the potential ecotoxicological risks have a lower magnitude.

The final destination of PhACs is biota. Although most of these compounds are not persistent, PhACs can be classified as "pseudo-persistent" because they continuously enter the aquatic environment. As a result, aquatic organisms are constantly exposed to a large variety of PPCPs and PhACs. Their ubiquitous presence causes chronic exposure and promotes bioaccumulation [6]. The most commonly studied biota samples are bivalves [69] and fish [70,71]. For instance, in some studies, mussels are even used as biological "samplers" for monitoring water quality. They are sedentary filter-feeders and can be exposed to contamination that is either dissolved in water or bound to particulates in the water column. Unluckily for mussels, in 1986 The U.S. National Oceanic and Atmospheric Administration (NOAA) lunched a specific monitoring program called "Mussel Watch Program". It was aimed to monitor contaminants of concern in bivalves (mussels and oysters) [72]. Samples from this program were used in a study by Dodder et al. (2014), where the occurrence of PPCPs, alkylphenols, polybrominated diphenyl ethers, pesticides and perfluorinated compounds was investigated. Results revealed that PPCPs, including PhACs, were the most dominant contaminant class in terms of absolute concentrations. While the usual suspects (diclofenac and carbamazepine) were not analyzed in this study, antibiotics (enrofloxacin, sulfamethazine and lomefloxacin) were frequently found in mussel tissue regardless of sampling location [73]. Wille et al. (2011) analyzed blue mussels (*Mytilus edulis*) caged at different stations in the Belgian coastal zone. The authors reported results for 11 PhACs. Five compounds were detected above the limit of quantification: ofloxacin, propranolol, salicylic acid, paracetamol and carbamazepine. The latter was found from 1 to 11 ng/g (dry weight) [74]. However, it impossible to determine the bioaccumulation potential of PhACs from occurrence data alone. To study the behaviour of these compounds, de Solla et al. (2016) conducted an in-depth study where bioaccumulation of 145 different PPCPs was investigated in wild and caged mussels (*Lasmigona costata*) near WWTP discharge site. It was concluded that bioaccumulation in the mussels correlated well with log K_{OC} (soil adsorption coefficient) and log K_{OW} (n-octanol/water partition coefficient). The highest bioaccumulation factors were found for non-polar antidepressants - amitriptyline and sertraline. Both of them were detected with high frequency and their concentrations varied in a range from around 5 to 80 ng/g (wet weight). As expected, highly polar PhACs like naproxen and metformin did not show any signs of persistence [75]. These results are broadly consistent with Valdes et al. (2016), where 20 PhACs, including carbamazepine and diclofenac, were analyzed in two fish species (*Gambusia affinis* and *Jenynsia multidentata*).A noticeable difference in the accumulation patterns was observed between both fish species. This observation suggests that different bioaccumulation pathways exist. Contrary to expectations, this study did not find traces of diclofenac in the analyzed samples. However, an opposite situation was noted for carbamazepine. The parent compound and two of its metabolites were monitored in gills, intestine, liver, brain and muscle of fish. Note: metabolites were not artificially added, nor detected in the exposure media during this study. Interestingly, carbamazepine and its epoxide were found in all analyzed organs, whilst hydroxylated metabolite was found only in muscle tissue and gills. In comparison to traditional persistent organic pollutants (log $K_{OW} > 3$), bioconcentration factors for carbamazepine are low.

Theoretically it is considered a non-bioaccumulating substance. However, the authors argue that this factor should not be used as a sole predictor of bioaccumulation potential [76]. Overall, many studies have shown that uptake of PhACs in biota is not a minor problem that can simply be swept under the rug. The majority of PhACs that enter marine and freshwater bodies can indeed be found in aquatic species. Meanwhile, there is still a large demand for research studies that could fully characterize the accumulation and metabolic processes in aquatic ecosystems continuously exposed to PhACs.

Taking everything into consideration, there is a common misconception that PhAC residues in the environment have no implications for human health. But it is not entirely true because such pollution can reach us through drinking water or by consumption of contaminated products (e.g. fish, molluscs, seaweeds, etc.). Therefore, high occurrence rates of PhACs in environmental compartments should not be considered a trivial issue created by environmental activists and scientific community, but it can soon enough negatively affect such sectors as healthcare (spread of antimicrobial resistance), food safety and limit the access to safe drinking water.

1.6. Ecotoxicological effects of PhACs the aquatic environment

Even though this dissertation is primarily focused on three specific topics related to PhACs, namely, their occurrence, analytical detection capabilities and removal, ecotoxicological aspects of these compounds should not be overlooked. It is the main reason why this topic is relevant at all. Based on reported environmental concentrations of PhACs in aquatic compartments, acute toxicity and short term adverse effects are rarely recorded for these substances. Yet, their ability to disrupt ecological processes in marine and, especially, freshwater ecosystems is not negligible [77]. The synthetic estrogen used in birth-control pills,17 alpha-ethynylestradiol (EE2), can be regarded as a prominent example. In 2007, a team of researchers from Department of Fisheries and Oceans (Canada) conducted a long-term study in an enclosed lake, where low concentration of EE2 (5-6 ng/L) was maintained throughout a period of seven years. Until the publication of this study, no conclusive evidence was found that EE2 is directly responsible for the induced abnormalities in certain fish populations in Asia. However, reported results indisputably showed that chronic exposure of fathead minnow (*Pimephales promelas)* to EE2 led to feminization of males. This phenomenon gradually decreased the reproductive success of fish, which eventually caused almost complete extinction of this species in the lake [78]. These devastating consequences were kicked off by only one specific compound. Even more alarming is the fact that the concentration level at which EE2 was present in water is incredibly low. Because it is below the limit of detection for most instrumental techniques, many multi-residue methods cannot detect it.,

In this context, another disastrous example must be mentioned. Although it is not directly related to the aquatic environment, it demonstrates how residues of PhACs can also alter terrestrial ecosystems. Since the 1990s vulture populations (*Gyps bengalensis, Gyps indicus, Gyps tenuirostris*) across the Indian subcontinent have been nearly wiped out. The current evidence indicates that the rapid decline of vultures in Southeast Asia is caused by toxic effects of diclofenac, which is used widely to treat inflammation in cattle. In several studies, it was confirmed that these scavenging birds die within a short time due to renal failure (kidney failure) after feeding on carcasses of livestock animals who have received a normal veterinary dose of diclofenac [79,80]. This finding once again reminds how fragile ecosystems are. A simple molecule, which is extensively used as a human and veterinary drug, can accidentally annihilate an entire population.

It must be noted that diclofenac and EE2 are extreme cases. In general, direct adverse effects of PhACs are not observed for marine and freshwater vertebrates at current exposure levels. However, behavioural distortions caused by PhACs can create a massive "domino-effect" reducing the survival potential of exposed species. For instance, Brodin et al. (2013) published a research article in Science, where behavioural abnormalities were investigated in wild European perch (*Perca fluviatilis*). In this study fish were subjected to moderate concentrations (1.8 µg/L) of oxazepam, a benzodiazepine anxiolytic drug wildly used to treat anxiety and insomnia. Although 1.8 µg/L might seem relatively high compared to expected environmental concentrations, the authors state that such conditions were selected to mimic oxazepam exposure levels nearby WWTP discharge sites. The exposed individuals displayed increased activity, reduced sociality and higher feeding rate. In the long term, these abnormalities can result in an accelerated depletion of food resources and negatively affect the spatial biodiversity of the exposed location [81]. The same drug has also been able to induce a similar effect in wild roach (*Rutilus rutilus*) at environmentally relevant concentrations below 1 µg/L [82]. Likewise, Martin et al. (2016) reported that another antidepressant (flouextine, 25 and 226 ng/L) alters antipredator behaviour in Eastern mosquitofish (*Gambusia holbrooki*). In other words, exposed individuals were more "careless". They entered the predator 'strike zone' more rapidly and did not utilize the usual strategy for predator avoidance as often as the control group [83]. One might argue that sub-lethal behavioural effects are trivial compared to acute toxicity or reproductive failure. Yet, such abnormalities can induce cascading indirect effects at all trophic levels within the habitat. To give a brief example, the previously described situation in India where diclofenac induced mass extinction of vulture communities has allowed feral dogs to scavenge on carcasses more frequently. As a consequence, the rate of human rabies infections from dog bites has increased in the past decade [84].

Numerous reports reveal that individual PhACs can have a serious ecological impact and may induce biological response in non-target organisms even at low exposure level. Besides, vertebrates (fish, mammals, birds, amphibians, etc.) are not the only ones that can be affected by these compounds. Aquatic plants, algae, microorganisms, crustaceans and microscopic animals such as nematodes and rotifers can also display negative side effects when exposed to PhACs [7]. The ecological effects of individual PhACs can be magnified when multiple PhACs (or other substances) are simultaneously present and create the so-called "cocktail effect". These effects are incredibly difficult to investigate and further research is needed to understand how complex mixtures of PPCPs affect the environment. Moreover, there is still a limited amount of data regarding long-term exposure, since it cannot be assessed via conventional short-term toxicity assessments [49,77].

1.7. Removal of PhACs during wastewater treatment processes

In recent decades, various technologies, including biological, physical and chemical processes have been extensively investigated for the removal of PhACs from domestic and industrial wastewater. WWTPs are the main units responsible for the efficient removal of pollution from incoming sewage. In most cases, the treatment of raw wastewater is performed in two stages. Primary treatment is designed to separate solids by the physical processes, e.g. flotation and sedimentation. Larger objects are retained by specific grids. Medium and small-sized solids are withdrawn in grit chambers and then directed to sedimentation tanks, where the elimination of solids continues. The parametric value of biochemical oxygen demand (BODs) and total suspended solids is usually halved during the primary treatment. Some pollutants which are bound to solids can be removed during this type of treatment, but colloidal and dissolved fraction stays largely unaffected. To remove the latter, secondary treatment is applied.

It utilizes different types of microorganisms in a controlled environment to remove pollutants employing biotransformation, air stripping, sorption and photo-transformation principles. To this day numerous secondary treatment technologies exist, e.g. application of activated sludge, membrane bioreactors (MBR), moving bed biofilm reactors (MBBR) and others. After the biological treatment the effluent can be either directed to secondary sedimentation tanks and discharged into the environment or subjected to more sophisticated pollutant removal technologies, namely, tertiary and quaternary treatment. The latter two are considered the best option for complete removal of PhACs and will be discussed later [85,86].

Although municipal WWTPs are effective and robust in many ways, they can achieve only partial removal of PhACs. The removal efficiency varies greatly depending on multiple factors. It is influenced by physico-chemical properties of individual compounds, environmental conditions, applied technologies and operational parameters (solids retention time and hydraulic retention time). Radjenović et al. (2009) compared the removal efficiency of 31 PhACs in a full-scale conventional WWTP (WWTP Terrassa, Spain) which utilizes activated sludge and two pilot-scale MBRs. Removal efficiencies for the full-scale unit ranged from 15.0% to 99.9%. The most problematic PhACs were diclofenac (NSAID), sotalol (beta-blocker) and fluoxetine (antidepressant). On the contrary, polar NSAIDs like ibuprofen and paracetamol were removed almost completely regardless of the type of treatment applied. The overall results suggested that MBR outperforms activated sludge treatment. The authors conclude that out of three dominant processes that occur simultaneously (biodegradation, sorption and abiotic degradation) removal of PhACs by sorption to sludge is a minor removal pathway. However, this process is not negligible because PhACs can still interact with suspended sludge through hydrophobic and electrostatic interactions and, in some cases, even chemically bind to bacterial proteins and nucleic acids [87]. In this context, the most important process is biodegradation. The predominant group of bacteria in sludge is heterotrophs. They mainly feed on organic molecules. While the inorganic matter is transformed by autotrophs, such as ammonia oxidizing bacteria. Removal of PhACs in WWTPs occurs by two biological pathways, i.e., co-metabolism, in which compounds are degraded by enzymes produced by microbial communities present in the sewage sludge or by substrate degradation in which microorganisms use organic compounds solely as a source of carbon and energy. Co-metabolism is considered to be the main biological pathway [88]. But then again, sorption processes and biotic removal of PhACs is highly dependent on the individual target compound. Salgado et al. (2012) showed that even compounds within one therapeutic group and similar physiochemical properties can exhibit very different removal rates. For instance, within the group of NSAIDs, diclofenac displayed low biological removal rate. Meanwhile, ibuprofen and ketoprofen were almost completely eliminated from the effluent [89]. This is not a random coincidence. Diclofenac has been identified as a highly problematic member of the PhACs. For that reason, it will be used in the next paragraph as a “case study” to briefly describe some of the most significant aspects of PhAC removal.

Diclofenac is slightly soluble in water and has a moderately low octanol–water coefficient. At neutral pH the carboxylic group of diclofenac has a negative charge. Thus, it repels the negatively charged sludge if conditions of the surrounding medium are not sufficiently acidic. According to Vieno et al. (2014), pH value is slightly lower during the primary treatment compared to secondary treatment with sludge [90]. This implies that diclofenac interacts with the sludge via adsorption only when pH is favourable. Unless such conditions are met, its adsorption to sludge is negligible. This phenomenon was confirmed by Urase et al. (2005), where removal of

diclofenac by sorption on activated sludge increased by more than 20 times when the medium was acidified to pH 4.4 [91]. The approach works well for a laboratory-scale experiment, but it cannot be practically applied on a full-scale WWTP. At least, not without major consequences that will negatively affect other removal processes. Hence, the estimated removal of diclofenac via solids rarely reaches 10% from the initial environmental load [90]. Efficient biodegradation requires that the substrate can reach the surface of the activated sludge. Numerous studies have shown that conventional application of activated sludge cannot biodegrade diclofenac, especially in anaerobic conditions. Under some circumstances, even higher concentrations of diclofenac can be observed in secondary effluent compared to untreated wastewater. According to Lee et al. (2012), this phenomenon prevails due to the deconjugation of glucuronide or sulphate conjugates of diclofenac [3].

Diclofenac is not the only compound that displays poor removal in conventional wastewater treatment practices. Other prominent examples that can be added to this list are, for example, carbamazepine, diazepam and clarithromycin [92]. In past decades, advancements of secondary treatment technologies like MBR and MBBR have greatly improved our capabilities to remove micropollutants from wastewater. MBR relies on membrane processes (e.g. microfiltration or ultrafiltration) which allows more control over activated sludge accelerating removal [93]. MBBRs rely on biofilms that are grown on small carriers. These carriers are suspended and mixed in a reactor during secondary treatment and can greatly increase the surface area for microbial communities [94]. Nonetheless, some WWTP utilize even more sophisticated techniques. The most promising solutions for the removal of PhACs have been summarized by Wang et al. (2016) and Cecconet et al. (2017). Examples of some tertiary and quaternary treatment methods along with a brief description are presented in Table 4 [95,96].

Table 4. **Advanced treatment techniques for removal PhACs from wastewater effluent**

Name		**Description** **Main benefits** **Main disadvantages**
Activated carbon, graphene, graphene oxide and carbon nanotubes		Removes PhACs on basis of π- π interactions (Van der Waals forces), hydrogen bonding and electrostatic interactions.
	+	High surface area and adsorption capacity for small molecules.
	-	Low adsorption capacity towards macromolecular substances
	-	Relatively high cost and difficult to recycle/regenerate
Ozonation		Applies non-selective oxidizing activity of hydroxyl radicals to eliminate pollutants.
	+	Widely used oxidation method in post-treatment, that can remove most PhACs with the removal efficiency more than 90%.
	-	Treatment can form toxic by-products.
Fenton oxidation, electro-Fenton and photo-Fenton oxidation		Uses metal-based catalysts (or iron salts) in combination with hydrogen peroxide to generate hydroxyl radicals and remove pollutants.
	+	Effective for large diapason of compounds, including macromolecules.
	-	Treatment can form toxic byproducts.
	-	High cost and complicated recycling process.

Table 4. continued

Name		Description
	+	**Main benefits**
	-	**Main disadvantages**
UV treatment		Dismantle chemical bonds of pollutants employing photolysis.
	+	Frequently applied when reclaimed water from WWTP is directly reused.
	-	Direct UV photolysis is not always effective for the removal of PhACs.
	-	A combination of UV and hydrogen peroxide can enhance the removal, but simultaneously produce toxic transformation products.
Ionizing irradiation (e.g. gamma irradiation, electron beams)		Creates reactive species through water radiolysis that react further with pollutants.
	+	Additional chemicals are not needed.
	+	Even the most persistent compounds can be degraded by this technique.
	-	Relatively expensive and gamma irradiation requires the use of radioisotopes.
Bioelectrochemical systems (microbial fuel cells and microbial electrolysis cells)		Microorganisms catalyse the red-ox reactions of organic and inorganic electron donors/acceptors, at their anodic and cathodic electrodes, which are separated by an ionic exchange membrane.
	+	A promising technique for the removal of PhACs.
	+	Is considered more sustainable in terms of energy demand.
	-	An emerging technology that lacks data for successful application in full-scale WWTPs.

All things considered, combined WWTP treatment techniques have demonstrated promising results in comparison to conventional secondary treatment. Advanced abiotic processes are slowly getting momentum and more frequently integrated into full-scale WWTPs to remove contaminants from secondary effluent. At the same time, novel biotechnologies can be incorporated to boost the efficiency of activated sludge, i.e. hybridized biomasses, tailor-made membranes, specific biofilm carriers and others. While many of these techniques have a bright future ahead, various implications still require focal attention in upcoming years, for example, possible toxicity of transformation products, sustainability and successful scale-up for full-scale applicability [96].

1.8. Analytical strategies for the determination of PhACs in environmental matrixes

Previous sections have provided some insight into the question of why PhACs are relevant from an environmental perspective. This section, in turn, is intended to give an overview of current methodologies for the determination of PhACs in environmental matrixes.

The analytical procedures to determine PhACs in environmental matrixes (e.g. surface water, wastewater, groundwater, sediments, biota, etc.) involve three critical steps - sample treatment, instrumental separation and detection. In modern applications, separation is mostly performed via gas chromatography (GC) or liquid chromatography (LC). The latter is considered the most popular option because the majority of PhACs are non-volatile and relatively polar. Although there are alternatives in terms of detection, almost all multi-residue methods rely solely on mass spectrometry (MS). All three aforementioned steps are equally important. A slight issue in one of them can completely disrupt the performance of the overall analytical method. In the next subsections, each analytical stage will be placed under the magnifying glass.

1.8.1. Sample treatment

Sample treatment, which usually involves homogenization, extraction and clean-up, is still a critical step despite the surge in advancements in the detection and separation technologies. The main goals for sample treatment are as follows: (i) efficiently extract the target analytes, (ii) remove interferences and (iii) obtain extracts

that are suitable for instrumental analysis. In general, environmental samples can be classified in three domains, i.e. water (groundwater, surface water), complex samples which do not originate from plants or animals (wastewater, sediments, soil, sludge, etc.) and so-called “biota” - animal or plant origin matrixes (fish, bivalve molluscs, aquatic plants, etc.). Each one of them requires a slightly different approach. For instance, surface water samples already contain small amount of interferences and therefore tedious sample preparation is not required. In contrast, samples of biological origin require more laborious treatment to remove interfering compounds and achieve sensitivity necessary for trace analysis [97].

The initial step in sample treatment for solid matrixes is homogenization and, in some cases, elimination of excess water. The latter is usually achieved by drying salts, e.g. Na_2SO_4 and $MgSO_4$, or by lyophilization. In addition to homogenization, enzymatic, alkaline and acidic digestion can also be a feasible option. Yet, digestion is scarcely applied for multi-residue methods, because some PhACs can readily decompose under digestive conditions [98]. Liquid matrixes do not require such pretreatment. In most situations, only filtration or centrifugation is necessary to remove suspended solids prior the extraction of target analytes.

After the pre-treatment, extraction of PhACs and selective sample clean-up must be carried out. Both of these steps are usually integrated into one procedure when analysing liquid samples. Hence, it is more convenient to discuss both extraction and clean-up together. In case of surface water, wastewater and groundwater, the most frequently applied techniques are as follows, solid phase extraction (SPE), dispersive liquid-liquid microextraction (DLLME), solid phase micro-extraction (SPME), stir bar sorptive extraction (SBSE) and liquid-liquid extraction (LLE) (see Table 5). The latter will not be discussed in detail since modern analytical methods (in the context of PhACs) scarcely use LLE, with exception of trace analysis of non-polar PhACs, e.g. determination of hormones via GC-MS.

Meanwhile, the extraction stage of solid samples is usually separated from clean-up. Commonly applied techniques involve conventional solid-liquid extraction (SLE), pressurized liquid extract (PLE), accelerated solvent extraction (ASE), Soxhlet extraction, microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). PLE, ASE and Soxhlet extraction are carried out at elevated temperature and/or pressure. MAE utilizes high frequency non-ionizing radiation (microwave energy) to irradiate the extraction mixture while UAE employs microwave energy for the same purpose. Either way, the goal is to promote the transfer of analytes from the sample matrix into the extraction solvent. All of these techniques are considered suitable for the extraction of PhACs from solid environmental matrixes. Nonetheless, the amount of co-extracted interferences and analyte recoveries may vary greatly between different methods even when the same extraction protocol is employed [99]. The techniques mentioned in the previous paragraph (Table 5.) can also be used for the treatment of extracts that are obtained from solid environmental samples. All of them are considered applicable, but SPE is the most popular approach among others.

Table 5. **Extraction and clean-up methods for determination of PhACs in aqueous environmental samples**

Name	Description	Ref.
SPE	In brief, SPE is a method used for the isolation and enrichment of selected analytes dissolved in a liquid phase. The sample is passed through a cartridge containing a specific stationary phase that retains analytes. SPE is the most extensively used sample preparation technique for PhAC analysis in aqueous matrixes. The advantages of SPE include simplicity, high enrichment factors and robustness. Besides, a large variety of stationary phases exists and enrichment of analytes can be achieved through different principles (e.g. normal phase, reversed phase, molecularly imprinted polymers (MIPs), ion exchange, etc.).	[100]
DLLME	DLLME is based on a ternary solvent system. It consists of an aqueous sample, dispersive solvent (e.g. methanol or acetonitrile) and extraction solvent (e.g. chloroform or dichloromethane). The dispersive solvent must be miscible with the organic extraction solvent. The extraction occurs when a mixture of both solvents is rapidly added to the aqueous sample. A cloudy state consisting of fine droplets is immediately formed and analytes are extracted into the extraction solvent, which is then separated via centrifugation.	[101]
DSPE	DSPE is categorized as an SPE technique. The main principles of this approach are similar to SPE. However, the stationary phase is not embedded in a specific cartridge but instead dispersed within the liquid sample. It can be applied for analyte enrichment or selective removal of interferences if target analytes do not exhibit affinity towards the stationary phase. In most cases, DSPE is used for matrixes that require relatively small volumes, e.g. wastewater, plasma and urine. Furthermore, materials like magnetic nanoparticles can be incorporated into the DSPE to increase surface area and ease the collection of dispersed material.	[102]
SPME	As the name suggests, SPME is closely related to SPE. It involves a tailor-made fiber that is coated with a stationary phase. The fiber is inserted into the sample and agitated for a brief period until extraction reaches its equilibrium. After that SPME device is transferred to an injection port where analytes are released into the instrumental system (e.g. GC-MS and LC-MS). In some situations, analytes are not directly desorbed from SPME but instead subjected to a further clean-up. This technique is usually applied for small sample volumes or pre-concentrated extracts.	[103]
SBSE	This technique employs a magnetic stir bar whose surface is coated with a specific stationary phase (e.g. polydimethylsiloxane). The bar is directly introduced into liquid sample media and stirred. The analytes are retained on its surface during stirring. After sorption, SBSE device is removed and the compounds can be desorbed. This technique can be applied for both small and large sample volumes. Furthermore, the surface of the bar can be functionalized according to method requirements.	[104]

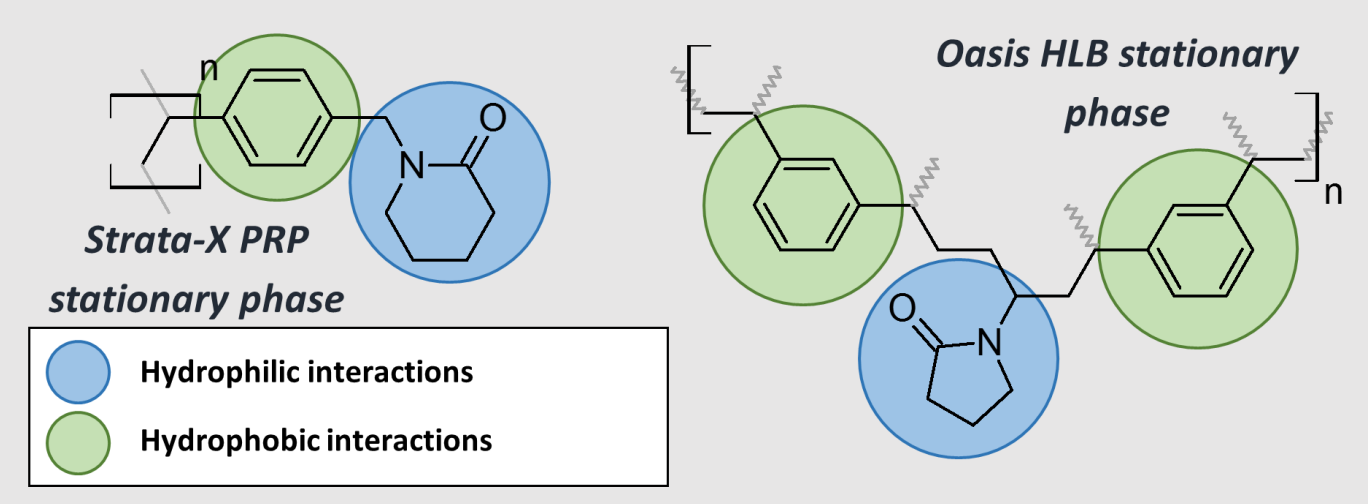


Figure 4. **SPE stationary phases of Oasis HLB and Strata-X PRP cartridges**

The most frequently applied SPE columns for multi-residue methods are Oasis HLB and Strata-X-PRP. The first consists of lipophilic divinylbenzene-vinylpyrrolidone and hydrophilic N-vinylpyrrolidone, while the latter is made of polydivinylbenzene resin containing piperidone groups (Figure 4). The hydrophilic moieties offer good wettability and high mass transfer rates from aqueous media, while the lipophilic ones provide retention of analytes via reversed phase principles. These properties allow the simultaneous isolation of compounds with various physico-chemical properties. Nonetheless, there are several alternatives available if target analytes are less versatile. For instance, MIPs are frequently applied for the analysis of hormones because almost all of these compounds share the same condensed ring system as they are mostly derived cholesterol. Therefore, structure-specific cavities can be incorporated in the sorbent to retain steroidal estrogens. Another example is acidic NSAIDs and aminoglycosides. The first group of substances contain a carboxylic group, while the latter are amino sugars. Thus, anion-exchange SPE can be used for NSAIDs while cation-exchange SPE for aminoglycosides. Also, SPE techniques can be easily automated and applied both offline and online [97,105]. While most of these extractions are performed in laboratories, SPE technology can also be used for field sampling. Several devices have been already designed for passive sampling of PhACs from surface waters, for instance, Chemcatcher®. These devices are submerged in water to retain pollutants from territories where otherwise large sample volumes would be required. Nevertheless, this sampling type is still semi-quantitative, because surrounding conditions significantly affect the performance of each passive sampler [106].

Another noteworthy method that can be used for the extraction of PhACs is QuEChERS. The abbreviation stands for “Quick, Easy, Cheap, Effective, Rugged and Safe”. It was developed in the early 2000s by Michelangelo Anastassiades during his post-doctoral visit at the Agricultural Research Service (a scientific in-house research agency of the US Department of Agriculture). This approach has revolutionized the field of pesticide residue analysis and suits as a great example of how simplicity can sometimes be the key to success [107]. In brief, QuEChERS method is based on the extraction with acetonitrile. The organic solvent is then separated by adding a mixture of salts (MgSO4, NaCl, e.g.). Afterwards, the freeze-out step can be incorporated to remove low solubility interferences (e.g. lipids and carbohydrates) or, alternatively, the crude extract can be additionally cleaned-up via DSPE procedure. Although QuEChERS is not considered highly selective, it is very useful for

multi-residue analysis and a growing number of studies have now begun to acknowledge this method as a good alternative to conventional SPE [108].

Additional clean-up can be required (e.g. silica gel or florisil based preparative chromatography, gel permeation chromatography, etc.) when analysing complicated sample matrixes. However, each additional clean-up step complicates the sample treatment procedure and makes the method more selective towards some analytes. Selectivity is mostly considered an advantage, but it can also become a drawback when different classes of PhACs are analysed within the same procedure because some of them may be lost due to an extensive clean-up. Foremost, several novel approaches have emerged, for instance, applications of ionic liquids, nanomaterial-based extraction, supramolecular solvents, etc. Yet, most of them are (i) still under development, (ii) prone to poor reproducibility and (iii) too complicated or costly to be used for routine analysis [98].

1.8.2. Chromatographic separation

Given the complexity of environmental samples, chromatographic separation is an essential part of PhAC analysis. As mentioned before, most of these substances are categorized as relatively polar, non-volatile and prone to thermal decomposition, thus separation is frequently achieved through LC rather than GC. Some of these limitations can be overcome by derivatization and, in a few instances, GC provides superior sensitivity which renders it more suitable for the determination of specific PhAC classes (e.g. steroidal estrogens and NSAIDs). Nonetheless, due to recent advances in UPLC based separation techniques, LC has become the primary choice for multi-residue methods. In general, contemporary applications depend on columns packed with sub-2 μm particles. This enables better chromatographic resolution and increased peak capacity. Meanwhile, older generation HPLC equipment continuously remains relevant because the availability of columns packed with core-shell particles. This technology allows maintaining high separation efficiency at relatively low back-pressures. Aside from the analytical benefits, the aforementioned techniques are also more environmentally friendly due to reduced solvent consumption and shorter analysis time. This aspect is often neglected and thus creates a paradox: researchers undertake studies to explore the realm of environmental pollutants but use methods that do not comply with the principles of green chemistry. Some practices that may be categorized as sustainable and have been adopted for the analysis of PhACs are: micro-flow and nano-flow LC applications [109], high-temperature LC, supercritical fluid chromatography with carbon dioxide [110] and substitution of LC with direct injection. The same principle also applies to reagents and materials, thus numerous promising alternatives have emerged, such as environmentally friendly mobile phases (e.g. acetone, ethanol and pure water) and reusable phase additives (e.g. ionic liquids) [111]. Altogether, these advances have made it possible to improve current analytical procedures with respect to their their environmental footprint.

Current trends in the analysis of PhACs indicate the growing importance of multi-residue methods. Taking into account the physico-chemical nature of target analytes, C8 and C18 bonded stationary phases are considered the first choice as they enable simultaneous separation of PhACs belonging to different therapeutic classes [112]. Although expanding the overall scope of the method is viewed as a benefit, it can also be considered a limitation in terms of selectivity. If alkyl bonded stationary phase cannot provide sufficient separation, other alternatives may be explored. For example, an enhanced selectivity can be achieved utilizing hydrophobic forces such as π-π interactions available through in π-conjugated systems. Surface functionalization of silica bond stationary phases is achieved by substituting octadecyl groups with phenyl moieties enabling superior retention of substances that

exhibit π-π interactions. It is an effective strategy that is suitable for various PhACs, such as NSAIDs, tetracycline antibiotics and benzodiazepines [113]. There is, of course, substances that cannot be retained by conventional reversed phase chromatography and require other approaches. For instance, aminoglycoside antibiotics are highly cationic, thus hydrophilic interaction chromatography (HILIC) must be used instead [114]. Furthermore, many PhACs are chiral, existing in the environment as a racemic mixture or a single enantiomer. Enantiospecific behaviour of chiral drugs is a fundamental aspect of their nature. Specific molecular recognition processes affect their fate in the environment and regulate the biological activity against living organisms. Despite these considerations, chirality remains largely neglected in the field of environmental analytical chemistry as the analysis of individual enantiomers requires enantiospecific separation [115]. Chiral stationary phases can resolve this issue and, as highlighted by Vazquez-Roig et al. (2014), provide new insights into enantiomer enrichment and stereoselective degradation that occurs in the environment and during wastewater treatment processes [116]. Moreover, a state-of-art analysis of PhACs may be carried out using two-dimensional (2D) LC techniques performed in either "heart-cut" or comprehensive modes. Hence, different stationary phases can be simultaneously utilized enabling unprecedented chromatographic separation [117]. 2D-LC is seldom used in this field due to demanding instrumental requirements and high complexity, but, nevertheless, it will have a bright future if these disputes can be resolved. Overall, the chromatographic tools discussed in this paragraph display several advantages, but their applicability is often limited to single residue (or single class) methods rather than multi-analyte methods.

Without a doubt, there is an extensive list of factors other than stationary phase that influence the overall performance of LC-based methods (e.g. injection and mobile phase constitution, mobile phase flow rate, injection volume, column temperature, mobile phase additives, gradient program etc.). Finding optimal chromatographic conditions is challenging. While there are some general "rules-of-thumb", empirical data are necessary to find the most suitable parametric values since each instrumental setup and target compound list is unique in its own right. Therefore, a more detailed discussion regarding these factors is presented in the results section.

1.8.3. Mass spectrometric detection

As the title suggests, this chapter of the thesis will be dedicated to MS as it is the most versatile analytical tool for monitoring organic pollutants in complex matrixes. Nevertheless, the detection of PhACs is not restricted to one particular methodology as numerous other techniques are available. Year after year, novel and inspiring methods emerge from the scientific literature promising to improve many aspects of environmental studies. However, only a small fraction of them withstand the test of time. A widely publicized example of fraudulent practice in analytical and bioanalytical chemistry occurred in 2015-2018, when a billion-dollar start-up "Theranos" was charged for alleged fraud. The company declared it can run over 200 tests on a few drops of blood using miniature testing devices that were, of course, undisclosed. Such unbelievably high testing capacity could easily fit in a plot from a science fiction novel. Luckily, these claims were found to be completely false and resulted in a massive scandal [118]. This case illustrates that the scientific community should remain sceptical when it comes to ludicrous claims and methods that are somewhat "too good to be true". Yet, there are plenty of positive examples that have proved to be reliable, accurate and applicable in this field. Some notable cases are: (i) enzyme-linked immunosorbent assays (e.g. steroid hormones in surface water) [119], (ii) biosensors based on microfluidic flow cells (diclofenac in WW) [120], (iii) capillary electrophoresis coupled with a diode array

detector (antibiotics in WW effluents from livestock farms) [121], (iv) HPLC coupled with a fluorescence detector (anti-cancer drugs in hospital effluents) [122] and (v) sensors based on molecularly-imprinted polymers doped with silver/gold nanoparticles that are analyzed using surface-enhanced Raman scattering (caffeine in WW) [123]. Nevertheless, the aforementioned methods are still in the early stages of development and so far, have not been able to demonstrate superior performance over their MS-based counterparts.

As mentioned before, most studies utilize LC-MS to resolve the analytical challenges presented by PhACs. To observe any m/z signals, analytes must first be ionized. Therefore, before we start discussing MS detection in more detail, this critical element has to be addressed. Electrospray ionization (ESI) source is considered the golden standard for polar pollutants and can cover a wide range of analytes in both polarities. However, over the last two decades, other options have emerged. For instance, Loffler et al. (2003) determined several acidic pharmaceuticals in sediments using atmospheric pressure chemical ionization (APCI) coupled with tandem mass spectrometry (MS/MS) [124]. A different study by Yamamoto et al. (2006) successfully obtained steroid hormone profiles in surface water samples using LC-MS/MS equipped with atmospheric pressure photoionization (APPI) source [125]. Their applicability is more specific, but both of these techniques are good candidates when conventional ESI cannot yield sufficient sensitivity, which is often the case in studies that focus on moderately non-polar drugs (e.g. fibrates, steroidal estrogens and carbamazepine). Besides, with slight modifications APCI can be easily coupled with GC or laser diode thermal desorption techniques, thus providing more versatility [126]. Meanwhile, ESI technology has been constantly upgraded to meet the changes in demand. Commercial availability of novel solutions, such as nano-ESI, high temperature ESI and hybrid ESI sources, have propelled this technique to the forefront of analytical chemistry. The main advantage of new generation ESI sources is that they provide greater room for customizability. In this way, the scope of the analysis can be extended, because the method becomes less restricted by the physico-chemical properties of the analyzed compounds. Another ionization approach that has recently gained considerable momentum is ambient mass spectrometry. This technique mostly applied in the field of bioanalytical chemistry, because a large number of samples can be analyzed without extensive pretreatment enabling rapid and automated MS-based imaging. In general, two ambient ionization types are used the most - direct analysis in real time (DART) and desorption electrospray ionization (DESI), where ionization is achieved by gas and aerosol, respectively. DESI has been used for direct analysis of carbamazepine in WW samples. The reported method allowed to achieve detection levels at low ng/L range, which is sufficient for PhAC analysis at environmentally relevant concentrations [127]. A more recent study applied DART technology to investigate testosterone traces in WW samples [128]. Despite their potential, these techniques are rarely applied in this field. However, we can expect ambient MS will gain greater appreciation in the following years, because miniaturized sampling devices are increasingly more accessible allowing to capture and preconcentrate pollutants on a specific surface or liquid medium, that can later be analyzed under ambient conditions. Besides, rapid screening of suspect contaminants is as relevant as ever, because the list of emerging pollutants is evolving rapidly [9].

Apart from the limitations imposed by the inertness of certain compounds that cause poor or non-existent ionization, matrix induced disturbances can be considered the central issue regarding ionization. In case of ESI, there are several hypotheses why matrix effects happen. The most widely accepted theory is that target compounds and co-eluting substances compete for available charge and the access to the surface of droplets formed from the

nebulizer tip. Thus, a higher amount of matrix components usually suppresses the analyte signal. Nevertheless, the opposite effect can also be observed in some instances (matrix enhancement effect) [129]. In comparison to ESI, APCI and APPI are less susceptible to ion suppression due to differences in ionization mechanisms. In APCI, gas phase ionization occurs in the region around the corona discharge needle, thus ionizable molecules compete only for the charge, but not for the surface of droplets as in the case of ESI. Meanwhile, APPI is mostly suitable only for nonpolar analytes that display poor ionization efficiency under ESI conditions. This leads to a fewer number of co-eluting compounds that can undergo ionization in APPI source and, consequently, matrix effect is less pronounced [130]. However, APCI and APPI have limited applicability. Hence, despite the underlying susceptibility to matrix effect, ESI remains the first-choice technique to study the environmental occurrence and behaviour of polar organic micropollutants such as PhACs.

Realistically speaking, complete elimination of matrix effect during ESI process cannot be achieved. In fact, co-eluting matrix components can cause several other issues, apart from suppression, e.g. negatively affect mass accuracy and reduce the quality of fragment spectra in MS/MS mode. Several solutions can be used to partially mitigate the impact of the matrix effect. These solutions can be categorized into two groups with respect to the expected outcome: those aimed to reduce the amount of co-eluting matrix components and those used to compensate for negative effects that influence the analytical response. In general, reduction of the matrix can be achieved in selective or non-selective manner. The latter usually means that the final extract is either diluted or smaller sample aliquots are used prior to extraction or clean-up. Whereas more selective removal of matrix interferences involves sophisticated clean-up protocols, use of mobile phase additives (e.g. buffer salts and mobile phase modifiers) and precise optimization of instrumental parameters (e.g. LC separation and ionization conditions) [131]. These measures are most successful when the analytical procedure is focused on a limited number of target analytes that, preferably, share similar physicochemical properties. In case of multi-residue methods, selective removal of matrix components is an ambiguous task and ultimately involves a compromise between the performance of the method (e.g. analyte recovery, sensitivity and scope) and matrix effect elimination. Thus, dilution and other non-selective ways are more frequently applied for the analysis of multi-class PhACs. If matrix effects cannot be eliminated by one of the above approaches appropriate calibration techniques must be applied. Such techniques are used to compensate for both matrix effects and analyte losses during sample preparation in quantitative LC-MS analyses. The best way is to use isotopically labelled surrogates or structurally similar unlabelled compounds for internal standardization. When internal standards are either not commercially available or too expensive, external calibration can be used. For this purpose, a blank sample matrix is spiked with known concentrations of target analytes, analyzed according to the sample preparation protocol and the obtained analytical responses are used to construct a calibration curve. This might seem like a universal solution for all methods, but there are several limitations. Firstly, the analyzed matrix should not contain target compounds. This, however, is rarely the case in environmental analysis, because many pollutants are known to occur ubiquitously in the environment. In situations where the selected matrix still contains the compounds of interest, blank subtraction can be used. Yet, an accurate quantification at trace levels is practically impossible when the blank sample contains high concentrations of target substances. To illustrate this, consider a situation when a WW sample that contains 500 ng/L of diclofenac is selected as a blank matrix for matrix-matched calibration. The lowest calibration level is 25 ng/L and the relative standard deviation (RSD) of the hypothetical

method is 10%. If we measure the blank sample and the first calibration point, the obtained analytical response falls within a range of 450-550 ng/L and 473-578 ng/L, respectively. Thus, blank subtraction at low concentration levels can produce extreme uncertainty and lead to erroneous interpretation of the results. Furthermore, the selected blank matrix must resemble the analyzed samples as closely as possible, while the samples themselves have to remain fairly constant in terms of composition. Apart from these approaches, the so-called "echo-peak technique" can also be considered a promising option in this context. The sample extract and a standard solution are injected sequentially one after another in the same chromatographic run. The standard peak elutes in close proximity with the sample peak, thus co-eluting matrix components affect both peaks in the same way. Hence, ion suppression or enhancement effects can be evaluated based on corresponding echo-peaks [132]. From this discussion, it is apparent that there is no universal solution to overcome the matrix effect in LC-MS applications. Nevertheless, its assessment is essential when analysing PhACs in complex environmental matrixes. Besides, no matter which technique is applied, it must be optimized to fit the requirements of the method and, most importantly, verified during the validation study.

Concerning MS techniques, different MS platforms are used in this field of study. To examine the current instrumental trends, a comprehensive literature survey was conducted summarizing twenty research articles (published between 2018 and 2019) that investigate the environmental occurrence of multi-class PhACs in environmental samples (e.g. WW, sludge, surface water, groundwater and biota). These methods are summarized in Table 6.

Table 6. **Procedures for determination of multi-class PhACs in environmental samples**

Instrumental setup	Sample treatment/clean-up	LC column (dimensions)	Matrix	N[a]	LOQ or MDL range	Ref.
UPLC-ESI-MS/MS (QqQ)	Filtration followed by "dilute-and-shoot" analysis approach	Zorbax Eclipse Plus-C18 (3.0 x 100 mm, 1.8 µm)	WWTP influents/effluents	33	LOQ: 2 - 37 ng/L	[133]
UPLC-ESI-Q-Orbitrap-HRMS	Filtration, addition of EDTA, alkalization and SPE clean-up (Oasis HLB)	Hypersil Gold C18 (2.1 × 50 mm, 1.9 µm)	WWTP influents/effluents and surface water	26	MDL: WWTP influents: 0.4 - 419 ng/L, WWTP effluents: 0.2 - 173 ng/L and surface water: 0.3 - 373 ng/L	[134]
HPLC-ESI-Q-Orbitrap-HRMS	Vacuum-assisted evaporative concentration	Atlantis T3 C18 (3.0 × 150 mm, 3.0 µm)	WWTP influents/effluents and surface water	265	MDL: WWTP influents: 0.9 - 5600 ng/L, WWTP effluents: 0.4 - 1400 ng/L and surface water: 0.3 - 900 ng/L	[135]
UPLC-ESI-MS/MS (QqQ)	Filtration addition of EDTA and SPE clean-up (Oasis HLB and Chromabonds HR-X SPE)	Zorbax Eclipse C18 (2.1 x 50 mm, 1.8 µm)	Groundwater and surface water	93	LOQ: 0.03 - 153 ng/L	[136]
UPLC-ESI-Q-TOF-HRMS	Filtration and SPE clean-up (Oasis HLB and Bond-Elut ENV)	BEH C18 (2.1 × 100 mm, 1.7 µm)	Surface water and drinking water	40	MDL: <0.1 - 86 ng/L	[137]
HPLC-ESI-MS/MS (QqQ)	Filtration and SPE clean-up (Oasis HLB and SPEC C18)	CORTECS C18 (2.1 x 75 mm, 2.7 µm)	WWTP influents/effluents	9	LOQ: 0.02 - 97 ng/L	[138]
UPLC-ESI-MS/MS (QqLIT)	Filtration followed by "dilute-and-shoot" approach or SPE clean-up (Oasis HLB)	Zorbax Eclipse C18 (2.1 x 50 mm, 1.8 µm)	WWTP influents/effluents	13	LOQ: 0.5 - 50 ng/L	[139]
UPLC-ESI-Q-TOF-HRMS	Filtration and SPE clean-up (Strata-X-PRP)	BEH Phenyl (2.1 × 50 mm, 1.7 µm)	WWTP effluents and surface water	88	MDL: 0.01 - 1 ng/L	[140]
UPLC-ESI-MS/MS (QqQ)	Filtration and SPE clean-up (Oasis HLB)	Intensity Solo C18 (2.1 x 100 mm, 2.0 µm)	WWTP influents/effluents and surface water	78	LOQ: 0.01 - 5 ng/L	[141]

Table 6. continued

Instrumental setup	Sample treatment/clean-up	LC column (dimensions)	Matrix	N[a]	LOQ or MDL range	Ref.
HPLC-ESI-MS/MS (QqQ)	Filtration, acidification, addition of EDTA followed by SPE clean-up (Oasis HLB, according to US EPA Method 1694)	Waters Xtera C18 (2.1 x 100 mm, 3.5 μm)	Groundwater and surface water	118[b]	MDL: 0.1 - 340 ng/L	[142]
UPLC-ESI-Q-Orbitrap-HRMS	Filtration, acidification, addition of EDTA and Online-SPE clean-up (C18)	CORTECS C18 (2.1 x 50 mm, 1.6 μm)	Groundwater and surface water	33	LOQ: 1 - 5.5 ng/L	[143]
UPLC-ESI-MS/MS (QqQ)	Filtration and SPE clean-up (Oasis HLB)	Unspecified C18 column (2.1 x 100 mm, 2.0 μm)	WWTP influents/effluents	78	MDL: 0.01 - 1.5 ng/L	[144]
UPLC-ESI-MS/MS (QqQ)	Extraction with pressurized hot water, dilution, filtration and SPE clean-up (Oasis HLB)	Unspecified C18 column (2.1 x 100 mm, 2.0 μm)	Wastewater irrigated soils	45	MDL: 0.01 - 0.83 ng/g (dry weight)	[144]
UPLC-ESI-MS/MS (QqQ)	Acidification, dilution and SPE clean-up (Oasis HLB)	Poroshell 120 SB-AQ (2.1 x 100 mm, 2.7 μm)	Fish and osprey plasma	21	MDL: 0.03 - 14 ng/mL	[145]
HPLC-ESI-MS/MS (QqLIT)	Filtration and SPE clean-up (Oasis HLB)	Purospher Star RP-18 (2.0 x 125 mm, 5.0 μm)	WWTP influents/effluents and surface water	35	LOQ: WWTP influents: 1 - 262 ng/L, WWTP effluents: 0.8 - 172 ng/L and surface water: 0.5 - 31 ng/L	[146]
UPLC-ESI-Q-Orbitrap-HRMS	Filtration, acidification, addition of EDTA and SPE clean-up (Oasis HLB and Speedisk SPE)	Hypersil Gold C18 (2.1 × 50 mm, 1.9 μm)	Surface water	52	MDL: <0.03 - 74 ng/L	[147]
UPLC-ESI-Q-Orbitrap-HRMS	Extraction with acetonitrile and ethyl acetate, evaporation and reconstruction in injection phase	Accucore RP C18 (2.1 × 100 mm, 2.6 μm)	Aquatic biota samples (fish, shrimps, crabs and mussels)	182	MDL: 1 - >50 ng/g (dry weight)	[148]
UPLC-ESI-Q-TOF-HRMS	Ultrasound assisted extraction followed by QuEChERS clean-up protocol	Kinetex EVO C18 (2.1 x 50 mm, 2.6 μm)	Fish muscle (salmon and chub)	27	LOQ: 0.8 - 85 ng/g (dry weight)	[149]
UPLC-ESI-MS/MS (QqQ)	Filtration and SPE clean-up (Oasis WAX)	Waters Acquity HSS T3 C18 (2.1 × 50 mm, 1.8 μm)	WWTP influents/effluents	9	LOQ: 2 - 160 ng/L	[5]
UPLC-ESI-MS/MS (QqQ)	Extraction with methanol, dilution, filtration and SPE clean-up (Oasis WAX)	Waters Acquity HSS T3 C18 (2.1 × 50 mm, 1.8 μm)	Suspended solids	9	LOQ: 4 - 86 ng/g (dry weight)	[5]
HPLC-ESI-Q-Orbitrap-HRMS	Extraction with acetonitrile and SPE clean-up (Oasis HLB)	Ascentis Express C18 (2.1 x 75 mm, 2.7 μm)	Fish muscle (tilapia, salmon and eel) and shrimps	25	MDL: 1 - 100 ng/g (dry weight)	[150]
HPLC-ESI-MS/MS (QqQ)	Filtration, acidification, addition of EDTA and SPE clean-up (Oasis HLB)	InfinityLab Poroshell 120 EC-C18 (3.0 × 50 mm, 2.7 μm)	WWTP influents/effluents	37	LOQ: 0.14–11 ng/L	[151]
HPLC-ESI-MS/MS (QqQ)	Extraction with acetonitrile, dilution, filtration, acidification, addition of EDTA and SPE clean-up (Oasis HLB)	InfinityLab Poroshell 120 EC-C18 (3.0 × 50 mm, 2.7 μm)	Sludge	37	LOQ: 0.65–17 ng/g (dry weight)	[151]

[a] The number of PhACs analyzed in the study.

[b] The study relied on several MS/MS acquisition methods.

As seen from Table 6, the most frequently applied MS platforms are triple quadrupole MS/MS instruments and high-resolution MS (HRMS) systems. Both provide different benefits and, in some instances, are even used together offering increased versatility and adaptability for the determination of PhACs [152]. By contrast, simpler mass analysers (i.e., low-resolution single quadrupole MS devices) are currently seldom used in this field, because of their inability to resolve target signals from interferences which is a critical aspect for trace level analysis of pollutants in complex matrixes. As mentioned above, both MS/MS and HRMS systems have different advantages, thus the purpose of the study is the primary factor for determining which analyser must be used to carry out instrumental detection. In general, the key performance parameters of each analyser include m/z

measurement accuracy, sensitivity, linear range, m/z range and scanning speed. MS/MS platforms are most suitable for trace level quantification studies since they provide increased sensitivity, wide linear range and speed. Two types of instrumental setups are commonly used: conventional triple quadrupole (QqQ) systems and hybrid linear ion traps (QqLIT), where the third quadrupole is substituted with linear ion trap (LIT) allowing additional fragmentation or accumulation of ions prior MS detection. Alongside hybrid LITs, there are also simpler LIT systems that can be considered a viable option for MS/MS measurements when ultra-high sensitivity is not required. Regardless of which MS/MS platform is used, several drawbacks should be addressed. Firstly, the analytical capability of MS/MS is constrained by the number of analytes that can be put in the acquisition method, because each MS/MS transition occupies a definite time interval. Therefore, only a limited number of transitions can be simultaneously measured in one chromatographic run. Data from Table 6 are in accordance with this notion, because the maximum number of different PhACs that were investigated by each study was higher for applications that used HRMS (N_{max} = 265 compounds) compared to those that applied MS/MS (N_{max} = 93 compounds). Measurements in full-MS mode can help to overcome this restriction, but, from an analytical standpoint, the obtained low-resolution full-MS data are deemed impractical for trace level analysis. Therefore, data acquisition is performed in single, multiple or parallel reaction monitoring modes to unlock the full potential of MS/MS. Additional pitfalls can arise from isobaric species that co-elute with target compounds and produce interfering product ions. Luckily, this problem can usually be resolved by optimizing the chromatographic separation method and sample preparation protocol or by selecting more characteristic ion transitions. Furthermore, screening of unknowns via MS/MS is inconvenient since data from low-resolution MS detectors cannot provide sufficient information for compound identification and high confidence structural elucidation [153].

HRMS is the most suitable technology to extend the scope of the analysis allowing to carry out more comprehensive quantitative and qualitative screening of PhACs. Typical HRMS instruments are hybrids which consist of a quadrupole (in some cases LIT), a collision cell and an analyser that provides high-resolving power, such as Orbitrap-MS, time-of-flight (TOF) and Fourier transform ion cyclotron resonance HRMS (FT-ICR-MS). These configurations are fundamental for obtaining more informative experimental data because they significantly extend the functionality of HRMS systems by allowing measurements in MS/MS and multistage fragmentation (MS^n) modes. Apart from the classical fragmentation of precursors via collision induced dissociation (CID) in the collision cell or ion trap, modern instruments can also be equipped with various complementary techniques that can dissociate molecules (e.g. in-source CID, in-cell CID, electron capture dissociation, sustained off-resonance irradiation, photodissociation and others). Moreover, modern HRMS instruments enable simultaneous acquisition of Full-MS data and all theoretical fragment-ion spectra (so called “SWATH” approach). Hence, targeted, un-targeted and retrospective screening can be incorporated in one chromatographic run as MS/MS spectra are recorded for all eluting analytes in a sample. For example, this technique was applied by Peña-Herrera et al. (2019) to analyze PhACs in fish muscle via HPLC-Q-TOF-HRMS, and over the past few years has gained increased attention among researchers [149]. Nevertheless, the main advantage of these platforms is high resolving power, because full-MS data and fragment features can be acquired with low-ppm mass accuracy over a wide m/z range. Thus, HRMS based analysis provides a vast amount of data that can be used to perform a variety of tasks: quantitative target analysis, non-target analysis, suspect screening, discovery of PhAC transformation products or

metabolites and retrospective analysis. Compared to low-resolution MS and MS/MS applications, HRMS can significantly extend the scope of analysis in terms of detectable analytes revealing formerly unnoticed PhAC related environmental issues. For instance, a recent study by Lee et al. (2020) reported an HPLC-Q-TOF-HRMS method in which simultaneous identification and semi-quantification was carried out for 484 micropollutants, including PhACs. Among the analyzed compounds, researchers found several PhACs with high occurrence rates, which have been previously overlooked due to instrumental limitations [154]. While HRMS instruments share many similarities, there are key differences among these analysers. In general, TOF systems demonstrate lower resolving power compared to their counterparts. The average resolving power ranges from 10,000 to 30,000 units, measured at full width at half maximum (FWHM) of the spectral peak. Even though some vendors manufacture high-end TOF instruments allowing enhanced performance (up to 80,000 units), they are mostly intended for bioanalytical research laboratories and used in combination with matrix-assisted laser desorption ionization technique. Meanwhile, Orbitrap and FT-ICR analysers are considered the top choice when it comes to state-of-the-art HRMS measurements. The latter is more suitable for the characterization of complex mixtures (e.g. dissolved organic matter and disinfection by-products) rather than single compounds. Furthermore, despite the remarkable resolving power (over 1,000,000), FT-ICR has not gained much attention in studies, which explore the presence of PhACs in environmental compartments. The reason for this is that extremely detailed MS data is not always necessary for the analysis of individual compounds [155]. The same cannot be said Orbitrap technology. Starting from 2005, when the first systems were made commercially available, their analytical capabilities have improved considerably and Orbitrap platforms have become an integral part of contemporary LC-MS laboratories [156]. Typical resolving power for these instruments range from 17,500 to 140,000 units and can be set by the user depending on the analytical requirements. Yet, higher resolving power requires longer acquisition times. Hence, scanning frequency is considerably lower (1.5 Hz) when the system is operated at its limits. At the same time, TOF instruments can resolve MS peaks with the same capacity regardless of the scanning speed [157]. Moreover, resolving power of Orbitrap and FT-ICR analysers decays when ions with higher m/z values are measured. Namely, resolving power of Orbitrap systems diminishes as the square root of m/z, while for FT-ICR this association is even more pronounced. The true nature of this phenomenon is complex but it can be simplified by looking at the way MS data are acquired. In both cases, measurements are based on frequency values of ion packets that undergo harmonic oscillations in the mass analyser. Both charge and mass of the ionized species have a direct impact on their trajectories and oscillatory behaviour in the analyser cell. This, of course, affects the frequency of each ion. Higher frequency allows ions to perform a full oscillation in a shorter time. Taking into account that MS measurements take place in a constant time interval, higher frequencies (lower m/z) can be measured with increased precision (i.e. better resolution) since they yield more data points [158]. Meanwhile, TOF analysers rely on a different principle to record spectra. In particular, accurate mass measurements are derived from time that is needed for equally accelerated ions to travel the distance of the flight tube and reach the detector. The velocity of each ion depends on m/z, but ion trajectories remain largely constant over the dynamic range. This process is significantly faster compared to the previously discussed frequency measurements. Hence, one scan is long enough to record data for the whole m/z diapason and the loss of resolution is no longer as pronounced at higher m/z values as it was in the previous case [157,159]. A comparison between all four MS platforms concerning resolving power is depicted in Figure 5. Deprotonated diclofenac (m/z 294.0094,

[M-H]$^-$) is used as a model substance to illustrate the benefits of HRMS. As can be seen, low resolution MS cannot provide sufficient separation to distinguish low abundance signals (Figure 5, A). TOF analyser can resolve the analyte signals, but isotopic fine structure remains unresolved (Figure 5, B). Only Orbitrap and FT-ICR systems can uncover the smallest details of low abundance isotopologues (Figure 5, C and D). Although these small MS features are mostly irrelevant for quantification studies, they are perceived as fundamental when it comes to successful molecular formula assignments in qualitative studies.

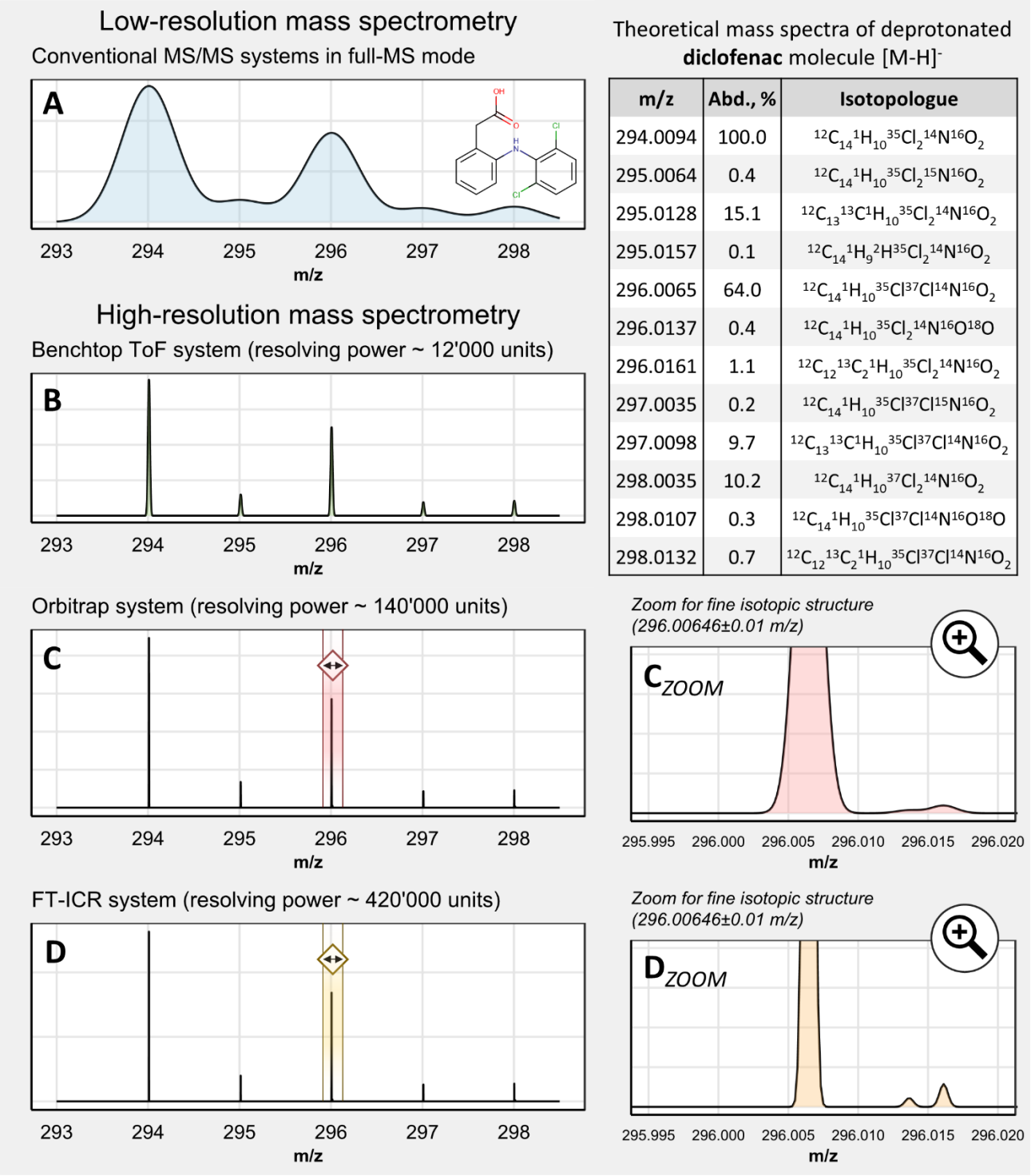


Figure 5. **Mass resolving capability of four MS analysers (MS/MS, TOF, Orbitrap and FT-ICR)**

Regardless of the analyser type, all HRMS experiments must be carried out with high mass accuracy. Typically values under 5 ppm are considered acceptable, but for Orbitrap and FT-ICR platforms sub-ppm accuracy is recommended to achieve the maximum performance and increased data quality. For this purpose, mass calibration is applied [160]. This usually involves measuring a set of reference masses (e.g. sodium formate clusters, Ultramark 1621 mixture and sodium trifluoroacetate clusters) that yield equally scattered analytical signals across the selected m/z range. Meanwhile, internal calibration and lock mass approaches are used to compensate for drift of mass accuracy that may occur during longer measurement series. The latter is accomplished by a continuous post-column addition of a known reference substance into the ionization source. This way m/z shifts can be adjusted for each of the acquired spectra. The internal calibration approach is similar, except that the calibrant is introduced into the source for a limited time at the beginning of MS acquisition method. Apart from mass calibration, careful attention must be paid to avoid oversaturation of the analyser cell. An excessive amount of ionic species can severely hurt detection capability through various detrimental effects (e.g. collisions between particles and space-charge effect). Samples have to be adequately diluted and ion accumulation time must be optimized to reduce these risks. To counter these issues, some vendors have incorporated specific control tools that regulate the ion transfer, preventing oversaturation of detector. For example, Orbitrap instruments rely on automatic gain control functionality which selectively accumulates ions based on the set threshold. However, it must be applied with great care, because co-eluting matrix components can drastically shorten accumulation times and negatively affect quantification [161–163].

Considering that PhACs are usually found at sub-μg/L concentrations in the environment, quantitative performance of the method is critical. Thus far, a number of studies have compared MS/MS and HRMS analysers with respect to sensitivity. Practically all of them revolve around Orbitrap systems since they are the only HRMS platforms that can achieve a similar sensitivity to that obtained by conventional QqQ and QqLIT MS/MS instruments [164,165]. For instance, Fedorova et al. (2013) investigated differences between LTQ-Orbitrap-HRMS and QqQ MS/MS for the analysis of 35 illicit drugs in WW samples. To improve the extent of the study, authors operated the HRMS system in full-MS and parallel reaction monitoring modes with a resolving power (at FWHM) of 70,000 and 17,500 units, respectively. The results of the study revealed that the HRMS method which was carried out in parallel reaction monitoring mode outperformed MS/MS. At the same time, a decrease of sensitivity was observed when operating the HRMS system under full-MS settings [166]. A note of caution is due here since parallel reaction monitoring mode can be applied to a certain extent in multi-residue methods. Similar to conventional QqQ MS/MS applications, only a limited number of transitions can be monitored simultaneously using this MS/MS technique as each overlapping MS/MS transition decreases the scanning frequency. Hence, when the number of analytes becomes too high, the obtained LC-MS chromatograms are not accurately reflected due to missing data points. This causes distorted chromatographic peaks and subsequently impairs the quantitative aspects of the method. Besides, non-target analysis or suspect screening cannot be accomplished using parallel reaction monitoring as this mode is not intended for the collection of full-MS data. A similar study was conducted by Herrero et al. (2014), where both QqQ MS/MS and Orbitrap-HRMS were used for the determination of veterinary drugs in WWTP effluents and influents. Instead of using parallel reaction monitoring mode, this study relied on a more wide-scope approach in which samples were analyzed using two alternating modes (full-MS and all ion fragmentation SWATH mode). This technique can be considered more suitable for HRMS applications

because it enables the full potential of HRMS capabilities. Furthermore, despite using a less sensitive approach, the results of the study show that Orbitrap (operated in full-MS/SWATH mode) was equally sensitive when compared with MS/MS method [167].

In order to verify the findings of these comparative studies, an additional investigation was performed in which the quantification limits reported by studies from Table 6 were evaluated. Methods were classified according to analyser type, and data for 16 PhACs was used to evaluate method sensitivity. As can be seen from Figure 6, MS/MS systems demonstrate slightly better performance in terms of sensitivity. This was especially noticeable for NSAIDs (ibuprofen and naproxen) and clarithromycin. Nevertheless, the difference is not overwhelming and can be greatly affected by numerous factors (e.g. sample preparation protocol, instrumental parameters, the condition of each instrument and the way quantitation limits are calculated). In general, this observation supports the evidence from other studies that modern HRMS platforms are suitable for conducting trace level quantification of PhACs in environmental samples [164–167].

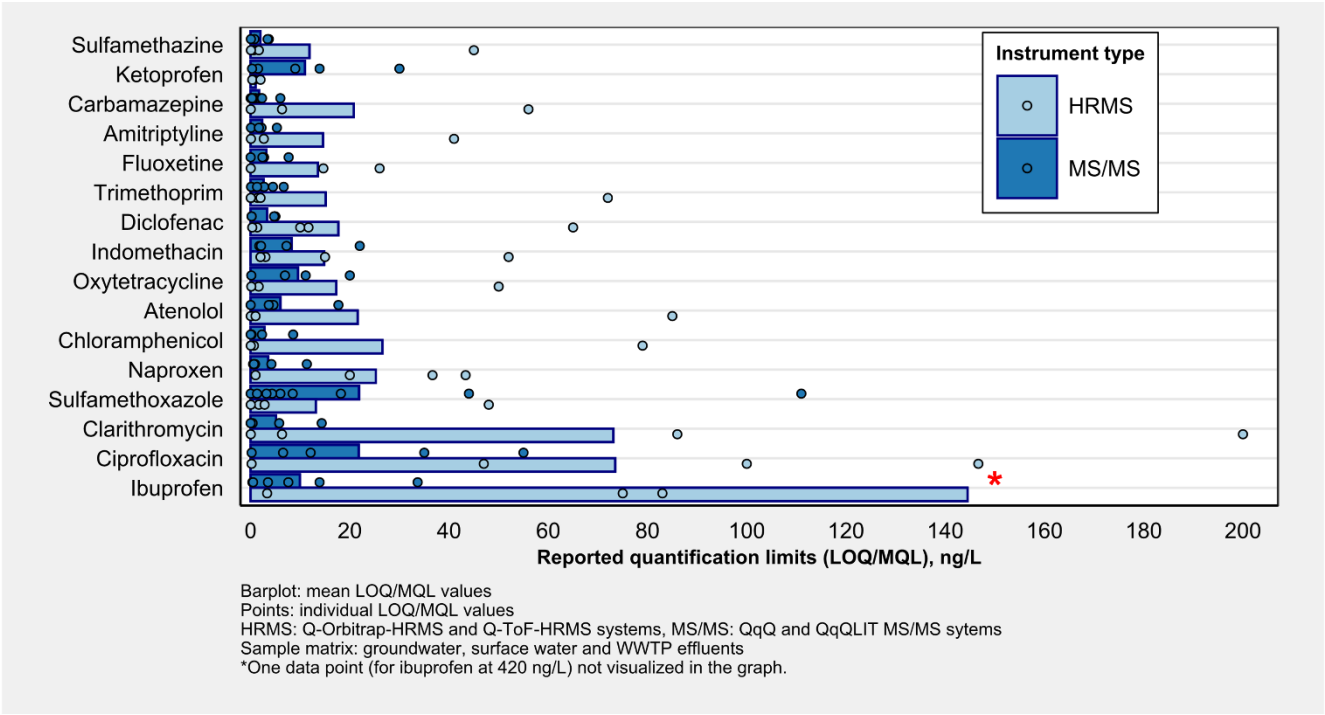


Figure 6. **Reported quantification limits from the literature for the analysis of PhACs in aquatic samples**

Overall, the topics discussed in this section offer a useful insight into some of the key aspects of LC-MS based analysis of PhACs. A detailed knowledge and understanding of the properties of confirmatory MS techniques are essential for choosing the most suitable approach as each MS analyser provides a distinct set of features (see Table 7). Yet, there are numerous other factors that should be considered during the method development phase. Thus, a more detailed commentary regarding the specifics of each technique will be discussed in the results section.

Table 7. **Comparison of typical MS systems (and FT-ICR) used for analysis of PhACs in environmental matrixes**

Parameter	MS analyser type			
	MS/MS	TOF	Orbitrap	FT-ICR
Resolving power	◇◇◇	◆◇◇	◆◆◇	◆◆◆
Sensitivity	◆◆◆	◆◇◇	◆◆◇	◆◇◇
Scanning speed	◆◆◇	◆◆◆	◆◆◇	◆◇◇
Scope of the analysis (number of compounds per one chromatographic run)	◆◇◇	◆◆◇	◆◆◆	◆◆◆
Cost	◆◇◇	◆◇◇	◆◆◇	◆◆◆
Robustness	◆◆◆	◆◆◇	◆◆◇	◆◇◇
Dynamic Range	◆◆◆	◆◇◇	◆◆◇	◆◇◇
Mass accuracy	✗	◆◇◇	◆◆◇	◆◆◆
Non-target analysis	✗	✓	✓	✓
Quantitative analysis	✓	✓	✓	✓
Very high - ◆◆◆, High - ◆◆◇, Medium - ◆◇◇, Low - ◇◇◇ Applicable - ✓, Not applicable - ✗				

1.8.4. Untargeted and suspect screening strategies of PhACs in environmental samples

Recent advancements in HRMS have allowed researchers to implement novel analytical strategies that involve non-target and suspect screening techniques. Typically, methods which are applied in this field rely on previously described TOF, Orbitrap and FT-ICR analysers as these HRMS technologies fulfil the following requirements for the analysis of unknowns: high resolving power, reasonable scanning frequency and ability to simultaneously acquire MS/MS and even MS^n spectra. Altogether, this significantly boosts the reliability of MS measurements and provides a solid ground for many analytical methods [168]. For instance, Singer et al. (2016) applied LC-Orbitrap-HRMS with incorporated data-dependent MS/MS acquisition to investigate more than 800 PhACs in WWTP effluents [169], whereas a more recent study reported an LC-Q-TOF-MS application that can perform suspect screening of more than 1000 PhACs and 250 metabolites in hospital sewage samples [170].

Methods that do not rely on reference standards (i.e. target methods) can be classified into two groups: (i) standard free suspect screening of known compounds and (ii) non-target screening of unknowns. The first method can be used for qualitative assessment of PhACs in a fashion that is similar to that utilized by targeted screening, whereas non-target screening is done without any a priori information and, thus, may be used for identification of complete unknowns, for example, previously unreported metabolites or transformation products of PhACs. From the perspective of data acquisition, both approaches require full-MS data and MS/MS information. The former is typically achieved through SWATH technique (all incoming ions are fragmented altogether) or data-dependent MS/MS acquisition in which a built-in algorithm automatically isolates and fragments the most abundant precursors that do not originate from background noise. At the same time, data processing steps are intrinsically different between non-target and suspect screening techniques (see Figure 7).

In case of suspect screening, a database of substances is constructed prior to instrumental analysis. It contains compound-specific information on each suspect (e.g. molecular formula, structure, exact m/z values of the expected precursor ions, isotopic pattern, possible MS/MS product ions, predicted retention time, etc.). These features can then be effectively used to confirm the presence of these substances in the analyzed samples. A typical

suspect screening workflow begins from full-MS data analysis, where extracted ion chromatograms are created for compounds of interest. If a chromatographic peak is found, then corresponding full-MS spectra is investigated in more detail. The measured accurate mass information and isotopic patterns are matched against reference data, while additional confirmation is achieved by comparing similarities between the measured MS/MS spectra and experimental library spectra (or *in-silico* generated MS/MS data). A suspect is considered putatively identified if the difference between expected and measured features meets the specified criteria [171].

On the contrary, the screening of unknowns is less straightforward and calls for in-depth analysis. The preliminary stage comprises data cleaning (e.g. filtering, smoothing and blank subtraction) and peak picking. This is achieved using peak-picking algorithms that automatically generate ion chromatograms for all MS traces. These peaks are then evaluated and filtered based on different constraints, such as S/N ratio, intensity threshold, peak symmetry and peak width. After the removal of non-compliant entries, peaks are investigated individually. The first step for tentative identification of unknowns is to obtain a correct elemental composition. It is done by heuristic filtering approach which relies on so-called "seven golden rules". This way, accurate mass data and corresponding isotopic patterns are examined in a trial-and-error manner to obtain the most fitting elemental composition [172]. For each molecular formula assignment, a list of candidate structures is retrieved from a database (e.g. EPA's CompTox, ChemSpider, PubChem, ChEBI and KEGG or an in-house compound list). At this stage, MS/MS data come into play. However, before any library search can take place, a set of reference spectra are required. MS/MS data can be obtained either from public MS data repositories (e.g. METLIN, MoNA and mzCloud) or generated for each structure using *in-silico* fragmentation tools. Finally, the measured product ions are matched against the reference fragmentation patterns of candidate structures and ranked according to an arbitrary similarity score. At this point, there is sufficient body of information that makes it possible to tentatively identify the unknown compound. The best-case scenario is when the assignment goes to the candidate that displays the highest scores in all instances (fragmentation features, accurate mass information and isotopic pattern). Yet, such circumstances are rarely encountered and manual examination of data is often required to establish the correct assignment [173].

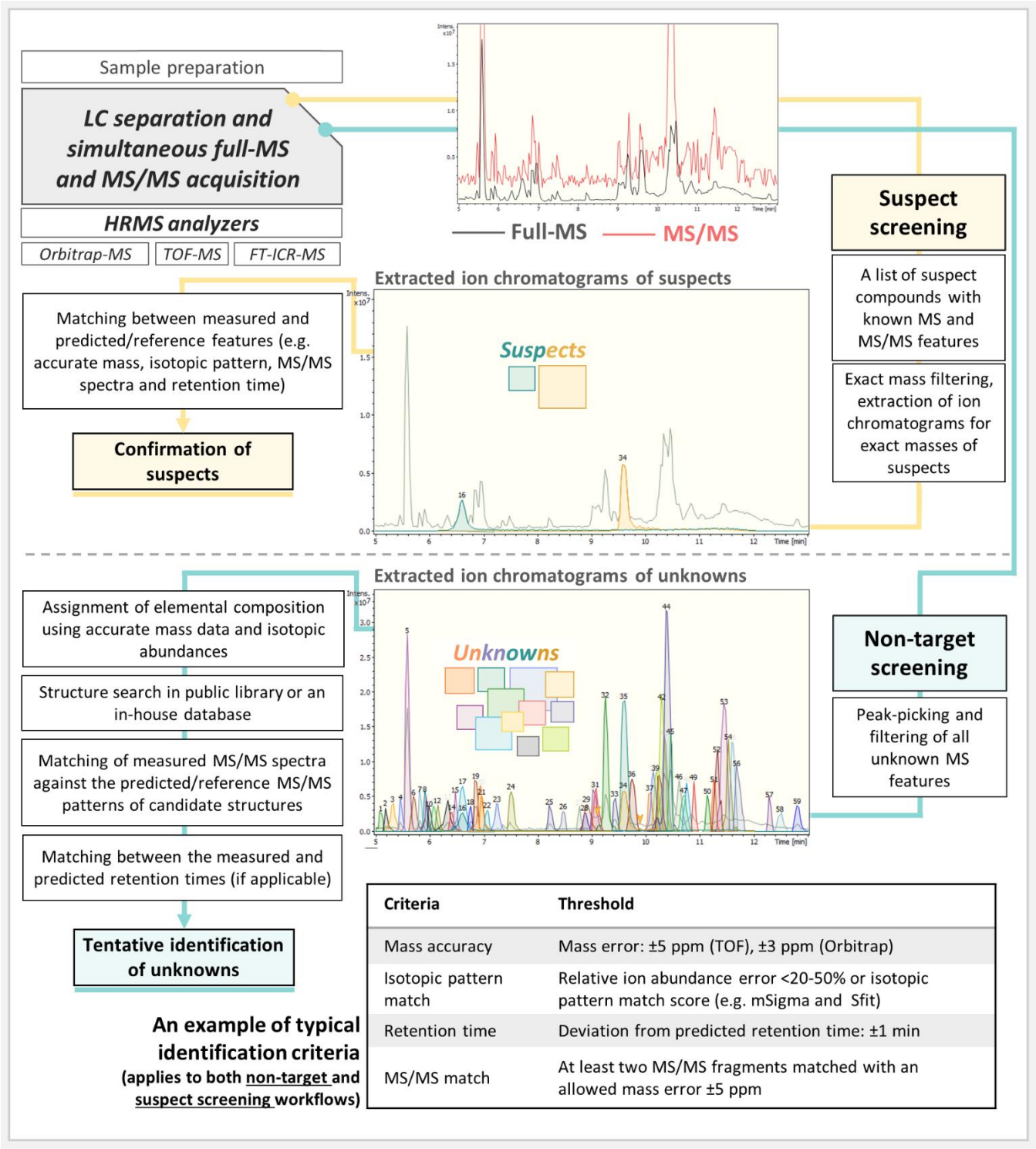


Figure 7. **Schematic depiction of non-target and suspect screening worflows**

Although this field is rapidly developing, many critical challenges remain. According to conclusions derived from the first collaborative non-target screening trial (run by the NORMAN Association), which investigated the presence of organic micropollutants such as pesticides, personal care products and PhACs, analytical methods applied in this field are already reasonably well harmonized. Meanwhile, data processing strategies show great disparity, which is especially pronounced in non-target methods [10]. To investigate the coherence between different software solutions, a recent study by Hohrenk et al. (2020) applied various data handling strategies for qualitative determination of PhACs and pesticides in WW samples. The study found out

that the overlap of features between four programs (MZmine2, enviMass, Compound Discoverer and XCMS online) in non-target mode was only around 10%. Besides, 40% and 55% of features did not match with any other program. Not surprisingly, more repeatable results were achieved using suspect screening strategy, where the average success rate between programs was higher, varying from 64% to 88% [174]. However, deficient extraction of MS features is not the only issue that needs consideration. The absence of reference data can be another obstacle increasing the rate of false positive matches in both approaches. Experimental MS/MS spectra are often not available, thus *in-silico* fragmentation tools have emerged as a suitable alternative to predict the fragmentation behaviour of candidate molecules. Some prominent examples that are frequently used in this field are: SIRIUS 4 [175], MetFrag [176] and CFM-ID [177]. Thus far several studies have explored whether in-silico fragmentation can produce reliable results for qualitative screening of PhACs and other small organic molecules in complex matrixes. In this context, Blaženović et al. (2017) compared four different *in-silico* fragmentation algorithms (MetFrag, CFM-ID, MAGMa+ and MS-FINDER) on a dataset used in 2016 CASMI (Critical Assessment of Small Molecule Identification) challenge. According to this study, the workflow that relied solely on experimental spectra yielded 60% correct hits. Meanwhile, a workflow that was additionally equipped with in-silico generated spectra (along with experimental) was able to achieve the overall success rate of 87% [178]. Apart from MS information, retention time is another component that is crucial for the elucidation of compounds. For targeted methods, a mismatch between the measured and expected retention times is considered an instant red flag. The same principle partially applies to non-target methods. Yet, it must be taken into account that even direct transfer of LC methods from laboratory to laboratory yields significant variance in retention times due to numerous reasons (e.g. deteriorated performance of the column, excess dead volume, variation of mobile phase composition, condition of LC hardware, etc.). Nevertheless, the retention order of analytes stays practically constant. Therefore, measuring the retention time of selected model compounds using the developed LC method can help to predict the behaviour of analytes that lack empirical data. Retention time prediction models have proved to be a useful technique for reference standard free analysis and, as highlighted by several studies, have shown solid performance in terms of prediction accuracy. For instance, McEachran et al. (2018) tested three prediction models: EPI Suite™ which is based solely on logP values and two more advanced models (ACD/ChromGenius and OPERA-RT). According to this study, an acceptable prediction accuracy (±15% chromatographic time window) was achieved for 95% of investigated compounds when using both of the advanced prediction models [179]. The above findings indicate that experimental and predicted characteristics complement each other and increase the overall success rates of qualitative methodologies.

HRMS based non-target and suspect screening strategies are attracting increased attention. During the last few years, these strategies have finally transitioned from the development stage to routine applications. Thus, it seems that qualitative HRMS-based methods are here to stay. In 2019, these state-of-the-art methods have already been used in numerous environmental monitoring studies, such as for short-term temporal monitoring of PhACs in surface waters [180], for determination of spatial patterns of new psychoactive substances [181] and for predicting the toxicity of previously unreported PhACs in sewage samples [182]. These are just a few examples of how reference standard free analysis can provide benefits that are difficult to achieve by targeted methodologies. Nevertheless, there is still a lot of work ahead, especially concerning effective LC-MS data handling practices and harmonized identification strategies.

1.9. Current trends and future perspectives

The literature review of this thesis sought to answer a number of relevant questions: Why should we be concerned about the presence of PhACs in the environment? Which analytical strategies may be used to enhance the current understanding of these elusive micropollutants? What are the pitfalls and limitations of current diagnostic tools? Which technique should be used to obtain the best results under given circumstances? With this in mind, there are many directions for future research that may prove useful.

Regardless of the detection method, sampling and sample preparation protocols have a significant impact on the results. An increasing amount of studies have demonstrated that passive sampling techniques can cover a broader range of analytes and provide enrichment of low abundance PhACs from aqueous matrixes that earlier remained mostly undetected because conventional grab-sampling is limited in terms of sample volume and can easily miss pollutants with temporal discharge patterns [183]. Moreover, passive sampling provides opportunities for citizen science and community-based environmental monitoring programs. For example, Newton et al. (2018) used point-of-use water filtration devices (Brita® filters) collected from individual households to investigate emerging organic pollutants in drinking water. The results of the study revealed the presence of several PhACs (e.g. simvastatin, a lipid-lowering medication, and norethisterone, a birth-control drug). Thus, suggesting that activated carbon filters obtained from commercial drinking water purification systems can be used for monitoring purposes [184]. Besides, future developments of miniature sensors can someday allow analysis of PhACs outside routine and research laboratories. For instance, a recent review article by Lu et al. (2020) which summarizes the latest advances in biosensors for the detection of estrogens reported that modern photoelectrochemical and electrochemical biosensors are capable of detecting 17b-estradiol in surface water samples at sub-ng/L levels, which is sufficient for environmental trace level analysis [179]. Yet, most of these miniaturized diagnostic tools are still under development and far from commercial availability. Meanwhile, the performance of sample preparation strategies, which are employed for MS-based detection methods, is continuously improved. Lipid rich matrixes (e.g. samples from fish and marine mammals) remain a challenge when analysing PhACs with high octanol-water partition coefficients. Hence, advanced techniques of sample extraction and clean-up are still needed to isolate non-polar PhACs from lipids [185,186].

A noteworthy MS technology that has not been covered in this thesis, but should be mentioned, is ion-mobility-MS which offers multi-dimensional separation of isomers and increased S/N. This technique is particularly suitable for the identification of PhAC transformation products and metabolites. For instance, Emhofer et al. (2019) used drift-tube ion-mobility Q-TOF-MS to characterize metabolites from three lipid-lowering drugs in plants after uptake from water. The proposed method was able to detect and tentatively identify 45 drug related compounds including numerous isomeric substances [187]. This is one of many examples demonstrating the effectiveness of ion-mobility-MS based applications and indicating that it can provide a platform for studying novel pollutants derived from PhACs. Besides, other complementary techniques (e.g. infrared multiple photon dissociation spectrometry and two-dimensional MS) are expected to be incorporated more frequently into MS-based multi-residue methods to expand analytical horizons.

As previously stated, reference standard free analytical methods are gaining momentum. The latest non-target and suspect screening developments are focused on improving the reliability of identification and peak picking workflows which ultimately reduces the rate of false positives and false negatives. Thus, novel prediction

models are being constantly developed to generate data that more closely matches experimental observations. Most prediction algorithms rely on machine-learning principles and require an enormous amount of experimental information to train the model. Therefore, availability of public MS/MS and MS^n data repositories is as relevant as ever. It is important to note that each model needs to be verified before it can be directly applied to the analysis of real samples. Several collaborative trials have already been conducted to assess the performance of different non-target and suspect screening workflows (mostly coordinated by NORMAN network laboratories in EU and Environmental Protection Agency in USA). Yet, given the growing popularity of such screening workflows, the number of ongoing collaborative trials is expected to increase to ensure the quality of results [10,188]. Meanwhile, the availability of certified reference materials for environmental non-target screening applications would allow direct in-house validation of these analytical procedures.

Another future challenge is to gain quantitative information from HRMS data without the use of reference standards [11]. Several attempts have been made to develop semi-quantitate methods that can predict compound-specific ionization efficiencies to estimate the concentration range of analyzed compounds. Although this approach has not been applied to investigate PhACs in environmental matrixes, a recent study successfully proved that standard free semi-quantification is possible for the screening of pesticides in food samples [189]. Therefore, it is foreseeable that this technique will likely gain popularity in this field.

As the number of studies that utilize HRMS continues to rise, the amount of data available to researchers is increasing. In the vast majority of cases, these studies focus on a specific group of compounds, but the data collected may also be useful for investigating other micropollutants. The first steps in this direction have been done by NORMAN Network who developed a platform (Digital Sample Freezing Platform) for archiving LC-HRMS data for the retrospective suspect screening of thousands of environmental pollutants including PhACs [190]. This platform can be used for storing, viewing and screening of substances in a much wider analytical window. Hence, data can be used for retrospective analysis to support regulatory environmental monitoring and influence policymaking processes for the management of PhACs and other emerging pollutants. While the project is still new, the first results are promising. For example, Angles et al. (2020) used this platform to reveal traces of previously ignored antibiotics and antifungal compounds in Bangladesh surface waters [191]. Moreover, these data can provide useful insights for epidemiological studies, because the presence of PhACs and their transformation products in environmental samples, especially raw WW, reflects many population-specific characteristics. Even though wastewater-based epidemiology is an emerging research field, it has already been effectively applied to study (i) drug consumption patterns, (ii) illicit drug use and (iii) prevalence of new psychoactive substances and designer drugs [192]. These are just a few examples of how MS-based mining of the chemical information contained in raw WW can assist researchers to better understand and evaluate complex factors of population health and behaviour.

In conclusion, the use of MS-based detection methods is expected to grow rapidly. The gap of sensitivity between MS/MS and HRMS systems will continue to narrow. This situation inevitably leads to the question: Is HRMS considered the new "gold-standard" for multi-analyte methods applied in environmental analytical chemistry? It seems that the paradigm shift is clearly under way and laboratories are gradually replacing QqQ MS/MS systems with TOF and Orbitrap analysers [193]. This might give the impression that the targeted low-resolution MS/MS methods are on the brink of extinction. The future will tell whether these two MS technologies

are going to co-exist. Meanwhile, my personal belief is that HRMS and MS/MS techniques will continue to be complementary rather than competing. Moreover, wide-scope screening workflows can be used effectively to identify knowledge gaps and prioritize PhACs of potential environmental concern. This information can then serve as a foundation for future monitoring programs and lead to better policy decisions. Besides, environmental pollution is a collective problem that affects the whole world. Evidence-based solutions are needed in all countries, regardless of their stage of economic development. Therefore, while science must move forward, budget-class MS equipment will remain relevant, especially in routine laboratories where ease of use, robustness and affordability are considered prerequisites for cost-efficient implementation of analytical procedures.

2. EXPERIMENTAL PART

2.1. Chemicals and materials

Analytical standards for the determination of aminoglycoside antibiotics were as follows: streptomycin sulfate (STP, 98.0%), neomycin sulfate (NEO, 90.0%), spectinomycin dihydrochloride hydrate (SPC, 98.0%), and gentamicin-2,5-sulfate hydrate (GEN, 96.5%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). While kanamycin sulfate (KAN, 99.0%) was obtained from Sigma–Aldrich (St. Louis, MO, USA) and dihydrostreptomycin sesquisulfate (DSTP, 98.0%) was supplied by Fluka (Buchs, Switzerland). All analytical standards used for multi-residue methods (PhACs by HPLC-Orbitrap-MS and DI-FT-ICR-HRMS) were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Fluka (Buchs, Switzerland). Meanwhile, analytical standards of for the determination of 12 acidic NSAIDs (carprofen, diclofenac, flufenamic acid, flunixin, ibuprofen, ketoprofen, mefenamic acid, meloxicam, naproxen, niflumic acid, tolfenamic acid, and vedaprofen) and internal standards (carprofen-d_3, diclofenac-$^{13}C_6$, flunixin-d_3, ibuprofend$_3$, meloxicam-d_3, tolfenamic acid-$^{13}C_6$, vedaprofend$_3$) were all of at least 94% purity, and were obtained from Sigma-Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer (Augsburg, Germany), and Witega Laboratorien Berlin-Adlershof GmbH (Berlin, Germany). Standard stock solutions of the aforementioned PhACs were individually prepared by dissolving the appropriate amount of the analytical standard in methanol or water (only aminoglycosides) to give a final concentration of around 1 mg/mL (calculated for base compound). For the reason that aminoglycosides can be easily adsorbed on polar surfaces as amber glass, all standard aminoglycoside stock and working solutions were stored at −18 °C in polypropylene tubes instead of glass containers. The working standard solutions of 0.1 mg/L were prepared by diluting the appropriate volumes of stock solutions in methanol. All the solutions were stored at -18 °C for up to 1 week or when the analytical response fell below 90% of the initial value obtained on the day of preparation.

In all instances, deionized water (18 MΩ cm) was generated by a Milli-Q water purification system (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile, HPLC-grade methanol, formic acid (≥98%), trichloroacetic acid (≥98%), sodium hydroxide (≥98%), EDTA disodium salt dihydrate (≥98.5%), and ammonium formate (≥99%) were obtained from Sigma–Aldrich. SPE experiments were performed on Strata-X PRP cartridges (200 mg/3 mL) obtained from Phenomenex (Torrance, CA, USA). Sorbents used for dSPE experiments were obtained from Phenomenex (Torrance, CA, USA) and were as follows: Sepra™ C18-E (Bulk Packing, 50 µm, 65A), Sepra™ PSA (Bulk Packing, 50 µm, 70A) and Strata™-X-A 33 µm Polymeric Strong Anion sorbent.

Multi-walled carbon nanotube agglomerates sold under the trademark Baytubes® C150P (further referred to as CNTs-1) were obtained from Bayer Material Science AG (Leverkusen, Germany). The main parameters of CNTs-1 were as follows: ≥ 95% purity, 4 nm internal diameter, 13 nm outer diameter and > 1 µm length. The reported specific surface area of CNTs-1 was 210 m^2/g [194]. Three other types of MWCNTs—commercially known as TNIM4 (referred to as CNTs-2), TNIMH4 (referred to as CNTs-3; with 2.48 wt% content of hydroxyl functional groups), and TNIMC4 (referred to as CNTs-4; with 1.55 wt% content of carboxyl functional groups)—were purchased from Chengdu Organic Chemicals Company (Sichuan Sheng, China). All of these MWCNTs were of > 95% purity, 5–10 nm internal diameter, 10–30 nm outer diameter, 10– 30 µm length and >110 m^2/g specific surface area according to the data provided by the manufacturer. All of the MWCNT materials were used without any additional chemical purification.

2.2. Samples

For determination of PhACs in WWTP influents using Orbitrap-MS a total of 21 samples of untreated WW (three samples per day during seven days of one week in April 2016) were collected at the central wastewater treatment plant (WWTP) "Daugavgriva" of the Riga city. Samples were collected in glass amber bottles and kept at +4ºC during transportation. Once delivered to the laboratory, the samples were filtered through 1.2 μm glass microfiber filters (GF/C, Whatman, UK) and extracted within 24 hrs. The same WWTP was used to obtain WW samples for studies that involved bioaugmentation and ionising radiation. The sampling time for these samples was May and June 2016 and the procedure for collection, transportation and storage was analogous to one described above.

For the determination of NSAIDs in surface water samples from the Daugava River in Riga (Latvia), ten samples were collected at different locations. Meanwhile, 14 samples (five surface water and nine tap water samples) were collected from different points in Oslo area (Norway). The samples were collected over a 3-month period during March–May, 2017 in 500 mL to 1 L pre-rinsed polypropylene bottles and kept at + 4 °C during the transportation. Once the samples were received at the laboratory, they were immediately filtered through 1.2 μm glass microfiber filters and stored at −18 °C until the day of sample preparation.

For FT-ICR-MS analysis a total of 72 samples (36 influents, 36 effluents) were collected from different WWTPs from Latvia during March and April 2019. Both effluents and influents were collected on the same day. Samples were collected in amber glass bottles, kept at +4 °C during transportation and on the day of the delivery were filtered through 1.2 μm glass microfiber filters (GF/C, Whatman, UK). After that, a 20 mL aliquot (3 replicates per sample) was transferred to a 50 mL polypropylene tube and stored at -18 °C in dark till the day of analysis.

For the determination of aminoglycosides, a total of 49 samples of honey were collected from different areas of the country of Georgia during the summer of 2016. An aliquot of approximately 25 mL was taken from each sample, transferred to a 50 mL polypropylene tube and stored at −18 °C till the day of analysis. Analysis of aminoglycoside antibiotics was also carried out for 21 raw WW samples (sample collection and storage is described in the 1st paragraph of this section).

2.3. Biodegradation experiments

Before incubation, WW samples were filtered and aerated for 15 min. Afterward, a 100 mL aliquot of WW was supplemented with microorganisms and/or nutrient composition according to the experiment setup reported by Muter et al. (2017) [195]. Incubation experiments were performed in 200 mL columns in triplicate, at 24 °C, with periodic agitation (once a day) for a period of 7 days. Sampling was performed after 1 h, 17 h, 48 h, and 168 h incubation. The nutrient composition consisted of 333 μL 30% sugar beet molasses containing 40% (w/w) sucrose (final concentration 0.1%), previously autoclaved for 20 min at 1 bar, and 500 μL cabbage leaf extract, prepared according to Muter et al. (2008) and sterilized by filtering through hydrophilic Minisart® Syringe Filter (Sartorius, Germany) [196]. Sludge-derived culturable bacteria and fungi were obtained by plating the activated sludge on Tryptone Glucose Yeast Extract Agar (TGA, Sifin, Germany) and Rose Bengal Agar with Chloramphenicol (Biolife, Italy), respectively. Bacteria and fungi were harvested after 48 h and 72 h, respectively, and the prepared suspensions contained 2.9×10^8 CFU/mL and 3.1×10^7 CFU/mL, respectively.

2.4. Irradiation experiments using ionising radiation

The linear particle accelerator ELU-4 (Thoriy Ltd., Russia) located in Salaspils (Institute of Chemical Physics, University of Latvia) was used for the irradiation of WW samples. The thickness of the sample layer in the plastic bags during the irradiation tests was approximately 2–3 mm. The samples were irradiated at ambient temperature by both electron beam and gamma radiation until the absorbed doses of 0.5, 1, 3, 5, 7, 10, 12, 15, 20, and 25 kGy were reached. Two different modes of electron beam treatment (referred to as EB_1 and EB_2) and two gamma-irradiation modes (denoted as G_1 and G_2) were applied to investigate the impact of ionising radiation on the wastewater treatment.

The electron beam radiation was generated by accelerated 5.0 MeV electron flux with 0.1 and 0.05 μA/cm^2 currents at solenoid current of 44 A and magnetron current of 0.16 A. In the EB_1 and EB_2 experiments, the samples were kept at 82 and 105 cm distances from the electron window for 1.5–75 and 3.0–150 s time periods depending on the irradiation dose, respectively and the does rates were 1200 and 600 kGy/h, respectively. The experiments were performed in duplicate by applying irradiation from both sides.

The electron beam radiation was converted into gamma rays by targeting the flux of accelerated electrons to water-cooled palladium plate converter (1.0 mm) that was kept at 5 cm distance from the electron window. The emitted gamma rays had continuous spectrum with the maximum energy of 5.0 MeV and the mean energy of 1.5 MeV. Fricke dosimeter was used to control the absorbed gamma ray doses. The conditions of G_1 and G_2 treatments were as follows: the samples were kept at 50 and 30 cm distances from the electron window from 80 up to 4000 s, and from 48 up to 2400 s depending on the absorbed dose, the dose rates were equal to 22.5 and 37.5 kGy/h, respectively. The temperature of the samples after irradiation was in the range of 18–28 °C. The samples were stored at −18 °C until the day of sample analysis.

2.5. Determination of PhACs by HPLC-Orbitrap-MS

2.5.1. Sample preparation and clean-up

Before SPE procedure, 20 μL of 0.5M Na_2EDTA solution and 100 μL of acetic acid were added to 200 mL of sample. Solid phase extraction was performed on Strata-X-PRP cartridges (200 mg/3 mL). Cartridges were conditioned with 3 mL of methanol and 3 mL of deionized water. The samples were loaded on SPE columns with a flow rate of 5 mL/min (approximately), the cartridges were dried for 30 min under vacuum and eluted with 6 mL of methanol. The obtained extracts were then evaporated to dryness under gentle nitrogen stream in water bath at 40ºC temperature. Finally, the dry residue was reconstituted in 100 μL of injection phase, which was water/methanol solution (80:20, v/v).

2.5.2. Instrumental analysis

The chromatographic separation of PhACs was carried out using an Accela 1250 UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) consisting of a degasser, a quaternary pump, a thermostatic autosampler, and a column oven. Chromatographic separation was performed using a Kinetex C18 analytical column (100 × 2.1 mm, 2.6 μm) obtained from Phenomenex (Torrance, CA, USA). The mobile phase consisting of 0.1% formic acid in water (A) and methanol (B) was delivered at the flow rate of 0.2 mL/min. The gradient program was as follows: 20% of B from 0 to 1.0 min, a gradual increase of B from 20% to 95% (1.0 to 5.0 min), keep B constant at 95% from 5.0 to 7.0 min, decrease B back to 20% from 7.0 to 7.1 min and finally re-equilibrate the column

with initial conditions at 20% of B from 7.0 to 10 min. The injection volume of sample aliquot was 5 µL. The column and autosampler were maintained at 40°C and 4°C, respectively.

The UHPLC system was coupled to a Q-Orbitrap-MS mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization probe operated in the positive and negative ionization modes. Nitrogen was used for spray stabilization, collision-induced dissociation experiments in the higher energy collision dissociation (HCD) cell and as the damping gas in the C-trap. The following ionization parameters were applied: heater temperature 300°C, electrospray voltage 2.8 kV, capillary temperature 250°C, sheath gas (N_2) 40 arbitrary units, auxiliary gas (N_2) 10 arbitrary units, and S-Lens RF level at 50 arbitrary units. The automatic gain control (AGC) was set to $3e^6$, the maximum injection time was set to 200 ms, and the number of micro-scans to be performed was set at 1 scan/s. Full-MS data both were acquired in both positive and negative modes at a mass resolving power of 70,000 FWHM. The m/z scan range was from 125 to 800 units.

For confirmatory purposes, a targeted MS/MS analysis was performed using a mass inclusion list which contained information about product ion mass, collision energies and the expected retention times of analytes (Table 8). In this acquisition mode, the Orbitrap-MS was operated again in both positive and negative modes at 17,500 FWHM. The AGC target was set to $2e^5$, the maximum ion injection time was set to 50 ms and the quadrupole isolation window was set to m/z 2. The collision energies (CE) were optimized for each target compound by introducing the working standard solution mixture at a concentration of 10 ng/µL via syringe pump at flow rate of 5 µL/min. The mass tolerance window was set to 5 ppm. All data processing was carried out using Xcalibur 2.2 software (Thermo Fisher Scientific).

Table 8. **Parameters for full-MS/dd-MS/MS analysis using Orbitrap-MS**

Analyte	Adduct	RT, min	Precursor mass, *m/z*	Product mass, *m/z*	Normalised CE
Acetaminophen	$[M+H]^+$	1.9	152.0706	110.0606	60
Atenolol	$[M+H]^+$	1.3	267.1703	145.0650	40
Atorvastatin	$[M+H]^+$	6.8	559.2603	440.2235	20
Azithromycin	$[M+H]^{2+}$	4.9	375.2615	158.1177	27
Caffeine	$[M+H]^+$	3.6	195.0877	138.0666	50
Carbamazepine	$[M+H]^+$	5.9	237.1022	194.0965	18
Ciprofloxacin	$[M+H]^+$	3.8	332.1405	288.1512	30
Clarithromycin	$[M+H]^+$	6.0	748.4842	590.3903	13
Diclofenac	$[M-H]^-$	7.1	294.0094	250.0193	10
Erythromycin	$[M+H]^+$	5.7	716,4580	558.3629	10
Fluoxetine	$[M+H]^+$	5.9	310.1413	148.1124	15
Gemfibrozil	$[M-H]^-$	7.6	249.1496	121.0644	10
Ibuprofen	$[M-H]^-$	7.2	205.1234	159.1166	10
Ketoprofen	$[M+H]^+$	6.5	255.1016	209.0961	20
Losartan	$[M+H]^+$	6.3	423.1695	207.0915	35
Metoprolol	$[M+H]^+$	4.4	268.1907	116.1072	14
Naproxen	$[M+H]^+$	6.6	231.1016	185.0961	20

Table 8. continued

Analyte	Adduct	RT, min	Precursor mass, *m/z*	Product mass, *m/z*	Normalised CE
Pravastatin	$[M-H]^-$	6.2	423.2388	129.0021	20
Propranolol	$[M+H]^+$	5.2	260.1645	116.1074	40
Simvastatin	$[M+H]^+$	7.9	419.2792	199.1487	17
Sulfamethoxazole	$[M+H]^+$	4.2	254.0594	156.0115	30
Trimethoprim	$[M+H]^+$	2.5	291.1452	123.0669	39
Valsartan	$[M+H]^+$	6.5	436.2343	291.1491	20
Xylazine	$[M+H]^+$	4.0	221.1107	164.0530	70

2.6. Determination of NSAIDs by HPLC-MS/MS

2.6.1. Sample preparation and clean-up

After the optimisation of sorption and desorption conditions, the final dSPE procedure was applied for the extraction of NSAIDs from samples. The pre-concentration procedure was carried out by adding an optimised content of CNTs-2 (20 mg) in glass tubes containing 100 mL of pre-filtered water samples. The samples were then acidified by adding hydrochloric acid (the obtained pH~2), the tubes containing samples were immediately capped, placed in the orbital bench-top shaker and shaken at ambient temperature for a 3-min period at the mixing speed of 300 rpm. After that, the samples were filtered through Durapore PVDF filters to remove sorbent particles, CNTs were placed in empty SPE tubes, washed with deionised water (3 mL) and dried for 5 min with air and 5 min with nitrogen. The extraction of NSAIDs was carried out using 10 mL of methanol containing 1% (v/v) of ammonium hydroxide. The extracts were evaporated to dryness and reconstituted in 1 mL of acetonitrile/water (10/90, v/v) followed by injection into the HPLC-MS/MS instrument.

2.6.2. Instrumental analysis

An Acquity HPLC system (Waters, USA) coupled to QqQ-MS/MS system QTrap 5500 (AB Sciex, USA) equipped with a Turbo Ion Spray electrospray (ESI) source was used for the sample analysis. Chromatographic separation was performed on a Luna C18 analytical column (100 × 4.6 mm, 2.6 μm) purchased from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 0.01% acetic acid in water (A) and 100% acetonitrile (B). The flow rate was 0.6 mL/min. The gradient program started with 40% of mobile phase B which was held constant from 0 to 0.5 min. The percentage of B was gradually raised from 40 to 80% (0.5 to 11 min) and then decreased back to 40% and held constant to re-equilibrate the system from 11 to 15 min. A 10 μL aliquot of the sample was injected, while the column compartment and autosampler temperatures were set at 30 and 4 °C, respectively. The QqQ-MS/MS detector was operated in turbo spray ESI-negative detection mode. The following parameters were applied for the analysis: ion spray voltage − 4.00 kV, source temperature - 300 °C, curtain gas nebulizer - 40 psi, ion source gas 1 - 50 psi and ion source gas 2 - 80 psi. The control of the instrument and the data processing were performed using the Analyst 1.6 software (AB Sciex, USA).

2.7. Determination of aminoglycosides by HPLC-Q-TOF-MS

2.7.1. Sample preparation and clean-up

Each sample of honey (5.00 ± 0.01 g) was weighed into a 50 mL polypropylene tube. The extraction solution (20 mL of 1% trichloroacetic acid in deionized water) was added, the mixture was vortexed for 1 min

and shaken for 10 min using a mechanical shaker. The pH of the mixture was then adjusted to 5.0 ±1.0 using 1.0 M NaOH solution. After that, the sample was centrifuged at 3500 rpm for 10 min and the supernatant was treated with SPE clean-up procedure using Strata-X-PRP cartridges (200 mg/3 mL). The cartridge was preconditioned with 3 mL of methanol and 3 mL of deionized water. After loading a 5 mL sample aliquot, the column was washed with 6 mL of water, dried for 5 min under vacuum, and eluted with 3 mL of 5% formic acid in deionised water (v/v) into a 15 mL polypropylene tube. Then 50 µL of 1.0 M ammonium formate solution was added to the final extract, the container was vortexed for approximately 10 s using orbital bench-top shaker and, finally, 250 µL of the final extract was transferred to an HPLC vial. The same procedure was applied for the analysis of WW samples. The only difference was that the initial sample volume was 200 mL.

2.7.2. Instrumental analysis

Chromatographic separation was achieved using a Dionex UltiMate 3000 rapid separation LC system (Thermo Scientific, Sunnyvale, CA, USA) comprising a binary high-pressure gradient pump, an autosampler, and a thermostatic column compartment. Chromatographic separation was archived using an Obelisc R (2.1 × 150 mm, 5 µm) analytical column obtained from SIELC Technologies (Prospect Heights, IL, USA). The mobile phase consisted of aqueous 1% formic acid solution v/v (A), acetonitrile (B), and deionised water (C). The flow rate was kept at 0.5 mL/min and the injection volume was 15 µL. The column compartment and autosampler temperatures were set to 30 and 14 °C, respectively. The following gradient conditions were used: 0–0.5 min, isocratic 0% A, 90% B, 10% C; 0.5–4.5 min, linear increase from 0 to 95% A, linear decrease from 90 to 5% B, linear decrease from 10 to 0% C; 4.5–9.0 min, isocratic 95% A, 5% B, 0% C; 9.0–9.1 min, return to the initial conditions and 9.1–15.0 min post-run equilibration at the initial conditions.

Mass spectra were acquired using a Compact Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an IonBooster (IB) high-temperature ESI source, which also was purchased from Bruker Daltonics. The instrument was equipped with a conventional ESI source for comparison during the method optimization stage. The mass calibration of the Q-TOF-MS instrument was performed before each sequence by direct infusion of aqueous solution of 1 mM sodium formate at the flow rate of 2 µL/min. The mass accuracy (5 ppm) during the sequence was maintained using the external calibration approach by injecting the aforementioned calibrant once every five samples using a 2 min long isocratic LC method that relied on the initial HPLC conditions that were used for the main method (0% A, 90% B, 10% C). The instrument was operated in positive ionization mode by alternating the acquisition between full-MS at the m/z range of 50–1000 and data-dependent MS/MS spectra using the scheduled precursor list (Table 9). All spectra were scanned at the rate of 2 spectra/s, and 3 MS/MS spectra were acquired for each compound with a cycle time of 2 s. The operating parameters of mass spectrometer were optimized as follows: the end plate offset was 500 V, dry gas temperature and flow rate were set at 200 °C and 3.0 L/min, respectively. When conventional ESI source was used, the capillary voltage was set at 4500 V and the dry gas flow rate was set at 10 L/min, while for IB, the capillary voltage was reduced to 1000 V and the dry gas flow rate was 3 L/min. The use of IB introduced two additional parameters that were absent for the standard ESI source: the charging voltage and vaporizer temperature that were set at 300 V and 350 °C, respectively. For instrument control and data acquisition, otofControl 4.0, HyStar 3.2 (Bruker Daltonics), and Chromeleon Xpress software (Thermo Scientific) were used, while DataAnalysis 4.3 software (Bruker Daltonics) was used for post-run mass calibration and data processing.

Table 9. **Compound list and optimized full-MS/dd-MS/MS parameters for the determination of aminoglycosides via HPLC-Q-TOF-MS**

Compound	Precursor	RT, min	Theoretical mass, m/z	Isolation width, m/z	CE, eV	MS/MS fragments – Q_1 and Q_2, m/z		Ratio Q_2/Q_1
Spectinomycin	$[M+H]^+$	5.45	351.1762	1.50	35	333.16	207.13	0.49±0.12
Streptomycin	$[M+H]^+$	6.00	582.2729	0.75	45	263.14	246.12	0.45±0.11
Dihydrostreptomycin	$[M+H]^+$	6.00	584.2886	0.75	45	263.15	246.12	0.27±0.07
Kanamycin	$[M+H]^+$	6.80	485.2453	1.50	20	163.11	205.12	0.37±0.09
Gentamicin C1	$[M+H]^+$	7.40	478.3235	1.50	30	157.13	322.20	0.23±0.06
Neomycin	$[M+H]^+$	7.60	615.3196	1.50	30	161.09	293.13	0.17±0.05

2.8. Determination of PhACs by DI-FT-ICR-HRMS via QuEChERS method

2.8.1. Sample preparation and clean-up

The polypropylene tube containing the frozen 20 mL aliquot of wastewater was uncapped, covered with an aluminium foil and freeze-dried using Benchtop "K" Series freeze dryer (VirTis, Gardiner, NY, USA). The freeze-dry process was paused for a while when the volume reached about 5 mL. Samples were then thawed, vortexed for 30 s, frozen and again subjected to freeze-dry procedure. The aforementioned step was included to ensure that all dry matter from the sample remains at the bottom of the container. After the drying procedure, 1 mL of acetonitrile/water (9:1, v/v) was added to the dry matter, vortexed for 1 min and sonicated for 10 min. The extract was then centrifuged for 10 min at 3500 rpm and carefully transferred to a 15 mL polypropylene tube, followed by addition of 1 mL of acetonitrile/water (1:9, v/v). Phase separation was induced by adding 500 mg of anhydrous magnesium sulphate to the extract. In order to minimize possible decomposition of target analytes due to exothermic dissolution of $MgSO_4$, the extract was cooled to +4 °C before the addition. After that, the sample was vortexed for 1 min, centrifuged for 5 min at 3500 rpm and an aliquot of 600 µL of the acetonitrile layer was transferred to a 2 mL V-shaped glass vial that contained 4 mg of dSPE sorbent (C18-E/Strata-X-A, 3:1, w/w). The mixture was immediately vortexed for 30 s and centrifuged for 5 min at 3500 rpm. Finally, a 100 µL aliquot was transferred to a fresh 2 mL amber glass vial, diluted with 1100 µL of acetonitrile/water (4:1, v/v), and analyzed within 24 h by FT-ICR-MS.

2.8.2. Instrumental analysis

The analysis was performed on a 7 T Bruker SolariX FT-ICR-MS system (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (ESI). Samples were introduced into the system by direct infusion via syringe pump at a flow rate of 5 µL/min. Spectra was acquired in both positive and negative ionization modes using a broadband mode (m/z range 50–1000). Full-MS data was acquired by accumulating 32 scans, whereas MS/MS spectra was acquired without multiple spectra accumulation. Time domain data size was set at 4 M for all modes. The observed resolving power (measured at FWHM) using these settings was around 490,000 at m/z 250 and 275,000 at m/z 500. Three fragment spectra were acquired per precursor at different collision energy (CE) values (5 V, 15V, and 25 V) using an isolation window of ±2.5 m/z. In order to obtain higher fragment ion intensities, ion accumulation time was increased to 500 ms, whereas for full-MS experiments this parameter was set to 100 ms and 20 ms for positive and negative ionisation modes, respectively. Main source parameters were as follows: nebulizer pressure 0.5 bar, dry gas flow rate 6 L/min, dry temperature 200 °C, and

capillary voltage 3.5 kV. The instrument was externally calibrated with sodium formate clusters, while lock mass was applied to maintain high mass accuracy. Data acquisition was performed using ftmsControl 2.2.0 (Bruker) and raw data processing was done in DataAnalysis 5.0 software (Bruker).

2.8.3. Suspect list

The initial list of suspects was derived from a publicly available database developed by German Federal Environmental Agency. The database is an outcome from a systematic review of 1,519 publications and comprises 178,708 data entries which summarize the occurrence data of 771 PhACs and their TPs [197]. In order to select compounds that can be investigated by this HRMS-based screening method the following filters were applied: (i) monoisotopic mass range from 100 to 1000 Da, (ii) literature source credibility "good", and (iii) at least one MS/MS spectra record available in MassBank of North America (MoNA) repository that has been obtained by ESI ionization. Entries that can be classified as salts or mixtures were converted back to their parent molecules. After the removal of non-compliant entries, ~600 unique suspects remained on the list. Next, each entry was supplemented with molecular information (e.g. molecular formula, SMILES and InChIKey) and full-MS features for both ionization modes, such as accurate mass and isotopic pattern. The latter two were calculated for $[M+H]^+$ and $[M-H]^-$ species using "enviPat" R package [198]. Each compound was matched against MoNA repository to obtain an MS/MS fingerprint. In total, more than 8000 experimental records were found for compounds on the list, and each spectrum was subsequently reprocessed in MetFrag, an *in-silico* fragmentation algorithm (mass error threshold ±5 ppm, relative abundance >10%). This step was implemented to annotate fragment ions and assign correct m/z values, because experimental data, for the most part, lack fragment formula annotations and may contain erroneous peaks due to poor mass accuracy or MS interferences. The obtained dataset with experimental MS/MS traces was then aggregated based on the corresponding precursor and polarity, and five most prevalent fragments were selected as the MS/MS fingerprint. However, it was not possible to obtain five features for numerous compounds due to limited data. Hence, a complementary approach was applied, which involved predicting MS/MS spectra via Competitive Fragmentation Modeling-ID (CFM-ID) [177]. Finally, fragments obtained by this manner were again processed similarly to experimental data and added to the database to improve the accuracy of the method, especially for suspects whose fingerprints contained less than five m/z values. The steps performed to prepare the suspect list are summarized in a flowchart (Figure 8, left side). The suspect database is available on request as a spreadsheet and contains the following information for each entry: molecular information (formula, SMILES, and InChIKey), full-MS traces (m/z values, isotopic pattern) and MS/MS traces (m/z values and corresponding fragment formulas for both experimental and predicted features). An example of one suspect list entry (carbamazepine) is depicted in Table 9.

Table 9. **An example (carbamazepine) of the information contained in each database record**

Name	Carbamazepine
MolecularFormula	$C_{15}H_{12}N_2O$
Monoisotopic Mass	236.095
InChI	InChI=1S/C15H12N2O/c16-15(18)17-13-7-3-1-5-11(13)9-10-12-6-2-4-8-14(12)17/h1-10H,(H2,16,18)
InChIKey1	FFGPTBGBLSHEPO-UHFFFAOYSA-N
SMILES	C1=CC=C2C(=C1)C=CC3=CC=CC=C3N2C(=O)N
Q1, m/z	237.1022
Q2, m/z	238.1056
Ratio Q2/Q1	16.29
Species	$[M+H]^+$
MS/MS fragment formulas (experimental)	$C_{14}H_{10}N$, $C_{13}H_9N$, $C_{14}H_{11}N$, $C_{13}H_9$, $C_{14}H_9N$
MS/MS fingerprint (experimental), m/z	192.0808, 179.0730, 193.0886, 165.0699, 191.0729
MS/MS fragment formulas (predicted)	$C_{14}H_{12}N$, $C_{15}H_{11}N_2$, $C_{15}H_{10}NO$, $C_{13}H_8N$, C_8H_5
MS/MS fingerprint (predicted), m/z	194.0964, 219.0917, 220.0757, 178.0651, 101.0386
Therapeutic group	Antiepileptic drugs
Type of Analyte	Parent substance

2.8.4. Target and suspect screening workflow

Initially, one full-MS spectrum (accumulation of 32 scans) was acquired for each ionization mode. Lock mass calibration and blank subtraction (from matrix-free reagent blank) were performed using Data Analysis 5.0. software (Bruker) and automated with VBScript (a subset of Microsoft's Visual BASIC). The peak list of all m/z values and corresponding intensities within the selected analyte range (100 – 1000 m/z) was exported as comma-separated values (CSV) and further processed in Rstudio environment (v. 1.1.463) with R (v. 3.5.3). Full-MS features were extracted and matched against the suspect list using the following criteria: accurate mass error for two most abundant ions (Q_1 and Q_2) ±1.25 ppm and relative error between theoretical and experimental ion ratios (±20% for suspect screening; from ±20% to ±50% for targeted screening). After this, compliant matches were further advanced to the next step – MS/MS acquisition. Three MS/MS spectra were acquired per precursor at different CE values (5, 15, and 25 V). In order to save time, minimize human error and make data handling easier, method files were created automatically via Rstudio environment by modifying a template method. In brief, the method consisted of stitched segments, where each segment acquires one scan per precursor using a defined CE value. For instance, if full-MS data yields 30 compliant matches from the suspect list in positive mode, an MS/MS acquisition method is automatically generated with 90 scan segments and immediately measured by the HRMS system. The set of measurements produces only one raw file, which is automatically processed by VBScript in DataAnalysis software, sliced into 90 individual scans, exported as separate CSV files, and, finally, evaluated using R. The same procedure applies for the negative mode. Next, features obtained from MS/MS data were matched against the suspect list database that contained accurate mass information of both experimental and predicted fragmentation fingerprints. If at least one fragment showed a match (mass accuracy ±2.5 ppm) then the suspect was considered to be tentatively identified.

The target analysis was carried out simultaneously with the suspect screening. Quantification was based on Q_1 ion intensities from full-MS data using a matrix-matched calibration. The total time of the workflow depended on the number of matching suspects. In particular, data processing (~5 min) and acquisition of two full-MS spectra (2*68 s) required approximately 7 min, while each MS/MS measurement extended the total time of the analysis by 4.5 s per precursor. Therefore, the average length of the workflow ranged from 10 to 15 min per sample. The outline of the workflow is depicted in a flowchart (Figure 8, right side), while a more detailed step-by-step guide is presented in Annex 1, while an example of measured full-MS and MS/MS data is depicted in Annex 2 (example compound – carbamazepine). R scripts applied in this study and an example dataset can be accessed online (https://github.com/ingusperkons/HRMS-screening-of-pharmaceuticals-in-wastewater).

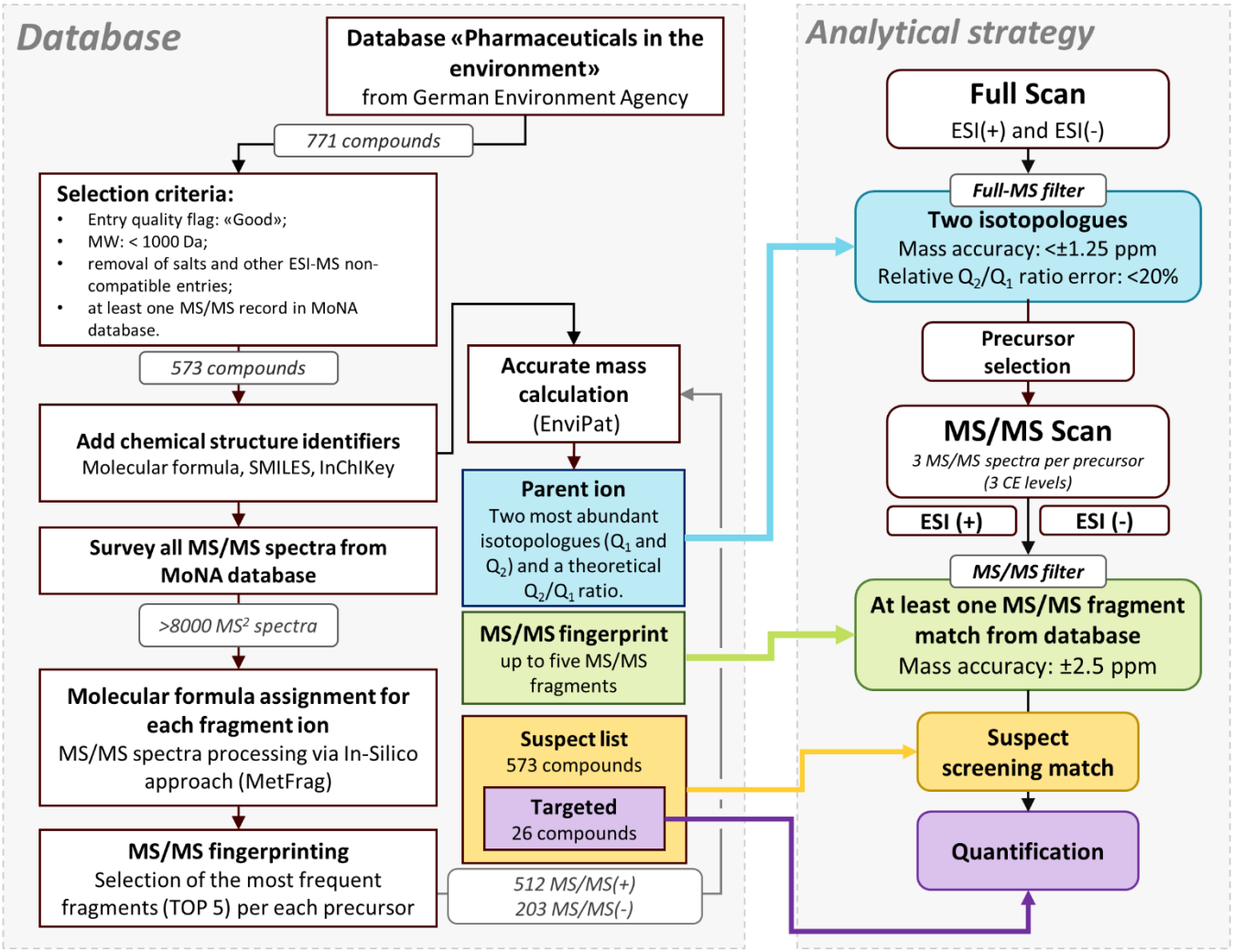


Figure 8. **A schematic overview of the screening workflow and the in-house database**

3. RESULTS AND DISCUSSION

3.1. Method optimization

3.1.1. Sample preparation protocol: determination of PhACs by HPLC-Orbitrap-MS

As already mentioned in the literature review, the most common technique for the isolation and enrichment of PhACs from various environmental matrices is solid phase extraction (SPE). This technique provides multiple benefits e.g. it is compatible with a wide range of sorbents and has lower solvent consumption compared to LLE. Besides, larger sample volumes can be loaded on the SPE cartridge allowing selective isolation of PhACs at sub-ng/L levels [199]. An SPE procedure was therefore developed and optimized for the analysis of WW samples.

Two different SPE sorbents were compared − the Strata-X-PRP and Strata C18 columns from Phenomenex. Pre-conditioning of columns, sample loading and analyte elution was performed under the same conditions for both column types. SPE cartridges were conditioned with 5 mL of methanol followed by 5 mL of water. The samples were loaded at a rate of 5 mL/min. Washing was carried out using 5 mL of water while 9 mL of methanol was used for elution of analytes. The obtained results expressed as extraction recoveries of the SPE process are presented in Figure 9. Slightly better recoveries were achieved for most of the compounds using the C18 sorbent. However, significant losses during the SPE procedure were observed for acetaminophen, azithromycin, sulfamethoxazole, and ciprofloxacin. The decrease in recovery that was noted for C18 sorbent could be attributed to the lack of the hydrophilic moieties in the sorbent, thus some PhACs, which have a low affinity towards hydrophobic C18 groups, are not sufficiently retained. Meanwhile, Strata-X-PRP is based on polydivinylbenzene resin that is functionalized with piperidone groups. The combination of hydrophilic and hydrophobic moieties offers good wettability and increase the hydrophilic interactions between the solutes and the stationary phase while maintaining retention of analytes via reversed phase principles. Therefore, Strata-X-PRP column was selected for the final SPE procedure since it gave significantly better recoveries for target PhACs.

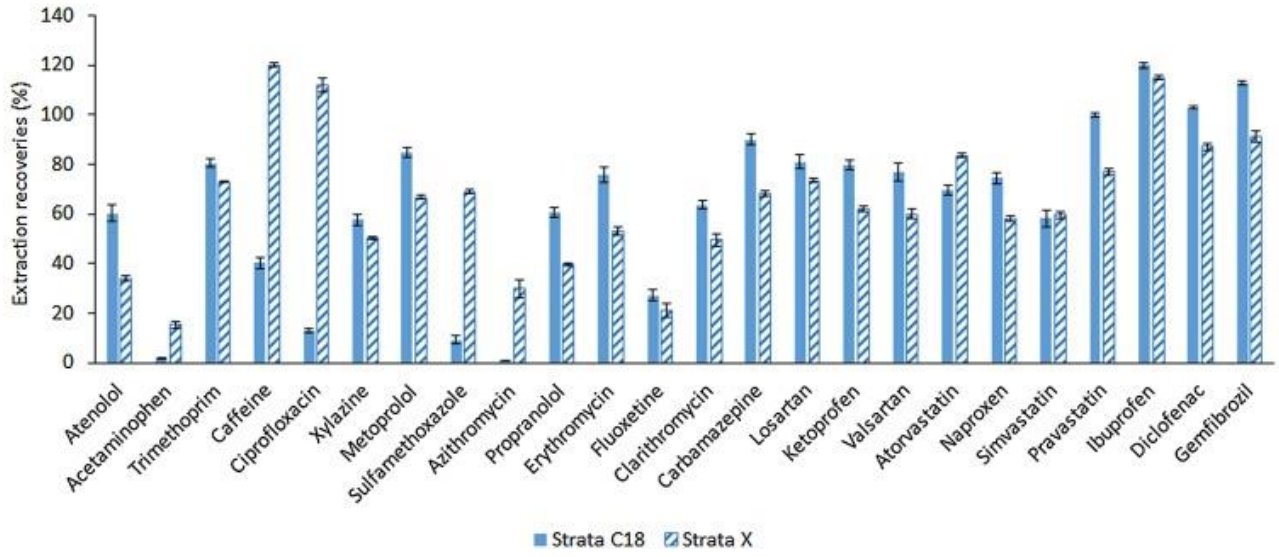


Figure 9. **Extraction recoveries (%) obtained for PhACs using Strata C18 and Strata-X-PRP SPE cartridges**

Further experiments involved selecting the most appropriate sorbent amount (100, 200 and 500 mg) for the SPE procedure. Critical differences in analyte recovery were noted for caffeine, ciprofloxacin, azithromycin and fluoxetine, with the highest enrichment yield obtained using 200 mg of sorbent, while significantly better acetaminophen recoveries were only achieved when using 500 mg of the sorbent. Although acetaminophen is a highly consumed drug in Latvia, the four former PhACs, especially ciprofloxacin and azithromycin, are considered more troublesome from the environmental perspective. Thus, based on these "opportunity costs", 200 mg of Strata-X-PRP sorbent was advanced for the main method.

The efficiency of analyte enrichment does not depend solely on the type of sorbent and can be influenced by several other factors. For instance, the pH of the sample plays a fundamental role in SPE-based extraction methods. This is especially important when extracting PhACs from aquatic matrixes since most of these compounds contain functional groups which can be protonated or deprotonated if the pH of the solution increases or decreases, respectively. Increased extraction efficiency was found for macrolide antibiotics, lipid regulators, and some NSAIDs when the samples were acidified to pH~ 3 prior SPE extraction. As anticipated, acidification did not improve the recoveries of all analytes since the PhACs selected within this study have very different chemical structures, and therefore it is difficult to develop extraction procedures that would be equally efficient. Furthermore, the addition of a chelating agent such as Na_2EDTA in water samples before the extraction step is recommended when analysing antibiotic residues of macrolide and fluoroquinolone groups in environmental samples. These antibiotics have a high tendency to complex with multivalent metal cations that are soluble in water, resulting in low extraction recoveries. Hence, the presence of a strong chelating agent is necessary for scavenging multivalent metal ions. Based on the results obtained from all optimisation experiments, the final protocol for extraction of selected PhACs in WW samples involved addition of Na_2EDTA to samples followed by acidification with acetic acid to pH 2.5.

3.1.2. Instrumental analysis: determination of PhACs by HPLC-Orbitrap-MS

Different mobile phases and mobile phase additives were compared to optimize the chromatographic separation and selectivity towards PhACs. Acetonitrile, methanol, and a mixture of both solvents were evaluated as organic mobile phases while deionized water without additives, buffered with 0.05 M ammonium formate and fortified with 0.1% formic acid were evaluated as aqueous mobile phases. All mobile phase combinations were examined for three different columns: Kinetex C18 (100 × 2.1 mm, 2.6 µm), Gemini – NX (150 × 2.0 mm, 3 µm) from Phenomenex and Hypersil GOLD (50 × 2.1 mm, 1.9 µm) from Thermo Fisher Scientific. Between all the combinations that were explored during the optimisation, the best peak shapes and responses in both positive and negative ionization modes were achieved using the Kinetex C18 (2.6 µm) and Hypersil GOLD (1.9 µm) columns when acidified water and methanol were used as organic and aquatic mobile phases, respectively. The efficiency of both columns was further investigated by analysing a WW sample spiked at 10 ng/L concentration and by calculating the plate number (N) and the retention factor (k). An example of the obtained chromatograms is shown in Figure 10. The number of theoretical plates characterizes the column efficiency and depends on column length and mobile phase flow rate. The values of N were calculated using the following equation:

$$N = \left(\frac{t_R}{w}\right)^2 \qquad (1)$$

, where t_R is retention time and w is the peak width.

The retention factor, which reflects interactions between the stationary phase and the eluent, was calculated using the following equation:

$$k = \left(\frac{t_R - t_0}{t_0}\right) \qquad (2)$$

, where t_R is the retention time and t_0 is the breakthrough time.

For most of the target analytes, much higher *N* values were observed on the Kinetex C18 column compared to Hypersil GOLD. Likewise, the calculated retention factor values were better for the Kinetex C18 column. Namely, the calculated *k* values for Kinetex C18 were in the range of 4 to 9, while for the Hypersil Gold column *k* values often exceeded 10 units (the preferred values of *k* are between 1 and 10). Therefore, the Kinetex C18 column was advanced further for the analysis of WW samples.

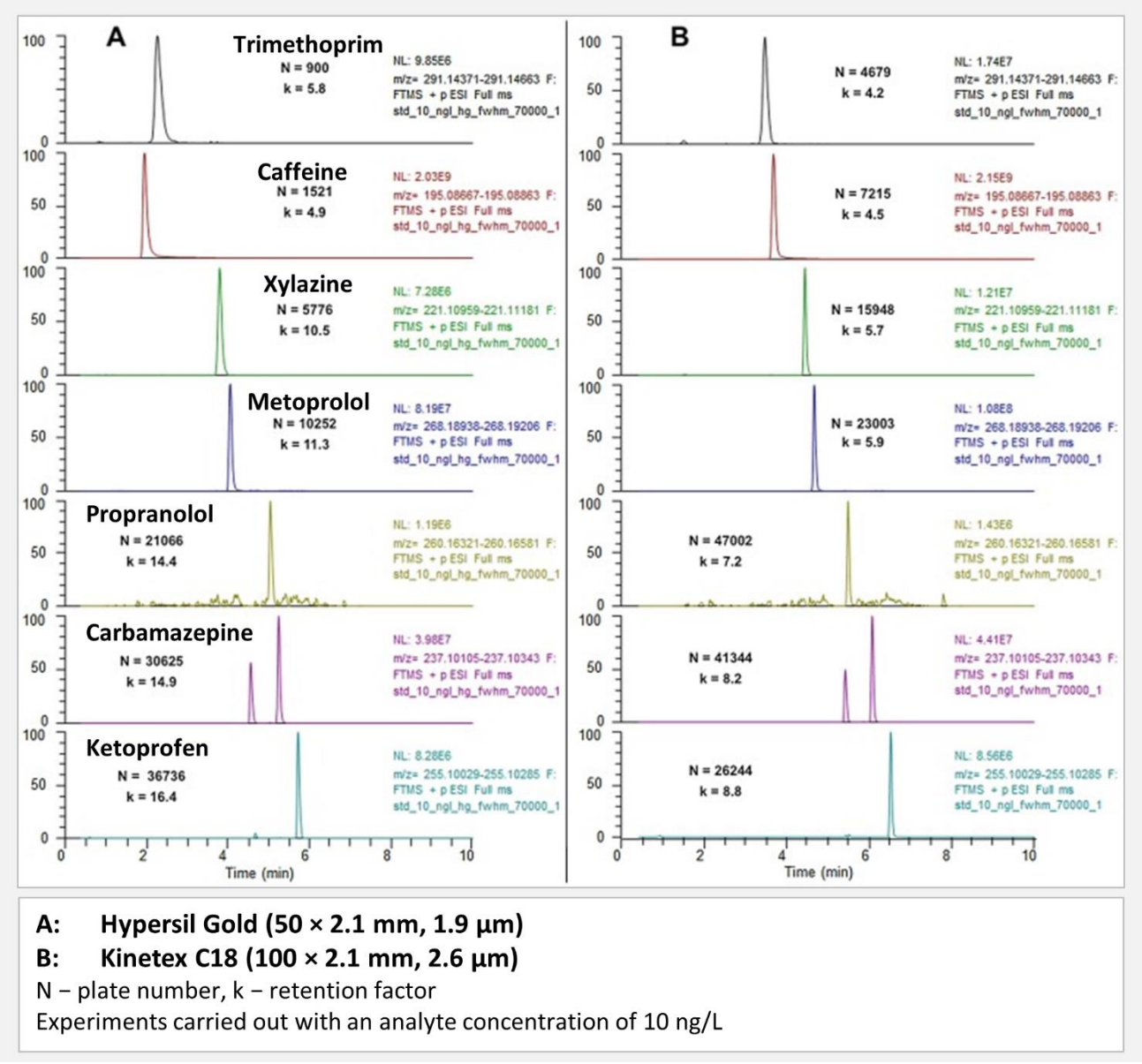
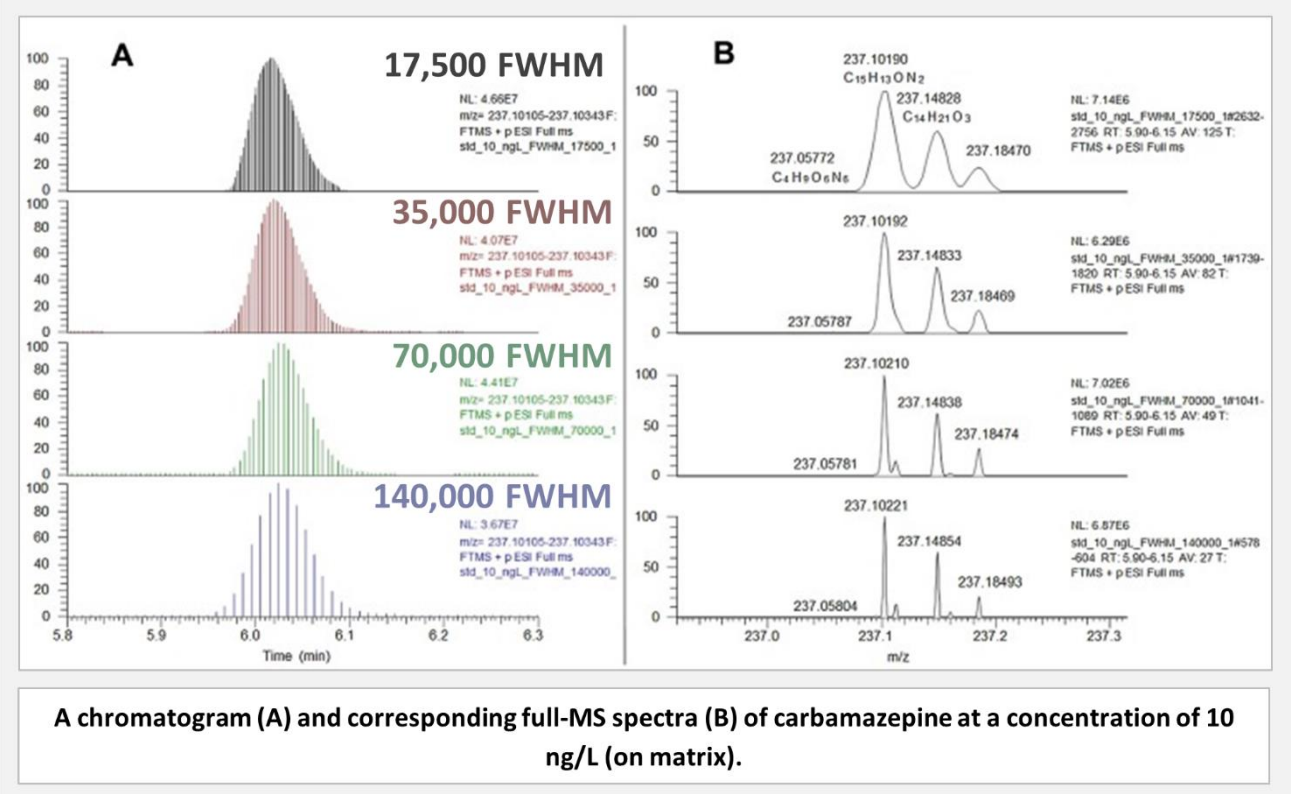


Figure 10. **Chromatograms of selected PhACs from the spiked wastewater sample using two LC columns**

The sensitivity and selectivity were compared for three scanning modes: full-MS, selected ion monitoring (SIM), and parallel reaction monitoring (PRM). Taking into account possible hindrances caused by co-eluting matrix components, the experiments were carried out on a spiked WW sample (10 ng/L). Overall, the values of S/N and the absolute areas of analyte peaks were superior in the full-MS mode. This observation might seem unexpected. Traditionally, PRM and targeted SIM modes are considered more sensitive because the quadrupole allows to transfer the ions of interest that fall within the defined m/z isolation window. Since co-eluting interferences are filtered out, higher amount of target ions are accumulated and injected in the analyser cell (within AGC limits). However, the performance of these modes deteriorates with an increasing number of analytes as PRM and SIM measurements are sequential, not simultaneous. When the number of analytes is too large, the scanning speed becomes unsuitable for HPLC compatibility and the AGC threshold, resolving power and/or accumulation time limits must be lowered, which in turn leads to decreased sensitivity and lower S/N ratio.

According to the criteria proposed by EU Commission Decision 2002/657/EC for both screening and confirmatory analytical methods, an HRMS full-MS mode with one precursor ion is considered suitable for screening purposes [200]. Meanwhile, confirmation methods cannot rely on full-MS data alone. Hence, a procedure was developed that incorporated MS/MS measurements as a part of dependent acquisition (full-MS/dd-MS/MS mode). This way product ion spectrum can be automatically obtained in accordance with the inclusion list that contains precursor masses.



A chromatogram (A) and corresponding full-MS spectra (B) of carbamazepine at a concentration of 10 ng/L (on matrix).

Figure 11. **Relationship between resolving power and scanning frequency on Orbitrap-MS**

Resolving power is one of the most critical parameters in the analysis of difficult matrices by HRMS techniques. It allows increasing both the selectivity and the scope of the method. Yet, excessively high resolution (such as 140,000 FWHM) would significantly affect the quality of LC-MS data due to the reduced scanning speed and fewer data points (Figure 11). Hence, this parameter must be pre-optimized before the method can be applied to sample analysis. A series of experiments were performed at four different resolution settings (17,500, 35,000,

70,000 and 140,000 FWHM) to explore the relationship between the quality of chromatograms and capability to resolve analyte response from interferences. As seen in Figure 11 (B), carbamazepine signal suffers from an overlapping interference, that remains unresolved until the resolution reaches 70,000 FWHM. When the resolution is set to the maximum value (140,000 FWHM), the separation between both m/z signals becomes even more pronounced. However, the number of data points per single LC-MS peak is lower and the quality of the peak shape deteriorates. Based on these considerations, the resolving power for full-MS mode was set at 70,000 FWHM, while 17,500 FWHM was found suitable for dd-MS/MS acquisition mode.

3.1.3. Sample preparation protocol: determination of aminoglycosides by HPLC-Q-TOF-MS

Several studies have been conducted to find the most efficient extraction procedure and SPE conditions for the determination of AGs in various matrices [201,202], indicating that the most promising sample preparation approach includes acidic sample treatment with TCA as protein precipitation agent, followed by an SPE clean-up. For protein-rich samples 5% TCA solution is most commonly applied. However, in this case the samples were treated with 1% solution of TCA, as honey and wastewater contains considerably lower amounts of protein than animal tissues or milk. Besides, excessive use of TCA could negatively affect the following SPE procedure according to a study by Wang et al. (2016) [202]. No other additives were selected for the proposed clean-up protocol, because the extraction yields during preliminary method development stages were not substantially affected by frequently used extraction medium enhancers such as EDTA and sodium formate.

The initial strategy for the proposed SPE procedure relied on a weak cation mixed-mode stationary phase (Strata-X-CW from Phenomenex), which is particularly designed for selective retention of strong bases. However, a generic elution approach with 1%, 5% and 10% of formic acid in three most common elution phases (methanol, water and acetonitrile) was able to yield satisfactory results only for STP, DSTP and SPC, while NEO, GEN, and KAN remained irreversibly bound to the weak cation stationary phase and apparently required stronger elution conditions that would not be compatible with the developed HPLC method. Alternatively, some authors [203,204] have reported different approach involving reversed-phase functionalized polymeric sorbent cartridges (Waters Oasis HLB). For that reason, a structurally similar SPE column (Strata-X-PRP from Phenomenex) was selected and evaluated for this study. The main difference between both SPE cartridges has been described in Section 1.8.1 (in the literature review). In brief, the Strata-X-PRP stationary phase contains piperidone moieties, whereas the Oasis HLB contains pyrrolidone groups. This slight difference should not significantly affect the general retention mechanisms, suggesting that the SPE procedure can be adapted from applications that use Oasis HLB (and vice versa). Therefore, 1 mL of aqueous 10% formic acid (v/v) followed by 3 mL of acetonitrile was used for the initial elution procedure (adapted from Dasenaki et al. (2016)) [203]. As expected, the results from elution profile analysis suggested that the aqueous formic acid alone was directly responsible for the elution of AGs, while the subsequent addition of acetonitrile increased the co-elution of matrix components. Taking into account the fact that IB source exhibits higher matrix suppression compared to conventional ESI, a decision was made to avoid using an additional organic solvent as an elution medium. A series of experiments with different volumes (2 mL, 3 mL, 4 mL, and 5 mL) of various formic acid mixtures (1%, 2.5%, 5%, and 10%, v/v) were conducted using a spiked honey sample (100 ng/g, adjusted to a pH of 7±1 with 1 M NaOH solution) to evaluate the SPE conditions in terms of analyte recovery. The highest recoveries were achieved with aqueous 10% formic acid (v/v), although only a small difference was observed between 2.5%, 5% and 10% in terms of analyte recovery. A volume of 3

mL proved to be sufficient for eluting the compounds of interest. Nevertheless, relatively low recoveries were observed for GEN and KAN (<50%). In order to address this issue, an additional series of experiments were carried out by extracting a honey sample at different pH values that was fortified with an AGs standard mixture (100 ng/g) with the previously selected SPE eluent. Results showed (Figure 12) that the target analytes can be separated in two distinct groups, where GEN and NEO exhibit the highest affinity towards the stationary phase at low pH range, while the rest of AGs were adsorbed better at neutral or slightly basic conditions. Fairly acceptable recoveries (ranging from 65% to 76%) were obtained at pH 5, and these were the conditions selected for the optimized method. One reason behind low extraction recoveries might be an oversaturation of SPE cartridge, because honey contains high levels of carbohydrates, which are also retained on the SPE cartridge during the clean-up procedure, thus occupying active sites and decreasing the absolute analyte recovery.

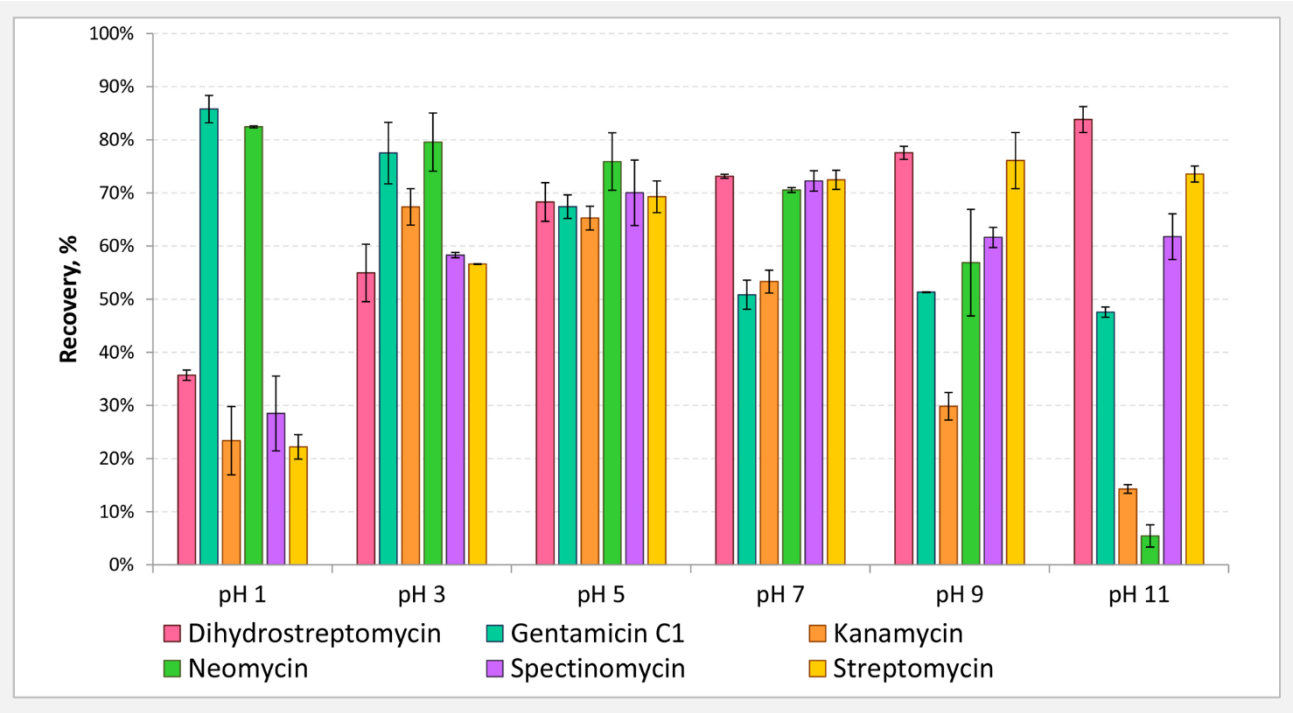


Figure 12. **Recoveries for blank sample spiked at 100 ng/g and extracted at different pH values (n=3)**

The matrix effect (ME) was evaluated by analysing identically prepared blank honey samples (n=5) that were fortified with known concentrations of AGs at the end of sample preparation procedure. The peak areas obtained for the target analytes were compared to ones obtained from the calibration standard at the corresponding concentration level. The ME values were calculated by the following formula:

$$ME\ (\%) = \frac{Peak\ area_{fortified\ honey\ sample}}{Peak\ area_{standard}} \cdot 100\% \qquad (3)$$

Signal enhancement was observed for GEN (110%), KAN (112%) and NEO (107%), while signal suppression occurred for STP (82%), DSTP (84%) and SPC (87%). Even though the sample extracts were already significantly diluted, ME still prevailed, pointing towards the necessity to apply the MMSCC approach to compensate for the loss of analytes and ME. The same approach was applied for WW analysis.

3.1.4. Instrumental analysis: determination of aminoglycosides by HPLC-Q-TOF-MS

Considering the highly polar nature of AGs and, thus, their poor retention on conventional reversed phase columns, it was decided to use a zwitterionic-type mixed-mode LC column - Obelisc R from SIELC Technologies. It has been previously applied for the analysis of some selected AGs by other authors. The column has shown superior separation performance due to the unique stationary phase containing cationic groups attached close to the silica surface and anionic groups that are separated by hydrophobic spacer [205,206]. The initial optimization experiments were carried out using a binary mobile phase consisting of 1% (v/v) formic acid in water (A) and 1% (v/v) formic acid in acetonitrile (B), using gradient conditions previously described by Diez et al. (2015) [205]. The elution was performed according to the following program: 0–4 min, 10–95% A; 4–5 min, 95% A; 5–8 min, 10% A. Although the obtained chromatogram displayed satisfactory peak symmetry for all target analytes, the relative separation between AGs was insufficient and all compounds were eluted in a narrow time interval. To maximize the differences of relative retention times, various gradient conditions were examined. A higher initial portion of acetonitrile and lower gradient slope were shown to produce a slightly better separation, at the same time increasing the peak width and enhanced peak tailing, especially for strongly retained compounds such as NEO and GEN, thus, impairing peak symmetry. As noted by Alechaga et al. (2014), the predominant separation mechanism in this case involved hydrophilic interactions between the stationary phase and AGs, namely, cation exchange occurring at the carboxyl sites of the column [207]. Furthermore, the elution order of AGs correlated with the number of protonation sites for each compound (including STP and DSTP where each guanidine group can add only one proton). This suggested that controlled protonation of the stationary phase might be the key for selective separation. For that reason, three mobile phase system consisting of aqueous 1% (v/v) formic acid (A), acetonitrile (B), and water (C) was tested in order to gradually change the mobile phase from neutral to acidic. This way at the beginning of the gradient program interactions between the basic functional groups of AGs and the stationary phase would be favored, while low pH of mobile phase at the end of the program would fully protonate the carboxyl sites and diminish the ion exchange interactions. However, peak symmetry problems were encountered during the optimization procedure (peak tailing, peak splitting and floating retention times) for early eluting compounds, especially for SPC. Based on the findings of Diez et al. (2015) where the author noted that ammonium counterions may disrupt the interactions between the charges in the Obelisc R stationary phase, further addition of ammonium formate was examined and showed that concentrations above 25 mM in the injection phase were sufficient to eliminate most of these disturbances [205]. As shown in Figure 13, this approach led to a considerably better relative separation of AGs in comparison to the previously described binary gradient program, while generally preserving peak symmetry although slight peak tailing for later eluting compounds (KAN, GEN and NEO) could still be observed to some extent.

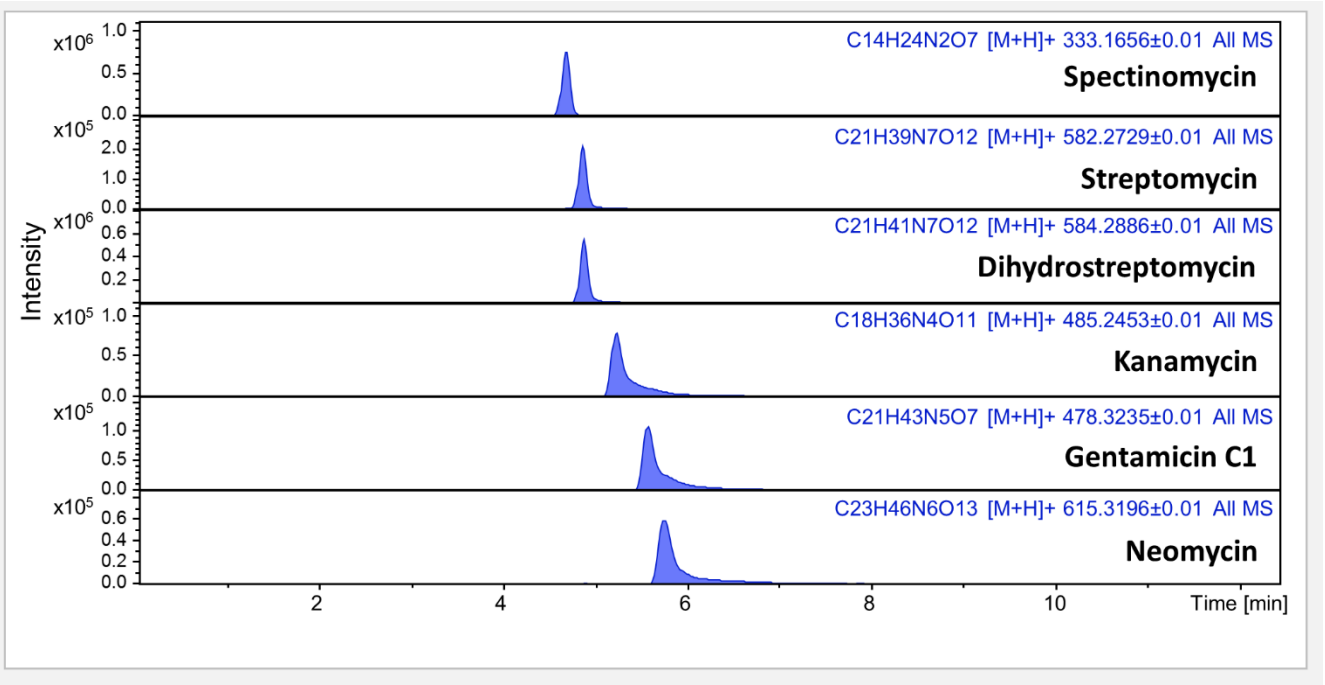
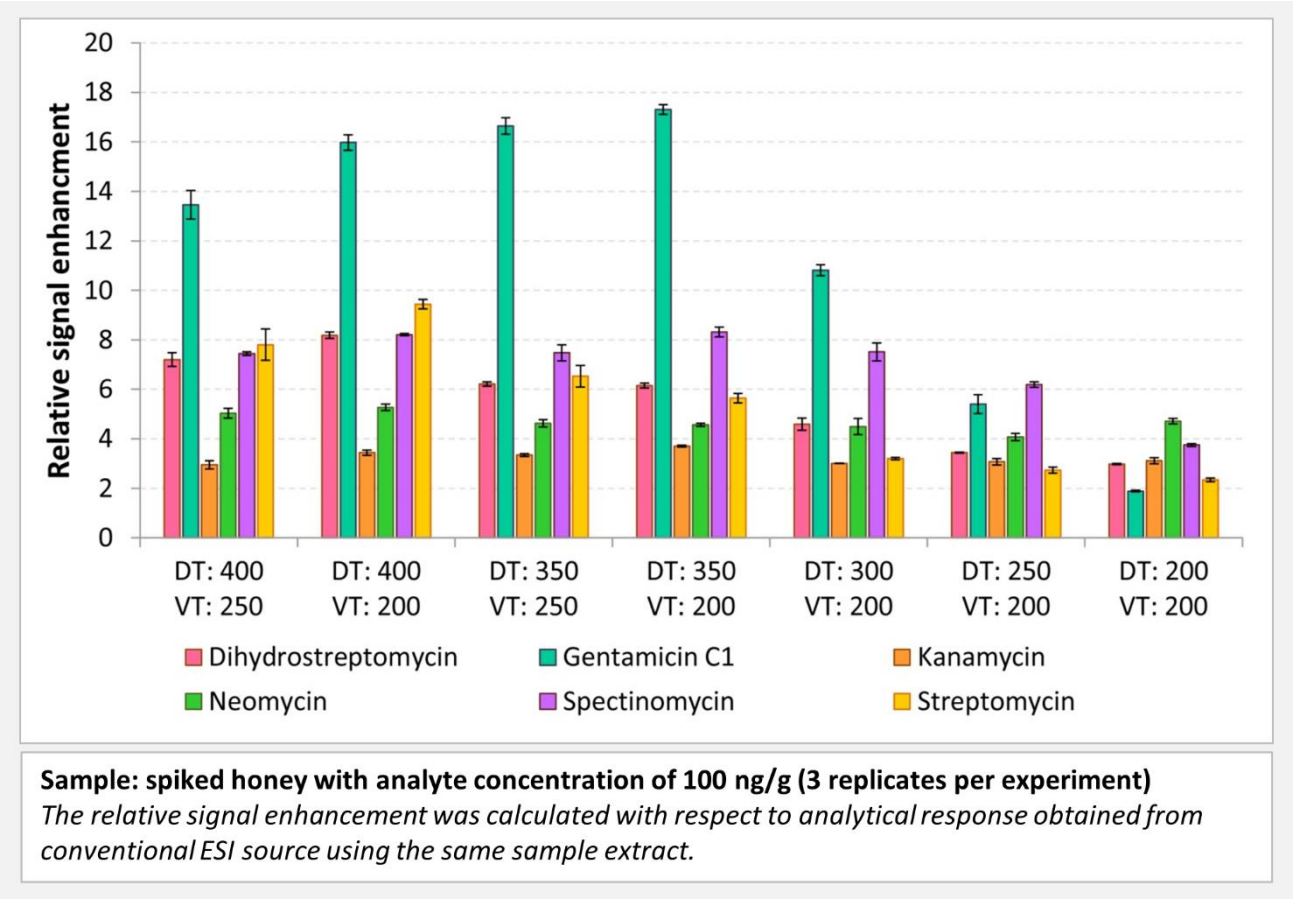


Figure 13. **Extracted ion chromatograms of $[M+H]^+$ species of AGs in a spiked honey sample (100 ng/g) using the fully optimized method**

One of the main goals was to investigate possibilities for enhancing the overall method sensitivity by applying the IonBooster™ (IB) high-temperature ESI source, which contains an additional heated spray zone and works under atmospheric pressure conditions. In previous works by Kempf et al. (2014) [208] and Chepyala et al. (2017) [209] IB was successfully used for the determination of psychotropic drugs, showing an enhanced ionization for most analytes, including polar compounds with high pKa values. This finding suggests that IB may have a potential for this particular application. Preliminary optimization revealed that only two parameters (drying temperature and vaporizer temperature) significantly affected the ionization efficiency, which was in agreement with the findings of other studies [208,209]. In order to evaluate the impact of IB temperature parameters, a series of optimization experiments were carried out by analysing a fortified honey sample with a concentration of 100 ng/g. An increase of drying temperature from 200 to 400°C led to signal enhancement for all target analytes (temperatures above 400°C were not tested as they exceeded the manufacturer's recommendations). Meanwhile, increasing the vaporizer temperature resulted in deterioration of the signal, indicating that thermal decomposition of analytes takes place within the IB source. The largest signal enhancement compared to conventional ESI source was observed for GEN (16-fold increase), followed by SPC, STP, and DSTP (at least 8-fold increase for each). Yet, the sensitivity for KAN (3-fold increase) and NEO (5-fold increase) remained almost constant during the optimization experiments as both temperature parameters did not have any notable impact on their ionization efficiency (Figure 14).



Sample: spiked honey with analyte concentration of 100 ng/g (3 replicates per experiment)
The relative signal enhancement was calculated with respect to analytical response obtained from conventional ESI source using the same sample extract.

Figure 14. **The impact of dry temperature (DT) and vaporizer temperature (VT) on the aminoglycoside signal enhancement**

To fulfil the identification point criterion specified in Commission Decision 2002/657/EC, that requires three points for group B substances in foodstuffs [200], a data-dependent full-MS/dd-MS/MS approach was again selected. In this case the number of identification points is higher than for previously described Orbitrap-MS method which was applied for the analysis of multi-class PhACs in WW samples. This difference can be attributed to the fact that honey is primarily classified as a foodstuff, thus other legislative measures are adopted for this type of matrix. Nevertheless, honey can be used for monitoring of environmental pollution. In this context residues of AGs are of high interest as these substances can are occasionally applied for the treatment of bacterial infections in apiculture and agriculture, particularly for American and European foulbrood disease and fire blight of pome fruits. Therefore, traces of AGs could be present in honey and honey-based products.

To gain more insight into the ionisation behaviour of AGs, a series of infusion experiments were carried out. Each compound was measured in full-MS and multiple reaction monitoring (MRM) modes to examine the ionization and fragmentation patterns, respectively. Doubly charged ions were observed for all analytes except for SPC. Nevertheless, MRM spectra revealed that singly charged species produced higher intensity fragmentation spectra and had more distinct fragmentation patterns. The Q-TOF instrument was therefore optimized to acquire the highest abundance of $[M+H]^+$ species and the collected data were used to set up a scheduled precursor list for the data-dependent full-MS/dd-MS/MS approach. Note: on Bruker instruments inclusion list is referred to as scheduled precursor list. The final optimized MS parameters can be found in Section 2.7.2 (in the experimental section). During the optimization study, it was noticed that only one parameter – the ion energy, which is the

energy difference between hexapole and quadrupole, was primarily responsible for the reduction of doubly charged species. A series of experiments were performed to optimize the this parameter by averaging 30-second intervals of full-MS spectra with a step of 2 eV and comparing the obtained intensities of $[M+H]^+$ and $[M+2H]^{2+}$ species. The most suitable values were found at 12 eV (for GEN, KAN and NEO) and 24 eV (for STP and DSTP), where the abundance of singly charged species exceed 95% and the absolute intensity of $[M+H]^+$ was close to the maximum for each compound (Figure 15). The optimized ion energy values were applied on a separate time segment, which was aligned with the retention time for each target analyte.

Three dd-MS/MS spectra were acquired per precursor in data-dependent full-MS/dd-MS/MS mode. To avoid faulty precursor selection, smart exclusion mode was enabled, meaning – the full-MS signal response of precursors was discriminated between steep and gradual (background noise). This way precursor selection was triggered only when its intensity started to form a noticeable LC-MS peak. Additionally, a precursor reconsideration algorithm was applied, e.g., if a precursor was already observed and the MS/MS spectrum was measured at an earlier retention time, the particular precursor was marked as found. However, if another full-MS peak for the same precursor was detected in the scheduled time interval, exclusion of precursor was reconsidered and an additional MS/MS analysis was performed. This approach helped preventing an un-triggered dd-MS/MS scan in the case of closely eluting isobaric compound. An example of full-MS/dd-MS/MS acquisition for STP and DSTP precursors is illustrated in Annex 3.

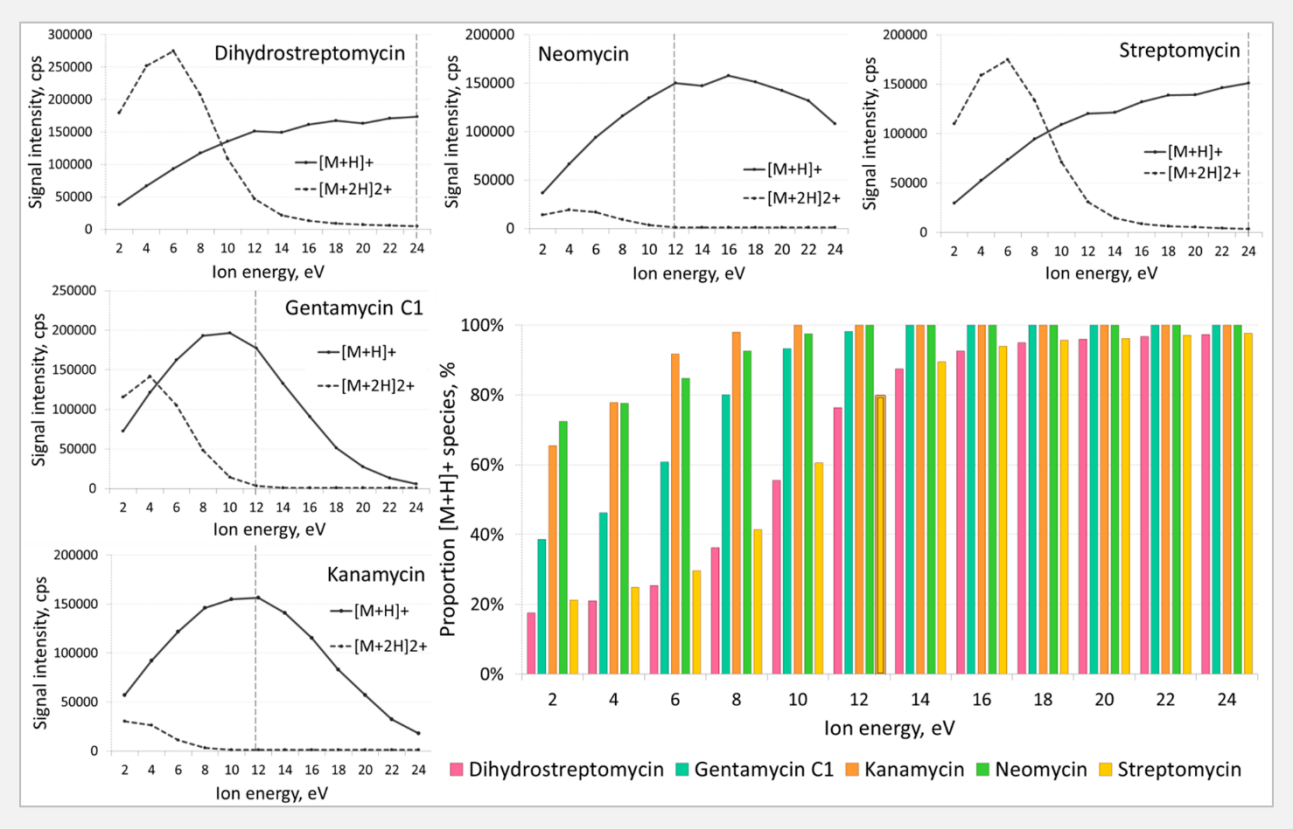


Figure 15. **The absolute intensities of singly- and doubly charged species for DSTP, GEN, KAN, NEO, and STP, and the relative abundance of $[M+H]^+$ ions (expressed as a proportion of the sum of $[M+H]^+$ and $[M+2H]^{2+}$) at different ion energy values**

3.1.5. Sample preparation protocol: determination of NSAIDs by HPLC-MS/MS

The main objective of this work was to develop a convenient and easily applicable method for the pre-concentration of NSAIDs from surface and tap water samples by using industrial grade multi-walled CNTs. Although analytical grade nanomaterial-based sorbents are available across the industry for more than two decades, their applicability in environmental analysis of PhACs has gained a momentum only recently. At the same time, industrial grade CNTs are typically less expensive compared to commercially available SPE sorbents based on other carbon nanomaterials.

To find the most suitable CNTs for the enrichment of NSAIDs, four commercially available industrial grade multi-walled carbon nanotubes with different characteristics, such as CNT agglomerates (CNTs-1), non-agglomerated CNTs (CNTs-2) and functionalised versions of CNTs-2 (CNTs-3 with hydroxy groups, and CNTs-4 with carboxyl groups), were examined. These materials have been widely used in materials science and applications for electronics. Nevertheless, the selected CNTs are available in sufficiently high purity and can be considered applicable to residue analysis [210,211].

While SPE procedure is often seen as the superior way to achieve efficient analyte extraction and clean-up from aquatic matrixes, some SPE procedures may be resource consuming and their efficiency strongly depends on the volume of the analysed sample, the complexity of analytes and the properties of stationary phase. Meanwhile, dSPE procedure can increase the interaction between the target compounds in the water and adsorbent layers, leading to enhanced adsorption. Previous studies that examined the applicability of CNTs in the field of residue analysis have concluded that pH and the functionalisation of CNTs are two most critical factors for successful enrichment of analytes [212]. Yet, a desorption step similar to those used in conventional SPE procedures should not be neglected, as it is essential to efficiently elute analytes retained on the CNTs. Taking into account the acidic nature of NSAID class drugs ($1 < pKa < 5$), the influence of pH on the sorption and desorption conditions was evaluated. Yet, it should be noted that these substances are aromatic compounds with various functionalities. This can lead to numerous interactions with CNTs (e.g. π–π electron- donor-acceptor interactions due to the presence of polarised amine groups, hydrophobic interactions with $-CF_3$ and chlorine substituents and hydrogen bonding interactions) [212]. For instance, Ma and Agarwal (2016) showed that four different adsorption mechanisms are simultaneously present during diclofenac adsorption on pristine multiwalled CNTs, such as electrostatic, hydrophobic, hydrogen bonding and Lewis acid-base interactions due to the presence of 2-carboxymethylphenyl, chlorine atoms and amino substituents in diclofenac [212].

To evaluate the selected CNTs for their suitability as dSPE sorbents for the enrichment of 12 different NSAIDs, a series of experiments were conducted using acidified and non-acidified water samples which were fortified with a known amount of NSAIDs (75 ng/mL). The adsorption efficiency and the amount of adsorbed target analytes were calculated according to the following equations:

$$Adsorption\ efficiency\ (\%) = (1 - \frac{C_0}{C_t}) \times 100 \qquad (4)$$

$$q_e = \frac{(C_0 - C_t) \times V}{W} \qquad (5)$$

, where C_o and C_t are the initial concentration (0.075 mg/L) and the concentration determined after the contact time, respectively. V is the volume of the solution (L), W is the amount of CNT adsorbent used (g), and q_e is the adsorption capacity i.e. the amount of each compound (mg/g) adsorbed by CNTs.

The acidified conditions showed a notable reduction in the concentrations of most NSAIDs in the test samples already after a contact time of 5 min. The adsorption efficiency ranged between 94 and 100% for almost all compounds except for flunixin and niflumic acid that yielded relatively lower adsorption efficiencies (65 to 75%). This observation is not unexpected. It is worth bearing in mind that selected NSAIDs were mostly in fully protonated form under these conditions (pH = 3). This way hydrogen bonding between the carboxyl groups and water is lower compared to deprotonated state which leads to enhanced affinity (via hydrophobic interactions) towards CNTs [213]. However, the two pyridine-3-carboxylic acid derivates (flunixin and niflumic acid) behave differently since they have lower pKa values compared to others. This is due to the sp^2 hybridized nitrogen located in the pyridine group that withdraws electrons from the aromatic ring and stabilizes carboxylate ion. An increased acidity (lower pKa) can, therefore, be observed. At low pH range, flunixin and niflumic acid exist in a zwitterionic state and/or in a fully protonated pyridinium form. The partition behaviour between these microspecies is pH-dependant. For this reason, an approximate estimation was carried out by calculating the relative distribution between species in MarvinSketch 20.4 software. As seen in Annex 4, these two NSAID class drugs exist mainly in zwitterionic state when the pH ranges from around 2 to 5. This explains the decrease in adsorption efficiency that was noticed during previous experiments in acidic media because zwitterions are less suspectable to hydrophobic interactions compared to uncharged molecules. Hence, incomplete adsorption on CNTs was noted.

The adsorption equilibrium between the analytes and CNTs was reached within an 8-h contact period under acidic conditions. To achieve full adsorption of all analytes under neutral conditions the contact period had to be increased to 24– 48 h. Furthermore, the effect of the amount of CNTs on the adsorption efficiency was examined for acidic conditions. The results showed that most NSAIDs are rapidly adsorbed even when the initial amount of CNTs is very low (5 mg). However, the sorbent load of 20 mg was selected as optimal due to the adsorption efficiency of flunixin and niflumic acid, which showed a slight dependence on CNTs amount. Interestingly, the adsorbed amount of NSAIDs on CNTs-2 increased faster than for CNTs-1, which can be attributed to the partial agglomeration of CNTs-1 requiring longer contact time to open active sites on the surface of CNTs-1.

Under neutral conditions, the interaction of analytes and CNTs (determined after 5 min contact period) decreased along with the decreasing CNT loading. An exception was noted in the case of vedaprofen. This compound showed good adsorption for both acidic and neutral conditions possibly due to the planar structure of the molecule and relatively high molecular surface area that facilitates interactions with CNTs. Compared to its

non-agglomerated counterparts, the CNTs-1 showed notably lower adsorption efficiency in the non-acidified solution. Meanwhile, functionalized CNTs (CNT-3 and CNT-4) displayed satisfactory adsorption for majority of the NSAIDs. However, some compounds required higher CNT loads for enhanced adsorption efficiency. For instance, the experimental averages of three replicate samples showed that only 3 to 42% and 2 to 64% of ibuprofen were adsorbed from neutral solutions when the loadings of CNT-3 and CNT-4 were increased from 5 to 50 mg, respectively. It can be seen that, irrespective of the type of CNT, acidic conditions are favored for the adsorption NSAIDs. Moreover, a decrease in pH of the solution may enhance negative charges arising on CNT surface, which may also facilitate the interaction between the surface of the sorbent and selected substances leading to better adsorption [212]. Based on these considerations, it was decided that acidic conditions are optimal for the adsorption of these analytes when using raw CNTs due to better adsorption of ibuprofen, flunixin, and niflumic acid.

The desorption experiments were conducted using all four types of the CNTs under both acidic and neutral conditions. The long-term study regarding the content of target analytes after equilibration for 7 days showed almost non-detectable levels of NSAIDs in the liquid phase, confirming the complete adsorption of analytes on the CNTs. Desorption of analytes was achieved by placing the sorbents in an empty SPE cartridge, washing with deionised water to clean the CNTs and drying. A solution consisting of 1% ammonium hydroxide in methanol, corresponding approximately to pH = 9, was used as an elution medium for preliminary desorption experiments. In brief, a 10 mL aliquot of desorption phase was added to the cartridge, kept for 5 min to wet the CNTs and eluted through the cartridges. The flow rate was regulated to around 2 mL per minute. The obtained extracts (after evaporation and reconstitution) were analysed by the developed LC-MS/MS method to evaluate the analyte recoveries. A significant difference (from 1 to 119%) in the analyte recoveries from CNTs between neutral and acidic conditions was found. As expected, the analyte desorption efficiency from neutral solutions confirmed the findings of adsorption experiments and the overall recoveries were lower, hence such conditions were not further explored. Meanwhile, the recovery of analytes from CNTs in acidic solutions was occasionally greater than 100% and elevated recoveries were noted for several analytes (flunixin, ketoprofen, mefenamic acid, niflumic acid, and vedaprofen). This could be attributed to the matrix effects and suggests that additional washing step might be necessary to remove matrix components. The recovery of all the NSAIDs from CNTs tested under acidic conditions followed the order CNTs-2 > CNTs-1 > CNTs-3 > CNTs-4. The recoveries for most of the compounds ranged from 40 to 115%. Low desorption efficiency was observed in the case of meloxicam and tolfenamic acid.

To further optimize the sample preparation method, a series of repeated adsorption/desorption experiments was performed for CNTs-2 and CNTs-1. The optimised preconcentration procedure based on the batch experiment results was used for acidified water samples (100 mL) followed by a brief adsorption (30 min duration) of analytes on 20 mg of CNTs-1 and CNTs-2. The used sample volume was suggested as optimal for the analysis of surface waters by Dahane et al. (2013) [214]. This study confirmed the previous results in which sufficient adsorption (adsorption efficiency 94 to 100%) of analytes was achieved on CNTs-2 and lower adsorption was only observed for flunixin and flufenamic acid on CNTs-1. Apart from 1% ammonium hydroxide solution in methanol, additional elution phases were also explored that consisted of pure methanol, acidified methanol and methanol with higher proportion of ammonium hydroxide. Elution media with higher pH values was investigated since the study by Dahane et al. (2013) reported that 10% ammonium hydroxide in methanol showed superiority over 1%

solution [214]. The desorption procedure was performed as described above with the exception that the prolonged contact period between the desorption solvent and analytes was excluded to reduce the amount of co-eluting matrix components. As anticipated, the desorption efficiency was higher for the CNTs-2 due to the higher free volume caused by wider ratio of nanoparticles compared to the denser packing of CNTs-1. The recovery of diclofenac and naproxen increased by almost 1.5 to 2 times when CNTs-2 were used, compared to that with CNTs-1. For other compounds, such as ibuprofen and vedaprofen, the difference between the two CNTs was not as pronounced. However, no significant difference was noticed between experiments at 5% and 10% of ammonium hydroxide solutions (Figure 16).

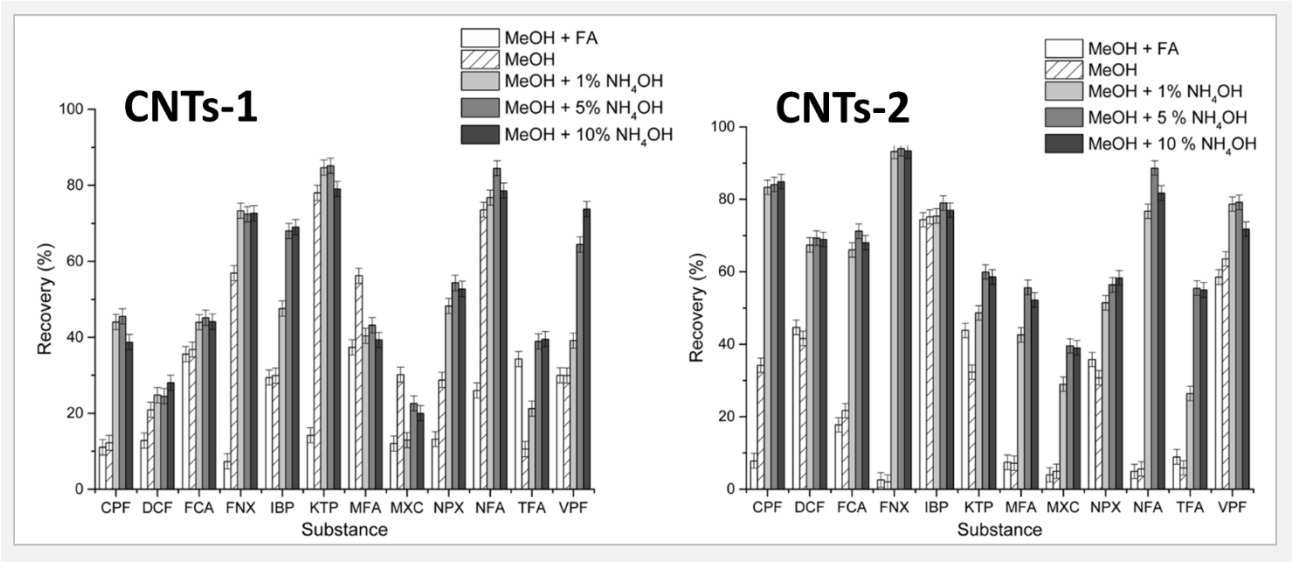


Figure 16. **Recovery of NSAIDs under different elution conditions**

Therefore, 5% (v/v) ammonium hydroxide in methanol was selected as the final desorption phase. The optimised dSPE procedure was applied for the study of real water samples. The sorbent/desorption solution combination for the recovery of retained analytes collected from real water samples (100 mL) was as follows: 20 mg of CNTs-2 and 10 mL of 5% (v/v) NH_4OH in methanol. The develop dSPE method allowed to recover between 70 and 94% of 7 out of 12 analytes including diclofenac (71 ± 1%) and ibuprofen (79 ± 1%). A sufficient but lower desorption was observed in the case of KTP, MFA, NPX, and TFA (60– 65%). Regrettably, one substance (meloxicam) showed relatively low desorption efficiency (40%). Such finding can be attributed to higher number of conjugated heterocycles compared to other NSAIDs which affects the van der Waals surface area of the compound and increases its affinity towards carbon nanotubes.

Finally, the possibility for the repeated use of the CNT sorbent (CNT-2) for the enrichment of analytes from water samples was evaluated. The results showed that for the majority of NSAIDs at least five repeated desorption cycles are necessary to completely remove any detectable traces of target compounds. Besides, an additional washing step should be incorporated to remove matrix components that cannot be eluted by 5% NH_4OH in methanol.

3.1.6. Instrumental analysis: determination of NSAIDs by HPLC-MS/MS

The LC -MS/MS method was optimised to ensure accurate qualitative/quantitative determination of the target analytes. Two MRM transitions were selected for each compound. Standard solutions of individual NSAIDs and internal standards were used to optimise the collision energy (CE) and other operational parameters of the ESI interface. Ionisation was achieved in negative ESI mode, which was found to be the most suitable due to the favorable ionisation of carboxyl groups of acidic NSAIDs [215].

The most intense transition (referred to as MRM_1) was selected for the quantification purposes, whereas the second transition (referred to as MRM_2) was used for qualitative confirmation. While the ratio of peak areas between the two transitions selected for quantification and qualification was calculated and applied as an additional parameter to confirm the presence of NSAIDs in surface and tap water samples and reduce the likelihood of false positive hits due to co-eluting isobaric substances.

A sufficient separation of analytes was gained by the optimal conditions including the application of internal standards to compensate for matrix effects. The extracted ion chromatograms for all of the 12 NSAID analytes and their internal standards are shown in Figure 17. The gradient program was optimized to enable acceptable separation for the analytes of highest environmental relevance (diclofenac, ibuprofen). Table 10 shows the optimised instrumental conditions including retention time, the two selected MRM transitions (MRM_1, MRM_2), and their transition collision energies (CE_1, CE_2) for target analytes. Unlike the other methods, an elaborated optimisation of instrumental parameters was not conducted for two reasons. Firstly, because analysis of NSAIDs via LC-MS/MS is a well-established procedure without much room for improvements. Secondly, the main purpose of this study was to develop a novel sample preparation procedure that relies on selective enrichment of NSAIDs by MWCNTs (discussed in the previous section).

Table 10. **The optimized MS conditions for analytes and internal standards**

Compound	**RT, min**	**MRM_1, *m/z***	**CE_1, eV**	**MRM_2, *m/z***	**CE_2, eV**	**Internal standard**
CPF	5.91	272→228	20	272→226	37	Carprofen-d_3
DCF	6.47	294→250	20	294→214	31	Diclofenac-$^{13}C_6$
FCA	7.74	280→236	24	280→176	40	Tolfenamic acid-$^{13}C_6$
FNX	5.21	295→251	25	295→191	40	Flunixin-d_3
IBP	6.75	205→159	10	205→161	14	Ibuprofen-d_3
KTP	4.48	253→209	11	253→197	13	Flunixin-d_3
MFA	7.81	240→196	20	240→180	35	Tolfenamic acid-$^{13}C_6$
MXC	4.28	350→146	28	350→286	20	Meloxicam-d_3
NPX	4.59	229→185	15	229→169	20	Carprofen-d_3
NFA	6.13	281→237	20	281→177	45	Ibuprofen-d_3
TFA	8.35	260→216	22	260→214	30	Tolfenamic acid-$^{13}C_6$
VPF	9.50	281→237	15	281→235	29	Vedaprofen-d_3
Carprofen-d_3	5.90	275→231	20	-	-	-
Diclofenac-$^{13}C_6$	6.48	300→256	20	-	-	-
Flunixin-d_3	5.21	298→254	25	-	-	-
Ibuprofen-d_3	6.75	208→161	12	-	-	-
Meloxicam-d_3	4.28	353→289	20	-	-	-
Tolfenamic acid- $^{13}C_6$	8.35	266→222	22	-	-	-
Vedaprofen-d_3	9.48	284→240	15	-	-	-

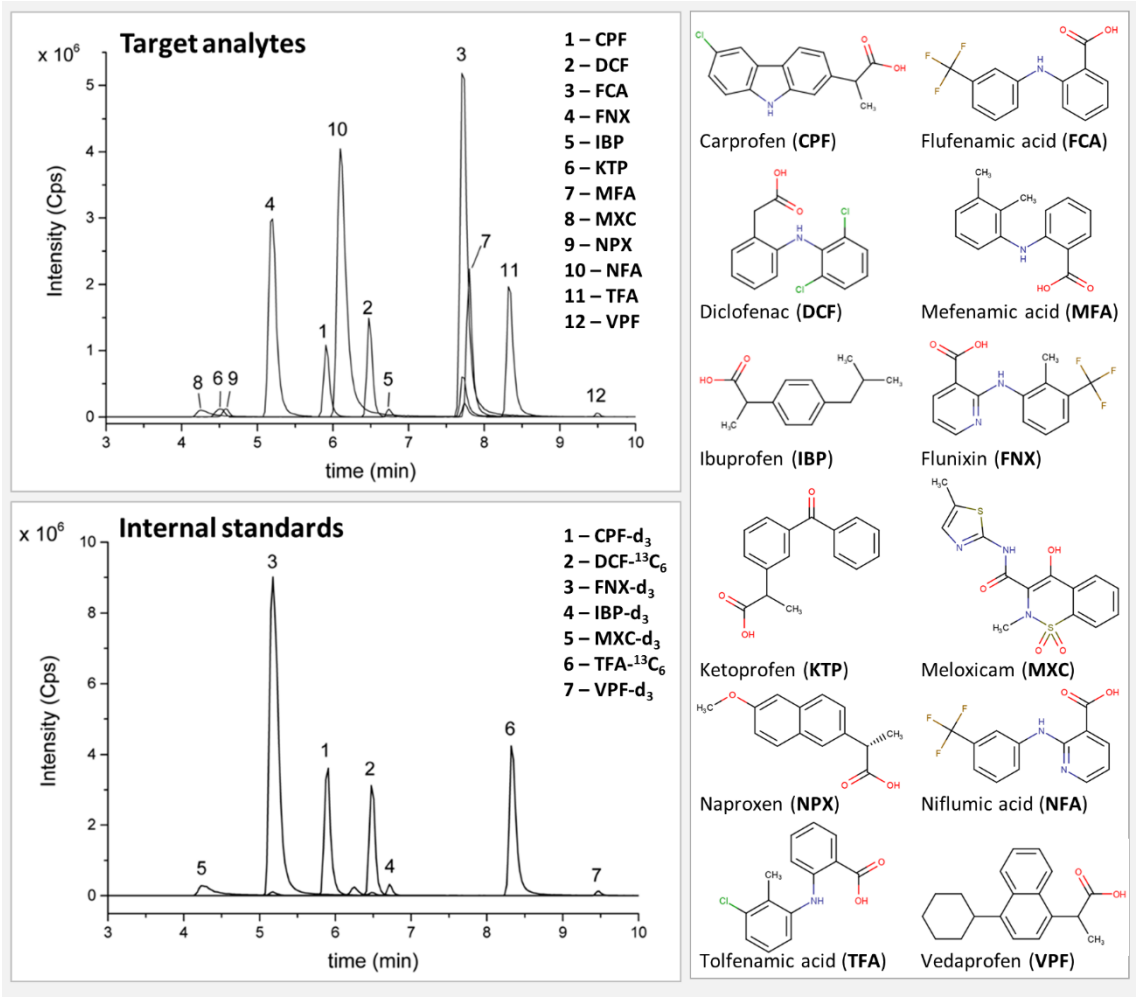


Figure 17. **Extracted ion chromatograms of the 12 NSAID analytes and their internal standards**

3.1.7. Sample preparation protocol: determination of PhACs by DI-FT-ICR-MS

Initial sample preparation tests were conducted with the conventional SPE technique that has been successfully applied in the Orbitrap-MS study, which was discussed in Section 3.1.1 [199]. Even though this practice is considered a gold standard and used in countless LC-MS based multi-analyte methods, it produced extremely high matrix suppression in the case of DI-HRMS even when high dilution factors were applied. Therefore, alternative sample preparation protocols were evaluated. In this context, QuEChERS (quick, easy, cheap, effective, rugged, and safe) was recognized as a potential substitute for SPE-based extraction. While this methodology is mainly used for pesticide residue analysis, it has recently gained increased attention in several in other areas [216]. For instance, QuEChERS protocol has been applied for the determination of 25 PhACs in sediment samples with satisfactory results (target compound recoveries between 64% to 101%) [217]. Nevertheless, QuEChERS is not considered a practical option when it comes to water samples, because only a limited sample volume can be extracted at once that rarely exceeds 10 mL. A way to overcome this limitation is freeze-drying. Again, it is not a common practice and precautions must be taken to avoid significant analyte losses. Yet, methodologies exist where freeze-drying technique has been applied to WW matrixes to extract compounds that show poor enrichment efficiencies via SPE-based extraction methods [218,219]. In addition, water removal allows to minimize co-extraction of matrix components, because only a limited fraction of dry matter dissolves in the extraction medium (acetonitrile–water). Based on these considerations, freeze-drying was used as a pre-

treatment step before the extraction stage and further clean-up using QuEChERS. Besides, the latter was modified to meet the needs of the developed FT-ICR-MS method.

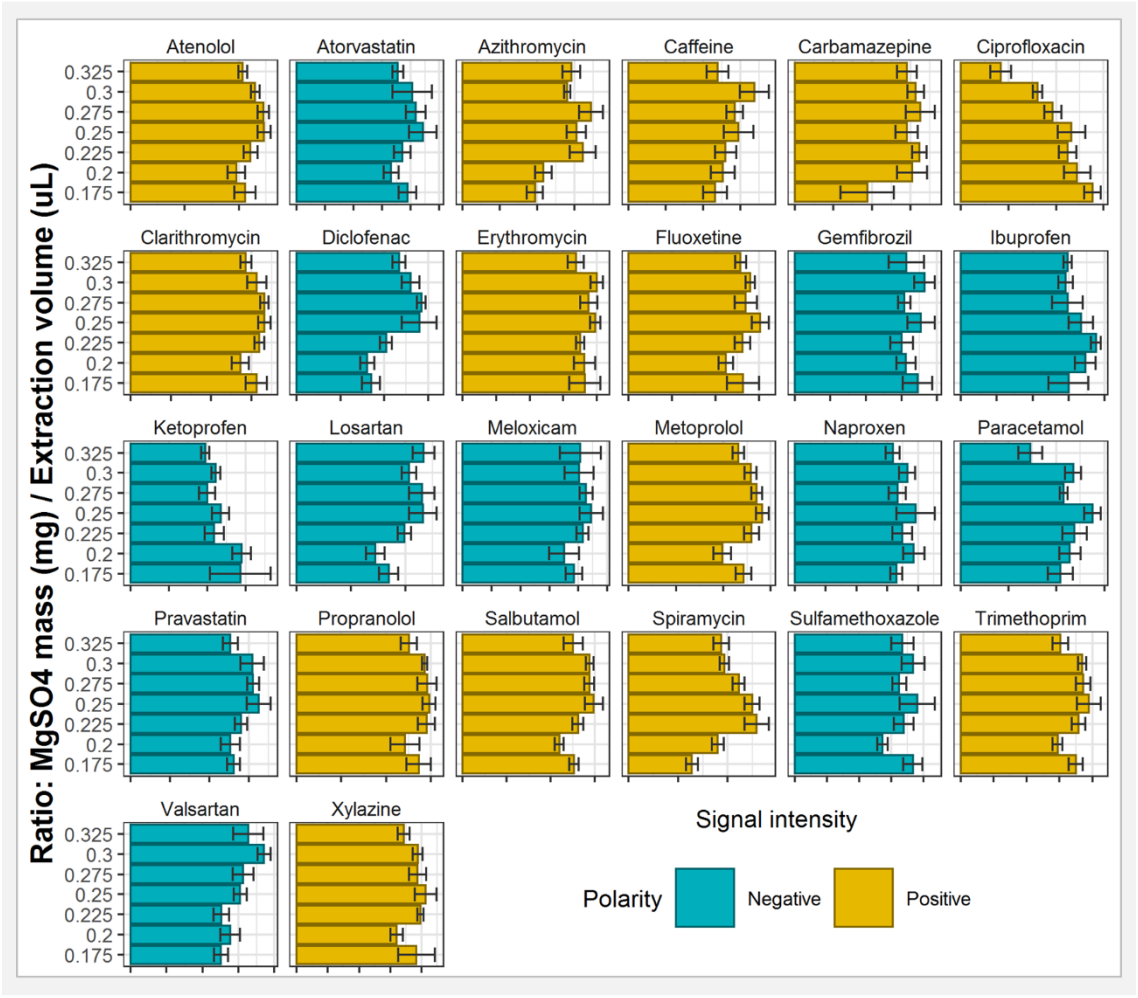


Figure 18. **The influence of MgSO4 amount on Q1 signal intensities of target PhACs**

Preliminary optimization was carried out without the presence of matrix to evaluate compatibility of QuEChERS salts and buffering agents with instrumentation. Two major issues were noted. Firstly, sodium chloride (NaCl) is partially transferred to acetonitrile during the phase separation step and consequently causes interfering MS signals that correspond to NaCl clusters: $[Na_nCl_{n+1}]^-$ and $[Na_{n+1}Cl_n]^+$. Secondly, citric acid produced an extremely intense peak at m/z 191.0197 in negative ionization mode. Neither NaCl nor citrate buffers impair analytical performance of LC-MS based applications, yet they can pose serious problems in DI-HRMS analysis (e.g. suppress ionization of analytes, oversaturate the ICR cell and promote the formation of additional adducts). Attempts were made to remove NaCl and citric acid residues from the final extract by evaporation and subsequent solvent exchange. For this purpose, dichloromethane, ethyl acetate, acetone and methyl tert-butyl ether were investigated, but, unfortunately, none of these solvents provided satisfactory results. Therefore, the use of NaCl and citrate salts was discarded and phase separation/extraction relied solely on $MgSO_4$. Next, the amount of anhydrous $MgSO_4$ was optimized. The most favourable conditions with respect to analyte recoveries were found when the ratio (mg/µL) between $MgSO_4$ and acetonitrile-water (1:1, v/v) was from 0.225 to 0.275 (Figure 18). The draft method was tested on WWTP influent samples and strong matrix suppression still prevailed. In order to minimize the co-extraction of matrix components from freeze-dried material, different acetonitrile-water compositions were investigated (50-100%). Results revealed that signal suppression decreased with higher

organic phase content, but, at the same time, several PhACs displayed poor extraction efficiency due to their relatively polar nature (e.g. caffeine, NSAIDs and macrolide antibiotics). Hence, acetonitrile-water (9:1, v/v) was selected as the best option and an additional sonification step was incorporated to assist more efficient analyte transfer to the extraction medium.

However, these efforts were not sufficient to counter the ion suppression, which particularly affected the negative ionization mode. While it is nearly impossible to pinpoint which substances cause this unfavourable situation, four ubiquitously present signals were noticed at m/z 297.1530, m/z 311.1686, m/z 325.1843 and m/z 339.1999 that displayed around 10-fold higher intensity than all the other full-MS peaks. A putative identification was carried out, suggesting that these interferences are anionic surfactants, in particular, linear alkylbenzene sulphonates ($C_nH_{2n-1}C_6H_5O_3S$, n=10-14). Yet, surfactant residues should not be considered the primary underlying cause of suppression since dissolved organic matter which can be found in wastewater comprises a complex mixture of organic substances with size up to 100 kDa. Therefore, further clean-up using dispersive SPE (dSPE) was evaluated. Preliminary dSPE experiments with various types of sorbent materials indicated that traditional dSPE sorbents (C18 and PSA) and anion exchange sorbent (Strata-X-A) might be improve the performance of the method. A series of experiments were carried out with various amounts of these dSPE sorbents (1, 3 and 5 mg) using fortified WWTP influent sample as target matrix. In addition, analogously prepared blank samples were also analyzed to assess how dSPE sorbents affect the recovery under conditions where the matrix is absent. In matrix-matched experiments C18 sorbent was able to reduce ion suppression for almost all target analytes, whereas PSA and Strata-X-A produced somewhat contradicting outcomes. PSA significantly worsened sensitivity of NSAIDs and other acidic PhACs. This effect was even more pronounced in spiked procedural blanks, because there is a negligible concentration of matrix substances, thus more binding sites are left unoccupied leading to even greater affinity towards acidic compounds. Since WWTP effluents contain much less organic matter than untreated influents, unintentional loss of analytes may occur and lead to underestimation of results. The same amount of Strata-X-A sorbent performed similarly to PSA, but the loss of acidic PhACs was much less pronounced. Besides, a Strata-X-A enabled removal of anionic surfactants. These differences between Strata-X-A and PSA sorbents are related to their structural characteristics. PSA contains two binding sites (primary and secondary amine), while Strata-X-A has only one quaternary amine moiety, therefore, the first exhibits higher overall ion-exchange capacity. Based on these findings, PSA was excluded, while the amount of C18 and Strata-X-A used in the final method was optimized to 3 mg and 1 mg, respectively.

Finally, dilution experiments were conducted to find the most appropriate dilution factor (1, 2, 4, 8, 12, 16 and 20). Matrix effect (ME) was calculated by dividing the analyte response in matrix-matched extract by the response in procedure blank and multiplying with 100% (3. Equation). Samples were fortified using post-extraction addition approach. A value of 100% indicates no effect meaning that the analytical response stays the same and is unaffected by matrix, whereas values below and above 100% indicate ionization suppression and ionization enhancement, respectively. Results showed that the final extract must be diluted with injection phase at least 12 times before stable ME can be obtained. At these conditions, the mean ME was 80.7 ± 24.5% (Figure 19). The worst suppression was observed for paracetamol (19%), naproxen (38%) and spiramycin (58%).

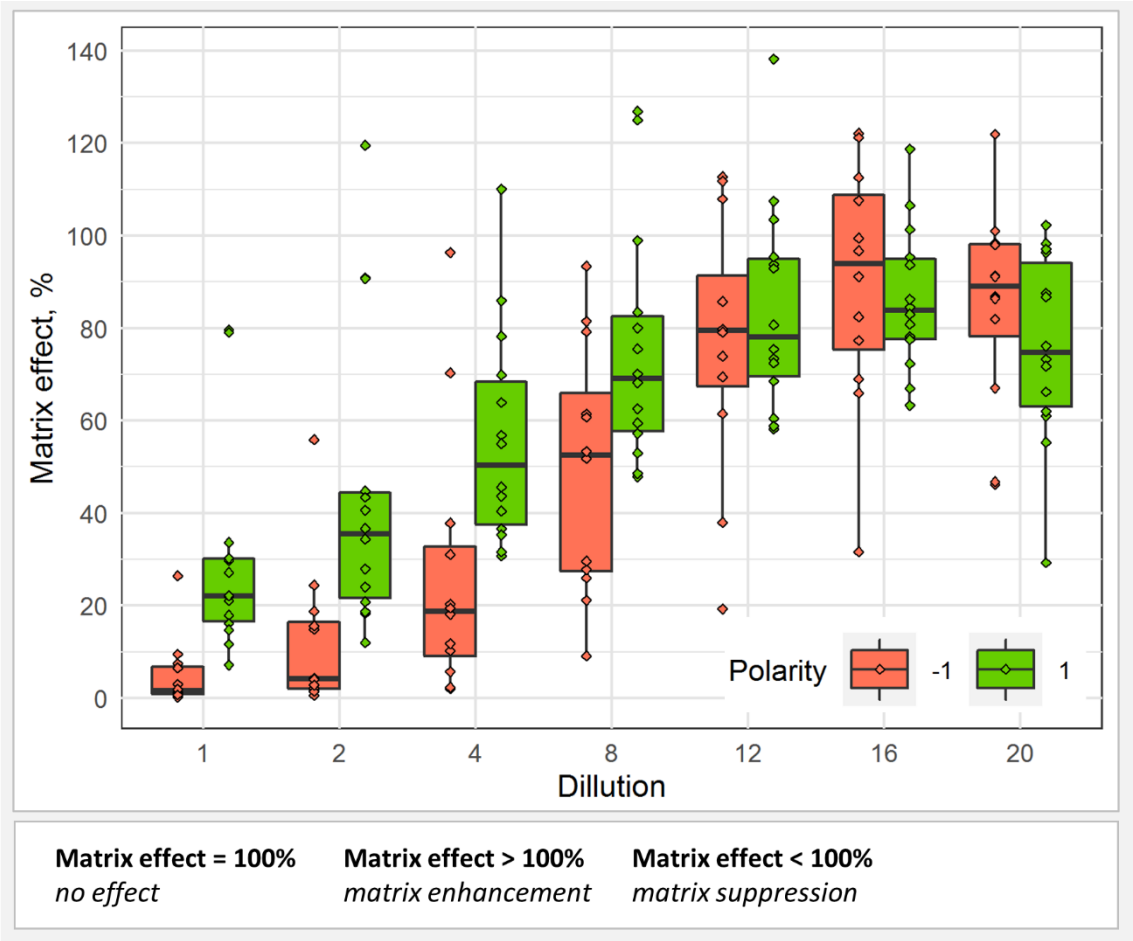


Figure 19. **Dilution factor of the final WWTP influent extract versus the observed matrix effect**

The developed method was further verified by determining the absolute recoveries of PhACs. This parameter was calculated by comparing the signal intensities of target analytes between procedural blanks (deionised water) that were spiked before and after the procedure. In addition, extracts obtained without dSPE clean-up were also evaluated in the same manner. As seen from Figure 20 (A), dSPE treatment still caused a significant loss of analytes, especially acidic PhACs. For instance, valsartan recovery decreased from 76% to 24% due to the clean-up procedure. Even though the absolute recovery was lower compared to extracts obtained by $MgSO_4$-assisted phase separation alone, dSPE clean-up step was able to provide an appropriate reduction of the matrix effect (Figure 20, B) and improve mass accuracy (Figure 20, C and D), allowing the extracts to be analyzed by direct infusion technique. Furthermore, dSPE procedure was able to supply more information regarding sample composition, increasing the total amount of full-MS signals by 11% and 6% for negative and positive ionization modes, respectively. Nevertheless, method matrix-matched standard calibration curve (MMSCC) approach had to be applied to compensate for analyte losses and improve method precision.

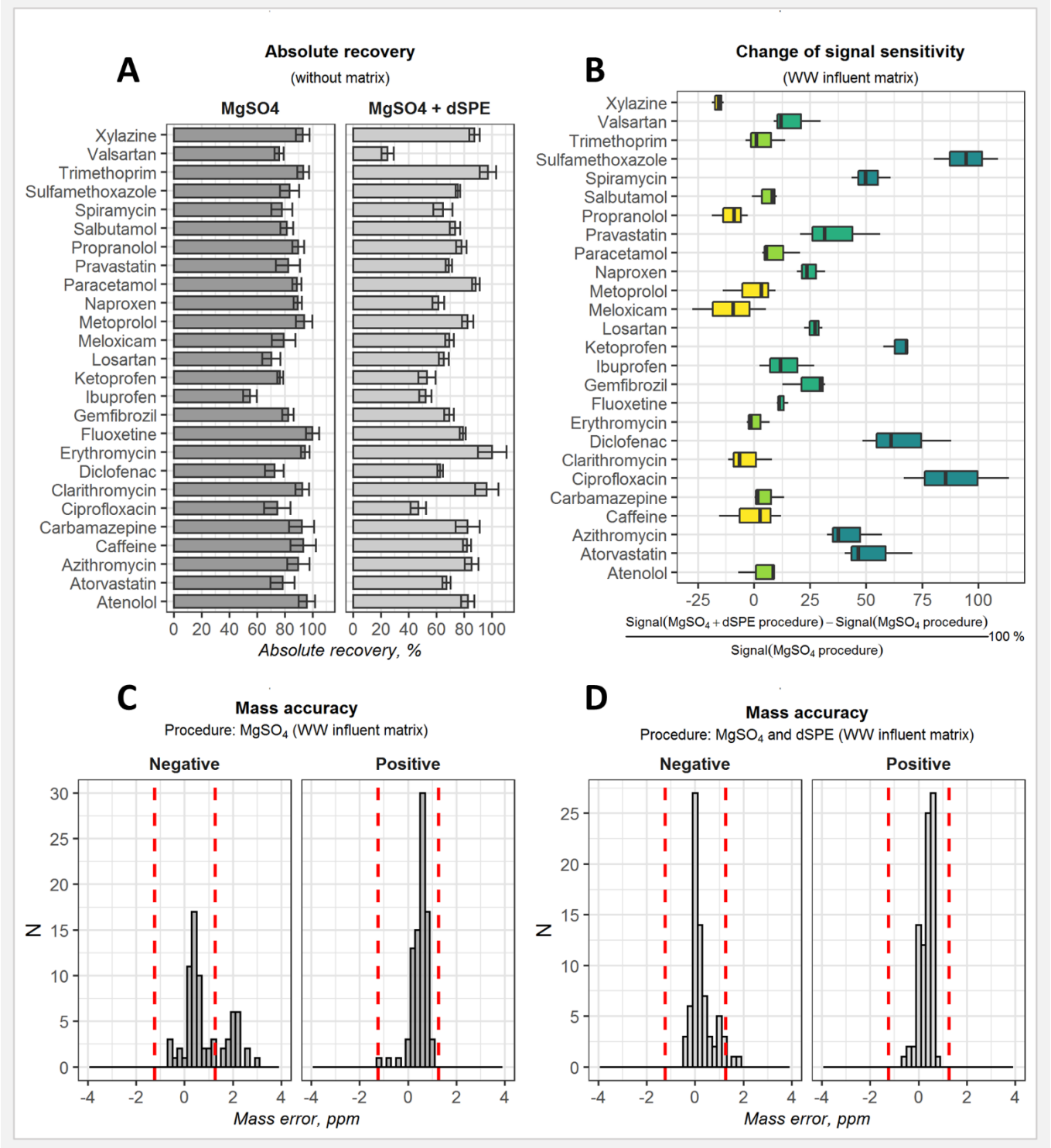


Figure 20. **Performance of the sample preparation protocol in terms of absolute recoveries (A), signal enhancement (B) and mass accuracy (C and D) for targeted PhACs**

3.1.8. Instrumental analysis: determination of PhACs by DI-FT-ICR-MS

The goal for the optimisation of FT-ICR-MS method was to achieve adequate sensitivity, signal stability and, most importantly, mass accuracy. Apart from the source parameters, the following instrumental factors were recognized as the most influential: flow rate, ion accumulation time, resolving power and injection phase. Special attention was paid to the injection phase because its composition plays a crucial role in the mitigation of matrix effect and can drastically affect ionization efficiency. First, experiments were conducted on procedural blanks that

were spiked with target PhACs after the sample preparation procedure to find the most suitable acetonitrile percentage (100%, 95%, 87%, 75%, 50%, 25% and 0%) in the injection phase (Figure 21). After that, most promising compositions were tested on matrix-matched samples to observe whether the ionization behaviour remains unchanged in the presence of matrix components. The results indicate that sensitivity increased along with the concentration of acetonitrile in the injection phase. Meanwhile, two measurements that were conducted with higher acetonitrile content (100% and 95%) produced a sudden drop of ionization efficiency (with exception to xylazine). This observation is in accordance with the hypothesis that hydrogen bonds play a crucial role in the formation of solvent shells that are necessary for solvation in ESI process. Therefore, pure aprotic polar solvents display poor results, when the amount of water (or any other proton donor) is insufficient [220]. Interestingly, the results from spiked procedure blanks and matrix-matched samples showed an equivalent trend. In both cases, acetonitrile concentrations at 87% and 75% displayed maximum sensitivity for almost all compounds, hence the injection phase was selected as 80% acetonitrile in water (v/v).

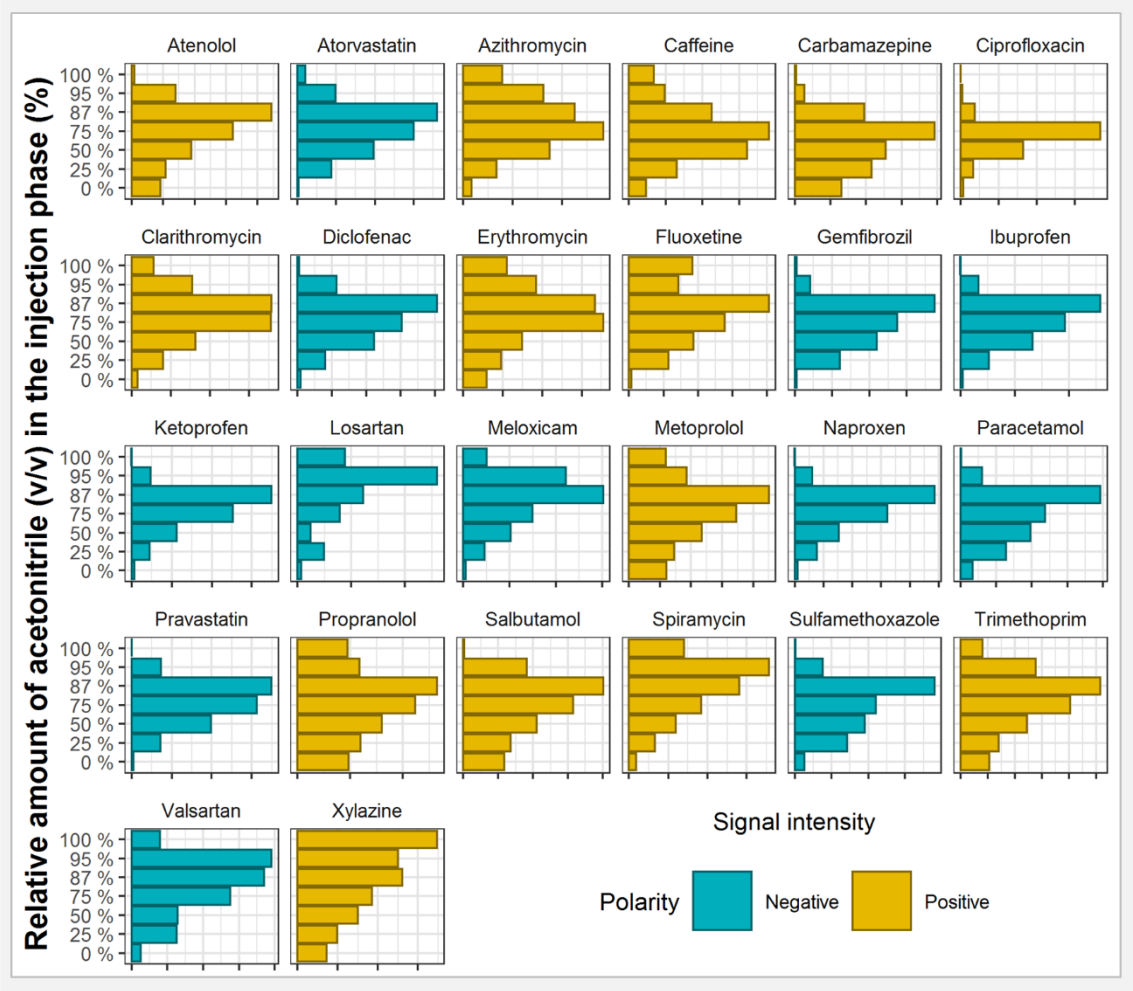


Figure 21. **A relation between injection phase composition and the observed Q_1 signal intensities on a pooled WWTP influent sample matrix**

The flow rate was optimized from 1 to 20 µL/min. As anticipated, lower flow rates produced higher sensitivity due to reduced sample dilution. At the same time, poor repeatability noticed when the flow rate was below 4 µL/min. The observed instability can be attributed to the instrumental configuration, because the applied ESI source is not particularly designed for infusion experiments at ultra-low flow rate, causing irregularities

during ionisation. According to specification, the equipped sprayer needle is not recommended for experiments involving flow rates below 2 µL/min. These considerations were taken into account and the flow rate for the main method was set at 5 µL/min.

Similar to Orbitrap-MS, prolonged accumulation of ions in the FT-ICR-MS system can cause an oversaturation of analyser cell that negatively impacts both sensitivity and mass accuracy. Fine tuning of ion accumulation time was done by analysing a fortified WWTP influent sample in triplicate at different parametric values (5, 10, 20, 35, 50, 75, 100, 150 and 200 ms). Signal responses followed a linear trend throughout the analysed range and an increased mass error was detected only starting from 150 ms in positive ESI mode. Meanwhile, negative ESI mode was adversely affected by oversaturation. A linear increase of signal intensities was not observed after 50 ms, whilst mass accuracy declined already at 35 ms. Hence, the final accumulation times were set at 100 ms and 20 ms for positive and negative ionization modes, respectively. The latter was maintained at 20 ms to reduce the impact of anionic surfactants on the full-MS spectra acquisition. Meanwhile, for MS/MS experiments, accumulation time was increased to 500 ms per scan, which was sufficient for obtaining fragment ion traces at the first calibration level.

Optimisation of CE values during MS/MS CID experiments were investigated by obtaining fragment spectra for target compounds at 5, 15, 25, 35 and 45 V. The obtained fragmentation patterns were matched against the database to find the most suitable CE value which yields reasonably characteristic fragmentation for all target compounds. Results revealed that, when CE was held at a constant value, the measured MS/MS spectra could not provide sufficiently rich information that can be used for library-based matching of product ion spectra. Therefore, an alternative solution was explored. The CE setting was adjusted for each precursor on an individual level using a linear function that takes into account the corresponding m/z value because the rule of thumb is that compounds with higher m/z values need higher CE for dissociation. While the quality of fragment spectra was slightly better compared to the previous experiments, several outliers were noticed that could not be matched with the library. Thus, MS/MS acquisition in the final method was carried out using three CE values (5 V, 15 V and 25 V) per precursor. Although additional measurements increase the total time of the analysis, this step was fundamental to improve the quality of MS/MS data.

Finally, transient size, which is directly linked to the resolving power, was deliberately kept at 4M. Resolving power of the HRMS system was around 490,000 and 275,000 at m/z 250 and m/z 500, respectively. The author is well aware that this value is lower than would be expected from this FT-ICR-MS system as the maximum transient size is 16M that would result in a much higher resolution. However, the rationale behind this decision is to show that the developed method is not necessarily limited to high magnetic field FT-ICR-MS systems and could be transferred to other HRMS platforms, most likely Orbitrap-MS.

3.2. Quality control, quality assurance and validation studies

3.2.1. Determination of PhACs by HPLC-Orbitrap-MS

The performance of the method was evaluated by estimating the linearity, recovery, repeatability (RSD_r, expressed as relative standard deviation), and selectivity. Experiments were carried out by analysing fortified samples at three concentration levels (5, 40 and 80 ng/L) with five replicates during a period of three days. Selectivity was tested by verifying the absence of interfering analytical signals at the expected retention time range for the analyte. ME were assessed to evaluate the degree of ion suppression or enhancement. The matrix effect was calculated by dividing the slopes of calibration curves from a pooled WWTP influent sample (so-called MMSCC approach) and the slopes of the procedure-matched calibration curves obtained from ultra-pure water. Again, a value of 100% indicates that there is no matrix effect, values >100% indicate enhancement, while values <100% indicate ion suppression.

The calibration curves that were prepared within the concentration range of 1-100 ng/L showed a good linearity, and the determination coefficients were higher than 0.992 for all PhACs included in this study. Repeatability of the method ranged from 7.0% to 42%, while the recovery ranged from 79% to 133% (Table 11).

Table 11. **Method validation parameters**

Compound	**RSD_r, %**	**Recovery, %**	**LOQ, ng/L**	**ME, %**
Acetaminophen	9.4	98	0.10	64
Atenolol	12	92	0.50	43
Atorvastatin	18	102	0.50	62
Azithromycin	27	126	1.0	104
Caffeine	42	132	0.10	125
Carbamazepine	6.4	102	0.01	82
Ciprofloxacin	35	115	1.0	130
Clarithromycin	12	87	0.01	77
Diclofenac	7.0	92	0.10	51
Erythromycin	33	90	0.10	71
Fluoxetine	31	86	0.50	44
Gemfibrozil	13	79	0.10	57
Ibuprofen	27	95	0.50	56
Ketoprofen	8.1	94	0.10	48
Losartan	16	91	0.05	46
Metoprolol	9.7	113	0.05	112
Naproxen	13	86	0.10	48
Pravastatin	6.7	78	0.01	39
Propranolol	22	109	0.01	82
Simvastatin	16	103	0.50	68
Sulfamethoxazole	10	94	0.05	35
Trimethoprim	8.4	133	0.01	104
Valsartan	15	97	0.10	74
Xylazine	11	109	0.010	63

The limit of quantification (LOQ) was empirically determined using a series of experiments that were spiked at ultra-low concentration levels. The lowest amount of an analyte in the sample for which the S/N ratio exceeded 10 was set as LOQ. The LOQ values varied from 0.010 to 1.0 ng/L. The obtained values were either equivalent or, in many instances, even lower compared to the LOQs of the same substances reported in other analytical methods for the quantification of PhACs in WW samples using LC-MS/MS [221–223] or LC-Q-TOF-MS [224].

The matrix effect of WW samples was evaluated in order to avoid inaccurate quantification since procedure matched calibration was conducted on deionised water, not WW. Taking into account the exceptionally high sensitivity of Orbitrap-MS, procedure-matched calibration seemed a more reasonable choice than MMSCC approach as it would cause a tremendous drop of accuracy at low concentration levels due to high background contamination of target analytes (reasons discussed in the fourth paragraph of Section 1.8.3). The results of ME study revealed that the majority of PhACs were subjected to ion suppression, except for azithromycin, caffeine, ciprofloxacin, metoprolol and trimethoprim, which showed signal enhancement. The ME values presented in Table 11 correspond to the WW used for the method validation and this parameter should be evaluated within each set of samples analyzed. Therefore, quality control samples (on various WWTP influent samples) were analysed within each batch and mean recoveries obtained from each sequence were applied for correcting the concentrations found in real samples.

3.2.2. Determination of aminoglycosides by HPLC-Q-TOF-MS

Validation was performed according to the guidelines laid down by Commission Decision 2002/657/EC, because honey is classified as food. The optimised version of the method was validated for decision limit (CCα), detection capability (CCβ), linearity, repeatability (RSD_r), within-laboratory reproducibility (RSD_{wR}), and recovery using the in-house validation concept. The experimental design of the validation and the necessary calculations were performed with InterVAL 3.3.2.4 software (QuoData, Dresden, Germany). Two leading factors were chosen for the validation design - the operator and the specific characteristics of sample matrix, as it was possible to divide the targeted sample batch into two separate groups based on their specific traits - low viscosity dark-toned honey and high viscosity light-toned honey. LOQs were established empirically by analysing a set of spiked honey samples with decreasing concentrations of AGs. The lowest observable concentration with the S/N ratio ≥10 was established as the LOQ. All intra-day and inter-day validation parameters were evaluated by the following experimental procedure: two distinctive honey samples (in random order) were spiked with five different concentrations (25, 50, 100, 250 and 500 ng/g) of AGs mixture in five replicates per level by two operators within a period of two days. The concentration in samples was calculated using MMSCC approach. In contrast to WW samples, selected PhACs are not ubiquitously present in honey, thus this technique does not require blank subtraction and can be applied without compromising method performance at low levels. To simplify validation experiments, the initial study design was slightly modified so that the assignment of operators would not be randomized between the days. Overall, 50 samples including the method matrix-matched calibration standards were analysed during the validation study. The results of the validation study are summarized in Table 12.

Table 12. **Validation data for the determination of AGs in honey**

Compound	LOQ (ng/g)	CCα (ng/g)	CCβ, (ng/g)	Linearity	Level, (ng/g)	RSD_r (%)	$RSD_{wR(n=2)}$ (%)	Accuracy (%)
DSTP	10	11.5	14.1	0.998	10	5.0	12.3	85.7
					50	5.3	7.2	98.0
					250	3.8	9.2	97.5
GEN	10	12.2	16.0	0.997	10	5.8	11.3	92.5
					50	5.6	10.7	97.6
					250	4.9	10.8	100.2
KAN	10	14.0	20.7	0.996	10	6.7	15.5	107.7
					50	6.7	9.8	96.8
					250	6.9	12.6	102.1
NEO	25	33.6	47.9	0.995	25	7.3	19.7	111.3
					100	7.1	16.3	88.0
					500	5.1	7.1	96.6
SPC	10	11.2	13.4	0.998	10	4.4	6.5	105.8
					50	4.3	7.6	103.9
					250	5.0	6.2	98.1
STP	10	11.3	13.5	0.998	10	5.0	7.3	86.2
					50	5.3	8.2	97.5
					250	5.9	7.8	98.4

As seen in Table 12., the highest LOQ was established for NEO. The sensitivity was poorer due to the broadening of chromatographic peaks. Decision limit (CCα[8])and detection capability (CCβ[9]) ranged from 11.2 to 33.6 ng/g and from 13.4 to 47.9 ng/g, respectively. The method showed satisfactory repeatability that remained almost constant for all target analytes throughout the validation study ($3.8\% \leq RSD_r \leq 7.3\%$), however, the within-laboratory reproducibility was considerably inferior, especially for NEO (19.7% for the lowest fortification level). Nevertheless, acceptable accuracy was achieved (from 86 to 111%), largely because of the MMSCC approach. The compound identification criteria, which were based on the MS/MS fragment ratios, were fulfilled for 48 out of 50 samples. Both outlier cases involved NEO. In particular, the fragment ion ratio of NEO exceeded the permitted range of 0.17±0.05 (Q1/Q2), suggesting that more distinctive fragmentation patterns might be required for this analyte. In general, the performance of the method in accordance to Commission Decision 2002/657/EC. However, objectively, the developed application might require additional improvements in the future to enhance the sensitivity towards NEO.

Additional validation for WW samples was not conducted. However, the performance of the method was verified by analysing a set of fortified WW samples at different concentrations (100, 250 and 500 ng/L). The obtained results indicated that the method shows similar performance characteristics for WW samples. The LOQ values for raw WW matrix ranged from 75 to 190 ng/L.

[8] Concentration level at which there is a probability α (in this case 5%) that a blank sample will yield a signal at this level or higher i.e. 95% propabilty that the observed signal is not noise.
[9] Concentration level at which there is a probability β (in this case 5%) that the measured concentration will yield an anlytical response which is above CCα level i.e. 95% propabilty that the observed signal will not be classified as undetected.

3.2.3. Determination of NSAIDs by HPLC-MS/MS

Validation study was carried out by analysing fortified surface water samples at two concentration levels (50 and 500 ng/L) with five replicates over a period of two days. ME was evaluated by the same approach as described in Section 3.2.1. The validation criteria of linearity, the mean recoveries, repeatability (RSDr, %), and instrumental sensitivity (the LOD and LOQ) are provided in Table 13.

Investigation of ME revealed that all of the analytes except IBP showed a notable ion suppression. The values were below − 20% for CPF, FNX, MFA and NFA. The ME values higher than 50% were displayed by four NSAIDs: DCF, NPX and VPF. Compared to Orbitrap-MS method, the matrix suppression was more pronounced. However, the MS/MS system which was used in this study has previously proved to be more suspectable to matrix effects [225]. Nevertheless, it was essential to use the internal standardisation technique to compensate for the suppressive effects of co-eluting substances and the analyte losses during adsorption and desorption procedures.

The linearity of the total method was evaluated using the determination coefficients for the linear regression calibration graphs. The R^2 values were above 0.99 for all of the analytes in the studied concentration range (from 1 to 1000 ng/L). The recoveries (abbreviated as "R" in Table 13.) and RSDr for all of the analytes were in the range from 65 to 120% and from 2 to 16%, respectively. The LOQ values of the developed LC-MS/MS method for most of the compounds were sufficiently low, ranging between 0.04 and 0.59 ng/L, except for NPX and VPF, both having the LOQ of 3.9 ng/L. The LOQs for IBP, KTP and DCF were very close to the developed Orbitrap-MS method, yet NPX showed a slightly lower performance regarding sensitivity.

Table 13. **Performance characteristics of the developed LC-MS/MS method**

Compound	R^2	**50 ng/L**		**500 ng/L**		**ME, %**	**LOD, ng/L**	**LOQ, ng/L**
		***R*, %**	**RSD_r, %**	***R*, %**	**RSD_r, %**			
CPF	0.9986	116	2	120	3	12	0.1	0.3
DCF	0.9991	106	3	95	4	50	0.1	0.3
FCA	0.9986	117	14	125	13	37	0.01	0.04
FNX	0.9973	93	7	94	3	19	0.09	0.3
IBP	0.9993	113	3	120	3	125	0.3	1.0
KTP	0.9979	99	3	95	3	30	0.05	0.2
MFA	0.9978	99	13	104	10	17	0.2	0.5
MXC	0.9976	99	5	66	4	33	0.2	0.6
NPX	0.9989	65	8	68	8	57	1.3	3.9
NFA	0.9980	79	9	82	7	11	0.04	0.1
TFA	0.9985	106	8	100	3	15	0.06	0.2
VPF	0.9994	113	10	117	7	75	1.3	3.9

3.2.4. Determination of PhACs by DI-FT-ICR-MS

In order to investigate the capability of the method and assign appropriate concentration levels for the validation, initial assessment of sensitivity was carried out by analysing target PhACs at various fortification levels via MMSCC approach. Decision limit (CCα) values were calculated based on the obtained calibration curve and corresponding signal-to-noise ratios (S/N). During this step, the typical S/N threshold was increased, since background noise produced by the FT-ICR-MS instrument is not negligible, especially for direct infusion analysis. According to Decision 2002/657/EC, CCα values are calculated at S/N of 3 [12]. However, for FT-ICR-MS instrumentation overly low S/N threshold can be a major cause of poor reproducibility [226], hence the S/N limit for the peak-picking algorithm was increased to 5. Furthermore, all full-MS data was recorded in profile mode to enable estimation of S/N during the post-processing stage. Taking into account that MS/MS spectra was acquired only for those compounds for which both ions were detected within the acceptable mass accuracy range (±1.25 ppm), CCα was therefore defined as the lowest concentration at which the least abundant analyte signal (Q_2) can be measured at S/N of 5 instead of 3. CCβ was calculated as follows: CCα value plus 1,64 times the standard deviation of the within-laboratory reproducibility that was obtained from the measured continent at the lowest validation level. Two ranges were used for the maximum permitted tolerance for ion abundance ratios (Q_2/Q_1). To reduce the number of false positives, suspect screening relied on a "strict" range which was set at 20% for all compounds. Meanwhile, "wide" range was used only in target screening and its limits were directly adapted from Decision 2002/657/EC (≥0.5 - 20%, 0.2 to 0.5 - 25%, 0.1 to 0.2 - 30% and ≤0.1 - 50%). For precursors that fulfilled full-MS identification criteria, MS/MS spectra was recorded at three CE levels due to reasons discussed in Section 3.1.8. and matched against the experimental and predicted MS/MS fingerprints. If at least one product ion feature within mass accuracy threshold (± 2.5 ppm) was detected, the suspect was reported as tentatively identified.

The target approach was validated using an in-house validation approach. A summarized overview for the acquired performance parameters is presented in Table 14. A detailed list of all investigated performance criteria is given in Annex 5-10. The screening detection limit (SDL) was established as the lowest level for which the most abundant ion (Q_1) was detected in all parallel measurements. The limit of identification (LOI) was set as the lowest level for which a compound fulfilled all identification criteria at "wide" range with a success rate of ≥90% [227]. From the perspective of quantitative analysis, the method can be considered sensitive and reliable only for limited number of analytes, because the obtained performance criteria were explicitly compound-specific. For example, atenolol, metoprolol and propranolol (beta-blockers) showed high sensitivity and satisfactory performance was met even at concentrations below 50 ng/L. On the contrary, low molecular weight PhACs such as paracetamol and naproxen required 10 times higher concentrations to meet the same criteria. Ion abundance ratio was recognized as the most critical factor for the successful identification of PhACs. For example, at the lowest fortification level (level A) only 17 out of 26 target compounds were able to meet this criterion with ≥90% success rate. Moreover, a slight bias towards negative residual error was observed for experimentally determined ratios. This observation hints that low abundance signals are occasionally rejected by the peak-picking algorithm since the analytical response fails to achieve the required S/N ratio. Thus, the accumulated full-MS spectrum displays systematically distorted isotopic patterns at low concentration levels. At the same time, the measured fragmentation features were in a good agreement with both experimental (MoNA) and predicted (CFM-ID

algorithm) MS/MS fingerprints. The total number of correct hits was slightly higher for experimental fragment features (94%) than for predicted (83%). On average 2.3 and 1.6 fragments were found for each compound in experimental and in-silico generated libraries, respectively. Only two substances could not be identified based on predicted spectra (clarithromycin and spiramycin). As anticipated, the experimental database showed higher success rate, yet in-silico generated fragmentation patterns were shown to be moderately accurate and therefore can be used as a complementary tool for the identification.

Table 14. **Main validation criteria and instrumental capabilities in terms of compliance rates that correspond to mass accuracy, ion ratio and MS^2 fingerprints**

Parameter	Conc. Level	Mean value	Median value	Range
Main validation criteria				
CCα, ng/L	-	128.4	65.5	18 - 693
CCβ, ng/L	-	234.2	98.0	27 - 1234
Coefficient of determination	-	0.983	0.98	0.95 - 0.99
RSD_{Sr}, %	A	16	16	5-38
	B	13	12	3 - 30
	C	12	10	5 - 29
RSD_{SwR}, %	A	23	21	8 - 61
	B	24	23	6 - 53
	C	20	17	7 - 51
Recovery, %	A	105.3	108	73 - 138
	B	101.1	102	75 - 121
	C	95.0	96	75 - 116
Mass accuracy and ion ratios				
Q_1 compliance rate (mass error ±1.25 ppm), %	A	97%	100%	75% - 100%
	B	98%	100%	75% - 100%
	C	100%	100%	92% - 100%
Q_2 compliance rate (mass error ±1.25 ppm), %	A	85%	100%	25% - 100%
	B	96%	100%	67% - 100%
	C	99%	100%	83% - 100%
Q_2/Q_1 ratio compliance rate (Q_2/Q_1 error ±20 %)	A	77%	96%	17% - 100%
	B	90%	100%	33% - 100%
	C	92%	100%	50% - 100%
MS^2 fingerprinting				
Experimental MS^2 fingerprint match (≥1 matched fragment, MoNA database)	A	87%	92%	33% - 100%
	B	96%	100%	75% - 100%
	C	100%	100%	100%
Predicted MS^2 fingerprint match (≥1 matched fragment, CFM-ID)	A	73%	92%	0% - 100%
	B	83%	100%	0% - 100%
	C	92%	100%	0% - 100%

Altogether, 73% of cases were successfully identified at a concentration close to CCβ while 89% and 94% success rate was observed for the two upper levels (Table 15). Only for 4 PhACs (azithromycin, spiramycin, sulfamethoxazole and valsartan) LOI could not be established due to insufficient success rate.

To verify the target screening method, one quality control sample (QC) was analyzed for each sample batch (10 samples per batch). The QC samples were obtained by fortifying WWTP influent and effluent aliquots at a

concentration that corresponded to the second validation level. Regrettably, the QC results were only partially consistent with the validation data and indicated that the method suffers from interferences more than previously thought. In particular, the maximum recovery during the validation study almost invariably stayed below 150%. Meanwhile, QC data displayed elevated recovery values in multiple occasions (14 from the total of 208 QC data points, Annex 11). This observation suggests that robustness was not properly assessed during the validation study, because all experiments (including MMSCC) were carried out on pooled WWTP influent and effluent matrixes, thus the inconsistency of matrix effects among diverse samples was not truly taken into account. The failure to accurately quantify some PhACs was thought to occur due to three major factors: unresolved interferences, poor performance of blank subtraction when high background levels of target PhACs was present in the selected QC sample matrixes and matrix induced enhancement/suppression of the ionization efficiency. Since QC samples were obtained from different WWTP influents and effluents, MMSCC calibration curves could not completely compensate the samples-specific matrix effects, causing potential errors in the quantification. Considering that it would be too laborious to prepare a calibration curve for each individual sample that fully compensates for these adverse effects, the developed method can only be considered semi-quantitative.

Table 15. **Successful identification rates of target PhACs using "strict" and "wide" identification thresholds**

Validation level	Q_1 detection rate (± 1.25 ppm)	Overall ID rate (strict)	Overall ID rate (wide)	≥75% ID rate (wide)	≥90% ID rate (wide)	100% ID rate (wide)	Compounds that failed to meet the specified identification criteria (success rate ≤90%, wide range)
Level A	97.1%	70.8%	73.4%	15/26	14/26	11/26	Azithromycin, caffeine, fluoxetine, gemfibrozil, ibuprofen, ketoprofen, meloxicam, naproxen, paracetamol, pravastatin, spiramycin, sulfamethoxazole and valsartan
Level B	98.4%	87.2%	88.5%	22/26	17/26	16/26	Azithromycin, gemfibrozil, meloxicam, paracetamol, pravastatin, spiramycin, sulfamethoxazole and valsartan
Level C	99.7%	91.7%	94.2%	25/26	22/26	18/26	Azithromycin, spiramycin, sulfamethoxazole and valsartan

Even highly selective HRMS applications, which involve chromatographic separation prior to MS detection, suffer from false positives on a regular basis. Therefore, careful attention must be paid to eliminate these risks, especially when the interpretation of results depends solely on MS data. First, procedure blanks (on deionised water) were used for blank subtraction to remove background peaks, originating from materials and reagents used in sample preparation. Next, ten different matrixes were analysed (e.g. fruits, vegetables, non-contaminated soil samples, and commodities of animal origin), which presumably should not contain detectable amounts of PhACs or their TPs. The samples were processed according to the workflow and suspect list entries that displayed a false positive match were flagged as non-compliant. A total of 39 suspects, including one target compound – gemfibrozil ($C_{15}H_{22}O_3$), were flagged by this approach and excluded from the sample data analysis. 74% of false positive hits belonged to suspects containing only C, H and O while additional 23% constituted of

C, H, O, and N. These findings are in accordance with the general observation that the absence of heteroatoms other than nitrogen and oxygen renders far more false positive hits in HRMS-based screening methods [228]. Furthermore, five WW samples that contained the highest number of suspect PhACs, were additionally investigated by HPLC-HRMS using the same sample preparation protocol (without the final dilution). The HPLC method was adapted from the previously developed HPLC-Orbitrap-MS method, while simultaneous acquisition of full-MS and fragment spectra was achieved by broadband collision-induced dissociation (bbCID) technique on the same FT-ICR-MS instrument in both polarities (transient size – 1M). Ion chromatograms were constructed using m/z values Q_1 ions and suspects that produced multiple chromatographic peaks were registered, indicating the presence of an interference. It must be noted that the HPLC analysis was not able to detect all expected suspect features. On average, 27% of the features that were found by direct infusion analysis remained undetected by the HPLC approach, indicating that accurate estimation of the actual rate of false positive hits may be problematic.

Finally, a possible source of interfering ion species was more closely examined for suspects, which were detected at least twice in the WW samples. Two public databases (Human Metabolome Database (HMDB) and Chemical Entities of Biological Interest (ChEBI)) were surveyed to extract information about molecular formula isomers, whereas isobaric species (mass accuracy range: ±2.5 ppm; elemental composition: C, H, N, and O; electron configuration: even; H/C ratio range: 0 to 3) were calculated using MolWeightToFormula (v. 3.1., Bruker) software. The obtained data were evaluated and compounds with a high likelihood of an interference, were dismissed from further analysis or classified as moderately susceptible to false-positive. The latter classification was introduced to ease the interpretation of results and highlight method limitations.

If the database search yielded a potential interference with the same molecular formula which could occur in WW samples due to anthropogenic or natural causes (e.g. simple peptides, human excretion products/metabolites, food and cosmetic ingredients, etc.), then moderate confidence level was assigned to the suspect. Meanwhile, compounds that yielded only one chromatographic peak in LC-MS analysis with MS/MS features that could not be matched against the reference fragmentation pattern, were discarded. If multiple peaks were detected and at least one of them was compatible in terms of MS/MS spectra then the suspect was not excluded. Moderate confidence level was also assigned to compounds which yielded ≥2 isobaric substances in MolWeightToFormula software within the set criteria. From 72 suspects that were detected at least twice in the samples, 24 were excluded due to concerns regarding high risk of false positive results, whereas 28 compounds were classified as moderately susceptible to false-positive (moderate confidence level).

A similar examination was carried out for all target analytes. Moderate confidence level was given to 15 target PhACs, while one compound was discarded from the targeted method. Specifically, a suspiciously large peak was found in the extracted ion chromatograms of gemfibrozil in 4 out of 5 samples. The corresponding MS/MS spectra had an uncharacteristic pattern, even though one fragment feature matched the database. Therefore, fortified QC sample was analysed by HPLC-FT-ICR-MS to obtain the actual retention time of this compound. A mismatch between retention times was observed, indicating that high detection frequency of gemfibrozil is possibly due to an isomeric interference. Moreover, the results of database query showed that gemfibrozil shares the same molecular formula of $C_{15}H_{22}O_3$ with octyl salicylate, a frequent ingredient of sunscreens and cosmetics. The measured MS/MS spectra was compared with several fragmentation patterns of octyl salicylate from MoNA database and significantly higher resemblance was found for salicylate than for the

target compound. These results are in accordance with conclusions derived from the analysis of non-wastewater samples, where several false positive matches were found for gemfibrozil.

Overall, a coherent relationship was observed that higher content of heteroatoms in the parent molecule, especially halogens, renders it less prone to interferences and at the same time results in a more distinguishable isotopic pattern. Thus, more reliable detection via DI-HRMS can be achieved.

3.3. Applicability of the developed methods

3.3.1. Application of HPLC-Orbitrap-MS for determination of PhACs in wastewater influents in WWTP "Daugavgriva"

The developed method was applied to the analysis of WWTP influent samples collected at the WW treatment plant "Daugavgriva" in April 2016. A total of 19 out of the selected 24 pharmaceuticals were detected in all of the samples. Traces of pravastatin were detected in 14 samples. No residues of fluoxetine, propranolol, gemfibrozil, and simvastatin were revealed (see Table 16).

Table 16. **The concentration of selected PhACs in wastewater samples from the WWTP "Daugavgriva"**

Analyte	Drug type	Concentration range, ng/L
Acetaminophen	Analgesic	1800-4200
Fluoxetine	Antidepressant	ND
Carbamazepine	Anti-epileptic	18-50
Xylazine	Veterinary sedative	2-180
Losartan	Anti-hypertensive	2-5
Valsartan	Anti-hypertensive	30-80
Caffeine	CNS stimulant	7000-12000
Ciprofloxacin	Fluoroquinolone antibiotic	250-400
Gemfibrozil	Lipid regulator	ND
Atorvastatin	Lipid regulator	3-10
Simvastatin	Lipid regulator	ND
Pravastatin	Lipid regulator	0.2-0.8
Azithromycin	Macrolide antibiotic	70-150
Erythromycin	Macrolide antibiotic	1-5
Clarithromycin	Macrolide antibiotic	1-21
Ketoprofen	NSAID	8-16
Naproxen	NSAID	9-20
Ibuprofen	NSAID	100-325
Diclofenac	NSAID	4-12
Sulfamethoxazole	Sulfanilamide antibiotic	50-120
Trimethoprim	Sulfanilamide antibiotic	15-43
Atenolol	β-blocker	50-150
Metoprolol	β-blocker	50-125
Propranolol	β-blocker	ND

The concentration range for most of the PhACs varied between 10 and 200 ng/L, while the concentrations for caffeine and acetaminophen were in low μg/L level, falling within a range of 7 – 12 μg/L and 1.8 – 4.2 μg/L, respectively. Due to their high abundance, caffeine and acetaminophen are recognised as chemical markers for water pollution by domestic wastewaters. The concentration of caffeine was almost constant for each day and slightly increased in samples collected in the evening. Taking into account the time that is necessary for WW to reach the WWTP, this trend could be attributed to higher consumption of caffeinated products in the morning. Acetaminophen levels in the samples typically were the highest at mid-day. Other studies have shown similar levels of caffeine and acetaminophen ranging from 5 to 192 μg/L and 1 to 52 μg/L, respectively [59,229–231].

Ciprofloxacin, which is the most consumed substance among fluoroquinolone class antibiotics in Latvia, was found at high levels within a range of 250 to 400 ng/L. High levels of ciprofloxacin have been previously reported in WW samples from a few ng/L in Asia up to >1 μg/L levels in UK [232].

Xylazine, a veterinary sedative used mostly for horses, was found at surprisingly high concentrations (50 – 150 ng/L) in 6 out of 7 days of sampling with an exception of the first day when the average concentration was 2.9 ng/L for three samples. Are there many horse pastures in Riga? No. In this case, the most likely reason for high levels of xylazine in WW is that a globally significant manufacturer of this compound is JSC Grindeks, located in Riga. Even though JSC Grindeks operates a state-of-the-art biological WWTP, xylazine is a small and relatively stable molecule that may partially escape bacterial degradation and enter the main sewage system. Previously it has been found in surface water samples at around 10 ng/L in Spain [233].

For NSAID class substances were determined during this study: ketoprofen, naproxen, ibuprofen and diclofenac. The highest concentrations were detected for ibuprofen at 326 ng/L, which is considerably lower compared to literature data where the levels of the parent compound (excluding its metabolites ibu-OH and ibu-CX) can be found in at levels equal to several μg/L [234]. Meanwhile, the concentration of other NSAIDs, including diclofenac, varied over a range of 4–20 ng/L, which is extremely low considering that these PhACs have high consumption rates, especially diclofenac [39].

A significant class of medicinal compounds is lipid regulators. Atorvastatin, a statin medication belonging to the class of lipid regulators, has a DDD value of 49, which makes it one of the most prescribed drugs in Latvia according to the statistics on the consumption of PhACs [39]. It was found in samples at the low concentration range of 3–10 ng/L. This can be attributed to rapid metabolism since it is efficiently metabolized to hydroxylated derivatives or beta-oxidation products and less than 2% of the active compound is excreted in urine [235].

As for the class of beta-blockers, atenolol and metoprolol were found at the range of 50–150 ng/L. The former is one of the most prescribed and used pharmaceuticals in Latvia with DDD index of 11. Atenolol and metoprolol have been reported to show inconsistently low removal rates by conventional WWTP and are usually removed via sorption processes [92]. Previous reports show that concentrations of β-blockers can vary from a few ng/L in WW samples from Spain up to levels that exceed several μg/L (e.g. 11 μg/L for atenolol in Korea) [230]. In general, the concentrations observed in samples are lower than anticipated, especially for NSAIDs. No indications of inaccurate quantification were found during the analysis of QC samples. Apart from quantification, these results can be attributed to several other causes: (i) grab sampling can miss high emission fluxes of PhACs, (ii) the consumption of PhACs is seasonal, thus the abundance of some PhACs is lower during the off-season and

(iii) the sampling process was not supervised by laboratory personnel, thus an unidentified error during the sampling or transportation could affect the final quality of results [59,61,236].

3.3.2. Application of HPLC-Orbitrap-MS for estimating removal of PhACs from municipal wastewaters using activated sludge and biostimulation

Biodegradation experiments were carried out using a pooled WWTP influent sample. Analysis of PhACs by previously described HPLC-Orbitrap-MS revealed the presence of 21 compounds with concentrations ranging from 13.2 ng/L to 52 µg/L (Figure 22). The highest concentration was observed for caffeine, which exceeded those of other pharmaceuticals by at least one order of magnitude. Other PhACs, that had a concentration above 1 µg/L, were: acetaminophen, naproxen, ibuprofen, xylazine, diclofenac, ciprofloxacin and valsartan (Figure 22). The majority of these compounds belong to the group of NSAIDs, except for the veterinary alpha-adrenergic agonist xylazine, the antibiotic ciprofloxacin and the angiotensin-receptor blocker valsartan. The concentrations in the pooled sample were significantly higher than detected in the previous section. However, it must be acknowledged that the sampling of the WW was performed at a different time period from WWTP "Daugavgriva". Besides, this time sampling and transportation were performed by laboratory personnel to ensure that samples are taken from the main receiver.

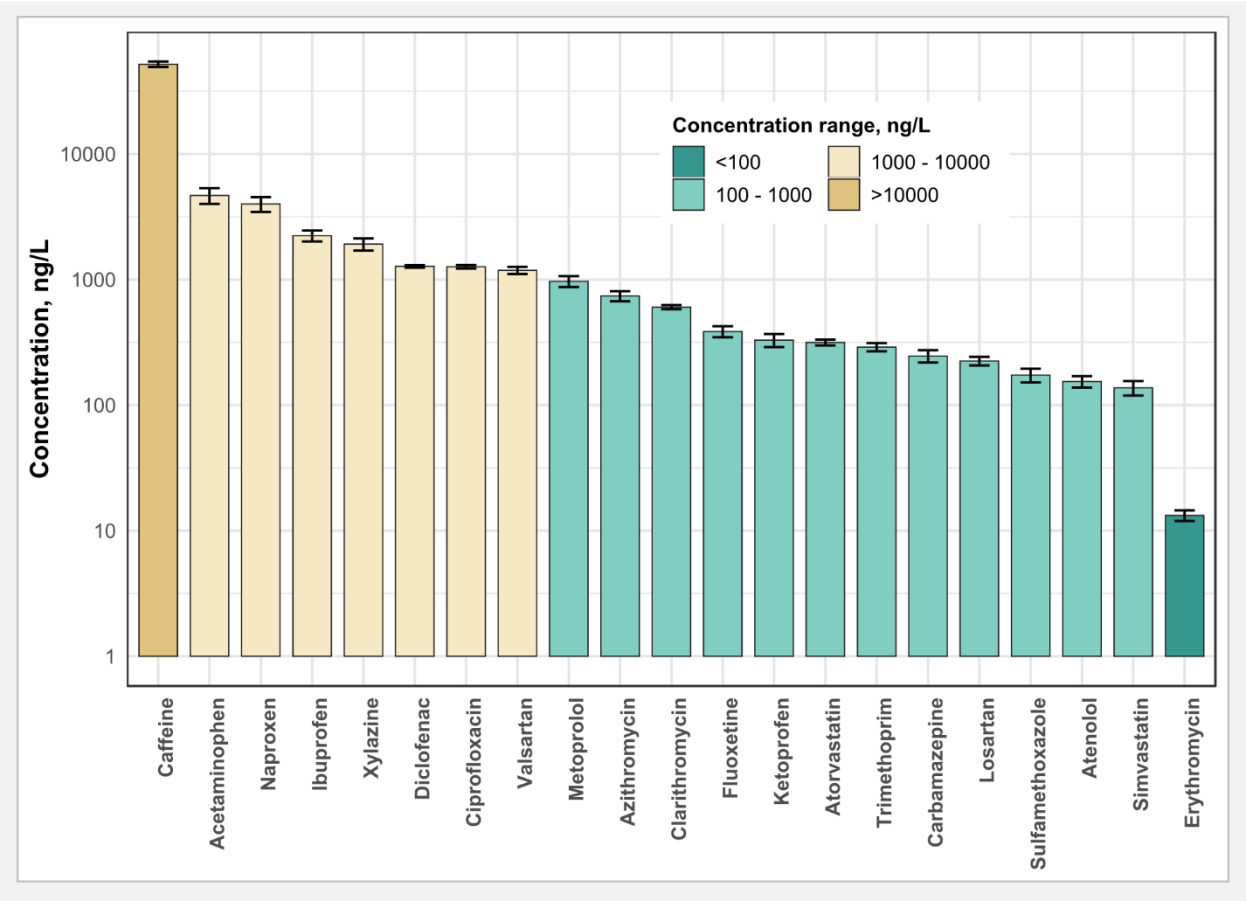


Figure 22. **PhAC concentrations in pooled wastewater sample**

In order to examine the biodegradation of PhACs, several treatment types were explored: no treatment (a nonaugmented control sample incubated for the same duration), treatment with activated sludge, treatment with sludge-derived culturable bacteria and treatment with sludge-derived culturable fungi. As expected, biodegradation was found to be compound-specific. Results from the control samples that were incubated for 17

hours showed that under nonaugmented conditions six compounds had poor removal efficiencies (below 20%). As seen from Figure 23, this effect was especially pronounced for diclofenac, ibuprofen and sulfamethoxazole which remained practically unchanged. Meanwhile, caffeine was removed effectively despite having extremely high initial concentration. Its removal efficiency ranged from 70% to 80% regardless of the treatment. Nevertheless, the obtained removal efficiency is slightly lower than anticipated, because caffeine is often characterized as an easily degradable compound which can be readily removed with 90% efficiency during conventional WWTP processes [237]. However, it must be noted that these experiments were performed under controlled conditions that do not completely simulate non-biological degradation processes (e.g. photolytic and oxidative degradation) that occur simultaneously in full-scale WWTPs.

Bioaugmentation with activated sludge stimulated the biodegradation process for 14 compounds. The activated sludge was especially efficient for trimethoprim and acetaminophen. Conversely, the addition of activated sludge to WW did not stimulate the removal of ciprofloxacin and sulfamethoxazole, while the addition of sludge-derived bacteria or fungi reduced the remaining concentration compared to control samples during the first 17 h from 65% to 30% and from 98% to 40%, respectively (Figure 23).

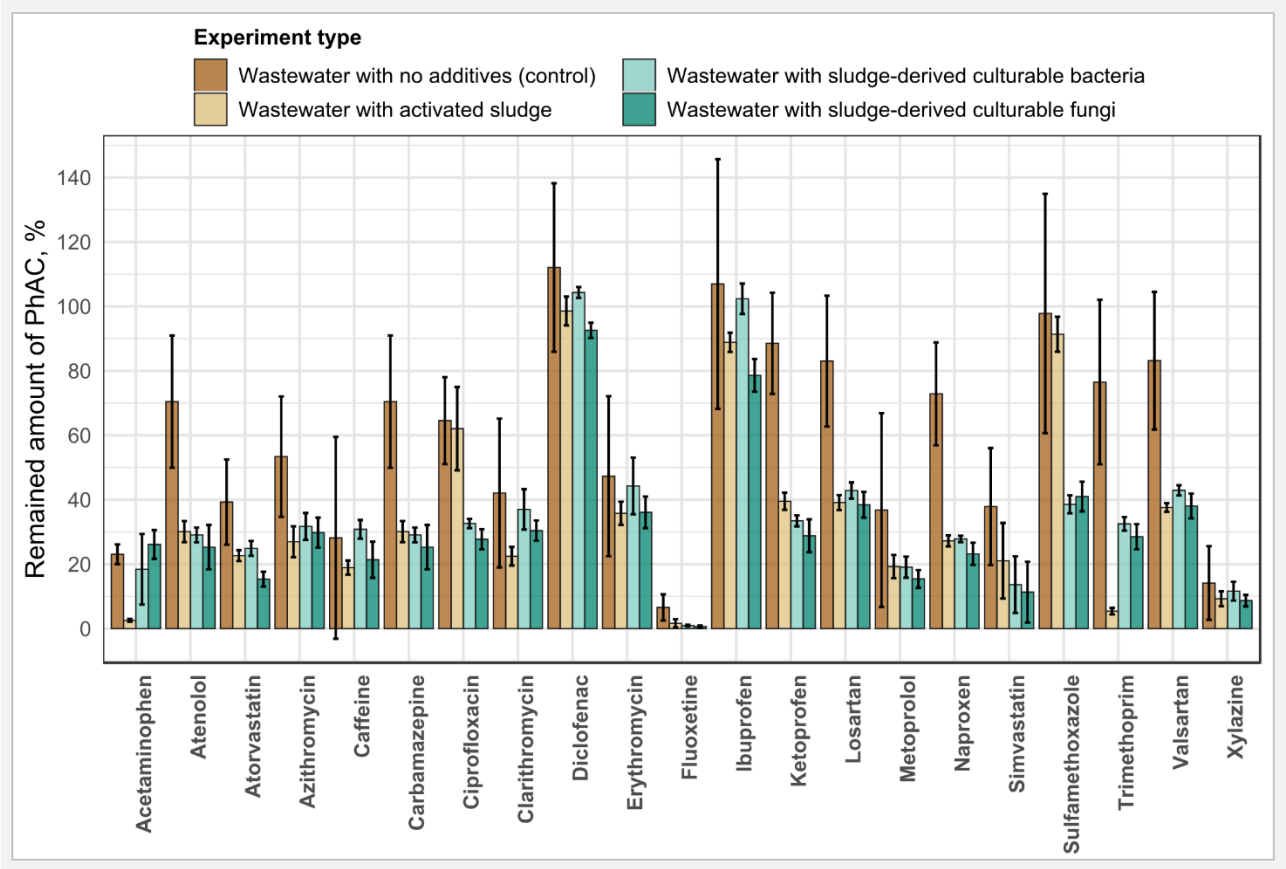


Figure 23. **The concentration of PhACs remaining in wastewater after incubation for 17 hours**

Carbamazepine, which has been recognized as problematic with regard to its elusive behaviour during WW treatment, showed somewhat satisfactory removal rates. After 17 hours carbamazepine concentration decreased by 30% in nonaugmented control sample, whereas around 70% removal was observed for all bioaugmented experiments. However, it is not clear if this result can be directly attributed to biotransformation processes since carbamazepine is relatively hydrophobic ($2 < \text{Log } D_{ow} < 3$) and considered to be a poorly biodegradable

compound (< 40%) [238]. Thus, the concentration of carbamazepine in samples could decrease due to its adsorption on suspended solids, followed by further removal from water by sedimentation [92].

Aside from these experiments, parallel biodegradation tests were carried out with extra nutrients to explore whether or not they have a stimulating effect on the removal of PhACs from WW. The nutrient solution comprised 30% sugar beet molasses containing 40% (w/w) sucrose. It was added to WW prior incubation. The final concentration of the added nutrient solution in WW was 0.1% (v/v). The addition of nutrients to WW changed the dynamics of the removal of PhACs. The most pronounced positive effect on the removal of nutrients was found for erythromycin. The degradation of sulfamethoxazole was also accelerated by the added nutrients, but this effect was detected only at the beginning of incubation, i.e. after 17 h. Most of the other PhACs detected in WW also were more rapidly removed in the presence of added nutrients. The residual concentration of PhACs in the non-stimulated WW was from 1.1 to 3.0 times higher than the concentration in the nutrient-stimulated WW. Another problem that emerged from the data was that some PCs were affected by the addition of nutrients in the opposite way. In particular, the removal of clarithromycin and atorvastatin was hindered in the presence of nutrients at the beginning of incubation, while for atenolol, xylazine, and carbamazepine this effect was observed after 48 h and 168 h.

Particular attention was paid to the biodegradation dynamics of PhACs having antimicrobial properties. Six compounds of those represented in the tested WW were selected for this analysis, i.e., azithromycin, ciprofloxacin, clarithromycin, erythromycin, sulfamethoxazole and trimethoprim. A gradual decrease of the concentration of all mentioned PhACs with exception to sulfamethoxazole was already observed during the first 17 h. As shown in Figure 23, sulfamethoxazole and trimethoprim after incubation for 17 h were comparatively more resistant to degradation, especially in the control samples as compared to the other four antimicrobials. Further incubation resulted in a gradual degradation of the remaining six antimicrobials, although a removal activity was dependent on the treatment type. For example, the remaining concentrations of trimethoprim after 17 h incubation without bioaugmentation and with activated sludge were 76.5 ± 25.5% and 5.4 ± 2.9%, respectively. The observed results are in accordance with the literature. For instance, a study by Petrović et al. (2005) reported that the mean degradation of sulfamethoxazole and trimethoprim in conventional WWTP was 35% and 75%, respectively [239].

Regardless of the treatment type, the removal of erythromycin was highly influenced by the presence of nutrients. Unlike most compounds, erythromycin showed rapid degradation during the first 17 hours, but its concentration stayed almost unchanged in experiments that were incubated for longer periods (Figure 24).

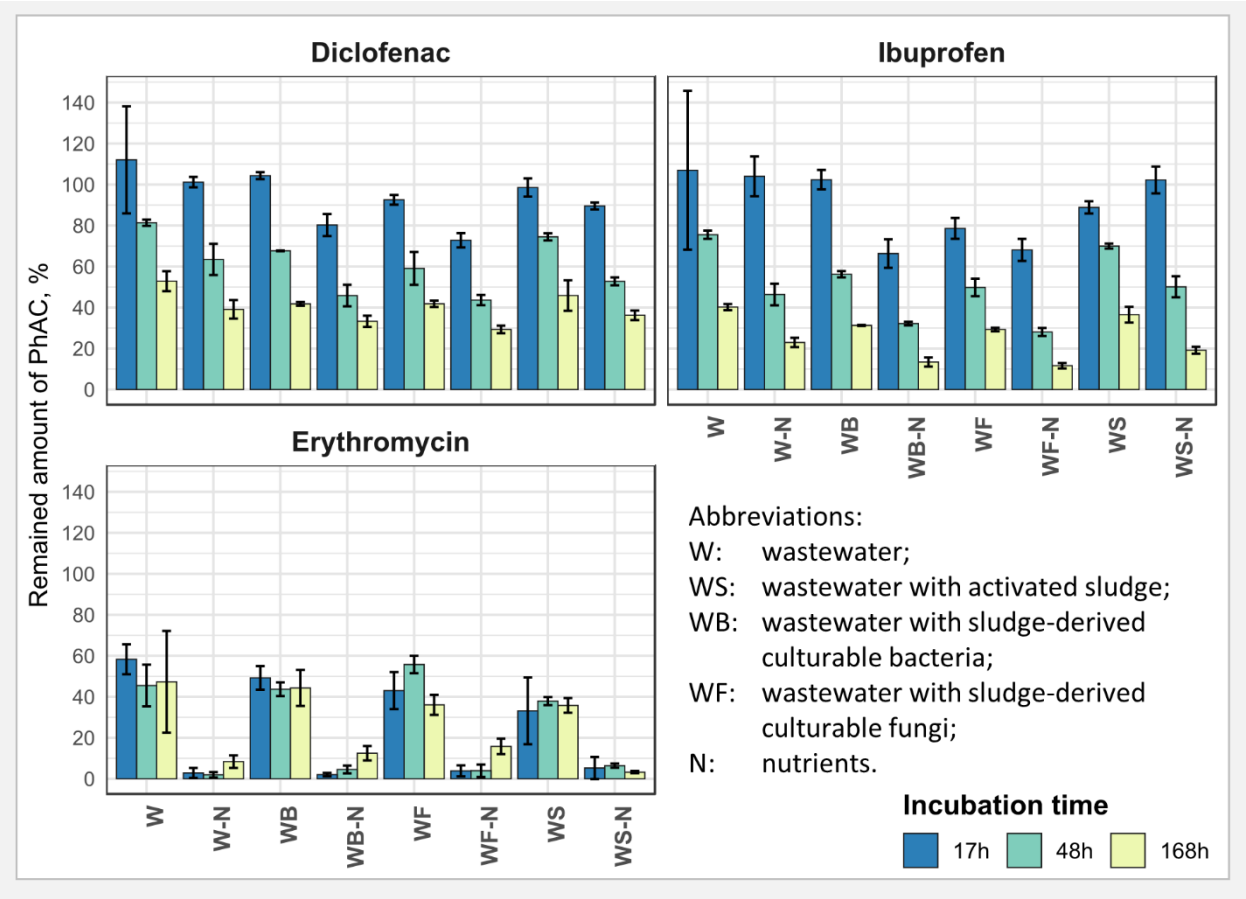


Figure 24. **The biodegradation of diclofenac, ibuprofen and erythromycin**

Ciprofloxacin was detected in WW samples at the concentration of 1265 ng/L, which was the highest level among all six antimicrobials. Previous studies have shown that this antibiotic has a high prevalence in sludge. For example, in a study about the removal of antibiotics from WWs in China, 19 antibiotics were detected in the untreated and treated WWs, with clarithromycin (6524 ng/L) and ofloxacin (5411 ng/L) being the most abundant [240].

The results of bioaugmentation show that biodegradation of ciprofloxacin was stimulated by nutrients, as well as sludge-derived bacteria and fungi. Surprisingly, the addition of intact activated sludge did not influence the removal of ciprofloxacin and the remaining concentration after 168 h incubation was the highest among the tested variants, i.e., 36.3% of the original concentration. Additional experiments using agar diffusion test revealed the abundance of ciprofloxacin-resistant bacteria in the activated sludge that was used for bioaugmented treatment. However, it is not clear whether this fact is attributable to the biodegradation dynamics or not. Besides, sorption of ciprofloxacin on sludge particles is considered to be the principal removal pathway, and its desorption from sludge might cause a variation of its concentration during the WWTP process [60,87,91].

As mentioned before, the comparison of different PhACs in WW by their biodegradability under the test conditions provided some unexpected results that must be discussed in greater detail. The worst removal rates were observed for diclofenac and ibuprofen. For instance, during the first 17 h of incubation, no degradation of these two compounds was observed in the control test and only a slight decrease (up to 20–30%) was detected in the bioaugmented WW samples (Figure 24). Nevertheless, further incubation resulted in gradual removal of these PhACs. After incubation for 168 h, the lowest remaining concentrations of ibuprofen among the tested types of

treatment were found to be in WW with activated sludge and nutrients (19%), WW with sludge-derived culturable bacteria and nutrients (13%), and WW with sludge-derived culturable fungi and nutrients (12%). As expected, the removal of diclofenac was slower, as compared to ibuprofen, and varied in the range from 29% to 53%, irrespectively of the type of treatment (Figure 24). The stimulating effect of nutrients on biodegradation was more pronounced for ibuprofen. Particularly, the ratio of the remaining ibuprofen in the non-stimulated vs. the nutrient-stimulated types of treatment gradually increased from [0.9 ÷ 1.5] to [1.4 ÷ 1.8] and [1.8 ÷ 2.5] after 17 h to 48 h and 168 h, respectively. Such a tendency was also observed for diclofenac, but to a lesser extent. Even though both compounds are aryl derivatives of propionic acid and classified as NSAIDs, they also have some dissimilarities. Namely, diclofenac has lower bioavailability, as compared to ibuprofen, because of the presence of chlorine substituents and two aromatic rings [90]. Hence, diclofenac exhibits lower susceptibility to biodegradation. Numerous studies have reported that diclofenac and ibuprofen cannot be completely removed during conventional treatment processes. According to the review article by Zhang et al. (2014), the removal efficiency of ibuprofen and diclofenac from the studies with aquatic plant-based systems was indicated as high (60%–80%) and moderate (40%–60%), respectively [238].

3.3.3. Application of HPLC-Orbitrap-MS for estimating removal of PhACs from municipal wastewaters using ionising radiation

The HPLC-Orbitrap-MS method was also used to study ionizing radiation induced degradation of PhACs in WW. Two different modes of electron beam treatment (denoted as EB_1 and EB_2) and two γ-irradiation modes (abbreviated as G_1 and G_2) were applied to better explore the impact of radiation on the WW treatment. The dose rate of electron flux applied for EB_1 and EB_2 treatment conditions differed by two times, whereas for G_1 and G_2, the difference was 1.7 times. Dependence of the absorbed dose on the irradiation source (accelerated electrons or gamma rays) was found in most cases. The dose rate increased in the order of $G_1 < G_2 < EB_1 < EB_2$, and the irradiation time was changed in the opposite direction. For example, to reach the absorbed dose of 5 kGy by the treatment of G_1, G_2, EB_1 and EB_2, the irradiation lasted 805, 480, 30, and 15 s, respectively. Thus, the irradiation by electron beam allowed to reduce the time of irradiation at maximum by more than 50 times compared to gamma radiation. However, the irradiation time and the absorbed dose showed a proportional effect on the degradation of most PhACs, when comparing the degradation induced by gamma radiation or accelerated electrons.

The monitoring of the PhAC concentrations after treatment with different radiation doses indicated that the degradation for most of the pharmaceuticals started rapidly at low irradiation doses of 0.5–3 kGy and reached the maximum extent of 84–100% after up to 5–7 kGy of radiation was absorbed by the solution (maximum extents of PhAC decomposition rates are compared in Table 17).

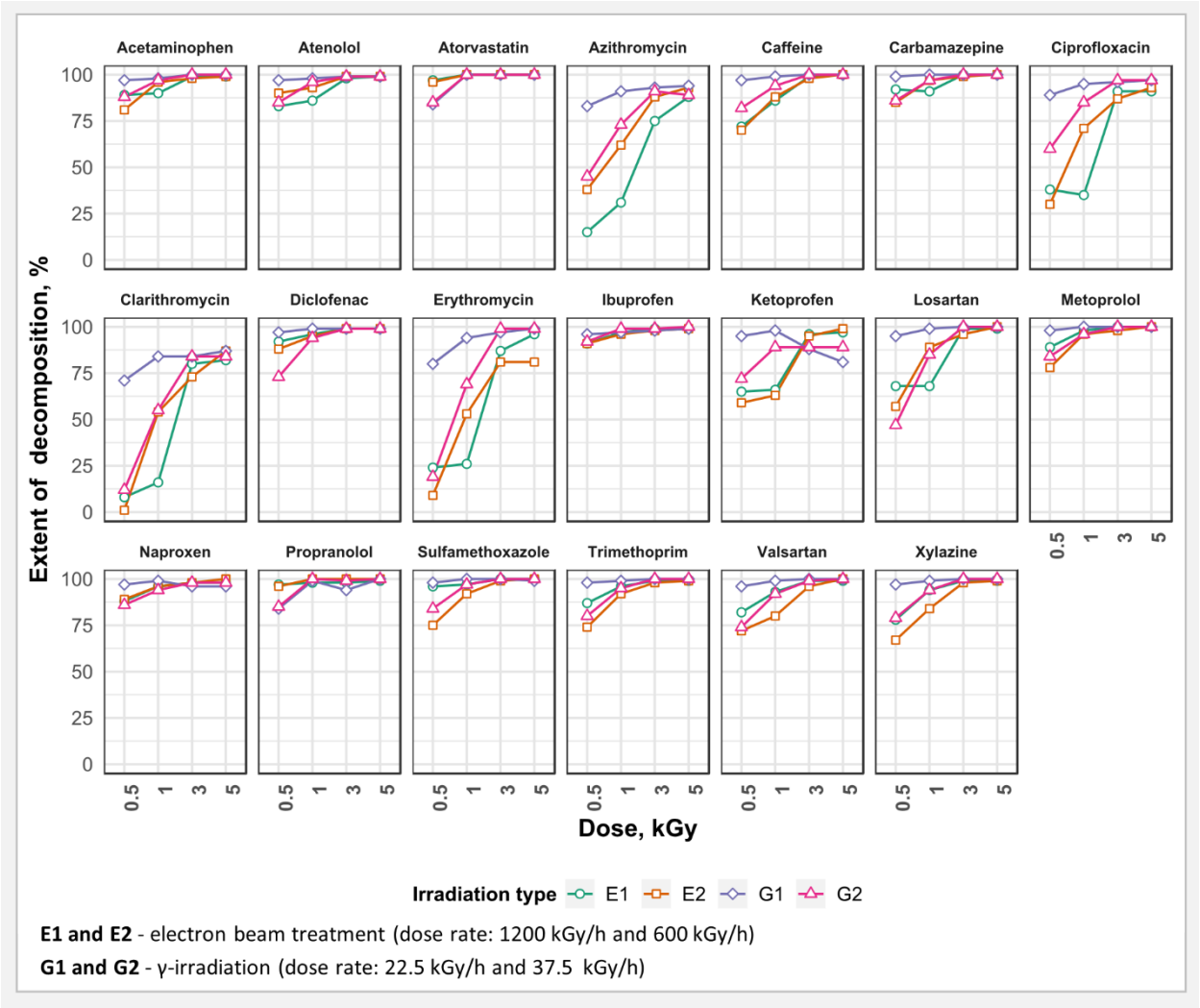


Figure 25. **Decomposition of PhACs under different electron beam and gamma radiation doses**

As seen in Figure 25, lower decomposition rates were observed for losartan, valsartan, ketoprofen, three macrolides and one fluoroquinolone - ciprofloxacin. The results for ciprofloxacin after the EB treatment at up to 5 kGy indicated the maximum decomposition yields of 91–93%, where the extent of decomposition increased up to 98% after gamma irradiation at the same absorbed dose. The decomposition efficiency of ciprofloxacin obtained in these experiments was lower than reported by Sayed et al. (2016), who achieved almost complete degradation of 4.6 mg/L ciprofloxacin solution at an absorbed dose of 0.87 kGy. However, that study was performed with ultrapure water, not WW [241].

The results revealed that three analysed macrolides remained at comparably high levels after the irradiation with up to 5 kGy absorbed doses, having the lowest degradation rates at the lowest applied irradiation dose of 0.5 kGy (see Figure 25). It may be predicted that the overall contamination of the analysed WW samples caused the low degradation yields and required an increase in the absorbed dose above 5 kGy to achieve effective decomposition of selected PhACs.

Among the four applied irradiation conditions, the gamma radiation treatment at dose rate of 22.5 kGy/h (G_1) provided the most prominent PhAC decomposition at the lowest applied irradiation dose (0.5 kGy), compared to the other applied irradiation conditions. The PhAC concentration levels after the proposed gamma irradiation at 0.5 kGy dose indicated >95% decomposition for twelve compounds (63% of the total PhAC contamination),

95% decomposition of three compounds (losartan, ibuprofen, and ketoprofen), <90% decomposition was determined in the case of the four macrolide antibiotics (71, 80, 83, and 89% for clarithromycin, erythromycin, azithromycin, and ciprofloxacin, respectively). The results indicated that the degradation extent of macrolides increased with the irradiation time. That was also the case with the degradation of NSAIDs and other PhACs, especially when comparing both EB treatment conditions. In the case of macrolides, this finding may be attributed to a complex decomposition mechanism involving attack by hydroxyl radicals at the glycosidic bonds of carbohydrate moieties, followed by lactone ring opening, as noted by Liu et al. (2014) [242].

Despite having high initial concentrations, caffeine and acetaminophen showed rapid degradation regardless of electron beam treatment type. These data are in accordance with earlier studies. For instance, Torun et al. (2014) reported degradation studies of highly concentrated (50 mg/L) aqueous solutions of caffeine, where caffeine was completely degraded by irradiation with ^{60}Co gamma radiation at up to 3 kGy doses, at the dose rate of 0.0156 Gy/s [243]. Whereas in 2015, Torun et al. (2015) reported similar observations of acetaminophen degradation by irradiation of aqueous solutions at up to 5 kGy absorbed doses of ^{60}Co gamma radiation [244]. In this study, caffeine was successfully degraded at similar irradiation doses. The concentration of caffeine decreased by 99.8 to 100% at EB dose of 5 kGy. Meanwhile, the degradation was complete already at 3 kGy irradiation under both gamma irradiation conditions. The same results were observed for acetaminophen, for which the degradation efficiencies during EB treatment at 5 kGy were already close to 99%, while almost complete degradation (99.9%) was achieved from gamma irradiation.

Table 17. **Maximum extent of PhAC decomposition (%) and the corresponding EB and gamma radiation doses**

Compound	EB radiation	Gamma radiation
Acetaminophen	99%; 3–5 kGy	100%; 5 kGy
Atenolol	99%; 3–5 kGy	99%; 3 kGy
Atorvastatin	100%; 1–5 kGy	100%; 1 kGy
Azithromycin	90–94; 5–15 kGy	91–94%; 3–5 kGy
Caffeine	99–100%; 3–5 kGy	100; 3 kGy
Carbamazepine	100%; 5 kGy	100%; 3 kGy
Ciprofloxacin	94%; 5–7 kGy	97%; 3–5 kGy
Clarithromycin	84–87%; 5–12 kGy	84–87; 3–5 kGy
Diclofenac	99%; 5 kGy	99%; 3 kGy
Erythromycin	81–97%; 3–5 kGy	99%; 3–7 kGy
Ibuprofen	99%; 5 kGy	99%; 5 kGy
Ketoprofen	99%; 5–7 kGy	89–98%; 1 kGy
Losartan	99–100%; 5 kGy	100%; 3 kGy
Metoprolol	100%; 5 kGy	100%; 3 kGy
Naproxen	98–99%; 5 kGy	100%; 3–5 kGy
Sulphamethoxazole	100%; 3–5 kGy	100%; 1–3 kGy
Trimethoprim	99%; 3–5 kGy	100%; 3–5 kGy
Valsartan	99–100%; 3–5 kGy	100%; 3–5 kGy
Xylazine	99%; 5 kGy	100%; 3 kGy

In order to determine the quantitative yields of PP degradation, the *G* values of decomposition were evaluated as functions of the irradiation conditions for two EB and two gamma ray treatments using the following equation:

$$G = \Delta C \times N_A / K \times D \qquad (6)$$

, where ΔC is the concentration of decomposed PhAC (mol/L), NA is the Avogadro's number 6.02 × 1023 (molecules mol−1), K (6.24 × 1019) is the conversion factor from kGy to 100 eV/L, D is the absorbed dose (kGy) and G is the decomposition yield of PhACs (μmol/J), considering that one molecule formed or decomposed per 100 eV of absorbed energy equals to 0.10364 μmol/J. The molar mass of each PhAC was used to calculate the molar concentrations in the samples.

The determined G values ranged from 10^{-2} to 10^{-10} μmol/J and showed a decrease with the absorbed dose due to effective degradation of most PhACs at 0.5–3 kGy doses of both by EB and gamma irradiation. As mentioned before, rather low decomposition yields for macrolides at 0.5–5 kGy absorbed doses were found.

3.3.4. Application of HPLC-Q-TOF-MS for determination of aminoglycosides

The developed method was applied for the determination of AGs in honey samples. All samples were analyzed in accordance with the previously described procedure. Quantification was performed using the MMSCC approach. MMSCC were prepared on the same day as the unknown samples using the same conditions. A total of 49 samples from various regions of Georgia and having different compositions were analyzed during the study. Residues of STP were detected in two samples (117 ng/g and 35 ng/g) and one sample contained GEN at the level of 32 ng/g. These results indicate that the presence of antimicrobial residues in honey remains an issue of food safety and environmental management. The same method was adapted for the analysis of WW samples. No traces of investigated AGs were found in these samples. This negative result can be attributed to the fact that only a minor fraction of administered AG dose is excreted in the parent form. Besides, the annual human consumption of AGs in Latvia is almost negligible (DDD per 1000 inhabitants per day < 0.1), suggesting that AG residues are unlikely to be detected in domestic WW samples.

3.3.5. Application of LC-MS/MS method for determination of NSAIDs in water samples

The elaborated dSPE protocol along with the developed LC-MS/MS method was applied for the analysis of 24 surface and tap water samples collected in Latvia and Norway. Only two NSAIDs (diclofenac and ibuprofen) were detected above LOQ limits in 14 of the analyzed samples. As seen in Table 17, diclofenac was found in two surface water samples – one from Norway (1.7 ng/L) and one from Latvia (8.4 ng/L). Meanwhile, the detection frequency of ibuprofen was much higher, especially in samples from Norway. Three surface water samples from the river Daugava contained traces of this NSAID (from 3.9 to 17 ng/L). All three samples were acquired from areas with high population density. Therefore, this finding supports the assumption that the concentration of human PhACs in surface water samples is directly linked to population density around the watercourse. For instance, Marsik et al. (2017) studied the occurrence of NSAIDs in the watercourses of Elbe basin in Czech Republic, showing that the higher levels of ibuprofen were detected near urban riverbank [245]. Nonetheless, the levels of both detected NSAIDs were lower than those reported for other European surface aquifers [1].

Another important finding was that 7 out of 9 tap water samples from Norway contained ibuprofen residues (from 1.2 to 9.2 ng/L). Those data are in accordance with the recent report by Cai et al. (2015), who determined ibuprofen concentrations in the range from < LOD to 17 ng/L at different stages of drinking water treatment plant [246]. While the detected concentration levels are well below therapeutic doses and predicted no-effect concentrations reported in the literature, the presence of NSAIDs (or any other PhAC) in drinking water is a concerning issue since effects of long-term exposure are largely unknown [247].

Table 17. **The concentrations of diclofenac and ibuprofen (ng/L) in the surface waters and tap water**

Country	Sampling region	Sample type	Diclofenac, ng/L	Ibuprofen, ng/L
Latvia	Ikskile	Surface water	*n.d.*	*n.d.*
Latvia	Saulkalne	Surface water	*n.d.*	*n.d.*
Latvia	Salaspils	Surface water	*n.d.*	**11**
Latvia	Darzini	Surface water	**8.4**	*n.d.*
Latvia	Kengarags	Surface water	*n.d.*	**3.9**
Latvia	Lucavsala	Surface water	*n.d.*	*n.d.*
Latvia	Kipsala	Surface water	*n.d.*	**17**
Latvia	Voleri-1	Surface water	*n.d.*	*n.d.*
Latvia	Voleri-2	Surface water	*n.d.*	*n.d.*
Latvia	Daugavgriva	Surface water	*n.d.*	*n.d.*
Norway	Svartkulp to Sognsvann	Surface water	*n.d.*	**1.6**
Norway	Sognsvannsbekken	Surface water	*n.d.*	*n.d.*
Norway	Akerselva Frysja	Surface water	*n.d.*	**1.0**
Norway	Akerselva Kuba	Surface water	*n.d.*	*n.d.*
Norway	Østensjø	Surface water	**1.7**	**5.1**
Norway	Tomter—Østold	Tap water	*n.d.*	*n.d.*
Norway	Vehus	Tap water	*n.d.*	*n.d.*
Norway	Ski	Tap water	*n.d.*	**1.7**
Norway	Bærum	Tap water	*n.d.*	**2.6**
Norway	Geilo	Tap water	*n.d.*	**1.6**
Norway	Oslo	Tap water	*n.d.*	**2.1**
Norway	Hemsedal	Tap water	*n.d.*	**9.2**
Norway	Oslo	Tap water	*n.d.*	**1.2**
Norway	Beito	Tap water	*n.d.*	**4.9**

3.3.6. Targeted screening of PhACs via DI-FT-ICR-MS in WWTP effluents and influents

The developed FT-ICR-MS method was applied for the analysis of 72 WW samples from 36 WWTPs in Latvia (see Annex 12). Out of 25 target PhACs, 20 and 24 different compounds were detected at least once in WWTP effluents and influents, respectively. Most frequently detected compounds in untreated WWTP influents were diclofenac (NSAID, 86%), metoprolol (beta-blocker, 78%), clarithromycin (macrolide antibiotic, 53%) and ibuprofen (NSAID, 47%). A similar pattern was observed for WWTP effluents. However, compounds that exhibit higher removal efficiency during municipal WW treatment processes were detected less frequently in the effluent samples [248]. For instance, considerably high levels (446 – 2183 ng/L) of paracetamol were detected in six influent samples, whereas this compound remained undetected in effluents. Similarly, ibuprofen, salbutamol and losartan showed lower detection frequencies in effluents. Meanwhile, some PhACs (e.g. diclofenac, erythromycin, clarithromycin, trimethoprim and carbamazepine) did not follow this trend. Erythromycin was the only compound that was detected more frequently in treated WW samples. This observation is not particularly surprising, because poor removal of erythromycin has been repeatedly reported in the literature [87]. Another key observation was the high prevalence of diclofenac in all sample types. This finding must be interpreted with caution as it may be associated with the detection method, because diclofenac is a particularly suitable analyte for HRMS-based applications as it bears two chlorines. Thus, a definite and abundant isotopic pattern can be obtained which enables higher sensitivity towards low abundance isotopologue signals. Nevertheless, frequent occurrence of diclofenac is not unusual, because it exhibits highly variable removal efficiencies in conventional WW treatment processes

and is considered as a contaminant of emerging concern [85]. A different explanation could be related to the sample origin. Almost all WWTPs in this study receive sewage from rural villages and small towns. The average age in these locations is relatively high with about 38 % of the population being over 55 years old [249]. Taking into account that diclofenac is administered not only for pain and inflammatory symptoms but also used for the treatment of arthrosis, which is a common condition among in older populations, higher detection rates of this NSAID can be anticipated [250]. This explanation also supports the high prevalence of beta-blockers in WW samples.

Table 18. **Occurrence and detection frequencies of target PhACs in WWTP influent and effluent samples**

Name	**WWTP Effluents**				**WWTP Influents**			
	Detection frequency, %	**Mean, ng/L**	**Median, ng/L**	**Range (min - max), ng/L**	**Detection frequency, %**	**Mean, ng/L**	**Median, ng/L**	**Range (min - max), ng/L**
Atenolol	36%	224	154	23 - 812	47%	250	59	16 - 1479
Atorvastatin	0%	*n.d.*	*n.d.*	*n.d.*	19%	146	159	39 - 284
Azithromycin	17%	57	50	26 - 108	25%	117	127	24 - 231
Caffeine	11%	355	368	165 - 520	28%	6835	2034	152 - 43922
Carbamazepine	22%	694	193	49 - 2171	28%	1599	262	111 - 8305
Ciprofloxacin	3%	108	108	108 - 108	3%	424	424	424 - 424
Clarithromycin	44%	379	269	64 - 1353	53%	453	152	17 - 2833
Diclofenac	78%	217	198	23 - 570	86%	570	253	27 - 3163
Erythromycin	19%	85	97	31 - 124	14%	98	68	51 - 196
Fluoxetine	6%	14	14	12 - 16	22%	12	12	9 - 16
Ibuprofen	17%	371	244	152 - 1066	47%	3317	658	108 - 28478
Ketoprofen	8%	1587	521	511 - 3728	22%	1731	733	377 - 9089
Losartan	6%	56	56	34 - 79	22%	242	89	46 - 1169
Meloxicam	3%	153	153	153 - 153	25%	107	24	13 - 658
Metoprolol	61%	168	96	5 - 735	78%	470	231	10 - 5523
Naproxen	14%	611	570	306 - 961	19%	1980	775	350 - 4283
Paracetamol	0%	*n.d.*	*n.d.*	*n.d.*	17%	1194	1276	446 - 2183
Pravastatin	19%	188	176	119 - 274	33%	335	360	41 - 535
Propranolol	31%	10	8	5 - 23	44%	13	8	4 - 46
Salbutamol	3%	31	31	31 - 31	22%	39	35	17 - 84
Spiramycin	0%	*n.d.*	*n.d.*	*n.d.*	0%	*n.d.*	*n.d.*	*n.d.*
Sulfamethoxazole	19%	214	119	60 - 676	25%	1499	321	88 - 11192
Trimethoprim	25%	52	49	12 - 95	28%	248	59	21 - 1650
Valsartan	0%	*n.d.*	*n.d.*	*n.d.*	22%	299	233	133 - 660
Xylazine	0%	*n.d.*	*n.d.*	*n.d.*	11%	13	13	7 - 20

As shown in Table 18, the concentration range of detected PhACs varied significantly. Some of this variability can be attributed to the instrumental capability, because sensitivity towards some analytes was insufficient (e.g. caffeine, valsartan, paracetamol and ciprofloxacin). Hence, lower detection rates along with elevated mean concentrations were obtained. For example, caffeine is nearly ubiquitously present in domestic WWs due to the high consumption of caffeinated beverages such as coffee, tea and soluble dietary supplements. It can even be used as an anthropogenic marker for environmental pollution [251]. However, caffeine was detected

at quantitative levels only in 28% and 11% of the WWTP influent and effluent samples, respectively. In general, these results do not show many atypical features and are in accordance with those reported in other European countries [252–255].

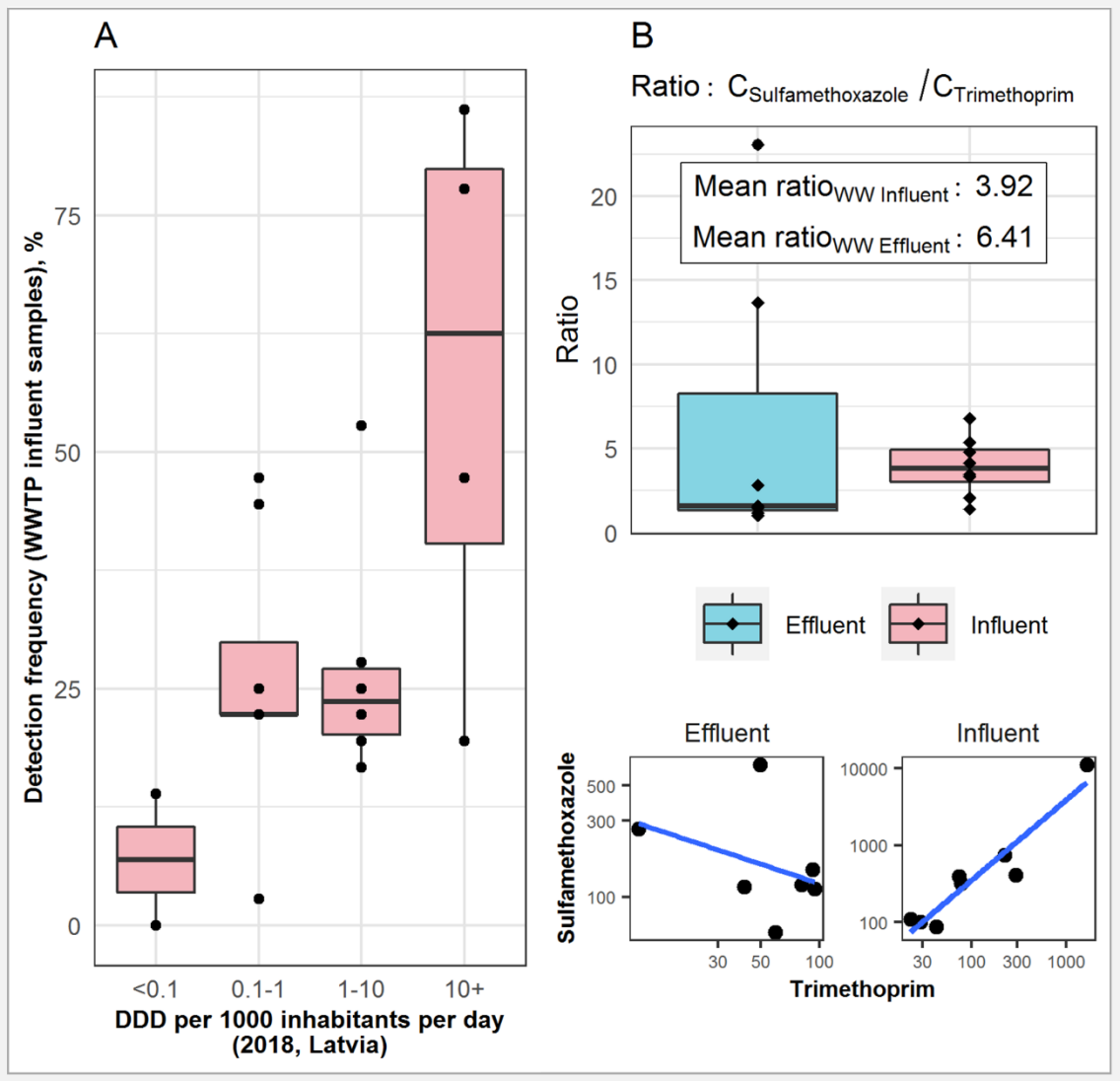


Figure 26. **Detection frequency of selected PhACs in WWTP influent samples (A) and the ratio between sulfamethoxazole and trimethoprim concentrations that were found in the analyzed WWTP influents and effluents**

To further verify the findings of this study, an assessment of the relationship between the obtained detection frequencies and national consumption of the individual PhACs was conducted. Consumption data were obtained from the annual report (data from 2018) by the State Agency of Medicines of Latvia and expressed as defined daily dose (DDD) per 1000 inhabitants per day [39]. For six target analytes information was not presented in the annual report. Figure 26 (A) reveals that, in most cases, PhACs which are consumed more frequently show higher detection rates in WWTP influents. Nevertheless, a disproportionately high occurrence can be seen for two beta-blockers (atenolol and propranolol), whilst atorvastatin, which had the highest DDD per 1000 inhabitants per day, was detected only in 19% of WWTP influent samples. The latter can be attributed to the fact that atorvastatin undergoes rapid metabolism and thus only a minor fraction of the administered dose is excreted in unchanged form [256]. Furthermore, an assessment of sulfamethoxazole/trimethoprim ratio was performed for samples that

contained both PhACs. There is evidence that the concentration ratio between these two compounds can be used as a marker to determine the main origin of WW, since there is a difference between human and veterinary dosage forms. In case of humans, the ratio between both antibiotics in the formulation is 5:1, while the typical ratio in WW samples ranges from 1.1 to 3.3 [257]. In this study, a consistent relationship was detected only in WWTP influents where the mean ratio was 3.92 (Figure 26, B). Meanwhile, WWTP effluents showed highly scattered ratios possibly due to different removal efficiencies of both antibiotics. Considering that all analyzed samples were from municipal WWTPs, these findings are in line with those of previous studies. Therefore, despite the semi-quantitative nature of the method, the presented results offer evidence that DI-HRMS can be applied for a rapid screening of PhACs in WW samples.

3.3.7. Suspect screening of PhACs via DI-FT-ICR-MS in WWTP effluents and influents

In total, 79 compounds from the suspect list were detected in the analyzed WW samples. 54 PhACs and 18 transformation products (TPs) were found in WWTP influent samples, while 38 PhACs and 16 TPs were detected in WWTP effluents. 15.1% (79/524) from total recall rate from the suspect list was Out of all compliant signals that were obtained from full-MS data, only 26% were discarded due to mismatch between the measured and theoretical MS/MS fingerprints, indicating that full-MS data provided by HRMS platforms may be rather reliable even without complementarity MS/MS analysis. However, fragment spectra matching provided an additional degree of safety, especially for compounds that are more suspectable to interferences. Experimental MS/MS features that were obtained from MoNA database once again displayed better accuracy, yielding a higher number of matched fragments in comparison to *in-silico* generated MS/MS features.

The relatively low prevalence of TPs in WW samples can be attributed to the composition of the suspect list, as it contained only 71 different TPs. Hence, it is not surprising that these substances were detected less frequently. Overall, the number of detected compounds varied to a great extent among the samples and was largely influenced by the load and WW origin. As shown in Figure 27 (B), the highest number of suspects was observed in samples that corresponded to a WWTP which receives sewage from urbanized areas and health care institutions. On the contrary, a lower prevalence of PhACs was found in WW samples from sparsely populated areas.

In order to assess temporal variability among PhACs, individual compounds were aggregated based on their therapeutic group and summarized according to their occurrence in WWTP influents. The classification was adapted from Beek et al. (2016) [197]. Temporal trends were assessed for 8 therapeutic classes, which showed the highest dominance in the analysed samples. The most frequently occurring therapeutic groups were: analgesics (mostly NSAIDs) > beta-blockers > antibiotics. From the data illustrated in Figure 27 (A), it is apparent that domestic WW from more populated areas and health care institutions contains higher diversity of PhAC residues. For instance, WW discharged from neuropsychology clinic (sample “N”) showed a considerably higher prevalence of antidepressants, anticonvulsants and psychiatric medication. Meanwhile, samples of non-specific origin yielded a higher incidence of most analgesics, antibiotics and beta-blockers. Taken together, these results suggest that the elaborated method can produce reliable results that are in accordance with the expected WW composition, hinting applicability in the field of wastewater-based epidemiology.

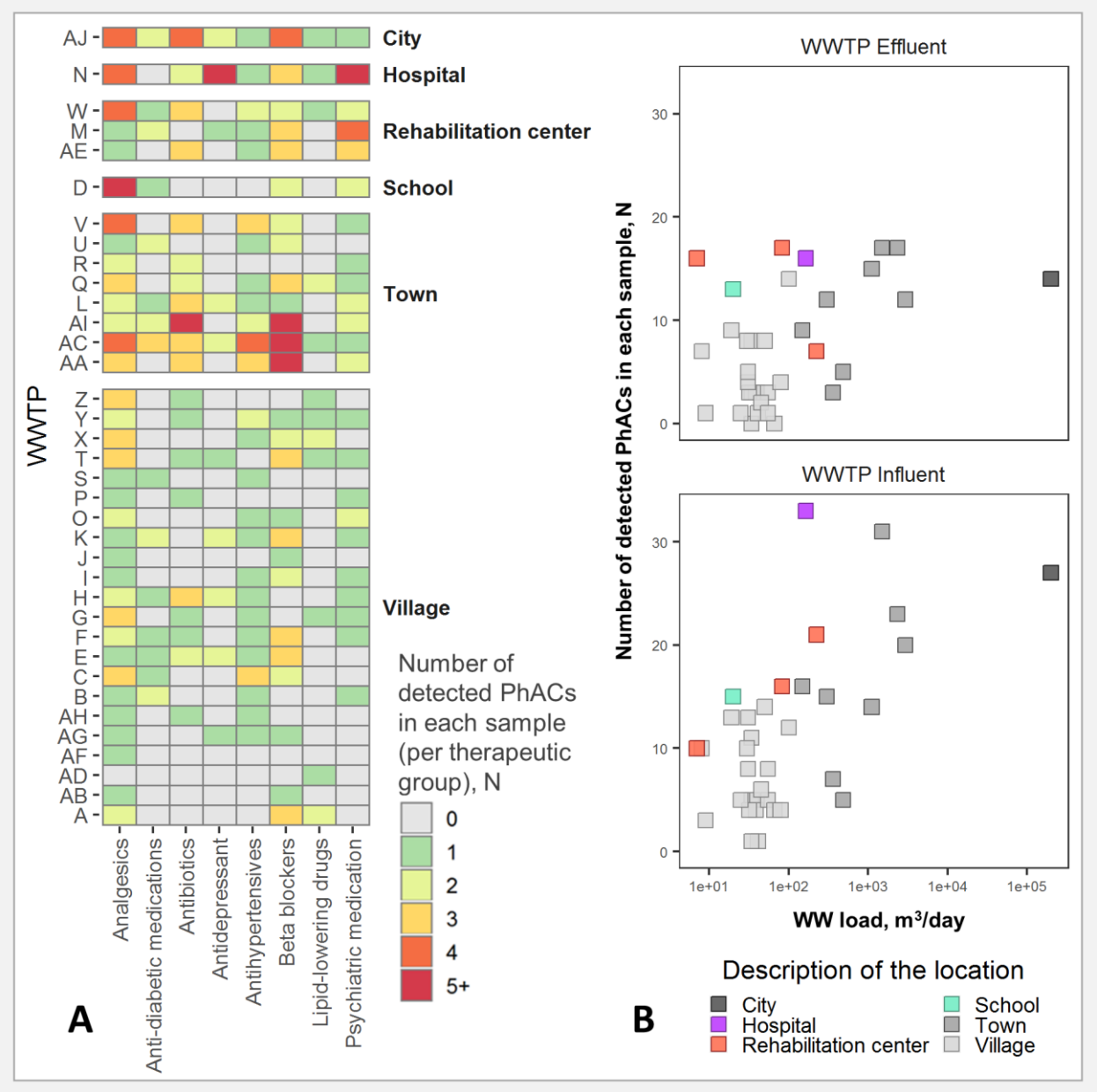


Figure 27. **Measured PhAC profiles from WWTP influent samples (A) and the overall prevalence PhACs among WWTP influents and effluents (sum of all compounds obtained from the suspect screening and targeted approach)**

Out of all suspect PhACs, telmisartan had the highest detection rate. It was found in 83% and 92% of the WWTP influent and effluent samples, respectively. This result may be explained by three reasons: (i) it is the most prescribed member among angiotensin II receptor blockers in Latvia, (ii) telmisartan is excreted almost entirely as an unchanged drug and (iii) it has a relatively long elimination half-life [258]. No interfering peaks were found for telmisartan signal in LC-HRMS analysis and the obtained spectra from bbCID fragmentation experiments also concurred with the experimental library spectra. Besides, other studies have also identified telmisartan as a problematic compound that exhibits poor removal efficiencies and high prevalence in both sewage and surface water samples in other European countries (e.g. Poland and Spain) [259,260]. Other PhACs which were repeatedly detected in WW samples are listed in Table 19.

Table 19. **A summary of PhACs and TPs from the suspect list that were detected most frequently in WWTP influents and effluents**

PhACs							
Name	**Molecular formula**	**Detection frequency [a], %**		**MS² features [b]**	**Conf. [c]**	**Possible interferences**	**DID [d]**
		Influent	**Effluent**				
Telmisartan	$C_{33}H_{30}N_4O_2$	83 (47)	92 (56)	1.7 (1.5)	H		5.5
Bisoprolol	$C_{18}H_{31}NO_4$	53 (36)	28 (22)	1.1 (1.0)	M	Nitrooctadecadienoic acids	19.2
Tramadol	$C_{16}H_{25}NO_2$	36 (36)	39 (36)	1.4 (1.4)	M	N/O-Desmethylvenlafaxine	1.5
Amisulpride	$C_{17}H_{27}N_3O_4S$	28 (28)	17 (17)	3.2 (3.2)	H		0.4
Oxcarbazepine	$C_{15}H_{12}N_2O_2$	28 (19)	14 (8)	2.3 (2.9)	M	Carbamazepine metabolites	0.4
Citalopram / Escitalopram	$C_{20}H_{21}FN_2O$	25 (3)	11 (0)	1.2 (1.0)	H		0.8 / 4.6
Sulpiride	$C_{15}H_{23}N_3O_4S$	25 (25)	25 (25)	1.9 (2.9)	H		0.2
Metformin	$C_4H_{11}N_5$	25 (25)	6 (6)	2.5 (1.5)	H		16.5
Rosuvastatin	$C_{22}H_{28}FN_3O_6S$	19 (14)	3 (3)	1.4 (1.6)	M	Isobaric substance $C_{33}H_{23}NO_3$	32.4
Sitagliptin	$C_{16}H_{15}F_6N_5O$	19 (22)	17 (19)	2.2 (1.5)	H		0.7
Lamotrigine	$C_9H_7Cl_2N_5$	17 (33)	19 (33)	2.1 (1.5)	H		0.8
Propafenone	$C_{21}H_{27}NO_3$	17 (17)	11 (8)	2.1 (1.1)	H		1.1
Transformation products of PhACs							
Name	**Molecular formula**	**Detection frequency [a], %**		**MS² features [b]**	**Conf. [c]**	**Possible interferences**	**Parent compound (corresponding DID [d])**
		Influent	**Effluent**				
Dextrorphan	$C_{17}H_{23}NO$	42 (0)	28 (0)	3.3 (0)	H		Dextromethorphan (0.1)
Hydroxydiclofenac	$C_{14}H_{11}Cl_2NO_3$	42 (39)	33 (28)	1.7 (1.7)	H		Diclofenac (18.1)
Carboxyibuprofen	$C_{13}H_{16}O_4$	36 (25)	28 (28)	1.1 (1.2)	M	Ethyl vanillin isobutyrate	Ibuprofen (23.7)
N,N-Didesmethylvenlafaxine	$C_{15}H_{23}NO_2$	36 (31)	39 (33)	1.1 (1.3)	M	O-desmethyltramadol	Venlafaxine (1.0)
N,O-Didesmethylvenlafaxine	$C_{15}H_{23}NO_2$	36 (31)	42 (36)	1.3 (1.1)	M	O-desmethyltramadol	Venlafaxine (1.0)
O-desmethyltramadol	$C_{15}H_{23}NO_2$	36 (31)	39 (36)	1.2 (1)	M	Didesmethylvenlafaxine	Tramadol (1.5)
O-desmethylvenlafaxine	$C_{16}H_{25}NO_2$	36 (39)	36 (33)	2 (1.3)	M	Tramadol	Venlafaxine (1.0)
N-Desmethylvenlafaxine	$C_{16}H_{25}NO_2$	33 (33)	39 (36)	1.2 (1.3)	M	Tramadol	Venlafaxine (1.0)
Acetoaminoantipyrine	$C_{13}H_{15}N_3O_2$	31 (31)	8 (3)	2.4 (1.5)	H		Metamizole (0.6)
Carbamazepine-2OH	$C_{15}H_{12}N_2O_2$	25 (22)	14 (8)	3.4 (3.3)	M	Oxcarbazepine	Carbamazepine (2.1)
Carbamazepine-10,11-epoxide	$C_{15}H_{12}N_2O_2$	22 (19)	8 (8)	2.8 (2.3)	M	Oxcarbazepine	Carbamazepine (2.1)

[a] Detection frequency obtained from the identification using experimental and predicted (in parentheses) MS/MS features

[b] Mean number of fragment signals matched per precursor using experimental and predicted (in parentheses) MS/MS features

[c] Identification confidence level ("H"- high and "M" - moderate)

[d] DDD per 1000 inhabitants per day (2018, Latvia)

Among them, several PhACs with high consumption volumes (bisoprolol, metformin and rosuvastatin) and substances that are predominantly excreted as parent compound (e.g. amisulpride, sulpiride and citalopram) were repeatedly detected. In fact, these two members of antipsychotic class PhACs, amisulpride and sulpiride, have been identified as pseudo-persistent pollutants. Meanwhile, lamotrigine (anticonvulsant) has shown to have higher concentrations in treated WW samples compared to raw influents, because of the deconjugation of the main human metabolite - lamotrigine-N2-glucuronide [261]. The data supported this phenomenon and higher Q_1 signal intensities were indeed found in WWTP effluents (Figure 28, A). High detection rates of tramadol and

oxcarbazepine have to be interpreted with caution. Although both PhACs are frequently reported by other studies, they are not well suited for our methodology. Oxcarbazepine signal can overlap with two major carbamazepine metabolites (carbamazepine-10,11-epoxide and 2-hydroxycarbamazepine) that share the same molecular formula and due to high consumption of the parent compound are co-occurring in WW samples. The same goes for tramadol which can suffer from interfering signals that are caused by venlafaxine metabolites (N- and O-desmethylvenlafaxine). Nevertheless, we did not discard those analytes, because LC-HRMS analysis revealed that parent PhACs and interfering TPs are present in WW samples.

Another bizarre finding was that dextrorphan, an active metabolite of dextromethorphan, was detected suspiciously often (42% of the WWTP influent samples). Even though the parent compound is marketed as a cough suppressant, it is also recognized as a recreational drug due to its side-effects. To avoid misuse of dextromethorphan it has been classified as a prescription drug in Latvia since 2009 and, as a consequence, its annual consumption has declined substantially and thus lower prevalence should be anticipated. These results may indicate that the actual use dextromethorphan containing medical products is higher than reflected by the official consumption statistics and, despite legislative measures, it is still available for users. Previous studies have also documented the presence of dextromethorphan metabolites in the environment [262,263]. Other exemplary TPs that were detected in our study were hydroxydiclofenac, carboxyibuprofen, didesmethylvenlafaxine, desmethyltramadol and carbamazepine metabolites. Among these compounds, only for hydroxydiclofenac "high" identification confidence was assigned. When compared to the parent molecule, traces of hydroxydiclofenac were found less often and in most cases with much lower Q_1 signal intensities. It has been shown that the ratio between the parent drug and hydroxylated metabolites is dependent on the route of administration. Topical applications yield significantly higher levels of the parent substance than hydroxylated metabolites [264]. Our results seem to be in accordance with this hypothesis, because topical diclofenac formulations (e.g. patches, creams and gels) are available over the counter in Latvia, while almost all oral dosage forms can be purchased only by prescription.

As mentioned in the previous section, 49 DDD of atorvastatin are consumed per 1000 inhabitants per day in Latvia. It is the most widely used PhAC among all prescription drugs. Yet, atorvastatin is extensively metabolised and only a small fraction is excreted unchanged (it was detected only in 19% of the WWTP influent samples via targeted approach). Therefore, a retrospective analysis was conducted by investigating the presence of main atorvastatin metabolites (e.g. atorvastatin lactone, hydroxyatorvastatin, hydroxyatorvastatin lactone and hydroxyatorvastatin glucuronide), which were not a part of the suspect database. Full-MS data were treated using identic isolation criteria as for the main method and the most abundant Q_1 ion of deprotonated and protonated species was used for comparison of signal intensities at negative and positive ionisation modes, respectively. Hydroxyatorvastatin was the only metabolite detected in WW samples. As seen in Figure 28 (B), the analytical response was higher for the metabolite compared to the parent drug. These data are in agreement with the findings of Langford and Thomas (2011), which showed that para- and ortho-hydroxyatorvastatin were detected at higher levels than atorvastatin in Norwegian aquatic environment [265]. However, a comparison of signal intensities should be interpreted with caution, since ionisation of each compound can vary, giving a misleading impression about the actual occurrence of compounds.

A similar retrospective analysis was carried out for carbamazepine and its metabolites (see Figure 28, C). The observed metabolite patterns showed a coherent trend. The analytical response of metabolites increased along

with increasing carbamazepine concentration. Besides, the most intense signals were found for samples AE, M and N, that received sewage from healthcare facilities (AE and M - rehabilitation centre, N - hospital).

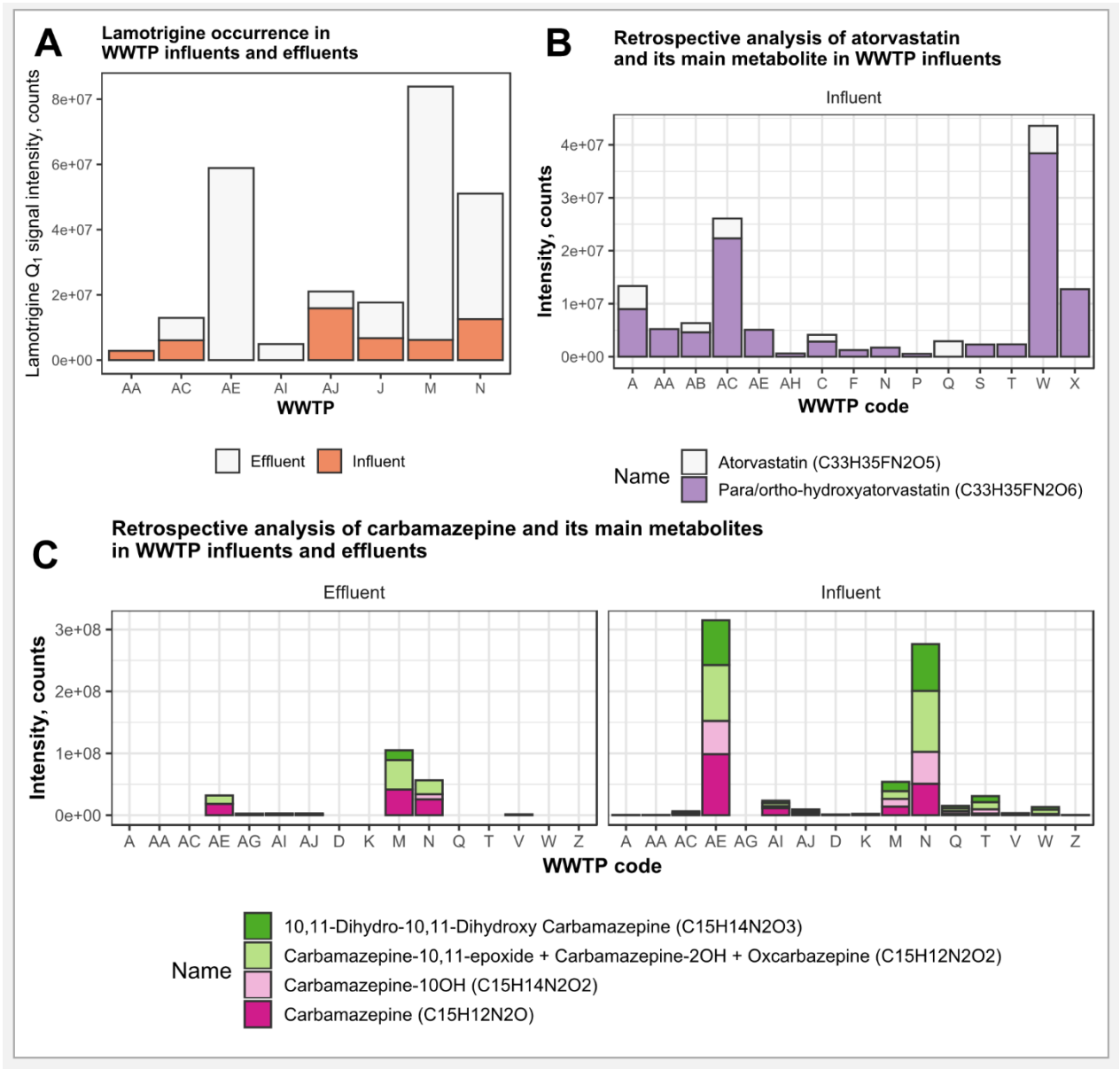


Figure 28. **Full-MS based retrospective analysis of selected PhACs and their TPs in WWTP influents and effluents: lamotrigine (A), atorvastatin (B) and carbamazepine (C)**

In general, lower detection rates were observed during reference standard-free screening of PhACs, because the majority of suspect signals were dismissed due to non-compliant Q_2/Q_1 ratios. As mentioned before, target analytes were qualified using "wide" ratio tolerance that ranged from 20% to 50%, whereas a strict tolerance limit (20%) was maintained for the qualification of suspects. This way reliability of the method may be increased, but only at the expense of sensitivity, which consequently caused lower detection frequencies and presumably narrowed the scope of the method. Nevertheless, the acquired results are comparable with LC-HRMS based screening methodologies, showing that high-throughput screening of WW samples via DI-HRMS is possible and can yield valuable information regarding the presence of PhACs and their TPs.

CONCLUSIONS

1. The developed analytical method based on SPE followed by LC-Q-Orbitrap-MS detection allowed to quantitively evaluate the presence of 24 emerging PhACs in wastewater samples. The method demonstrated high selectivity and sensitivity, providing limits of quantification for selected compounds from 0.01 to 1.0 ng/L and analyte recovery from 77 to 133%. Besides, optimization of resolving power showed that sufficient detection frequency can be obtained during data-dependent acquisition of full-MS and MS/MS spectra at resolving power of 70,000 and 17,500, respectively.

2. The LC-Q-Orbitrap-MS method was applied for the quantification of PhACs in wastewater samples collected in Riga, Latvia. The results revealed the presence of 20 selected pharmaceuticals. The highest concentration was found for caffeine and acetaminophen (paracetamol) with average concentrations exceeding several μg/L. With respect to antibiotics, the highest levels were found for ciprofloxacin, azithromycin and sulfamethoxazole.

3. A novel LC-Q-TOF-MS method was developed for a comprehensive residue analysis of six aminoglycoside antibiotics. Although TOF analysers are considered less sensitive, the obtained sensitivity (LOQ ranged from 50 to 125 pg per injection) was comparable with the conventional QqQ MS/MS approach. In addition, a unique three mobile phase system that involved a gradual decrease of pH was found to be the most suitable for separation of target aminoglycosides using a zwitterionic-type mixed-mode LC column. Meanwhile, the application of high-temperature ESI source enhanced ionization efficiency of target analytes compared to conventional ESI source. Moreover, this approach was able to eliminate $[M+Na]^+$, $[M+NH_4]^+$, and $[M+K]^+$ adducts and reduced the amount of doubly charged species which are recognized as a significant issue during ESI-assisted ionization of aminoglycosides.

4. A unique sample preparation procedure based on a modified QuEChERs approach was developed for the extraction of multi-class PhACs from raw and treated wastewater samples. Freeze-drying was found to be an efficient sample pre-treatment approach that allowed to minimize sample volume prior to the extraction without significant loss of analytes. Several dSPE clean-up strategies were explored during the development stage and the best performance was found when C18 and strong anion exchange sorbents were applied simultaneously. This approach allowed to minimize the extent of matrix induced ion suppression and enabled comprehensive screening of PhACs via DI-ESI-FT-ICR-HRMS method.

5. An innovative suspect and target screening methodology using direct infusion FT-ICR-HRMS was developed for semi-quantitative analysis of 26 compounds and qualitative screening of more than 500 PhACs and their transformation products using a custom-made suspect database. Overall, a total of 79 suspects and 24 target compounds were detected in 72 wastewater samples from Latvia. The results indicate that the applied resolving power (490,000 at m/z 250 and 275,000 at m/z 500) is sufficient for screening purposes and thus the method is not limited to ICR-HRMS systems, but can also be adapted to new generation Orbitrap mass analysers. The main advantages of the method are as follows: it is rapid, it can be easily automated and it practically does not require laborious post-processing steps which are an essential part for most LC-HRMS screening methods (e.g. peak picking and integration, linking full-MS data with MS/MS traces, manual identification of suspects etc.). Furthermore, a potentially wider scope of PhACs can be covered as both hydrophobic and hydrophilic PhACs can

be analyzed simultaneously, because limitations set by conventional reversed-phase chromatography are non-existent. The results indicate that the method is suitable for screening of PhACs that contain at least two different heteroatoms. Even higher selectivity can be achieved towards halogenated PhACs that can produce a distinct isotopic pattern, such as diclofenac and losartan.

6. The developed LC-MS/MS method was used to explore the applicability of carbon nanotubes as an alternative dSPE sorbent for the extraction of 12 acidic NSAIDs from aquatic samples. Results showed that adsorption of NSAIDs occurs most readily when target compounds are in the protonated state, thus samples required acidification prior the extraction. A contact period of 30 min was sufficient to achieve almost complete adsorption for all investigated NSAIDs (95 to 100%). Meanwhile, only a partial release of target analytes was achieved and required a highly alkaline elution medium (5% of ammonium hydroxide in methanol, *v*/*v*). The final recoveries for the developed dSPE method ranged from 60 to 94% for 11 out of 12 target analytes. Only meloxicam showed unsatisfactory desorption efficiency (40%) due to a higher number of conjugated heterocycles compared to other NSAIDs which affects the van der Waals surface area of the compound and increases its affinity towards carbon nanotubes.

7. The developed LC-Q-Orbitrap-MS method was used to assess removal of PhACs from wastewater by biological processes (activated sludge, sludge-derived bacteria and fungi). Results revealed that treatment with activated sludge for 17 hours reduced the initial concentrations below 40% for all except four PhACs (two antibiotics and two NSAIDs). The highest removal efficiency (>95%) was observed for trimethoprim and acetaminophen. Meanwhile, sulfamethoxazole and ciprofloxacin underwent biodegradation only when additional bioaugmentation was performed with sludge-derived bacteria and fungi. An important issue that emerged from the data was that two NSAID class PhACs showed exceptionally poor removal potential compared to other PhACs. Overall, the results show that bioaugmentation can be used to enhance PhAC degradation. Yet, removal efficiency strongly depends upon the incubation time and compound specific physico-chemical characteristics that promote/hinder biotransformations, oxidative processes and sorption.

8. The developed LC-Q-Orbitrap-MS method was used to assess the effectiveness of ionizing radiation as a way to remove PhAC residues from WWTP influents. Experiments with real sludge samples exposed to gamma and electron beam radiation revealed that the majority of PhAC can be degraded with >90% efficiency at 0.5–3 kGy absorbed radiation doses. Only macrolide antibiotics and one fluoroquinolone (ciprofloxacin) showed higher stability. Nevertheless, exposure to higher doses (≥5 kGy) caused almost complete elimination of these compounds. In general, both irradiation approaches yielded satisfactory results. However, the electron beam technique was able to achieve the same extent of degradation 50 times faster compared to gamma radiation.

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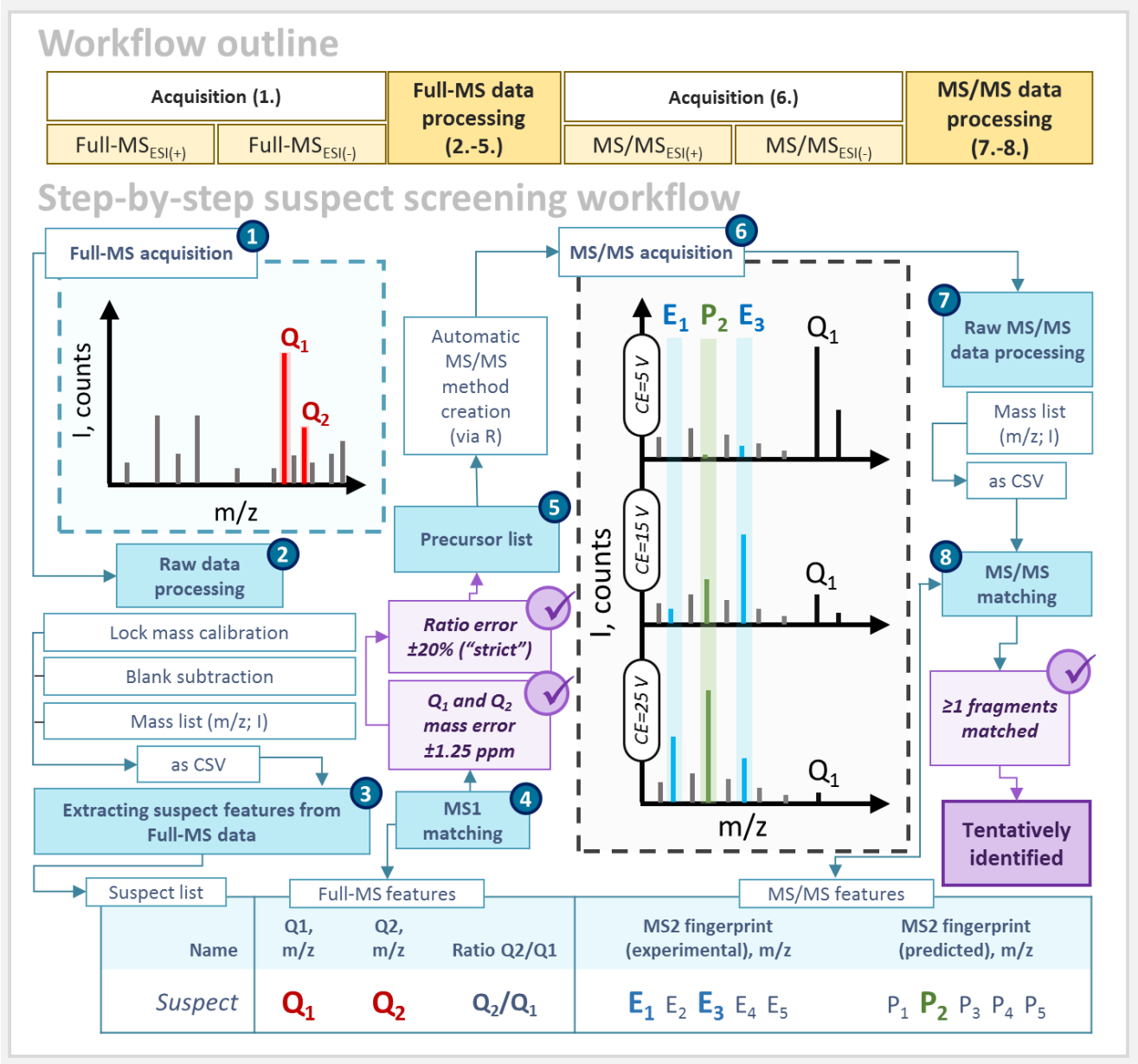
ANNEXES

Annex 1

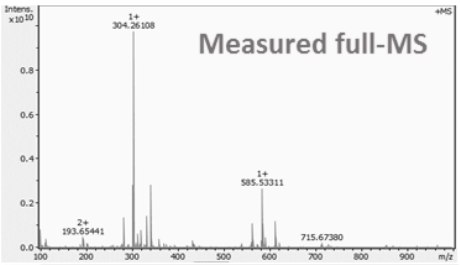
A schematic step-by-step workflow of the developed screening procedure on FT-ICR-MS



An example of tentative carbamazepine identification using the developed FT-ICR-MS method

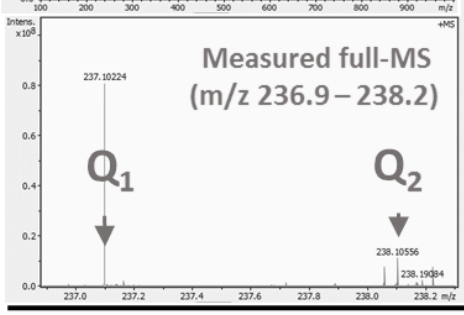
Suspect list input (information for full-MS)

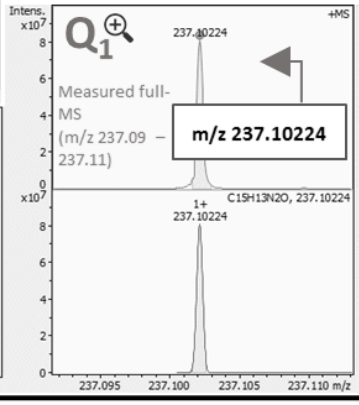
Name	Molecular Formula	Q1, m/z	Q2, m/z	Ratio Q2/Q1
Carbamazepine	$C_{15}H_{12}N_2O$	237.1022	238.1056	16.3

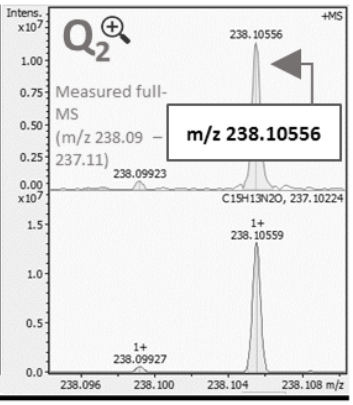


Measured ratio (Q2/Q1)	Theoretical ratio (Q2/Q1)	Ratio error,%
14.10	16.3	-13.5

Mass accuracy: <0.5 ppm for both ions







Suspect list input (information for MS/MS)

MS/MS fragment formulas (MoNA library)

$C_{14}H_{10}N$; $C_{13}H_9N$; $C_{14}H_{11}N$; $C_{13}H_9$; $C_{14}H_9N$

MS/MS fingerprint, m/z (found, ~~not found~~)

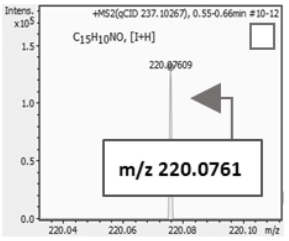
192.0808 | 179.0730 | 193.0886 | 165.0699 | 191.0730

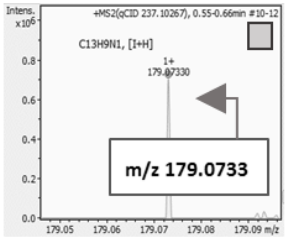
MS/MS fragment formulas (CFM-ID generated)

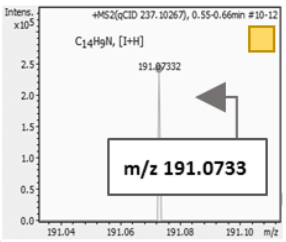
$C_{14}H_{12}N$; $C_{15}H_{11}N_2$; $C_{15}H_{10}NO$; $C_{13}H_8N$; C_8H_5

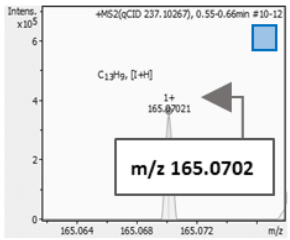
MS/MS fingerprint, m/z (found, ~~not found~~)

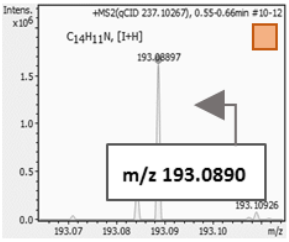
~~194.0964~~ | ~~219.0917~~ | 220.0757 | 178.0651 | ~~101.0386~~

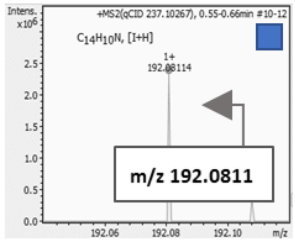


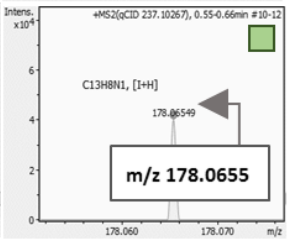










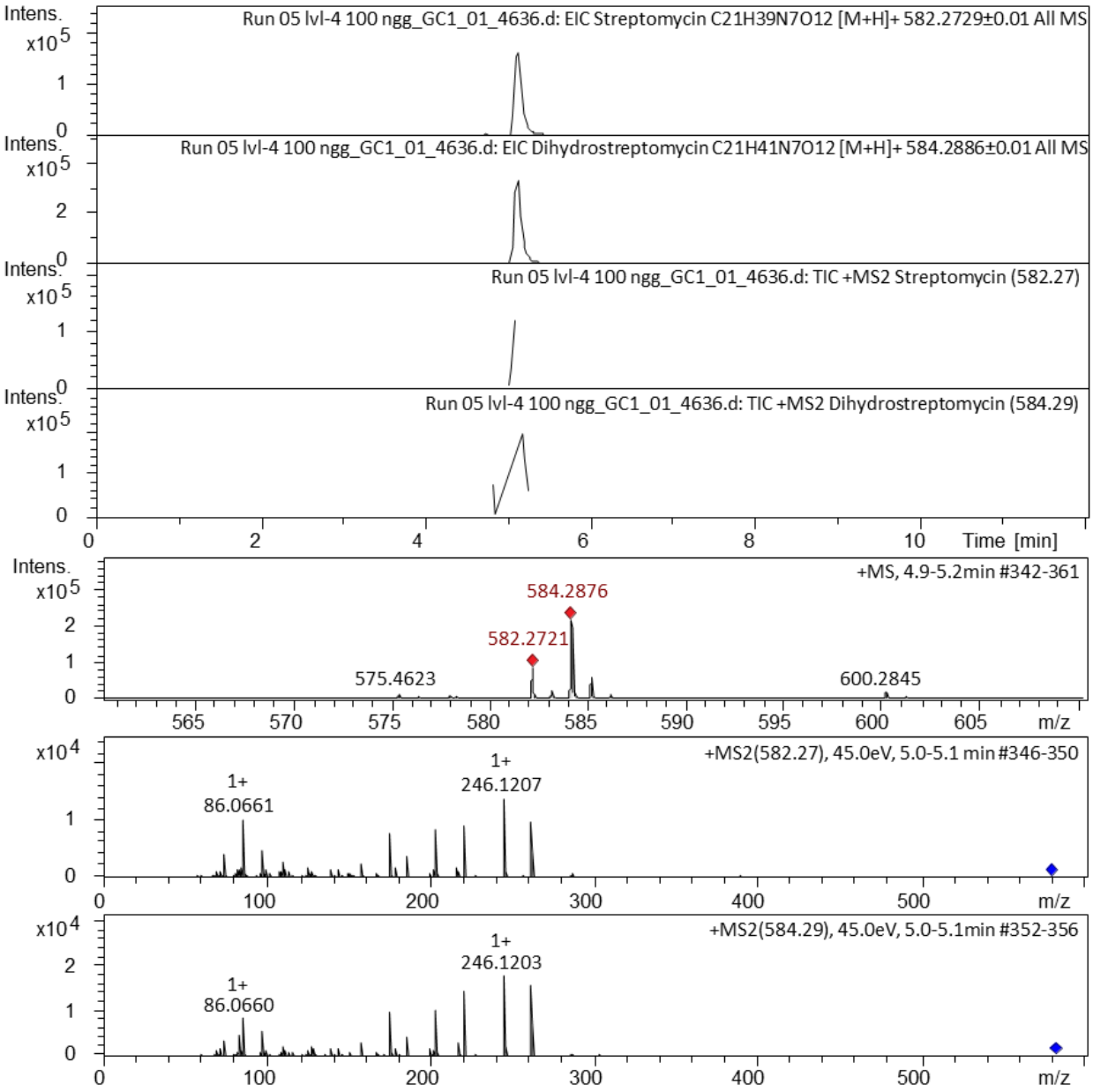


Matched fragments (MoNA library)

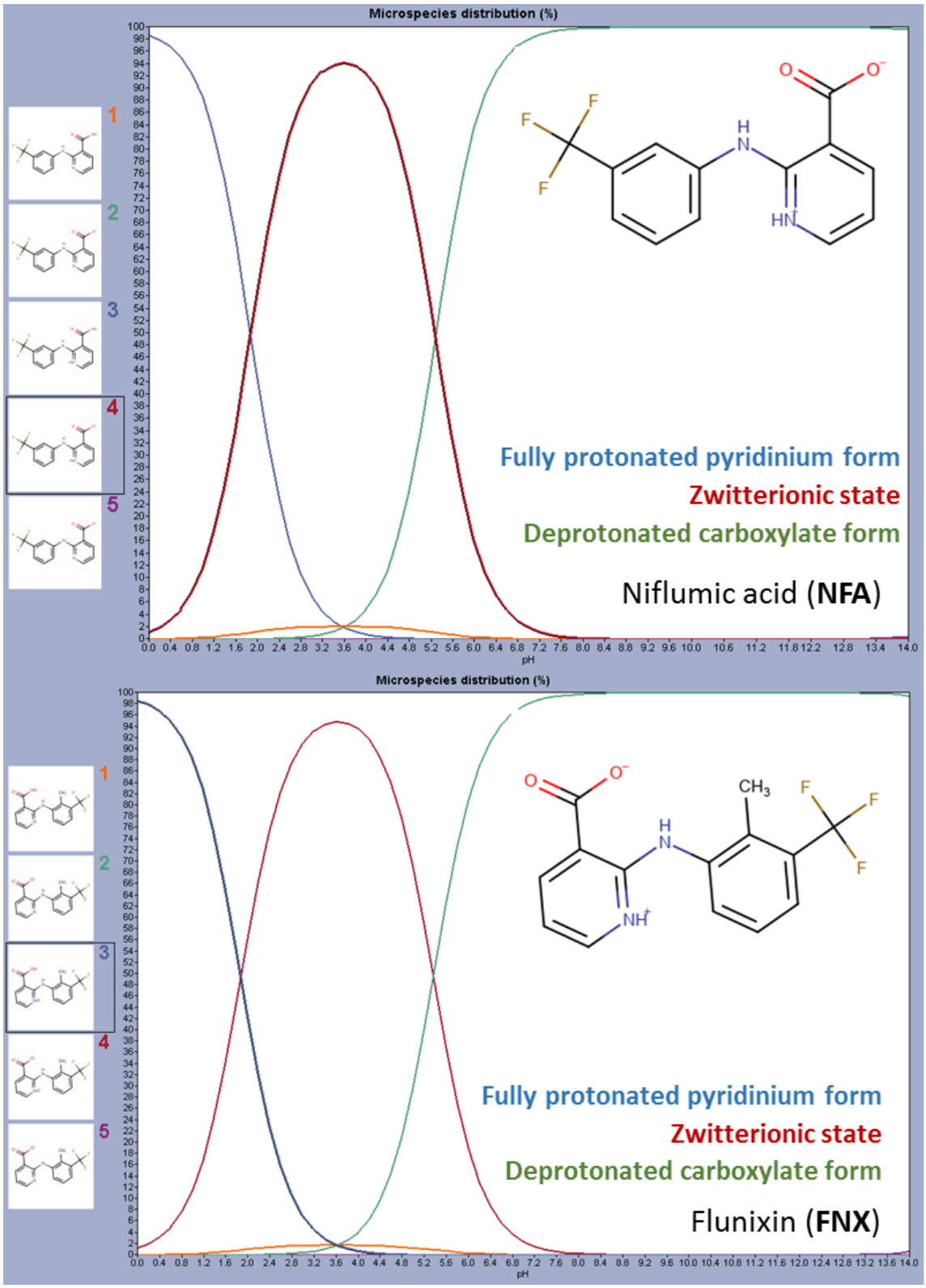
Matched fragments (predicted)

Annex 3

An example of full-MS/dd-MS/MS spectra for a fortified honey sample taken from the validation study (100 ng/g of STP and DSTP). Extracted ion chromatograms are shown for $[M+H]^+$ species of STP and DSTP with the corresponding total ion chromatograms for each of the dd-MS/MS traces. The averaged full-MS spectra for both precursors and the fragment dd-MS/MS spectra are illustrated below.



Microspecies distribution of niflumic acid and flunixin under different pH values (generated by MarvinSketch 20.4. software)



Annex 5

Main validation parameters (sensitivity and detection capabilities) of the developed FT-ICR-MS method

Name	CCα, ng/L	CCβ, ng/L	SDL, ng/L	LOI, ng/L	Coefficient of determination	Range, ng/L	Validation levels A/B/C, ng/L
Atenolol	28	35	50	50	0.99	50-1000	50/250/500
Atorvastatin	94	120	100	100	0.99	100-2000	100/500/1000
Azithromycin	66	97	100	>1000	0.98	100-2000	100/500/1000
Caffeine	544	904	500	500	0.99	500-10000	500/2500/5000
Carbamazepine	76	99	100	100	0.99	100-2000	100/500/1000
Ciprofloxacin	220	310	250	250	0.98	250-5000	250/1000/2000
Clarithromycin	37	60	50	50	0.97	50-1000	50/250/500
Diclofenac	20	38	50	50	0.99	50-1000	50/250/500
Erythromycin	28	59	100	100	0.98	100-2000	100/500/1000
Fluoxetine	28	35	50	250	0.99	50-1000	50/250/500
Gemfibrozil	74	115	100	1000	0.98	100-2000	100/500/1000
Ibuprofen	122	274	1000	1000	0.98	250-5000	250/1000/2000
Ketoprofen	171	290	250	1000	0.99	250-5000	250/1000/2000
Losartan	33	46	50	50	0.98	50-1000	50/250/500
Meloxicam	49	168	250	2000	0.99	250-5000	250/1000/2000
Metoprolol	23	32	50	50	0.99	50-1000	50/250/500
Naproxen	266	602	500	5000	0.98	500-10000	500/2500/5000
Paracetamol	693	1234	1000	5000	0.98	1000-10000	1000/2500/5000
Pravastatin	65	81	100	1000	0.98	100-2000	100/500/1000
Propranolol	19	27	50	50	0.99	50-1000	50/250/500
Salbutamol	26	37	50	50	0.99	50-1000	50/250/500
Spiramycin	120	260	>2000	>2000	0.95	250-5000	250/1000/2000
Sulfamethoxazole	222	304	250	>2000	0.98	250-5000	250/1000/2000
Trimethoprim	18	54	100	100	0.98	100-2000	100/500/1000
Valsartan	274	775	500	>5000	0.97	500-10000	500/2500/5000
Xylazine	22	32	50	50	0.99	50-1000	50/250/500

Recovery, repeatability (RSD_r) and between-day reproducibility (RSD_{wR}) of target PhACs obtained by FT-ICR-MS method

Name	***Level A***			***Level B***			***Level C***		
	RSD_r, %	**RSD_{wR}, %**	**Recovery, %**	**RSD_r, %**	**RSD_{wR}, %**	**Recovery, %**	**RSD_r, %**	**RSD_{wR}, %**	**Recovery, %**
Atenolol	7	9	118	3	8	101	6	7	96
Atorvastatin	15	16	120	6	13	120	10	16	98
Azithromycin	13	19	135	13	17	93	17	21	101
Caffeine	32	44	78	18	35	89	17	30	84
Carbamazepine	13	14	118	11	13	89	16	27	95
Ciprofloxacin	18	22	74	23	53	88	21	24	75
Clarithromycin	17	28	138	16	23	104	9	12	111
Diclofenac	19	22	118	10	10	105	8	14	84
Erythromycin	18	19	73	22	31	95	13	25	87
Fluoxetine	6	8	126	5	15	101	7	15	87
Gemfibrozil	10	25	131	4	11	110	8	10	103
Ibuprofen	17	37	83	13	37	108	8	18	104
Ketoprofen	23	29	106	30	50	121	16	29	112
Losartan	12	16	80	11	22	112	7	8	89
Meloxicam	26	29	78	14	32	102	11	23	97
Metoprolol	9	11	115	4	6	105	5	9	96
Naproxen	27	41	129	19	39	111	12	27	94
Paracetamol	23	33	121	18	24	117	16	33	91
Pravastatin	6	10	107	11	25	97	8	17	116
Propranolol	5	10	95	5	6	89	9	12	94
Salbutamol	9	13	87	8	23	118	5	9	102
Spiramycin	22	34	86	18	29	75	16	28	83
Sulfamethoxazole	15	20	121	22	31	112	15	16	98
Trimethoprim	18	22	109	11	15	94	6	7	87
Valsartan	38	61	106	23	46	91	29	51	85
Xylazine	9	12	87	6	12	81	10	21	101

Mass accuracy: Q_1 performance characteristics from the FT-ICR-MS method's validation dataset

Name	Level A			Level B			Level C		
	Mean error, ppm	SD, ppm	Comp. rate[a], %	Mean error, ppm	SD, ppm	Comp. rate[a], %	Mean error, ppm	SD, ppm	Comp. rate[a], %
Atenolol	0.13	0.02	100%	0.12	0.00	100%	0.13	0.02	100%
Atorvastatin	-0.03	0.37	100%	0.05	0.33	100%	0.08	0.32	100%
Azithromycin	-0.19	0.78	83%	-0.11	0.30	83%	-0.16	0.19	100%
Caffeine	-0.04	0.03	100%	-0.01	0.02	100%	0.01	0.02	100%
Carbamazepine	0.11	0.02	100%	0.12	0.02	100%	0.12	0.02	100%
Ciprofloxacin	0.07	0.02	100%	0.07	0.01	100%	0.07	0.01	100%
Clarithromycin	-0.07	0.04	100%	-0.08	0.03	100%	-0.08	0.02	100%
Diclofenac	-0.60	0.24	100%	-0.40	0.24	100%	-0.25	0.23	100%
Erythromycin	0.47	0.08	100%	0.39	0.05	100%	0.37	0.02	100%
Fluoxetine	0.10	0.02	100%	0.11	0.01	100%	0.10	0.02	100%
Gemfibrozil	0.34	0.49	100%	0.29	0.46	100%	0.14	0.51	100%
Ibuprofen	-0.26	0.49	75%	0.00	0.32	100%	-0.01	0.29	100%
Ketoprofen	0.32	0.54	100%	0.29	0.51	100%	0.30	0.50	100%
Losartan	-0.27	0.10	100%	-0.25	0.06	100%	-0.22	0.04	100%
Meloxicam	-0.34	0.17	100%	-0.25	0.10	100%	-0.25	0.09	100%
Metoprolol	0.11	0.01	100%	0.13	0.02	100%	0.13	0.02	100%
Naproxen	-0.46	0.66	83%	0.21	0.42	100%	0.21	0.42	100%
Paracetamol	-0.03	0.18	100%	-0.05	0.15	100%	-0.05	0.17	100%
Pravastatin	-0.24	0.09	100%	-0.21	0.06	100%	-0.16	0.05	100%
Propranolol	0.01	0.01	100%	0.01	0.01	100%	0.01	0.01	100%
Salbutamol	-0.05	0.02	100%	-0.03	0.02	100%	0.00	0.02	100%
Spiramycin	-0.57	0.37	83%	-1.05	0.10	75%	-1.04	0.19	92%
Sulfamethoxazole	0.32	0.53	100%	0.30	0.50	100%	0.33	0.52	100%
Trimethoprim	-0.05	0.03	100%	-0.03	0.01	100%	-0.02	0.01	100%
Valsartan	-0.15	0.14	100%	-0.13	0.15	100%	-0.13	0.11	100%
Xylazine	0.00	0.02	100%	0.02	0.01	100%	0.02	0.00	100%

[a] Compliance rate.

Mass accuracy: Q_2 performance characteristics from the FT-ICR-MS method's validation dataset

Name	Level A			Level B			Level C		
	Mean error, ppm	SD, ppm	Comp. rate[a], %	Mean error, ppm	SD, ppm	Comp. rate[a], %	Mean error, ppm	SD, ppm	Comp. rate[a], %
Atenolol	0.00	0.03	100%	0.03	0.02	100%	0.05	0.02	100%
Atorvastatin	0.17	0.33	100%	0.09	0.32	100%	0.13	0.33	100%
Azithromycin	0.34	0.58	50%	-1.08	0.12	67%	-0.32	0.34	100%
Caffeine	-0.03	0.04	100%	0.01	0.04	100%	0.02	0.03	100%
Carbamazepine	0.01	0.05	100%	0.03	0.03	100%	0.05	0.02	100%
Ciprofloxacin	0.05	0.03	100%	0.04	0.03	100%	0.06	0.03	100%
Clarithromycin	-0.10	0.09	100%	-0.12	0.06	100%	-0.12	0.03	100%
Diclofenac	-0.36	0.41	100%	-0.29	0.23	100%	-0.18	0.18	100%
Erythromycin	0.41	0.11	100%	0.28	0.04	100%	0.25	0.04	100%
Fluoxetine	0.09	0.04	100%	0.10	0.03	100%	0.10	0.02	100%
Gemfibrozil	-0.25	0.66	92%	-0.02	0.61	100%	0.12	0.56	100%
Ibuprofen	-0.03	0.32	42%	0.10	0.34	100%	0.11	0.35	100%
Ketoprofen	-0.84	0.31	50%	0.02	0.59	100%	-0.11	0.63	100%
Losartan	-0.37	0.20	100%	-0.30	0.09	100%	-0.26	0.06	100%
Meloxicam	-0.23	0.31	100%	-0.32	0.11	100%	-0.30	0.12	100%
Metoprolol	-0.08	0.03	100%	-0.07	0.02	100%	-0.05	0.02	100%
Naproxen	0.45	0.39	25%	0.09	0.50	100%	0.07	0.50	100%
Paracetamol	0.45	0.17	25%	-0.52	0.55	67%	0.08	0.13	100%
Pravastatin	-0.29	0.14	100%	-0.27	0.07	100%	-0.24	0.06	100%
Propranolol	0.11	0.02	100%	0.12	0.02	100%	0.10	0.02	100%
Salbutamol	0.02	0.03	100%	0.03	0.03	100%	0.05	0.02	100%
Spiramycin	-0.30	0.78	42%	-0.25	0.47	75%	-0.32	0.46	83%
Sulfamethoxazole	-0.61	0.61	92%	-0.72	0.51	92%	-0.29	0.70	100%
Trimethoprim	0.09	0.02	100%	0.09	0.02	100%	0.10	0.02	100%
Valsartan	-0.07	0.26	83%	-0.09	0.19	100%	-0.03	0.27	100%
Xylazine	0.00	0.03	100%	0.02	0.02	100%	0.03	0.02	100%

[a] Compliance rate.

Ion ratio: Q_2/Q_1 performance characteristics from the FT-ICR-MS method's validation dataset

Name	Level A			Level B			Level C		
	Mean ratio	Mean error, %	Comp. rate (wide)[a], %	Mean ratio	Mean error, %	Comp. rate (wide) [a], %	Mean ratio	Mean error, %	Comp. rate (wide) [a], %
Atenolol	15.2	-0.5	100%	14.9	-2.8	100%	14.9	-2.4	100%
Atorvastatin	37.0	1.8	83% (100%)	33.9	-6.8	100%	34.4	-5.3	100%
Azithromycin	40.9	6.8	25%	42.3	-2.1	83%	40.6	-6.0	58% (75%)
Caffeine	9.2	6.2	92%	8.6	-0.6	100%	8.3	-5.0	100%
Carbamazepine	16.3	-0.1	100%	16.4	0.5	100%	16.2	-0.3	100%
Ciprofloxacin	18.1	-2.4	100%	18.1	-2.4	100%	18.7	0.9	100%
Clarithromycin	40.9	-4.5	100%	40.7	-5.0	100%	40.5	-5.4	100%
Diclofenac	61.7	-4.2	100%	59.8	-7.1	100%	56.0	-13.0	92%
Erythromycin	40.1	-3.9	100%	39.4	-5.6	100%	38.7	-7.3	100%
Fluoxetine	17.8	-3.4	100%	18.3	-1.0	100%	18.0	-2.6	100%
Gemfibrozil	15.7	-4.1	75%	16.0	-2.2	83%	15.8	-3.5	83% (92%)
Ibuprofen	15.6	10.2	25% (42%)	13.8	-2.1	100%	13.3	-5.8	100%
Ketoprofen	17.5	0.3	25%	17.2	-1.8	92%	17.1	-2.2	83% (100%)
Losartan	32.1	-0.1	100%	32.4	0.6	100%	32.0	-0.5	100%
Meloxicam	16.1	4.8	92%	15.3	-0.2	100%	14.9	-2.7	100%
Metoprolol	16.0	-2.7	100%	15.7	-4.2	100%	15.3	-6.6	100%
Naproxen	14.6	-4.8	25%	15.2	-0.7	83%	15.5	1.2	92%
Paracetamol	8.6	-1.6	17% (25%)	9.3	6.6	42% (67%)	8.6	-1.1	75% (92%)
Pravastatin	26.3	4.2	92%	24.4	-3.6	100%	23.7	-6.2	100%
Propranolol	17.2	-1.1	100%	16.5	-5.2	100%	16.1	-7.4	100%
Salbutamol	14.0	-1.8	100%	13.8	-2.8	100%	13.4	-5.7	100%
Spiramycin	44.3	-8.9	33%	40.4	-17.1	33%	43.2	-11.4	50%
Sulfamethoxazole	11.3	3.2	58% (67%)	11.7	6.5	50%	11.0	0.4	75%
Trimethoprim	15.0	-2.2	100%	14.2	-7.2	100%	13.7	-10.7	100%
Valsartan	25.6	-1.7	50% (75%)	27.7	6.2	83% (92%)	26.3	0.9	75% (83%)
Xylazine	12.7	-2.6	100%	12.2	-6.4	100%	11.8	-9.3	100%

[a] Compliance rate using "strict" and "wide" ratio limits. The latter is given in parenthesis. If the compliance rates between "strict" and "wide" limits are equal, only one value is given.

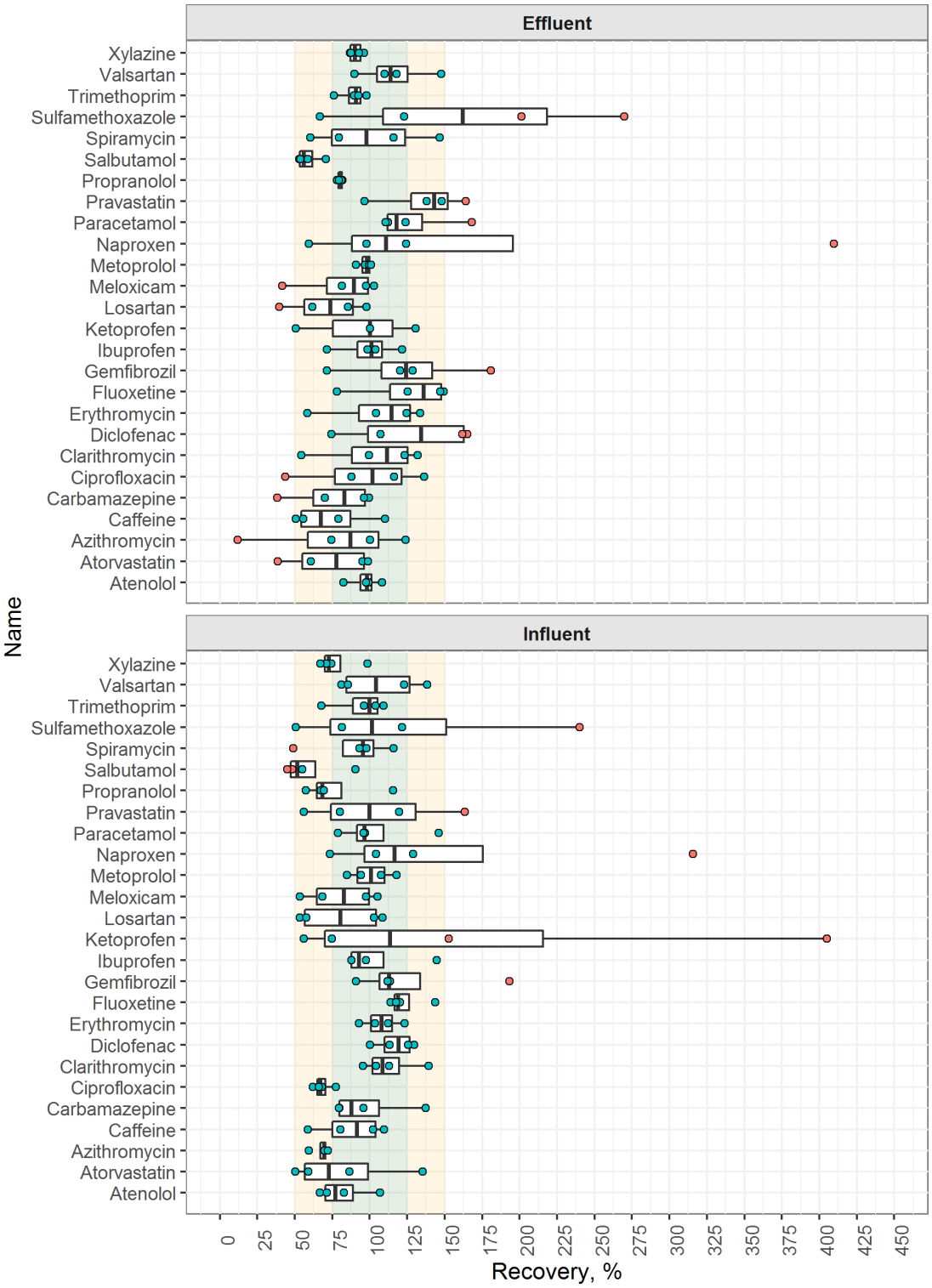
MS/MS fingerprinting: experimental (MoNA database) and predicted (CFM-ID) MS/MS spectra performance characteristics from the FT-ICR-MS method's validation dataset

Name	Level A				Level B				Level C			
	MoNA (experimental fingerprint)		CFM-ID (predicted fingerprint)		MoNA (experimental fingerprint)		CFM-ID (predicted fingerprint)		MoNA (experimental fingerprint)		CFM-ID (predicted fingerprint)	
	Range (max N)[b]	Comp. rate[a], %	Range (max N)[b]	Comp. rate[a], %	Range (max N)[b]	Comp. rate[a], %	Range (max N)[b]	Comp. rate[a], %	Range (max N)[b]	Comp. rate[a], %	Range (max N)[b]	Comp. rate[a], %
Atenolol	2 (5)	100%	1 (5)	100%	2 (5)	100%	1-2 (5)	100%	2-3 (5)	100%	2-3 (5)	100%
Atorvastatin	2 (2)	92%	1 (5)	92%	2 (2)	100%	1 (5)	100%	2 (2)	100%	1 (5)	100%
Azithromycin	3 (5)	83%	1 (5)	75%	3-4 (5)	100%	1-2 (5)	100%	4 (5)	100%	2-3 (5)	100%
Caffeine	1-2 (4)	75%	1-2 (5)	92%	2-3 (4)	100%	2 (5)	100%	2 (4)	100%	2 (5)	100%
Carbamazepine	2-3 (5)	100%	1 (5)	100%	5 (5)	100%	1-2 (5)	100%	5 (5)	100%	2 (5)	100%
Ciprofloxacin	1-2 (5)	92%	1-2 (5)	92%	1 (5)	92%	1 (5)	100%	1 (5)	100%	1 (5)	100%
Clarithromycin	3 (5)	100%	0 (5)	0%	3 (5)	100%	0 (5)	0%	3 (5)	100%	0 (5)	0%
Diclofenac	2 (3)	100%	2 (5)	100%	2 (3)	100%	2 (5)	100%	2 (3)	100%	2 (5)	100%
Erythromycin	2-3 (5)	100%	1 (5)	100%	3 (5)	100%	2 (5)	100%	3 (5)	100%	2 (5)	100%
Fluoxetine	1 (5)	33%	1 (5)	33%	1 (5)	100%	1 (5)	100%	1 (5)	100%	1 (5)	100%
Gemfibrozil	1 (1)	92%	2 (5)	92%	1 (1)	100%	2 (5)	100%	1 (1)	100%	2 (5)	100%
Ibuprofen	1 (1)	100%	1 (5)	100%	1 (1)	100%	1 (5)	92%	1 (1)	100%	2 (5)	100%
Ketoprofen	2 (2)	83%	1 (5)	92%	2 (2)	100%	1 (5)	100%	2 (2)	100%	1 (5)	100%
Losartan	2-3 (3)	100%	1-3 (5)	100%	3 (3)	100%	3 (5)	100%	3 (3)	100%	3 (5)	100%
Meloxicam	3 (3)	58%	2-3 (5)	58%	3 (3)	75%	2-3 (5)	67%	3 (3)	100%	2-3 (5)	100%
Metoprolol	4 (5)	100%	1 (5)	100%	4 (5)	100%	1-2 (5)	100%	4 (5)	100%	2 (5)	100%
Naproxen	1 (1)	92%	2-3 (5)	92%	1 (1)	100%	2 (5)	92%	1 (1)	100%	3 (5)	100%
Paracetamol	1 (1)	83%	0 (5)	0%	1 (1)	100%	1 (5)	75%	1 (1)	100%	1 (5)	100%
Pravastatin	3 (5)	67%	1 (5)	67%	3 (5)	75%	0 (5)	25%	3 (5)	100%	1 (5)	100%
Propranolol	3 (5)	100%	0 (5)	17%	3 (5)	100%	1 (5)	100%	3 (5)	100%	1-2 (5)	100%
Salbutamol	2 (3)	100%	3 (5)	100%	2 (3)	100%	4 (5)	100%	2 (3)	100%	4 (5)	100%
Spiramycin	1-2 (5)	75%	0 (5)	0%	2 (5)	100%	0 (5)	0%	2 (5)	100%	0 (5)	0%
Sulfamethoxazole	1-2 (2)	67%	1-2 (5)	67%	2 (2)	75%	0 (5)	42%	2 (2)	100%	1 (5)	100%
Trimethoprim	4 (5)	100%	2 (5)	100%	4 (5)	100%	2-3 (5)	100%	4 (5)	100%	3 (5)	100%
Valsartan	1 (2)	58%	1-2 (5)	58%	1 (2)	75%	0 (5)	67%	1 (2)	100%	1-2 (5)	100%
Xylazine	2-3 (3)	100%	1 (5)	67%	3 (3)	100%	1 (5)	100%	3 (3)	100%	1 (5)	100%

[a] Compliance rate.

[b] The range of detected MS/MS fragments that matched the MS/MS features in the suspect list. The maximum number MS/MS traces that were available for matching are given in parenthesis.

Overview of the recovery values obtained from QC samples that were measured in-between batches during FT-ICR-MS measurements



Sampling locations of WWTPs that were used to study the occurance of PhACs in WWTP influents and effluents via FT-ICR-MS